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Evidence for Lamprey GnRH-I and -III-like Molecules in the Brains of the Southern Hemisphere Lampreys *Geotria australis* and *Mordacia mordax*

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The present study has characterized gonadotropic releasing hormone (GnRH)-like molecules in the brains of representatives of the two southern hemisphere families of lampreys, Geotriidae and Mordaciidae. Chromatographic and immunocytochemical evidence showed that the brains of *Geotria australis* and *Mordacia mordax* contain two forms of GnRH-like molecules. These two forms correspond to lamprey GnRH-I and -III, which were first sequenced from the brain of the anadromous sea lamprey *Petromyzon marinus*, a representative of the family Petromyzontidae that is found only in the northern hemisphere. In chromatographic studies (HPLC) using lamprey GnRH-I and -III antiserum, two early eluting GnRH forms coeluted with synthetic lamprey GnRH-I and -III standards. Our studies thus indicate that, despite their apparently long period of separation, the three families of extant lampreys have each retained both of the lamprey GnRH (-I and -III forms) molecules. Moreover, immunocytochemical localization of lamprey GnRH indicated that the pattern of its distribution in the adult brain of at least one of these southern hemisphere lampreys (*G. australis*) is similar to that previously described for *P. marinus.* Distribution of GnRH in the brain of larval *G. australis* was not as extensive as that in larval *P. marinus*, which may account for the later gonadal development in the former species. The fact that lamprey GnRH-I and -III are the dominant GnRH forms in all three families of lampreys implies that these neurohormones have an ancient origin.

Key Words: GnRH; lamprey; *Geotria; Mordacia;* evolution; reproduction; Agnatha.

A key neuroendocrine function of the hypothalamus in all vertebrates is the release of the decapeptide gonadotropin-releasing hormone (GnRH) which, in turn, acts on the pituitary gland, regulating the pituitary–gonadal axis. Currently, 11 primary structures of GnRHs have been determined in various vertebrates (Sower, 1997; Carolsfeld *et al.,* 2000). Included in this group are the structures of GnRHs of three fish species of the Agnathans and Chrondrichthyes: an agnathan, the sea lamprey, *Petromyzon marinus* (lamprey GnRH-I and -III; Gorbman, 1965), an elasmobranch, the spiny dogfish, *Squalus acanthias* (dogfish GnRH and chicken GnRH-II; Lovejoy *et al.,* 1992); and a holocephalan, the ratfish, *Hydrolagus colliei* (chicken GnRH-II; Lovejoy *et al.,* 1991). The primary structure of lamprey GnRH-III, which differs in three amino acids from that of lamprey GnRH-I, is more closely related to the other members of the GnRH family than is lamprey GnRH-I. Lamprey GnRH-III has 80% amino acid sequence identity with chicken GnRH-II and dogfish GnRH, 70% identity with catfish GnRH-I, lamprey GnRH-I, and salmon GnRH, and 60% identity with mammalian GnRH and chicken GnRH-I (Sower *et al.,* 1993).

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Fossil evidence suggests that the earliest vertebrates (agnathans) evolved over 470 million years ago (Forey and Janvier, 1993). The living agnathans are thus of particular importance in understanding hypothalamic–pituitary relationships since they represent the oldest lineage of vertebrates. The agnathans are classified into two groups, myxinoids (hagfish) and petromyzontids (lamprey). The lampreys are represented in the contemporary fauna by three families (Potter, 1980). All 34 species of holarctic lampreys are placed in the Petromyzontidae, whereas the 4 species of southern hemisphere lampreys are allocated to either the Geotriidae or the Mordaciidae. It has been proposed that all extant lampreys evolved from a form similar to the holarctic species *Ichthyomyzon unicuspis* (Hubbs and Potter, 1971). *P. marinus* is considered to be more closely related to *Ichthyomyzon* species than are the Geotriidae and Mordaciidae, which are believed to have separated from *Ichthyomyzon* early and subsequently to have undergone considerable change and specialization.

Lampreys are the most basal vertebrates to clearly demonstrate roles for multiple GnRH molecules acting as neurohormones involved in reproductive activity. Both lamprey GnRH-I and -III have been shown to induce steroidogenesis and spermiation and/or ovulation in adult *Petromyzon* (Sower, 1989, 1990, 1997). Furthermore, in lampreys undergoing metamorphosis, there was a demonstrated increase in brain lamprey GnRH-I and -III which coincided with the acceleration of gonadal maturation (Youson and Sower, 1991). In immunocytochemistry studies, both lamprey GnRH-I and -III immunoreaction were found in the cell bodies in the rostral hypothalamus and preoptic area in larval (Tobet *et al.*, 1995; Wright *et al.*, 1994) and adult (Nozaki *et al.*, 1999) sea lampreys. We have suggested that, in the larval stage, most of the immunoreactive (ir)-GnRH is lamprey GnRH-III, indicating that GnRH-III may be the more active form during gonadal maturation. These data suggest that the structure and function of the GnRHs in vertebrates appear to be highly conserved throughout vertebrate evolution.

The aim of this study was to characterize the forms of GnRH in the brains of the southern hemisphere lampreys *Geotria australis* and *Mordacia mordax*, using high-perfomance liquid chromatography (HPLC), followed by radioimmunoassay (RIA) with specific antisera raised against known GnRHs, a method which has been used to separate and identify the GnRHs in the brains of many other vertebrates. The characteristics of GnRH of the above two species were compared with those of *P. marinus* to ascertain whether representatives of the three families of lamprey contain the same or different forms of the hormone. The second aim of the study was to determine the distribution of GnRH in the brains of larval and adult *G. australis* and to compare these with the same life cycle stages of *P. marinus.*

METHODS

Lampreys

Larval (premetamorphic stage) and adult *G. australis* were collected from rivers in southwestern Australia. *M. mordax* larvae were collected from rivers in southeastern Australia. Brains from 10 adult *G. australis* and 6 larval *M. mordax* were rapidly dissected out and immediately frozen on dry ice for later hormone extraction and assay. Two adult brains and three larval heads from *G. australis* were fixed in Bouin's fixative and later dehydrated in a graded alcohol series, cleared, and embedded in paraffin. Serial sections were cut at 10-μm thickness in either the transverse or the sagittal plane and collected on poly-l-lysine-coated glass slides for immunocytochemistry. There were insufficient specimens to prepare sections of the brains of *M. mordax.*

Extraction, HPLC, and RIA

Frozen brains were extracted using the method described by Yu *et al.* (1987) and Fahien and Sower (1990). Briefly, brains were weighed and then homogenized in 2.0 M ice-cold acetic acid. The homogenates were centrifuged and the supernatants were dried on a refrigerated vacuum centrifuge, reconstituted in deionized and filtered water, and recentrifuged. The extract was filtered through a 0.45-μm Arco LC disposable filter and then injected into a 20-μl loop on a high-performance liquid chromatography system that consisted of a Perkin-Elmer Series 100 pump with a C18 reverse-phase column. The isocratic mobile phase consisted of 7.40 g ammonium acetate and 3.04 g citric acid in 19% acetonitrile/water. The final pH was ad-

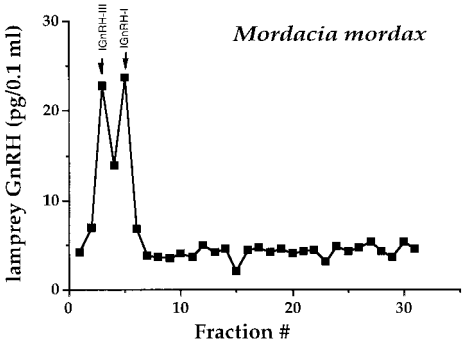

FIG. 1. Reverse-phase HPLC of *Mordacia mordax* brain extract and radioimmunoassay with lamprey GnRH antiserum 1467. Arrows indicate peaks of assayed synthetic standards of lamprey GnRH-III and lamprey GnRH-I.

justed to 4.62 with phosphoric acid. The flow rate was 0.5 ml/min. Synthetic mammalian GnRH, chicken GnRH-I and -II, salmon GnRH, dogfish GnRH, and lamprey GnRH-I and -II standards were chromatographed in parallel on the same HPLC system.

Radioimmunoassay was performed as previously described in Stopa *et al.* (1988), Fahien and Sower (1990), and Robinson *et al.* (2000). Synthetic mammalian GnRH and lamprey GnRH-I were used as the iodinated ligand and standard. The antisera were used at dilutions of 1:20,000 for mammalian RIA (R1245) and 1:10,000 for lamprey RIA (1467). The lamprey GnRH antibody (1467) binding ranged between 33 and 58%. The mammalian GnRH antibody (R1245) binding ranged between 39 and 44%. Antiserum R1245 demonstrates cross-reactivities of 65, 19.5, 4.16, and $<0.00001\%$ for chicken GnRH I, salmon GnRH, chicken GnRH II, and lamprey GnRH-I, respectively (Calvin *et al.*, 1993). Antiserum 1467 has cross-reactivities of 7.3% with lamprey GnRH-III and less than 0.03, 0.02, and 0.01% cross-reactivity for chicken GnRH-II, mammalian GnRH, and chicken GnRH-I, respectively (Sower *et al.*, 1993).

Immunocyctochemistry

Deparaffinized, rehydrated sections were treated with 1% hydrogen peroxide in methyl alcohol to block endogenous peroxidase activity. After being rinsed in running water, the sections were exposed to cold 2.5% trypsin for 3 min and again washed in running water before they were incubated with 0.5% casein solution for 30 min, washed in phosphate-buffered saline (PBS), and incubated with normal serum for 30 min, to reduce nonspecific binding. Slides were then transferred to humid incubation chambers and incubated overnight at 4° with primary antiserum (1:50,000 dilution of antiserum 3952 generated toward lamprey GnRH-III, with cross-reactivity with lamprey GnRH-I) (Sower *et al.*, 1993). The resulting antigen–antibody complex was then visualized using a commercially available biotinylated peroxidase procedure (Vectastain ABC kit, Vector Lab, Inc., Burlington, CA). The specificity of the antiserum was demonstrated by treating one to two sections on each slide with normal rabbit serum (diluted 1:50,000) in place of the primary antiserum. These control sections contained no immunoreactivity.

RESULTS

RIA and Chromatography

The elution times of fractions from *G. australis* and *M. mordax* brains were compared to the five synthetic standards eluted with an identical HPLC system. The elution profiles of the GnRH standards were eluted in the following order: lamprey-III, chicken-I/mammal, lamprey-I, chicken-III, and salmon.

Two ir-GnRH peaks were detected using AB1467 in *M. mordax* (Fig. 1). One peak eluted in the same position as synthetic lamprey GnRH-I. The second peak coeluted with synthetic lamprey GnRH-III. Similar peaks were noted with *G. australis* samples (Fig. 2).

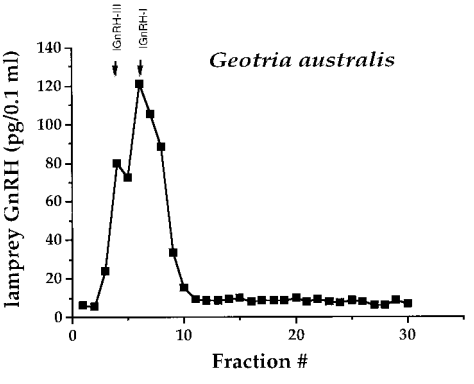

FIG. 2. Reverse-phase HPLC of *Geotria australis* brain extract and radioimmunoassay with lamprey GnRH antiserum 1467. Arrows indicate peaks of assayed synthetic standards of lamprey GnRH-III and lamprey GnRH-I.

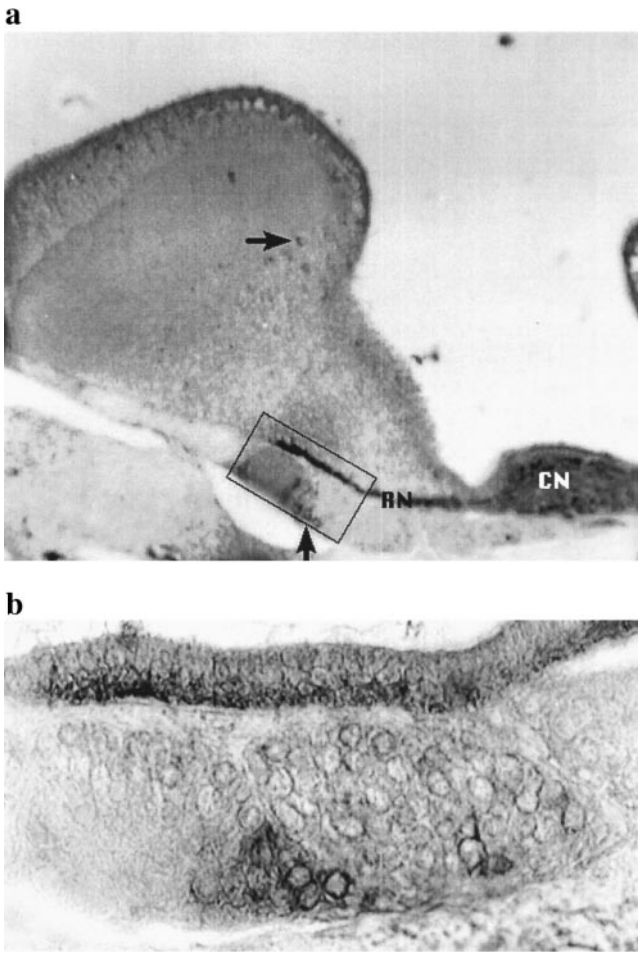

FIG. 3. (a) Parasagittal section through the hypothalamus and pituitary of a *Geotria australis* larva, showing ir-GnRH cell bodies in the hypothalamus (arrow) and in the pituitary (arrow). Fiber tracts between the cell bodies and the neurohypophysis are more lateral than those in this section. Fibers containing ir-GnRH collect into fascicles in the rostral neurohypophysis and completely fill the caudal neurohypophysis. (b) High-power view of the pituitary boxed in (a) showing ir-GnRH cell bodies.

Immunocytochemistry

Geotria australis larvae. Cells and fibers containing ir-GnRH were detected in the brains and pituitary of each of the three larval lampreys examined. Ir-GnRH cell bodies were observed only in the most dorsal region of the preoptic area (POA), close to the third ventricle of the brain, and in several discrete groups in the adenohypophysis (Fig. 3). Ir-GnRH-containing fiber tracts could be traced from the cell bodies in the POA through the hypothalamus (Fig. 4) to the rostral neurohypophysis and then into the entire caudal neurohypophysis, where the ir-GnRH-containing fibers collected into fascicles.

Geotria australis adults. The majority of the ir-GnRH cell bodies were located in the dorsal and ventral areas of the POA (Fig. 5). In addition, there was a medial group of ir-GnRH cell bodies immediately caudal and dorsal to the optic chiasma, bordering the third ventricle (Figs. 5 and 7). These cell bodies possessed axons which projected to, and directly contacted, the third ventricle (Fig. 7). They may also have been bipolar, possessing a second axon which projected back into the hypothalamus. A third group of ir-GnRH cell bodies was present in the medial hypothalamus (Fig. 5). As in the larvae, there were also groups of ir-GnRH cells in the ventral adenohypophysis (proximal region only) (Fig. 6).

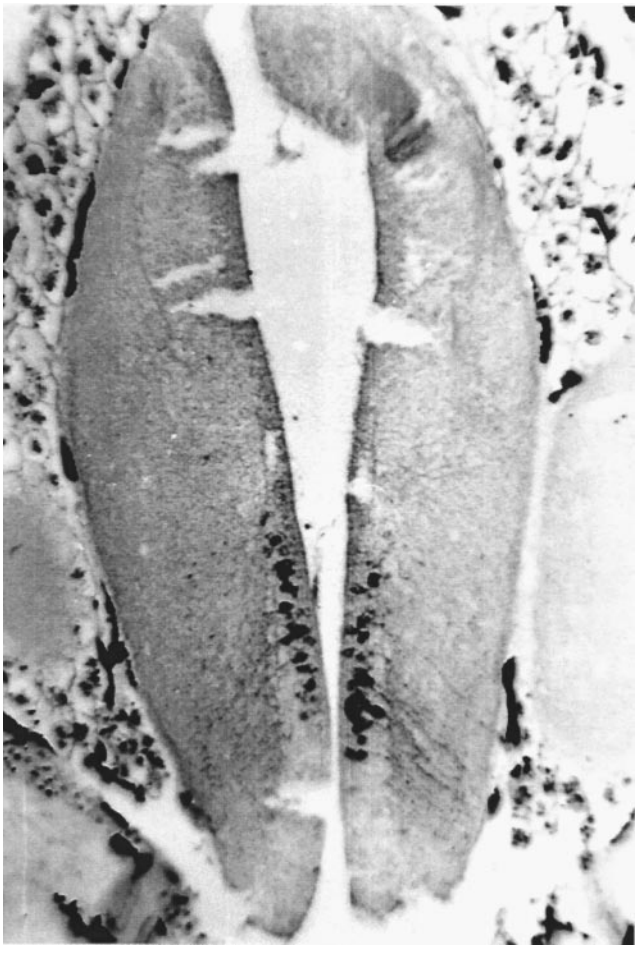

FIG. 4. Transverse section through the hypothalamus of a *G. australis* larva showing ir-GnRH cell bodies among the ependymal cells lining the third ventricle, with fiber tracts coursing toward the neurohypophysis, which is more caudal than this section.

Ir-GnRH fibers were immunostained far more extensively in the brains of the adults than in the brains of the larvae. Dense tracts of ir-GnRH fibers could be traced from the cell bodies in the POA to well-organized preoptico–hypophyseal tracts projecting in an arc laterally and ventrally toward the rostral neurohypophysis where the immunoreactivity collected into fascicles. These fascicles of ir-GnRH completely filled the caudal neurohypophysis. The ir-GnRH fascicles of the rostral neurohypophysis also projected between the proximal pars distalis and the pars intermedia in conjunction with the appearance of ir-GnRH cells in the proximal pars distalis of the adenohypophysis (Fig. 6).

In addition to the well-organized preoptico–hypothalamo projections, many ir-GnRH fibers extended from the cell bodies in the preoptic nucleus forward into the caudal ventral region of the olfactory lobes (Fig. 5), where some ir-fascicles, similar to those found in the neurohypophysis, were observed. Some ir-GnRH fibers extended dorsally into the habenular region just caudal to the pineal. There were also numerous extrahypothalamic projections extending backward through the medial hypothalamus into the midbrain. A few of these projections even extended beyond the medulla.

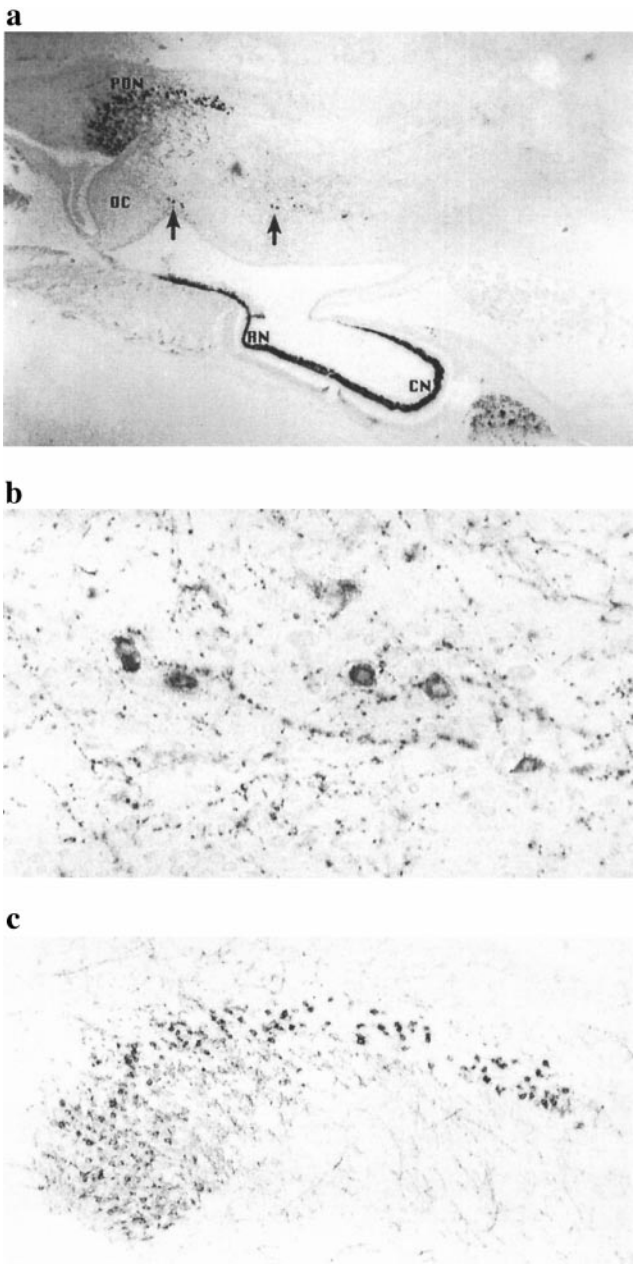

FIG. 5. (a) Parasagittal section through the hypothalamus and pituitary of an adult *G. australis.* Strong ir-GnRH is seen in the cell bodies of the preoptic nucleus (PON) and fascicles within rostral (RN) and caudal neurohypophysis (CN). RN is near the ventricle midsaggital but not lateral. A few ir-GnRH cell bodies are also seen in the ventral hypothalamus just caudal to the optic chiasma (OC) (arrow) and in the medial hypothalamus (arrow). Ir-GnRH fiber tracts can be seen extending from the PON cell bodies forward toward the olfactory lobe and backward into the hypothalamus and beyond to the midbrain. (b) High-power view of the ir-GnRH cell bodies arrowed in (a) in the ventral hypothalamus. (c) Higher-power view of the ir-GnRH cell bodies and fibers in the PON.

DISCUSSION

The present study has characterized gonadotropin-releasing hormone-like molecules in the brains of representatives of the two southern hemisphere families of lampreys, Geotriidae and Mordaciidae. Our chromatographic and immunocytochemical studies show that, despite an apparently long separation of the three extant families of lampreys, the brains of representatives from each of the families have the same GnRH molecules (lamprey GnRH-I and -III). In the chromatography studies, there were two major ir-GnRH peaks in both *M. mordax* and *G. australis* corresponding to synthetic lamprey GnRH-III and -I standards. Since lampreys are one of the only two surviving groups of the agnathan radiation, our findings suggest that at least one of these forms of lamprey GnRH is likely to be ancestral to the other forms found in the gnathostome vertebrates. Lamprey GnRH-III is

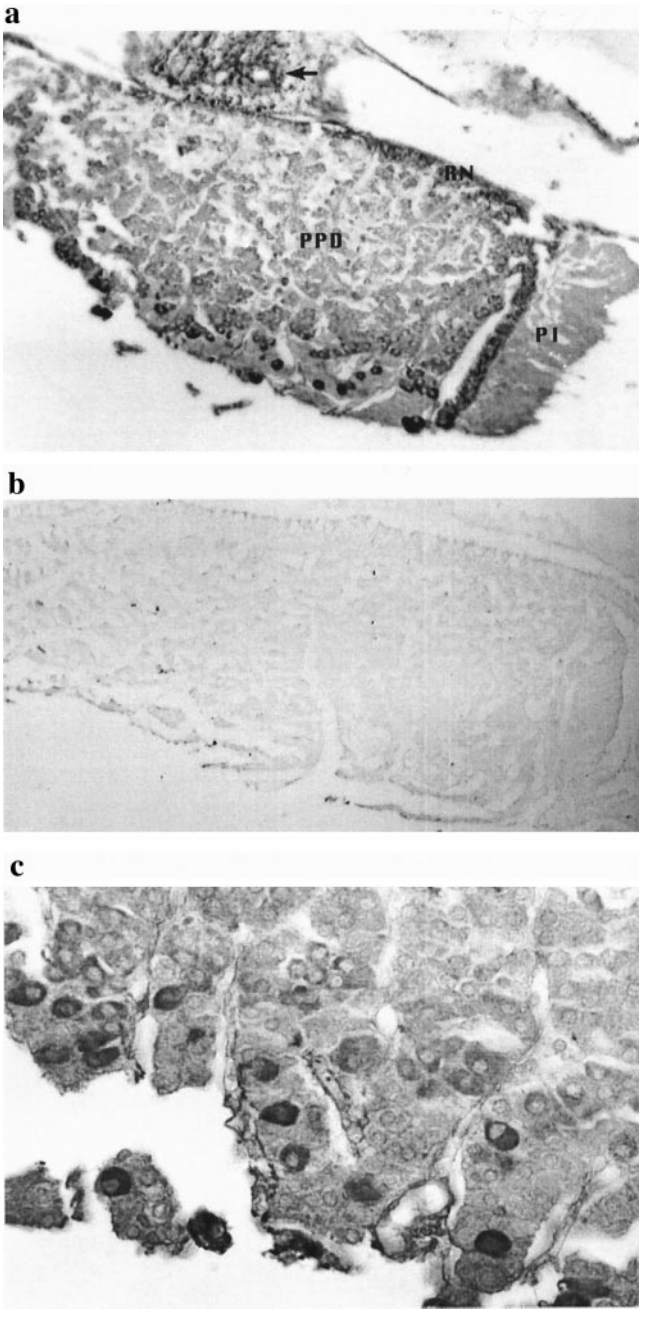

FIG. 6. (a) Ir-GnRH cells in the ventral proximal pars distalis of an adult *G. australis.* This section also shows ir-fibers of the preoptico–hypothalamo tract (arrow) as it converges into the rostral neurohypophysis which extends between the pars distalis and the pars intermedia. (b) Control section of the ventral proximal pars distalis devoid of any ir-GnRH cells. (c) High-power view of ir-GnRH cells in the ventral proximal pars distalis.

more closely related than lamprey GnRH-I to the other members of the GnRH family, sharing 80% homology with chicken GnRH-II, which has been proposed as the ancestral gnathostome form of GnRH (King and Millar, 1992).

Our immunocytochemical studies are also consistent with the presence of both lamprey GnRH-I and -III in the brain of *G. australis,* as has been shown for the holarctic lampreys *P. marinus* (King *et al.,* 1988) and *Lampetra tridentata* (Nozaki *et al.,* 1984). Moreover, there is a similar distribution pattern of ir-GnRH cell bodies and fibers in adult specimens from both families. The situation in the larvae, however, is somewhat different. Wright *et al.* (1994) and Tobet *et al.* (1995) have studied the distribution of lamprey GnRH in the brain of larval and metamorphosing *P. marinus.* Whereas this study on *G. australis* found some of the ir-GnRH cell bodies and tracts described by these workers, they were less widely distributed, being confined to the dorsal POA with simple tracts projecting only to the rostral and caudal neurohypophysis. The more extensive tracts described for *P. marinus* larvae were found only in the adult *G. australis.* Of significance is the late development of the gonads in *G. australis* as compared to *P. marinus.* In large, premetamorphic *G. australis,* the testis is still small and apparently undifferentiated with only a few germ cells present among numerous somatic cells (Hardisty *et al.,* 1986). The late development of the gonads in *G. australis* correlates positively with the much reduced distribution of lamprey GnRH in the brain of the larvae, compared to the adults.

A more intriguing difference between *G. australis* and *P. marinus* is the presence of ir-GnRH cells in the

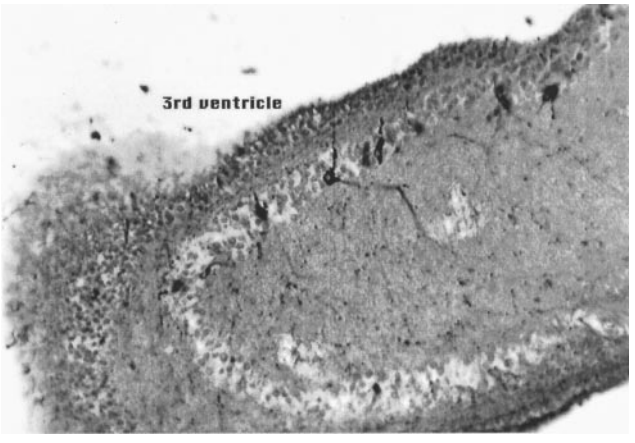

FIG. 7. Sagittal section through part of the anterior hypothalamus of adult *G. australis* showing ir-GnRH cell bodies bordering the third ventricle. Note fibers extending from cell bodies toward the third ventricle and into the body of the hypothalamus.

adenohypophysis of both larvae and adults of *G. australis*. There is no mention of these cells in any of the *P. marinus* or *L. tridentata* studies (King *et al.*, 1988; Nozaki *et al.*, 1984; Tobet *et al.*, 1995; Wright *et al.*, 1994), which suggests that they were not observed. The significance of these cells in *G. australis* remains to be investigated. In *P. marinus*, immunocytochemical studies showed that the principal direction of distribution of axons from the nucleus preopticus GnRH cells was toward the neurohypophysis via a recognizable tract (Nozaki *et al.*, 1999). This resulted in a heavily stained dorsal layer of the two-layered neurohypophysis. Thus, if the function of the GnRH is to release gonadotropin from the adjacent adenohypophysis, it must diffuse through the ventral layer of the neurohypophysis to reach the GTH cells. Unlike most vertebrates, a distinct vascular or neural link between the hypothalamus and the adenohypophysis has not been observed in either lamprey or hagfish (Gorbman, 1965). However, there is evidence to support the concept of hypothalamic control of adenohypophysial function by diffusion of the neurohormones from the neurohypophysis to the pars distalis of the adenohypophysis (King *et al.*, 1988; Nozaki *et al.*, 1984, 1994). Based on these immunocytochemistry studies, lamprey GnRH-I and -III neurons project their fibers primarily into the neurohypophysis from the preoptic region. It is hypothesized that neurosecretory peptides like gonadotropin-releasing hormone can diffuse from the brain (neurohypophysis) to the adenohypophysis, either directly or by an additional route via secretion into the third ventricle to be transported by tanycytes to the adenohypophysis, and regulate secretory activity in lampreys. The discovery of ir-GnRH cells in the pituitary of *G. australis* in the present study may indicate an additional regulatory pathway that is unknown.

This study suggests that lamprey GnRH-I and -III may be the dominant GnRH forms in all species of lampreys, which, in turn, suggests either that they are the ancestral agnathan forms of GnRH or that they evolved early in the lamprey lineage. In two recent studies using antibodies to lamprey GnRH (lGnRH-III), as well as other GnRH antibodies, immunoreactive GnRH was detected in the brain of the Atlantic hagfish, *Myxine glutinosa* (Sower *et al.*, 1995), and Pacific hagfish, *Eptatretus stouti* (Braun *et al.*, 1995). In the Pacific hagfish studies, two GnRH systems were proposed, one system which is widely diffuse throughout the brain and another which is restricted to the preoptic–neurohypophysial system (Braun *et al.*, 1995). These two distinct GnRH systems proposed in Pacific hagfish were identified using a salmon GnRH antibody (PBL-49) (Braun *et al.*, 1995). This antiserum displayed a differential affinity for the two systems, indicating that the two systems differ in the amount or identity of GnRH. In an unpublished study using another salmon antiserum, J. C. King, E. L. Anthony, and S. A. Sower also detected ir-salmon GnRH in the mid- and hindbrain of Atlantic hagfish. The chromatography data of Sower *et al.* (1995) support the evidence that there are at least two forms of GnRH in hagfish brain, although in this same study, the immunocytochemical data demonstrated the presence and distribution of only lamprey GnRH-III-like immunoreactivity in the neurohypophysis of the hagfish. The studies in hagfish, from both HPLC-RIA and immunocytochemistry, clearly suggest that a lamprey GnRH-III-like molecule is present in the hagfish brain (Sower *et al.*, 1995). Therefore, the data from the present study, as well as the cited hagfish studies, provide strong evidence for the conservation of lamprey GnRH-III in agnathans.

In this paper, agnathans are considered to be paraphyletic in origin, with the modern agnathans classified into two groups, myxinoids (hagfish) and petromyzontids (lamprey) (Forey, 1984; Forey and Janvier, 1993). Recent paleontological analyses of modern groups have also suggested that the jawed vertebrates are more closely related to lampreys than either is to the hagfishes (Forey and Janvier, 1993). The interrelationships of the extant and extinct early evolved vertebrates have been a subject of extensive review, particularly in light of more recent information and discoveries of extinct agnathans (reviewed in Forey and Janvier, 1993; Pough *et al.*, 1996). More extensive determination of hormonal and developmental genes may help in resolving the phylogenetic relationships among hagfish, lampreys, and jawed vertebrates. The findings from the present study support the concept that gene duplication occurred close to the origin of the vertebrates, before the origin of the lampreys.

The conservation of GnRH form among all lampreys provides a strong basis for investigating other similarities and differences in the reproductive axis of the three families of extant lampreys. This should

prove to be a fruitful means of discerning ancestral patterns in the control of reproduction in vertebrates.

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