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Gonadotropin-Releasing Hormones in the Brain and Pituitary of the Teleost, the White Sucker

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The present study investigated GnRH forms within the brain of a representative of the order Cypriniformes, the white sucker, *Catostomus commersoni*, using HPLC, RIA, and immunocytochemistry. Several immunoreactive (ir) GnRH forms were identified in the brain of the white sucker by chromatography and radioimmunoassay, including ir-salmon GnRH, ir-lamprey GnRH-I and -III, and ir-chicken GnRH-II. Results from immunocytochemical studies were consistent with multiple GnRH forms distributed in different patterns, particularly for fibers. Neuronal perikarya containing ir-salmon GnRH and ir-lamprey-like GnRH were found laterally within the preoptic area and rostral hypothalamus. Cells containing exclusively ir-salmon GnRH appeared slightly more rostrally, but in the same region. Fibers containing ir-salmon GnRH and ir-lamprey-like GnRH were seen throughout the caudal telencephalon and extended into the diencephalon, toward the pituitary. Fibers containing ir-chicken-II-like GnRH were also seen in the caudal telencephalon, but were concentrated more dorsally in the diencephalon. Within the pituitary, fibers containing ir-salmon GnRH and ir-lamprey-like GnRH entered the neurohypophysis, but differed in their destinations. Fibers containing ir-salmon GnRH remained within the neurohypophysis, while fibers containing ir-lamprey-like GnRH targeted adenohypophyseal tissue. These findings are consistent with the hypothesis that multiple GnRH forms with multiple functions exist within the brain and pituitary of teleosts and provide further evidence of a lamprey-like GnRH within an early evolved teleost species.

Gonadotropin-releasing hormone (GnRH) released from the hypothalamus is the major neuroendocrine regulator of the pituitary-gonadal axis in vertebrates. To date, the primary structures for 10 vertebrate GnRHs in various vertebrate species and 2 in invertebrates have been determined (Jimenez-Linan *et al.*, 1997; Sherwood *et al.*, 1997; Sower, 1997). Since GnRH was first isolated from the brain of a mammal, the pig (Matsuo *et al.*, 1971), the GnRH family has expanded and the primary structure of at least one unique GnRH has been determined from representative species from all classes of vertebrates. All GnRH molecular forms consist of 10 amino acid residues and are considered to be highly conserved, with sequence identities ranging between 50 and 90% (Sower, 1997). Conserved features of the GnRH molecule are the identical N- and C-termini, and the same amino acid residues at positions 1, 4, 9, and 10.

In recent years, it has been shown that in vertebrates at least two different GnRH forms are expressed within

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the brain of a single species, generally one GnRH functions as a neurohormone regulating the pituitary in mediating the release of gonadotropin, and the other form may have a neurotransmitter / neuromodulatory function and is generally localized in areas outside the hypothalamus or midbrain regions (Muske, 1993). Some species, such as the dogfish, may contain as many as seven GnRH isoforms (Powell *et al.,* 1986). Chicken GnRH-II is the most ubiquitous, present in the brain in all vertebrate classes except agnathans. However, in mammals, only a limited number of species (monotremes, marsupials, and primitive eutherians) have been shown to contain more than one form of GnRH, using indirect methods of HPLC, RIA, and ICC. In these species, the second form identified was chicken GnRH-II. The only direct evidence of GnRHs other than the mammalian form has been by identification of complementary DNA in three species: the tree shrew, in which two prepro-GnRH mRNAs to mammalian and chicken GnRH-II (Kasten *et al.,* 1996) were identified; the guinea pig, in which a prepro-GnRH encodes for a new form of GnRH called guinea pig GnRH (Jimenez-Linan *et al.,* 1997); and most recently in the human, in which individual genes for mammal GnRH and chicken GnRH-II were demonstrated (White *et al.,* 1998).

Within the vertebrate class, Osteichthyes, the primary structures of GnRHs that have been identified include chicken GnRH-II, salmon GnRH, mammalian GnRH, catfish GnRH, and seabream GnRH. There are various classification systems of fish, but in this paper, we will use the classification system described by Colbert and Morales (1991). Within the class of Osteichthyes are three divisions or infraclasses. These include Chondrostei which are considered the most primitive and include sturgeons, paddlefish, and bichirs; Neopterygii (Holostei) which are considered intermediate and include garpikes and bowfins; and Neopterygii (Teleostei) which include most of the bony fish and are considered more advanced. There are further divisions within the teleost group which include the earlier evolved, intermediate, and most advanced. In the earlier evolved Chondrostei, the mammalian GnRH primary structure was determined in Russian sturgeon, *Acipenser gueldenstaedti* (mammalian GnRH) (Lescheid *et al.,* 1995). In the early evolved teleosts, the primary structures have been determined in Thai and African catfish, *Clarias macrocephalus* and *C. gariepinus* (chicken GnRH-II and catfish GnRH) (Bogerd *et al.,* 1992; Ngamvongchon *et al.,* 1992), and in chum salmon, *Oncorhynchus keta* (salmon GnRH) (Sherwood *et al.,* 1983). In the intermediately evolved teleosts, the primary structure has been determined in codfish, *Gadus morhua morhua* (salmon GnRH) (Wu *et al.,* 1986). Lastly, in the more advanced teleosts, the primary structures of GnRH have been determined in seabream, *Sparus aurata* (chicken GnRH-II, salmon and seabream GnRH) (Powell *et al.,* 1994), and African cichlid, *Haplochromis burtoni* (seabream GnRH) (Powell *et al.,* 1995). Additionally, by isolating and cloning prepro-GnRH cDNA, genes have been identified encoding prepro-salmon(s) GnRH in a variety of salmon and trout species (*Oncorhynchus masu, O. tshawytscha, O. mykiss, Salmo salar, S. trutta,* and *Salvelinus fontinalis*) (Klungland *et al.,* 1992a,b; Suzuki *et al.,* 1992), prepro-sGnRH in red seabream (*Pagrus major*) (Okuzawa *et al.,* 1994), prepro-chicken(c) GnRH-II and -catfish GnRH in African catfish (*C. gariepinus*) (Bogerd *et al.,* 1994), and prepro-cGnRH-II, -sGnRH, and -seabream GnRH in African cichlid (*H. burtoni*) (White *et al.,* 1995).

Using indirect methods including a combination of high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) procedures, immunoreactive (ir) chicken-GnRH-I and -II, and salmon, catfish, seabream, and mammalian GnRH have been identified in fish from several teleost families (King and Millar, 1997; Powell *et al.,* 1995; Sherwood *et al.,* 1994). The most widespread forms that occurred were ir-chicken GnRH-II and ir-salmon GnRH. A number of unidentified ir-GnRH forms have been demonstrated as well. However, of particular interest is the first demonstration of ir-lamprey GnRH-I in brains of five advanced teleost marine perciform species, bluefin tuna (*Thunnus thynnus*), black seabream (*Acanthopagrus schlegeli*), red seabream (*Pagrus major*), red spotted grouper (*Epinephelus akaara*), and Japanese flounder (*Paralichthys olivaceus*) (Okuzawa *et al.,* 1993), and in the pituitary of an intermediate evolved teleost, the platyfish (*Xiphophorus maculatus*) of the order Cyprinodontiformes (Magliulo-Cepriano *et al.,* 1994). There have been no studies reported on the possible presence of ir-lamprey GnRH in an early evolved teleost. Therefore, the objective of the present investigation was to characterize immunoreactive forms of GnRH using

immunocytochemistry, chromatography, and immunological methods within the brain and pituitary of white sucker (*Catostomus commersoni*), a cyprinid, which is an early evolved teleost considered to be at the very base of the euteleostean radiation.

METHODS

Animals

Adult white suckers, average length 36 cm, were purchased from New Hampshire bait shops, caught with nets set in the Oyster River, Durham, New Hampshire, and Pleasant Lake, Northwood, New Hampshire, or received by overnight delivery from Hammond Bay Biological Station, Millersburg, Michigan. Sampling occurred at three different times of the year: (1) mid-January / late-February, (2) mid-April, and (3) late-September / early-October. White suckers were maintained for no more than 24 hr in a freshwater flow-through system supplied with ambient local reservoir water before being killed. For chromatographic and radioimmunological studies, whole brains were dissected immediately following decapitation, frozen on dry ice, and stored at −80°C until extracted and assayed. The mean weight of the brains was 0.63 g ($n = 9$). For immunocytochemical studies, animals were anesthetized using 0.1% tricaine methanesulfonate and killed by decapitation. Brains ($n = 15$) and pituitaries ($n = 4$) were dissected in cold 0.05 M phosphate-buffered saline (PBS) and immersion-fixed overnight at 4°C in 2% acrolein / 0.1 M phosphate buffer, pH 7.4 (King *et al.*, 1988). All tissues were transferred to 0.05 M PBS and stored at 4°C until sectioning and immunocytochemical processing (see below).

Extraction and HPLC

Frozen brains were extracted as described by Yu *et al.* (1987) and Fahien and Sower (1990) and eluted on a HPLC system following the methods of Fahien and Sower (1990) and Calvin *et al.* (1993). Briefly, the extract was filtered using an ACRO LC 13 (0.45 μm) filter and then injected into a 20-μL loop on a Perkin-Elmer HPLC system with a Pecosphere 3CR C18 (0.46 × 8.3 cm) reverse-phase column. The isocratic phase consisted of 7.40 g ammonium acetate and 3.04 g citric acid in 1 L of 19% acetonitrile / water (final pH adjusted to 4.6 with phosphoric acid) (Stopa *et al.*, 1988). The flow rate was 2 mL / min with fractions collected every 18 s for the first 34 fractions and then every minute for subsequent fractions.

Synthetic mammalian GnRH, chicken GnRH-I and -II, salmon GnRH, and lamprey GnRH-I and -III standards (0.5 μg / ml) were chromatographed in parallel on the same HPLC system. HPLC-grade water was injected onto the column after each standard and sample fractions were collected and assayed for GnRH.

Radioimmunoassay

Radioimmunoassays were performed as previously described by Stopa *et al.* (1988) and Fahien and Sower (1990) using synthetic mammalian GnRH or lamprey GnRH-I as the radioiodinated tracer and standard. The antisera were used at dilutions of 1:100,000 for mammal RIA (R1245), 1:25,000 for lamprey-I RIA (1467), and 1:16,000 for lamprey-III RIA (3952). The mammalian antiserum (R1245) binding ranged between 39 and 44%, lamprey-I antiserum (1467) binding ranged between 33 and 58%, and lamprey-III antiserum (3952) binding ranged between 40 and 45%. Antiserum R1245 has cross-reactivities of 65, 19.5, and 4.16% for chicken GnRH-I, salmon GnRH, chicken GnRH-II, respectively, and <0.00001% lamprey GnRH-I and lamprey GnRH-III (Calvin *et al.*, 1993; Sower, unpublished). Antiserum 1467 has cross-reactivities of 7.3% with lamprey GnRH-III and less than 0.03, 0.02, and 0.01% for chicken GnRH-II, mammal GnRH, and chicken GnRH-I, respectively (Sower *et al.*, 1994). Antiserum 3952 has cross-reactivities of >100% with lamprey GnRH-I, and <0.01% for mammal GnRH, chicken GnRH-I, chicken GnRH-II, and salmon GnRH (Table 1).

Immunocytochemistry

Antisera were generated against (1) salmon GnRH, Lots 432, 1667, and 1668, provided by Dr. Judy King, (2) lamprey GnRH-I, Lots 1467 and 21-134, provided by Drs. Judy King and Stacia Sower respectively, (3) lamprey GnRH-III, Lot 3952, provided by Dr. Stacia Sower, and (4) chicken GnRH-II antisera provided by Dr. James Millam (Millam *et al.*, 1993) and Lot 675,

TABLE 1
Cross-Reactivity of GnRH in Radioimmunoassay

GnRH	Antiserum	% Cross-reactivity					
		cGnRH-I	cGnRH-II	Salmon	lGnRH-I	lGnRH-III	mGnRH
mGnRH	R1245	65	4.16	19.5	<0.00001	ND[a]	100
lGnRH-I	1467	<0.01	<0.03	ND	100	7.3	<0.02
lGnRH-III	3952	<0.01	<0.01	ND	100	100	<0.01

[a] ND, nondetectable.

provided by Dr. Judy King, respectively. All antisera were diluted in 1.0% BSA/0.05 M PBS/0.3% Triton X-100, pH 7.4, and used as follows: (1) antiserum 432 at 1/1000, antiserum 1667 at 1/4000, and antiserum 1668 at 1/1000 and 1/2000, (2) antiserum 1467 at 1/5000 and antiserum 21-134 at 1/2000, (3) antiserum 3952 at 1/10,000, and (4) chicken GnRH-II antiserum at 1/2000 to 1/5000 and antiserum 675 at 1/1000. Negative controls were preabsorptions as follows: (1) antiserum 1667 with 20 or 40 μM sGnRH and 20 μM lamprey GnRH-III, and antiserum 1668 with 40 μM sGnRH, (2) antiserum 1467 with 40 μM lamprey GnRH-I, 40 μM lamprey GnRH-III, or 40 μM sGnRH, and antiserum 21-134 with 40 μM chicken GnRH-II, (3) antiserum 3952 with 20 or 40 μM lamprey GnRH-III, 20 or 40 μM sGnRH, 40 μM chicken GnRH-II, and 50 μM somatostatin, and (4) chicken GnRH-II antiserum with 40 μM chicken GnRH-II and 40 μM lamprey GnRH-III.

Table 2 shows general immunoreactivity and specific preabsorption data for the antisera used in the present study. Preabsorption data revealed that antisera 1667 and 1668 were selective for salmon GnRH. While preabsorption of both antisera with salmon peptide abolished immunoreactivity, preabsorption of 1667 with lamprey GnRH-III did not abolish immunoreactivity. Preabsorption of antisera 21-134 and 3952 with chicken GnRH-II and chicken GnRH-II antiserum with lamprey GnRH-III was performed because of the similarity of the two peptides, which differ by only two amino acids (Fig. 1). Preabsorption of antiserum 3952 with somatostatin was performed because previous studies with lamprey GnRH antisera have shown cross-reactivity with this peptide (Stopa *et al.*, 1988). All preabsorptions of these antisera abolished their immunoreactivities except for preabsorption of antiserum 3952 with somatostatin. Since preabsorption data for antisera 1467, 21-134, 3952, and anti-chicken GnRH-II suggested that these antisera could not differentiate among multiple forms, specificity was inferred by differential distributions of immunoreaction product in both the brain and the pituitary (see Results).

Immunoreaction products associated with lamprey

TABLE 2
Preabsorption Study Results in the Brain and Pituitary of the White Sucker

Antisera against	Lot No.	Brain or pituitary	Immunoreactivity				
			Without preabsorption	Preabsorption with			
				sGnRH	lGnRH-I	lGnRH-III	cGnRH-II
sGnRH	1667	Brain	+++	− (Abolished)	ND	+++	ND
		Pituitary	++	− (Abolished)	ND	+	ND
sGnRH	1668	Brain	+++	− (Abolished)	ND	ND	ND
lGnRH-I	1467	Brain	+++	− (Abolished)	− (Abolished)	−	ND
lGnRH-I	21–134	Brain	+++	ND	ND	ND	− (Abolished)
lGnRH-III	3952	Brain	+++	− (Abolished)	ND	− (Abolished)	− (Abolished)
		Pituitary	+++	− (Abolished)	ND	− (Abolished)	ND
cGnRH-II	unnamed	Brain	+++	ND	ND	− (Abolished)	− (Abolished)

Note. +++, intense immunoreaction; ++, moderate immunoreaction; +, faint immunoreaction; −, abolished.

The GnRH Family of Peptides

GnRH	1	2	3	4	5	6	7	8	9	10
Vertebrate										
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Guinea Pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH_2
Chicken-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
Sea Bream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2
Chicken-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2
Lamprey-III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2
Lamprey-I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2
Invertebrate										
Tunicate-I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH_2
Tunicate-II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH_2

FIG. 1. Primary amino acid structures of the 10 vertebrate and 2 invertebrate GnRH molecules. Underlined amino acids represent differences in forms compared to mammal GnRH.

and chicken-II GnRH-directed antisera are referred to as ir-lamprey-like and ir-chicken-II-like to indicate the presumed identity of the immunoreactive elements. Immunoreaction products associated with salmon GnRH-directed antisera are referred to as ir-sGnRH because the HPLC data and the immunocytochemical preabsorption data corroborated its identity.

Immunocytochemical analyses were adapted from procedures described previously (Tobet *et al.,* 1995). Briefly, adult white sucker brains and pituitaries fixed in 2% acrolein / 0.1 M phosphate buffer (pH 7.4) were sectioned at a thickness of 50 μm and bathed in plastic containers with nitex mesh bottoms, immersed in 0.05 M PBS. Sections were placed sequentially into three separate containers so that adjacent sections could be incubated with different primary antisera. Sections were then sequentially pretreated with 0.1 M glycine, 0.5% sodium borohydride, 5% normal goat serum (NGS) / 0.3% Triton X-100 / 1% hydrogen peroxide with avidin blocking reagent (Vector Laboratories), and 5% NGS / 0.3% Triton X-100 with biotin blocking reagent (Vector Laboratories), all made in PBS at 4°C. Incubation with primary antisera was performed at 4°C for a duration of 1 or 2 nights. Sections were then washed in 1% NGS / 0.02% Triton X-100 / PBS at RT, and incubated for 2 h at RT in anti-rabbit biotinylated secondary antiserum (Vector Laboratories). Washes were repeated with 0.02% Triton X-100 / PBS; sections were incubated for 1 h at RT in ABC reagent (Vector Laboratories), and then washed in Tris-buffered-saline (TBS; 0.05 M, pH 7.4, at RT). Visualization was achieved by a dark gray / black precipitate resulting from reaction with 0.025% 3,3′-diaminobenzidine (DAB) / 0.02% nickel ammonium sulfate / TBS with 0.02% hydrogen peroxide for 5 min.

An estimate of the number of neuronal perikarya containing ir-GnRH was obtained by counting the number of neurons in one-third of the sections from each brain. For consistency of counting, estimates of the number of neuronal perikarya containing ir-sGnRH were based on sections immunoreactive with antisera 1667 or 1668, and the number of lamprey-like GnRH neurons was based on sections immunoreactive for antiserum 3952.

RESULTS

Chromatography and RIA

Elution profiles of six synthetic GnRHs chromatographed in parallel to one another on the same HPLC system were determined by RIA and occurred in the following order: (1) lamprey GnRH-III and mammalian and chicken GnRH-I (not shown), (2) lamprey GnRH-I, (3) chicken GnRH-II, and (4) salmon GnRH. In the white sucker brain, mammalian GnRH antiserum 1245 detected four immunoreactive peaks (Fig. 2). The major peak coeluted with chicken GnRH-II, a lesser peak coeluted with salmon GnRH, and two other peaks could not be identified. Lamprey GnRH-III antiserum 3952 detected immunoreactive peaks which coeluted with lamprey GnRH-III, lamprey GnRH-I (possibly), chicken GnRH-II, an unknown and salmon GnRH. Lamprey GnRH-I antiserum 1467 detected one immunoreactive peak (Fig. 2), which coeluted with lamprey GnRH-I.

Immunocytochemistry

In the white sucker brain, the majority of neuronal perikarya containing ir-salmon and ir-lamprey-like GnRH were found laterally in the preoptic area, often close to the optic tracts as they emerged from the optic chiasm (Figs. 3 and 4). Estimates of cell number taken from animals in similar conditions (acrolein fixed from mid-January/late-February) indicate that the number of neurons containing ir-salmon GnRH or ir-lamprey was small, in a range less than 200 cells total (per one-third brain: ir-salmon GnRH, mean = 64.1 ± 12.7 SEM, based on $n = 4$ and mean = 21.0 based on two animals for ir-lamprey GnRH). Differences in the distributions of the immunoreaction products using antisera directed against salmon or lamprey GnRH were observed, with neurons containing ir-lamprey-like GnRH not as widespread in the rostrocaudal

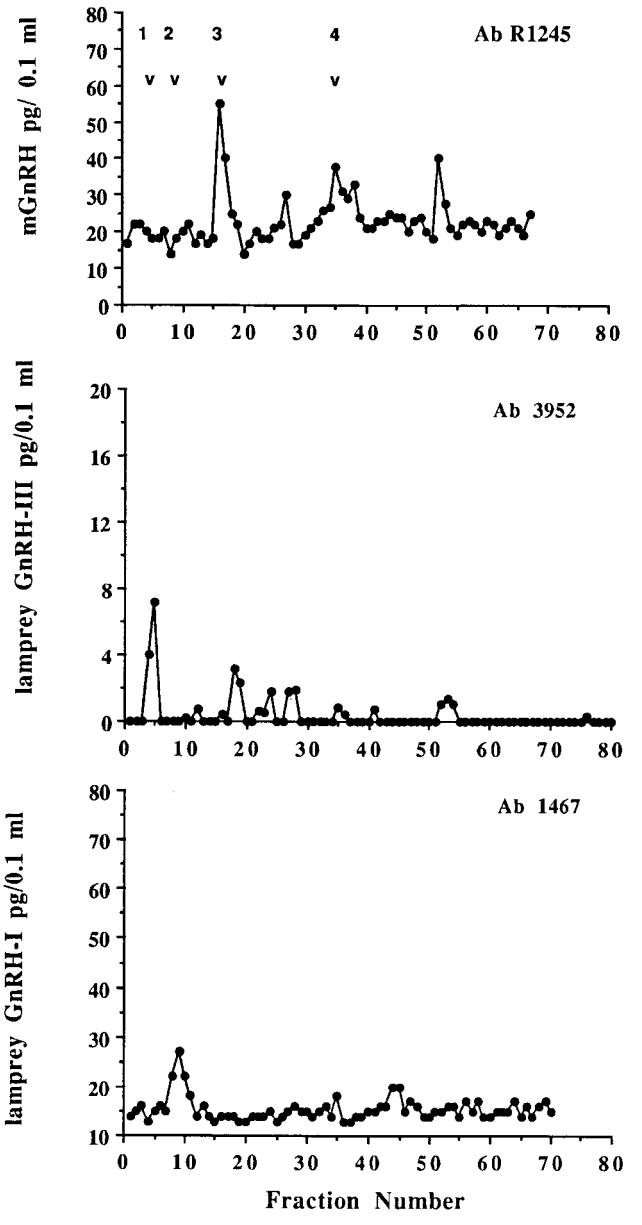
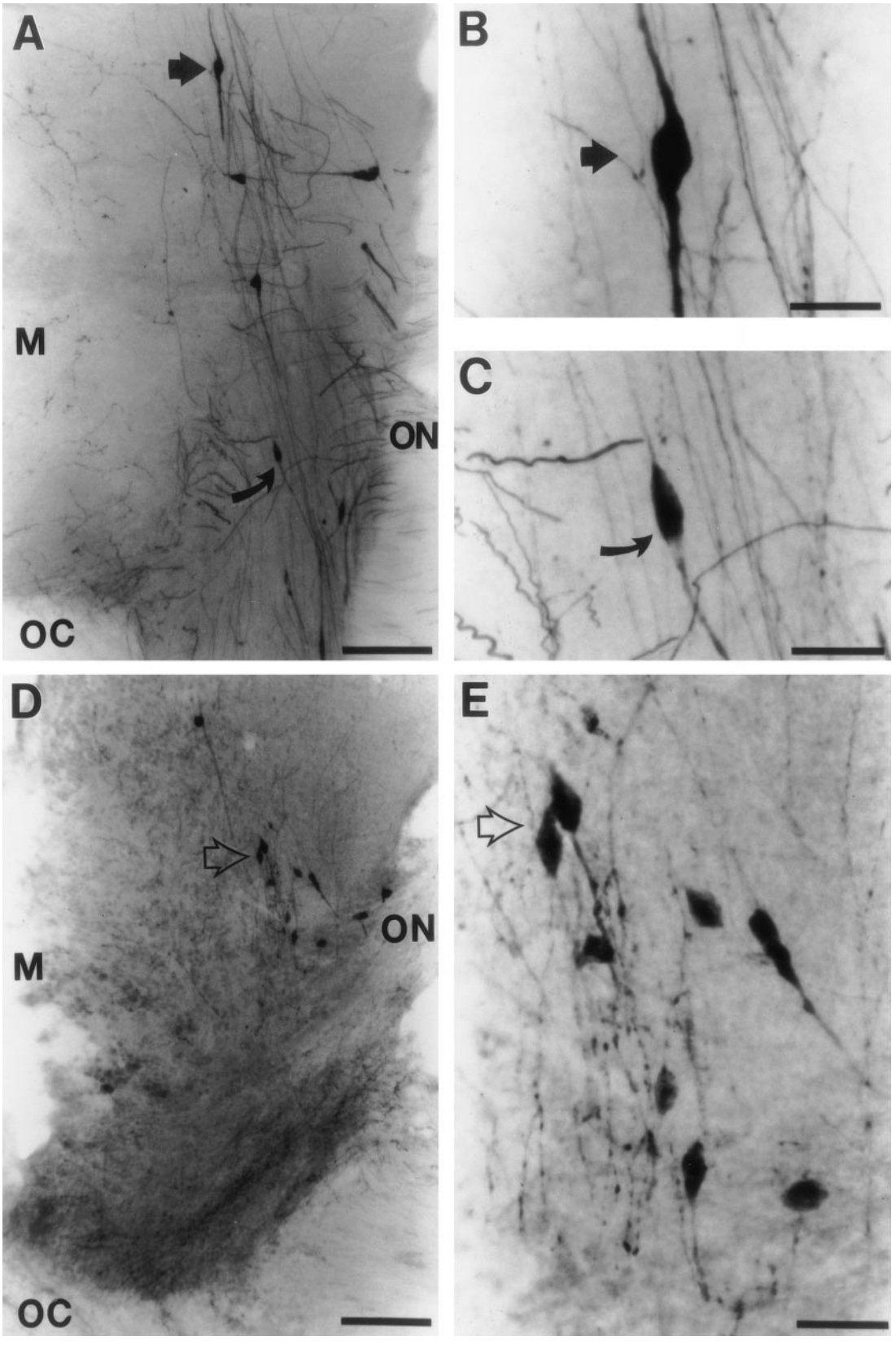


FIG. 2. Reverse-phase HPLC of adult white sucker brain extract and radioimmunoassay with mammalian GnRH antiserum R1245, lamprey GnRH-I antiserum 1467, and lamprey GnRH-III antiserum 3952. Arrows indicate assayed synthetic standards in the order: (1) lamprey GnRH-III, mammalian and chicken GnRH-I (not shown), (2) lamprey GnRH-I, (3) chicken GnRH-II, and (4) salmon GnRH.

FIG. 3. Photomicrographs illustrating the differential morphology of neuronal perikarya containing ir-salmon GnRH (A–C) and ir-lamprey GnRH (D, E) in the preoptic area of adult white sucker brain. In A–C, a horizontal section was incubated with antiserum 1667, and in D and E a horizontal section was incubated with antiserum 3952. In A and B a large black arrow indicates a larger ir-salmon GnRH-containing neuron, and in A and C a curved black arrow indicates a smaller ir-salmon GnRH-containing neuron. In D and E a large open arrow indicates smaller ir-lamprey GnRH-containing neurons. M, medial; OC, optic chiasm; ON, optic nerve. Scale bars: A and D, 100 mm. Scale bars: B, C, and E, 25 mm.



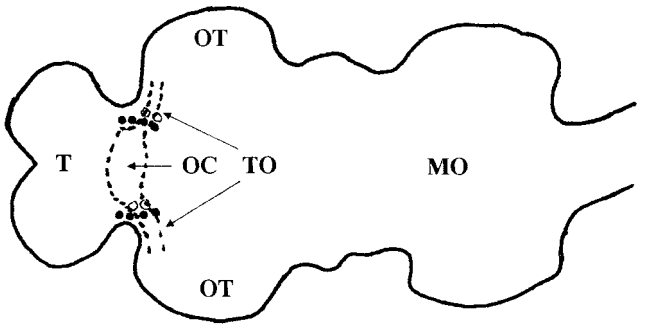


FIG. 4. Horizontal schematic of ir-salmon and ir-lamprey GnRH neurons within the brain of adult white sucker. Black circles represent perikarya within the plane of view and open circles indicate perikarya above the plane of view. T, telencephalon; OC, optic chiasm; TO, tractus opticus; OT, optic tectum; MO, medulla oblongata.

dimension as perikarya containing ir-salmon GnRH. Neurons containing ir-salmon GnRH were notably larger than those containing ir-lamprey-like GnRH (Fig. 3), and this was further indicated when a random sample of cells was measured for maximal diameter (ir-salmon GnRH, mean = 24.3 ± 3.1 mm, $n = 9$, versus ir-lamprey-like GnRH, mean = 16.3 ± 1.1 mm, $n = 15$; a significant difference by t test, $P < 0.05$). Whether this was due to a difference in abundance of peptide being reflected in a difference in the amount of reaction product filling the respective cells could not be determined. Neurons containing ir-lamprey-like GnRH were predominantly restricted to the caudal portion of the cell group (Figs. 3D and 3E). Perikarya containing ir-chicken-II-like GnRH were not reliably visualized, perhaps due to continual transport of the peptide into neuronal fibers.

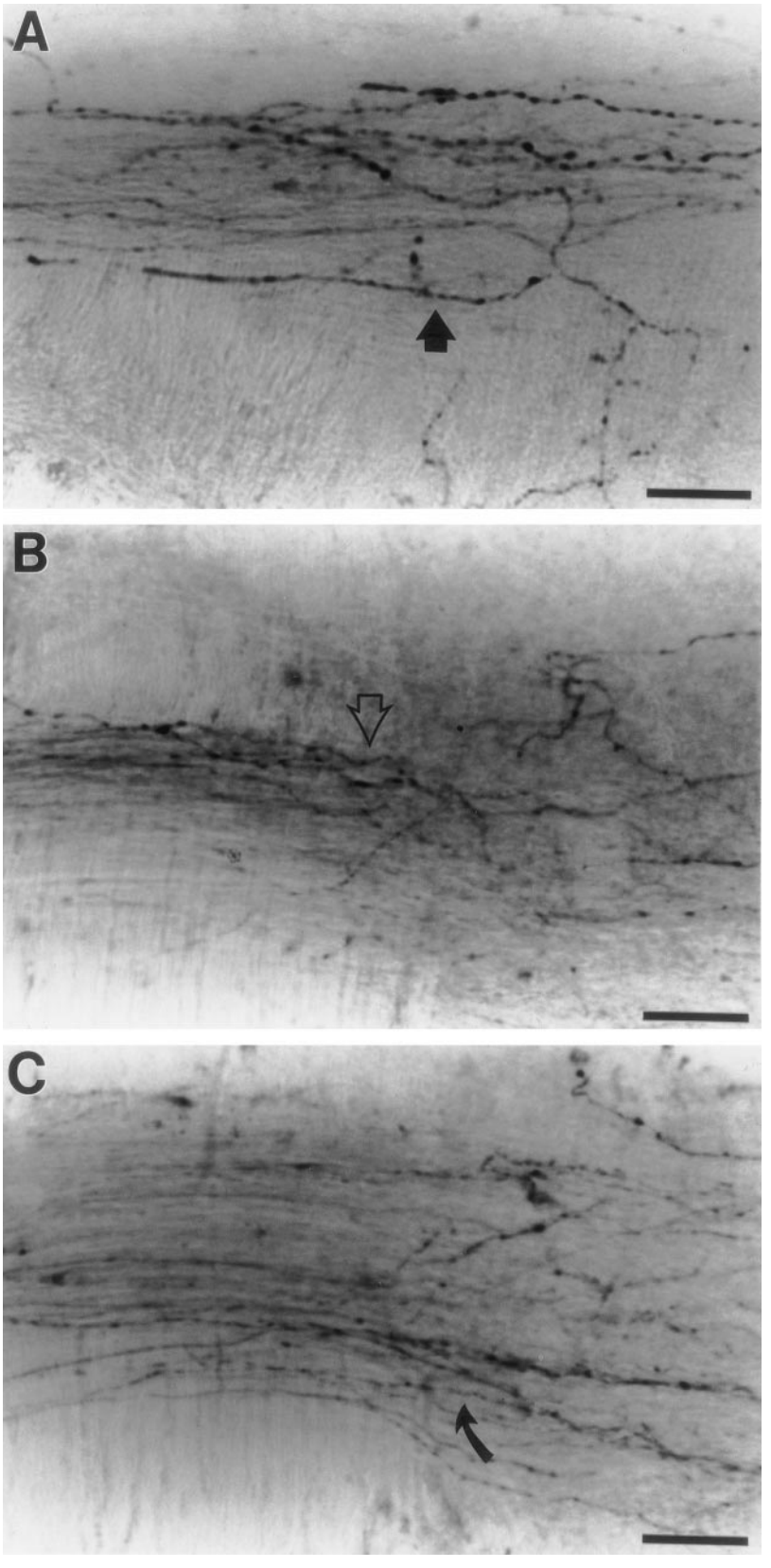
Although immunoreactive perikarya were limited to the lateral preoptic area, immunoreactive fibers were widely distributed and observed as rostrally as the olfactory bulbs. Fibers containing ir-salmon and ir-lamprey-like GnRH (Figs. 5A, 5B, and 6a) streamed rostrally and caudally from the location of their neuronal origin, fanning throughout the caudal telencephalon and into the ventral diencephalon, toward the pituitary. Similarly, fibers containing ir-chicken-II-like GnRH (Figs. 5C and 6b) were found throughout the caudal telencephalon, but caudal distributions remained concentrated in the dorsal portion of the diencephalon. All immunoreactive fiber types extended minimally into the optic tectum (Fig. 6).

In the white sucker pituitary, fibers containing ir-salmon and ir-lamprey-like GnRH were seen entering the neurohypophysis, differing in their apparent targets. Fibers containing ir-salmon GnRH remained within the neurohypophysis (Figs. 7A and 8a), while fibers containing ir-lamprey GnRH showed this pattern and also entered adenohypophysial regions (Figs. 7B and 8b). Fibers containing ir-chicken-II-like GnRH were not found in the pituitary of the white sucker.

DISCUSSION

Consistent with previous studies in teleost fish, the present data demonstrate four different forms of GnRH (chicken GnRH-II, salmon GnRH, lamprey GnRH-I, and lamprey GnRH-III) and two unknown GnRHs in the brain of the white sucker. These are the first studies to demonstrate ir-lamprey GnRH in an early evolved teleost species. Two of the peaks detected by radioimmunoassay using the mammalian antiserum corresponded to synthetic chicken GnRH-II and salmon GnRH standards, while the other immunoreactive peaks could not be identified. A major ir-GnRH peak coeluted with lamprey GnRH-I as determined by HPLC and RIA using the lamprey GnRH-I antiserum 1467. Another major peak coeluting with lamprey GnRH-III was shown using a lamprey GnRH-III antiserum. These findings are consistent with the hypothesis that multiple GnRH forms with multiple functions exist within the brain and provide further evidence of lamprey-like GnRH in a teleost (Magliulo-Cepriano *et al.*, 1994; Okuzawa *et al.*, 1993).

FIG. 5. Photomicrographs illustrating fibers containing (A) ir-salmon GnRH, (B) ir-lamprey GnRH, and (C) ir-chicken GnRH-II, within the caudal telencephalon of adult white sucker brain. Sagittal sections were incubated with antiserum 1667 in A, antiserum 3952 in B, and chicken GnRH-II antiserum in C. In A, large black arrow indicates an ir-salmon GnRH fiber. In B, large open arrow indicates an ir-lamprey GnRH fiber. In C, curved black arrow indicates an ir-chicken GnRH-II fiber. Scale bars: A–C, 25 mm.



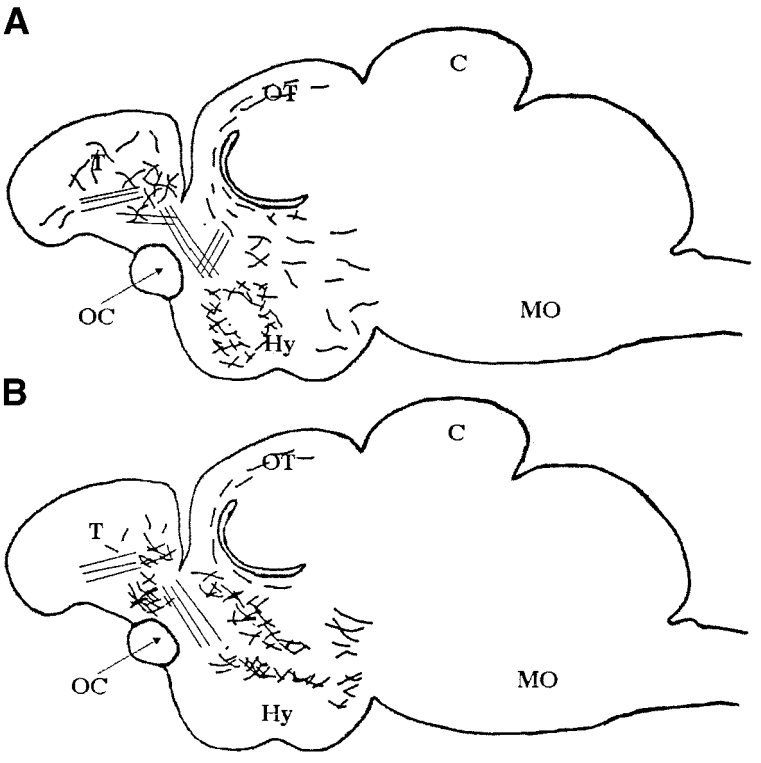


FIG. 6. Midline sagittal schematic of (a) parallel ir-salmon and ir-lamprey GnRH fiber systems, and (b) ir-chicken GnRH-II GnRH fiber system, within the brain of adult white sucker. T, telencephalon; OC, optic chiasm; Hy, hypothalamus; OT, optic tectum; C, cerebellum; MO, medulla oblogata.

Immunocytochemical studies of the white sucker brain and pituitary were consistent with the above chromatographic findings. The immunocytochemical data could not determine definitively the nature of the major lamprey GnRH-like form. The HPLC data suggested the major lamprey GnRH-like forms are both lamprey GnRH-I and lamprey GnRH-III, whereas the most effective antiserum in immunocytochemistry was directed against lamprey GnRH-III. Although, this antiserum was very selective for lamprey GnRH-III in larval lamprey based on similar preabsorption experiments (Tobet *et al.*, 1995), it did not show similar selective activity in the white sucker of the current study. Thus, in this study, both ir-lamprey GnRH forms will simply be referred to as ir-lamprey-like GnRH.

Neurons containing either ir-salmon or ir-lamprey-like GnRH were distributed similarly in the lateral preoptic area, differing from the medial localization found in many other vertebrates (Muske, 1993). Differences between the ir-salmon and ir-lamprey-like GnRH systems in the white sucker are likely localized in different cells. Thus, there was a slightly wider and more rostral distribution of ir-salmon perikarya in the brain, and a difference in apparent sizes of the two cell types. Neurons containing ir-salmon GnRH included a group of larger rostral cells exclusively containing ir-salmon GnRH. Neurons containing ir-lamprey-like GnRH were smaller in general compared to ir-salmon-containing neurons. It is not likely that antiserum 3952 immunoreaction product was due to chicken GnRH-II, considering the difference between the dorsal distribution of ir-chicken-II-like GnRH fibers and the ventral distribution of ir-lamprey-like GnRH fibers. Neurons containing ir-chicken-II-like GnRH were not reliably visualized by anti-chicken GnRH-II antisera. Our finding of ir-lamprey-like GnRH in the white sucker brain by immunocytochemistry contrasts the lack of detectable lamprey GnRH by similar methods in the brain of a later evolved (intermediate) teleost, the platyfish (Magliulo-Cepriano *et al.*, 1994).

Fibers containing ir-salmon and ir-lamprey-like GnRH coursed rostrally throughout the caudal telen-

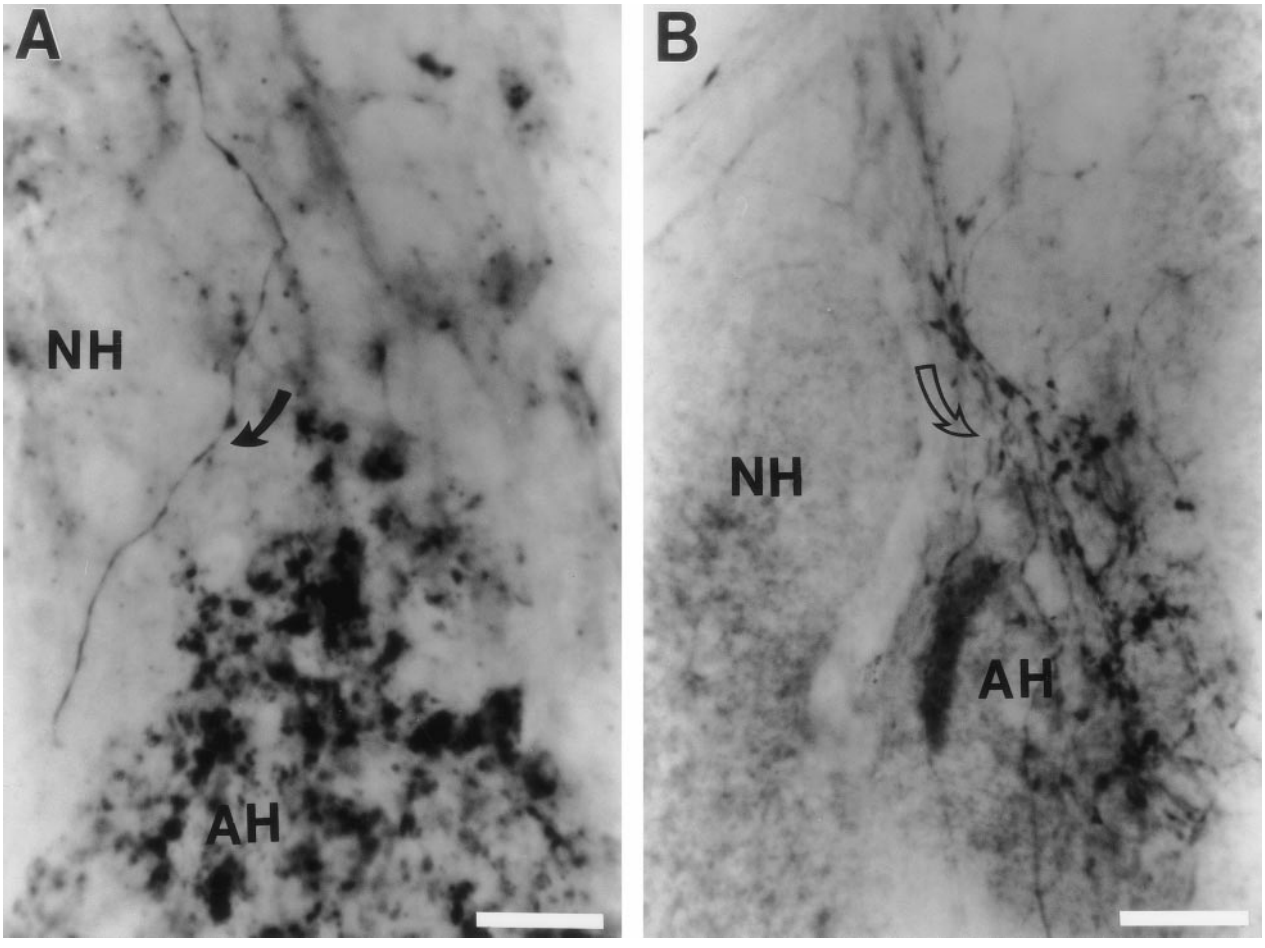


FIG. 7. Photomicrographs illustrating the differential targets of (A) ir-salmon GnRH-containing fibers and (B) ir-lamprey GnRH-containing fibers in the pituitary of adult white sucker. In A, a coronal section was incubated with antiserum 1667, and the curved black arrow indicates an ir-salmon GnRH-containing fiber steering clear of the adenohypophysis (AH) and remaining within the neurohypophysis (NH). In B, a sagittal section was incubated with antiserum 3952, and the curved open arrow indicates ir-lamprey GnRH-containing fibers turning and entering the adenohypophysis (AH) from the neurohypophysis (NH). Reaction product not in fibers is nonspecific. Scale bars: A and B, 25 mm.

cephalon and caudally into the ventral diencephalon. In contrast, fibers containing ir-chicken-II-like GnRH were distributed in the dorsal diencephalon, in addition to regions of the telencephalon. All ir-GnRH fibers were found minimally in the optic tectum. The dissimilarity between the distributions of neurons and fibers containing ir-salmon and ir-lamprey-like GnRH and fibers containing ir-chicken-II-like GnRH seems to be consistent with findings in other teleosts, in which separate preoptic-hypophysial and dorsal midbrain systems exist (Amano *et al.*, 1991; Coe *et al.*, 1992; Lepretre *et al.*, 1993; Oka and Matsushima, 1993; Sherwood *et al.*, 1994). However, the verity of a midbrain GnRH system existing in the white sucker remains to be seen given the lack of detectable ir-chicken-II-like GnRH perikarya in any region.

Although detection of neurons containing ir-salmon or ir-lamprey-like GnRH was more successful than detection of neurons containing ir-chicken-II-like GnRH, visualization of all types of ir-GnRH perikarya proved difficult at times. A number of factors may have influenced these observations, including the animals' sex, reproductive status, physiological status, and health. Sex was not determined while sampling fish for experiments and therefore no observations of sexual differences in GnRH distributions were made. However, sex differences in GnRH systems have been noted in the African cichlid (*Haplochromis burtoni*) (Davis and

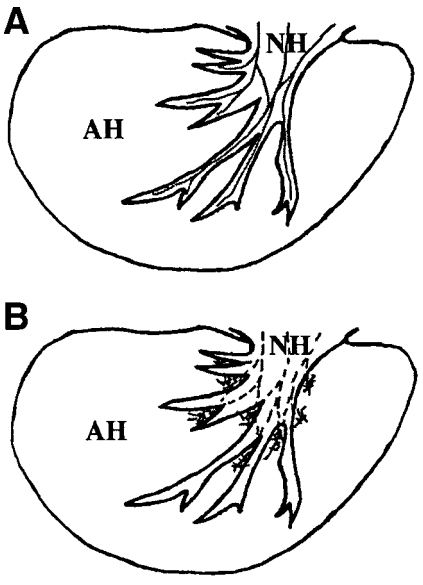


FIG. 8. Sagittal schematic of (a) ir-salmon GnRH fibers (solid lines), and (b) ir-lamprey GnRH fibers (dashed lines), in the pituitary of adult white sucker. NH, neurohypophysis; AH, adenohypophysis.

Fernald, 1990; Francis *et al.*, 1992; White and Fernald, 1993). Additionally, sampling occurred at three different points in the reproductive cycle of the white sucker: (1) mid-January / late-February—reproductive dormancy, (2) mid-April—spawning migration, and (3) late-September / early-October—reproductive dormancy (25). Though sampling during the spawning migration of the white sucker was intended to increase the likelihood of visualizing GnRH neurons at a time when they might maximally produce the decapeptide, no increase in immunoreactivity was noted.

Direct innervation of the pituitary by fibers of hypothalamic GnRH neurons is characteristic of the class Osteichthyes (Muske, 1993; Nozaki *et al.*, 1984; Schreibman *et al.*, 1979). Both ir-salmon and ir-lamprey-like GnRH fibers entered the neurohypophysis of the white sucker, but differed in their distribution. In the pituitary, fibers containing ir-salmon GnRH remained within the neurohypophysis, while those containing ir-lamprey-like GnRH appeared to target the adenohypophysis. Magliulo-Cepriano *et al.* (1994) found ir-lamprey-like GnRH within gonadotropes of the platyfish, but could find no evidence of a delivery system for the peptide from the brain. In contrast, the present study reveals an ir-lamprey-like GnRH preoptic-hypophysial neural tract and indicates a possible hypophysiotropic role for lamprey-like GnRH in the white sucker. Colocalization of multiple GnRH forms within the pituitary has been shown for chicken-II and catfish GnRHs in the African catfish (*Clarias gariepinus*) (Schultz *et al.*, 1993) and for chicken-II and salmon GnRHs in the platyfish (Magliulo-Cepriano *et al.*, 1994), possibly indicating a cooperative mechanism for gonadotropin release within these species. Whether salmon and lamprey GnRHs act separately or in concert within the pituitary of the white sucker is unknown.

Fibers containing ir-chicken-II-like GnRH were absent from the pituitary of the white sucker. Though other immunocytochemical studies have shown ir-chicken-II-like GnRH within the pituitary of other teleost species (Magliulo-Cepriano *et al.*, 1994; Schultz *et al.*, 1993), the present experiments are consistent with findings in the masu salmon (Amano *et al.*, 1991). Combined with the dorsal distribution of ir-chicken-II-like GnRH fibers in the diencephalon, this evidence leads one to doubt that chicken GnRH-II is an effector of gonadotropin release within this species. Other possible roles for GnRH peptides include functions as neurotransmitters and neuromodulators (Oka and Matsushima, 1993; Okuzawa *et al.*, 1990; Yu *et al.*, 1991). Chicken GnRH-II may serve one or both of these functions in the white sucker brain.

In summary, HPLC and RIA analyses demonstrated that multiple forms of GnRH were present in the white sucker brain and pituitary, including lamprey-like forms. Complementary immunocytochemical studies were undertaken demonstrating that localization of ir-salmon and ir-chicken GnRH-II could be distinguished from ir-lamprey GnRH based on differential distributions using partially selective antisera. Thus, it is likely that the multiple forms demonstrated by HPLC and RIA are localized in different cell populations in the white sucker brain and project differently to the pituitary. The results of the present study strengthen the body of evidence suggesting lamprey GnRH forms occur in an early evolved teleost that is at the very base of the euteleostean radiation and more recently evolved than the Agnatha.

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