

# Multiple Molecular Forms of Gonadotropin-Releasing Hormone in the Brain of an Elasmobranch: Evidence for IR-Lamprey GnRH

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CALVIN, J. L., C. H. SLATER, T. G. BOLDUC, A. P. LAUDANO AND S. A. SOWER. *Multiple molecular forms of gonadotropin-releasing hormone in the brain of an elasmobranch: Evidence for IR-lamprey GnRH*. PEPTIDES 14(4) 725-729, 1993.—These studies investigated brains of skate, *Raja erinacea* (order Rajiformes, class Chondrichthyes), for gonadotropin-releasing hormone (GnRH) peptides by chromatography and immunoreactivity with region-specific antisera raised against mammalian GnRH and lamprey GnRH. The region-specific antibody to lamprey GnRH-I was produced following conjugation to bovine serum albumin using the bis-diazotized benzidine method. This antibody was characterized by assaying a range of increasing dilutions of the known vertebrate GnRHs, as well as analogs to lamprey GnRH-I. Two analogs, lamprey [Phe<sup>2</sup>]GnRH-I and lamprey [Leu<sup>7</sup>]GnRH-I, were synthesized by solid phase peptide synthesis using a benzhydrylamine resin as the supporting medium and purified by chromatography. This antibody demonstrated less than 0.01% cross-reactivity with all GnRH peptides tested, suggesting a highly specific antibody with a region of amino acids 2-8 that appears essential for binding. In the skate brain, five immunoreactive (IR) GnRH forms were detected, four of which eluted in the same positions as synthetic mammal and chicken GnRH-I (which coelute): lamprey GnRH-I, salmon and chicken GnRH-II, and one that was an unidentified form. A minor peak coeluted with lamprey GnRH-III. The major form in the skate brain is considered to have eluted with synthetic mammalian GnRH. These studies confirm an earlier report of an IR-mammalian GnRH peptide and provide new evidence for IR-lamprey GnRH in the brain of an elasmobranch.

Gonadotropin-releasing hormone      Elasmobranch      Mammalian GnRH      Lamprey GnRH

CHROMATOGRAPHIC and immunological studies using antibodies to the known forms of vertebrate gonadotropin-releasing hormone (GnRH) have established that there are two or more forms of immunoreactive (IR) GnRH present within brains of representative vertebrate species (7). The structures of GnRH have been determined in three species of primitive fishes: an agnathan—sea lamprey (*Petromyzon marinus*) (24,25); an elasmobranch—spiny dogfish shark (*Squalus acanthias*) (12); and a holecephalan—ratfish (*Hydrolagus colliiei*) (14). The primary structures of GnRH that have been determined are as follows: mammal (2,16); salmon (22); two forms in catfish (catfish-I and chicken GnRH-II) (20); two forms in chicken-I (8,9,18) and -II (17); two forms in the alligator (chicken-I and -II) (13); two forms in dogfish (dogfish GnRH and chicken GnRH-II) (12); one form in ratfish (chicken GnRH-II) (14); and two forms in lamprey (lamprey GnRH-I and GnRH-III) (24,25).

In chondrichthyans, there are limited studies on the nature of GnRH molecular forms in brains. Powell et al. (21) dem-

onstrated seven GnRH molecular forms in the extracts from brains of dogfish (*Poroderma africanum*) in the order of Squaliformes (class Chondrichthyes), three of which coeluted with synthetic mammalian GnRH, chicken GnRH-II, and salmon GnRH. The other four IR-GnRH peptides are considered novel forms. The dominant form of an immunoreactive GnRH-like peptide in brains from spiny dogfish shark and ratfish eluted with synthetic chicken GnRH-II (23). More recently, the dominant form in ratfish was sequenced and verified to be chicken GnRH-II (14). In spiny dogfish shark, Lovejoy et al. (15) demonstrated four forms of IR-GnRH in both brain and terminal nerve extracts, three of which coeluted with synthetic mammalian GnRH, chicken GnRH-II, and salmon GnRH. The other form that eluted between chicken GnRH-II and salmon GnRH was considered a novel form. Two of these forms have now been sequenced; one is a novel form, dogfish GnRH, and the other form is chicken GnRH-II (12). There have been no reported studies on GnRH

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forms in the brains of fish in another order of the class Chondrichthyes: order Rajiformes.

Until recently, lamprey GnRH had not been detected in the brain of any species of fish other than the lamprey. Immunoreactive lamprey GnRH-I, as well as IR-salmon GnRH, has been detected in the brain of white sucker (11), a member of the order Cypriniformes, one of the more primitive orders of teleosts. Immunoreactive lamprey GnRH-I was also identified in the hypothalamus and median eminence of the human brain by a combination of immunocytochemistry, high performance liquid chromatography (HPLC), and radioimmunoassay (27)

The objective of this study was to determine the nature of GnRH in the brains of skate (order Rajiformes, class Chondrichthyes) using specific antibodies to mammalian GnRH and lamprey GnRH-I. In this paper, we have generated a highly specific antiserum against lamprey GnRH-I followed by the development of sensitive and specific radioimmunoassay for lamprey GnRH-I.

#### METHOD

##### *Preparation of Antiserum*

Synthetic  $^{14}\text{C}$ -lamprey GnRH-I (kindly supplied by Dr. Russell Doolittle, U. of San Diego) was purified by HPLC and conjugated to bovine serum albumin (BSA) using the bis-diazotized benzidine (BDB) method described by Ellinwood et al. (4). This procedure preferentially conjugates the lamprey GnRH to BSA at either the histidine or tyrosine residues in positions 2 and 3, respectively. Based on calculations from TCA precipitation, 11.3 molecules of lamprey GnRH were conjugated to each BSA molecule.

For the initial injection into a rabbit, 200  $\mu\text{g}$  (0.522 ml) of BSA-lamprey GnRH was mixed with 1.0 ml of complete Freund's adjuvant and 0.48 ml phosphate buffered saline (PBS). The total volume of 2.0 ml was injected subcutaneously at several sites using one male rabbit. There were five subsequent injections every 2 weeks using incomplete Freund's adjuvant. A pre-experimental bleed was taken prior to immunization and an initial bleed was taken 5 weeks after the first injection. One week after the fifth injection, post bleeds were taken every other week. Each bleed was assayed to determine binding using a lamprey GnRH radioimmunoassay procedure, as described by Stopa et al. (27) and Fahien and Sower (5).

##### *Peptide Synthesis*

Lamprey [Phe $^2$ ]GnRH-I and lamprey [Leu $^7$ ]GnRH-I were synthesized by solid phase peptide synthesis procedures developed by Bruce Merrifield (26). Because GnRH is an amide, a benzhydrylamine resin was required as the supporting medium. Amino acids were coupled with either 1,3-dicyclohexylcarbodiimide (DCC) or symmetrical anhydride. In the DCC coupling, 6 mmol of boc-pGlu-dicyclohexylamine (DCHA) was suspended in 12.4 ml of ice-cold ethyl acetate (100 g/20 g derivative) in a separatory funnel. Following addition of 1.2 equivalents of ice-cold sulfuric acid, the mixture was shaken until the derivative dissolved. The ethyl acetate layer was removed, 10.0 ml of ice-cold deionized ultrafiltered water was added, and the mixture was extracted twice with 20 ml of ethyl acetate. The ethyl acetate layers were combined and washed twice with 20 ml of ice-cold deionized ultrafiltered water. Residual water was removed from the extract by the addition of 5.0 g of magnesium sulfate. After shaking for 15 min, the magnesium sulfate was removed by filtration using a Whatman #1 filter. The solution was aliquoted and lyophilized on a speed-vac concentrator (Savant Speed-Vac,

Farmingdale, NY). After the addition of each amino acid, a ninhydrin test was used to determine coupling efficiencies (6). Based on these assays, all coupling efficiencies were greater than 99.4%. The GnRH also has a characteristic pyroglutamic (pGlu) residue. Before coupling the boc-pyroglutamic dicyclohexylamine, it was desalted to convert the residue to the free amino acid boc derivative.

Following the final deprotection of the synthesized peptide resin, the peptide was cleaved from the resin by large-scale trifluoromethanesulfonic acid (TFMSA) cleavage (1). Lamprey [Phe $^2$ ]GnRH-I was deformedylated at the Trp residue using a guanidine/ethanolamine method developed by Applied Biosystems (3).

The synthetic peptides were purified to apparent homogeneity by chromatography on a  $2.5 \times 100\text{-cm}$  volume of Sephadex G-10 (and Sephadex G-15) (Sigma, St. Louis, MO) equilibrated with 2 M acetic acid at a flow rate of 2 ml/min. Fractions were further purified by QAE-Sephadex A-25 chromatography (10 ml 0.2% formic acid/pyridine, pH 3.3; 5 ml of 80% acetonitrile at a flow rate of 4 ml/min). The structures of the peptides were confirmed by amino acid analyses performed by UNH's Instrumentation Center.

##### *Animals*

Sexually mature skate (*Raja erinacea*) were collected by otter trawl from the Gulf of Maine between Portsmouth and the Isles of Shoals, NH. Whole brains were collected from freshly killed fish and frozen on dry ice and stored at  $-80^\circ\text{C}$  until extracted and assayed.

##### *Extraction and HPLC*

Frozen brains were extracted using the method described by Yu et al. (28) and Fahien and Sower (5). Briefly, brains were weighed, then homogenized in 2.0 M ice-cold acetic acid with a polytron. The homogenates were centrifuged, the supernatants dried on a refrigerated vacuum centrifuge and reconstituted in millipore-filtered water, and recentrifuged. The extract was filtered through a 0.45-mm Arco LC 13 disposable filter and then injected into a 20- $\mu\text{l}$  loop on an HPLC system that consisted of a Perkin and Elmer series 100 pump with a Pecosphere 3CR C18 (0.46  $\times$  8.3 cm) reverse-phase column (5). The isocratic mobile phase consisted of 7.40 g ammonium acetate and 3.04 g citric acid in 1 l of 19% acetonitrile/water (final pH was adjusted to 4.6 with phosphoric acid) (27). The flow rate was 2 ml/min with fractions collected every 18 s for the first 10.2 min and then collected every 1 min.

Synthetic mammalian GnRH, chicken GnRH-I and -II, salmon GnRH, and lamprey GnRH-I and -III standards were chromatographed on the same HPLC system.

##### *Radioimmunoassay*

Radioimmunoassay (RIA) was performed as previously described in Stopa et al. (27) and Fahien and Sower (5) using synthetic mammalian GnRH or lamprey GnRH as the iodinated ligand and standard. The antisera, R1245 and 21-134, were used at dilutions of 1:20,000 for mammalian RIA and 1:100,000 for lamprey RIA, respectively. The antibody binding ranged between 35–43% for 21-134 of  $^{125}\text{I}$ -lamprey GnRH and 51–66% for R1245 of  $^{125}\text{I}$ -mammalian GnRH. R1245 has a specificity that recognizes the entire molecule with cross-reactivities of 65, 19.5, 4.16, and <0.01% for chicken GnRH-I, salmon GnRH, chicken GnRH-II, and lamprey, respectively.

### Antibody Characterization

The antibody produced against lamprey GnRH-I (21-134) was characterized by assaying a range of increasing dilutions of several analogs to the conjugated peptide. Standard curves were used with different GnRHs: chicken-I and -II, salmon [D-Ala<sup>6</sup>], mammal, lamprey [Phe<sup>2</sup>], and lamprey [Leu<sup>7</sup>] with lamprey GnRH-I as the internal standard. Standards ranged from 0 to 2500 pg, and percent cross-reactivity was determined. Each assay was triplicated and each individual dilution was duplicated.

### RESULTS

The lamprey GnRH antibody (21-134) demonstrated less than 0.01% cross-reactivity with all GnRH peptides tested, thereby suggesting a highly specific antibody (Table 1).

The elution times of fractions from the skate brains were compared to the five synthetic standards eluted with an identical HPLC system. The elution profiles of the GnRH standards are shown by arrows in Fig. 1 in the following order: lamprey-III, chicken-I/mammal, lamprey, chicken-II, and salmon.

Three IR-GnRH peaks were detected using Ab 1245 (Fig. 1). Two peaks eluted in the same positions as synthetic mammalian/chicken GnRH-I and salmon GnRH. The major peak was considered mammalian GnRH. A third peak was a late-eluting form that did not elute with any of the known standards. A minor IR-GnRH peak was noted that coeluted earlier with synthetic lamprey GnRH-III.

Three IR-GnRH peaks were detected using Ab 21-134, which eluted in the same position as synthetic lamprey, chicken GnRH-II, and salmon GnRH (Fig. 1).

### DISCUSSION

The present study is the first report on IR-GnRH forms in the brains of skate. The data from this study indicate that brains from skate contain GnRH peptides similar to mammalian GnRH (possibly chicken GnRH-I), chicken GnRH-II, lamprey GnRH-I, and salmon GnRH, as well as at least one novel form. These data are similar to earlier studies in another order in the class of Chondrichthyes (order Squaliformes) by Powell et al. (21) and Lovejoy et al. (12,15), who demonstrated several forms of immunoreactive GnRH brains of the dogfish and spiny dogfish shark, including three similar to mammalian GnRH, chicken GnRH-II, and salmon GnRH. Based on these cited studies, the identified peak that coeluted with the synthetic mammalian and chicken GnRH-I was considered to be mammalian GnRH. It is not clear from the present study if the unidentified form is similar to the newly identified dogfish GnRH because of the

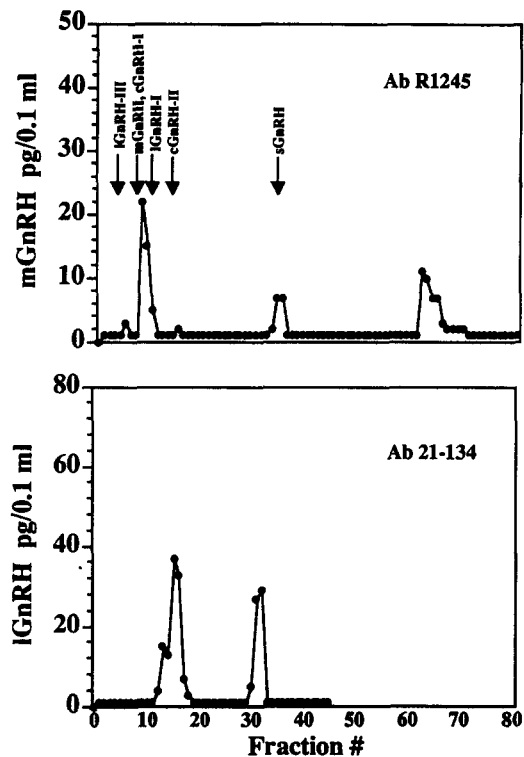


FIG. 1. Reverse-phase HPLC of *R. erinacea* brain extract and radioimmunoassay with mammalian GnRH antiserum R1245 (top) and lamprey GnRH antiserum Ab 21-134 (bottom). Arrows indicate peaks of assayed synthetic standards in the order of lamprey GnRH-III, mammal and chicken GnRH-I; lamprey GnRH-I; chicken GnRH-II and salmon GnRH.

different HPLC systems that were employed and the current unavailability of dogfish GnRH.

The lamprey GnRH antibody (21-134) demonstrated less than 0.01% cross-reactivity with all GnRH peptides tested, suggesting a highly specific antibody. Mammalian GnRH, chicken-I and -II GnRH, and salmon GnRH failed to cross-react significantly with 21-134. Two analogs of lamprey and one analog of mammalian GnRH also failed to cross-react with 21-134. These included analogs of lamprey GnRH-I in which His<sup>2</sup> was replaced by Phe (lamprey [Phe<sup>2</sup>]GnRH-I) and a substitution of Trp<sup>7</sup> by Leu (lamprey [Leu<sup>7</sup>]GnRH-I). The mammalian analog in which Gly<sup>6</sup> was substituted by Ala (mammalian [D-Ala<sup>6</sup>]GnRH) also showed no cross-reactivity. These data demonstrate a highly specific antibody for lamprey GnRH, which is sensitive to single amino acid substitutions. From the limited number of analogs tested, a region of amino acids 2-8 appears essential for binding to the antibody 21-134.

The present study and recent data from Stopa et al. (27), who demonstrated IR-lamprey GnRH-I in the human hypothalamus and median eminence, suggest that a lamprey or lamprey-like GnRH molecular form may have been retained in some species of vertebrates during 500 million years of evolution and is not restricted to the oldest and most primitive class. The class Chondrichthyes evolved from the mainstream of vertebrate evolution about 400 million years ago. The Rajiformes (skates and rays) diverged from the other elasmobranchs (Squaliformes: sharks) by approximately 100 million years. To date, IR-lamprey GnRH-I has only been identified in the skate and not in the

TABLE 1

CROSS-REACTIVITY OF GnRH AND ANALOGS WITH 21-134 ANTISERUM

Compound	Relative Activity
Lamprey GnRH	1.00
Mammalian GnRH	<0.00001
Chicken GnRH-I	<0.00001
Chicken GnRH-II	<0.00001
Salmon GnRH	<0.00001
Lamprey [Phe <sup>2</sup> ]GnRH	<0.00001
Lamprey [Leu <sup>7</sup> ]GnRH	<0.00001
Mammalian [D-Ala <sup>6</sup> ]GnRH	<0.00001

sharks. These data would suggest that lamprey GnRH has been retained in this order of fish. Lamprey GnRH-III has 80% identity with chicken GnRH-II and dogfish GnRH; 70% identity with catfish GnRH-I, lamprey GnRH-I, and salmon GnRH; and 60% identity with mammal GnRH and chicken GnRH-I (25). Lamprey GnRH-III is more closely related to the other members of the GnRH family compared to lamprey GnRH-I; this would suggest that there is still the possibility that shark brains do contain lamprey GnRH, based on the number of GnRH forms that have been detected by chromatographical and immunological methods and the similarity of lamprey GnRH-III with dogfish GnRH and chicken GnRH-II. This will remain an unresolved issue until all GnRH forms in brains of representative species of Chondrichthyes have been sequenced.

Multiple molecular forms of GnRH within single species have been documented throughout the vertebrate classes. Lamprey brains contain three unique forms of the molecule. The amino acid sequence is known for two forms (lamprey GnRH-I and lamprey GnRH-III), but only the amino acid composition for the second form (lamprey GnRH-II) (24,25). The few representative species (sharks only) of elasmobranch fish that had previously been examined exhibit several forms of GnRH. Studies of the dogfish, *P. africanum*, have demonstrated IR-salmon GnRH-I, mammalian GnRH, and chicken GnRH-II, as well as unique forms (21). The dominant form of an immunoreactive GnRH-like peptide in brains from spiny dogfish shark (*S. acanthias*) and ratfish eluted with synthetic chicken GnRH-II (23). More recently, this dominant form in ratfish was sequenced and verified to be chicken GnRH-II (14). Four forms of IR-GnRH were demonstrated in both brain and terminal nerve extracts in the spiny dogfish shark, three of which coeluted with synthetic mammalian GnRH, chicken GnRH-II, and salmon GnRH (15). The other form, which eluted between chicken GnRH-II and salmon GnRH, was considered a novel form. This novel form (dogfish GnRH), as well as chicken GnRH-II in spiny dogfish shark, has just been recently sequenced (4).

The functional significance of multiple forms of GnRH within the brain and in extrahypothalamic locations within the same

species is presently under study. The presence of immunoreactive GnRH in extrahypothalamic brain regions is well documented, which is suggestive of multiple functions such as neurotransmitters and/or neuromodulators. Different forms of GnRH within the hypothalamic region have suggested that gonadotropin secretion is probably regulated by a dual mechanism (28). More recent evidence in the chicken indicates that while both chicken GnRHs induce gonadotropin secretion, chicken GnRH-II is 10 times more potent than chicken GnRH-I (19). The GnRH-I in lamprey appears to be more restricted in its distribution in the brain compared to GnRH in mammal brains (10), suggesting a more restricted function. However, the function of the different forms of GnRH in sharks is not yet known. Lovejoy et al (12) demonstrated that dogfish GnRH stimulated the release of gonadotropin and growth hormone from goldfish pituitary fragments. As in the brain, Lovejoy et al. (15) demonstrated all four forms of IR-GnRH in the terminal nerve of the dogfish shark. Multiple molecular GnRHs found within the hypothalamus of a single species may perform separate regulatory control over different pituitary hormones (10,29). Chicken GnRH-II has been found in the brains of many phylogenetic diverse species and has been suggested to be an early evolved and well-conserved form of the molecule (15,21). This is supported by evidence that salmon GnRH and chicken GnRH-II immunoreactive structures are anatomically separate in the goldfish brain (29). However, the function of chicken GnRH-II in elasmobranchs is unknown.

Evidence of the existence of multiple forms of GnRH within the brain of a single species continues to increase. These studies confirm an earlier report of an IR-mammal GnRH peptide and provide new evidence for IR-lamprey GnRH-I in the brain of an elasmobranch.

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