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# Differential Distribution of Gonadotropin-Releasing Hormone-Immunoreactive Neurons in the Stingray Brain: Functional and Evolutionary Considerations

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**Gonadotropin-releasing hormone (GnRH) is a neuropeptide that occurs in multiple structural forms among vertebrate species. Bony fishes, amphibians, reptiles, birds, and mammals express different forms of GnRH in the forebrain and endocrine regions of the hypothalamus which regulate the release of reproductive gonadotropins from the pituitary. In contrast, previous studies on bony fishes and tetrapods have localized the chicken GnRH-II (cGnRH-II) nucleus in the midbrain tegmentum and, combined with cladistic analyses, indicate that cGnRH-II is the most conserved form throughout vertebrate evolution. However, in elasmobranch fishes, the neuroanatomical distribution of cGnRH-II and dogfish GnRH (dfGnRH) cells and their relative projections in the brain are unknown. We used high-performance liquid chromatography and radioimmunoassay to test for differential distributions of various GnRH forms in tissues from the terminal nerve (TN) ganglia, preoptic area, and midbrain of the Atlantic stingray, *Dasyatis sabina*. These experiments identified major peaks that coelute with cGnRH-II and dfGnRH, minor peaks that coelute with lamprey GnRH-III (lGnRH-III), and unknown forms. Immunocytochemistry experiments on brain sections show that dfGnRH-immunoreactive (-ir) cell bodies are localized in the TN ganglia, the caudal ventral telencephalon, and the preoptic area. Axons of these cells project to regions of the hypothalamus and pituitary, diencephalic centers of sensory and behavioral integration, and the midbrain. A large, discrete, bilateral column of cGnRH-II-ir neurons in the midbrain tegmentum has sparse axonal projections to the hypothalamus and regions of the pituitary but numerous projections to sensory processing centers in the midbrain and hindbrain. Immunocytochemical and chromatographic data are consistent with the presence of lGnRH-III and other GnRH forms in the TN that differ from dfGnRH and cGnRH-II. This is the first study that shows differential distribution of cGnRH-II and dfGnRH in the elasmobranch brain and supports the hypothesis of divergent function of GnRH variants related to gonadotropin control and neuromodulation of sensory function.**

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*Key Words:* **elasmobranch; GnRH; gonadotropin-releasing hormone; immunocytochemistry; neuromodulation; octavolateral; stingray; reproduction.**

Gonadotropin-releasing hormone (GnRH) is a neuropeptide best known for its regulation of gonadotropin release from the pituitary and occurs in at least 10 different forms among vertebrates. The primary amino acid structures are identified for mammal GnRH (mGnRH) in mammals (Matsuo *et al.,* 1971;

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Burgus *et al.,* 1972) and amphibians (Conlon *et al.,* 1993); chicken GnRH-I (cGnRH-I) in the alligator and chicken (Miyamoto *et al.,* 1983; Lovejoy *et al.,* 1991a); chicken GnRH-II (cGnRH-II) in the chicken, alligator, amphibian, teleost, ratfish, and shark (King and Millar, 1982, 1986; Miyamoto *et al.,* 1984; Lovejoy *et al.,* 1991a,b,c; Ngamvongchon *et al.,* 1992; Powell *et al.,* 1994); catfish (cfGnRH), salmon (sGnRH), and seabream variants in various bony fishes (Sherwood *et al.,* 1983; Ngamvongchon *et al.,* 1992; Powell *et al.,* 1994); dogfish (dfGnRH) in the dogfish shark (Lovejoy *et al.,* 1992b); and lamprey GnRH-I (lGnRH-I) and -III (lGnRH-III) in the lamprey (Sherwood *et al.,* 1986b; Sower *et al.,* 1993). At least 1 GnRH form, which varies across taxa, is usually concentrated in neurons of the terminal nerve (TN)[3] and/or septo–preoptic areas (POA) of the forebrain and projects to the pituitary. These different forms of GnRH in the TN and forebrain may regulate gamete development and reproductive behavior (Muske, 1993; King and Millar, 1995).

In contrast, chicken GnRH-II is considered to be the most evolutionarily conserved variant because of its widespread distribution among gnathostome vertebrates (King and Millar, 1995) and support from cladistic studies on fishes and tetrapods (Grober *et al.,* 1995; Dores *et al.,* 1996). Many vertebrate taxa have cGnRH-II immunoreactive (-ir) cell bodies in brain regions caudal to the hypothalamus, especially in the midbrain tegmentum (Muske, 1993; King and Millar, 1995). This separate population of GnRH-containing cells projects primarily to regions such as the midbrain, hindbrain, and spinal cord (e.g., Lepretre *et al.,* 1993; Yamamoto *et al.,* 1995; Rosen *et al.,* 1997) which are not directly involved in the hypothalamic–pituitary–gonadal axis of hormonal regulation. The extrahypothalamic location and widespread projections of the tegmentum cGnRH-II form may represent an important neuromodulatory or neurotransmitter system. For example, GnRH neurons that project to sensory processing centers may function to integrate sensory cues during various reproductive behaviors (Muske, 1993; Oka and Matsushima, 1993; Wright and Demski, 1993; Montero *et al.,* 1994; King and Millar, 1995; Yamamoto *et al.,* 1995). However, physiological effects of cGnRH-II upon sensory processing centers are unexplored.

Despite the large body of work on the forms and distribution of GnRH variants among vertebrates, the taxonomic survey is incomplete. Previous immunocytochemistry (ICC) studies have described differential distribution of GnRH variants in teleosts (Yu *et al.,* 1988; Amano *et al.,* 1991; Montero *et al.,* 1994; Yamamoto *et al.,* 1995; Zandbergen *et al.,* 1995; Robinson *et al.,* 1999), sturgeon (Lepretre *et al.,* 1993), amphibians (Muske and Moore, 1994; Iela *et al.,* 1996; Pinelli *et al.,* 1997), reptiles (Tsai and Licht, 1993), birds (Katz *et al.,* 1990), and mammals (Dellovade *et al.,* 1993). However, only one study attempted to localize different GnRH variants in an elasmobranch (*Scyliorhinus canicula*) but did not identify the presence of a midbrain GnRH-ir cell group (D'Antonio *et al.,* 1995). The taxonomic position of elasmobranch fishes presents a unique opportunity to study the evolution and function of GnRH forms in relation to both the agnathans (which do not contain cGnRH-II) and the bony fishes. The purpose of this study was to determine whether the distribution of cGnRH-II and other GnRH forms is consistent with the general pattern seen in other vertebrate classes. Our results show that, in the Atlantic stingray (*Dasyatis sabina*), cGnRH-II is expressed by cells in the midbrain tegmentum, whereas dfGnRH is present in the TN, forebrain, and preoptic area. The different neuroanatomical distributions of the cGnRH-II and dfGnRH-immunoreactive (-ir) cells, and their respective projections to the midbrain–hindbrain–spinal cord and TN/forebrain, are consistent with separate sensory/behavioral processing and reproductive functions in the elasmobranch brain.

[3] Abbreviations used: 3V, third ventricle; 4V, fourth ventricle; AC, anterior commissure; BF, basal forebrain bundle; BV, blood vessel; CE, cerebellum; CT, connective tissue; DDP, descussatio dorsalis pallii; DON, dorsal octaval nucleus; DP, dorsal pallium; EMT, eminentia thalami; G2, ganglion 2 of terminal nerve; G3, ganglion 3 of terminal nerve; HA, habenula; HP, hypothalamus; LP, lateral pallium; M, medulla; ML, median lobe of pituitary; MLF, medial longitudinal fasciculus; MON, medial octaval nucleus; MR, mammillary recess; N2, optic nerve; N8, acoustic nerve; NIL, neurointermediate lobe of pituitary; OB, olfactory bulb; OC, optic chiasm; OE, olfactory epithelium; OLT, olfactory tract; OTR, optic tract; PC, posterior commissure; PG, periventricular gray; POA, preoptic area; RL, rostral lobe of pituitary; SV, saccus vasculosus; T, tectum; TEG, tegmentum; TEL, telencephalon; TL, caudal ventral telencephalon; TN, terminal nerve; VIT, ventriculus impar telencephali; WB, white body.

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## METHODS

### *Animals and Tissue Preparation*

Mature male (22–25 cm disk width) Atlantic stingrays, *D. sabina,* were collected via dip net or seine from the Banana River, Florida at the site of previous reproductive studies (Maruska *et al.,* 1996; Tricas *et al.,* 2000). For high-performance liquid chomatography (HPLC) and radioimmunoassay (RIA) analyses, animals were euthanized on ice in the field; the brains were removed, transported on ice back to the laboratory, and stored at −80°C. For immunocytochemistry experiments, rays were captured, placed in transport coolers, and returned to the lab. Individuals were then anesthetized with MS-222, immobilized with 0.5 cc pancuronium bromide, perfused transcardially with saline (0.9% NaCl), and fixed (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were removed from the chondrocranium, postfixed for 6–12 h, washed in phosphate buffer (0.1 M PB), and cryoprotected in 30% sucrose in 0.1 M PB for 1.5–2 days or stored in 0.1 M PB with 0.2% sodium azide for later use. Brains were embedded in OCT medium, sectioned in the transverse, sagittal, or horizontal plane on a freezing microtome at 40 $\mu$m, and collected onto gelatin-coated or Superfrost slides. In some cases, sections were cut at 28–34 $\mu$m and placed alternately upon two to four slides so that multiple antibodies could be applied to adjacent brain sections.

### *Extraction, HPLC, and Radioimmunoassay*

The white body (WB), preoptic area, and midbrain tegmentum regions from five male stingrays collected in December 1996 were pooled and chromatographed on both an isocratic and a gradient HPLC system followed by dfGnRH, lGnRH-III, and mGnRH radioimmunoassays. Multiple HPLC and RIA analyses were used to increase the confidence of the elution profiles used to identify specific GnRH variants within different brain regions and to support the immunocytochemical results.

Frozen brains were dissected into three separate regions for HPLC/RIA analyses: (1) white body sample of the terminal nerve, including a rostral portion of the lateral pallium, the adjacent second terminal nerve ganglion (G2), and a portion of the olfactory tract; (2) preoptic area, sampled from the posterior margin of the lateral pallium caudal to the anterior hypothalamus; and (3) midbrain tegmentum, sampled from the midhypothalamus caudal to the level at the posterior margin of the tectum (tectum removed). Brain regions from the five individuals were pooled, homogenized in 2.0 M ice-cold acetic acid, and centrifuged. Supernatants were dried on a refrigerated vacuum centrifuge, reconstituted in filtered water, and recentrifuged. Extracts were then filtered through a 0.45-mm Acro LC 13 filter and injected into a 20-$\mu$l loop on a Perkin–Elmer Series 100 pump HPLC system with a Pecosphere 3CR (0.46 × 8.3 cm) reverse-phase column. The isocratic mobile phase was 19% acetonitrile HPLC buffer adjusted to a flow rate of 2 ml/min. Forty 21-s fractions were collected for each sample. For the gradient HPLC system (Applied Biosystems), the extract was filtered with an Acro LC 13 (0.45-$\mu$m) filter, injected into a 2-ml loop system, and then pumped at a flow rate of 1.0 ml/min onto a 0.46 × 25 cm Vydac 215TP54 C4 column (Separations Group, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid. The concentration of acetonitrile in the eluting solvent was raised to 50% (vol/vol) over 60 min using a linear gradient and then raised to 100% (vol/vol) over 5 min. Then, 1-ml fractions were collected. Synthetic mGnRH, cGnRH-I and -II, lGnRH-I and -III, sGnRH, and dfGnRH standards were chromatographed on both the Perkin–Elmer and the Applied Biosystems HPLC systems.

Radioimmunoassay was performed as previously described by Fahien and Sower (1990) using synthetic lGnRH-I as the iodinated ligand and standard. Synthetic lGnRH-I was iodinated using a modification of the chloramine-T method and purified by Sephadex and Sep-Pak chromatography. The pooled brain regions from December were assayed with the dfGnRH antibody 7CR-10 (provided by N. Sherwood), lGnRH-III antibody 3952 (provided by S. Sower), and mGnRH antibody R1245 (provided by T. Nett). Final dilutions for these assays were 1:36,000 (7CR-10), 1:80,000 (3952), and 1:100,000 (R1245). Cross-reactivity data for these antibodies determined by RIA are listed in Table 1.

### *Immunocytochemistry*

Brain sections were washed in 0.05 M phosphate-buffered saline (PBS) and incubated in 3.0% normal

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**TABLE 1**
Cross-Reactivities of GnRH Antisera

| Antiserum | GnRH cross-reactivity (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | CI | CII | S | DF | LI | LIII | Mh | SB | TI | TII | CF |
| 675 | <0.01 | <0.01 | 100 | <0.01 | ND | <0.01 | ND | ND | ND | ND | ND | ND |
| 1668 | <0.01 | <0.01 | 0.7 | 100 | ND | 0.4 | ND | ND | ND | ND | ND | ND |
| 3952 | <0.01 | <0.01 | <0.01 | <0.01 | 6.3 | 123 | 100 | ND | ND | ND | ND | ND |
| R1245 | 100 | 65 | 4.16 | 19.5 | ND | <0.00001 | ND | ND | ND | ND | ND | ND |
| Adams-100 | <0.01 | <0.01 | 100 | 0.05 | 2.0 | 2.1 | 4.7 | <0.13 | <0.03 | <0.13 | <0.12 | <0.12 |
| 7CR-10 | <0.03 | <0.03 | 100 | 84.8 | 25.0 | 6.0 | 12.6 | <0.04 | <0.03 | 0.02 | <0.03 | <0.03 |

*Note.* Characteristics determined by RIA analysis (provided by N. Sherwood, J. King, and S. Sower). M, mammal; CI, chicken I; CII, chicken II; S, salmon; DF, dogfish; LI, lamprey I; LIII, lamprey III; Mh, hydroxyproline mammalian; SB, seabream; IT, tunicate I; TII, tunicate II; CF, catfish; ND, not determined.

goat serum in PBS with 0.3% Triton X-100 (PBSTX) for 30 min followed by incubation with different primary antisera diluted in PBSTX at 1:5000 for 18 h at room temperature in a sealed, humidified chamber. In some cases, an avidin–biotin blocker (Vector) was employed to reduce excessive background stain. The antisera were 7CR-10 (anti-dfGnRH) (gift from Nancy Sherwood), Adams-100 (anti-cGnRH-II) (gift from Tom Adams), 675 (anti-cGnRH-II), 1668 (anti-sGnRH), and 3952 (anti-lGnRH-III), all prepared in the rabbit. Complete cross-reactivity data of these antisera determined by RIA are listed in Table 1. However, because cross-reactivities in RIA cannot be directly applied to immunocytochemistry, these data are listed for reference purposes to characterize the antisera. Anti-sGnRH 1668 was selected as a possible anti-dfGnRH surrogate because sGnRH and dfGnRH differ by only one amino acid. Anti-lGnRH-III 3952 was chosen after preliminary HPLC/RIA indicated the possible presence of lGnRH-III in pooled stingray brains. Sections were washed in PBS, incubated in biotinylated goat-anti rabbit secondary antibody (Vector ABC kit) for 1 h, washed in PBS, treated with an endogenous peroxidase quenching step (0.5% $H_2O_2$ in PBS) for 10–20 min, and washed in PBS again. Slides were then incubated for 0.5 h in a humidified chamber with the avidin–HRP complex (Vector ABC kit), washed in PBS, and reacted with DAB–$NiCl_2$ chromogen substrate (Vector) for 5–7 min, which turns immunoreactive material dark purple or black. Sections were counterstained in 0.1% methyl green (0.5 min), rinsed in distilled $H_2O$, dehydrated in an ethanol series, cleared in xylene, and coverslipped.

Negative controls included (1) omission of primary antibody, (2) preabsorption of primary antisera with its peptide counterpart, (3) omission of secondary antibody, and (4) incubation of untreated tissue in chromogen. Positive controls included gourami, *Colisa lalia,* brain tissue known to contain immunoreactive cGnRH-II cell bodies in the midbrain tegmentum (Yamamoto *et al.,* 1995) and lamprey, *Petromyzon marinus,* brain tissue known to contain only lamprey forms of GnRH (King *et al.,* 1988; Sower *et al.,* 1993). Antibody cross-reactivity was tested by preabsorption of primary antibodies overnight at 4°C with cGnRH-II, dfGnRH, and lGnRH-III peptides (8 $\mu$M final concentration).

GnRH-ir cell measurements were taken from cross sections of the midbrain tegmentum, preoptic area, and white body of four male stingrays collected in February 1997. Eight to 10 somata from each brain area in each animal were randomly selected and measured with an ocular micrometer along the longest cell axis. Only cells with clearly defined boundaries were included in these measurements. Bipolar and monopolar cells occurred in different brain regions; thus, there was no confusion regarding which cell type was measured (see Results).

## RESULTS

### Chromatography and Radioimmunoassay

Chromatographic and radioimmunoassay analyses detected at least five different GnRH forms within the

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**TABLE 2**
Summary of HPLC/RIA Experiments to Identify GnRH Variants in the Brain of the Atlantic Stingray, *Dasyatis sabina*

| HPLC system/antibody | White body[a] | Preoptic area | Midbrain |
|---|---|---|---|
| Isocratic HPLC | | | |
| Anti-dfGnRH | DF, CII, S | DF, CII, S? | CII, DF |
| Anti-lGnRH-III | LIII, DF? | LIII, DF | LIII |
| Anti-mGnRH | CII | DF? | CII |
| Gradient HPLC | | | |
| Anti-dfGnRH | DF, CII, S, Unknown | DF, CII | CII, DF, Unknown |
| Anti-lGnRH-III | DF, LIII, Unknown | LIII, Unknown | LIII, Unknown |
| Anti-mGnRH | DF | CII | CII |

*Note.* Results are summarized for both the gradient and the isocratic HPLC systems. CII, chicken II; S, salmon; DF, dogfish; LIII, lamprey III. ?, peak concentrations measured were <10 pg/0.1 ml.
[a] White body samples contained part of lateral pallium, G2, and olfactory tract.

white body, preoptic area, and midbrain tegmentum of the Atlantic stingray, *D. sabina* (Table 2). The anti-dfGnRH 7CR-10, anti-lGnRH-III 3952, and anti-mGnRH R1245 all detected immunoreactive peaks in every brain region on both isocratic and gradient HPLC fractions (Table 2). Immunoreactive peaks that eluted in the same position as those of synthetic dfGnRH, cGnRH-II, lGnRH-III, and sGnRH standards, as well as unidentified peaks were detected (Fig. 1). These unknown forms were identified using the 3952 and 7CR-10 antibodies. The 3952 antibody, which is highly specific for lamprey GnRH forms in RIA (Table 1), identified a novel form that does not coelute with any known form of GnRH (Figs. 1B, 1D, and 1E). The 7CR-10 antibody is less specific, can bind with several GnRH forms, and may have also labeled a second novel GnRH variant (Fig. 1E). The concentrations of immunoreactive GnRH detected with the anti-mGnRH R1245 were generally below 2.0 pg/0.1 ml. Therefore, anti-mGnRH R1245 assays were not considered as accurate as anti-dfGnRH and anti-lGnRH-III assays and were not included in the analyses.

In the white body, anti-dfGnRH 7CR-10 detected three ir peaks which coeluted with dfGnRH, cGnRH-II, and sGnRH standards on the isocratic system. On the gradient HPLC system, four ir peaks eluted in the same positions as those of sGnRH, dfGnRH, cGnRH-II, and an early unidentified peak (Fig. 1A). Anti-lGnRH-III 3952 detected ir peaks which coeluted with lGnRH-III and possibly dfGnRH on the isocratic system. On the gradient system, two ir peaks eluted in the same positions as those of lGnRH-III and dfGnRH. In addition, a third unknown form did not elute in the same position as any of our GnRH standards (Fig. 1B). The major GnRH-ir peak in the white body coeluted with dfGnRH at a concentration of approximately 25 pg/0.1 ml from the gradient system fractions (Fig. 1A). Thus, the white body likely contains multiple GnRH forms that coelute with synthetic dfGnRH, sGnRH, lGnRH-III, cGnRH-II, and an unknown variant (Table 2).

In the preoptic area, anti-dfGnRH 7CR-10 detected two GnRH-ir peaks which coeluted with synthetic cGnRH-II and dfGnRH on the gradient system (Fig. 1C). On the isocratic system, anti-dfGnRH 7CR-10 showed major ir peaks that coeluted with cGnRH-II and dfGnRH and a minor peak with sGnRH. Anti-lGnRH-III 3952 detected two ir peaks on both the isocratic and the gradient HPLC systems which coeluted with lGnRH-III and dfGnRH on the former and an unknown and lGnRH-III on the latter (Fig. 1D). The major GnRH-ir peak in the preoptic area coeluted with dfGnRH on the gradient system at a concentration of about 200 pg/0.1 ml (Fig. 1C). The preoptic area likely also contains multiple forms of GnRH that coelute with synthetic dfGnRH, lGnRH-III, sGnRH, and cGnRH-II. In addition, a possible novel form was detected in the POA (Fig. 1D and Table 2).

In the midbrain tegmentum, the isocratic and gradient HPLC showed two major anti-dfGnRH 7CR-10 peaks which coeluted with cGnRH-II and dfGnRH peptides (Fig. 1E). In addition, the peak at 50 pg/0.1 ml that eluted at fraction 28 did not coelute with any known GnRH standard and thus represents an unknown form. A second unknown form is indicated on the gradient HPLC using anti-lGnRH-III 3952 by the

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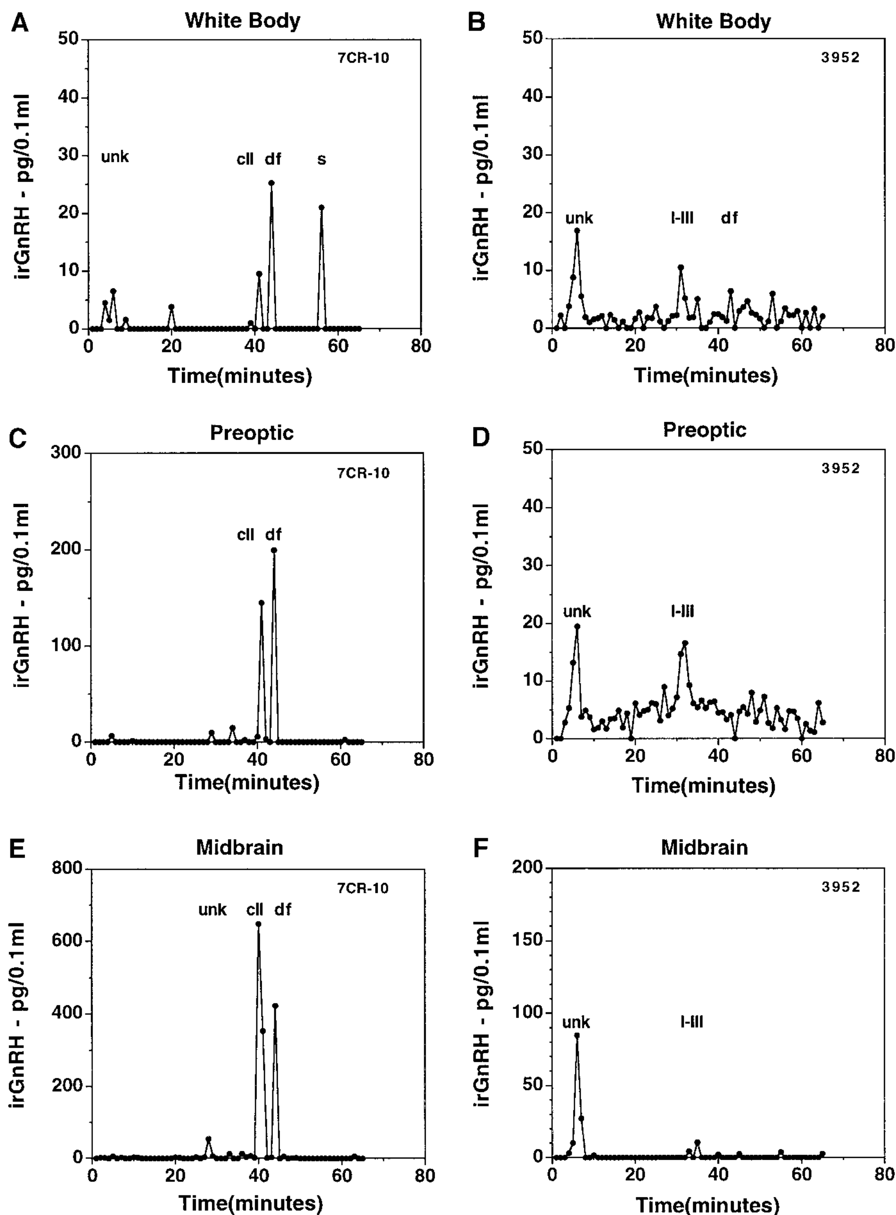


**FIG. 1.** Gradient HPLC elution and radioimmunoassay profiles of putative GnRH decapeptides extracted from the white body, preoptic, and midbrain regions of the stingray, *Dasyatis sabina.* (A) White body with 7CR-10 shows ir peaks that align with chicken GnRH-II (cII), dogfish GnRH (df), and salmon GnRH (s) peptides and an unknown (unk) form. (B) White body with 3952 shows ir peaks that align with lamprey III (l-III) and dogfish peptides and the unknown form in A. (C) Preoptic with 7CR-10 shows ir peaks that align with chicken II and dogfish peptides. (D) Preoptic with 3952 shows ir peaks that align with lamprey III peptide and the unknown form. (E) Midbrain with 7CR-10 shows prominent ir peaks that align with chicken II and dogfish peptides and a second unknown at fraction 28. (F) Midbrain with 3952 shows ir peaks that align with lamprey III peptide and an unknown form.

major peak at fraction 6 (~85 pg/0.1 ml) (Fig. 1F). In comparison, anti-lGnRH-III 3952 applied to isocratic fractions showed a major ir peak that coeluted with the lGnRH-III standard. The greatest concentration of GnRH-ir recorded in any brain region was detected in the midbrain tegmentum on the gradient HPLC system with the anti-dfGnRH (~600 pg/0.1 ml) and is likely the cGnRH-II variant (Fig. 1E). These results indicate cGnRH-II is the dominant midbrain form, with additional GnRH variants that co-

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**TABLE 3**
Morphological Features and Dimensions of GnRH-ir Cell Bodies in the Brain of the Atlantic Stingray, *Dasyatis sabina*

| | Tegmentum | Preoptic area | White body |
|---|---|---|---|
| Monopolar | − | + | + |
| Bipolar | + | − | − |
| Multipolar | + | − | − |
| Soma diameter ($\mu$m) | 28.6 ± 1.4 | 19.9 ± 2.7 | 19.8 ± 5.8 |
| Number of cells | $n = 40$ | $n = 36$ | $n = 56$ |
| GnRH-ir | cGnRH-II | dfGnRH | dfGnRH/Unknown |

*Note.* Measurements given as mean and standard deviations. +, presence; −, absence. Data obtained from four mature males, 22–25 cm disk width.

elute with dfGnRH and lGnRH-III. In addition, the HPLC/RIA analyses indicate the possible presence of additional, unknown forms in the midbrain (Figs. 1E and 1F and Table 2).

## Immunocytochemistry

GnRH-ir somata were found in three distinct brain regions in *D. sabina:* the terminal nerve ganglia, the ventral telencephalon and preoptic area, and the midline of the midbrain tegmentum. These neuronal groups were distinguished by size, morphology of the cell body, and immunoreactivity (Tables 3 and 4).

***GnRH-like immunoreactivity in the peripheral terminal nerve.*** The terminal nerve extends from the olfactory bulbs to the rostral telencephalon parallel and medial to the olfactory tract. Elasmobranch fishes are the only taxonomic group in which the TN is completely distinct from the adjacent olfactory tract (Demski *et al.,* 1987). The TN of *D. sabina* contains three distinct ganglia which are located along the rostral forebrain and associated olfactory structures (Fig. 2), similar to the round stingray, *Urolophus halleri* (Demski *et al.,* 1987). The third TN ganglion, G3, sits on the dorsal medial surface of the olfactory bulb (Figs. 2 and 3A), whereas the first TN ganglion, WB, and second TN ganglion, G2, are located on the olfactory tract (OLT) near the anterior telencephalon (Figs. 2 and 5A). All of the ganglia contain numerous cells that are immunoreactive to most GnRH antisera. These cells are monopolar and organized in dense clusters that are difficult to enumerate (Figs. 3B, 5A, and 5D). All measurements were taken from isolated somata found in the white body (Table 3). The mean diameter of white body somata was 19.8 ± 5.8 $\mu$m SD ($n$ = 56 cells, 4 animals).

Differences in immunoreactivity of various antisera were compared simultaneously with alternate brain sections from the same animal. Anti-lGnRH-III 3952

**TABLE 4**
Reaction of GnRH-ir Neurons to Preabsorption with Various GnRH Peptides in the Brain of the Atlantic Stingray, *Dasyatis sabina*

| Antiserum (antigen) | Preabsorbed peptide | TEG | POA/TL | WB/G2 | G3 |
|---|---|---|---|---|---|
| 7CR-10 (dfGnRH) | none | + | + | + | + |
| | cGnRH-II | − | − | − | − |
| | dfGnRH | − | − | − | − |
| | lGnRH-III | − | − | − | − |
| Adams-100 (cGnRH-II) | none | + | + | + | ND |
| | cGnRH-II | − | − | − | ND |
| | dfGnRH | + | * | − | − |
| 675 (cGnRH-II) | none | + | + | −/+* | −/+* |
| | cGnRH-II | − | − | − | − |
| | dfGnRH | − | − | − | − |
| 1668 (sGnRH) | none | −/+ | + | + | + |
| | cGnRH-II | − | −/+* | −/+* | ND |
| | dfGnRH | − | − | − | − |
| 3952 (lGnRH-III) | none | + | * | ++ | ++ |
| | cGnRH-II | − | −/+* | ++ | ++ |
| | dfGnRH | + | −/+* | −/+ | ++ |

*Note.* TEG, tegmentum; POA, preoptic area; TL, caudal ventral telencephalon; WB/G2, white body and second TN ganglia; G3, distal terminal nerve ganglion; ++, intense positive reaction; +, positive reaction; −/+, weak reaction; −, negative reaction; *, fibers only; ND, not determined.

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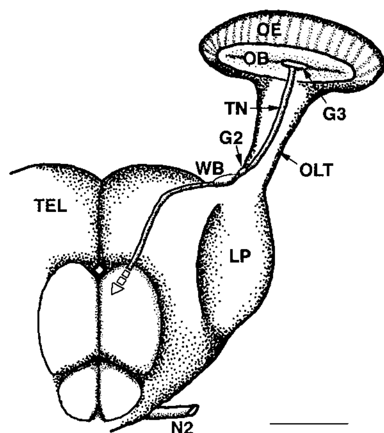


**FIG. 2.** Schematic drawing of the terminal nerve and ganglia on the dorsal forebrain in *Dasyatis sabina.* The terminal nerve (TN) runs along the olfactory tract (OLT) and the dorsal surface of the telencephalon (TEL) and has three ganglia. The white body (WB) and ganglion 2 (G2) are located between the OLT and the rostral TEL, and ganglion 3 (G3) is positioned on the dorsal surface of the olfactory bulb (OB). The TN enters the brain at the level of the rostral medial pallium, descends (broken line) to the caudal ventral telencephalon, and enters the ventral hypothalamic lobes. OE, olfactory epithelium; LP, lateral pallium; N2, optic nerve. Scale bar, 1 cm.

intensely labeled cells and fibers in G3, anti-sGnRH and anti-dfGnRH moderately labeled both cells and fibers, and anti-cGnRH-II 675 weakly labeled only a few fibers (Table 4). Immunoreactive axons in the olfactory bulb were more numerous with anti-lGnRH-III than anti-df GnRH or anti-sGnRH, and large varicose fibers within the connective tissue between the olfactory bulb and the olfactory epithelium were found only with anti-lGnRH-III (Fig. 3C). Anti-dfGnRH preabsorbed with either dfGnRH, cGnRH-II, or lGnRH-III peptide completely abolished any label which indicates its high cross-reactivity with those forms. Anti-dfGnRH 7CR-10 was applied to lamprey, *P. marinus,* brain sections to confirm that 7CR-10 indeed labels lGnRH-III, as only lamprey forms of GnRH can be detected in *P. marinus* brain tissue. These results indicate that 7CR-10 likely detects a lGnRH-III-like peptide in G3. Furthermore, anti-lGnRH-III intensely labels more cells and fibers in G2 than does 7CR-10, which indicates the presence of another form of GnRH (possibly an unknown form) that is different from the lGnRH-III-like variant (Figs. 4A and 4C). Also, it appears that neither dfGnRH nor cGnRH-II is expressed in G3 because preabsorption of anti-lGnRH-III with either peptide does not decrease the intense immunoreactivity in this ganglion (Table 4).

GnRH immunoreactivity in the WB and G2 differed from that in G3 (Table 4). Anti-lGnRH-III labeled cells

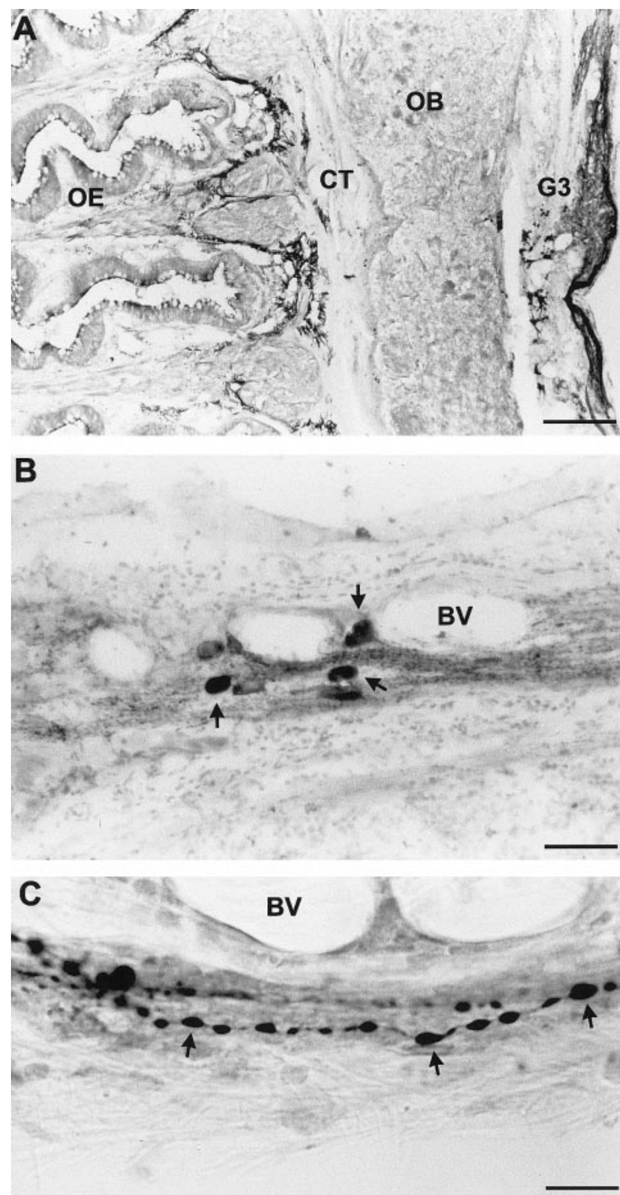


**FIG. 3.** GnRH-ir neurons in olfactory bulb and third ganglion of the terminal nerve in *Dasyatis sabina.* (A) Longitudinal section through the olfactory capsule shows close proximity of densely labeled G3 to the olfactory bulb (OB), connective tissue (CT), and olfactory epithelium (OE). Scale bar, 200 $\mu$m. (B) GnRH-ir somata (arrows) within G3 and closely associated blood vessels (BV). Scale bar, 60 $\mu$m. (C) Large varicosities of GnRH-ir fibers (arrows) within connective tissue between OB and OE. Scale bar, 20 $\mu$m.

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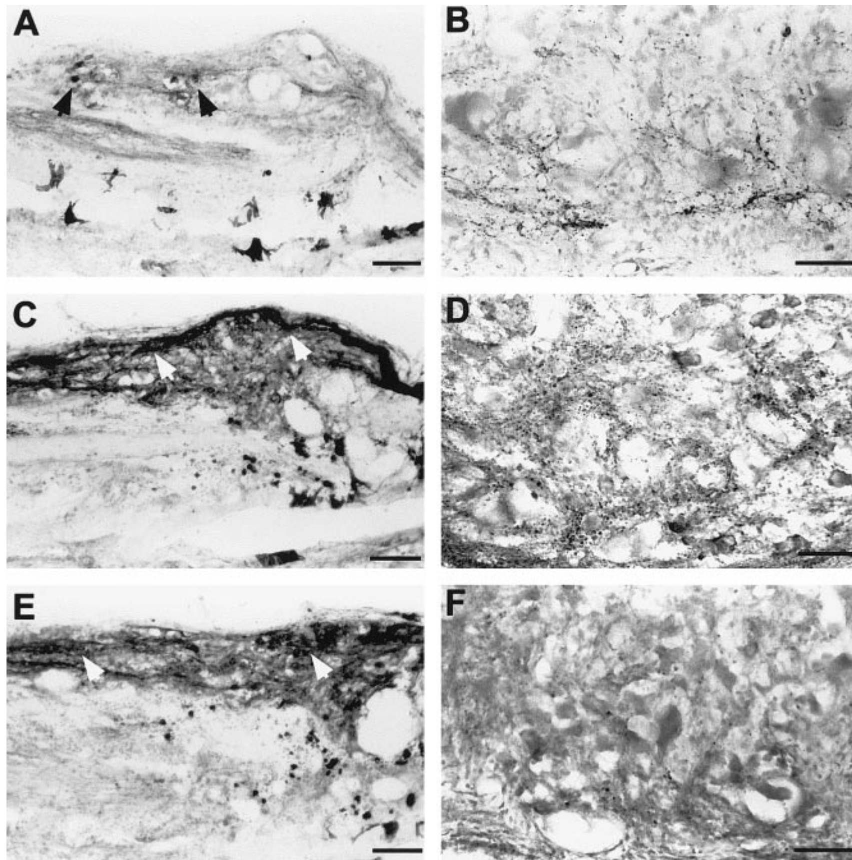


**FIG. 4.** Immunoreactivity of the third ganglion near the olfactory bulb and white body of the stingray terminal nerve. (A) Treatment of the third ganglion (G3) with anti-dfGnRH shows weak immunoreactivity (arrows). (B) Similarly, treatment of the white body (WB) with anti-dfGnRH also shows weak immunoreactivity. (C) In comparison to the weak immunoreactivity to anti-dfGnRH, treatment of G3 with anti-lamprey GnRH-III shows intense immunoreactivity (arrows). (D) Treatment of WB with anti-lamprey GnRH-III also shows intense immunoreactivity. (E) Treatment of G3 with anti-lamprey GnRH-III preabsorbed with dfGnRH peptide shows intense immunoreactivity (arrows) similar to that in C. (F) Treatment of WB with anti-lamprey GnRH-III preabsorbed with dfGnRH peptide shows weaker immunoreactivity than that observed in D. These experiments are consistent with the possible expression of lamprey GnRH III in G3 and WB. Treatments were performed on alternate sections from the same animal. All scale bars, 100 $\mu$m.

and fibers in the WB/G2 more intensely than did any other antiserum (Fig. 4D). However, when preabsorbed with dfGnRH peptide, this immunoreactivity was greatly reduced (Fig. 4F) and preabsorption with cGnRH-II showed no effect (Table 4). Anti-dfGnRH, anti-cGnRH-II Adams-100, and anti-sGnRH all labeled cells and fibers in WB/G2. However, preabsorption of these antisera with dfGnRH abolished all immunoreaction. Preabsorption of anti-sGnRH with cGnRH-II peptide still labeled fibers in WB. Anti-cGnRH-II 675 exhibited the weakest reaction in the WB and labeled only sparse fibers. As expected, experiments with anti-cGnRH-II Adams-100, anti-cGnRH-II 675, and anti-dfGnRH preabsorbed with cGnRH-II peptide resulted in complete loss of label due to their high cross-reactivity with cGnRH-II (Table 4). These results indicate that there are likely multiple forms of GnRH in the WB/G2 ganglia and one of the forms exhibits dfGnRH-like immunoreactivity. Chicken GnRH-II somata are likely not present in WB/G2.

***GnRH-like immunoreactivity in the forebrain.*** The TN is a conspicuous thick tract of GnRH-ir fibers that enters the forebrain in two distinct areas. One tract travels on the dorsal medial surface of the forebrain from the WB to the dorsal medial telencephalon (Figs. 2 and 5A–5C). This tract then descends to the ventral medial telencephalon and preoptic area (Figs. 6D, 7A, and 7B), hypothalamus, optic chiasm, and

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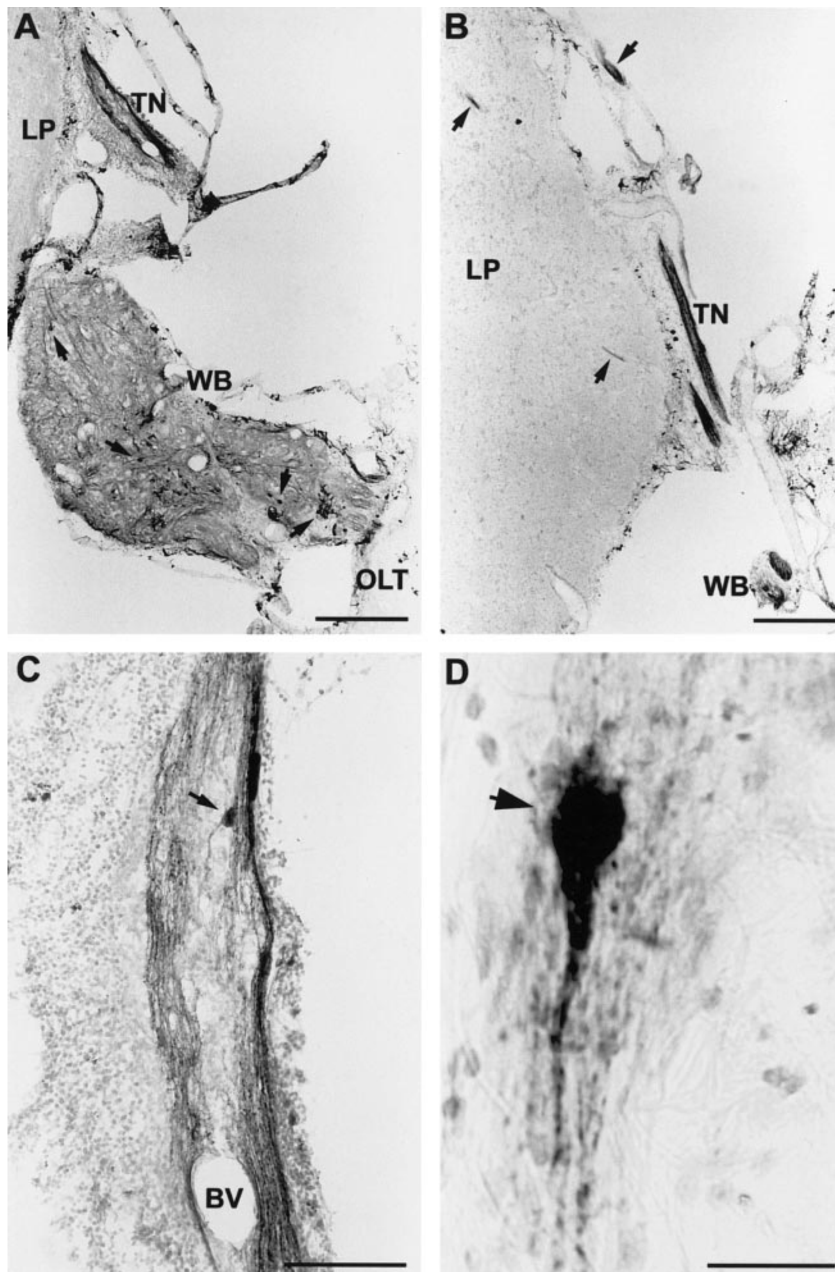


**FIG. 5.** GnRH immunoreactivity in the proximal terminal nerve ganglia of the stingray. (A) Transverse section shows GnRH-ir somata (arrows) in the white body (WB) of the terminal nerve (TN) at the junction of the olfactory tract (OLT) and lateral pallium (LP). Scale bar, 40 $\mu$m. (B) Transverse section at the junction of the WB and LP shows several branches of the TN (arrows) which course dorsomedially through the telencephalon. Scale bar, 400 $\mu$m. (C) Higher magnification of the TN tract on the dorsal surface of the rostral telencephalon shown in A. Note the monopolar cell (arrow) within this tract near a blood vessel (BV). Scale bar, 100 $\mu$m. (D) A monopolar GnRH-ir soma (arrow) and axon in the white body of the terminal nerve. Scale bar, 20 $\mu$m.

possibly other brain regions. As it descends into the ventral forebrain, the route of the TN appears to follow an important fiber tract, the *tractus pallii,* which originates in the roof of the telencephalon and connects the telencephalic pallial regions to hypothalamic areas (Smeets *et al.,* 1983). Several GnRH-ir somata are found scattered along the length of the TN tract but are neither well organized nor clearly associated with

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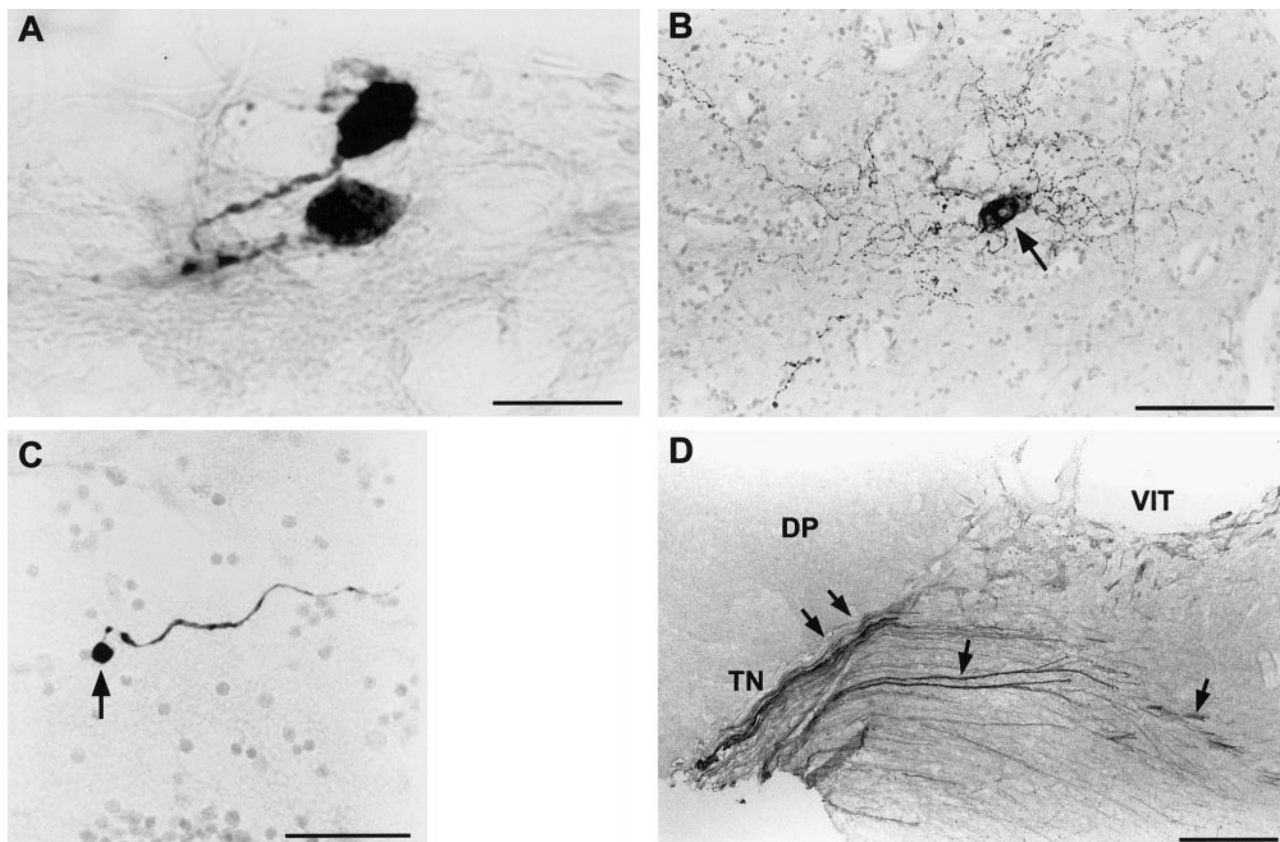


**FIG. 6.** GnRH immunoreactivity in the dorsal forebrain of the stingray. (A) Sagittal section through the dorsal lateral pallium shows two monopolar GnRH-ir somata near the surface. Scale bar, 20 μm. (B) A GnRH-ir large soma (arrow) and numerous fibers with varicosities in the dorsal pallium. Scale bar, 100 μm. (C) Thick GnRH-ir fiber with terminal ending (arrow) near pallial midline. Scale bar, 40 μm. (D) Sagittal section through the caudal telencephalon shows the thick dfGnRH-ir terminal nerve tract (TN) and branches (arrows) that descend ventrally from the dorsal medial pallium (DP) beneath the ventricular impar telencephali (VIT). Scale bar, 400 μm.

any particular telencephalic nuclei. Many fibers and cells in this tract are closely associated with blood vessels (Fig. 5C). The second branch of the dense GnRH-ir TN tract enters the rostral ventral telencephalon from the junction of the olfactory tract and medial forebrain and travels dorsolaterally into the lateral pallium (Fig. 5B). Also, complexes of varicose fibers, which may have numerous *en passant* synapses or axon terminations, are located within the dorsal terminal nerve tract and along the caudal lateral pallium (Figs. 6B–6D). A few GnRH-ir cells are located in the region of the lateral pallium just under the dura on the dorsal and ventral surface of the brain (Figs. 6A and 6B). Generally, the immunoreactivity in the TN tract within the telencephalon is similar to that found in the WB/G2. Terminal nerve fibers within the forebrain likely contain dfGnRH because immunoreactivity in the prominent descending tracts (Fig. 6D) was completely abolished when all antisera were preabsorbed with dfGnRH peptide. TN tracts outside the brain (along the olfactory tract and on the dorsal aspect of the telencephalon) probably contain multiple forms of GnRH, as some fibers were still evident after anti-lGnRH-III was preabsorbed with dfGnRH peptide. The occasional cells seen in the dorsal pallial regions were labeled by anti-dfGnRH, anti-cGnRH-II Adams-100, and anti-lGnRH-III. However, these cells were too uncommon to reliably perform controlled preabsorption experiments and to determine their GnRH form.

A small group of less than 50 GnRH-ir cells is scattered in the ventral preoptic area from just above the optic chiasm to the caudal telencephalon (Fig. 8). The preoptic GnRH-ir neurons have a monopolar, oval or

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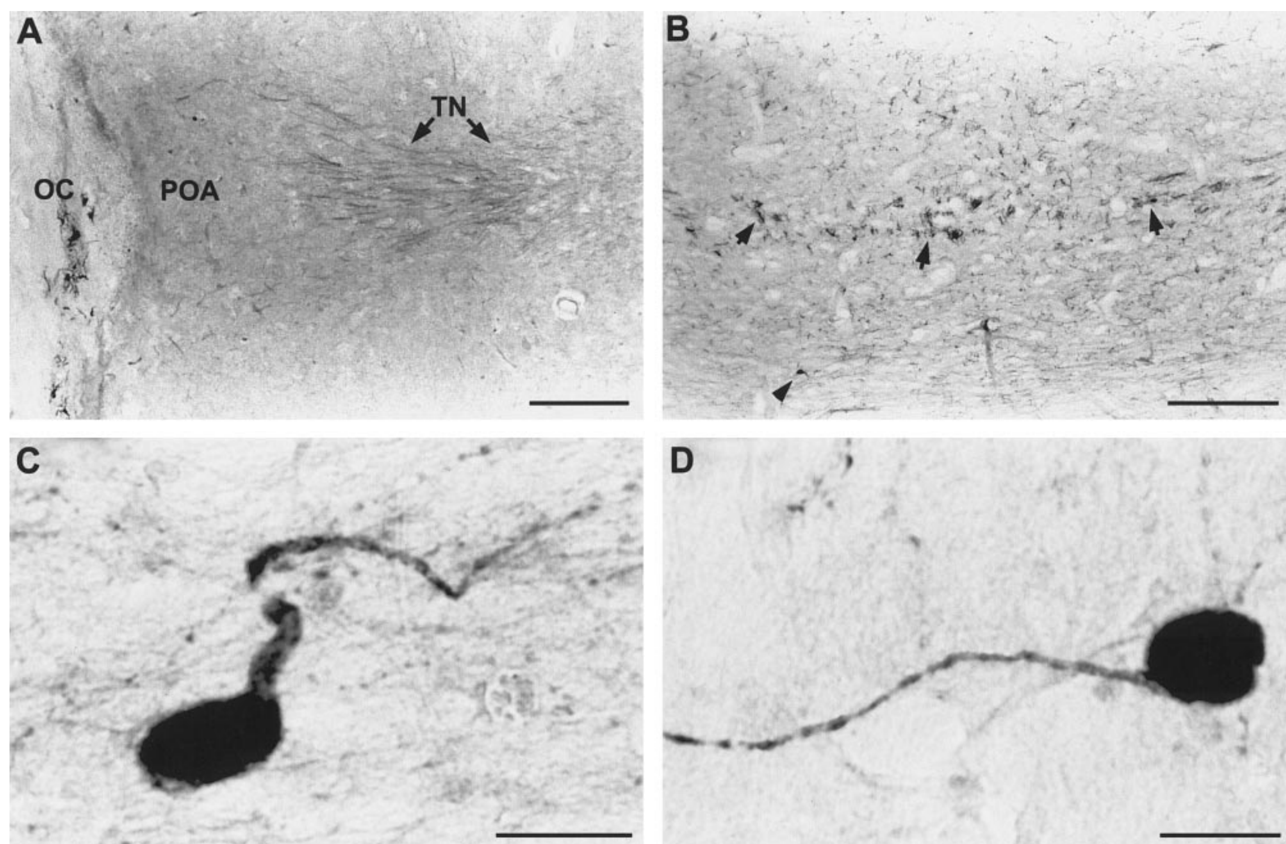


**FIG. 7.** GnRH immunoreactivity in the ventral telencephalon of the stingray. (A) Horizontal section through the preoptic area (POA) of the terminal nerve (TN) fiber tract immediately rostral to the optic chiasm (OC). Scale bar, 400 $\mu$m. (B) Transverse section through the caudal telencephalon shows the thick ventral fiber tracts of TN (arrows) and few immunoreactive somata (arrowhead). Scale bar, 200 $\mu$m. (C) Representative dfGnRH-ir monopolar cell in the preoptic area. Scale bar, 20 $\mu$m. (D) Monopolar GnRH-ir soma and axon in the hypothalamus. Scale bar, 20 $\mu$m.

round cell body, approximately 20 $\mu$m in diameter ($n$ = 36 cells, 4 animals) with a single, thick process (Table 3 and Fig. 7C). A few cells of the same morphology and size were found in the hypothalamus and caudal diencephalon and are thought to be an extension of this group (Fig. 7D). GnRH-ir fibers are found throughout the length of the ventral telencephalon but are concentrated rostrally. Many GnRH-ir fibers are found in the ventral region of the POA below and around the ventricle, just above the optic chiasm and laterally along the optic tract (Figs. 7A and 7B). These GnRH-ir fibers often terminate near the ventral ventricular surface and only sparse fibers are found dorsal to the ventricular area. A dense accumulation of varicose fibers is seen ventrally in the inferior lobe of the hypothalamus and several ir fibers penetrate the anterior median eminence. Immunolabeling in the ventral forebrain was similar to that in the WB/G2, except for evidence of cGnRH-II projections in the forebrain (Table 4). Anti-cGnRH-II Adams-100 labeled some fibers when preabsorbed with dfGnRH, whereas anti-cGnRH-II 675 labeled fibers only when not preabsorbed. Anti-lGnRH-III showed an overall decrease in immunoreactivity in the forebrain compared to the TN complex. When this antibody was preabsorbed with cGnRH-II, few or no fibers were seen in the caudal telencephalon/rostral diencephalon, whereas some fibers were seen more rostrally in the forebrain. Conversely, preabsorption of anti-lGnRH-III with dfGnRH showed more fibers in the caudal than in the rostral forebrain. Also, anti-sGnRH labeled some fibers when it was preabsorbed with cGnRH-II, whereas immunoreactivity was abolished when it was preabsorbed with dfGnRH (Table 4). The

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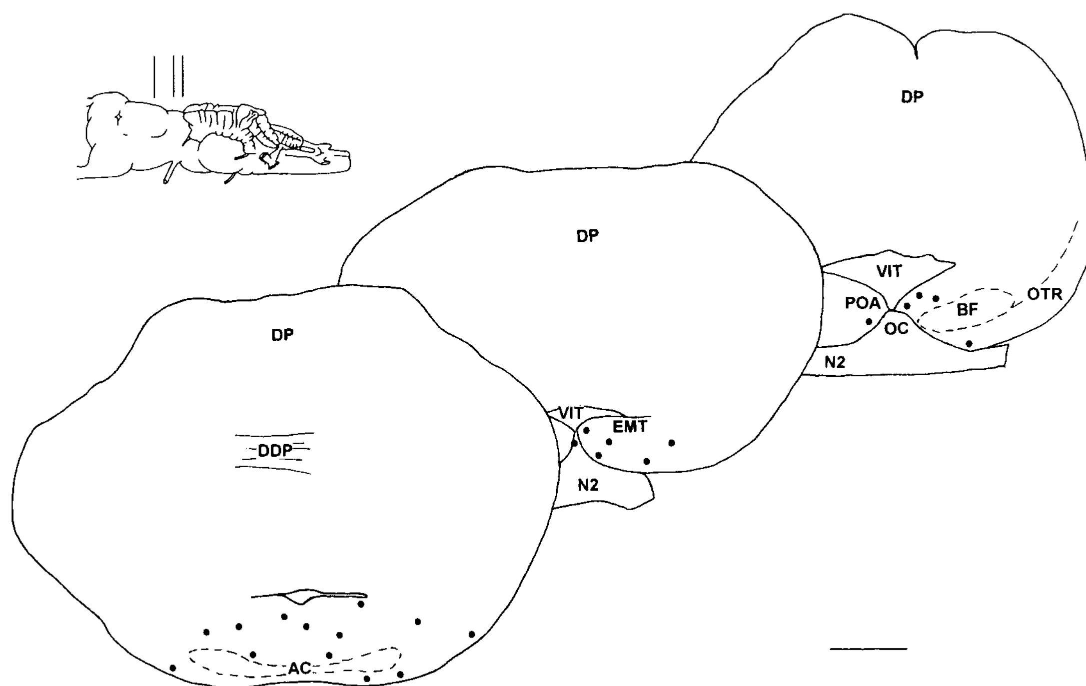


**FIG. 8.** Rostrocaudal camera lucida series of three transverse sections that show the distribution of dogfish GnRH-ir somata in the preoptic area of the stingray. Immunoreactive somata (dots) were found in the ventral preoptic area (POA) from the optic chiasm (OC) rostral to the anterior commissure (AC). This figure is a composite from multiple animals and the total number of somata observed in any single fish was variable. Inset of stingray brain shows location of each section. BF, basal forebrain bundle; DDP, descusatio dorsalis pallii; DP, dorsal pallium; EMT, eminentia thalami; N2, optic nerve; OTR, optic tract; VIT, ventricular impar telencephali. Scale bar, 2.0 mm.

prominent GnRH-ir fiber tracts in the rostral ventral medial TEL and POA (Figs. 6D, 7A, and 7B) are most likely projections from the TN, because immunoreactivity is greatly reduced when all antisera are preabsorbed with dfGnRH. However, there is evidence for multiple forms of GnRH in the diencephalon and caudal forebrain (Table 4). The similarities in GnRH-ir soma size, morphology, and immunoreactive properties of the TN WB/G2 group compared to those of the ventral telencephalic group supports the hypothesis that they both express dfGnRH. Figure 9 summarizes the distribution of dfGnRH-ir cells and fibers in the parasagittal plane.

***GnRH immunoreactivity in the midbrain, cerebellum, and hindbrain.*** A large group of approximately 800 GnRH-ir cell bodies extends from the oculomotor nucleus to the posterior commissure (PC). Cells are distributed along the midline of the midbrain tegmentum, below the third ventricle, and between the tracts of the medial longitudinal fasciculus (MLF) (Figs. 10A and 11). The location of this group is almost identical to that described for GnRH-ir cells in the elasmobranch tegmentum by Wright and Demski (1991). These somata are fusiform, bipolar or multipolar with a mean major diameter of 28.6 ± 1.4 $\mu$m SD ($n = 40$ cells, 4 animals) (Table 3 and Fig. 10B). Anti-cGnRH-II Adams-100, anti-cGnRH-II 675, and anti-dfGnRH 7CR-10, all known to have high reactivity to cGnRH-II in RIA, label these cells intensely. Anti-lGnRH-III also labels these midbrain cells. In contrast, anti-sGnRH shows weak reactivity in a limited number of cell bodies in this region. All antisera preabsorbed with cGnRH-II peptide failed to label the tegmental somata (Table 4). However, anti-lGnRH-III labeled cells and fibers in WB and G3 with no decrease in immunoreactivity, and anti-sGnRH labeled fibers in the ventral forebrain and WB when preabsorbed with cGnRH-II peptide. In comparison with other antisera that detect dfGnRH (7CR-10, 1668, 675), the anti-cGnRH-II Adams-100 and anti-lGnRH-III preabsorbed with dfGnRH peptide still labeled this large midbrain cell group, which provides evidence that tegmental so-

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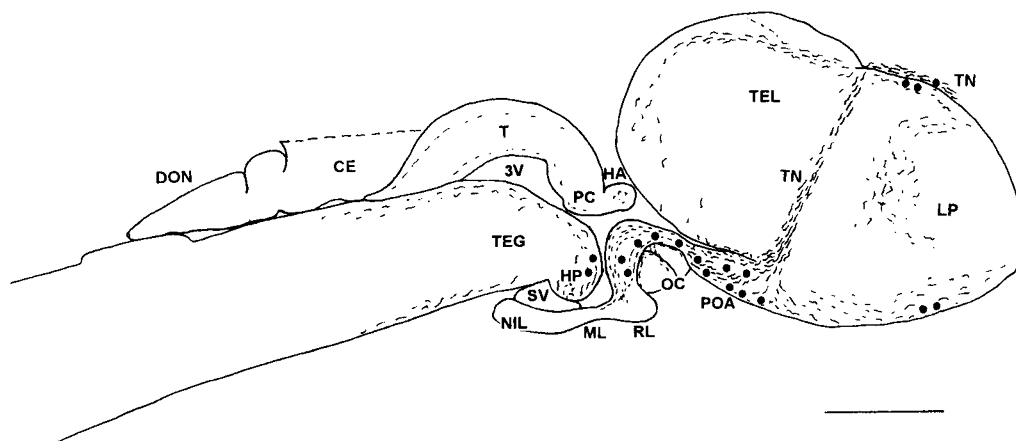


**FIG. 9.** Diagrammatic parasagittal representation of dogfish GnRH-ir somata and fibers in the stingray brain. Dogfish GnRH-immunoreactive somata (dots) are located near the base of the terminal nerve (TN), the rostral and caudal ventral telencephalon (TEL), preoptic area (POA), and hypothalamus (HP). Immunoreactive fiber tracts were also identified in the lateral pallium (LP), dorsal pallium, tegmentum (TEG), and tectum (T). Dorsal portion of the corpus cerebellum (CE) above dashed line is not shown. 3V, third ventricle; DON, dorsal octaval nucleus; HA, habenula; ML, median lobe of pituitary; NIL, neurointermediate lobe of pituitary; OC, optic chiasm; PC, posterior commissure; RL, rostral lobe of pituitary; SV, saccus vasculosus. Ventral lobe of pituitary is not shown. Scale bar, 0.5 cm.

mata likely contain the cGnRH-II variant and not df-GnRH (Table 4). Figure 11 summarizes the distribution of cGnRH-II-ir fibers and cell bodies in the parasagittal plane.

Beaded GnRH-ir fibers in the caudal midbrain first appear in the periventricular gray (PG) which surrounds the third ventricle (3V). Some of these beaded fibers are diffuse and extend dorsally toward the tectum, whereas others course laterally into the lateral mesencephalic nucleus (LMN) and the medial mesencephalic nucleus (MMN). Distinct GnRH-ir fiber tracts are apparent in periventricular and central tectal zones (Fig. 11). Medial sections show that fibers often terminate near the third ventricle. Immunoreactive fibers along the midline and ventral tegmentum are more concentrated, whereas lateral (LMN, MMN) and superficial tectal fibers are more diffuse. GnRH-ir cells and fibers in this area of the tegmentum are often closely associated with blood vessels (Fig. 10A). Relatively long beaded fibers traverse the ventral tegmentum just above the saccus vasculosus in the rostral midbrain. Further, complexes of thick fibers with swellings are seen throughout the mammillary recess (MR) (Fig. 10E). GnRH-ir fibers concentrated in the hypothalamus and median eminence also projected to the saccus vasculosus (SV) and the neurointermediate lobe of the pituitary (NIL) (Fig. 11). We also identified numerous cGnRH-II-ir fibers in the corpus cerebellum but did not examine the specific projections within this region of the brain.

A dense GnRH-ir fiber tract in the interpeduncular areas (IP) projects caudal toward the hindbrain and spinal cord (Fig. 11). Another caudal projection of GnRH-ir fibers from the midbrain tegmentum follows the ventricular periphery toward sensory processing regions in the brainstem (Fig. 10D). Concentrated GnRH-ir fibers run transversely in the isthmus toward the electrosensory dorsal octavolateral nucleus (DON) and the mechanosensory medial octavolateral nucleus (MON). GnRH-ir fibers also occur around the cerebellar crest at the dorsal nucleus periphery and in branches of the eighth cranial nerve (Fig. 10C). Anti-sGnRH weakly labeled GnRH-ir fibers in the midbrain and hindbrain regions compared to anti-dfGnRH and anti-cGnRH-II (both Adams-100 and 675). Preabsorption of all antisera with cGnRH-II peptide completely abolished all immunoreactivity in this area. However, anti-cGnRH-II Adams-100 preabsorbed with dfGnRH peptide still labeled numerous fibers in the lower brain regions, whereas immunoreactivity with all other antisera was abolished under these same conditions. These results provide strong evidence that most GnRH-ir fibers in the hindbrain originate from the cGnRH-II-ir tegmental cell group.

There appears to be overlap among dfGnRH and cGnRH-II fiber pathways in the rostral midbrain and

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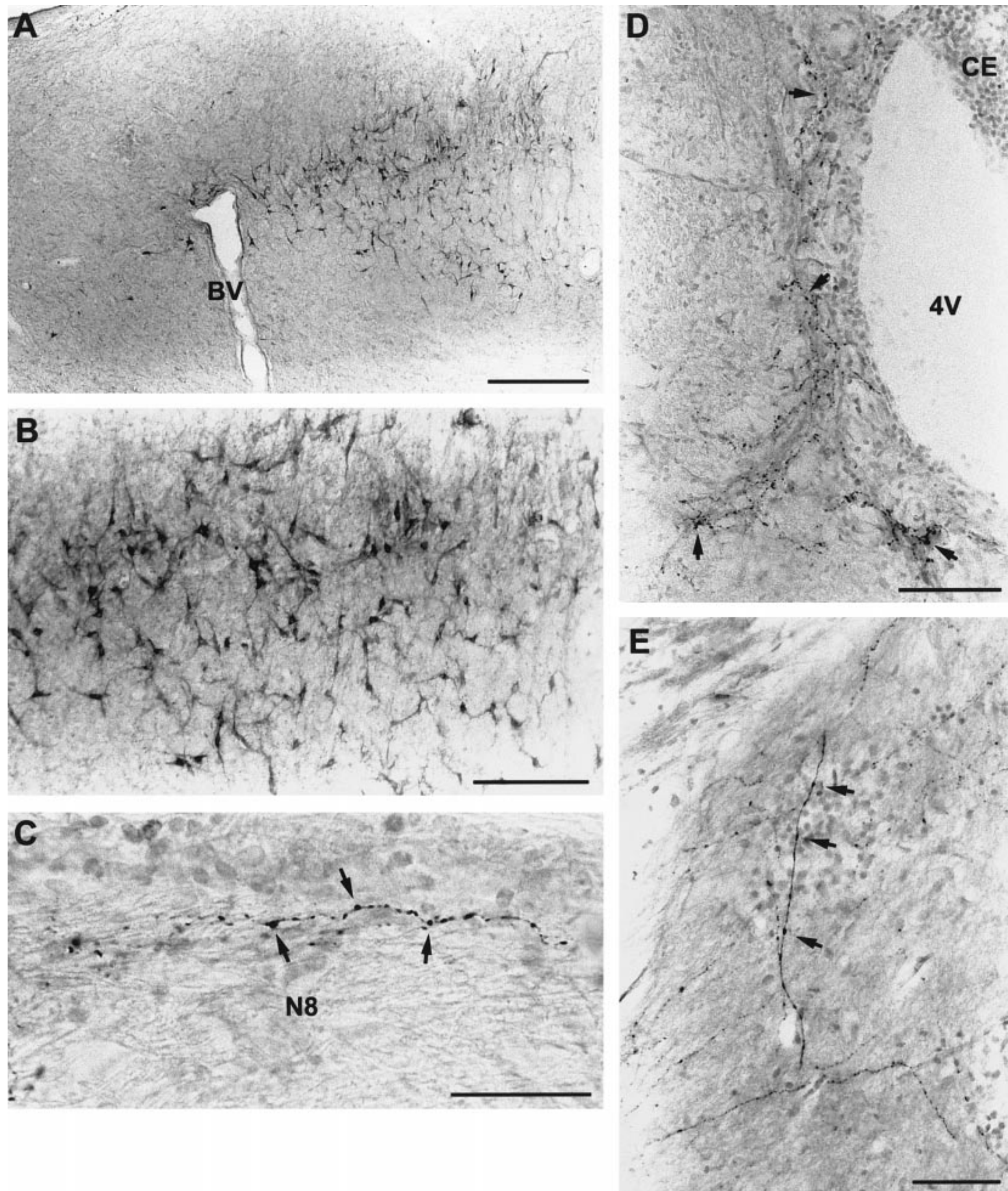


**FIG. 10.** GnRH immunoreactivity in the midbrain tegmentum and hindbrain of the stingray. (A) The tegmental cGnRH-II-ir cell group is located near the midsagittal plane. Note prominent blood vessel (BV) which runs dorsoventrally through the tegmentum in close association with immunoreactive somata and fibers. Scale bar, 400 $\mu$m. (B) cGnRH-II-ir somata have either bipolar or multipolar morphologies. Scale bar, 200 $\mu$m. (C) Sagittal section through the eight nerve (N8) as it enters the brainstem shows immunoreactive axons with swellings (arrows). Scale bar, 25 $\mu$m. (D) Transverse section of the rostral hindbrain shows immunoreactive fibers (arrows) near the lateral wall of the fourth ventricle (4V) below the cerebellum (CE). Scale bar, 200 $\mu$m. (E) GnRH-ir fibers in the mammillary recess have large swellings (arrows) which may be *en passant* synapses. Scale bar, 10 $\mu$m.

diencephalon as determined by ICC and HPLC/RIA analyses (Figs. 1, 9, and 11). Chicken GnRH-II-ir fibers were identified in the median eminence, neurointermediate lobe of the pituitary, and hypothalamus. However, cGnRH-II-ir fibers were clearly more concentrated in the midbrain and hindbrain regions than were dfGnRH-ir fibers. In contrast, dfGnRH-ir fibers were more concentrated in the forebrain areas (Figs. 9

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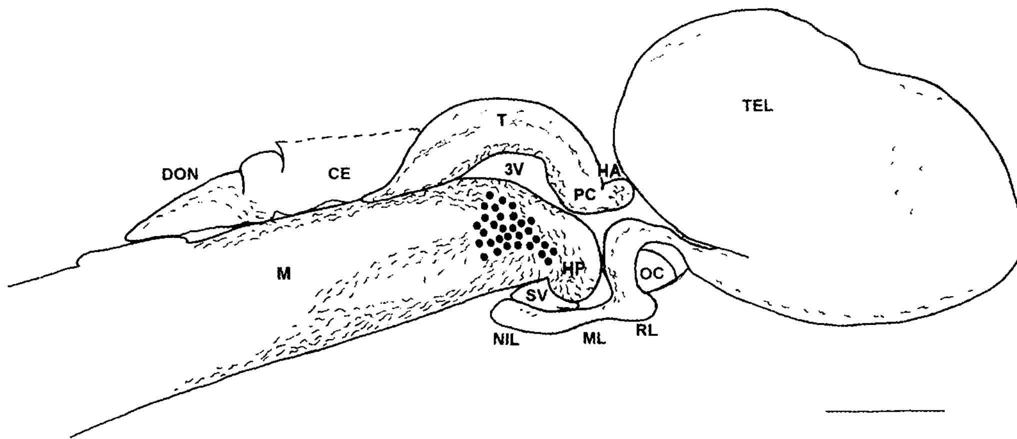


**FIG. 11.** Diagrammatic parasagittal representation of chicken GnRH-II-ir somata and fibers in the stingray brain. Approximately 800 cGnRH-II-ir somata (dots) form a large group along the tegmentum midline that extends from the oculomotor nucleus to the posterior commissure (PC). The most prominent axon projections (lines) observed were to the preoptic area, hypothalamus (HP), habenula (HA), tectum (T), dorsal octaval nucleus (DON), cerebellum (not shown), and ventral medulla (M). Corpus of CE is not illustrated above dashed line. 3V, third ventricle; ML, median lobe of pituitary; NIL, neurointermediate lobe of pituitary; OC, optic chiasm; RL, rostral lobe of pituitary; SV, saccus vasculosus; TEL, telencephalon. Ventral lobe of pituitary is not shown. Scale bar, 0.5 cm.

and 11). Because of the high cross-reactivity properties of the polyclonal antibodies used in this study, we cannot discount the possible presence of other GnRH variants such as lGnRH-III, sGnRH, or novel forms in the stingray brain.

## DISCUSSION

This study supports the prediction that dfGnRH and cGnRH-II neurons form separate and distinct populations in the brain of the Atlantic stingray, *D. sabina.* In addition, we provide evidence for a distinct GnRH-ir cell population in G3 of the terminal nerve, which although clearly not conclusive, is nonetheless consistent with the presence of the lGnRH-III form. HPLC and RIA analyses using the highly lamprey-GnRH-specific 3952 antibody indicate that lGnRH-III may also occur in the white body, preoptic, and midbrain regions. Although GnRH-ir fibers occur throughout most areas of the brain, GnRH-ir cell bodies are concentrated in the terminal nerve ganglia, the caudal ventral telencephalon and preoptic area, and the midbrain tegmentum. Dogfish GnRH-ir cell bodies occur in the proximal terminal nerve ganglia and forebrain areas. Demski *et al.* (1997) reported a large scattered population of GnRH-ir cells in the basal forebrain area of the skate. These may be dfGnRH-containing cells. Chicken GnRH-II-ir somata are limited to the midbrain tegmentum along the midline below the third ventricle between the tracts of the MLF. This is the same midbrain group originally described by Wright and Demski (1991) in the round stingray, *U. halleri,* thornback guitarfish, *Platyrhinoidis triseriata,* and the leopard shark, *Triakis semifasciata.* However, their study employed a nonspecific anti-sGnRH which labeled multiple forms of the GnRH decapeptide. Thus, the present study is the first to identify the cGnRH-II form in the midbrain tegmental cell group of an elasmobranch fish.

To date D'Antonio *et al.* (1995) is the only other study that attempted to localize different forms of GnRH in the elasmobranch brain. Their description of GnRH-ir fibers in the postchiasmatic areas and hypothalamus of the spotted dogfish, *Scyliorhinus canicula,* is similar to that found in *D. sabina.* However, D'Antonio *et al.* (1995) reported that all GnRH-ir somata were restricted to the telencephalon and diencephalon. GnRH-ir cells labeled by all three antisera (anti-cGnRH-II, anti-mGnRH, and anti-sGnRH) were localized in the POA and some mGnRH-ir cells were found in the telencephalon. HPLC/RIA data in *S. canicula* indicate the presence of cGnRH-II in the hindbrain and cGnRH-II, dfGnRH, and an unknown form in the mesencephalon, but no immunoreactive somata

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or axons were found in these areas. Likewise, GnRH-ir cells were not observed in the midbrain of the spiny dogfish, *Squalus acanthias,* nor in that of the black skate, *Bathyraja kincaidii,* in ICC experiments that used a broad-based antibody (Lovejoy *et al.,* 1992a). Thus, the apparent lack of GnRH-ir midbrain cells or fibers in these species may be due to specific antisera characteristics or possible seasonal expression of cGnRH-II by the midbrain cell group.

Other studies that demonstrate differential distribution of GnRH variants within a single species identified GnRH forms that differ by at least two amino acids (e.g., Lepetre *et al.,* 1993; Yamamoto *et al.,* 1995; Pinelli *et al.,* 1997; Robinson *et al.,* 1999), and problems with antisera cross-reactivity were probably minor. In contrast, cGnRH-II and dfGnRH differ by only one amino acid at position 8 and are especially susceptible to significant cross-reactivity when polyclonal antibodies are used (Tables 2 and 4). Anti-GnRH cross-reactivities determined by RIA are not directly applicable to immunocytochemistry experiments (Larsson, 1988). However, the technique for preabsorption of anti-GnRH sera with a homologous or heterologous peptide is similar to that used for RIA in that the peptide antigen is not bound within tissue. This technique proved useful for binding the subpopulation of the antibodies that is specific to the preabsorption peptide and eliminates labeling of that peptide in the tissue. Thus, immunocytochemical results from application of various preabsorbed antibodies to specific brain tissues provided more confidence in our identification of the GnRH variants. Nonetheless, ultimate confirmation of each variant will require peptide or gene sequence analyses.

### *The Distribution of GnRH Neurons in the Stingray Terminal Nerve and Forebrain*

Immunocytochemical results indicate that G3 in the stingray contains multiple forms of GnRH, one of which has lGnRH-III-like immunoreactivity. However, HPLC/RIA analyses of G3 are not available to support this finding. The intense immunoreactive label from anti-lGnRH-III in this ganglion persists when this antibody was preabsorbed with either dfGnRH or cGnRH-II peptide (Table 4 and Figs. 4A, 4C, and 4E). Thus, it is likely that neither dfGnRH nor cGnRH-II is expressed in this distal ganglion. In addition, application of anti-dfGnRH 7CR-10 labels lamprey GnRH cells in the lamprey brain; therefore, 7CR-10 may also have labeled a lamprey form in the stingray G3. One previous study found evidence for lGnRH-I through HPLC/RIA analysis in elasmobranch brain tissue (Calvin *et al.,* 1993), and several hypotheses for the evolution and phylogenetic distribution of GnRH were proposed (Muske, 1993; King and Millar, 1995; Grober *et al.,* 1995; Dores *et al.,* 1996). However, many of these models do not include lamprey GnRH variants, in part due to the fact that most studies have not examined for the presence or absence of lamprey forms using the appropriate antiserum either in immunocytochemistry or in HPLC/RIA. Furthermore, G3 was not assayed in any studies in which different forms of GnRH were identified; thus, any forms that may be specific to this structure could be overlooked.

Lamprey GnRH-III has 80% identity with cGnRH-II and dfGnRH, and all are considered to be closely related to the ancestral molecule (Sower *et al.,* 1993). Lamprey GnRH-III, cGnRH-II, and dfGnRH occur in species that represent the two oldest vertebrate lineages. In Atlantic hagfish, chromatographic and immunocytochemical evidence showed that the neurohypophysis contains a GnRH-like molecule that is closely related to lGnRH-III (Sower *et al.,* 1995). In other immunocytochemical studies using the Pacific hagfish, *Eptatretus stouti,* Braun *et al.* (1995) suggested that there are two GnRH systems in hagfish, one system which is widely diffuse throughout the brain and another which is restricted to the preoptic–neurohypophysial system. Thus, modern hagfish may have retained one or more of the early or stem GnRH forms. Lamprey GnRH-I was documented in five derived perciform fishes (Okuzawa *et al.,* 1993), the less recently derived cyprinodontiform platyfish, *Xiphophorus maculatus* (Magliulo-Cepriano *et al.,* 1994), and an early evolved teleost, the white sucker, *Catostomus commersoni* (Robinson *et al.,* 1999). Therefore, it is possible that lGnRH-III, cGnRH-II, and dfGnRH have coevolved and are coexpressed in the elasmobranchs, as the Chondrichthyes is the oldest taxon in which the cGnRH-II variant was identified. However, the phylogenetic relationships among fish taxa based upon GnRH (e.g., Grober *et al.,* 1995; Dores *et al.,* 1996) and other molecular analyses (e.g., Rasmussen and Arnason, 1999) are controversial and await confirmation. Nevertheless, recent *in vitro* work on the rat hemipi-

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tuitary indicates that lGnRH-III has a powerful and specific effect on follicle-stimulating hormone release but not upon luteinizing hormone release (Yu *et al.,* 1997). Although definitive identification of lGnRH-III in the stingray remains uncertain until it is sequenced, the existence of multiple GnRH-ir forms in the stingray forebrain and pituitary raises the possibility that each variant serves a distinct role in regulation of different pituitary gonadotrophs.

This study shows that dfGnRH is most likely the primary form contained in the proximal TN and forebrain of the stingray. However, chromatographic and RIA analyses of the stingray WB indicate the presence of at least four different GnRH variants that coelute with dfGnRH, sGnRH, lGnRH-III, and cGnRH-II standards and possibly an unknown form (Fig. 1 and Table 2). Lovejoy *et al.* (1992c) demonstrated up to four forms of GnRH in the terminal nerve of the spiny dogfish, *S. acanthias,* which include a dfGnRH-ir and a cGnRH-II-ir form. Because that study employed only HPLC/RIA analyses, localization of different GnRH cell types in the TN ganglia of the dogfish was not demonstrated. Our study provides immunocytochemical evidence for multiple forms of GnRH in the stingray TN and forebrain. First, anti-cGnRH-II 675 weakly labeled fibers in both the WB and the G2 ganglia. Second, application of anti-cGnRH-II Adams-100 preabsorbed with dfGnRH peptide labeled only a few fibers in the TEL and POA but no cell bodies in the TN ganglia. Thus, it is unlikely that cGnRH-II is a major form in the forebrain or TN of the stingray. Experiments in which anti-lGnRH-III was preabsorbed with dfGnRH showed reduced WB immunoreactivity. Whereas unpreabsorbed anti-sGnRH 1668 preferentially labeled somata and fibers in the forebrain and TN (Table 4), immunoreactivity was completely abolished in all TN ganglia, POA, and TEL when preabsorbed with dfGnRH. These findings support the prominent presence of dfGnRH but cannot reject the possible presence of sGnRH, lGnRH-III, or lGnRH-like forms in the stingray TN and forebrain.

### *The Distribution of GnRH Neurons in the Stingray Midbrain and Hindbrain*

Our results show that cGnRH-II is the dominant form in the midbrain and hindbrain of the stingray. HPLC/RIA analyses clearly show major peaks that coelute with cGnRH-II peptide (Fig. 1). These experiments also show the presence of other forms that coelute with dfGnRH, lGnRH-III, and unknown forms. However, these latter variants occur in low concentrations compared to cGnRH-II and most likely result from fiber projections through the midbrain that originate from GnRH-ir cells in other brain regions. Our immunocytochemistry experiments support the dominance of cGnRH-II in the midbrain and hindbrain. Although anti-cGnRH-II Adams-100 has a low RIA cross-reactivity with dfGnRH, it still intensely labels both dfGnRH and cGnRH-II somata and fibers (Tables 2 and 4). Preabsorption of anti-cGnRH-II Adams-100 with dfGnRH peptide eliminated binding to dfGnRH and resulted in a more specific cGnRH-II reaction product. Separate experiments in which each antiserum was preabsorbed with cGnRH-II peptide completely abolished labeling of tegmental cells. Additional experiments in which anti-lGnRH-III was preabsorbed with dfGnRH showed tegmental cells labeled as intensely as unpreabsorbed treatments. Using such preabsorption techniques, cGnRH-II-ir cells were shown to be restricted to the midbrain of the stingray (Fig. 11). The results of these experiments support the hypothesis that tegmental cells express cGnRH-II in the elasmobranch brain and are consistent with cGnRH-II expression in the tegmentum of other vertebrate classes (for review see Muske, 1993).

Midbrain tegmental cells in *D. sabina* that express cGnRH-II were located along the midline of the tegmentum, below the third ventricle, between tracts of the MLF, and in the region of the interpeduncular nucleus. This large group of cells, which recently was named the midbrain parasagittal nucleus by Demski *et al.* (1997), projects to many regions of the central nervous system, especially those involved in sensory processing and sensory integration. There is a prominent projection of fibers to the electrosensory DON and mechanosensory MON in the brainstem. Beaded cGnRH-II-ir fibers form an axonal layer between the peripheral zone, which contains electrosensory principal cells, and the molecular layer that originates from granule cells in the caudal cerebellum (Krebs and Tricas, 1997). There are also segregated projections to lamina of the tectum, which is known to integrate visual, electrosensory, and mechanosensory information. Other cGnRH-II projections are found to the cerebellum, reticular formation, and spinal cord. In addi-

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tion, we present immunocytochemical evidence for cGnRH-II projections to the hypothalamus, median eminence, neurointermediate lobe of the pituitary, habenula, and telencephalon (Fig. 11).

### *Possible Functions of GnRH Variants in the Stingray Brain*

The pathway by which GnRH reaches the gonadotropic cells of the pituitary is still enigmatic. The elasmobranch pituitary is unique among vertebrates in that it is subdivided into the neurointermediate, median, rostral, and ventral lobes. Early experiments on the spotted dogfish, *S. canicula,* showed that the ventral lobe is the primary gonadotropic region in the pituitary, whereas the neurointermediate lobe shows weaker gonadotropic effects on steroidogenesis (Sumpter *et al.,* 1978). Unlike other vertebrates, there is not a well-defined vascular or neural pathway between the hypothalamus and the primary gonadotropic region of the pituitary (see Dodd, 1983 for review). Our immunocytochemical experiments using both anti-dfGnRH and anti-cGnRH-II show fiber projections to the rostral, median, and neurointermediate (but not ventral) lobes. While some lobes may be regulated by direct GnRH neural pathways, the lack of GnRH innervation to the ventral lobe is consistent with some other transport method.

Other possible pathways of GnRH transport to the pituitary include the brain circulation and the cerebrospinal fluid (CSF). Transport of GnRH from the TN to the ventral lobe via the cerebral circulation (Demski *et al.,* 1987; Wright and Demski, 1993) is supported by observations of dense-cored vesicles in the TN that are closely associated with endothelial cells of blood vessels (Demski and Fields, 1988). However, electron microscopic identification of dense-cored vesicles must be coupled with immunocytochemistry to verify the presence of GnRH in these areas of vesicular activity. Stimulation of the peripheral TN trunk in *D. sabina* increased GnRH levels in the CSF of the fourth ventricle (Moeller and Meredith, 1998) and supports the idea that GnRH could be released directly from the TN into the CSF. However, the exact GnRH variants in the cerebral circulation or CSF were not determined, and the natural stimulus that would cause TN excitation in the stingray remains unknown. In addition, any possible action upon the pituitary by GnRH via these pathways remains to be demonstrated.

The temporal expression of reproductive peptides in the brain must also be considered when attempting to determine the function of GnRH nuclei. Variation in GnRH cell body size, number, and expression that are related to reproduction and life history stages are known for teleost fishes (e.g., Sherwood *et al.,* 1993; Francis *et al.,* 1993; Grober *et al.,* 1994). In adult male *D. sabina,* GnRH-ir somata in the basal TEL and POA showed the greatest variation in cell number compared to other GnRH-ir cell groups. Animals collected in the late summer (late July) and during the peak mating period (February) showed up to 50 GnRH-ir POA cells, whereas stingrays collected in the nonmating season (April–June) showed no more than 5 GnRH-ir POA somata (Tricas *et al.,* unpublished data). No changes in the number of GnRH-ir cells were observed for the terminal nerve or tegmental GnRH cell groups. The reproductive season for *D. sabina* begins with sperm production (Maruska *et al.,* 1996) and a concurrent increase in serum androgen production in August (Tricas *et al.,* 2000). It is therefore likely that the POA dfGnRH cells are involved with gonadotropin release to stimulate gonadal recrudescence. Based upon relatively few GnRH-ir somata in the POA, Demski (1989) suggested that the elasmobranch reproductive cycle is controlled by GnRH produced in the TN which reaches the ventral lobe of the pituitary via the blood circulation. An alternative explanation for the relatively low numbers of GnRH-ir POA cells is that the reproductive cycle is controlled by the periodic expression of GnRH. Further research is needed to define seasonal patterns of GnRH expression in elasmobranch fishes.

The TN in elasmobranchs may serve to integrate environmental cues with reproductive behaviors (Demski, 1989). Our immunocytochemical and HPLC/RIA results show multiple GnRH variants in the TN. Previous electrophysiological evidence also shows multiple cell types in the elasmobranch WB, and some cells may receive different inputs from peripheral or central pathways (White and Meredith, 1987). Thus, the elasmobranch terminal nerve system appears to serve a complex function. The reproductive season for *D. sabina* begins in late August when male gonads begin to enlarge and female oocytes begin vitellogenesis. Male rays aggressively court and mate

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with multiple females for the following 7 months until females ovulate near the end of March (Maruska *et al.,* 1996; Kajiura *et al.,* 2000). Different GnRH forms within the TN ganglia may act through efferent and afferent pathways and coordinate reproductive processes at physiological and behavioral levels. For instance, gonad recrudescence in many fishes is known to be under photoperiod and temperature control, and similar associations are seen in *D. sabina* (Tricas *et al.,* 2000). Such environmental cues may activate gonadotropin release in the stingray pituitary directly via TN afferents that stimulate gonadotroph cells or indirectly by TN afferents that stimulate GnRH production in the POA. The subsequent increase in androgen levels probably promotes the aggressive biting and chasing in the reproductive behaviors of *D. sabina* and may stimulate the protracted 7- to 8-month preovulatory mating in this species (Maruska *et al.,* 1996; Kajiura and Tricas, 1996; Tricas *et al.,* 2000). The location of GnRH-ir somata in G3 at the level of the olfactory bulb is consistent with possible modulation of olfactory sensitivity to pheromones during mating. In addition, Demski *et al.* (1997) described efferent projections of putative TN GnRH-ir fibers to the retina of the skate and suggested a neuromodulatory action on retina cells. Therefore, the various TN and forebrain GnRH systems in the stingray most likely activate gametogenesis and steroidogenesis and integrate sensory systems with reproductive behaviors.

Results from the present study indicate that dfGnRH, lGnRH-III-like, and cGnRH-II-ir neurons can be distinguished immunocytochemically, spatially, and morphologically in the elasmobranch brain (Table 3). Several GnRH-ir cell populations in derived teleosts can be distinguished on the basis of cell body shape, size, location, immunoreactivity, and gene expression (White *et al.,* 1995; Yamamoto *et al.,* 1995). These similarities were also found among stingray POA, WB, and G2 GnRH-ir cells and were distinct from those found in the midbrain. This observation is consistent with the proposal that the septo–preoptic and TN cells represent a single GnRH system (Muske, 1993). Septo–preoptic and TN GnRH neurons have a common embryonic origin in the olfactory placode in some mammals (Schwanzel-Fukuda and Pfaff, 1991), birds (Murakami *et al.,* 1991), and amphibians and project to the same brain areas, supporting the anatomical evidence of a single system (Muske, 1993). Cell bodies of the elasmobranch septal regions vary in their organization and location among species and are currently undefined in *D. sabina.* Thus, we were unable to resolve the specific neuroanatomical locations of GnRH-ir cell bodies in this region of the forebrain. However, the neuroanatomical location of GnRH-ir cells within regions associated with the limbic system indicates a possible role in the integration of olfactory information with sexual behaviors.

In contrast to other vertebrates that have a distinct cGnRH-II nucleus in the tegmentum, we have demonstrated that, in the stingray, cGnRH-II cells are distributed as a large column of cells that extends from the oculomotor nucleus through part of the interpeduncular nucleus to the posterior commissure. Wright and Demski (1991) reported tegmental cell GnRH-ir fibers in the fasciculus retroflexus that connects the habenula with the interpeduncular nucleus. The interpeduncular nucleus in vertebrates is thought to integrate limbic olfactory input with sensory information and to receive information from the habenula (see Butler and Hodos, 1996). Thus, one function of the cGnRH-II tegmental cells may be modulation of the epithalamus, which regulates circadian rhythms in response to light cycles. Clearly, more anatomical and neurophysiological experiments are needed.

Muske (1993) proposed that the ancestral vertebrate GnRH condition is a large widespread posterior system which could serve all basic GnRH functions, such as regulation of the brain–pituitary gonadal axis, integration of sensory cues, and synchronization of reproductive behaviors. The cGnRH-II neuronal system in the stingray projects to many sensory processing and integrative centers, which include the electrosensory DON and mechanosensory MON regions of the hindbrain. The presence of GnRH-ir fibers in this area is consistent with a sensory neuromodulator function for cGnRH-II. Tricas *et al.* (1995) demonstrated that the male round stingray, *U. halleri,* employs its electrosense to locate buried females during the mating season. Thus, the projection of GnRH-ir fibers into the primary electrosensory processing center (DON) in *D. sabina* may alter the sensitivity of the system during the mating season. Similarly, Rosen *et al.* (1997) report cGnRH-II projections in hindbrain sensory processing regions in the green anole, *Anolis carolinensis,* which may function to facilitate courtship behavior. Also, cGnRH-II functions as a coneurotransmitter via a spe-

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cific cGnRH-II receptor in amphibian sympathetic ganglia (Troskie *et al.,* 1997). The presence of cGnRH-II fibers and terminals near motor neurons in the spinal cord of the elasmobranch (Wright and Demski, 1991) and amphibian (Chartrel *et al.,* 1998) indicates that the cGnRH-II variant may influence locomotor information associated with reproductive behavior. The evidence across vertebrate taxa is consistent with the hypothesis that cGnRH-II functions as a neuromodulator or neurotransmitter due to its anatomical location and projections that are distinct from brain centers known to control gametogenesis and steroidogenesis.

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