Histol Histopath (1993) 8: 133-138

Histology and Histopathology

Gonadotrophin-releasing hormone immunoreactivity in the brain of the tropical freshwater fish, *Pygocentrus notatus* (Teleostei-Characidae)

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Summary. The distribution of GnRH in the brain of the teleost *Pygocentrus notatus* was demonstrated with the avidin-biotin peroxidase immunocytochemical method using highly specific antibody against synthetic mammalian GnRH. Optimal immunoreaction was obtained using: 1) Bouin's fluid for fixation; 2) repeated incubation with primary antiserum; 3) the use of a detergent in the dilution buffer; 4) the high sensitivity of the avidin-biotin immunoperoxidase method with the cobalt intensification of 3-3'diaminobenzidine tetrahydrochloride; and 5) the use of primary antibody with high specificity. GnRH-immunoreactive (GnRH-ir) in cells and/or axons was observed in all main brain regions. In the forebrain, GnRH-ir was located in a network extending from the caudal part of the olfactory bulb to the telencephalon. GnRH-ir fibres were also observed in the optic tectum, cerebellum and hypothalamus. Two groups of neuronal cell bodies were identified. One group was located in the antero-ventral telencephalon corresponding to the nucleus olfacto-retinalis. The second group was found in the rostrodorsal hypothalamus. No GnRH-ir material was detected in the pituitary gland, thus confirming the results of previous studies on brain GnRH-ir distribution obtained by radioimmunoanalysis in this species. These results demonstrate a high degree of similarity between the GnRH systems of *P. notatus* and other teleost species.

Key words: Hypothalamus, GnRH Immunoreactivity, Brain Teleostei, Immunocytochemistry, Telencephalon

Introduction

In recent years there has been a growing interest in the distribution of gonadotrophin-releasing hormones (GnRH) in the brain of teleost fish. The mapping of this system in teleosts, however, has been restricted in temperate species (rainbow trout, *Salmo gairdneri*: Goos and Murathanoglu, 1977; carp, *Cyprinus carpio*: Nozaki and Kobayashi, 1979; Pan et al., 1979; platyfish, *Xiphophorus maculatus*: Schreibman et al., 1979, 1982; Münz et al., 1981, 1982; Halpern-Sebold and Schereibman, 1983; three-spined stickle-back, *Gastorosteus aculeatus*: Borg et al., 1982; goldfish, *Carassius auratus*: Kah et al., 1984, 1986; Stell et al., 1984; eel, *Anguilla rostrata*: Grober et al., 1987), so far there have been few reports (Goos et al., 1985) on the distribution of GnRH in the brain of tropical fishes. In spite of species variations, a general pattern of GnRH immunoreactivity can be recognized. However, more studies on teleosts from different taxonomic groups would be necessary before a more comprehensible picture can be completed.

In previous studies, using radioimmunoassay, we have shown the presence of GnRH in brain extracts of *Pygocentrus notatus* (Gentile et al., 1986; Marcano et al., 1989). This fish is a seasonal breeder widely distributed in rivers and lagoons of the Venezuelan plains. Under natural conditions, it shows a pattern of gonadal maturation starting a few months before the beginning of the rainy season, thus the maximal gonadosomatic index is achieved during the dry season (Gentile, 1983; Cáceres-Dittmar, 1989). This reproductive cycle behaviour pattern is shared by many species of the Characidae family of great interest in aquaculture.

Many investigations have used antibodies against mammalian Gonadotrophin hormone-releasing hormone (mGnRH) to demonstrate its presence in the brain of several teleost species (for reviews see Peter, 1983; Demski, 1984; Goos et al., 1985). In the present study, using an immunoperoxidase method, we provide a more complete assessment of the anatomical distribution of GnRH immunoreactivity (GnRH-ir) perikarya and nerve fibres in the brain and pituitary of *P. notatus*.

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Materials and methods

Experimental animals

Adult male and female *P. notatus* (standard length ≥ 15 cm) were collected from lagoons and ponds of the Guarico river and its tributaries (Guarico and Apure States) and transported in aerated tanks to the laboratory. The animals were captured at the end of the dry season, which coincides with high gonadosomatic index and the increase of GnRH in most brain regions (Gentile et al., 1986). The sex of the fish was determined by visual inspection of the gonads.

Immunocytochemistry

Several protocols were tried out to achieve material preservation of GnRH-ir. The protocol given below was the one found to give an optimal tissue preservation, cutting qualities and preservation of antigenicity. Eleven brains were fixed in saturated picric acid aqueous solution and 40% paraformaldehyde (3:1) for 5 hours (fixation with 4% paraformaldehyde either by perfusion (4 brains) or immersion (3 brains) gave suboptimal results). After fixation, the brains with the pituitaries attached were embedded in paraffin, and sectioned on a microtome at 10 µm. The sections were collected on poly-L-lysine-coated slides, de-paraffinized in two changes of xylene and rehydrated in Phosphate Buffer Saline (PBS) 0.1M, pH 7.2. Immunostaining was performed at room temperature using a modified avidin-biotin immunoperoxidase technique (Tapia et al., 1988).

The sections were incubated for 30 min in normal goat serum (NGS 1:30), then incubated for 3 h with rabbit anti-GnRH (antibody 635-5 kindly provided by L. Jennes, Ohio State University, USA) raised against synthetic mGnRH (Jennes and Stumpf, 1983) and diluted 1:5000 in PBS containing 0.1% Triton X-100. Antibody was replaced hourly (Gu et al., 1983). After a 5 min rinse in PBS, the sections were incubated for 1 h in biotinylated donkey-anti-rabbit IgG (BDAR, Amersham, U.K.) diluted 1:100 in PBS (final concentration, 50 µg/ml). Following a 5 min rinse in PBS, the sections were incubated for 30 min in avidin-biotin complex (ABC) (Vector Laboratories) diluted 1:100 in PBS and rinsed for 5 min in PBS. The sections then were treated for 10 min with a solution containing 90 µM of hydrogen peroxide, 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, U.S.A.) at a final concentration of 1.40 mM and 0.84 mM cobalt chloride in PBS, or alternatively 3-amino-9 ethylcarbazone (final concentration 0.88 mM) dissolved in 50 mM N,N-dimethylformamide in 0.1M acetate buffer, pH 5.2. The sections were thoroughly rinsed in tap water, counterstained with eosin on Meyer's haematoxylin, and mounted in DPX or glycerin-gelatin.

The specificity of the immunostaining technique used was tested by incubating the sections in the presence of decreasing concentrations of the primary antiserum, which resulted in a gradual decrease and an eventual disappearance of the staining. Other control experiments consisted of: 1) pre-absorption of GnRH antiserum with synthetic mGnRH (10 µg/ml) for 24 hours at 4 °C; 2) omission of single step in the immunocytochemical procedure; 3) replacement of the primary antiserum by normal rabbit serum or a non-related antiserum.

Results

General observations

The major anatomical features of the brain of

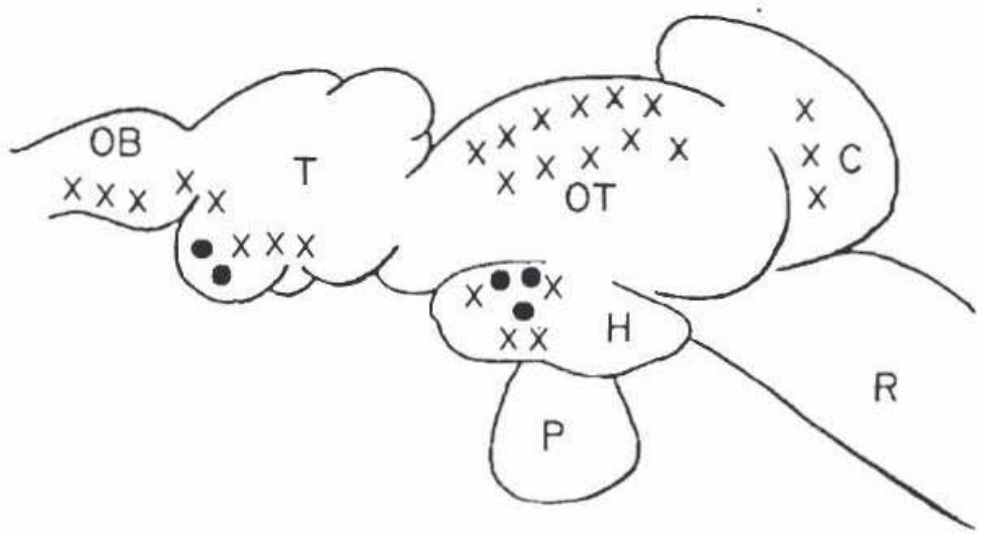

Fig. 1. Diagrammatic representation of the saggital section through the brain of the freshwater fish *Pygocentrus notatus*, showing the distribution of GnRH-immunoreactive cell (•) and fibres (x). OB, olfactory bulb; T, telencephalon; OT, optic tectum; H, hypothalamus; C, cerebellum; R, rhombencephalon; and P, pituitary gland.

Pygocentrus notatus are shown in Fig. 1. Note that the olfactory bulbs (OB) did not seem to be interconnected by peduncles or olfactory tracts like in other teleosts. The optic tectum (OT) was relatively large, and the torus semicircularis (tsc) was well developed (Fig. 2). The cerebellum was small in *P. notatus* and extended rostrally over the caudal part of the OT. Figure 1 also shows the relatively large size of the pituitary gland.

After incubation of the tissue sections with GnRH antiserum, distinctly stained neuronal somata and axons were observed in most parts of the brain. Some regions were densely innervated by GnRH-ir axons (Fig. 1).

The density of immunoreactive fibres, as well as the intensity of the immunoreaction, was strongly dependent on the treatment of the tissue. To obtain optimal immunoreaction, it was imperative to use 1) Bouin's fluid for fixation, 2) repeated incubation with primary antiserum, 3) a detergent in the dilution buffer, 4) the high sensitivity of the avidin-biotin immunoperoxidase method with the cobalt intensification of the DAB, and 5) primary antibody with high specificity.

Telencephalon and olfactory bulbs

A dense network of positive fibres was observed extending caudally from the ventral parts of the olfactory bulb to the antero-ventral telencephalon (Fig. 1). In both regions abundant but disperse GnRH-ir fibres were also present (Fig. 3). In the antero-ventral telencephalon one group of GnRH-ir was located in an area corresponding

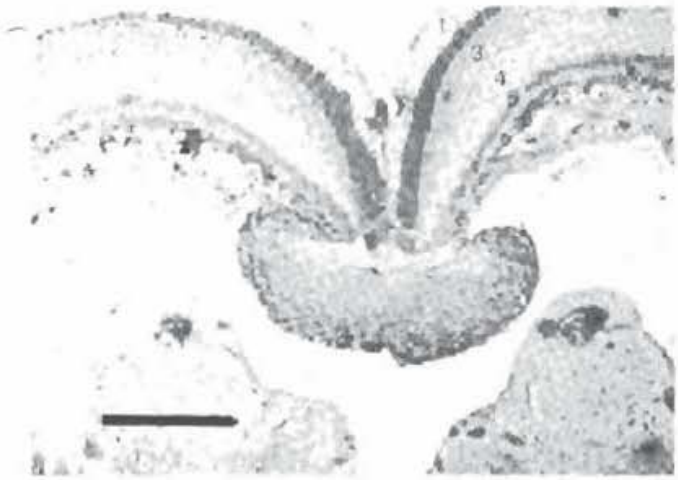

Fig. 2. Immunoreactive mGnRH fibres in the optic tectum of *Pygocentrus notatus*. Cross-section. 1. Stratum marginale, 2. Stratum opticum, 3. Stratum fibrosum et griseum superficiale, 4. Stratum griseum centrale, 5. Stratum album centrale, 6. Stratum periventriculare. Avidin-Biotin immunoperoxidase technique. Bar: 150 µm

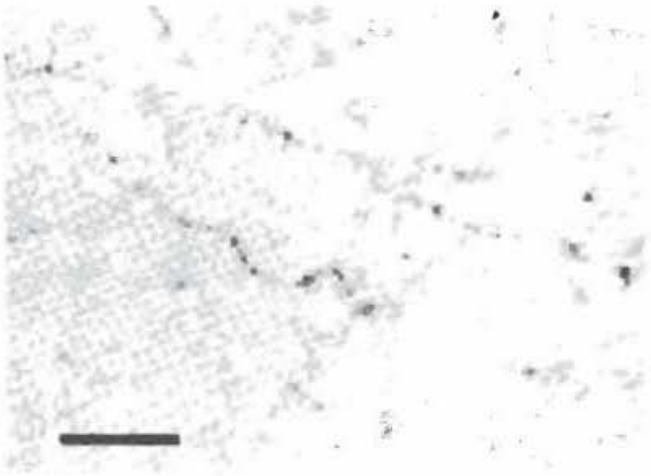

Fig. 3. mGnRH-immunoreactive fibres in the telencephalon. Longitudinal section. Avidin-biotin immunoperoxidase technique. Bar: 10 µm

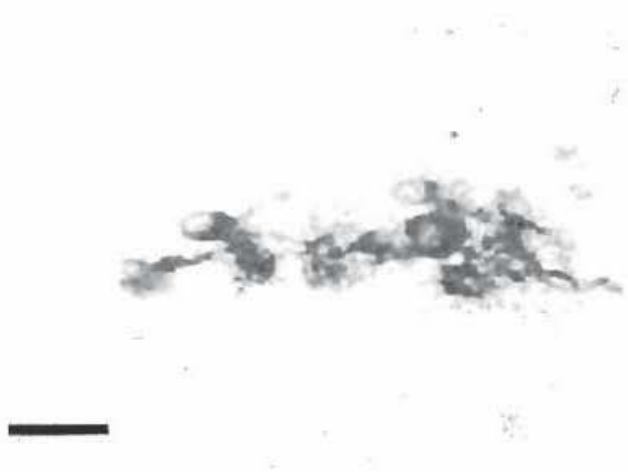

Fig. 4. GnRH immunoreactive cell bodies in the rostroventral area of the telencephalon. Avidin-biotin immunoperoxidase technique. Bar: 5 µm

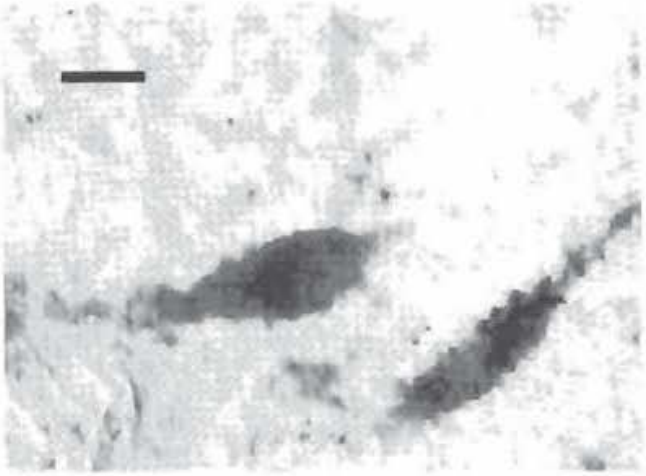

Fig. 5. GnRH immunoreactive cell bodies in the hypothalamus. Avidin-biotin immunoperoxidase technique. Bar: 5 µm

to the *nucleus olfactoretinalis* (NOR). These neurons showed a tendency to be spherical in shape (Fig. 4).

Optic tectum and Cerebellum

GnRH-ir axons were observed in only three layers of the tectum opticum (Fig. 2). The highest densities were associated with the *stratum fibrosum et griseum superficialis* and the *stratum album centrale*. Moderate numbers of axons were observed in the *stratum griseum centrale*. A dense innervation of GnRH-ir axons was observed in the central region of the cerebellum.

Hypothalamus and Pituitary gland

Numerous GnRH-ir fibres were observed in most parts of the hypothalamus (Fig. 1). The highest densities were observed in the anterior hypothalamus. Intensely immunoreactive neurons were identified around the preoptic area. The neurons seemed to be bipolar (Fig. 5), elongated, and oriented along the rostrocaudal axis of the brain. In the present study, no GnRH-immunoreactivity was observed in the pituitary gland.

Discussion

The present results indicate a similar pattern of distribution of GnRH to that seen in other teleosts. The immunocytochemical characterization of GnRH showed abundant immunoreactivity in the telencephalon and hypothalamus of *P. notatus*. This immunoreactivity was mainly located in fibres along the brain. Immunostaining of perikarya was limited to two neuronal cell groups, one corresponding anatomically to the NOR and the second group located in the anterodorsal hypothalamus.

The presence of a GnRH-containing group of cells related to both olfactory and visual systems was first reported in the platyfish by Münz et al. (1981, 1982). According to these authors, such a system is present only in teleost species having olfactory bulbs closely attached to the telencephalon (Münz et al., 1982). *Pygocentrus notatus* has nonpedunculated olfactory bulbs and the group of GnRH-ir cells located at the junction between the olfactory bulbs and the telencephalon may correspond to the NOR, as was also found in the platyfish, (Münz et al., 1981, 1982), the guppy, *Poecilia reticulata* (Zentel et al., 1987), and the sole, *Solea solea* (Núñez-Rodríguez et al., 1985). Breton et al. (1986) described GnRH perikarya in the olfactory tract and bulbs, the ventral telencephalon, the preoptic region and the basal hypothalamus of the brown trout, *Salmo trutta*. In the rainbow trout, *Salmo gairdneri*, GnRH perikarya was localized exclusively in the nucleus preopticus (Schäfer et al., 1989). The evidence indicates that the NOR interconnects the optic and olfactory systems both anatomically and functionally (Schreibman and Margolis-Nunno, 1987).

High densities of GnRH-ir fibres are present in some layers of the optic tectum. This brain structure is highly developed in *P. notatus*. It is well known that the size of tectum varies in different taxonomic groups (Nieuwenhuys, 1982) and is relatively very large in highly visual teleost like *Salmo*. This may be related to the development of brain areas with visual input. In most teleosts the tectum opticum does not only represent the largest but also the most highly differentiated centre of the brain. The presence of GnRH-ir in the tectum opticum has been shown in other teleost species (Subhedar and Krishna, 1988). Further experimental work is required to clarify the functional significance of the presence of GnRH-ir in the tectum opticum.

The presence of cell bodies in the preoptic area has been reported in the goldfish using both m-GnRH (Kah et al., 1984) and salmon GnRH (Kah et al., 1986). Moreover, immunoreactive cell bodies have also been observed in the African catfish nucleus preopticus. Our observations confirm these data.

The present immunocytochemical analysis did not demonstrate GnRH-ir material in the pituitary. However, GnRH-ir has been reported in the pituitary gland of different teleost species (Dubois et al., 1979; Goos et al., 1985; Subhedar and Krishna, 1988). Recently, Schäfer et al. (1989) have shown that the bulk of GnRH-positive material is found in the neural intermediate lobule, whereas the pituitary pars distalis contains much less immunoreactive material. The differences between previous reports on the presence of GnRH-ir in the pituitary gland and our results, is probably related to the existence of a different type of GnRH in this site, as has been shown in the brain of other teleosts (Barnett et al., 1982; Sherwood et al., 1983, 1984, 1989; King and Millar, 1985; Powell et al., 1986) or perhaps may be due to methodological variations.

It has been shown that the hypothalamic and extrahypothalamic brain regions containing GnRH-ir are involved in the control of reproduction in teleosts (Ball 1981; Peter, 1983, Demski, 1984). Furthermore, areas with visual input, such as the optic tectum, as well as areas related to olfactory and other chemosensory functions contain GnRH-ir fibres.

Although this study does not show an overlapping in the distribution of GnRH and dopamine, it would be interesting to relate this finding to the distribution of dopamine in the same species (Guerrero et al., 1990). The dopamine content in the brain of *P. notatus* coincides with the localization of GnRH. The areas where this coincidence occurs include the hypothalamus and the telencephalon. Such observations further substantiate the importance of interactions between the neural circuits for the two systems involved in regulation of reproductive activity and behaviour, which may also involve dopamine control of GnRH release.

In conclusion, the present results and those observed in other teleosts demonstrate a high degree of similarity between the GnRH systems in teleost fish. In addition, they provide information concerning the organization of GnRH systems in the *Pygocentrus notatus* brain. Finally, this work supports the earlier studies on the distribution

of GnRH by radioimmunoassay in the brain of *P. notatus* (Gentile et al., 1986; Marcano et al., 1989).

Acknowledgements. We are grateful to Dr. L. Jennes for providing the GnRH antibody, Dr. Neil Lynch for revision of the manuscript, Miss Dilia Hernández for excellent typing services and Mr Alfonso Sierra for the drawings. We also thank MSc. Otto Castillo, MSc. Miguel Fontaine and other members of FONAIAP and Miss Cruz Delina Cáceres for their valuable help in the collection of *P. notatus*. This work was supported by grants from CONICIT S1-1623 and CDCH 10-11-2357-90.

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Accepted September 2, 1992

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