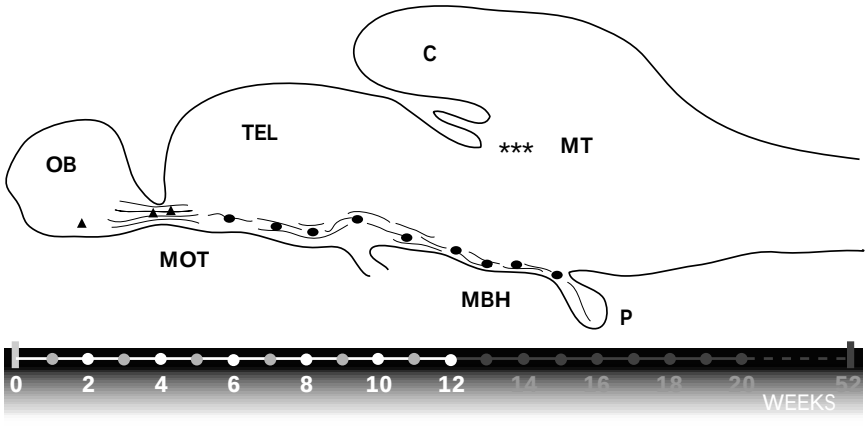
Chapter 2

Two different GnRH forms within three distinct GnRH neuron populations in the brain of the African catfish (*Clarias gariepinus*): early development of the GnRH system

Eline A.Dubois, Matthijs A.Zandbergen, Jan Peute, Henk J.Th.Goos.

J. Comp. Neurol. (submitted)



Two different GnRH forms within three distinct GnRH neuron populations in the brain of the African catfish *(Clarias gariepinus)*: early development of the GnRH system

Abstract

The early development of both the catfish gonadotropin-releasing hormone (cfGnRH)- and the chickenGnRH-II (cGnRH-II)-system was investigated in African catfish by immunocytochemistry using antibodies against the GnRH associated peptide (GAP) of the respective preprohormones. Weakly cfGAP-immunoreactive (ir) neurons and fibers were present at 2 weeks post hatching (ph), but only in the ventral telencephalon and pituitary. Two weeks later, cfGnRH fibers and neurons were also observed in more caudal and in more rostral brain areas, i.e. in olfactory bulb, olfactory tract, ventral telencephalon, dorsal to the optic chiasm, ventral hypothalamus and pituitary. At 10-12 weeks ph, i.e. when puberty starts, the immunocytochemical cfGnRH pattern in the brain was comparable with that in adults. Based on differences in temporal and spatial appearance and morphology, two distinct cfGnRH populations were identified in the ventral forebrain: a population innervating the pituitary (ventral forebrain system) and a so-called terminal nerve (TN) population. DiI tracing studies revealed that the TN population has no connections with the pituitary. The cGnRH-II system is present from 2 weeks post hatching onwards. The cGnRH-II neurons are localized in the midbrain tegmentum and only their size and staining intensity increase during development. Comparison of GnRH systems amongst vertebrates may be the basis to propose a hypothesis on the evolution and the function of the different molecular forms of GnRH and of the neurons that produce these peptides. We hypothesize, that during fish evolution the cGnRH-II system evolved as a separate system in the midbrain and that salmon GnRH became evident in the TN population. African catfish is a less advanced teleost species with cfGnRH as forms for both the ventral forebrain system and the TN population.

Introduction

Gonadotropin-releasing hormone (GnRH) is the main regulator of

gonadotropin release from the pituitary. Generally two or three different forms of GnRH are present in the brain of a given species, distributed over specific brain areas (Amano et al. 1997). The species-specific GnRH is localized in the ventral forebrain (mainly in the pre-optic area (POA) and hypothalamus), the conserved cGnRH-II neurons are concentrated in the midbrain tegmentum; the third form, if present, is localized in the terminal nerve (TN; Fernald and White 1999).

The origin and embryonic development of the ventral forebrain GnRH system in vertebrates has been and still is a main point of interest. GnRH neurons that innervate the pituitary arise from the olfactory placode and migrate into the brain during embryonic development (reviewed by Sherwood et al. 1997; Schwanzel-Fukuda 1997). In the newt, ablation of the olfactory placode early in development resulted in the absence of the GnRH neurons in the brain (Murakami et al. 1992). In mammals, the migration of these neurons was studied in the mouse (Schwanzel-Fukuda and Pfaff 1989), rat (Jennes 1989) and rhesus macaque (Ronnekleiv and Resko 1990). Without exception, these studies demonstrated the earliest GnRH-immunoreactivity in the olfactory region. Later during gestation, GnRH immunoreactivity is found in more caudal brain areas and finally in the caudal hypothalamus. Also in teleosts the ontogeny and migration of the ventral forebrain GnRH neurons were thoroughly investigated (Halpern-Sebold and Schreibman 1983; Chiba et al. 1994; Parhar et al. 1995a; Feist and Schreck 1996; Parhar 1998; Amano et al. 1998). The first GnRH neurons were also shown to be present in the olfactory region, while later in development the neurons appeared in more caudal regions (ventral telencephalon, POA, medial basal hypothalamus (MBH), pituitary).

The origin of the cGnRH-II containing neurons in the midbrain is less clear. Ontogenetic studies showed that these cells are present in the midbrain very early in development (Amano et al. 1998; Parhar et al. 1998; White and Fernald 1998). There is no evidence for a migration of these neurons; at least they do not originate from the olfactory placode (Northcutt and Muske 1994). The germinal zone near the third ventricle (White and Fernald 1998) or the ventricular ependyma (Parhar 1999) are mentioned as possible sites of origin.

The discovery of a third GnRH form in the terminal nerve (TN) of several modern teleosts (Powell et al. 1994; White et al. 1995; Parhar 1998; Montaner et al. 2000), reactivated the discussion as to the origin of the different GnRH type neurons. Parhar (1999) hypothesized that the GnRH neurons in the POA and hypothalamus would derive from the basal diencephalon directly, whereas the TN neurons originate from the olfactory placode.

In a species like the red seabream (*Pagrus major*), the midbrain GnRH neurons express cGnRH-II and the TN neurons contain salmonGnRH (sGnRH), while the species specific seabreamGnRH (sbGnRH) is found in the

POA GnRH neurons (Okuzawa et al. 1997). In the African catfish, only two forms of GnRH are found (Bogerd et al. 1994): the species specific catfishGnRH (cfGnRH) and cGnRH-II. Using the highly specific antibodies against the respective GnRH associated peptides (GAPs; Zandbergen et al. 1995), in adult catfish cfGnRH neurons were found along the entire ventral forebrain, including the olfactory bulb and MOT, while cGnRH-II, as in all other vertebrates studied to date, was localized in the midbrain tegmentum.

There is no information yet as whether the two GnRH forms identified in the African catfish represent also two populations of GnRH neurons. This question was approached in the present study by monitoring the early development of the cfGnRH and cGnRH-II system. Additional DiI tracing studies were performed in order to establish the possible connection between resp. cf- and cGnRH-II neuron populations and the pituitary. As a consequence of the results obtained in this study, we propose a scheme for the evolution of GnRH forms and their specific localization throughout the Fishes.

Materials and methods

Animals

African catfish were bred and raised in the hatchery of the research group of Comparative Endocrinology, University of Utrecht. The fish were kept in copper-free recirculating water of 26°C and fed ad libitum with *Artemia* for the first week and thereafter with food pellets (Trouvit, Putten, the Netherlands). Brain and pituitary samples were taken at 1, 2, 4, 6, 8, 10, 12, and 14 weeks post hatching (ph). Around 8 weeks ph, the sex of the fish could be determined and from then on only males were studied. The testes were sampled in order to determine the stage of spermatogenesis. For the DiI tracing study, fish of 7 weeks ph were used.

Immunocytochemistry

Fixation of brain and pituitary tissue and the immunocytochemical procedure were described before (Zandbergen et al. 1995). In brief, after overnight fixation in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4), the tissues were rinsed in graded sucrose solutions and frozen. Sagittal sections were cut in a cryostat and mounted on glass slides.

CfGnRH neurons and fibers were determined by anti-cfGAP antibodies (1:500 diluted) as first antibody, and peroxidase-conjugated anti-rat IgG (1:500, Sigma). CGnRH-II neurons were labeled with anti-cIIGAP antibodies (1:500 diluted), followed by goat-anti-rabbit (Sigma, 1:50 diluted) and rabbit peroxidase-labeled anti-peroxidase (Sigma, 1:100 diluted). The peroxidase was visual-

ized with a 4-chloro-1-naphtol and hydrogen peroxidase solution or with the glucose oxidase-DAB-nickel method (Shu et al. 1988). Pre-immune serum or buffer in stead of the first antibody were used in control reactions.

DiI tracing study

Seven-week-old fish were decapitated and the lower jaw was removed. The brain was ventrally approached by carefully removing the sphenoid bone covering the brain from the olfactory bulb till the spinal cord. A microcrystal of DiI (1-1'-dioctadecyl-3,3,3',3'-tetramethylindocarbicyanin perchlorate, Molecular probes Inc., OR, USA) was implanted with an elongated glass pipette either in the junction of the olfactory bulb and medial olfactory tract (MOT) or in the distal part of the pituitary. The brain was then covered with 2% agar and immersed in 4% paraformaldehyde in 0.1M phosphate buffer for 2 to 3 weeks at 37°C. Then the brains were dissected from the remaining skull bones, embedded in agar and cut in 100 μm sections on a vibratome (Leica, Nussloch, Germany). The sections were mounted on glass slides and coverslipped in glycerol: gelatin (1:1). The sections were viewed under a Leica fluorescence microscope using a rhodamine filter.

Testis histology

The testes of the fish between 8 and 14 weeks ph were fixed in Bouin. After dehydration in graded ethanol, the testes were embedded in paraffin. Sections of 7 μm were mounted on slides and stained with haemalum-eosin. The stages of spermatogenesis were classified after Schulz et al. (1994): stage I, spermatogonia; stage II, spermatogonia and spermatocytes; stage III, spermatogonia, spermatocytes and spermatids; stage IV, all germ cells including spermatozoa. The onset of puberty is marked by the transition of spermatogonia (stage I) to spermatocytes (stage II).

Results

Immunocytochemistry

Both cfGAP- and cIIGAP-immunoreactivity were detected in catfish brains from 2 weeks ph onwards (Table 1). At the age of 2 weeks incidentally small cfGnRH neurons (Fig. 1A) and short cfGnRH fibers were observed in the Vv. Occasionally, a weak cfGnRH fiber was stained in the developing pituitary. No immunoreactivity was found in the olfactory region. At 4 and 6 weeks ph, the intensity, number and size of the cfGnRH neurons and fibers had increased. Moreover, the cfGnRH fibers and neurons were now distributed over a larger area: they were present in the nucleus preopticus periventricularis (NPP) and

Table I Summary of the spatial and temporal expression pattern of cfGnRH and cGnRH-II in the African catfish, as compared with the adult situation (indicated as +++). The absence of GnRH-ir is marked by (-); the first appearance is indicated by (+); more developed immunoreactive neurons and fibers are marked by (++) and a fully developed area with neurons and fibers similar to the adult situation is indicated by (+++). The cGnRH-II system has no ir-fibers. N.D.: not determined.

age	TN population cfGnRH		ventral forebrain population cfGnRH					midbrain population cGnRH-II
(weeks)	OB	MOT	Vv	NPP	NAP	MBH	pituitary	midbrain
1	-	-	-	-	-	-	-	-
2	-	-	+	-	-	-	+	++
4>6	+	+	++	+	+	+	+	+++
8	++	++	+++	++	++	++	+	+++
10>12	n.d.	+++	+++	+++	+++	+++	+	+++
14	n.d.	+++	+++	+++	+++	+++	++	+++
adult	+++	+++	+++	+++	+++	+++	+++	+++

nucleus anterioris periventricularis (NAP), and in a lesser extent in the MBH. Now, also weakly stained small cfGnRH neurons were present in the olfactory bulb (OB) and in the MOT. At 6 weeks ph these neurons and fibers were scattered over the ventral forebrain, but still not forming a continuum from anterior till posterior (Table 1, Fig. 1B) as was described for the adult catfish (Zandbergen et al. 1995).

From 8 weeks ph on, the sex of the fish could be determined and then only males were investigated. The testes of these fish contained spermatogonial stem cells and spermatogonia (stage I). At this age, the cfGnRH neurons and fibers became more abundant and the adult pattern of cfGnRH neurons, reaching from the olfactory bulb till pituitary was now established.

Catfish of 10 and 12 weeks ph were still in stage I of spermatogenesis; the 14 week-old fish were in stage II (actual pubertal fish). The distribution of the cfGnRH fibers and neurons was similar (unaltered) in the fish of 10 till 14 weeks ph, but the frequency of fibers had further increased (Fig. 1C, D, F, G). From 10-12 weeks ph onwards the appearance of the cfGnRH system in the brain is comparable to the situation in the adult (Table 1). The penetration of cfGnRH fibers into the PPD (proximal pars distalis) became evident at 12 weeks, followed by a finer branching at 14 weeks, when the fibers came in the vicinity of the gonadotropic cells (Fig. 1H).

The strongly stained cfGnRH neurons in the ventral forebrain are unipolar or bipolar in shape and measure about 16 μm in length and 8 μm in height. The cfGnRH fibers show many varicosities due to locally accumulated protein. The cfGnRH neurons in the OB and MOT display a different morphology, characterized by a smaller size (mean diameter of 7 μm), a round shape and a weaker staining intensity. In the MOT, these neurons are clustered and mostly surrounded by an intensively staining immunoreactive bundle of cfGnRH fibers (Fig. 1E, 4).

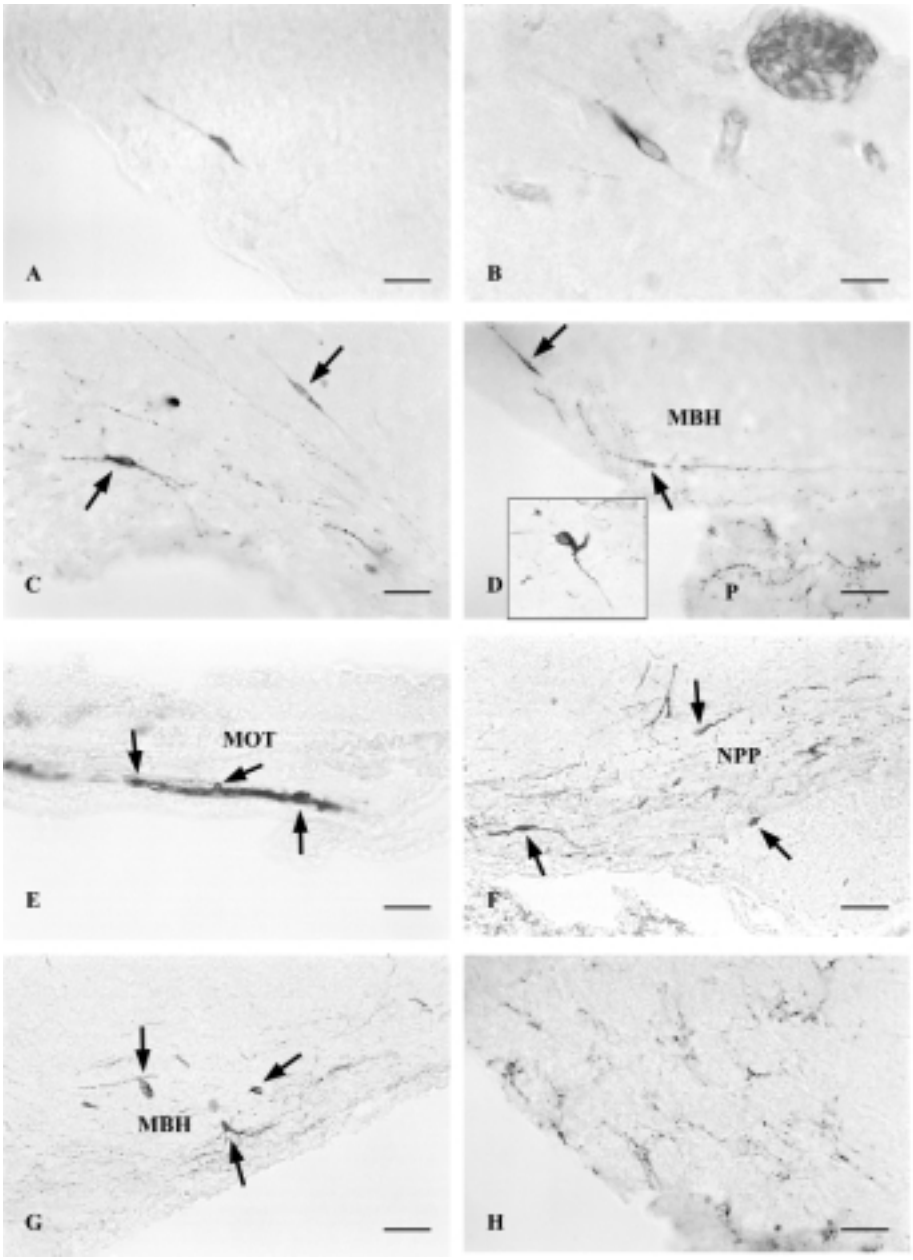


Fig. 1 Early development of the cfGnRH system in the ventral forebrain visualized by anti-cfGAP antibodies. A: cfGAP-ir neurons in the VV of at 2 weeks. B: cfGAP-ir neuron in the NAP of a 6 week-old fish. C: cfGAP-ir neurons and fibers above the chiasm, in the NAP in a 10 week-old fish. D: Overview of MBH and part of the pituitary in a 10 week-old fish. Inset: cfGAP-ir neuron in the MBH. E: cfGAP-ir fiber bundle in the MOT with cfGAP-ir neurons of a 12 week-old fish. F: cfGAP-ir fibers and neurons in the NPP of a 12 week-old fish. G: cfGAP-ir fibers and neurons in the MBH in a fish of 12 weeks. H: cfGAP-ir fiber network in the pituitary of a 14 week-old fish. MOT: medial olfactory tract, MBH: medial basal hypothalamus, NPP: nucleus preopticus periventricularis, P: pituitary. Arrows point at cfGAP-ir neurons. Scale bars: 23 μm (A), 14 μm (B), 36 μm (C, E, H), 59 μm (D, F, G), and 20 μm (inset D).

The cGnRH-II neurons are already present at 2 weeks ph and equal the number found in adult fish (Table 1). During development only their size and ir-staining intensity increase (Fig. 2). The cIIGAP-immunoreactivity was restricted to neurons in the synencephalic area of the midbrain tegmentum (MT); cGnRH-II fibers were not observed in the MT or any other area of the brain, including the pituitary. The cGnRH-II neurons appeared as large cells with an irregular shape (mean diameter between 25-40 μm) and they were often observed in close proximity of blood vessels and near the wall of the third ventricle.

DiI tracing study

By implanting a DiI microcrystal in the caudal pituitary the dye diffused throughout the MBH and more rostrally, it labeled neurons both in the dorso-medial and ventro-lateral nucleus preopticus (NPO, Fig. 3A and B). Near the midline of the brain DiI-labeled fibers passed over the optic chiasm (Fig. 3C), and ended within the Vv (Fig. 3D). In more rostral areas, including the MOT and the OB, no DiI-labeling was observed. Furthermore, the midbrain area including the cGnRH-II cells remained unlabeled.

With the approach from the other side - implantation of DiI in the bulb - the telencephalon (TEL) was heavily labeled (Fig. 3E). In caudal direction fibers labeled with DiI could be followed up to the MBH. The entire pituitary, however, was completely devoid of any DiI labeled fiber (Fig. 3F).

Discussion

In the African catfish, three distinct populations of GnRH neurons were identified in the present study, whereas only two different forms (cfGnRH and cGnRH-II) have been characterized (Bogerd et al. 1994). Chicken GnRH-II ir neurons are localized in the MT, whereas the cfGnRH is distributed over two

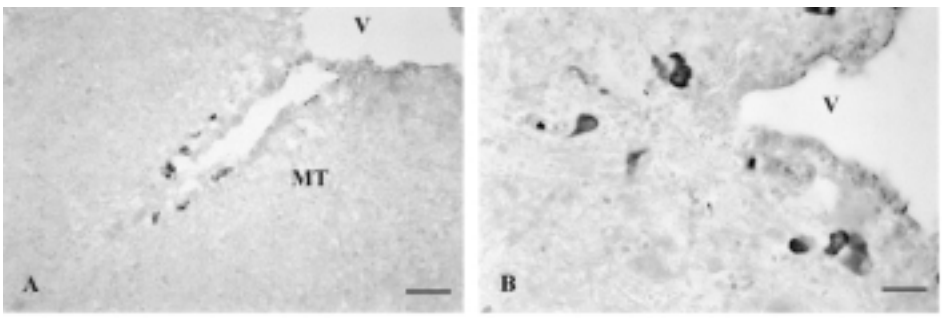


Fig. 2 cIIGAP-ir neurons in the midbrain tegmentum in a 6 week-old fish (A) and a 14 week-old fish (B). V: third ventricle, MT: midbrain tegmentum. The bars represent 71 μm in (A) and 36 μm in (B).

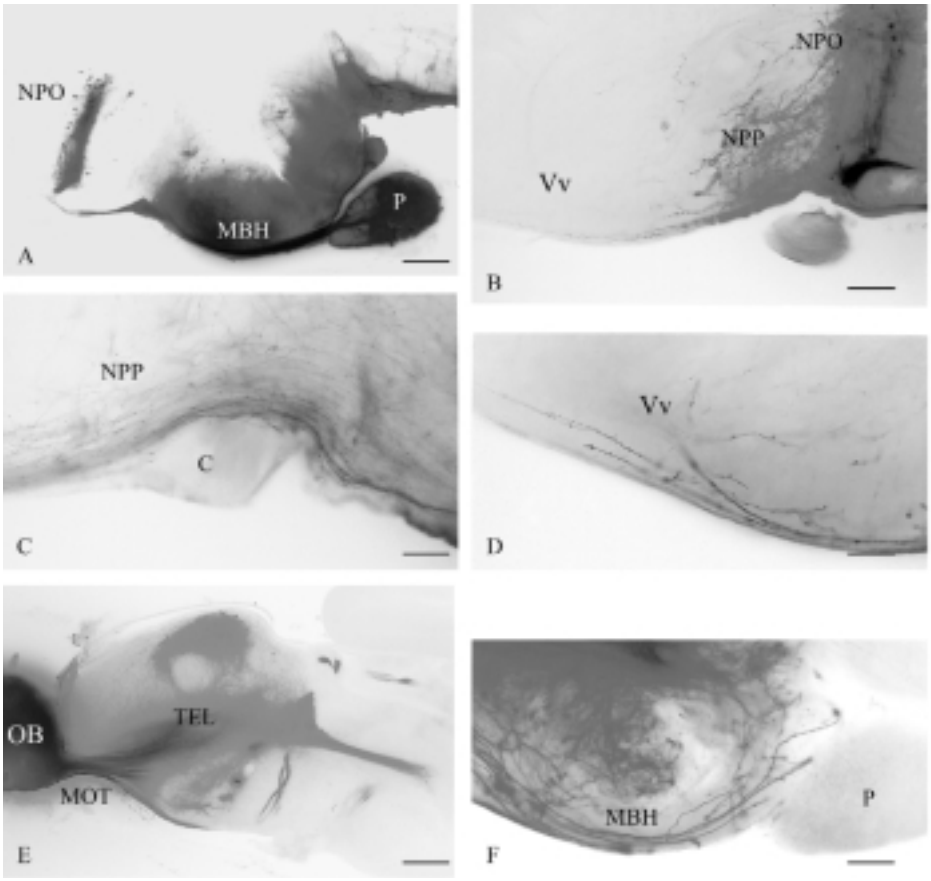


Fig.3 Microphotographs showing brains of 7 week-old catfish 3 weeks after implantation of a DiI microcrystal in the pituitary (A, B, C, and D) or olfactory bulb (E and F). A: Overview of the DiI implantation site (pituitary) and its extensive diffusion in the MBH and NPO. B: Enlargement of the NPO and NPP and small DiI labeled fibers in the Vv. C: A more medial section compared to B showing DiI labeled fibers dorsally from the optic chiasm and within the NPP. D: Small DiI labeled fibers in the Vv. E: Overview of the DiI microcrystal implantation in the OB and its extensive diffusion in the TEL. F: Enlargement of the MBH showing extensive DiI labeled fibers. Note that no DiI labeled fibers are present in the pituitary. Scale bars: 270 μm in A, E; 140 mm in B, F; 70 mm in C, D. C: optic chiasm, MBH: medial basal hypothalamus, MOT: medial olfactory tract, NPO: nucleus preopticus, NPP: nucleus preopticus periventricularis, OB: olfactory bulb, P: pituitary, TEL: telencephalon.

populations of neurosecretory neurons: the TN neuronal population and the GnRH ir neurons in the ventral forebrain, respectively. Based on differences in morphology and timing of first appearance, and on the absence of a direct connection with the pituitary, the TN population is considered to be different from the cfGnRH neurons in the ventral forebrain. The early development of the cfGnRH and cGnRH-II systems in the African catfish is summarized in Table 1 and schematic drawings are shown in Fig. 4.

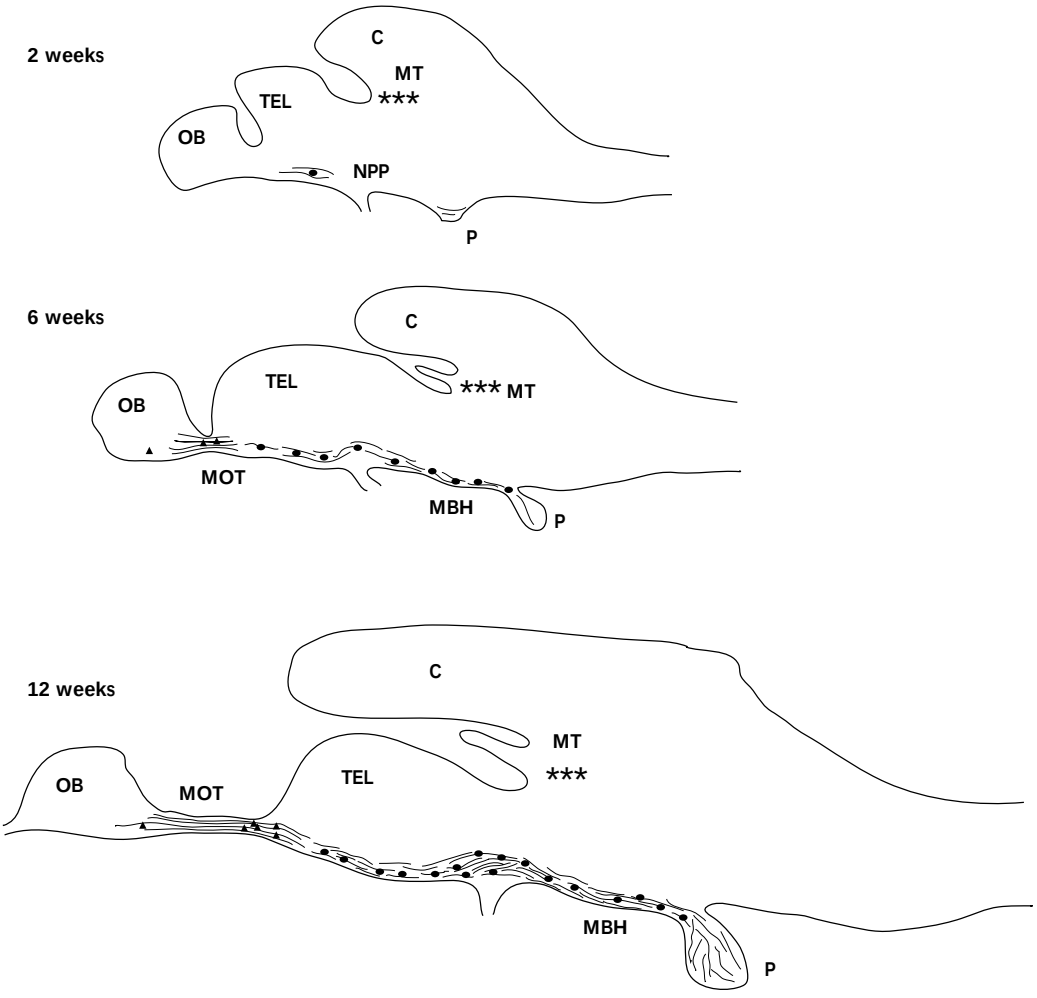


Fig. 4 Schematic representations of the GnRH systems in the African catfish at 2, 6 and 12 weeks ph. cGnRH-II system in the MT (*), cfGnRH system in the TN (▲) and ventral forebrain area (●).

Ventral forebrain population

The first cfGnRH immunoreactivity was observed 2 weeks ph. CatfishGAP-immunoreactivity was then only weakly present in the ventral telencephalon (Vv) and in the pituitary. Two to four weeks later cfGAP-immunoreactivity could be detected in all areas that have been described earlier for adult catfish, including the TN (Zandbergen et al. 1995). The intensity of the immunoreactivity and the extension of the fiber network develop further until 10-12 weeks ph.

Based on our observations, we can not conclude whether cfGnRH neurons in the ventral forebrain of the African catfish migrate to their final destination, like it has been described in other vertebrates (See Introduction). An earlier study on the African catfish (Dubois et al. 1998) showed that the distribution of the cfGnRH neurons over the ventral forebrain shifted in caudal direction between 4 and 6 weeks ph. Thus, it is conceivable that migration of cfGnRH did occur between 2 and 6 weeks ph. Although it has been hypothesized that the ventral forebrain neurons originate from the olfactory placode, we cannot provide evidence for this hypothesis, since the first detectable cfGnRH immunoreactivity is (already) localized in the Vv. Another theory proposes that the ventral diencephalon might be the site of origin for the pituitary innervating GnRH neurons (Parhar 1999). In catfish such a site would be localized more rostrally, because the cfGnRH neurons appear first in the Vv.

TN population

In many teleost species the first GnRH-ir cells are found in the olfactory placode and subsequently in the TN (Chiba et al. 1994; Parhar et al. 1995a; Amano et al. 1998; Parhar et al. 1998). The present study, however, shows that in the African catfish it are the ventral forebrain cfGnRH neurons that appear first (at around 2 weeks ph) and that it takes another 2 weeks before the cfGnRH cells in the TN could be demonstrated. Since in fish of 1 and 2 weeks ph, cfGnRH-ir cells were not detectable in the olfactory bulb or MOT, we have no evidence for the origin of the TN cfGnRH-ir neurons in the olfactory placode.

The TN neurons in teleosts are considered to be a distinct population, not only because they generally develop at an earlier stage than the ventral forebrain GnRH neurons, but also because in some cases they contain a form of GnRH that is different from that in the ventral forebrain GnRH neurons e.g. (Parhar 1997; Carolsfeld et al. 2000). In addition they have a different morphology compared to the ventral forebrain GnRH neurons (Oka and Ichikawa 1992; Kim et al. 1995a; Chiba et al. 1996b), they are differentially regulated by gonadal steroid hormones (Amano et al. 1991; Parhar and Sakuma 1997) and they are supposed to have a different function (Oka 1992; Kobayashi et al. 1994; Yamamoto et al. 1995; Nevitt et al. 1995).

In the African catfish, both the TN GnRH neurons and the ventral forebrain GnRH neurons contain cfGnRH and thus can not be distinguished from each other by their GnRH content. However, also in this species they seem to be a population of GnRH neurons that is distinct from the ventral forebrain GnRH neurons. This assumption is based on the stage of development during which the TN GnRH neurons can be detected by their immunoreactivity, which is different from the ventral forebrain GnRH neurons, although the

sequence is opposite compared to other teleost species studied so far. The TN neurons differ from the GnRH neurons in the ventral forebrain system also in morphology regarding the size (usually smaller), shape (round versus elongated) and distribution (clustered versus scattered). DiI, implanted in the olfactory bulb, only reached the TN GnRH neurons, while the pituitary implanted dye reached the GnRH neurons in the ventral forebrain and not the TN neurons.

Thus, although all GnRH neurons in the ventral brain of the African catfish produce only one form of GnRH, that is cfGnRH, there is good evidence that also in this species we are dealing with two distinct GnRH neuronal populations.

The function of the TN GnRH neurons is not as clear as of the hypophysiotropic GnRH neurons. This study shows that the TN GnRH neurons widely project over different brain areas, but that they have no connection with the pituitary. Similar results were obtained with tracing studies in the dwarf gourami (*Colisa lalia*; Yamamoto et al. 1995) and goldfish (*Carassius auratus*; Bartheld and Meyer 1986; Anglade et al. 1993). Since the TN GnRH neurons are obviously not hypophysiotropic, other possible functions have been investigated (Kobayashi et al. 1994; Yamamoto et al. 1995; Amano et al. 1997). Lesions of the TN in dwarf gourami inhibited certain aspects of reproductive behavior (Yamamoto et al. 1997), whereas in the goldfish the GnRH levels in the hypothalamus were decreased (Kobayashi et al. 1994; Kim et al. 1995b). Thus, it is conceivable that TN GnRH acts as a neuromodulator, functionally related with reproductive behavior (Parhar et al. 1998).

Midbrain tegmentum population

Two weeks after hatching the cGnRH-II system appears and at 6 weeks ph the morphology of the cGnRH-II neurons is comparable with that in adults (Zandbergen et al. 1995). From the present study no firm conclusions can be drawn as to the possible origin of the cGnRH-II neurons. Probably, these neurons derive from the zone near the ventricular wall (White and Fernald 1998; Parhar 1999). The early mature state of the cGnRH-II system indicates an important function already during early development. The present study shows that the cGnRH-II neurons do not have a neuronal connection with the pituitary, excluding a direct hypophysiotropic function. Other actions of cGnRH-II are summarized as a neuromodulator or neurotransmitter (Montero and Dufour 1996).

In the African catfish, antibodies against cIIGAP only labeled perikarya; no immunoreactive fibers were observed. This is in contrast with studies on these cells in other teleosts (Amano et al. 1991; Yamamoto et al. 1995; Kim et al. 1995a; Montero and Dufour 1996; Parhar 1998), which showed an extensive

fiber pattern over the entire brain using an anti-cGnRH-II antibody. Three explanations are possible: (1) the cGnRH-II neurons in the catfish do not have extensions or (2) the cIIGAP is not transported into the fibers or in undetectable amounts. However, neither the antibody against cGnRH-II could label cGnRH-II fibers in the MT (Zandbergen et al. 1995). And (3) sexual behavior can influence GnRH systems in content, cell number, and size (Rissman 1996; Rissman et al. 1997). The fact that African catfish in captivity do not display reproductive behavior might cause inactivity of the cGnRH-II system.

GnRH evolution in teleosts

Fish represent the most interesting vertebrate group in GnRH evolution, since they display 10 of the 15 different GnRH forms that have been discovered until now. Primitive and less advanced fish species possess two different GnRH forms, whereas 3 forms are identified in most modern teleosts (Table 2). The forms of GnRH and their localization can be classified into five groups (Table 2). The Agnathans (1) possess only their own lampreyGnRH, whereas all other fish species carry the conserved cGnRH-II form. The Chondrichtyes (2) have dogfishGnRH and the primitive teleosts (3) like the sturgeon (*Ascipenser transmontanus*), reedfish (*Calamoichthys calabaricus*) and eel (*Anguilla anguilla*) carry the mammalian form. The third group including the more advanced teleosts (4) catfish, salmon (*Oncorhynchus nerka*) and goldfish (*Carassius auratus*) display variability in their GnRHs, with salmonGnRH as most important form. The herring (*Clupea harengus pallasi*) is the exception in this group, expressing two different forms in resp. the ventral forebrain and the TN. The last group is formed by the recently evolved teleosts (5), which express salmonGnRH in the TN and seabreamGnRH in the ventral forebrain and pituitary. In the medaka (*Oryzias latipes*) and pejerry (*Odentesthes bonariensis*) sGnRH is expressed in the TN as well, but they have medakaGnRH in the ventral forebrain (Table 2). The discovery of eventual new GnRH forms could shed new light on the phylogeny of GnRH in fishes.

The development of the TN neurons is closely related with the GnRH evolution. It has been shown that lampreys lack a TN population (Eisthen and Northcutt 1996). In the Chondrichtyes, primitive fish species and more advanced teleosts the TN neurons are mostly similar in size or smaller than the ventral forebrain GnRH neurons (Leprêtre et al. 1993; Montero et al. 1994; Wright and Demski 1996), whereas the TN neurons are larger in advanced and modern species (Francis et al. 1994; Kim et al. 1995a; Chiba et al. 1996b; White and Fernald 1998). The TN population shows a clear developmental pattern

> Table II Schematic distribution of GnRH forms over fish species categorized by their distinct population: midbrain tegmentum population (MT), terminal nerve population (TN), and ventral forebrain population. L: lampreyGnRH, cII: cGnRH-II, df: dogfishGnRH, m: mammalianGnRH, s: salmonGnRH, cf: catfishGnRH, sb: seabreamGnRH, md: medakaGnRH.

			MT	TN	ventral forebrain
1	**Agnatha**				
	Lamprey	Sower et al.1993			L
	Petromyzon marinus	Sherwood et al. 1986			
	Hagfish	Sower et al. 1995			LIII?
	Myxine glutinosa				
2	**Chondrichtyes**				
	Holocephali				
	Ratfish	Lovejoy et al. 1992	cII		
	Hydrolagus colliei				
	Elasmobranchii				
	Atlantic stingray	Forlano et al. 2000	cII	df	df
	Dasyatis sabina				
	Dogfish	Lovejoy et al. 1992	cII	df	
	Squalus acanthias				
3	**Actinopterygii**				
	Acipenseriformes				
	Sturgeon	Sherwood et al. 1991	cII	m	m
	Acipenser transmontanus				
	Polypteriformes				
	Reedfish	Sherwood et al. 1991	cII	m	m
	Calamoichthys calabaricus				
	Lepisosteiformes				
	Alligator gar	Sherwood et al. 1991	cII	m	m
	Lepisosteus spatula				
	Anguilliformes				
	Eel	King et al. 1991	cII	m	m
	Anguilla anguilla				
4	**Clupeiformes**				
	Herring	Carolsfeld et al. 2000	cII	s	h
	Clupea harengus pallasi				
	Salmoniformes				
	Sockeye salmon	Parhar et al. 1995a	cII	s	s
	Oncorhynchus nerka				
	Cypriniformes				
	White Sucker	Robinson et al. 1995	cII	s	s
	Catastomus commersoni				
	Zebrafish	Powell et al. 1996a	cII	s	s
	Brachydanio rerio				
	Goldfish	Lin en Peter 1997	cII	s	s
	Carassius auratus				
	Siluriformes				
	African catfish	Bogerd et al. 1994	cII	cf	cf
	Clarias gariepinus				
5	**Characiformes**				
	Pacu	Powell et al. 1997a	cII	s	sb
	Piaractus mesopotamicus				
	Cyprinodontiformes				
	Medaka	Okubo et al. 2000	cII	s	md
	Oryzias latipes				
	Atheriniformes				
	Pejerry	Montaner et al. 2000	cII	s	md
	Odentesthes bonariensis				
	Scorpaeniformes				
	Rockfish	Powell et al. 1996a	cII	s	sb
	Sebastes rastrelliger				
	Perciformes				
	Gilthead seabream	Gothilf et al. 1996	cII	s	sb
	Sparus aurata				
	Red seabream	Okuzawa et al. 1997	cII	s	sb
	Pagrus major				
	Striped bass	Chow et al. 1998	cII	s	sb
	Morone saxatilis				
	Cichlid	White et al. 1995	cII	s	sb
	Haplochromis burtoni				
	Tilapia	Parhar 1997	cII	s	sb
	Oreochromis mossambicus				

during fish evolution which parallels the evolution of GnRHs. Obviously the GnRH neurons in the TN obtained a specialized function - related with olfaction and reproductive behavior - during evolution, which favored a GnRH form different from the one expressed in the ventral forebrain in order to fulfill its function.

Similarly as the cGnRH-II has become the GnRH form that is expressed during evolution in the midbrain from Chondrichtyes onwards (Lovejoy et al. 1992; Forlano et al. 2000) till mammals, the sGnRH seems to have claimed this role in the TN population in the modern fish species. However, until now no sGnRH neurons in the TN have been characterized in higher evolved vertebrates, which could indicate that sGnRH in the TN is a short evolutionary lineage restricted to modern fishes. On the other hand, the discovery of this extra TN-GnRH in the near future within higher vertebrates can not be excluded.

Acknowledgments

This study was part of the masters program of Mrs. Pauline van der Voort. Mr. Frits Kindt of the Department of Image Processing and Design is acknowledged for his advice and help with the preparation of the photographs.