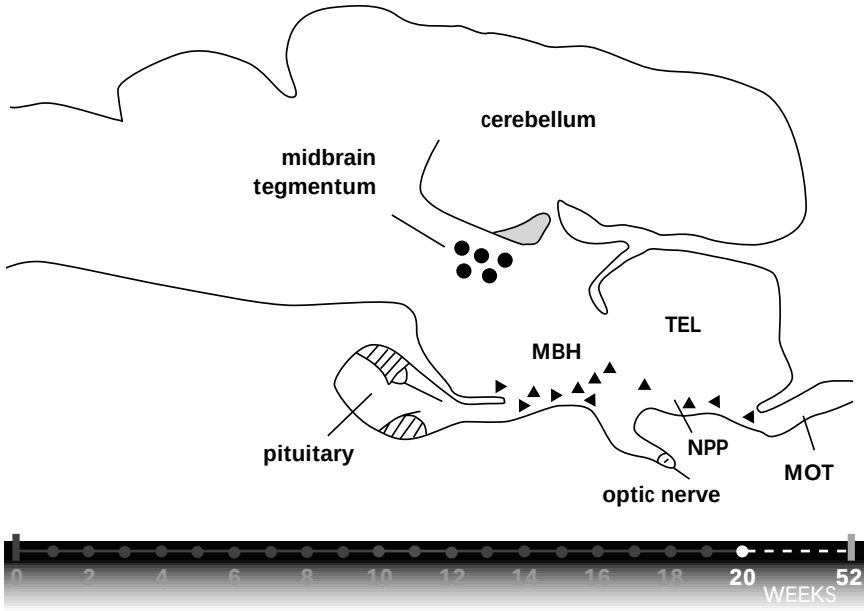
Chapter 6

Functional morphology of cGnRH-II cells in the midbrain of a teleost fish, *Clarias gariepinus*

Johannes Meek, Eline A. Dubois, Matthijs A. Zandbergen, Roelie de Boer-van Huizen, Jan Peute.

J. Comp. Neurol. (submitted)



Functional morphology of cGnRH-II cells in the midbrain of a teleost fish, *Clarias gariepinus*

Abstract

The present study aims at a morphological analysis of the chickenGnRH-II immunoreactive cells in the midbrain of the African catfish, *Clarias gariepinus*, in order to investigate whether their structural properties and relations might provide clues to understand their functional significance. For this purpose, cGnRH-II cells were identified using a specific antibody against cGnRH-II-associated peptide (cIIGAP) and analyzed using light and electron microscopical techniques. For comparison, neurons of the neighboring nucleus of the fasciculus longitudinalis medialis (nflm) were analyzed as well.

ChickenGnRH-II cells are exclusively located in the synencephalon, which previously was considered as the rostral part of the midbrain tegmentum, but presently as the most caudal part of the prosencephalon. ChickenGnRH-II cells have no cIIGAP immunoreactive processes outside the confines of the synencephalon, which suggests that they do not project to the pituitary or other brain regions. There are about 100 cGnRH-II cells per fish, all located at the interface between the so-called subventricular reticulum in the midline region and the more laterally located neuropyl which embeds the nflm. Each cGnRH-II cell contains an indentated nucleus with one nucleolus, a paranuclear cap of Nissl substance (rough endoplasmatic reticulum: RER) and two distinct cytoplasmatic compartments: one subdivision characterized by the occurrence of small dense-cored vesicles and many Golgi-cisterns and a second subdivision containing densely and regularly packed lamellae of RER.

They have only a few synaptic contacts on their surface but instead several close and large apposition regions with other GnRH cells as well as processes of nflm neurons. They are always in contact with dilated extracellular spaces of the subventricular synencephalic reticulum. Our results suggest that chickenGnRH-II cells are controlled by the neurons of the nflm via dendro-somatic appositions and that they have no axons. They probably release their products into the extracellular spaces of the subventricular reticulum, from which they may diffuse into the ventricular fluid and/or the bloodstream. Since the midbrain GnRH system is highly conserved and basically similar in all classes of vertebrates, the same probably holds for other vertebrates. The function of chickenGnRH II remains enigmatic.

Introduction

Gonadotropin-releasing hormone (GnRH) producing cells occur in the brain of all vertebrates and consist of two or three populations, located in the terminal nerve ganglion, the ventral forebrain and the rostral midbrain (Fernald and White 1999). Those in the ventral forebrain innervate the pituitary and stimulate the gonadotropic cells to produce and release their hormone, which in turn stimulates gonadal cells to secrete sex steroids and to produce germ cells (reviewed by (Montero and Dufour 1996; Amano et al. 1997). The amino acid sequence of GnRHs produced in the forebrain varies substantially in different vertebrate species and classes (Powell et al. 1996b; Carolsfeld et al. 2000; Yoo et al. 2000; Okubo et al. 2000a).

In contrast to the GnRH produced by the forebrain, the GnRH produced by the midbrain is the same in all vertebrates investigated until now. It has been called chickenGnRH-II (cGnRH-II), since its composition has been analyzed for the first time from homogenates of the chicken brain (Miyamoto et al.1984). However, the function of cGnRH-II as produced by the midbrain cells is enigmatic. Suggestions that have been put forward in the literature vary from neurotransmitter or neuromodulator (Montero and Dufour 1996) to facilitator in sexual behavior (Rissman 1996; Rissman et al. 1997).

Immunohistochemical studies have shown that cGnRH-II cells are always localized in the rostrodorsal part of the midbrain tegmentum (Muske, 1997). In this region also the nucleus of the fasciculus longitudinalis medialis (nflm) is located, which gives rise to descending projections into the spinal cord via the fasciculus longitudinalis medialis (flm), a distinct fiber tract in the mediodorsal part of the mid- and hindbrain tegmentum (Meek and Nieuwenhuys 1998). Recent embryogenetic research has revealed that this rostral midbrain tegmental region, also indicated as the synencephalon, the place where the di- and mesencephalon fuse, in fact originates from the most caudal segment of the forebrain or prosencephalon, indicated as the first prosomere or P1 (Nieuwenhuys 1998). In addition to the cGnRH-II and nflm cells, most of the pretectal cell groups originate from this prosencephalic segment (Nieuwenhuys 1998).

Several studies have reported the presence or cGnRH-II immunoreactive fibers in different parts of the brain, suggesting widespread projections of the midbrain cGnRH-II cells (Rissman et al. 1995; Yamamoto et al. 1995; Parhar et al. 1996b; Robinson et al. 2000). However, recent demonstrations of unspecificity and cross reactivity of the antibodies used with other types of GnRH might throw some doubt on this conclusion (Zandbergen et al. 1995). To avoid misinterpretations due to immuno-unspecificity, we developed antibodies against GnRH associated peptides (GAPs) in catfish, which are highly specific for either catfish GnRH producing neurons as present in the forebrain or

cGnRH-II producing neurons as present in the midbrain cells of catfish (Zandbergen et al. 1995; Dubois et al. 1998). The specificity of the GAP antibodies was confirmed by *in situ* hybridization (Zandbergen et al. 1995).

It spite of the absence of unequivocally demonstrated projections of midbrain cGnRH-II cells to the pituitary (Anglade et al. 1993; Yamamoto et al. 1995; Parhar et al. 1996), in vitro experiments show that cGnRH-II is about 100 times more potent to stimulate GTH release from the pituitary of catfish than catfish GnRH (Schulz et al. 1993; Bosma et al. 2000). This suggests that midbrain GnRH cells still might have an important function in the regulation of GTH. If that is true, cGnRH-II has to be released into the blood to reach the pituitary. One goal of the present study is to analyze whether there are special blood vessels that connect the midbrain with the pituitary.

In order to unravel the possible function of cGnRH-II, we started a detailed light, confocal and electron microscopical analysis of the cGnRH-II cells in the African catfish, with particular attention to morphological aspects that might point to the functional significance of these cells and there intriguing location in the synencephalon. More specific, we tried to identify sites of cGnRH-II release into blood vessels, extracellular spaces or the ventricular fluid, we compared cGnRH-II cells in animals of different sex and size; and we analyzed the synaptic input and other contacts of cGnRH-II cells. For comparison, the same aspects were studied for nflm cells, which have a similar size as cGnRH-II cells in catfish and show a codistribution in the synencephalon.

Materials and methods

Animals and surgery

Eight fish (six males and two females) of the species *Clarias gariepinus* bred in the hatchery of the Department of Experimental Zoology, were used for immunohistochemistry. The fish ranged in length from 25 to 30 cm and were at the young adult stage (8 months of age). Before perfusion with fixatives in preparation for histology, fish were deeply anesthetized with 2% phenoxyethanol in 0.9% NaCl. Animal housing and all procedures were carried out in accordance with Dutch and international guidelines.

CIIGAP immunohistochemistry

After anesthesia, fish were perfused with 0.9% NaCl followed by either 2% paraformaldehyde and 2% glutaraldehyde in 100 mM phosphate buffer (PB), pH 7.4, 2% paraformaldehyde and 0.2% glutaraldehyde in the same buffer, or 4% paraformaldehyde without glutaraldehyde in the same buffer. After perfusion, the brain was removed, immersed for 3 h in the same fixative, and serial 50 μm or 200 μm thick sections were cut in the transverse plane with a

vibratome. Alternate sections were treated for light and electron microscopy.

For light microscopy (LM), 50 μm thick sections were rinsed three times for 20 min in Tris buffered saline (TBS) at pH 7.35, preincubated in 0.1% bovine serum albumin (BSA) in TBS with 0.1% Triton X-100 and incubated overnight at room temperature in anti-cIIGAP diluted 1:16.000 in preincubation medium. The preparation and specificity of the antibody has been described earlier (Zandbergen et al. 1995). After three times rinsing for 20 min in TBS, the sections were incubated for 90 min in a solution containing a biotinylated donkey-anti-rabbit antibody diluted 1: 1500 in TBS. The sections were rinsed three times for 20 min in TBS, transferred for 60 min to a solution containing Avidin-Biotin Complex (Vector standard ABC kit) diluted 1: 800 together with 0.1% BSA in TBS, followed by three 20 min rinses in TBS. Next, the sections were preincubated for 10 min in a solution containing 20 mg DAB and 300 mg nickel-ammonium sulfate per 100 ml of 0.05 M Tris, pH 7.6, followed by incubation for 5-10 min in the same solution after addition of 10 μl 30% H_2O_2. After two rinses in PBS, sections were dehydrated, some were counterstained with cresyl violet, and all were mounted in Entellan. The 200 μm thick vibratome sections were labeled with anti-cIIGAP and anti-rabbit-FITC (Sigma, St. Louis, MO) as previously described (Dubois et al. 1998). These sections were investigated in a Leica confocal laser-scanning microscope.

For electron microscopy (EM), 50 μm thick vibratome sections were treated similarly as described above for light microscopy, but without Triton-X-100 and nickel-intensification. Instead, the DAB deposit was intensified using the gold-substituted silver peroxidase (GSSP) method (Pol and Gorcs 1986). For this purpose, sections were rinsed twice for 20 min in 2% sodium acetate and bleached for 3 to 4 h in 10% thioglycolic acid. After four 20 minute rinses in sodium acetate, they were developed for a maximum of 8 min in a mixture containing 10 ml solution A (5% sodium carbonate), 10 ml B (consisting of 0.5 g silver nitrate, 0.5 g ammonium nitrate and 2.5 g tungstosilic acid in 250 ml distilled water) and 40 μl C (37% formaldehyde in water). Development was stopped by immersion for 2 min in 1% acetic acid. After three 20 minute rinses, the silver precipitate was substituted by gold during incubation for 8 minutes in 0.05% chloroauric acid ($H_4AuCl_4.xH_2O$; BDH Chemicals). After two 20 minute rinses in sodium acetate, sections were dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon between a slide and coverslip that were coated with Repelcoatr, thus allowing the easy removal of selected sections at a later stage. Selected 50 μm sections were remounted on prepolymerized Epon blocks and 80 nm ultrathin sections were cut with a Reichert Ultracut-E. These were contrasted with uranyl acetate and lead citrate and studied in a Philips EM 301.

Quantitative light microscopy

To analyze the location, distribution, number and size of cGnRH-II cells, series of 15 μm thick serial paraffin sections of four brains were used. These were already made in 1979 for the preparation of the book of Nieuwenhuys et al. (Meek and Nieuwenhuys 1998) and gratefully used for the present study. It concerned brains obtained form specimens of 10.5 (Cl 1), 24 (Cl 2), 29 (Cl 3) and 35 (Cl 4) cm length, unfortunately of unknown sex and age. They were sectioned transversely (Cl 1, 2 and 4) or horizontally (Cl 3), and stained according to Nissl, Kluver-Barrara or Bodian to visualize cells, cells and myelinated fibers, and myelinated as well as unmyelinated fibers, respectively (Donkelaar and Nicholson 1998). Most sections were stained according to Bodian, and in these sections cGnRH-II cells could not be distinguished from neurons of the nflm. However, in Nissl- and Kluver-Barrera stained sections, cGnRH-II cells could be unequivocally identified and distinguished from nflm neurons (see Results). Consequently, these were used for light microscopical analysis and quantification of cGnRH-II and nflm cells.

The numbers and average sizes of cGnRH-II cells identified in paraffin sections were estimated from cells that contain a nucleolus. The nucleolus has an average position in the middle of the cell body and thus the transsection of the cell body that contains the nucleolus gives the best estimation of the cell diameter (Albers et al. 1988). Moreover, the nucleolus does not split during paraffin sectioning (Vogels et al. 1990), which prevents double counts of the same neuron. The number of cells was estimated by division of the counted number of cells (with a nucleolus) by the number of sections analyzed and subsequent multiplication with the total number of 15 μm thick paraffin sections containing cGnRH-II or nflm cells (20 in Cl 1 and Cl 2, 30 in Cl 3 and Cl 4). In immunohistochemically treated vibratome sections, the nucleolus was not visible. Consequently, for this material diameter estimations were based on cells with a nucleus, whereas estimations of numbers could not be made on the basis of this material.

Results

Light microscopical characterization of cGnRH-II cells

ChickenGnRH-II cells in the midbrain of the African catfish are characterized by the following light microscopical features: they have a large nucleus with one nucleolus and a distinct cap of Nissl substance (Fig. 1A). Their cytoplasm consists of two separated compartments: one densely filled with intensely staining Nissl substance and another without any Nissl substance. These features clearly differ from the neighboring and sometimes even intermingled neurons of the nflm, which have no nuclear Nissl cap and no separate cyto-

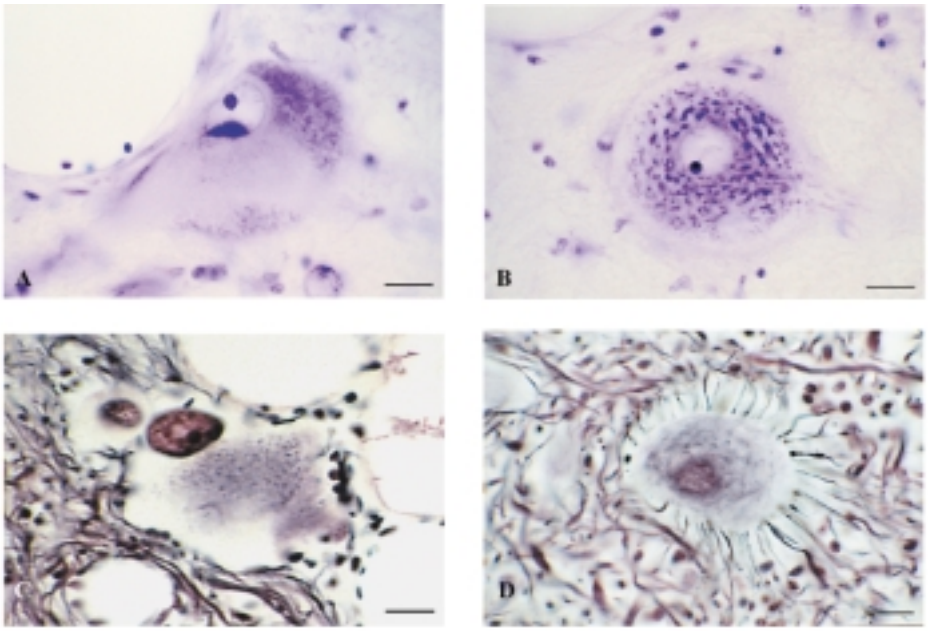


Fig.1 Light microscopical pictures of cGnRH-II cells and nflm cells in the African catfish. A: a Nissl-stained cGnRH-II cell with the nucleus, nucleolus, Nissl cap, and two cytoplasmatic compartments, one filled with Nissl substance and the other without. B: a FLM cell with the nucleus and nucleolus and patchy distributed Nissl substance. C: Bodian stained cGnRH-II cell and D: Bodian stained nflm cell with dense innervation. Scale bars represent 16 μm.

plasmatic compartments, but evenly distributed 'floes' of Nissl substance, similar to almost all large motorneurons in the brain (Fig. 1B). An additional distinguishing feature of nflm cells is their dense innervation, as clearly visible in Bodian stains (Figs. 1C,D).

Immunohistochemical processing of brain sections with our specific antibody against cIIGAP reproducibly stained the cGnRH-II cells in the synencephalon of different animals, without any staining of other cells in the brain of catfish (Fig. 2A). Likewise, no labeled fibers were observed in any part of the brain, including the synencephalon itself, suggesting that cGnRH-II cells do not have axonal projections. We exclusively observed short, dendrite-like processes originating from cGnRH-II cells (Figs. 2A, B).

Location of cGnRH-II cells

Serial transverse sections show that all cGnRH-II cells in catfish are located very close to the midline, caudoventrally to the caudodorsal wall of the infundibulum (Figs. 3A and 5). The nflm has the same rostrocaudal extension as the cGnRH-II cell population, but is located somewhat more laterally. The midline region of the synencephalon below the ventral midline fissure of the ventricle has a reticular appearance with many extracellular spaces, and will be designated as the subventricular synencephalic reticulum (Figs. 3A, B, 4A, 5). It

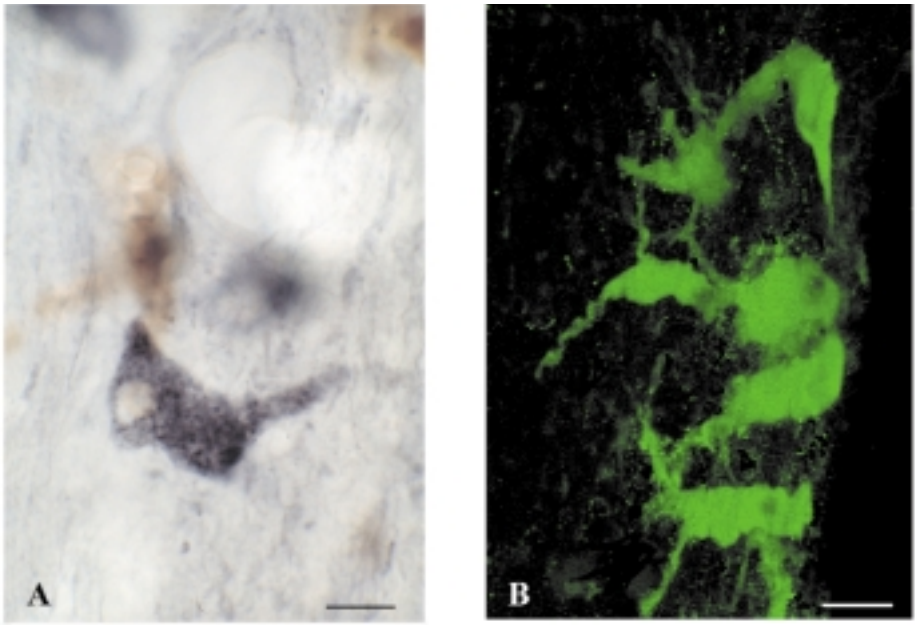


Fig. 2 cGnRH-II cells labeled with anti-cIIGAP. A: LM picture of a cIIGAP-labeled cGnRH-II cell in the midbrain. B: CLSM image of cGnRH-II cells in the midbrain composed of 32 projections over 40 μm. Only short dendritic extensions of cGnRH-II cells are visible. Scale bars represent 16 μm in A and 20 μm in B.

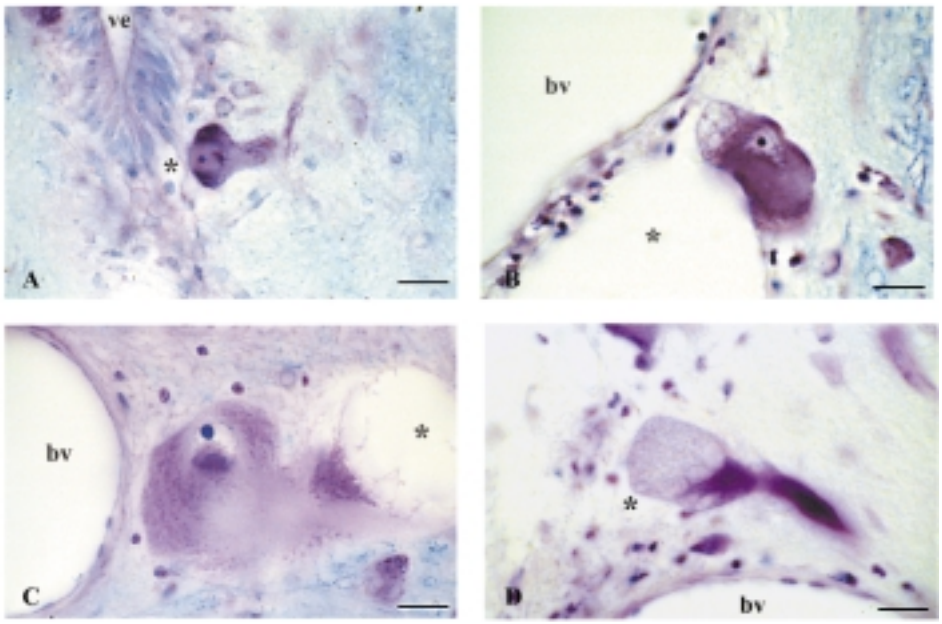


Fig. 4 A-D: cGnRH-II cells of different size, as present in Cl 1 (A), Cl 4 (B), Cl 2 (C), showing distinct relations with dilated extracellular spaces (*). Figure D shows a process of a cGnRH-II cell of Cl 2, of which the cell body was located in a neighboring section filled with Nissl substance and containing vacuolated cytoplasm in the distal end. Bv: blood vessel, ve: ventricle, sr: subventricular reticulum. Scale bars represent 16 μm.

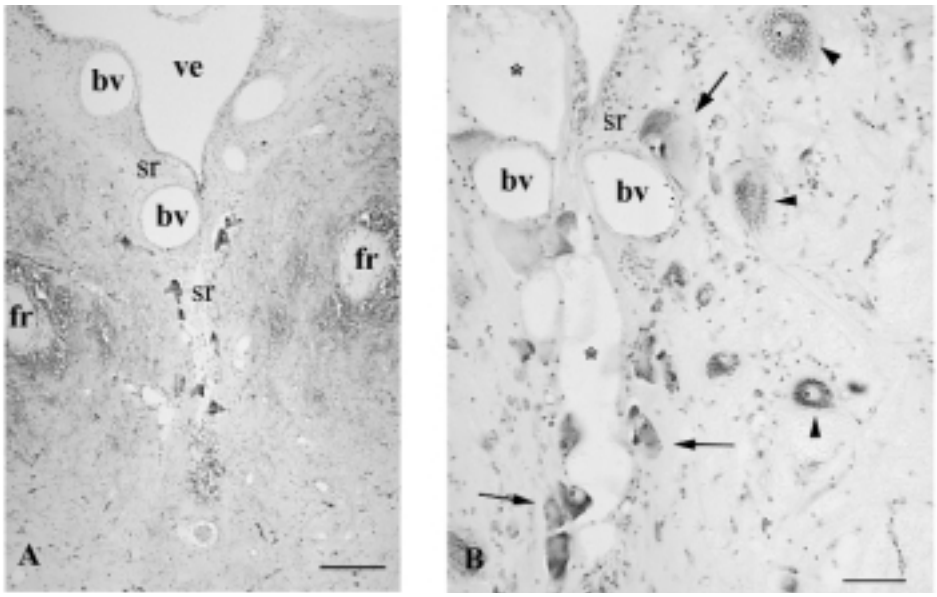


Fig. 3 Light microscopical pictures showing the localization of cGnRH-II cells. A: low magnification of a transverse section through the synencephalon of Cl 4 with cGnRH-II cells situated lateral to the midline of the brain. B: Nissl stained section of the synencephalon of Cl 2 with examples of cGnRH-II cells (arrows) and more laterally localized FLM cells (arrowheads). Bv: blood vessel, ve: ventricle, fr: fasciculus retroflexus, sr: subventricular reticulum, *: dilated extracellular space. Scale bars represent 160 μm in A, 64 μm in B

does not contain neurons, but exclusively glial processes and -cells. The more laterally located neuropyl is more densely stained in light microscopical sections and does not contain extracellular spaces. Instead, it is composed of intermingled dendritic and axonal processes with mutual synaptic contacts as well as small neurons; elements that are absent in the subventricular reticulum. ChickenGnRH-II cells appear to be precisely located at the interface between the subventricular reticulum in the midline region and the more laterally located neuropyl, which embeds the neurons of the nflm (Figs. 3B and 5 IV).

Two large veins course horizontally through the caudal portion of the population of cGnRH-II cells (Figs. 3A, B, 5 III). They collect blood from the pretectal region and fuse into a large, unpaired vein that courses dorso-ventrally at the caudal pole of the nflm and the cGnRH-II cell population, just rostral to the oculomotor nucleus. This midline vein empties caudal in the venous sinus that surrounds the pituitary.

Size and number of cGnRH-II cells

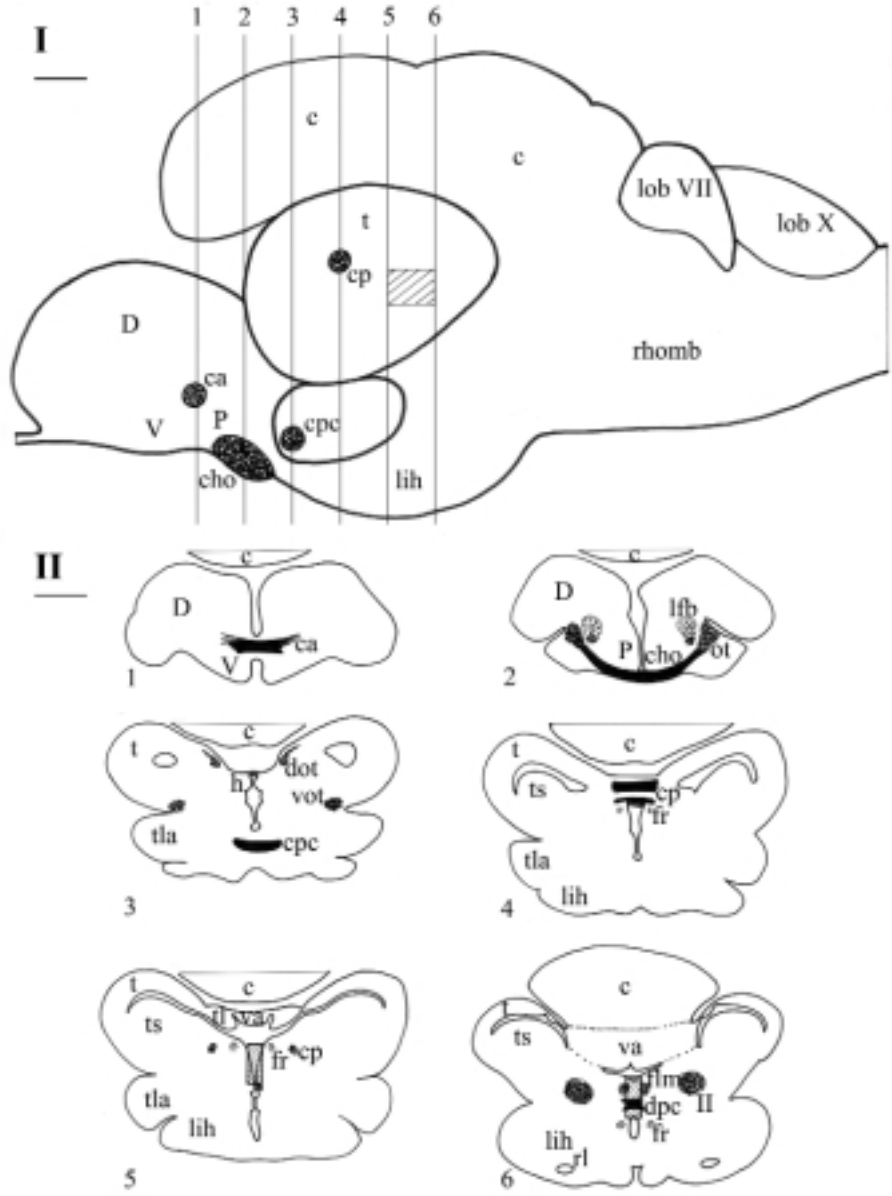
The diameter of unequivocally identified cGnRH-II cells in catfish varies from 10 to 70 μm, with a nuclear diameter varying accordingly from 7 to 21 μm (Fig. 4). This implies a difference in cytoplasmatic volume of about 500 times (i.e. from about 500 to 25000 μm^3). The differences in size of GnRH cells are

not randomly distributed, but show both sex- and size-related trends. Comparison of cGnRH-II cells in immunohistochemically treated vibratome sections obtained from male and female catfishes of 25 cm length (age about 8 months) revealed that cGnRH-II cells in males are larger than in females of similar size and age (average diameters 22.4 ±1.0 μm and 17.9 ±0.9 μm, respectively). Comparison of Nissl stained paraffin sections of four animals of different size (with unfortunately unknown sex and age, see methods), showed that the average cell diameters of cGnRH-II cells varied from 16.5 μm in a catfish of 10.5 cm length to 24.6 μm and 26.9 μm in two fishes of 29 and 35 cm length, respectively. The largest cGnRH-II cells in these fishes were 25, 37 and 40 μm in diameter, respectively. The fourth specimen in our collection of Nissl stained brain sections was an animal of 25cm length with extremely large cGnRH-II cells. Their average diameter was 48 μm, while the largest cells had diameters of 70 μm. This large average size was correlated with extremely large and widely extending extracellular spaces in the subventricular reticulum (Fig. 5 III: Cl 2)

The average size of the neurons of the nflm did not show changes that were correlated to those of the cGnRH-II cells or animal size, but was similar in all four Nissl stained brains analyzed (33.5 to 36 μm), including the brain with the extremely large cGnRH-II cells. The estimated number of cGnRH-II cells in different animals was about 100 (50 on each side) without significant differences between animals of different sex or size. In contrast, the estimated number of unequivocally identifiable nflm cells varied from 55 in the animal of 10.5 cm length to 90, 110 and 130 in the animals of 24, 29 and 35 cm length, respectively.

Electron microscopical characterization of cGnRH-II cells

Electron microscopical analysis of cGnRH-II and nflm cells corroborated the light microscopical characteristics described above (Fig. 6). The Nissl cap of the nucleus appears to represent a separate compartment of rough endoplasmatic reticulum (RER), located in an indentation of the nucleus (Figs. 6A, 7A). The Nissl-rich compartment of the cytoplasm contains densely and regularly packed lamellae of RER (Fig 7B), whereas the Nissl-free part of the cytoplasm is characterized by the presence of many Golgi cisterns and small dense-cored vesicles (Figs. 7C,D). In relatively small cGnRH-II cells, the Golgi-and vesicle-rich compartment is located around the nucleus, whereas the Nissl-rich part is located peripherally (Figs. 7B,E). In larger cells, the distribution is more irregular, since the Golgi-and vesicle rich cytoplasmatic compartment penetrates the peripherally located RER compartment. As a result the latter is distributed in clusters along the cellular surface (Fig. 6A, 7B, 8A,E). In addition to a nucleolus, the nucleus of GnRH cells appears to contain a spherical region with less dense chromatin than the rest of the nucleus and as large as the nucleolus (Fig.



7E). It is noteworthy to mention the absence of heterochromatin. As already observed in the light microscope, neurons of the nflm have their RER and Golgi cisterns codistributed throughout the cytoplasm (Fig. 6B). They do not contain dense cored-vesicles (Fig. 6D).

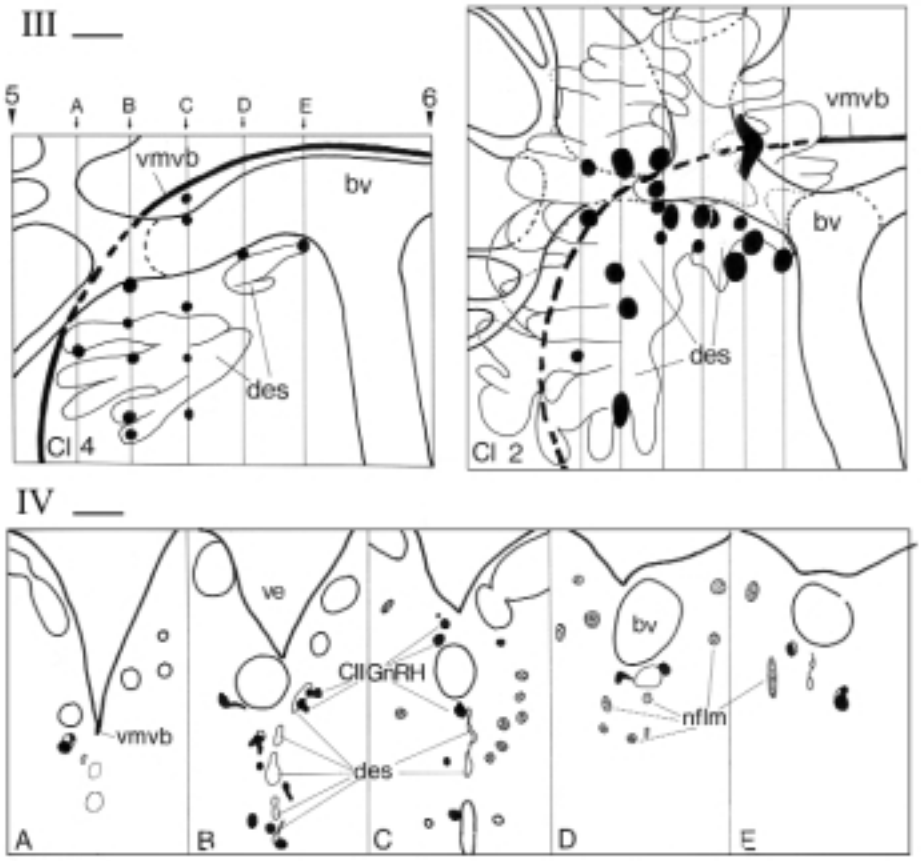


Fig. 5 Drawings of transverse sections and reconstructed lateral views of the brain (I and II) and the synencephalic region that contains the cGnRH-II cells (III and IV) of the African catfish. I: Lateral view of the brain of Clarias gariepinus in which lines 1 to 6 indicate the levels of the transverse sections shown in II. II: rostral-caudal series of transverse sections. Hatched areas indicate the rostral and caudal level of the reconstruction of Cl 4 shown in III, Cl4. III: Reconstructed lateral views of the brain area containing cGnRH-II cells of two different animals, Cl4 and Cl2. A to E indicate the level of the transverse sections of Cl 4, as shown in part IV, on which the reconstruction was based. IV: Series of rostro-caudal sections through the cGnRH-II region of Cl4. CGnRH-II cells are drawn in black and nflm cells in gray. Scale bars represent 1mm in I, 2mm in II, 237 μm in III and 190 μm in IV. Bv: blood vessel, c: cerebellum, ca: commissura anterior, cho: chiasma opticum, cp: commissura posterior, cpc: commissura postchiasmatica, D: area dorsalis telencephali, des: dilated extracellular space, dot: dorsal optic tract, dpc: decussatio pedunculi cerebellaris, flm: fasciculus longitudinalis medialis, fr: fasciculus retroflexus, h: habenula, ll: lemniscus lateralis, lfb: lateral forebrain bundle, lih: lobus inferior hypothalami, Lob VII: lobus fascialis, Lob X: lobus vagi, nflm: nucleus of the fasciculus longitudinalis medialis, ot: optic tract, p: pre-optic area, rhomb: rhombencephalon, rl: recessus lateralis, t: tectum mesencephali, tl: torus longitudinalis, tla: torus lateralis, ts: torus semicircularis, V: area ventralis telencephali, va: valvula cerebelli, ve: ventricle, vmvb: ventro-medial ventricular boundary, vot: ventral optic tract.

Membrane specializations and contacts of cGnRH-II cells

ChickenGnRH-II cells in catfish have only few synaptic contacts on their soma and dendrite-like process (Fig. 8A). The number of contacts per somatic profile studied varied from zero to only three. This strongly differs from nflm cells,

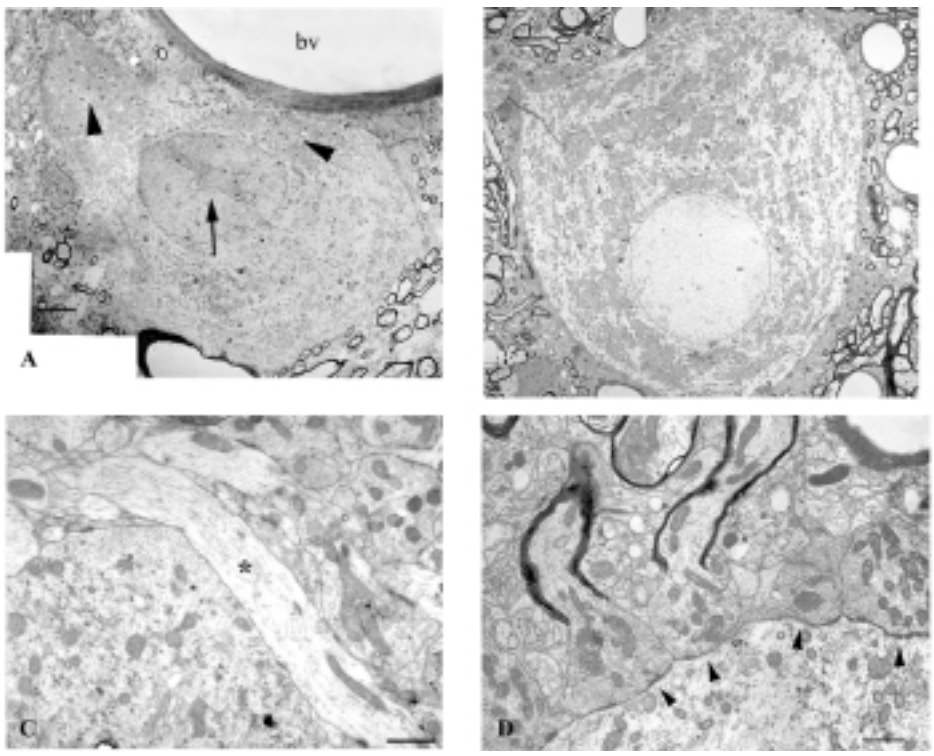


Fig. 6 Electron micrographs of cGnRH-II and nflm cells as observed in immunohistochemically treated midbrain tissue. In this material DAB precipitate indicates the presence of cGnRH-II immunoreactivity and is visible as black spots or small holes (when the precipitate has disappeared because of the ultrathin section procedure). A: Low magnification of a cGnRH-II cell, with an indented nucleus with included an RER-cap (arrow) and RER-rich (arrowhead) and RER-poor cytoplasmatic compartments. B: Low magnification of an nflm cell showing the nucleus and cytoplasm with a patchy distribution of RER. C: Detail of a cGnRH-II cell and its close apposition with a process of an nflm cell (*). D: Detail showing the dense innervation of an nflm cell. Arrows indicate synaptic contacts with small flattened vesicles and symmetric membrane specializations. Two presynaptic elements can be observed to originate from a myelinated axon. Bv: blood vessel. Scale bars are 6.8 μm in A and B, 1 μm in C, and 0.8 μm in D.

which are very densely covered with inhibitory (i.e. flattened vesicles containing) synaptic contacts, which originate from myelinated fibers (Fig. 6D). Most of the presynaptic profiles contacting cGnRH-II cells were quite large and contained flattened vesicles, thus being probably inhibitory. A few presynaptic profiles contacting cGnRH-II cells contained small dense-cored vesicles (Fig. 8A).

Both cGnRH-II cells and nflm cells show appositions with capillaries without clear specializations. The capillary endothelial cells and their basal lamina were always intact at sites of appositions and the apposing cells did not show any specialization or indication of uptake or release at these sites (Figs. 6A, 8B). The frequency of occurrence of appositions between capillaries and cGnRH-II or FLM cells was similar and seemed to be not larger than could be

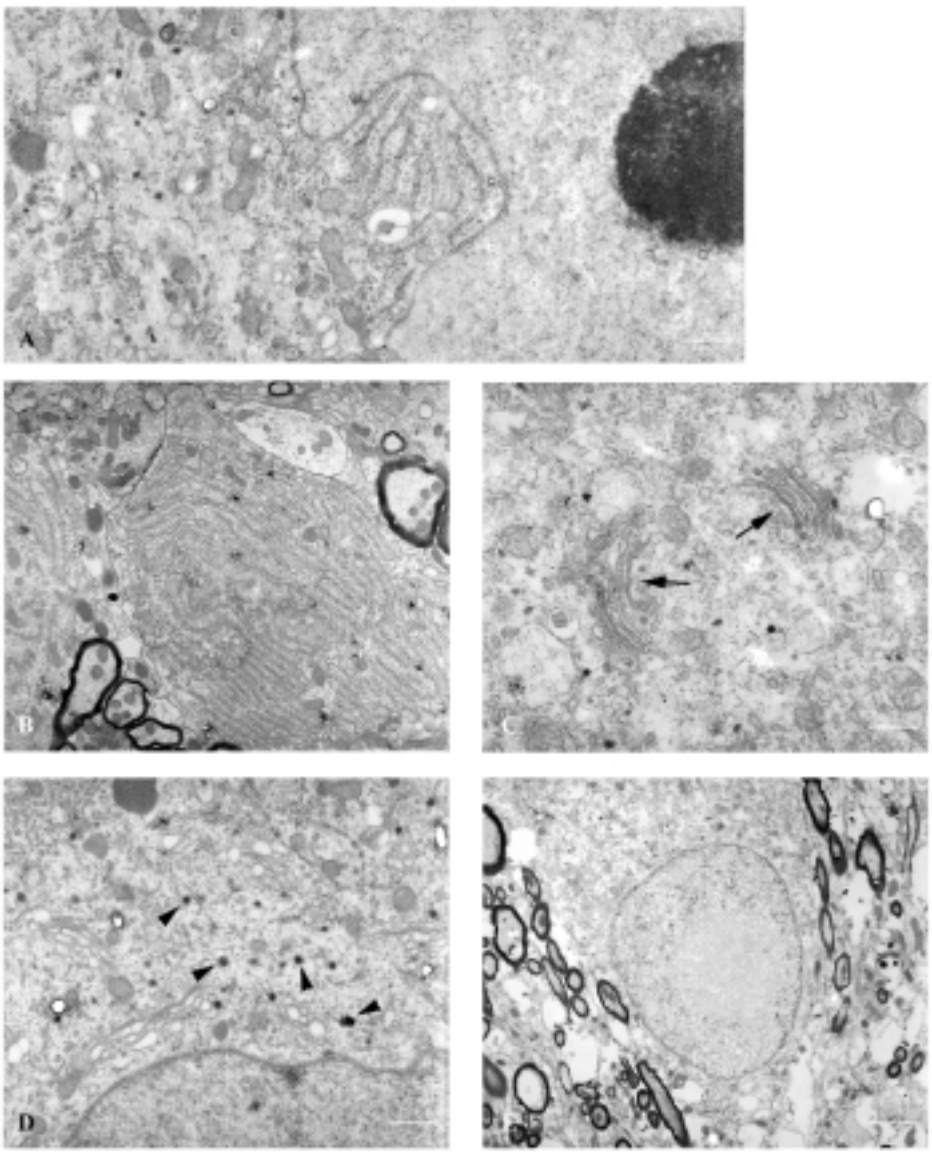


Fig. 7 Electron microscopical pictures of the ultrastructural characteristics of cGnRH-II cells. A: A part of the nucleus (with nucleolus) with an indentation filled with RER. B: Cytoplasmatic compartment densely filled with RER. C: Golgi-apparatus (arrows) as present in the granular compartment of the cytoplasm. D: Granular compartment with some dense cored vesicles (arrowheads) located between the nucleus (bottom) and the RER compartment (top). E: The part of the nucleus of a cGnRH-II cell that contains a light chromatin region. Scale bars are: 0.8 μm. in A and D, 1.3 μm in B, 0.6 μm in C, 2.4 μm in E.

expected from a random distribution of capillaries and cells. The cGnRH-II cells apposing the large veins that run through the synencephalon (Figs. 4D, 6A) equally showed no specialization at their apposition sites. ChickenGnRH-II cells were always separated from the content of the veins by a layer of

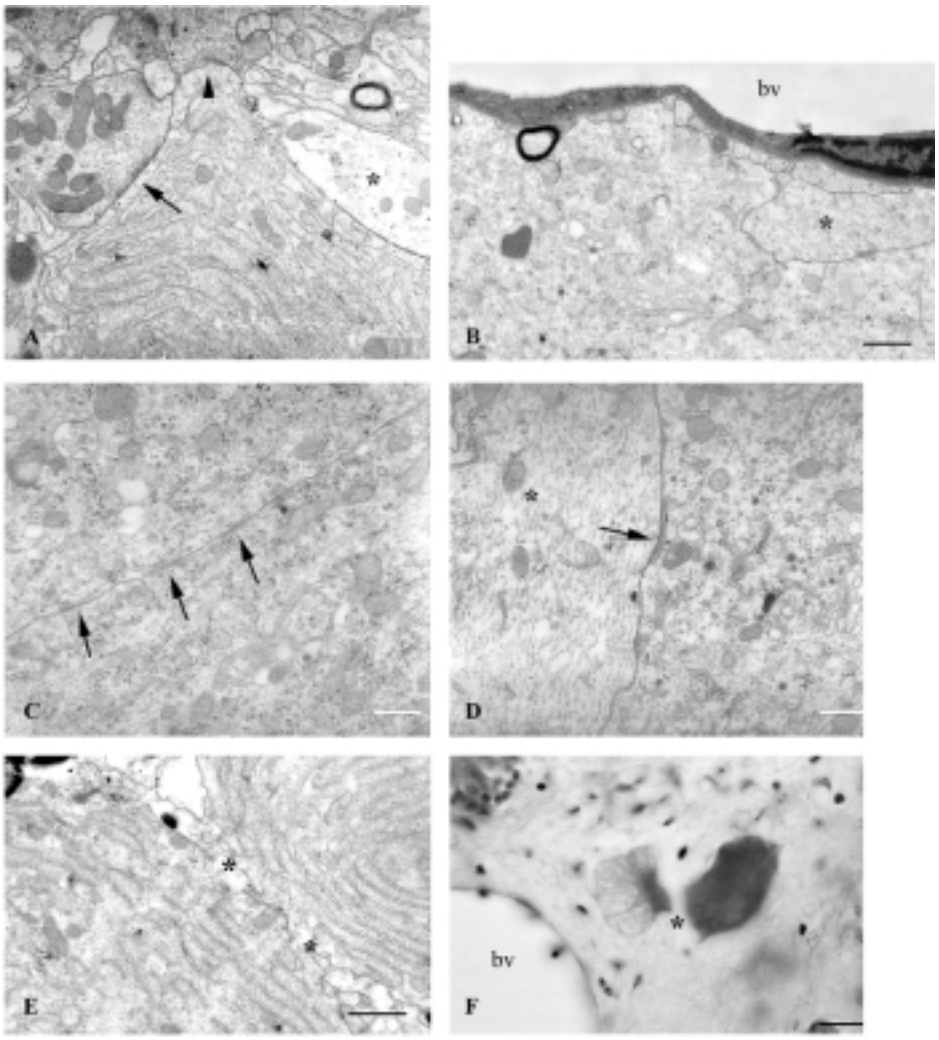


Fig. 8 Electron microscopical pictures of contacts of cGnRH-II cells. A: Presumed inhibitory synapse with a presynaptic element containing small flattened vesicles (arrow) and a synapse of which the presynaptic element contains dense cored vesicles (arrowhead). Asterisks indicates an nflm process. B: A cGnRH-II cell in close apposition to a blood vessel and an nflm process (*). C: Close apposition between two cGnRH-II cells with subsurface lamellae (arrows). D: Close apposition with a subsurface lamella (arrow) between a cGnRH-II cell and an nflm process (*). E: Two neighboring cGnRH-II RER containing compartments, of which the cellular membranes are disrupted and which are separated by presumed shrunken extracellular space (*). F: LM photograph of a similar configuration as shown in E, in which the extracellular space (*) is dilated. Scale bars: 1 μm. in A, 0.8 μm in B, 0.6 μm in C, D, and E, 16 μm in F.

endothelium and a basal lamina (Fig. 8B).

ChickenGnRH-II cells may have very close and large appositions with each other as well as with processes of nflm neurons (Figs. 6C, 8A-D), and their frequency of occurrence is much higher than expected on the basis of random

locations and orientations. Several cGnRH-II cells had such an intense mutual contact that their boundaries could not be detected in the light microscope. It was only detected in the EM that certain cGnRH-II cell configurations in fact represented two or three apposing cells. Such intense contact zones often showed sites with subsurface lamellae close to the cell membrane (Fig. 8C). Apposition zones with processes of nflm cells are less extensive compared to those with other cGnRH-II cells, but equally close, and equally show subsurface lamellae (Fig. 8D). In electronmicroscopical material, by far the largest part of the surface of cGnRH-II cells is surrounded by glial tissue of the subventricular reticulum.

In light microscopical sections fixed with Bouin fixative, virtually all cGnRH-II cells are in contact with dilated extracellular spaces of the subventricular reticulum. The degree of dilation of these spaces as well as their extension is correlated with the size of the GnRH cells in the different animals investigated (Figs. 4, 5 III). LM observations suggest apocrine release of material from cGnRH-II cells into these spaces, since cGnRH-II cells show vacuoles and other deviations in cytoplasmatic content and structure at sites of protrusions into the dilated extracellular spaces (Figs. 4B, D, 8F). Sometimes protrusions of GnRH cells almost completely surround dilated extracellular spaces (Fig. 4C). Neurons of the nflm or other neurons in the synencephalon or elsewhere never showed such features. The presumed electron microscopical image of the relation between GnRH cells and dilated extracellular space is shown in figure 8E. As will be discussed in the discussion section, we presume that the dilated extracellular spaces visible in Bodian stained sections are shrunken in EM material due to fixation with glutaraldehyde.

Discussion

The present study is, apart from a short note by Miller and Kriebel (1985), the first report of the qualitative and quantitative morphological and ultrastructural features of midbrain GnRH cells in vertebrates and has revealed a number of interesting morphological characteristics of cGnRH-II cells in the catfish, *Clarias gariepinus*. These morphological characteristics will be discussed in view of their significance for a better understanding of their possible function in catfish. For this purpose, we will focus on the question whether cGnRH-II cells have axons or not, and on the possible significance of the relations of cGnRH-II cells with dilated extracellular spaces and with nflm cells. Finally, we will put forward and discuss a new hypothesis concerning cGnRH-II cells on the basis of our morphological findings. Since the structure of the GnRH produced by midbrain GnRH cells as well as the location of these cells is highly conserved throughout the vertebrate kingdom, the resulting conclusions will

probably hold for all vertebrates.

Do chickenGnRH-II cells have axons?

The present results showed no signs of axons of cGnRH- II cells, which is in sharp contrast with several previous studies that described extensive cGnRH-II immunoreactive axonal projections in the brain of several teleosts, including the masu salmon (*Oncorhynchus masou*; Amano et al. 1991), the dwarf gourami (*Colisa lalia*; Yamamoto et al. 1995), the tilapia (*Oreochromis mossambicus*; Parhar 1998), the sockeye salmon (*Oncorhynchus nerka*; Parhar et al. 1996a), and the medaka (*Oryzias latipes*; Parhar et al. 1998). The most likely basis for this discrepancy is the use of different antibodies. We used an anti-cIIGAP antibody in the present study, which is very specific, whereas the older studies just mentioned all used anti cGnRH-II antibodies. One possibility to be considered is that cIIGAP might not be present in axons, when cGnRH-II would be fully and exclusively produced and already be spliced from its GAP in the somatic and dendritic cytoplasm. However, this would be in strong contrast to catfishGAP, which is not only found in the basal forebrain cell bodies, but also in their axons coursing to the pituitary and several brain centers, as well as in their terminals in the pituitary (Zandbergen et al. 1995). Moreover, also with an antibody against cGnRH-II itself we equally did not observe immunoreactive axons originating from cGnRH-II cells (Zandbergen et al. 1995). So, we consider it most likely that cGnRH-II cells in catfish indeed have no axons, and that previous suggestions of axonal projections of cGnRH-II cells are probably due to cross reactivity of the antibodies used. All antibodies raised against cGnRH-II show cross-reactivity with other GnRHs, as described in several studies (Zandbergen et al. 1995; Yamamoto et al. 1995; Parhar et al. 1996b). Consequently, the identity of the GnRHs produced by different cell populations could only be determined by means of *in situ* hybridization (Zandbergen et al. 1995; Parhar et al. 1996a; Parhar 1998; White and Fernald 1998). The presumed origin of GnRH fibers from cGnRH-II cells, however, was mainly concluded from their immunoreactive staining and their persistence after ablation of the terminal nerve ganglion (Yamamoto et al. 1995). We suggest that the latter has led to misinterpretations due to cross-reactivity, in spite of the careful experiments and considerations of the investigators. Also our electron microscopical and CLSM observations tend to support the absence of cGnRH-II axons, since we exclusively observed dendrite like processes originating from cGnRH-II cells.

The presumed absence of axons, added to the absence of significant excitatory synaptic input, raises the question whether cGnRH-II midbrain cells are, similar to basal forebrain GnRH cells, neurons, or represent a type of endocrine or paracrine cell located in the brain. The subcellular characteristics as monitored by electron microscopy allow for both interpretations. However,

endocrine cells with a diameter of 70 µm have not been observed in vertebrates until now, whereas neurons of this size are rather frequent, including, for example, the neurons of the nflm. The origin of cGnRH-II cells from the germinal zone of the third ventricle (Parhar et al. 1998; White and Fernald 1998) also suggests a neuronal origin. On the other hand, the absence of axons and the presumed secretion of products from the soma and the short processes of cGnRH-II cells, to be discussed below, would argue for an endocrine character of cGnRH-II cells.

In view of the presumed absence of axons of cGnRH II cells, including the absence of projections to the pituitary, it remains intriguing that cGnRH-II appears to be 100 times more potent to stimulate the pituitary than catfish GnRH (Schulz et al. 1993; Bosma et al. 2000). The present study strongly suggests that a direct (portal) vascular connection between cGnRH-II cells and the pituitary does not exist. Although the present study shows that there is consistently an identifiable vein transversing the cGnRH-II cell population, cGnRH-II cells do neither have a preferential location around this vein nor have specialized contact sites or release sites with this vein in cases of (presumed incidental) contact. Although this vein opens into the sinuses that surround the pituitary, there is no indication of any special functional connection between the pituitary and this sinus, other than that the pituitary veins end into this sinus, similar to the cGnRH-II passing vein. Of course, it remains well possible that cGnRH-II diffuses into the vascular system via the extracellular spaces of the subventricular reticulum (see also below). ChickenGnRH-II can reach the pituitary ultimately after having passed the body circulation, but we could not find a morphological substrate for a more direct and specialized contact between cGnRH-II cells in the midbrain and the pituitary.

Do cGnRH-II cells release their products into the surrounding extracellular spaces?

A striking feature in our light microscopical material was the relation of cGnRH-II cells with dilated extracellular spaces, which were larger and more extending in catfish with larger cGnRH-II cells (Cl 4). The fact that these spaces were absent in our EM material might be due to considerable shrinkage and disappearance of extracellular spaces by using glutaraldehyde to improve ultrastructure. In contrast, perfusion and fixation with Bouin fixative preserves the dimensions and cellular relations almost precisely as shown in our LM material.

The relation of cGnRH-II cells with extracellular spaces and the correlation of their sizes strongly suggest secretion of cGnRH-II and maybe other products into the dilated extracellular spaces of the synencephalon. However, the mechanisms involved in such a secretion are unclear. In the EM we did not observe any sign of exocytosis or accumulation of dense cored vesicles along

the surface membrane. In the LM, we observed features that suggest the dissolution and secretion of large parts of the peripheral dendrites or peripheral cellular cytoplasm as a whole into the dilated extracellular spaces (Fig. 4). Such a process also occurs in e.g. secretory cells lining breast cysts, where it has been indicated as apocrine secretion (Malatesta et al. 1998). ChickenGnRH II probably diffuses into the ventricle, from where it can be transported to other brain regions. In addition, some cGnRH-II might move into the blood vessels traversing the subventricular reticulum, in order to influence the pituitary and/or other tissues in the periphery.

The peripherally located RER might well play a role in restoration of the cellular membrane and protection of the cytoplasm from damage during secretion of large parts of the granular cytoplasmatic compartment. Membrane formation and restoration is a function generally attributed to RER. In contrast, the perinuclear RER probably plays a role in the translation of GnRH and associated proteins. A nuclear indentation filled with RER, as observed in cGnRH-II cells, is also observed in e.g. Purkinje cells (Monteiro et al. 1992) and in the TN GnRH neurons of a salmonid (Chiba et al. 1996b Oka and Ichikawa 1991) and suggest a high degree of transscriptional and translational activity. This is in accordance with the strong *in situ* hybridization signal for cGnRH-II (Zandbergen et al. 1995). Irregularly shaped nuclei with deep indentation and the absence of heterochromatin have also been described in apocrine breast cells and indicate a high metabolic rate (Malatesta et al. 1998).

By considering the morphological variability observed at different stages in the life of a cGnRH-II cell, the following picture emerges: cGnRH-II cells gradually grow from about 12 μm in diameter in 6 week-old fish (5 cm in length; Dubois et al. 1998) via 16.5 μm in diameter at a fish length of 10 cm (Cl 1 of the present study) to about 20μm in diameter in 12 week-old fish of 12 cm long (Dubois et al. 1998). At the age of 8 month (which is related to a body size of about 25 cm in our lab conditions) their diameter is still about 20 μm and somewhat larger in males than in females (present study; about 22 μm and 18 μm, respectively). At this stage, the extracellular spaces start to develop together with, or perhaps as the result of secretion of cGnRH II. In still larger, thus presumably older fish (Cl 4 of the present study) or under certain circumstances (Cl 2 of the present study) the production and secretion of cGnRH may dramatically increase under the influence of until now unknown circumstances.

Do nflm cells excite cGnRH-II cells?

The nucleus of the fasciculus longitudinalis medialis (nflm) is the most rostral nucleus of a diverse set of brainstem nuclei that project to the spinal cord (Hlavacek et al. 1984; Oka et al. 1986; Prasado Rao et al. 1987; Behrend and Donicht 1990; Meek and Nieuwenhuys 1998). It is present in all vertebrates

and consistently located in the ventral part of the synencephalon or first prosomere (Meek and Nieuwenhuys 1998; Nieuwenhuys 1998). ChickenGnRH-II cells are consistently located dorsomedial to the nflm in all vertebrates, and consequently this phylogenetically old synencephalic couple is well conserved throughout the evolution of vertebrates. The present study suggests that they are not incidentally colocalized in the synencephalon, but have a functional interrelationship, since they have specialized dendro-somatic appositions.

The appositions between cGnRH-II cells and nflm processes do not show signs of synaptic specializations, such as membrane thickenings or synaptic vesicles. In contrast, they show no other features than a very close proximity over extensive areas, with some subsurface lamellae. Exactly the same type of apposition is present between neighboring cGnRH-II cells. We suggest that these appositions establish ephaptic contacts between the apposing elements. An ephaptic contact allows for mutual influences of excitable membranes by means of electric field effects (Faber and Korn 1989; Jefferys 1995; Han et al. 2000; Meek et al. 2000). In this way, GnRH cells might influence nflm cells or vice versa. We presume that nflm dendrites excite cGnRH-II cells, since other stimulatory influences on cGnRH-II cells, either synaptic or humoral, have not been observed or described until now. In contrast, nflm cells are richly innervated and integrate a variety of inputs (Meek and Nieuwenhuys 1998) and the present study). Conceivably, they do not only integrate these inputs to influence the spinal cord, but also to regulate the activity of cGnRH-II cells.

The function of the nflm and its spinal projection is completely unknown, and consequently we can only speculate about the conditions under which the nflm might excite or trigger cGnRH-II cells. Their relation with cGnRH-II cells might suggest a function in the regulation of sexually related motor behavior or, conversely, the inhibition of undesired movements during sexual behavior. It is clear that more knowledge is necessary about the afferent and efferent connections and the function of the nflm before a hypothesis can be developed concerning the presumed influence of the nflm on the activity of cGnRH-II cells.

Concluding remarks (functional considerations)

On the basis of our observations and the discussion above, we have developed the following hypothesis: cGnRH-II cells have no axons, but release their products in the surrounding extracellular spaces of the subventricular synencephalic reticulum, and are stimulated by nflm cells via dendro-somatic appositions.

To date, we do not yet know the function cGnRH-II in the brain of catfish or other vertebrates. The presumed release into extracellular spaces suggests paracrine functions in the regulation of brain regions in the nearby environment of cGnRH-II cells, including the nflm. However, by diffusion into the

ventricular fluid other brain regions may be influenced as well, while diffusion into the blood stream allows for influences on peripheral organs, including the pituitary. The high sensitivity of pituitary GTH cells for very low concentrations of cGnRH-II suggests that the pituitary indeed is also regulated by cGnRH-II. Presumably, cGnRH-II plays a role in the correlated regulation of behavioral as well as (neuro)endocrine aspects of sexual behavior, but further research is necessary to unravel the precise functional mechanisms and significance of cGnRH-II release in vertebrates