Development of gonadotropin-releasing hormone systems in the male African catfish, *Clarias gariepinus*

Ontwikkeling van gonadotropin-releasing hormone systemen in de mannelijke Afrikaanse meerval, *Clarias gariepinus*

(With a summary in English)

(Met een samenvatting in het Nederlands)

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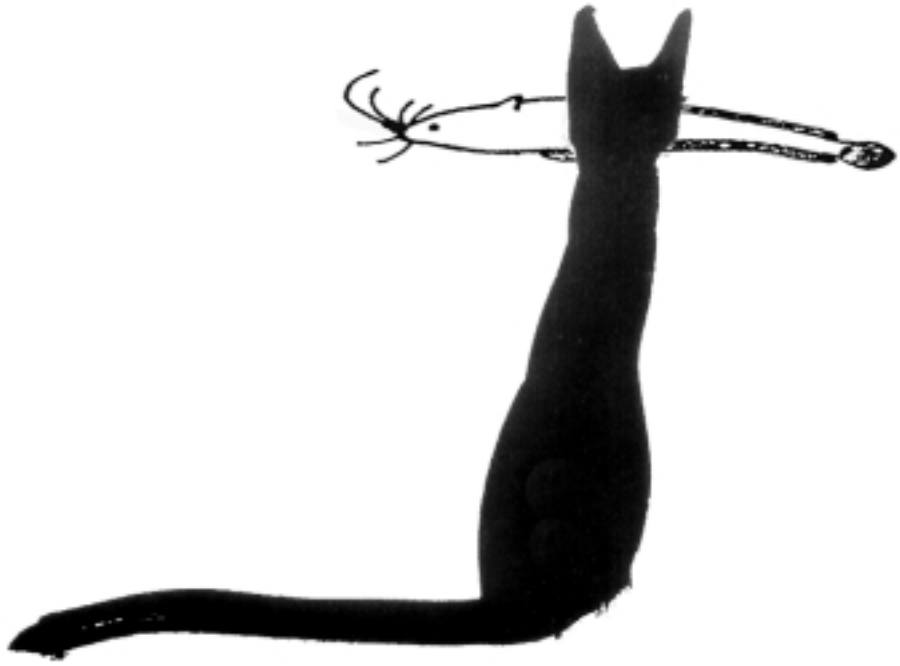


Non dico mai bugie;
ma la veritá non a tutti
Pietro Sarpi

Contents

Summary

Reproductive processes are mainly regulated by the brain-pituitary-gonad axis (BPG-axis). Gonadotropin-releasing hormone (GnRH) neurons localized in the brain release their hormone GnRH, which allows the release of gonadotropic hormone by gonadotropic cells in the pituitary. Gonadotropic hormone, in turn, regulates the production of sex steroids and germ cells in the gonads. The steroids complete the dynamic BPG-axis by exerting feedback effects at the level of the brain, pituitary and gonads.

The transition period of a juvenile, inactive axis towards a mature, functional system is known as puberty. Several concepts deal with the question how the onset of puberty is regulated. The prevailing "missing-link" concept assumes that one or more components of the axis are absent or not functional before puberty. For our animal model, the African catfish, *Clarias gariepinus*, it has been proposed that sex steroids initiate and/or accelerate pubertal development. However, based on earlier findings, it was suggested that the "missing link" could also be localized at a higher level of the BPG-axis, i.e. at the GnRH system in the brain.

Most vertebrates express two forms of GnRH: a species-specific form and cGnRH-II. The latter is identified in all vertebrates and is well conserved throughout evolution. The species-specific GnRH with a hypophysiotropic function, varies amongst species, but represents a separate lineage in GnRH genealogy. The African catfish carries two forms of GnRH in the brain: catfishGnRH (cfGnRH) in neurons dispersed over the entire ventral forebrain, from the olfactory bulb till the pituitary and cGnRH-II expressed by cells in the midbrain.

In order to test the hypothesis that sex steroids and/or a functional GnRH system are important determinants in the onset of puberty in the African catfish, we studied the normal development of the GnRH system in the brain and the effects of certain steroids on this development. Our results revealed that the cfGnRH system in the ventral forebrain achieves its morphological adult status just at the onset of puberty, probably after a migratory route, which originates in the olfactory placode, similar as in other vertebrates. The innervation of the gonadotropic cells in the pituitary by cfGnRH fibers

is, however, not completed before the end of puberty. A second population of neurons also expressing cfGnRH was identified in the most rostral region of the telencephalon, in the terminal nerve ganglion (TN). Because of its different morphology, later appearance during development and the absence of a connection with the pituitary, the TN population was considered as a separate category of cfGnRH neurons. In modern fish species the TN population expresses a third form of GnRH, which also favors a specific functional identity.

In contrast to the cfGnRH system in the ventral forebrain, the cGnRH-II system displays an adult pattern already before the onset of puberty and, moreover, has no morphological connection with the pituitary. Nevertheless, cGnRH-II is a potent releaser of gonadotropic hormone *in vitro*. In order to understand more of the functional significance of the cGnRH-II system, we studied its structural properties and neuroanatomical environment. Microscopical studies revealed that cGnRH-II cells in the midbrain have no axons and are scarcely innervated by neurons of the nucleus fasciculus longitudinalis medialis. Furthermore, the cGnRH-II cells showed many characteristics of high metabolic activity, but clear evidence for cGnRH-II release could not be obtained, despite their close apposition to capillaries. On the other hand, cGnRH-II cells display striking contacts with dilated extracellular spaces in the subventricular reticulum, suggesting a release into the cerebrospinal fluid.

Since only the ventral forebrain cfGnRH system is directly connected with the pituitary, steroid effects on this system were investigated. For this study we selected three developmental periods: the immature, juvenile period (2-6 weeks of age), the onset of puberty (10-12 weeks of age) and the actual pubertal period (12-17 weeks of age). It was found that testosterone (T) stimulated the immunoreactivity and the peptide content of cfGnRH neurons during these three periods. In addition, in juvenile fish T treatment also resulted in a significant increase in the number of cfGnRH neurons. Other sex steroids, like 11 β-hydroxyandrostenedione (OHA) or 11-ketotestosterone (11KT), which are strong stimulators of testicular development and spermatogenesis, did not (or hardly) affect the cfGnRH system in the African catfish. The development of the cGnRH-II system in the midbrain remained unchanged after T administration.

The effects of T on the cfGnRH system are probably mediated via its aromatization product estradiol (E_2), similarly as T exerts its stimulatory effect on the gonadotropic cells. Indeed, aromatase, the enzyme responsible for the conversion of T into E_2 is present in the brain areas where cfGnRH neurons are localized.

The present thesis shows that specific sex steroid (T and E_2) are important for the recruitment of cfGnRH neurons and for the increase in the

cfGnRH content in brain and pituitary by stimulating the cfGnRH production and/or by inhibiting its release. Thus, in the African catfish, T is a serious candidate for the "missing-link", since it is required for activating the cfGnRH neurons in the preparation for the onset of puberty.

Another candidate for the "missing-link" is the actual released amount of cfGnRH that is available for the gonadotropic cells at the onset of puberty. It has been demonstrated *in vitro* that gonadotropic cells are able to respond adequately upon GnRH stimulation at this stage. Moreover, pituitary incubation studies revealed that suitable amounts of endogenous cfGnRH are present in the axon terminals. We hypothesize that functional contact between the first cfGnRH terminals in the pituitary and the gonadotropic cells is required for the initiation of puberty. Once this switch is turned on, the three levels of the BPG-axis simultaneously display their maturational processes: innervation of the pituitary by cfGnRH fibers, development of gonadotropic cells and the first wave of spermatogenesis.

Abbreviations

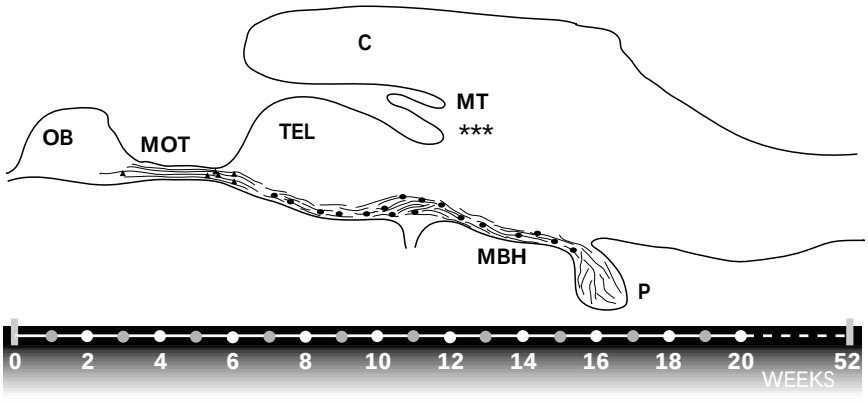
A list of the commonly used abbreviations

cf-:	catfish
cI-:	chicken-I
cII-:	chicken-II
CLSM:	confocal laser scanning microscopy
df-:	dogfish
E_2:	estradiol
Flm:	fasciculus longitudinalis medialis
GAP:	gonadotropin-releasing hormone associated peptide
GnRH:	gonadotropin-releasing hormone
gp-:	guinea pig
GTH:	gonadotropic hormone
h-:	herring
ir:	immunoreactive
(11)KT:	11-ketotestosterone
L-:	lamprey
LH:	luteinizing hormone
m-:	mammalian
MB:	midbrain
MBH:	medial basal hypothalamus
md-:	medaka
MOT:	medial olfactory tract
MT:	midbrain tegmentum
NAP:	nucleus anterioris periventricularis
Nflm:	nucleus of the fasciculus longitudinalis medialis
NIL:	neurointermediate lobe
NPP:	nucleus preopticus periventricularis
OB:	olfactory bulb
OHA:	11β-hydroxyandrostenedione
P:	pituitary
POA:	pre-optic area

PPD:	proximal pars distalis
r-:	rana
RER:	rough endoplasmatic reticulum
RIA:	radioimmunoassay
s-:	salmon
sb-:	seabream
SEM:	standard error of the mean
T:	testosterone
t-:	tunicate
Tel:	telencephalon
TN:	terminal nerve
Vv:	area ventralis of the ventral telencephalon

Chapter 1

Introduction



Introduction

Reproduction is a fascinating and complex biological process, essential for the survival of each species. At every conception, sets of genes are recombined to form a new and unique individual. Sexual reproduction requires adequate behavioural and physiological preparation of both future parents. Without synchronised gamete formation, partner recognition, and relevant sexual behaviour, reproduction is bound to be unsuccessful. Moreover, optimal environmental conditions such as food availability, season, temperature are required for raising offspring. Neural, neuro-endocrine and endocrine networks integrate the internal and external cues into a co-ordinated action of the reproductive system in both partners.

The brain-pituitary-gonad axis (BPG-axis) is the physiological system that is most directly involved in the control of reproduction in vertebrates (Fig. 1). At the level of the brain neurons are present which produce the neuropeptide gonadotropin-releasing hormone (GnRH). GnRH reaches the gonadotropic cells either indirectly via the portal system (in tetrapods) or directly near the axon endings (in most teleosts). In the pituitary, GnRH stimulates the synthesis and release of the gonadotropic hormones FSH (follicle stimulating hormone) and LH (luteinizing hormone). Both hormones are secreted into the bloodstream and transported to the gonads, where they fulfil two main functions: stimulation of synthesis and release of gonadal hormones and formation of gametes. The gonadal hormones are responsible for germ cell development, but they also control the regulation of the reproductive axis, by exerting feedback actions on the three levels of the axis. Whether this feedback is positive or negative mainly depends on the maturational and functional state of the axis.

The difference between a juvenile and an adult in terms of reproductive capacity is based on the developmental status of the BPG-axis. The period of transition of the juvenile, inactive BPG-axis into a functionally active axis is known as pubertal development or puberty. The endocrine control of the BPG axis maturation is only partly understood (Schulz and Goos 1999).

Several hypotheses are related with the endocrine control of pubertal development. One hypothesis, i.e. the “missing link concept” (Goos 1993) is based on the assumption that before puberty part of the BPG axis is still not

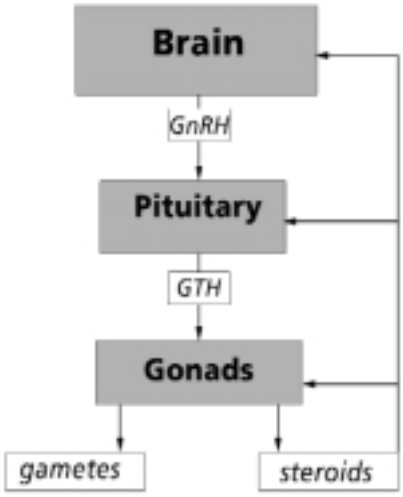


Fig. 1 Schematic representation of the brain-pituitary-gonad axis. In grey the organs, in white the hormones produced by each level of the axis, and the gametes. GnRH: gonadotropin-releasing hormone, GTH: gonadotropic hormone.

functional or missing. Another hypothesis, the so-called gonadostat concept (Olster and Foster 1986; Tang et al. 1997), can be considered as a specific interpretation of the "missing-link" concept. This second hypothesis states that the GnRH system in the brain of juveniles is inhibited by gonadal steroids. Pubertal development starts when the GnRH system looses its sensitivity for the negative feedback of the steroids; subsequently, GnRH is released and in turn triggers the maturation of the pituitary and gonads. The gonadostat concept was mainly designed for mammals, including humans, and focuses on the initiation of the pulsatile release of GnRH at the start of puberty. A third hypothesis can also be considered as a modification of the missing-link concept and is mostly applied to fish, but may be valid for other vertebrates as well. This hypothesis states that pre-pubertal gonadal steroids exert a morphogenetic action on several parts of the BPG-axis, thus initiating and/or accelerating the onset of puberty (Xiong et al. 1994; Amano et al. 1997). So, actually the steroids are considered as missing links in this hypothesis.

Based on earlier studies by the research group for Comparative Endocrinology (Cavaco et al. 1998b; Schulz and Goos 1999) we have adopted the concept of morphogenetic action by gonadal steroid hormones. It was shown that gonadal steroid hormones indeed stimulate testicular function and the gonadotropic capacity of the pituitary in the male African catfish (*Clarias gariepinus*). Moreover, it was demonstrated that in this species each of the gonadal steroid hormones has its own domain of action within the hypophyseal-gonadal axis (for review, see Cavaco 1998). In the present thesis the effects of testicular steroid hormones on the developing GnRH system in male African catfish are described.

GnRH: the forms and the gene

The decapeptide GnRH is of great importance in all vertebrates, as it forms an essential link in the integration of the external and internal stimuli in the control of reproduction. The brain integrates the available information and transfers the signal to the GnRH neurons when to release their GnRH to allow an adequate response by the reproductive axis. Since GnRH plays this pivotal role in all vertebrates, it is well understandable that the peptide is highly conserved throughout evolution.

The first reports about the existence of a GnRH-like substance date from 1971 (Matsuo et al. 1971; Amoss et al. 1971; Baba et al. 1971). Since this neuropeptide was first identified in mammals (pig and sheep), it is now known as mammalianGnRH (mGnRH). The original name was luteinizing hormone releasing hormone (LHRH), referring to its stimulatory effect on LH release. We now know that also FSH release is stimulated by the peptide, hence its more general name GnRH. Until now 15 forms of GnRH have been identified in various vertebrates and in a protochordate (Table I). There is some debate about the nomenclature of GnRHs, but most commonly, all GnRHs are named after the species in which they were first discovered. Six GnRHs were isolated from fish species: salmonGnRH (sGnRH; Sherwood et al. 1983), catfishGnRH (cfGnRH; Bogerd et al. 1994), dogfishGnRH (dfGnRH; Lovejoy et al. 1992), seabreamGnRH (sbGnRH; Powell et al. 1994), herringGnRH (hGnRH; Carolsfeld et al. 2000), and medakaGnRH (mdGnRH; Okubo et al. 2000a). Primitive species as the lamprey and the protochordate *Ciona intestinalis* have their own forms of GnRH: lampreyGnRH I and III (Sherwood et al. 1986; Sower et al. 1993) and tunicateGnRH I and II respectively (Powell et al. 1996; Di Fiore et al. 2000). ChickenGnRH I (cGnRH-I; King and Millar 1982) and chickenGnRH-II (cGnRH-II; Miyamoto et al. 1984) were both first characterised in chicken. Apart from the common mGnRH, recently the guinea pigGnRH (gpGnRH) was shown as a novel and alternative form of GnRH in this mammalian species (Jimenez-Linan et al. 1997). The most recent finding of a novel GnRH was made in an amphibian, the frog (*Rana dybowskii*): ranaGnRH (rGnRH; Yoo et al. 2000).

When comparing the amino acid sequences of the different GnRHs (Sherwood 1987), it appears that positions 1, 4, 9 and 10 are conserved, suggesting their importance for the biological function of the peptide (Table I). Proline and glycine at the C-terminus protect the hormone against degradation, whereas the stability of the GnRH conformation is mainly due to histidine and proline at positions 2 and 9, respectively. The assumed β-turn within the peptide is thought to be between positions 5 and 6. The most variable positions 5 till 8 play a role in receptor binding (Blomenröhr 2000).

Table I Amino acid sequences of the 15 different GnRH forms. Amino acids listed in bold differ from the cGnRH-II sequence.

GnRH	1	2	3	4	5	6	7	8	9	10	
Chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2	(Miyamoto et al. 1984)
Mammalian	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Arg**	Pro	Gly-NH_2	(Matsuo et al. 1971; Baba et al. 1971)
Rana	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Trp**	Pro	Gly-NH_2	(Yoo et al. 2000)
Catfish	pGlu	His	Trp	Ser	His	Gly	**Leu**	**Asn**	Pro	Gly-NH_2	(Bogerd et al. 1994)
Salmon	pGlu	His	Trp	Ser	**Tyr**	Gly	**Trp**	**Leu**	Pro	Gly-NH_2	(Sherwood et al. 1983)
Herring	pGlu	His	Trp	Ser	His	Gly	**Leu**	**Ser**	Pro	Gly-NH_2	(Carolsfeld et al. 2000)
Medaka	pGlu	His	Trp	Ser	**Phe**	Gly	**Leu**	**Ser**	Pro	Gly-NH_2	(Okubo et al. 2000a)
Seabream	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Ser**	Pro	Gly-NH_2	(Powell et al. 1994)
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	**Leu**	Pro	Gly-NH_2	(Lovejoy et al. 1992)
Chicken I	pGlu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Gln**	Pro	Gly-NH_2	(King and Millar 1982)
Guinea pig	pGlu	**Tyr**	Trp	Ser	**Tyr**	Gly	**Val**	**Arg**	Pro	Gly-NH_2	(Jimenez-Linan et al. 1997)
Lamprey I	pGlu	His	**Tyr**	Ser	**Leu**	**Glu**	Trp	**Lys**	Pro	Gly-NH_2	(Sower et al. 1993)
Lamprey III	pGlu	His	Trp	Ser	His	**Asp**	Trp	**Lys**	Pro	Gly-NH_2	(Sower et al. 1993)
Tunicate I	pGlu	His	Trp	Ser	**Asp**	**Tyr**	**Phe**	**Lys**	Pro	Gly-NH_2	(Di Fiore et al. 2000)
Tunicate II	pGlu	His	Trp	Ser	**Leu**	**Cys**	**His**	**Ala**	Pro	Gly-NH_2	(Di Fiore et al. 2000)

The GnRH gene consists of 3 introns and 4 exons (Fig. 2). The second, third and part of the fourth exon encode for the pre-pro-hormone, which contains a signal peptide (21-23 amino acids), the GnRH itself (10 amino acids), a cleavage site (Gly-Lys-Arg) and the GnRH-associated peptide (GAP, 40-60 amino acids) (King and Millar 1992; King and Millar 1997). The sequence of the second exon is the most conserved, while the other exons show high variability. As a consequence, the signal peptides and the GnRHs are well conserved, but the GAPs show less homology amongst species.

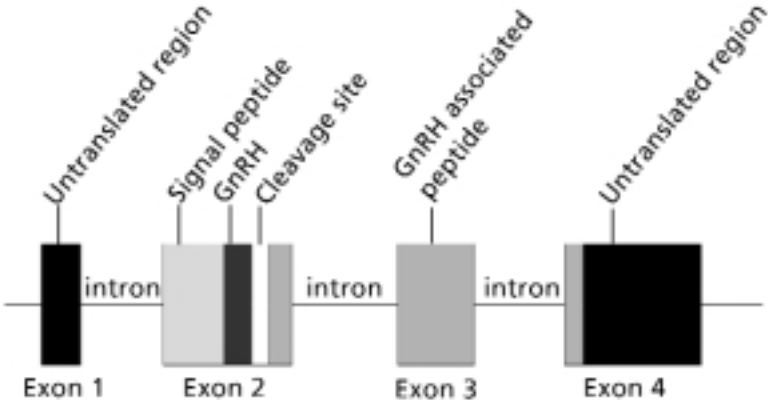


Fig. 2 Schematic representation of the GnRH gene, consisting of 4 exons and 3 introns. The translated region consists of the signal peptide, GnRH, a cleavage site, and the GnRH-associated peptide.

GnRH: phylogeny and evolutionary aspects

The phylogenetic distribution of GnRHs is extensively investigated, as shown in Table II. In all classes of vertebrates and in a few invertebrates the presence of certain GnRH forms was established. Without any doubt GnRH is an old and well-conserved peptide, since it already controls reproductive functions in molluscs (Zhang et al. 2000) and protochordates (King and Millar 1992; Fernald and White 1999).

In general, all investigated species to date possess 2 or 3 different forms of GnRH (Table II). The most conserved form of GnRH is cGnRH-II and it coexists in all classes of vertebrates from the Chondrichtyes onwards, together with a species-specific GnRH and a possible third form. The two or three forms of GnRH coexisting in one species are transcribed from different genes. The species-specific forms vary e.g. cGnRH-I in birds, hGnRH in herring, dfGnRH in sharks, sGnRH in salmonids and mGnRH in most mammals. However, if a third form is present, as shown for "modern" fishes, it is always the sGnRH form (Table II).

As mentioned earlier, cGnRH-II is supposed to have appeared early in vertebrate evolution, and hence it is thought to represent the most conservative GnRH lineage (Table III) (King and Millar 1992). In addition, it is hypothesised that a second, variable lineage exists with mGnRH, cGnRH-I and the "fish" GnRHs (cfGnRH, sGnRH and dfGnRH; King and Millar 1992). The presence of two or more forms of GnRH within one species suggests that both lineage's are probably derived from gene duplication in early vertebrate evolution (King and Millar 1992; Sherwood et al. 1993; Montero and Dufour 1996). Moreover, the similar architecture of the GnRH genes is important evidence for the gene duplication hypothesis (Parhar 1999). The GnRHs from lamprey and tunicate do not clearly fit in the two lineage's model (Millar et al. 1997).

GnRH: localisation and origin

In general, all vertebrate species have at least two forms of GnRH (cGnRH-II and the species-specific GnRH), or even three forms as discovered in modern fish. The two or three GnRH forms not only are differentially localised in the brain (Fig. 3); they have different embryonic origins as well.

The cells that express cGnRH-II are clustered in a distinct nucleus at the fusion site of the anterior midbrain and posterior diencephalon (synencephalon). The ventricular ependyma or germinal zone is the putative embryonic origin of these cells, since they first appear in this region in the developing brain (Parhar 1998; White and Fernald 1998; Parhar 1999).

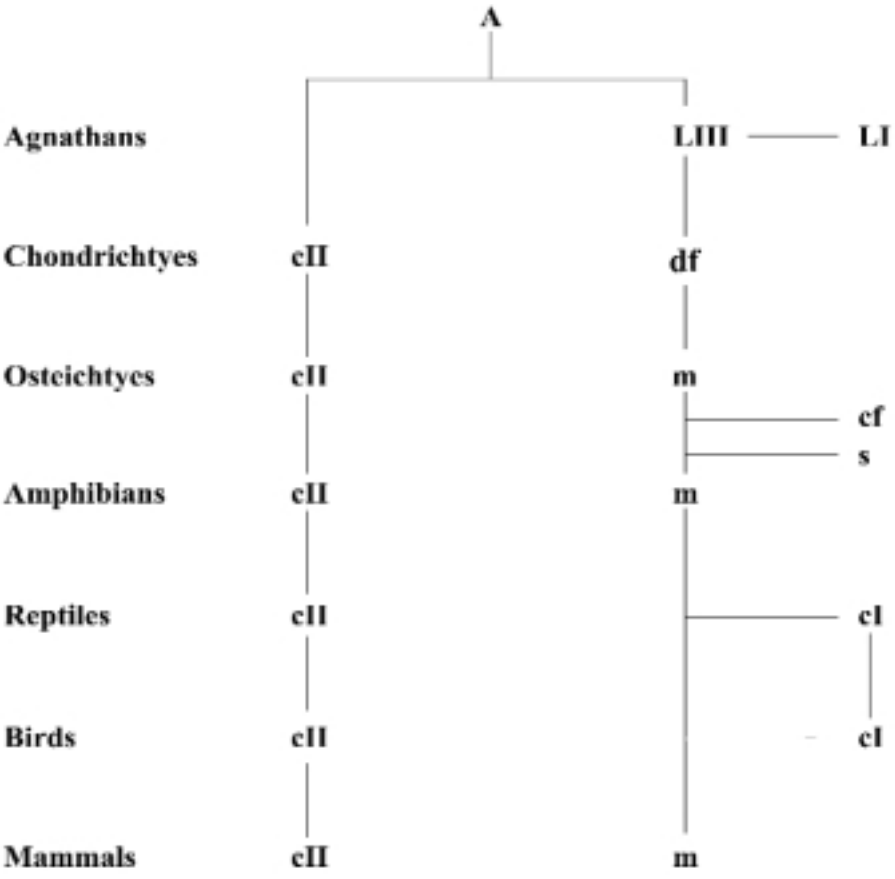
The species-specific GnRH in species carrying only two forms is localised

Table II Phylogenetic distribution of different GnRH forms. cII: chickenGnRH-II; df: dogfishGnRH; m: mammalianGnRH; h: herringGnRH; cf: catfishGnRH, s: salmonGnRH; md: medakaGnRH; sb: seabreamGnRH; r: ranaGnRH; gp: guinea pigGnRH; cI: chickenGnRH-I; t: tunicateGnRH (I and II) and L: lampreyGnRH (I and III).

		cII	df	m	h	cf	s	md	sb	r	gp	cI	t	L
Invertebrates														
Mollusk	Zhang et al. 2000			m?									tI?	
Aplysia californica														
Protochordate														
Tunicate	Di Fiore et al. 2000			m?									tI+II	
Ciona intestinalis	Powell et al. 1996													
Agnatha														
Lamprey	Sower et al.1993													LI+III
Petromyzon marinus	Sherwood et al. 1986													
Hagfish	Sower et al. 1995													LIII
Myxine glutinosa														
Chondrichtyes														
Ratfish	Lovejoy et al. 1992	cII												
Hydrolagus colliei														
Atlantic stingray	Forlano et al. 2000	cII	df											LIII?
Dasyatis sabina														
Dogfish	Lovejoy et al. 1992	cII	df											
Squalus acanthias														
Sarcopterygii														
Lungfish	King et al. 1995	cII		m										
Protopterus annectens														
Actinopterygii														
Sturgeon	Sherwood et al. 1991	cII		m										
Acipenser transmontanus														
Reedfish	Sherwood et al. 1991	cII		m										
Calamoichthys calabaricus														
Eel	King et al., 1991	cII		m										
Anguilla anguilla														
Herring	Carolsfeld et al. 2000	cII			h									
Clupea harengus pallasi														
Sockeye salmon	Parhar et al. 1995	cII					s							
Oncorhynchus nerka														
Goldfish	Lin en Peter 1997	cII					s							
Carsasius auratus														
African catfish	Bogerd et al. 1994	cII				cf								
Clarias gariepinus														
Pacu	Powell et al. 1997	cII					s		sb					
Piaractus mesopotamicus														
Medaka	Okubo et al. 2000	cII					s	md						
Oryzias latipes														
Pejerry	Montaner et al. 2000	cII					s	md						
Odentesthes bonariensis														
Red seabream	Okuzawa et al. 1997	cII					s		sb					
Pagrus major														
Cichlid	White et al. 1995	cII					s		sb					
Haplochromis burtoni														
Tilapia	Parhar 1997	cII					s		sb					
Oreochromis mossambicus														
Amphibians														
Frog	Conlon et al. 1993	cII		m										
Rana ridibunda														
Newt	Muske and Moore 1994	cII		m										
Taricha granulosa														
Frog	Yoo et al. 2000	cII		m						r				
Rana dybowskii														

		cII	df	m	h	cf	s	md	sb	r	gp	cl	t	L
Reptiles														
Turtle	Sherwood and	cII										cl		
Pseudemys srcipta	Whittier 1988													
American alligator	Lovejoy et al. 1991	cII										cl		
Alligator mississippiensis														
Lizard	D'Aniello et al. 1994	cII										cl		
Podarcis s. sicula														
Birds														
Chicken	Dunn and Millam 1998	cII										cl		
Gallus domesticus														
Turkey	Millam et al. 1993	cII										cl		
Gallus meleagris														
Mammals														
Tree shrew	Kasten et al. 1996	cII		m										
Tupaia glis belangeri														
Guinea pig	Jimenez-Linan et al. 1997	cII									gp			
Macaque	Latimer et al. 2000	cII		m										
Macaca mulatta														
Human	White et al. 1998	cII		m										

Table III Hypothetical evolutionary scheme for GnRH genealogy with two main lineages: the GnRH-II and the mammalianGnRH lineage (Sherwood et al. 1997; King and Millar 1997). A: ancestral form, cII: chicken-II, LIII: lampreyIII, LI: lampreyI, df: dogfish, cf: catfish, s: salmon and cI: chicken-I.



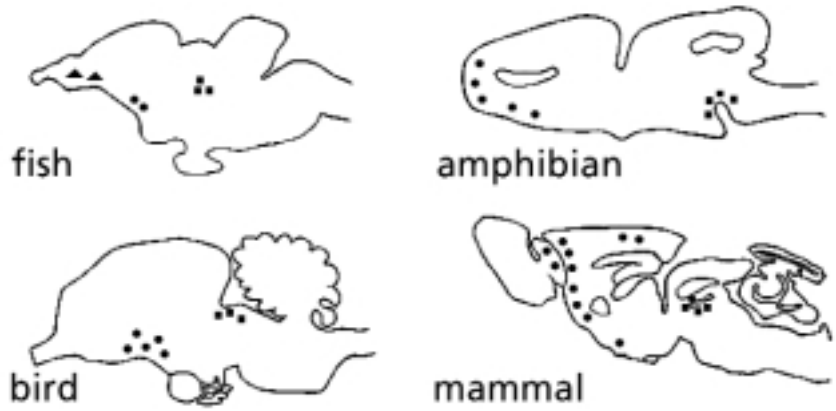


Fig. 3 Schematic drawings of GnRH localisation in the brain of a fish (White et al. 1995), an amphibian (Muske 1993), a bird (Mikami et al. 1988) and a mammal (Rissman et al. 1995). The species specific GnRH or GnRH1 (●) is localised in the ventral forebrain; cGnRH-II or GnRH2 (■) is situated in the midbrain area; salmonGnRH neurons (▲) are present in the terminal nerve of some teleosts.

in the ventral forebrain, mostly restricted to the pre-optic area (POA), basal hypothalamus, and pituitary. Because neurons of this system were also observed during development in the terminal nerve (TN) and the olfactory bulb (OB), it has been suggested that the neurons originate from the olfactory region. Moreover, a rare disease, the Kallmann's syndrome which is characterised by hypogonadotropic hypogonadism combined with the disability to smell, suggested an ontogenetic liaison between the olfactory system and reproductive brain areas (Schwanzel-Fukuda et al. 1992; Parhar et al. 1995b; Quinton et al. 1996). The syndrome is caused by a failure in the olfactory bulbs, impairing the outgrowth of the olfactory nerve into the brain. It was hypothesised that the GnRH neurons of this system originate from the olfactory placode and that they migrate during development into the brain in the direction of the hypothalamus and the pituitary. Many studies monitored the migration of the GnRH neurons during early development in all classes of vertebrates: salmon (Chiba et al. 1994; Parhar et al. 1995a; Amano et al. 1998) and platyfish (*Xiphophorus maculatus*; Halpern-Sebold and Schreibman 1983), various frog species (Muske and Moore 1990; Di Fiore et al. 1996), lizard (D'Aniello et al. 1994), chicken (Sullivan and Silverman 1993), rat (Jennes 1989) and rhesus macaque (Ronnekleiv and Resko 1990). Murakami et al. (1992) provided firm evidence for this hypothesis by showing the absence of GnRH neurons in the brain after ablation of the olfactory placode.

Some neurons stop their migration earlier than others, resulting in a scat-

tered rostro-caudal distribution of neurons over the entire migratory pathway (OB, TN, ventral telencephalon, pre-optic area, and medial basal hypothalamus) (Schwanzel-Fukuda and Pfaff 1989; Schwanzel-Fukuda and Pfaff 1990; Schwanzel-Fukuda 1999). The distribution of the GnRH neurons over the ventral forebrain can be scattered like in eel (Montero et al. 1994), catfish (Zandbergen et al. 1995) and salmon (Amano et al. 1997), or clustered in separate nuclei as seen in the mammalian (King and Anthony 1984), the avian (Dunn and Millam 1998) and the frog brain (Conlon et al. 1993).

Quanbeck et al. (1997) characterised early and late migrating GnRH neurons in the rhesus monkey. Both groups originate from the olfactory placode, but they differ in timing of migration, morphology and final destination. Functionally different GnRH sub-populations could also be distinguished on the basis of differential regulation by steroids, (Amano et al. 1994; Herbison 1998) or by photoperiod (Amano et al. 1995) .

One GnRH sub-population, called the TN ganglion, is localised at the junction of the olfactory nerve and the telencephalon and is present in most vertebrates. This population has no hypophysiotropic function, but it is probably involved in reproductive behaviour in relation to olfaction (Yamamoto et al. 1997).

In teleosts with three different forms of GnRH i.e., the sea bream (*Sparus aurata*; Powell et al. 1994), tilapia (*Oreochromis mossambicus*; Parhar 1998), pacu (*Piaractus mesopotamicus*; Powell et al. 1997, and the African cichlid *(Haplochromis burtoni*; White et al. 1995), cGnRH-II, sbGnRH and sGnRH are localised in respectively the midbrain, the POA/hypothalamus, and the terminal nerve. Thus, the species-specific form is restricted to the POA. The neurons producing the species-specific GnRH innervate the pituitary and thus are functionally connected with the neuroendocrine control of reproduction. It is not clear yet whether both the TN and the POA/hypothalamus neurons are derived from the olfactory placode. Parhar et al. (Parhar 1998; Parhar et al. 1998) hypothesised that indeed the GnRH neurons in the terminal nerve arise from the olfactory placode, whereas GnRH neurons in the POA originate in the basal telencephalon.

With the appearance of a third form in the brain, coexisting with cGnRH-II and the species-specific form, the terminology of the forms and systems has become rather complex. Therefore Fernald and White (1999) proposed a new nomenclature. GnRH1 is the species-specific form and regulates pituitary LH release; GnRH2 is the conserved cGnRH-II in the midbrain. If sGnRH is present in the terminal nerve, it is referred to as GnRH3. In the present thesis, however, the original nomenclature (cfGnRH and cGnRH-II) will be used, since the recent alternative is still poorly accepted in literature.

GnRHs: functions and targets

The gonadotropic cells in the pituitary are the main targets of the species specific GnRH in the ventral forebrain. The pituitary is an endocrine gland apposed to and morphologically connected with the ventral diencephalon and consisting of a nervous and an endocrine part. This organ plays a.o. an important role in development and function of gonads, thyroid and adrenals. In teleosts, the gonadotropic cells are situated in the proximal pars distalis (PPD), together with TSH cells and somatotrophs.

The anatomical basis for hypothalamic control, i.e. how the releasing hormones reach their target cells in the pituitary, differs between tetrapods and fish. In tetrapods, hypothalamic neurons have their synaptic endings on the portal vessels in the median eminence, whereas in salmonids hypothalamic axons terminate on the basal membrane between the neurohypophysis and the partes intermedia and distalis (Parhar and Iwata 1994). In other teleosts the endocrine cells in the pituitary are directly innervated by hypothalamic fibers (Peter et al. 1990), but some doubt exists as to the true synaptic nature of the GnRH axon - gonadotropic cell contact (Peute et al. 1984; Peute et al. 1987).

Apart from the gonadotrophs, the GnRH axons may have more targets in the fish pituitary. GnRH has been shown to control the secretory activity of the growth hormone and the somatolactin cells in trout (*Oncorhynchus mykiss*; Parhar and Iwata 1994) and in goldfish (*Carassius auratus*; Marchant et al. 1989). In tilapia it has been demonstrated that also prolactin cells are under GnRH regulation (Weber et al. 1997), as are the thyroid stimulating hormone cells in carp (Roy et al. 2000).

An effective approach for studying the target sites of GnRH is by localising the expression of its receptor. The GnRH receptor (GnRH-R) is G-protein-coupled and characterised by 7 transmembrane helices. The presence of two or three forms of GnRH in a given species supposes the existence of as much different receptors (King and Millar 1997; Troskie et al. 1998). Indeed, two different forms of GnRH-R were cloned and identified in goldfish (Illing et al. 1999), the African catfish (Blomenröhr 2000) and zebrafish (Troskie et al. 1998), but only one GnRH-R was cloned from the eel pituitary (Okubo et al. 2000b). The different GnRH-Rs in a species are encoded by different genes that are apparently expressed independently from each other. GnRH-Rs are expressed not only in the pituitary, but also in a wide variety of organs such as various brain regions, ovary and testis, but also in liver, eye and olfactory epithelium (Bogerd, personal communication). The function of GnRH in these organs is, however, mostly unknown.

To date, the presence of GnRH-Rs in the gonads and the possible function of GnRH in testis and ovary is subject of many investigations. GnRH-

receptor expression was found in the testes of the eel (Okubo et al. 2000b), in the testes and ovaries of the goldfish (Yu et al. 1998) and in the ovaries of the rat (Kogo et al. 1999). Recent studies also revealed local expression of GnRH in the gonads (Yu et al. 1998; Schalburg and Sherwood 1999; Schalburg et al. 1999). Therefore, GnRH could fulfil an autocrine or paracrine function in the gonads (Pati and Habibi 1998; Pati and Habibi 2000).

The presence of cGnRH-II in all vertebrate classes implies an important function, which is still not well determined. A role as neurotransmitter or neuromodulator was suggested (King and Millar 1992; Montero and Dufour 1996), but there are also indications that cGnRH-II is involved in the regulation of sexual behaviour (Maney et al. 1997; Volkoff and Peter 1999).

Although cGnRH-II under experimental conditions is able to release LH/FSH from the pituitary (and usually it is even more potent than the species specific GnRH), its physiological relevance is still unclear. Moreover, for a number of species it could not be demonstrated if and how cGnRH-II reaches the pituitary. In birds and mammals no hypophyseal innervation by cGnRH-II fibers was observed (Dellovade et al. 1993; Dunn and Millam 1998). In some teleosts, however, cGnRH-II has been shown to be present in the pituitary, although in small amounts (Yu et al. 1988; Schulz et al. 1993). Fibers containing cGnRH-II could not be shown in the pituitary of African catfish (Zandbergen et al. 1995) or salmon (Amano et al. 1991), suggesting that cGnRH-II might reach the pituitary via the bloodstream or the cerebrospinal fluid. The presence of GnRH binding proteins in the serum of goldfish (Huang et al. 1991a) suggests that the peptide may indeed be transported via the circulation (Huang and Peter 1988; Huang et al. 1991a; Huang et al. 1991b).

GnRH: its regulation

The GnRH system in the brain is influenced by many physiological systems in order to control reproduction. Hence, various substances are involved in the regulation of GnRH expression and release: leptin (Cunningham et al. 1999; Foster and Nagatani 1999), neuropeptide Y (NPY; Li et al. 1999), β-endorphin (Kandeel and Swerdloff 1997; Sarkar and Subhedar 2000), dopamine (DA; Timmers and Lambert 1989), serotonin (Khan and Thomas 1993), glutamate and γ-amino butyric acid (GABA; Feleder et al. 1996; Fueshko et al. 1998), and last but not least: steroid hormones. The effects of e.g. photoperiod, sexual behaviour, stress and gonadal maturation on the GnRH system are all mediated via one or more of these substances or via yet unknown signal molecules.

The focus of the present thesis is on the regulatory role of steroids on the development of the cerebral GnRH system. Earlier studies indicated that

steroids may affect hypothalamic GnRH content and/or expression. In mammals, controversial results were obtained after steroid treatment or steroid withdrawal (orchidectomy/ ovariectomy) (Kalra and Kalra 1989; Gore and Roberts 1997; Kim et al. 1997). For example, in one study on rats the expression of mGnRH in the POA increased after castration, while T or estradiol (E_2) replacement restored normal levels (Spratt and Herbison 1997). However, in another study in the rat, GnRH mRNA levels were decreased after castration (Rothfeld et al. 1987). These contradictory results could be explained by differences in the duration or the timing (ovarian cycle in females) of the treatment, the dose of the steroid, as well as the type of steroid.

In fish, a more uniform picture of the regulation of the development and activity of the ventral forebrain GnRH system is available. Mostly, the effects of three different steroids e.g. testosterone (T), 11-ketotestosterone (11KT) and estradiol (E_2) have been investigated. T and E_2 play important roles in sex differentiation and reproductive development of fish. The effects of T or E_2 in immature fish are stimulatory as they increase brain and pituitary GnRH content (Schreibman et al. 1986; Goos et al. 1986; Montero et al. 1995; Breton and Sambroni 1996), the number of GnRH neurons (Amano et al. 1994), and/or GnRH mRNA levels (Soga et al. 1998). The GnRH cell populations, however, which are influenced by T depend on the species. In the rainbow trout, T caused an increase in GnRH content in both the ventral telencephalon and POA (Breton and Sambroni 1996), whereas in the platyfish only the GnRH content of the nucleus olfacto-retinalis (NOR) had increased (Schreibman et al. 1986). 11KT is the main circulating androgen in most teleosts. It plays an important role in the control of spermatogenesis. Although less intensively investigated, there is one study that shows a positive effect of 11KT on the number of GnRH neurons in tilapia (*Thalassoma bifascatum*; Grober et al. 1991).

Since the GnRH neurons are able to react to steroids, it is to be expected that GnRH neurons contain estrogen and/or androgen receptors (ARs and ERs). The GnRH gene promotor should contain an estrogen or androgen responsive element. In 1993 an estrogen responsive element (ERE) has been discovered on the promotor of the salmon GnRH gene (Klungland et al. 1993). ERs were identified in fish brain (Ma et al. 2000; Pakdel et al. 2000), but not in co-localisation with GnRH neurons (Navas et al. 1995; Kah et al. 1997), although in brain regions very close to these cells (ventral TEL, POA, MBH). This is in contrast to mammals: GnRH and ERs have been shown to be co-localised in the rat POA (Butler et al. 1999; Skynner et al. 1999).

At present, androgen receptors have less intensively been examined in fish, as their cloning only started in the late nineties. A single AR has been sequenced in the red seabream (*Pagrus major*; Touhata et al. 1999), two iso-

forms have been characterised in the rainbow trout (Takeo and Yamashita 1999) and two distinct forms have been identified in Atlantic croaker (*Micropogonias undulatus*; Sperry and Thomas 1999) and eel (*Anguilla japonica*; Ikeuchi et al. 1999). A localisation study in goldfish brain demonstrated the presence of AR in telencephalon, POA, diencephalon and midbrain (Gelinas and Callard 1997). These AR-positive brain areas correspond with the GnRH localisation, but co-localisation at the cellular level has not been examined.

The animal model: the African catfish, *Clarias gariepinus*

In 1982, the African catfish was introduced in the Research Group for Comparative Endocrinology. Since then, much knowledge, especially regarding its reproductive development, was collected. The African catfish is a convenient animal for model studies because it is easy to raise (Leeuw et al. 1985) and it can be kept in high densities in aquaria. After hatching, the African catfish grows rapidly, and even before the age of puberty reaches a size that allows many experimental procedures like blood sampling, dissecting pituitary, brain and testis, castration, and injecting or implanting hormones. Furthermore, the African catfish has low requirements considering water- and food quality, although it is sensitive to changes in water temperature, resulting in fungus infections.

In our facilities, we breed African catfish by artificial induction of ovulation and fertilisation. Female catfish are injected with pituitary extract to induce ovulation. Ovulation occurs about 12 hrs after injection and thousands of matured eggs are collected by stripping. A male fish is sacrificed to obtain sperm. About one day after fertilisation the eggs hatch and free-swimming larvae start feeding after one day. After 4 weeks, the developing gonads differentiate into ovaries in the female, while the testicular differentiation occurs two weeks later (Hurk et al. 1989). This thesis concentrates on male catfish, therefore only the spermatogenesis will be considered here. The first wave of spermatogenesis starts at 10 weeks of age and is completed at 24 weeks of age (Cavaco 1998; Schulz et al. 1999). After completion of the first wave of spermatogenesis the adolescent stage is achieved. Under hatchery conditions, a catfish is considered to be adult and fully mature after about one year (Schulz et al. 1997b ; Fig. 4).

In the African catfish two distinct forms of GnRH have been characterised: cfGnRH and cGnRH-II (Bogerd et al. 1994). Both GnRHs have been localised by immunocytochemical techniques and their mRNA expression pattern was shown after *in situ* hybridisation (Zandbergen et al. 1995). Since

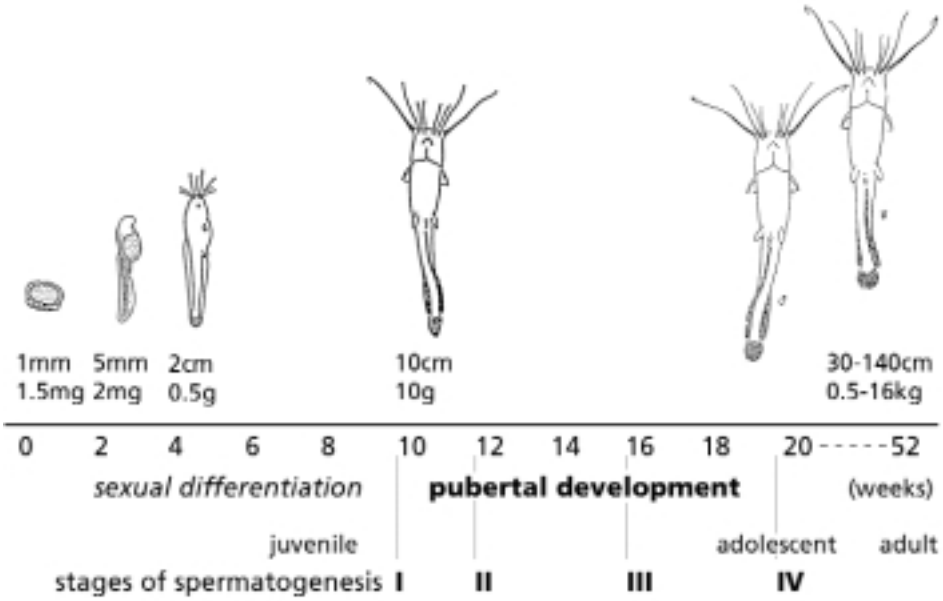


Fig. 4 Life span of the African catfish from the fertilised egg till the adult state at 52 weeks. The four stages of spermatogenesis during the pubertal development are abbreviated as: stage I: spermatogonia, stage II: spermatogonia and spermatocytes, stage III: spermatogonia, spermatocytes, and spermatids, stage IV: all germ cells including spermatozoa.

cfGnRH and cGnRH-II only differ by two amino acids, cross-reactions of the antibodies against GnRH often occur. In order to avoid cross-reactivity, we used antibodies raised against the two respective GAPs, which show less homogeneity in their amino acid sequence.

CatfishGnRH perikarya were localised in the ventral forebrain (olfactory bulb, medial olfactory tract, ventral area of the telencephalon, pre-optic area, nucleus anterioris periventricularis and medial basal hypothalamus) (Fig. 5). Fibers were shown in the same regions, forming a continuum from the olfactory bulb till the pituitary. The most extensive fiber pattern was observed dorsal from the optic chiasm, in the MBH and in the pituitary. Fibers of different origin and character, including the cfGnRH fibers from the ventral forebrain, innervate the gonadotropic cells in the PPD of the pituitary (Peute et al. 1984; Peute et al. 1987).

ChickenGnRH-II immunoreactivity was localised in large cells in the midbrain tegmentum dorsally in the diencephalon. Neither with anti-cGnRH-II nor with anti-cIIGAP any extensions of these large cells could be labelled (Zandbergen et al. 1995). The cGnRH-II cells apparently have no direct connection with the pituitary via axons, but cGnRH-II may be transported to the pituitary via the circulation or the cerebrospinal fluid.

The amount of cfGnRH in the pituitary is about 700 times higher than

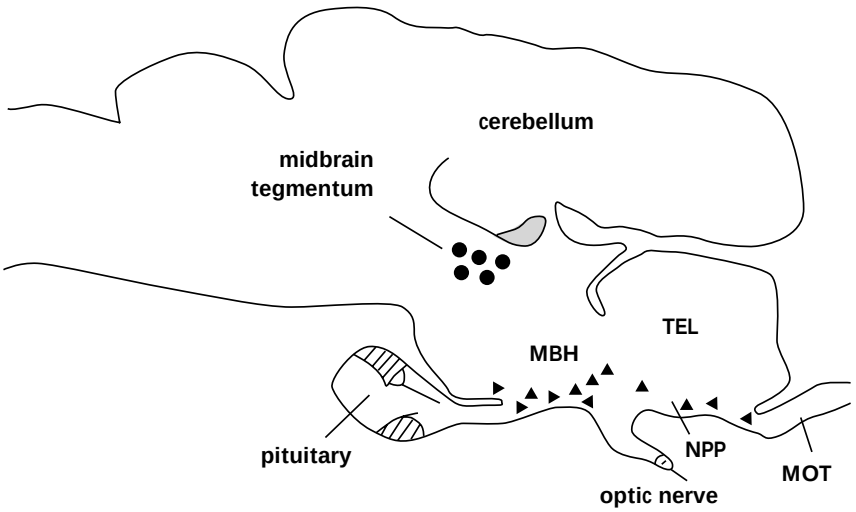


Fig. 5 Schematic drawing of the localisation of GnRH neurons in the African catfish: cfGnRH neurons (() in the ventral forebrain and cGnRH-II cells (() in the midbrain tegmentum. The hatched area in the pituitary represents the proximal pars distalis, where the gonadotropic cells are situated.

the amount of cGnRH-II, i.e. 12690 pg of cfGnRH and 18 pg of cGnRH-II (Goos et al. 1997). The brain of adult, male fish, however, contained 625 pg of cfGnRH and 3225 pg of cGnRH-II. Since in adult catfish over 90% of the cfGnRH is present in the pituitary gland, it was suggested, that the cfGnRH is mainly stored in the nerve endings in the vicinity of the gonadotropic cells.

Puberty in the African catfish

Puberty in the male African catfish is defined as the onset of meiosis in the testes, characterised by the appearance of spermatocytes at 12 weeks of age (Cavaco et al. 1998b). After spermatogonial proliferation (stage I), the spermatocytes form the first stage in germ cell differentiation. This stage will be referred to as stage II of spermatogenesis. Around 16 weeks of age the first spermatids are observed (stage III). The first wave of spermatogenesis is completed by the appearance of spermatozoa (stage IV). The four stages and their time of appearance in development are depicted in Figs. 6 and 4, respectively.

Gonadal steroids may be the key players in the onset of puberty, since they have been shown to stimulate the maturation of the GnRH system in the brain (Amano et al. 1997), the gonadotrophs in the pituitary (Xiong et al. 1994) and the testicular development (Miura et al. 1991). In the African catfish, effects of steroid hormones on the maturation of the pituitary and testes were examined in fish of 10 weeks of age by 2-week treatments. The 11β-oxygenated andro-

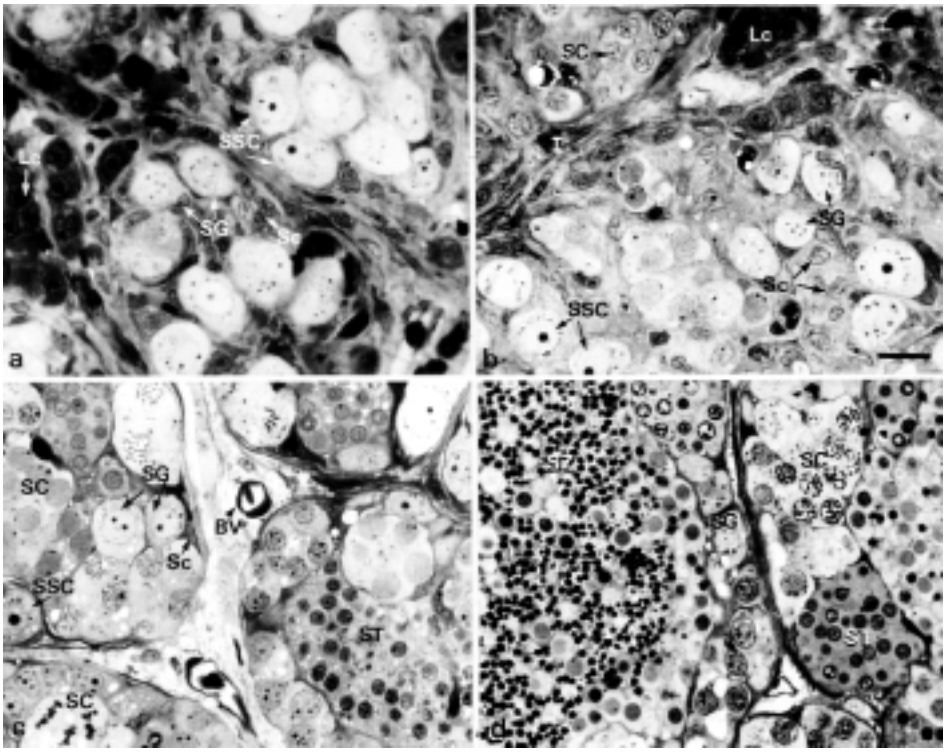


Fig. 6 Micrographs of the four stages of spermatogenesis in the African catfish. a) stage I, spermatogonia; b) stage II, spermatogonia and spermatocytes; c) stage III, spermatogonia, spermatocytes, and spermatids; d) stage IV, all germ cells including spermatozoa. Lc: Leydig cell, SSC: spermatogonial stem cell, SG: spermatogonia, Sc: Sertoli cell, I: interstitium, BV: blood vessel, SC: spermotocytes, ST: spermatids, SZ: spermatozoa, L: lumen. Adapted from (Cavaco et al. 1996).

gens 11β-hydroxyandrostenedione (OHA) and its conversion product 11-ketotestosterone (11KT) stimulate spermatogenesis, testicular development and the appearance of secondary sex characteristics (Cavaco et al. 1998b). However, at the pituitary level these 11-oxygenated steroids inhibit the gonadotropic cells to mature (Cavaco et al. 1998b). On the other hand, aromatizable androgens, like androstenedione and testosterone, and estradiol induce the maturation of pituitary gonadotrophs, by stimulating GTH gene expression, storage of GTH, and inhibition the GTH release, while neither T nor E_2 stimulated spermatogenesis or testicular development (Cavaco 1998). So, each group of steroids has its own domain of action within the BPG-axis and is apparently involved in a discriminatory way in the control of pubertal development. Hence, Schulz and Goos (1999) proposed that a "tightly balanced production" of both 11-oxygenated and aromatizable androgens is necessary for the maturation of the pituitary-testis axis.

Scope of the thesis

The research group for Comparative Endocrinology concentrates on the hormonal control of pubertal development in fish, with the African catfish as model. The BPG axis is the main neuro-endocrine system regulating reproduction; hence its development is crucial for the acquirement of sexual maturity. Before puberty, the pituitary and gonads are already able to respond to GnRH and GTH, respectively (Schulz et al. 1994; Schulz et al. 1995). Therefore it is assumed that the regulatory processes initiating puberty are operating at a supra-hypophyseal level. On the other hand, gonadal steroids may play a key-role in pubertal development, since they stimulate pituitary and testis maturation.

This thesis deals with two main questions:

(1) How do the two GnRH systems in the African catfish develop and are gonadal steroids in control of this development?

(2) How is the development of the two GnRH systems related to the onset of puberty of the African catfish?

Starting point was the discovery of two GnRH forms in the catfish (Bogerd et al. 1994) and their localisation in adults (Zandbergen et al. 1995). Chapter 2 describes the early morphological development of the catfish GnRH and cGnRH-II system, respectively. With a tracing study the projections into brain and towards the pituitary of both systems were visualised.

The control of the development by steroid hormones and the functional characteristics of the cfGnRH system are investigated in chapters 3 to 5. Since the ventral forebrain cfGnRH, by the nature of its projections, could be assigned as the hypophysiotropic system, it was supposed to have a key position in the maturation of the BPG-axis. It is therefore that we focussed on the cfGnRH system rather than on the cGnRH-II system in these experiments. First, the effect of steroids on the GnRH systems in immature catfish (2-6 weeks of age) was examined (Chapter 3). The innervation of the pituitary and its steroidal control between 12 and 17 weeks of age were studied in Chapter 4. Castration experiments and steroid replacements were performed in order to investigate effects on the cfGnRH system in the brain and on the development of the cfGnRH fiber network in the pituitary. Chapter 5 deals with the control of steroids on the GnRH system at the onset of puberty (10-12 weeks of age). This chapter also reviews the findings of chapters 3 and 4, providing a total picture of steroid regulation of the GnRH system during development (2-20 weeks).

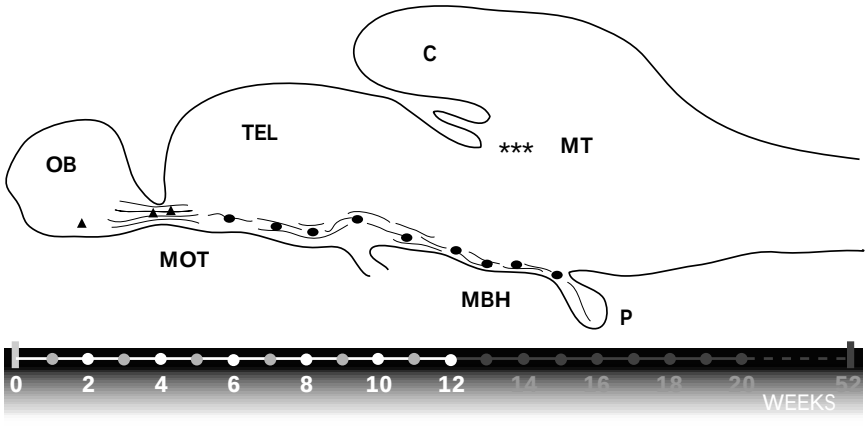
Chapter 6 contains a morphological description of the cGnRH-II cells. In species studied to date, the function of the midbrain cGnRH-II neurons remained unsolved. In contrast to the cfGnRH cells in the ventral forebrain in our experiments, the cGnRH-II cells did not show any reaction to the applied hormone treatments. Nevertheless, cGnRH-II is a very potent GTH releasing hormone (about 100 times more potent than cfGnRH; Schulz et al. 1993). Moreover, cGnRH-II is present in a small but relevant amount in the pituitary (Goos et al. 1997). In a morphological study, describing the histological structure and the ultrastructure of the cGnRH-II neurons, we pinpointed at possible release sites near blood capillaries or subventricular spaces.

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).

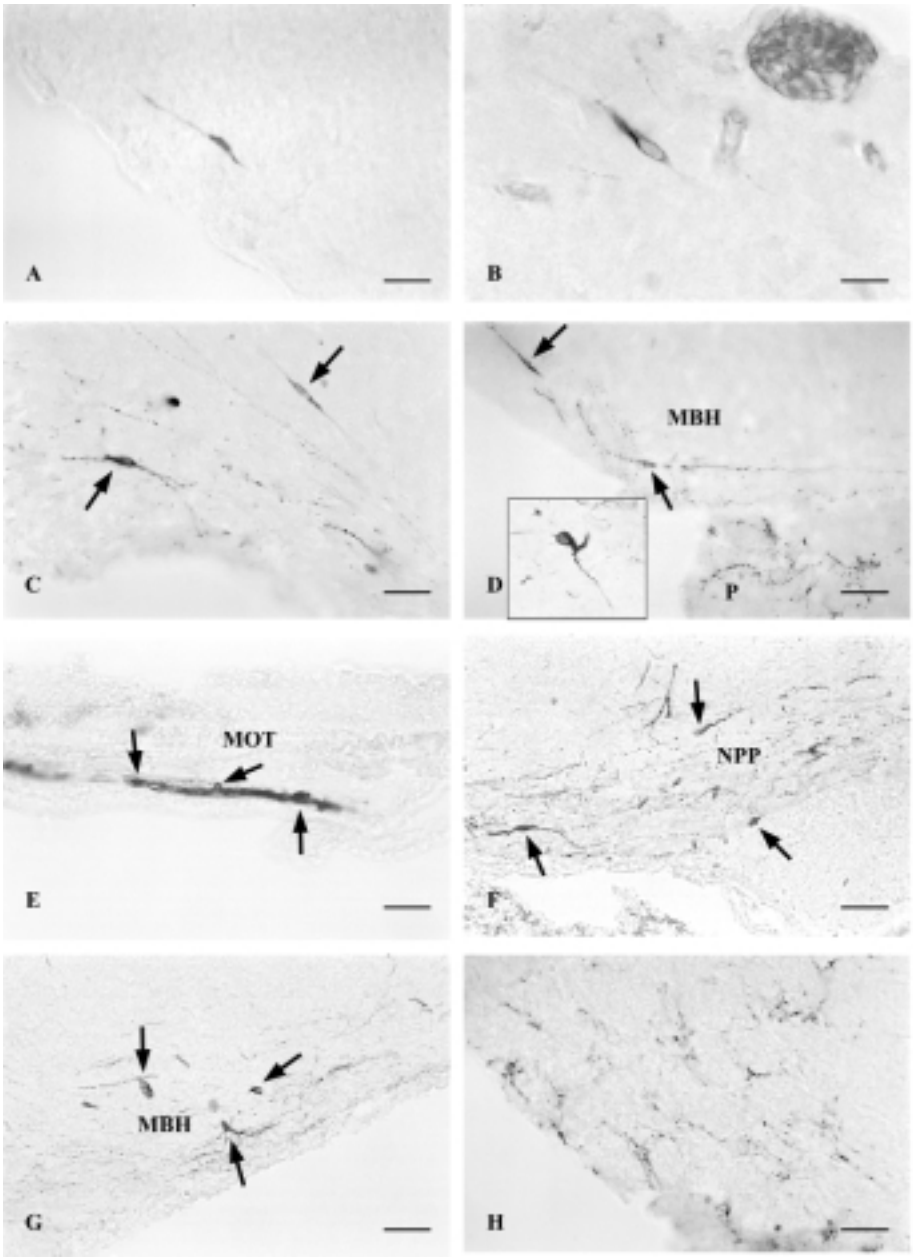


Fig. 1 Early development of the cfGnRH system in the ventral forebrain visualized by anti-cfGAP antibodies. A: cfGAP-ir neurons in the VV of at 2 weeks. B: cfGAP-ir neuron in the NAP of a 6 week-old fish. C: cfGAP-ir neurons and fibers above the chiasm, in the NAP in a 10 week-old fish. D: Overview of 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

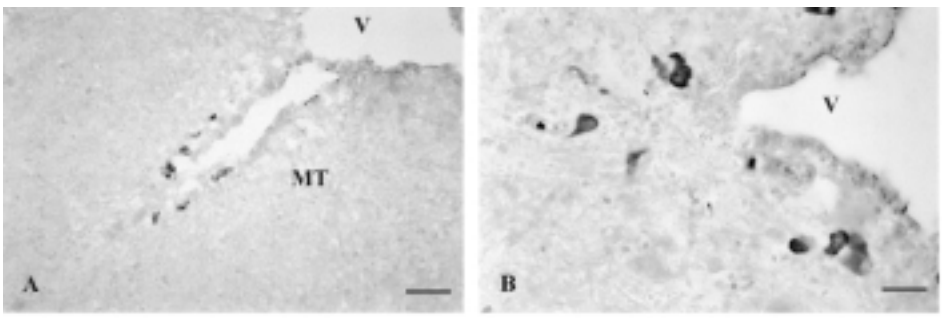


Fig. 2 cIIGAP-ir neurons in the midbrain tegmentum in a 6 week-old fish (A) and a 14 week-old fish (B). V: third ventricle, MT: midbrain tegmentum. The bars represent 71 μm in (A) and 36 μm in (B).

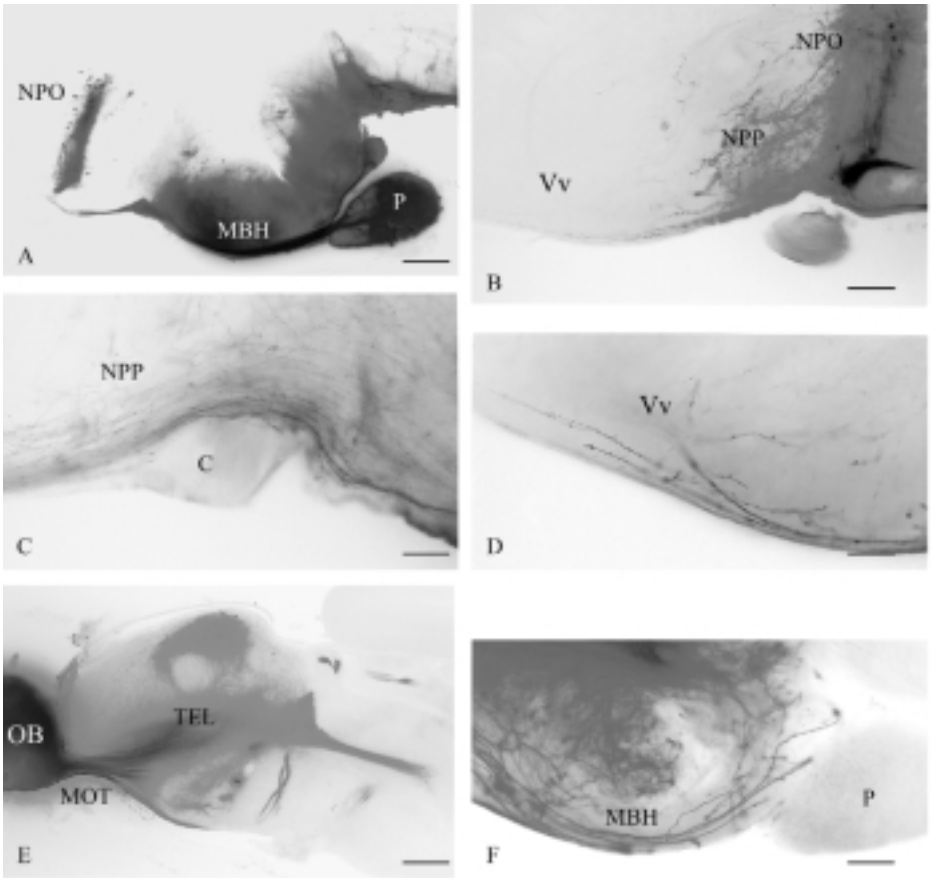


Fig.3 Microphotographs showing brains of 7 week-old catfish 3 weeks after implantation of a DiI microcrystal in the pituitary (A, B, C, and D) or olfactory bulb (E and F). A: Overview of the DiI implantation site (pituitary) and its extensive diffusion in the MBH and NPO. B: Enlargement of the NPO and 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.

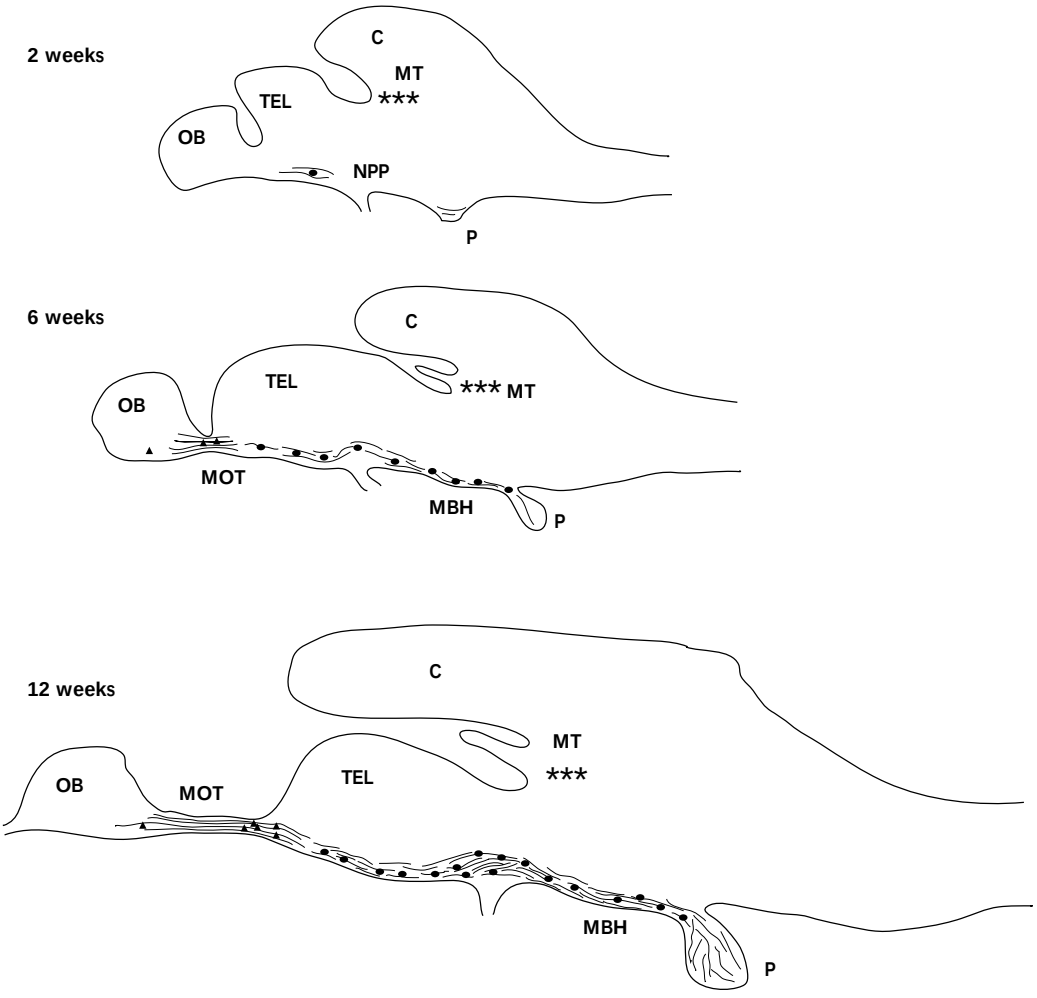


Fig. 4 Schematic representations of the GnRH systems in the African catfish at 2, 6 and 12 weeks ph. cGnRH-II system in the MT (*), cfGnRH system in the TN (▲) and ventral forebrain area (●).

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

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Chapter 3

Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish (*Clarias gariepinus*)

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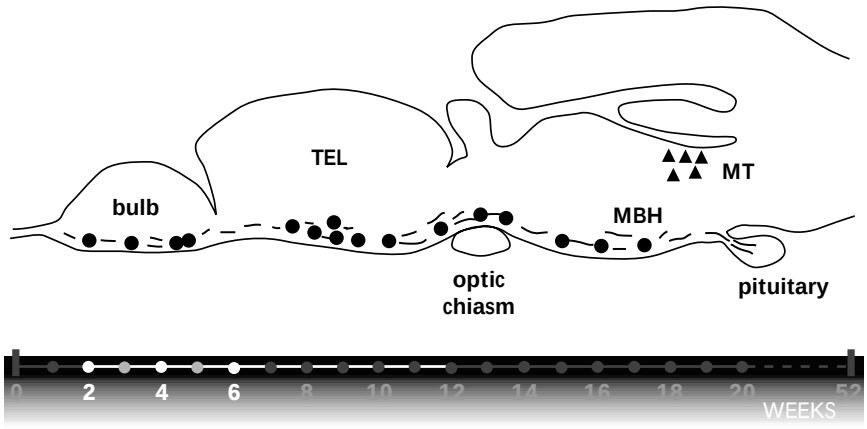

Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish *(Clarias gariepinus)*

Abstract

The effects of two endogenous steroids on the maturation of the catfish GnRH and the chicken GnRH-II system in the African catfish were investigated. Immature fish (two weeks of age, which is before sexual differentiation, thus male and female genotypes present) were fed with food pellets containing either testosterone (T), 11β-hydroxyandrostenedione (OHA) or no steroid (control). After two and four weeks of treatment, the effects on the two GnRH systems were investigated immunocytochemically, using specific antibodies against the respective GnRH associated peptides (GAPs). By means of fluorescence microscopy the number of GnRH perikarya and the cell surfaces were determined. Confocal laser scanning microscopy was applied to verify spatial distribution and staining intensity. After two weeks of treatment no difference in any of the parameters between the groups was observed. However, four weeks T treatment resulted in significantly more cfGnRH-ir perikarya in the brain as compared to the OHA- and control groups. In addition, in the T group the number of immunoreactive fibers was markedly higher and the staining of the perikarya and axons was more intense. The distribution of cfGnRH-ir neurons over the ventral forebrain differed between the two age groups: in four week old fish, the largest concentration of neurons was localised in the ventral telencephalon, while two weeks later the number of neurons in the supra-optic area had markedly increased, suggesting that the cfGnRH system is still undergoing developmental changes during this period. In six weeks old fish the average volume of the cfGnRH perikarya (expressed as surface size in the microscopical sections) in both the OHA- and T group was significantly bigger than that in the control group. The cGnRH-II-ir neurons in the midbrain tegmentum showed strong immunoreactivity in all groups, both treated and non-treated. In contrast to the cfGnRH neurons, the staining intensity and the number of cGnRH-II neurons did not change after steroid treatment. The results of this study show that T is able to accelerate the development of the cfGnRH system, whereas OHA has only minimal effects; the cGnRH-II system develops independent from these steroids.

Introduction

Gonadotropin-releasing hormone (GnRH) is considered to be one of the most prominent neuroendocrine factors that control the release of gonadotropins from the pituitary gonadotrophs. Until now ten molecular forms of GnRH have been identified in vertebrates (King and Millar 1992; Montero and Dufour 1996; Jimenez-Linan et al. 1997) and recently two forms were discovered in a protochordate (Powell et al. 1996b). The chicken GnRH-II (cGnRH-II) is considered to be the evolutionary oldest form since it is present in all classes of gnathostomes (reviewed by King and Millar 1992; Montero and Dufour 1996). In all vertebrates, including higher evolved mammals as recently shown by Lescheid (1997), two, or even three different forms of GnRH are expressed, with cGnRH-II being invariably one of the two. In general, the two forms of GnRH are differentially localised and they appear to have different functions and ontogeny. The cGnRH-II is expressed in the midbrain, whereas the other form of GnRH is localised in the ventral forebrain system. By its localisation and the projections of axons towards the median eminence (and towards the gonadotrophs in pituitary in most fish species), the latter GnRH system apparently is the most prominent one in regulating gonadotropin release. It is generally assumed that it originates from the olfactory placode (Schwanzel-Fukuda and Pfaff 1989; Northcutt and Muske 1994) and that the GnRH neurons migrate into the ventral forebrain. This process has been described e.g. in the mouse (Wray et al. 1989), chicken (Sullivan and Silverman 1993), rhesus macaque (Ronnekleiv and Resko 1990), chum salmon (*Oncorhynchus keta*; Chiba et al. 1994), platyfish (*Xiphophorus maculatus*;Halpern-Sebold and Schreibman, 1983), and sockeye salmon (*Oncorhynchus nerka*; Parhar et al. 1995a). The ontogeny and the function of the midbrain GnRH system remain unclear to date.

The regulation of the spatial, temporal and functional development of the GnRH system is still poorly understood. It is hypothesised that steroids can initiate and/or accelerate the maturation of the brain-pituitary-gonad axis (Goos 1993), which has been studied in several teleost species. In rainbow trout (*Oncorhynchus mykiss*; Goos et al. 1986; Breton and Sambroni 1996) and masu salmon (*Oncorhynchus masou*; Amano et al. 1994) testosterone (T) stimulated the GnRH synthesis and content in the forebrain. In the nucleus olfactoretinalis (NOR) of the platyfish (Schreibman et al. 1986) T caused an increase in the intensity of GnRH immunostaining. In the female silver eel (*Anguilla anguilla*; Dufour et al. 1985; Montero et al. 1995) T had no effect on the ventral forebrain system, but increased the cGnRH-II levels in the midbrain. In the cichlid *Haplochromis burtoni* (Francis et al. 1994; Soma et al. 1996) T induced

shrinkage of the preoptic GnRH cell soma, which had increased in size after castration. In general, T exerts a positive effect on GnRH expression. The effect of another important steroid in male teleosts, 11-ketotestosterone (11KT), was similar to that of T in the platyfish (Schreibman et al. 1986) and in *Haplochromis burtoni* (Soma et al. 1996). Also estrogens have been shown to positively affect the GnRH content of the forebrain. Estradiol (E_2) caused brain (but not hypothalamus) and pituitary GnRH content to increase in the triploid rainbow trout (Breton and Sambroni 1996). The forebrain GnRH content of the female eel (Montero et al. 1995) also increased after E_2 treatment. The differential effects of sex steroids in the studies mentioned above may be due to differences in dose, way of administration, age and species. Most of these studies were performed at the stage that the GnRH system was already developed, but not yet fully mature.

In the African catfish (*Clarias gariepinus*), catfish GnRH (cfGnRH)) and cGnRH-II were identified and characterised (Bogerd et al. 1994). Both GnRH systems were localised in adults by immunocytochemistry and *in situ* hybridisation using antibodies and probes respectively against the GnRH associated peptides (GAPs; Zandbergen et al. 1995). In order to avoid cross-reactions, these authors recommend the use of antibodies against the two GAPs rather than antibodies against the GnRHs only, because the GAPs strongly differ in amino acid composition. Since cfGAP immunoreactivity and cfGnRH immunoreactivity are equally localised, we will further refer to cfGnRH-ir.

Furthermore, the effects of two gonadal steroids, T and 11β-hydroxyandrostenedione (OHA, the main testicular product and precursor for hepatic conversion to 11KT; Cavaco et al. 1997) on the developing GnRH systems were investigated in immature catfish of undifferentiated sex. As regards the effects of these steroid hormones on the pituitary-gonad axis, related studies have shown that T has its domain of action in the gonadotrophs by stimulating GTH gene expression and release, while on the testicular level the 11-oxygenated steroids affect spermatogenesis and testicular growth (Cavaco et al. 1998b). In the present study we focus on the effects of these steroids at the level of the brain. Starting at two weeks post hatching, the fish were fed with food pellets containing T or OHA. After two and four weeks of treatment, brain samples were processed for cfGnRH-GAP and cGnRH-II-GAP immunocytochemistry and morphometric analysis. In order to compare the GnRH system with a fully developed GnRH system, we also subjected fish of 12 weeks to morphometric analysis. Fish of this age are at the onset of puberty (spermatocytes present in the testes) and have a fully differentiated GnRH system in the brain (unpublished results).

Materials and Methods

Animals and experimental design

The African catfish were raised in the hatchery of the Department of Experimental Zoology, University of Utrecht as described by de Leeuw et al. (1985a), except that pituitary extract instead of human chorionic gonadotropin was applied to induce ovulation. Two weeks after hatching, 200 fish (approximately 1.5 cm in length) were randomly taken and divided into three groups (for control, OHA and T treatment, respectively) and kept in 180 l aquaria at 28.5 ± 1.5°C in copper-free running water (0.8 l/min) and a 14 h daily light period. After two days of normal feeding and acclimation to the new environment, feeding with steroid containing pellets (Trouvit, Putten, the Netherlands) started. The dose for both steroids was set at 50 mg/kg food pellets, which induces a significant increase in plasma levels (Cavaco, unpublished results). The fish were fed three times a day on a ratio of 20% of their body weight at the beginning of the experiment, gradually decreasing to 8% at six weeks of age. Steroid containing pellets were prepared as follows. T (Merck) and OHA (Sigma) were dissolved in 100% ethanol (50mg/100ml) while stirring for 1 h. The steroid containing ethanol or the ethanol without steroid (for control pellets) was sprayed over the food pellets, mixed thoroughly and subsequently allowed to evaporate during 48 h. The pellets were kept at 4°C until use. At four weeks (after two weeks treatment, average length of the fish 2 cm) and six weeks (four weeks treatment, ± 4 cm) of age 25 fish per group were sampled. In addition, 5 untreated male catfish of 12 weeks old were selected. The fish were killed by decapitation and the brain with the pituitary was carefully dissected.

Confocal Laser Scanning Microscopy (CLSM)

To obtain a general overview of the cfGnRH system, the brains were prepared for CLSM and fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 5 h at room temperature. Then the tissues were rinsed in 0.1M phosphate buffer containing 10% sucrose for 16 h at 4°C. Sections of 100μm were cut on a vibratome (Leica VT 1000S, Nussloch, Germany), collected in 0.1M PBS in a 48 well plate (Costar, Cambridge, MA, USA) and treated as free-floating sections. The sections were rinsed in 1% non-fat dried milk and 1% Triton X-100 in PBS for 4 h at 4°C. The antibody, anti-cfGAP raised in rat, was diluted 1:500 in 1% acetylated bovine serum albumin (BSAc, Aurion, Wageningen, the Netherlands) and 0.3% Triton X-100 in PBS. After 72 h gently shaking at 4°C, the sections were washed three times for 1 hour each in BSAc-Triton-PBS. The secondary antiserum, anti-rat IgG-FITC (Sigma, St. Louis, MO, USA) was diluted 1:320 in the same buffer. The sections were incubated for 24 h at 4°C

in the dark. After rinsing two times for 1 hour each in BSAc-Triton-PBS, the sections were mounted on slides and coverslipped with antifading solution mowiol (Aldrich, Steinheim, Germany)/ p-phenylenediamine free base (PPD, Sigma, 1mg/ml). The specificity of the antiserum was controlled by applying preimmune serum or buffer only.

The sections were analysed by a Leica upright confocal laser scanning microscope with an argon/krypton laser. The objective 25x with a pinhole of 170 μm diameter was applied. The scanned images were processed by the Leica TCSNT software program. The intensity of the immunoreactivity was estimated in a blind test procedure.

Immunofluorescence light microscopy

The brains were kept overnight in fixative (4% paraformaldehyde, 0.2% picric acid in 0.1M phosphate buffer, pH 7.4), were rinsed three times for 1 hour each in 0.1M phosphate buffer and subsequently carried through a series of increasing sucrose gradients (5% and 10%) in the same buffer for 2 h each. After 16 h in 20% sucrose solution at 4°C, the brains were embedded in Tissue Teck and quickly frozen (-20°C). In a cryostat (Leica), transversal and sagittal sections of 12μm were cut, attached and dried on gelatine coated slides and kept at -20°C till immunocytochemistry was performed. Antisera, raised against cfGnRH associated peptide (rat anti-cfGAP) and cGnRH-II associated peptide (rabbit anti-cGAP-II) respectively, were used according to Zandbergen et al. (1995). As secondary antisera, respectively anti-rat IgG-FITC conjugate (Sigma) diluted 1:320 or anti-rabbit IgG-FITC conjugate (Sigma) diluted 1:200 were used. The incubations with the secondary antisera were performed in the dark at room temperature for 90 minutes. After rinsing two times for 10 minutes in Coons buffer containing 1% BSAc, the slides were coverslipped with mowiol/PPD (1mg/ml). The GnRH-ir systems were quantitatively analysed by counting cfGnRH-ir neurons in every alternate section, and cGnRH-II-ir neurons in every fourth section.

Data sampling and statistics

The results are expressed as mean ± standard error of the mean (SEM) per brain. The data (number of ir-cfGnRH and ir-cGnRH-II neurons and cell surface) were processed for statistical analysis by one-way ANOVA followed by Fisher's least significant difference test ($p<0.05$). For determination of the surface of cfGnRH-ir perikarya, the cells were randomly selected from micrographs (magnification of 295x) taken from the 12μm sections. From each ellipse-shaped perikaryon the maximum and minimum diameter were measured and used in the formula $\pi.r1.r2$ (surface of an ellipse).

Results

The small body size of the four and six week old fish did not allow to take blood samples for steroid measurements in order to verify the steroid uptake by the hormone treated animals. However, some macroscopical features showed remarkable differences between steroid treated animals and controls, which can be considered as a proof for hormone uptake. In addition, T treated fish had a stronger musculature and harder bone mass as compared to the two other groups. Indeed, only in the T group, the MOT was more elongated due to migration of the olfactory bulb in rostral direction, which is comparable to the situation in adult catfish. No macroscopically visible effects of OHA were observed at six weeks of age. However, OHA treated animals showed male sex reversal at later ages.

CLSM

The control situation of the cfGnRH immunoreactivity in the forebrain is represented in Fig. 1. At the age of 4 weeks cfGnRH-ir perikarya were present along the medial ventral forebrain, extending in a continuum from the olfactory bulb till the pituitary. In the olfactory bulb a few weakly stained neurons were observed, in addition to dispersed neurons in the MOT between the olfactory bulb and the Vv. CfGnRH-ir fibers were present in the same areas as the perikarya: olfactory bulb, MOT, Vv, NPP, dorsally of the optic chiasm, NAP, MBH and pituitary. The situation in the steroid treated groups did not differ from the control group at this age.

In the 6 week old catfish brain, the immunoreactivity and the number of cfGnRH-ir fibers had increased compared to the situation at 4 weeks. Fig. 2 shows the 3D images in an area of the ventral forebrain of the treated and non-treated groups at 6 weeks. The T group shows the most intense immunoreactivity and the highest number of ir-fibers, followed by the OHA group and the control.

Quantitative analysis of cfGnRH immunoreactivity

The average number of cfGnRH-ir neurons in the entire brain in the 4 weeks control fish was 118.8 ± 15.0 (mean ± SEM). The majority of the ir-perikarya was concentrated in the rostral areas: bulb, MOT and especially in the Vv. The mean cell surface of the cfGnRH-ir cells in the 4 weeks control groups measured 24.9 ± 1.6 μm^2.

At 6 weeks, the average cell surface had increased significantly in the control group (36.9 ± 2.9 μm^2, Fig. 4). The average number of cfGnRH-ir neurons had not changed (124.4 ± 23.2, Fig.3). However, compared to the 4 weeks group, the larger concentration of ir-cfGnRH neurons was localised now more caudally in the brain, especially in the supra-optic area, i.e. NPP-NAP and in

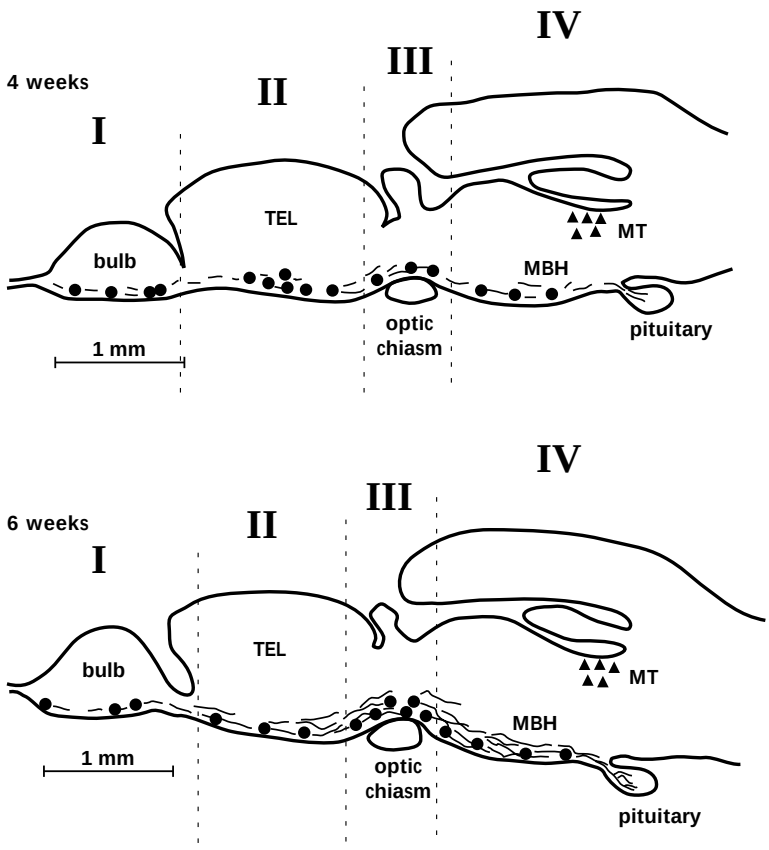

Fig. 1. Schematic drawings of the catfish brain at 4 and 6 weeks. The cfGnRH neurons (●) and fibers are localized along the entire ventral forebrain; the cGnRH-II neurons (▲) form a distinct nucleus in the MT. I-IV correspond with the subsequent brain parts in Fig. 4.

the MBH (Fig. 5), whereas the number of ir-neurons in the bulb-MOT and Vv had decreased.

After two weeks of steroid treatment, no significant differences with the control group could be established, neither in the number of cfGnRH-ir neurons and their surface, nor in the distribution of ir-neurons over the forebrain (Figs. 3, 4 and 5). However, after 4 weeks treatment with T, the number of cfGnRH-ir neurons had doubled to 274.0 ± 29.2 (Fig.3), which was significantly more than in the two other groups. Four weeks treatment with OHA had no significant effect on the number of cfGnRH-ir neurons (184.0 ± 24.8) in comparison with the control group.

Between 4 and 6 weeks the size of the cfGnRH-ir neurons increased in the control group. In addition to this age-related difference, the cfGnRH-ir perikarya of the two steroid treated groups increased significantly more in size as compared to the increase between the control groups (Fig. 4).

The distribution of cfGnRH-ir neurons over the ventral forebrain in the

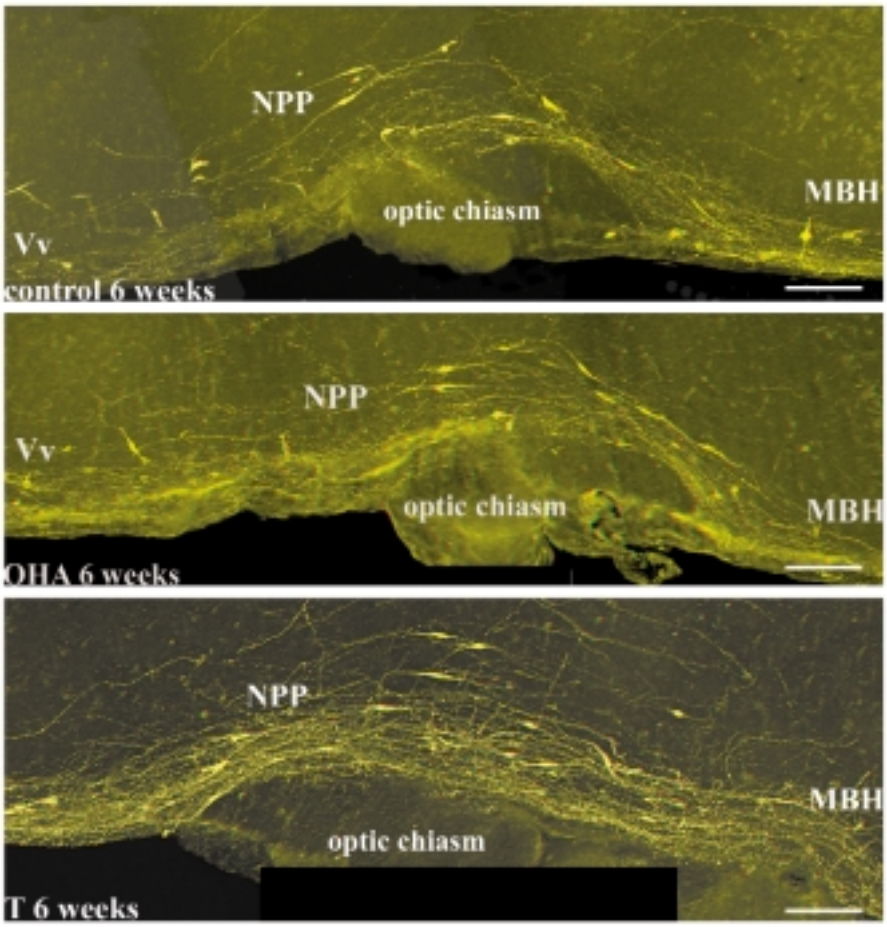

Fig. 2. 3D images obtained by confocal laser scanning microscopy. Sagittal vibratome sections (100μm) of the ventral forebrain of the three groups (control, OHA, and T) at 6 weeks are shown from the caudal part of the Vv until the beginning of the MBH. An objective of 25x and a pinhole of 170μm were applied. Scale bar represents 100 μm.

two OHA groups showed the same pattern as in the control groups at 4 and 6 weeks (Fig. 5). However, the pattern in the T group had changed, i.e., the number of cfGnRH-ir neurons along the entire ventral forebrain had increased, whereas in the control and OHA treated groups the number of neurons in the bulb-MOT and Vv had decreased.

In 12 week old catfish, 343.2 ± 15.4 cfGnRH neurons were counted in the ventral forebrain. Fifty percent of these neurons were localised in the area above the chiasm (NPO-NPP), while thirty percent was positioned in the MBH. At this age a cluster of cfGnRH neurons was observed in the MOT where it enters the telencephalon. The mean cell surface measured 112.3 ± 20.4 μm^2.

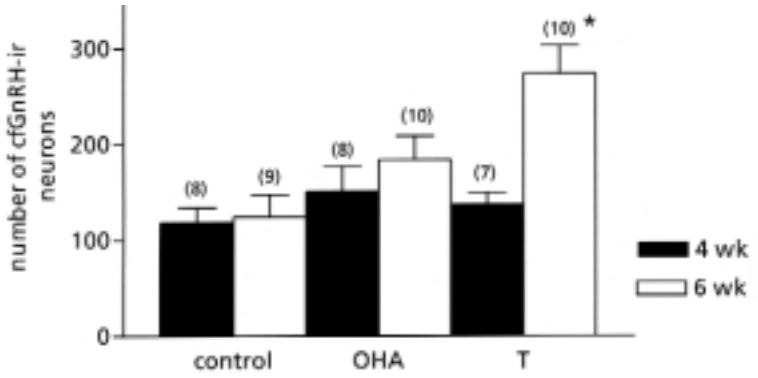

Fig. 3. Numbers of cfGnRH-ir neurons (mean (SEM) in the brain after administration of food pellets containing no steroid (control), OHA, or T at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, white bars). N is between 7 and 10. * Significantly different from all other groups.

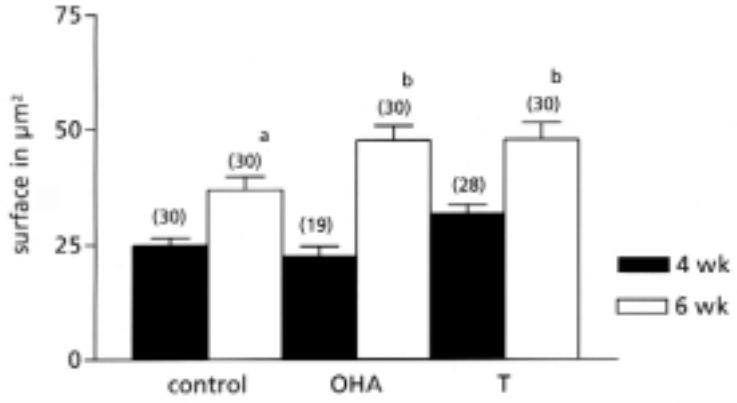

Fig. 4. Surfaces of cfGnRH-ir neurons (in μm^2, mean ± SEM) in the ventral forebrain after feeding food pellets containing no steroid (control), OHA, or T at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, white bars). N is between 19 and 30. a Significantly different from 4 weeks. b Significantly different from 4 weeks and from the control of 6 weeks.

Quantitative analysis of cGnRH-II immunoreactivity

Perikarya, immunoreactive for cGnRH-II were localised in a paired nucleus in the MT (Fig. 1). Generally, only ir-cell bodies without fibers were detected. These cell bodies were much larger than the cfGnRH neurons and measured up to 125 μm^2. The shape of the cGnRH-II-ir neurons was irregular as compared to the round or ellipsoid shape of the cfGnRH neurons. Delicate fibers were occasionally encountered throughout the brain, but not in the pituitary. In the control fish at 4 weeks of age the total number of cGnRH-II-ir neurons in the brain was 101.2 ± 8.4 and at 6 weeks 135.2 ± 6.4 (Fig. 6). The

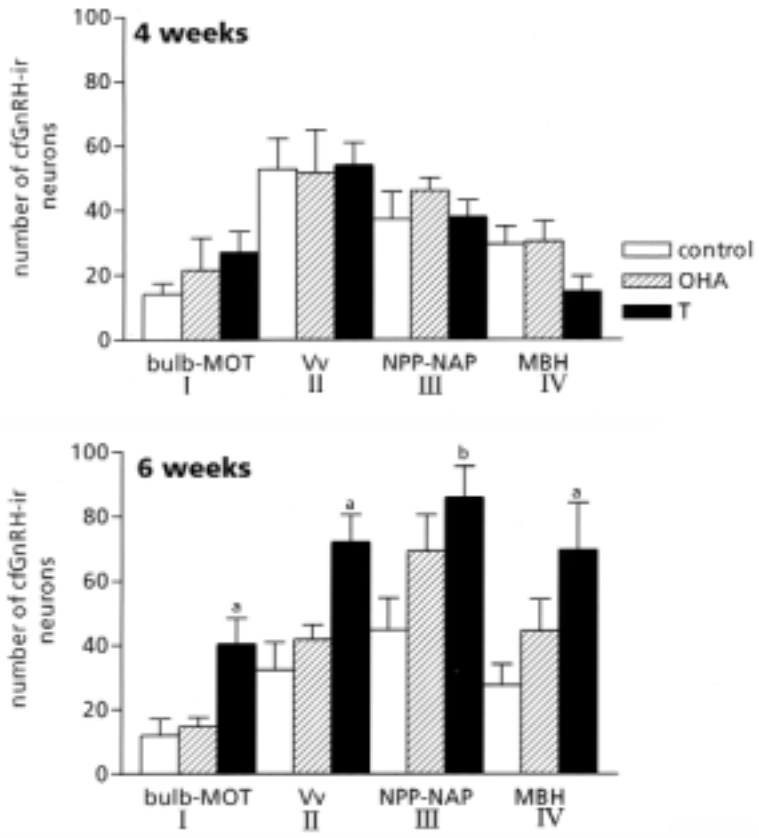

Fig. 5. Numbers of cfGnRH-ir neurons (mean ± SEM) in the ventral forebrain, subdivided in four subsequent brain parts (bulb-MOT, Vv, NPP-NAP, MBH) after treatment with food pellets containing no steroid (control, white bars), OHA (hatched bars), or T (black bars) at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, grey bars). The Roman numbers correspond with those in Fig. 1. * Significantly different from 4 weeks. [a] Significantly different from control and OHA of 6 weeks. [b] Significantly different from control.

number of neurons in the steroid treated groups did not differ significantly from that in the control fish: 99.2 ± 10.0 (OHA, 4 weeks), 135.2 ± 15.6 (OHA, 6 weeks), 117.2 ± 13.6 (T, 4 weeks) and 153.6 ± 15.6 (T, 6 weeks Fig. 6).

In 12 week old catfish the number of cGnRH-II-ir neurons had increased to 149.3 ± 16.5. Moreover, the cell surface had drastically augmented (302.8 ± 38.5 μm^2).

Discussion

In the present study, the effects of OHA and T on the development of the two GnRH systems in immature catfish were investigated. Both the cfGnRH and the cGnRH-II system were studied by means of immunocytochemistry and

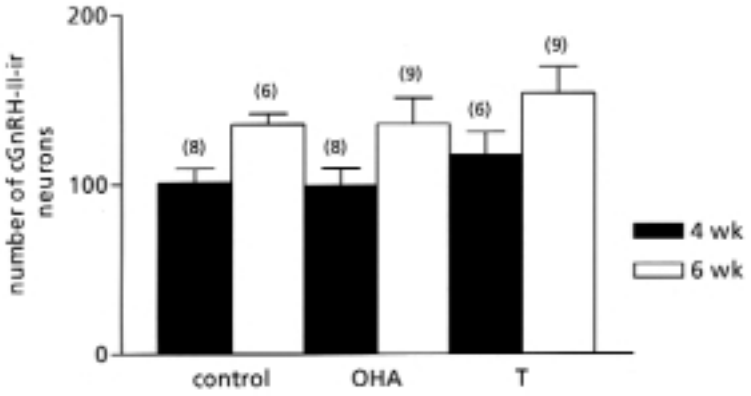

Fig. 6. Numbers of cGnRH-II-ir neurons (mean ±SEM) in the brain after administration of food pellets containing no steroid (control), OHA, or T at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, white bars). N is between 6 and 9.

morphometric analysis at four and six weeks of age and after treatment for two, respectively four weeks with OHA or T. We will first consider the changes during normal development between the ages of 4 and 6 weeks. The 3D micrographs obtained by CLSM (Fig. 2) show a continuum of cfGnRH-ir neurons over the entire ventral forebrain. No nuclei of GnRH neurons were encountered in fish of 4 and 6 weeks of age, not even in the terminal nerve ganglion (TN ganglion) or NOR, where others found GnRH nuclei (Halpern-Sebold and Schreibman, 1983; Parhar et al.1996a; Amano et al.1997). However, at the age of 12 weeks we do find a nucleus of cfGnRH-ir neurons localised in the MOT entering the telencephalon, which is comparable to the NOR or TN ganglion.

At 4 weeks the cfGnRH neurons were weakly immunoreactive, irrespective of the treatment, indicating that these neurons did not yet display full synthetic activity. At 6 weeks of age, however, the cfGnRH neurons are further differentiated; i.e. the immunoreactivity, cell size and number of fibers had increased. This development even continues to later stages, since in catfish of 12 weeks, the cfGnRH neurons - when fully developed - are even larger. This is in contrast with a study of Ronnekleiv and Resko (1990), who claimed that GnRH neurons in the rhesus monkey (*Macaca mulatta*) were already completely differentiated even before migration to their final destination.

In the control animals, the total number of cfGnRH-ir neurons did not alter between four and six weeks of age. Their distribution over the ventral forebrain, however, appeared to shift. At 4 weeks the highest number of cfGnRH-ir neurons was found in the Vv, while at 6 and 12 weeks the largest accumulation of neurons was found in the NPP-NAP and MBH. Two mechanisms can explain this shift in cfGnRH-ir neuron distribution: 1) cfGnRH-ir

neurons in the Vv disappear due to dedifferentiation, inactivation or apoptosis, while simultaneously immature neurons in the NPP-NAP and MBH differentiate into synthetically active cfGnRH neurons or 2) cfGnRH-ir neurons migrate from the Vv in caudal direction to NPP-NAP and MBH. Schwanzel-Fukuda and Pfaff (1990) showed similar changes in distribution patterns of neurons over the different brain areas during ontogeny of the ventral forebrain GnRH system in the mouse. Thus also in the catfish, migration of cfGnRH neurons from the ventral telencephalon towards the hypothalamic area is a possible explanation for the observed shift within the neuron population. The embryonic origin of the cfGnRH neurons was not investigated in the African catfish, but it might be the olfactory placode, since this is the origin of the ventral GnRH system in teleosts studied to date (see review Amano et al.1997).

Two weeks treatment with OHA or T did not cause any significant changes in number and distribution of cfGnRH neurons. Either the period of two weeks treatment was too short to increase the amount of circulating steroid to an appropriate level, or the cfGnRH neurons were not yet susceptible for the steroid.

Pronounced effects of T were visible at the age of six weeks, i.e. after four weeks of treatment. First, the number of cfGnRH-ir neurons had doubled, but not yet reached the level of cfGnRH neurons in 12 week old fish. (Schreibman et al. 1986; Goos et al. 1986; Breton and Sambroni 1996)The increase in number of GnRH neurons, normally occurring during development from juvenile to adult, is most likely due to recruitment of hitherto undifferentiated ("sleeping") neurons. It does not seem to be caused by mitotic division of neuronal elements, since dividing GnRH neurons have neither been observed in the present study, nor by Schwanzel-Fukuda and Pfaff (1990) in the mouse. We propose the existence of two subpopulations of cfGnRH neurons: 1) autonomously developing neurons and 2) neurons that require a certain level of T for their differentiation. Accordingly, the treatment with T in the present experiment may have caused extra recruitment of cfGnRH neurons of the second category.

The effect of T on the number of cfGnRH-ir neurons was not restricted to a specific area, but recorded along the entire ventral forebrain. In other teleosts, the effects of T (or its aromatised product E_2) were mostly restricted to the preoptic area, e.g. in masu salmon (Amano et al. 1994), *Haplochromis burtoni* (Soma et al. 1996) and rainbow trout (Breton and Sambroni 1996). The present observation, that T influenced cfGnRH neurons along the entire ventral forebrain, might be due to the suggestion that most neurons were not yet at their final destination. We hypothesise, that T-sensitive GnRH neurons are initially dispersed over the entire ventral forebrain, but cluster in specific areas during later development.

Second, following T treatment the overall immunofluorescence intensity had increased both in the neurons and in the fibers, which might be caused by an increase in the synthesis of the peptide and/or to an inhibition of its release. To date there are strong indications that steroids mostly act on the synthesis of GnRH rather than on its release (Montero et al. 1995).

Third, cfGnRH cell size was significantly larger compared to the control groups, though not as large as in pubertal fish of 12 weeks old. This is in contrast with the observation by Soma et al. (1996) and Francis et al. (1993), who showed an increase in cell size following castration and a reduction after subsequent T treatment. However, these observations were made in sexually mature fish with a well differentiated GnRH system. Thus, the nature of the T feedback might be dependent on the developmental stage of the GnRH system.

How T acts on GnRH neurons and whether its action is directly or indirectly via other neurons is not clear yet. Soma et al. (1996) have suggested that in *H. burtoni* androgens can affect GnRH cell size directly and without the need of aromatisation. However, androgen receptors in GnRH neurons or androgen-responsive elements within the promoter region of the GnRH gene have not been reported in teleosts. It has been suggested by a number of investigators, that T has to be aromatised to E_2 before it can cause its effects (Montero et al. 1995). Although estrogen responsive element-like motifs have been demonstrated on the GnRH promotor of the Atlantic salmon (Klungland et al. 1993), estrogen receptors have not been demonstrated on GnRH neurons in teleosts (Navas et al. 1995). Moreover, Parhar et al. (1996a) were not able to demonstrate an estrogen effect on the GnRH expression in tilapia (*Oreochromis mossambicus*). To determine whether T acts directly on the GnRH neurons, i.e. without the need of aromatisation, further experiments with non-aromatizable androgens, such as dihydrotestosterone and aromatase inhibitors will be carried out.

The only significant effect of OHA was an increase of the cfGnRH neuronal size. Since OHA can be converted into 11KT in the liver (Cavaco et al. 1997), and this hepatic capacity is already present in juvenile male catfish (Cavaco 1998), it is not clear whether the observed effect is due to OHA, 11KT or both. In *H. burtoni* (Soma et al. 1996) 11KT induced the same effects as T on the GnRH system, and in the platyfish (Schreibman et al. 1986) 11KT was even more effective than T. In the African catfish, OHA and 11KT display different effects as compared to T on the testicular and the hypophysial level (Cavaco et al. 1998b). The 11-oxygenated androgens stimulate testis growth and spermatogenesis in prepubertal catfish and have only minor effects on the pituitary; aromatizable androgens, like T and androstenedione (and E_2) have their domain of action in the pituitary, inducing gonadotroph maturation and GTH gene expression (Cavaco et al. 1997).

The cGnRH-II system seems to develop gradually during the juvenile stages regarding neuron number and cell surface. In the present study the development of the cGnRH-II system in the MT was neither affected by T nor by OHA treatment. Except for a negative feedback action of androgens in the European eel (Montero et al. 1995), no regulation of the cGnRH-II system by gonadal steroids has been reported to date. Moreover, a hypophysiotropic function of the midbrain cGnRH-II system is still questionable (Montero and Dufour 1996) and a possible innervation of the pituitary gonadotrophs in teleosts by cGnRH-II fibers has only been reported for the goldfish (Anglade et al. 1993). In several other teleost species, among them the African catfish (Zandbergen et al. 1995), no axonal connection between the neurons in the MT and the pituitary has been observed. It can, however, not be excluded that cGnRH-II may reach the gonadotrophs either via the circulation (Huang et al. 1991a) or via the cerebrospinal fluid (Skinner et al. 1998).

In conclusion, this study showed that T stimulates the development of the cfGnRH system in the African catfish, possibly by accelerating the recruitment of undifferentiated GnRH neurons. Moreover, T enhanced cfGnRH immunoreactivity, most probably by increasing the synthesis of the peptide. Finally, treatment with T resulted in a larger size of the GnRH neurons, which was also obtained with OHA. Furthermore, this study showed that these two native steroids did not affect the development and the histological appearance of the cGnRH-II system.

Acknowledgments

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Chapter 4

Gonadotropin-releasing hormone fibers innervate the pituitary of the male African catfish (*Clarias gariepinus*) during puberty

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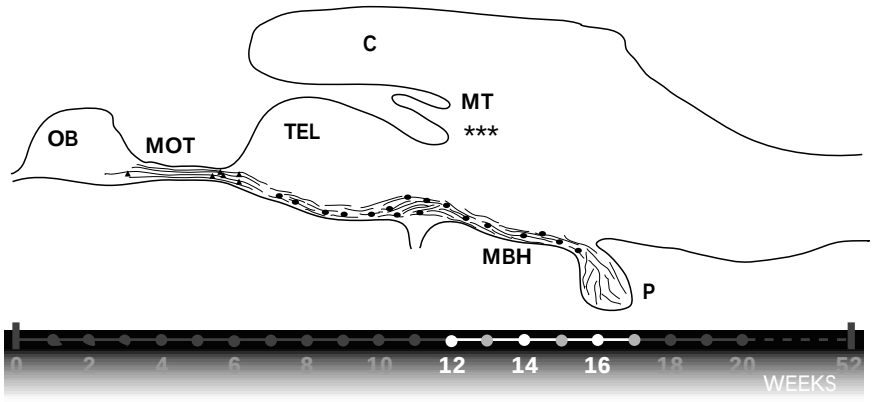


Gonadotropin-releasing hormone fibers innervate the pituitary of the male African catfish (Clarias gariepinus) during puberty

Abstract

The development of the catfish gonadotropin-releasing hormone (cfGnRH) fiber network in the pituitary of male African catfish (*Clarias gariepinus*) was investigated in relation to puberty. Double immunolabeling studied by confocal laser scanning microscopy revealed a concomitant development of gonadotrophs and pituitary cfGnRH innervation during the first wave of spermatogenesis. Catfish GnRH-immunoreactive fibers in the proximal pars distalis (PPD) of the pituitary were initially observed at the age of 10 weeks (onset of spermatogonial proliferation) and gradually reached the adult pattern at the age of 20 weeks (spermatozoa present in the testis). The content of cfGnRH associated peptide (cfGAP, part of the prohormone) in the pituitary similarly increased during puberty. At the electron microscopical level, fibers containing cfGAP-ir granules came into close proximity of the gonadotrophs at 18 weeks of age. *In vitro* studies indicated a progressively increasing basal and cfGnRH-stimulated luteinizing hormone (LH) secretion during pubertal development. The LH secretion patterns were similar in response to exogenous cfGnRH (0.1 μM) or to endogenous cfGnRH, the release of which was induced by forskolin (1 μM). Castration experiments demonstrated that the innervation of the pituitary with cfGnRH fibers continued after surgery, accompanied by an increase in the cfGAP levels. However, gonadotroph development was retarded, suggesting a differential regulation of the two maturational processes. Since testosterone stimulates both processes, other testicular factors may also be involved. Puberty-associated changes in LH release patterns appear to reflect changes in the GnRH sensitivity and in the pool of releasable LH, while the availability of cfGnRH does not appear to be a limiting factor.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons in vertebrates are divided over two or three systems, which differ in embryological origin, anatomy and expressed form of GnRH. The ontogeny and development of the ventral forebrain system are well documented (Francis et al. 1994). It is generally accepted

that these GnRH neurons originate from the olfactory placode (Schwanzel-Fukuda and Pfaff 1990). Their migration into the brain and their pathway through the medial ventral forebrain has been described for several teleost species (Francis et al. 1994; Chiba et al. 1994; Parhar et al. 1995a; Feist and Schreck 1996; Amano et al. 1998; Habibi and Huggard 1998; Parhar et al. 1998).

The best-documented function of the ventral forebrain GnRH system is the stimulation of luteinizing hormone (LH) release. The structural basis of this process in vertebrates was reviewed by Sower (1998). In tetrapods, GnRH is released into the capillaries of the median eminence and then transported to the gonadotrophs via the portal vessels. In teleosts, however, GnRH fibers either enter the pars nervosa (salmonids: Parhar and Iwata 1994) or penetrate the proximal pars distalis (PPD, most other teleosts: Peter et al. 1990).

Little is known about the temporal and spatial aspects of the development of the GnRH innervation in the pituitary of teleosts during pubertal maturation. Since the control of the GnRH system over gonadotropin release becomes potentially operational when the gonadotrophs are contacted by cfGnRH fibers, the development of the fiber network may be a key process for establishing the GnRH control.

Sexual steroid hormones are considered to play an important role in the onset of puberty (Goos 1993) and many studies have been performed to investigate their effects on the GnRH system and the pituitary gonadotrophs. In rainbow trout (*Oncorhynchus mykiss*) and African catfish, pituitary LH contents were strongly elevated after treatment with aromatizable androgens and estradiol (Crim et al. 1981; Magri et al. 1985; Goos et al. 1986; Schulz et al. 1994; Cavaco et al. 1998; Schulz and Goos 1999). Testosterone (T) effectively stimulates the GnRH system in several teleost species (Goos et al. 1986; Amano et al. 1994; Breton and Sambroni 1996; Dubois et al. 1998) while estradiol increased GnRH levels in the pituitary of the European eel, *Anguilla anguilla* (Montero et al. 1995).

In the present study we focused on the maturation of the GnRH fiber network in the pituitary of the African catfish. Two forms of GnRH have been identified in this species (Bogerd et al. 1994): chicken GnRH-II (cGnRH-II), restricted to the area of the midbrain tegmentum and catfish GnRH (cfGnRH), localized in neurons and fibers spread over the medial ventral forebrain from the olfactory bulb till the gonadotropic cell area in the pituitary (Zandbergen et al. 1995). The development of the neurons of the ventral forebrain GnRH system was investigated in a previous study (Dubois et al. 1998). It was shown that the adult pattern of cfGnRH neuron and fiber distribution in the brain is already achieved at the onset of puberty (10-12 weeks of age, onset of spermatogonial proliferation) when, however, only a few cfGnRH fibers were observed in the neurointermediate lobe (NIL) and the PPD. This

finding suggests a later development of the GnRH fiber network in the pituitary compared to the cfGnRH neurons and their axons in the brain.

We describe here the structural development of the cfGnRH neuronal network in the pituitary and its cfGnRH-associated peptide (cfGAP) content in relation to gonadotroph maturation and spermatogenesis. The role of testicular hormones in these processes was studied by castration and T replacement experiments. The functional development of the GnRH-LH system was investigated using *in vitro* pituitary incubations.

Materials and Methods

Animals

African catfish were raised in the hatchery of the Department of Experimental Zoology, University of Utrecht. The animals were kept in a copper-free recirculating water system with a temperature of 25 ± 2ºC, 14h daily light period and food pellets (Trouvit, Putten, the Netherlands) were provided ad libitum. For the electron microscopic study male fish of 10, 14 and 18 weeks of age were used; for the confocal laser scanning microscopy (CLSM) male fish of 10, 12, 16, 20 weeks of age, and adults were investigated. Since cfGnRH cannot be labeled with radioactive iodine and attempts with other cfGnRH-immunoassay methods were not successful, a quantitative analysis of cfGAP in the pituitary was performed by radioimmunoassay (RIA); for this purpose pituitaries of 10-, 12-, 16-, and 20-weeks-old fish were used. The fish were sacrificed by decapitation; the pituitary and testes were dissected and fixed for immunocytochemistry (CLSM or EM) and histology, respectively. For the *in vitro* studies on LH secretion, pituitaries were used of males of 9, 13, 16, and 20 weeks of age.

Immunocytochemistry for CLSM and EM

The cfGnRH fiber network and the gonadotrophs in the pituitary were visualized by immunofluorescent double labeling, followed by CLSM. The fixation and immunofluorescence procedures were performed according to Dubois et al. (1998). The cfGnRH fibers were labeled with anti-cfGnRH-associated peptide (anti-cfGAP, 1:500 (Zandbergen et al. 1995)) and anti-rat IgG-TRITC (1:320, Sigma, St. Louis, MO) or with anti-cfGnRH (1:1000) and anti-rabbit IgG-FITC (1:200, Sigma, St. Louis, MO); the gonadotrophs were labeled with anti-α,βLH (1:5000) according to Zandbergen et al. (1993) and anti-rabbit IgG-FITC.

The ultrastructure of the cfGnRH fibers and their morphological relationship with the gonadotrophs were studied by electron microscopy. Cryosubstitution and immunocytochemistry for EM were performed as

described by Zandbergen et al. (1992). Anti-cfGAP (Zandbergen et al. 1995) and goat-anti-rabbit-gold 10nm (Aurion, Wageningen, The Netherlands) were applied as the primary and secondary antiserum, respectively.

Testicular histology

To determine the stages of spermatogenesis, testes were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4), dehydrated in graded ethanol and embedded in paraffin. Sections of 7μm were cut and stained with hematoxilin-eosin. Four stages of germ cell development were distinguished: stage I (onset of puberty, spermatogonia present in the testis), stage II (spermatogonia and spermatocytes), stage III (spermatogonia, spermatocytes and spermatids), and stage IV (all germ cells, including spermatozoa; Schulz et al. 1994).

Pituitary incubation studies

Pituitaries from 9-, 13-, 16- and 20-week-old fish were used to test the LH release response of the maturing gonadotrophs. The release of LH was stimulated by a moderate dose (0.1 μM) of exogenous cfGnRH, or by evoking the release of endogenous cfGnRH from the cfGnRH-containing fibers in the pituitary tissue fragments with 1 μM forskolin (Rebers et al. 2000a). The general incubation conditions were as described in Schulz et al. (1995). In brief, pituitaries of 9- and 13-week-old fish were incubated *in toto* while the glands of 16- and 20 week-old fish were divided in two parts. After an overnight preincubation, the incubation was continued with fresh medium for 3 h to determine basal LH secretion. The incubation was continued for another 3 h with medium containing 0.1 μM cfGnRH or 1 μM forskolin. In this way, each pituitary could serve as its own control (basal *versus* stimulated LH secretion). The LH release in the medium was measured by RIA.

Castration and testosterone replacement

To investigate the involvement of testicular hormones in the development of GnRH innervation and maturation of the gonadotrophs, a castration experiment, including T replacement, was carried out. Fish of 12 weeks of age were anaesthetized with 0.03% tricaine methane sulphonate (TMS, Cresent Research Chemicals, Phoenix, AZ, USA), followed by application of 2% phenoxyethanol (Sigma, St. Louis, MO, USA) on the gills (300 μl on each side). A ca. 3-cm long incision was made to open the body cavity. The testes were removed (castrated fish, C) or left in place (sham-operated fish, S). The incision was sutured with silk and disinfected with potassium permanganate solution (1000 ppm). The fish were allowed to recover by rinsing the gills for 5-10 minutes with running tap water (26° C). The fish were then kept in aquaria for 3 weeks. Feeding was resumed after the closure of the wound (after 2 days). At

15 weeks of age the first two groups (15 C and 15 S) were sampled: the fish were weighed, blood was taken from the caudal vein, followed by decapitation and collection of the pituitary for immunocytochemistry or determination of the LH content. The testes of the sham-operated fish were dissected and weighed in order to determine the gonadosomatic index, which represents the percentage of testis weight over body weight. On the same day, the remaining fish were divided in 3 groups (17C, 17S and 17C+T) and each fish received a silastic pellet containing T (40 µg/g BW, 17C+T) or no steroid (17C and 17S). The pellets were implanted via a small incision in the body cavity. Two weeks after implantation the fish were sampled as described above. At 12 weeks of age, a start control group was sampled.

Radioimmunoassays

In order to provide quantitative data on the cfGnRH content in the pituitary, homogenates were measured by a cfGAP-RIA. For that purpose the first 25 amino acids of GAP were synthesized (GAP25, American Peptide Company, CA, USA) and used as standard and for labeling with ^{125}I. Pituitaries from the castration experiment and pituitaries sampled during puberty (10, 12, 16, and 20 weeks) were homogenized in 0.3 ml RIA buffer (0.05 M barbituric acid (Merck)/5,5-diethylbarbituric acid sodium salt (Fluka), 0.1% NaN_3 (Merck), and 0.2% BSA, pH 8.6. After sonicating 1 min, the homogenates were centrifuged for 30 min at 5000g at 4°C; the supernatant was used in the RIA. The radioiodination of GAP was performed according to the method of Okuzawa et al. (1990). The standards were dissolved in RIA buffer ranging from 100pg to 25600 pg/tube. An antiserum against GAP25 raised in rabbit (63-3) was used in a 400-fold dilution together with 1% normal rabbit serum, in order to achieve 30% binding of the labeled GAP. Of samples and standards, 50µl was assayed in duplicate, and incubated overnight at 4°C in the presence of 50µl of diluted first antibody (63-3) and 100µl RIA buffer. The 2nd day 12500cpm in 100µl of label was added to each tube and incubated under the same conditions overnight. The next day the second antibody, goat-anti-rabbit, diluted 40-fold in RIA buffer was added. After an overnight incubation at 4(C, 2ml RIA buffer was added and the tubes were centrifuged for 30 min at 5000rpm. The supernatant was aspirated and the pellet was washed with 1ml RIA buffer and centrifuged under the same conditions. The supernatant was aspirated again and the precipitate was counted in a gamma counter. The cfGAP-RIA was validated by examining the parallelism of pituitary extract from adult catfish. No cross-reactions with cfGnRH, cGnRH-II or LH were observed; the interassay difference was below 10%. The protein determination was performed according to Bradford (1976).

LH was measured in the medium from the *in vitro* incubations and in the pituitaries from the castration experiment. For the LH content, the pituitaries

were homogenized and centrifuged (Schulz et al. 1997), and LH was quantified in the supernatant using the assay system described by Goos et al. (1986). T levels in the plasma were measured by RIA (Schulz et al. 1994) to check the effect of the T implantation.

Statistics

All results are given as means ± SEM. The data of the *in vitro* incubations are also presented as stimulation quotient, calculated as the stimulated LH secretion divided by the basal LH secretion for each individual pituitary. The effects of 0.1 μM cfGnRH or 1 μM forskolin on LH secretion was analyzed by testing if the stimulation quotient differed significantly from a population mean of 1 (one-sample t test). All other data were analyzed by one-way ANOVA followed by Fisher's least significant difference test ($p<0.05$).

Results

Pubertal development of the cfGnRH system in the pituitary

Anti-cfGAP is localized similarly to anti-cfGnRH in pituitary sections (Fig. 1A, B) as shown in pituitaries of 20-week-old fish. In Fig. 1C, D the adult situation of the pituitary innervation by cfGAP-ir fibers is depicted. The LH-ir cells are fully mature and the cfGAP-ir fibers form a widely distributed network within the PPD, and in intimate contact with the LH-ir cells.

At the onset of puberty (10 weeks of age, testis in stage I), only a few thick cfGAP-ir fiber bundles and a relatively small number of LH-ir cells were present in the pituitary (Fig. 2A). The ir-bundles were observed in the NIL and in the periphery of the developing pars distalis. The cfGAP immunoreactivity was most intense in the characteristic Herring-bodies in the nerve fibers. LH-ir cells were small, irregular in shape and scattered over the PPD and cfGAP-ir fibers were rarely found in their immediate vicinity (Fig. 2B).

Two weeks later (12 weeks, beginning of stage II of spermatogenesis), the cfGAP-ir fibers were still concentrated in numerous, strongly immunoreactive nerve bundles (Fig. 2C, D). They were orientated towards and now also found within the PPD where they surrounded groups of gonadotrophs. At the age of 16 weeks (stage III of spermatogenesis), the gonadotrophs were no longer organized in distinct groups, but evenly distributed throughout the PPD (Fig. 2E); an increasing number of cfGAP-ir fibers were located in close proximity to gonadotrophs and the formation of a widespread fiber network is initiated (Fig. 2F).

In fish of 20 weeks old (stage IV of spermatogenesis), an extensive network of mostly delicate cfGAP-ir fibers surrounded the gonadotrophs throughout the entire PPD (Fig. 2G). The gonadotrophs had increased in size

and were less irregular in shape than at 12 and 16 weeks of age (Fig. 2H). At this age, the PPD of catfish of this age resembled histologically the adult pattern (compare with Fig 1C, D), i.e. the gonadotrophs attained the adult size and round shape, and the majority of the cells was in close vicinity of cfGAP-ir fibers.

In addition to the histological observations, the cfGAP content of the pituitaries at the same time points was determined. The cfGAP levels are also shown in Fig. 2, both as cfGAP per pituitary (Fig. 2I) and as cfGAP per mg protein (Fig. 2K). At 10 weeks of age an average of 167 ±15 pg cfGAP per pituitary was measured, which increased during puberty to 696 ±101 pg at 20 weeks of age. The rise of cfGAP between 12 weeks (278 ±28 pg) and 16 weeks of age (649 ±75 pg) was significant. The content expressed as cfGAP per mg protein follows the same pattern as cfGAP per pituitary (Fig. 2I, K).

The ultrastructure of the 10-week-old pituitary showed many undifferentiated cells in addition to differentiated somatotrophs (Fig 3A). Occasionally, a cell with weakly LH-ir secretory granules was observed. CfGAP-ir fibers with secretory vesicles were only encountered in the NIL and grouped in thin bundles (Fig. 3B). At this stage no cfGAP-ir fibers were found in the proximity of the few identifiable gonadotrophs.

In 14 week-old catfish (stage II of spermatogenesis) the three different lobes of the pituitary (NIL, rostral pars distalis and PPD) could clearly be distinguished. The number of differentiated gonadotrophs had increased as compared to the 10-week-old fish and now cfGAP-ir fibers were occasionally observed in their vicinity. The PPD of 18-week-old fish at stage III of testicular development, no longer exhibited undifferentiated glandular cells. The cfGAP-ir fibers were frequently found in the vicinity of the gonadotrophs and also of the neighboring somatotrophs (Fig. 3C,D). No synaptic contacts between fibers and gonadotrophs were observed.

Pituitary incubation studies

Basal and stimulated LH release increased during puberty (Fig. 4 a-c). Administration of forskolin (1 μM) elevated LH release, as did cfGnRH (0.1 μM), but more inconsistently and to a lesser extent, in particular when calculated as stimulation ratios (Fig. 4c). Both cfGnRH and forskolin treatment resulted in stimulation quotients significantly higher than 1 in all experiments. The response patterns were similar, with a transient increase in GnRH sensitivity at 16 weeks of age, irrespective of eliciting LH release by exogenous cfGnRH or by releasing endogenous cfGnRH by forskolin.

Castration experiment

The absence of testicular tissue in the castrated fish was checked at autopsy. Castration resulted in a significant decrease of T plasma levels at 15 weeks (S15

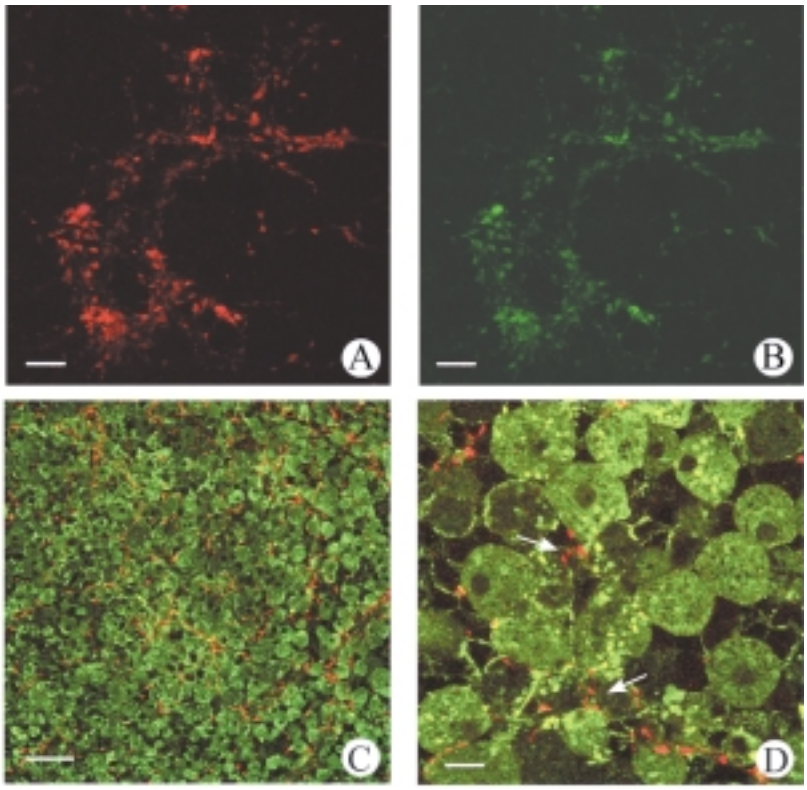


Fig. 1. CLSM images showing cfGnRH or cfGAP and LH immunoreactivity in the pituitary. **A** 20 weeks old, the cfGAP immunoreactivity (red) and **B** cfGnRH (green) immunoreactivity colocalize in the proximal pars distalis; scale bar represents 10μm. **C, D** adult pituitary with cfGAP-immunoreactivity in red and LH-immunoreactivity in green. **C** overview of the PPD (bar 50 μm) and **D** detail of the PPD (bar 10 μm). The arrows point at delicate cfGAP-ir fibers.

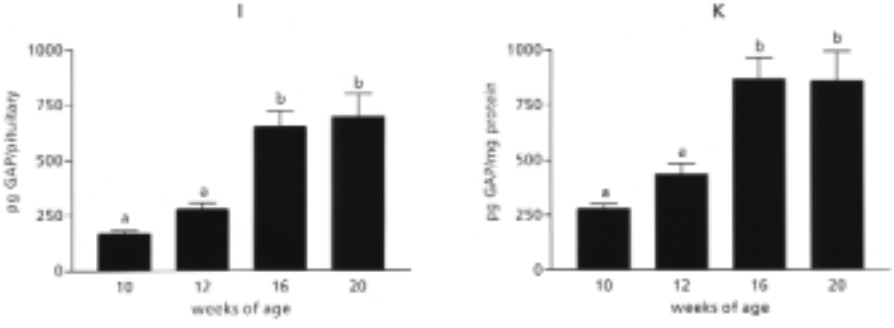
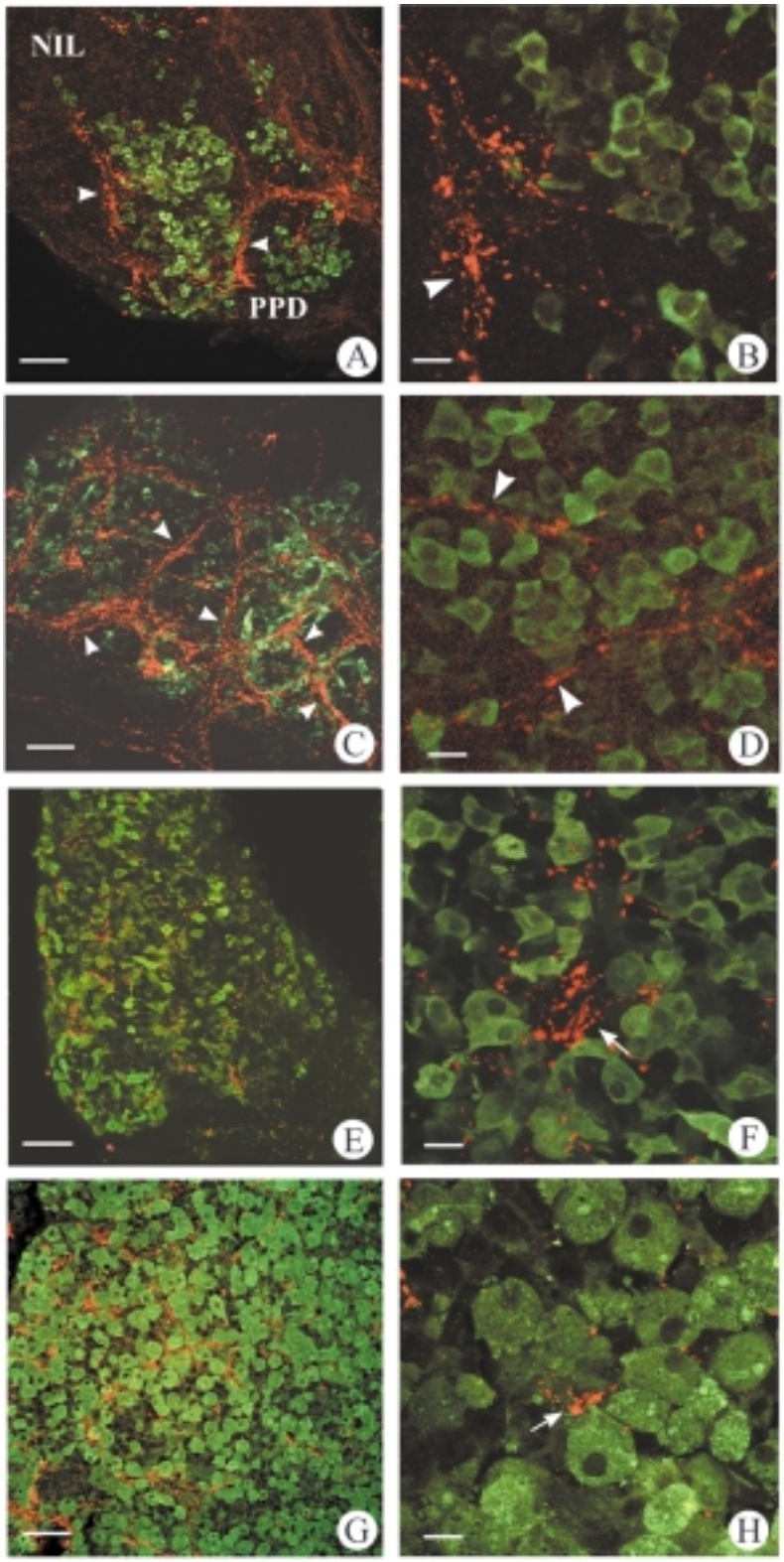


Fig. 2. Pubertal development of the cfGnRH system in the pituitary represented as CLSM images of cfGAP-ir fibers and LH-ir cells (left), and as cfGAP content of the pituitary (right) at 10, 12, 16 and 20 weeks of age. **A-H** Developmental series of cfGAP immunoreactivity (red) and LH immunoreactivity (green) in the pituitary of African catfish from 10 till 20 weeks of age. The left column (**A, C, E, G**) shows an overview of the pituitary (bar 50μm) of 10, 12, 16 and 20 weeks of age, respectively. The right column (**B, D, F, H**) represents the gonadotrophs and cfGAP-ir fibers in more detail (bar 10μm). **A, B** 10 week-old. **C, D** 12 weeks old. **E, F** 16 week-old. **G, H** 20 week-old. NIL: neurointermediate lobe, PPD: proximal pars distalis. Arrowheads indicate large cfGAP-ir nerve bundles, arrows point at delicate cfGAP-ir fibers. The graphs on the right represent **I** the amount of GAP (in pg) per pituitary and **K** the amount of GAP (in pg) per mg protein. Bars sharing the same letter are not significantly different ($p<0.05$, ANOVA, Fisher's least significant difference test).


NIL
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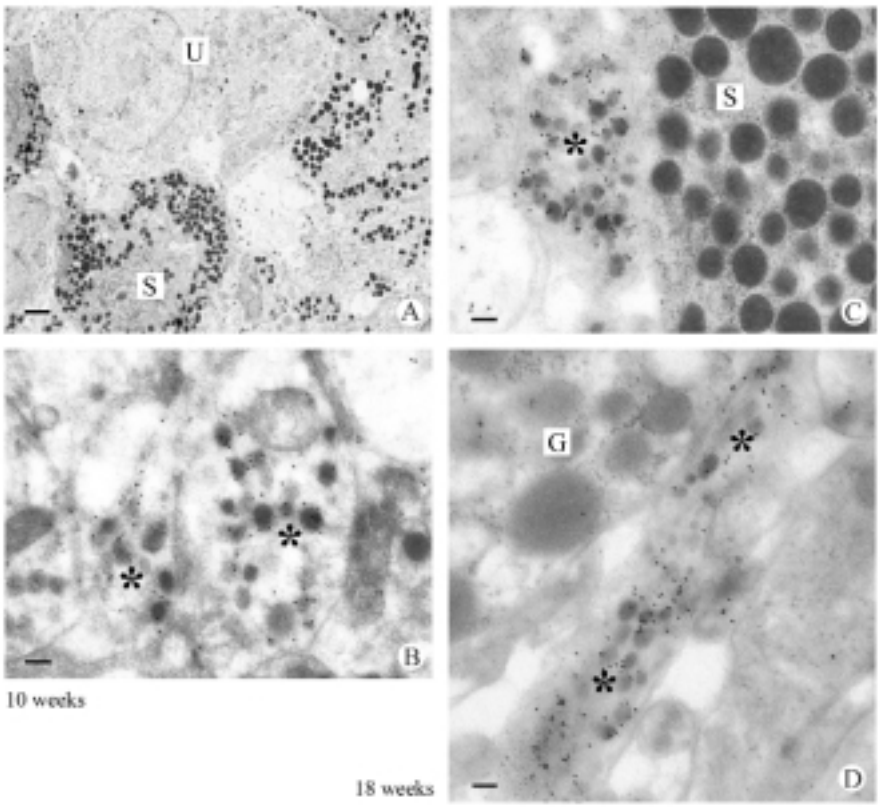

Fig. 3. Electron microscopic images of the pituitary. A Overview of the pituitary in 10-week-old African catfish, which shows somatotrophs (S) and undifferentiated cells (U). Bar 700 nm. B Two axons labeled with anti-cfGAP and 10 nm gold in the NIL of 10-week-old catfish; scale bar 90 nm. C An anti-cfGAP labeled axon in close apposition of a somatotroph (S) in an 18-week-old fish; scale bar 80 nm. D An anti-cfGAP labeled fiber in close vicinity of a gonadotroph (G) in a catfish of 18 weeks of age; scale bar 90 nm. The anti-cfGAP labeled axons are indicated with an asterisk.

0.9 ± 0.2 ng/ml, C15 0.1 ± 0.08 ng/ml) and 17 weeks (S17 2.5 ± 0.2 ng/ml, C17 0.7 ± 0.2 ng/ml), 3 and 5 weeks respectively after surgery. Implantation of T 3 weeks after castration led to strongly increased plasma T 2 weeks later (19.2 ± 1.8 ng/ml, a level comparable to that of adult fish (Schulz et al. 1994)).

Three weeks of castration had a clear morphological effect on the gonadotrophs; the LH immunoreactivity and cell size were decreased and the gonadotrophs were irregularly shaped compared to the cells in the sham-operated fish (Figs. 5 A, B). This was also reflected in the pituitary LH content (Fig. 6), castrated fish exhibiting significantly lower LH contents than sham-operated ones of the same age. Castrated fish with a T pellet (17C+T) displayed pituitary LH contents similar to sham-operated fish of 17 weeks.

In contrast, the cfGAP innervation pattern was not affected by castration. The immunoreactivity and the distribution of the fibers were similar in sham-operated and castrated fish (Figs. 5 C, D). However, T treatment of castrated fish

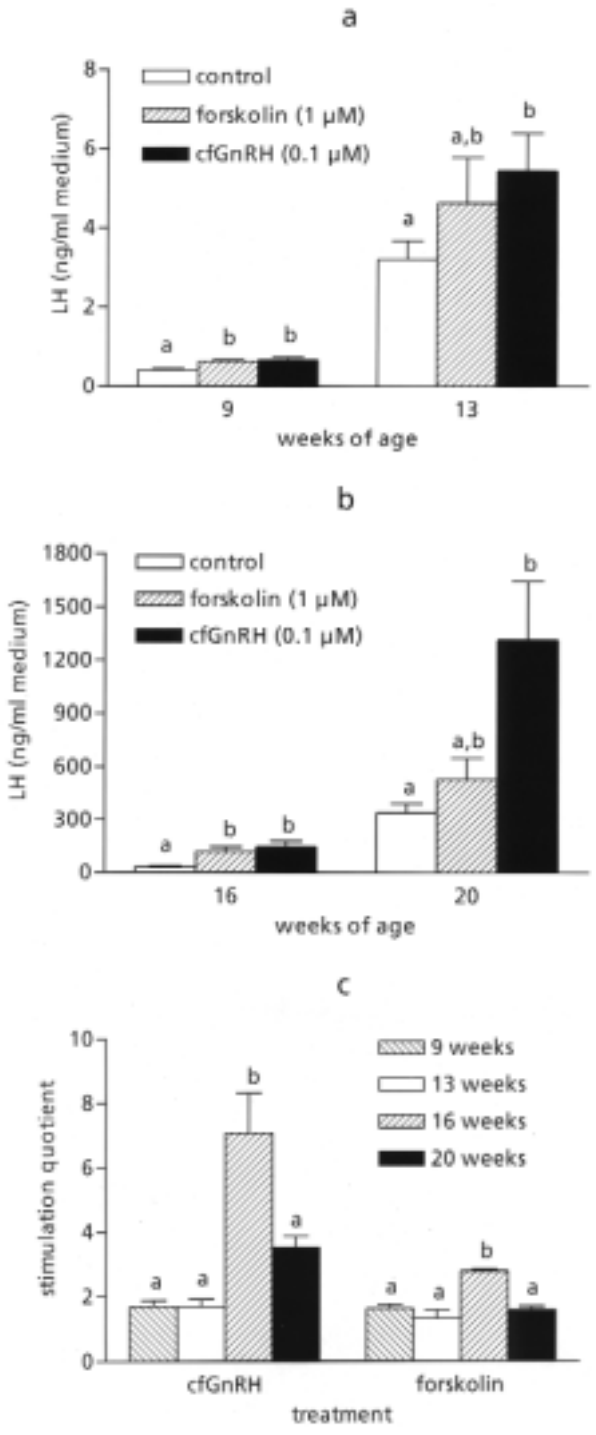

Fig. 4. ***In vitro*** **secretion of LH by pituitaries of African catfish of 9 and 13 (a) and 16 and 20 weeks of age (b). Basal LH secretion was determined after 3 h incubation in the absence of test substances (open columns; n=10-12), stimulated LH secretion was determined after another 3 h incubation in the presence of 1 µM forskolin (hatched columns) or 0.1 µm cfGnRH (black columns). (c) The stimulation quotient (stimulated LH secretion divided by basal LH secretion of a given pituitary) of cfGnRH- or forskolin-stimulated LH release during puberty. In all cases the stimulation quotient was significantly higher than 1.**

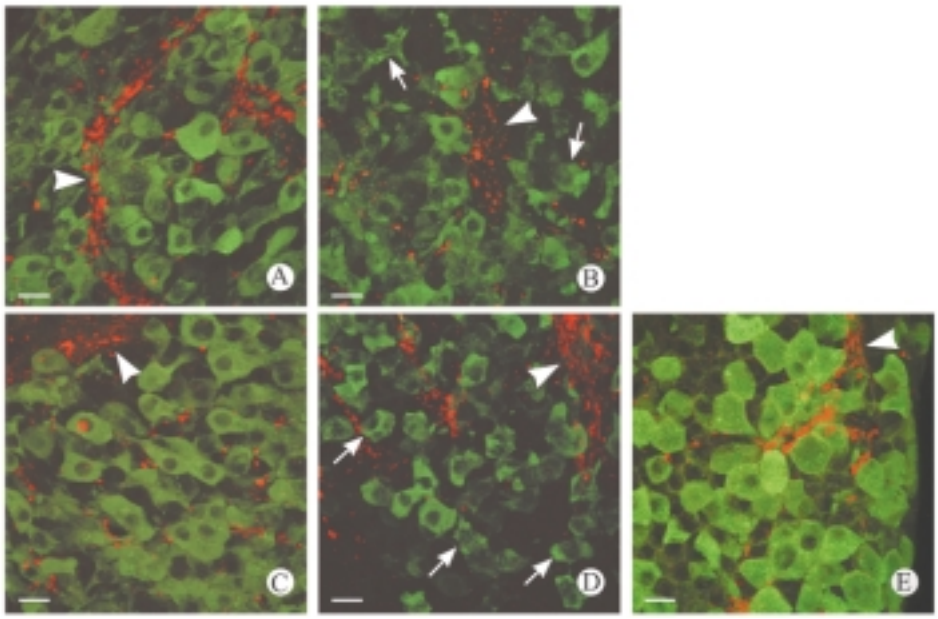

Fig. 5. CLSM images of pituitaries showing cfGAP immunoreactivity in red and LH-immunoreactivity in green. **A, B** pituitaries of shams and castrated fish at 15 weeks, i.e. 3 weeks after surgery. **C, D** pituitaries of shams and castrated fish at 17 weeks, i.e. 5 weeks after surgery and implanted with a control pellet at 15 weeks. **E** Pituitary of a castrated fish at 17 weeks, i.e. 5 weeks after surgery and implanted at 15 weeks with a pellet containing T. bar 10μm. The arrowheads point at the large immunoreactive nerve bundles, the arrows indicate the gonadotrophs that are irregularly shaped after castration.

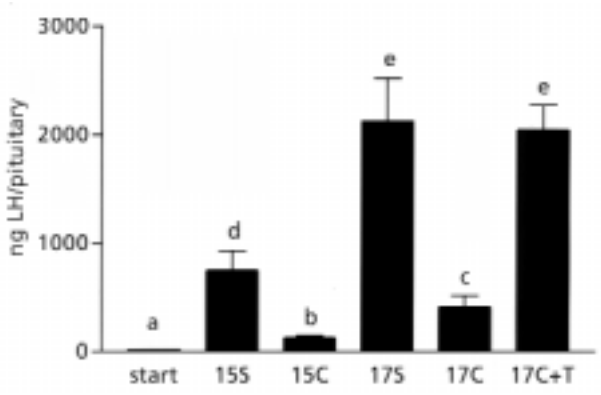

Fig. 6. LH content in sham-operated (15S, 17S), castrated (15C, 17C), and castrated and T-implanted (17C+T) fish. Bars sharing the same letter are not significantly different ($p<0.05$, ANOVA, Fisher's least significant difference test).

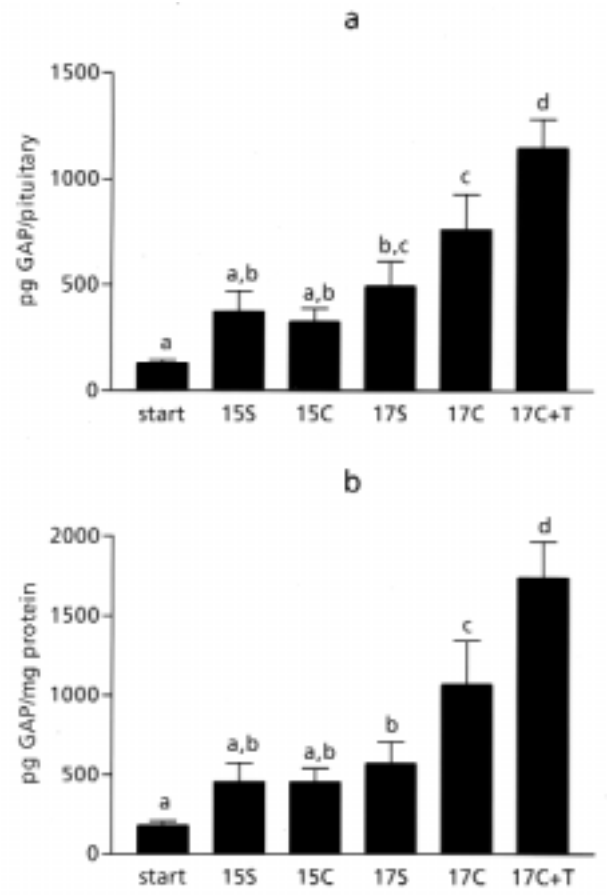

Fig. 7. GAP content in the pituitary of sham-operated (15S, 17S), castrated (15C, 17C), and castrated and testosterone-implanted (17C+T) fish. a the amount of GAP (in pg) per pituitary and b the amount of GAP (in pg) per mg protein. Bars sharing the same letter are not significantly different ($p<0.05$, ANOVA, Fisher's least significant difference test).

resulted in a stronger labeling of the cfGAP fibers and also of the gonadotrophs (Fig. 5E).

The data obtained by the cfGAP RIA showed that 5 weeks of castration resulted (Fig. 7a) in an increased hypophyseal cfGAP content (17C: 761 ±167 pg cfGAP) when compared to the sham-operated fish (17S: 492 ±119 pg cfGAP); this difference was significant when expressed per mg protein (Fig. 7b). The cfGAP contents were even more elevated in the combination of castration and T replacement (17C+T: 1144 ±135 pg).

The sham-operated fish showed a normal testicular development during the experiment as indicated by the increase in GSI from start control (12 weeks; 0.011 ± 2.10^{-3}) to 15 weeks (15S; 0.033 ± 5.10^{-3}) and a further significant increase at the age of 17 weeks (17S; 0.11 ±0.02).

Discussion

The structural development of the cfGnRH fiber network in the pituitary of the African catfish can be subdivided into four phases. The process starts with the appearance of immunoreactive cfGAP-ir fibers in the neurohypophysis at the age of 10-11 weeks. This is followed by a penetration of the fibers into the developing proximal pars distalis where the first gonadotrophs are differentiated. Next, more gonadotrophs are recruited and immunoreactive cfGAP-ir fibers surround clusters of gonadotrophs. The development is completed when numerous delicate branches invade the intercellular spaces between the gonadotrophs and appear in the immediate vicinity of the cells. Then, at about 20 weeks of age a situation is reached which is comparable with the adult status (Zandbergen et al. 1995). While the cfGnRH neurons in the ventral forebrain are completely developed at 12 weeks of age (Dubois et al. 1998), the present data demonstrate that the morphological development of the pituitary cfGnRH innervation requires another eight weeks. This development is completed in parallel to the first wave of spermatogenesis.

These histological observations are supported by the data of the cfGAP contents in the pituitary, i.e. the appearance of numerous, delicate fibers at 16 weeks is accompanied by a significant increase in cfGAP content.

The development of the GnRH-network in the pituitary of the African catfish parallels the main phases of testicular development, as described by Schulz et al. (1994). Also in the platyfish (*Xiphophorus maculatus*, Halpern-Sebold and Schreibman (1983) observed the first GnRH-immunoreactivity in the pars distalis at the onset of puberty, followed by completion of the network at the time that sexual maturity was established. A similar correlation between GnRH network formation in the (neuro-) hypophysis and gonadal maturation was described in salmonids (Parhar et al. 1995a; Amano et al. 1998).

The development of the gonadotrophs in the pituitary of the African catfish, as described in the present study, corresponds with earlier observations (Zandbergen et al. 1993; Schulz et al. 1997b). During puberty, these cells show a gradual increase in size, number and LH-immunoreactivity, probably forming the basis for the increasing amounts of LH released. The present results also indicate that between 18 and 20 weeks (stage IV of spermatogenesis) the spatial relation between cfGnRH-fibers and gonadotropic cells in the PPD reaches the mature state as described by Peute et al. (1987). Synaptic endings of cfGnRH-fibers on the gonadotrophs could not be demonstrated, due to the absence of osmium tetroxide in the cryosubstitution procedure. In conventially fixed pituitaries, however, it was shown that fibers with similar granules as those labeled with anti-cfGAP were in synaptic contact with gonadotrophs (Peute et al. 1984; Peute et al. 1987). It is not clear yet whether cfGnRH is

released in the intercellular space close to gonadotrophs or is transferred directly via synaptic contacts. As discussed in the following section, endogenous cfGnRH is able to release LH already in stage I of spermatogenesis.

In order to assess functional consequences of the morphological development of the pituitary innervation, we studied cfGnRH- and forskolin-stimulated LH release. We have found recently that cfGnRH-containing nerve fibers release cfGnRH following stimulation of the adenylate cyclase (Rebers et al. 2000a). The pituitary incubation experiments made use of this observation to study LH release in response to endogenous cfGnRH, and by comparing this release pattern to the one in response to exogenous cfGnRH. As in previous studies, pituitaries were sensitive to GnRH at all ages studied, and basal and stimulated LH secretion increased during puberty, reflecting the maturation of the gonadotrophs (Schulz et al. 1995; Schulz et al. 1997b). The similarity of the LH release patterns in response to exogenous *versus* endogenous cfGnRH (Fig. 3c) suggests that the amount of cfGnRH in the vicinity of the gonadotrophs is unlikely to be a limiting or determining factor for the LH release pattern observed. The latter, therefore, appears to be determined rather by the gonadotrophs´ sensitivity towards GnRH stimuli. This is currently studied by analyzing GnRH receptor expression.

The results of the castration experiment suggest that the structural development of the GnRH innervation pattern during puberty is not dependent on gonadal hormones. The increase in cfGAP content after castration, however, provides evidence for the presence of an inhibiting factor originating from the testes. This unknown factor is probably not T, since replacement of the latter caused a stronger immunoreactivity of the cfGAP fibers and further increased the cfGAP content. The mechanism by which T, or its aromatization product E_2, increases cfGAP levels in the pituitary is unclear, but most likely T/E_2 either enhances the GnRH synthesis and/or inhibits its release (Dubois et al. 1998).

The maturation of the gonadotrophs is dependent on the presence of the testis. The decrease in pituitary LH content after castration and its reincrease following T treatment correspond with earlier findings in the African catfish (Cavaco et al. 1995; Schulz and Goos 1999) and indicates that T plays an important role with regard to LH synthesis. Actually T is first converted into E_2via aromatase that is present in the gonadotrophs (Leeuw et al. 1985b). Also in other teleosts a positive feedback of T on gonadotroph development and LH synthesis has been reported (Gielen et al. 1982; Magri et al. 1985; Breton et al. 1997; Habibi and Huggard 1998; Dickey and Swanson 1998; Borg et al. 1998).

In summary, the present findings demonstrate a gradual invasion of the cfGnRH fibers into the PPD of the pituitary during the first wave of spermatogenesis, accompanied by an increase of the cfGAP content. The cfGnRH fiber network develops in synchrony with the maturation of the gonadotrophs.

However, these two processes can be dissociated by castration: gonadotroph maturation is inhibited, while cfGAP levels in the pituitary are increased. In a previous study, we demonstrated that T stimulates the maturation of the cfGnRH perikarya in the ventral forebrain in 2-6 week-old catfish (Dubois et al. 1998). The present study, again, revealed that both the cfGAP content and the immunoreactivity in the pituitary were increased after T replacement. So, the development of the cfGnRH-LH system is under multifactorial control, with T as the main stimulatory factor for each of the components. Finally, we propose that the maturation of a functional cfGnRH-LH system is determined by (at least) three factors: the availability of cfGnRH in the vicinity of the gonadotrophs, the gonadotrophs' GnRH responsiveness, and finally the LH synthesis/release capacity of these cells.

Acknowledgements

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Chapter 5

Gonadal steroids and the maturation of the species specific gonadotropin-releasing hormone system in brain and pituitary of the male African catfish (*Clarias gariepinus*)

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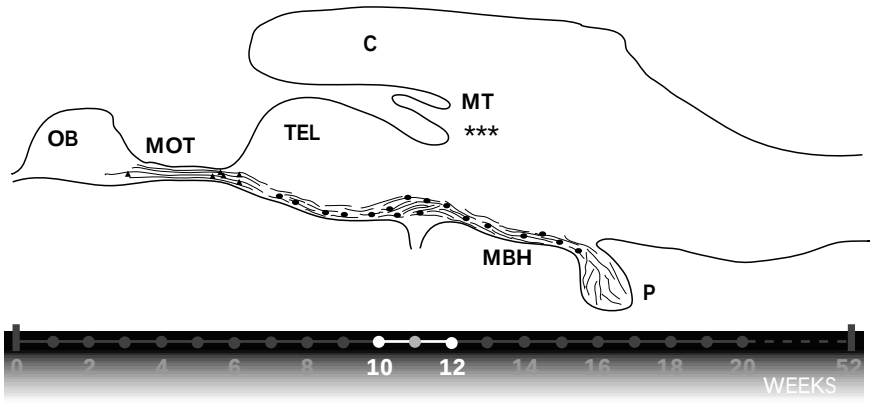


Gonadal steroids and the maturation of the species specific gonadotropin-releasing hormone system in brain and pituitary of the male African catfish *(Clarias gariepinus)*

Abstract

The effect of testosterone (T), 11-ketotestosterone (KT) and estradiol (E_2) on the development of the catfish gonadotropin-releasing hormone system (cfGnRH) of male African catfish (*Clarias gariepinus*), at the onset of puberty (between 10 and 12 weeks post hatching [ph]) was investigated. The cfGnRH neurons, located in the ventral forebrain, were visualized by immunofluorescence and their numbers were determined and the amounts of cfGnRH-associated peptide (cfGAP) in the pituitary were measured by RIA. Steroid treatments did not significantly alter the numbers of immunoreactive GnRH neurons. However, T and E_2 caused an increase in the amount of GnRH, demonstrated by the intensity of the immunostaining of GnRH neurons and fibers in the brain and the amount of cfGAP in the pituitary. Treatment with KT, the main circulating androgen in adult male catfish, did neither change the number of cfGnRH neurons, nor elevated the cfGnRH content in the pituitary. In previous experiments with younger, prepubertal fish (2-6 weeks ph), T caused an elevation of the number of cfGnRH neurons to the same level as present in pubertal fish of 12-14 weeks. We conclude that the onset of puberty in the male African catfish coincides with the completion of the - steroid dependent - structural maturation of the cfGnRH system in the brain. T and/or E_2, however, still are able to exert a positive influence on the amounts of cfGnRH during the later stages of pubertal development, thus still playing a role in the control of the cfGnRH system.

Introduction

To date 15 different forms of gonadotropin releasing hormone (GnRH) have been identified (Powell et al. 1996b; Carolsfeld et al. 2000; Yoo et al. 2000; Okubo et al. 2000a; Montaner et al. 2000). In most species two forms are expressed in the brain, but it was recently shown that a third GnRH exists in modern teleost species (Amano et al. 1997; Parhar et al. 1998; Fernald and White 1999). The two (or three) forms not only differ in amino acid sequence,

but also in embryonic origin, localization and function. The species specific GnRH (also indicated as GnRH1; Fernald and White 1999) is localized in the ventral forebrain/hypothalamus; its most obvious function is to control hypophyseal gonadotropin release. The neurons that produce this GnRH originate from the olfactory placode (King and Millar 1992; Sherwood et al. 1993; Parhar 1999; Fernald and White 1999). GnRH2 is the highly conserved chickenGnRH-II (cGnRH-II), present in all vertebrates and localized in large neurons in the midbrain; its function is still under debate (King and Millar 1992; Fernald and White 1999). The third form (GnRH3), as found in modern teleost species, is expressed in the terminal nerve, olfactory bulbs and rostral telencephalon (Parhar 1999; Fernald and White 1999).

The functional development of the GnRH1 system in immature teleosts is under stimulatory control of gonadal steroids, especially testosterone (T). This has been demonstrated for the masu salmon (*Oncorhynchus masou*; Amano et al. 1994), rainbow trout (*Oncorhynchus mykiss*; Goos et al. 1986; Breton and Sambroni 1996), platyfish (*Xiphophorus maculatus*; Schreibman et al. 1986) and African catfish (*Clarias gariepinus*; Dubois et al. 1998). In the European eel (*Anguilla anguilla*), estradiol (E_2) alone or in combination with T was equally effective (Montero et al. 1995).

In the African catfish two different forms of GnRH have been identified: catfish GnRH (cfGnRH = GnRH1) and cGnRH-II (Bogerd et al. 1994). CatfishGnRH is distributed over the entire ventral forebrain i.e., from olfactory bulb till pituitary, whereas cGnRH-II was exclusively found in the midbrain tegmentum (Zandbergen et al. 1995). We previously investigated the effects of T and 11β-hydroxyandrostenedione (OHA) on the development of the cfGnRH system in the brain of prepubertal catfish (2-6 weeks of age, gonads still undifferentiated; Dubois et al. 1998). T treatment resulted both in an increase in number of cfGnRH neurons and the intensity of the immunostaining, whereas OHA had a weak positive effect on the cell size only.

The period of pubertal development in the male African catfish spans the time between the first meiotic division in the testes (between 10-12 weeks of age) and the completion of the first wave of spermatogenesis (20-24 weeks of age) and covers the functional development of brain-pituitary-gonad axis.

In a previous study we demonstrated that the cfGnRH system in the brain hardly develops any further during the process of puberty (Dubois et al. 2000). However, we have shown that the prepubertal development of the cfGnRH system is accelerated by T treatment (Dubois et al. 1998). Therefore, we hypothesized that the cfGnRH neurons are already structurally differentiated at the onset of puberty. In the present study, we investigated whether gonadal steroid hormones still have an effect on the number of cfGnRH neurons and the cfGnRH content in the pituitary at the onset of pubertal development. To that end we studied the effects of three different steroid hormones on the

cfGnRH system of the male African catfish at the onset of puberty by determining the numbers, size and immunoreactivity of cfGnRH neurons, and by quantifying the cfGnRH levels in the pituitary by radioimmunoassay.

Materials and methods

African catfish were raised in the aquarium of the Research Group for Comparative Endocrinology by artificial fertilization. At the age of 10 weeks males received silastic pellet implants (by making a small incision in the abdominal cavity) containing 30μg/g body weight of T, 11-ketotestosterone (KT), E_2 or no steroid (control), respectively. After 2 weeks blood samples were taken. The fish were killed by decapitation and brain, pituitary and testes were sampled. Per experimental group 5 brains and attached pituitaries were fixed for immunocytochemistry as described earlier (Dubois et al. 1998). In brief, the tissues were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, cryoprotected in graded sucrose and frozen. After sectioning, the highly specific anti-cfGAP (Zandbergen et al. 1995) against cfGnRH-associated peptide was applied to stain the cfGnRH neurons. The numbers of cfGnRH-ir neurons were counted in every alternate section and their size was measured (Dubois et al. 1998). Furthermore, the differences in staining intensity were established in a double-blind test. The remaining pituitaries (n=15-20) were processed for cfGAP determination by RIA. This assay has been developed previously (Dubois et al. 2000) for two reasons. Antisera against GAP are more specific compared to GnRH-antisera. Moreover, cfGnRH does not contain a tyrosine residue and is therefore hard to use as label in an iodine-based RIA. Testicular histology was studied as described before (Dubois et al. 2000) in order to determine the stage of spermatogenesis. Stage I indicates the presence of spermatogonia in the testes; stage II is characterized by spermatogonia and spermatocytes in the testes; in stage III also spermatids are present; stage IV, all stages of spermatogenesis. The plasma of each group was analyzed, in order to check the effect of the implantation on steroid plasma levels (Schulz et al. 1994). All data were analyzed by one-way ANOVA followed by Fisher's least significant difference test ($p<0.05$) and their results are given as means ± SEM.

Results

All steroid implantations caused increased plasma levels (Fig. 1). The T implantation (Fig.1a) resulted in a 16-fold increase of the T levels in the plasma. The level of E_2 was 7 times higher in the E_2-treated group than in the control fish (Fig.1b) and the KT levels had increased 20-fold after KT treatment (Fig.1c).

Histological analysis of the testes showed that spermatogenesis was advanced in fish treated with 11KT (all in stage III), while 40% of the controls, T- and E_2-treated fish were in stage I and 60% in stage II.

The steroid treatments neither affected the total number of cfGnRH neurons in the brain (Fig.2) nor the number per specific brain region (telencephalon, suprachiasmatic area and medial basal hypothalamus; data not shown). Similarly, no change in the size of the cfGnRH neurons was observed after any of the steroid treatments.

The intensity of the immunostaining in the T-treated group, however, was stronger than in all other groups; the E_2 and KT groups were not affected in this respect. In Fig. 3 the suprachiasmatic area of a control fish (Fig. 3A) and a T-treated fish (Fig. 3B) are depicted, the latter showing both higher density of cfGnRH fibers and a more intense immunofluorescence of the individual fibers and neurons.

The quantification of cfGnRH in the pituitary revealed a significant increase after T (347 (±37pg GAP/mg protein) and E_2 (278 ±29pg GAP/mg protein) treatment versus controls (204 ±26pg GAP/mg protein); treatment with KT had no effect (171 ±14pg, Fig.4).

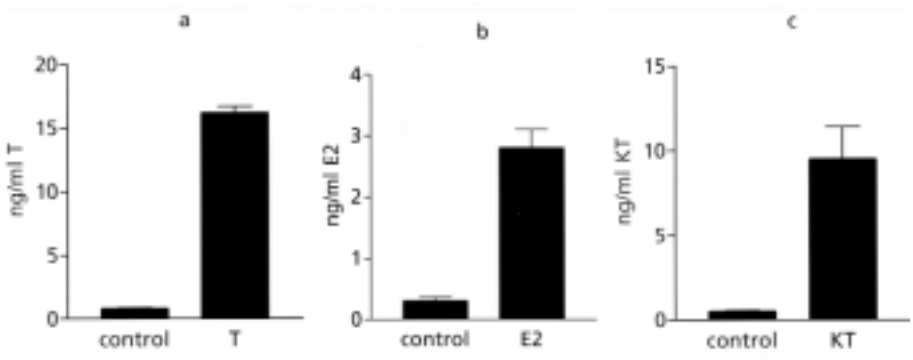


Fig. 1 Plasma levels of T (a), E_2 (b), and KT (c) in male African catfish of 12 weeks of age after a two-week treatment with the respective steroid (right) and the control group (left). The data are plotted as mean ±SEM ng steroid/ml plasma; n=15-20.

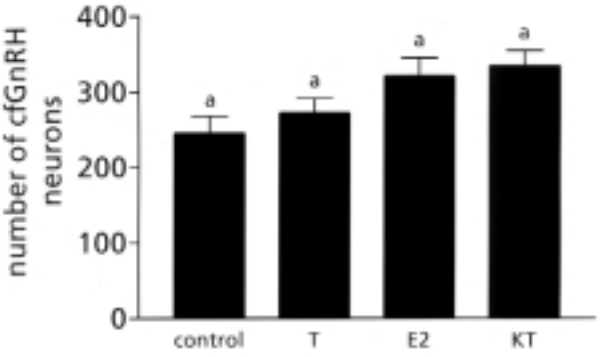


Fig. 2 Total number of cfGnRH neurons in the brain of African catfish after two weeks of steroid treatment. Mean ±SEM; n=5. No significant differences after Fisher's least significant difference test. Groups sharing the same letter are not significantly different.

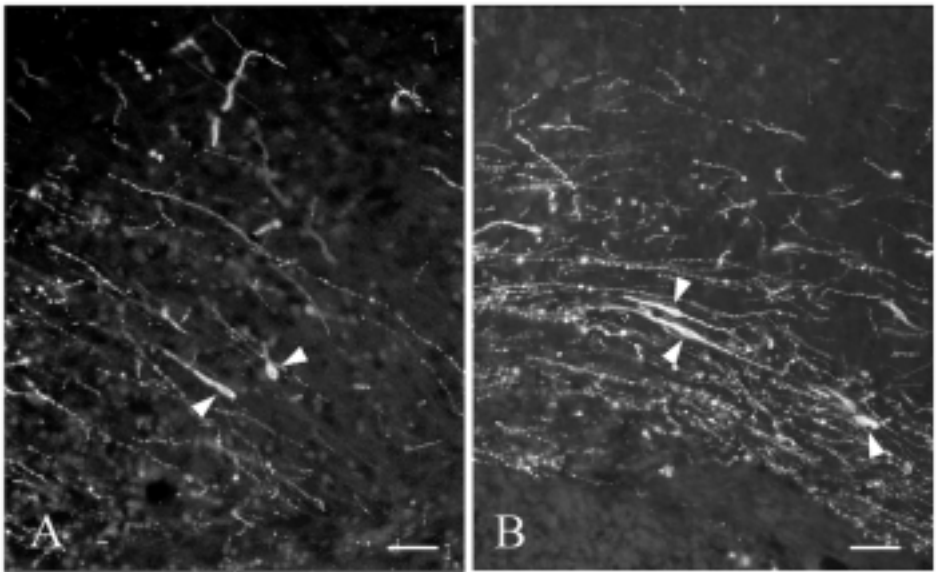


Fig. 3 Images obtained by immunofluorescence microscopy. CfGnRH neurons and fibers in the suprachiasmatic area of control fish (A) and T treated fish (B) of 12 weeks of age. The arrows point at the cfGnRH cell bodies, scalebar = 50 μm.

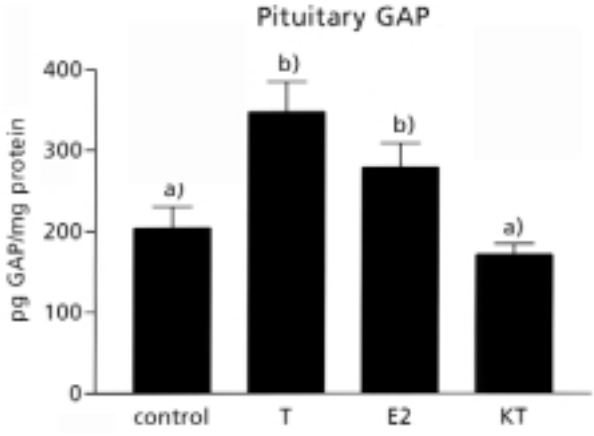


Fig. 4 CatfishGnRH content in the pituitary after two weeks of steroid treatment in male African catfish. The data are plotted as mean ±SEM pg cfGAP per mg protein; n=15-20. Groups with the same letter are not significantly different.

Discussion

After the steroid implantations, the plasma levels of resp. T, E_2 and 11KT were all significantly elevated. Although the induced steroid levels are high as compared to the control values, they are within the physiological range of the more advanced developmental stages (20-40 week-old adolescent male catfish; Schulz et al. 1994). On the testicular level, 11KT advanced the stage of spermatogenesis, whereas T and E_2 had no effect on the spermatogenesis. These

findings correspond with earlier studies in the African catfish (Cavaco et al. 1998b).

Exogenous gonadal steroids accelerate the maturation of the brain-pituitary-gonad axis, as shown in many teleost species (Nagahama 1994; Xiong et al. 1994; Amano et al. 1997). Sexual maturation in the male African catfish starts with the differentiation of the testis at six weeks of age (Hurk et al. 1989). The first appearance of spermatocytes in the testes around week 12 marks the onset of puberty (Schulz et al. 1999). At the level of the brain, cfGnRH was first observed in the ventral telencephalon at two weeks of age (Dubois et al. 1998). Ten weeks later, at the onset of puberty, the perikarya and fibers of the cfGnRH system are present in the brain from olfactory bulb till pituitary. The formation of the cfGnRH fiber network around the gonadotrophs in the pituitary, however, develops later during puberty between 10 and 20 weeks of age (Dubois et al. 2000).

In pubertal African catfish, pituitary and testes are susceptible to different native steroid hormones. Testicular development is stimulated by members of the 11-oxygenated androgens: 11β-hydroxyandrostenedione (OHA) and 11-ketotestosterone (KT) (Cavaco et al. 1998b). OHA is the main testicular product, which is converted in the liver into KT, the main androgen in the plasma (Cavaco et al. 1997). Another group of steroids e.g. aromatizable androgens like T and androstenedione, as well as E_2, were shown to have their domain of action on the gonadotrophs in the pituitary (Cavaco et al. 1998a; Cavaco et al. 1998b). Thus, these two groups of steroids act in different domains of the BPG-axis.

The question which type of steroid plays a dominant role in the maturation of the cfGnRH system is still under debate. In three successive studies, we have investigated the effects of gonadal steroids on the maturation of the cfGnRH system in brain and pituitary during three developmental periods: in prepubertal fish. Fish were studied from 2 till 6 weeks of age (Dubois et al. 1998), at the onset of puberty between 10 and 12 weeks of age (the present study) and during later puberty, up to 17 weeks (Dubois et al. 2000). As parameters, the number of cfGnRH neurons in the brain, the content of cfGnRH in the pituitary and the overall immunostaining intensity were used.

The 11-oxygenated steroids, powerful in stimulating the testicular development, appeared not to be involved in the maturation of the cfGnRH system in any stage of pubertal development. Testosterone, however, has a stimulating effect on cfGnRH immunoreactivity in brain and pituitary in all three age groups. The size of the cfGnRH neurons could be altered by steroid treatment in prepubertal catfish, but could not be influenced anymore at the onset of puberty. In contrast to the African catfish, the GnRH cell size in the adult cichlid (*Haplochromis burtoni*) can be regulated by castration or steroid replacement (Soma et al. 1996).

Also the increase in the *number* of cfGnRH neurons due to T was only observed in the prepubertal fish, i.e. between 2 and 6 weeks of age (Dubois et al. 1998). At later stages of pubertal development, the number of neurons is no longer susceptible to changes in T levels, which indicates that already at the onset of puberty the program of cfGnRH neuron recruitment is completed.

The content of cfGnRH in the pituitary increased after T or E_2 treatment in fish at the onset of puberty. These levels equal the amounts of cfGnRH as measured in the non-stimulated pituitary at the age of 14 weeks (stage 2-3 of spermatogenesis; Dubois et al. 2000). In late pubertal fish (17 weeks old; Dubois et al. 2000), the cfGnRH content in the pituitary can still be increased by T. The stimulatory effect of T or E_2 on the content of GnRH was also observed in the trout (Goos et al. 1986; Breton and Sambroni 1996), eel (Montero et al. 1995), and platyfish (Schreibman et al. 1986).

Thus, the structure of the cfGnRH system is morphologically differentiated at this stage, whereas the amount of cfGnRH peptide within the neurons and fibers is still under control of steroid hormones.

Whether T has a direct effect on the GnRH neurons is not known. The presence of androgen receptors in the goldfish brain has been reported (Gelinas and Callard 1997), but not (yet) in the brain of African catfish. An indirect effect of T, via aromatization to E_2, is also feasible. At least aromatase is present in regions of the catfish brain, including the preoptic area and basal hypothalamus (Timmers et al. 1987), where numerous GnRH neurons are located. Likewise, the maturation of the gonadotrophs in the pituitary is dependent on the aromatization of T to E_2 (Rebers et al. 2000b).

The overall increase in GnRH staining intensity and content after T treatment can be explained in two ways: (1) T stimulates the synthesis of cfGnRH or (2) this steroid inhibits the release of cfGnRH, thus causing an accumulation. Results of other studies in teleosts provide evidence for increase in GnRH numbers (Amano et al. 1994) and content (Schreibman et al. 1986; Goos et al. 1986; Montero et al. 1995; Breton and Sambroni 1996), but not for a direct increase in GnRH synthesis after T treatment. In the African catfish, there is some evidence for an inhibition of the GnRH release after the elevation of T plasma levels rather than increase synthesis, since T treatment resulted in a decrease in LH plasma levels (Cavaco et al. 1998a).

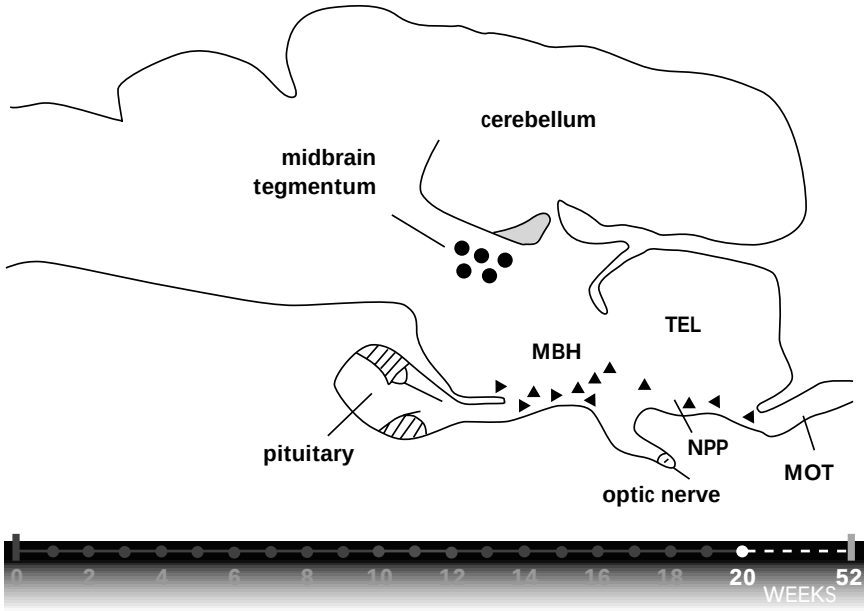
Summarizing, we conclude that the structural development of the cfGnRH neurons system in the ventral forebrain of the African catfish is under stimulatory control of T only until the onset of puberty. During puberty, however, T and/or E_2 still influence the amounts of cfGnRH within both perikarya and fibers, thus indicating their role in the control of the cfGnRH system.

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-

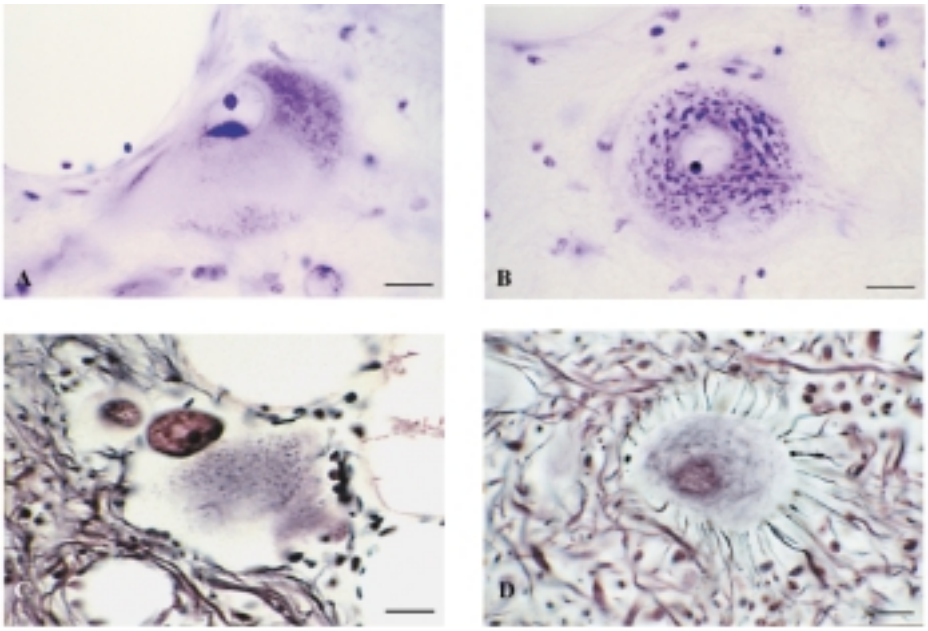


Fig.1 Light microscopical pictures of cGnRH-II cells and nflm cells in the African catfish. A: a Nissl-stained cGnRH-II cell with the nucleus, nucleolus, Nissl cap, and two cytoplasmatic compartments, one filled with Nissl substance and the other without. B: a FLM cell with the nucleus and nucleolus 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

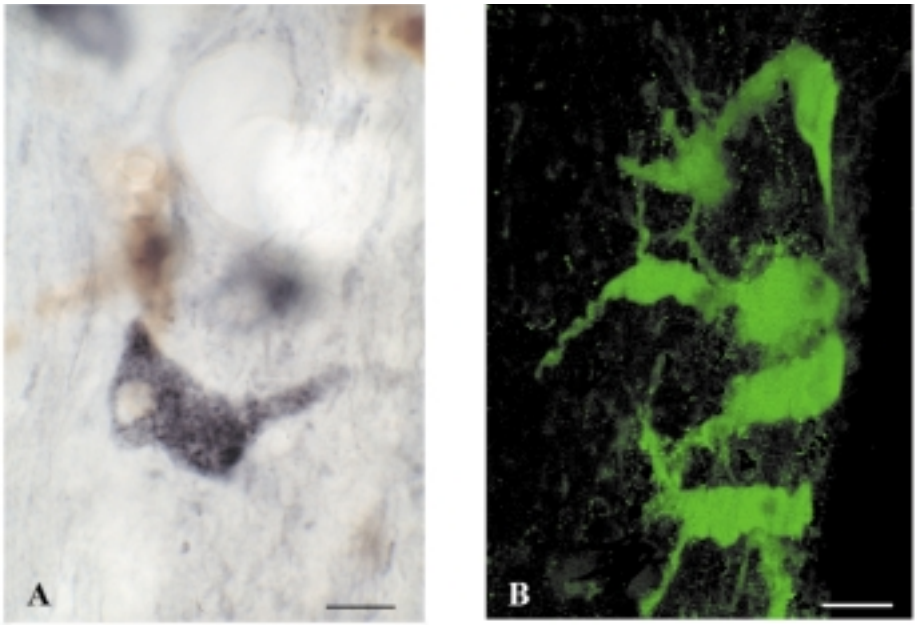


Fig. 2 cGnRH-II cells labeled with anti-cIIGAP. A: LM picture of a cIIGAP-labeled cGnRH-II cell in the midbrain. B: CLSM image of cGnRH-II cells in the midbrain composed of 32 projections over 40 μm. Only short dendritic extensions of cGnRH-II cells are visible. Scale bars represent 16 μm in A and 20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

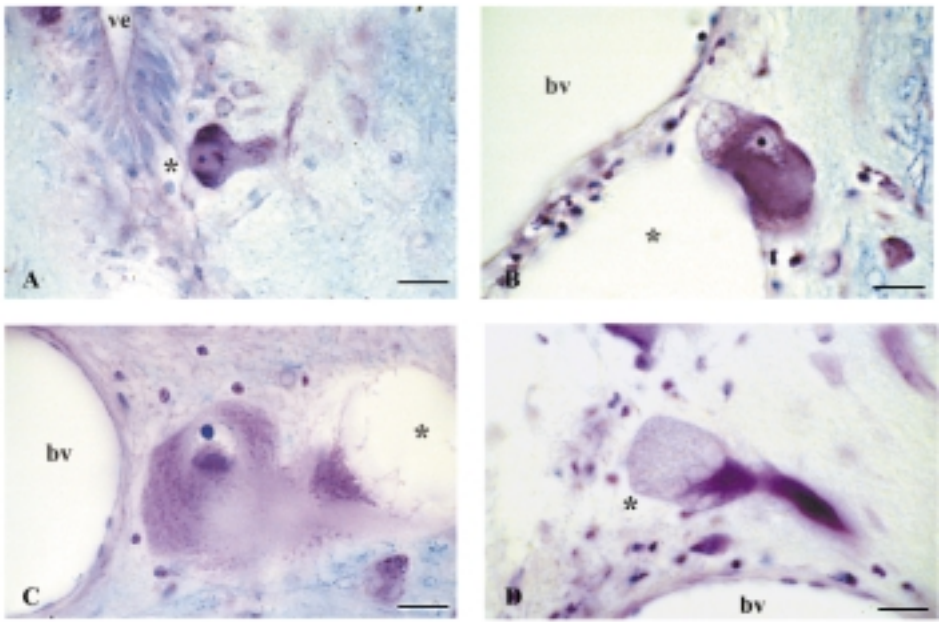


Fig. 4 A-D: cGnRH-II cells of different size, as present in Cl 1 (A), Cl 4 (B), Cl 2 (C), showing distinct relations with dilated extracellular spaces (*). Figure D shows a process of a cGnRH-II cell of Cl 2, of which the cell body was located in a neighboring section filled with Nissl substance and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

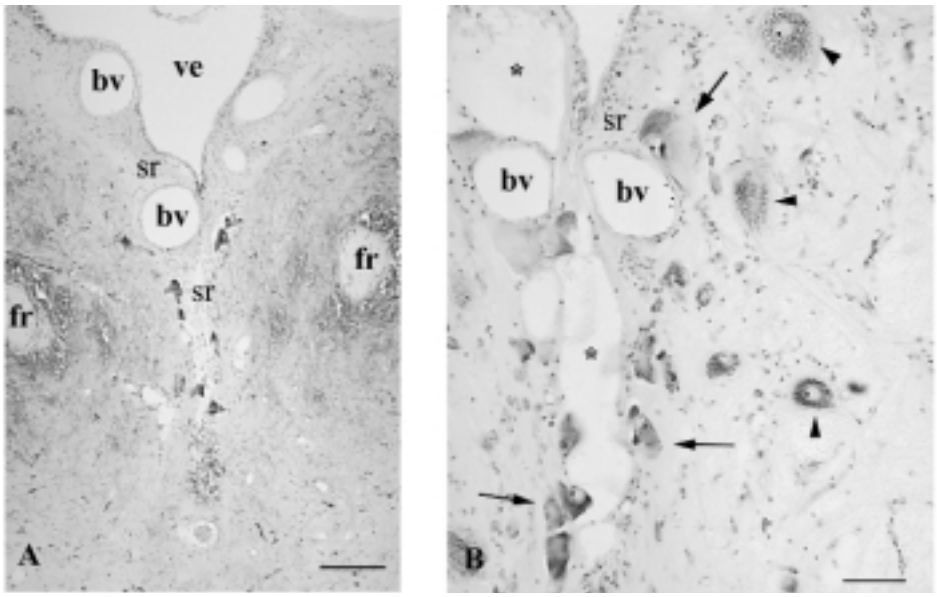


Fig. 3 Light microscopical pictures showing the localization of cGnRH-II cells. A: low magnification of a transverse section through the synencephalon of Cl 4 with cGnRH-II cells situated lateral to the midline of the brain. B: Nissl stained section of the synencephalon of Cl 2 with examples of cGnRH-II 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

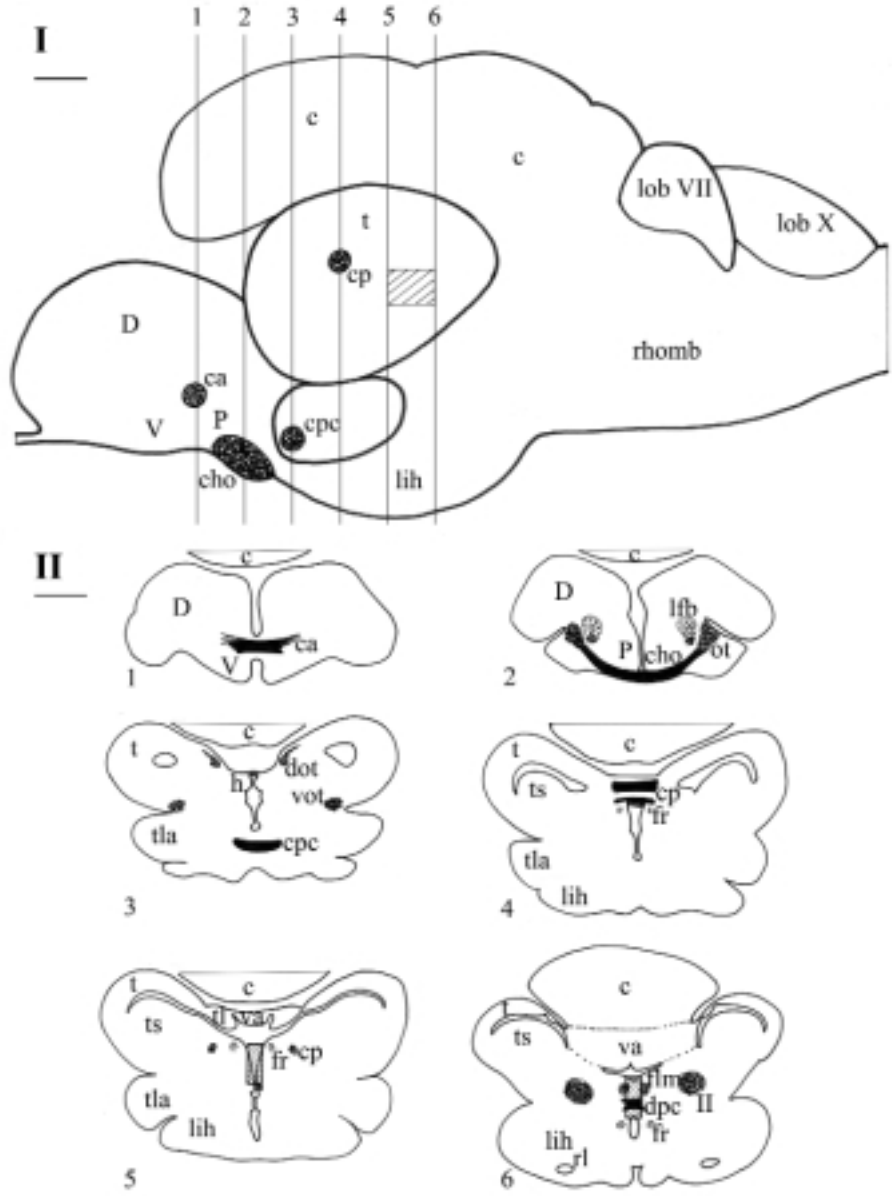
The diameter of unequivocally identified cGnRH-II cells in catfish varies from 10 to 70 μm, with a nuclear diameter varying accordingly from 7 to 21 μm (Fig. 4). This implies a difference in cytoplasmatic volume of about 500 times (i.e. from about 500 to 25000 μm^3). The differences in size of GnRH cells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

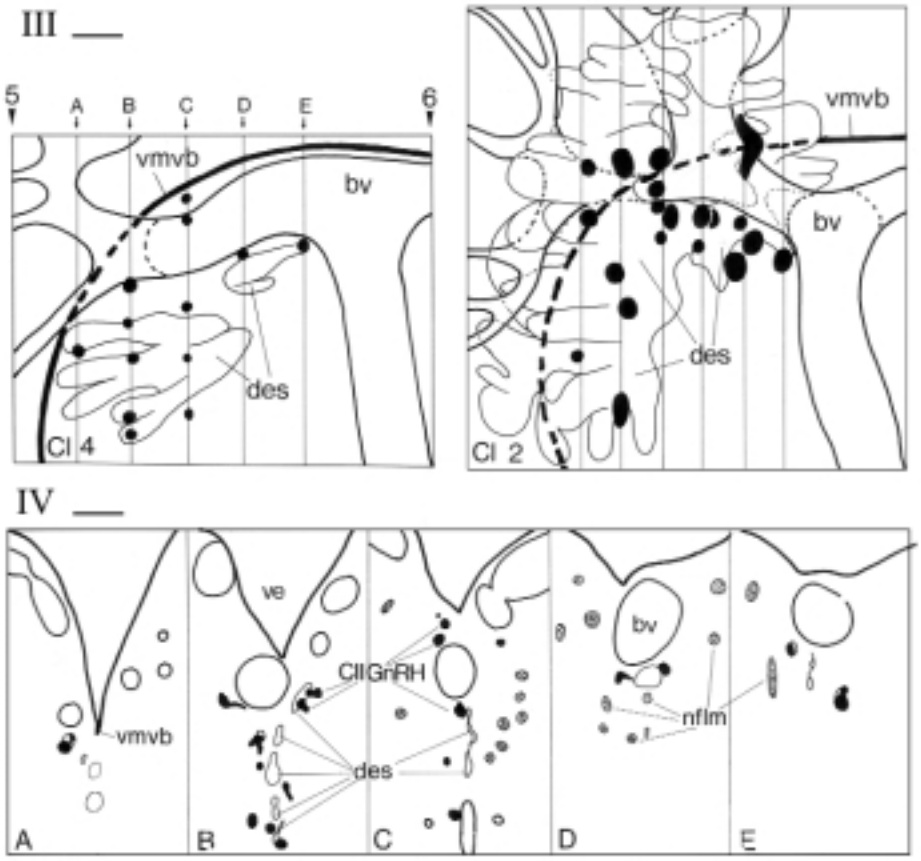


Fig. 5 Drawings of transverse sections and reconstructed lateral views of the brain (I and II) and the synencephalic region that contains the cGnRH-II cells (III and IV) of the African catfish. I: Lateral view of the brain of Clarias gariepinus in which lines 1 to 6 indicate the levels of the transverse 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,

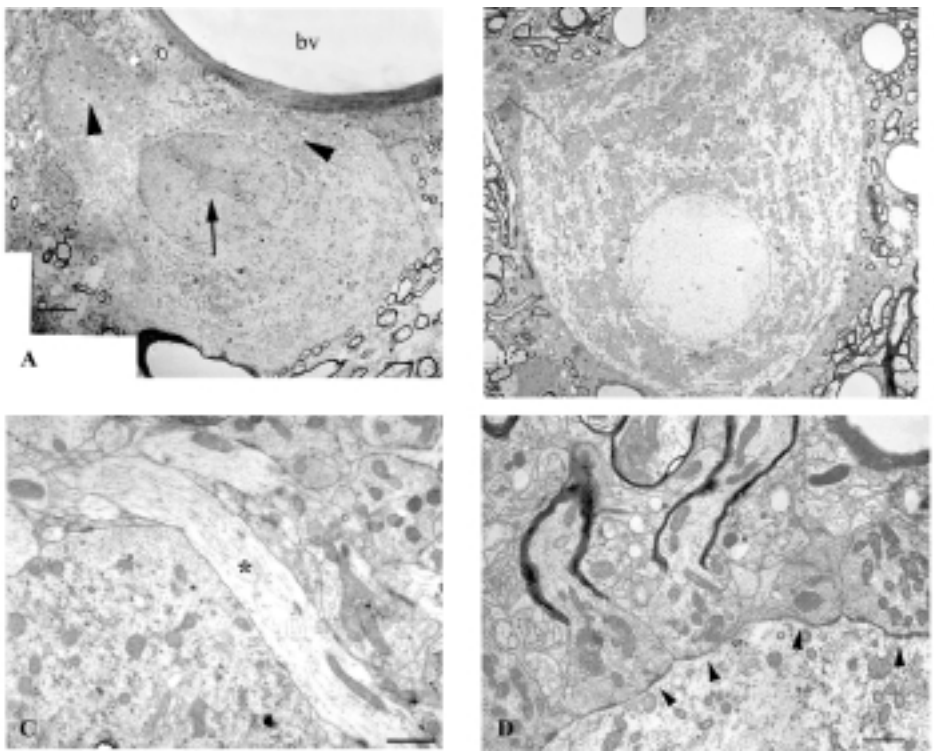


Fig. 6 Electron micrographs of cGnRH-II and nflm cells as observed in immunohistochemically treated midbrain tissue. In this material DAB precipitate indicates the presence of cGnRH-II immunoreactivity and is visible as black spots or small holes (when the precipitate has disappeared because of the ultrathin 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

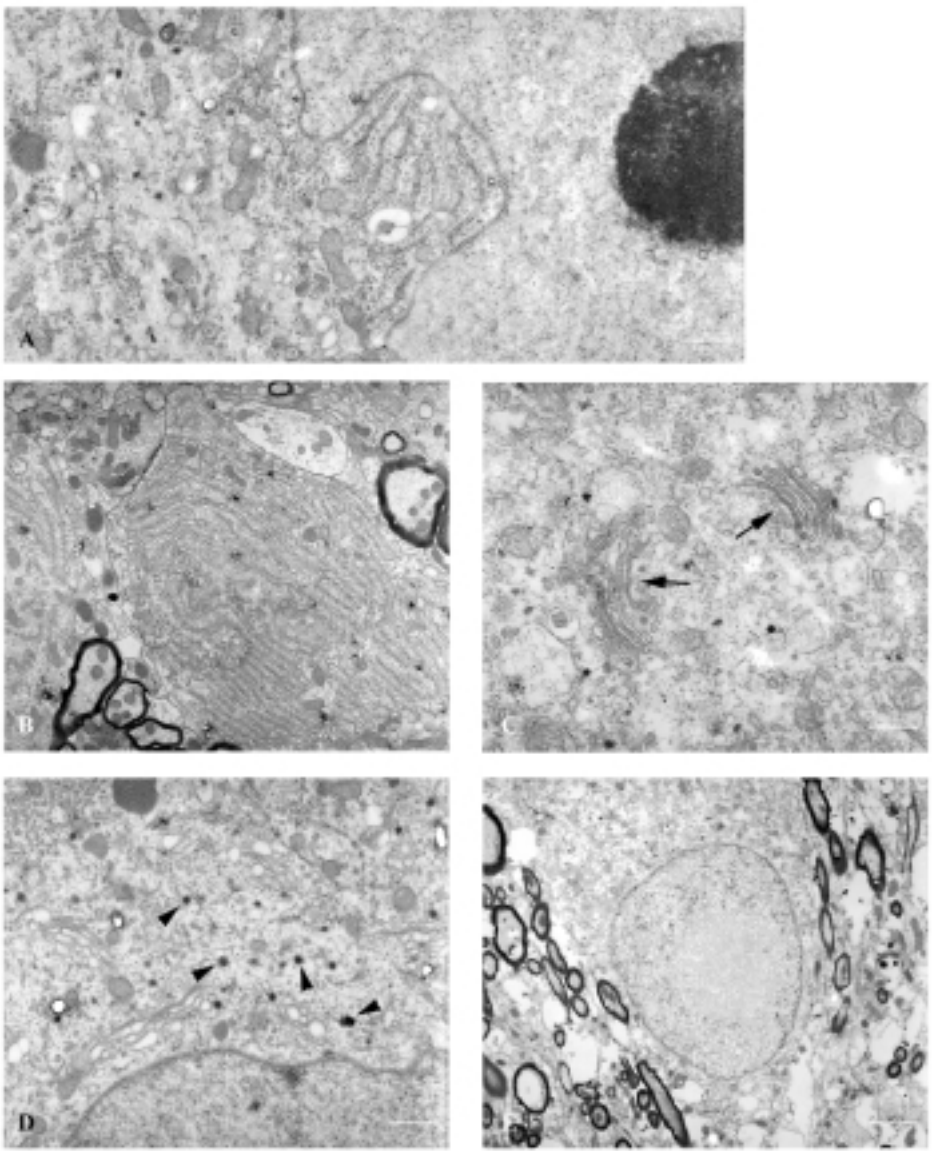


Fig. 7 Electron microscopical pictures of the ultrastructural characteristics of cGnRH-II cells. A: A part of the nucleus (with nucleolus) with an indentation filled with RER. B: Cytoplasmatic compartment densely filled with RER. C: Golgi-apparatus (arrows) as present in the granular compartment of the 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

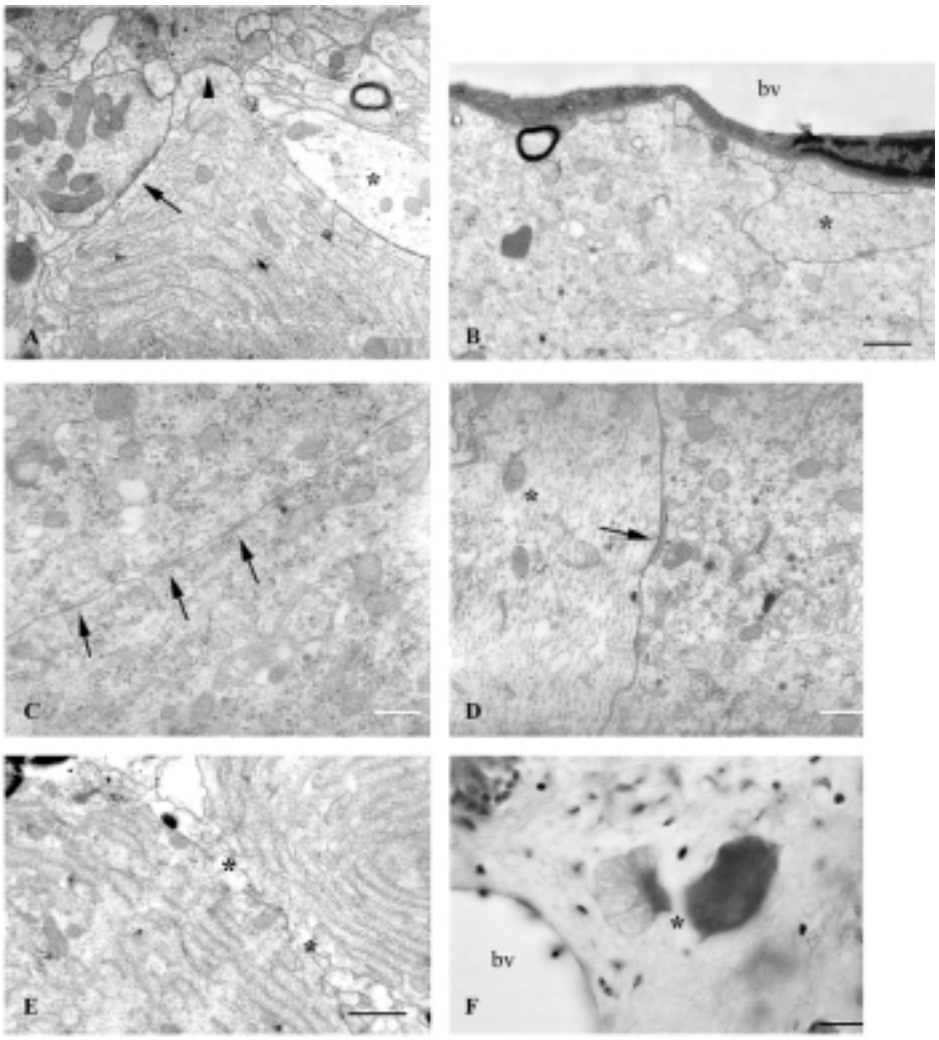


Fig. 8 Electron microscopical pictures of contacts of cGnRH-II cells. A: Presumed inhibitory synapse with a presynaptic element containing small flattened vesicles (arrow) and a synapse of which the presynaptic element contains dense cored vesicles (arrowhead). Asterisks indicates an nflm process. B: 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)

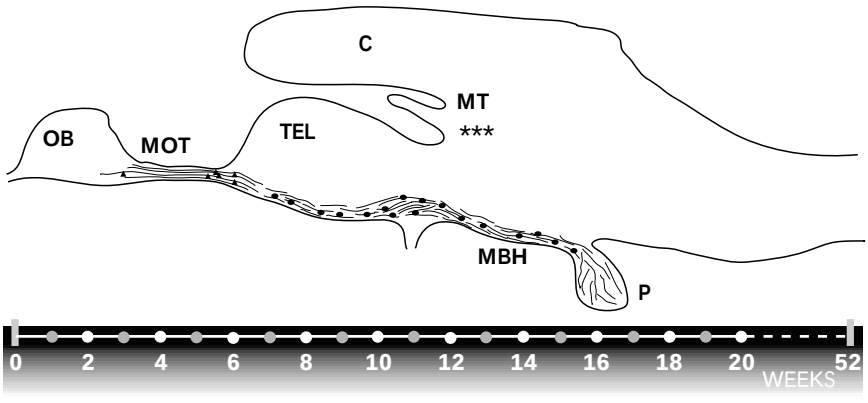
On the basis of our observations and the discussion above, we have developed the following hypothesis: cGnRH-II cells have no axons, but release their products in the surrounding extracellular spaces of the subventricular synencephalic reticulum, and are stimulated by nflm cells via dendro-somatic appositions.

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

Chapter 7

Summarizing discussion



Summarizing discussion

In this thesis, the investigations on the development of the gonadotropin-releasing hormone (GnRH) systems and its regulation by steroid hormones are described. There were two reasons to undertake this study.

First, we wanted to compare the development of the GnRH systems of the African catfish with that in other teleost species because of an evolutionary interest.

In most vertebrates, two or three different forms of GnRH can be distinguished. They have different functions, are localized in different brain areas and are of different embryological origin. The African catfish expresses two forms of GnRH (chicken GnRH-II and catfish specific GnRH, cfGnRH), while teleosts that appeared more recently in evolution mostly carry three forms of GnRH.

Comparing the GnRH systems of the African catfish with those of other teleosts, one can ask several questions. How does the catfish compensate for this lack of a third form? Are the embryonic origin, development and function of the GnRH systems of the catfish comparable to other teleosts? Do steroids play a role in the development of the GnRH systems and if so, what kind of steroids and in which phase of the development? And if we compare the fifteen forms of GnRH that have been identified till now, what does that tell us about the molecular evolution of the catfish GnRHs?

The second set of questions concerns the functional role of the catfish GnRH system and its steroidal control of development at the onset of puberty in the male African catfish.

During the last decade, the research group for Comparative Endocrinology assembled a wealth of knowledge on the (pre)pubertal condition of gonadotropic cells, testicular development and circulating (steroid) hormones in the African catfish, but information on the development of the GnRH system was still lacking. Therefore we decided to study this GnRH system as part of the brain-pituitary-gonad axis, with special emphasis on its spatio-temporal development. Furthermore, effects of steroid hormones on the development of the GnRH system were investigated in order to test the hypothesis that the steroid hormones and the GnRH system are intimately involved in the initiation of pubertal development (Goos 1993).

Two GnRH forms, three GnRH populations (chapter 2)

Generally two forms of GnRH are expressed in each vertebrate species: cGnRH-II in the midbrain and a species-specific GnRH in the ventral forebrain (Montero and Dufour 1996; Amano et al. 1997). Also the African catfish expresses two forms of GnRH (cGnRH-II and cfGnRH), like most primitive and less advanced teleosts. Modern teleosts, on the other hand, display a third GnRH form in the terminal nerve (TN), in most cases identified as salmon GnRH. Reinvestigation of the evolutionary older teleost species revealed that they also have a distinct GnRH population in the TN. However, these cells express the same GnRH as present in the ventral forebrain (for review see chapter 2). Indeed, in the African catfish we could identify a population of cfGnRH neurons in the TN as well. These neurons were shown to be different from the cfGnRH neurons in the ventral forebrain: not only by a different localization, but also on the base of a different morphology, distribution, and of a later time of appearance during ontogeny. Such a distinct TN GnRH neuron population seems only to be present in teleosts. In higher vertebrates the TN neurons are present, but they are not considered to be part of the ventral forebrain GnRH system, rather than a distinct population (Yellon and Newman 1991; Smith et al. 1997; Rastogi et al. 1998).

In most studies, it has been shown that the GnRH neurons in the TN have no axonal connection with the pituitary, so it is unlikely that these cells have a direct hypophysiotropic function. Their localization in close vicinity of olfactory areas, however, suggests a neuromodulatory function in integrating olfactory signals - which may be evoked by e.g. pheromones - into the BPG axis.

It is now generally accepted that the ventral forebrain GnRH neurons - but not the cGnRH-II cells in the midbrain tegmentum - originate from the olfactory placode (see Introduction). We now propose that also TN GnRH neurons originate from the olfactory placode and that they reach their final destination on the border between the olfactory bulb and the telencephalon after only a short migratory route via the terminal nerve. Obviously, cells in the olfactory placode are designated to become either TN neurons or ventral forebrain neurons and they are differentially programmed concerning the timing and distance of migration.

The position of catfishGnRH in the evolutionary GnRH tree

The presence of several molecular forms of GnRH within one species, their phylogenetic distribution, and the recent discovery of new forms (Jimenez-Linan et al. 1997; Carolsfeld et al. 2000; Yoo et al. 2000; Okubo et al. 2000a), tempt to propose a new evolutionary GnRH tree (Fig. 1). The construction of such a tree starts with a study of the phylogenetic distribution of GnRHs (see Introduction) and by comparing amino acid sequences.

As in a former model by King and Millar (see Introduction), two main lin-

eages are discerned in the hypothetical evolutionary GnRH tree: the cGnRH-II lineage and the mammalian lineage. Extending that model with more recently obtained data, we propose the following hypothetical tree, which now also includes the position of cfGnRH (Fig. 1). ChickenGnRH-II is probably preceded by the tunicate and lamprey GnRHs, of which lGnRH-III is the putative ancestor of cGnRH-II. DogfishGnRH is probably derived from the cGnRH-II lineage, since these two only differ in one amino acid. Similarly, the seabream and medaka GnRHs and those of higher vertebrates (rana, chickenI and guinea pig) are closely related to mGnRH and thus probably originate from the mGnRH lineage.

Comparing the amino acids on positions 5 and 7, the cGnRH-II lineage is characterized by a histidine on position 5, while a tyrosine residue on this position dominates in the mGnRH line. At position 7 we find tryptophane in the cGnRH-II lineage and leucine in the mGnRH line. These two characteristics confirm the genealogy of respectively lampreyGnRH-III and dfGnRH in the cGnRH-II lineage, and sb-, md-, r-, cI-, and gpGnRH in the mammalian lineage (Fig. 1). The histidine on position 5 and the leucine on position 7 in catfish GnRH and also in herring GnRH do not provide an extra clue for a place in either lineage. The same holds for salmonGnRH, which carries a tyrosine on position 5 and a tryptophane on position 7 (Fig. 1). According to a closer homology with other fish GnRHs like sbGnRH and mdGnRH, we fit cfGnRH and sGnRH in the mammalian lineage. Moreover, when regarding localization and function of the different GnRH forms within the Osteichtyes, it appears that h-, cf-, s-, sb-, and mdGnRH are localized in the ventral forebrain and have a hypophysiotropic function similar to the other GnRHs in the mGnRH lineage. This also favors their place in the mGnRH line. However, since salmonGnRH expressed in the TN of modern evolved teleosts has obtained another function, an independent sGnRH lineage might exist (chapter 2).

Development and steroidal control of the cfGnRH system in ventral forebrain and pituitary (chapters 2-5)

The first appearance of cfGnRH in the ventral forebrain of African catfish was observed 2 weeks of age, whereas the TN GnRH neuron population could not be distinguished until 4 weeks of age. At six weeks of age, cfGnRH neurons were present in the TN ganglion, the ventral telencephalon, and in the preoptic and caudal areas of the basal hypothalamus. Between week 10 and 12 morphology and distribution of the ventral forebrain GnRH system are similar to those in adult catfish: i.e. cfGnRH neurons are evenly distributed over the ventral forebrain from the olfactory bulb till the pituitary, accompanied by many varicose fibers in the medial ventral forebrain. The number of cfGnRH neurons increases rapidly during development until approximately 12 weeks of

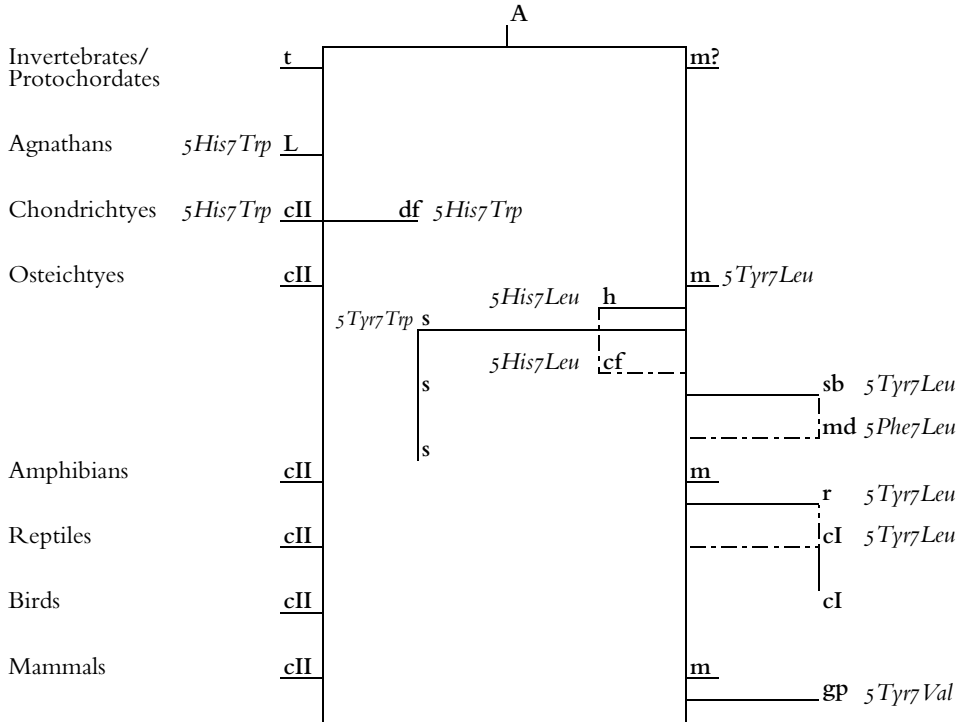


Fig. 1 Hypothetical evolutionary tree for GnRH. Two main lineages are discerned: the cGnRH-II lineage involves cGnRH-II, dfGnRH and the older lamprey and tunicate GnRHs. A histidine on position 5 and a tryptophane on position 7 characterize the GnRH in this lineage. The second line is called the mammalianGnRH lineage to which mGnRH, hGnRH, cfGnRH, sGnRH, sbGnRH, mdGnRH, rGnRH, cGnRH-I and gpGnRH belong. The amino acid on position 5 is mostly a tyrosine, while a leucine takes position 7. Dashed lines indicate uncertain origin. A: ancestral form, t: tunicateGnRH, L: lampreyGnRH, cII: chickenGnRH-II, df: dogfishGnRH, m: mammalianGnRH, h: herringGnRH, cf: catfishGnRH, s: salmonGnRH, sb: seabreamGnRH, md: medakaGnRH, r: ranaGnRH, cI: chickenGnRH-I, gp: guinea pigGnRH.

age, which is similar to the adult situation. At this stage the cfGnRH fiber network in the pituitary has not yet developed. The innervation of the pituitary occurs between week 10 and week 20, and can be subdivided in 4 phases. The process starts with the appearance of cfGnRH fibers in the pituitary and ends with the completion of a fine and highly branched cfGnRH fiber network, which is comparable with the adult state. We also demonstrated that the cfGnRH (we related these to the cfGAP levels) in the pituitary gradually rise during puberty, with a strong and significant increase between week 12 and 16.

The development of the ventral forebrain GnRH system in the African catfish precedes gonadal development. In this respect the situation in the catfish is comparable with that in other teleosts (Schreibman et al. 1986; Amano et al. 1998), although the absolute time of GnRH system development varies between species (Parhar et al. 1995b).

The effects of steroids on the GnRH system development were tested during three periods: between 2 and 6 weeks (juvenile period), between 10 and 12 weeks (at the onset of puberty), and between 12 and 17 weeks (pubertal period).

Testosterone (T), and to a lesser extent estradiol (E_2), increased both the intensity of the cfGnRH immunostaining and the content of cfGAP in the pituitary in all three periods. This effect can be due to two different actions of T: 1) an increase the synthesis of cfGnRH by stimulating transcription and/or translation or 2) inhibition of the cfGnRH release.

We propose that T mainly acts indirectly via E_2, since aromatases are present in brain regions with cfGnRH neurons (Timmers et al. 1987). The effect of E_2 must then be mediated by estrogen receptors and estrogen responsive elements on the GnRH gene, which were already demonstrated in the brain of several teleosts (see Introduction). However, we cannot exclude the possibility of a direct T effect via androgen receptors.

The effect of T on the number of cfGnRH neurons in the ventral forebrain only occurred when fish were treated in the juvenile period. All ventral forebrain areas were affected alike, suggesting that an elevation of the T levels had no effect on the caudal migration of the GnRH neurons. Alternatively, we propose that treatment with T in the juvenile stage causes recruitment of cells that are bound to become cfGnRH neurons later during normal development. Thus, T "wakes up" undifferentiated cfGnRH neurons and accelerates their development. Later, at the onset of puberty, exogenous T can not longer have such an effect, because all potential cfGnRH neurons were already differentiated at that time.

In other teleosts, T is also a strong stimulator for the differentiation of the ventral forebrain GnRH system (Amano et al. 1994). The role of 11-oxygenated steroids seems to be less important: in a cichlid (Soma et al. 1996) and bluehead wrasse (*Thalassoma bifasciatum;* Grober et al. 1991) minor effects of 11-ketotestosterone (11-KT) on the development of the GnRH system were observed.

Contrary to the effects of steroids on cfGnRH neurons of the ventral forebrain system, cGnRH-II cells in the midbrain were not affected in African catfish. Also in other teleosts no effects on cGnRH-II cells were observed (Soga et al. 1998; Parhar 1998), except for the eel (Montero et al. 1995).

ChickenGnRH-II cells in the midbrain: features and development (chapter 6)

The functional significance of the cGnRH-II system in the midbrain, present in all vertebrate species that have been investigated so far, is still unknown. All experimental treatments in our own studies and in others did not provide clear information about the function of these neurons. We anticipated that an

anatomical study of these cells and their environment could yield data about the ultrastructure and axonal projection that might allow some new suggestions. We expected answers to questions like how and where do the cGnRH-II cells release their product and to which medium (other neurons, cerebrospinal fluid, bloodstream); is there a direct or indirect connection to the pituitary, since we know that cGnRH-II is such a potent LH releaser?

A detailed morphological study revealed many ultrastructural characteristics of high metabolic activity. The most striking feature was the absence of any axon, which makes these cells different in function from other GnRH neurons. Synaptic contacts were scarce and the innervation seems to be limited to axons of the nucleus fasciculus longitudinalis medialis. ChickenGnRH-II cells always are localized in close vicinity of extracellular spaces in the subventricular reticulum, into which they might release their product.

Thus, although this morphological study revealed interesting information, we have to conclude that it did not give us the final clue as to functional significance of cGnRH-II cells. Several studies mention their possible role in reproductive behavior (Rissman 1996; Rissman et al. 1997). However, the catfish we used for our studies were kept in captivity, where they do not expose sexual behavior. Therefore, they may not be the best model to investigate this function.

GnRH system, steroid hormones and puberty (chapter 3, 4, 5)

The present thesis revealed three interesting features of the development of the GnRH system in relation to puberty.

1) Both the cGnRH-II and the cfGnRH system in the brain have achieved their morphological adult state just before the onset of puberty. The steroid hormone T (and E_2) is probably required for the recruitment of potential cfGnRH neurons and thus for completion of the cfGnRH system. In addition, T causes an increase in cfGnRH immunoreactivity and pituitary cfGAP content. The cGnRH-II system seems to develop independently from steroid hormones, and probably has no role in the onset of puberty, since it seems to be fully mature several weeks before the onset of puberty.

2) The cfGnRH fiber network in the pituitary reaches its final innervating pattern not before the end of puberty. Thus the question was put forward whether the innervation of the pituitary is limiting to the onset of puberty. Already at the onset of gonadal pubertal development, cfGnRH fibers surround islands of gonadotropic cells. As gonadal development proceeds, gradually more gonadotropic cells are contacted by cfGnRH fibers. It is, however, uncertain whether or not cfGnRH is effectively released at the contact sites at this stage. Since we are not able to measure the release of cfGnRH in the pituitary, an indirect method was applied. In chapter 4, a pituitary incubation experiment is described, revealing that during puberty the LH response to

endogenous and exogenous cfGnRH is similar. So, the available amount of cfGnRH in the vicinity of gonadotropic cells is not likely to be the limiting factor, but rather the ability of the cfGnRH terminals to release cfGnRH.

3) The maturation of the gonadotropic cells and the first wave of spermatogenesis both parallel the process of innervation of the pituitary by cfGnRH fibers. In the following model we propose that, once the switch is turned on, all three levels of the BPG-axis simultaneously start with their final maturation.

Model

Puberty is referred to as the onset of spermatogonial multiplication. Based on the results obtained by the present and an earlier study (Cavaco 1998), we propose that steroid hormones are the key players for the onset of puberty in the African catfish. The actions of steroid hormones and GnRHs at the onset of puberty are depicted in the model, represented by Fig. 2.

The testes of immature catfish produce significant amounts of steroids, resulting in low plasma levels of OHA, 11KT, androstenedione and T (Schulz et al. 1999). The 11-oxygenated steroids (OHA and 11KT) stimulate testicular development and initiate spermatogenesis (Cavaco et al. 1998b). Aromatizable androgens, represented by T have their domain of action on the brain-pituitary level and stimulate the maturation of gonadotropic cells in the pituitary, after being aromatized into E_2 (Cavaco 1998; Cavaco et al. 1998b). At the level of the brain, the effects of T are also stimulatory: T recruits cfGnRH neurons and induces an increase of the amount of GnRH within the cells. This stimulation of GnRH neuron recruitment was observed in immature males and females, suggesting that the action of T is sex independent. T, however, seems also to have inhibitory effects, that is on the release of cfGnRH. This presumed inhibitory action of T was deduced from the simultaneously decreased LH plasma levels under T treatment. Since *in vitro* T stimulates the release of LH from gonadotropic cells (Rebers et al. 2000b), the *in vivo* inhibition of LH release is probably localized at the supra-hypophyseal level.

Schulz et al. (1995) have shown that the LH content of gonadotropic cells *in vitro* is releasable when such cells are challenged with the native GnRHs (cfGnRH or cGnRH-II, the latter being the most potent releasing peptide). It means that rather the availability of a LH-release inducing hormone and not the responsiveness of the gonadotropic cells or their LH release capacity is the limiting factor for the maturation of the BPG-axis. The switch-on (a relevant amount of cfGnRH in the vicinity of the gonadotropic cells) is probably early at the onset of puberty, when cfGnRH fibers start to enter the proximal pars distalis. From this point on, the BPG-axis at all levels starts to mature: cfGnRH innervation in the pituitary, maturation of the gonadotropic cells and the first wave of spermatogenesis in the testes.

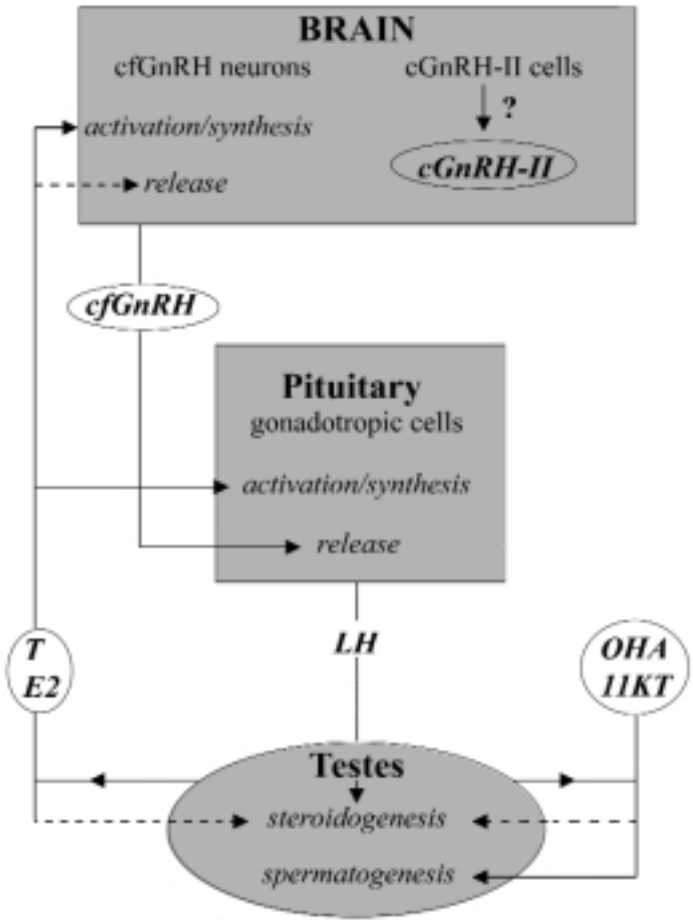


Fig. 2 Hypothetical model for the actions of the steroids OHA and 11KT, and T, and GnRHs on different levels of the BPG-axis at the onset of puberty. Fat lines: stimulatory action, dashed lines: inhibitory action, ?: presumed action. For further explanation see text. OHA: 11β-hydroxyandrostenedione, 11KT: 11-ketotestosterone, T: testosterone, E_2: estradiol.

References

Albers FJ, Meek J, Nieuwenhuys R. 1988 Morphometric parameters of the superior colliculus of albino and pigmented rats. J Comp Neurol 274, 357-370.

Amano M, Oka Y, Aida K, Okumoto N, Kawashima S, Hasegawa Y. 1991 Immunocytochemical demonstration of salmon GnRH and chicken GnRH- II in the brain of masu salmon, *Oncorhynchus masou*. J Comp Neurol 314, 587-597.

Amano M, Hyodo S, Urano A, Okumoto N, Kitamura S, Ikuta K, Suzuki Y, Aida K. 1994 Activation of salmon gonadotropin-releasing hormone synthesis by 17α-methyltestosterone administration in yearling masu salmon, *Oncorhynchus masou*. Gen Comp Endocrinol 95, 374-380.

Amano M, Hyodo S, Ikuta K, Suzuki Y, Urano A, Aida K. 1995 Short photoperiod accelerates preoptic and ventral telencephalic salmon GnRH synthesis and precocious maturation in underyearling male masu salmon. Gen Comp Endocrinol 99, 22-27.

Amano M, Urano A, Aida K. 1997 Distribution and function of gonadotropin-releasing hormone (GnRH) in the teleost brain. Zool Sci 14, 1-11.

Amano M, Oka Y, Kitamura S, Ikuta K, Aida K. 1998 Ontogenic development of salmon GnRH and chicken GnRH-II systems in the brain of masu salmon (*Oncorhynchus masou*). Cell Tissue Res 293, 427-434.

Amoss M, Burgus R, Blackwell R, Vale W, Fellows R, Guillemin R. 1971 Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. Biochem Biophys Res Commun 44, 205-210.

Anglade I, Zandbergen MA, Kah O. 1993 Origin of the pituitary innervation in the goldfish. Cell Tissue Res 273, 345-355.

Baba Y, Matsuo H, Schally AV. 1971 Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. Biochem Biophys Res Commun 44, 459-463.

Bartheld CSv and Meyer DL. 1986 Tracing of single fibers of the nervus terminalis in the goldfish brain. Cell Tissue Res 245, 143-158.

Behrend K and Donicht M. 1990 Descending connections from the brain stem to the spinal cord in the electric fish *Eigenmannia*. Quantitative description based on retrograde horseradish peroxidase and fluorescent dye transport. Brain Behav Evol 35, 227-239.

Blomenröhr M. 2000 Differences in structure-function relations between nonmammalian and mammalian GnRH receptors: what we have learnt from African catfish GnRH receptors.

PhD Thesis, Utrecht, the Netherlands.

Bogerd J, Zandbergen MA, Andersson E, Goos HJTh. 1994 Isolation, characterization and expression of cDNAs encoding the catfish- type and chicken-II-type gonadotropin-releasing-hormone precursors in the African catfish. Eur J Biochem 222, 541-549.

Borg B, Antonopoulou E, Mayer I, Andersson E, Berglund I, Swanson P. 1998 Effects of gonadectomy and androgen treatments on pituitary and plasma levels of gonadotropins in mature male Atlantic salmon, *Salmo salar*, parr- positive feedback control of both gonadotropins. Biol Reprod 58, 814-820.

Bosma PT, Rebers FEM, Dijk Wv, Willems PHGM, Goos HJTh, Schulz RW. 2000 Inhibitory and stimulatory interactions between endogenous gonadotropin-releasing hormones in the African catfish (*Clarias gariepinus*). Biol Reprod 62, 731-738.

Bradford MM. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.

Breton B and Sambroni E. 1996 Steroid activation of the brain-pituitary complex gonadotropic function in the triploid rainbow trout *Oncorhynchus mykiss*. Gen Comp Endocrinol 101, 155-164.

Breton B, Sambroni E, Govoroun M, Weil C. 1997 Effects of steroids on GTH I and GTH II secretion and pituitary concentration in the immature rainbow trout *Oncorhynchus mykiss*. C R Acad Sci Paris - Life Sci 320, 783-789.

Butler JA, Sjoberg M, Coen CW. 1999 Evidence for oestrogen receptor α-immunoreactivity in gonadotropin-releasing hormone-expressing neurones. J Neuroendocrinol 11, 331-335.

Carolsfeld J, Powell JFF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE, Sherwood NM. 2000 Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. Endocrinol 141 (2), 505-512.

Cavaco JEB., Schulz RW, Trudeau VL, Lambert JGD, Goos HJTh. 1995 Sexual steroids and regulation of puberty in male African catfish (*Clarias gariepinus*). Proceedings of the fifth international symposium on the reproductive endocrinology of fish. Goetz FW and Thomas P (Eds) Austin, TX, USA, pp 360.

Cavaco JEB, Lambert JGD, Schulz RW, Goos HJTh. 1996 Pubertal development of male African catfish, *Clarias gariepinus*. *In vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol Biochem 16, 129-138.

Cavaco JEB, Vischer HF, Lambert JGD, Goos HJTh, Schulz RW. 1997 Mismatch between patterns of circulating and testicular androgens in African catfish, *Clarias gariepinus*. Fish Physiol Biochem 17, 155-162.

Cavaco JEB. 1998 Sexual steroid hormones participate in the control of puberty in male African catfish, *Clarias gariepinus*. PhD Thesis, Utrecht, the Netherlands.

Cavaco JEB, Huck JHJ, Peute J, Schulz RW, Goos HJTh. 1998a Steroid hormones and gonadotroph activation in juvenile male African catfish (*Clarias gariepinus*). Thesis, chapter 6 , 115-135, see above.

Cavaco JEB, Vilrokx C, Trudeau VL, Schulz RW, Goos HJTh. 1998b Sex steroids and the initiation of puberty in male African catfish, *Clarias gariepinus*. Am J Physiol 275, 1793-1802.

Chiba A, Shunya O, Honma Y. 1994 Ontogenetic development of gonadotropin-releasing hormone-like immunoreactive neurons in the brain of the chum salmon, *Oncorhynchus keta*. Neurosci Letters 178, 51-54.

Chiba A, Sohn YC, Honma Y. 1996a Distribution of neuropeptide Y and gonadotropin-releasing hormone immunoreactivities in the brain and hypophysis of the ayu, *Plecoglossus altivelis* (Teleostei). Arch Histol Cytol 59 (2), 137-148.

Chiba A, Sohn YC, Honma Y. 1996b Immunohistochemical and ultrastructural characterization of the terminal nerve ganglion cells of the Ayu, *Plecoglossus altivelis* (Salmoniformes, Teleostei). Anat Rec 246 (4), 549-556.

Chow M, Kight KE, Gothilf Y, Alok D, Stubblefield J, Zohar Y. 1998 Multiple GnRHs present in a teleost species are encoded by separate genes: analysis of the sbGnRH and cGnRH-II genes from the striped bass, *Morone saxatilis*. J Mol Endocrinol 21, 277-289.

Conlon JM, Collin F, Chiang YC, Sower SA, Vaudry H. 1993 Two molecular forms of gonadotropin-releasing hormone from the brain of the frog, *Rana ridibunda*: purification, characterization, and distribution. Endocrinol 132, 2117-2123.

Crim LW, Peter RE, Billard R. 1981 Onset of gonadotropic hormone accumulation in the immature trout pituitary gland in response to estrogen or aromatizable androgen steroid hormones. Gen Comp Endocrinol 44, 374-381.

Crowley WF and Jameson JL. 1992 Clinical counterpoint: gonadotropin-releasing hormone deficiency: perspectives from clinical investigation. Endocrine Reviews 13, 635-640.

Cunningham MJ, Clifton DK, Steiner RA. 1999 Leptin's actions on the reproductive axis: perspectives and mechanisms. Biol Reprod 60, 216-222.

D'Aniello B, Pinelli C, King JA, Rastogi RK. 1994 Neuroanatomical organization of GnRH neuronal sytems in the lizard (*Podarcis s. sicula*) brain during development. Brain Res 657, 221-226.

Dellovade TL, King JA, Millar RP, Rissman EF. 1993 Presence and differential distribution of distinct forms of immunoreactive gonadotropin-releasing hormone in the musk shrew brain. Neuroendocrinol 58, 166-177.

Di Fiore MM, King JA, D'Aniello B, Rastogi RK. 1996 Immunoreactive mammalian and chicken-II GnRHs in *Rana esculenta* brain during development. Reg Peptides 62, 119-124.

Di Fiore MM, Rastogi RK, Ceciliani F, Messi E, Botte V, Botte L, Pinelli C, D'Aniello B, D'Aniello A. 2000 Mammalian and chicken I forms of gonadotropin-releasing hormone in the gonads of a protochordate, *Ciona intestinalis*. PNAS 97 (5), 2343-2348.

Dickey JT and Swanson P. 1998 Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). J Mol Endocrinol 21, 291-306.

Donkelaar HJt and Nicholson C. 1998 Notes on techniques. In: The central nervous system in vertebrates. Nieuwenhuys R, Donkelaar HJt, Nicholson C (Eds): Vol. 1, pp 327-356.

Dubois EA, Florijn MA, Zandbergen MA, Peute J, Goos HJTh. 1998 Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish (*Clarias gariepinus*). Gen Comp Endocrinol 112, 383-393. Chapter 3

Dubois EA, Zandbergen MA, Peute J, Hassing I, Dijk Wv, Schulz RW, Goos HJTh. 2000 Gonadotropin-releasing hormone fibers innervate the pituitary of the male African catfish

(*Clarias gariepinus*) during puberty. Neuroendocrinol 72, 252-262. Chapter 4

Dufour S, Fontaine YA, Kerdelhue B. 1985 Increase in brain and pituitary radioimmunoassayable gonadotropin releasing hormone (GnRH) in the European silver eel treated with sexual steroid or human chorionic gonadotropin. Neuropeptides 6, 495-502.

Dunn IC and Millam JR. 1998 Gonadotropin releasing hormone: forms and functions in birds. Poultry and avian biology reviews 9 (2), 61-85.

Eisthen HL and Northcutt RG. 1996 Silver Lampreys (*Ichthyomyzon unicuspis*) lack a gonadotropin-releasing hormone- and FMRFamide- immunoreactive terminal nerve. J Comp Neurol 370, 159-172.

Faber DS and Korn H. 1989 Electric field effects: Their relevance in central neural networks. Physiol Rev 63, 821-863.

Feist G and Schreck CB. 1996 Brain-pituitary-gonadal axis during early development and sexual differentiation in the rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 102, 394-409.

Feleder C, Jarry H, Leonhardt S, Wuttke W, Moguilevsky JA. 1996 The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. Neuroendocrinol 64 (4), 305-312.

Fernald RD and White RB. 1999 Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front Neuroendocrin 20, 224-240.

Forlano PM, Maruska KP, Sower SA, King JA, Tricas TC. 2000 Differential distribution of gonadotropin-releasing hormone-immunoreactive neurons in the stingray brain: functional and evolutionary considerations. Gen Comp Endocrinol 118, 226-248.

Foster DL and Nagatani S. 1999 Physiological perspectives on leptin as a regulator of reproduction: role in timing puberty. Biol Reprod 60, 205-215.

Francis RC, Soma K, Fernald RD. 1993 Social regulation of the brain-pituitary-gonadal axis. PNAS 90, 7794-7798.

Francis RC, Lee HN, Fernald RD. 1994 Ontogeny of gonadotropin releasing hormone-containing neurons in the teleost brain. Dev Brain Res 78, 151-160.

Fueshko SM, Key S, Wray S. 1998 GABA inhibits migration of luteinizing hormone-releasing hormone neurons in embryonic olfactory explants. J Neurosci 18 (7), 2560-2569.

Gelinas D and Callard GV. 1997 Immunolocalization of aromatase- and androgen receptor-positive neurons in the goldfish brain. Gen Comp Endocrinol 106, 155-168.

Gielen JT, Goos HJTh, Peute J, Bosch Jvd, Oordt PGWJv. 1982 The brain-pituitary-gonadal axis in the rainbow trout, *Salmo gairdneri*: gonadal hormones and the maturation of gonadotropic cells. Cell Tissue Res 225, 45-56.

Goos HJTh, Leeuw Rd, Cook H, Oordt PGWJv. 1986 Gonadotropic hormone-releasing hormone (GnRH) bioactivity in the brain of the immature rainbow trout, *Salmo gairdneri*: the effect of testosterone. Gen Comp Endocrinol 64, 80-84.

Goos HJTh. 1993 Pubertal development: big questions, small answers. Cellular communication in reproduction. Facchinetti F, Henderson IW, Pierantoni R, Polzenetti-Magni AM (Eds),

Bristol UK: Journal of Endocrinology Ltd. pp 11-20.

Goos HJTh, Bosma PT, Bogerd J, Tensen C, Li KW, Zandbergen MA, Schulz RW. 1997 Gonadotropin-releasing hormones in the African catfish: molecular forms, localization, potency and receptors. Fish Physiol Biochem 17, 45-51.

Gore AC and Roberts JL. 1997 Regulation of gonadotropin-releasing hormone gene expression *in vivo* and *in vitro*. Frontiers in Neuroendocrinol 18, 209-245.

Gothilf Y, Munoz-Cueto JA, Sagrillo CA, Selmanoff M, Chen TT, Kah O, Elizur A, Zohar Y. 1996 Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): Complementary deoxyribonucleic acid characterization and brain localization. Biol Reprod 55 (3), 636-645.

Grober MS, Jackson IMD, Bass AH. 1991 Gonadal steroids affect LHRH preoptic cell number in a sex/role changing fish. J Neurobiol 22 (7), 734-741.

Grober MS, Fox SH, Laughlin C, Bass AH. 1994 GnRH cell size and number in a teleost fish with two male reproductive morphs: sexual maturation, final sexual status and body size allomatry. Brain Behav Evol 43, 61-78.

Habibi HR and Huggard DL. 1998 Testosterone regulation of gonadotropin production in goldfish. Comp Biochem Physiol 119, 339-344.

Halpern-Sebold LR and Schreibman MP. 1983 Ontogeny of centers containing luteinizing hormone-releasing hormone in the brain of platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. Cell Tissue Res 229, 75-84.

Han VZ, Grant K, Bell CC. 2000 Rapid activation of GABAergic interneurons and evidence for calcium independent release of GABA in the mormyrid electrosensory lobe. J Neurophysiol 83, 1592-1604.

Herbison AE, Robinson JE, Skinner DC. 1993 Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone. Neuroendocrinol 57, 751-759.

Herbison A. 1998 Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. Endocrine Rev 19 (3), 302-330.

Hlavacek M, Tahar M, Libouban S, Szabo T. 1984 The momyrid brainstem. I. Distribution of brainstem neurons projecting to the spinal cord in *Gnathonemus petersii*, an HRP study. J Hirnforsch 6, 603-615.

Huang YP and Peter RE. 1988 Evidence for a gonadotropin-releasing hormone binding protein in goldfish (*Carassius auratus*) serum. Gen Comp Endocrinol 69, 308-316.

Huang YP, Guo DC, Peter RE. 1991a Isolation and characterization of a gonadotropin-releasing hormone binding protein in goldfish serum. Gen Comp Endocrinol 84, 58-66.

Huang YP, Trudeau VL, Peter RE. 1991b Evaluation of a specific gonadotropin-releasing hormone binding protein in the serum of goldfish: a study on the influence of sex, season, GnRH injection and estradiol treatment *in vivo*. Gen Comp Endocrinol 84, 76-82.

Hurk Rvd, Richter CJJ, Janssen-Dommerholt C. 1989 Effects of 17α-methyltestosterone and 11β-hydroxyandrostenedione on gonad differentiation in the African catfish, *Clarias gariepinus*. Aquaculture 83, 179-191.

Ikeuchi T, Todo T, Kobayashi T, Nagahama Y. 1999 cDNA cloning of a novel androgen receptor subtype. J Biol Chem 274, 25205-25209.

Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE, Millar RP. 1999 Two gonadotropin-releasing hormone receptor subtypes with distinct selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). PNAS 96, 2526-2531.

Jefferys JGR. 1995 Nonsynaptic modulation of neuronal activity in the brain: electric current and extracellular ions. Physiol Rev 75, 689-723.

Jennes L. 1989 Prenatal development of the gonadotropin-releasing hormone- containing systems in the brain. Brain Res 482, 97-108.

Jimenez-Linan M, Rubin BS, King JC. 1997 Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. Endocrinol 138, 4123-4130.

Kah O, Anglade I, Linard B, Pakdel F, Salbert G, Bailhache T, Ducouret B, Saligaut C, Le Goff Y, Jego P. 1997 Estrogen receptors in the brain-pituitary complex and the neuroendocrine regulation of gonadotropin release in rainbow trout. Fish Physiol Biochem 17, 53-62.

Kalra SP and Kalra PS. 1989 Do testosterone and estradiol-17β enforce inhibition or stimulation of luteinizing hormone-releasing hormone secretion? Biol Reprod 41, 559-570.

Kandeel FR and Swerdloff RS. 1997 The interaction between beta-endorphin and gonadal steroids in regulation of luteinizing hormone (LH) secretion and sex steroid regulation of LH and proopiomelanocortin peptide secretion by individual pituitary cells. Endocrinol 138 (2), 649-656.

Kasten TL, White SA, Norton TT, Bond CT, Adelman JP, Fernald RD. 1996 Characterization of two new preproGnRH mRNAs in the tree shrew: first direct evidence for mesencephalic GnRH gene expression in a placental mammal. Gen Comp Endocrinol 104, 7-19.

Khan IA and Thomas P. 1993 Immunocytochemical localization of serotonin and gonadotropin-releasing hormone in the brain and pituitary gland of the Atlantic croaker *Micropogonias undulatus*. Gen Comp Endocrinol 91, 167-180.

Kim K, Seong JY, Kang SS, Sun W. 1997 Neuroendocrine regulation of GnRH gene expression. In: GnRH neurons: gene to behavior. Parhar IS and Sakuma Y (Eds) Tokyo: Brain Shuppan, pp 401-427.

Kim MH, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashima S, Suzuki Y, Aida K. 1995a Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. J Comp Neurol 356, 72-82.

Kim MH, Kobayashi M, Aida K. 1995b Changes in brain GnRH after olfactory tract section in female goldfish. Fisheries Science 61 (4), 614-617.

King JA and Millar RP. 1982 Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization. J Biol Chem 257, 10729-10732.

King JC and Anthony ELP. 1984 LHRH neurons and their projections in humans and other animals: species comparisons. Peptides 5 (Suppl. 1), 195-207.

King JA, Dufour S, Fontaine YA, Millar RP. 1990 Chromatographic and immunological eidence for mammalian GnRH and chicken GnRH-II in eel (*Anguilla anguilla*) brain and pitu-

itary. Peptides 11, 507-514.

King JA and Millar RP. 1992 Evolution of gonadotropin-releasing hormones. TEM 3, 339-346.

King JA, Millar RP, Vallarino M, Pierantoni R. 1995 Localization and characterization of gonadotropin-releasing hormones in the brain, gonads, and plasma of a dipnoi (lungfish, *Protopterus annectens*). Reg Peptides 57 (2), 163-174.

King JA and Millar RP. 1997 Coordinated evolution of GnRHs and their receptors. In: GnRH neurons: gene to behavior. Parhar IS and Sakuma Y (Eds) Tokyo: Brain Shuppan, pp 51-77.

Klungland H, Andersen O, Kisen G, Aleström P, Tora L. 1993 Estrogen receptor binds to the salmon GnRH gene in a region with long palindromic sequences. Mol Cell Endocrinol 95, 147-154.

Kobayashi M, Amano M, Kim MH, Furukawa K, Hasegawa Y, Aida K. 1994 Gonadotropin-releasing hormones of terminal nerve origin are not essential to ovarian development and ovulation in goldfish. Gen Comp Endocrinol 95, 192-200.

Kogo H, Fujimoto T, Park MK, Mori T. 1999 Gonadotropin-releasing hormone receptor mRNA expression in the ovaries of neonatal and adult rat. Cells Tissues Organs 164, 14-22.

Korytko AI, Marcelino J, Blank JL. 1995 Differential testicular responses to short daylength in deer mice are reflected by regional and morphological differences in the GnRH neuronal system. Brain Res 685, 135-142.

Latimer VS, Rodrigues SM, Garyfallou VT, Kohama SG, White RB, Fernald RD, Urbanski HF. 2000 Two molecular forms of gonadotropin-releasing hormone (GnRH-I and GnRH-II) are expressed by two separate populations of cells in the rhesus macaque hypothalamus. Mol Brain Res 75, 287-292.

Leeuw Rd, Resink JW, Rooyakkers EJM, Goos HJTh. 1985a Pimozide modulates the luteinizing hormone-releasing hormone effect on gonadotrophin release in the African catfish, *Clarias lazera*. Gen Comp Endocrinol 58, 120-127.

Leeuw Rd, Smit-van Dijk W, Zigterman JWJ, Loo JCMvd, Lambert JGD, Goos HJTh. 1985b Aromatase, estrogen 2-hydroxylase, and catechol-O-methyltransferase activity in isolated, culture gonadotropic cells of mature African catfish, *Clarias gariepinus* (Burchell). Gen Comp Endocrinol 60, 171-177.

Leprêtre E, Anglade I, Williot P, Vandesande F, Tramu G, Kah O. 1993 Comparative distribution of mammalian GnRH (gonadotropin- releasing hormone) and chicken GnRH-II in the brain of the immature Siberian sturgeon (*Acipenser baeri*). J Comp Neurol 337, 568-583.

Lescheid DW, Terasawa E, Abler LA, Urbanski HF, Warby CM, Millar RP, Sherwood NM. 1997 A second form of gonadotropin-releasing hormone (GnRH) with characteristics of chicken GnRH-II is present in the primate brain. Endocrinol 138, 5618-5629.

Li C, Chen P, Smith MS. 1999 Morphological evidence for direct interaction between arcuate nucleus neuropeptide Y (NPY) neurons and gonadotropin-releasing hormone neurons and the possible involvement of NPY Y1 receptors. Endocrinol 140, 5382-5390.

Lin XW and Peter RE. 1997 Cloning and expression pattern of a second [$His^5Trp^7Tyr^8$]gonadotropin-releasing hormone (chickenGnRH-II) mRNA in goldfish: evidence for two distinct genes. Gen Comp Endocrinol 170, 262-272.

Lovejoy DA, Fischer WH, Parker DB, McRory JE, Park M, Lance V, Swanson P, Rivier JE, Sherwood NM. 1991a Primary structure of two forms of gonadotropin-releasing hormone from brains of the American alligator (*Alligator mississippiensis*). Reg Peptides 33, 105-116.

Lovejoy DA, Sherwood NM, Fischer WH, Jackson BC, Rivier JE, Lee T. 1991b Primary structure of gonadotropin-releasing hormone from the brain of a holocephalan (ratfish: *Hydrolagus colliei*). Gen Comp Endocrinol 82, 152-161.

Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM. 1992 Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. PNAS 89, 6373-6377.

Magri MH, Solari A, Billard R, Reinaud P. 1985 Influence of testosterone on precocious sexual development in immature rainbow trout. Gen Comp Endocrinol 57, 411-421.

Malatesta M, Mannello F, Sebastiani M, Cardinali A, Marcheggiani F, Reno F, Gazzanelli G. 1998 Ultrastructural characterization and biochemical profile of human gross cystic breast disease. Breast Cancer Res Tre 48, 211-219.

Maney DL, Richardson RD, Wingfield JC. 1997 Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow. Horm Behav 32, 11-18.

Marchant TA, Chang JP, Nahorniak CS, Peter RE. 1989 Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinol 124 (5), 2509-2518.

Matsuo H, Baba Y, Nair NMG, Arimura A, Schally AV. 1971 Structure of the porcine LH- and FSH- releasing hormone. I. The proposed amino acid sequence. Biochem Biophys Res Commun 43, 1334-1339.

Meek J and Nieuwenhuys R. 1998 (The brains of) Holosteans and Teleosts. In: The central nervous system of vertebrates. Nieuwenhuys R, Donkelaar HJt, Nicholson C (Eds) : Vol. 2, pp 759-938.

Meek J, Hafmans THM, Han VZ, Bell CC, Grant K. 2000 Myelinated dendrites in the mormyrid electrosensory lobe. J Comp Neurol in press.

Mikami S, Yamada S, Hasegawa Y, Miyamoto K. 1988 Localization of avian LHRH-immunoreactive neurons in the hypothalamus of the domestic fowl, *Gallus domesticus*, and the Japanese quail, *Coturnix coturnix*. Cell Tissue Res 251, 51-58.

Millam JR, Faris PL, Youngren OM, El Halawani ME, Hartman BK. 1993 Immunohistochemical localization of chicken gonadotropin- releasing hormones I and II (cGnRH I and II) in turkey hen brain. J Comp Neurol 333, 68-82.

Millar R. P., Troskie B, Sun Y.M., Ott T, Wakefield I, Myburgh D, Pawson A, Davidson JS, Flanagan C, Katz A, Hapgood J, Illing N, Weinstein H, Sealfon SC, Peter RE, Terasawa E, King JA. 1997 Plasticity in the structural and functional evolution of GnRH: a peptide for all seasons. Proceedings of the XIII international congress of comparative endocrinology. Yokohama, Japan, pp 15-27.

Miller KE and Kriebel RM. 1985 Electron microscopic identification of mesencephalic neu-

rosecretory cells in teleosts. Cell Tissue Res 242, 445-448.

Miura T, Yamauchi K, Takahashi H, Nagahama Y. 1991 Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japenese eel (*Anguilla japonica*). PNAS 88, 5774-5778.

Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H. 1984 Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. PNAS 81, 3874-3878.

Montaner A. D., Park MK, Fischer WH, Craig AG, Chang JP, Somoza GM, Rivier JE, Sherwood NM. 2000 Identification of a new GnRH form in pejerry, *Odentesthes bonariensis*. 4th International symposium on fish endocrinology. Swanson P and Dickhoff WW (Eds) Seattle,WA, pp 74.

Monteiro RAF, Rocha E, Marini-Abreu MM. 1992 Quantitative age-releated changes on nuclear invaginations of neocerebellar Purkinje cells. Neuroreport 3, 1089-1092.

Montero M, Vidal B, King JA, Tramu G, Vandesande F, Dufour S, Kah O. 1994 Immunocytochemical localization of mammalian GnRH (gonadotropin-releasing hormone) and chicken GnRH-II in the brain of the European silver eel (*Anguilla anguilla* L.). J Chem Neuroanat 7 (4), 227-241.

Montero M, Le Belle N, King JA, Millar RP, Dufour S. 1995 Differential regulation of the two forms of gonadotropin- releasing hormone (mGnRH and cGnRH-II) by sex steroids in the European silver eel (*Anguilla anguilla*). Neuroendocrinol 61, 525-535.

Montero M and Dufour S. 1996 Gonadotropin-releasing hormones (GnRH) in fishes: evolutionary data on their structure, localization, regulation, and function. Zool Stud 35 (3), 149-160.

Murakami S, Kikuyama S, Arai Y. 1992 The origin of the luteinizing hormone-releasing hormone (LHRH) neurons in newts (*Cynops pyrrhogaster*): the effect of olfactory placode ablation. Cell Tissue Res 269, 21-27.

Muske LE and Moore FL. 1990 Ontogeny of immunoreactive gonadotropin-releasing hormone neuronal systems in amphibians. Brain Res 534, 177-187.

Muske LE. 1993 Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. Brain Behav Evol 42, 215-230.

Muske LE and Moore FL. 1994 Antibodies against different forms of GnRH distinguish different populations of cells and axonal pathways in a urodele amphibian, *Taricha granulosa*. J Comp Neurol 345, 139-147.

Muske LE. 1997 Onotogeny, phylogeny and neuroanatomical organization of multiple molecular forms of GnRH. In: GnRH neurons: gene to behavior. Parhar IS and Sakuma Y (Eds) Tokyo: Brain Shuppan, pp 145-179.

Nagahama Y. 1994 Endocrine regulation of gametogenesis in fish. Int J Dev Biol 38, 217-229.

Navas JM, Anglade I, Bailhache T, Pakdel F, Breton B, Jégo P, Kah O. 1995 Do gonadotropin-releasing hormone neurons express estrogen receptors in the rainbow trout? A double immunohistochemical study. J Comp Neurol 363, 461-474.

Nevitt GA, Grober MS, Marchaterre MA, Bass AH. 1995 GnRH-like immunoreactivity in the

peripheral olfactory system and forebrain of Atlantic salmon (*Salmo salar*): a reassessment at multiple life history stages. Brain Behav Evol 45, 350-358.

Nieuwenhuys R. 1998 Morphogenesis and general structure. In: The central nervous system of vertebrates. Nieuwenhuys R, Donkelaar HJt, Nicholson C (Eds) :Vol. 1, pp 159-228.

Northcutt RG and Muske LE. 1994 Multiple embryonic origins of gonadotropin-releasing hormone (GnRH) immunoreactive neurons. Dev Brain Res 78, 279-290.

Oka Y, Satou M, Ueda K. 1986 Descending pathways to the spinal cord in the hime salmon (landlocked red salmon, *Oncorhynchus nerka*). J Comp Neurol 254, 91-103.

Oka Y and Ichikawa M. 1991 Ultrastructure of the ganglion cells of the terminal nerve in the dwarf gourami (*Colisa lalia*). J Comp Neurol 304, 161-171.

Oka Y and Ichikawa M. 1992 Ultrastructural characterization of gonadotropin-releasing hormone (GnRH)-immunoreactive terminal nerve cells in the dwarf gourami. Neuroscience Letters 140, 200-202.

Oka Y. 1992 Gonadotropin-releasing hormone (GnRH) cells of the terminal nerve as a model neuromodulator system. Neuroscience Letters 142, 119-122.

Okubo K, Amano M, Yoshiura Y, Suetake H, Aida K. 2000a A novel from of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun 276 (1), 298-303.

Okubo K, Suetake H, Usami T, Aida K. 2000b Molecular cloning and tissue-specific expression of a gonadotropin-releasing hormone receptor in the japanese eel. Gen Comp Endocrinol 119, 181-192.

Okuzawa K, Amano M, Kobayashi M, Aida K, Hanyu I, Hasegawa Y, Miyamoto K. 1990 Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. Gen Comp Endocrinol 80, 116-126.

Okuzawa K, Granneman J, Bogerd J, Goos HJTh, Zohar Y, Kagawa H. 1997 Distinct expression of GnRH genes in the red seabream. Fish Physiol Biochem 17, 71-79.

Olster DH and Foster DL. 1986 Control of gonadotropin secretion in the male during puberty. A decrease in response to steroid inhibitory feedback in the absence of an increase in steroid-independent drive in the sheep. Endocrinol 118, 2225-2234.

Pakdel F, Metivier R, Flouriot G, Valotaire Y. 2000 Two estrogen receptor (ER) isoforms with different estrogen dependencies are generated from the trout ER gene. Endocrinol 141, 571-580.

Parhar IS and Iwata M. 1994 Gonadotropin releasing hormone (GnRH) neurons project to growth hormone and somatolactin cells in the steelhead trout. Histochem 102, 195-203.

Parhar IS, Iwata M, Pfaff DW, Schwanzel-Fukuda M. 1995a Embryonic development of gonadotropin-releasing hormone neurons in the sockeye salmon. J Comp Neurol 362, 256-270.

Parhar I, Pfaff D, Schwanzel-Fukuda M. 1995b Genes and behavior as studied through gonadotropin-releasing hormone (GnRH) neurons: comparative and functional aspects. Cell Mol Neurobiol 15, 107-116.

Parhar I, Soga T, Sakuma Y. 1996a *In situ* hybridization for two differentially expressed GnRH genes following estrogen and triiodothyronine treatment in the brains of juvenile tilapia (cichlid). Neuroscience Letters 218, 135-138.

Parhar IS, Pfaff DW, Schwanzel-Fukuda M. 1996b Gonadotropin-releasing hormone gene expression in teleosts. Mol Brain Res 41 (1-2), 216-227.

Parhar IS and Sakuma Y. 1997 Regulation of forebrain and midbrain GnRH neurons in juvenile teleosts. Fish Physiol Biochem 17, 81-84.

Parhar IS, Soga T, Ishikawa Y, Nagahama Y, Sakuma Y. 1998 Neurons synthesizing gonadotropin-releasing hormone mRNA subtypes have multiple developmental origins in the medaka. J Comp Neurol 401, 217-226.

Parhar IS. 1998 GnRH in Tilapia: three genes, three origins and their roles. In: GnRH neurons, gene to behavior. Parhar IS and Sakuma Y (Eds) Tokyo: Brain Shuppan, pp 99-122.

Parhar IS. 1999 Multiple gonadotropin-releasing hormone neuronal systems in vertebrates. Kor J Biol Sci 3, 1-7.

Pati D and Habibi HR. 1998 Presence of salmon gonadotropin-releasing hormone (GnRH) and compounds with GnRH-like activity in the ovary of goldfish. Endocrinol 139, 2015-2024.

Pati D and Habibi HR. 2000 Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis. Mol Cell Endocrinol 160, 75-88.

Peter RE, Yu KL, Marchant TA, Rosenblum PM. 1990 Direct neural regulation of the teleost adenohypophysis. J Exp Zool Suppl 4, 84-89.

Peute J, Leeuw Rd, Goos HJTh, Oordt PGWJv. 1984 Ultrastructure and immunolabeling of gonadotropes and thyrotropes in the pituitary of African catfish, *Clarias gariepinus*. Cell Tissue Res 238, 95-103.

Peute J, Schild RG, Schild VA, Buijs RM, Asselt LACv, Oordt PGWJv. 1987 Immunocytochemical evidence for peptidergic (GnRH) and dopaminergic innervation of gonadotropic cells in the pituitary of the African catfish, *Clarias gariepinus*. Gen Comp Endocrinol 67, 303-310.

Pol ANvd and Gorcs T. 1986 Synaptic relationships between neurons containing vasopressin, gastrin-releasing peptide, vasoactive intestinal polypeptide, and glutamate decarboxylase immunoreactivity in the suprachiasmatic nucleus: Dual ultrastructural immunocytochemistry with gold-substituted silver peroxidase. J Comp Neurol 252, 507-521.

Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA, Sherwood NM. 1994 Three forms of gonadotropin-releasing hormone characterized from brains of one species. PNAS 91, 12081-12085.

Powell JFF, Krueckl SL, Collins PM, Sherwood NM. 1996a Molecular forms of GnRH in three model fishes: rockfish, medaka and zebrafish. J Endocrinol 150, 17-23.

Powell JFF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM. 1996b Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. PNAS 93, 10461-10464.

Powell JFF, Standen EM, Carolsfeld J, Borella MI, Gazola R, Fischer WH, Park M, Craig AG, Warby CM, Rivier JE, Val-Sella MV, Sherwood NM. 1997 Primary structure of three forms

of gonadotropin-releasing hormone (GnRH) from the pacu brain. Reg Peptides 68, 189-195.

Prasado Rao DP, Jadhao AG, Sharma SC. 1987 Descending projection neurons to the spinal cord of the goldfish. J Comp Neurol 265, 96-108.

Quanbeck C, Sherwood NM, Millar RP, Terasawa E. 1997 Two populations of luteinizing hormone-releasing hormone neurons in the forebrain of the rhesus macaque during embryonic development. J Comp Neurol 380, 293-309.

Quinton R, Duke VM, Dezoysa PA, Bouloux PMG. 1996 The neurobiology of Kallmann's syndrome. Human Reproduction 11 (7 Suppl.), 121-127.

Rastogi RK, Meyer DL, Pinelli C, Fiorentino M, D'Aniello B. 1998 Comparative analysis of GnRH neuronal systems in the amphibian brain. Gen Comp Endocrinol 112, 330-345.

Rebers FEM, Bosma PT, Dijk Wv, Goos HJTh, Schulz RW. 2000a GnRH stimulates LH release directly via inositol phosphate and indirectly via cAMP in African catfish. Am J Physiol 278, 1572-1578.

Rebers FEM, Hassing GAM, Zandbergen MA, Goos HJTh, Schulz RW. 2000b Regulation of steady-state luteinizing hormone messenger ribonucleic acid levels, de novo synthesis, and release by sex steroids in primary pituitary cell cultures of male African catfish, *Clarias gariepinus*. Biol Reprod 62, 864-872.

Rissman EF, Alones VE, Craig-Veit CB, Millam JR. 1995 Distribution of chicken-II gonadotropin-releasing hormone in mammalian brain. J Comp Neurol 357, 524-531.

Rissman EF. 1996 Behavioral regulation of gonadotropin-releasing hormone. Biol Reprod 54, 413-419.

Rissman EF, Li X, King JA, Millar RP. 1997 Behavioral regulation of gonadotropin-releasing hormone production. Brain Res Bull 44 (4), 459-464.

Robinson TC, Tobet SA, Chase C, Waldron T, Sower SA. 2000 Gonadotropin-releasing hormones in the brain and pituitary of the teleost, the white sucker. Gen Comp Endocrinol 117, 381-394.

Ronnekleiv OK and Resko JA. 1990 Ontogeny of gonadotropin-releasing hormone-containing neurons in early fetal development of rhesus macaques. Endocrinol 126, 498-511.

Rothfeld JM, Hejtmancik JF, Conn PM, Pfaff DW. 1987 LHRH messenger RNA in neurons in the intact and castrate male rat forebrain, studied by *in situ* hybridization. Exp Brain Res 67, 113-118.

Roy P, Datta M, Dasgupta S, Bhattacharya S. 2000 Gonadotropin-releasing hormone stimulates thyroid activity in a freshwater murrel, *Channa gachau* (ham.), and carps, *catla catla* (ham.) and *Cirrhinus mrigala* (ham.). Gen Comp Endocrinol 117 (3), 456-463.

Sarkar S and Subhedar N. 2000 β-endorphin and gonadotropin-releasing hormone in the forebrain and pituitary of the female catfish, *Clarias batrachus*: double-immunolabeling study. Gen Comp Endocrinol 118, 39-47.

Schalburg KRv and Sherwood NM. 1999 Regulation and expression of gonadotropin-releasing hormone gene differs in brain and gonads in rainbow trout. Endocrinol 140, 3012-3024.

Schalburg KRv, Warby CM, Sherwood NM. 1999 Evidence for gonadotropin-releasing hormone

peptides in the ovary and testis of rainbow trout. Biol Reprod 60, 1338-1344.

Schreibman MP, Margolis-Nunno H, Halpern-Sebold LR, Goos HJTh, Perlman PW. 1986 The influence of androgen administration on the structure and function of the brain-pituitary-gonad axis of sexually immature platyfish, *Xiphophorus maculatus*. Cell Tissue Res 245, 519-524.

Schulz RW, Bosma PT, Zandbergen MA, Sanden MCAvd, Dijk Wv, Peute J, Bogerd J, Goos HJTh. 1993 Two gonadotropin-releasing hormones in the African catfish, *Clarias gariepinus*: localization, pituitary receptor binding, and gonadotropin release activity. Endocrinol 133, 1569-1577.

Schulz RW, Corput Lvd, Janssen-Dommerholt J, Goos HJTh. 1994 Sexual steroids during puberty in male African catfish (*Clarias gariepinus*): serum levels and gonadotropin-stimulated testicular secretion in vitro. J Comp Physiol 164, 195-205.

Schulz RW, Renes IB, Zandbergen MA, Dijk Wv, Peute J, Goos HJTh. 1995 Pubertal development of male African catfish (*Clarias gariepinus*). Pituitary ultrastructure and responsiveness to gonadotropin-releasing hormone. Biol Reprod 53, 940-950.

Schulz RW, Wind Fvd, Janssen-Dommerholt C, Peute J, Mylonas CC, Zohar Y, Swanson P, Goos HJTh. 1997a Modulation of testicular androgen production in adolescent African catfish (*Clarias gariepinus*). Gen Comp Endocrinol 108, 56-66.

Schulz RW, Zandbergen MA, Peute J, Bogerd J, Dijk Wv, Goos HJTh. 1997b Pituitary gonadotrophs are strongly activated at the beginning of spermatogenesis in African catfish, *Clarias gariepinus*. Biol Reprod 57, 139-147.

Schulz RW and Goos HJTh. 1999 Puberty in male fish: concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). Aquaculture 177, 5-12.

Schulz RW, Lubberink K, Zandbergen MA, Janssen-Dommerholt C, Peute J, Goos HJTh. 1999 Testicular responsiveness to gonadotropic hormone *in vitro* and Leydig and Sertoli cell ultrastructure during pubertal development of male African catfish (*Clarias gariepinus*). Fish Physiol Biochem 15, 243-254.

Schwanzel-Fukuda M and Pfaff DW. 1989 Origin of luteinizing hormone-releasing hormone neurons. Nature 338, 161-164.

Schwanzel-Fukuda M and Pfaff DW. 1990 The migration of luteinizing hormone-releasing hormone (LHRH) neurons from the medial olfactory placode into the medial basal forebrain. Experientia 46, 956-962.

Schwanzel-Fukuda M, Jorgenson KL, Bergen HT, Weesner GD, Pfaff DW. 1992 Biology of normal luteinizing hormone-releasing hormone neurons during and after their migration from olfactory placode. Endocrine Reviews 13, 623-634.

Schwanzel-Fukuda M. 1997 The origin and migration of LHRH neurons in mammals: a comparison between species including humans. In: GnRH neurons: gene to behavior. Parhar IS and Sakuma Y (Eds) Tokyo: Brain Shuppan, pp 221-242.

Schwanzel-Fukuda M. 1999 Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. Microsc Res Tech 44, 2-10.

Sherwood NM, Eiden L, Rivier JE, Spiess J, Vale W. 1983 Characterization of a teleost

gonadotropin-releasing hormone. PNAS 80, 2794-2798.

Sherwood NM, Sower SA, Marshak DR, Fraser BA, Brownstein MJ. 1986 Primary structure of gonadotropin-releasing hormone from lamprey brain. J Biol Chem 261, 4812-4819.

Sherwood N. 1987 The GnRH family of peptides. TINS 10, 129-132.

Sherwood NM and Whittier JM. 1988 Gonadotropin-releasing hormone from brains of reptiles: turtles (*Pseudemys scripta)* and snakes (*Thamnophis sirtalis parietalis*). Gen Comp Endocrinol 69 (3), 319-327.

Sherwood NM, Doroshov S, Lance V. 1991 Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). Gen Comp Endocrinol 84, 44-57.

Sherwood NM, Lovejoy DA, Coe IR. 1993 Origin of mammalian gonadotropin-releasing hormones. Endocrine Rev 14, 241-254.

Sherwood NM, Schalburg KRv, Lescheid DW. 1997 Origin and evolution of GnRH in vertebrates and invertebrates. In: GnRH neurons: gene to behavior. Parhar IS and Sakuma Y (Eds) Tokyo: Brain Shuppan, pp 3-25.

Shu S, Ju G, Fan L. 1988 The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Letters 85, 169-171.

Skinner DC, Caraty A, Evans NP. 1998 Does gonadotropin-releasing hormone in the cerebrospinal fluid modulate luteinizing hormone release? Neuroendocrinol 67, 37-44.

Skynner MJ, Sim JA, Herbison AE. 1999 Detection of estrogen receptor α and β messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons. Endocrinol 140, 5195-5201.

Smith MT, Moore FL, Mason RT. 1997 Neuroanatomical distribution of chicken-I gonadotropin-releasing hormone (cGnRH-I) in the brain of the male red-sided garter snake. Brain Behav Evol 49, 137-148.

Soga T, Sakuma Y, Parhar IS. 1998 Testosterone differentially regulates the expression of GnRH messenger RNAs in the terminal nerve, preoptic and midbrain of male tilapia. Mol Brain Res 60, 13-20.

Soma KK, Francis RC, Wingfield JC, Fernald RD. 1996 Androgen regulation of hypothalamic neurons containing gonadotropin-releasing hormone in a cichlid fish: Integration with social cues. Horm Behav 30 (3), 216-226.

Sower SA, Chiang YC, Lovas S, Conlon JM. 1993 Primary structure and biological activity of a third gonadotropin- releasing hormone from lamprey brain. Endocrinol 132, 1125-1131.

Sower SA, Nozaki M, Knox CJ, Gorbman A. 1995 The ocurrence and distribution of GnRH in the brain of Atlantic hagfish, an agnathan, determined by chromatography and immunocytochemistry. Gen Comp Endocrinol 97, 300-307.

Sower SA. 1998 Brain and pituitary hormones of lampreys, recent findings and their evolutionary significance. Am Zool 38, 15-38.

Sperry TS and Thomas P. 1999 Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinol

140, 1602-1611.

Spratt DP and Herbison AE. 1997 Regulation of preoptic area gonadotrophin-releasing hormone (GnRH) mRNA expression by gonadal steroids in the long-term gonadectomized male rat. Mol Brain Res 47, 125-133.

Sullivan KA and Silverman AJ. 1993 The ontogeny of gonadotropin-releasing hormone neurons in the chick. Neuroendocrinol 58, 597-608.

Takeo J and Yamashita S. 1999 Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. J Biol Chem 274, 5674-5680.

Tang YP, Kashon ML, Sisk CL. 1997 Brain region-specific regulation of luteinizing hormone-releasing hormone messenger ribonucleic acid in the male ferret: interactions between pubertal maturation and testosterone. Endocrinol 138, 4740-4747.

Timmers RJM, Lambert JGD, Peute J, Vullings HGB, Oordt PGWJv. 1987 Localization of aromatase in the brain of the male African catfish, *Clarias gariepinus* (Burchell), by microdissection and biochemical identification. J Comp Neurol 258, 368-377.

Timmers RJM and Lambert JGD. 1989 Catechol-O-methyltransferase in the brain of the male African catfish, *Clarias gariepinus*; distribution and significance for the metabolism of catecholestrogens and dopamine. Fish Physiol Biochem 7, 201-210.

Touhata K, Kinoshita M, Tokuda Y, Toyohara H, Sakaguchi M, Yokoyama Y, Yamashita S. 1999 Sequence and expression of a cDNA encoding the red seabream androgen receptor. Biochim Biophys Acta 1450, 481-485.

Troskie B, Illing N, Rumbak E, Sun YM, Hapgood J, Sealfon SC, Conklin D, Millar RP. 1998 Identification of three putative GnRH receptor subtypes in vertebrates. Gen Comp Endocrinol 112, 296-302.

Vogels OMJ, Broere CAJ, Laak HJt, Donkelaar HJt. 1990 Cell loss and shrinkage in the nucleus basalis of meynert's complex in Altzheimer's disease. Neurobiol Aging 11, 3-13.

Volkoff H and Peter RE. 1999 Actions of two forms of gonadotropin-releasing hormone and a GnRH antagonist on spawning behavior of the goldfish *Carassius auratus*. Gen Comp Endocrinol 116, 347-355.

Weber GM, Powell JFF, Park M, Fischer WH, Craig AG, Rivier JE, Nanakorn U, Parhar IS, Ngamvongchon S, Grau EG, Sherwood NM. 1997 Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. J Endocrinol 155, 121-132.

White RB, Eisen JA, Kasten TL, Fernald RD. 1998 Second gene for gonadotropin-releasing hormone in humans. PNAS 95, 305-309.

White RB and Fernald RD. 1998 Ontogeny of gonadotropin-releasing hormone (GnRH) gene expression reveals a distinct origin for GnRH-containing neurons in the midbrain. Gen Comp Endocrinol 112, 322-329.

White SA, Kasten TL, Bond CT, Adelman JP, Fernald RD. 1995 Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. PNAS 92, 8363-8367.

Wray S, Nieburgs A, Elkabes S. 1989 Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. Dev Brain Res 46, 309-318.

Wright DE and Demski LS. 1996 Organization of GnRH and FRMF-amide systems in two primitive bony fishes (Order Polypteriformes). Brain Behav Evol 47, 267-278.

Xiong F, Liu D, Le Drean Y, Elsholtz HP, Hew CL. 1994 Differential recruitment of steroid hormone response elements may indicate the expression of the pituitary gonadotropin II β-subunit gene during salmon maturation. Mol Endocrinol 8, 782-793.

Yamamoto N, Oka Y, Amano M, Aida K, Hasegawa Y, Kawashima S. 1995 Multiple gonadotropin-releasing hormone (GnRH)-immunoreactive systems in the brain of the dwarf gourami: *Colisa lalia*: immunohistochemistry and radioimmunoassay. J Comp Neurol 355, 354-368.

Yamamoto N, Oka Y, Kawashima S. 1997 Lesions of gonadotropin-releasing hormone-immunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. Neuroendocrinol 65, 403-412.

Yellon SM and Newman SW. 1991 A developmental study of the gonadotropin-releasing hormone neuronal system during sexual maturation in the male Djungarian hamster. Biol Reprod 45, 440-446.

Yoo MS, Kang HM, Choi HS, Kim JW, Troskie BE, Millar RP, Kwon HB. 2000 Molecular cloning, distribution and pharmalogical characterization of a novel gonadotropin-releasing hormone ([Trp8]GnRH) in frog brain. Mol Cell Endocrinol 164, 197-204.

Yu KL, Sherwood NM, Peter RE. 1988 Differential distribution of two molecular forms of gonadotropin- releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides 9, 625-630.

Yu KL, He ML, Chik CC, Lin XW, Chang JP, Peter RE. 1998 mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. Gen Comp Endocrinol 112, 303-311.

Zandbergen MA, Peute J, Verkley AJ, Goos HJTh. 1992 Application of cryosubstitution in neurohormone- and neurotransmitter-immunocytochemistry. Histochemistry 97, 133-139.

Zandbergen MA, Branden CAVvd, Schulz RW, Janssen-Dommerholt J, Ruijter JM, Goos HJTh, Peute J. 1993 GTH-cells in the pituitary of the African catfish, *Clarias gariepinus*, during gonadal maturation: an immuno-electron microscopical study. Fish Physiol Biochem 11 (1-6), 255-263.

Zandbergen MA, Kah O, Bogerd J, Peute J, Goos HJTh. 1995 Expression and distribution of two gonadotropin-releasing hormones in the catfish brain. Neuroendocrinol 62, 571-578.

Zhang L, Wayne NL, Sherwood NM, Postigo HR, Tsai PS. 2000 Biological and immunological characterization of multiple GnRH in a opisthobranch mollusk, *Aplysia californica*. Gen Comp Endocrinol 117, 77-89.

Samenvatting

Vissen, puberteit, hormonen, castreren…….. Dat zijn volgens mij de steekwoorden die de leek zou noemen, als ik hem zou vragen waar mijn promotieonderzoek over ging. Toch zou ik graag iets meer uitleggen over deze begrippen en hun onderlinge samenhang. Omdat het lezen van alle hoofdstukken voor de meesten van jullie misschien teveel gevraagd is, heb ik mijn bevindingen van de afgelopen 4 jaar hier in wat begrijpelijker taal neergezet. Als jullie dit onderzoek dan aanspreekt, zou ik aanraden zeker ook de Inleiding en Samenvattende Discussie te lezen.

Om met de vissen te beginnen: het proefdier in dit onderzoek is de Afrikaanse meerval met de Latijnse naam *Clarias gariepinus*. In de Inleiding staat een tekening van dit dier afgebeeld. Het proefdier is goed te hanteren, groeit snel en is geschikt voor allerlei praktische handelingen. Binnen de onderzoeksgroep Vergelijkende Endocrinologie is een grote hoeveelheid kennis opgebouwd over voortplanting en puberteit van de meerval.

Puberteit bij de mens is de levensperiode waarin de rijping van de geslachtorganen begint en ook de periode van geestelijke ontwikkeling. Biologisch gezien is puberteit de periode die begint met de ontwikkeling en productie van kiemcellen en eindigt met rijpe eicellen of zaadcellen en dus de mogelijkheid tot succesvol voortplanten.

Het voortplantingssyteem bestaat uit 3 belangrijke stations: een onderdeel van de hersenen, de hypofyse (een hormoonklier, die onder aan de hersenen hangt) en de geslachtsorganen. Omdat dit onderzoek gedaan is aan mannelijke meervallen, zal ik verder alleen het mannelijke geslachtsorgaan noemen, de testes. In de bodem van de hersenen liggen gespecializeerde zenuwcellen die het hormoon gonadotropin-releasing hormone, oftewel GnRH, produceren. Deze zenuwcellen geven hun hormoon GnRH af aan de hypofyse (het 2de station), die daarop gonadotropine afgeeft aan de bloedbaan. Hiermee is de naam van GnRH verklaard. Gonadotropine is een verzamelnaam voor follikel stimulerend hormoon (FSH) en luteinizerend hormoon (LH). In de testes verzorgt gonadotropine de aanmaak van zaadcellen en steroid hormonen. De hersen-hypofyse-testes as is een dynamisch systeem, omdat de steroid hormonen een positieve of negatieve (afhankelijk van de levensfase) terugkoppeling kunnen

hebben op elk station. Om een voorbeeld te geven, het steroid hormoon testosteron stimuleert de aanmaak van gonadotropine in de hypofyse van puberende meervallen.

Het hierboven beschreven voortplantingssysteem is weergegeven zoals het functioneert in een volwassen individu. Vóór de puberteit zijn de verschillende stations echter nog niet rijp en is het systeem nog niet actief. De rijping van het voortplantingssyteem is een complex proces, waarvan de precieze werking nog onbekend is. Er is een hypothese die suggereert dat één of meer componenten van het systeem ontbreken voor de puberteit. Kandidaat voor deze zogenaamde "missing link" zijn de steroid hormonen uit de testes. Er is al aangetoond dat behandeling van vissen met bepaalde steroiden aan het begin van de puberteit de rijping van de hypofyse stimuleert en het gehalte aan gonadotropine doet stijgen. Weer andere steroiden bevorderen alleen de productie van zaadcellen in de testes. Er zijn echter ook aanwijzingen, dat de "missing link" is gelegen in de zenuwcellen die GnRH maken.

In mijn onderzoek heb ik de ontwikkeling van deze GnRH zenuwcellen in de hersenen gevolgd en heb ik het eventuele effect van steroid hormonen op deze ontwikkeling onderzocht d.m.v. behandeling met deze hormonen of door het weghalen ervan (castratie). De GnRH cellen kunnen onder de microscoop bekeken worden, nadat ze zijn aangekleurd met speciale antilichamen.

De resultaten zijn als volgt: het systeem van GnRH cellen in de hersenen ziet er qua ontwikkeling volwassen uit op het moment dat de puberteit begint. Echter, de zenuweindigingen van deze cellen zijn dan nog lang niet in de buurt van de gonadotropine cellen in de hypofyse, waar ze het GnRH moeten afgeven. Dit proces van ingroeien van de zenuwuitlopers is pas voltooid aan het eind van de puberteit. Het is ook de vraag of de GnRH cellen aan het begin van de puberteit wel voldoende GnRH afgeven. Verder bleek, dat bepaalde steroid hormonen inderdaad nodig zijn voor de normale ontwikkeling van de GnRH cellen.

We kunnen dus concluderen, dat steroid hormonen essentieel zijn voor de activatie van de GnRH cellen. We stellen dat de afgifte van voldoende GnRH aan de hypofyse net voor de puberteit, de aanzet is voor de rijping van alle stations van het voortplantingssysteem en dus de “missing link” is.

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Henk, mijn promotor. Dank je wel voor het behouden van overzicht in mijn promotieonderzoek. De door jou ingestelde brainstorm-dagen waren in dat opzicht zeer nuttig. Als ik dacht de vraagstelling duidelijk voor ogen te hebben, kon jij deze toch altijd nog veel meer verduidelijken. Ik vind het jammer, dat we niet meer zijn toegekomen aan de regulatie van de GnRH receptor. Moeder Natuur en de krappe tijd noopten ons om de experimenten af te blazen. Buiten het werk om, vond ik het eten na het labuitje bij jou thuis altijd een hoogtepunt. Gewoon gezellig, met medewerkers, AIO's en studenten onder elkaar.

Ine en Maartje, mijn paranimfen. Ine, ik wist al tijdens mijn derde AIO-jaar, dat ik jou wilde vragen om mijn paranimf te worden. Gewoon, omdat je een collega bent, die ik graag mag en omdat je me aan mijn moeder doet denken (mag ik dit wel zeggen?). Het toeval wilde, dat je zelfs echt in mijn project betrokken raakte, omdat er GAP-RIA's opgezet moesten worden. We vertellen niemand, dat de GAP-RIA het nu niet meer wil doen. Maartje, als lessenaargenoot en bestuursmaatje in het USConcert zijn we al jaren goede vriendinnen. Dankzij jouw onvoorwaardelijk steun en aanwezigheid tijdens mijn moeilijke periode, ben ik er weer bovenop gekomen. Ik ben reuzeblij, dat je paranimf wilde worden. En nu je weet hoe meerval smaakt, ben je goed in het onderwerp ingewijd.

Een AIO kan eigenlijk niet zonder hulp van studenten in zijn/haar project, zo ook ik niet. Bedankt Pauline van der Voort (niet echt mijn student, maar wel het voorwerk gedaan), Aletta Florijn, Claudia Leemereise, Siona Slob (nu ook voor het AIO-schap gevallen), Anne Hindriks en Meike Vos. Jullie bijdragen waren soms zelfs zo belangrijk, dat een aantal van jullie mede-auteur geworden is van een publicatie. Ik bedank jullie ook voor de gezellige tijd op het lab. Ik heb veel van jullie geleerd; ik hoop, dat jullie van mij ook wat hebben opgestoken!

Mijn kamergenoten van Z208. Drie lotgenoten (AIO's) en een analiste, allen van het vrouwelijke geslacht op één kamer, dat kon nooit wat worden dachten de meesten. Er is inderdaad veel en gezellig gekletst, maar zeker ook hard gewerkt. Marion, jij was als eerste van ons aan de beurt om te promoveren en dus een voorbeeld. We zijn qua karakter misschien elkaars tegenpolen, maar we konden goed praten over het werk, gezondheid, huizen, kinderen etc. Dat de (degelijke Duitse?) thermosfles voor de thee kapot ging aan het eind van jouw promotieperiode, was iets te symbolisch voor je vertrek. Cristina, thank you for a nice time in our room. Phone calls from Portugal and travel agencies, computer problems, scientific problems; these things created a lively

atmosphere. Good luck with your last part of the thesis. Marjolein, ik heb met plezier genoten van al je spannende reisverhalen. Marja, nog maar kort kamergenoot, maar gelukkig heb je een nieuwe waterkoker en thermoskan meegebracht.

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Veel dank ben ik verschuldigd aan de dierverzorgers: meneer van Oostrum, Henk, Job en Ko. Jullie hebben alle meervallen voor mijn experimenten gekweekt. Tijdens de experimenten hebben jullie ze goed in de gaten gehouden en ook vaak gevoerd. Henk, ik wil jou speciaal bedanken voor de prettige samenwerking en je kundige kijk op de gezondheid van de vissen. Zonder een gezond proefdier begin je niets!

De medewerkers van de fotografie, Frits, Piet, Wil en Ronald, wil ik graag bedanken voor het ontwikkelen van alle films, het leren van Photoshop, het printen van figuren en het scannen van vele dia's en negatieven. De laatste tijd kwam ik bijna dagelijks binnenstormen om nog even een figuur te wijzigen of een printje te maken. Het was geen probleem, het leek zelfs of jullie op me rekenden, want er was altijd wel een computer vrij of even tijd voor een printje (en een praatje). Dankzij jullie training kan ik me nu goed redden in Photoshop, maar met een simpel trucje van Piet of Frits gaat het toch altijd weer beter. Ik heb zo heel wat foto's "wat aardiger gemaakt om naar te kijken". Maar of ik echt zo'n grote Manipulator ben..... Frits, reserveer je alvast een startplaats voor de Pheidippidesloop?

Verder bedank ik de overige medewerkers van Z3; Maarten Terlou, Marcel Kramer en de medewerkers van Vormgeving. Een extra dank-je-wel voor Emy Franck voor de layout en cover van het proefschrift.

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Buiten het lab nam het Utrechtsch Studenten Concert een belangrijke plaats in. Meespelen in een orkest is een bijzondere ervaring: inspanning en ontspanning tegelijk. Bedankt orkestgenoten, voor de ontzettend leuke en leerzame tijd naast studie en werk.

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Bedankt!

Eline

Publication list

Dubois EA, Zandbergen MA, Peute J, Goos HJTh. 1998. Effects of gonadal steroids on the developing GnRH system in juvenile African catfish (*Clarias gariepinus*). 19th Conference of European Comparative Endocrinologists, 1-4 September 1998, Nijmegen, the Netherlands. p20. (Poster)

Dubois EA, Zandbergen MA, Peute J, Goos HJTh. 1998. Development of the hypophysial innervation by GnRH neurons in African catfish (*Clarias gariepinus*). Folia Endocrinologica Japonica :The 4th International Congress of Neuroendocrinology, 11-16 October 1998, Kitakyushu, Japan. 74: 473. (Poster)

Dubois EA, Florijn MA, Zandbergen MA, Peute J, Goos HJTh. 1998. Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish (*Clarias gariepinus*). Gen. Comp. Endocrinol. 112:383-393.

Dubois EA, Zandbergen MA, Peute J, Goos HJTh. 1999. What is the role of pituitary innervation by GnRH fibers in the onset of puberty in the African catfish (*Clarias gariepinus*)? The 5th International Conference on the Control of the Onset of Puberty, 26-28 September 1999, Liege, Belgium. p 47. (Poster)

Dubois EA, Slob S, Hassing I, Zandbergen MA, Peute J, Goos HJTh. 2000. Differential regulation of the catfish gonadotropin-releasing hormone system by steroids in juvenile and pubertal African catfish (*Clarias gariepinus*). 4th International Symposium on Fish Endocrinology, July 31 through August 3, 2000, Seattle, WA, USA. p 23. (Presentation)

Dubois EA, Zandbergen MA, Peute J, Hassing I, van Dijk W, Schulz RW, Goos HJTh. 2000. Gonadotropin-releasing hormone fibers innervate the pituitary of male African catfish (*Clarias gariepinus*) during puberty. Neuroendocrinol. 72:252-262.

Dubois EA, Slob S, Zandbergen MA, Peute J, Goos HJTh. 2001. Gonadal steroids and the maturation of the species-specific gonadotropin-releasing hormone system in brain and pituitary of the male African catfish (*Clarias gariepinus*). Comp. Biochem. Physiol. In press.

Dubois EA, Zandbergen MA, Peute J, Goos HJTh. 2001. Two different GnRH forms within three distinct populations in the brain of the African catfish (*Clarias gariepinus*): early development of the GnRH system. Submitted.

Meek J, Dubois EA, Zandbergen MA, de Boer- van Huizen R, Peute J. 2001. Functional morphology of cGnRH-II cells in the midbrain of a teleost fish, *Clarias gariepinus*. Submitted.

Curriculum vitae

De schrijver van dit proefschrift werd op 12 maart 1974 te 's Gravenhage geboren. Na het behalen van het gymnasium diploma aan het Gymnasium Haganum te Den Haag in 1992, werd in datzelfde jaar begonnen met de studie biologie aan de Universiteit Utrecht. Tijdens de studie biologie werd een onderzoeksstage gedaan bij het Nederlands Instituut voor Drugs en Doping Research (NIDDR) onder supervisie van Dr. D de Boer. Het stage betrof het opzetten van een detectiemethode voor het zwangerschapshormoon (hCG) in urine ten bate van dopingonderzoek en het bestuderen van effecten van dit hormoon op de zg. testosteron/epitestosteron-ratio. Een tweede onderzoeksstage werd uitgevoerd bij de onderzoeksgroep Vergelijkende Endocrinologie onder leiding van Dr. J.E.B. Cavaco, waarbij effecten van steroid hormonen op de testes van juveniele Afrikaanse meervallen werden onderzocht. De afstudeerscriptie werd geschreven bij dezelfde onderzoeksgroep, ditmaal begeleid door Prof. Dr. H.J.Th. Goos, met als onderwerp het gonadotropin-releasing hormone. Na het behalen van het doctoraal biologie in augustus 1996, werd in september van datzelfde jaar begonnen met het promotieonderzoek beschreven in dit proefschrift wederom bij de onderzoeksgroep Vergelijkende Endocrinologie met Prof. Dr. H.J.Th. Goos als promotor. Tijdens het promotieonderzoek werden diploma's gehaald voor het werken met radioactieve stoffen (Stralinghygiene, 4B) en met proefdieren (art. 9 onderzoeker). Tijdens studie en promotieonderzoek deed de schrijfster veel ervaring op in organisatie, beleid en bestuur tijdens de uitvoering van diverse bestuursfuncties bij het Utrechtsch Studenten Concert en het Koordinerend Orgaan van Studenten Muziekgezelschappen te Utrecht.