Chapter 1

Introduction

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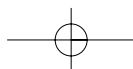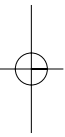
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Introduction
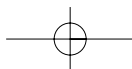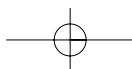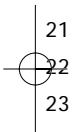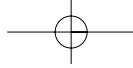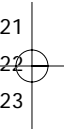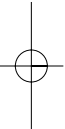
Reproduction is a fascinating and complex biological process, essential for the survival of each species. At every conception, sets of genes are recombined to form a new and unique individual. Sexual reproduction requires adequate behavioural and physiological preparation of both future parents. Without 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.
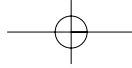
The brain-pituitary-gonad axis (BPG-axis) is the physiological system that is most directly involved in the control of reproduction in vertebrates (Fig. 1). At the level of the brain neurons are present which produce the neuropeptide gonadotropin-releasing hormone (GnRH). GnRH reaches the gonadotropic 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
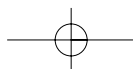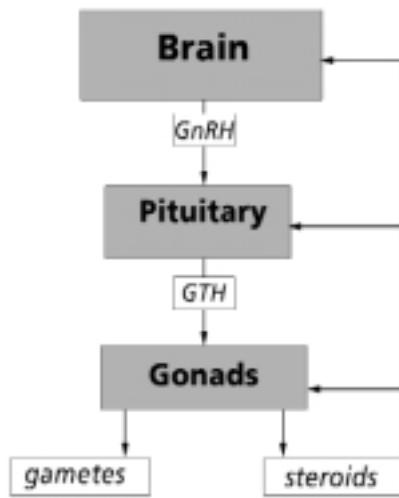
Fig. 1 Schematic representation of the brain-pituitary-gonad axis. In grey the organs, in white the hormones produced by each level of the axis, and the gametes. GnRH: gonadotropin-releasing hormone, GTH: gonadotropic hormone.

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.
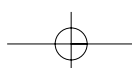
GnRH: the forms and the gene

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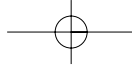
Table I Amino acid sequences of the 15 different GnRH forms. Amino acids listed in bold differ from the cGnRH-II sequence.

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.
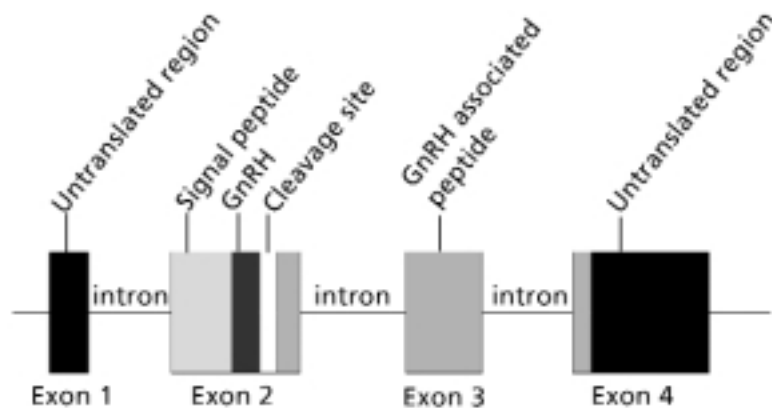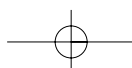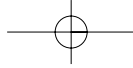

Fig. 2 Schematic representation of the GnRH gene, consisting of 4 exons and 3 introns. The translated region consists of the signal peptide, GnRH, a cleavage site, and the GnRH-associated peptide.

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
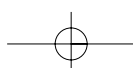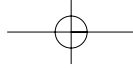
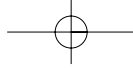**Table II** Phylogenetic distribution of different GnRH forms. cII: chickenGnRH-II; df: dogfishGnRH; m: mammalianGnRH; h: herringGnRH; cf: catfishGnRH, s: salmonGnRH; md: medakaGnRH; sb: seabreamGnRH; r: ranaGnRH; gp: guinea pigGnRH; cI: chickenGnRH-I; t: tunicateGnRH (I and II) and L: lampreyGnRH (I and III).
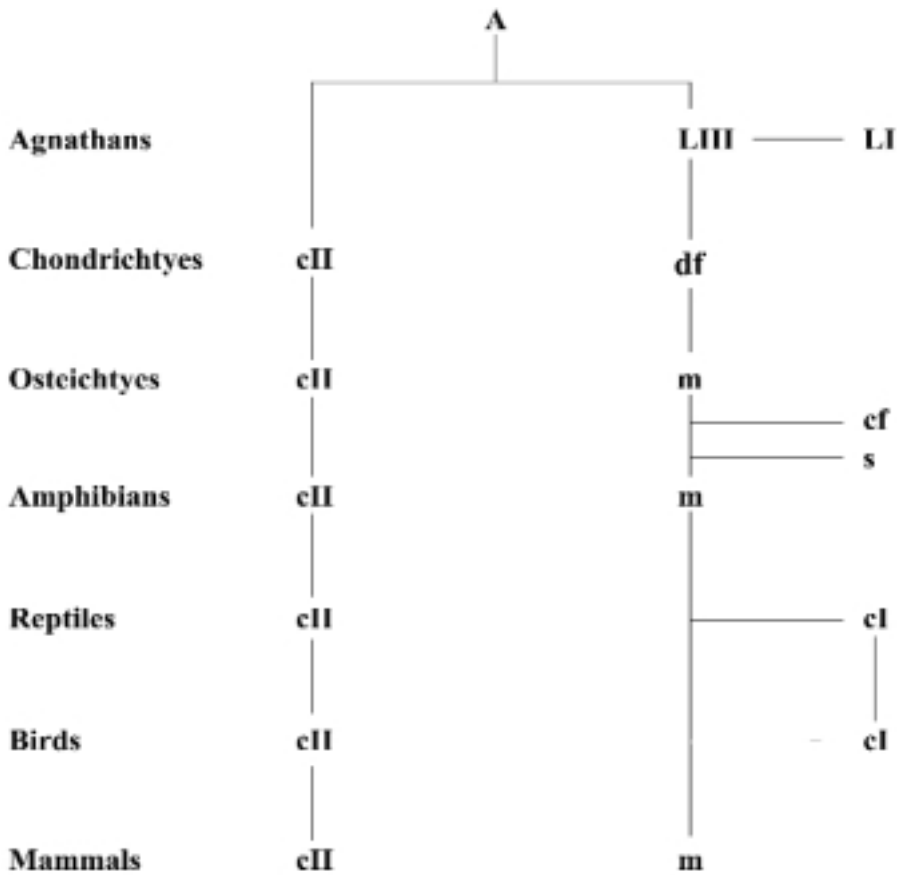
		cII	df	m	h	cf	s	md	sb	r	gp	cI	t	L
Invertebrates														
Mollusk	Zhang et al. 2000			m?									tI?	
Aplysia californica														
Protochordate														
Tunicate	Di Fiore et al. 2000			m?									tI+II	
Ciona intestinalis	Powell et al. 1996													
Agnatha														
Lamprey	Sower 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														
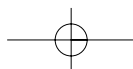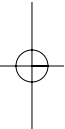
			cll	df	m	h	cf	s	md	sb	r	gp	cl	t	L
Reptiles															
Turtle	Sherwood and	cll											cl		
Pseudemys srcipta	Whittier 1988														
American alligator	Lovejoy et al. 1991	cll											cl		
Alligator mississippiensis															
Lizard	D'Aniello et al. 1994	cll											cl		
Podarcis s. sicula															
Birds															
Chicken	Dunn and Millam 1998	cll											cl		
Gallus domesticus															
Turkey	Millam et al. 1993	cll											cl		
Gallus meleagris															
Mammals															
Tree shrew	Kasten et al. 1996	cll		m											
Tupaia glis belangeri															
Guinea pig	Jimenez-Linan et al. 1997	cll									gp				
Macaque	Latimer et al. 2000	cll		m											
Macaca mulatta															
Human	White et al. 1998	cll		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, cll: chicken-II, LIII: lampreyIII, LI: lampreyI, df: dogfish, cf: catfish, s: salmon and cl: chicken-I.

A

Agnathans   LIII —— LI

Chondrichtyes   cII   df

Osteichtyes   cII   m
                                  cf
                                  s
Amphibians   cII   m

Reptiles   cII   cl

Birds   cII   cl

Mammals   cII   m
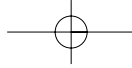
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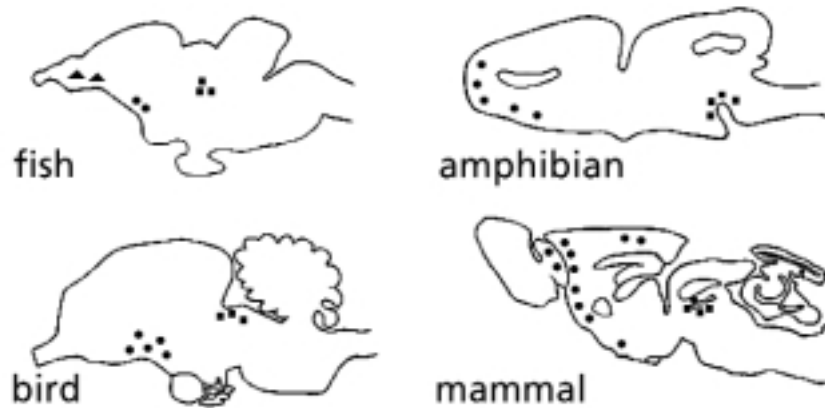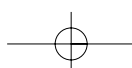26
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Fig. 3 Schematic drawings of GnRH localisation in the brain of a fish (White et al. 1995), an amphibian (Muske 1993), a bird (Mikami et al. 1988) and a mammal (Rissman et al. 1995). The species specific GnRH or GnRH1 (●) is localised in the ventral 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 sep-
arate nuclei as seen in the mammalian (King and Anthony 1984), the avian
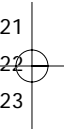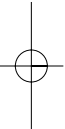(Dunn and Millam 1998) and the frog brain (Conlon et al. 1993).

Quanbeck et al. (1997) characterised early and late migrating GnRH neu-
rons 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 proba-
bly 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 ter-
minal nerve. Thus, the species-specific form is restricted to the POA. The neu-
rons 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) pro-
posed 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.
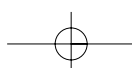
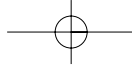## GnRHs: functions and targets

The gonadotropic cells in the pituitary are the main targets of the species spe-
cific 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 impor-
tant 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 hor-
mones 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 hor-
mone cells in carp (Roy et al. 2000).

An effective approach for studying the target sites of GnRH is by localis-
ing the expression of its receptor. The GnRH receptor (GnRH-R) is G-pro-
tein-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 func-
tion 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
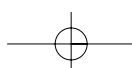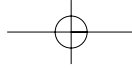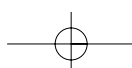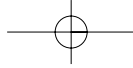
steroids may affect hypothalamic GnRH content and/or expression. In mammals, controversial results were obtained after steroid treatment or steroid withdrawal (orchidectomy/ ovariectomy) (Kalra and Kalra 1989; Gore and Roberts 1997; Kim et al. 1997). For example, in one study on rats the expression of 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
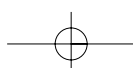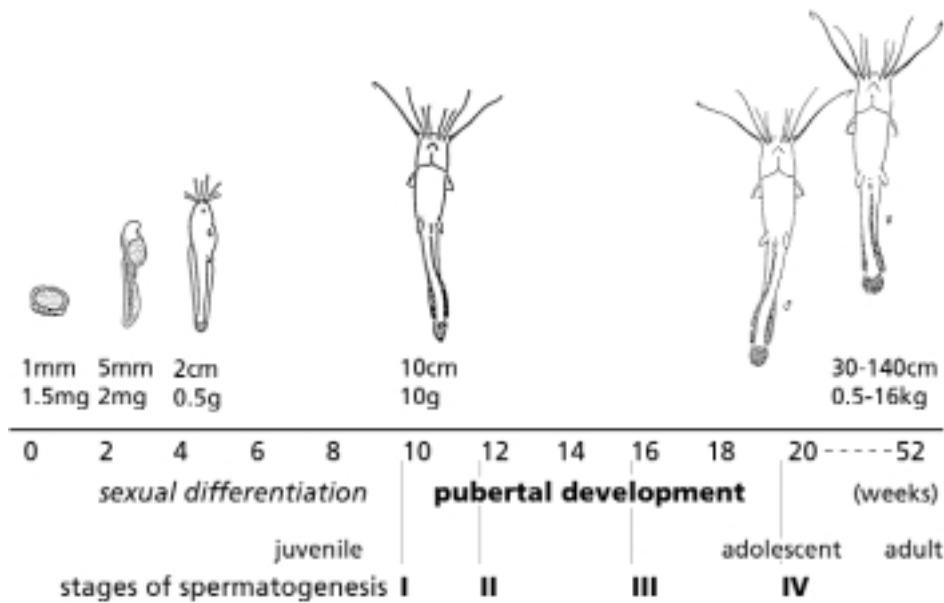
Fig. 4 Life span of the African catfish from the fertilised egg till the adult state at 52 weeks. The four stages of spermatogenesis during the pubertal development are abbreviated as: stage I: spermatogonia, stage II: spermatogonia and spermatocytes, stage III: spermatogonia, spermatocytes, and spermatids, 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.

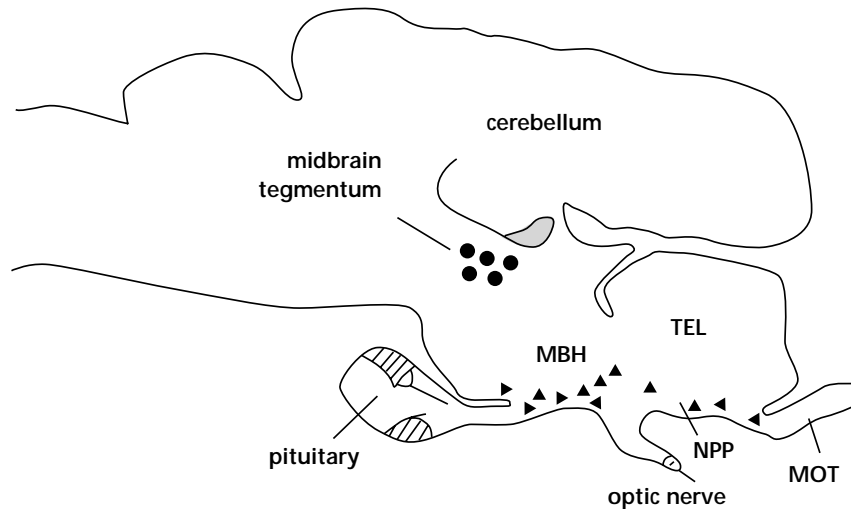The amount of cfGnRH in the pituitary is about 700 times higher than

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 sperma-tocytes 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 sper-matids 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-
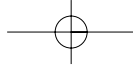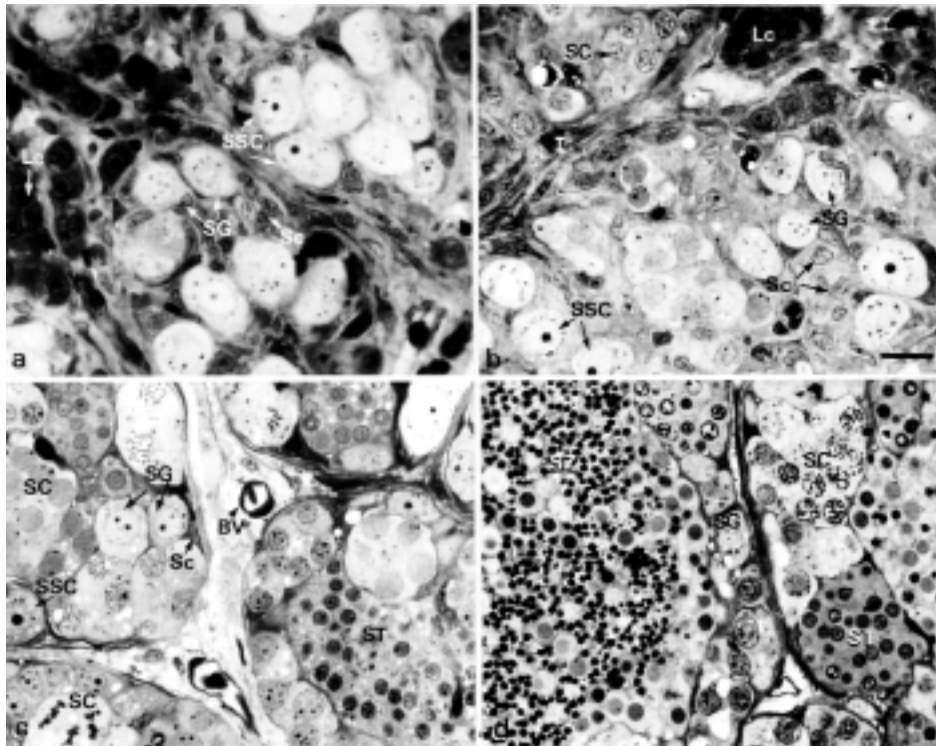
Fig. 6 Micrographs of the four stages of spermatogenesis in the African catfish. a) stage I, spermatogonia; b) stage II, spermatogonia and spermatocytes; c) stage III, spermatogonia, spermatocytes, and spermatids; d) stage IV, all germ cells including spermatozoa. Lc: Leydig cell, SSC: spermatogonial 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).
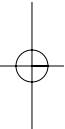
gens 11β-hydroxyandrostenedione (OHA) and its conversion product 11-keto-testosterone (11KT) stimulate spermatogenesis, testicular development and the appearance of secondary sex characteristics (Cavaco et al. 1998b). However, at the pituitary level these 11-oxygenated steroids inhibit the gonadotropic 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.
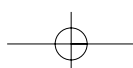
Scope of the thesis

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).
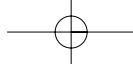
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      Chapter 6 contains a morphological description of the cGnRH-II cells. In species studied to date, the function of the midbrain cGnRH-II neurons remained unsolved. In contrast to the cfGnRH cells in the ventral forebrain in our experiments, the 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.