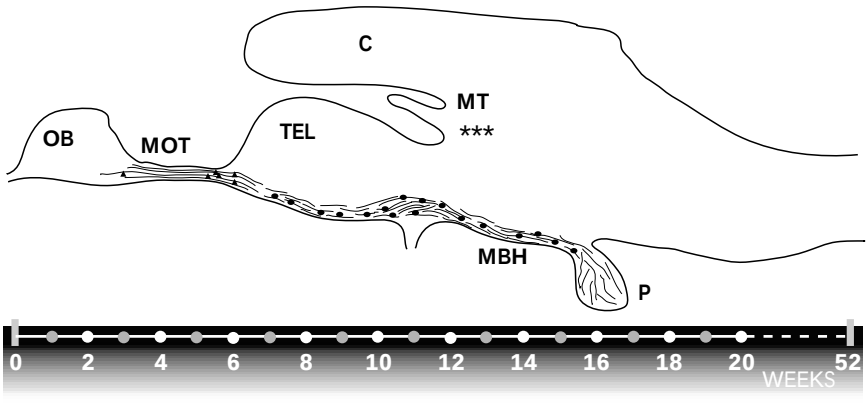
Chapter 7

# 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

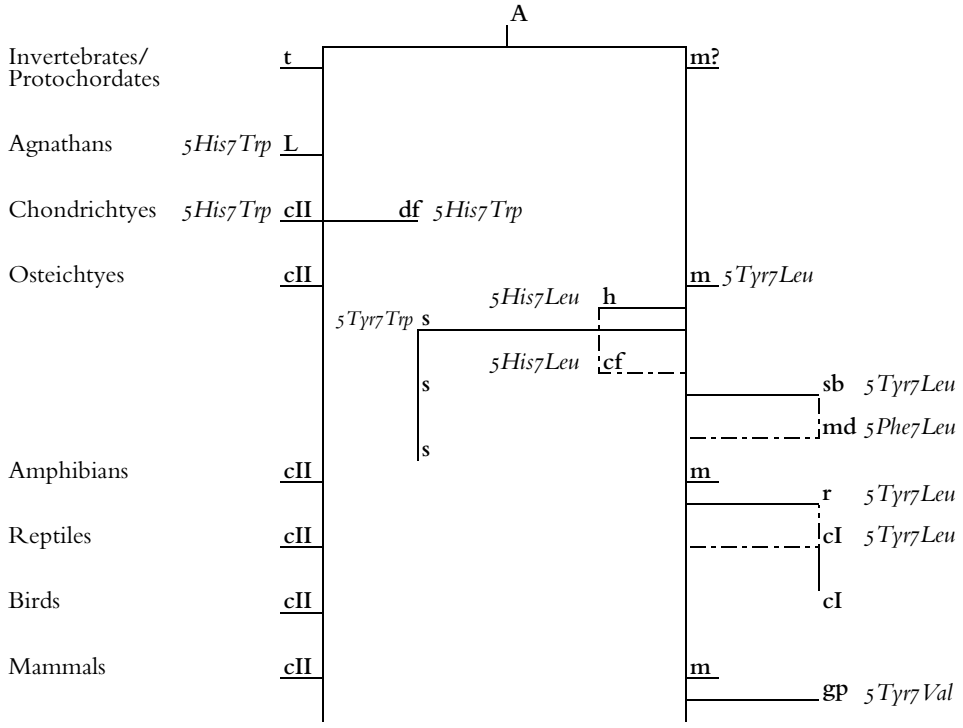



**Fig. 1 Hypothetical evolutionary tree for GnRH. Two main lineages are discerned: the cGnRH-II lineage involves cGnRH-II, dfGnRH and the older lamprey and tunicate GnRHs. A histidine on position 5 and a tryptophane on position 7 characterize the GnRH in this lineage. The second line is called the mammalianGnRH 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.

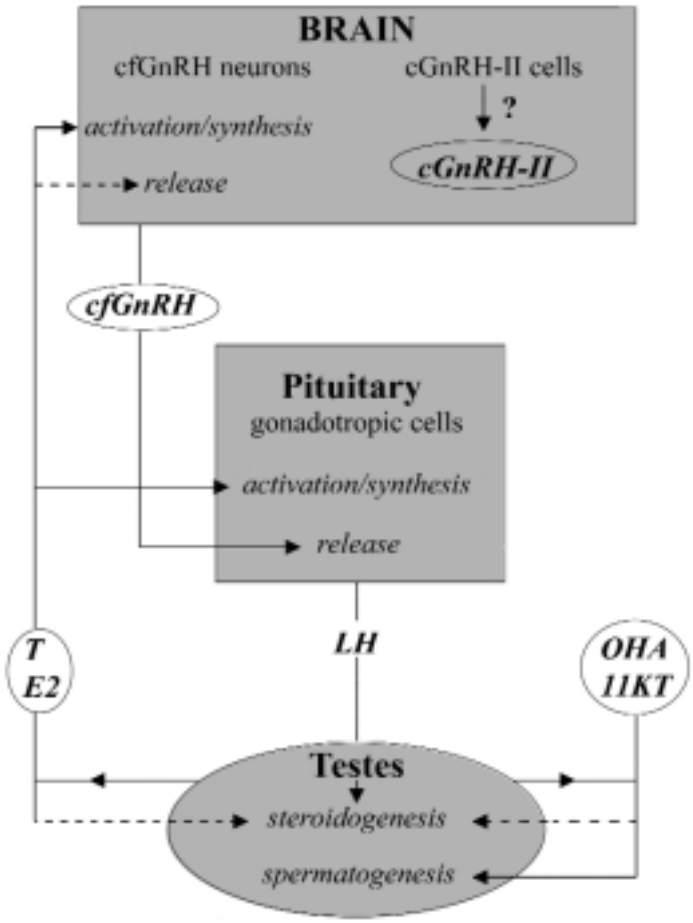



**Fig. 2** Hypothetical model for the actions of the steroids OHA and 11KT, and T, and GnRHs on different levels of the BPG-axis at the onset of puberty. Fat lines: stimulatory action, dashed lines: inhibitory action, ?: presumed action. For further explanation see text. OHA: 11β-hydroxyandrostenedione,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