Chapter 3

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

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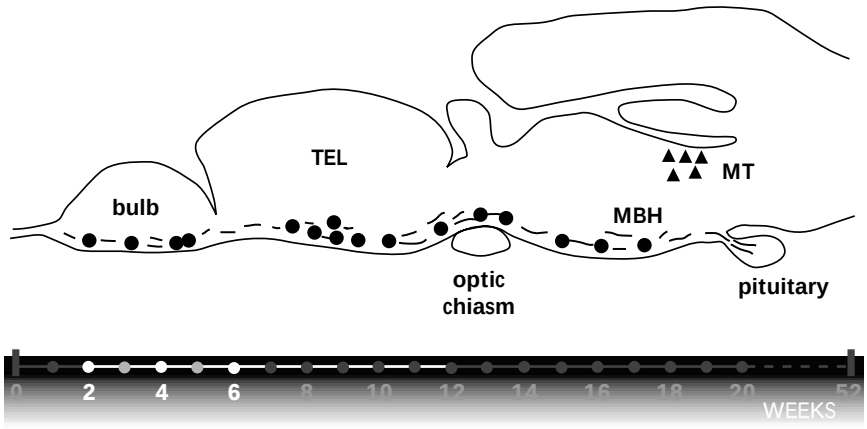

Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish *(Clarias gariepinus)*

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

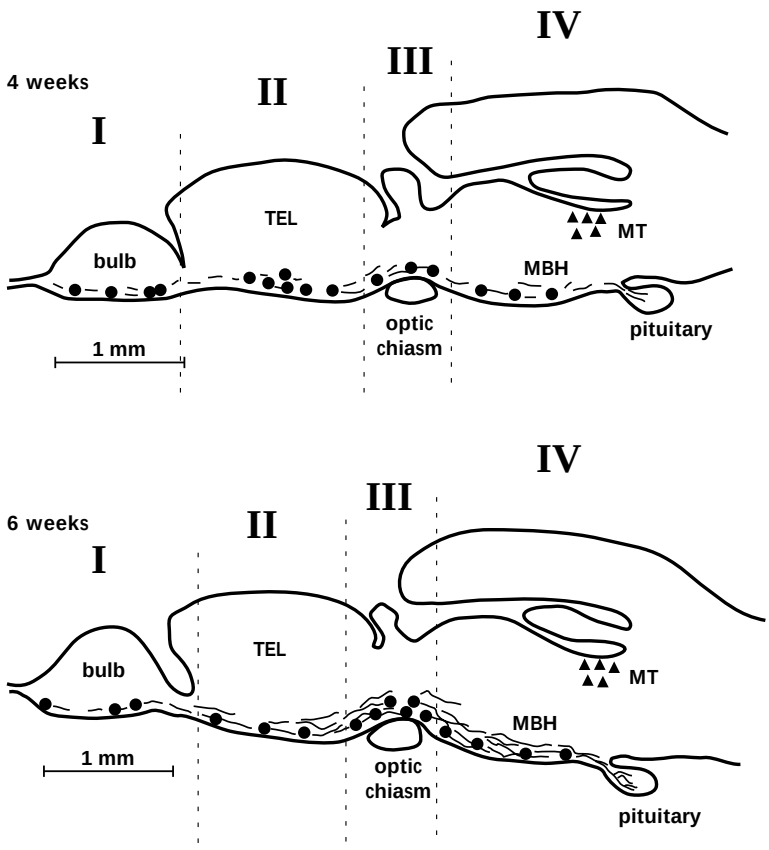

Fig. 1. Schematic drawings of the catfish brain at 4 and 6 weeks. The cfGnRH neurons (●) and fibers are localized along the entire ventral forebrain; the cGnRH-II neurons (▲) form a distinct nucleus in the MT. I-IV correspond with the subsequent brain parts in Fig. 4.

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

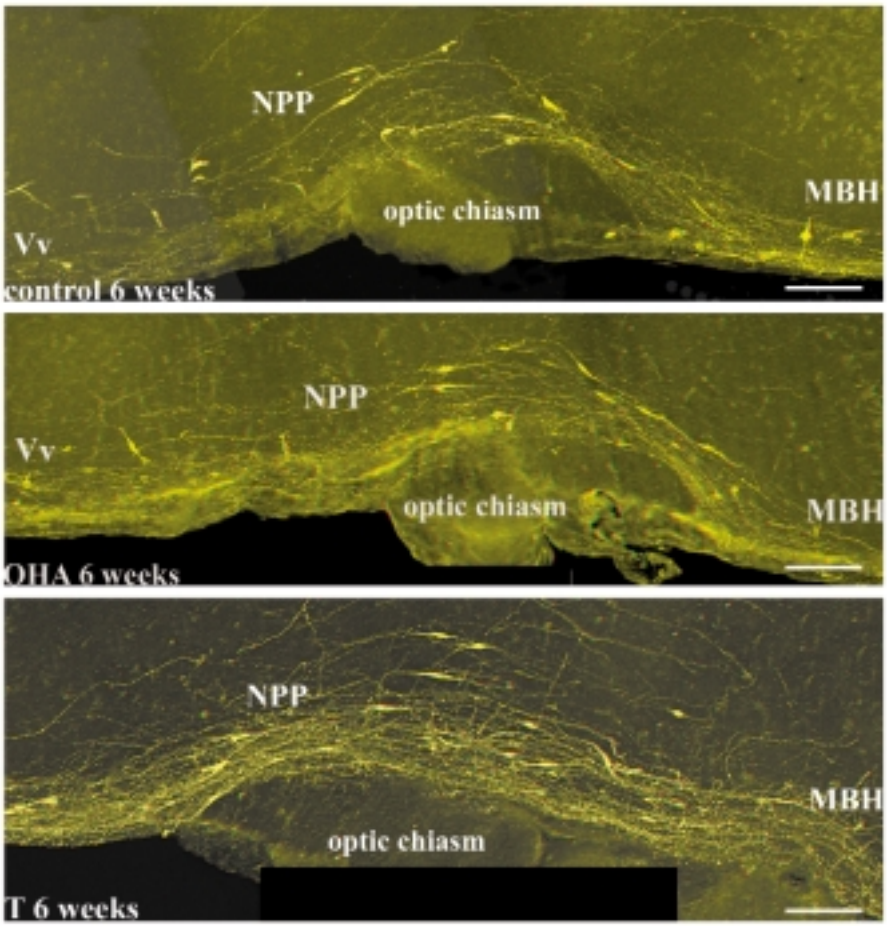

Fig. 2. 3D images obtained by confocal laser scanning microscopy. Sagittal vibratome sections (100μm) of the ventral forebrain of the three groups (control, OHA, and T) at 6 weeks are shown from the caudal part of the Vv until the beginning of the MBH. An objective of 25x and a pinhole of 170μm were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

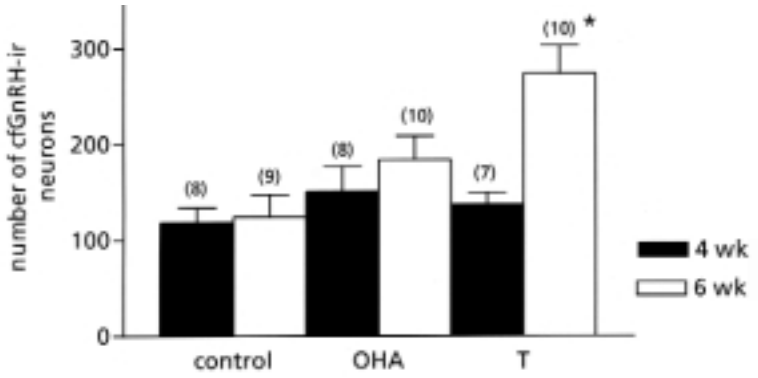

Fig. 3. Numbers of cfGnRH-ir neurons (mean (SEM) in the brain after administration of food pellets containing no steroid (control), OHA, or T at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, white bars). N is between 7 and 10. * Significantly different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

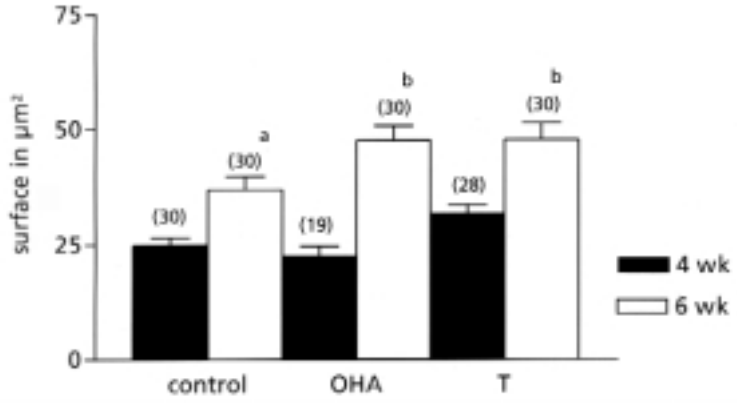

Fig. 4. Surfaces of cfGnRH-ir neurons (in μm^2, mean ± SEM) in the ventral forebrain after feeding food pellets containing no steroid (control), OHA, or T at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, white bars). N is between 19 and 30. a Significantly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

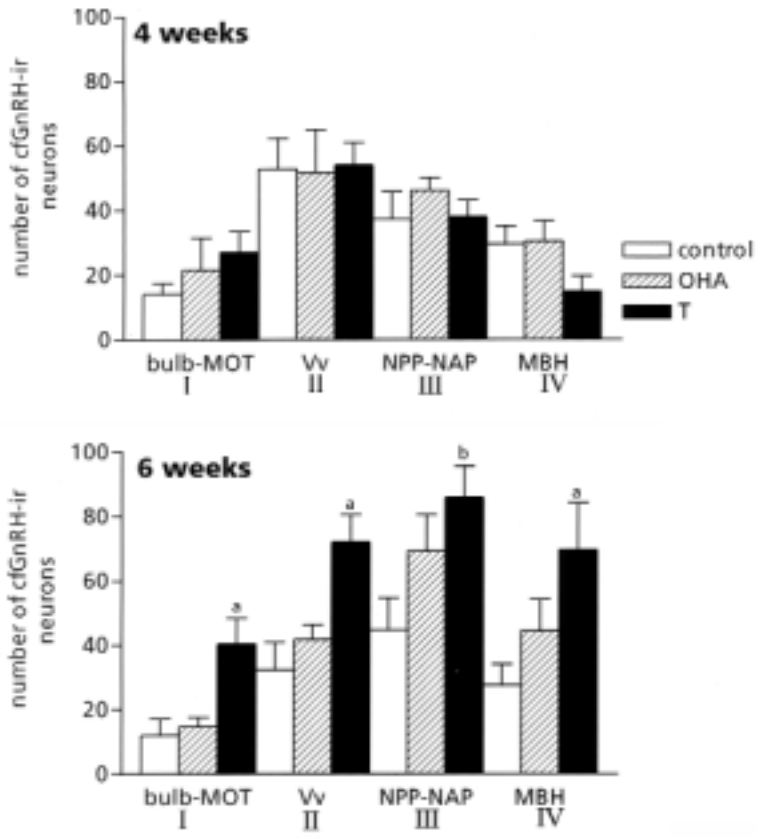

Fig. 5. Numbers of cfGnRH-ir neurons (mean ± SEM) in the ventral forebrain, subdivided in four subsequent brain parts (bulb-MOT, Vv, NPP-NAP, MBH) after treatment with food pellets containing no steroid (control, white bars), OHA (hatched bars), or T (black bars) at, respectively, 4 weeks (2 weeks of 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

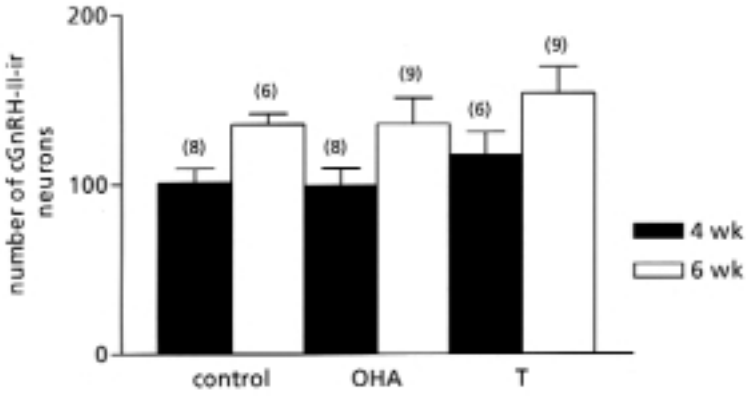

Fig. 6. Numbers of cGnRH-II-ir neurons (mean ±SEM) in the brain after administration of food pellets containing no steroid (control), OHA, or T at, respectively, 4 weeks (2 weeks of treatment, black bars) and 6 weeks of age (4 weeks of treatment, white bars). N is between 6 and 9.

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.

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