Chapter 4

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

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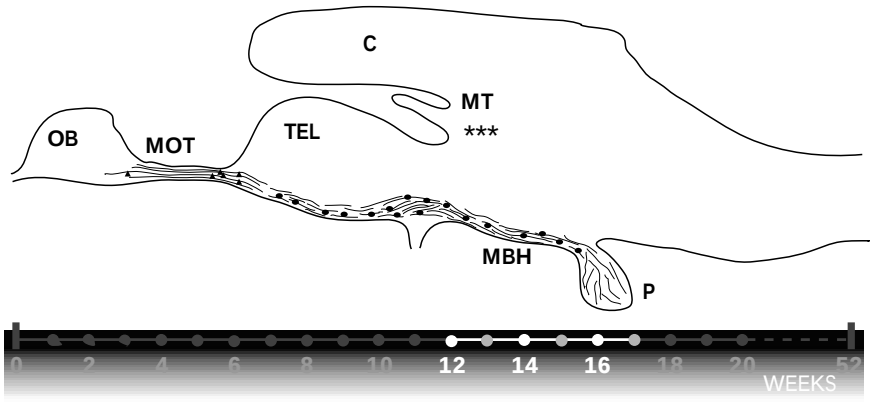


Gonadotropin-releasing hormone fibers innervate the pituitary of the male African catfish (Clarias gariepinus) during puberty

Abstract

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

Introduction

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

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

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

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

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

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

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

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

Materials and Methods

Animals

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

Immunocytochemistry for CLSM and EM

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

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

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

Testicular histology

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

Pituitary incubation studies

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

Castration and testosterone replacement

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

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

Radioimmunoassays

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

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

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

Statistics

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

Results

Pubertal development of the cfGnRH system in the pituitary

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

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

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

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

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

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

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

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

Pituitary incubation studies

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

Castration experiment

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

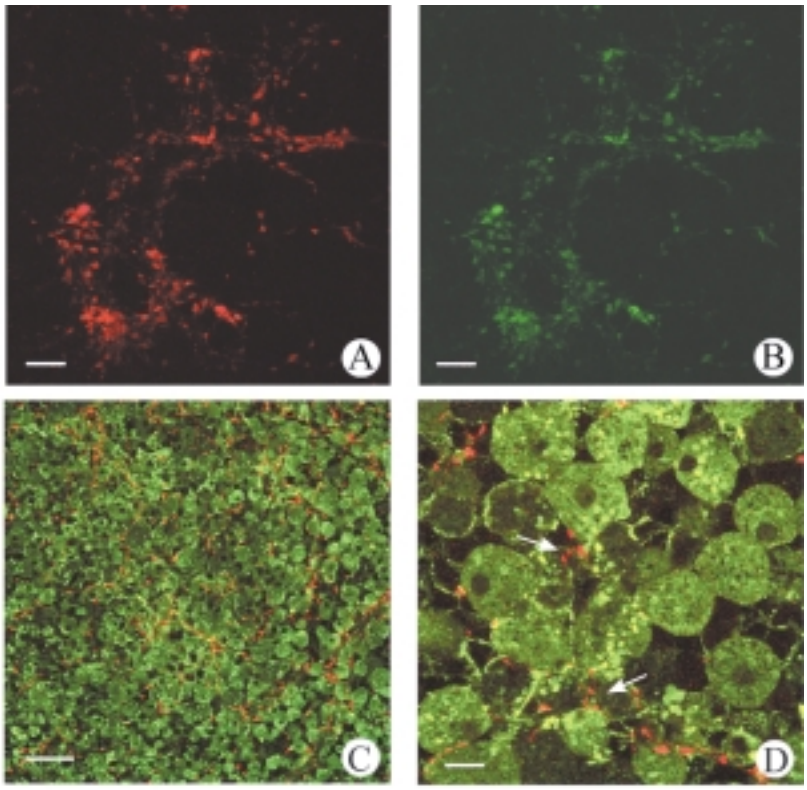


Fig. 1. CLSM images showing cfGnRH or cfGAP and LH immunoreactivity in the pituitary. **A** 20 weeks old, the cfGAP immunoreactivity (red) and **B** cfGnRH (green) immunoreactivity colocalize in the proximal pars distalis; scale bar represents 10μm. **C, D** adult pituitary with cfGAP-immunoreactivity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

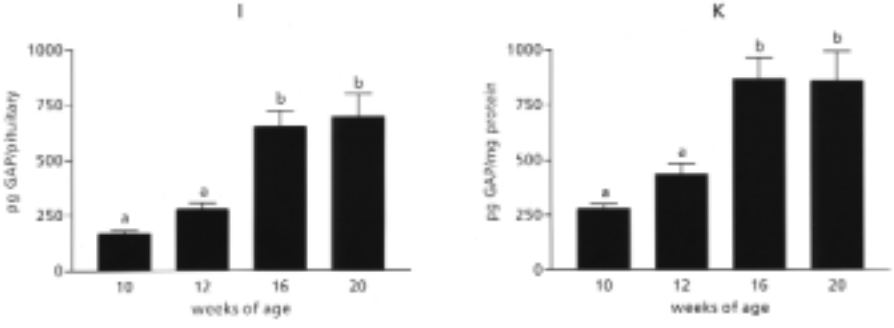
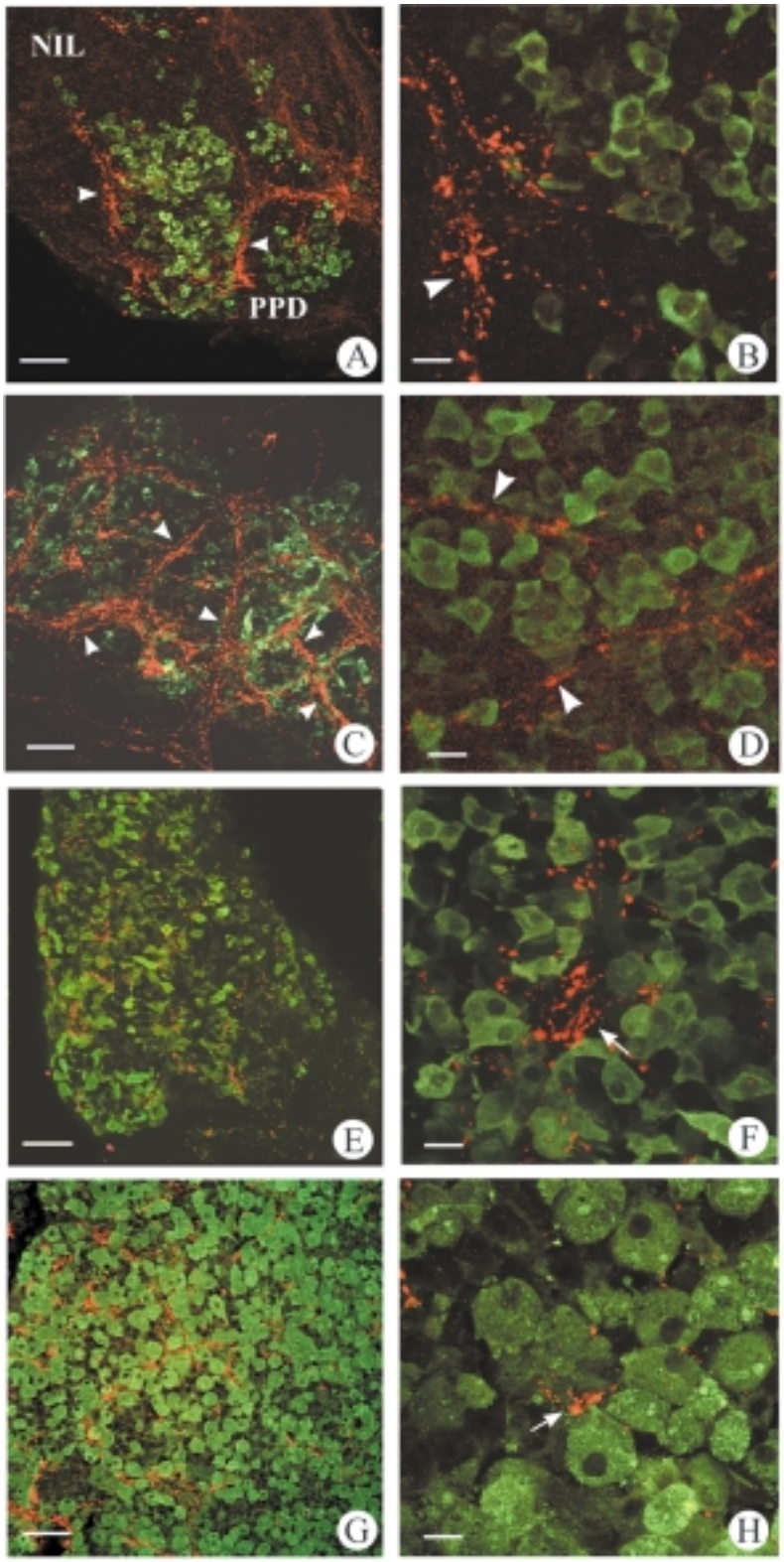


Fig. 2. Pubertal development of the cfGnRH system in the pituitary represented as CLSM images of cfGAP-ir fibers and LH-ir cells (left), and as cfGAP content of the pituitary (right) at 10, 12, 16 and 20 weeks of age. **A-H** Developmental series of cfGAP immunoreactivity (red) and LH immunoreactivity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


NIL
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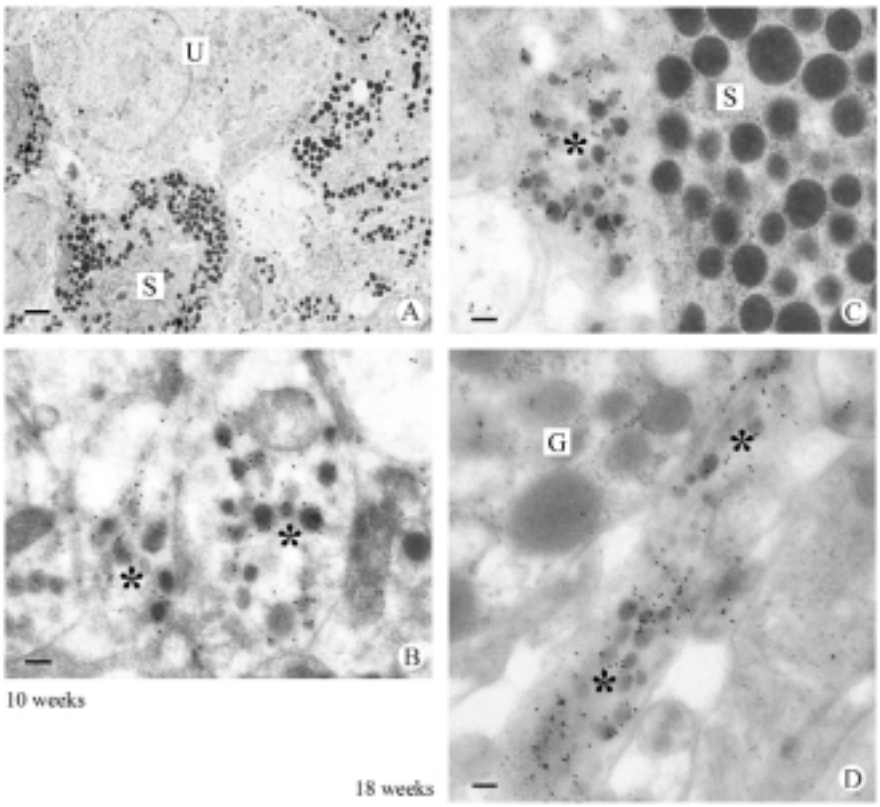

Fig. 3. Electron microscopic images of the pituitary. A Overview of the pituitary in 10-week-old African catfish, which shows somatotrophs (S) and undifferentiated cells (U). Bar 700 nm. B Two axons labeled with anti-cfGAP and 10 nm gold in the NIL of 10-week-old catfish; scale bar 90 nm. C An anti-cfGAP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

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

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

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

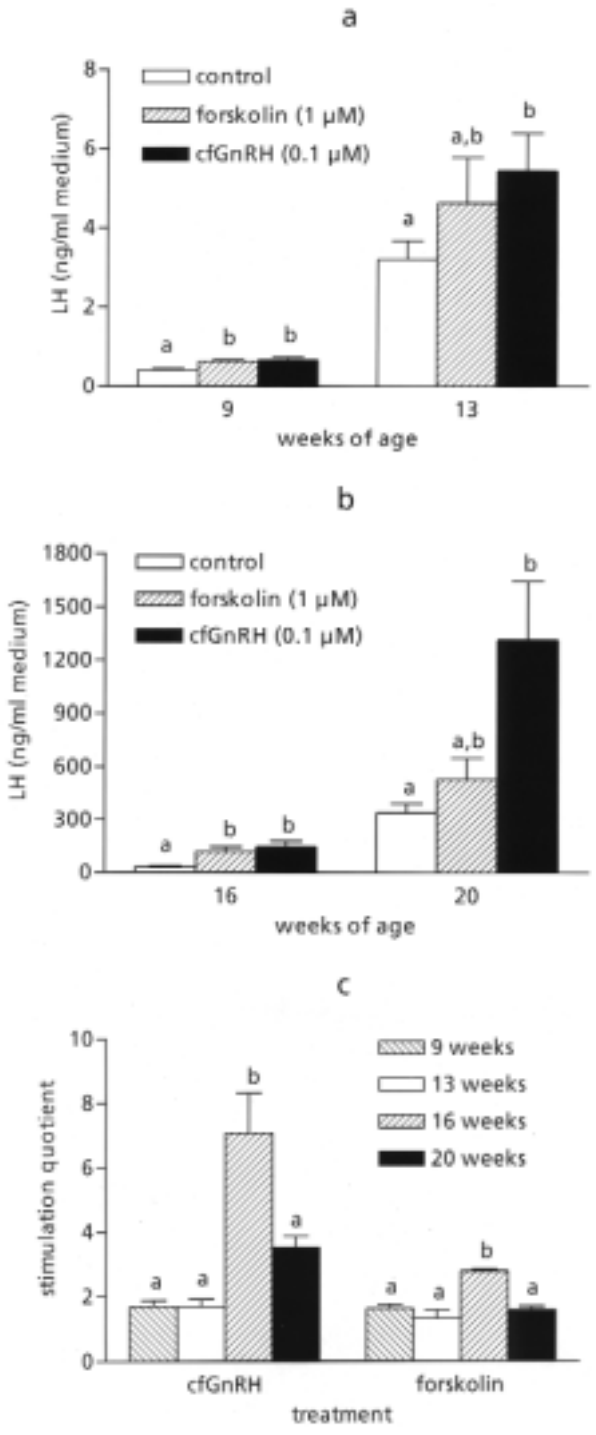

Fig. 4. ***In vitro*** **secretion of LH by pituitaries of African catfish of 9 and 13 (a) and 16 and 20 weeks of age (b). Basal LH secretion was determined after 3 h incubation in the absence of test substances (open columns; n=10-12), stimulated LH secretion was determined after another 3 h incubation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

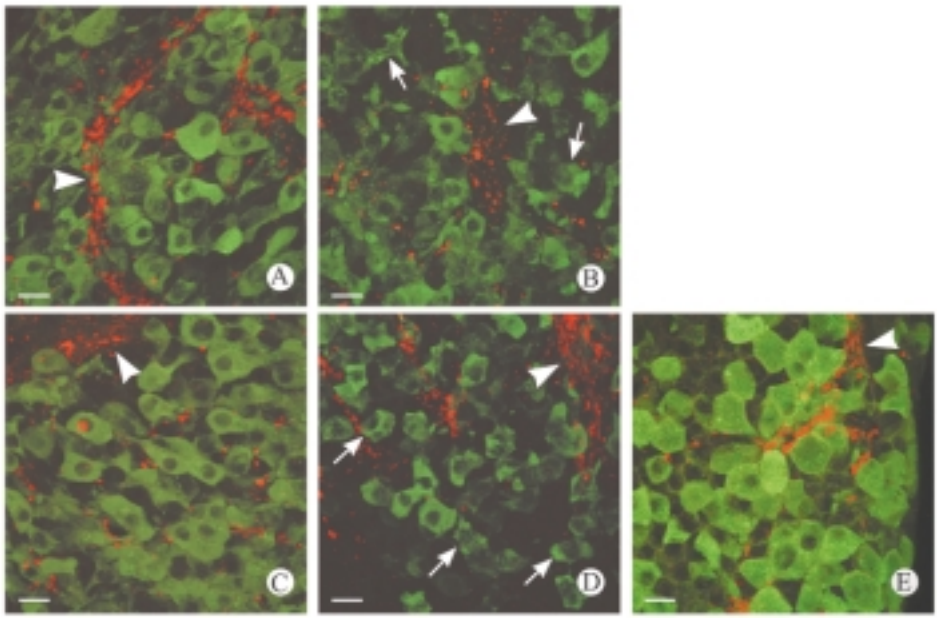

Fig. 5. CLSM images of pituitaries showing cfGAP immunoreactivity in red and LH-immunoreactivity in green. **A, B** pituitaries of shams and castrated fish at 15 weeks, i.e. 3 weeks after surgery. **C, D** pituitaries of shams and castrated fish at 17 weeks, i.e. 5 weeks after surgery and implanted with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

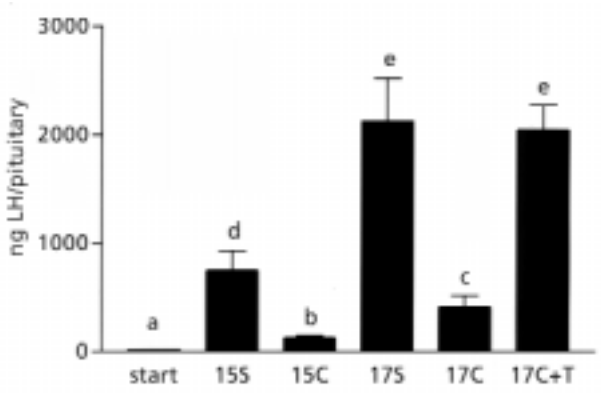

Fig. 6. LH content in sham-operated (15S, 17S), castrated (15C, 17C), and castrated and T-implanted (17C+T) fish. Bars sharing the same letter are not significantly different ($p<0.05$, ANOVA, Fisher's least significant difference test).

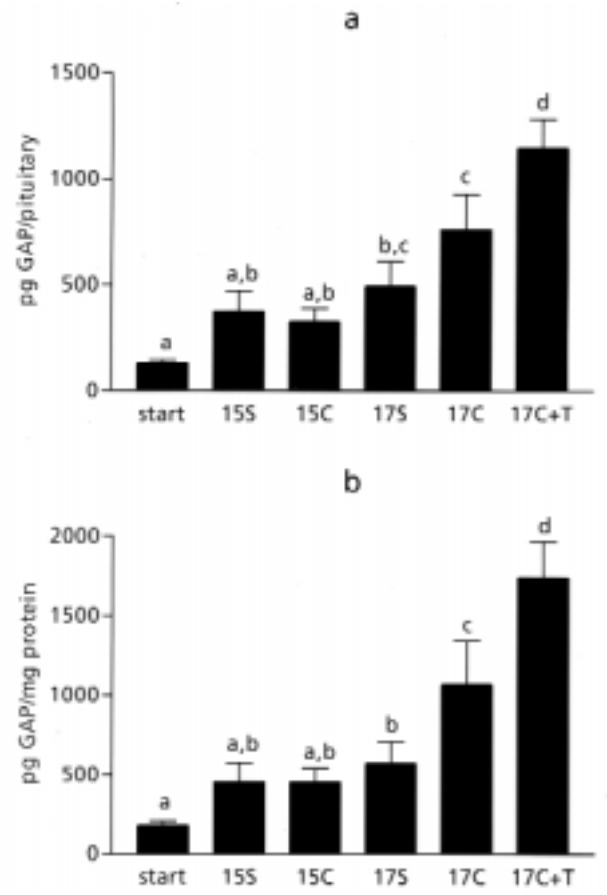

Fig. 7. GAP content in the pituitary of sham-operated (15S, 17S), castrated (15C, 17C), and castrated and testosterone-implanted (17C+T) fish. a the amount of GAP (in pg) per pituitary and b the amount of GAP (in pg) per mg protein. Bars sharing the same letter are not significantly different ($p<0.05$,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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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