
**REGULATION OF FISH GONADOTROPINS**

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Abstract

In spite of the taxonomic distance between fish and mammals, their different mode of reproduction, and the peculiar morphology of the hypothalamo-hypophyseal connection, the two classes share many similarities in gonadotropin regulation. This review describes regulatory mechanisms that have been conserved throughout vertebrate evolution from fish to mammals, and indicate those specific to bony fish (teleosts).

Although teleosts lack a hypophyseal portal system, neurohormones similar to those of mammals are carried by nerve fibers from the preoptic region to ramify within the distal pituitary. Fish possess two gonadotropins similar to FSH and LH in other vertebrates. While FSHβ of ancient fish is basically similar to that of higher vertebrates, changes which involve variation in the number of cysteins and glycosylation sites have occurred in its sequence during teleost evolution.

GnRH stimulates expression of the shared α (GPα) subunit gene and those of FSHβ and LHβ subunits as well as their secretion. The transduction pathways of its signal leading to LH release involve Ca$^{2+}$, phospholipase C (PLC), inositol trisphosphate (IP3), diacylglycerol (DAG), arachidonic acid, and protein kinase C (PKC). Reports in goldfish imply the involvement of Na$^+$, K$^+$ and nitric oxide too. In certain fish (tilapia) there is evidence for the involvement of adenylate cyclase, cAMP and protein kinase A (PKA) as an integral part of the transduction.

Dopamine (DA) acting through DA D$_2$ type receptors may inhibit LH release in many fish species (especially cyprinids). DA inhibition sites were located distal to activation of PKC or PKA, and were found in tilapia to be reversed in vitro by ionomycin or arachidonic acid. DA does not affect the release of FSH in trout.

GnRH increases the steady-state levels of GPα, LHβ and FSHβ mRNAs. PACAP38 and NPY potentiate GnRH effect on gonadotropic cells, and also act directly on the pituitary cells to co-regulate the expression of gonadotropin subunit genes. Whereas PACAP increases all three subunits, NPY regulates only GPα and LHβ mRNAs having no effect on that of FSHβ.

The effect of these peptides on the expression of the gonadotropin subunit genes is transduced differentially; GnRH regulates GPα and LHβ via PKC-ERK and PKA-ERK cascades, while affecting the FSHβ transcript through a PKA-dependent but ERK-independent cascade. The signals of both NPY and PACAP are transduced via PKC and PKA, each converging at the ERK level. However, NPY regulates only GPα and LHβ subunit genes while PACAP regulates the FSHβ subunit as well.

Like those of the mammalian counterparts, the csLHβ gene promoter is driven by a strong proximal tripartite element to which three different transcription factors bind. These include Sf-1 and Pitx-1 as in mammals, but the function of the Egr-1 appears to have been replaced by the estrogen receptor (ER).

Functional studies of the tilapia FSHβ 5’ flanking region-deletion series positioned the GnRH responsive region between (-821) to (-1211), spanning the canonical AP1 and CRE motifs. Further deletion between (-1211) to (-1065), which eliminates the AP1 motif, resulted in a partial loss of the response. This implicates the involvement of both elements, CRE as well as AP1, in conferring GnRH responsiveness of the tFSHβ promoter.

Dependent on the species, gender and maturity stage, testosterone, 11-ketotestosterone and estradiol-17β have been implicated in the regulation of gonadotropin subunit genes. High transcript levels of LHβ are associated with high levels of these steroids while those of FSHβ are reduced when exposed to high steroid levels. Gonadal or hypophyseal activin participates in the regulation of FSHβ mRNA, and in goldfish can also reduce the mRNA of LHβ.
I. Introduction

Bony fish are the most varied and certainly the most numerous among the vertebrate classes (more than 20,000 species). Early actinopterygian fish made their first appearance as an evolutionary line distinct from cartilaginous fish during the Devonian era some 400 million years ago, and the progression of true bony fish (Teleostei) has been taking place as from the Cretaceous period till present. Adaptive radiation and speciation within this group occurred throughout the Cenozoic era, at the time when the progressive radiation took place among mammals (Colbert et al., 2001).

In spite of the great taxonomic distance between these vertebrate classes, the hypothalamo-hypophyseal-gonadal axis in fish is operating in a manner similar to that in mammals. The hypothalamo-hypophyseal portal system is absent in teleost fish; nevertheless, hypothalamic nerve fibers run from the hypothalamus through the pituitary stalk discharging their neurohormones in a close proximity to the secretory cells in the distal pituitary. The various cell types in the teleost pars distalis are arranged in typical clusters that may facilitate the transfer of specific neurohormones to the respective cell type. Lactotropes occupy the main volume of the rostral pars distalis, whereas the somatotropes are arranged in palisades surrounding the finger-like ramification of the nerve fibers entering the proximal pars distalis. Cells exhibiting FSHβ mRNA in the pituitary of tilapia and salmon occupy a position adjacent to the somatotropes, whereas the cells exhibiting LHβ mRNA occupy a more peripheral position of the proximal pars distalis (Nozaki et al., 1990, Melamed et al., 1998; Elizur et al., 2000, Yaron et al., 2001; Zmora et al., 2002, Fig. 1).

Fig. 1 near here (ISH of tilapia pituitary)

Most fish are oviparous although several species exhibit viviparity; gestation can take place within the ovarian cavity or even within the ovarian follicle. Fish with synchronous ovaries may spawn only once in their lifetime (e.g., Atlantic salmon) or once in a season (carp). In these fish, the vitellogenic phase is distinct from the final maturation phase (reviewed by Yaron, 1995). The former is characterized by high levels of FSH and estradiol regulating the synthesis of vitellogenins and zona radiata proteins in the liver and their incorporation in the ovary. (Fig. 2A). In the final maturation stage, the post-vitellogenic oocytes resume meiosis under the regulation of LH, the maturation inducing progestational steroid (e.g., 17α, 20β-dihydroxy-4-pregnen-3-one) and the maturation- inducing factor (a complex consisting of cdc2-kinase and cyclin B; reviewed by Nagahama et al., 1993; Nagahama, 1997; Fig. 2B).

FIG. 2 near here (hormones involved in the vitellogenic and periovulatory phases)

In contrast, many fish posses an asynchronous (or group- synchronous) ovary containing, at any time during the breeding season, several generations of oocytes at various stages of development. Accordingly, these fish exhibit spawning cycles of various durations (e.g., 24 h in the gilthead seabream or 28 days as in tilapia). In these fish the vitellogenic stage of one oocyte generation may coincide with the final maturation of a more advanced generation making the sequence of hormone profiles more complex. It is not surprising; therefore, that such a variety in reproductive strategies is associated with considerable temporal differences in the gonadotropic hormone and sex steroid profiles.

Endocrine regulation of gonadal development in fish is further elaborated by their flexibility of sex determination and sex differentiation. A considerable number of fish species are hermaphrodites, normally changing their phenotypic sex throughout their life cycle. Some
are protandrous like the gilthead seabream (*Sparus aurata*), which begins his adult life as a phenotypic male and at the age of 3 y becomes a phenotypic female. Other hermaphrodites, such as the grouper (*Epinephelus spp.*) are protogynous, beginning their adult life as phenotypic females and become functional males after several years. Even in non-hermaphrodites (gonochoristic) fish sex differentiation is rather labile and can change naturally according to the ambient temperature, population density, or due to the presence or absence of the opposite sex. Furthermore, sex differentiation in gonochoristic fish can be easily manipulated by feeding fry with androgens for masculinization and estrogens for feminization (reviewed by Devlin and Nagahama, 2002).

In all vertebrates species studied so far, the brain produces two gonadotropin-releasing hormones, and in certain teleosts it produces up to three distinct forms. Recent molecular analyses have demonstrated three GnRH monophyletic lineages sharing sequence identities as well as characteristic expression sites in the brain (reviewed by Fernald and White, 1999; Dubois et al., 2002). The GnRH1 lineage includes the mammalian GnRH (mGnRH), chicken-I GnRH (cGnRH-I), and many novel forms found in fish, namely, sea bream GnRH (sbGnRH), catfish GnRH (cfGnRH); herring GnRH (hGnRH); medaka GnRH (mdGnRH); and dogfish GnRH (dfGnRH). The GnRH2 group encompasses the more conserved form found in all studied vertebrates, the chicken II (cGnRH-II), while the GnRH3 group contains the salmon (sGnRH) form identified only in teleost fish.

A growing body of evidence supports the direct involvement of GnRH1 family members in the control of gonadotropin secretion. Specific immunoassays developed to quantify peptide levels of each of the three GnRH forms characterizing perciform fish (i.e. cGnRH-II, sGnRH and sbGnRH), indicated that sbGnRH peptide is the most abundant form in the pituitary of sexually mature fish (Powell et al., 1994; Holland et al., 1998), and that daily surges of sbGnRH (represented by increase in sbGnRH mRNA levels) paralleled preovulatory LH secretion and synthesis in spawning seabream females (Gothilf et al., 1997). Immunocytochemical and *in situ* hybridization studies demonstrated exclusive or predominant expression of sbGnRH in the preoptic area, which innervates the pituitary [gilthead seabream (Gothilf et al., 1996); tilapia, (Parhar, 1998); European seabass (Gonzalez-Martinez et al. 2001, 2002; Zmora et al., 2002); and barfin flounder, *Verasper moseri* (Amano et al., 2002)]. sGnRH-producing cells were found to occur in the ventromedial olfactory bulbs and the terminal nerve, while cGnRH-II-producing cells occur in the midbrain tegmentum and generally have no axons running ventrally to the pituitary or to the hypophysiotropic region of the hypothalamus. In spite of some spatial overlapping found in the European sea bass (*Dicentrarchus labrax*) of sGnRH associated peptide (sGAP) and sbGAP mRNAs (Gonzalez-Martinez et al., 2001), it is generally accepted that in perciform fish only sbGnRH is directly involved in the control of gonadotropin secretion (Gothilf et al., 1996).

**II. Duality of fish gonadotropins**

### A. GtH I and GtH II

Until the mid 1980’s all the physiological functions associated with gonadotropins, namely, the control of steroid production, vitellogenesis, final oocyte maturation (FOM) in the female and spermatogenesis and spermiation in the male, were ascribed to a single gonadotropin (GtH; reviewed by Burzawa Gerard, 1982). However, this concept was changed in the late eighties of the previous century following the discovery by Kawauchi and coworkers of two distinct gonadotropins (GtH I and GtH II) in the salmon. GtH II was previously known as the common GtH (Suzuki et al, 1988 a,b,c; Kawauchi et al., 1989; reviewed by Swanson, 1991). As in the mammalian counterparts, both GtHs are heterodimeric glycoproteins, sharing an identical α subunit and distinct β subunits which confers the biological specificity of the respective GtH. Based on the similarity between the sequences of
GtH Iβ and FSH β on the one hand, and GtH IIβ and LHβ on the other hand (e.g. Quérat, 1994, 1995), a resolution was adopted at the Sixth International Symposium on the Reproductive Physiology of Fish, Bergen 1999 to use the terms FSH for fish GtH I, and LH for fish GtH II. This resolution was further corroborated by the recent extensive analysis of gonadotropin subunit in all gnathostomes (Quérat et al., 2001).

B. Isolation of two gonadotropins and cloning of subunit cDNAs

Two distinct gonadotropic peptides have been isolated so far from the chum salmon, *Oncorhynchus keta*, (Suzuki et al., 1998 a,b,c); coho salmon, *O. kisutch* (Swanson et al., 1991), Atlantic croacker, *Microgogias undulatus* (Copeland and Thomas, 1993); common carp, *Cyprinus carpio* (Van Der Kraak et al., 1992); bonito, *Katsuwonus pelamis* (Koide et al., 1993); red seabream, *Pagrus major* (Tanaka et al., 1993); tuna, *Thunnus obesus* (Okada et al., 1994), rainbow trout, *Oncorhynchus mykiss* (Prat et al., 1996), Mediterranean yellowtail, *Seriola dumerilii* (Garcia-Hernandez et al., 1997) and Atlantic halibut, *Hippoglossus hippocliluces* (Weltzien et al., submitted a). In addition, fragmented peptides with amino acids corresponding to the known subunit cDNA sequences of the mummichog (*Fundulus heteroclitus*) were prepared. Using antisera against these peptides, the native LH and FSH of the fish have been isolated (Shimizu and Yamashita, 2002).

The use of molecular biology cloning techniques extended further the evidence for gonadotropin duality in fish. Thus far, cDNA sequences encoding gonadotropin subunits were isolated and characterized from more than 19 fish species (Table I), representing seven teleostean orders (Anguilliformes, Cypriniformes, Siluriformes, Cyprinodontiformes, Salmoniformes, Perciformes and Pleuronectiformes) as well as the more ancient taxa of bony fish, the Chondrosteans, and cartilagenous fish, the Elasmobranchs. Therefore, gonadotropin duality should be considered as a ubiquitous feature among fish.

C. Evolutionary considerations of fish gonadotropin subunits

Phylogenetic analyses based on amino acid sequences of vertebrate’s gonadotropin subunits, derived from either chemically isolated peptides or deduced from cDNAs, revealed monophylegetic lineage for each subunit (Li and Ford, 1998, Quérat et al., 2000).

Among gonadotropin subunits, the sequences of GPOα-subunit show the highest degree of conservation across vertebrates. Sequence comparisons exhibited similarities ranging from 60% to 90%, largely due to the carboxyl-terminus half, which is the most conserved part of the molecule (Quérat, 1994). Nevertheless, unlike the situation in tetrapods, two types of α-subunit were identified in the pituitary of certain cyprinid and salmonid species, namely, *Cyprinus carpio* (Y. –S. Chang et al., 1988), *Carassius auratus* (Kobayashi et al., 1997), *Oncorhynchus keta* (Itoh et al., 1990) and *O. kisutch* (Dickey and Swanson, 2000). The α-subunit duplicity may be attributed to the tetraploid status exhibited by certain cyprinid and salmonid fish species (Larhammar and Riserger, 1994; Allendorf and Thorgaard, 1984, respectively).

In contrast, the gonadotropin β-subunits of fish appears to be subjected to different structural constrains during the course of evolution as compared to that of tetrapods. High degree of similarity is displayed by tetrapod FSHβ sequences (especially among mammals), while the cognate LHβ-subunits show accelerated rate of evolution, which apparently parallels the appearance of chorionic gonadotropins (Wallis, 2001). Conversely, fish FSH β-subunits are more divergent than LH β-subunits (average identities of 53% and 67%, respectively) mainly due to a rapid change which occurred during the evolution of teleosts in general, and that of
perciform species in particular (Kato et al., 1993; Hassin et al., 1995, Rosenfeld et al., unpublished). Specifically, the N-terminal of teleosts FSHβ sequences exhibits an unexpected divergence at sites that are most conserved in FSHβ of other vertebrates, namely, asparagine (N) the potential glycosylation site, and cysteine (Cys). It would appear that the common pattern of 12 Cys and 2 N-linked glycosylation sites, which characterizes all tetrapod FSHβ subunits, is conserved in dogfish and sturgeon, representing ancient fish groups (Chondrichthyes and Chondrostei, respectively), but occurs only in eels among true bony fish (Teleostei). In other teleosts, FSHβ sequences can be segregated into two groups: one consists of sequences from fish representing the superorder Ostariophysi (catfish and cyprinids) comprising 13 Cys residues. The additional Cys at the N-terminal is probably due to relocation of signal peptide cleavage site. The other consists of sequences of salmonid and perciform fish, comprising only a single N-linked glycosylation site and 12 cysteines. The second N residue and the fourth Cys residue were replaced by other amino acids due to non-synonymous substitutions (Fig. 3). Towards the development of the latter pattern, two partial repetitions through time were recognized within the FSHβ sequences of conger eels and cyprinid species, both tend to show examples of parallel evolution. It was suggested that positive selection coupled to functional constraints underlie the exclusive pattern of salmonids and perciforms FSH genes (Rosenfeld et al, unpublished).

Fig. 3. near here (Evolution of FSHβ gene – Hanna)

D. Functional considerations

1. Salmonid model for duality in gonadotropic function

Both FSH and LH are equally potent in stimulating estradiol secretion from the vitellogenic ovary of amago salmon (Oncorhynchus rhodurus) and coho salmon (Oncorhynchus kisutch), but LH is more potent in stimulating secretion of the maturation-inducing steroid (MIS; 17α, 20β-dihydroxy-4-pregnen-3-one) from postvitellogenic oocytes (Suzuki et al., 1988c, Swanson et al., 1991). In situ hybridization and immunocytochemical studies have shown that FSH producing cells are prevalent in the pituitaries of immature fish and in fish during the vitellogenic stage, while LH-producing cells become numerous towards final oocyte maturation (FOM; Naito et al., 1991). FSH, but not LH, stimulates the incorporation of vitellogenin into the ovaries of rainbow trout (Tyler et al., 1991, 1997). In coho salmon, only FSH is present in the blood of immature fish, its levels increase during the vitellogenic phase and drop during the approach to FOM and spawning (reviewed by Kawauchi et al., 1989; Swanson et al., 1991). Duality in gonadotropic function was also found in males; the sensitivity of salmon testicular tissue to the steroidogenic effect of LH increases during the progress of spermatogenesis. However, the production of MIS in response to FSH declines at later stages of spermatogenesis (Stages IV to V; Planas and Swanson, 1995). In the rainbow trout, pituitary FSHβ mRNA levels increase during early stages of gametogenesis while LHβ is weakly expressed in both males and females. FSHβ and LHβ mRNA levels increase together at later stages of gametogenesis (prespermiation and spermiation in males and around ovluation in females), with LHβ predominating (Weil et al., 1995). These results are corroborated by actual determination of FSH and LH in the circulation of the trout. FSH is present in the circulation throughout vitellogenesis, or at least early vitellogenesis, and surges again during oocyte maturation, together with the surge of LH (Prat et al., 1996; Breton et al., 1998). FSH appears in immature male trout a year before spermiation and again during the final stage of testicular growth (Prat et al., 1996).
ovulation, FSH will increase in trouts only when ovulated eggs evacuate the body cavity (Breton et al., 1998).

2. Functional gonadotropic duality in non-salmonid fish

The levels of FSHβ mRNA in the Japanese conger (Conger myriaster) peak during the primary yolk globule phase and then decrease, while those of LHβ show dramatic increase with the progression of ovarian development (Kajimura et al., 2001b). In the Japanese eel (Anguilla japonica), pituitary FSHβ mRNA levels were high in immature fish, however, when fish are injected with gonadotropin preparation (which results in gonadal development), the levels declined and remained very low until after ovulation. However, mRNA levels of LHβ in these fish were very low initially and increased markedly with ovarian maturation and ovulation. The differential expression of FSHβ and LHβ genes during induced maturation led to the suggestion that the two gonadotropins are synthesized sequentially and have separate roles relating to reproductive events in the Japanese eel (Suetake et al., 2002). Taken together, it would appear that the pattern of FSH and LH in these anguilliform fish is not much different from the salmonid model.

The mRNA levels of FSHβ in maturing male striped bass (Morone saxatilis) were elevated during early spermatogenesis, whereas those of LHβ mRNA levels peaked during spermiation. The appearance of spermatozoa in the testis was associated with a decrease in FSH mRNA and a rise in that of LH mRNA (Hassin et al., 2000).

The biological activity of FSH and LH in the common carp (Cyprinus carpio) were found to be similar in stimulating steroid secretion from the ovary and in stimulating in vitro oocyte maturation, LH being more effective than FSH (Van Der Kraak et al., 1992). A similar pattern was found in the tuna (Thunnus obesus) where both FSH and LH stimulated in vitro production of estradiol-17β and testosterone in ovarian follicles, with the response to LH being significantly greater than to FSH (Okada et al., 1994). Preliminary experiments in the red seabream (Pagrus major) showed that FSH and LH are active in two homologous bioassays: in vitro oocyte maturation and/or in vitro estradiol-17β production assays (Tanaka et al., 1993). A more detailed study indicated that although both gonadotropins were able to stimulate the production of 11-ketotestosterone from the homologous testis, in the ovaries LH but not FSH was able to induce in vitro oocyte maturation and the competence to respond to the maturation-inducing steroid (Kagawa et al., 1998).

In contrast to the situation in salmonids, with FSH predominating throughout the vitellogenic phase or throughout the cycle, and LH increasing only towards ovulation, in gilthead seabream and goldfish the mRNAs of both FSHβ and LHβ fluctuate in parallel. Both are low in immature fish, both increase in maturing fish, they are maximal in mature fish and decrease in sexually regressed fish At all phases the mRNA levels of LHβ are much higher than those of FSHβ (Elizur et al., 1995; Yoshiura et al., 1997; Sohn et al., 1999b).

The contrast between the salmonid model and the situation in gilthead seabream and goldfish was attributed to the their different mode of reproduction. Salmonids, such as trout or coho salmon, are synchronous spawners whereby all oocytes develop and ovulate simultaneously. The gilthead seabream and goldfish are multispawner; their asynchronous ovary contains several generations of oocytes, each at a different stage of development, and synthesis of both FSH and LH β subunits are most likely to be required for different stages of vitellogenesis as well as for final oocyte maturation which occur simultaneously (Gothilf et al., 1997; Yoshiura et al., 1997; Sohn et al., 1999b). A similar situation was reported in
another multispawning fish, the blue gourami (*Trichogaster trichopterus*) where the mRNA levels of FSHβ and LHβ increase in parallel during vitellogenesis (Jackson et al., 1999).

In spite of being a synchronous spawner, the LHβ gene in the black carp (*Mylopharyngodon piceus*) is already expressed in the pituitary 4 years before reaching sexual maturity, an observation that does not conform with the salmonid model (Gur et al., 2000b). Similarly, LHβ is expressed and LH is present in both the pituitary and plasma of immature common carp. Although FSHβ gene is present in carp and other fish, its functional significance can vary among fish, either in accordance to their mode of reproduction or along taxonomic lines. It is possible that in cyprinid fish some functions of the FSH are taken over by the more abundant gonadotropin, namely LH.

The increase in FSHβ transcripts in male goldfish during the breeding season was less pronounced than in females. The non-synchronous pattern of expression of the two β subunits in male goldfish was explained by the constantly high GSI in the mature stage of the testis throughout the year with only a gradual increase in androgen levels during the spawning season (Sohn et al., 1999b).

Sexual dimorphism in the expression of FSH and LH β-subunits has been reported in seabreams. In the gilthead seabream (*Sparus aurata*) absolute levels of FSHβ mRNA in pituitaries of males were higher than in females while those of LHβ were generally higher in females (Elizur et al., 1995). In the red seabream (*Pagrus major*) FSHβ mRNA levels in males increase parallel to gonadal development, whereas those of females remained low throughout sexual maturation. In contrast, LHβ mRNA levels of both sexes are maintained at high levels from the beginning of gametogenesis to spawning season (Gen et al., 2000).

### III. Regulation of gonadotropin release

In fish the hypothalamus exerts its regulation on the release of GtH via several neurohormones such as GnRH in its various forms, dopamine, PACAP, norepinephrin, neuropeptide Y, serotonin, and to a lesser extent also via nicotine, bombesin, cholecystokinin and galanine (reviewed by Van der Kraak et al., 1998). Most of the studies on the hypothalamic regulation on fish gonadotropin have focused on GnRH.

The GnRH receptor belongs to the rhodopsin/β-adrenergic-like family of G protein-coupled receptors (GPCR) that mediate their intracellular actions through the activation of one or more guanine nucleotide-binding signal transducing proteins (G proteins; Strader et al., 1995). The receptor is composed of a single polypeptide chain that traverses the lipid bilayer seven times, forming alternating extracellular and intracellular sequences oriented to form a ligand-binding domain.

The currently known receptors of GnRH (GnRH-R) can be broadly divided into two groups (Sealfon et al., 1997). The type I GnRH-Rs that do not posses C-terminal intracellular tail, have greater affinity for mammalian GnRH (GnRH I) than for other forms of the peptide and occurs in mammals. GnRH-Rs of Type II have a C-terminal tail of varying length and have greater affinity for the cGnRH-II (GnRH II); they occur in non-mammalian vertebrates (catfish, goldfish, Xenopus, striped bass, chicken etc.; Mc Ardle et al., 2002).

It has been shown that the intracellular domains of the rhodopsin/β-adrenergic-like family of GPCRs, particularly the regions closest to the plasma membrane in intracellular loops 2 (2i) and 3 (3i) as well as some specific regions located in the membrane-proximal portion of the carboxy-terminus, are important sites for G protein coupling and specificity determination (O’Dowd et al., 1988; Moro et al., 1993). Studies on the mouse and the human GnRH-R indicated that loop 2i is a critical element in determining the Gq11-mediated
transduction mechanism of this receptor (Sealfon et al., 1997). However, given the functional similarity among the members of this GPCR subfamily, it is also likely that other intracellular domains may be required for optimal signal transduction (Sealfon et al., 1997).

The following paragraph will review the available information on the ways in which these signals mediate the GnRH effect on GtH release in the fish pituitary. The signal transduction pathways leading to the release of gonadotropins in goldfish as a model has been reviewed recently in an extensive manner (Chang et al., 2000). Therefore, the following account will only summarize its main findings and add data on the situation in other fish that differs from the above model. It should be noted that thus far gonadotropin release in all studies on signal transduction in fish was measured using a single gonadotropin standard: carp GtH (cGtH) for goldfish and tilapia GtH (taGtH) for tilapia, both correspond to the respective LHs. However, for the sake of accuracy, we will use the term GtH in the section dealing with the regulation of gonadotropin release.

A. Protein kinase C and Calcium

Evidence on the involvement of protein kinase C (PKC) and Ca$^{2+}$ in the transduction of GnRH signal derived initially from studies on the pituitary of tilapia (Levavi-Sivan and Yaron, 1989) and goldfish (Chang et al., 1991). In both fish GtH release in response to PKC activators is dependent on extracellular Ca$^{2+}$, and is subjected to modulation by L-type voltage sensitive Ca$^{2+}$ channels (VSCC) (Levavi-Sivan and Yaron, 1989; Chang et al., 1996, see Fig. 4). In mammals, GnRH utilizes types α and β-II of PKC for stimulation of LH secretion from rat pituitary cells (Naor et al., 1989). However, so far there is no information about the PKC subtypes involved in GtH release or synthesis in fish.

B. Ca$^{2+}$, PLC and IP$_3$

Extracellular calcium and its influx through VSCC are essential for both the acute and prolonged GtH response to sGnRH in the murrel (Jamaluddin et al., 1989), catfish (Van-Asselt et al., 1990), tilapia (Levavi-Sivan and Yaron, 1993), and goldfish (Chang et al., 1996). The acute GtH response can be attenuated by the use of Ca$^{2+}$-deficient medium, the inorganic Ca$^{2+}$ channel blocker CoCl$_2$, or the organic Ca$^{2+}$ channel inhibitor nifedipine in all fish tested (tilapia: Levavi-Sivan and Yaron, 1989; murrel: Mukhopadhyay et al., 1995; goldfish: Jobin et al., 1996). An important element in the GnRH-induced activation of VSCC and Ca$^{2+}$ influx in the rat gonadotrope model is the Ca$^{2+}$-sensitive K+ channels (Stojilkovic and Catt, 1995). However, these channels could not be detected in goldfish gonadotropes (Van Goor et al., 1996). The long-term GtH response to cGnRH-II in this fish is more sensitive to the reduction of Ca$^{2+}$ concentration in the medium and to VSCC inhibitors than that elicited by sGnRH. Although both GnRH forms activate PLC and increase total IP$_3$ formation in dispersed pituitary cells, or in mammalian cells expressing cloned goldfish GnRH receptors, only sGnRH significantly elevates IP$_3$ levels in the native system. Surprisingly, GnRH-stimulated GtH release is not affected by pretreatment with thapsigargin, a Ca$^{2+}$/ATPase inhibitor known to interfere with replenishing IP$_3$-sensitive Ca$^{2+}$ stores in this fish (reviewed by Chang et al., 2000). Whether other thapsigargin-insensitive intracellular Ca$^{2+}$ stores are involved in mediating GnRH-stimulated GtH release requires further investigation. More information is also required to determine how these different Ca$^{2+}$ stores are regulated, and how they link to Ca$^{2+}$ entry events through the plasma membrane in fish gonadotropes. While in mammalian gonadotropes GnRH elevates [Ca$^{2+}$]i mostly by acting on internal Ca$^{2+}$ sequestering stores, in goldfish GnRH-stimulated gonadotropes rapidly increase Ca$^{2+}$ entry to enhance their [Ca$^{2+}$]i levels. (Mollard and Kah, 1996).

C. Calmodulin
In mammals, gonadotropin release is initiated by increased intracellular calcium resulting from its mobilization from intracellular stores and influx through receptor-operated channels. Increase in intracellular calcium, the presence of both calmodulin (CaM) and calmodulin binding-proteins in the pituitary, and the fact that calmodulin inhibitors abolish LH release, suggest that formation of Ca$^{2+}$-calmodulin complexes is an important intermediate step in the signaling pathway of LH release (reviewed by Waters and Conn, 1991). Extracellular addition of calmodulin enhanced the Ca$^{2+}$-dependent GtH response to GnRH in pituitary cells of the Indian murrel (*Channa punctatus*); this was totally dependent on the presence of Ca$^{2+}$. It was also shown that CaM binding proteins are present in murrel pituitary membranes (Mukhopadhyay et al., 1995). CaM and calmodulin-dependent protein kinase II (CaM kinase II) were reported to participate in mediating sGnRH and cGnRH-II effects on GtH secretion in goldfish (Chang et al., 2000). Both appear to participate in the long-term phase, but not in the acute phase of GnRH-stimulated LH release in goldfish (Jobin et al., 1996). However, murrel GnRH and Ca$^{2+}$ stimulated cellular CaM-kinase II activity within 5 min after stimulation of pituitary cells (Mukhopadhyay et al., 1997). In addition, a differential sensitivity of the actions of various forms of GnRH to modulation by CaM and CaM kinase inhibitors was reported in the goldfish, whereby cGnRH-II effects being more sensitive than sGnRH (Jobin et al., 1996).

**D. Extracellular sodium**

Extracellular Na$^+$ was reported to be involved in the acute and prolonged sGnRH and cGnRH-II stimulation of GTH-II release in goldfish. TTX-sensitive Na$^+$ channels, although present in goldfish gonadotropes, are not involved in mediating GtH release responses, but amiloride-sensitive Na$^+$/H$^+$ antiports are required for these activities (reviewed by Chang et al., 2000).

**E. Arachidonic acid**

Mobilization of arachidonic acid (AA) via the activation of phospholipase A$_2$ (PLA$_2$) is an important step in GnRH-stimulated GtH release in both goldfish (Chang et al., 1996) and tilapia (Levavi-Sivan and Yaron, 1993; Levavi-Sivan et al., 1995, Fig. 4). In goldfish, the diacylglycerol (DAG) lipase inhibitor, U-57908, did not decrease sGnRH- and cGnRH-II-induced GtH secretion. On the other hand, the PLA$_2$ inhibitors, bromophenacyl bromide (BPB), chloroquine, and quinacrine, reduced sGnRH-elicited, but not cGnRH-II-stimulated release. The addition of AA reversed the inhibitory action of BPB on sGnRH-elicited release. sGnRH-induced AA mobilization probably involves activation of PLA$_2$ but not DAG lipase. These results also support the hypothesis that AA signaling component is much less important in mediating the long-term cGnRH-II-stimulated GtH secretion, as compared to sGnRH-elicited release (Chang et al., 1994).

**F. Nitric oxide**

The presence of nitric oxide synthase (NOS)-like immunoreactivity has been demonstrated in goldfish gonadotropes by immunocytochemistry (Chang et al., 2000). Addition of NO donor and treatment with cGMP analog increases GtH secretion from goldfish pituitary cells. It was concluded that the NO/cGMP pathway interacts with other second messenger systems in the control of GtH release. The specific nature of such interaction is not clear yet.

**G. Cyclic AMP**
It is well established that most of the biological actions of GnRH in mammalian pituitary are mediated by Gq/11-coupled pathways, leading to elevation of IP$_3$, DAG and intracellular Ca$^{2+}$ (Naor, 1990). Nevertheless, several studies have shown that GnRH stimulates cAMP production in mixed pituitary cell cultures concomitantly with increasing the release of gonadotropins, suggesting that cAMP signaling might also mediate physiological responses in the gonadotropes (Bourne and Baldwin, 1987; van der Merwe et al., 1994). Furthermore, expression of mammalian GnRH receptor on GH3 (Kuphal et al., 1994), COS-7 (Arora et al., 1998) or CHO (Nelson et al., 1999) cell lines, demonstrated that GnRHa induced release of cAMP. This concept has been corroborated by the findings that the increased level of cAMP was achieved through activation of Gs protein and adenylate cyclase (AC; Liu et al., 2002) and that the highest abundance of Gs$\alpha$ protein is in the pituitary gonadotropes (Wilson et al., 1994).

In tilapia too the addition of forskolin or dbcAMP to pituitary fragments or dispersed pituitary cells was followed by an increase in GtH release. Moreover, addition of GnRH to the medium resulted in increased cAMP levels in primary culture of dispersed tilapia pituitary cells concomitantly with augmented GtH release (Levavi-Sivan and Yaron, 1992; Fig. 4). Furthermore, the PKA inhibitor H89 significantly reduced the GnRH-stimulated LH release from primary culture of tilapia pituitary cells, albeit inhibition of PKC in the same experiment was much more effective in reducing LH release (Melamed et al., 1996). In contrast, GnRH-stimulated LH release in the African catfish was not associated with elevated cAMP levels, and forskolin had no effect on GtH release in primary culture of pituitary cells. However, when pituitary fragments of this fish were used, cAMP was elevated by GnRH (Rebers et al., 2000a). It is important to note that in 293T cells, transiently transfected with catfish GnRH receptor, the administration of GnRH resulted in increased concentrations of cAMP, that were abolished in the presence of GnRH antagonist (Blomenröhr et al., 1997).

Despite the fact that stimulation of the cAMP-dependent cascade can lead to GtH release, cAMP is not directly mediating sGnRH or cGnRH-II stimulation of GtH release in goldfish. sGnRH and cGnRH-II stimulation of the release were not accompanied by elevations in cAMP production (Chang et al., 1992). Moreover, blockade of PKA with H89 had no significant effects on GtH response; 8-Br-cAMP- or forskolin-elicited GtH release were at least additive to the responses elicited after activation of PKC or AA (Chang et al., 1996). In perforated-patch, voltage-clamp studies, cAMP increased the magnitude of Ca$^{2+}$ currents in identified goldfish gonadotropes (Chang et al., 2000).

Another view on the possible role of cAMP is reported in the murrel where a clear correlation was found between cAMP level, Ca$^{2+}$, CaM and phosphodiesterase (PDE) activity. The removal of Ca$^{2+}$ from the incubation medium drastically reduced the stimulatory effect of GnRH on cAMP (Mukhopadhyay et al., 1995). It was suggested that a rise in Ca$^{2+}$ results in an increase in CaM, which, in turn, stimulates phosphodiesterase activity thus augmenting the degradation of cAMP (Mukhopadhyay et al., 1997).

H. Dopaminergic inhibition of GtH release

Dopamine (DA) inhibits basal and GnRH induced GtH release in a number of fish species both in vivo and in vitro (Chang et al., 1984a,b). The DA inhibition is especially significant in cyprinid fish. Spawning induction in cyprinid fish in aquaculture requires the use of DA D$_2$ antagonists (pimozide, domperidone or metoclopramide) to promote a significant surge of LH that will lead to successful ovulation (reviewed by Yaron, 1995). In rainbow trout too treatment with pimozide or $\alpha$-methyl-p-tyrosine (MPT), an inhibitor of catecholamine synthesis, increased blood LH levels of sham-operated vitellogenic fish.
Nevertheless, these drugs were not able to significantly change blood FSH levels in these fish (Saligaut et al., 1998).

Dopaminergic inhibition of basal and GnRH-stimulated LH release is mediated by dopamine D$_2$ receptors. A long-term action of DA is the reduction the number of GnRH receptors in the pituitary (De Leeuw et al., 1984; reviewed by Van Der Kraak et al., 1998) and to inhibit GnRH release (Peter et al., 1991). The acute direct effect of DA inhibition of GtH release was traced along several transduction cascades in tilapia (Fig. 4). DA was found to reduce both basal and GnRH$\alpha$-stimulated GtH release from perfused pituitary fragments, without altering the formation of cAMP. DA was found to inhibit GtH responses to forskolin and cAMP analogs tilapia as well as in goldfish pituitary cells. It was concluded that dopaminergic inhibition is located at a site distal to the formation of cAMP in both tilapia and goldfish (Levavi-Sivan et al., 1995; Chang et al., 2000).

Fig. 4. near here (Sites of dopaminergic inhibition)

Activating PKC either by DiC8 or TPA could not reverse the inhibition of GtH release from cultured of tilapia pituitary cells by specific DA D$_2$ agonists. This indicated a site for DA action distal to PKC. However, the stimulatory effect of arachidonic acid on the release in perfusion and culture was not reduced by DA or by its agonists, which suggests a site for DA action proximal to AA formation (Levavi-Sivan et al., 1995). In goldfish DA D$_2$ agonists have been shown to attenuate the GnRH-induced elevation of IP levels and [Ca$^{2+}$] in pituitary cells. The D$_2$ inhibition of GtH release in goldfish is reversed by the ionophore A23187 (Chang et al., 1993) while in tilapia the release is reversed by the ionophore, ionomycin (capable of releasing Ca$^{2+}$ from intracellular sources) but not by A23187 that causes Ca$^{2+}$ influx. This would indicate that although DA may inhibit Ca$^{2+}$ influx, it does not affect GtH release in response to intracellular mobilization of the ion (Levavi-Sivan et al., 1995, Fig. 4). In both goldfish and tilapia pituitary cells, the PKC-stimulated GtH release is reduced by DA while the AA-stimulated release is reversed (Chang et al., 1992, 1993, 2000; Levavi-Sivan et al., 1995). Moreover, DA decreased the magnitude and slowed the activation kinetics of ionic current trough VSCC in identified goldfish gonadotropes (Van Goor et al., 1998).

In spite of its strong inhibitory effect on GtH release, dopamine does not interfere with the stimulatory effect of GnRH on LH$\beta$ gene expression in tilapia (Melamed et al., 1996).

Most of the research on the signal transduction of GnRH toward GtH release was conducted on goldfish and tilapia. Evidently, reports on the transduction of the GnRH signal and DA sites of inhibition differ in goldfish and in tilapia. It is unlikely that these differences emerge from reproductive differences in these fish since both are asynchronous spawners. It is possible that these differences emerge from variance between the species belonging to different orders (Cypriniformes and Perciformes), or perhaps due to different methodologies utilized. Evidently further research is still required on these topics to clarify the above differences. Furthermore, the transduction of GnRH signal affecting the release of FSH is a topic that has been held back hitherto by the lack of appropriate FSH$\beta$ standards and antisera. Evidently, this topic requires urgent attention.

I. $\gamma$-aminobutyric acid (GABA)

GABA-containing nerve fibers originating from the preoptic region or from the nucleus lateralis tuberis in the hypothalamus reach close proximity to gonadotropic cells in the pituitary of goldfish. Furthermore, injection of GABA results in increased release of LH in regressed or early maturing fish. GABA or its agonists can stimulate LH release from
perifused pituitary slices (containing intact nerve endings) but not from dispersed pituitary cells. It was suggested, therefore, that GABA's major effect is stimulating the release of GnRH from nerve endings in the pituitary thus indirectly affect the gonadotropes (Kah et al., 1992, 1993). However, it has been suggested that GABA can act directly on the gonadotrophs too by elevating intracellular Ca\(^{2+}\) (Käh et al., 1993).

Immunohistochemical studies showing the presence of glutamate decarboxylase-positive fibers in the neurohypophyseal digitations adjacent to the gonadotropic cells provided morphological clue for the possibility that this amino acid is capable of interacting with the gonadotropes of rainbow trout too. Indeed, experiments \textit{in vivo} and \textit{in vitro} showed that GABA has an overall stimulatory effect on the secretion of FSH and LH in this fish. The extent of the stimulation was found to be dependent on the gender and reproductive stage of the fish and was more obvious in facilitating the effect of GnRH (Mañanós et al., 1999).

Also in the catfish, \textit{Heteropneustes fossilis} GABA was found to stimulate LH secretion but this effect could be seen only in the prespawning phase and in fish treated with GnRH, or with pimozide to reduce dopaminergic inhibition of (Joy et al., 1999).

**Effects of gonadal steroids**

Castrated Atlantic salmon (\textit{Salmo salar}) male parr showed a decrease in pituitary and plasma FSH and LH indicating positive testicular feedback on both FSH and LH. 11-ketoandrostenedione (1-KT) showed an increase in plasma and pituitary FSH levels, whereas testosterone (T) implants suppressed FSH in July, prior to the spawning season, and stimulated it in November, at the end of the spawning season (Borg et al., 1998). Implanting T-containing capsules into post-breeding Atlantic salmon prevented the normal decline in plasma LH levels without affecting those of FSH (Antonopoulou et al., 1999a). These results demonstrate clearly that the effect of steroids on FSH can be positive or negative dependent on the reproductive phase. FSH plasma levels in coho salmon decreased in response to T and E2 administration, while the progestogen 17α, 20β-dihydroxy-4-pregnen-3-one was ineffective. At the age and reproductive phase examined LH was undetectable in the circulation. (Dickey and Swanson, 1998).

Implantation of T, although not affecting basal LH levels, did potentiate LH release in response to GnRH superactive analog in sexually recrudescent and preovulatory female common carp (\textit{Cyprinus carpio}) and Chinese loach (\textit{Paramisgurnus dabryanus}). These findings demonstrate that T, but not estradiol, can increase pituitary responsiveness to exogenous GnRH in sexually recrudescent and sexually mature female carp and loach (Trudeau et al., 1991). These results are corroborated by experiments on isolated pituitary cells. Overnight exposure to T of primary culture pituitary cells taken from female goldfish at all reproductive phases did not affect basal LH release, but increased its response to 30 min pulses of sGnRH or cGnRH II. These results indicate a direct positive influence of T on GnRH-stimulated LH release (Lo and Chang, 1998). As T is known to stimulate the expression of LHβ gene in goldfish (e.g. Khakoo et al., 1994; Sohn et al., 1998b) and the accumulation of LH in the pituitary in trout (Breton et al., 1997), it is possible that the augmented response to GnRH merely reflects the increased size of LH pool stored in the pituitary cells. It is worth noting that the lack of estradiol potency in these experiments are in contrast to the findings in many other fish that the administered androgen is aromatized in the treated fish to estradiol and that fish gonadotrophs may possess aromatizing capability (Melamed et al., 2000).

Nevertheless, not all fish can aromatize the exogenous T, as seen in the protandrous
black porgy (Acanthopagrus schlegeli) where plasma LH was similar in control and T injected fish whereas E2-injected fish had a significantly higher LH level in the circulation (Lee et al., 1999; Du et al., 2001).

As gonadotropin release is dependent on the amount of hormone produced and stored in the gonadotrophs, the effect of steroids on gonadotropins is further elaborated in section IV C (Effect of steroid hormones on GtH subunit expression).

### IV. Regulation of gonadotropin subunit genes

#### A. Effect of hypothalamic peptides on GtH subunit mRNA

1. GnRH

   a. Physiological consideration

      In sexually regressed goldfish, sGnRH treatment increased the mRNA levels of Gπα and while the same dose of cGnRH-II treatment was without effect. However, in sexually mature fish both cGnRH-II and sGnRH stimulated the expression of the two subunits genes, with cGnRH-II exerting a greater effect on LHβ mRNA indicating the dependence of the response to GnRH on gonadal stage and the greater potency of cGnRH II (Khakoo et al., 1994). In a subsequent study cGnRH II was found to be more effective than sGnRH on FSHβ expression as well (Klausen et al., 2001).

      A direct effect of sGnRH on LHβ mRNA was demonstrated in cultured pituitary cells of tilapia hybrids (Oreochromis niloticus x O. aureus). A time-response study indicated an initial increase in the transcripts 12 h after exposure to the neuropeptide and further elevation occurred after 18 and 24 h (Melamed et al., 1996). In a subsequent study in tilapia, sGnRH led after 24 h, to a 3.7 increase in Gπα mRNA, 2.7-fold in LHβ mRNA and only 1.7 folds in that of FSHβ (Gur et al., 2000a).

      These results are in line with the finding in the striped bass (Morone saxatilis) in which the response of Gπα and LHβ mRNAs of maturing males to GnRH implantation was higher than that of FSHβ (Hassin et al., 1998), and with the results in goldfish where the response of LHβ mRNA was, in general, higher than that of FSHβ either in vivo, in perfusion of pituitary fragments or in culture of dispersed pituitary cells (Klausen et al., 2001). Similarly, implanting GnRHa in pre-spawning homing sockeye salmon resulted in increased mRNA levels of Gπα and LHβ but not those of FSHβ (Kitahashi et al., 1998). Apparently, opposite results were obtained in pituitary cell culture of two year-old coho salmon (Oncorhynchus kisutch) where exposure to sGnRH resulted in increased gene and protein expression of the two Gπα subunits (α1 and α2) but not in that of LHβ. It was assumed that the donor fish were at a stage too early for LH production, which normally appears only when fish approach spawning (Dickey and Swanson, 2000) as was seen in the case of the pre-spawning sockeye salmon.

      A study was designed in our laboratory to reveal whether gonadotropic response to sGnRH in the common carp (Cyprinus carpio) changes during sexual ontogeny, and whether the responses of FSHβ and LHβ subunits are uniform or differential. Fish were injected with sGnRHa (25 µg/kg) and were sampled 6, 12 or 24 h later. Juvenile fish did not respond at all to sGnRHa. In maturing females, FSHβ mRNA increased three-fold while that of LHβ increased twofold. In maturing males FSHβ mRNA did not change, and only a slight increase occurred in that of LHβ. In postvitellogenic mature females there was no response of FSHβ mRNA while that of LHβ increased dramatically. In spermatizing males at the same age mRNA of both FSHβ and LHβ increased following sGnRHa injection. Immunoreactive LH was present in the pituitary and plasma of all fish examined but in juvenile fish it did not change following sGnRH
injection. In maturing and mature fish sGnRHa administration to both genders was followed by a marked increase in circulating LH concomitant with a decrease in its pituitary content, indicating the limited amount of the hormone stored in the gland. It was concluded that the response of gonadotropin subunit mRNAs in the common carp is differential depending on the gender and the phase of sexual ontogeny (Kandel-Kfir et al., 2002).

In the goldfish too, differential regulation of GtH subunits by GnRH was reported. Pituitary cells of immature fish did not respond at all to the peptide, in pituitary cells of mature fish only FSHβ mRNA increased in response to the peptide while in those of regressed fish FSHβ mRNA decreased and LHβ mRNA showed no response (Sohn et al., 2001).

Differential regulation of GtH subunits was reported in the European sea bass too where injection of GnRHa resulted in increased mRNA levels of both GPα and LHβ in fish previously primed with sex steroids while no such effect could be seen on that of FSHβ (Mateos et al. 2002). The lack of FSHβ response to GnRH in the European sea bass is somewhat surprising since other perciform fish (e.g., striped bass and tilapia) do increase their FSHβ mRNA in response to GnRH. This insensitivity could be due to the early phase in the sexual ontogeny of the experimental sea bass which were three years old but still prepubertal.

In the African catfish (Clarias gariepinus) injection of catfish (cf)GnRH or cGnRH II resulted in increased levels of GPα and LHβ mRNAs 8 h after injection, following an initial drop in the expression levels at 2 h. However, in contrast to the foregoing information, none of the GnRHs could stimulate the transcription of the subunit mRNAs when given directly to pituitary cells in culture. Furthermore, higher concentrations of cfGnRH resulted in a decrease in the transcript level. As castrated fish did not respond to GnRH in vivo, it was concluded that although these GnRHs stimulated LH release in the catfish, they had no direct effect on GPα and LHβ mRNAs and that the effect seen in vivo is due to a surge in gonadal steroids such as 11-ketotestosterone that had been shown to stimulate LHβ subunit gene transcription (Rebers et al., 2002).

The foregoing account does not allow final conclusions as to the differential response of GtH subunits to GnRH. Information on additional fish species and exact gonadal phase is still needed. Nevertheless, it would appear that the type of the GtH subunit gene preferentially expressed in response to GnRH is dependent on the gender and reproductive phase of the fish. Steroids secreted by the gonads are likely to play an important role in the manifold response to GnRH, while temporal distribution and levels of GnRH-receptors and their transduction pathways too take part in this differential response.

b. GnRH signal transduction

The signaling leading to nuclear effects of G-protein-coupled receptors (GPCRs), such as the GnRH receptor, is conveyed by a family of cytosolic protein kinases collectively known as the mitogen-activated protein kinase (MAPK) cascades (Seger and Krebs, 1995; Kraus et al., 2001). Members of this family include two forms of the extracellular signal-regulated kinase (ERK1/2), the jun N-terminal kinases (JNK1/2/3), the p38MAPK and the big MAPK (ERK5). The ability of the activated MAPKs to translocate to the nucleus and trigger gene expression enables the formation of a signaling pathway leading from the receptor on the cell membrane to the nucleus (reviewed by Shacham et al., 2001; Naor et al., 2000). The ubiquitous MAPK family is involved in various cellular functions such as cell growth, differentiation, transformation, cell cycle and apoptosis. In mammals, several pathways have been characterized connecting G-protein coupled receptors (GPCRs) to MAPK activation (reviewed by Kraus et al., 2001).

In pituitary-derived cells, GnRH was found to activate, to various extents, all four MAPK cascades by a protein kinase C (PKC)- and tyrosine-kinase dependent mechanisms (Naor et al., 2000). The activation of ERK (p42 and p44 MAPKs) by GnRH has been studied as a model system for GnRH signaling in the pituitary and was reported to be involved in
GnRH-induced gonadotropin gene expression in mammals (Roberson et al., 1995; Reiss et al., 1997; Naor et al., 2000). GnRH was found to stimulate ERK1/2 in the rat pituitary, and was implicated in the α-subunit gene expression (Reiss et al., 1997; Naor et al., 2000). The GnRH activation of salmon LHβ promoter transected into αT3-1 cells could be totally suppressed in the presence of MEK inhibitor PD-098059 (PD; Ando et al., 2001). Ca^{2+}, PKC and tyrosine kinase too were found to mediate GnRH-induced ERK activation, with PKC being both essential and sufficient for this function (Reiss et al., 1997). It was further suggested that most of GnRH signaling toward ERK is mediated via the protein tyrosine kinase (PTK)/Ras-independent pathway, which could also include direct activation of Raf1 by PKC. GnRH-induced ERK activation also requires Ras activation via PKC, Src and transactivation of EGF receptor (Grosse et al., 2000). Following this paradigm, it was hypothesized that GnRH stimulation of gonadotropin subunit gene expression in fish will also involve the MAPK cascade. Indeed, exposure of tilapia pituitary cells to sGnRH elevated the phosphorylated levels of ERK (pERK). This effect was found to involve PKC and PKA both are known to mediate GnRH-induced GtH subunit gene expression too (Melamed et al., 1996; Gur et al., 2001b; reviewed by Yaron et al., 2001). Nevertheless, inhibition of PKC by GF109203X (GF) or that of MAPK kinase (MEK) by PD, although suppressing the ERK levels and GPα and LHβ transcript levels, did not affect the mRNA level of FSHβ. These results indicate that GnRH regulates GPα and LHβ transcription in tilapia via PKC and PKA, both converging at the ERK level, whereas the regulation of FSHβ transcription by GnRH is carried out via PKA-dependent, but PKC and ERK-independent pathways (Gur et al., 2002a; Figs. 5a,b and 7). These results differ from the situation in mammalian models where PD at a similar concentration (50 μM) was able to block the mRNA response of FSHβ and GPα to GnRH given in pulses to pituitary cells of male rats (Haisenleder et al., 1998). Moreover, in the mammalian LβT2 cell line, inhibition of MAPK by U0126 abolished the induction of ovine FSHβ promoter by GnRH but not by the PKC stimulator TPA, suggesting that GnRH and TPA induce the activity of the oFSHβ promoter through different, although possibly overlapping, pools of PKC isoforms (Vasilyev et al., 2002).

The foregoing comparison portrays the divergence between certain mammalian models and the regulation of the teleost gonadotropin subunits through shared transduction systems. The uncoupling of cAMP-PKA and MAPK pathways in mediating GnRH-induced FSHβ gene expression in tilapia, in contrast to their involvement in the expression of the GPα and LHβ genes, illustrates a mechanism by which the differential regulation of gonadotropin subunits is achieved utilizing alternative transduction pathways. PKC-ERK and PKA-ERK cascades operate in elevating GPα and LHβ mRNAs, whereas induction of FSHβ transcription is PKC- and/or ERK-independent being directly regulated through cAMP-PKA (Gur et al., 2001b; 2002a). It is possible that GnRH action on FSHβ gene expression in the fish is exerted directly via PKA-CREB-CRE pathway. A cAMP response element (CRE) was detected together with multiple AP1 sites on the 5’ flanking region (FR) of the FSHβ gene of tilapia (Fig. 5b and 7b). Furthermore, functional analysis of the promoter revealed that the CRE motif, positioned at −1062 bp upstream from the start site, may play a central role in its expression by regulating the tilapia FSHβ gene in a positive manner or negative manners (Rosenfeld et al., 2001 and unpublished). Nevertheless, these results do not exclude possible involvement of GF-insensitive PKC isoforms, or the possibility that MAPK cascades other than MEK-ERK (e.g. JNK, P38 and BMK) mediate FSHβ transcription. Indeed, in the LβT2 cells, GnRH differentially activates the ERK and JNK cascades, with JNK being essential for rat LHβ promoter activity (Yokoi et al., 2000; Harris et al., 2002).
Apart from GnRH, which is the primary and best-known regulator of gonadotrope activity, the pituitary adenylate cyclase-activating polypeptide (PACAP) and neuropeptide Y (NPY) are of particular importance among the hypothalamic peptides. Both were found to act as gonadotropin secretagogues in mammals (e.g. Kalra and Crowley, 1992; Rawling and Hezareh, 1996; Sherwood et al., 2000) and in teleosts (Peng et al., 1993a; Wong et al., 2000; Chang et al., 2001). Although conveyed directly by hypothalamic nerve fibers, the main effects ascribed to these peptides in fish are GnRH release and GnRH-induced LH release, while evidence for their direct effects on the pituitary cells is scarce (reviewed by Evans, 1999 and Sherwood et al., 2000).

2. Pituitary Adenylate Cyclase Activating Peptide (PACAP)

a. Physiological considerations

PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family (Miyata et al., 1989, 1990). Two biologically potent forms of PACAP were found in mammals: a 38 amino acid form (PACAP38) and a shorter form (PACAP27) (Miyata et al., 1990). Due to the presence of two PACAP receptor types (PAC1 and VPAC2) in mammalian gonadotropes, PACAP has the potential to regulate several intracellular signaling mechanisms, i.e., PLC, Ca\(^{2+}\) and cAMP-PKA (reviewed by Sherwood et al., 2000; and Vaudry et al., 2000). In the rat gonadotropes, PACAP alters gonadotropin mRNA levels and the release of LH, FSH and \(\alpha\)-subunit, probably via the PAC1 receptor type (Tsujii et al., 1994, 1995). An indirect inhibitory effect of PACAP on FSH\(\beta\) mRNA by increasing the expression of follistatin was reported in \(\alpha\)T3-1 cell line (Winters et al., 1997). However, the most studied function of PACAP in fish is the stimulation of growth hormone (GH) release as demonstrated in trout (Parker et al., 1997), goldfish (Wong et al., 1998), European eel (Montero et al., 1998) and turbot (Rousseau et al., 2001). It is considered as the main GH releasing hormone (GHRH) at least in cyprinids and the turbot (Wong et al., 2000; Rousseau et al., 2001, respectively).

In goldfish, the GH-releasing action of PACAP is mediated via pituitary PAC1 receptors coupled to the adenylate cyclase-cAMP- protein kinase A and phospholipase C -IP3 -protein kinase C pathways. It was suggested that increased Ca\(^{2+}\) influx and activation of Ca\(^{2+}\)-calmodulin protein kinase II are involved too. In addition to its effect on GH release, PACAP was also found to modestly stimulate the release of LH and to augment the release of LH in response to GnRH, its signal being transduced through cAMP-PKA cascade (Chang et al., 2001).

The effect of PACAP on gonadotropin subunit gene expression has been studied in tilapia. Pituitary cells exposed to PACAP38 showed an increase in the mRNA levels of GP\(\alpha\), FSH\(\beta\) and LH\(\beta\) subunits. As these effects could be suppressed by either the PKA inhibitor H89 or the PKC inhibitor GF109201X (GF), they appear to be mediated via both PKA and PKC pathways (Fig. 5a,b; Gur et al., 2002b). PACAP38 also augmented the transcription-response of GP\(\alpha\) to GnRH (Gur et al., 2001a), which is in line with the results showing that this peptide can interact with GnRH in stimulating release of both gonadotropins from rat anterior pituitary cell culture (Burrin et al., 1998) and from the goldfish pituitary (Chang et al., 2001). Nevertheless, the signaling pathways of PACAP downstream from PKA and PKC, in conjunction with the expression of gonadotropin subunit genes in either mammals or fish, needed further elucidation.

b. PACAP signal transduction

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PACAP38 (0.001-10 nM) was found to increase the levels of pERK in primary culture of pituitary cells and the mRNA levels of GPα, FSHβ and LHβ. Furthermore, a decrease in pERK levels was noted in the presence of graded concentration of H89, GF, or the MAPK kinase (MEK) inhibitor PD. The suppression of these kinases was associated with a decrease in the mRNA levels of GPα, FSHβ and LHβ subunits (Gur et al., 2002b). These results do not conform with the effect of PACAP (100 nM) on mammalian pituitary cell line αT3-1 where the resulting activation of ERK or its inhibition are associated with DNA synthesis and cell proliferation rather then with transcriptional activation of human αGSU- luciferase reporter construct (Fowkes et al., 2001). This apparent discrepancy between PACAP effects in tilapia and mammalian cell line can be attributed to species as well as nature of cells. It may also point to the involvement of other signaling pathways, not yet examined, in PACAP’s effects on transcription as opposed to those on expression.

3. Neuropeptide Y (NPY)

a. Physiological considerations

NPY belongs to a pancreatic polypeptide family. Its structure in fish was deduced and characterized by peptide purification or cDNA cloning from neural tissue in the rainbow trout, Oncorhynchus mykiss, cod, Gadus morhua (Jensen and Conlon, 1992), goldfish, Carassius auratus (Blomqvist et al., 1992), and sea bass, Dicentrarchus labrax (Cerdá-Reverter et al., 1998), or by genomic DNA in the zebrafish (Cerdá-Reverter and Larhammar, 2000). NPY producing sites in the goldfish were located mainly in the ventral telencephalon, preoptic area, olfactory bulbs, and various thalamic regions, as well as in the hypothalamus, optic tectum and locus coeruleus (Peng et al., 1994). NPY immunoreactivity was detected also in the pituitary of platyfish, Xiphophorus maculatus (Magliulo-Cepriano and Schreibman, 1993), ayu, Plecoglossus altivelis (Chiba et al., 1996), lungfish, Protopterus annectens (Vallarino et al., 1998), and Senegalese sole, Solea senegalensis (Rodriguez-Gomez et al., 2001). Double immunostaining study in the ayu indicated a close proximity of GnRH-positive nerve fibers and fibers containing NPY suggesting a correlative involvement of NPY and GnRH in the regulation of the pituitary gonadotropic function (Chiba et al., 1996).

b. NPY signal transduction

Peptides of the NPY-family are known to exert their biological actions via G-protein-coupled receptors (GPCRs) segregated into subtypes Y1-Y5. In mammals, NPY was reported to act via Y1 receptor to physiologically amplify preovulatory LH surge (Leupen et al., 1997), and activation of Y4-subtype in rat was implicated in LH and FSH secretion (Jain et al., 1999 and Raposinho et al., 1999, respectively). NPY-effects on gonadotropins have been studied only in the context of NPY-induced GnRH release. This effect is mediated in mammals by the Y1 receptor subtype, which is coupled to phosphoinositide turnover and mobilization of intracellular Ca2+ (Kalra and Crowley, 1992). A similar effect on GnRH release and LH secretion was ascribed to NPY in rainbow trout (Breton et al., 1990; Danger et al., 1991) and goldfish (Peng et al., 1993a). It was suggested that NPY-stimulated LH secretion in teleosts probably involves presynaptic actions mediated by the Y2-like receptors, as well as by a direct postsynaptic action on pituitary cells via the Y1-like receptors (Cerdá-Reverter and Larhammar, 2000). Nevertheless, until recently hardly any information was available on the signaling pathways mediating a direct action of NPY on pituitary cells in fish or mammals, nor on its effect on gonadotropin subunit gene expression.

Our recent studies in tilapia showed that exposing pituitary cells in primary culture to NPY (0.1 nM) increased GPα mRNA levels. This effect could be suppressed by GF and to a lesser extent by H89. It was concluded, therefore, that the effect of NPY on the transcription of this subunit is mediated mainly by PKC with a secondary role for the PKA pathway. In addition, NPY was found to augment the GnRH-stimulated increase in mRNA level of this
subunit (Gur et al., 2001a). Further studies in tilapia demonstrated that NPY regulates the transcript level of LHβ, with both PKA and PKC mediating its effect. As the effect of NPY on the transcript level of Gα and LHβ subunit could be suppressed by the MEK inhibitor PD, it was suggested that PKA and PKC pathways stimulated by NPY in tilapia pituitary converge downstream at the MEK-ERK cascade (Gur et al., 2002b).

It should be emphasized, however, that NPY had no effect at all on the mRNA level of the FSHβ (Gur et al., 2002b; Figs 5b). Similar results have been reported in sheep where NPY infused in vivo to prepubertal female lambs resulted in elevated frequency of cells exhibiting LH immunoreactivity and increased levels of LHβ mRNA but had no effect on FSH cells nor on the FSHβ mRNA levels (Wankowska et al., 2002).

Fig. 5a,b. near here (Coordinate regulation of Gα, LHβ and FSHβ genes in tilapia)

4. Signal transduction of hypothalamic peptides – multiple pathways and signal specificity

It has been previously proposed that activation of multitude of pathways by the same receptor results from receptor microaggregation mobilizing several forms of G proteins (Gs, Gq, etc), each coupled to a different transduction pathway (Stanislaus et al., 1998). Such a mechanism may explain the fact that binding of GnRH can activate the PLC-phosphoinositides-DAG-PKC-Raf-MEK-ERK cascade and at the same time stimulate the adenylyl cyclase-cAMP-PKA cascade as seen in the foregoing results in tilapia.

As shown above, the signals of all hypothalamic peptides examined in tilapia (GnRH, PACAP38 and NPY) evoke the same transduction pathways: PKC and PKA, yet the regulation of FSHβ gene, although affected by PKA, is refractory to NPY (Gur et al., 2002b). This exemplifies the phenomenon of signal specificity, which is considered to be a major problem in comprehending signal transduction in general (Naor et al., 2000). This is also reflected by the observation in tilapia that ERK mediates PACAP- but not GnRH- stimulated FSHβ gene expression (Gur et al., 2002a). As PKC in tilapia has a major role in NPY-stimulation of ERK, whereas PKA was predominant in PACAP activation of ERK (Gur et al., 2002b), it is possible that the kinetics and the ratio of the various MAPK cascades activated by GnRH and PACAP differ from the activation of NPY, resulting in signal specificity.

Another cellular mechanism that can explain such signal specificity is the presence of scaffold proteins (such as β arrestin) that can form a module of specific kinases spatially isolated from other cascades in the cytosol. The role traditionally assigned to β arrestins is preventing further signaling via GPCRs and recruiting clathrin and adapter protein2 (AP-2) that serves to target activated receptors to endocytic vesicles, internalization and desensitization which involves activation of dynamin too. Recently, novel scaffolding and signaling functions of these molecules have been uncovered. β arrestins were also found to function as molecular adapters in the modulation of several GPCRs signaling cascades, namely that of JNK and that of ERK and to involve activation of dynamin (Miller and Lefkowitz, 2001). If, indeed, each of the receptors of GnRH, PACAP and NPY form a spatially separated module consisting of the scaffold protein combined with its specific kinases or adapter proteins, it may explain the phenomenon of signal specificity seen in the above experiments in tilapia. It should be also noted that GnRH receptors lacking the C-terminal tail present only in mammals are unlikely to bind β-arrestin (MacArdle et al., 2002). However, the teleost GnRH-R that posses a C-terminal tail, may have the ability to bind β-arrestin and form separate compartments for each signaling cascade leading from the receptor to the specific MAPK.
From the results achieved in tilapia pituitary cells as a model for the more evolved teleost fish, it would appear that the MAPK (ERK) pathway plays a pivotal role in the differential regulation of the gonadotropin subunit gene expression by the hypothalamic peptides. Nevertheless, the role of other MAPK pathways (e.g. JNK, p38 MAPK and BMK) in gonadotropin regulation still needs to be clarified.

B. Regulatory elements on the subunit promoter
1. The LHβ gene promoter

While considerable research has been carried out on the mammalian LHβ gene promoters, only five LHβ teleost promoters have been isolated so far: those of Chinook salmon (Oncorhynchus tshawytscha; Xiong and Hew 1991; Xiong et al., 1994a), common carp (Cyprinus carpio; Y.-S. Chang et al., 1992), goldfish (Carassius auratus; Sohn et al., 1999a), black carp (Mylopharyngodon piceus; Elizur et al., 2000) and tilapia (Oreochromis mossambicus; Rosenfeld et al., 1997, Elizur et al., 2000). Comparison of these sequences has revealed that the cyprinid species share a particularly high degree of conservation (about 90% sequence identity).

Sequence analysis of the 5'FR of these genes has demonstrated the presence of potential promoter elements (i.e. TATA box and CAAT) as well as several cis-acting motifs, some of which have been shown to be essential in the basal and stimulated transcription of mammalian LHβ genes. For example, a comparison of the piscine LHβ promoters with those of mammals reveals that all proximal promoters contain putative Sf-1 and Pitx-1 binding sites, but unlike their mammalian counterparts, the teleost proximal promoters do not contain an early growth response factor 1 (Egr-1) response element. However, functional studies of these promoters have, so far, been restricted to the Chinook salmon LHβ (csLHβ) gene.

Like those of the mammalian counterparts, the csLHβ gene promoter is driven by a strong proximal tripartite element to which three different transcription factors bind. These include Sf-1 and Pitx-1 as in mammals, but the function of the Egr-1 appears to have been replaced by the estrogen receptor (ER). A functional Egr-1 RE is lacking on the proximal promoter and co-transfection of an Egr-1 expression vector in transient transfection studies fails to stimulate promoter activity (Melamed et al., unpublished). Clearly these three factors have a synergistic interaction on the csLHβ promoter, which parallels that of Egr-1, Sf-1 and Pitx-1 on the mammalian promoters (Melamed et al., 2002; Le Dréan et al., 1996, 1997; Tremblay and Drouin, 1999). Interestingly, however, this is the only piscine LHβ gene so far examined that contains this full palindromic sequence, albeit imperfect, although in other species, half sites are often found.

The lack of role for Egr-1 in regulation of the csLHβ gene is indeed surprising, given the crucial role that it has been attributed with in the regulation of the LHβ gene in mammals, and its presumptive role in mediating the GnRH effect (Tremblay and Drouin, 1999; Dorn et al., 1999; Halvorson et al., 1999; Call and Wolfe, 2002). Egr-1 mRNA is clearly up regulated in response to GnRH, and the protein is probably also phosphorylated (Tremblay and Drouin, 1999). This phosphorylation may not be required for Egr-1 binding to the target DNA, but rather for its interactions with other proteins (Melamed et al., unpublished). In contrast, the ways in which Sf-1 and Pitx-1 mediate the GnRH effect are not entirely clear, as neither appears to be transcriptionally stimulated by GnRH (Tremblay and Drouin, 1999). However both are phosphorylated on binding to the promoter, Sf-1 on one residue and Pitx-1 on three residues, which may perhaps be mediated by GnRH-activated MAPK (Hammer et al., 1999; Melamed et al., 2002 and unpublished;) and thus be a part of the regulatory mechanism.

The role of ER in regulation of the csLHβ gene is, however, in keeping with observations that ER acts as a transactivator, even in the absence of a ligand (Hall et al.,
The ER can be activated in mammalian gonadotropes by PACAP or directly by PKA or PKC; all of these effects are independent of E\textsubscript{2}. There is also clear evidence that the GnRH signaling pathways activate the ER independently of E\textsubscript{2} while the addition of E\textsubscript{2} has a synergistic effect (Demay et al., 2001; Schreihofer et al., 2001). Furthermore, it has been suggested that the synergistic interaction between PKA and ER results from a functional interaction between CREB and ER, which occurs even when a CRE is not present, and that CREB is essential for the PKA activation of ER, possibly through stabilizing interaction of the ER with coactivators such as CREB binding protein (CBP; Lazennec et al., 2001).

Despite the ability of the csLH\textbeta proximal promoter to activate transcription, the longer 3.3 kb promoter is significantly more responsive to GnRH, apparently due to the interaction of distal enhancers with the proximal regulatory region. The upstream promoter contains an additional distal ERE which interacts with the proximal ERE, and also appears to mediate the GnRH effect (Liu et al., 1995; Melamed et al., unpublished). This interaction is facilitated by a series of four Pitx-1 binding sites located between -1366 and -1506 bp from the transcriptional start site. These elements are bound by Pitx-1, which dimerizes, and so induces bending of the target DNA (Melamed et al., 2002; Fig. 6). Similar far-upstream regions of mammalian LH\beta gene promoters have not been studied (e.g. Quirk et al., 2001 Weck et al., 2000).

Fig. 6. near here (Regulation of LH promoter)

Also prominent on the csLH\textbeta gene promoter is the presence of a powerful silencer, which reduces activity of the promoter to around 4% that of the minimal 39 bp promoter when transfected into heterologous cells. The silencer comprises an 18 bp core sequence close to the transcriptional start site, and binds a protein that has yet to be identified. However, this protein is not detected in mature gonadotropes and on transfection, where the silencer is barely active, suggesting that the silencer binding protein contributes to the cell-specific expression of the LH\beta gene (Melamed et al., unpublished). Earlier studies revealed that in heterologous HeLa cells the activity of the silencer could be repressed through the pERE, but this effect was more pronounced when both EREs were present and were in close proximity to each other. In primary culture of trout pituitary cells, the promoter responded to E\textsubscript{2} only in cells from juvenile or maturing but not in those from spawning fish (Xiong et al., 1994b). It has been suggested therefore, that functional interaction between the ER and the silencer binding proteins might regulate expression of this gene in the immature fish (Xiong et al., 1994a).

Other mammalian or teleost LH\beta gene promoters do not contain sequences of high similarity to that of the csLH\beta silencer, however, not enough studies have been carried out to verify whether sequences sharing a degree of similarity, close to the transcriptional start site, might impart a silencing effect. Once the protein is identified, it will be possible to elucidate how this silencer binding protein fits into the overall picture of regulation of the csLH\beta gene, and how this type of regulation has developed through evolution.

2. The FSH\beta gene promoter

In comparison with other gonadotropin subunit genes, our understanding of the endocrine regulation of the FSH\beta gene is lagging. This is especially true for teleosts, where the FSH\beta gene has been reported so far from only three fish species: tilapia (\textit{O. mossambicus}; Rosenfeld et al., 1997; Rosenfeld et al., 2001), goldfish (\textit{Carassius auratus}; Sohn et al., 1998a) and black carp (\textit{Mylopharyngodon piceus}; Elizur et al., 2000).

Comparison of FSH\beta 5' flanking region (5' FR) revealed that they all share several putative response elements, such as a GSE, CRE, half sites of ERE, and activating protein 1 response element (AP1), the recognition site for the Fos and Jun transcription factors (Fig.
7A,B). Similar to the situation in the LHβ promoter regions, sequence conservation (above 80%) characterizes the FSHβ 5′ FR of the cyprinid species. A relatively distal location (-97 bp) of the TATA box distinguishes the tilapia FSHβ (tFSHβ) promoter from that of other vertebrates. This untypical location of the TATA box may interfere with uniform transcription of the gene from a single start site, and is possibly the reason for its mRNA multiplicity. It was suggested, therefore, that the motifs of Sp1 and/or GC box, identified at the respective loci of (-190 bp) and (-75 bp) within the tFSHβ 5′ FR, are potential initiators for transcription, resembling the situation in many TATA-less promoters (Rosenfeld et al., 2001).

Functional studies of FSHβ genes are of a preliminary nature and have been reported so far only in tilapia. The isolated 1.7 kb fragment of the tFSHβ 5′ FR fused to the firefly luciferase (LUC) gene has directed an efficient basal expression in cultured tilapia pituitary cells (Rosenfeld et al., 2001). Sequential deletion analysis confined its minimal promoter to a proximal fragment, spanning 600 bp upstream to the CAP site. This 0.6 kb fragment, which includes a potential GSE and two tandem AP1 motifs, induced similar LUC activity as compared to the 1.7 kb fragment. Interestingly, the array of two tandem AP1 motifs was also found in the proximal promoter of the ovine FSHβ (oFSH) gene (Strahl et al., 1997). In the latter, each of the two AP1 motifs was found to act independently or in cooperation to induce basal transcription of the oFSHβ gene.

Further characterization of the tFSHβ 5′ FR showed that the region between (-600 bp) to (-1211 bp) contains enhancing element/s, while the region between (-1211 bp) to (-1389 bp) contains silencing element/s (Rosenfeld et al., 2001). It was postulated that a putative CRE motif, found at (-1063 bp), and upstream negative element/s (yet to be verified) function in unison to repress tFSHβ basal transcription (Fig. 7B). A similar negative regulation, which involves a CRE motif and, at least, two other elements within an upstream segment, was reported in the major histocompatibility class I promoter (Kirshner et al., 2000). Another potential regulator of tFSHβ basal expression is the canonical AP1 motif located at (-1111 bp). The ability of such a motif to enhance basal transcription independently, or in synergism with a CRE motif, was previously demonstrated for the tyrosine hydroxylase gene (Ghee et al., 1998).

Unlike the α subunit gene that is expressed in both gonadotropes as well as in the thyrotropes, the expression of the FSHβ and LHβ subunit genes is restricted to the gonadotropes only (reviewed by Albanese et al., 1996). Furthermore, in the fish studied so far, the expression of each β subunit is restricted to a specific cell type in the pituitary (trout, Nozaki et al., 1990; tilapia: Melamed et al., 1998; sea bream: Elizur et al., 2000). Despite this restriction, the 1.7 kb of the tFSHβ promoter successfully induced considerable basal expression in several heterologous non-gonadotrope cultured cells, including COS-7, TO2 and EPC. It is not entirely clear yet whether the non-specific expression occurs due to the absence of cis-regulatory elements within the tested promoter region, or the lack of trans-acting gonadotrope-specific factors within the tested cell-lines. Stepwise deletion analysis demonstrated a differential expression pattern in which the 0.6 kb of the tFSHβ promoter directed significantly enhanced LUC activity (1.5-3 fold) compared to the 1.7 kb or the 1.2 kb fragments, which did not differ from each other. These results are not in line with the pattern obtained with the homologous pituitary cells, which may implicate dissimilar repertoire of basal transcription factors operating in the heterologous cell-lines and the homologous pituitary cells (Rosenfeld et al., unpublished).

The 1.7 kb tFSHβ promoter successfully directed GnRH-induced transcription in a dose-dependent manner with a maximal 1.5-2 fold increase in LUC activity in transfected pituitary cells of tilapia in primary culture (Rosenfeld et al., 2001). Parallel in vitro studies, aimed to validate the effect of GnRH on transcript levels of tilapia gonadotropin subunits, demonstrated
a similar fold induction of tFSHβ mRNAs (Gur et al., 2001b; see section IV,A). This would indicate a direct GnRH regulation of tilapia FSHβ at the level of gene expression rather than at the level of transcript stability.

Functional studies of the tFSHβ 5′FR-deletion series positioned the GnRH responsive region between (-821 bp) to (-1211 bp), spanning the canonical AP1 (-1111 bp) and CRE (-1063 bp) motifs. Further deletion between (-1211 bp) to (-1065 bp), which eliminates the AP1 motif, resulted in a partial loss of the response. This implicates the involvement of both elements, CRE as well as AP1, in conferring GnRH responsiveness of the tFSHβ promoter (Fig. 7B; Rosenfeld et al., unpublished). Recently, it was shown that GnRH responsiveness of the oFSHβ gene in LβT2 cells is driven by two distal promoter regions, which associate with one or more proximal promoter elements. No consensus sequences for these regulatory elements have been established yet (Vasilyev et al., 2002).

Place Fig. 7A, B. near here (Basal and GnRH-stimulated expression of FSHβ gene in tilapia)

Transient transfection studies performed in αT3 cell line showed that activation of PKA with forskolin resulted in a 2.8 fold induction of LUC activity driven by the tFSHβ promoter. A lower extended increase in LUC activity (1.5 fold) was obtained by activating PKC with TPA (Rosenfeld et al., unpublished). The ability of forskolin and TPA to stimulate tFSHβ promoter, coupled with the role of AP1 and CRE motifs in acquiring responsiveness to GnRH, suggest the involvement of both PKC and PKA pathways in GnRH stimulation of tFSHβ promoter (Fig. 7B). The involvement of cAMP-PKA system in transducing GnRH signaling toward FSHβ gene transcription is corroborated by other experiments in tilapia pituitary employing a PKA inhibitor (see section IV, A, 1). Nevertheless, PKC-ERK pathway was not found to be involved in this gene transcription in these studies. Another possibility still to be explored is that the effect of PKA on FSHβ gene transcription is conveyed through other MAPK cascades such as JNK affecting AP1 (Figs. 7A,B).

C. Sex steroids and GtH subunit transcripts

1. Studies in tilapia

Information on the impact of sex steroids on GtH subunit mRNA in tilapia has been derived from observations in fish at different stages of gonadal development, exhibiting varying levels of circulating sex steroids and from in vivo and in vitro experiments where fish or pituitary cells were exposed to sex steroids.

Such observations showed that high levels of FSHβ mRNA were seen only in fish with relatively low levels of sex steroids (~1 ng/ml T in males or E2 in females). In fish with higher or lower steroid levels, the levels of FSHβ mRNA were low. In fish with higher steroid levels, the LHβ transcript levels were also high, and in regressed male fish, a positive linear correlation was seen between the levels of LHβ mRNA in the pituitary and circulating T levels (Melamed et al., 2000).

In order to reveal whether the steroids affect the GtH subunit gene transcription directly at the pituitary level, glands were excised from tilapia at various stages of their reproductive phase and their cells were dispersed and cultured. In pituitary cells from immature males the FSHβ transcript increased in response to 10 nM T given for 36-48 h. In cells from maturing males only exposure to low doses of T (10-100 pM) caused an increase in FSHβ mRNA levels (Melamed et al., 1997). In fish at the end of the spawning season levels of the FSHβ mRNA decreased following exposure to higher doses of the steroids (T at 10 and 100 nM and E2 at 1-100 nM). The addition of T or E2 (10 pM-100 nM) did not result in any change in LHβ mRNA (Melamed et al., 2000). Cultured pituitary cells taken from regressed fish and exposed to T (0.1-10 nM) showed a dose-dependent increase in the mRNA of LHβ level (up
to 450%). However, no change was seen in the levels of FSHβ mRNA. Exposure of the cells to 11-ketotestosterone (KT) at the same concentrations was not followed by any change in the mRNA levels either of FSHβ or of LHβ (Melamed et al., 1998).

To study the effect of T on Gpα subunit, pituitary cells were taken from immature male tilapia or mature fish at the beginning of the reproductive season and cultured for 3 days. They were then exposed to graded doses of T (0.01-100 nM). Gpα mRNA levels increased only in cells taken from the fish with the more developed gonads (Gur et al., 2000a).

The presented results indicate that T has an effect similar to that of E2 on the transcription of GtH subunits, and that the non-aromatizable androgen lacks such effect. This implies that the effect of T in tilapia pituitary is exerted via its aromatization to E2. When dispersed pituitary cells were separated on density gradient, aromatase activity was localized to the gonadotropin-enriched fraction and was not present in the fraction containing the somatotropes (Melamed et al., 1999).

2. Studies in other fish

Chronic implantation of T into 2-y old juvenile sturgeons (Acipenser transmontanus) belonging to the ancient osteichthyan group, Chondrostei, resulted in increased levels of the pituitary content of both LH and FSH (Pavlick and Moberg, 1997). T, other aromatizable androgens or estradiol increased the mRNA levels of LHβ in goldfish (Khakoo et al., 1994; Huggard et al., 1996; Sohn et al., 1998b; Kobayashi et al., 2000b), coho salmon (Dickey and Swanson, 1998), European eel (Quérat et al., 1991) and European sea bass (Mateos et al., 2002). Similarly, LH pituitary content increased following treatment with T or E2 in rainbow trout (Breton et al., 1997), Parr Atlantic salmon (Antonopoulou et al., 1999a), coho salmon (Dickey and Swanson, 1998) and European sea bass (Mateos et al., 2002).

Castration of male Parr Atlantic salmon reduced pituitary LH content but treatment with T increased the content, an effect that was attenuated when T treatment was combined with the aromatase inhibitor ATD. These results indicate the presence of an aromatase-dependent positive feedback of T on LH. However, the non-aromatizable androgen, 11-ketotestosterone also had a stimulatory effect on LH, although weaker than that of T (Antonopoulou et al., 1999a).

Experiments in the protandrous black porgy, Acanthopagrus schlegeli using androgen combined with aromatase inhibitors ATD or fadrozole indicated that LH secretion is positively regulated by an estrogen-specific effect in both phenotypic females or males. Nevertheless, gonadal stage had significant effects on the responsiveness of LH to E, stimulation in males (Lee et al., 2001).

Treatment with physiological doses of E2 stimulates the expression of Gpα, FSHβ and LHβ genes both in vivo and in vitro in goldfish and this effect is seen in both early and late phases of gonadal recrudescence. Furthermore, GtH protein (probably LH) is increased dramatically in pituitary fragments exposed to E2. However, this study was carried out in groups of mixed sex, and sexual dimorphism in the response could not be distinguished (Huggard-Nelson et al., 2002). LHβ transcript and LH de novo synthesis were elevated by exposure of African catfish pituitary cells to T or E2 (Rebers et al., 1997, 2000b).

In immature male striped bass (Morone saxatilis), the combination of T and GnRHα stimulated a three- to fivefold increase in the mRNA levels of FSHβ and LHβ, but the same treatment had no effect on gonadotropin gene expression in maturing males indicating a differential response at various stages of maturity (Hassin et al., 2000). It is possible that the effect of T treatment in immature male is more pronounced than in maturing males already producing their own androgens. A peculiar and still unexplained phenomena was seen in the
striped bass where administration of dopamine receptor D2 antagonist, pimozide, to immature males suppressed the stimulatory effect of GnRHa and T on FSHβ and LHβ mRNA levels (Hassin et al., 2000). Whether this effect is a genuine action of dopamine is still to be explored, however, exposure of pituitary cells of another perciform fish, tilapia to dopamine had no effect on the expression of LHβ although it strongly suppressed its release (Melamed et al., 1966).

Similar to the situation in tilapia, transcript levels of FSHβ were reduced by T or E2 treatment in coho salmon (Dickey and Swanson, 1998), in juvenile or early recrudescing goldfish fed or goldfish implanted steroid-containing pellets (Sohn et al., 1998b, 2000; Kobayashi et al., 2000), and in European sea bass implanted for 12 days with pellets containing E2, T or dihydrotestosterone (DHT; Mateos et al., 2002). Thirty days after implantation of E2 in trout, FSH pituitary content declined (Breton et al., 1997). Removing the source of sex steroids by castration resulted in a dramatic increase in pituitary FSH content in mature parr Atlantic salmon (Salmo salar) in summer indicating a predominantly negative feedback of gonadal hormone(s) on FSH. (Antonopoulou et al., 1999a). The elevation of FSHβ mRNA in castrated goldfish could be reversed by T, E or KT indicating in this fish too a negative regulation of FSH by gonadal steroids (Kobayashi et al., 2000).

Implantation for 12 days of pellets containing E2, T or DHT resulted in an increase in the GPα mRNA of the European sea bass (Mateos et al., 2002). Chronic in vivo treatment with T or E2 was followed by an increase in GPα mRNA in European eel (Anguilla anguilla; Counis et al., 1987; Quérat et al., 1991). However, addition of T or E2 to eel pituitary cell culture for 24, 72 h or even 13 days failed to increase GPα mRNA. These results are in contrast with the increased transcript levels in African catfish (Clarias gariepinus) pituitary cell culture exposed to T for 48 h, with such increase being abated by the aromatase inhibitor, ATD (Rebers et al., 2000b). Another study in African catfish shows that castration reduced but did not abolish the maturation-associated elevation in pituitary LH content. Treatment with estradiol or testosterone but not with 11-oxygenated androgens resulted in increased levels of LHβ mRNA and pituitary content of LH with only marginal effect on GPα mRNA (Cavaco et al., 2001).

The situation in goldfish is controversial. In sexually immature fish, T or 11-hydroxyandrostenedione at physiological doses increased GPα mRNA while high, non-physiological doses of both androgens given in vivo was unstimulatory or even suppressed the transcript levels. However, when such high doses were given to mature fish, the GPα transcript level did increase. T was also effective when given continuously to pituitary fragments in perfusion (Huggard et al., 1996). It should be noted, however, that such perfused pituitary fragments contain nerve fibers carrying hypothalamic hormones that may be sensitive to the presence of the steroids. In another study on goldfish implanted for 2 weeks with T, E2 or KT (Sohn et al., 1998b) or in juvenile goldfish fed with these steroids, no change could be noted in the level of GPα transcript (Kobayashi et al., 2000). Area the effects androgenic or estrogenic?

In most fish species the effect of sex steroids on GtH subunit gene expression or on LH content is attributed to E2 because administration of non-aromatizable androgens or abating aromatase activity abolished the steroid effect (Melamed et al., 1998; Antonopoulou et al., 1999b; Rebers et al, 2000b, Cavaco et al., 2001). The situation in the European sea bass (Dicentrarchus labrax) differs inasmuch as the non aromatizable androgen DHT implanted for 12 days caused an increased mRNA levels of both GPα and LHβ and a decrease in that of FSHβ much the same as did estradiol or testosterone (Mateos et al., 2002). In the European eel (Anguilla anguilla) T elevates LHβ mRNA and LH but not GPα expression. Similar effects can also be induced by non-aromatizable androgens but not by estradiol, indicating an
androgen-specific effect in this fish (Huang et al., 1997). There is no agreement about the situation in goldfish as the non-aromatizable androgen 11-hydroxyandrosterone was effective in increasing both GPα and LHβ mRNAs (Huggard et al., 1996). In another study 11-KT failed to increase the levels of these transcripts whereas T and E2 were effective (Sohn et al., 1998b; Kobayashi et al., 2000).

In conclusion, the foregoing account, and the review by Dufour et al. (2000) show that a considerable volume of information has been accumulated on the stimulatory effect of T and E2 on LHβ mRNA and on LH pituitary content and release. Most sources report the results of steroid treatment in vivo. Such route of administration cannot accurately target the site of the steroid effect or its mode of action. Therefore, reports on steroid effect on GtH content or mRNA achieved in vitro may not match, or even contradict results obtained in vivo (see Huang et al., 1997; Rebers et al., 1997, 2000b). Specifically, these studies cannot distinguish between direct effect of the steroids on the pituitary and possible indirect effects mediated by hypothalamic or other agents (reviewed by Van Der Kraak et al. 1998). For example, E2 alone or in combination with androgens elevates brain and pituitary mGnRH levels which will also lead to increase in pituitary LH content (Montero et al., 1995). In addition, sex steroids are able to stimulate the release of other hypophysiotropic hormones, such as NPY, which may exert their own effect on the pituitary or on GnRH release (Peng et al., 1993b). T and E2 can potentiate in vitro the stimulation of LH release (and FSH to some extent) by GABA (Mañanós et al., 1999). In addition E2 can modulate monoamine oxidase to alter hypothalamic serotonin that may alter gonadotropin secretion (Senthilkumaran and Joy, 1994). It is not clear yet whether such effects also involve increased subunit gene transcription.

The foregoing account on the physiological effects of gonadal steroids, especially of estrogens or aromatizable androgens is in line with the multitude of ERE in the promoter of fish LH and FSH β genes. It is not clear yet how non-aromatizable androgens exert their effect on these genes and how the seasonal changes and sex differential effects are modulated in fish. Another point to be addressed in the future is the effect of membrane estrogen receptors and their downstream cascades involving the PKA-CREB-CRE pathway (see section IV, B, 1).

D. Gonadal peptides

The presence of a non-steroidal gonadal product that can affect gonadotropic secretion was initially reported in tilapia (Rubin-Kedem et al., 1989). Since then, the studies of Dr. W. Ge and co-workers established the immunocytochemical presence of activin βA and activin βB in the granulosa cells and previtellogenic oocytes in the ovary, and in the testicular interstitial and Sertoli cells of the goldfish. Strong immunoreactive α subunit was localized only in mature sperm cells leading to the conclusion that the goldfish gonads may produce mainly activin rather than inhibin-like protein. The cDNAs of goldfish activin βA and βB subunits have been cloned and expressed in CHO cell line, and goldfish recombinant activin A and B has been produced. In addition, the full-length cDNA for a type II and several type I activin receptors have been cloned (reviewed by Ge, 2000). The cDNA of follistatin (Bauer et al., 1998), and a novel member of the TGF-β family, antivin, (Thisse and Thisse, 1999) have been cloned from the zebrafish. It was concluded that the latter may serve as an activin antagonist by competing with its binding to the Type II activin receptors, thus acting in an analogous way to inhibin in mammals (reviewed by Ge, 2000).

Activin βA and βB were localized immunocytochemically in the somatotropes within the proximal pars distalis of goldfish pituitary (Ge and Peter, 1994). Furthermore, activin as well as activin receptors were found to be expressed in the pituitary of this fish (Ge et al., 1997) suggesting certain paracrine and autocrine functions for activin within the pituitary (Ge, 2000).
Early studies on goldfish indicated that porcine activin A, but also inhibin A stimulates the release of LH from cultured pituitary cells of goldfish (Ge et al., 1992). More recent studies using the recombinant goldfish activin B given in culture of pituitary cells led to increased levels of FSHβ mRNA but had the opposite effect on that of the LHβ (Yam et al., 1999). The discrepancy between the earlier finding on activin stimulation of LH release and the latter findings on its inhibitory effect was attributed to possible seasonal variation in the gonadotropin sensitivity to activin (Ge, 2000).

In postovulatory rainbow trout, non-steroidal factor(s) in the ovarian fluid were found to inhibit FSH release but stimulate the secretion of LH (Chyb et al., 1999). It was suggested that this effect is due to the presence of an inhibin/activin-like factor in the ovarian fluid because recombinant inhibin was found, in a subsequent study, to inhibit FSH and to stimulate LH release from cultured pituitary cells of the trout (Chyb and Breton, 2000).

Preliminary results in tilapia hybrids (Oreochromis niloticus x O. aureus) have shown that recombinant human activin A added to cultured pituitary cells at 0.5-20 ng/ml for 48 h led to an increase of all gonadotropin subunit mRNAs. FSHβ mRNA increased more than 24-fold at the maximal concentration of 20 ng/ml while that of LHβ increased up to 12-fold. The least affected was GPa mRNA that increased only 2.5 fold (Yaron et al., 2001). The discrepancy between the results in tilapia and goldfish can be attributed to the different type of activin (A or B) used in these studies, to the heterologous source of the peptide (human) used in tilapia or to an inherent difference between the fish species.

Pituitary cells of coho salmon too responded to recombinant human activin A by increase in mRNA of both GPa (α1 and α2) and of FSHβ with no effect on LHβ (Davies et al., 2000). However, the donor fish in this study were immature males, a stage at which LHβ mRNA did not increase even in response to GnRH (Dickey and Swanson, 2000).

A 48-h exposure of cultured pituitary cells of tilapia hybrids to porcine inhibin (0.5-20 ng/ml) resulted only in a marginal decrease in the mRNA levels of GPa and FSHβ. The response of LHβ was more complex: at inhibin concentrations of 0.5 and 1 ng/ml there was a dose-dependent increase of up to 5-fold in the transcript, whereas at higher concentrations of the peptide the stimulatory effect was reduced considerably (Yaron et al., 2001).

Due to the contradictory results, it would appear that more information is required in other fish regarding the effect of these gonadal peptides on gonadotropin subunit genes with respect to the gonadal developmental stage before any generalization can be drawn.

V. Conclusions

The data presented in this review indicate that expression of each gonadotropin subunit responds differently to the various regulatory hormones. In most cases, the responses of LHβ and GPa are similar, whereas that of FSHβ differs. It is suggested that transduction of GnRH signal leading to the increased expression of LHβ and GPa is mainly mediated through PKC and MAPK cascades, whereas that of FSHβ is through cAMP-PKA. Promoter analysis of the csLH and tFSHβ generally corroborates this hypothesis: MAPK phosphorylates Sf-1 and possibly ER that bind to the csLHβ promoter, while PKA may be responsible for CREB binding to the tFSH CRE. Other hypothalamic regulators such as NPY and PACAP are able to stimulate expression of LHβ and GPa, but only PACAP stimulates also FSHβ.

Responses in LHβ and FSHβ gene expression to gonadal steroids appear also to be differential: high transcript levels of FSHβ is associated with relatively low testosterone or estradiol levels, while high levels of LHβ transcript is associated with high levels of these steroids. It should be noted, however, that our own research and that of others have indicated that the response of the pituitary gonadotropes to hypothalamic or gonadal hormones is dependent on the maturation stage of the treated fish or the donor fish. This has hindered
research particularly in asynchronous spawners in which the precise reproductive state is often difficult to evaluate and cannot be synchronized to produce homologous groups of experimental animals. This may also explain some of the apparently conflicting results from groups working on different species.

The research described here has done much to illuminate the regulatory mechanisms of the teleost pituitary gonadotropins at the molecular level. However, dramatic increases in gonadotropin production in the maturing fish have not yet been entirely recapitulated in vitro. This clearly indicates that there are additional elements at work, individual or combinatorial, which are as yet elusive. It is the hope of the authors that the above account will stimulate research into this important field of comparative endocrinology that can also benefit the development of fish culture.

VI. REFERENCES


their second messengers on the mRNA levels of gonadotropin IIβ subunit and growth hormone in the teleost fish, tilapia. *Neuroendocrinology* **64**, 320-328


pituitary αT3-1 cell line: differential roles of calcium and protein kinase C. *Endocrinology* **138**, 1673-1682.


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Fig. 1.

(a) Localization of FSHβ and (b) LHβ subunit mRNAs in the proximal pars distalis of the pituitary of tilapia. *In situ* hybridization was carried out using a digoxigenin (DIG)-labeled cDNA probes as in Melamed et al. (1998). FSH, FSHβ; LH, LHβ; N, ramification of nerve fibers within the proximal pars distalis; S, somatotropes; Scale, 200 µm. (From Yaron et al., 2001, permission not yet granted by Elsevier, ZY).
A. Schematic presentation of the endocrine regulation during the vitellogenic phase in fish reproduction. During this phase the release of FSH, under GnRH stimulation, promotes the secretion of estradiol from the ovarian follicles. Estradiol stimulates the synthesis of vitellogenin and zona radiata proteins that are sequestered by the follicles under the regulation of FSH and are incorporated in the oocytes as the yolk and the zona radiata.

B. Schematic presentation of the endocrine regulation of the final oocyte maturation in a cyprinid fish. During this phase LH release is stimulated by GnRH, and probably by suppression of dopamine inhibition. Postvitellogenic ovarian follicles respond by secretion of the maturation-inducing steroid, 17α, 20β-dihydroxy-4-pregnen-3-one (17,20-P). This progestogen stimulates, in a paracrine manner, the synthesis of cyclin B, that together cdc2-kinase form the maturation-inducing factor (MPF). The final maturation of the oocytes, resumption of meiosis and ovulation are depicted as can be seen in fish oocytes by the migration of the germinal vesicle and its breakdown.
Fig. 3

Left panel: Multiple sequence alignment of the FSHβ N-terminal in bony fish (Osteichthyes). Sequences are aligned from the first amino acid of the mature peptide. Gaps (shown by asterisk) were introduced to maximize alignment. The Cys residues are marked with white letters on black background, and putative glycosylation sites are marked with gray background. Arrows indicate the Cys and N residues that were subjected to positive selection during the evolution of teleosts.

Right panel: A proposed evolutionary model for the FSHβ molecules in bony fish (Osteichthyes). Gray boxes represent branches exhibiting semi-parallelism with the salmoniformes/Perciformes branch.
Sites of dopaminergic inhibition of gonadotropin (LH) release located experimentally in tilapia pituitary cells. Dopamine was found to inhibit GtH release at a site distal to Ca$^{2+}$ influx, and at sites distal to protein kinase C (PKC) and protein kinase A (PKA). Arachidonic acid or Ca$^{2+}$ mobilization from intracellular sources generated by ionomycin, could circumvent the effect of dopamine.

[Ca$^{2+}$]e, extracellular Ca$^{2+}$; [Ca$^{2+}$]i, cytosolic Ca$^{2+}$; CaM, calmodulin; CaM-K, calmodulin-dependent kinase; DAG, diacylglycerol; IP3, inositol trisphosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; PLA2, phospholipase A2; PLC, phospholipase C.
a. Proposed mechanism for coordinated regulation of tilapia GPα and LHβ subunit gene expression by GnRH, PACAP and NPY. These 3 peptides bind to different GPCRs. Activation of G_q stimulates phospholipase C (PLC) activity to generate inositol trisphosphate (IP_3) and diacylglycerol (DAG). Increase in the levels of these second messengers and cytosolic Ca^{2+} leads to activation of protein kinase C (PKC). Parallel activation of G_s stimulates adenylate cyclase (AC) activity resulting in cAMP formation and activation of protein kinase A (PKA). Both PKC and PKA converge at the level of RAF to phosphorylate MAPK kinase (MEK) and subsequently the extracellular signal-regulated kinase (ERK). This MAPK enters the nucleus and regulates directly or indirectly transcription factors (X), currently unknown for tilapia GPα and LHβ subunit promoters.

b. Proposed mechanism for coordinated regulation of tilapia FSHβ subunit gene expression by GnRH and PACAP. GnRH and PACAP bind to the respective GPCRs. GnRH activated G_s and stimulated AC activity resulting in cAMP formation and activation of PKA. PKA, via phosphorylation of cAMP-response-element-binding protein (CREB) binds and activates cAMP-response element (CRE) located on the FSHβ promoter or phosphorylates other MAPK cascades (e.g., JNK) that, in turn, binds and activates AP-1 sites on the promoter. PACAP, by binding to its GPCR, activates both PKC and PKA that converge at the level of RAF to phosphorylate MEK and subsequently ERK. This MAPK enters the nucleus and regulates, directly or indirectly, currently unknown transcription factors (X) or the CRE in the tilapia FSHβ subunit promoter. Presumed pathways are designated by fragmented arrows.
Regulation of csLHβ promoter activity. The proximal promoter binds Sf-1, Pitx-1 and ER which interact synergistically and presumably recruit a cofactor complex to interact with the general transcription factors (GTFs). The Sf-1 and Pitx-1 are both phosphorylated, possibly in response to GnRH. Further upstream, Pitx-1 also homodimerizes and binds to multiple response elements which induces conformational change in the DNA, to facilitate the interaction between proteins binding the distal ERE and the proximal promoter. The actions of the proximal silencer (pSil) are evident only in non gonadotrope cells.
A. Basal Expression

B. GnRH-stimulated expression

Fig. 7. Proposed model of basal and GnRH stimulated tFSHβ gene expression.

A. The transcription factors involved in basal tFSHβ gene expression are shown bound to their cognate cis-elements in the tFSHβ promoter.

B. GnRH-stimulated transcription factors and their putative signaling pathways. The abbreviations are as follows: AP-1, activating protein-1; CREB, cAMP-response element binding protein; DAG, diacylglycerol; IP₃, inositol trisphosphate; JNK, Jun N-terminal kinase; PKA, protein kinase A; PKC, protein kinase C. (+) and (-) represent positive and negative trans-acting factors, respectively. (?) Represents an unknown motive/factor which negatively regulates basal expression of tFSHβ.