FISH GONADOTROPINS AND GnRH's: FUNDAMENTAL ASPECTS AND PRACTICAL APPLICATIONS

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Since long it has been established that the pituitary of tetrapods secretes two gonadotropins: follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Lich et al., 1977; Pierce and Parson, 1981). The main functions of FSH and LH are the control of gametogenesis and sex hormone synthesis respectively. However, there are examples that this distinction is not as strictly as that, for example, the function of FSH in the onset of luteinisation and the consequent progesterone secretion by the mammalian corpus luteum.

LH and FSH, and in addition thyroid stimulating hormone (TSH) are glycoproteins, consisting of two noncovalently bound, chemically distinct subunits, referred to as α and β. The α subunit is common to all three hormones, while the β-subunit is hormone specific (Papkoff, 1972).

The respective β-subunit does not exhibit any biological activity unless bound to an α-subunit; the biological specificity depends on the β-subunit.

FISH GONADOTROPINS: HOMOLOGY WITH MAMMALIAN DUALITY IN GONADOTROPINS

Fish pituitaries have been considered for many years to produce only one gonadotropic hormone. This hormone closely resembled FSH and LH and combined the two main functions (Burzawa-Gérard and Fontaine, 1972; Burzawa-Gérard, 1982). Variations in chemical composition have been observed, but were interpreted as heterogenetic forms of carbohydrate composition of one and the same hormone (Huang et al., 1981) and the existence of a single GTH in teleosts was generally

accepted. Results from morphological studies with respect to the existence of one or two types of gonadotropic cells were controversial. Some authors described only a single gonadotropic cell type, others found two types. Differential forms of gonadotropic cells often have been interpreted as functional stages of the same cell type (Van Oordt and Peute, 1983).

Over the last twenty years many attempts have been made to purify GTH from teleost pituitaries and to elucidate its chemical structure and its biological activities (Buzawa-Gérard, 1971; Donaldson *et al.*, 1972; Idler *et al.*, 1975a, 1975b, 1975c; Breton *et al.*, 1976; Yoneda and Yamazaki, 1976; Farmer and Papkoff, 1977; Kobayashi *et al.*, 1985; Goos *et al.*, 1986a; Ng and Idler, 1979; Kawauchi *et al.*, 1987). A number of these studies report on the purification of a single GTH.

The concept of duality was put forward again by the studies of Idler and co-workers, who claimed to have separated two types of GTHs from several teleost species by using affinity chromatography on concanavalin A-sepharose (Con-A) (Idler *et al.*, 1975c; Ng and Idler, 1979). The fraction, referred to as Con-A I, not absorbed on Con-A (carbohydrate poor), stimulated yolk incorporation, whereas the Con-A absorbed fraction (carbohydrate rich, Con-A II) induced oocyte maturation and ovulation. The vitellogenic Con-A I GTH appeared not to be homologous with LH and FSH; it does not consist of two subunits and lacks the glycoprotein character of the mammalian gonadotropins. Thus, this concept of duality is different from that of LH-FSH relationship.

Ando and Ishii (1988) reported an indirect evidence with regard to duality in teleost GTH. They purified two types of GTH from the pituitary gland of yellow fin tuna; one is active in tuna, while the other is inactive in tuna but active in goby.

Physicochemical evidence

A detailed physicochemical and biological characterisation of glycoproteins extracted from chum salmon (*Oncorhynchus keta*) pituitaries was carried out by Suzuki *et al.* (1988a). Glycoproteins were classified into two groups by molecular weight, isoelectric point, amino acid composition and N-terminal residues. The gonadotropic activities of the two groups of glycoproteins were demonstrated *in vivo* by the effect on gonadal growth of juvenile rainbow trout (*Salmo gairdneri*). The

two glycoproteins were designated as GTH I and GTH II, respectively. GTH I and GTH II appeared to consist of two subunits each (Suzuki *et al.*, 1988b), this being the first characteristic for homology with the mammalian gonadotropins. GTH I is characterised by two forms of the α-subunit and one β-subunit, while GTH II has only one form of the α-subunit (which is identical to the the GTH Iα2) but two variants of the β-subunit (only two substitutions difference). Chum salmon pituitaries characteristically produce two variants of other hormones (STH, LTH, POMC) with over 95% similarity, probably introduced by tetraploid salmonids.

GTH Iβ has only 31% sequence identity with GTH IIβ (Itoh *et al.*, 1988). This degree of sequence homology is comparable with the homology between mammalian LHβ and FSHβ. The deduced amino acid sequence from the nucleotide sequence of the cDNA's encoding for α2, Iβ and IIβ are consistent with the results obtained from sequence analysis of the polypeptides (Itoh *et al.*, 1988). Thus, it appears that salmon GTH I and GTH II are two distinct hormones and not variations of one GTH.

As a result of these studies the concept of duality of teleost GTHs

has now at least a sound chemical base.

It remains questionable, whether in species where till now only one GTH has been isolated a second form will appear to be present. It seems obvious, however, that in cases where only one GTH has been isolated, this is most comparable with the salmon-GTH II. This not only follows from comparisons of amino acid sequences (Trinh *et al.*, 1986 for chinook salmon, *Oncorhynchus tschawytscha;* Jolles *et al.*, 1977 for carp, *Cyprinus carpio*, Koide *et al.*, 1991 for the African catfish, *Clarias gariepinus*), but also from comparisons of nucleotide sequences of cDNA encoding for GTHβ-subunits (Trinh *et al.*, 1986; Itoh *et al.*, 1988; Chang *et al.*, 1990). Radioimmunoassays, based on these GTHs might not be specific for GTH II, but usually measure a GTH which is able to induce maturation and ovulation (*i. e.* Goos *et al.*, 1986a; Copeland and Thomas, 1989).

Immunocytochemical localisation

The presence of two gonadotropins in salmon is not only suggested by the detection of two chemically different glycoproteins with homology to mammalian FSH and LH. Immunocytochemically, using antibodies against coho or

chum salmon GTH I and GTH II β-subunits, in the rainbow trout, the chum salmon and the Atlantic salmon (*Salmo salar*) two distinct immunoreactive cell types could be demonstrated in the proximal pars distalis. No co-localization of GTH I and GTH II was observed (Nozaki et al., 1990a). In rainbow trout it was found that GTH I immuno-reactive cells are the only gonadotrops in the period prior to puberty. GTH II cells appeared with the onset of vitellogenesis and spermatogenesis. At the time of final reproductive maturation both cell types were present, but the GHT II cells dominated (Nozaki, 1990b). The GTH II cells appear to correspond to previously described teleost gonadotropic cells (Van Oordt and Peute, 1983).

Biological activity

Although radio-immunoassays, developed for GTH I and GTH II, show considerable cross-reactivities (Suzuki et al., 1988c), pituitary and plasma GTH concentrations during ontogeny and the reproductive cycle and after challenging with D-Ala[6]-LHRH, suggest indeed that GTH I is predominantly present during prepuberal stages, while GTH II is synthesised and released during vitellogenesis and spermatogenesis and final maturation. Data about the biological activity of the two salmon GTHs are still limited. Their competence to stimulate estradiol and $17\alpha, 20\beta$-dihydroxy-4-pregnene-3-one ($17\alpha, 20\beta$-P) synthesis by amago salmon ovarian follicles has been tested. Both GTHs stimulate estradiol synthesis equally, but the competence of GTH II to stimulate 17α-20β-P production is higher than that of GTH I (Suzuki et al., 1988d), suggesting once more the maturational role of GTH II.

The investigations on salmon gonadotropins have shown that two gonadotropins are present in these species. It does not mean, however, that the concept of dual control of gonadal functions, as in mammals, has been established for teleosts now. It has to be investigated whether the exsistence of two forms of gonadotropic hormone is a common feature of all teleosts. Furthermore, till now no real distinction between functions of the two GTHs could be made.

FUTURE RESEARCH ON TELEOST GONADOTROPINS

Further evaluation of the concept of duality of gonadotropins: presence of GTH I and II

For species, from which sufficient

pituitary preparations are available (usually commercial fish species) a systematic search should be carried out to the presence of two gonadotropins, distinguishable by physico-chemical characteristics, as has been undertaken for salmon species. At present some of such studies are already in advanced stages, *i. e.* for the African catfish, *Clarias gariepinus* (Koide *et al.*, 1991).

Once specific antibodies will have been raised against such highly purified hormones these can be applied for immuno-cytochemical studies on pituitaries which are either too small to supply sufficient pituitary material for purification of the hormones, or are not easily accessible. The localisation of immuno-reactive GTHs can than be studied in order to investigate whether more than one type of gonadotropic cell can be distinguished. Given the strong homology between the gonadotropins described so far, it has to be expected that even heterologues immuno-cytochemistry will allow acceptable conclusions.

Molecular cloning has been carried out for a number of maturational gonadotropins (*i. e.* for carp, Chang *et al.*, 1990; for Pacific chinook salmon, Trinh *et al.*, 1986). If DNA probes are made available, the localisation and synthesis of⋅ GTHs during development to sexual maturity and the reproductive cycle can be studied in terms of GTH gene expression, even in very small fish in which plasma levels of pituitary hormone concentrations can not be measured by immuno-assays. Examples of such species, from which the development to puberty and the annual reproductive cycle has been studied extensively, but where the investigations of the gonadotropic activity of the pituitary necessarily have been limited to morphological studies are the platyfish, *Xiphophorus maculatus* (Schreibman *et al.*, 1986) and the stickle-back, *Gasterosteus aculeatus* (Borg, 1987).

Biological significance

So far, GTH I from salmon has been considered to be involved in gonadal development and vitellogenesis, whereas GTH II is referred to as the maturational hormone, controlling final oocyte maturation, ovulation and spermiation (Dickhoff *et al.*, 1990). This is only based, however, on the time sequence in appearance during pre-puberal development. There are no conclusive data on a difference in function, neither during development to sexual

maturation, nor during the reproductive cycle, apart from a relative difference in capacity to stimulate the synthesis of the maturation inducing hormone, $17\alpha, 20\beta$-P.

Physiological studies have to be initiated to investigate whether each of the two chemically different forms of GTHs indeed have their own specific function. These will imply the search for specific target tissues, receptor studies in these tissues and their specific responses.

Applied research: induction of sexual maturation

Studies on the duality of hypophysial control of gonadal functions not only are of importance from the fundamental point of view, but might also have practical implications. Till now artificial induction of reproduction always included the induction of final oocyte maturation and ovulation and in the case of males, induction of spermiation. Fish are treated either with gonadotropin releasing hormone (GnRH) analogous, in many cases in combination with a dopamine-D_2-receptor antagonist, or with more or less purified gonadotropin preparations. The aim of these applications is either to release hypophysial maturational gonadotropin or to apply it directly, in order to simulate the synthesis of maturation inducing ovarian steroids. In other protocols such steroids (17α, 20β-P or its precursor 17α-hydroxyprogesteron) were applied directly (c.f. Goos et al., 1987; Richter et al., 1987). Such treatments are restricted to fish species in which the development of oocytes through vitellogenic stages takes place spontaneously and the artificial induction only includes final maturation and ovulation of post-vitellogenic eggs. Some commercially interesting fish species, however, like the European and Japanese eels (Anguilla anguilla and Anguilla japonica), do not develop to sexual maturity under conditions of captivity. Under natural circumstances, sexual maturation starts at the final stage of migration from fresh water back to the sea and maturation is supposed to occur near the spawning grounds, presumably the Sargasso Sea and the Chinese Sea, respectively. Once the seaward-migrating, sexually maturing eels leave the rivers and estuaries, they are effectively lost to researchers and sexual mature eels have never been caught. So sexual maturation has to be mimicked in the laboratory in order to obtain animals that can be used for artificial propagation.

Attempts for controlled reproduc-

tion have been made for a long time for the European eel (Fontaine *et al.*, 1964; Boetius and Boetius, 1980; Prokhorchik *et al.*, 1987). Although ovarian growth, vitellogenesis and ovulation could be induced, viable eggs were never obtained. Attempts with the Japanese eel have been more successful. Yamauchi and collaborators succeeded to obtain fertilised eggs. The larvae hatched, but never survived for more than ten days (Yamauchi *et al.*, 1976). In a few more experiments larvae could be obtained (Matsumura, 1979), but always the survival rates were very poor.

In all experiments carried out so far, sexual maturation in eels was induced by injection of rather high dosages of HCG, carp, salmon or trout pituitary extracts. Most investigators ascribed the poor survival rate of the eel larvae to insufficient vitellogenesis, maybe leading to incomplete development of the gastro-intestinal tract of the embryos (personal communications) and the inability of the larvae to feed themselves. It might be expected that all above mentioned hormone preparations, used to induce sexul maturation of female eels, will contain mainly maturational gonadotropin since the pituitaries were

obtained from adult, sexually,mature fish. If, however, GTH I is the gonadotropin predominanty involved in the control of gonadal devolopment, sexual maturation and vitellogenesis, it might be hypothesised that the poor results with artificial propagation of the eel are due to the use of the inappropriate hormone: mainly GTH II in stead of GTH I.

Therefore, for the development of techniques for the induction of sexual maturation it is of great importance that the specific role of the presumed GTHs in this process will be clarified, that more information becomes available on the regulation of synthesis and release of the GTHs and that eventually GTH I preparations will be available.

The latter might prove to be difficult to achieve on a commercial base, but some experimental data suggest that long term treatment with androgens results in synthesis and release of "developmental GTH" : GTH I. Advanced development of gonadotropic cells in the juvenile pituitary, accompanied by GTH synthesis, could be achieved by androgen treatment by Schreibman *et al.* (1986) in the platyfish and by Crim and Evans (1979) and Gielen *et al.* (1982) in the rainbow trout. In their experiments with the trout, Gielen and Goos (1984)

demonstrated that the short term effect of androgens on GTH synthesis is a direct one, not necessarily mediated by a gonadotropic hormone-releasing hormone (GnRH) release. A longer lasting androgen treatment of juvenile rainbow trout resulted in precocious sexual maturation (Crim and Evans, 1982). Goos et al. (1986b) demonstrated that such a treatment caused enhanced GTH plasma levels and increased hypothalamic GnRH bioactivity. The assays used in these studies to measure hypophysial and plasma GTH concentrations were evidently not designed to measure GTH I or GTH II specifically. The experiments, therefore should be repeated, using a GTH I specific immuno-assay, to test whether indeed androgens in juveniles specifically stimulate synthesis and release of GTH I, thus causing precocious sexual maturation.

GONADOTROPIC HORMONE-RELEASING HORMONES (GnRHs)

Introduction

It is well established that in vertebrates gonadotropin secretion is under control of hypothalamic hormones, all belonging to one peptide family, the gonadotropin releasing hormones (GnRHs). The structure of five of these peptides has been identified: all are deca-peptides. The mammalian GnRH, the first member of the family, was found in porcine and ovine hypothalamus (Burgus et al., 1972). Two more GnRHs were isolated from chicken hypothalamus (chicken GnRH I and II) (King and Millar, 1982; Miyamoto et al., 1984) one from salmon brain (Sherwood et al., 1983) and one from lamprey brain (Sherwood et al., 1986).

GnRH in teleosts

In teleost fish, multiple forms of GnRH are present within tissue of a single species. Salmon-GnRH has been found in combination with chicken I or chicken II GnRH and also a number of unidentified GnRH-like peptides have been detected (King and Millar, 1985; Powell et al., 1986; Sherwood et al., 1984, Sherwood et al., 1989). In the European eel, chicken II and mammalian GnRH is present (King et al., 1990). In contrast, in mammals only one GnRH could be demonstrated (King et al., 1988).

In mammals it has been shown that GnRH is a cleavage product from a precursor molecule. This molecule comprises the GnRH se-

quence, followed by a 56-amino acid peptide (Adelman et al., 1986). This peptide, termed as GAP (GnRH associated peptide) has been shown to be a potent inhibitor of prolactine release in rat and human. Planas et al. (1990) demonstrated an inhibitory effect of human GAP on total prolactine release from tilapia pituitaries in vitro, but a stimulatory action on the release of newly synthesised prolactine.

All known GnRHs have the N-terminus and the COOH-terminus in common (pGlu-His and Pro-GlyNH$_2$, respectively). Both termini are important for receptor binding and receptor activation. Indeed, all GnRHs and their analogous with identical N- and C-terminus have gonadotropin releasing activity in teleosts. However, receptor affinity, and accordingly biological activity, might be different (De Leeuw et al., 1988b; Habibi et al., 1989a). Surprisingly, the native forms for a certain species are not necessarily the most active ones. For example in the African catfish, salmon GnRH isn't, but chicken II is present. However, sGnRH has higher receptor affinity compared to chicken II and the ED$_{50}$ is more than ten times lower (Goos et al., in preparation).

Biological significance of multiple forms of GnRH

The biological significance of multiple forms of GnRH in the brain is not clear. Studies in chicken (Sharp et al., 1990) revealed that chicken GnRH I, in contrast to chicken II, is closely related to reproductive functions. So it seems that in this species, as in mammals two gonadotropins are under control of a single GnRH. In fish the situation might be different. Although GnRHs are found in different brain areas, suggesting more functions than the control of gonadotropin secretion, in teleosts usually both GnRH's are found in nerve endings in the pituitary near the gonadotropic cells (Kah et al., 1986; Yu et al., 1987, 1988; Okuzawa et al., 1990). In the African catfish, catfish GnRH I and II are even co-localised within the same secretory granules in neurosecretory fibres in the gonadotropic area of the pituitary (Zandbergen et al., 1991). It is not known what this means for the the control of the secretion of the gonadotropic hormones. In goldfish, it was shown that GnRH not only regulates gonadotropin secretion, but also the secretion of growth hormone (Marchant et al., 1989). The presence of immuno-reactive GnRH in different

brain areas suggests a neurotrans-
mitter function of the peptides. In
this respect, it has been postulated
that GnRH in the olfactory tract
may serve the transmisson of sex
pheromonal stimuli (c.f. Resink,
1989).

GnRH action and its relation to other hypophysiotropic and peripheral hormones

GnRH binds specifically to re-
ceptors in the pituitary. Habibi et al.
(1987) found two GnRH receptors in
the goldfish hypophysis while De
Leeuw et al. (1988a) and Anderson
et al. (1989) described a single bind-
ing site in the pituitary of the Afri-
can catfish and the stickle-back,
respectively.

Dopamine interferes with the
action of GnRH on its hypophysial
targets (i.e. Chang and Peter, 1983;
De Leeuw et al., 1985). Via D$_2$ type
of dopamine receptors it inhibits the
spontaneous or GnRH stimulated
GTH release (Omuljaniuk et al.,
1987; Van Asselt et al., 1988). In
goldfish and the African catfish
there is evidence that dopamine
acts via a down regulation of GnRH
receptors. It has been known for
a long time that gonadal steroids
have a negative feedback action on
gonadotropin release. An important

pathway in the mechanism of
action of gonadotropin secretion
regulating hormones such as gonadal
steroids and hypothalamic dopamine
on hypophysial gonadotropin output
involves a regulation of GnRH
receptor activity (Habibi et al.,
1989b).

Investigations on the mechanism
of action of GnRH have shown that
this is a calcium and protein kinase C
dependent process (Van Asselt et al.,
1989; Chang et al., 1990, 1991). Unlike
in mammals, cAMP appears to be
involved too in GnRH action (Goos
et al., 1991; Levavi-Sivan and Yaron,
1991).

With regard to the regulation of
the secretion of GnRH it has been
known for some time that in mam-
mals GnRH release is under opiod
and catecholamine control (Kalra
and Kalra, 1983; Baraclough and
Wise, 1982). Recently, Yu et al.
(1991) have shown in in vitro ex-
periments that in the goldfish GnRH
release from the hypothalamus and
pituitary is inhibited by dopamine
but stimulated by serotonin. It is
not clear yet whether dopamine not
only inhibits GnRH release but also
GnRH gene expression.

Practical applications

The understanding of the dual

control (by GnRH and dopamine) of the gonadotropin secretion has had important consequences for the development of techniques for the artificial induction of ovulation in fish that do not ovulate spontaneously in captivity. Stable GnRH analogous, in combination with dopamine receptor antagonists are being used and commercially available for the propagation of species like carp, loach and catfish. The GnRH analogous usually are synthetic mammalian or salmon GnRH, with modifications in the sixth position to prevent enzymatic breakdown and thus enlarging the half-life time. A commonly used dopamine receptor antagonist is Domperidone, but also Pimozide or Sulperide are very effective. Such technique can be used in species where post-vitellogenic eggs are readily available and only the final maturation and ovulation has to be induced. However, in species where not only the pre-ovulatory GTH surge fails to occur, but also the gonadal development is impaired, long term treatment with GnRH might be successful as was shown by Lee *et al.* (1986) for the milkfish, *Chanos chanos*. Crim *et al.* (1986) used longer GnRH treatments to synchronise reproductive cycles in the female Atlantic salmon, *Salmo*

salar (Zohar, unpublished data).

Future developments will probably more be directed to fundamental than to applied aspects on GnRH. Protocols for the induction of oocyte maturation and ovulation in fish with post-vitellogenic eggs might need adaptation when used in new species.

For species where artificial reproduction not only includes the induction of final maturation and ovulation, but also gonadal development as in eels, fully effective hormone treatments still have to be developed. GnRH applications could be part of such treatments as shown in recent experiments with Japanese eels (Hirose, personal communication). Female eels were treated for several weeks with silver carp pituitary extracts. As soon as body weight started to increase, due to hydration of the oocytes, the fish were injected with LHRH analog, which caused rapid ovulation of viable eggs.

There are, however, many fundamental questions left.

Localisation of GnRH system in the brain

Immuno-cytochemical studies have revealed a rather heterogenous

distributional pattern of GnRH. However, from studies in platyfish, *Xiphophorus maculatus* (Schreibman et al., 1986), stickle-back (Borg et al., 1982) and goldfish (Kah et al., 1986), it appeared that the antero-ventral preoptic region and the posterior part of the nucleus lateralis tuberis are possible sites of GnRH origin. However, the antiserum used can influence the results of immuno-cyto-chemical studies. Goos et al. (1985) found with an anti-mammalian-GnRH, immuno-reactivity in numerous neurons of the preoptic nucleus of the African catfish. Using, however a highly specific antibody to chicken II GnRH, one of the native forms of GnRH in the catfish, only a few neurons in the caudal part of the telencephalon showed a reaction. Both antisera show strong reactivity in the same neuro-secretory fibres in the proximal pars distalis of the pituitary. Although immuno-cyto-chemistry has shown its value for the detection of GnRH, the results are controversial. *In situ* hybridisation with the cDNAs encoding for GnRHs not only will solve the question as where GnRHs originate from, but also will give more information about GnRH gene expression under normal physiological and experimental conditions.

Mechanisms of interaction of GnRH and other hormones, *i. e.* dopamine and sex steroids

The mechanism of action of dopamine and sex steroids on GnRH-stimulated GTH release is partly understood. For both dopamine and gonadal steroids it has been shown that they act at the level of GnRH receptor binding, both causing a decrease in GnRH binding capacity (De Leeuw et al., 1988a, 1989b; Habibi et al., 1989b).

GnRH action depends on extracellular calcium and protein kinase C. It has to be investigated however, whether dopamine blocks Ca-influx or acts at the level of protein kinase C.

cAMP is involved in the action of GnRH in teleosts (Goos et al., 1991; Levavi-Sivan and Yaron, 1991). Although dopamine *in vitro* completely blocked the release of GTH from catfish pituitaries, it had no influence on cAMP generation.

Preliminary experiments in African catfish have shown that inositol phosphate metabolism might be involved in the action of GnRH. It still has to be investigated whether dopamine interferes at this level.

Unpublished data on the effect of GnRH in the African catfish have

demonstrated that GnRH not only stimulates the release of GTH but also its synthesis and that the last synthesised GTH is being released first ("Last in, first out"-hypothesis). We have no data yet on the effect of dopamine on the GnRH induced GTH synthesis.

Indeed, there are many questions left with regard to the mechanism of action of GnRH and its relation to other hormones and neuro-transmitters.

In more than fifteen species it has been shown now that multiple forms of GnRH are present and it has to be expected that there will be more of this in the near future. From the evolutionary point of view interesting speculations can be made. The physiological significance of GnRHs however, apart from their hypophysiotropic action, awaits clarification. It might be a working hypothesis to assume that GnRH-like peptides, and maybe other peptides, co-expressed as part of the same pro-hormone molecule, all belong to one peptide family, the members of which each have their specific function in reproduction.

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