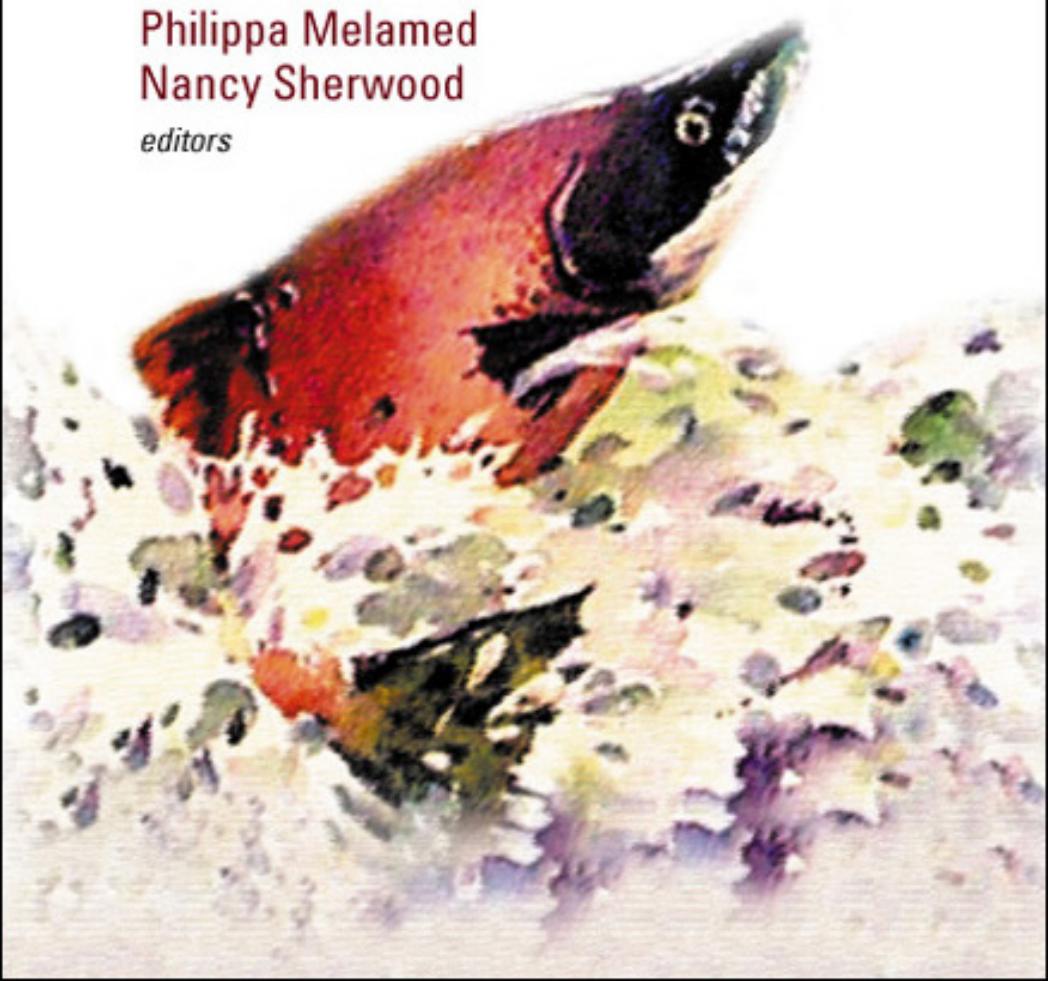


Hormones and their Receptors in Fish Reproduction

Philippa Melamed
Nancy Sherwood
editors





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Preface

In this monograph we focus on the molecular mechanisms through which hormones regulate reproductive activity and function in fish. Over the past decade or so this field has changed dramatically. Although it was known for many years that GnRH promotes reproductive activity by stimulating production and release of the pituitary gonadotropins, recent discoveries of multiple forms of GnRH with wide expression patterns have complicated the picture. Cloning and localization studies of the various isoforms have revealed that two or three forms are expressed in the brain alone, but only one of these is found in the preoptic area and this comprises the major regulator of the gonadotropins. This has led to interesting studies on the differential regulation of expression of these genes (Sherwood and Adams). At the pituitary gonadotrope, the GnRH binds a seven-transmembrane G-protein coupled receptor which was seen in mammals to lack the C-terminal intracellular tail. This was shown to affect its internalization, but not its desensitization. The cloning of the catfish GnRH-R that does contain a C-terminal intracellular tail, as do other non-mammalian receptors, and subsequent structure-function studies have done much to elucidate the receptor-ligand interactions and pharmacology of these receptors (Blomenröhr *et al.*).

We now know that the pituitary produces two gonadotropins, but it was only in the late 1980s and early 1990s that the cDNAs encoding the

teleost equivalent of the follicle stimulating hormone (FSH), initially known as GtH I, and luteinizing hormone (LH or GtH II), were first isolated. Further studies in additional species showed that the structures of the two fish gonadotropins largely parallel the mammalian LH and FSH, while their circulating levels and actions indicate they are true homologs. This has established the duality of gonadotropins in teleost fish and opened up new areas of research into the mechanisms through which they are differentially regulated. Molecular analysis of the regulatory regions of these genes has been somewhat elusive, owing to difficulties in isolating these genes, but the field is rapidly advancing. The Chinook salmon LH β gene promoter has been particularly well studied and shows interesting variations from the conserved mammalian LH β genes, while similar studies are underway on the FSH β gene (Chong *et al.*). A part of the differential regulation of the gonadotropins undoubtedly comes from opposing effects of activin which stimulates FSH β expression and, perhaps uniquely in fish, has an inhibitory effect on the LH. At the level of the gonad, the activin β A is stimulated by the gonadotropins and, in turn, it powerfully stimulates oocyte maturation (Ge).

The gonadotropins stimulate steroidogenesis through an elaborate pathway of steroidogenic enzymes. The starting point of this pathway is the StAR enzyme which mobilizes cholesterol and is expressed in a wide range of tissues. In mammals StAR expression results from activation of cAMP, although this may differ in teleosts. As the fish progress through reproductive development, the steroid production shifts due to alterations in the levels of the relevant steroidogenic enzymes from androgen/estrogen production to producing primarily progestogens (Young *et al.*). The resulting circulating steroids stimulate gametogenesis, gamete release and they affect reproductive behavior. These effects are mediated through specific steroid hormone receptors, of which the estrogen receptor (ER) has been studied in most detail. In fact, two distinct estrogen receptors and several isoforms are present in fish, and many show tissue restricted expression. In this way estrogen can exert specific effects on its target genes, some of which are mediated through synergistic interactions of ER with factors activated by alternatively stimulated pathways (Menuet *et al.*).

In females, a vital role of estrogen is stimulation of the production of vitellogenin which provides nutrition for early development of the larvae. Although this gene is highly sensitive to stimulation by estrogen, the ER binding sites differ considerably from the consensus, yet are clearly involved in transactivation by ER. Subsequent to its production, the vitellogenin must then be transported into the oocytes where the protein is cleaved into yolk proteins for consumption by the young (Ding). In some teleost species, as in mammals, estrogen also stimulates production of prolactin which has diverse roles in reproduction of mammals and fish, the latter of which have only partially been elucidated but may include regulating reproductive behavior and steroidogenesis (Le Rouzic and Prunet).

Although this field is constantly expanding and no work could present a complete picture, this volume presents an up-to-date picture of some of the recent advances in molecular mechanisms involved in regulating fish reproduction and will be of interest those working in or new to the field.

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Chapter 1

Gonadotropin-Releasing Hormone in Fish: Evolution, Expression and Regulation of the GnRH Gene

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Abstract

Fish, the largest group of vertebrates, use diverse reproductive strategies. Considerable advances have been made in understanding the structure and role of GnRH, the main hormone regulating reproduction. Twenty four distinct peptide structures are known for GnRH from octopus to human. Genes and cDNAs have been cloned and sequenced for both invertebrates and vertebrates. Genomic studies are being used to verify the number and type of GnRH forms and to look for novel forms. Mapping of the evolutionary pathway of GnRH orthologs and paralogs has just begun, but is advancing with the combined use of structures for the peptide, cDNA and gene structures. Phylogenetic analysis, distribution patterns and linkage studies in individual species will help to define the relationships and possibly the orthologs for the GnRH molecule. Expression studies suggest that GnRH is widely expressed outside of the classical brain areas of the olfactory brain, telencephalon, preoptic area and midbrain. An emerging area of interest in fish reproduction is GnRH gene regulation in which upstream and downstream promoters, transcription factors, cell-specific expression and duplicate genes have been studied.

Introduction

Reproduction in fish has been of interest since ancient times, but only recently with the advent of protein chemistry and molecular biology have we been able to identify molecules such as GnRH that control reproduction and coordinate reproduction with the environment. Fish have always posed a special challenge for the study of reproduction because there are about 25,000 species and they have diverse strategies to produce offspring. Consequently, a few species such as salmon and goldfish were selected for study as representative fish. However, the introduction of genomics and proteomics has begun to change this scenario. Now, the full genome (complete DNA sequence) of fugu and partial genomes of zebrafish, Atlantic salmon, tilapia and medaka are available with the promise of more fish genomes to come.

Molecular biology and genomics have revolutionized the study of fish reproduction as to the structure of hormones and their receptors, intracellular signaling molecules and enzymes for synthesis of steroids. In parallel, microarrays (gene chips) are being developed for obtaining mRNA expression profiles in response to the presence or absence of hormones in a variety of tissues and stages. To date, microarrays have been prepared to determine changes in expression of hundreds or thousands of genes concurrently during development in zebrafish¹ or adaptation to environmental changes in zebrafish,¹ mudsuckers² and channel catfish.³ In addition, microarrays for other fish including Atlantic salmon are available, which is the initial step in examining expression of genes in tissues related to reproduction.

The goal of functional genomics is to establish the function of individual genes and gene networks within a cell or tissue. In endocrinology, the identification of hormone-responsive transcriptomes is important. Thus, to understand the function of a reproductive hormone and its receptor it is necessary to identify the network of genes that are activated or repressed in a specific tissue. The hormone may bind to its receptor in the plasma membrane, but the cascade of factors that are activated including transcription factors that bind to specific genes can be identified. This gives a clue not only to the function of the hormone,

but also to the function and regulation of the target genes and their role in a network. To date, this global approach has been applied predominately in mammals, but the same principles apply to fish. For mammals, a database (Ovarian Kaleidoscope Database-II) has been prepared for genes identified specifically in the ovary.⁴ Information is available on each identified gene as to function, chromosome location, expression in specific types of ovarian cells, alternative splicing and mutant phenotypes.

An example of a genomics approach is the study of the effect of GnRH on one of its receptors in a pituitary cell line. Stuart Sealfon and coworkers have studied in detail the effects of GnRH on cell signaling and transcription factors using microarray analysis.⁵ Likewise, proteomics (identification of proteins expressed simultaneously in a specific tissue at a specific stage) has become important in mammalian studies, but remains to be tapped by fish studies to identify concurrent proteins or transcription factors that increase or decrease in response to stimuli such as GnRH. Also, proteomics is useful in identifying protein products that result from alternative splicing and posttranslational modifications, both of which lead to an increase in protein diversity. The percentage of splicing for fish transcripts is not known yet, but about 35–50% of human genes have spliced transcripts.⁶ Thus, the protein profile can give a fuller picture than mRNA alone of cell function. In short, the proteome is much larger than the transcriptome.

GnRH Structure in Fish

GnRH in fish, as in other vertebrates, is a ten-amino acid peptide made primarily in nerve cells in the brain. In addition, GnRH peptides have been reported in ovary and testis of fish and in ovary, testis, mammary gland and placenta of mammals. The structure of GnRH in jawless fish, cartilaginous fish and bony fish has been determined to date primarily by protein chemistry and molecular biology. Fish share two forms of GnRH (mammalian (m)GnRH and chicken (c)GnRH-II) with other vertebrates, but additionally have nine forms of GnRH that are distinct to fish. The eleven GnRH structures determined for fish are shown

in the context of other vertebrates and invertebrates (Fig. 1). The conclusion from examining GnRH structures is that they are highly conserved and follow directly from the nine GnRHs recently identified in protochordates (tunicates or sea squirts).⁷ The GnRH identified in octopus is 12 amino acids (Fig. 1), but is likely part of the GnRH family.⁸

1 2 3 4 5 6 7 8 9 10

Mammal	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂
Whitefish	pGlu-His-Trp-Ser-Tyr-Gly- <u>Met</u> - <u>Asn</u> -Pro-Gly-NH ₂
Salmon	pGlu-His-Trp-Ser-Tyr-Gly- <u>Trp</u> - <u>Leu</u> -Pro-Gly-NH ₂
Sea Bream	pGlu-His-Trp-Ser-Tyr-Gly-Leu- <u>Ser</u> -Pro-Gly-NH ₂
Pejerrey	pGlu-His-Trp-Ser- <u>Phe</u> -Gly-Leu- <u>Ser</u> -Pro-Gly-NH ₂
Herring	pGlu-His-Trp-Ser- <u>His</u> -Gly-Leu- <u>Ser</u> -Pro-Gly-NH ₂
Catfish	pGlu-His-Trp-Ser- <u>His</u> -Gly-Leu- <u>Asn</u> -Pro-Gly-NH ₂
Dogfish	pGlu-His-Trp-Ser- <u>His</u> -Gly- <u>Trp</u> - <u>Leu</u> -Pro-Gly-NH ₂
Chicken-II	pGlu-His-Trp-Ser- <u>His</u> -Gly- <u>Trp</u> - <u>Tyr</u> -Pro-Gly-NH ₂
Lamprey-III	pGlu-His-Trp-Ser- <u>His</u> - <u>Asp</u> - <u>Trp</u> - <u>Lys</u> -Pro-Gly-NH ₂
Lamprey-I	pGlu-His- <u>Tyr</u> -Ser- <u>Leu</u> -Glu- <u>Trp</u> - <u>Lys</u> -Pro-Gly-NH ₂

Guinea Pig	pGlu- <u>Tyr</u> -Trp-Ser-Tyr-Gly- <u>Val</u> -Arg-Pro-Gly-NH ₂
Chicken-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu- <u>Gln</u> -Pro-Gly-NH ₂
Frog	pGlu-His-Trp-Ser-Tyr-Gly-Leu- <u>Trp</u> -Pro-Gly-NH ₂
Tunicate-1	pGlu-His-Trp-Ser- <u>Asp</u> - <u>Tyr</u> - <u>Phe</u> - <u>Lys</u> -Pro-Gly-NH ₂
Tunicate-2	pGlu-His-Trp-Ser- <u>Leu</u> - <u>Cys</u> - <u>His</u> - <u>Ala</u> -Pro-Gly-NH ₂
Tunicate-3	pGlu-His-Trp-Ser-Tyr- <u>Glu</u> - <u>Phe</u> - <u>Met</u> -Pro-Gly-NH ₂
Tunicate-4	pGlu-His-Trp-Ser- <u>Asn</u> - <u>Gln</u> -Leu- <u>Thr</u> -Pro-Gly-NH ₂
Tunicate-5	pGlu-His-Trp-Ser-Tyr- <u>Glu</u> - <u>Tyr</u> - <u>Met</u> -Pro-Gly-NH ₂
Tunicate-6	pGlu-His-Trp-Ser- <u>Lys</u> -Gly- <u>Tyr</u> - <u>Ser</u> -Pro-Gly-NH ₂
Tunicate-7	pGlu-His-Trp-Ser-Tyr- <u>Ala</u> -Leu- <u>Ser</u> -Pro-Gly-NH ₂
Tunicate-8	pGlu-His-Trp-Ser- <u>Leu</u> - <u>Ala</u> -Leu- <u>Ser</u> -Pro-Gly-NH ₂
Tunicate-9	pGlu-His-Trp-Ser- <u>Asn</u> - <u>Lys</u> -Leu- <u>Ala</u> -Pro-Gly-NH ₂
Octopus	
	pGlu- <u>Asn</u> - <u>Tyr</u> -His- <u>Phe</u> -Ser- <u>Asn</u> -Gly- <u>Trp</u> - <u>His</u> -Pro-Gly-NH ₂

Fig. 1 Structure of the 24 known GnRH forms. Amino acids that are different from the mammalian GnRH form are underlined. GnRH forms that have been identified in fish are boxed.

Genomics and GnRH in Fish

The sequencing of the complete genome of two species of pufferfish (*Fugu*) allows us for the first time to determine with near certainty the total number of distinct GnRH peptides within a single fish species and the copy number for each gene. In pufferfish (*Fugu rubripes*) we have identified only one copy of each of three forms of GnRH in the genome: seabream (sb)GnRH, salmon (s)GnRH and chicken (c)GnRH-II (personal observation). Thus, analysis of the genome confirms earlier studies showing that (1) many teleosts have three forms of GnRH, (2) each form of GnRH is encoded by a separate gene and (3) *Fugu* has the same three forms of GnRH (sbGnRH, sGnRH, cGnRH-II) as other fish in the same order.

Only the partial genome of zebrafish (*Danio rerio*) has been sequenced to date, but it confirms that only two forms of GnRH are present: sGnRH and cGnRH-II.⁹ Additionally, the genes for these two GnRHs have been isolated and sequenced.^{10,11} To date, only one copy of each zebrafish GnRH gene has been identified in genome analysis or experimentally.

The genome for Atlantic salmon (*Salmo salar*) is in the process of being sequenced. To date, only one copy of each gene for sGnRH and cGnRH-II has been identified.

Evolution of Fish and the GnRH Gene

A current hypothesis for evolution of fish is that these early vertebrates are descendants of ancestral urochordates (tunicates) or cephalochordates (e.g. *Amphioxus*). Two duplications of the entire genome are thought to have occurred early in fish evolution.^{12,13} The exact timing of these duplications is still controversial, but there are arguments suggesting the events happened near the origin of ancestral hagfish and lamprey.¹⁴ The additional DNA is proposed to be the source of expansion from single genes to gene families, creating animals with greater complexity of structural and regulatory molecules. However, many genes after the duplication were lost as functional genes, likely by rearrangement or mutation.

At present only two forms of GnRH have been identified by structure in single species of jawless fish (lamprey), cartilaginous fish (ratfish and dogfish) and early evolving bony fish (sturgeon). The primary structure of these

GnRHs has been sequenced using protein chemistry, plus the cDNA for lamprey GnRH-I has been identified. Lamprey and dogfish GnRHs appear to be restricted to the jawless and cartilaginous classes of fishes, respectively, if the criterion for identification is structure. Lamprey and salmon GnRH were reported to be present in humans using immunological techniques, but are not encoded in the human genome. Thus, only mGnRH (early bony fish, eel and butterfly fish) and cGnRH-II (cartilaginous and all bony fish including teleosts) are shared by some fish and tetrapods.

Teleosts present a more complicated, but interesting story. It is proposed that a further duplication of the genome occurred somewhere preceding or during early teleostean evolution.¹⁵ One proof rests with the analysis of Hox genes. Early chordates (tunicates and *Amphioxus*) have only one cluster of Hox genes; coelacanth and tetrapods (including humans) have four clusters,¹⁶ but some teleosts such as zebrafish have seven clusters on separate chromosomes.^{16,17} It remains to be determined whether a third genome duplication occurred early in teleosts but subsequently led to a reduction in Hox clusters to seven in zebrafish and to four in *Fugu*, medaka and striped bass or only certain groups of fish had a third duplication of the genome.¹⁵

An attempt to match the phylogenetic events of the GnRH gene with the Hox cluster is not straightforward. One might predict that tunicates would have a single form of GnRH on a single gene, but the complete genome for each of two tunicates, *Ciona intestinalis* and *Ciona savignyi*, shows that each species has two GnRH genes and each gene encodes three GnRH peptides in tandem.⁷ The structure of the tunicate GnRHs is ten amino acids and has 60–80% identity to those in the vertebrate GnRH family. So, duplication of the region encoding GnRH and the full gene must have occurred in the stem line of the tunicates after ancestral vertebrates and tunicates separated about 600 million years ago. In lamprey, sharks and early bony fish (sturgeon), only two GnRH peptides have been identified within each species, even though sturgeon are polyploid. Alas, neither the cDNAs nor genes for GnRH have been isolated for any jawless or cartilaginous fish except for lamprey GnRH.¹⁸ In theory, one might expect eight forms of GnRH in teleost fish (zebrafish have seven Hox clusters), but only three distinct forms of GnRH have been identified to date in any one species, including *Fugu*, after a genome search (Fig. 2).

A further duplication of the genome beyond that of other teleosts is thought to have occurred in certain orders of teleost fish including the Salmoniformes (e.g. whitefish, grayling, salmon and trout), Cypriniformes (e.g. goldfish and common carp) and Siluriformes (catfish). For a long time, only two forms of GnRH could be identified in these fish. However, it was shown recently that a basal group of salmonids, whitefish, have three forms,¹⁹ whereas in the later evolving salmonids such as sockeye, chum, and rainbow trout, only two forms of GnRH have been identified. Another type of proof for the additional duplication of salmonids and goldfish is duplicate copies of GnRH genes. Three species of salmon have two copies of the gene encoding sGnRH²⁰⁻²⁴ and goldfish have two copies of the gene encoding cGnRH-II.^{25,26} These duplicate genes encode identical peptides, but vary in nucleotides for the signal peptide, gene-associated peptide (GAP) and untranslated regions. If present, duplicate copies should be easier to find when the genomes for these fish are available.

Phylogenetic Analysis of GnRH Genes

All of the distinct GnRH forms in fish to date have been identified using protein sequencing after purification with antibodies. These eleven fish GnRH forms include lamprey GnRH-I,²⁷ lamprey GnRH-III,²⁸ dogfish,²⁹ herring,³⁰ whitefish,¹⁹ salmon,³¹ catfish,^{32,33} pejerrey,³⁴ sea bream,³⁵ chicken GnRH-II^{29,30,32-38} and mammal.³⁹ The latter two forms were first isolated as proteins from chicken⁴⁰ and pig,⁴¹ respectively. Some of these same eleven GnRH peptides were also isolated from other species of fish by protein and/or cDNA sequencing (Fig. 2). However, a primary structure of only ten amino acids does not contain enough information to prepare a useful phylogenetic tree.

Many vertebrate species share the known forms of GnRH, as there are only 14 distinct vertebrate forms identified to date. In a number of species, the cDNAs have been determined for nine of the 11 known GnRHs; only lamprey GnRH-III and dfGnRH remain to be isolated and cloned, as shown in Fig. 2. The translated portion of the precursor (pre-proGnRH) molecule does contain sufficient information to prepare a phylogenetic tree (Fig. 3).

Class	Name	cDNA/gene gnrh 1,2,3	Genus species	oGnRH	tGnRH	IGnRH	dGnRH
Mollusca	Octopus	*	<i>Octopus vulgaris</i>		9 forms		
Ascidioaceans	tunicates		<i>Chelyosoma productum</i>		2/9 forms		
		*	<i>Ciona intestinalis</i>		6/9 forms	IGnRH	
		*	<i>Ciona savignyi</i>		5/9 forms	2 forms	
Agnatha	lamprey	*	<i>Petromyzon marinus</i>				
Chondrichthyes	dogfish		<i>Squalus acanthias</i>				
	ratfish		<i>Hydrologus collettei</i>				
Osteichthyes	sturgeon		<i>Acipenser gueldenstaedti</i>				
teleosts	Japanese eel	**	<i>Anguilla japonica</i>				
	arawana	**	<i>Scleropages jardini</i>				
	herring	*	<i>Clupea harengus pallasi</i>				
	shad	*	<i>Alosa sapidissima</i>				
	whitefish	***	<i>Coregonus clupeaformis</i>				
	sockeye salmon	*	<i>Oncorhynchus nerka</i>				
	coho salmon	*	<i>Oncorhynchus kisutch</i>				
	chum salmon	*	<i>Oncorhynchus keta</i>				
	chinook salmon	*	<i>Oncorhynchus tshawytscha</i>				
	masu	*	<i>Oncorhynchus masou</i>				
	rainbow trout	**	<i>Oncorhynchus mykiss</i>				
	Atlantic salmon	*	<i>Salmo salar</i>				
	brown trout	*	<i>Salmo trutta</i>				
	brook trout	*	<i>Salvelinus fontinalis</i>				
	goldfish	**	<i>Carassius auratus</i>				
	roach	**	<i>Rutilus rutilus</i>				
	zebrafish	**	<i>Danio rerio</i>				
	pacu		<i>Piaractus mesopotamicus</i>				
	African catfish	**	<i>Clarias gariepinus</i>				
	plainfin midshipman	*	<i>Porichthys notatus</i>				
	pejerrey		<i>Odontesthes bonariensis</i>				
	medaka	***	<i>Oryzias latipes</i>				
	gilthead seabream	***	<i>Sparus aurata</i>				
	red seabream	*	<i>Pagrus major</i>				
	African cichlid	***	<i>Haplochromis burtoni</i>				
	tilapia	*	<i>Oreochromis mossambicus</i>				
	tilapia	*	<i>Oreochromis niloticus</i>				
	striped sea bass	***	<i>Morone saxatilis</i>				
	European sea bass	***	<i>Dicentrarchus labrax</i>				
	barfin flounder	***	<i>Verasper moseri</i>				
	pufferfish	***	<i>Fugu rubripes</i>				
	freshwater pufferfish	***	<i>Tetraodon nigroviridis</i>				
Amphibia	frog	*	<i>Rana dybowskii</i>				
	frog		<i>Rana ridibunda</i>				
	bull frog	**	<i>Rana catesbeiana</i>				
	frog	*	<i>Xenopus laevis</i>				
Reptilia	alligator		<i>Alligator mississippiensis</i>				
Aves	chicken	*	<i>Gallus domesticus</i>				
Mammalia	mouse	*	<i>Mus musculus</i>				
	rat	*	<i>Rattus norvegicus</i>				
	guinea pig	*	<i>Cavia porcellus</i>				
	sheep		<i>Ovis aries</i>				
	pig		<i>Sus scrofa</i>				
	musk shrew	**	<i>Suncus murinus</i>				
	tree shrew	**	<i>Tupaia glis belangeri</i>				
	rhesus monkey	**	<i>Macaca mulatta</i>				
	human	**	<i>Homo sapiens</i>				

Fig. 2 Species in which GnRH structure is determined by protein, cDNA or gene. A colored box indicates the form identified in each species and an asterisk (*) indicates if a cDNA or gene has been isolated (gnrh2 = cGnRH-II, gnrh3 = sGnRH, gnrh1 = all other forms).

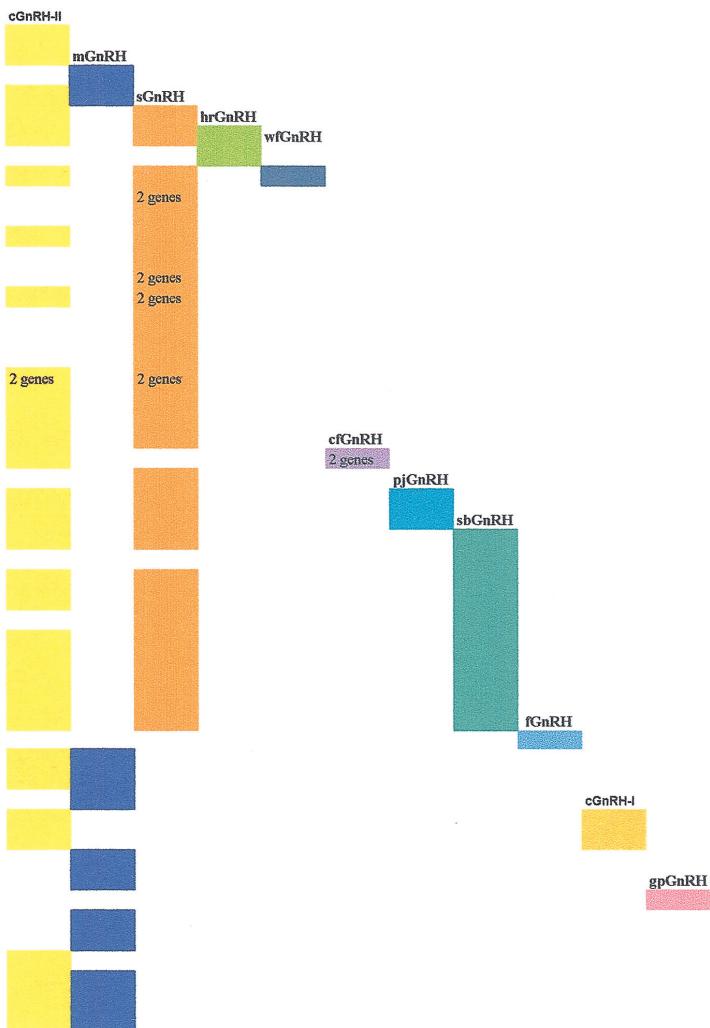


Fig. 2 (Continued)

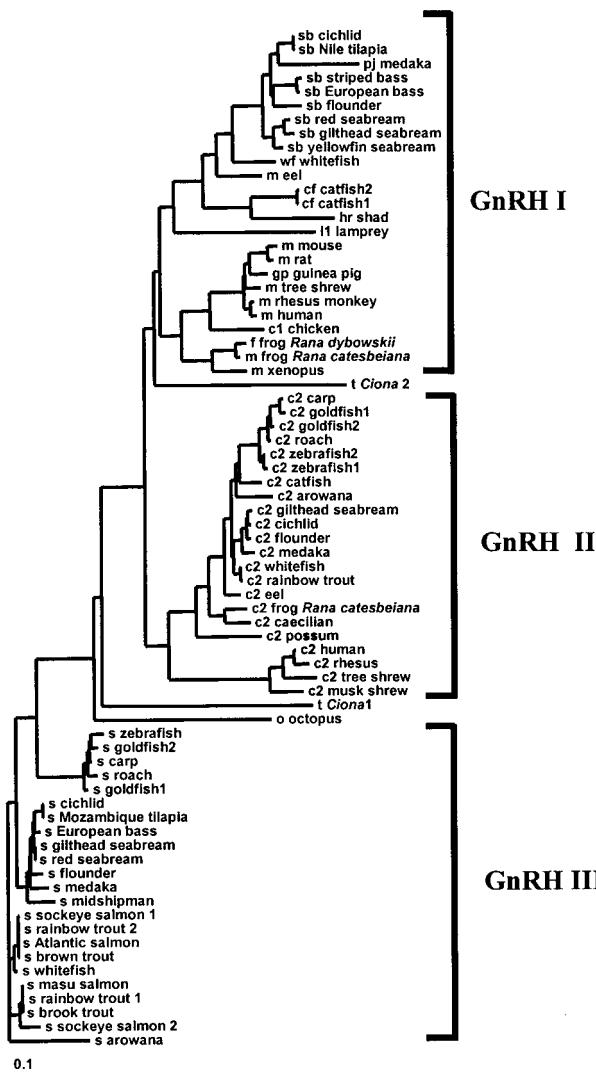


Fig. 3 Phylogenetic relationship of precursors derived from known DNA sequences encoding gonadotropin-releasing hormone (GnRH). The relationship was generated with CLUSTAL W and the unrooted tree was generated using Treeview version 1.5.2. The scale bar represents the estimated evolutionary distance as 0.1 amino acid substitutions per site. In alphabetical order the precursors, species and accession numbers are listed. Adapted from Vickers *et al.*, *Biol. Reprod.* **70**: 1136–1146, 2004.

chicken (c)GnRH-I		
c1 chicken (<i>Gallus gallus</i>)		X69491
chicken (c)GnRH-II		
c2 arowana (<i>Scleropages jardinii</i>)		AB047326
c2 caecilian (<i>Typhlonectes natans</i>)		AF167558
c2 carp (<i>Cyprinus carpio</i>)		AY147400
c2 catfish (<i>Clarias gariepinus</i>)		X78047
c2 cichlid (<i>Haplochromis burtoni</i>)		AF076962
c2 eel (<i>Anguilla japonica</i>)		AB026990
c2 European bass (<i>Dicentrarchus labrax</i>)		AF224281
c2 flounder (<i>Verasper moseri</i>)		AB066359
c2 frog (<i>Rana catesbeiana</i>)		AF186096
c2 gilthead seabream (<i>Sparus aurata</i>)		U30325
c2 goldfish 1 (<i>Carassius auratus</i>)		U40665
c2 goldfish 2		U40568
c2 human (<i>Homo sapiens</i>)		NM001501
c2 medaka (<i>Oryzias latipes</i>)		AB041334
c2 musk/house shrew (<i>Suncus murinus</i>)		AF107315
c2 possum (<i>Trichosurus vulpecula</i>)		AF193516
c2 rainbow trout (<i>Oncorhynchus mykiss</i>)		AF125973
c2 rhesus monkey (<i>Macaca mulatta</i>)		AF097356
c2 roach (<i>Rutilus rutilus</i>) cGnRH-II		U60668
c2 striped bass (<i>Morone saxatilis</i>)		AF056313
c2 tree shrew (<i>Tupaia glis</i>)		U63327
c2 whitefish (<i>Coregonus clupeaformis</i>)		AY245102
c2 zebrafish 1 (<i>Danio rerio</i>)		AF511531
c2 zebrafish 2		AY094357
catfish (cf)GnRH		
cf catfish 1		X78049
cf catfish 2		X78048
frog (f)GnRH		
f frog (<i>Rana dybowskii</i>)		AF139911
guinea pig (gp)GnRH		
gp guinea pig (<i>Cavia porcellus</i>)		AF033346
herring (hr)GnRH		
hr shad (<i>Alosa sapidissima</i>)		AF536381
lamprey (l)GnRH-I		
ll lamprey (<i>Petromyzon marinus</i>)		AF144481
mammalian (m)GnRH		
m eel		AB026989
m frog (<i>Rana catesbeiana</i>)		AF188754
m human		NM000825
m mouse (<i>Mus musculus</i>)		M14872
m rat (<i>Rattus norvegicus</i>)		NM012767
m rhesus monkey		Dong <i>et al.</i> , 1996

m	tree shrew	U63326
m	<i>Xenopus laevis</i>	L28040
octopus (oc)GnRH		
oc	octopus (<i>Octopus vulgaris</i>)	AB037165
pejerrey (pj)GnRH		
pj	medaka	AB041336
salmon (s)GnRH		
s	arowana	AB047325
s	Atlantic salmon (<i>Salmo salar</i>)	X79709
s	brook trout (<i>Salvelinus fontinalis</i>)	X79712
s	brown trout (<i>Salmo trutta</i>)	X79713
s	carp	AF521130
s	cichlid	AF076961
s	European seabass	AF224280
s	flounder	AB066358
s	gilthead seabream	U30311
s	goldfish sGnRH1	U30301
s	goldfish sGnRH2	AB017271
s	masu salmon (<i>Oncorhynchus masou</i>)	S44614
s	midshipman (<i>Porichthys notatus</i>)	S79620
s	medaka	AB041335
s	Mozambique tilapia (<i>Oreochromis mossambicus</i>)	AY167989
s	Nile tilapia (<i>Oreochromis niloticus</i>)	AF467291
s	rainbow trout 1	AF232212
s	rainbow trout 2	AF232213
s	red seabream (<i>Pagrus major</i>)	D26108
s	roach	U60667
s	sockeye salmon 1 (<i>Oncorhynchus nerka</i>)	D31868
s	sockeye salmon 2	D31869
s	whitefish	AY245103
s	zebrafish	AJ304429
seabream (sb)GnRH		
sb	cichlid (<i>H. burtoni</i>)	F076961
sb	European bass	AF224279
sb	flounder	AB066360
sb	gilthead seabream	U30320
sb	Nile tilapia	AF467291
sb	red seabream	D86582
sb	striped bass	AF056314
sb	yellowfin seabream (<i>Acanthopagrus latus</i>)	AB089312
tunicate (t) GnRHs		
t	<i>Ciona</i> 1	AY204706
t	<i>Ciona</i> 2	AY204708
whitefish (wf)GnRH		
whitefish		AY245104

Grober and coworkers⁴² did the original phylogenetic analysis of preproGnRH sequences. Phylogenetic analysis using parsimony (PAUP) was used to produce an unrooted 50% majority rule consensus tree. They analyzed 18 GnRH cDNA sequences and found groupings for mGnRH (including cGnRH-I), sGnRH, cfGnRH and cGnRH-II. Three years later, an updated version of an unrooted neighbor-joining phylogenetic tree of preproGnRH precursor nucleotides was published⁴³ in which 12 additional sequences were included. The two catfish GnRHs, expressed in the anterior brain, were grouped with the cGnRH-II forms found in the midbrain. This is a dilemma as cfGnRH precursors have a higher sequence identity with the GnRH I than GnRH II precursors.

In the present review we have included a further update with a total of 73 preproGnRH sequences. Figure 3 shows that one group, often referred to as GnRH I, includes mGnRH, gpGnRH, cGnRH-I, fGnRH, sbGnRH, cfGnRH, wfGnRH, hrGnRH and pjGnRH (which is identical to the medaka peptide).⁴⁴ Another group includes all the cGnRH-II precursors and a third includes the sGnRH precursor molecules. The cfGnRH precursor has been placed in different groupings in the past, but fits with GnRH I precursors in our analysis. This is logical as cfGnRH has the same function and location as other GnRHs in the GnRH I group. The oldest GnRHs (octopus, tunicate) do not fall clearly within a group.

Orthologs and Paralogs

We can trace the evolutionary roots of human genes using conservation of sequence and gene order on individual chromosomes. Comparative analyses can be based on protein sequence, DNA sequence and linkage of genes on chromosomes. Identification of hormones or receptors that are orthologs as opposed to paralogs is necessary to establish the evolutionary pathway for these molecules. Orthologous genes in different species evolved from a common ancestral gene by speciation. Orthologs usually retain the same function over a long period of

evolution.^{45–47} Hence, orthologous genes do not have major changes in structure or function when a new species evolves, whereas the paralogous gene results from duplication of the gene or genome within a species. A paralog may have related or different functions. Thus, the ortholog continues to encode a product that subserves the primary function, whereas the paralog has the freedom of a duplicated gene to encode a product with altered structure and function provided the gene product can still bind to a receptor. Paralogous genes can evolve due to duplication of the whole genome, partial genome or individual genes.

Linkage of genes on chromosomes can be compared between species to identify orthologs provided gene order is retained. Linkage analysis is helpful in animals that are phylogenetically close, such as mammals or even between mammals and fish for some genes that have conservation of gene order in chromosome segments (synteny). However, linkage analysis of phylogenetically distant animals, such as tunicates and fish or tunicates and humans, may not be useful, as it is likely that the gene localization on chromosomes is unrelated.^{4,7}

The GnRH gene provides an excellent example of where linkage analysis is useful and where it is not useful. One theory is that orthologous GnRH-coding genes can be identified between species if they retain the same upstream gene in different species. The nearest upstream gene to the three forms of GnRH in fish (medaka) and the two forms of GnRH in humans was determined for each GnRH gene.⁴⁸ A highly conserved protein (FLJ20038) precedes the medaka form of GnRH (mdGnRH) and human GnRH (mGnRH). In contrast, protein tyrosine phosphatase alpha (PTP α) is the upstream neighbor for the gene encoding cGnRH-II in both species, and PTP ϵ precedes sGnRH in medaka. Further support for this approach is that the mdGnRH and mGnRH are both in the same brain location and function as the releaser of gonadotropins from the pituitary, as expected for orthologs. In contrast, cGnRH-II is found primarily in the midbrain in fish and mammals, although the function is not clear. The sGnRH gene originated in teleost fish, and has not been detected in any tetrapods. Linkage analysis appears to identify the mGnRH and cGnRH-II precursors that preceded the split between bony fish and tetrapods.

Linkage analysis has not been helpful in identifying the GnRH ortholog in the protochordate, *C. intestinalis*. An ancestral protochordate, possibly a tunicate, is thought to have given rise to vertebrates. Two genes, each encoding three distinct forms of GnRH in tandem, have been identified for *C. intestinalis*.⁷ The six forms of GnRH are 60–80% identical in amino acids compared to human GnRH (mGnRH) and 60–70% identical to the cGnRH-II peptide. This structural similarity, along with their ability to induce spawning in *C. intestinalis*, make them members of the GnRH family. However, linkage analysis using the nearest upstream gene to identify the GnRH ortholog for vertebrates does not work. The upstream genes associated with GnRH-encoding genes in a fish and human, that is FLJ20038, PTP α and PTP ϵ , can be identified in the *C. intestinalis* genome, but these genes are not upstream of the identified GnRH genes. The gene order that was conserved in fish and mammals is not present in tunicates. Rather, it appears that the chromosomes have undergone changes, which could include duplication, fusion, translocation, inversion and other events known to occur in evolution.⁶ Thus, the original GnRH orthology in invertebrates has not been identified.

Expression of GnRH in Fish Brain

In fish brain, GnRH is expressed very early in development as GnRH neurons are born and migrate to form populations in three to four general regions: the preoptic area that innervates the pituitary, the midbrain, the terminal nerve, as well as other areas in the forebrain. In some fish species, cells congregate into more distinct populations, whereas in other species the cells tend to form a weakly linked continuum of neurons in most of the areas represented. The notable exception to this is midbrain neurons, which typically form a discrete population.

Beside the number and location of GnRH neuron populations, there is also more than one form of GnRH expressed in fish brain. Fish brain contains at least two forms of GnRH peptide and many species contain three forms; each form is produced from a different gene. The

identification and number of forms for each fish species was discussed above, whereas the evolutionary origin of the cells that make up these populations in fish will be discussed below. Most studies strongly suggest that each neuron produces only one form of GnRH although the possibility remains that one neuron may produce more than one form of GnRH. Furthermore, tetraploid fish may have retained a duplicate copy of any of the GnRH genes.

Typically, teleost fish have genes for transcription of at least cGnRH-II and sGnRH and a third, more variable form. Generally speaking, the teleosts that express only two forms of GnRH are often tetraploid. The second copy of a gene for sGnRH is not restrained so that the regulatory region can change to alter expression in different GnRH cell populations. Sockeye salmon neurons express two sGnRH genes, sGnRH-1 and -2, and coexpress these genes in a continuum in olfactory nerve, olfactory bulbs, ventral telencephalon, preoptic area and the hypothalamus.⁴⁹ The GnRH neurons in the terminal nerve have widely distributed axons in the forebrain, and may coordinate some behavior with sensory input, although the function is not clear.⁵⁰

In teleost bony fish, the neurons of the preoptic area contain either sGnRH or, if the species expresses a third GnRH form (e.g. sbGnRH), the third form is expressed instead of sGnRH. This GnRH form constitutes the main hypophysiotrophic factor governing the release of gonadotropins from the pituitary gonadotropes, and these neurons innervate the pituitary.⁵¹ This is supported by studies that show the highest content of pituitary GnRH is the form that is found in the preoptic neurons.^{30,52,53}

The midbrain neuron population in fish is believed to be exclusively cGnRH-II-producing neurons in all the fish studied to date. These cells are suggested to play a role in reproductive behavior.

GnRH in Gonads of Fish

GnRH is best known in vertebrates for its expression in neurons and role in stimulating the release of gonadotropins from the pituitary. However, expression of GnRH and GnRH receptors is not confined

to the brain and pituitary but is widespread in peripheral tissues. Two sites of interest are the ovary and testis because they express both GnRH and GnRH receptors.

GnRH mRNA Expression in Fish Gonads

The identification of GnRH synthesis in gonads and study of the function of GnRH in gonads is a relatively new direction. The first evidence for expression of GnRH in gonads was GnRH mRNA in rat ovary.⁵⁴ This was followed by confirmation of the presence of GnRH in rat ovary^{55,56} and identification in rat testis,^{56,57} as well as identification of GnRH mRNA in human ovary or testis,⁵⁷⁻⁵⁹ monkey testis⁶⁰ and chicken testis.⁶¹ In guinea pig ovary or testis, gpGnRH mRNA was not detected by an RNase protection assay.⁶² Overall these findings were likely the impetus for fish researchers to include gonads in their tissue expression studies for GnRH. The first report of GnRH gene expression in the gonads of fish using Northern analysis was sGnRH mRNA in ovary and testis of adult midshipman (*Porichthys notatus*).⁴² Since that report in midshipman, GnRH mRNAs for a number of the GnRH forms found in fish have been detected in the gonads of a number of species: sGnRH, cGnRH-II and sbGnRH, by PCR only in testis of the cichlid, *Haplochromis burtoni*;⁶³ sGnRH (mRNA-1 and mRNA-2) and cGnRH-II by sequencing in ovary and testis of rainbow trout;^{23,24,64} sGnRH mRNA-2 in ovary and testis of sockeye salmon by sequencing;²³ lamprey GnRH-I by Northern analysis in adult sea lamprey testis but not ovary.¹⁸

In the tetraploid rainbow trout, sockeye salmon and masu salmon there are two genes that code for sGnRH, sGnRH-1 and -2. From the age of five months the sGnRH mRNA-2 was expressed intermittently in the gonads of rainbow trout throughout the first two years of life.²⁴ Rainbow trout and salmon sexually mature during their third year of life. Rainbow trout continue to express sGnRH-1 and -2 in their brain in each month of their third year, and expression of the two genes continues to be intermittent in the ovary and testis.⁶⁴ Both sGnRH-1 and -2 genes are expressed in ovary and testis in the third year, but sGnRH genes were not expressed in every month, and the

genes were not always expressed at the same time. An upstream promoter is not used in rainbow trout gonads (or brain) for the sGnRH-1 gene, whereas use of an upstream promoter or a downstream promoter for sGnRH gene-2 results in two different transcripts. Maturing ovaries and testes produce a number of alternatively-spliced sGnRH transcripts, resulting from intron retention.^{24,64-66} Introns were also retained in transcripts for sbGnRH and cGnRH-II, but not sGnRH, in seabream ovary.⁶⁷ The significance of the alternatively spliced transcripts is not known. In some instances the retention of an intron would mean the introduction of a premature stop codon, resulting in a shortened GAP. If a normal GAP is required for proper processing of the precursor then production of the mature GnRH peptide is jeopardized.

GnRH Protein in Fish Gonads

The presence of GnRH mRNA transcripts in fish gonads supports a potential autocrine or paracrine role for GnRH in fish reproduction, whereas the presence of mature peptide solidifies that role. Since gonadal GnRH cDNA has been demonstrated in a number of vertebrate species, one might expect to find the translated mature peptide product in the gonad, perhaps at levels that coincide with the GnRH gene expression. However, for all the vertebrates studied, mature GnRH peptides have been identified only in gonads of two species of fish: goldfish and rainbow trout. sGnRH was isolated and sequenced from the goldfish ovary,⁶⁸ and identified by radioimmunoassay only in mature 18- to 20-month old rainbow trout ovary.⁶⁹ More cGnRH-II was found than sGnRH in rainbow trout ovary, and cGnRH-II was also found in 17- to 20-month old rainbow trout testis.⁶⁹ Evidence for GnRH-like activity inferring mature GnRH peptide has been reported for two other species. Extracts from the ovaries of African catfish⁷⁰ and seabream⁷¹ stimulated gonadotropin release from pituitary, suggesting the presence of a GnRH or GnRH-like material in the ovaries of these species.

The presence of GnRH mRNA in gonads does not necessarily indicate the production of GnRH peptide. GnRH peptide was not

detected in the gonads at any time in adult rainbow trout during their third year leading to spawning even though transcripts for both of the salmon genes were detected.⁶⁴ These data suggest that peripheral GnRH production is important in the early development and maturation of the gonads of fish, but are not required at least in large quantities when the fish have reached maturity even though the GnRH genes continue to be expressed. It also suggests that in the brain, cells will store GnRH for a period of time, whereas GnRH production in cells of peripheral tissues such as the gonads may undergo rapid turnover and therefore make identification of peptide in these tissues difficult. Because of this, GnRH gene expression may not be a reliable predictor for the presence of GnRH in gonads. Instead, gene expression may simply represent a potential for mature GnRH peptide production, in which the ultimate production is under the control of local gonadal regulatory influences, including transcription and translation factors, as well as other autocrine and paracrine peptides.

GnRH-Receptor Expression in Fish Gonads

The production of a ligand by peripheral tissues requires the presence of a receptor on the same tissue for GnRH to exert an autocrine or paracrine action. GnRH acts by binding to seven-transmembrane G protein coupled-receptors on the surface of the target cell, ultimately activating second-messenger systems on the inside of the cell. The pathways activated by GnRH receptors that have been studied mainly are the inositol triphosphate (IP_3) and cAMP pathways. Expression of GnRH receptors on gonadal cells have been shown in a number of vertebrates including adult human and mature male rat testis⁵⁷ and in chicken testis.⁶¹ Binding sites for GnRH have been identified on the ovaries and the testes of a number of fish species including African catfish,⁷⁰ seabream,⁷¹ and lamprey.⁷² In addition, GnRH receptors are being cloned in an increasing number of fish species and the expression pattern for these receptors in gonads is being determined in some of these species. GnRH receptors have been identified by Northern analysis

or RT-PCR in goldfish testis⁷³ and ovary,⁷⁴ Japanese eel testis,⁷⁵ adult rainbow trout ovary,⁷⁶ *H. burtoni* testis,⁷⁷ and African catfish testis and ovary.⁷⁸ Overall, these data support binding studies that show binding of GnRH ligand to gonads. The expression of the receptor genes implies, as for the ligand genes, that the gene product is a translated mature protein receptor expressed on the cell surface in the gonads. However the presence of these receptors as mature protein embedded in a cell membrane remains to be confirmed by either immunocytochemistry or by Western analysis.

Role(s) of GnRH in the Gonads

The exact role of GnRH in peripheral sites is not well understood. Obviously the function is not the production of GnRH for the release of gonadotropins from the pituitary. Evidence is accumulating from mammalian and fish studies that GnRH is one of a number of regulatory peptides that may have unique actions in the ovary and testis compared to their action in the brain-pituitary axis.

In vitro studies have identified possible actions for cGnRH-II even at low concentrations (10^{-9} M) in baboon ovary cells that include inhibition of progesterone release.⁷⁹ GnRH at a high concentration (10^{-2} M) stimulated meiotic maturation of follicle-enclosed oocytes from rats.⁸⁰ A few studies, mainly by Habibi and colleagues, have examined effects of GnRH peptide on fish ovary *in vitro*. A number of GnRH peptides (sGnRH, cGnRH-I and -II, lGnRH-I and -III, and mGnRH) each stimulate goldfish oocyte meiosis, but block gonadotropin-induced meiosis and testosterone production.⁶⁸ In a seabream follicle assay, sGnRH stimulated meiosis whereas cGnRH-II did not (sbGnRH was not tested).⁶⁷ Furthermore, in ovarian tissue isolated from lamprey, lGnRH-III at 10^{-3} to 10^{-5} M caused an increase in estradiol production, but did not cause release of progesterone.⁷² Differences in these studies may result from the maturity level of the follicles, or species differences in GnRH receptors present on the follicles that are mediating the effects to the cells. However these discrepancies among species should not overshadow the strong evidence for local actions of GnRH peptide in the ovary.

Actions by GnRH in the gonads are not limited to the ovary. Studies of the mammalian and amphibian testis show that GnRH may play a significant role in testis physiology. Treatment of hypophysectomized rats with 10–200 µg GnRH or GnRH analog per animal inhibited reproduction and in *in vitro* studies ($\geq 10^{-9}$ M) GnRH or GnRH analogs inhibited *in vitro* production of gonadotropin-induced androgen.⁸¹ The effect is mediated through testis GnRH receptors, because infusion of a GnRH receptor antagonist *in vivo* for seven days in adult and immature rat testis reduced the expression of receptors for GnRH, LH, FSH and lactogen, as well as the testis content of testosterone.⁸² The action of GnRH in the fish testis is still under study. Lamprey GnRH-III increased the level of estradiol in lamprey testis when added at 100 ng/ml and caused increased levels of both estradiol and progesterone at 1000 ng/ml.⁷² Each of sGnRH and cGnRH-II increased apoptosis in goldfish testis slices at physiological levels (10^{-8} M and 10^{-7} M).⁸³ A more complete understanding of the actions and mechanisms of GnRH peptide in fish gonads may be provided by more concentrated efforts and including techniques such as microarray and proteomic analysis.

GnRH in Early Fish Development

GnRH neurons have been studied in the early developmental stages of vertebrates to understand the embryological origin of GnRH neurons. Researchers can track the origin and migration of these young neurons, because they produce mature GnRH peptide. The development of neurons producing immunoreactive mGnRH has been well studied in mammals in which neurons are derived from the olfactory placode and migrate into the brain in the prenatal stage.⁸⁴

Also, the GnRH system is expressed in non-neural tissues in early vertebrate development. Humans express GnRH in the placenta⁸⁵ and the fetal brain.⁸⁶ Fetal rats express GnRH mRNA and GnRH receptor mRNA as early as 14.5 days post conception in the testis and by 15.5 or 18.5 days post conception in the ovary for the receptor and ligand, respectively.⁵⁶

Adult female rats express GnRH in the mammary gland when lactating⁸⁷ and juvenile male rats increased the amount of GnRH mRNA and peptide from low but detectable levels from postnatal day 8.⁸⁸

Fish are included in this poorly understood developmental expression of GnRH. Eggs and whole embryos of fish express GnRH early in development. Some of the earliest studies of immunoreactive GnRH neurons in fish were in the platyfish in which the pattern of sequential development of three immunoreactive GnRH cell populations in the brain was described.⁸⁹ The various populations of GnRH neurons in fish brains are thought to have different embryological origins. In fish, the early expression, origin, and fate of GnRH neurons have been studied primarily in salmon, seabass, cichlid and zebrafish.

Mature GnRH peptide is produced at very early stages since immunocytochemical studies showed chum salmon to have immunoreactive GnRH cells in the olfactory placode at 16⁹⁰ to 20 days⁹¹ after fertilization. These early studies in chum salmon pointed to the olfactory placode as the origin for a GnRH cell population in fish that is found later in the olfactory nerve and forebrain regions. The data were supported by studies in a number of other salmon species. The same origin and fate of GnRH immunoreactive cells was seen in sockeye salmon based on a population of cells first detected 19 days after fertilization and *in situ* hybridization detected sGnRH mRNA in these cells at 72 days after fertilization.⁹² sGnRH mRNA has been detected as early as 10 and 30 days after fertilization in whole embryos of rainbow trout and sockeye salmon, respectively.²³ sGnRH neurons are expressed in olfactory epithelium and olfactory nerve at 40 days after fertilization in masu salmon⁹³ and migrate to populated regions from the olfactory nerve to the ventral telencephalon and preoptic area.⁵³

A system consisting of three populations of GnRH cells in fish is likely common across the phylogenetic range in teleost fish. Japanese eel brains show immunoreactive GnRH in embryos of 5 cm length in the olfactory bulbs, preoptic area and midbrain tegmentum.⁹⁴ In medaka, some cells with GnRH-immunoreactive neurons were seen one day after fertilization — one to two immunoreactive sGnRH cells at the base of the olfactory placodes, and cGnRH-II-immunoreactive

neurons were in the midbrain tegmentum, near the ventricular wall.⁹⁵ By day 17, sGnRH immunoreactive and cGnRH-II immunoreactive neurons were seen in the nucleus olfactoretinalis and midbrain tegmentum, respectively. Notably, in the adult medaka, the midbrain was immunoreactive to sGnRH and mGnRH antibody. However, *in situ* hybridization with probes specific to sGnRH and cGnRH-II mRNA correlated with the antibody results. Since that study, a third form of GnRH (pjGnRH or mdGnRH)⁴⁴ has been identified in medaka and it cross-reacts with the GF-6 antibody.³⁴ This form groups closely with the GnRH 1 based on the preproGnRH sequence and is possibly involved in GnRH release in medaka. The cells in the forebrain identified by Parhar and colleagues using GF-6 antibody may have been sGnRH neurons, mdGnRH neurons, or a mixture of the two types of GnRH-expressing neurons. Evidence from medaka for a ventricular origin of the midbrain population of cells is supported by recent *in situ* hybridization studies of the developing GnRH systems in the European sea bass. In sea bass, cGnRH-II mRNA-expressing cells appeared in the germinal zone of the third ventricle four days after hatching.⁹⁶ Together, studies in salmon and sea bass suggest that forebrain, preoptic area, and terminal nerve GnRH neurons have a common embryological origin in the olfactory placode, whether the species in question has one or two different GnRH forms making up these cell populations.^{53,96}

More recently, studies in zebrafish reveal that the neurons for the different GnRH populations are born in regions distinct from olfactory regions in the developing neural plate.⁹⁷ Whitlock and colleagues⁹⁷ hypothesize that the cells that become the terminal nerve population arise from the cranial neural crest, whereas the hypothalamic neuronal population arises from the adenohypophyseal region of the developing anterior neural plate.

Further study of the ontogeny of cells producing different forms of GnRH will help to identify reasons for the different times of birth of GnRH neurons. It will also further our understanding of non-gonadotropic functions of GnRH in early development, as well as to further characterize GnRH systems in vertebrates including fish, and provide more clues to determine the evolution of different GnRH systems in fish.

GnRH Gene Regulation

Regulation is a broad term that can refer to gene transcription, RNA splicing, mRNA stability, translation of mRNA or release of the peptide from the cell. This section is concerned with the factors that control transcription of the GnRH genes. The transcription of each GnRH gene is controlled as to the cell type in which GnRH is expressed, the pattern of expression with time and the number of transcripts. Because the two or three types of GnRH in a single brain are expressed in cells in different locations and are expressed in different amounts and stages, one can predict that the genes will have some differences in the promoters and 5' flanking regions. GnRH orthologs with similar expression characteristics and functions in evolution of the vertebrates might have more highly conserved regulatory regions for their genes. GnRH paralogs may have less conserved gene promoters if compared to orthologs. Regulation is likely to be similar for genes in groups I and III (forebrain GnRH) as opposed to group II (midbrain GnRH) in Fig. 3.

GnRH Gene Regulation in Mammals

The model GnRH gene in which regulation has been most fully examined is the mGnRH gene in mice, rats and humans. Because GnRH neurons are few in number, small and scattered in the basal forebrain and hypothalamus, many regulatory studies have been done in immortalized GnRH cells developed from mouse brain tissue. Human and murine promoters have been tested in these immortalized cells resulting in the identification of a number of transcription factors, both positive and negative; these studies have been reviewed.^{98,99}

A transcription factor that was identified as important for differentiation of GnRH neurons early in development of mammals is the homeobox gene, Gsh-1.⁹⁹ Other examples of transcription factors for human mGnRH are Oct-1, Brn-2 and Oct-6; the latter inhibits the GnRH gene.¹⁰⁰⁻¹⁰² Steroid receptors such as the glucocorticoid receptor (GR), and estrogen receptors (ER α and ER β) are reported to alter GnRH transcription. The glucocorticoid receptor binds to the promoter and can increase or decrease

transcription of GnRH genes depending on the presence of Oct-1. It is not settled whether the ER effects result from direct receptor binding to the GnRH promoter or indirect tethering of the receptor to another transcription factor. Also, the steroids may act on other cells that make synaptic contact with GnRH neurons.

It is clear that there are differences in the organization of the GnRH promoter even for the human and rat promoters for mGnRH in that the promoter region that controls cell-specific expression in the brain is within the first 1 kb (-992 to -763 bp) in human GnRH gene,¹⁰⁰ but is upstream (-1571 to -1863) in the rat promoter.¹⁰³ The latter sequence, which is critical for neuron-specific expression in the rat GnRH promoter, is not present in the human promoter.¹⁰⁴ The similarity of nucleotides in the human and rat mGnRH promoter involves some distal regions and the region adjacent to the transcription start site (-343 to -1 bp).¹⁰⁴ The second human GnRH gene (GnRH2) encoding cGnRH-II is in a different group (Fig. 3) and has not been studied as completely as mGnRH. The cGnRH-II promoter has consensus sites for binding cAMP binding protein (CREB), which has been shown to be a functional site.¹⁰⁵ Additionally, Oct-1, Oct-6 (Tst-1) and Jun/Fos are consensus sites in the human gene encoding cGnRH-II.⁷ Thus, as expected the regulatory regions for the human mGnRH of the GnRH I group and cGnRH-II of the GnRH II group encoding genes are quite different.

Primate GnRH genes, but not mouse or rat genes, have two promoters, upstream and downstream. For example, the human gene encoding mGnRH uses the downstream promoter for expression in the brain, but uses the downstream and upstream promoter (-579) for expression in reproductive tissues such as the testis, ovary, mammary gland, placenta and in placental and breast tumor cell lines.^{58,60,106} This arrangement of two promoters for one gene is similar to that in the salmon.

GnRH Gene Regulation in Fish

The conservation of GnRH regulatory regions can be compared for genes encoding different forms of GnRH among species. In fish, eleven

GnRH forms have been identified, but the 5' flanking (regulatory) sequences for the genes are reported for only four forms. The sGnRH gene is known for Atlantic salmon,¹⁰⁷ sockeye salmon (two genes),^{21,23} masu salmon (two genes),²² rainbow trout (two genes),²⁴ zebrafish,¹⁰ medaka,⁴⁸ cichlid¹⁰⁸ and *Fugu* (see *Fugu* genome). The sbGnRH gene is reported for striped bass,¹⁰⁹ cichlid,¹⁰⁸ tilapia¹¹⁰ and *Fugu* (see genome). The cGnRH-II gene is reported for striped bass,¹⁰⁹ medaka,⁴⁸ cichlid,¹⁰⁸ zebrafish¹¹ and *Fugu* (see genome). The mdGnRH gene is only known for medaka.⁴⁸ In medaka fish, the sGnRH, cGnRH-II and medaka GnRH genes have been isolated and sequenced as noted above, but analysis of the 5'flanking region for identification of response elements that could potentially bind transcription factors was not reported.⁴⁸ Likewise, the 5'flanking region for the sGnRH gene in zebrafish has been sequenced, but not analyzed. In *Fugu* fish, three GnRH genes can be identified within the genome, but neither experimental isolation nor analysis of the 5'flanking region have been reported. Nonetheless, the other 14 genes listed above are sufficient to gain some insight into the regulation of GnRH genes in fish.

Experimental studies have shown that specific GnRH promoters have transcriptional activity in fish. Only a single promoter has been reported for most fish GnRH genes. For example, in zebrafish the single sGnRH gene expresses a reporter gene in the expected brain region.¹⁰ Gene regulation in tetraploid salmon is interesting in that two genes with distinct promoter regions encode the sGnRH gene (sGnRH gene-1 and gene-2), although only one copy of the cGnRH-II gene has been detected to date. Only the sGnRH gene-2 has an upstream and downstream promoter in Atlantic salmon, sockeye salmon, masu salmon and rainbow trout. This is similar to the identification of two promoters in the single copy mGnRH gene in primates.^{60,102,106} Also of interest in the fish genes is the presence of over 1 kb between the upstream and downstream promoter in Atlantic and masu salmon compared to the sockeye and rainbow trout promoters. In Atlantic salmon, the upstream and downstream promoters for sGnRH were shown to activate transcription when placed individually in a construct with a lacZ reporter gene, and injected into zebrafish fertilized eggs. However, only the

downstream promoter drove expression in appropriate brain cells in zebrafish embryos suggesting that the downstream promoter can direct cell-specific expression.¹¹¹ The masu salmon promoter for sGnRH gene-2 was not analyzed for promoter activity, but the entire upstream region has high homology with that of Atlantic salmon.²² In sockeye salmon and rainbow trout, transcripts were isolated from gonads and/or brain showing that both promoters are used; the upstream promoter results in a longer transcript due to a longer 5'UTR and retention of the first intron in some transcripts.^{21,23,64}

The function of the upstream and downstream promoters appears to be related to tissue-specific expression. In rainbow trout during year 1 and year 2, sGnRH gene-1 and gene-2 were transcribed in both the brain and gonads from the downstream promoter and the upstream promoter in gene-2 was used in testes and ovary.²⁴ However, rainbow trout during the final year of maturation (year 3) additionally used the upstream promoter for brain transcripts in certain months preceding spawning.⁶⁴ Thus, the function of the upstream promoter remains to be determined as to whether there are differences in species, type of GnRH, neural or non-neural tissue or pattern during the reproductive cycle.

Complete analysis of the promoter, enhancer and silencer regions in the 5' flanking region with identification of transcription factors that regulate GnRH genes using experimental methods is rare for fish. The first report of experimental data was based on the sGnRH gene in Atlantic salmon^{112,113} in which the human estrogen receptor was shown to bind to specific sites at ~1.5 kb upstream of the transcription start site. The two binding sites were described as estrogen response element (ERE)-like motifs in which each motif had two palindromic ERE half-sites with 8 or 9 nucleotides between them. The proof of specific binding was by footprinting and gel shift assays. Another experimental study showed that all of the promoter-enhancer region could be deleted beyond -981 nt and yet retain full expression of a reporter gene in zebrafish embryos.¹⁰ However, a greater deletion beginning at -928 prevents expression of the reporter gene, as the putative enhancer is removed. These experimental results suggested that the region from -928 to -981 is an enhancer region that may bind Oct-1, CREB and

Sp-1. It remains to be shown that these transcription factors bind to the region using footprinting, gel shift and other assays.

Most of the information about regulation of the GnRH genes in fish is derived from *in silico* analysis in which putative transcription factor binding sites are identified by computer analysis of consensus sites. This method is based on data showing that regulatory elements in the promoters and enhancers of genes can be defined by their DNA sequences and position relative to coding sequences. For the GnRH genes in fish, 14 promoter-enhancer regions have been examined with *in silico* methods: three sGnRH gene1, six sGnRH gene2, three sbGnRH, and two cGnRH-II genes.

The putative transcription factors identified were CREB/ATF (sGnRH gene1 and gene2, sbGnRH and cGnRH-II genes); GATA (sGnRH gene1 and 2), Jun/Fos (sGnRH gene1 and 2, sbGnRH gene and cGnRH-II genes), Brn-2 (sGnRH gene 1 and 2 and cGnRH-II gene), Oct-1 (sGnRH genes 1 and 2, sbGnRH gene and cGnRH-II gene), Pit-1 (sGnRH gene2, sbGnRH gene and cGnRH-II gene), and Sp-1 (sGnRH gene2 and sbGnRH gene). The consensus sites that might bind to these factors in specific GnRH genes were not reported for all the species studied. In addition, some steroid response elements were detected that might be bound by the estrogen receptor (sGnRH genes1 and 2, sbGnRH gene and cGnRH-II gene), the progesterone receptor (sGnRH gene2 and sbGnRH gene), and glucocorticoid receptor (sGnRH genes1 and 2, sbGnRH and cGnRH-II genes). The ERE is interesting in that Alestrom's group first noticed that there were ERE half sites, ERE-like motifs, and a large palindromic cruciform structure.

A considerable amount of experimental research is needed to verify whether the consensus sites are functional in fish. Ideally, regulatory regions need to be compared for GnRH genes in the three groups (Fig. 3) in species throughout evolution. Already a number of consensus sites have been noted between human GnRH genes and salmon or tunicate GnRH genes.^{7,24} And yet the region that regulates cell-specific expression in the rat and human mGnRH promoter can be distinct in location and sequence. It is also possible that the response elements within the promoters may be somewhat different in tunicates, fish and mammals. To date, the response elements have been defined by studies in mammals.

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References

1. Ton C, Stamatou D, Dzau VJ and Liew CC (2002). Construction of a zebrafish cDNA microarray: gene expression profiling of the zebrafish during development. *Biochem. Biophys. Res. Commun.* **296**: 1134–1142.
2. Gracey AY, Troll JV and Somero GN (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc. Natl. Acad. Sci. USA* **98**: 1993–1998.
3. Ju Z, Dunham RA and Liu Z (2002). Differential gene expression in the brain of channel catfish (*Ictalurus punctatus*) in response to cold acclimation. *Mol. Genet. Genomics* **268**: 87–95.
4. Ben-Shlomo I, Vitt UA and Hsueh AJW (2002). Perspective: The ovarian kaleidoscope database-II. Functional genomic analysis of an organ-specific database. *Endocrinology* **143**: 2041–2044.
5. Wurmbach E, Yuen T, Ebersole BJ and Sealfon SC (2001). Gonadotropin-releasing hormone receptor-coupled gene network organization. *J. Biol. Chem.* **276**: 47195–47201.
6. Leo CP, Hsu SY and Hsueh AJW (2002). Hormonal genomics. *Endocrine Rev.* **23**: 369–381.
7. Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE and Sherwood NM (2003). Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protostome, *Ciona intestinalis*. *Endocrinology* **144**: 1907–1919.
8. Iwakoshi E, Takuwa-Kuroda K, Fujisawa Y, Hisada M, Ukena K, Tsutsui K and Minakata H (2002). Isolation and characterization of a GnRH-like peptide from *Octopus vulgaris*. *Biochem. Biophys. Res. Commun.* **291**: 1187–1193.
9. Powell JFF, Krueckl SL, Collins PM and Sherwood NM (1996). Molecular forms of GnRH in three model fishes: Rockfish, medaka and zebrafish. *J. Endocrinol.* **150**: 17–23.

10. Torgersen J, Nourizadeh-Lillabadi R, Husebye H and Aleström P (2002). *In silico* and *in situ* characterization of the zebrafish (*Danio rerio*) gnrh3 (sGnRH) gene. *BMC Genomics* **3**: 25–36.
11. Steven C, Lehnert N, Kight K, Ijiri S, Harris WA and Zohar Y (2003). Molecular characterization of the GnRH system in zebrafish (*Danio rerio*): Cloning of GnRH-II, adult brain expression patterns and pituitary content of GnRH-I and GnRH-II. *Gen. Comp. Endocrinol.* **133**: 27–37.
12. Ohno S (1970). *Evolution by Gene Duplication*. Springer, Berlin.
13. Ohno S (1998). The notion of the Cambrian pananimalia genome and a genomic difference that separated vertebrates from invertebrates. In: Muller WEG (ed.), *Molecular Evolution: Towards the Origin of Metazoa*. Vol. 21. *Progress in Molecular and Subcellular Biology Series*, Springer, Berlin, pp. 97–117.
14. Furlong RF and Holland PWH (2002). Were vertebrates octoploid? *Phil. Trans. R. Soc. Lond. B* **357**: 531–544.
15. Stellwag EJ (1999). Hox gene duplication in fish. *Sem. Cell Dev. Biol.* **10**: 531–540.
16. Koh EGL, Lam K, Christoffels A, Erdmann MV, Brenner S and Venkatesh B (2003). Hox gene clusters in the Indonesian coelacanth, *Latimeria menadoensis*. *Proc. Natl. Acad. Sci. USA* **100**: 1084–1088.
17. Amores A, Force A, Yan Y-L, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang Y-L, Westerfield M, Ekker M and Postlethwait JH (1998). Zebrafish *hox* clusters and vertebrate genome evolution. *Science* **282**: 1711–1714.
18. Suzuki K, Gamble RL and Sower SA (2000). Multiple transcripts encoding lamprey gonadotropin-releasing hormone-I precursors. *J. Mol. Endocrinol.* **24**: 365–376.
19. Adams BA, Vickers ED, Warby C, Park M, Fischer WH, Craig AG, Rivier JE and Sherwood NM (2002). Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, *Coregonus clupeaformis*. *Biol. Reprod.* **67**: 232–239.
20. Ashihara M, Suzuki M, Kubokawa K, Yoshiura Y, Kobayashi M, Urano U and Aida K (1995). Two differing precursor genes for the salmon-type gonadotropin-releasing hormone exist in salmonids. *J. Mol. Endocrinol.* **15**: 1–9.
21. Coe IR, von Schalburg KR and Sherwood NM (1995). Characterization of the Pacific salmon gonadotropin-releasing hormone gene, copy number and transcription start site. *Mol. Cell. Endocrinol.* **115**: 113–122.

22. Higa M, Kitahashi T, Sasaki Y, Okada H and Ando H (1997). Distinct promoter sequences of two precursor genes for salmon gonadotropin-releasing hormone in masu salmon. *J. Mol. Endocrinol.* **19**: 149–161.
23. von Schalburg KR, Harrower WL and Sherwood NM (1999). Regulation and expression of gonadotropin-releasing hormone in salmon embryo and gonad. *Mol. Cell. Endocrinol.* **157**: 41–54.
24. von Schalburg KR and Sherwood NM (1999). Regulation and expression of gonadotropin-releasing hormone gene differs in brain and gonads in rainbow trout. *Endocrinology* **140**: 3012–3024.
25. Lin X-W and Peter RE (1996). Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. *Gen. Comp. Endocrinol.* **101**: 282–296.
26. Lin X-W and Peter RE (1997). Cloning and expression pattern of a second [His⁵Trp⁷Tyr⁸] gonadotropin-releasing hormone (chicken GnRH-II) mRNA in goldfish: Evidence for two distinct genes. *Gen. Comp. Endocrinol.* **107**: 262–272.
27. Sherwood NM, Sower SA, Marshak DR, Fraser BA and Brownstein MJ (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem.* **261**: 4812–4819.
28. Sower SA, Chiang Y-C, Lovas S and Conlon JM (1993). Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* **132**: 1125–1131.
29. Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE and Sherwood NM (1992). Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Natl. Acad. Sci. USA* **89**: 6373–6377.
30. Carolsfeld J, Powell JFF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE and Sherwood NM (2000). Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. *Endocrinology* **141**: 505–512.
31. Sherwood N, Eiden L, Brownstein M, Spiess J, Rivier J and Vale W (1983). Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci. USA* **80**: 2794–2798.
32. Ngamvongchon S, Lovejoy DA, Fischer WH, Craig AG, Nahorniak CS, Peter RE, Rivier JE and Sherwood NM (1992). Primary structures of

- two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. *Mol. Cell. Neurosci.* **3**: 17–22.
- 33. Bogerd J, Li KW, Janssen-Dommerholt C and Goos H (1992). Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). *Biochem. Biophys. Res. Commun.* **187**: 127–134.
 - 34. Montaner AD, Park MK, Fischer WH, Craig AG, Chang JP, Somoza GM, Rivier JE and Sherwood NM (2001). Primary structure of a novel gonadotropin-releasing hormone in the brain of a teleost, pejerrey. *Endocrinology* **142**: 1453–1460.
 - 35. Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA and Sherwood NM (1994). Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proc. Natl. Acad. Sci. USA* **91**: 12081–12085.
 - 36. Lovejoy DA, Sherwood NM, Fischer WH, Jackson BC, Rivier JE and Lee T (1991). Primary structure of gonadotropin-releasing hormone from the brain of a holocephalan (Ratfish: *Hydrolagus colliei*). *Gen. Comp. Endocrinol.* **82**: 152–161.
 - 37. Weber GM, Powell JFF, Park M, Fischer WH, Craig AG, Rivier JE, Nanakorn U, Parhar IS, Ngamvongchon S, Grau EG and Sherwood NM (1997). Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. *J. Endocrinol.* **155**: 121–132.
 - 38. Powell JFF, Standen EM, Carolsfeld J, Borella MI, Gazola R, Fischer WH, Park M, Craig AG, Warby CM, Rivier JE, Val-Sella MV and Sherwood NM (1997). Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. *Regul. Pept.* **68**: 189–195.
 - 39. Lescheid DW, Powell JFF, Fischer WH, Park M, Craig A, Bukovskaya O, Barannikova IA and Sherwood NM (1995). Mammalian gonadotropin-releasing hormone (GnRH) identified by primary structure in Russian sturgeon, *Acipenser gueldenstaedti*. *Regul. Pept.* **55**: 299–309.
 - 40. Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K and Matsuo M (1984). Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proc. Natl. Acad. Sci. USA* **81**: 3874–3878.

41. Matsuo H, Baba Y, Nair RMG, Arimura A and Schally AV (1971). Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem. Biophys. Res. Commun.* **43**: 1334–1339.
42. Grober MS, Myers TR, Marchaterre MA, Bass AH and Myers DA (1995). Structure, localization, and molecular phylogeny of a GnRH cDNA from a paracanthopterygian fish, the plainfin midshipman (*Porichthys notatus*). *Gen. Comp. Endocrinol.* **99**: 85–99.
43. White RB, Eisen JA, Kasten TL and Fernald RD (1998). Second gene for gonadotropin releasing hormone in humans. *Proc. Natl. Acad. Sci. USA* **95**: 305–309.
44. Okubo K, Amano M, Yoshiura Y, Suetake H and Aida K (2000). A novel form of gonadotropin-releasing hormone in medaka, *Oryzias latipes*. *Biochem. Biophys. Res. Commun.* **276**: 298–303.
45. Henikoff S, Greene EA, Pietrokowski S, Bork P, Attwood TK and Hood L (1997). Gene families: The taxonomy of protein paralogs and chimeras. *Science* **278**: 609–614.
46. Tatusov RL, Koonin EV and Lipman DJ (1997). A genomic perspective on protein families. *Science* **278**: 631–637.
47. Lee Y, Sultana R, Pertea G, Cho J, Karamycheva S, Tsai J, Parvizi B, Cheung F, Antonescu V, White J, Holt I, Liang F and Quackenbush J (2002). Cross-referencing eukaryotic genomes: TIGR orthologous gene alignments (TOGA). *Genome Res.* **12**: 493–502.
48. Okubo K, Mitani H, Naruse K, Kondo M, Shima A, Tanaka M, Asakawa S, Shimizu N, Yoshiura Y and Aida K (2002). Structural characterization of GnRH loci in the medaka genome. *Gene* **293**: 181–189.
49. Amano M, Ashihara M-O, Yoshiura Y, Kitamura S, Ikuta K and Aida K (1998). Two differing salmon GnRH precursor mRNAs are co-expressed in the brain of sockeye salmon (*Oncorhynchus nerka*). *Cell Tissue Res.* **292**: 267–273.
50. Oka Y (2002). Physiology and release activity of GnRH neurons. *Prog. Brain Res.* **141**: 259–281.
51. Kah O, Breton B, Dulka JG, Nunez-Rodriguez J, Peter RE, Corrigan A, Rivier JE and Vale WW (1986). A reinvestigation of the Gn-RH (gonadotropin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. *Cell Tissue Res.* **244**: 327–337.

52. Collins PM, O'Neill DF, Barron BR, Moore RK and Sherwood NM (2001). Gonadotropin-releasing hormone content in the brain and pituitary of male and female grass rockfish (*Sebastes rastrelliger*) in relation to seasonal changes in reproductive status. *Biol. Reprod.* **65**: 173–179.
53. Amano M, Okubo K, Ikuta K, Kitamura S, Okuzawa K, Ymada H, Aida K and Yamamori K (2002). Ontogenetic origin of salmon GnRH neurons in the ventral telencephalon and the preoptic area in masu salmon. *Gen. Comp. Endocrinol.* **127**: 256–262.
54. Oikawa M, Dargan C, Ny T and Hsueh AJW (1990). Expression of gonadotropin-releasing hormone and prothymosin-alpha messenger ribonucleic acid in the ovary. *Endocrinology* **127**: 2350–2356.
55. Clayton RN, Eccleston L, Gossar F, Thalbard JC and Morel G (1992). Rat granulosa cells express the gonadotropin-releasing hormone gene: evidence from *in-situ* hybridization histochemistry. *J. Mol. Endocrinol.* **9**: 189–195.
56. Botte M-C, Chamagne A-M, Carre M-C, Counis R and Kottler M-L (1998). Fetal expression of GnRH and GnRH receptor genes in rat testis and ovary. *J. Endocrinol.* **159**: 179–189.
57. Bahk JY, Hyun JS, Chung SH, Lee H, Kim MO, Lee BH and Choi WS (1995). Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis. *J. Urology* **154**: 1958–1961.
58. Dong K-W, Yu K-L and Roberts JL (1993). Identification of a major upstream transcription start site for the human progonadotropin-releasing hormone gene used in reproductive tissues and cell lines. *Mol. Endocrinol.* **7**: 1654–1666.
59. Peng C, Fan NC, Ligier M, Vaanannen J and Leung PCK (1994). Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. *Endocrinology* **135**: 1740–1746.
60. Dong K-W, Deval P, Zeng Z, Gordon K, Williams RF, Hodgen GD *et al.* (1996). Multiple transcription start sites for the GnRH gene in rhesus and cynomolgus monkeys: A non-human primate model for studying GnRH gene regulation. *Mol. Cell. Endocrinol.* **117**: 121–130.
61. Sun Y-M, Dunn IC, Baines E, Talbot RT, Illing N, Millar RP and Sharp PJ (2001). Distribution and regulation by oestrogen of fully processed and variant transcripts of gonadotropin releasing hormone I and

- gonadotropin releasing hormone receptor mRNAs in the male chicken. *J. Neuroendocrinol.* **13:** 37–49.
- 62. Jimenez-Linan M, Rubin BS and King JC (1997). Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. *Endocrinology* **138:** 4123–4130.
 - 63. White RB and Fernald RD (1998). Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. *Gen. Comp. Endocrinol.* **112:** 17–25.
 - 64. Gray SL, Adams BA, Warby CM, von Schalburg KR and Sherwood NM (2002). Transcription and translation of the salmon gonadotropin-releasing hormone genes in brain and gonads of sexually maturing rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod.* **67:** 1621–1627.
 - 65. Uzbekova S, Ferriere F, Guiguen Y, Bailhache T, Breton B and Lareyre J-J (2001). Stage-dependent and alternative splicing of sGnRH messengers in rainbow trout testis during spermatogenesis. *Mol. Reprod. Devel.* **59:** 1–10.
 - 66. Uzbekova S, Lareyre J-J, Madigou T, Davail B, Jalabert B and Breton B (2002). Expression of prepro-GnRH and GnRH receptor messengers in rainbow trout ovary depends on the stage of ovarian follicular development. *Mol. Reprod. Devel.* **62:** 47–56.
 - 67. Nabissi M, Soverchia L, Polzonetti-Magni AM and Habibi HR (2000). Differential splicing of three gonadotropin-releasing hormone transcripts in the ovary of seabream (*Sparus aurata*). *Biol. Reprod.* **62:** 1329–1334.
 - 68. Pati D and Habibi HR (2000). Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis. *Mol. Cell. Endocrinol.* **160:** 75–88.
 - 69. von Schalburg KR, Warby CM and Sherwood NM (1999). Evidence for gonadotropin-releasing hormone peptides in the ovary and testis of rainbow trout. *Biol. Reprod.* **60:** 1338–1344.
 - 70. Habibi HR, Pati D, Ouwens M and Goos HJT (1994). Presence of gonadotropin-releasing hormone (GnRH) binding sites and compounds with GnRH-like activity in the ovary of the African catfish, *Clarias gariepinus*. *Biol. Reprod.* **50:** 643–652.
 - 71. Nabissi M, Pati D, Polzonetti-Magni AM and Habibi HR (1997). Presence and activity of compounds with GnRH-like activity in the ovary of

- seabream *Sparus aurata*. *Am. J. Physiol. Reg. Int. Comp. Physiol.* **272**: R111–R117.
72. Gazourian L, Deragon KL, Chase CF, Pati D, Habibi HR and Sower SA (1997). Characteristics of GnRH binding in the gonads and effects of lamprey GnRH-I and -III on reproduction in the adult sea lamprey. *Gen. Comp. Endocrinol.* **108**: 327–339.
73. Yu K-L, He M-L, Chik C-C, Lin X-W, Chang JP and Peter RE (1998). mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. *Gen. Comp. Endocrinol.* **112**: 303–311.
74. Illing N, Troskie BE, Nahornisak CS, Hapgood JP, Peter RE and Millar RP (1999). Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). *Proc. Natl. Acad. Sci. USA* **96**: 2526–2531.
75. Okubo K, Suetake H, Usami T and Aida K (2000). Molecular cloning and tissue-specific expression of a gonadotropin-releasing hormone receptor in the Japanese eel. *Gen. Comp. Endocrinol.* **119**: 181–192.
76. Madigou T, Mananos-Sanchez E, Hulshof S, Anglade I, Zanuy S and Kah O (2000). Cloning, tissue distribution, and central expression of the gonadotropin-releasing hormone receptor in the rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod.* **63**: 1857–1866.
77. Robison RR, White RB, Illing N, Troskie BE, Morley M, Millar RP and Fernald RD (2001). Gonadotropin releasing hormone receptor in the teleost *Haplochromis burtoni*: Structure, location, and function. *Endocrinology* **142**: 1737–1743.
78. Bogerd J, Diepenbroek WB, Hund E, van Oosterhout F, Teves AC, Leurs R and Blomenrohr M (2002). Two gonadotropin-releasing hormone receptors in the African catfish: No differences in ligand selectivity, but differences in tissue distribution. *Endocrinology* **143**: 4673–4682.
79. Siler-Khodr TM, Grayson M and Eddy CA (2003). Action of Gonadotropin-releasing hormone II on the baboon ovary. *Biol. Reprod.* **68**: 1150–1156.
80. Hillensjo T and LeMaire WJ (1980). Gonadotropin releasing hormone agonists stimulate meiotic maturation of follicle-enclosed rat oocytes *in vitro*. *Nature* **287**: 145–146.
81. Gnessi L, Fabbri A and Spera G (1997). Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environments. *Endo. Rev.* **18**: 541–609.

82. Huhtaniemi IT, Nikula H, Detta A, Stewart JM and Clayton RN (1987). Blockade of rat testicular gonadotropin-releasing hormone (GnRH) receptors by infusion of a GnRH antagonist has no major effects on Leydig cell function *in vivo*. *Mol. Cell. Endocrinol.* **49**: 89–97.
83. Andreu-Vieyra CV and Habibi HR (2001). Effects of salmon GnRH and chicken GnRH-II on testicular apoptosis in goldfish (*Carassius auratus*). *Comp. Biochem. Physiol. B* **129**: 483–487.
84. Wray S (2002). Development of gonadotropin-releasing hormone-1 neurons. *Front. Neuroendocrinol.* **23**: 292–316.
85. Seeburg PH and Adelman JP (1984). Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. *Nature* **311**: 666–668.
86. Aubert ML, Grumbach MM and Kaplan SL (1977). The ontogenesis of human fetal hormones IV. *J. Clin. Endocrinol. Metab.* **44**: 1130–1141.
87. Palmon A, Aroya NB, Tel-Or S, Burstein Y, Fridkin M and Koch Y (1994). The gene for the neuropeptide gonadotropin-releasing hormone is expressed in the mammary gland of lactating rats. *Proc. Natl. Acad. Sci. USA* **91**: 4994–4996.
88. Dutlow CM, Rachman J, Jacobs TW and Millar RP (1992). Prepubertal increases in gonadotropin-releasing hormone mRNA, gonadotropin-releasing hormone precursor, and subsequent maturation of precursor processing in male rats. *J. Clin. Invest.* **90**: 2496–2501.
89. Halpern-Sebold LR and Schreibman MP (1983). Ontogeny of centers containing luteinizing hormone-releasing hormone in the brain of platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. *Cell Tissue Res.* **229**: 75–84.
90. Chiba A, Oka S and Honma Y (1994). Ontogenetic development of gonadotropin-releasing hormone-like immunoreactive neurons in the brain of the chum salmon, *Oncorhynchus keta*. *Neurosci. Lett.* **178**: 51–54.
91. Parhar IS and Iwata M (1996). Intracerebral expression of gonadotropin-releasing hormone and growth hormone-releasing hormone is delayed until smoltification in the salmon. *Neurosci. Res.* **26**: 299–308.
92. Parhar IS, Iwata M, Pfaff DW and Schwanzel-Pukuda M (1995). Embryonic development of gonadotropin-releasing hormone neurons in the sockeye salmon. *J. Comp. Neurol.* **362**: 256–270.
93. Amano M, Oka Y, Kitamura S, Ikuta K and Aida K (1998). Ontogenetic development of salmon GnRH and chicken GnRH-II systems in the brain of masu salmon (*Oncorhynchus masou*). *Cell Tissue Res.* **293**: 427–434.

94. Chiba H, Nakamura M, Iwata M, Sakuma Y, Yamauchi K and Parhar IS (1999). Development and differentiation of gonadotropin hormone-releasing hormone neuronal systems and testes in the Japanese eel (*Anguilla japonica*). *Gen. Comp. Endocrinol.* **114**: 449–459.
95. Parhar IS, Soga T, Ishikawa Y, Nagahama Y and Sakuma Y (1998). Neurons synthesizing gonadotropin-releasing hormone mRNA subtypes have multiple developmental origins in the medaka. *J. Comp. Neurol.* **401**: 217–226.
96. Gonzalez-Martinez D, Zmora N, Zanuy S, Sarasquete C, Elizur A, Kah O and Munoz-Cueto JA (2002). Developmental expression of three different prepro-GnRH (Gonadotropin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). *J. Chem. Neuroanat.* **23**: 255–267.
97. Whitlock KE, Wolf CD and Boyce ML (2003). Gonadotropin-releasing hormone (GnRH) cells arise from cranial neural crest and adenohypophyseal regions of the neural plate in the zebrafish, *Danio rerio*. *Dev. Biol.* **257**: 140–152.
98. Nelson SB, Eraly SA and Mellon PL (1998). The GnRH promoter: Target of transcription factors, hormones, and signaling pathways. *Mol. Cell. Endocrinol.* **140**: 151–155.
99. Burbach JPH (2002). Regulation of gene promoters of hypothalamic peptides. *Front. Neuroendocrinol.* **23**: 342–369.
100. Wolfe A, Kim HH, Tobet S, Stafford DE and Radovick S (2002). Identification of a discrete promoter region of the human GnRH gene that is sufficient for directing neuron-specific expression: A role for POU homeodomain transcription factors. *Mol. Endocrinol.* **16**: 435–449.
101. Wierman ME, Xiong X, Kepa JK, Spaulding AJ, Jacobsen BM, Fang Z, Nilaver G and Ojeda SR (1997). Repression of gonadotropin-releasing hormone promoter activity by the POU homeodomain transcription factor SCIP/Oct-6/Tst-1: A regulatory mechanism of phenotype expression? *Mol. Cell. Biol.* **17**: 1652–1665.
102. Dong KW, Zheng HM, Wen ZY and Chen ZG (2001). The POU homeodomain protein Oct-1 binds Cis-regulatory element essential for the human GnRH upstream promoter activity in JEG-3 cells. *J. Clin. Endocrinol. Metab.* **86**: 2838–2844.

103. Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP and Mellon PL (1995). A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. *Mol. Endocrinol.* **9:** 467–477.
104. Kepa JK, Spaulding AJ, Jacobsen BM, Fang Z, Xiong X, Radovick S and Wierman ME (1996). Structure of the distal human gonadotropin releasing hormone (hGnRH) gene promoter and functional analysis in GT1-7 neuronal cells. *Nucleic Acids Res.* **24:** 3614–3620.
105. Chen A, Laskar-Levy O, Ben-Aroya N and Koch Y (2001). Transcriptional regulation of the human GnRH-II gene is mediated by a putative cAMP response element. *Endocrinology* **142:** 3483–3492.
106. Dong K-W, Yu K-L, Chen Z-G, Chen Y-D and Roberts JL (1997). Characterization of multiple promoters directing tissue-specific expression of the human gonadotropin-releasing hormone gene. *Endocrinology* **138:** 2754–2762.
107. Klungland H, Lorens JB, Andersen Ø, Kisen GØ and Aleström P (1992). The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. *Mol. Cell. Endocrinol.* **84:** 167–174.
108. White RB and Fernald RD (1998). Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. *Gen. Comp. Endocrinol.* **112:** 17–25.
109. Chow MM, Kight KE, Gothilf Y, Alok D, Stubblefield J and Zohar Y (1998). Multiple GnRHs present in a teleost species are encoded by separate genes: analysis of the sbGnRH and cGnRH-II genes from the striped bass, *Morone saxatilis*. *J. Mol. Endocrinol.* **21:** 277–289.
110. Farahmand H, Rahman MA, Sohm F, Hwang G-L and Maclean N (2003). Isolation and expression of tilapia (*Oreochromis niloticus*) serine 8-type GnRH coding and regulatory sequences. *Gene* **304:** 97–106.
111. Husebye H, Collas P and Aleström P (1997). A functional study of the salmon GnRH promoter. *Mol. Mar. Biol. Biotech.* **6:** 357–363.
112. Klungland H, Andersen Ø, Kisen G, Aleström P and Tora L (1993). Estrogen receptor binds to the salmon GnRH gene in a region with long palindromic sequences. *Mol. Cell. Endocrinol.* **95:** 147–154.
113. Klungland H, Andersen Ø and Aleström P (1992). The salmon gonadotropin-releasing hormone encoding gene in salmonids. *Mol. Mar. Biol. Biotech.* **1:** 420–425.

Chapter 2

GnRH Receptors in Fish: Differences in Structure-Function Relations between Mammalian and Non-mammalian GnRH Receptors

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Abstract

We have learnt from fish that the existence of multiple forms of GnRH and GnRH receptors in a single species is a general phenomenon amongst vertebrates. Moreover, the cloning of fish GnRH receptors revealed that differences between mammalian and nonmammalian GnRH receptors in their pharmacology and regulation are accompanied by striking structural variations. Here, the results of studies on mammalian and catfish GnRH receptor expression, regulation, activation, and ligand binding are summarized. To this end, mammalian-nonmammalian chimeric GnRH receptors, site-directed mutagenesis of GnRH receptors,

various GnRH analogs and a three-dimensional model of the receptor-ligand complex were used. As a result of these studies we understand better the relationship between structure and function of GnRH receptors in general.

Mammalian GnRH Receptors

The actions of gonadotropin-releasing hormone (GnRH) and its analogs have been investigated by binding and signal transduction studies *in vivo* and *in vitro* (tissue fragments, and isolated and cultured gonadotropes). The impetus for recent progress in the understanding of the molecular mechanism of GnRH action, however, was provided by the isolation of GnRH receptor cDNAs. Expression of these GnRH receptor cDNAs in cell lines provided the tools for studies on the structure-function relation of GnRH receptors concerning receptor-ligand interaction, receptor activation and signaling, and receptor regulation. Moreover, it became possible to study the spatiotemporal expression pattern of the mRNAs that code for GnRH receptors.

Cloning of Mammalian GnRH Receptors

In 1992, the cDNA encoding the mouse GnRH receptor was the first to be cloned.¹ To this end, two degenerate oligonucleotide primers, based on amino acid sequences conserved among G protein-coupled receptors (GPCRs), were used in a polymerase chain reaction (PCR) with cDNA from a murine gonadotrope tumor cell line (α T3-1 cells) as template. The identity of the GnRH receptor was confirmed by *Xenopus* oocyte expression. In the same year, the mouse GnRH receptor was also identified by *Xenopus* oocyte expression cloning.² Once the mouse GnRH receptor sequence was known, homologous cDNAs were cloned from pituitaries of five additional mammalian species, using primers based on the mouse receptor sequence: rat,^{3,4} human,⁵ sheep,^{6,7} cow⁸ and pig.⁹ The cDNAs of the GnRH receptor isolated from human breast and ovarian tumors¹⁰ and from rat gonads¹¹ were identical to the pituitary GnRH receptor cDNAs of the corresponding species.

In 1994, the gene of the human GnRH receptor, spanning over 18.9 kb, which is interrupted by 2 introns of 4.2 and 5.0 kb, was isolated.¹²

Localization of Mammalian GnRH Receptors

To determine the anatomical site of action of GnRH peptides, receptor autoradiography was performed from the late 70's onwards. To this end, stable GnRH analogs, like Buserelin, were labeled with radioactive iodine (¹²⁵I), which allows tissue localization. At that time, it was surprising that specific GnRH-binding sites are not restricted to pituitary gonadotropes. GnRH-binding sites were also detected in hypothalamic, GnRH-secreting neurons and in other parts of the rat brain.¹³ Moreover, GnRH has been demonstrated to bind specifically to Leydig but not Sertoli cells in the testis,¹⁴ to granulosa and luteal cells in the ovary,¹⁵ to the placenta,¹⁶ to tumors of the breast,¹⁷ to the thymus,¹⁸ and to the adrenal cortex.¹⁹ So far, no antibody that specifically recognizes the GnRH receptor has been reported.²⁰ After the discovery of the GnRH receptor DNA sequences, however, it became possible to determine which tissues express the mRNA coding for GnRH receptors using Northern blot and reverse transcription (RT)-PCR techniques, and to localize with *in situ* hybridization which cell types express the GnRH receptor mRNA. Northern blot analysis revealed GnRH receptor mRNA expression in the pituitary, testicular Leydig cells and ovary, whereas no hybridization signal was found in liver, adrenal, kidney, spleen, lung and placenta in rat.^{2,4} The absence of hybridization in adrenal and placental tissue might be due to the detection limit of this technique. Using RT-PCR, the pituitary,²¹ hypothalamus²² and several human cancer cell lines²³ have been reported to express GnRH receptor mRNA. Results of recent *in situ* hybridization studies showed a large degree of overlap in the localization of the GnRH receptor mRNA-expressing neurons with those identified by autoradiography.¹³

In summary, the coexistence of GnRHs and their receptors in neuronal and peripheral tissues supports the view that GnRHs may act as neurotransmitter and/or neuromodulator in the brain and as a hormone, with an autocrine-paracrine function in gonadal and other peripheral tissues, in addition to their established neuroendocrine role in the pituitary.

Structure of Mammalian GnRH Receptors

Mammalian GnRH receptors consist of a single polypeptide chain of 327 (mouse, rat) and 328 (human, sheep, cow, pig) amino acids, and exhibit high homology (>85% amino acid identity) among themselves. Their calculated molecular mass based on the deduced amino acid sequence is approximately 38 kDa, while the determined mass by photoaffinity labeling is 50 to 60 kDa.²⁴ The difference is due to N-linked glycosylation of the receptor.²⁵ GnRH receptors contain an extracellular amino-terminal segment and seven hydrophobic domains, each of which probably forms a membrane-spanning α -helix (TM), connected by extracellular and intracellular loops (Fig. 1).¹ This organization is a characteristic feature of receptors belonging to the family of GPCRs. The seven TMs of rhodopsin and of adrenergic receptors are arranged as a closed loop in the counterclockwise direction from TM 1 to TM 7 when viewed from the extracellular surface.²⁶ However, GnRH receptors have relatively little sequence homology with other GPCRs, apart from oxytocin and vasopressin receptors with which they share about 25% homology.²⁷ Nevertheless, GnRH receptors contain a number of amino acid residues that are highly conserved throughout the rhodopsin group of GPCRs. They include Asn⁵³ in TM 1, Leu⁸⁰ and Leu⁸³ in TM 2, Cys¹¹⁴, Ile¹³⁵-Ser-X-Asp-Arg¹³⁹-X-X-X-Ile¹⁴³ in TM 3, Trp¹⁶⁴, Ser¹⁶⁷ and Pro¹⁷³ in TM 4, Phe²¹⁹-X-X-Pro²²², Met²²⁶ and Ile²³³ in TM 5, Lys²⁶⁶ and Phe²⁷⁵-X-X-Cys-Trp-X-Pro-Tyr²⁸² in TM 6, and Phe³¹⁰, Asn³¹⁴ and Pro³¹⁹-X-X-Tyr³²² in TM 7 (Fig. 1).^{28,29} On the other hand, some features conserved among GPCRs are modified in mammalian GnRH receptors (Fig. 1).^{29,30}

The most striking alteration of mammalian GnRH receptors is the absence of the intracellular C-terminal domain. Moreover, the highly conserved DRY-triplet adjacent to TM 3 is changed into a DRS-triplet. Substitution of Ser¹⁴⁰ of the DRS motif in the mouse GnRH receptor by Tyr did not affect G-protein coupling but significantly increased receptor affinity and internalization rate.³¹ In addition, mammalian GnRH receptors have an Asn⁸⁷ residue instead of a conserved Asp residue in TM 2 and an Asp^{318/319} residue instead of a conserved Asn

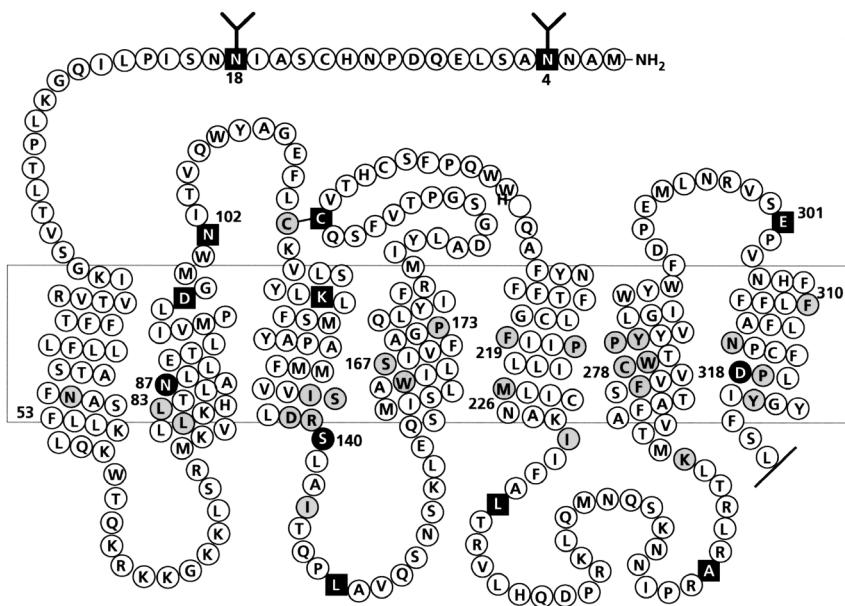


Fig. 1 Schematic representation of the mouse GnRH receptor. Amino acid motifs that are highly conserved among GPCRs are indicated by grey circles. Residues that are highly conserved among GPCRs but different in mammalian GnRH receptors are indicated by black circles. Other residues that affect receptor function are marked by black squares (see text). (Adapted with permission from Ref. 29. © 1997 *Journals of Reproduction and Fertility*.)

residue in TM 7. The functional significance of this apparent interchange of conserved residues was studied in mouse and rat mutant GnRH receptors.^{32–34} A close proximity between Asn⁸⁷ in TM 2 and Asp³¹⁸ in TM 7 was considered to be necessary for maintaining the functional integrity of the GnRH receptor, since the Asp⁸⁷ mutant GnRH receptor lost GnRH binding capacity while the reciprocal Asp⁸⁷Asn³¹⁸ mutant receptor regained wild-type receptor properties.³² For GPCRs in general, it has been assumed that conserved polar TM residues create a hydrogen bond network in the receptor center.³⁵ Furthermore, a disulfide bridge between a highly conserved Cys residue at the extracellular end of TM 3 with one of the Cys residues in the second extracellular loop probably

stabilizes the conformation of GPCRs.²⁸ For the rat and the human GnRH receptor it has indeed been demonstrated that a disulfide bridge between Cys¹¹⁴ and Cys¹⁹⁶ is important for the general structure of the receptor.^{20,36} Moreover, in agreement with reports on other GPCRs, N-linked glycosylation of the extracellular amino-terminal domain (Fig. 1) seems to be important for proper receptor folding and surface expression.²⁵

Receptor-Ligand Interaction

Knowledge about the precise interaction of GnRH receptors with their ligands provides the basis for the rational design of novel peptide and nonpeptide GnRH analogs. Direct structural data available for GPCRs was limited to the low-resolution structures of bovine and frog rhodopsin.^{37,38} Investigations of the binding pocket of GPCRs have relied on indirect approaches, particularly the study of the functional effects of site-directed mutagenesis and the construction of computational three-dimensional molecular models of the ligand-receptor complex. Through these approaches, side chains required for high-affinity binding of peptide ligands have been identified in both the extracellular and transmembrane domains.³⁹ Recently, the crystal structure of bovine rhodopsin was determined,⁴⁰ which will allow much more detailed structural studies in the future.

The binding pocket for small biogenic amines is the best characterized.^{39,41} Asp¹¹³ in TM 3 of the β -adrenergic receptor has been reported to be the most important interaction point with the amine group in the ligand. GnRH receptors have a conserved Lys¹²¹ residue at the homologous position in TM 3 (Fig. 1). The effects of a series of mutations at this locus in the human GnRH receptor have indicated that a charge-strengthened hydrogen bond donor is required at this locus for high affinity agonist binding but not for high affinity antagonist binding.⁴² Based on the available structure-activity data of GnRH analogs, His² in the ligand is a potential candidate for interaction with this locus. Moreover, His² of GnRH has been implicated in a

high-affinity interaction with Asp⁹⁸ in TM 2.⁴³ But the ligand-binding pocket of GnRH receptors is larger than those for the biogenic amines, which bind solely to residues in the TM bundle. The Asn¹⁰² residue in the extracellular loop 1 of the human GnRH receptor⁴⁴ and the Glu³⁰¹ residue in the extracellular loop 3 of the mouse GnRH receptor⁴⁵ have been demonstrated to participate in the ligand-binding pocket for GnRHs as well (Fig. 1). Mutation of Asn¹⁰² to Ala revealed that Asn¹⁰² is a critical residue determining the transduction of GnRH potency in the receptor specifically for ligands with C-terminal glycinamide, while ligands with C-terminal ethylamide are less dependent on Asn¹⁰².⁴⁴ Therefore, the Asn¹⁰² side chain is supposed to form a hydrogen bond with the C-terminal amide moiety of GnRH. Moreover, it was known that mammalian GnRH receptors have a high selectivity for the basic Arg⁸ residue of mammalian GnRH.⁴⁶ While systematically mutating conserved acidic residues in the extracellular and transmembrane domains of the mouse GnRH receptor, the Glu³⁰¹ residue in the extracellular loop 3 has been identified to specifically discriminate between basic and nonbasic residues at position 8 of the ligand.⁴⁵ Thus, Glu³⁰¹ residue in TM 3 of the mouse GnRH receptor plays a role in the recognition of Arg⁸ in the ligand.

Nonmammalian GnRH receptors, on the contrary, have previously been shown to be promiscuous in interacting with most of the GnRHs. All nonmammalian GnRH receptors seem to have a preference for chicken GnRH-II (cGnRH-II), although mammalian GnRH receptors are also substantially activated by cGnRH-II.^{46,47} Therefore, it is desirable to obtain information on the sequence of nonmammalian GnRH receptors in order to get insight into structural homologies and differences between mammalian and nonmammalian GnRH receptors. Such knowledge will help to enhance our understanding of GnRH receptor structure and its ligand-binding site.

Receptor Activation and Signaling

Binding of a ligand to a GPCR leads to the transduction of the signal of the ligand from the external environment of the cell to G proteins,

present at the intracellular environment of the cell, that will, in turn, communicate with intracellular effectors.⁴⁸ The intracellular signaling events after stimulation of mammalian GnRH receptors, expressed in either gonadotropes, pituitary cell lines or heterologous cell systems, have been reviewed extensively.^{27,49-51} Briefly, GnRH receptors are coupled to G proteins of the G_{q/11} family. Next, phospholipase C (PLC) activation, mediated by these G proteins, leads to an elevation of the second messengers inositol 1,4,5-triphosphate (IP) and diacylglycerol which, in turn, are required for protein kinase C (PKC) activation and calcium mobilization. The latter are necessary for the GnRH-induced release of gonadotropins. Activation of phospholipase A₂ and of phospholipase D is also involved in this process, as demonstrated by the liberation of arachidonic acid and phosphatidic acid. Differential cross-talk of calcium, arachidonic acid and PKC also activates downstream elements like the mitogen-activated protein kinase cascade. This cascade mediates the activation of transcription factors such as c-fos and c-jun and as such regulates gonadotropin gene expression. In addition, GnRH-induced elevation of cyclic 3',5'-adenosine monophosphate (cAMP) levels have been reported in selected cell lines, but not in gonadotropes. Recently, it has been demonstrated that this event is not due to GnRH receptor coupling to G_s proteins, but cAMP formation is rather initiated downstream of the receptor/G protein coupling.⁵²

The mechanism of GPCR activation by agonists has been reviewed.⁵³⁻⁵⁵ The most widely accepted model used to describe agonist activation of GPCRs is the ternary complex model, which accounts for the cooperative interaction between receptor, G protein and agonist.⁵⁶ This model has been extended to accommodate constitutively active receptors and inverse agonists.⁵⁷ The revised model proposes that GPCRs exist in equilibrium between two functionally distinct states: an inactive receptor conformation and an active one. Agonist binding shifts the equilibrium towards the active receptor conformation. The latter state has the highest affinity for agonists, and is the only form that can bind G proteins. The shift is supposed to be caused by changes in TM hydrogen bonds which subsequently result in TM reorganization by their rotation. Thereby, selected residues will be hidden whereas others,

important for G-protein interaction, become exposed. Residues that are overall conserved among GPCRs (Fig. 1) are considered to be involved in this intramolecular signal propagation.²⁹

The Asp and Arg residues of the DRY motif at the junction of TM 3 and the intracellular loop (IL) 2 are highly conserved among GPCRs. Studies on the mouse GnRH receptor revealed that mutants, in which Asp¹³⁸ was replaced by Asn or Glu, exhibited an augmented IP response, whereas substitution of Arg¹³⁹ by Gln, Ala or Ser resulted in an impaired IP response.⁵⁸ In addition, the Asp³¹⁸ residue of the mouse GnRH receptor has been implicated in efficient PLC coupling.⁵⁸ For many GPCRs, the Asp residue that is involved in forming the TM 2/TM 7 microdomain has been reported to be required for efficient coupling. Yet, in the GnRH receptor, the requirement for an acidic side chain seems to be fixed at the TM 7 locus, whereas other GPCRs contain the Asp residue in the TM 2.⁵⁹ Recently, these findings were combined in a model of GnRH receptor activation.⁶⁰ That model predicts that the orientation of the highly conserved Arg¹³⁹ side chain is constrained in the inactive receptor state by an ionic interaction with the neighboring conserved residue Asp¹³⁸. During activation, Asp¹³⁸ becomes protonated, and the Arg side chain is released. The conserved bulky side chain of Ile143 modulates the positioning of the Arg side chain by keeping it away from the cytoplasmic aqueous medium and thereby promotes the interaction with Asp³¹⁸ that characterizes the active state of the receptor.⁶⁰ In addition, another highly conserved aromatic moiety in the DPLIY motif in TM 7 has also been implicated in the process of GnRH receptor activation.⁶¹

Domains critical for interaction with the G protein have been localized to the second and third cytoplasmic loops, particularly in their N- and C-terminal regions, and sometimes in the membrane proximal regions of the C-terminal tail.⁶² For the GnRH receptor, Leu¹⁴⁷³¹ and Arg¹⁴⁵³⁰ in IL 2, the IL 3 in general^{63,64} and particularly residues Leu²³⁷⁶⁵ and Ala²⁶¹⁶⁶ of IL 3 have been reported to be important for agonist induced signal propagation. But also IL 1 of the mouse GnRH receptor seems to play a role in receptor signaling.⁶⁷ The heterogeneity in the amino acid sequence and size of ILs for various

GPCRs, however, suggests that the secondary structure rather than the primary sequence and/or the length of the loops is important in determining G-protein coupling and activation.⁶²

It is remarkable that GnRH antagonists designed for mammals do not necessarily have antagonistic effects on nonmammalian pituitaries.⁶⁸ Differences in the pharmacology of GnRH receptors in vertebrate species³⁰ are considered to provide additional information about structural requirements for receptor activation. Moreover, mammalian and nonmammalian GnRH receptors are both stimulated by GnRHs. Therefore, nonmammalian receptors are likely to represent a good tool for checking models of GnRH activation which are based on effects of mammalian GnRH receptor mutagenesis.

Receptor Regulation

As a living cell has to integrate a variety of stimuli, it is important that the responsiveness of any cell is tightly controlled. A major mechanism involved in the control of GPCRs is receptor desensitization, which is characterized by an attenuated receptor responsiveness as a consequence of prolonged exposure to agonists. However, a distinction has to be made between the following three processes: rapid receptor desensitization, receptor internalization or sequestration and receptor downregulation (Fig. 2).⁶⁹

Rapid desensitization involves the functional uncoupling of receptors from their effector systems within seconds to minutes after agonist binding. Agonist-specific (homologous) desensitization is characterized by phosphorylation of agonist-occupied receptors by specific GPCR kinases and subsequent binding of an arrestin protein, which sterically hinders receptor coupling to a G protein.⁷⁰ During heterologous desensitization, second messenger-stimulated kinases, e.g. protein kinase A (PKA) and PKC, phosphorylate any GPCR containing appropriate PKA and/or PKC consensus phosphorylation sites. The phosphorylation sites for both second messenger-stimulated kinases and GPCR kinases are often found in the C-terminal tail or within the third intracellular loop.⁷¹ It is of interest that mammalian GnRH receptors completely

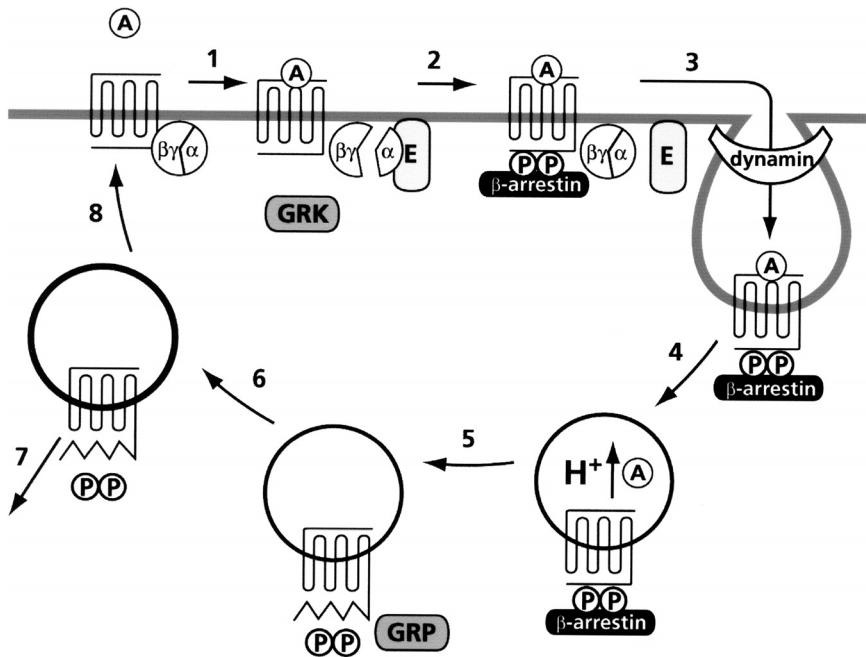


Fig. 2 General scheme for desensitization and cycling of GPCRs. Binding of the agonist (A) not only causes activation of the effector (E) but also facilitates phosphorylation of the receptor [often by GPCR kinases (GRKs) on specific sites within the C-terminal tail], which enables binding of β-arrestin (1–2). β-arrestin prevents effector activation and targets the desensitized receptor for internalization via clathrin-coated vesicles which are pinched off from the plasma membrane by dynamin (2–3). The receptor is then dephosphorylated by a GPCR phosphatase (PRP), a process that may be facilitated by acidification of the vesicle lumen and consequent alteration of receptor conformation (4–6). The receptor can then be targeted to lysosomes for degradation (7) or, after dephosphorylation (resensitization), recycled to the cell surface (8). Note that GPCRs can also be internalized by bulk endocytosis and by mechanisms independent of receptor phosphorylation. (Reprinted with permission from Ref. 76. © 1999 Elsevier Science Ireland Ltd.)

lack the C-terminal tail and only contain a relatively short third intracellular loop.³⁰ However, sustained exposure of gonadotropes to GnRH reduces GnRH-stimulated gonadotropin secretion, a fact that is exploited in the major clinical applications of GnRH analogs. Recently, it has been demonstrated that the rat GnRH receptor is not

phosphorylated in an agonist-dependent manner⁷² and that it is resistant to rapid desensitization of PLC signaling in α T3-1, COS-7 and HEK 293 cells.⁷³⁻⁷⁵ The desensitization of gonadotropin secretion is rather due to post-receptor mechanisms, namely the downregulation of IP₃ receptors and consequent desensitization of GnRH effects on cytosolic calcium.⁷⁶

Besides an uncoupling of GPCRs from their respective G proteins, agonists can also promote the internalization of receptors within minutes to hours. β -arrestin targets desensitized receptors to clathrin-coated pits, from where they are pinched off in vesicles mediated by the GTPase dynamin. Receptor internalization can either lead to receptor resensitization via receptor dephosphorylation and subsequent recycling to the plasma membrane, or receptors are targeted to lysosomes for proteolytic degradation resulting in receptor downregulation. Once receptors are downregulated, recovery needs *de novo* synthesis of receptor proteins.⁷⁰ Similar to rapid homologous desensitization, the process of receptor internalization depends on agonist-induced phosphorylation of serine/threonine residues, predominantly located in the C-terminal tail.^{77,78} The rat GnRH receptor, missing this tail and being resistant to agonist-dependent phosphorylation,⁷² has been demonstrated to be poorly internalized compared to the thyrotropin-releasing hormone (TRH) receptor, that contains a C-terminal tail.⁷⁵ Agonist-induced internalization of the rat GnRH receptor seems to be β -arrestin independent, but dynamin dependent.^{75,79,80}

Receptor downregulation (degradation) and upregulation (*de novo* synthesis) account for marked changes in GnRH receptor number that occur in a number of different endocrine states including development, estrous cycle, lactation, castration, hormone replacement and aging.⁸¹

Comparison of Structure-Function Relations between the Catfish GnRH Receptor and its Mammalian Counterparts

The African catfish GnRH receptor was the first GnRH receptor characterized from a nonmammalian vertebrate.⁸² Surprisingly, the catfish

GnRH receptor differs from its mammalian counterparts in containing an intracellular C-terminal tail and in having Asp residues in TM 2 and 7 (Fig. 3). Subsequently, the cloning of chicken,⁸³ goldfish,⁸⁴ bullfrog,⁸⁵ *Xenopus laevis*,⁸⁶ *Seriola dumerilii* (personal observation) and red seabream (personal observation) GnRH receptors revealed that all nonmammalian GnRH receptors have a C-terminal tail and the Asp/Asp motif in TM 2 and 7. Thus, these unique features described for mammalian GnRH receptors are not found in their nonmammalian counterparts.

Next to structural variations, differences between mammalian and nonmammalian GnRH receptors were found in their pharmacology and their regulation. In the following sections, the results of studies on catfish and mammalian GnRH receptor expression, regulation, activation, and ligand binding will be summarized. To this end, mammalian-nonmammalian chimeric GnRH receptors, site-directed mutagenesis of GnRH receptors, various GnRH analogs and a three-dimensional model of the receptor-ligand complex were used.

The Carboxyl-Terminal Tail

In many GPCRs, the intracellular C-terminal tail is important for receptor expression^{87,88} and for regulatory processes, e.g. agonist-induced phosphorylation and subsequent receptor desensitization and internalization.^{70,89} The presence of the C-terminal tail in nonmammalian GnRH receptors has enabled studies on the role of this C-terminal tail in these receptors as well as the functional consequences of its absence in mammalian GnRH receptors.

Progressive truncations of the C-terminal tail decreased cell surface expression of the catfish GnRH receptor,⁹⁰ whereas the addition of the catfish C-terminal tail to the naturally tailless rat GnRH receptor resulted in elevated levels of receptor expression at the cell surface.⁹¹ The enhanced expression of mammalian GnRH receptors, fused to the C-terminal tail of the catfish GnRH receptor, in transfected cells could be of value in screening for therapeutically useful GnRH analogs. However, in mammalian gonadotropes, the expression level of the naturally tailless GnRH receptor seems to be sufficient, since it has

been demonstrated that occupancy of 20% of GnRH receptors can evoke 80% of the biological response.⁹² In addition to enhancing mammalian GnRH receptor expression, the C-terminal tail of the catfish GnRH receptor has also been used as a linker between the rat GnRH receptor and the green fluorescent protein in order to visualize receptor localization in living cells.⁹³

Furthermore, it was demonstrated that the catfish GnRH receptor is susceptible to agonist-induced phosphorylation and that Ser³⁶³ in the C-terminal tail is the major phospho-acceptor site in this process.⁹⁰ Mammalian GnRH receptors, on the contrary, are resistant to agonist-dependent phosphorylation due to the lack of a C-terminal tail.⁷² This was substantiated by comparing wild-type mammalian GnRH receptors with chimeras of the mammalian GnRH receptor fused to the C-terminal tail of either the mammalian TRH receptor or the catfish GnRH receptor: only the latter two are phosphorylated in an agonist-dependent manner.⁷² Thus, the presence of a functional C-terminal tail confers agonist-induced GnRH receptor phosphorylation.

Agonist-induced phosphorylation seems to be a prerequisite for desensitization of the catfish GnRH receptor. It undergoes rapid desensitization of the IP response in HEK 293 and COS-7 cells,^{72,75} whereas the tailless rat GnRH receptor showed no desensitization of the IP response within seconds to minutes in αT3-1, HEK 293 or COS-7 cells.^{72,75} Addition of the C-terminal tail of either the catfish GnRH receptor or the rat TRH receptor to the rat GnRH receptor resulted in rapid desensitization of the IP response.^{72,75} Possibly the pattern of GnRH release demands GnRH receptors that are susceptible to desensitization in the catfish pituitary, where GnRH is locally secreted in the vicinity of the gonadotropes, whereas in mammals GnRH is released into the portal system in a pulsatile fashion. Still, mammalian gonadotropin secretion is desensitized upon continuous GnRH stimulation, because the absence of mammalian GnRH receptor desensitization is compensated by a desensitizable postreceptor mechanism, i.e. the down regulation of IP₃ receptors and consequent desensitization of GnRH effects on cytosolic calcium.⁷⁶ On the other hand, it has not been investigated properly whether catfish gonadotropes

show rapid desensitization of the gonadotropin secretion as a result of the fact that catfish GnRH receptors are susceptible to desensitization within seconds to minutes. It has been demonstrated that continuous administration of 10 μ M mammalian GnRH (mGnRH) analog to perfused catfish pituitaries resulted in a constant amount of released gonadotropins measured in 10-min interval fractions.⁹⁴ This finding might indicate that catfish gonadotropes are not susceptible to desensitization. However, rapid desensitization already occurs within seconds to minutes and a study monitoring gonadotropin secretion during the first 10 minutes has never been performed on catfish gonadotropes.

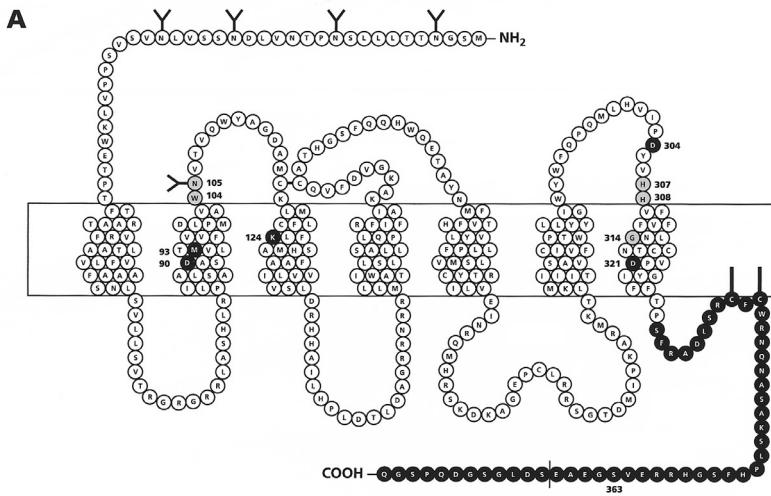
The process of agonist-induced internalization of the catfish GnRH receptor also depends on receptor phosphorylation, since the removal of the phosphorylation site resulted in impaired receptor internalization compared to the wild-type receptor.⁹⁰ However, truncated catfish GnRH receptors, still containing the phosphorylation site, also showed slower internalization kinetics than the wild-type receptor. The last 12 amino acids of the C-terminal tail seemed to be important for interaction with accessory proteins like β -arrestin and as such influence agonist-dependent receptor internalization.⁹⁰ The rat GnRH receptor, on the contrary, has been demonstrated to internalize in a β -arrestin-independent, but dynamin-dependent, manner.^{79,80} Rat GnRH receptor internalization kinetics are slower than those of the rat TRH receptor and of the catfish GnRH receptor, but an enhanced internalization rate was achieved under conditions of high β -arrestin when the rat TRH receptor C-terminal tail was added to the rat GnRH receptor.⁷⁵ The β -arrestin-independent part of the catfish GnRH receptor internalization is similar to that of the rat GnRH receptor.⁷² Apart from the C-terminal tail, that is important for β -arrestin-dependent internalization, other residues in the catfish GnRH receptor protein may be involved in the β -arrestin-independent part of receptor internalization. Studies on mutant mammalian GnRH receptors have provided evidence for the importance of conserved amino acids in the DRY/S triplet in the second intracellular loop, a conserved apolar amino acid in the third intracellular loop, and of aromatic amino acids in TM 7 in the mammalian GnRH

receptor internalization.^{31,58,61,65} Homologous residues in the catfish GnRH receptor may also be involved in the β -arrestin-independent part of catfish GnRH receptor internalization.

Residues in TM 2 and TM 7

The apparent interchange of otherwise highly conserved Asp and Asn residues in TM 2 and 7 of the mammalian GnRH receptors raised the possibility that these two residues interact with each other bringing TM 2 and 7 into close proximity. Studies on mutant mouse GnRH receptors indeed indicated a functional and spatial relationship of these two side chains.³² Data from all other GPCRs studied are in accordance with a spatial proximity of the conserved TM 2 and TM 7 side chains.⁵⁹ The nonmammalian GnRH receptors, however, have two Asp residues at the homologous positions (Fig. 3). Possibly, the presence of the two Asp residues in nonmammalian GnRH receptors represents an evolutionary intermediate between the conserved Asp/Asn arrangement found in most GPCRs and the Asn/Asp motif of mammalian GnRH receptors. Indeed, crystallographic studies have demonstrated that Asp side chains can occur in spatial proximity within proteins and can form hydrogen bonds when one of the Asp side chains is protonated (Fig. 1).^{95,96}

It has been reported that Asp⁹⁰ in TM 2 of the catfish GnRH receptor is implicated in receptor protein expression at the cell surface.⁹⁷ This is in accordance with findings on the mouse GnRH receptor that also requires the Asn residue at the homologous position in TM 2 for receptor expression.⁵⁸ Furthermore, it has been demonstrated for the catfish GnRH receptor that a negatively charged residue in this part of TM 2 is important for ligand binding and signaling.⁹⁷ It is more likely that Asp⁹⁰ is necessary for a proper receptor conformation than for direct interaction with the ligand. Based on a model for the human GnRH receptor it was speculated that Asp⁹⁰ in TM 2 interacts with Lys¹²⁴ in TM 3 and as such stabilizes the inactive state of the catfish GnRH receptor. However, the role of these two residues in catfish GnRH receptor binding and activation is rather independent.⁹⁷

**B**

	10	9	8	7	6	5	4	3	2	1
cfGnRH	G-NH ₂	P	N	L	G	H	S	W	H	pE
cGnRH-II	G-NH ₂	P	Y	W	G	H	S	W	H	pE
mGnRH	G-NH ₂	P	R	L	G	Y	S	W	H	pE

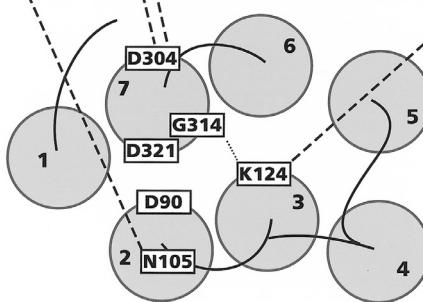


Fig. 3 (A) Schematic side view of the catfish GnRH receptor in the plasma membrane. Black circles indicate residues that have been investigated by site-directed mutagenesis, grey circles indicate residues that have been identified by three-dimensional molecular models for the binding of cGnRH-II or cGnRH-II-R8 to the catfish GnRH receptor.

(B) Schematic top view of the catfish GnRH receptor as seen from the extracellular side with each helix represented by a circle and the extracellular interconnecting loops (ELs)

The acidic side chain of Asp³²¹ in TM 7 does not seem to play a role in either ligand binding or efficient receptor coupling to G proteins.⁹⁸ The homologous Asp³¹⁸ residue in the mouse GnRH receptor, on the contrary, has been implicated in efficient PLC coupling.⁵⁹ Mammalian GnRH receptors have obviously fixed the requirement for an acidic side chain in TM 7, in contrast to most other GPCRs that require an acidic side chain in TM 2 for receptor activation.⁵⁹

In conclusion, it seems that the acidic side chain of Asp⁹⁰ in TM 2 of the catfish GnRH receptor is important for catfish receptor functioning, whereas Asp³²¹ in TM 7 is likely to be protonated. As such, TM 2 and TM 7 of the catfish GnRH receptor are capable of coming into close proximity, as has been postulated for mammalian GnRH receptors as well as for other GPCRs.

Receptor-Ligand Interactions

Mammalian GnRH receptors have a high affinity for GnRH peptides with a positively charged Arg on position 8.⁴⁶ It has been demonstrated that the negatively charged Glu³⁰¹ of the mouse GnRH receptor plays a role in the recognition of Arg⁸ in the ligand.⁴⁵ The catfish GnRH

Fig. 3 (Continued)

and the N-terminal domain as solid lines. The helices are organized sequentially in a counterclockwise fashion.

Extracellular loops 1 and 2 are joined by a disulfide bridge. D³²¹ (TM 7) is supposed to be protonated, thereby allowing an interaction with D⁹⁰ in TM 2. Moreover, K¹²⁴ and G³¹⁴ interact with each other in a way that TM 3 and 7 come into close proximity. M⁹³ (TM 2) sterically hinders an interaction of D⁹⁰ (TM 2) and K¹²⁴ (TM 3). N¹⁰⁵ (EL 1) interacts with the carboxyl-terminal G¹⁰-NH₂, K¹²⁴ (TM 3) with the amino-terminal pE¹, and D³⁰⁴ (EL 3) with R⁸ of mGnRH as well as with Y⁸ of cGnRH-II. The aromatic ring of Y⁸ of cGnRH-II is embedded in a pocket formed by the aromatic side chains of residues W¹⁰⁴, H¹⁰⁷ and H¹⁰⁸ in the receptor. Agonist binding to the receptor triggers phosphorylation of S³⁶³ in the intracellular carboxyl-terminal tail. S³⁶³ and the last twelve amino acid residues of the C-terminal tail are important for β-arrestin-dependent receptor internalization. In addition, the C-terminal tail plays a role in cell surface expression of the receptor and ligand binding.

receptor contains an Asp residue at the position homologous to Glu³⁰¹ in the mouse GnRH receptor.⁸² However, this nonmammalian GnRH receptor does not show a high affinity for mGnRH that contains an Arg on position 8.⁹⁸ To our surprise, mutagenesis studies and a three-dimensional molecular model of the catfish GnRH receptor having cGnRH-II-R8 docked in the binding pocket revealed that Asp³⁰⁴ of the catfish GnRH receptor nevertheless recognizes Arg⁸ in mGnRH and in chimeric GnRH analogs (catfish GnRH-R8 [cfGnRH-R8], cGnRH-II-R8, cGnRH-II-dW6,R8; Fig. 3).⁹⁹ It is likely that the low affinity of the catfish GnRH receptor for mGnRH is due to an unfavorable fit of residue 5 (Tyr) and/or residue 7 (Leu) of this ligand into the binding pocket of the catfish GnRH receptor rather than to the inability of this receptor to specifically recognize Arg⁸ in mGnRH.

Instead of a preference for mGnRH, the catfish GnRH receptor like other nonmammalian GnRH receptors, shows a high affinity for cGnRH-II.³⁰ Using native and chimeric GnRH analogs varying in positions 5, 7 and 8, we identified His⁵, Trp⁷ and Tyr⁸ as features of cGnRH-II conferring specificity for the catfish GnRH receptor.⁹⁹ Results of binding studies with the native and chimeric peptides on D304A and D304N mutant catfish GnRH receptors suggested that Asp³⁰⁴ is not important for the recognition of Tyr⁸ in cGnRH-II.⁹⁹ However, the molecular model for binding of cGnRH-II to the catfish GnRH receptor indicated that Tyr⁸ is able to form a hydrogen bond with Asp³⁰⁴ in the wild-type receptor (Fig. 3).⁹⁹ But in the absence of Asp³⁰⁴, Tyr⁸ can also form a hydrogen bond with Ala¹⁹⁵, and the aromatic ring of Tyr⁸ is embedded in a pocket formed by the aromatic rings of residues Trp¹⁰⁴ in TM 2 and His³⁰⁷ and His³⁰⁸ in TM 7 (Fig. 3).⁹⁹ Therefore Asp³⁰⁴ is less important for the recognition of Tyr⁸ in cGnRH-II than of Arg⁸ in mGnRH.

Molecular dynamic simulations further illustrate that His⁵ of cGnRH-II is in close proximity with Gln²⁹⁵ and His³⁰⁷ of the catfish GnRH receptor,⁹⁹ whereas Tyr⁵ of mGnRH interacts with Asp²⁹³ (homologous to Gln²⁹⁵ of the catfish GnRH receptor) in the human GnRH receptor.¹⁰⁰ The Trp⁷ residue of cGnRH-II is embedded in a hydrophobic pocket formed by residues of TM 3, 4 and 7 together with residues of the second extracellular loop.⁹⁹

In addition, binding studies on a Lys¹²⁴-mutant catfish GnRH receptor⁹⁷ and molecular models for the binding of cGnRH-II to the catfish GnRH receptor⁹⁷ implicated Lys¹²⁴ (TM 3) as contact site for the amino-terminal pGlu¹ and Asn¹⁰⁵ (EL 1) for the carboxyl-terminal Gly¹⁰-NH₂ (Fig. 3). Moreover, cGnRH-II binds to the catfish GnRH receptor in a constrained β-turn conformation, with Gly⁵ facing to the entrance of the binding pocket.⁹⁹ These results are in accordance with findings on mGnRH binding to mammalian GnRH receptors.^{42,44,100}

Several GnRHs and Several GnRH Receptors in a Single Species

Extensive gene duplication did occur during the evolution of vertebrates and contributed to increasing complexity and functional diversity of regulatory processes in organisms.¹⁰¹ Gene duplication yields the possibility for a given species to synthesize an increasing number of bioactive peptides as well as an increasing number of receptors for these peptides, and allows co-evolution of ligand-receptor pairs. For example, the genes coding for the peptides vasopressin and oxytocin seem to have arisen by duplication of a common ancestral gene, since they are not only similar in sequence but also share a similar intron-exon organization and are situated next to each other in the genome.¹⁰² In addition, separate receptors exist for vasopressin and oxytocin. A second example involves human chorionic gonadotropin and luteinizing hormone that both bind selectively to the luteinizing hormone receptor, whereas follicle-stimulating hormone interacts exclusively with the follicle-stimulating hormone receptor.¹⁰³ These authors suggested that gene duplication of both the gene for the primordial glycoprotein as well as the gene for its receptor, was followed by sequence divergence resulting in sequences responsible for the specific interaction and preventing binding of an inappropriate ligand. For the examples mentioned above, gene duplication culminated in the gain of new biological functions and a greater functional diversity. However, evolution is a dynamic, ongoing process and therefore several aspects

of what we study may represent functional redundancy. Knockout mice of the interferon receptor type 1 or type 2, for example, are still functional, since the related protein functionally compensates for the absence of the other one.¹⁰⁴ Having multiple receptors for a given peptide or multiple peptides for a given receptor also provides the advantage of protection against lethal mutations and enables the accumulation of spontaneous mutations without comprising the organism's survival. Changes in the coding region of a receptor might lead to different subtypes with distinct ligand selectivity, rates of desensitization/internalization or differential coupling to effector systems. Variations in the regulatory region, on the other hand, may lead to changes in spatiotemporal expression or regulation pattern.¹⁰⁵

For the GnRH peptide, three basic forms seem to have evolved from an ancestral gene. In order to facilitate comparative studies, Fernald and White¹⁰⁶ suggested a new nomenclature: the gene encoding the hypothalamic form, which is variable among species, should be called GnRH1, the conserved mesencephalic form GnRH2 and the conserved telencephalic form GnRH3. The corresponding peptides should be named GnRH for mGnRH and all other forms should indicate the difference compared to GnRH, such that GnRH2 codes for [His⁵Trp⁷Tyr⁸]GnRH (formerly named cGnRH-II) and GnRH3 codes for [Trp⁷Leu⁸]GnRH (formerly named salmon GnRH; sGnRH). In the catfish brain, two forms of GnRH have been identified, namely [His⁵Leu⁷Asn⁸]GnRH (formerly named cfGnRH) produced in neurons of the ventral forebrain and [His⁵Trp⁷Tyr⁸]GnRH synthesized in neurons of the midbrain tegmentum.^{107,108}

After the first indications that two GnRH receptor genes exist in a single species,¹⁰⁹ two different GnRH receptor subtypes were identified in goldfish. Using a similar cloning strategy, we were also able to identify and characterize a second GnRH receptor subtype from catfish.¹¹⁰ Both catfish receptor subtypes had a binding preference for [His⁵Trp⁷Tyr⁸]GnRH compared to [His⁵Leu⁷Asn⁸]GnRH. Moreover, none of the two endogenous ligands yielded different IP or cAMP responses in cells expressing either the catfish GnRH receptor 1 or 2.¹¹⁰ However, studies on the highly homologous goldfish GnRH receptors

revealed that cells expressing the goldfish GnRH receptor A generated IPs with up to 100-fold lower EC₅₀ values in response to native ligands compared to the goldfish receptor B.⁸⁴ In that respect, the two goldfish GnRH receptors seem to have evolved differently compared to the two catfish GnRH receptors. Other aspects of receptor functioning, e.g. the rate of receptor desensitization and internalization, and the coupling to other signaling routes like the mitogen-activated protein kinase cascade or the arachidonic acid pathway, have not been tested yet for either the catfish or the goldfish GnRH receptors.

Next to mammalian species, the goldfish has been studied most extensively with regard to GnRH receptor localization. In accordance to the situation in mammals, GnRH-binding sites and mRNA encoding GnRH receptors have been detected in gonadotropes, in different extrapituitary regions of the brain, in ovary and in testis of the goldfish. However, GnRH receptor detection in somatotropes, liver and kidney of the goldfish differs from reports on mammalian GnRH receptor expression.^{84,111–113} The two goldfish GnRH receptors are differentially expressed in brain, ovary and liver.⁸⁴ For example, in the goldfish ovary, only the receptor subtype A is expressed, together with [Trp⁷Leu⁸]GnRH¹¹⁴ which is more potent in stimulating this receptor type than receptor subtype B. The co-expression in the same (or nearby) tissue of a specific receptor subtype with a specific ligand might indicate the evolution of a coordinated functional unit.

The question remains as to which tissues or cell types express the two catfish GnRH receptor subtypes (as well as their endogenous ligands) and whether their expression is differentially regulated in a number of different endocrine states. We know that, in contrast to goldfish, GnRH receptors in the pituitary of the catfish are restricted to the gonadotropes.¹¹⁵ Moreover, GnRH-binding sites have been reported in catfish ovary, together with compounds displaying GnRH-like activity.¹¹⁶ To get more detailed information, studies on the spatiotemporal expression of the two catfish GnRH receptors as well as their ligands have recently been performed using real-time quantitative PCR.¹¹⁰

In conclusion, specialization after gene duplication depends on the adaptive abilities of the organism as well as the selective pressure of

its environment. The GnRH-GnRH receptor system in the goldfish seems to have evolved differently compared to that of the catfish. Speculations on the existence of a second GnRH receptor subtype in humans have recently been confirmed by a patent application on such a receptor. Characterization of the pharmacological properties and the identification of the sites of expression of this second type of GnRH receptor in humans will be important for the application of various GnRH analogs in the clinic.

And Finally, Have We Learnt Anything from Fish?

Indeed we did. We have learnt that the existence of multiple forms of GnRH and GnRH receptors in a single species is a general phenomenon amongst vertebrates and we now understand better the relationship between structure and function of GnRH receptors in general.

Multiple Forms of GnRH and GnRH Receptors in a Single Species

The identification of several forms of GnRH and of several types of GnRH receptor present in a single fish species initiated more detailed research in other species including mammals. This led to the discovery of three basic forms of GnRH: a hypothalamic form of variable sequence, the highly conserved cGnRH-II and the telencephalic salmon GnRH. Moreover, three classes of GnRH receptor have been identified, possibly having co-evolved in conjunction with their ligands. Detailed studies on co-localization of specific forms of GnRH with specific types of GnRH receptor,¹¹⁰ ligand selectivity and selected usage of signal transduction pathways are currently being performed, and should help to define the precise function of the different GnRH systems.

GnRH Receptors: Structure-Function Relationship

Several GnRH receptors from mammalian species have been cloned before. They all differ from other GPCRs in missing the cytoplasmic

C-terminal domain and in showing an exchange of two otherwise highly conserved residues in TM 2 and 7. The catfish GnRH receptor was the first nonmammalian one that was cloned. It revealed quite a surprising structure compared to its mammalian counterparts. The presence of a C-terminal tail, and of a configuration in TM 2 and 7 that is intermediate between the one in mammalian GnRH receptors and other GPCRs, stimulated studies on the structure-function relationship of these striking structural features.

We have learnt that the C-terminal tail is important for a high receptor expression in the plasma membrane. Yet, the low expression level of GnRH receptors in mammalian gonadotrophs still seems to be sufficient for proper GnRH signalling. Moreover, this domain is a target of agonist-induced receptor phosphorylation, a prerequisite for receptor desensitization and subsequent receptor internalization in an arrestin-dependent manner. Now we understand why desensitization of the gonadotropin release from mammalian gonadotrophs in response to a prolonged GnRH stimulus is achieved by desensitization of the downstream IP₃ receptor/calcium system. That is because the GnRH receptor cannot be desensitized by phosphorylation and this lack has to be compensated for otherwise.

Furthermore, the high affinity of nonmammalian GnRH receptors for cGnRH-II and their low affinity for mammalian GnRH helped us in identifying residues conferring ligand selectivity. Moreover, nonmammalian GnRH receptors are a good tool to check molecular models of mammalian GnRH receptors, as they predict for receptor conformation, ligand interaction and receptor activation. For example, the presumed close proximity between TM 2 and 7 in mammalian GnRH receptors is not in contradiction with the two adjacent Asp residues in nonmammalian GnRH receptors, if one assumes the protonation of one of these Asp residues. The model made it acceptable that the necessity of an acidic residue in TM 7 for G-protein coupling has been passed to the acidic side chain in TM 2 of mammalian GnRH receptors. On the other hand, the predicted relationship of a negatively charged residue in TM 2 with a positively charged residue in TM 3 in GnRH receptor activation could not be confirmed with experimental data of the catfish GnRH receptor.

In addition, it became clear that the low affinity of nonmammalian GnRH receptors for mammalian GnRH is not due to a lack of specific recognition of Arg⁸ in mammalian GnRH. It rather results from the unfavorable fit of Tyr⁵ and/or Leu⁷ of this ligand into the binding pocket of nonmammalian GnRH receptors. Last but not least, using native and chimeric GnRHs, the residues His⁵, Trp⁷ and Tyr⁸ of cGnRH-II have been identified as mediating the high affinity of the catfish GnRH receptor for cGnRH-II.

References

1. Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B and Sealton SC (1992). Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. *Mol. Endocrinol.* **6:** 1163–1169.
2. Reinhart J, Mertz LM and Catt KJ (1992). Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **267:** 21281–21284.
3. Eidne KA, Sellar RE, Couper G, Anderson L and Taylor PL (1992). Molecular cloning and characterization of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. *Mol. Cell. Endocrinol.* **90:** R5–R9.
4. Kaiser UB, Zhao D, Cardona GR and Chin WW (1992). Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. *Biochem. Biophys. Res. Commun.* **189:** 1645–1652.
5. Chi L, Zhou W, Prokhozhan A, Flanagan C, Davidson JS, Golembio M, Illing N, Millar RP and Sealton SC (1993). Cloning and characterization of the human GnRH receptor. *Mol. Cell. Endocrinol.* **91:** R1–R6.
6. Brooks J, Taylor PL, Saunders PTK, Eidne KA, Struthers WJ and McNeilly AS (1993). Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. *Mol. Cell. Endocrinol.* **94:** R23–R27.
7. Illing N, Jacobs GFM, Becker II, Flanagan CA, Davidson JS, Eales A, Zhou W, Sealton SC and Millar RP (1993). Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors. *Biochem. Biophys. Res. Com.* **196:** 745–751.

8. Kakar SS, Rahe CH and Neill JD (1993). Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin-releasing hormone (GnRH). *Domest. Anim. Endocrinol.* **10**: 335–342.
9. Weesner GD and Matteri RL (1994). Rapid communication: nucleotide sequence of luteinizing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary. *J. Anim. Sci.* **72**: 1911–1911.
10. Kakar SS, Grizzle WE and Neill JD (1994). The nucleotide sequences of human GnRH receptors in breast and ovarian tumors are identical with that found in pituitary. *Mol. Cell. Endocrinol.* **106**: 145–149.
11. Moumni M, Kottler ML and Counis R (1994). Nucleotide sequence analysis of mRNAs predicts that rat pituitary and gonadal gonadotropin-releasing homone receptor proteins have identical primary structure. *Biochem. Biophys. Res. Com.* **200**: 1359–1366.
12. Fan NC, Jeung E, Peng C, Olofsson JI, Krisinger J and Leung PCK (1994). The human gonadotropin-releasing hormone (GnRH) receptor gene: cloning, genomic organization and chromosomal assignment. *Mol. Cell. Endocrinol.* **103**: R1–R6.
13. Jennes L, Eyigor O, Janovick JA and Conn PM (1997). Brain gonadotropin-releasing hormone receptors: localization and regulation. *Recent. Prog. Horm. Res.* **52**: 475–491.
14. Bourne GA, Refiani S, Payne AH and Marshall JC (1980). Testicular GnRH receptors: characterization and localization on interstitial tissue. *J. Clin. Endocrinol. Metab.* **51**: 407–409.
15. Hazum E and Nimrod A (1982). Photoaffinity labeling and fluorescence distribution studies of gonadotropin-releasing hormone receptors in ovarian granulosa cells. *Proc. Natl. Acad. Sci. USA* **79**: 1747–1750.
16. Bramley TA, McPhie CA and Menzies GS (1992). Human placental gonadotropin-releasing hormone (GnRH) binding sites: I Characterization, properties and ligand specificity. *Placenta* **13**: 555–581.
17. Eidne KA, Flanagan CA, Harris N and Millar RP (1987). Gonadotropin-releasing hormone (GnRH)-binding sites in human breast cancer cell lines and inhibitory effects of GnRH antagonists. *J. Clin. Endocrinol. Metab.* **64**: 425–432.
18. Machetti B, Guarcello V, Morale M, Bartoloni G, Farinella Z, Cordaro S and Scapagnini U (1989). Luteinizing hormone-releasing hormone binding sites in the rat thymus: Characteristics and biological function. *Endocrinology* **125**: 1025–1036.

19. Eidne KA, Hendricks D and Millar RP (1985). Demonstration of a 60K molecular weight luteinizing hormone-releasing hormone receptor in solubilized adrenal membranes by a ligand-immunoblotting technique. *Endocrinology* **116**: 1792–1795.
20. Cook JV and Eidne KA (1997). An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation. *Endocrinology* **138**: 2800–2806.
21. Sanno N, Jin L, Qian X, Osamura RY, Scheithauer BW, Kovacs K and Lloyd RV (1997). Gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor messenger ribonucleic acids expression in nontumorous and neoplastic pituitaries. *J. Clin. Endocrinol. Metab.* **82**: 1974–1982.
22. Seong JY, Kang SS, Kam K, Han YG, Kwon HB, Ryu K and Kim K (1998). Differential regulation of gonadotropin-releasing hormone (GnRH) receptor expression in the posterior mediobasal hypothalamus by steroid hormones: Implication of GnRH neuronal activity. *Mol. Brain Res.* **53**: 226–235.
23. Yin H, Cheng KW, Hwa HL, Peng C, Auersperg N and Leung PK (1998). Expression of the messenger RNA for gonadotropin-releasing hormone and its receptor in human cancer cell lines. *Life Sci.* **62**: 2015–2023.
24. E Hazum (1983). Photoaffinity labeling of peptide hormone receptors. *Endocr. Rev.* **4**: 352–362.
25. Davidson JS, Flanagan CA, Zhou W, Becker II, Elario R, Emeran W, Sealfon SC and Millar RP (1995). Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: Role in receptor expression but not ligand binding. *Mol. Cell. Endocrinol.* **107**: 241–245.
26. Ji TH, Grossmann M and Ji I (1998). G protein-coupled receptors: I. Diversity of receptor-ligand interactions. *J. Biol. Chem.* **273**: 17299–17302.
27. Stojilkovic SS, Reinhart J and Catt KJ (1994). Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. *Endocr. Rev.* **15**: 462–497.
28. Baldwin J (1993). The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12**: 1693–1703.
29. Flanagan CA, Millar RP and Illing N (1997). Advances in understanding gonadotrophin-releasing hormone receptor structure and ligand interactions. *Rev. Reprod.* **2**: 113–120.

30. Sealfon SC, Weinstein H and Millar RP (1997). Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocr. Rev.* **18**: 180–205.
31. Arora KK, Sakai A and Catt KJ (1995). Effect of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **270**: 22820–22826.
32. Zhou W, Flanagan C, Ballesteros JA, Konicka K, Davidson JS, Weinstein H, Millar RP and Sealfon SC (1994). A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor. *Mol. Pharmacol.* **45**: 165–170.
33. Cook JV, Faccenda E, Anderson L, Couper GG, Eidne KA and Taylor PL (1993). Effects of Asn87 and Asp318 mutation on ligand binding and signal transduction in the rat GnRH receptor. *J. Endocrinol.* **139**: R1–R4.
34. Awara WM, Guo C and Conn PM (1996). Effects of Asn318 and Asp87Asn318 mutations on signal transduction by the gonadotropin-releasing hormone receptor. *Endocrinology* **137**: 655–662.
35. Schwartz TW (1996). Molecular structure of G-protein coupled receptors. In: Foreman JC and Johansen T (eds.) *Textbook of Receptor Pharmacology*, CRC Press Inc, pp. 65–84.
36. Davidson JS, Assefa D, Pawson A, Davies P, Hapgood J, Becker I, Flanagan C, Roeske RW and Millar R (1997). Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: Localization of attachment site to Cys residue in N-terminal segment. *Biochemistry* **36**: 12881–12889.
37. Baldwin J, Schertler GFX and Unger VM (1997). An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein coupled receptors. *J. Mol. Biol.* **272**: 144–154.
38. Unger VM and Schertler GFX (1995). Low resolution structure of bovine rhodopsin determined by electron cryo-microscopy. *Biophys. J.* **68**: 1776–1786.
39. Strader CD, Fong TM, Tota MR and Underwood D (1994). Structure and function of G protein-coupled receptors. *Animal Reprod. Sci.* **63**: 101–132.
40. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Trong IL, Teller DC, Okada T, Stenkamp RE, Yamamoto M and

- Miyano M (2000). Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289**: 739–745.
41. Fraser CM, Lee NH, Pellegrino SM and Kerlavage AR (1994). Molecular properties and regulation of G protein-coupled receptors. *Prog. Nucleic Acid Res. Mol. Biol.* **49**: 113–156.
42. Zhou W, Rodic V, Kitanovic S, Flanagan CA, Chi L, Weinstein H, Maayani S, Millar RP and Sealfon SC (1995). A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites. *J. Biol. Chem.* **270**: 18853–18857.
43. Rodic V, Flanagan C, Millar R, Konvicka K, Weinstein H and Sealfon SC (1996). Role of Asp2.61 (98) in agonist complexing with the human gonadotropin-releasing hormone receptor. *Soc. Neurosci. Abst.* **1302**.
44. Davidson JS, McArdle CA, Davies P, Elario R, Flanagan CA and Millar RP (1996). Asn(102) of the gonadotropin releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide. *J. Biol. Chem.* **271**: 15510–15514.
45. Flanagan CA, Becker II, Davidson JS, Wakefield IK, Zhou W, Sealfon SC and Millar RP (1994). Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone. *J. Biol. Chem.* **36**: 22636–22641.
46. Millar RP, Flanagan C, Milton RC and King JA (1989). Chimeric analogues of vertebrate gonadotropin-releasing hormones comprising substitutions of the variant amino acids in position 5, 7, and 8. *J. Biol. Chem.* **264**: 21007–21013.
47. Millar RP, Troskie B, Sun Z-M, Ott T, Wakefield I, Myburgh D, Pawson A, Davidson JS, Flanagan C, Katz A, Hapgood J, Illing N, Weinstein H, Sealfon SC, Peter RE, Terasawa E and King JA (1997). Plasticity in the structural and functional evolution of GnRH: a peptide for all seasons. In: Kawashima S and Kikuyama S (eds.) *Advances in Comparative Endocrinology*, Proceedings of the XIIIth International Congress of Comparative Endocrinology, Monduzzi Editore S.p.A., Bologna, pp. 15–27.
48. Gudermann T, Schöneberg T and Schultz G (1997). Functional and structural complexity of signal transduction via G protein-coupled receptors. *Annu. Rev. Neurosci.* **20**: 399–427.
49. Kaiser UB, Conn PM and Chin WW (1997). Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. *Endocr. Rev.* **18**: 46–70.

50. Naor Z, Harris D and Shacham S (1998). Mechanism of GnRH receptor signaling: Combinatorial cross-talk of Ca^{2+} and protein kinase C. *Front. Neuroendocrinol.* **19**: 1–19.
51. Shacham S, Cheifetz MN, Lewy H, Ashkenazi IE, Becker OM, Seger R and Naor Z (1999). Mechanism of GnRH receptor signaling: from the membrane to the nucleus. *Ann. Endocrinol.* **60**: 79–88.
52. Grosse R, Schmid A, Schöneberg T, Herrlich A, Muhn P, Schultz G and Gudermann T (2000). Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to $G_{q/11}$ proteins. *J. Biol. Chem.* **275**: 9193–9200.
53. Gether U and Kobilka BK (1998). G protein receptors: II. Mechanism of agonist activation. *J. Biol. Chem.* **273**: 17979–17982.
54. Wess J (1997). G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *EASEB J.* **11**: 346–354.
55. Kenakin T (1993). Stimulus-response mechanisms. In *Pharmacologic Analysis of Drug-Receptor Interaction*, Anonymous, Raven Press, New York, pp. 39–68.
56. De Lean A, Stadel J and Lefkowitz RJ (1980). A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase coupled β -adrenergic receptor. *J. Biol. Chem.* **271**: 15510–15514.
57. Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993). A mutation-induced activated state of the β_2 -adrenergic receptor. *J. Biol. Chem.* **268**: 4625–4636.
58. Arora KK, Cheng ZY and Catt KJ (1997). Mutations of the conserved DRS motif in the second intracellular loop of the gonadotropin-releasing hormone receptor affect expression, activation, and internalization. *Mol. Endocrinol.* **11**: 1203–1212.
59. Flanagan CA, Zhou W, Chi L, Yuen T, Rodic V, Robertson D, Johnson M, Holland P, Millar RP, Weinstein H, Mitchell R and Sealfon SC (1999). The functional microdomain in transmembrane helices 2 and 7 regulates expression, activation, and coupling pathways of the gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **274**: 28880–28886.
60. Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H and Sealfon SC (1998). Functional microdomains in G protein-coupled receptors — The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **273**: 10445–10453.

61. Arora KK, Cheng ZY and Catt KJ (1996). Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. *Mol. Endocrinol.* **10**: 979–986.
62. Wess J (1998). Molecular basis of receptor/G protein-coupling selectivity. *Pharmacol. Ther.* **80**: 231–264.
63. Ulloaaguirre A, Stanislaus D, Arora V, Vaananen J, Brothers S, Janovick JA and Conn PM (1998). The third intracellular loop of the rat gonadotropin-releasing hormone receptor couples the receptor to G(s)- and G(q/11)-mediated signal transduction pathways: Evidence from loop fragment transfection in GGH(3) cells. *Endocrinology* **139**: 2472–2478.
64. Grosse R, Schoneberg T, Schultz G and Gudermann T (1997). Inhibition of gonadotropin-releasing hormone receptor signaling by expression of a splice variant of the human receptor. *Mol. Endocrinol.* **11**: 1305–1318.
65. Chung H-O, Yang Q, Catt KJ and Arora KK (1999). Expression and function of the gonadotropin-releasing hormone receptor are dependent on a conserved apolar amino acid in the third intracellular loop. *J. Biol. Chem.* **274**: 35756–35762.
66. Myburgh DB, Millar RP and Hapgood JP (1998). Alanine-261 in intracellular loop III of the human gonadotropin-releasing hormone receptor is crucial for G-protein coupling and receptor internalization. *Biochem. J.* **331**: 893–896.
67. Arora KK, Krsmanovic LZ, Mores N, O'Farrell H and Catt KJ (1998). Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **273**: 25581–25586.
68. Jacobs GFM, Flanagan CA, Roeske RW and Millar RP (1995). Agonist activity of mammalian gonadotropin-releasing antagonists in chicken gonadotropes reflects marked differences in vertebrate gonadotropin-releasing receptors. *Mol. Cell. Endocrinol.* **108**: 107–113.
69. Lohse MJ (1993). Molecular mechanisms of membrane receptor desensitization. *Biochem. Biophys. Acta* **1179**: 171–188.
70. Lefkowitz RJ (1998). G protein-coupled receptors: III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* **273**: 18677–18680.
71. Tobin AB (1997). Phosphorylation of phospholipase C-coupled receptors. *Pharmacol. Ther.* **75**: 135–151.

72. Willars GB, Heding A, Vrecl M, Sellar R, Blomenröhr M, Nahorski SR and Eidne KA (1999). Lack of a C-terminal tail in mammalian gonadotropin-releasing hormone receptor confers resistance to agonist-dependent phosphorylation and rapid desensitization. *J. Biol. Chem.* **274**: 30146–30153.
73. Anderson L, McGregor A, Cook JV, Chivers E and Eidne KA (1995). Rapid desensitization of GnRH-stimulated intracellular signalling events in alpha T3-1 and HEK-293 cells expressing the GnRH receptor. *Endocrinology* **136**: 5228–5231.
74. Willars GB, McArdle CA and Nahorski SR (1998). Acute desensitization of phospholipase C-coupled muscarinic M3 receptors but not gonadotropin-releasing hormone receptors co-expressed in αT3-1 cells: Implication for mechanisms of rapid desensitization. *Biochem. J.* **333**: 301–308.
75. Heding A, Vrecl M, Bogerd J, McGregor A, Sellar R, Taylor PL and Eidne KA (1998). Gonadotropin-releasing hormone receptors with intracellular carboxyl-terminal tails undergo acute desensitization of total inositol phosphate production and exhibit accelerated internalization kinetics. *J. Biol. Chem.* **273**: 11472–11477.
76. McArdle CA, Davidson JS and Willars GB (1999). The tail of the gonadotrophin-releasing hormone receptor: desensitization at, and distal to, G protein-coupled receptors. *Mol. Cell. Endocrinol.* **151**: 129–136.
77. Lefkowitz RJ, Hausdorff WP and Caron MG (1998). Role of phosphorylation in desensitization of the β-adrenoceptor. *TIPS* **11**: 190–194.
78. Zhang J, Ferguson SS, Barak LS, Aber MJ, Giros B, Lefkowitz RJ and Caron MG (1997). Molecular mechanisms of G protein-coupled receptor signaling: role of G protein-coupled receptor kinases and arrestins in receptor desensitization and resensitization. *Receptors Channels* **5**: 193–199.
79. Vrecl M, Anderson L, Hanyaloglu A, McGregor AM, Groarke AD, Milligan G, Taylor PL and Eidne KA (1998). Agonist-induced endocytosis and recycling of the gonadotropin releasing hormone receptor: Effect of beta-arrestin on internalization kinetics. *Mol. Endocrinol.* **12**: 1818–1829.
80. Heding A, Vrecl M, Hanyaloglu AC, Sellar R, Taylor PL and Eidne KA (2000). The rat gonadotropin-releasing hormone receptor internalizes via a beta-arrestin-independent, but dynamin- dependent, pathway: Addition of a carboxyl-terminal tail confers beta-arrestin dependency. *Endocrinology* **141**: 299–306.

81. Shulman DI and Bercu BB (1999). Molecular biology of gonadotropin-releasing hormone and the gonadotropin-releasing hormone receptor. *Mol. Cell. Pediatric. Endocrinol.* **10**: 179–189.
82. Tensen CP, Okuzawa K, Blomenröhr M, Rebers F, Leurs R, Bogerd J, Schulz R and Goos H (1997). Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor. *Eur. J. Biochem.* **243**: 134–140.
83. Troskie BE, Sun Y, Hapgood J, Sealfon SC, Illing N and Millar RP (1997). Mammalian GnRH receptor functional features revealed by comparative sequences of goldfish, frog and chicken receptors. Program of the 79th Annual Meeting of the Endocrine Society (Abstract).
84. Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE and Millar RP (1999). Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). *Proc. Natl. Acad. Sci. USA* **96**: 2526–2531.
85. Wang L, Bogerd J, Choi HS, Soh JM, Chun SY, Seong JY, Blomenröhr M, Troskie BE, Millar RP and Kwon HB (2000). Three distinct types of gonadotropin-releasing hormone receptors characterized in the bullfrog. *Proc. Natl. Acad. Sci. USA* **98**: 361–366.
86. Troskie BE, Hapgood JP, Millar RP and Illing N (2000). Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor expressed in the pituitary and midbrain of *Xenopus laevis*. *Endocrinology* **141**: 1764–1771.
87. Oksche A, Dehe M, Schuelein R, Wiesner B and Rosenthal W (1998). Folding and cell surface expression of the vasopressin V₂ receptor: Requirement of the intracellular C-terminus. *FEBS Lett.* **424**: 57–62.
88. Iida-Klein A, Guo J, Xie LY, Jüppner H, Potts JT, Kronenberg HM, Brinhurst FR, Abou-Samra AB and Segre GV (1995). Truncation of the carboxyl-terminal region of the rat parathyroid hormone (PTH)/PTH-related peptide receptor enhances PTH stimulation of adenylyl cyclase but not phospholipase C. *J. Biol. Chem.* **270**: 8458–8465.
89. Ferguson SSG, Barak LS, Zhang J and Caron MG (1996). G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. *Can. J. Physiol. Pharmacol.* **74**: 1095–1110.
90. Blomenröhr M, Heding A, Sellar R, Leurs R, Bogerd J, Eidne KA and Willars GB (1999). Pivotal role for the cytoplasmic carboxyl-terminal tail

- of a nonmammalian gonadotropin-releasing hormone receptor in cell surface expression, ligand binding, and receptor phosphorylation and internalization. *Mol. Pharmacol.* **56**: 1229–1237.
91. Lin X, Janovick JA, Brothers S, Blomenröh M, Bogerd J and Conn PM (1998). Addition of catfish gonadotropin-releasing hormone (GnRH) receptor intracellular carboxyl-terminal tail to rat GnRH receptor alters receptor expression and regulation. *Mol. Endocrinol.* **12**: 161–171.
 92. Naor Z, Clayton RN and Catt KJ (1980). Characterization of gonadotropin-releasing hormone receptors in cultured rat pituitary cells. *Endocrinology* **107**: 1144–1152.
 93. Cornea A, Janovick JA, Lin X and Conn PM (1999). Simultaneous and independent visualization of the gonadotropin-releasing hormone receptor and its ligand: evidence for independent processing and recycling in living cells. *Endocrinology* **140**: 4272–4280.
 94. De Leeuw R, Th Goos HJ and Van Oordt PGWJ (1986). The dopaminergic inhibition of the gonadotropin-releasing hormone-induced gonadotropin release: An *in vitro* study with fragments and cell suspensions from pituitaries of the African catfish, *Clarias gariepinus*. *Gen. Comp. Endocrinol.* **63**: 171–177.
 95. Harrison RW and Weber IT (1994). Molecular dynamics simulations of HIV-1 protease with the peptide substrate. *Protein Eng.* **7**: 1353–1363.
 96. Davies DR (1990). The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Biophys. Chem.* **19**: 189–215.
 97. Blomenröh M, Kühne R, Hund E, Leurs R, Bogerd J and Ter Laak T (2001). Proper receptor signaling in a mutant catfish gonadotropin-releasing hormone receptor lacking the highly conserved Asp⁹⁰ residue. *FEBS Lett.* **501**: 131–134.
 98. Blomenröh M, Bogerd J, Leurs R, Schulz RW, Tensen CP, Zandbergen MA and Goos HT (1997). Differences in structure-function relations between nonmammalian and mammalian gonadotropin-releasing hormone receptors. *Biochem. Biophys. Res. Comm.* **238**: 517–522.
 99. Blomenröh M, Ter Laak T, Kühne R, Beyermann M, Hund E, Bogerd J and Leurs R (2002). Chimaeric gonadotropin-releasing hormone (GnRH) peptides with improved affinity for the catfish (*Clarias gariepinus*) GnRH receptor. *Biochem. J.* **361**: 515–523.

100. Hoffmann SH, Ter Laak AM, Kühne R, Reilander H and Beckers T (2000). Residues within transmembrane helices 2 and 5 of the human gonadotropin-releasing hormone receptor contribute to agonist and antagonist binding. *Mol. Endocrinol.* **14**: 1099–1115.
101. Holland PWH (1999). Gene duplication: Past, present and future. *Semin. Cell Dev. Biol.* **10**: 541–547.
102. Mohr E, Meyerhof W and Richter D (1995). Vasopressin and oxytocin: Molecular biology and evolution of the peptide hormones and their receptors. *Vitamins and Hormones* **51**: 235–266.
103. Moyle WR, Campbell RK, Myers RV, Bernard MP, Han Y and Wang X (1994). Co-evolution of ligand-receptor pairs. *Nature* **368**: 251–255.
104. Müller U (1999). Ten years of gene targeting: Targeted mouse mutants, from vector design to phenotype analysis. *Mech. Dev.* **82**: 3–21.
105. Darlison MG and Richter D (1999). The ‘chicken and egg’ problem of co-evolution of peptides and their cognate receptors: Which came first? In: Richter D (ed.) *Regulatory Peptides and Cognate Receptors*. Springer Verlag, Berlin, pp. 1–11.
106. Fernald RD and White RB (1999). Gonadotropin-releasing hormone genes: Phylogeny, structure, and functions. *Front. Neuroendocrinol.* **20**: 224–240.
107. Bogerd J, Zandbergen T, Andersson E and Goos H (1994). Isolation, characterization and expression of cDNA encoding the catfish-type and chicken-II-type gonadotropin-releasing hormone precursors in the African catfish. *Eur. J. Biochem.* **222**: 541–549.
108. Zandbergen MA, Kah O, Bogerd J, Peute J and Goos HJT (1995). Expression and distribution of two gonadotropin-releasing hormones in the catfish brain. *Neuroendocrinol.* **62**: 571–578.
109. Troskie B, Illing N, Rumback E, Sun Y-M, Hapgood J, Sealfon SC, Conklin D and Millar PR (1998). Identification of three putative GnRH receptor subtypes in vertebrates. *Gen. Comp. Endocrinol.* **112**: 296–302.
110. Bogerd J, Diepenbroek WB, Hund E, Van Oosterhout F, Teves ACC, Leurs R and Blomenröhr M (2002). Two gonadotropin-releasing hormone receptors in the African catfish: No differences in ligand selectivity, but differences in tissue distribution. *Endocrinology* **143**: 4673–4682.

111. Habibi HR, Peter RE, Sokolowska M, Rivier JE and Vale WW (1987). Characterization of gonadotropin-releasing hormone (GnRH) binding to pituitary receptors in goldfish (*Carassius auratus*). *Biol. Reprod.* **36**: 844–853.
112. Habibi HR and Pati D (1993). Extrapituitary gonadotropin-releasing hormone (GnRH) binding sites in goldfish. *Fish Phys. Biochem.* **11**: 1–6.
113. Yu KL, Chik CC, Lin XW, Chang JP and Peter RE (1998). mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. *Gen. Comp. Endocrinol.* **112**: 303–311.
114. Pati D and Habibi HR (1998). Presence of salmon gonadotropin-releasing hormone (GnRH) and compounds with GnRH-like activity in the ovary of goldfish. *Endocrinology* **139**: 2015–2024.
115. Bosma PT, Kolk SM, Rebers F, Lescroart O, Roelants I, Willems PHGM and Schulz RW (1997). Gonadotrophs but not somatotrophs carry gonadotrophin-releasing hormone receptors: Receptor localization, intracellular calcium, and gonadotrophin and GH release. *J. Endocrinol.* **152**: 437–446.
116. Habibi HR, Pati D, Ouwens M and Goos HJT (1994). Presence of gonadotropin-releasing hormone (GnRH) binding sites and compounds with GnRH-like activity in the ovary of African catfish, *Clarias gariepinus*. *Biol. Reprod.* **50**: 643–652.

Chapter 3

Molecular Regulation of Gonadotropin Gene Expression in Teleosts

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Abstract

The gonadotropin genes, follicle stimulating hormone (FSH) and luteinizing hormone (LH), are heterodimers comprising a common α and a distinct β subunit; the latter confers biological specificity. The genes encoding the LH β subunit in various teleost fish were first isolated in the early 1990s and considerable study has been carried out to elucidate basal and gonadotropin releasing hormone (GnRH)-mediated mechanisms of activation, most notably in the Chinook salmon. At the same time, the effects of various hormones and signaling pathways on cDNA levels in several species, most notably tilapia, were measured. In contrast, the gene encoding the FSH β subunit has been more elusive, mainly due to its lack of conservation, particularly in the size of the first intron. However, tilapia, goldfish and Chinook salmon FSH β genes have been isolated recently, and some functional studies have been carried out, as well as studies on the signaling pathways mediating GnRH effects. This chapter will review the work carried out on both LH and FSH β subunit genes, and will compare the findings with the gene regulatory mechanisms elucidated for mammalian species.

Overview

Transcription of the gonadotropins, in fish as in mammals, is regulated through hypothalamic, paracrine and systemic factors including gonadal

steroids and peptides. Numerous studies have been carried out to determine the effects of these various factors both *in vivo* and *in vitro* on the endogenous LH β and FSH β mRNA levels, as well as on circulating levels of the hormones.¹⁻³ However, studies addressing the molecular mechanisms through which these factors regulate LH β and FSH β gene expression at the level of the promoters have been limited to the Chinook salmon and tilapia. This study will review our own findings on the functional analysis of these gonadotropin gene promoters, and propose models of the integrated molecular mechanisms regulating their activity, with reference to studies carried out on other species and in mammals.

Sequence Analysis: Teleost and Mammalian

The LH β Gene

The first teleost LH β subunit gene to be isolated and sequenced was that of the Chinook salmon, *Oncorhynchus tshawytscha* (csGtHII β or csLH β) by Xiong and Hew in 1991.⁴ This gene appears in the genome in a single copy and, like its mammalian homologs, contains three exons and two introns; the first intron is up-stream of the translational start site (Fig. 1a). This gene was seen to undergo alternative splicing to produce two transcripts varying by 12 nucleotides, but differential functions of these isoforms have not been shown.⁴ In 1992 and 1999 respectively, the promoter sequences of common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) LH β genes were published. A single gene was identified for goldfish and two LH β genes were found in common carp, which encode the same protein. These genes share similar structure to the mammalian LH β genes, in regards to number and position of introns and exons, and in the cDNA sequences, although the fish genes are generally shorter (Fig. 1a).^{5,6} All four genes contain TATA box sequences, which are located 21 to 25 base pairs (bp) from the transcriptional start site. However, the 5' flanking regions of the cyprinid genes show little similarity with that of the csLH β gene.

The lack of similarity between teleost LH β promoter sequences, or between these and the mammalian homologs is in contrast to the

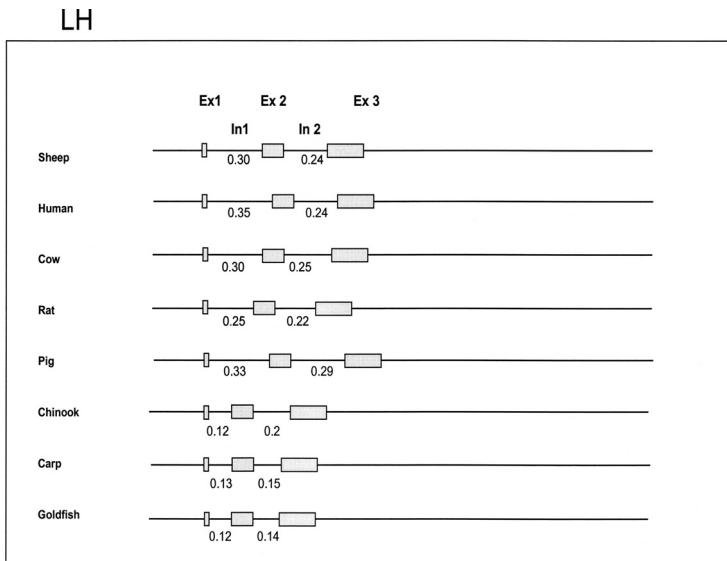


Fig. 1a Comparison of LH β subunit genes: in mammals and fish, the LH β gene contains three exons (Ex: boxes) and two introns (In), which are largely well conserved regarding both position and size (shown in kb).

high degree of identity amongst the mammalian LH β proximal promoters.^{7,8} However, common to all of these proximal promoters are putative Sf-1 and Pitx-1 binding sites (Fig. 1b). The mammalian LH β promoter also contains a highly conserved Egr-1 response element (RE), which is reported to be crucial for activity of the LH β gene promoter activity^{8–10} yet is absent in all teleost LH β promoters reported so far. However, the teleost LH β proximal promoters all appear to contain near-palindromic estrogen response elements (EREs; Fig. 1b), which likely indicates their importance in regulating transcriptional activity of this gene (see below). The proximal 289 bp of the 5' flanking region in the csLH β is sufficient to drive transcription and to respond to gonadotropin releasing hormone (GnRH), overcoming the actions of a proximal silencer, but only in gonadotropes (Melamed *et al.*, personal observation). However, there appears to be an additional element further

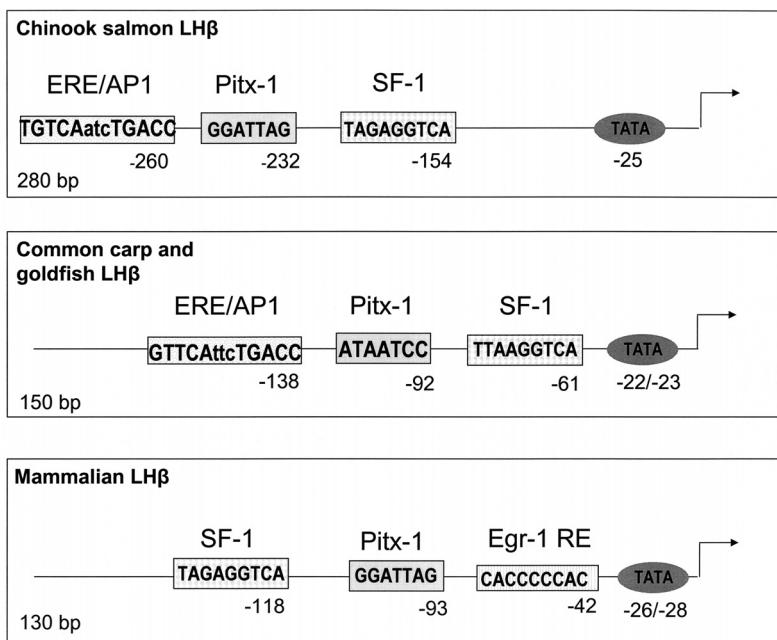


Fig. 1b Response elements (REs) on the proximal promoters of the Chinook salmon, common carp, goldfish and a typical mammalian LH β gene. The mammalian proximal promoter is highly conserved in all species studied so far, showing Sf-1, Pitx-1 and Egr-1 REs that are functional and synergistic. Fewer fish LH β genes are available for comparison, but that of Chinook salmon contains functional Sf-1, Pitx-1 REs and a near palindromic ERE; these three elements appear conserved in the common carp and goldfish, although the Pitx-1 RE is in reverse orientation.

up-stream of 447 bp that prevents a GnRH response unless sequences up to and including the distal ERE are present.⁷

The FSH β Gene

FSH β gene promoters have been published for goldfish and tilapia, and recently we have also cloned that of the Chinook salmon (Chong and Melamed, personal observation).¹¹⁻¹² Promoter regions of all of

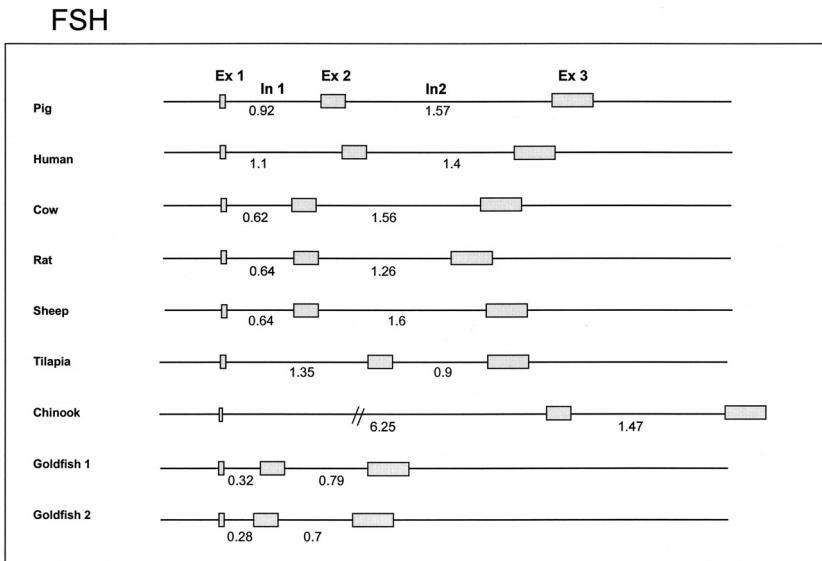


Fig. 2a Comparison of FSH β subunit genes: the FSH β genes all contain three exons and two introns, but the sizes of the introns vary widely. Abbreviations as in Fig. 1a.

these sequences vary widely between species and even the “duplicated” goldfish FSH β genes are quite divergent beyond 300 bp upstream of the transcriptional start site.¹²

All of these genes contain large first introns, while the first exons are particularly short: in the Chinook salmon (csFSH β) the first intron is over 6.2 kb while the first exon is just 27 bp (Fig. 2a). Although the csFSH β has just one transcriptional start-site, the tilapia gene contains multiple start-sites (Chong and Melamed, personal observation).¹³ Of the three species, only the goldfish proximal promoter sequences contain consensus TATA boxes, located at -26 bp. The tilapia FSH β TATA box is contained further upstream at -91 bp, while the Chinook salmon FSH (csFSH) β gene proximal promoter contains only a TATA box in reverse orientation, at -43 bp. This indicates further the likelihood that divergent mechanisms regulate transcription of these

homologous genes, possibly relying considerably on other ubiquitous transcription factors to recruit the general transcription machinery.

Transient transfection studies using the tilapia gene promoter have revealed that, unlike the LH β promoter, the FSH β promoter is very active even in non-gonadotrope cells. Further, studies using truncated tilapia FSH β promoter constructs revealed that regions activating optimal levels of transcription are located 337 bp upstream of the transcriptional start site, which contains putative response elements for transcription factors such as AP-1, Pitx-1 and CREB, some of which have been implicated in the regulation of mammalian FSH β genes (Fig. 2b). Further up-stream, a silencer is present which represses transcriptional activity by about 90%.¹³ Similar functional studies have not yet been

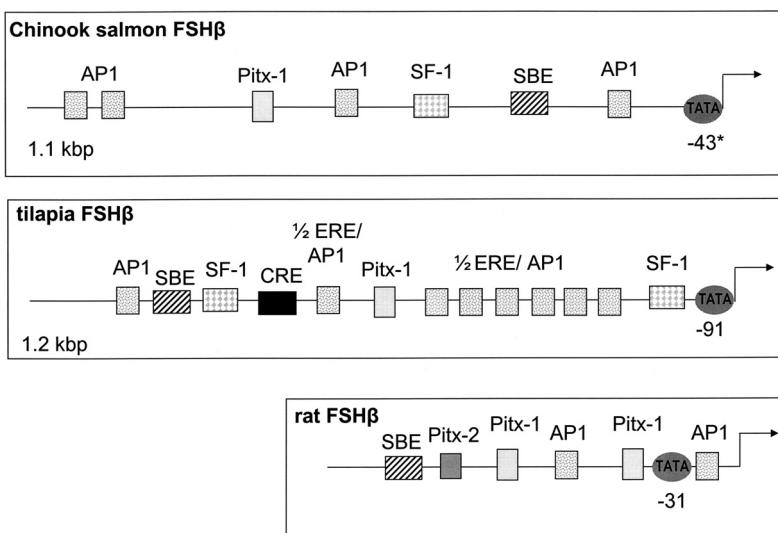


Fig. 2b Putative response elements on the promoters of the Chinook salmon (1.1 kb), tilapia (1.2 kb), and those that have been shown to be functional on the rat (260 bp) FSH β gene promoters. Data taken from references as cited in the text, while putative Pitx-1 sites and SBE on the tilapia gene were identified independently. (*) The TATA box on the csFSH β is in the reverse orientation.

carried out using the csFSH β gene promoter, although interestingly the first intron was found to drive expression of a reporter gene in both gonadotrope and non-gonadotrope cells, and is even responsive to GnRH (Chong and Melamed, personal observation). The significance of this has yet to be determined.

Convergence of Endocrine Regulated Signals in the Nucleus

Gonadotropin-releasing Hormone

GnRH is the primary hypothalamic stimulator of LH and possibly also FSH synthesis and release in fish, as in mammals. Its actions have been shown numerous times through *in vivo* injections, in primary cultures of teleost pituitary cells in tilapia and goldfish,^{1,14–17} and also in transfection studies using the csLH β and the tilapia FSH β gene promoters fused to reporter genes.^{7,13}

The actions of GnRH in mammals and fish are mediated through binding a membrane-bound G-protein coupled receptor which, through Gq, activates phospholipase C stimulating production of inositol triphosphate (IP₃) and diacylglycerol (DAG). The latter then stimulates protein kinase C (PKC) which activates the mitogen activated kinases (MAPKs) ERK and JNK (Figs. 3a,b).^{18–20} The roles of these kinases in mediating the GnRH effect on LH β and FSH β transcripts have been demonstrated extensively in mammals^{21,22} and have also been shown in tilapia through use of specific inhibitors.^{15,16}

Also, in the teleost pituitary, GnRH is able to enhance cAMP production^{23,24} through Gs, although this may not be universal to all species.²⁵ Elevation of cAMP levels has been shown to lead to an increase in LH β and FSH β mRNA levels in mammals and tilapia; in addition, the role of protein kinase A (PKA) in mediating the GnRH effect on these transcript levels has been demonstrated in tilapia.^{15,17,26} The stimulation of PKA by cAMP appears to stimulate a pathway interacting with that activated by PKC, while also reducing rates of the tilapia

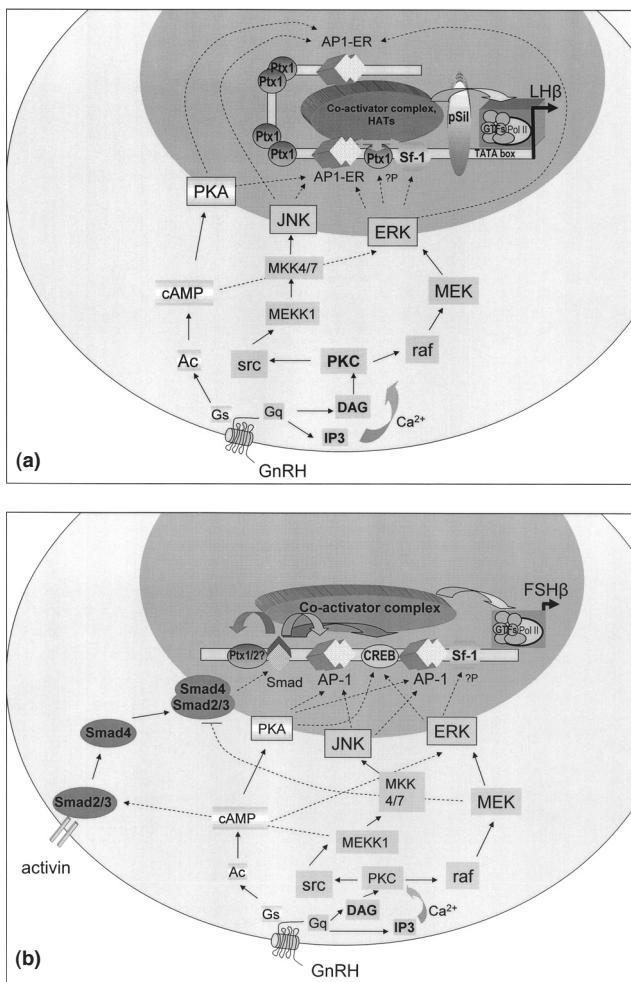


Fig. 3 Schematic model representing the mechanisms through which (a) the csLH β and (b) the teleost FSH β gene promoters may be stimulated. Intracellular pathways activated by GnRH have been described in detail by Naor *et al.*²² and specifically in fish by Yaron *et al.*³ while those activated by activin are summarized by Zhang and Derrinck³⁶; both shown in solid lines. Possible modes of action that have yet to be demonstrated specifically for activation of these genes are depicted in dashed lines. Figure 3a is based primarily on research on the csLH β , while Fig. 3b represents findings from preliminary research on mammalian and fish FSH β promoter activation (see text for details), and not all factors may necessarily be involved in a single species.

LH β transcript degradation.^{15,17,23} Interestingly, elevation of cAMP levels in tilapia pituitary cells increased levels of ERK1/2, although it is not clear whether this effect is specific to the gonadotropes.²⁷ The GnRH activation of MAPKs via pertussis toxin-sensitive G-protein or specifically via PKA has been reported previously in mammalian α T3-1 gonadotropes and in GGH3 cells stably transfected with the GnRH-R, respectively.^{28,29}

Activation of these various kinases leads to phosphorylation and subsequent translocation and/or activation of the nuclear transcription factors. Although few of these actions are well understood, the MAPK proteins are known to phosphorylate DNA binding factors such as c-fos, c-jun (the AP-1 factors), Egr-1, Sf-1, and estrogen receptor (ER) in response to GnRH (Koh and Melamed, personal observation).^{20,30} Similarly, c-fos, c-jun and ER α are also phosphorylated by PKA and PKC (Figs. 3a and b).^{31,32} These modifications and their potential effects on LH β and FSH β gene transcription are discussed below.

Activin

Both activin and the related bone morphogenic proteins (BMPs) 6 and 7 have been shown to activate the mammalian FSH β gene.^{33,34} The former is produced in the pituitary as well as the gonads, its activity being tightly controlled by the binding protein follistatin, and the receptor antagonist, inhibin (see Chapter 5). Both activin and the BMPs are members of the TGF β family of growth factors that utilize a series of Smad proteins; the latter, as a result of phosphorylation, enter the nucleus and activate transcription (Fig. 3b). The binding site for the Smad proteins is only four bp long and the specificity of action results from interactions either as a homodimer on a palindromic binding element (SBE), or with other transcription factors such as FAST1, FAST2, CREB, TFE3, c-jun and c-fos which bind adjacent sites.³⁵⁻³⁸ Many of these factors are activated by other known pathways of signal transduction, allowing for functional cross-talk between the signaling activated by different factors.³⁶

Other Hypothalamic Factors

There is evidence that other hypothalamic factors such as PACAP and NPY may also stimulate transcription of the LH β gene, both in mammals and teleosts.^{39–43} Although much of this action may be through effects on GnRH expression and release, immuno-reactive NPY is detected in the teleost pituitary and PACAP receptors are present specifically on the gonadotropes in mammals.^{42,44} Furthermore, studies in tilapia pituitary cells confirmed a direct effect for both regulatory peptides on the LH β subunit gene, and just PACAP on the FSH β subunit gene; both effects are apparently mediated through the PKC-MAPK and the PKA-MAPK pathways.²⁷

Dopamine is a major inhibitor of LH release in many teleosts, especially the cyprinids. Relief of its effect is essential for spawning to occur and, accordingly, dopamine antagonists are frequently included in spawning inducing agents.⁴⁵ Dopaminergic inhibition of basal and GnRH-stimulated LH release is mediated by dopamine D₂ receptors at a site distal to the formation of cAMP and the action of PKC.⁴⁶ However, dopamine does not appear to affect LH synthesis and no changes in transcript levels were noted in primary tilapia pituitary cells after 4–36 h dopamine exposure.¹⁵

Gonadal Steroids

Gonadal steroids, especially estrogen are known to regulate LH expression in an important feedback role, exerting both positive and negative effects at different stages of the reproductive cycle. These effects involve multiple sites along the hypothalamic-pituitary axis and include regulation of GnRH expression.^{47,48} However, exposure of teleost primary pituitary cells to estrogen also affects LH β and FSH β mRNA levels directly; the type and magnitude of effect seemingly depends on the reproductive stage of the fish.^{49,50} In tilapia primary pituitary cells, testosterone has similar effects to those of E₂ on LH β mRNA levels, which appear to result from aromatization of testosterone.⁵⁰ Aromatase activity is present in the gonadotropes of tilapia and African catfish.^{51,52} Thus, in addition to estrogen's feedback mechanism originating in the

gonads, E₂ may also be produced locally in the pituitary gonadotropes to exert an autocrine effect.

Mechanisms of E₂ action at the level of the LH β gene promoter have been studied for the Chinook salmon, and the csLH β promoter is highly responsive to E₂.⁵³ The pathways through which estrogen exerts its effects via the various estrogen receptors and EREs are reviewed in Chapter 7. Functional EREs are not commonly found on mammalian LH β gene promoters, although a functional ERE was demonstrated on the rat LH β gene distal promoter, at 1159 bp from the start site.⁵⁴ This appears to be in contrast with the situation in fish, where in goldfish, common carp and the Chinook salmon LH β genes, an almost full palindromic ERE is found on the proximal promoter, although this has only been shown to be functional for the csLH β .⁵³ Given that the effects of estrogen receptor appear to be entirely dependent on E₂, the liganded estrogen receptor may comprise an important factor in regulation of this gene in teleosts (see below).

In contrast to the stimulatory effects of estrogens and aromatizable androgens, in mammals non-aromatizable androgens exert a direct inhibitory effect on LH β gene transcription. Ligand-bound androgen receptor (AR), but not ER, effectively suppressed activity of the bovine LH β promoter, which could be overcome by over-expression of Egr-1 or Pitx-1, or of a Sf-1 mutant that lacked the ligand binding domain (LBD). It was demonstrated that this repressive effect of AR was exerted through binding the Sf-1 LBD which prevents interaction of Sf-1 with Egr-1 or Pitx-1.⁵⁵

DNA-Binding Proteins: Activation and Combinatorial Actions

In Regulating the LH β Gene

The gonadotrope specific element and Sf-1 are crucial for LH β gene expression

The gonadotrope specific element (GSE) was first identified as being crucial to gonadotropin α subunit gene activation, and its binding protein

was isolated and identified as the orphan nuclear receptor, Sf-1; so named because of its presence in the steroidogenic tissues.^{56, 57} Its principle role in the adrenal gland and gonads is to co-ordinate expression of the cytochrome P450 steroid hydroxylases.⁵⁸ However it also has a crucial role in regulation of the LH β subunit gene and disruption of the expression of the FTZ-F1 gene that encodes Sf-1 leads to loss of the LH β transcripts, as well as those of FSH β and the GnRH-R.⁵⁹

A crucial role for Sf-1 in teleost LH β gene activation has been demonstrated for csLH β : deletion of the proximal GSE reduced basal transcriptional activity to 4% that of the intact promoter, and also abolished the GnRH response (Melamed *et al.*, personal observation).⁶⁰ Over-expression studies have confirmed the ability of Sf-1 to transactivate this gene when Sf-1 was over-expressed alone, or together with ER and/or Pitx-1.^{7,61}

Studies on mammalian Sf-1 have shown that it is probably not transcriptionally up-regulated by GnRH, but may be phosphorylated by MAPK.^{8,30} The phosphorylation of Ser203 mimics the stabilizing effects of a ligand on the LBD of this orphan receptor, and increases its interaction with the cofactors GRIP1 and SMRT.^{30,62} This serine residue was shown to be essential for maximal Sf-1 transactivation of a reporter gene fused to regions of the *Müllerian inhibiting substance* or *P450 21-hydroxylase* promoters.³⁰ However the role of this phosphorylation has yet to be demonstrated specifically in the regulation of LH β gene expression.

The transcriptional activity of Sf-1 *in vivo* was also shown to be regulated through its acetylation, which was carried out *in vitro* by GCN5. Hyperacetylation by treatment with trichostatin A led to translocation of Sf-1 into the cytoplasm which is concomitant with an increase in its half-life.⁶³ The mechanisms regulating acetylation of Sf-1 are as yet unknown.

The presence of multiple Sf-1 isoforms has been demonstrated for several teleost species. Zebrafish appear to contain at least three isoforms, two of which result from alternative splicing of a single gene; one of the latter isoforms serves as an antagonist as it lacks the C-terminal activation function 2 (AF2) core. The isoforms show varying patterns of expression both temporally and spatially, but all share almost complete

identity with the mouse Sf-1 in the conserved Ftz box, and over 90% identity in the DNA binding region.⁶⁴⁻⁶⁶ In the Chinook salmon we have also partially identified four different isoforms in various reproductive tissues, one of which was expressed predominantly in the pituitary, while the others were preferentially expressed in the ovary or the liver (Melamed and Hew, personal observation). The importance of subtle differences in these isoforms is not yet clear. However, it was noted that over expression of mammalian Sf-1 or zFF1A increased csLH β transcription to similar degrees either alone, or in combination with ER.⁶⁴

Pitx-1 interacts with Sf-1 and is crucial for the LH β response to GnRH

Pitx-1, a homeodomain transcription factor related to bicoid, appears crucial for gonadotrope cell differentiation and for gonadotropin gene expression, as well as being involved in hind limb development. It is expressed throughout pituitary organogenesis and knock-out of the Pitx-1 gene revealed considerable reduction in LH β , FSH β and TSH β expression levels, and a reduction in the numbers of gonadotropes.⁶⁷⁻⁶⁹ Binding sites for Pitx-1 are found on genes encoding most of the pituitary hormones, and site-directed mutagenesis studies on some of these have revealed their essential roles in promoter activity. Transactivation by Pitx-1 commonly involves dimerization with other specific transcription factors.⁷⁰⁻⁷²

For the csLH β gene, deletion of the proximal Pitx-1 RE (at 232 bp from the start site) did not affect basal transcription, but abolished responsiveness to GnRH.⁷ Although over-expression of Pitx-1 alone is not able to activate the csLH β proximal promoter, we have shown that it interacts synergistically with Sf-1 or with Sf-1 and ER.⁷ In mammals, Pitx-1 was reported to potentiate the effect of Sf-1 through interacting with its ligand binding domain; the binding facilitates activity of the activation domain, similar to the action of a “true” ligand, and without absolutely requiring a Pitx-1 RE.^{8,70} However, for the csLH β promoter this may not be the case as the GnRH response of the csLH β

requires the intact Pitx-1 binding site.⁷ It would thus seem reasonable to hypothesize that the role of Pitx-1 on the csLH β proximal promoter is either through helping recruit common or interacting cofactors, or through direct interaction with the general transcription factors (see below).

Pitx-1 itself is not transcriptionally up-regulated by GnRH.⁸ However, it does contain a number of putative phosphorylation sites for MAPK, PKC and PKA. Using a novel protein chip technique coupled with mass spectrometry, we were able to show that the Pitx-1 from L β T2 nuclear extracts binds the Pitx-1 RE and is approximately 240 Da larger than the predicted size, indicating three phosphorylated residues.⁷ The identities of the phosphorylated residues and the kinases responsible still have to be ascertained.

Has the role of Egr-1 in mediating the GnRH effect on the LH β in mammals been replaced in fish by AP-1 and/or ER?

Despite its highly conserved presence on the mammalian LH β gene promoters, Egr-1 REs are not found on proximal promoters of any of the published fish LH β genes (Fig. 2a), and over expression of Egr-1 in COS 1 cells has no effect on csLH β promoter activity (Melamed, personal observation). This is surprising given the crucial role Egr-1 plays in mediating the GnRH response in mammals. Egr-1, which is barely detectable in unstimulated cells, is both transcriptionally up-regulated and phosphorylated in response to GnRH. It is thought that these two actions allow for the crucial synergistic interaction of Egr-1 with Sf-1 and Pitx-1.^{8,10}

Given that fish LH β gene promoters are equally responsive to GnRH as their mammalian homologs, it seems likely that another factor has taken on this role of mediator of the GnRH signal in the nucleus. Because near-consensus EREs are found on all of the published fish LH β proximal promoters and, like the Egr-1, ER interacts synergistically with Sf-1 and Pitx-1,⁷ we hypothesized that the ER may have adopted this role. Indeed, the ER α can be phosphorylated by MAPK and PKC, and the ERE appears to have a crucial role in mediating the

GnRH response. However, further studies showed that the ERE is not essential for the interaction of ER α with Sf-1 and Pitx-1, and that c-jun can also activate the csLH β promoter through this ERE, in an effect that is additive to that of Sf-1 and Pitx-1 (Koh and Melamed, personal observation).

GnRH potently stimulates expression of c-jun and c-fos.^{20,32} Therefore, it is likely that the GnRH increases binding of the AP-1 factors to the ERE and that the ER acts to induce LH β transactivation through a mechanism independent of the ERE. ER α has previously been reported to transactivate genes through interactions with AP-1 in the absence of an ERE,⁷³ so these mechanisms are clearly not mutually exclusive and may operate differently throughout the reproductive cycle of the fish (Koh and Melamed, personal observation).

Pitx-1, ER/AP-1 and other factors may form an upstream enhanceosome on the csLH β gene

Although 300 bp of the proximal csLH β promoter are sufficient to impart GnRH responsiveness, the longer 3.3 kb promoter is clearly the most responsive. A distal ERE (dERE) is present at -2659 bp, which is able to interact functionally with the proximal ERE,⁵³ and we have shown that the dERE is involved in mediating part of the GnRH effect, suggesting that the proteins binding these distal and proximal regions of the promoter interact through a common complex (Fig. 3; Melamed *et al.*, personal observation). However, as the factors binding the proximal ERE have yet to be identified, components of this enhanceosome could reasonably comprise ER and/or AP-1 factors.

Moreover, an upstream series of four Pitx-1 REs (between 1366 and 1506 bp from the start site) is also essential to both the GnRH effect and the basal transcription activity. These binding sites appear to work in tandem: we have demonstrated that Pitx-1 can homodimerize to induce conformational change in the target DNA. Given that truncated promoter constructs containing this region, but not the dERE, failed to impart GnRH responsiveness, it is likely that the Pitx-1 acts specifically as an architectural protein to allow interactions between the

distal enhanceosome with the proximal promoter and associated transcription factors or cofactors (Fig. 3a).⁷

In Regulating the FSH β Gene

AP-1 binding sites/EREs are conserved on FSH β gene promoters across the species

The presence of two functional AP-1 sites on the ovine FSH β promoter was reported, and these were shown to be required and sufficient to confer GnRH responsiveness in transfected HeLa cells.^{74,75} The presence of one or more these elements appears well conserved across the species (Fig. 2b).^{12,75} Given that c-jun and c-fos are transcriptionally activated and phosphorylated in response to GnRH, this dimer is a likely mediator in the GnRH stimulatory effect on the FSH β gene. The same group later published studies on transgenic mice in which deletion of the AP-1 binding site abolished the GnRH response, but did not affect that of activin, indicating that AP-1 specifically mediates the GnRH effect *in vivo*. However basal FSH levels in these mice, and also the response to treatment with GnRH antagonists were similar as in the wild type animals, suggesting that GnRH is regulating the FSH through a mechanism that does not require the AP-1 sites. It was hypothesized that this relates to global changes in activity of the gonadotropes, rather than direct effects on the FSH β gene.³³

Preliminary studies on the csFSH β gene indicate that over-expression of c-jun stimulates dramatically the activity of the first intron to drive expression of a reporter gene, and that this effect is mediated in part though an AP-1 binding site (Chong and Melamed, personal observation). However, ER α was also able to increase activity of this region, an effect requiring the same AP-1 binding site and a direct repeat of two half EREs, with a 7 bp spacer (Chong and Melamed, personal observation). As ER and AP-1 factors are known to interact with each other at AP-1 binding sites, further studies are required to elucidate the possible roles and mechanisms through which these factors activate expression of the teleost FSH β genes.

Do Pitx-1 and Sf-1 have a direct role in activating the FSH β gene?

Potential Pitx-1 and Sf-1 binding elements (RGATTA and RAGGYC), are found on both Chinook salmon and tilapia FSH β gene proximal promoters in the above and/or the reverse sequence. On the rat FSH β proximal promoter, Pitx-1 was shown to have a role in basal transcriptional activity, although its binding site was not essential to promoter activity: over-expression of Pitx-1 was still able to stimulate activity of the reporter gene after mutation of the two identified REs.⁷⁶ This suggests either the presence of additional unidentified binding sites, or an indirect effect, possibly through activation of a different DNA binding factor. Interestingly, the authors also found that the effect of over-expression of Pitx-1 was synergistic with stimulation by GnRH.⁷⁶ The authors conclude that the Pitx-1 is interacting with the GnRH stimulated pathway; this interaction is likely given the association of Pitx-1 in the GnRH-mediated effect on the LH β genes.

The mammalian FSH β gene promoters do not contain a consensus GSE, although Sf-1 gene knock-out has a dramatic effect on FSH β gene expression, suggesting an indirect mechanism of Sf-1 action on the FSH β gene⁵⁹. Whether Sf-1 binds directly to the teleost FSH β gene promoter is not yet known, but given that Pitx-1 and Sf-1 interact synergistically to activate the LH β gene, it is possible that a similar mechanism could activate the teleost FSH β genes, possibly in response to GnRH activated signals. However, the large and unconserved distances between these putative response elements, and the lack of homogeneity in the organization of the proximal promoters across the species make understanding the molecular mechanisms involved complex, and emphasize the need for more experimental data.

Direct effects of Smad proteins and their interacting partners

On the rat FSH promoter, the combination of Smad3 and Smad4, in L β T2 cells exposed also to activin, resulted in an increase in promoter activity by over 70-fold, while Smad2, with or without Smad3, had no effect. The region of the rat FSH β gene promoter responsive to activin

was recently identified as a palindromic Smad binding element (SBE: GTCTAGAC) that binds Smad4 and is required for the effect of activin. An adjacent Pitx2c binding site was also essential for the activin effect.³⁸ A putative SBE is found on the proximal promoters of both Chinook salmon and tilapia FSH β , although in both genes, the first half of the palindrome is in the reverse sequence (AGACAGAC). Interestingly, our studies on the regulatory role of the csFSH β first intron, showed that over-expression of Smad1 in COS 1 cells was highly stimulatory to expression of the reporter gene (Chong and Melamed, personal observation).

Silencers

The proximal promoter of the csLH β contains a powerful silencer. This region, located at -39 to -95 from the transcriptional start site, inhibits transcription in a large number of cell lines, with promoter activity dropping to 4% that of the minimal 39 bp promoter. Most of this activity is attributed to the sequence -58 to -76 bp, and it can largely be overcome by distal regions of the promoter (Melamed *et al.*, personal observation).⁷⁷ The silencer is considerably less potent in gonadotrope-derived cell lines such as α T3-1 or L β T2 cells, or in primary pituitary cells from the *pars distalis*, than in heterologous cell lines. Further, we have demonstrated that the core region of the silencer binds proteins in nuclear extracts of primary pituitary *pars intermedia* trout cells, but not those from the *pars distalis* (Melamed *et al.*, personal observation). As yet we have been unable to identify the silencer binding protein which means we are not able to understand how this element fits in with the overall regulation of the LH β gene transcription. Furthermore, the lack of obvious homology between the csLH β silencer and sequences on LH β genes from other species leaves us to conjecture about its place in the evolutionary development of the LH β genes.

The promoter of the tilapia FSH β gene also contains a silencer, located between 1211 and 1389 bp from the transcriptional start site. In primary tilapia pituitary cells, inclusion of this region reduced basal promoter activity by nearly 90%. Although the silencer sequence was

not identified, it was speculated that the downstream CRE might bind a CREB repressive variant such as ICER, which could interact with the upstream region to depress promoter activity.¹³ Interestingly, the region of the promoter containing the silencer includes a TC-rich sequence with some homology to a consensus silencer sequence compiled from observations in other species.⁷⁸ This consensus silencer sequence, whose function is known to be position independent, is also found in the proximal region of the csFSH β gene promoter, although its function has yet to be tested.

Recruitment of Coactivators and Chromatin Remodeling

For neither the LH β nor the FSH β gene have any of the relevant cofactors been identified unambiguously in fish or mammals. Coactivators are essential in the initiation of transcription both for their role in making the chromatin accessible to other DNA binding factors and the general transcription factors, and in stabilizing binding of the general transcriptional machinery to the promoter. Recruitment of the coactivators is often promoter specific, arising from combinatorial recruitment through several DNA binding factors.⁷⁹ For example, Sf-1 has been shown to interact with CBP, p300, p/CIP, SRC-1, GCN5 and TIF2 on a variety of promoters.^{63,80,81} Moreover, the activity of regulated kinases, such as PKA, appears to have some influence on the particular coactivators recruited.⁸²

ER also is reported to interact with diverse proteins, including a variety of coactivators and co-repressors, and these interacting partners clearly differ in diverse cell types. The best validated of these ER-interacting cofactors are SRC-1, TIF2 and AIB1, all of which interact with the AF-2 domain of ER and serve to recruit additional factors such as CBP, P300 and pCAF.⁸³ These recruited factors all contain intrinsic acetyl transferase activity, through which they reduce the interaction of N-terminal histone tails with the DNA, so facilitating transcription by allowing access to other proteins including the general

transcription factors. In addition, ER α was recently shown to interact with protein arginine methyl transferase 2, which enhanced ER α transactivation.⁸⁴ Although the precise role for methylation in transcriptional activation is not well understood, it has been hypothesized that combinations of these covalent modifications of the histone tails form a histone code which is read and interpreted by other proteins, leading to formation at the promoter of distinct protein complexes that activate specific downstream events.⁸⁵ ER α also interacts with the TRAP220 class of proteins which form part of the mediator complex and interact directly with the general transcriptional machinery.⁸⁶

Although synergistic transcription factors often recruit the same cofactors, in our hands Ptx-1 was unable to bind either CBP or p300, and its role in the tripartite complex on the LH β proximal promoter remains unclear.⁷ This is in contrast with the report that the HOX homeodomain can interact with CBP and p300, and in so doing, it interrupts the histone acetyl transferase activity and prevents binding of the HOX to the target gene.⁸⁷ However, this implies that HOX factors would repress transcription of genes in their chromatin template, which is clearly not the case, arguing for tight regulation of interacting partners, presumably through post-translational modifications.⁷ Clearly in order to determine the proteins interacting with Pitx-1, screening experiments are required. Notably however, the Ptx-1 related protein, bicoid, binds directly to TFIID through which it recruits the TBP-TAF complex to the promoter and so directs transcriptional activation.⁸⁸

A wealth of evidence indicates the importance of combinatorial actions to recruit cofactors in a gene-specific manner, such that interacting proteins determined by *in vitro* techniques may well not be indicative of the relevant interacting partners *in vivo*, and do little to explain the intricacies of transcriptional activation at a particular gene locus. Clearly, the coactivators involved specifically in transcription of the LH β and FSH β genes have yet to be elucidated. Moreover, the importance of covalent modifications of the histone tails in regulating gonadotropin gene expression has not yet been demonstrated. Nevertheless, our own preliminary experiments indicate that both LH β and FSH β are highly repressed at certain stages of development by

histone deacetylation, which can be alleviated in part by GnRH (Abdul Kadir and Melamed, personal observation). The challenge remains to identify the pathways through which binding of specific transcription factors is able to recruit the cofactors, remodel the chromatin and allow transcriptional activation to occur as a result of hormonal stimulus.

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References

1. Melamed P, Rosenfeld H, Elizur A and Yaron Z (1998). Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. *Comp. Biochem. Physiol. C* **119**: 325–338.
2. Devlin RH and Nagahama Y (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **208**: 91–364.
3. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A and Levavi-Sivan B (2003). Regulation of fish gonadotropins. *Int. Rev. Cytol.* **225**: 131–185.
4. Xiong F and Hew CL (1991). Chinook salmon (*Oncorhynchus tshawytscha*) gonadotropin II B-subunit gene encodes multiple messenger ribonucleic acids. *Can. J. Zool.* **69**: 2572–2578.
5. Chang YS, Huang FL and Lo TB (1992). Isolation and sequence analysis of carp gonadotropin β-subunit gene. *Mol. Mar. Biol. Biotechnol.* **1**: 97–105.
6. Sohn YC, Yoshiura Y, Suetake H, Kobayashi M and Aida K (1999). Nucleotide sequence of gonadotropin IIβ subunit gene in goldfish. *Fisheries Sci.* **65**: 800–801.
7. Melamed P, Koh M, Preklathan P, Bei L and Hew CL (2002). Multiple mechanisms for Pitx-1 transactivation of a luteinizing hormone β subunit gene. *J. Biol. Chem.* **277**: 26200–26207.

8. Tremblay JJ and Drouin J (1999). Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and Sf-1 to enhance luteinizing hormone β gene transcription. *Mol. Cell. Biol.* **19**: 2567–2576.
9. Dorn C, Ou QL, Svaren J, Crawford PA and Sadovsky Y (1999). Activation of luteinizing hormone β gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. *J. Biol. Chem.* **274**: 13870–13876.
10. Halvorson LM, Kaiser UB and Chin WW (1999). The protein kinase C system acts through the early growth response protein 1 to increase LH beta gene expression in synergy with steroidogenic factor-1. *Mol. Endocrinol.* **13**: 106–116.
11. Rosenfeld H, Levavi-Sivan B, Melamed P, Yaron Z and Elizur A (1997). The GtH β subunits of tilapia: gene cloning and expression. *Fish Physiol. Biochem.* **17**: 85–92.
12. Sohn YC, Suetake H, Yoshiura Y, Kobayashi M and Aida K (1998). Structural and expression analysis of gonadotropin I β subunit genes in goldfish (*Carassius auratus*). *Gene* **222**: 257–267.
13. Rosenfeld H, Levavi-Sivan B, Gur G, Melamed P, Meiri I, Yaron Z and Elizur A (2001). Characterization of the tilapia FSH β gene and analysis of its 5' flanking region. *Comp. Biochem. Physiol. B* **129**: 389–398.
14. Khakoo Z, Bhatia A, Gedamu L and Habibi HR (1994). Functional specificity for salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH II coupled to gonadotropin release and subunit messenger ribonucleic acid level in the goldfish pituitary. *Endocrinology* **134**: 838–847.
15. Melamed P, Gur G, Elizur A, Rosenfeld H, Sivan B, Rentier-Delrue F and Yaron Z (1996). Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II β subunit and growth hormone in the teleost fish, tilapia. *Neuroendocrinology* **64**: 320–328.
16. Gur G, Bonfil D, Safarian H, Naor Z and Yaron Z (2001). GnRH receptor signaling in tilapia pituitary cells: role of mitogen-activated protein kinase (MAPK). *Comp. Biochem. Physiol. B* **129**: 517–524.
17. Gur G, Bonfil D, Safarian H, Naor Z and Yaron Z (2002). GnRH signaling pathways regulate differentially the tilapia gonadotropin subunit genes. *Mol. Cell Endocrinol.* **189**: 125–134.

18. Roberson MS, Zhang T, Li HL and Mulvaney JM (1999). A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone alpha-subunit promoter by gonadotropin-releasing hormone. *Endocrinology* **140**: 1310–1318.
19. Yokoi T, Ohmichi M, Tasaka K, Kimura A, Kanda Y, Hayakawa J, Tahara M, Hisamoto K, Kurachi H and Murata Y (2000). Activation of the luteinizing hormone β promoter by gonadotropin-releasing hormone requires c-Jun NH₂-terminal protein kinase. *J. Biol. Chem.* **275**: 21639–21647.
20. Liu FJ, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM and Webster NJG (2002). Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L β T2 cells. *J. Biol. Chem.* **77**: 32099–32108.
21. Reiss N, Levi LN, Shacham S, Harris D, Seger R and Naor Z (1997). Mechanism of mitogen-activated protein kinase activation by gonadotropin-releasing hormone in the pituitary α T3-1 cell line: differential roles of calcium and protein kinase C. *Endocrinology* **138**: 1673–1682.
22. Naor Z, Benard O and Seger R (2000). Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. *Trends Endocrinol. Metab.* **11**: 91–99.
23. Levavi-Sivan B and Yaron Z (1992). Involvement of cyclic adenosine monophosphate in the stimulation of gonadotropin secretion from the pituitary of a teleost fish, tilapia. *Mol. Cell. Endocrinol.* **85**: 175–182.
24. Mukhopadhyay B, Biswas R and Bhattacharya S (1995). Gonadotropin releasing hormone stimulation of cyclic 3',5'-AMP in the pituitary cell of a teleost (*Channa punctatus*, Bloch) requires extracellular calcium: its relationship to gonadotropin release. *Gen. Comp. Endocrinol.* **97**: 353–365.
25. Chang JP, Jobin RM and Wong AOL (1993). Intracellular mechanism mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus*. *Fish Physiol. Biochem.* **11**: 25–33.
26. Ishizaka K, Tsujii T and Winters SJ (1993). Evidence for a role for the cyclic adenosine 3',5'-AMP monophosphate/protein kinase-A pathway in regulation of the gonadotropin subunit messenger ribonucleic acids. *Endocrinology* **133**: 2040–2048.
27. Gur G, Bonfil D, Safarian H, Naor Z and Yaron Z (2002). Pituitary adenylate cyclase activating polypeptide and neuropeptide Y regulation of gonadotropin subunit gene expression in tilapia: Role of PKC, PKA and ERK. *Neuroendocrinology* **75**: 164–174.

28. Sim PJ, Wolbers WB and Mitchell R (1995). Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein-kinase-C and a pertussis-toxin-sensitive G-protein. *Mol. Cell. Endocrinol.* **112**: 257–263.
29. Han XB and Conn PM (1999). The role of protein kinases A and C pathways in the regulation of mitogen-activated protein kinase activation in response to gonadotropin-releasing hormone receptor activation. *Endocrinology* **140**: 2241–2251.
30. Hammer GD, Krylova I, Zhang Y, Darimont BD, Simpson K, Weigel NL and Ingraham HA (1999). Phosphorylation of nuclear receptor SF-1 modulates cofactor recruitment: Integration of hormone signaling in reproduction and stress. *Mol. Cell* **3**: 521–526.
31. Curran T and Franzia BR (1988). Fos and Jun — the AP-1 connection. *Cell* **55**: 395–397.
32. Cesnjaj M, Catt KJ and Stojilkovic SS (1994). Coordinate actions of calcium and protein kinase-C in the expression of primary response genes in pituitary gonadotropes. *Endocrinology* **135**: 692–701.
33. Huang HJ, Sebastian J, Strahl BD, Wu JC and Miller WL (2001). Transcriptional regulation of the ovine follicle-stimulating hormone- β gene by activin and gonadotropin-releasing hormone (GnRH): Involvement of two proximal activator sites for GnRH stimulation. *Endocrinology* **142**: 2267–2274.
34. Huang HJ, Wu JC, Su P, Zhirnov O and Miller WL (2001). A novel role for bone morphogenic proteins in the synthesis of follicle-stimulating hormone. *Endocrinology* **142**: 2275–2283.
35. Liu B, Dou CL, Prabhu L and Lai E (1999). FAST-2 is a mammalian winged-helix protein which mediates transforming growth factor β signals. *Mol. Cell Biol.* **19**: 424–430.
36. Zhang Y and Deryck R (1999). Regulation of smad signaling by protein associations and signaling crosstalk. *Trends Cell Biol.* **9**: 274–279.
37. Hua X, Liu X, Ansari XD and Lodish HF (1998). Synergistic cooperation of TFE3 and Smad proteins in the TGF- β -induced transcription of the plasminogen activator inhibitor-1 gene. *Genes Dev.* **12**: 3084–3095.
38. Suszko MI, Lo DJ, Suh H, Camper SA and Woodruff TK (2003). Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. *Mol. Endocrinol.* **17**: 318–332.
39. Kalra SP and Crowley WR (1992). Neuropeptide-Y: a novel neuroendocrine peptide in the control of pituitary hormone secretion, and its relation to luteinizing hormone. *Front. Neuroendocrin.* **13**: 1–46.

40. Peng C, Chang JP, Yu KL, Wong AOL, van Goor F, Peter RE and Rivier JE (1993). Neuropeptide-Y stimulates growth-hormone and gonadotropin-II secretion in the goldfish pituitary — involvement of both presynaptic and pituitary cell actions *Endocrinology* **132**: 1820–1829.
41. Rawlings SR (1996). Pituitary adenylate cyclase-activating polypeptide regulates $[Ca^{2+}]_i$ and electrical activity in pituitary cells through cell type-specific mechanisms. *Trends Endocrinol. Metab.* **7**: 374–378.
42. Sherwood NM, Krueckl SL and McRory JE (2000). The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr. Rev.* **21**: 619–670.
43. Wong AOL, Li WS, Lee EKY, Leung MY, Tse LY, Chow BKC, Lin HR and Chang JP (2000). Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochem. Cell Biol.* **78**: 329–343.
44. Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A and Vaudry H (2000). Pituitary adenylate cyclase-activating polypeptide and its receptors: From structure to functions *Pharmacol. Rev.* **52**: 269–324.
45. Yaron Z, Levavi-Sivan B, Melamed P, Rosenfeld H and Elizur A (1998). Second messengers involved in the response of gonadotropin hormone (GtH) cells in fish: GtH release and GtH II β mRNA levels. *Ann. N.Y. Acad. Sci.* **839**: 254–259.
46. Levavi-Sivan B, Ofir M and Yaron Z (1995). Possible sites of dopaminergic inhibition of gonadotropin release from the pituitary of a teleost fish, tilapia. *Mol. Cell. Endocrinol.* **109**: 87–95.
47. Gharib SD, Wierman ME, Shupnik MA and Chin WW (1990). Molecular biology of the pituitary gonadotropins. *Endocr. Rev.* **11**: 177–199.
48. Shupnik MA (1996). Gonadal hormone feedback on pituitary gonadotropin genes. *Trends Endocrinol. Metab.* **7**: 272–276.
49. Yaron Z, Gur G, Melamed P, Rosenfeld H, Levavi-Sivan B and Elizur A (2001). Regulation of Gonadotropin subunit genes in tilapia. *Comp. Biochem. Physiol. B* **129**: 489–502.
50. Melamed P, Gur G, Rosenfeld H, Elizur A, Schulz RW and Yaron Z (2000). Reproductive development of male and female tilapia hybrids (*Oreochromis niloticus* \times *O. aureus*) and changes in mRNA levels of gonadotropin (GtH) I β and II β subunits. *J. Exp. Zool.* **286**: 64–75.
51. Melamed P, Gur G, Rosenfeld H, Elizur A and Yaron Z (1999). Possible interactions between gonadotrophs and somatotrophs in the pituitary of

- tilapia: apparent roles for insulin-like growth factor I and estradiol. *Endocrinology* **140**: 1183–1191.
- 52. de Leeuw R, Th Goos HJ, Peute J, van Pelt AMM, Burzawa-Gerard E and van Oordt PGWJ (1984). Isolation of gonadotropes from the pituitary of the African catfish, *Clarias lazera*. Morphological and physiological characterization of the purified cells. *Cell Tissue Res.* **236**: 669–675.
 - 53. Liu D, Xiong F and Hew CL (1995). Functional analysis of estrogen responsive elements in Chinook salmon (*Oncorhynchus tshawytscha*) gonadotropin II β subunit gene. *Endocrinology* **136**: 3486–3493.
 - 54. Shupnik MA and Rosenzweig BA (1991). Identification of an estrogen responsive element in the rat LH- β gene — DNA: estrogen receptor interactions and functional analysis. *J. Biol. Chem.* **266**: 17084–17091.
 - 55. Jorgensen JS and Nilson JH (2001). AR suppresses transcription of the LH beta subunit by interacting with steroidogenic factor-1. *Mol. Endocrinol.* **15**: 1505–1516.
 - 56. Horn F, Windle JJ, Barnhart KM and Mellon PL (1992). Tissue-specific gene-expression in the pituitary: the glycoprotein hormone α subunit gene is regulated by a gonadotrope specific protein. *Mol. Cell. Biol.* **12**: 2143–2153.
 - 57. Barnhart KM and Mellon PL (1994). The orphan nuclear receptor, steroidogenic factor-I, regulates the glycoprotein hormone α -subunit gene in pituitary gonadotropes. *Mol. Endocrinol.* **8**: 878–885.
 - 58. Ikeda Y, Lala DS, Luo X, Kim E, Moisan MP and Parker KL (1993). Characterization of the mouse FTZ-F1 gene, which encodes an essential regulator of steroid hydroxylase gene expression. *Mol. Endocrinol.* **7**: 852–860.
 - 59. Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen WH, Nachtigal MW, Abbud R, Nilson JH and Parker KL (1994). The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev.* **8**: 2302–2312.
 - 60. Le Dréan Y, Liu D, Wong AOL, Xiong F and Hew CL (1996). Steroidogenic factor 1 and estradiol receptor act in synergism to regulate the expression of the salmon gonadotropin II β subunit gene. *Mol. Endocrinol.* **10**: 217–229.
 - 61. Le Dréan Y, Liu D, Xiong F and Hew CL (1997). Presence of *cis*-acting elements on gonadotropin gene promoters in diverse species dictates the selective recruitment of different transcription factors by steroidogenic factor-1. *Mol. Cell. Endocrinol.* **135**: 31–40.

62. Desclozeaux M, Krylova IN, Horn F, Fletterick RJ and Ingraham HA (2002). Phosphorylation and intramolecular stabilization of the ligand binding domain in the nuclear receptor steroidogenic factor 1. *Mol. Cell. Biol.* **22:** 7193–7203.
63. Jacob AL, Lund J, Martinez P and Hedin L (2001). Acetylation of steroidogenic factor 1 protein regulates its transcriptional activity and recruits the coactivator GCN5. *J. Biol. Chem.* **276:** 37659–37664.
64. Liu D, Le Dréan Y, Ekker M, Xiong F and Hew CL (1997). Teleost FTZ-F1 homolog and its splicing variant determine the expression of the salmon gonadotropin II β subunit gene. *Mol. Endocrinol.* **11:** 877–890.
65. Chai C and Chan WK (2000). Developmental expression of a novel Ftz-F1 homolog, *ffl1b* (NR5A4), in the zebrafish *Danio rerio*. *Mech. Dev.* **91:** 421–426.
66. Von Hofsten J, Jones I, Karlsson K and Olsen PE (2001). Developmental expression patterns of FTZ-F1 homologues in zebrafish (*Danio rerio*). *Gen. Comp. Endocrinol.* **121:** 146–155.
67. Lanctôt C, Moreau A, Chamberland M, Tremblay ML and Drouin J (1997). The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124:** 2807–2817.
68. Drouin J, Lamolet B, Lamonerie T, Lanctôt C and Tremblay JJ (1998). The PTX family of homeodomain transcription factors during pituitary developments. *Mol. Cell. Endocrinol.* **140:** 31–36.
69. Szeto DP, Rodriguez-Esteban C, Ryan AK, O'Connell SM, Liu R, Kioussi C, Gleberman AS, Izpisua-Belmonte JC and Rosenfeld MG (1999). Role of Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. *Genes Dev.* **13:** 484–494.
70. Tremblay JJ, Marcil A, Gauthier Y and Drouin J (1999). Ptx-1 regulates SF-1 activity by an interaction that mimics the role of the ligand-binding domain. *EMBO J.* **18:** 3431–3441.
71. Poulin G, Lebel M, Chamberland M, Paradis FW and Drouin J (2000). Specific protein-protein interaction between basic helix-loop-helix transcription factors and homeoproteins of the Pitx family. *Mol. Cell. Biol.* **20:** 4826–4837.
72. Quirk CC, Lozada KL, Keri R and Nilson JH (2001). A single Pitx1 binding site is essential for activity of the LH β promoter in transgenic mice. *Mol. Endocrinol.* **15:** 734–746.

73. Hall JM, Couse JF and Korach KS (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J. Biol. Chem.* **276**: 36869–36872.
74. Strahl BD, Huang HJ, Pederson NR, Wu J, Gosh BR and Miller WL (1997). Two proximal activating protein-1 binding sites are sufficient to stimulate transcription of the ovine follicle-stimulating hormone- β gene. *Endocrinology* **138**: 2621–2631.
75. Strahl BD, Huang HJ, Sebastian J, Ghosh BR and Miller WL (1998). Transcriptional activation of the ovine follicle stimulating hormone β -subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. *Endocrinology* **139**: 4455–4465.
76. Zakaria MM, Jeong KH, Lacza C and Kaiser UB (2002). Pituitary homeobox 1 activates the rat FSH beta (rFSH beta) gene through both direct and indirect interactions with the rFSH beta gene promoter. *Mol. Endocrinol.* **16**: 1840–1852.
77. Xiong F, Liu D, Elsholtz HP and Hew CL (1994). The Chinook salmon gonadotropin II β gene contains a strong minimal promoter with a proximal negative element. *Mol. Endocrinol.* **8**: 771–781.
78. Baniahmad A, Muller M, Steiner Ch and Renkawitz R (1987). Activity of two different silencer elements of the chicken lysozyme gene can be compensated by enhancer elements. *EMBO J.* **6**: 2297–2303.
79. McKenna NJ and O'Malley BW (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**: 465–474.
80. Crawford PA, Polish JA, Ganpule G and Sadovsky Y (1997). The activation function-2 hexamer of steroidogenic factor-1 is required, but not sufficient for potentiation by SRC-1. *Mol. Endocrinol.* **11**: 1626–1635.
81. Borud B, Hoang T, Bakke M, Jacob AL, Lund J and Mellgren G (2002). The nuclear receptor coactivators p300/CBP/Cointegrator-Associated Protein (p/CIP) and Transcription Intermediary Factor 2 (TIF2) differentially regulate PKA-stimulated transcriptional activity of steroidogenic factor 1. *Mol. Endocrinol.* **16**: 757–773.
82. Lund J, Borud JB, Mellgren G, Aesoy R, Hoang TY, Jacob AL and Bakke M (2002). Differential regulation of SF-1 cofactor interactions. *Endocrine Res.* **28**: 505–513.
83. McDonnell DP and Norris DJ (2002). Connections and regulation of the human estrogen receptor. *Science* **296**: 1642–1644.

84. Qi C, Chang J, Zhu YW, Yeldandi AV, Rao SM and Zhu YJ (2002). Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. *J. Biol. Chem.* **277**: 28624–28630.
85. Strahl BD and Allis CD (2000). The language of covalent histone modifications. *Nature* **403**: 41–45.
86. Kang YK, Guermah M, Yuna CX and Roeder RG (2002). The TRAP/mediator coactivator complex interacts directly with estrogen receptors alpha and beta through the TRAP220 subunit and directly enhances estrogen receptor function *in vitro*. *Proc. Natl. Acad. Sci. USA* **99**: 2642–2647.
87. Shen WF, Krishnan K, Lawrence HJ and Largman C (2001). The HOX homeodomain protein block CBP histone acetyltransferase activity. *Mol. Cell. Biol.* **21**: 7509–7522.
88. Sauer F, Hanse SK and Tjian R (1995). DNA-template and activator-coactivator requirements for transcriptional synergism by *Drosophila* bicoid. *Science* **270**: 1825–1828.

Evidence for Pleiotropic Effects of Prolactin in Teleost Fish

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Abstract

In vertebrates, prolactin (PRL) is considered a pleiotropic hormone with more than 300 distinct physiological actions already described, including effects on growth, development, reproduction, lactation, water and electrolyte balance, immunoregulation, and behavior. In fish, PRL is considered to be the fresh-water adapting hormone, given its ability to regulate the hydromineral balance in teleosts. Although PRL involvement in osmoregulation is well documented, little information is available concerning other effects of the hormone in fish. This chapter will summarize the non-osmoregulatory roles of PRL and will focus on new information that allows a better understanding of the pleiotropic effects of PRL in fish. We will show how characterization of the prolactin receptor (PRL-R) in euryhaline and stenohaline teleosts and expression of the receptor in different organs suggest involvement in different functions. We also summarize older and more recent studies of prolactin's role in functions such as reproduction, immunoregulation and stress.

Introduction

Prolactin (PRL) is probably one of the most versatile hormones produced by the vertebrate pituitary. First known as the pituitary

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hormone of lactation in mammals,^{1,2} PRL has been characterized in more than 300 separate actions among vertebrates.^{3,4} Pleiotropic actions of PRL can be classified in different areas: growth and development; endocrinology and metabolism; hydromineral balance; brain and behavior; reproduction and immunoregulation.⁴

In teleost fish, PRL is considered to be primarily responsible for the control of hydromineral balance in a hypoosmotic environment and most of the PRL studies in fish have studied its role in water and ion regulation.^{5–8} PRL regulation of hydromineral balance was first studied in the killifish *Fundulus heteroclitus*⁹ and these results were further expanded to other euryhaline fishes such as tilapia. Thus, killifish and tilapia (*Oreochromis mossambicus* and *O. niloticus*) are strictly dependent on PRL for their survival in fresh water (FW). Hypophysectomy in this hypoosmotic environment leads to death within a few days due to a dramatic disequilibrium of the hydromineral balance. Only PRL injections can restore ionic levels in these species.^{9–12} Inversely, in euryhaline fish developing in seawater (SW) or brackish water (BW), PRL injections inhibit gill Na⁺ extrusion, leading to a pronounced imbalance of water and ion exchange.^{13,14} However, this strict PRL dependency for survival in hypoosmotic environment cannot be generalized and in other euryhaline fish species such as salmonids, the osmoregulatory roles of PRL are less clear. Although plasma PRL levels decrease after direct transfer from FW to SW,^{15–18} hypophysectomy does not impair survival in FW, with only slight disequilibrium of the hydromineral balance.^{19,20} Moreover, PRL alone has no effect on SW adaptation in salmon but is able to antagonize the SW-adapting effect observed after growth hormone (GH) treatment.²¹ Nevertheless, PRL is generally described as the hormone of fish for adaptation to a hypoosmotic environment or for antagonistic effects on adaptation to a hyperosmotic environment.

The central role of PRL in osmoregulation is well documented and numerous reviews have been devoted to the subject.^{5,6,8} However, little information is available concerning other effects of the hormone in fish. The present review will focus on non-osmoregulatory roles of PRL and will focus on new information for a better understanding of the pleiotropic effects of PRL in fish. We will investigate how the

characterization of prolactin receptors (PRL-R) in euryhaline and stenohaline teleosts and tissue distribution of receptors suggest PRL involvement in different functions. We also summarize older and more recent studies of prolactin's role in functions such as reproduction, immunoregulation and stress.

PRL and Reproduction

In mammals, numerous studies have described the diverse reproductive actions of PRL, some of which may be stimulated by locally produced PRL.^{4,22,23} In contrast, information concerning the role of PRL in fish is more limited and a complete view of PRL reproductive actions is lacking. However, recent data on expression of PRL and its receptors in relation to reproduction should reinstate the interest of studying PRL and reproduction in fish.

PRL Effects on Steroidogenesis

The most clear-cut effects of PRL on reproduction concern steroidogenesis, as have been shown using ovine (o)PRL in *Aequidens pulcher* females, chum salmon (s)PRL in hypophysectomized *Fundulus heteroclitus* males, tilapia (ti)PRL in *Poecilia reticulata* females and both tiPRLs in *O. mossambicus* males.^{24–27} Rubin and Specker²⁷ reported an increased testosterone production in courting tilapia. However, there was no consistent pattern of changes in PRL levels during the reproductive cycle in female tilapia *O. mossambicus*.²⁸ Tan *et al.*²⁶ reported that tiPRL stimulated estradiol (E_2) secretion by oocytes of the guppy in four developmental stages: perinucleolar, yolk-vesicle, and early and late vitellogenic. Galas and Epler²⁹ recently reported that PRL significantly suppressed steroid secretion by follicular cells before ovulation and in the course of vitellogenesis in the rainbow trout. However, Young *et al.*³⁰ did not observe any effects of PRL on E_2 production by cultured full-grown immature, mid-vitellogenic follicles of amago salmon. Similarly, carp PRL was ineffective in stimulating E_2 secretion by vitellogenic

follicles.³¹ Altogether, these data do not allow a generalization of PRL effects on steroidogenesis in all fish species studied so far.

Despite the absence of clear effects of PRL on steroidogenesis, numerous studies on teleost fish show that estrogens may regulate PRL cell activity suggesting a positive feedback action *in vivo* and *in vitro*. E₂ stimulates PRL synthesis in tilapia³² and markedly activates PRL cells in mudsucker *Gillichthys mirabilis*³³ and in adult male and female eels.^{33–35} A dose-dependent increase in pituitary synthesis of two forms of PRL was also demonstrated in E₂-treated tilapia *in vivo*.³⁶ In seabream, whereas E₂ significantly increased PRL in juvenile fish, it caused a drastic reduction in PRL gene expression in adults.³⁷

Moreover, *in vitro* studies using organ-cultured pituitary from adult male tilapia indicated that E₂ treatment stimulates PRL synthesis,³² spontaneous PRL release^{38,39} and promotes stimulation of PRL release by TRH.³⁸ Interestingly, both PRL isoforms can play a role in the endocrine regulation of vitellogenesis in tilapia.⁴⁰

However, similar effects of E₂ on PRL expression and release were not observed in other species. Studies in rainbow trout indicate that plasma PRL levels do not significantly change during vitellogenesis when large increases in plasma E₂ levels are observed.⁷ Similarly, Le Goff *et al.*⁴¹ showed that *in vivo* application of E₂ treatment in rainbow trout neither modifies pituitary PRL and PRL mRNA contents nor induces changes in serum PRL concentration whatever the stage studied (immature fish, mature male or mature female ovariectomized fish). Both Northern blot analysis and *in situ* hybridization demonstrated that functional estrogen receptors are absent or undetectable in the lactotropes of rainbow trout.⁴¹ A similar situation has been described in catfish in which immunocytochemical studies localized E₂ mainly in gonadotropes whereas no significant signal was observed in lactotropes.⁴²

PRL and Maternal Behavior

Although in mammals and birds there is some evidence for PRL action on parental behavior^{4,43} and more specifically on maternal behavior,^{44–47} the endocrine control of parental behavior in some fish species has been

a matter of controversy. A prolactin-like hormone has been suggested to play a role in the regulation of parental care in substrate-spawning species of cichlids⁴⁸ and in some other teleosts (*Gasterosteus aculeatus*, *Lepidchromis macrochirus*).^{49,50} In male stickleback, PRL is involved in fanning behavior (refreshment of the water around eggs and newly hatched fry). First, PRL cell activity was reported to be enhanced during the period of about 7 days when the males take care of the eggs in the nests and spend much time fanning.⁵¹ Second, administration of homologous PRL was reported to significantly increase the level of fanning behavior from 6 to 12 days after administration.⁴⁹ Preliminary results on the mouthbreeding cichlid *O. mossambicus* have shown that prolactin secretion is unchanged during the different stages of the mouthbreeding cycle.⁵¹ Recently, the PRL isoform tiPRL_{II} has been suggested to be involved in the control of maternal behavior in incubating female tilapia, as the plasma concentrations of tiPRL_{II} exhibit significant variations during this period.⁴⁰ Thus PRL is probably involved in the control of fanning behavior and possibly other aspects of parental care, but perhaps not in the control of parental care in general.

PRL-R, PRL and the Gonads

Many data have accumulated showing the expression of PRL-R in reproductive tissues in fish. Initial binding studies performed in teleosts have been carried out using oPRL. Specific binding of ¹²⁵I-oPRL to membranes of tilapia ovarian and testicular cells was detected by Edery *et al.*⁵² More recently, molecular characterization of PRL-R in different teleost species and expression of receptors in gonads has suggested direct PRL effects in fish reproduction (Table 1). PRL-R transcripts are expressed in ovary and testis of stenohaline fishes such as goldfish and seabream,^{53,54} whereas expression of PRL-R in the ovary was established in the euryhaline species, tilapia and rainbow trout, using Northern-blot analyses of immature gonads.^{55,56} Although PRL-R expression has been demonstrated in both testis and ovary of tilapia, no transcript was detected in testis of rainbow trout. In Japanese flounder, tissue distribution analyses using both Northern-blot and RNase-protection assays revealed expression of PRL-R transcripts in

Table 1 Tissue distribution of the PRL-R in different fish species. X: mRNA detected; nd: not detected; nt: not tested.

Species		Goldfish ⁵³	Tilapia ^{55,111,112}	Rainbow trout (unpublished observations) ¹¹³	Japanese flounder ⁵⁷	Sea bream ^{37,54}
Tissue						
Brain	X	X		X	nd	X
Gills	X	X	X		X	X
Gut	X	X	X		X	X
Kidney	X	X	X		X	X
Liver	X	nd	X		nd	X
Muscle	nt	nd	nd		X	nd
Heart	X	nd	nd		X	nd
Bone	nt	nt	nt		nt	nd
Head kidney	nt	X	X		nt	nt
Lymphocytes	nt	X	nt		nt	nt
Skin	nt	X	nd		nt	X
Spleen	nt	X	X		X	nt
Testis	X	X	nd		X	X
Ovary	X	X	X		nd	X
Larvae	nt	nt	nt		nt	X

testis but not in ovary of mature fish.⁵⁷ Overall, it is difficult to draw a clear picture of PRL-R expression in fish gonads on the basis of these divergent results obtained in different species, from mature or immature animals, and at different stages of gametogenesis. A complete analysis of transcripts expression during spermatogenesis/ovogenesis in several species is necessary for a more thorough understanding.

In mammals, the presence of short and long forms of PRL-R has been characterized and related to regulation of PRL actions in reproduction.^{58,59} This work was initiated in seabream in which sbPRL-R expression was detected in spermatogonia and oocytes,³⁷ and four PRL-R transcripts were characterized at 2.8; 1.9; 1.3 and 1.1 kb. The presence of numerous PRL-R transcripts in this species is in direct

Table 2 PRL-R transcripts sizes expressed in different species of vertebrates. Symbols: *: transcript size suggesting short form; **: truncated isoform.

Species	Size of the transcripts encoding PRL-R	
	Long form	Short and/or truncated form
Human ¹¹⁴	10.5; 6.2; 3.4; 2.7	
Bear ¹¹⁵	10; 3.7; 2.6	
Bull ¹¹⁶	4.4; 3.8; 2.6	
Deer ¹¹⁷	12.2; 9.5; 7.1; 3.5; 2.5	1.7**
Ram/Sheep ^{61,118,119}	13; 12; 10.2; 4.5; 3.2	1.6*
Rabbit ^{120,121}	10.5; 6.2; 3.4; 2.7	
Rat ^{63,122}	9.7; 5.5; 4.6; 3.5; 2.5	2.1; 1.8
Mouse ⁶⁰	10.0; 9.0; 8.3	4.2; 3.5; 2.4; 1.4
Turkey ¹²³	3.0	
Chicken ^{62,124}	11.7; 4.6; 3.6; 2.0	1.7**; 1.2**
Xenopus ¹²⁵	8; 3	
Tilapia ^{55,111,112}	3.2	
Goldfish ⁵³	4.6; 3.5	
Rainbow trout (unpublished observations) ¹¹³	3.4	
Japanese flounder ⁵⁷	2.8; 1.9; 1.3; 1.1	
Seabream ^{37,54}	2.8; 1.9	1.3*; 1.1*

contrast with previous observations in tilapia and rainbow trout in which a single transcript was found in both male and female gonads (Table 2).^{55,56} Expression of several transcripts in gonads is more common in higher vertebrates including mouse, ram and chicken.^{60–62} Moreover, the expression of different sizes of transcripts in seabream,³⁷ suggests expression of a shorter isoform of PRL-R. This is the only information in fish suggesting the existence of a short form of the PRL-R. In mammals both short and long forms have been characterized in gonads.^{63,64}

However, it is still not known precisely how the regulation of PRL-R expression affects fish reproduction and whether PRL is involved to the same degree in females and males. In higher vertebrates, studies undertaken on homozygous PRL-R knockout mice did reveal differences

between males and females. Homozygous (-/-) female mice are completely sterile,⁶⁵⁻⁶⁸ whereas homozygous (-/-) male mice show no major defect in fertility, implying that PRL is not a key hormone in the control of male fertility.⁶⁸ Finally, local synthesis of PRL has been observed in goldfish and in seabream gonads, which reinforces the possibility of PRL acting as a reproductive hormone.^{69,70}

PRL and Immune Functions

In mammals, PRL is a common mediator of the immunoneuroendocrine network in which nervous, endocrine, and immune systems communicate.⁷¹ PRL plays a significant role in regulation of the humoral and cellular immune responses in physiological as well as pathological states, such as autoimmune diseases.^{72,73} PRL is more particularly required for mitogenic-stimulated proliferation of lymphocytes.^{74,75} Moreover, PRL-R is expressed on T and B lymphocytes but both long- and short-forms of PRL-R are also detected in thymus, spleen, lymph nodes and bone marrow of rodents.^{76,77}

In fish, PRL-R transcripts are detected by Northern blot in various organs implicated in immunoregulation: in spleen of tilapia; rainbow trout and Japanese flounder, and in head kidney of tilapia and rainbow trout (unpublished observations).^{55,57} PRL-R transcripts are also expressed in lymphocytes of tilapia and rainbow trout (unpublished observations).⁷⁸ This pattern of PRL-R distribution strongly suggests a direct involvement of PRL in fish immunoregulation. Moreover, extrapituitary expression of mRNA for two PRLs was detected in lymphoid tissues and cells such as head kidney, spleen, intestine and leukocytes from peripheral blood and head kidney in tilapia and in rainbow trout.^{78,79} Altogether, these data suggest that PRL plays a paracrine and/or autocrine role in regulating immune responses in fish, a picture well established in mammals.

Furthermore, there is now an expanding set of results demonstrating the immunoregulatory effects of PRL in fish. The first experiments in the gulf killifish (*Fundulus grandis*) using oPRL were reported to modify allograft rejection, which is related to the specific cellular immune

system.⁸⁰ Moreover, decreased levels of plasma PRL followed by a reduction of antibody production to the virulent bacteria, *Yersinia ruckeri*, were observed in rainbow trout transferred from fresh water to seawater, suggesting a stimulatory role of PRL in antibody production.⁸¹

More recently, prolactin was shown to induce a direct proliferative response on chum salmon peripheral blood leukocytes *in vitro*.⁸² At the same time, Sakai *et al.* reported an elevation of respiratory burst activity of phagocytic cells in the presence of PRL *in vitro*.⁸³ In juvenile silver seabream, daily injections of PRL for 7 days induced significant increases in percent phagocytosis and phagocytic index of macrophages.⁸⁴ In this study, PRL injection also resulted in elevated blood lymphocyte counts.⁸⁴ PRL implants were able to restore plasma IgM levels of hypophysectomized rainbow trout.⁸⁵ Finally, treatment with tiPRLs (PRL₁₇₇ and PRL₁₈₈) was shown to enhance superoxide anion (O₂⁻) production *in vitro* in head kidney leukocytes of tilapia in a dose-related manner.⁷⁸ All together, these recent data strongly suggest that PRL acts as an enhancer of the immune response in fish immunoregulation.

PRL and Stress

Several experiments undertaken on teleost fish suggest that PRL could modulate the response to stress, but other results appear to be partially contradictory. Prolactin is probably implicated in the control of physiological adaptation to sublethal acid stress in tilapia, as its secretion was reported to increase markedly during acid exposure.⁸⁶ Avella *et al.*⁸⁷ reported that in coho salmon chronic confinement stress caused a gradual increase in the plasma PRL, which lagged behind the cortisol response, whereas acute stress by handling, which induced a transient increase in cortisol, had no apparent effect on PRL. They also showed that confinement appeared to be more stressful in fish directly transferred from salt water than in fresh water-adapted fish. However, Pottinger *et al.*⁸⁸ reported a reduction in plasma PRL in confined rainbow trout, whereas Kakizawa *et al.*⁸⁹ could not demonstrate a change in plasma PRL in rainbow trout after acute lowering of the water level and chasing. Interestingly, these conditions were shown to stimulate plasma cortisol,

somatolactin and growth hormone levels. However, in rainbow trout stressed by confinement, Le Rouzic and Prunet (unpublished observations) observed an increase in PRL-R transcripts in gills but not in intestine and kidney. This suggests that the role of PRL in stress response in fish is more complicated than initially anticipated and deserves further studies.

PRL Effects on Metabolism

PRL is clearly involved in the regulation of metabolism in higher vertebrates,⁴ whereas in fish, PRL effects have been reported on lipid stores (for goldfish and kokanee salmon)^{90,91} and also on glucose reserves (for catfish and tilapia).^{92,93} Ovine PRL treatment could also contribute to lipolysis in coho salmon during smoltification, in brown muscle and liver.⁹⁴ However, this lipolytic effect in salmon appears to be dependent on the timing of the oPRL treatment.⁹⁵ The same observation was made in cyprinodontid fishes in which an injection performed in the morning stimulated lypolysis but the same injection later during the day led to an opposite effect.^{96,97} However, metabolic effects of PRL on liver using oPRL are not without criticism. Indeed, Dauder *et al.*⁹⁸ using membrane extracts from tilapia liver observed that tiGH was able to compete with oPRL during binding studies, suggesting the possibility that oPRL binds the GH-R, so the effects of oPRL on liver could in fact be GH-like.

Moreover, in fish, Northern-blot analyses and RNase-protection assays performed in various species failed to detect any significant mRNA expression in liver (tilapia; Japanese flounder; rainbow trout: unpublished observations).^{55,57} In two other species (goldfish and seabream), PRL-R expression was hardly detectable in liver after a RT-PCR amplification coupled with Southern-blot analyses.^{53,54} It is important to note that this is a very sensitive technique that allows detection of minute amounts of transcript, and it should be considered whether so few copies of a transcript in a tissue are of any physiological relevance. All together, this absence or low expression of PRL-R in fish liver is in complete

opposition with the very high expression of hepatic PRL-R in mammals.⁹⁹

Other PRL Effects

It has been reported that PRL acts on epithelial cell proliferation in various fish species.^{100–102} Also, in these species, it has been observed that PRL injection enhances mucus cell proliferation. Likewise, this proliferation is observed in goldfish and European eel.^{103,104} An increase of epithelial cell number and mucus quantity could contribute to establishing a physical barrier limiting water and ion exchange at the skin surface.^{105,106} Mucus production could also contribute to protecting the fish from external pathogens.

Other PRL effects in specific functions are actually too fragmentary to be discussed here. However, *in vivo* and *in vitro* experiments suggest PRL involvement in skin pigmentation is linked to fish behavior.^{107,108}

Conclusion

The recent development of new molecular tools which allow both study of PRL-R gene expression and local synthesis of PRL hormones has opened the way for further studies to characterize the pleiotropic effects of this hormone in fish. The present data confirm roles of PRL, which have been widely illustrated in higher vertebrates. The difficulty in observing clear-cut effects of PRL in fish may be related to PRL acting in synergy/antagonism with other hormones. Thus, the osmoregulatory actions of PRL that are mandatory for freshwater survival in several fish species, are probably an exception and for most of its other regulatory roles, PRL actions should be analyzed in relation to other hormonal factors, such as corticosteroids. Finally, a description of the short-form of PRL-R recently identified in humans and rodent^{109,110} is still lacking in fish. This certainly deserves further investigations, as this new form of receptor has been suggested to act as a ‘decoy’ receptor, which would allow the tissue to adapt to high levels of PRL.⁵⁸

References

1. Stricker P and Grueter R (1928). Action du lobe antérieure de l'hypophyse sur la montée laiteuse. *C. R. Soc. Biol.* **99**: 1978–1980.
2. Riddle O, Bates RW and Dykshorn SW (1933). The preparation, identification and assay of prolactin-a hormone of the anterior pituitary. *Am. J. Physiol.* **105**: 191–216.
3. Nicoll CS and Bern HA (1972). On the actions of PRL among the vertebrates: is there a common denominator? In: Wolstenholme GEW and Knight K (eds), *Lactogenic Hormones*, Churchill Livingstone, London, pp. 229–337.
4. Bole-Feysot C, Goffin V, Edery M, Binart N and Kelly PA (1998). Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev.* **19**: 225–268.
5. Loretz CA and Bern HA (1982). Prolactin and osmoregulation in vertebrates. An update. *Neuroendocrinol.* **35**: 292–304.
6. Hirano T, Ogasawara T, Bolton JP, Collie NL, Hasegawa S and Iwata M (1986). Osmoregulatory role of prolactin in lower vertebrates. In: Kirsch R and Lahliou B (eds), *Comparative Physiology of Environmental Adaptations*, Vol 1, Karger, Basel, pp. 112–124.
7. Prunet P, Avella M, Fostier A, Björnsson BT, Boeuf G and Haux C (1990). Roles of prolactin in salmonids. *Prog. Clin. Biol. Res.* **342**: 547–552.
8. Manzon LA (2002). The role of prolactin in fish osmoregulation: a review. *Gen. Comp. Endocrinol.* **125**: 291–310.
9. Pickford GE and Philipps JG (1959). Prolactin, a factor in promoting survival of hypophysectomized killifish in fresh water. *Science* **130**: 454–455.
10. Handin RI, Nandi J and Bern HA (1964). Effects of hypophysectomy on survival and on thyroid and interrenal histology of cichlid teleosts. *Gen. Comp. Endocrinol.* **63**: 309–317.
11. Dharmamba M, Handin RI, Nandi J and Bern HA (1967). Effect of prolactin on freshwater survival and on plasma osmotic pressure of hypophysectomized *Tilapia mossambica*. *Gen. Comp. Endocrinol.* **9**: 295–302.
12. Dharmamba M and Maetz J (1972). Effects of hypophysectomy and prolactin on the sodium balance of *Tilapia mossambica* in fresh water. *Gen. Comp. Endocrinol.* **19**: 175–183.

13. Dharmamba M and Maetz J (1976). Branchial sodium exchange in seawater-adapted *Tilapia mossambica*: effects of prolactin and hypophysectomy. *J. Endocrinol.* **70**: 293–299.
14. Aupérin B, Rentier-Delrue F, Martial JA and Prunet P (1994). Evidence that two tilapia (*Oreochromis niloticus*) prolactins have different osmoregulatory functions during adaptation to a hyperosmotic environment. *J. Mol. Endocrinol.* **12**: 13–24.
15. Prunet P, Boeuf G and Houdebine LM (1985). Plasma and pituitary prolactin levels in rainbow trout during adaptation to different salinities. *J. Exp. Zool.* **235**: 187–196.
16. Hasegawa S, Hirano T, Ogasawara T, Iwata M, Akiyama T and Arai S (1987). Osmoregulatory ability of chum salmon, *Oncorhynchus keta*, reared in fresh water for prolonged periods. *Fish Physiol. Biochem.* **4**: 101–110.
17. Young G, Björnsson BT, Prunet P, Lin RJ and Bern HA (1989). Smoltification and seawater adaptation in coho salmon (*Oncorhynchus kisutch*): plasma prolactin, growth hormone, thyroid hormones and cortisol. *Gen. Comp. Endocrinol.* **74**: 335–345.
18. Yada T, Takahashi A and Hirano T (1991). Seasonal changes in seawater adaptability and plasma levels of prolactin and growth hormone in landlocked sockeye salmon (*Oncorhynchus nerka*) and amago salmon (*Oncorhynchus rhodurus*). *Gen. Comp. Endocrinol.* **82**: 33–44.
19. Komourdjian MP (1984). Monovalent plasma ion regulation in the hypophysectomized Atlantic salmon. *Gen. Comp. Endocrinol.* **56**: 278–282.
20. Björnsson BT and Hanson T (1983). Effects of hypophysectomy on the plasma ionic osmotic balance in rainbow trout, *Salmo gairdneri*. *Gen. Comp. Endocrinol.* **49**: 240–247.
21. Madsen SS and Bern HA (1992). Antagonism of prolactin and growth hormone: impact on seawater adaptation in two salmonids, *Salmo trutta* and *Oncorhynchus mykiss*. *Zool. Sci.* **9**: 775–784.
22. Untergasser P, Kranewitter W, Schwarzler P, Madersbacher S, Dirnhofer S and Berger P (1997). Organ-specific expression pattern of the human growth hormone/placental lactogen gene-cluster in the testis. *Mol. Cell. Endocrinol.* **130**: 53–60.
23. Schwarzler P, Untergasser G, Hermann M, Dirnhofer S, Abendstein B and Berger P (1997). Prolactin gene expression and prolactin protein in premenopausal and postmenopausal human ovaries. *Fertil. Steril.* **68**: 696–701.

24. Blüm V and Weber KM (1968). The influence of prolactin on the activity of steroid-3-beta-ol dehydrogenase in the ovaries of the cichlid fish *Aequidens pulcher*. *Experientia* **24**: 1259–1260.
25. Singh H, Griffith RW, Takahashi A, Kawauchi H, Thomas P and Stegeman JJ (1988). Regulation of gonadal steroidogenesis in *Fundulus heteroclitus* by recombinant salmon growth hormone and purified salmon prolactin. *Gen. Comp. Endocrinol.* **72**: 144–153.
26. Tan CH, Wong LY, Pang MK and Lam TJ (1988). Tilapia prolactin stimulates estradiol 17 β synthesis *in vitro* in vitellogenic oocytes of the guppy *Poecilia reticulata*. *J. Exp. Zool.* **248**: 361–364.
27. Rubin DA and Specker JL (1992). *In vitro* effects of homologous prolactins on testosterone production by testes of tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* **87**: 189–196.
28. Weber GRM and Grau EG (1999). Changes in serum concentrations and pituitary content of the two prolactins and growth hormone during the reproductive cycle in female tilapia, *Oreochromis mossambicus*, compared with changes during fasting. *Comp. Biochem. Biophys. C Pharmacol. Toxicol. Endocrinol.* **124**: 323–335.
29. Galas J and Epler P (2002). Does prolactin affect steroid secretion by isolated rainbow trout ovarian cells? *Comp. Biochem. Physiol. B* **132**: 287–297.
30. Young G, Ueda H and Nagahama Y (1983). Estradiol-17 β and 17 α -, 20 β -dihydroxy-4-pregn-3-one production by isolated ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) in response to mammalian pituitary and placental hormones and salmon gonadotrophin. *Gen. Comp. Endocrinol.* **79**: 233–239.
31. Van der Kraak G, Rosenblum PM and Peter RE (1990). Growth hormone-dependent potentiation of gonadotropin-stimulated steroid production by ovarian follicles of the goldfish. *Gen. Comp. Endocrinol.* **79**: 233–239.
32. Wigham RS, Nishioka RS and Bern HA (1977). Factors affecting *in vitro* activity of prolactin cells in the euryhaline teleost *Sarotherodon mossambicus* (*Tilapia mossambica*). *Gen. Comp. Endocrinol.* **32**: 120–131.
33. Nagahama Y, Nishioka NS, Bern HA and Gunther RL (1975). Control of prolactin secretion in teleosts, with special reference to *Gillichthys mirabilis* and *Tilapia mossambica*. *Gen. Comp. Endocrinol.* **25**: 166–188.

34. Olivereau M and Olivereau J (1979). Effect of estradiol-17 beta on the cytology of the liver, gonads and pituitary, and on plasma electrolytes in the female freshwater eel. *Cell Tissue Res.* **199:** 431–454.
35. Olivereau M, Dubourg P, Chambolle P and Olivereau J (1986). Effects of estradiol and mammalian LHRH on the ultrastructure of the pars distalis of the eel. *Cell Tissue Res.* **246:** 425–437.
36. Poh L-H, Munro AD and Tan CH (1997). The effects of estradiol on the prolactin and growth hormone content of the pituitary of the tilapia, *Oreochromis mossambicus*, with observations on the incidence of black males. *Zool. Sci.* **14:** 979–986.
37. Cavaco JEB, Santos CRA, Ingleton PM, Canario AVM and Power DM (2003). Quantification of prolactin (PRL) and PRL receptor messenger RNA in gilthead seabream (*Sparus aurata*) after treatment with estradiol-17 β . *Biol. Reprod.* **68:** 588–594.
38. Barry T and Grau EG (1986). Estradiol-17 β and thyrotropin-releasing hormone stimulate prolactin release from the pituitary gland of a teleost fish *in vitro*. *Gen. Comp. Endocrinol.* **62:** 306–314.
39. Weber GM, Powell JFF, Park M, Fischer WH, Criag AG, Rivier JE, Nankorn U, Parhar IS, Ngamvongchon S, Grau EG and Sherwood NM (1997). Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. *J. Endocrinol.* **155:** 121–132.
40. Tacon P, Baroiller JF, Le Bail PY, Prunet P and Jalabert B (2000). Effect of egg deprivation on sex steroids, gonadotropin, prolactin, and growth hormone profiles during the reproductive cycle of the mouthbrooding cichlid fish *Oreochromis niloticus*. *Gen. Comp. Endocrinol.* **117:** 54–65.
41. Le Goff P, Salbert G, Prunet P, Saligaut C, Th. Bjornsson B, Haux C and Valotaire Y (1992). Absence of direct regulation of prolactin cells by estradiol-17 β in rainbow trout (*Oncorhynchus mykiss*). *Mol. Cell. Endocrinol.* **90:** 133–139.
42. Peute J, Schulz R, Glazenburg K, Lambert JGD and Blüm V (1989). Pituitary steroids in two teleost species: immunohistological and biochemical studies. *Gen. Comp. Endocrinol.* **76:** 63–72.
43. Freeman ME, Kanyicska B, Lerant A and Nagy G (2000). Prolactin: Structure, function, and regulation of secretion. *Physiol. Rev.* **80:** 1523–1631.

44. Bridges RS (1985). Prolactin stimulation of maternal behavior in female rats. *Science* **227**: 782–784.
45. Bridges RS, Robertson MC, Shiu RPC, Friesen HG, Stuer AM and Mann PE (1996). Endocrine communication between conceptus and mother: placental lactogen stimulation of maternal behavior. *Neuroendocrinol.* **64**: 57–64.
46. Lucas BK, Ormandy CJ, Binart N, Bridges RS and Kelly PA (1998). Null mutation of the prolactin receptor gene produces a defect in maternal behavior. *Endocrinology* **139**: 4102–4107.
47. Sakaguchi K, Tanaka M, Ohkubo T, Doh-Ura L, Fujikawa T, Sudo S and Nakashima K (1996). Induction of brain prolactin receptor long-form mRNA expression and maternal behavior in pup-contacted male rats: promotion by prolactin administration and suppression by female contact. *Neuroendocrinol.* **63**: 559–568.
48. Blüm V and Fiedler K (1965). Hormonal control of reproductive behaviour in some cichlid fish. *Gen. Comp. Endocrinol.* **5**: 186–196.
49. De Ruiter AJH, Wendelaar Bonga SE, Slijkhuis H and Baggerman B (1986). The effect of prolactin on fanning behavior in the male three-spined stickleback, *Gasterosteus aculeatus* L. *Gen. Comp. Endocrinol.* **64**: 273–283.
50. Kindler PM, Bahr JM, Gross MR and Philipp DP (1991). Hormonal regulation of parental care behaviour in nesting male Bluegills: do the effects of bromocriptine suggest a role for prolactin? *Physiol. Zool.* **64**: 310–322.
51. Slijkhuis H, De Ruiter AJH, Baggerman B and Wendelaar Bonga SE (1984). Parental fanning behavior and prolactin cell activity in the male three-spined stickleback *Gasterosteus aculeatus* L. *Gen. Comp. Endocrinol.* **54**: 297–307.
52. Edery M, Young G, Bern HA and Steiny S (1984). Prolactin receptors in tilapia (*Sarotherodon mossambicus*) tissues: binding studies using ^{125}I -labeled ovine prolactin. *Gen. Comp. Endocrinol.* **56**: 19–23.
53. Tse DLY, Chow BKC, Chan CB, Lee LTO and Cheng CHK (2000). Molecular cloning and expression studies of a prolactin receptor in goldfish (*Carassius auratus*). *Life Sci.* **66**: 593–605.
54. Santos CRA, Ingleton PM, Cavaco JEB, Kelly PA, Edery M and Power DM (2001). Cloning, characterization, and tissue distribution of

- prolactin receptor in the seabream (*Sparus aurata*). *Gen. Comp. Endocrinol.* **121**: 32–47.
55. Sandra O, Le Rouzic P, Cauty C, Edery M and Prunet P (2000). Expression of the prolactin receptor (tiPRL-R) gene in tilapia *Oreochromis niloticus*: tissue distribution and cellular localization in osmoregulatory organs. *J. Mol. Endocrinol.* **24**: 215–224.
56. Prunet P, Sandra O, Le Rouzic P, Marchand O and Laudet V (2000). Molecular characterization of the prolactin receptor in two fish species, tilapia *Oreochromis niloticus* and rainbow trout *Oncorhynchus mykiss*: a comparative approach. *Can. J. Physiol. Pharmacol.* **78**: 1086–1096.
57. Higashimoto Y, Nakao N, Ohkubo T, Tanaka M and Nakashima K (2001). Structure and tissue distribution of prolactin receptor mRNA in Japanese flounder (*Paralichthys olivaceus*): conserved and preferential expression in osmoregulatory organs. *Gen. Comp. Endocrinol.* **123**: 170–179.
58. Horseman ND (2002). Prolactin receptor diversity in humans: novel isoforms suggest general principles. *Trends Endocrinol. Metab.* **13**: 7–8.
59. Schuler LA, Nagel RJ, Gao J, Horseman ND and Kessler MA (1997). Prolactin receptor heterogeneity in bovine fetal and maternal tissues. *Endocrinology* **138**: 3187–3194.
60. Buck K, Vanek M, Groner B and Ball RK (1992). Multiple forms of prolactin receptor messenger ribonucleic acid are specifically expressed and regulated in murine tissues and mammary cell line HC11. *Endocrinology* **130**: 1108–1114.
61. Jabbour HN and Lincoln GA (1999). Prolactin expression in the testis of the ram: localization, functional activation and the influence of gonadotrophins. *Mol. Cell. Endocrinol.* **148**: 151–161.
62. Mao JNC, Burnside J, Li L, Tang J, Davolos C and Cogburn LA (1999). Characterization of unique truncated prolactin receptor transcripts, corresponding to the intracellular domain, in the testis of the sexually mature chicken. *Endocrinology* **140**: 1165–1174.
63. Shirota M, Banville D, Ali S, Jolicoeur C, Boutin JM, Djiane J and Kelly PA (1990). Expression of two prolactin receptor in rat ovary and liver. *Mol. Endocrinol.* **4**: 1136–1142.
64. Clarke DL, Arey BJ and Linzer DIH (1993). Prolactin receptor messenger ribonucleic acid expression in the ovary during the rat estrous cycle. *Endocrinology* **133**: 2594–2603.

65. Binart N, Helloco C, Ormandy CJ, Barra J, Clement-Lacroix P, Baran N and Kelly PA (2000). Rescue of preimplantory egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration. *Endocrinology* **141**: 2691–2697.
66. Reese J, Binart N, Brown N, Ma WG, Paria BC, Das SK, Kelly PA and Dey SK (2000). Implantation and decidualization defects in prolactin receptor (PRLR)-deficient mice are mediated by ovarian but not uterine PRLR. *Endocrinology* **141**: 1872–1881.
67. Kelly PA, Binart N, Lucas B, Bouchard B and Goffin V (2001). Implication of multiple phenotypes observed in prolactin receptor knockout mice. *Front. Neuroendocrinol.* **22**: 140–145.
68. Goffin V, Binart N, Touraine P and Kelly PA (2002). Prolactin: The new biology of an old hormone. *Ann. Rev. Physiol.* **64**: 47–67.
69. Imaoka T, Matsuda M and Mori T (2000). Extrpituitary expression of the prolactin gene in the goldfish, African clawed frog and mouse. *Zool. Sci.* **17**: 791–796.
70. Santos CR, Brinca L, Ingleton PM and Power DM (1999). Cloning, expression, and tissue localisation of prolactin in adult sea bream (*Sparus aurata*). *Gen. Comp. Endocrinol.* **114**: 57–66.
71. Goffin V, Bouchard B, Ormandy CJ, Weimann E, Ferrag F, Touraine P, Bole-Feysot C, Maaskant RA, Clement-Lacroix P, Edery M, Binart N and Kelly PA (1998). Prolactin: a hormone at the crossroads of neuroimmunoendocrinology. *Ann. N. Y. Acad. Sci.* **840**: 498–509.
72. Walker SE, Allen SH and McMurray RW (1993). Prolactin and autoimmune disease. *Trends Endocrinol. Metab.* **4**: 147–151.
73. Neidhart M (1998). Prolactin in autoimmune diseases. *Proc. Soc. Exp. Biol. Med.* **217**: 408–419.
74. Hiestand PC, Mekler P, Nordmann R, Grieder A and Permmongkol C (1986). Prolactin as a modulator of lymphocyte responsiveness provides a possible mechanism of action for cyclosporine. *Proc. Natl. Acad. Sci. USA* **83**: 2599–2603.
75. Hartmann DP, Holaday JW and Bernton EW (1989). Inhibition of lymphocyte proliferation by antibodies to prolactin. *FASEB J.* **3**: 2194–2202.
76. Touraine P, Leite DMM, Dardenne M and Kelly PA (1994). Expression of short and long forms of prolactin receptors in murine lymphoid tissues. *Mol. Cell. Endocrinol.* **104**: 183–190.

77. Leite DMM, Touraine P, Gagnerault MC, Savino W, Kelly PA and Dardenne M (1995). Prolactin receptors in immune system. *Ann. Endocrinol.* **56**: 567–570.
78. Yada T, Uchida K, Kajimura S, Azuma T, Hirano T and Grau EG (2002). Immunomodulatory effects of prolactin and growth hormone in the tilapia, *Oreochromis mossambicus*. *J. Endocrinol.* **173**: 483–492.
79. Yada T and Azuma T (2002). Hypophysectomy depresses immune functions in rainbow trout. *Comp. Biochem. Physiol. C* **131**: 93–100.
80. Nevid NM and Meier AH (1985). Timed daily administrations of hormones and antagonists of neuroendocrine receptors alter day-night rhythms of allograft rejection in the gulf killifish, *Fundulus grandis*. *Gen. Comp. Endocrinol.* **97**: 327–339.
81. Betouille S, Troutaud D, Khan N and Deschaux P (1995). Réponse anticorps, cortisolémie et prolactinémie chez la truite arc-en-ciel. *C. R. Acad. Sci. Paris* **318**: 677–681.
82. Sakai M, Kobayashi M and Kawauchi H (1996). Mitogenic effect of growth hormone and prolactin on chum salmon *Oncorhynchus keta* leucocytes *in vitro*. *Vet. Immunol. Immunopathol.* **53**: 185–189.
83. Sakai M, Kobayashi M and Kawauchi H (1996). *In vitro* activation of fish phagocytic cells by GH, prolactin and somatolactin. *J. Endocrinol.* **151**: 113–118.
84. Narnaware YK, Kelly SP and Woo NYS (1998). Stimulation of macrophage phagocytosis and lymphocyte count by exogenous prolactin administration in silver sea bream (*Sparus sarba*) adapted to hyper- and hypo-osmotic salinities. *Vet. Immunol. Immunopathol.* **61**: 387–391.
85. Yada T, Nagae M, Moriyama S and Azuma T (1999). Effects of prolactin and growth hormone on plasma immunoglobulin M levels of hypophysectomized rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **115**: 46–52.
86. Wendelaar Bonga SE, van der Meij JC and Flik G (1984). Prolactin and acid stress in the teleost *Oreochromis* (formerly *Sarotherodon*) *mossambicus*. *Gen. Comp. Endocrinol.* **55**: 323–332.
87. Avella M, Schreck CB and Prunet P (1991). Plasma prolactin and cortisol concentrations of stressed coho salmon, *Oncorhynchus kisutch*, in fresh water or salt water. *Gen. Comp. Endocrinol.* **81**: 21–27.

88. Pottinger TG, Prunet P and Pickering AD (1992). The effects of confinement stress on circulating prolactin levels in rainbow trout (*Oncorhynchus mykiss*) in fresh water. *Gen. Comp. Endocrinol.* **88**: 454–460.
89. Kakizawa S, Kaneko T, Hasegawa S and Hirano T (1995). Effects of feeding, fasting, background adaptation, acute stress, and exhaustive exercise on the plasma somatolactin concentrations in rainbow trout. *Gen. Comp. Endocrinol.* **98**: 137–146.
90. Minick MC and Chavin W (1970). Effect of pituitary hormones upon serum free fatty acids in goldfish (*Carassius auratus L.*). *Am. Zool.* **9**: 1082.
91. McKeown BA, Leatherland JF and John TM (1975). The effect of growth hormone and prolactin on the mobilization of free fatty acids and glucose in the kokanee salmon, *Oncorhynchus nerka*. *Comp. Biochem. Physiol. B* **50**: 425–430.
92. Singh AK (1981). Effect of prolaction in the tissue glycogen and lipid content in the catfish *Heteropneutes fossilis* (Bloch). *Indian J. Exp. Biol.* **19**: 425–427.
93. Vijayan M, Morgan J, Sakamoto T, Grau E and Iwama G (1996). Food-deprivation affects seawater acclimation in tilapia: hormonal and metabolic changes. *J. Exp. Biol.* **199**: 2467–2475.
94. Sheridan MA (1986). Effects of thyroxin, cortisol, growth hormone and prolactin on lipid metabolism of coho salmon, *Oncorhyncus kisutch*, during smoltification. *Gen. Comp. Endocrinol.* **64**: 220–238.
95. Leatherland JF, McKeown BA and John TM (1974). Circadian rhythm of plasma prolactin, growth hormone, glucose and free fatty acids in juvenile kokanee salmon, *Oncorhynchus nerka*. *Comp. Biochem. Physiol.* **47**: 821–828.
96. De Vlaming VL and Sage M (1972). Diurnal variation in fattening response to prolactin treatment in two cyprinodontid fishes, *Cyprinodon variquatus* and *Fundulus similis*. *Contrib. Mar. Sci.* **16**: 59–63.
97. Ensor DM (1978). *Comparative Endocrinology of Prolactin*, Chapman and Hall, London.
98. Dauder S, Young G, Hass L and Bern HA (1990). Prolactin receptors in liver, kidney and gill of the tilapia (*Oreochromis mossambicus*): characterization and effect of salinity on specific binding of iodinated ovine prolactin. *Gen. Comp. Endocrinol.* **77**: 368–377.

99. Nagano M and Kelly PA (1994). Tissue distribution and regulation of rat prolactin receptor gene expression: quantitative analysis by polymerase chain reaction. *J. Biol. Chem.* **269**: 13337–13345.
100. Mattheij JAM and Stroband HWJ (1971). The effects of osmotic experiments and prolactin on the mucous cells in the skin and the ionocytes in the gills of the teleost *Cichlasoma biocellatum*. *Z. Zellforsch* **121**: 93–101.
101. Schwerdtferger WK (1979). Morphometrical studies of the ultrastructure of the epidermis of the guppy, *Poecilia reticulata* Peters, following adaptation to seawater and treatment with prolactin. *Gen. Comp. Endocrinol.* **38**: 476–483.
102. Wendelaar-Bonga SE and Meis S (1981). Effects of external osmolality, calcium and prolactin on growth and differentiation of the epidermal cells of the cichlid teleost *Sarotherodon mossambicus*. *Cell Tissue Res.* **221**: 109–123.
103. Ogawa M (1970). Effects of prolactin on the epidermal mucous cells of the goldfish *Carassius auratus* L. *Can. J. Zool.* **48**: 501–503.
104. Olivereau M and Olivereau J (1971). Influence de l'hypophysectomie et d'un traitement prolactinique sur les cellules à mucus de la branchie chez l'anguille. *C. R. Soc. Biol.* **165**: 2267–2271.
105. Marshall WS (1976). Effects of hypophysectomy and ovine prolactin on the epithelial mucus-secreting cells of the Pacific staghorn sculpin, *Leptocottus armatus* (Teleostei: Cotidae). *Can. J. Zool.* **54**: 1604–1609.
106. Shephard KL (1981). The influence of mucus on the diffusion of water across fish epidermis. *Physiol. Zool.* **54**: 23–34.
107. Kitta K, Makino M, Oshima N and Bern HA (1993). Effects of prolactins on the chromatophores of the tilapia, *Oreochromis niloticus*. *Gen. Comp. Endocrinol.* **92**: 355–365.
108. Oshima N, Makino M, Iwamuro S and Bern HA (1996). Pigment dispersion by prolactin in cultured xantophores and erythrophores of some fish species. *J. Exp. Biol.* **275**: 45–52.
109. Das R and Vonderhaar BK (1995). Transduction of prolactin's (PRL) growth signal through both long and short forms of the PRL receptor. *Mol. Endocrinol.* **9**: 1750–1759.
110. Hu ZZ, Meng J and Dufau ML (2001). Isolation and characterization of two novel forms of the human prolactin receptor generated by

- alternative splicing of a newly identified exon 11. *J. Biol. Chem.* **276**: 41086–41094.
111. Sandra O, Sohm F, de Luze A, Prunet P, Edery M and Kelly PA (1995). Expression cloning of a cDNA encoding a fish prolactin receptor. *Proc. Natl. Acad. Sci. USA* **92**: 6037–6041.
 112. Sandra O, Le Rouzic P, Rentier-Delrue F and Prunet P (2001). Transfer of tilapia (*Oreochromis niloticus*) to a hyperosmotic environment is associated with sustained expression of prolactin receptor in intestine, gill and kidney. *Gen. Comp. Endocrinol.* **123**: 295–307.
 113. Le Rouzic P, Sandra O, Grosclaude J, Rentier-Delrue F, Jolois O, Tujague M, Pakdel F, Sandowski Y, Cohen Y, Gertler A and Prunet P (2001). Evidence of rainbow trout interaction with its receptor through unstable homodimerisation. *Mol. Cell. Endocrinol.* **172**: 105–113.
 114. Boutin JM, Edery M, Shirota M, Jolicoeur C, Lesueur L, Ali S, Gould D, Djiane J and Kelly PA (1989). Identification of cDNA encoding a long form of prolactin receptor in human hepatoma and breast cancer cells. *Mol. Endocrinol.* **9**: 1455–1461.
 115. Howell-Skalla L, Bunick D, Bleck G, Nelson RA and Bahr JM (2000). Cloning and sequence analysis of the extracellular region of the polar bear (*Ursus maritimus*) luteinizing hormone receptor (LH_r), follicle stimulating hormone receptor (FSH_r), and prolactin receptor (PRL_r) genes and their expression in the testis of the black bear (*Ursus americanus*). *Mol. Reprod. Dev.* **55**: 136–145.
 116. Scott P, Kessler MA and Schuler LA (1992). Molecular cloning of the bovine prolactin receptor and distribution of prolactin and growth hormone receptor transcripts in fetal and utero-placental tissues. *Mol. Cell. Endocrinol.* **89**: 47–58.
 117. Clarke LA, Edery M, Loudon ASI, Randall VA, Postel-Vinay MC, Kelly PA and Jabbour HN (1995). Expression of the prolactin receptor gene during the breeding and non-breeding seasons in red deer (*Cervus elaphus*): evidence of the expression of two forms in the testis. *J. Endocrinol.* **146**: 313–321.
 118. Cassy S, Charlier M, Belair L, Guillomot M, Charron G, Bloch B and Djiane J (1998). Developmental expression and localization of the prolactin receptor (PRL-R) gene in the ewe mammary gland during pregnancy and lactation: estimation of the ratio of the two forms of the PRL-R messenger ribonucleic acid. *Biol. Reprod.* **58**: 1290–1296.

119. Tortonese DJ, Brooks J, Ingleton PM and McNeilly AS (1998). Detection of prolactin receptor gene expression in the sheep pituitary gland and visualization of the specific translation of the signal in gonadotrophs. *Endocrinology* **139**: 5213–5223.
120. Dusanter-Fourt I, Gaye P, Belair L, Petridou P, Kelly PA and Djiane J (1991). Prolactin receptor gene expression in the rabbit: identification, characterization and tissue distribution of several prolactin receptor messenger RNAs encoding a unique precursor. *Mol. Cell. Endocrinol.* **77**: 181–192 (1991).
121. Dusanter-Fourt I, Belair L, Gespach C and Djiane J (1992). Expression of the prolactin (PRL) receptor gene and PRL-binding sites in rabbit intestinal epithelial cells. *Endocrinology* **130**: 2877–2882.
122. Hu ZZ and Dufau ML (1991). Multiple and differential regulation of ovarian prolactin receptor messenger RNAs and their expression. *Biophys. Biochem. Res. Commun.* **181**: 219–225.
123. Zhou JF, Zadworny D, Guéméné D and Kuhnlein U (1996). Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in *Meleagris gallopavo*. *Biol. Reprod.* **55**: 1081–1090.
124. Tanaka M, Maeda K, Okubo T and Nakashima K (1992). Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. *Biochem. Biophys. Res. Commun.* **188**: 490–496.
125. Yamamoto T, Nakayama Y, Tajima T, Abe S and Kawahara A (2000). Cloning of a cDNA for Xenopus prolactin receptor and its metamorphic expression profile. *Dev. Growth Differ.* **42**: 167–174.

Chapter 5

Activin and Its Receptors in Fish Reproduction

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Abstract

Activin is a dimeric growth factor belonging to the transforming growth factor β (TGF- β) superfamily. Since its first identification and purification in 1986, activin and its related proteins have been firmly established as important regulators of mammalian reproduction. In fish, most of the members of activin family have been identified, as well as the activin receptors and intracellular signaling molecules; they are expressed at multiple levels of the brain-hypophysial-gonadal axis. In the pituitary, activin stimulates goldfish FSH β but suppresses LH β expression; the effects can be abolished by follistatin, an activin-binding protein. In the ovary, activin subunits are localized in the follicle cells and the pre-vitellogenic oocytes. Recombinant human activin A and goldfish activin B both enhance final oocyte maturation and have a powerful effect on the development of oocyte maturational competence in the zebrafish, most likely by acting directly on the oocyte. Gonadotropin(s) stimulates activin β A and follistatin expression in zebrafish follicle cells through cAMP-PKA-dependent pathways. However, gonadotropin(s) suppresses activin β B expression through cAMP-dependent but PKA-independent pathways. The ovarian activin system is also influenced by other local ovarian factors such as epidermal growth factor (EGF), transforming growth factor α (TGF- α) and pituitary adenylate cyclase-activating polypeptide (PACAP). In the testis, activin β B is expressed by the Sertoli cells of the Japanese eel and its expression is up-regulated by hCG.

These lines of evidence in fish point to activin as an important paracrine and autocrine regulator at multiple sites of the reproductive axis in both males and females.

Introduction

Vertebrate reproduction is controlled by a complex regulatory axis involving the brain, pituitary and gonads. The major hormones released by each level of this axis have been well characterized in all groups of vertebrates including fish. However, mounting evidence shows that in addition to the classical hormones or neurohormones, a variety of peptide growth factors are involved in the regulation of vertebrate reproduction at different levels of the axis. Among these growth factors, the activin family is one of the best characterized.

Activin ($\beta\alpha\beta\alpha$, $\beta\alpha\beta\beta$ and $\beta\beta\beta\beta$) is a dimeric protein that structurally belongs to the transforming growth factor β (TGF- β) superfamily. The activities of activin are generally antagonized by inhibin, a heterodimer of a novel α subunit and a β subunit ($\alpha\beta\alpha$ and $\alpha\beta\beta$). Inhibin and activin are closely related by sharing the same β subunits.¹ Both proteins, particularly activin, have wide spatial distribution.² In most tissues, activin functions as a paracrine and autocrine factor to regulate local physiological events. Follistatin is a single chain protein with biological activities similar to those of inhibin, but it is structurally unrelated to activin and inhibin.³ All three proteins were originally identified as gonadal non-steroidal factors that specifically regulate pituitary follicle-stimulating hormone (FSH) secretion without significant effects on luteinizing hormone (LH). Activin stimulates, whereas inhibin and follistatin suppress FSH secretion. Therefore, they play important roles in the differential regulation of the two gonadotropins.⁴⁻¹¹ In addition to stimulating FSH production, compelling evidence has implicated activin in a vast array of physiological and developmental processes, particularly reproduction. These functions include secretion and expression of preoptic gonadotropin-releasing hormone (GnRH)^{12,13} and pituitary growth hormone (GH),^{14,15} gonadal steroidogenesis and

gametogenesis,^{16–19} oxytocin release from the posterior pituitary,²⁰ and mesoderm formation during embryonic development.^{21,22}

Activin exerts its actions through binding to its specific receptors. Two proteins of 50–60 kD and 70–80 kD that bind activin were initially identified by affinity-labeling. The proteins were termed activin type I (ActRI) and type II (ActRII) receptors, respectively.²³ The first activin receptor (type IIA) was cloned in the mouse with an expression cloning strategy.²⁴ Subsequent studies have revealed different variants of activin type I and type II receptors in the same or different species.^{25,26} Both activin type I and type II receptors are transmembrane kinases with a single cross-membrane domain.^{24–26} However, unlike the receptors of many other growth factors, the kinases of activin receptors are serine/threonine rather than tyrosine-specific.²⁷ Two distinct but closely related subtypes of activin type II receptor have been identified in the same species and were termed type IIA and type IIB, respectively.²⁵ Similarly, two subtypes of the type I receptor, type IA (ALK2) and type IB (ALK4), have also been demonstrated in activin signaling.^{26,28,29} It has been well documented that neither type I nor type II alone can activate activin signal transduction pathways. According to the current hypothesis, activin initiates its signaling by first binding to a type II receptor followed by recruiting and activating a type I receptor to form an activin-type I-type II heteromeric complex. It is the type I receptor in the complex that, in turn, activates the signal transduction pathways, which involve a series of intracellular signaling proteins called Smads.³⁰ Eight Smad proteins, Smad1–8, have been identified in vertebrates. They are divided into three functional classes: the receptor-regulated Smads (R-Smads, Smad1, 2, 3, 5 and 8), the common Smads (Co-Smads, Smad4) and the inhibitory Smads (I-Smads, Smad6 and 7).³¹ Among the receptor-regulated Smads, Smad2 and 3 have been demonstrated in activin and TGF- β signaling, whereas others are involved in the signaling of other TGF- β family members such as bone morphogenetic proteins (BMP). Upon activation by the type II receptor through phosphorylation, the activin type I receptor first activates Smad2 or 3, which then recruits the common Smad4 to form a complex. The complex then moves into the nucleus to regulate the expression of

target genes. The inhibitory Smads are not directly involved in activin signal transduction; instead, they act antagonistically to block the signaling of activin and other TGF- β family members in the target cells. Therefore, they serve a potentially negative role in regulating cell responsiveness to activin and related proteins.³²

In sharp contrast to activin receptors, studies on inhibin receptors have generated puzzling results despite tremendous efforts. Although activin mechanisms of action are well characterized, the receptors and signaling mechanisms of inhibin have not been as well defined. This has led to an interesting hypothesis that inhibin may not have its own receptors, but probably functions as a natural antagonist to compete with activin for the same receptors.^{33–35} Inhibin probably binds to specific activin type II receptors through its β subunit as does activin. However, inhibin's distinct α subunit prevents its association with the type I receptor, a step important for activin signaling. Although this idea is supported by the evidence that inhibin has binding affinity for cloned activin type II receptors, the affinity is significantly lower than that with activin.^{24,25} The low affinity between inhibin and activin receptors cannot account for the potent effects of inhibin in its antagonism of activin in various bioassays, and this discrepancy has been the major driving force for the non-stop search for specific inhibin receptors. These efforts have led to the discovery of two inhibin coreceptors, betaglycan and inhibin-binding protein (InhBP), which represents the most exciting development in recent studies of activin and inhibin. Betaglycan, previously referred to as TGF- β type III receptor, is a membrane protein that is not involved in signal transduction. It was recently demonstrated to bind inhibin with high affinity, and this binding dramatically increases the binding affinity of inhibin for activin type II receptors, which enhances its competition with activin.^{36,37} In contrast, InhBP specifically targets activin type IB receptor (ALK4), and their association provides a docking site for inhibin to bind the type I receptor, which disrupts the formation of the activin-receptor complex.^{38,39}

Compared with studies on activin and inhibin, information on the function of follistatin was limited until 1990 when follistatin was found,

surprisingly, to be a specific binding protein of activin.⁴⁰ Follistatin is a powerful inhibitor of activin due to its extremely high affinity for activin, which makes their binding virtually irreversible.³

The wide spatial distribution and functional relationship of activin, inhibin and follistatin strongly suggest that these molecules form an intrinsic regulatory system that functions in a variety of organ systems. Activin is a central player of the system, whereas inhibin and follistatin play important roles to fine-tune the activities of activin by either blocking activin receptors or neutralizing activin. The unique activin-inhibin-follistatin regulatory system is now one of the most active research areas in reproductive physiology, and the role of activin as a major regulator of the brain-hypophysial-gonadal axis has been well established in mammals.

Although activin and its receptors have been extensively examined in mammals, very limited studies have been carried out in other vertebrates such as fish on the involvement of activin in reproductive functions. This chapter provides a brief summary and update on our recent work and that of others on the activin system in fish and its role in the regulation of reproduction.⁴¹

Activin-Inhibin-Follistatin Regulatory System in Fish

Activin, Inhibin and Follistatin in Fish

The existence of the activin family in fish was first demonstrated by cloning the mature region of both activin β A and β B subunits in the goldfish.⁴² Since then, we and others have cloned full-length cDNAs of activin β A and β B subunits from the goldfish (*Carassius auratus*),^{43,44} zebrafish (*Danio rerio*)^{45,46} and Japanese eel (*Anguilla japonica*) (GenBank accession No.: AB025356). DNA fragments for activin subunits have also been isolated in the rainbow trout (*Oncorhynchus mykiss*),⁴⁷ common carp (*Cyprinus carpio*), medaka (*Oryzias latipes*),⁴⁸ killifish (*Fundulus heteroclitus*) (GenBank accession No.: AF503775) and red sea bream (*Pagrus major*) (GenBank accession No.: AB006786).

In the goldfish, activin β A and β B subunits are synthesized as precursors of 404 and 392 amino acids, respectively, with only 44% amino acid identity over the entire protein and 60% over the C-terminal mature region. When compared with that of tetrapods, the mature peptide of goldfish β B subunit shares more than 93% amino acid sequence identity with its counterpart in mammals, which makes activin β B one of the most conserved regulatory proteins in vertebrates. Interestingly, in contrast to β B, fish activin β A exhibits more sequence variation from that of other vertebrates. The mature goldfish β A subunit has 81% amino acid sequence identity with human and *Xenopus* β A subunits.^{43,44} The difference in structural conservation between activin β A and β B suggests that activin β B subunit has been under higher selective pressure during vertebrate evolution, and the β A and β B subunits may each have distinct biological functions. Both activin subunits are expressed in a variety of tissues including the brain, pituitary, ovary and testis.^{43,44} We have confirmed the functional identity of cloned goldfish activin β A and β B by expressing the proteins in Chinese hamster ovary (CHO) cells.^{43,44} Specific activin activity in the culture medium from the transfected CHO cells was assayed with F5-5 cells, a mouse leukemia cell line that responds to activin by differentiating into hemoglobin-producing cells.⁴⁹ We have thereafter established several stable CHO cell lines that produce recombinant goldfish activin A (β A β A) and B (β B β B) with biological activities.

As an important member of the activin family, inhibin functions as a natural antagonist of activin in mammals. However, there has been no convincing evidence for its existence in fish despite the observations that mammalian inhibin has biological activities in fish pituitary⁵⁰ and ovary.⁵¹ Recently, a homolog of inhibin α subunit was cloned in the rainbow trout. The mature region of the molecule exhibits 50–60% sequence identity with the counterpart of mammals.⁵² However, the functional identity of this putative fish inhibin-specific subunit needs to be verified, preferably with the recombinant protein.

Interestingly, a novel member of the TGF- β family, antivin, was recently identified in the zebrafish, and it is structurally related to mouse Lefty gene.⁵³ Studies on antivin and its interaction with activin and

activin receptors during embryonic development suggest that this novel protein probably functions as an antagonist of activin. Antivin may compete with activin for activin type II receptors, but it cannot activate the type I receptors.⁵³ If proved, this mechanism would be analogous to that of inhibin in mammals.

The presence of follistatin, an activin-binding protein, in fish has been demonstrated by cloning its cDNA in the zebrafish. The full-length zebrafish follistatin cDNA codes for a protein of 321 amino acids with a typical signal peptide at the N-terminal region. Fish follistatin shows 74–79% sequence identity with that of mouse and *Xenopus*.⁵⁴ The cloning of follistatin opens the door to understanding its physiological relevance in fish reproduction.

Activin Receptors and Smads in Fish

Activin signals through specific type I (IA and IB) and type II (IIA and IIB) receptors, which activate intracellular Smad proteins (Smad2, 3 and 4). Using RT-PCR and library screening, we first cloned a full-length cDNA for activin type IIB receptor in the goldfish.⁵⁵ The protein of goldfish activin type IIB receptor (ActRIIB) has a putative signal peptide of 22 amino acids. The rest of the protein contains a relatively small N-terminal extracellular domain (109 amino acids), a transmembrane domain (27 amino acids) and a long C-terminal intracellular domain (346 amino acids). The extracellular domain contains two potential N-linked glycosylation sites (NRS, NSS) and the characteristic Cys box near the transmembrane domain. Goldfish ActRIIB shares about 70 to 80% amino acid identity with that of other vertebrates, with the intracellular domain being slightly more conserved. The intracellular domain contains two consensus sequences, DFKSRN and GTRRYMAPE, which are conserved for serine kinases.⁵⁶ The binding properties of goldfish ActRIIB were analyzed by expressing the receptor in COS cells followed by specific activin binding and affinity labeling.⁵⁵ In addition to goldfish, an activin type IIB receptor⁵⁷ and a type IB-like receptor (TARAM-A)⁵⁸ have also been cloned in the zebrafish. The zebrafish ActRIIB shows wide tissue distribution including the ovary,

testis and brain.⁵⁷ The wide tissue distribution of both activin and its receptors indicates that the activin system functions at multiple levels of the fish brain-hypophysial-gonadal axis.

Recently, the major intracellular Smad proteins that are involved in activin signaling have been cloned in the zebrafish including Smad2 and Smad3 (R-Smads), Smad4 (Co-Smad)⁵⁹ and Smad7 (I-Smad).⁶⁰ Zebrafish Smad2 and Smad3 are highly conserved with more than 90% sequence identity with their mammalian counterparts, whereas Smad4 exhibits higher sequence variation with 76% identity between zebrafish and humans.⁵⁹ Zebrafish Smad7 is 81% identical with amphibian and mammalian Smad7 but much less similar to Smad6.⁶⁰ Most of the information on these zebrafish Smads concerns their functions in embryonic development. Very little is known about the role of Smads in activin signaling in fish reproduction.

Activin and Its Receptors in Fish Reproduction

Activin Regulation of Gonadotropin Expression and Secretion

In fish, two chemically distinct gonadotropins, FSH (previously named GTH-I) and LH (previously named GTH-II), have been purified and characterized in a number of species including the chum salmon (*Oncorhynchus keta*),⁶¹ coho salmon (*Oncorhynchus kisutch*)⁶² and common carp.⁶³ Similar to gonadotropins in tetrapods, fish FSH and LH share a common α subunit, but each has a unique β subunit that determines the specificity of hormone actions. The duality of fish gonadotropins has been confirmed by molecular cloning of FSH β and LH β subunits in a variety of teleosts.⁶⁴⁻⁶⁹ Although fish FSH and LH exhibit similar activities *in vitro*,^{63,70} they have distinct patterns of expression and secretion during the reproductive cycle. The level of FSH is high during gonadal growth, whereas LH production surges before maturation.^{71,72} This is supported by the functional differences reported for FSH and LH. In the rainbow trout, FSH but not LH stimulates vitellogenin uptake by the growing oocytes both *in vivo* and

in vitro.⁷³ On the other hand, LH induces final oocyte maturation in the red seabream, whereas FSH has no effect.⁷⁴ With the increasing understanding of FSH and LH biosynthesis and their functions during fish reproductive cycle, one of the questions to be answered is how these two gonadotropins are differentially regulated.

In mammals, it is well documented that activin and its related proteins, inhibin and follistatin, play critical roles in the differential regulation of FSH and LH secretion and expression.¹ In fish, although goldfish has been an outstanding model for neuroendocrine and endocrine regulation of LH secretion and the activin system is well documented in this species,^{41,55,75} there has been no information on goldfish FSH secretion and its regulation, mainly due to the lack of a specific FSH radioimmunoassay. Recently, both FSH β and LH β have been cloned in the goldfish,⁶⁴ which provides a useful tool to study their regulation at the transcriptional level.

Using an *in vitro* static culture of dispersed pituitary cells, we have recently examined the effects of recombinant goldfish activin B and human activin A on the expression of goldfish FSH β and LH β mRNA. Interestingly, both activin B and activin A have inverse effects on the mRNA levels of FSH β and LH β subunits. Activin significantly stimulates FSH β but suppresses LH β expression in a clear dose-dependent manner, and goldfish activin B seems to be more potent than human activin A.^{76,77} The stimulation of FSH β by activin is consistent with that in mammals. However, the inhibition of basal LH β expression by activin is novel and has not been reported in other vertebrates, although activin stimulates ovine FSH secretion but inhibits GnRH-induced LH release.⁷⁸ The effects of activin on the two gonadotropin β subunits can both be abolished by co-treatment with recombinant human follistatin.^{76,77} The novel inhibitory effect of activin on LH β expression seems to contradict our previous observation that mammalian activin stimulated goldfish LH secretion *in vitro*.⁵⁰ The cause for the discrepancy is unclear. There is a possibility that activin may have different effects on LH at transcriptional and secretion levels. A study was recently carried out in a hybrid tilapia (*Oreochromis niloticus* \times *O. aureus*) using human activin A; however, different from the inverse effects we have demonstrated in

the goldfish, human activin A was shown to stimulate the expression of both FSH β and LH β in tilapia.⁷⁹ The discrepancy between the two studies may be due to different species or physiological states of fish used in the experiments. Our finding that activin exerts inverse effects on the expression of FSH β and LH β subunits in the goldfish seems to support a hypothesis recently proposed by Breton and his colleagues.^{80,81} They observed in the rainbow trout that the secretion of FSH and LH changed in an opposite way depending on whether or not the ovulated eggs were kept inside the ovarian cavity or stripped. They postulated that certain factors exist in the ovary that have opposite effects on the two gonadotropins,⁸⁰ and that these factors are likely non-steroidal in nature and are present in the ovarian fluid.⁸¹ Although more work needs to be done to elucidate the identity of the hypothetical ovarian factors in the rainbow trout, activin could be an interesting candidate based on our observations in the goldfish.

In a preliminary study, we noticed a clear seasonal variation of gonadotropin responsiveness to activin, particularly by LH β .⁸² The activin inhibition of goldfish LH β mRNA was prominent during sexual regression; however, the inhibition seemed to diminish when the fish approached gonadal maturity. This observation has led us to hypothesize that there might be a decreased inhibition of LH β by activin during gonadal maturation, either due to decreased activin biosynthesis or pituitary LH responsiveness. This diminished activin inhibition may contribute to the high levels of LH before ovulation. This idea is still hypothetical and needs to be evaluated by further investigations.

One of the issues we have been addressing is the source of activin that acts in the pituitary. Our previous immunocytochemical staining⁸³ and RT-PCR assay⁴³ showed that activin subunits are expressed in the goldfish pituitary. Recently, we have further demonstrated that activin receptors (type I and type II) and follistatin are all expressed in the goldfish pituitary.⁷⁷ These lines of evidence strongly suggest that activin serves as a paracrine and autocrine factor in the goldfish pituitary. To test the functionality of the intrapituitary activin system, we have studied the effects of recombinant follistatin on the basal expression of FSH β and LH β . Follistatin not only reverses the effects of exogenous activin,

but also has inverse effects on the basal levels of FSH β and LH β , and its effects are opposite to those of activin.⁷⁷ These results indicate that the pituitary-derived activin molecules play important roles in regulating the basal expression of FSH and LH, and that the activin system in the pituitary may serve as an important regulatory point for other endocrine and neuroendocrine factors that control the biosynthesis of the two gonadotropins.

The Activin System and Its Regulation in the Ovary

Expression and localization of activin and its receptors in the ovary

Activin was initially purified from the ovary of mammals as a pituitary FSH stimulator.^{9,84} However, studies in mammals have provided clear evidence for paracrine and autocrine functions of activin in the gonads.⁸⁵ Using Northern blot analysis or RT-PCR, we have demonstrated the expression of activin β A and β B in a variety of goldfish tissues including the ovary, testis, pituitary and brain, strongly implicating activin in the regulation of reproductive functions in fish at different levels of the brain-hypophysial-gonadal axis.^{43,44} In the goldfish ovary, follicle cells exhibit strong immunoreaction for both activin β A and β B. Interestingly, previtellogenic oocytes also show a strong reaction for activin β A and β B.⁸⁶ The unique distribution of activin subunits in the ovary has also been confirmed in the ovary of medaka and thin-lipped grey mullet (*Liza ramada*).^{45,87} A recent study in the rainbow trout showed that activin β A and β B are both expressed in the thecal cells of the follicles.⁵² Collectively, these lines of evidence point to activin as a local regulator in the fish ovary. This is supported by the cloning and localization of activin receptors in the ovary.^{55,57} Our recent experiments have shown that both activin type IIA and IIB receptors are expressed in the cultured goldfish ovarian follicle cells (So and Ge, personal observation). In the zebrafish, the expression of activin type IIA receptor in the ovarian follicle cells is stimulated by human chorionic gonadotropin (hCG) and the drugs that increase intracellular cAMP levels (8-Br-cAMP, forskolin and IBMX).⁸⁸

The expression of zebrafish activin type IIB receptor has also been localized in the oocytes of different stages.⁵⁷ Despite these observations, the exact roles of activin in the fish ovary remain largely unknown.

Effects of activin on oocyte maturation

Using zebrafish as a model, we have investigated the involvement of activin in final oocyte maturation. Zebrafish provides an outstanding model in that large number of ovarian follicles at different stages can be easily obtained every day. Using an *in vitro* follicle incubation, we have tested the effect of recombinant goldfish activin B on the maturation rate of full-grown follicles. Activin B significantly promotes oocyte maturation in a clear time- and dose-dependent manner. The effect of activin can be blocked by co-incubation with recombinant human follistatin.⁸⁹ Interestingly, follistatin also suppresses the hCG-induced oocyte maturation, implying that activin may mediate gonadotropin actions in the zebrafish ovary. The induction of zebrafish oocyte maturation by activin seems to involve changes at the transcriptional level because the effect can be blocked by actinomycin D.⁸⁹ A similar effect of activin on zebrafish oocyte maturation has also been observed with recombinant human activin A.⁵¹ This is different from a previous study in the red seabream that reported no effect of bovine activin A on the oocyte maturation.⁹⁰ The reason for the discrepancy between the two studies is unclear.

According to the “two-stage” model proposed for gonadotropin-induced fish oocyte maturation,⁹¹ LH promotes oocyte maturational competence (OMC) (responsiveness to maturation-inducing hormone, MIH) in the first stage and stimulates MIH production in the second stage. Because LH does not act directly on the oocytes, its effect on OMC is likely mediated by local ovarian factors. To investigate if activin is involved in the process, we have examined the effects of recombinant goldfish activin B and human activin A on the development of OMC in the mid-vitellogenic zebrafish follicles. Both forms of activin exhibit powerful effects on OMC in these vitellogenic follicles, and their effects are as potent as that of hCG; again, follistatin inhibits the effects of

activin and hCG, suggesting a role for activin in gonadotropin actions.⁹² One interesting question to address is whether activin enhances OMC by direct action on the oocyte or indirect action through the mediation of the follicle cells. A direct action by activin on the oocyte is strongly supported by our recent evidence that activin β A and β B are predominantly expressed in the follicle cells of full-grown follicles, whereas the entire activin signaling system, including activin type I and II receptors, Smad2, 3, 4 and 7, is abundantly expressed in the oocyte.⁹³ Interestingly, the oocyte also expresses high level of follistatin as compared with that in the follicle cells, suggesting a potential negative feedback mechanism for the oocyte to signal the follicle cells.⁹³ The possibility that activin may modulate oocyte maturation through follicle cells cannot be ruled out because activin receptors and Smad proteins are also expressed in the cultured follicle cells.^{88,89,93} Studies on the involvement of activin in the secretion of MIH (17β , 20β -dihydroxy-4-pregnen-3-one, DHP) from the granulosa cells and its precursor 17α -hydroxyprogesterone from the theca cells would shed light on this issue. However, a recent study from our laboratory showed no effect of activin on the expression of a carbonyl reductase-like 20β -hydroxysteroid dehydrogenase (20β -HSD) in the zebrafish ovary, the enzyme that converts 17α -hydroxyprogesterone to DHP.⁹⁴ In agreement, recombinant goldfish activin B has little or slightly inhibitory effect on the conversion of 17α -hydroxyprogesterone to DHP by the full-grown immature goldfish follicles.⁹⁵ More studies are needed to elucidate the regulatory role of the local activin system in oocyte maturation and other ovarian functions.

Interaction of activin system and other local factors in the ovary

Fish ovary is an extremely dynamic organ that undergoes continuous cyclic morphological and physiological changes. Similar to that of mammals, the regulation of fish ovarian functions involves not only pituitary gonadotropins (FSH and LH) and other endocrine hormones, but also a complex local network that mainly includes growth factors and cytokines. The roles of a number of growth factors in fish ovary have been investigated, including

insulin-like growth factor-I (IGF-I)^{90,96–100} and epidermal growth factor (EGF).^{101–103} We have recently demonstrated that EGF and transforming growth factor- α (TGF- α) both have a potent stimulatory effect on the final maturation of zebrafish oocytes, and their effects are dependent on mRNA transcription and protein translation. Interestingly, the effects of both factors can be completely abolished by follistatin, suggesting a role for activin in the regulatory pathway of EGF in the ovary. This hypothesis is strongly supported by the evidence that EGF and TGF- α significantly increase the expression of activin β A and β B subunits as well as activin type IIA receptor but suppress that of follistatin in the follicle cells.^{104,105} In addition to EGF and TGF- α , other local factors may also function by impacting the ovarian activin system. Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide initially identified in the hypothalamus for its induction of cAMP generation in the pituitary, is widely expressed in a variety of tissues.¹⁰⁶ In the goldfish and zebrafish, the expression of PACAP has been demonstrated in the ovary;^{106–108} however, its roles in fish ovary remain to be evaluated. Our recent data showed that synthetic zebrafish PACAP promotes oocyte maturation, but it also significantly stimulates follistatin expression in the cultured zebrafish follicle cells.¹⁰⁸ This result is consistent with our observation that follistatin expression in the zebrafish follicle cells is enhanced by increasing intracellular cAMP levels.¹⁰⁹

Regulation of activin, follistatin and activin receptors in the ovary

In the mammalian ovary, the expression of the activin system including its receptors and binding protein follistatin, is controlled by a variety of endocrine and paracrine factors.^{85,110} In the ovary of fish, the activin system seems to play a critical role in the regulatory pathways of a number of factors including pituitary gonadotropin(s) and local EGF, TGF- α and PACAP. To understand how activin is related to endocrine hormones and local factors in controlling ovarian functions, we have investigated the regulation of activin subunits, follistatin and activin receptor (type IIA) in the zebrafish follicle cells by gonadotropins and other ovarian factors. Gonadotropins (hCG and goldfish pituitary extract)

significantly increase the mRNA levels of activin β A, type IIA receptor and follistatin in a time- and dose-dependent manner.^{46,88,109} The stimulation of activin β A and follistatin by hCG is mediated by the cAMP-PKA pathway because all the drugs (db-cAMP, 8-Br-cAMP, forskolin and IBMX) that increase the intracellular cAMP level mimic the effects of gonadotropins, and the effects of hCG and forskolin can be blocked by H89, a PKA inhibitor.^{46,88,109} In contrast to activin β A, the expression of activin β B is significantly suppressed by both hCG and goldfish pituitary extract. Although the suppression can be mimicked by db-cAMP, forskolin and IBMX, it cannot be reversed by H89.⁴⁶ These findings suggest that one or both pituitary gonadotropins differentially regulate the two activin subunits β A and β B in the zebrafish ovary through cAMP-PKA-dependent and cAMP-dependent but PKA-independent pathways, respectively. The identity of the PKA-independent pathway that leads to the inhibition of activin β B remains unknown and is currently under investigation in our laboratory. Studies on the expression profiles of activin β A and β B in the ovarian cycle and their correlation with that of the pituitary gonadotropin(s) would provide important clues to the physiological relevance of these *in vitro* findings.

The Activin System and Its Regulation in the Testis

The importance of the activin system in fish gonads has also been evidenced in the testis. In the Japanese eel, an activin β B subunit cDNA was cloned by subtractive hybridization. A single injection of hCG significantly stimulated activin β B expression in the eel testis, and the expression has been localized to the Sertoli cells.¹¹¹ As a downstream mediator of gonadotropin(s), activin B seems to play a critical role in promoting spermatogenesis both *in vivo* and *in vitro*.^{112,113} However, a recent study in the rainbow trout demonstrated no effect of activin on the proliferation of spermatogonia *in vitro*.¹¹⁴ More studies using different model species may shed light on this issue. The regulatory role of activin in the eel testis seems to be analogous to that in the ovary, suggesting similar endocrine and paracrine/autocrine mechanisms for the regulation of gametogenesis in both male and female teleosts.

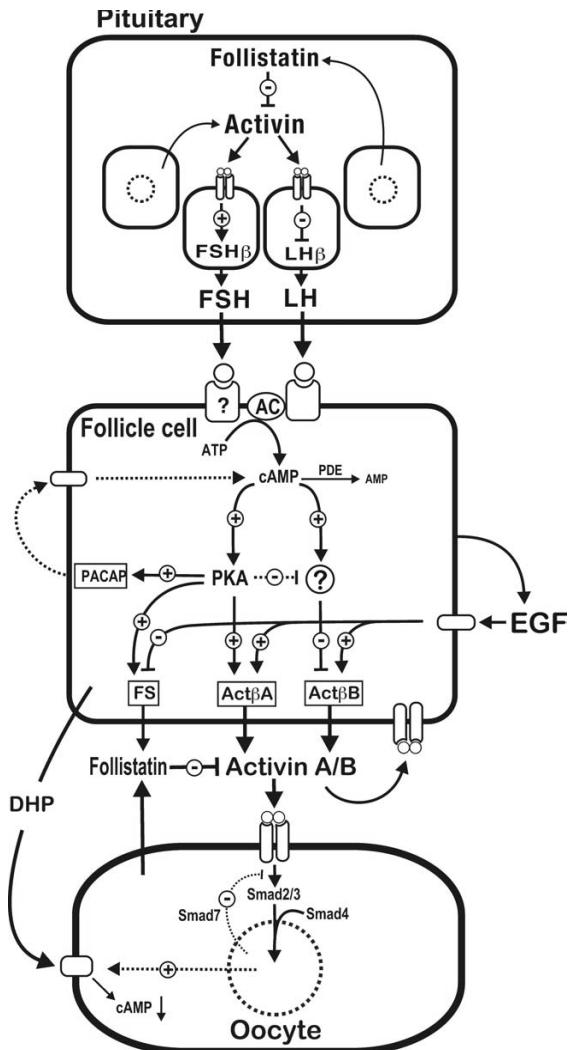


Fig. 1 Schematic representation of activin, its receptors and follistatin in fish pituitary and ovary. FSH, follicle-stimulating hormone; LH, luteinizing hormone; EGF, epidermal growth factor; PACAP, pituitary adenylate cyclase-activating polypeptide; FS, follistatin; Act β A, activin β A subunit; Act β B, activin β B subunit; DHP, 17 α , 20 β -dihydroxy-4-pregnen-3-one; AC, adenylate cyclase; cAMP, cyclic adenosine 3', 5'-monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; +, stimulation; -, inhibition. The pathways in dotted lines need to be further confirmed.

Concluding Remarks

Activin and its associated proteins exhibit an extremely wide range of biological activities, and activin involvement in reproductive regulation appears to be highly conserved across vertebrates. Compared with that in mammals and *Xenopus*, the information on the activin system and its functions in fish is still scanty. Most studies in the past years in fish have been focused on identification of activin family members using molecular biology and immunocytochemical staining approaches. With most components of activin and its signaling system identified in major fish models such as goldfish, zebrafish and rainbow trout, the future work on activin and its related proteins should be focused more on the functional aspects of the system in the regulation of fish reproduction and development. All evidence from our research and that of others clearly indicates that activin and its associated proteins play diverse roles at multiple levels of the fish reproductive system including the pituitary and gonads (Fig. 1). Considering the advantages offered by fish models, it is anticipated that studies in fish on the activin system will not only enhance our understanding of fish reproduction and its regulation, but also provide valuable information on the structural and functional evolution of this critically important family of proteins in vertebrates.

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References

1. Ying S-Y (1988). Inhibins, activins, and follistatins: gonadal proteins modulating the secretin of follicle-stimulating hormone. *Endocr. Rev.* **9**: 267–293.

2. Meunier H, Rivier C, Evans RM and Vale W (1988). Gonadal and extragonadal expression of inhibin α , β A, and β B subunits in various tissues predicts diverse functions. *Proc. Natl. Acad. Sci. USA* **85**: 247–251.
3. Phillips DJ and de Kretser DM (1998). Follistatin: a multifunctional regulatory protein. *Front. Neuroendocrinol.* **19**: 287–322.
4. Ling N, Ying S-Y, Ueno N, Esch F, Denoroy L and Guillemin R (1985). Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* **82**: 7217–7221.
5. Miyamoto K, Hasegawa Y, Fukuda M, Momura M, Igarashi M, Kangawa K and Matsuo H (1985). Isolation of porcine follicular fluid inhibin of 32K daltons. *Biochem. Biophys. Res. Commun.* **129**: 396–403.
6. Robertson DM, Foulds LM, Leversha L, Morgan FJ, Hearn MTW, Burger HG, Wettenhall REH and de Kretser DM (1985). Isolation of inhibin from bovine follicular fluid. *Biochem. Biophys. Res. Commun.* **126**: 220–226.
7. Rivier J, Spiess J, McClintock R, Vaughan J and Vale W (1985). Purification and partial characterization of inhibin from porcine follicular fluid. *Biochem. Biophys. Res. Commun.* **133**: 120–127.
8. Ueno N, Ling N, Ying SY, Esch F, Shimasaki S and Guillemin R (1987). Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proc. Natl. Acad. Sci. USA* **84**: 8282–8286.
9. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D and Spiess J (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**: 776–779.
10. Esch FS, Shimasaki S, Mercado M, Cooksey K, Ling N, Ying S, Ueno N and Guillemin R (1987). Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. *Mol. Endocrinol.* **1**: 849–855.
11. Robertson DM, Klein R, de Vos FL and McLachlan RI (1987). The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochem. Biophys. Res. Commun.* **149**: 744–749.
12. Calogero AE, Burrello N, Ossino AM, Polosa P and D'Agata R (1998). Activin-A stimulates hypothalamic gonadotropin-releasing hormone release by the explanted male rat hypothalamus: interaction with inhibin and androgens. *J. Endocrinol.* **156**: 269–274.

13. MacConell LA, Lawson MA, Mellon PL and Roberts VJ (1999). Activin A regulation of gonadotropin-releasing hormone synthesis and release *in vitro*. *Neuroendocrinology* **70**: 246–254.
14. Bilezikjian LM, Corrigan AZ and Vale W (1990). Activin-A modulates growth hormone secretion from cultures of rat anterior pituitary cells. *Endocrinology* **126**: 2369–2376.
15. Struthers RS, Gaddy-Kurten D and Vale WW (1992). Activin inhibits binding of transcription factor Pit-1 to the growth hormone promoter. *Proc. Natl. Acad. Sci. USA* **89**: 11451–11455.
16. Hsueh AJ, Dahl KD, Vaughan J, Tucker E, Rivier J, Bardin CW and Vale W (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. *Proc. Natl. Acad. Sci. USA* **84**: 5082–5086.
17. Sadatsuki M, Tsutsumi O, Yamada R, Muramatsu M and Taketani Y (1993). Local regulatory effects of activin A and follistatin on meiotic maturation of rat oocytes. *Biochem. Biophys. Res. Commun.* **196**: 388–395.
18. Rombauts L, Vanmontfort D, Decuyper E and Verhoeven G (1996). Inhibin and activin have antagonistic paracrine effects on gonadal steroidogenesis during the development of the chicken embryo. *Biol. Reprod.* **54**: 1229–1237.
19. Mather JP, Attie KM, Woodruff TK, Rice GC and Phillips DM (1990). Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. *Endocrinology* **127**: 3206–3214.
20. Sawchenko PE, Plotsky PM, Pfeiffer SW, Cunningham Jr. ET, Vaughan J, Rivier J and Vale W (1988). Inhibin β in central neural pathways involved in the control of oxytocin secretion. *Nature* **334**: 615–617.
21. Smith JC, Price BMJ, Van Nimmen K and Huylebroeck D (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature* **345**: 729–731.
22. van den Eijnden-Van Raaij AJM, van Zoelent EJ, van Nimmen K, Koster CH, Snoek GT, Durston AJ and Huylebroeck D (1990). Activin-like factor from a *Xenopus laevis* cell line responsible for mesoderm induction. *Nature* **345**: 732–734.
23. Mathews LS (1994). Activin receptors and cellular signaling by the receptor serine kinase family. *Endocr. Rev.* **15**: 310–325.
24. Mathews LS and Vale WW (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* **65**: 973–982.

25. Attisano L, Wrana JL, Cheifetz S and Massagué J (1992). Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell* **68**: 97–108.
26. ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K and Heldin CH (1994). Characterization of type I receptors for transforming growth factor- β and activin. *Science* **264**: 101–104.
27. Massagué J (1992). Receptors for the TGF- β family. *Cell* **69**: 1067–1070.
28. Tsuchida K, Mathews LS and Vale WW (1993). Cloning and characterization of a transmembrane serine kinase that acts as an activin type I receptor. *Proc. Natl. Acad. Sci. USA* **90**: 11242–11246.
29. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH and Miyazono K (1994). Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J. Biol. Chem.* **269**: 16985–16988.
30. Pangas SA and Woodruff TK (2000). Activin signal transduction pathways. *Trends Endocrinol. Metab.* **11**: 309–314.
31. Wrana J (2000). Regulation of Smad activity. *Cell* **100**: 189–192.
32. Massagué J and Chen Y-G (2000). Controlling TGF- β signaling. *Genes Dev.* **14**: 627–644.
33. Donaldson CJ, Vaughan JM, Corrigan AZ, Fischer WH and Vale WW (1999). Activin and inhibin binding to the soluble extracellular domain of activin receptor II. *Endocrinology* **140**: 1760–1766.
34. Lebrun JJ and Vale WW (1997). Activin and inhibin have antagonistic effects on ligand-dependent heteromerization of the type I and type II activin receptors and human erythroid differentiation. *Mol. Cell. Biol.* **17**: 1682–1691.
35. Martens JW, de Winter JP, Timmerman MA, McLuskey A, van Schaik RH, Themmen AP and de Jong FH (1997). Inhibin interferes with activin signaling at the level of the activin receptor complex in Chinese hamster ovary cells. *Endocrinology* **138**: 2928–2936.
36. Gray PC, Bilezikian LM and Vale W (2001). Antagonism of activin by inhibin and inhibin receptors: a functional role for betaglycan-glycan. *Mol. Cell. Endocrinol.* **180**: 47–53.
37. Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilezikian LM and Vale W (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature* **404**: 411–414.

38. Chapman SC and Woodruff TK (2001). Modulation of activin signal transduction by inhibin B and inhibin-binding protein (INhBP). *Mol. Endocrinol.* **15**: 668–679.
39. Bernard DJ, Chapman SC and Woodruff TK (2001). An emerging role for co-receptors in inhibin signal transduction. *Mol. Cell. Endocrinol.* **180**: 55–62.
40. Nakamura T, Takio K, Eto Y, Shibai H, Titani K and Sugino H (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**: 836–838.
41. Ge W (2000). Roles of the activin regulatory system in fish reproduction. *Can. J. Physiol. Pharmacol.* **78**: 1077–1185.
42. Ge W, Gallin WJ, Strobeck C and Peter RE (1993). Cloning and sequencing of goldfish activin subunit genes: strong structural conservation during vertebrate evolution. *Biochem. Biophys. Res. Commun.* **193**: 711–717.
43. Ge W, Miura T, Kobayashi H, Peter RE and Nagahama Y (1997). Cloning of cDNA for goldfish activin β B subunit, and the expression of its mRNA in gonadal and non-gonadal tissues. *J. Mol. Endocrinol.* **19**: 37–45.
44. Yam KM, Yu KL and Ge W (1999). Cloning and characterization of goldfish activin β A subunit. *Mol. Cell. Endocrinol.* **154**: 45–54.
45. Wittbrodt J and Rosa FM (1994). Disruption of mesoderm and axis formation in fish by ectopic expression of activin variants: the role of maternal activin. *Genes Dev.* **8**: 1448–1462.
46. Wang Y and Ge W (2003). Involvement of cyclic adenosine 3', 5'-monophosphate in the differential regulation of activin β A and β B expression by gonadotropin in the zebrafish ovarian follicle cells. *Endocrinology* **144**: 491–499.
47. Tada T, Hirono I and Aoki T (1998). Structure and expression of activin genes in rainbow trout. *Mol. Mar. Biol. Biotechnol.* **7**: 72–77.
48. Tada T, Hirono I, Aoki T and Takashima F (1998). Cloning and sequencing of carp and medaka activin subunit genes. *Fish. Sci.* **64**: 680–685.
49. Eto Y, Tsuji T, Takezawa M, Takano S, Yokogawa Y and Shibai H (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. *Biochem. Biophys. Res. Commun.* **142**: 1095–1103.
50. Ge W, Chang JP, Peter RE, Vaughan J, Rivier J and Vale W (1992). Effects of porcine follicular fluid, inhibin-A, and activin-A on goldfish gonadotropin release *in vitro*. *Endocrinology* **131**: 1922–1929.

51. Wu T, Patel H, Mukai S, Melino C, Garg R, Ni X, Chang J and Peng C (2000). Activin, inhibin, and follistatin in zebrafish ovary: expression and role in oocyte maturation. *Biol. Reprod.* **62**: 1585–1592.
52. Tada T, Endo M, Hirono I, Takashima F and Aoki T (2002). Differential expression and cellular localization of activin and inhibin mRNA in the rainbow trout ovary and testis. *Gen. Comp. Endocrinol.* **125**: 142–149.
53. Thisse C and Thisse B (1999). Antivin, a novel and divergent member of the TGF β superfamily, negatively regulates mesoderm induction. *Development* **126**: 229–240.
54. Bauer H, Meier A, Hild M, Economides SSA, Hazelett D, Harland RM and Hammerschmidt M (1998). Follistatin and noggin are excluded from the zebrafish organizer. *Dev. Biol.* **204**: 488–507.
55. Ge W, Tanaka M, Yoshikuni M, Eto Y and Nagahama Y (1997). Cloning and characterization of goldfish activin type IIB receptor. *J. Mol. Endocrinol.* **19**: 47–57.
56. Hanks SK, Quinn AM and Hunter T (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**: 42–52.
57. Garg RR, Bally-Cuif L, Lee SE, Gong Z, Ni X, Hew CL and Peng C (1999). Cloning of zebrafish activin type IIB receptor (ActRIIB) cDNA and mRNA expression of ActRIIB in embryos and adult tissues. *Mol. Cell. Endocrinol.* **153**: 169–181.
58. Yelick PC, Abduljabbar TS and Stashenko P (1998). zALK-8, a novel type I serine/threonine kinase receptor, is expressed throughout early zebrafish development. *Dev. Dyn.* **211**: 352–361.
59. Dick A, Mayr T, Bauer H, Meider A and Hammerschmidt M (2000). Cloning and characterization of zebrafish smad2, smad3 and smad4. *Gene* **246**: 69–80.
60. Pogoda HM and Meyer D (2002). Zebrafish Smad7 is regulated by Smad3 and BMP signals. *Dev. Dyn.* **224**: 334–349.
61. Suzuki K, Kawauchi K and Nagahama Y (1988). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. *Gen. Comp. Endocrinol.* **71**: 292–301.
62. Swanson P, Suzuki K, Kawauchi H and Dickhoff WW (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol. Reprod.* **44**: 29–38.

63. Van Der Kraak G, Suzuki K, Peter RE, Itoh H and Kawauchi H (1992). Properties of common carp gonadotropin I and gonadotropin II. *Gen. Comp. Endocrinol.* **85**: 217–229.
64. Yoshiura Y, Kobayashi M, Kato Y and Aida K (1997). Molecular cloning of the cDNAs encoding two gonadotropin β subunits (GTH-I β and -II β) from the goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* **105**: 379–389.
65. Lin YWP, Rupnow BA, Price DA, Greenberg RM and Wallace RA (1992). *Fundulus heteroclitus* gonadotropins. 3. Cloning and sequencing of gonadotropic hormone (GTH) I and II β -subunits using the polymerase chain reaction. *Mol. Cell. Endocrinol.* **85**: 127–139.
66. Elizur A, Zmora N, Rosenfeld H, Meiri I, Hassin S, Gordin H and Zohar Y (1996). Gonadotropins β -GtHI and β -GtHII from the gilthead seabream, *Sparus aurata*. *Gen. Comp. Endocrinol.* **102**: 39–46.
67. Hassin S, Elizur A and Zohar Y (1995). Molecular cloning and sequence analysis of striped bass (*Morone saxatilis*) gonadotrophin-I and -II subunits. *J. Mol. Endocrinol.* **15**: 23–35.
68. Kato Y, Gen K, Maruyama O, Tomizawa K and Kato T (1993). Molecular cloning of cDNAs encoding two gonadotrophin beta subunits (GTH-I β and -II β) from the masu salmon, *Oncorhynchus masou*: rapid divergence of the GTH-I β gene. *J. Mol. Endocrinol.* **11**: 275–282.
69. Sekine S, Saito A, Itoh H, Kawauchi H and Itoh S (1989). Molecular cloning and sequence analysis of chum salmon gonadotropin cDNAs. *Proc. Natl. Acad. Sci. USA* **86**: 8645–8649.
70. Suzuki K, Nagahama Y and Kawauchi H (1988). Steroidogenic activities of two distinct salmon gonadotropins. *Gen. Comp. Endocrinol.* **71**: 452–458.
71. Weil C, Bougoussa-Houadec M, Gallais C, Itoh S, Sekine S and Valotaire Y (1995). Preliminary evidence suggesting variations of GtH 1 and GtH 2 mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **100**: 327–333.
72. Swanson P (1991). Salmon gonadotropins: reconciling old and new ideas. In: Scott AP, Sumpter JP, Kime DE and Rolfe MS (eds.), *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*, FishSymp91, Sheffield, pp. 2–7.
73. Tyler CR, Sumpter JP, Kawauchi H and Swanson P (1991). Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **84**: 291–299.

74. Kagawa H, Tanaka H, Okuzawa K and Kobayashi M (1998). GTH II but not GTH I induces final maturation and the development of maturational competence of oocytes of red seabream *in vitro*. *Gen. Comp. Endocrinol.* **112**: 80–88.
75. Ge W, Peter RE and Nagahama Y (1997). Activin and its receptors in the goldfish. *Fish Physiol. Biochem.* **17**: 143–153.
76. Yam KM, Yoshiura Y, Kobayashi M and Ge W (1999). Recombinant goldfish activin B stimulates gonadotropin-I β but inhibits gonadotropin-II β expression in the goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* **116**: 81–89.
77. Yuen CW and Ge W (2004). Follistatin suppresses FSH β but increases LH β expression in the goldfish—evidence for an activin-mediated autocrine/paracrine system in fish pituitary. *Gen. Comp. Endocrinol.* **135**: 108–115.
78. Muttukrishna S and Knight PG (1991). Inverse effects of activin and inhibin on the synthesis and secretion of FSH and LH by ovine pituitary cells *in vitro*. *J. Mol. Endocrinol.* **6**: 171–178.
79. Yaron Z, Gur G, Melamed P, Rosenfeld H, Levavi-Sivan B and Elizur A (2001). Regulation of gonadotropin subunit genes in tilapia. *Comp. Biochem. Physiol. B* **129**: 489–502.
80. Breton B, Govoroun M and Mikolajczyk T (1998). GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. *Gen. Comp. Endocrinol.* **111**: 38–50.
81. Chyb J, Mikolajczyk T and Breton B (1999). Post-ovulatory secretion of pituitary gonadotropins Gth I and Gth II in the rainbow trout (*Oncorhynchus mykiss*): regulation by steroids and possible role of non-steroidal gonadal factors. *J. Endocrinol.* **163**: 87–97.
82. Yuen CW and Ge W (2000). Seasonal variation of activin-regulated goldfish pituitary GTH-I and GTH-II expression and evidence for the involvement of gonadal steroids. In: *The Fourth International Symposium on Fish Endocrinology*, Seattle, Washington, USA, p.86.
83. Ge W and Peter RE (1994). Activin-like peptides in somatotrophs and activin stimulation of growth hormone release in goldfish. *Gen. Comp. Endocrinol.* **95**: 213–221.
84. Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M and Guillemin R (1986). Pituitary FSH is released by a heterodimer of the β -subunits from the two forms of inhibin. *Nature* **321**: 779–782.

85. Mather JP, Moore A and Li RH (1997). Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. *Proc. Soc. Exp. Biol. Med.* **215**: 209–222.
86. Ge W, Cook H, Peter RE, Vaughan J and Vale W (1993). Immunocytochemical evidence for the presence of inhibin and activin-like proteins and their localization in goldfish gonads. *Gen. Comp. Endocrinol.* **89**: 333–340.
87. Mousa MA and Mousa SA (2003). Immunohistochemical localization of inhibin and activin-like proteins in the brain, pituitary gland, and the ovary of thin-lipped grey mullet, *Liza ramada* (Risso). *Gen. Comp. Endocrinol.* **132**: 434–443.
88. Pang Y and Ge W (2002). Gonadotropin regulation of activin β A and activin type IIA receptor expression in the ovarian follicle cells of the zebrafish, *Danio rerio*. *Mol. Cell. Endocrinol.* **188**: 195–205.
89. Pang Y and Ge W (1999). Activin stimulation of zebrafish oocyte maturation *in vitro* and its potential role in mediating gonadotropin-induced oocyte maturation. *Biol. Reprod.* **61**: 987–992.
90. Kagawa H, Kobayashi M, Hasegawa Y and Aida K (1994). Insulin and insulin-like growth factors I and II induce final maturation of oocytes of red seabream, *Pagrus major*, *in vitro*. *Gen. Comp. Endocrinol.* **95**: 293–300.
91. Patiño R, Yoshizaki G, Thomas P and Kagawa H (2001). Gonadotropic control of ovarian follicle maturation: the two-stage concept and its mechanisms. *Comp. Biochem. Physiol. B* **129**: 427–439.
92. Pang Y and Ge W (2002). Gonadotropin and activin enhance maturational competence of oocytes in the zebrafish (*Danio rerio*). *Biol. Reprod.* **66**: 259–265.
93. Wang Y and Ge W (2003). Spatial expression patterns of activin and its signalling system in the zebrafish ovarian follicle: evidence for paracrine action of activin on the oocytes. *Biol. Reprod.* **69**: 1998–2006.
94. Wang Y and Ge W (2002). Cloning of zebrafish ovarian carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase and characterization of its spatial and temporal expression. *Gen. Comp. Endocrinol.* **127**: 209–216.
95. Calp MK, Matsumoto JA and Van Der Kraak G (2003). Activin and transforming growth factor- β as local regulators of ovarian steroidogenesis in the goldfish. *Gen. Comp. Endocrinol.* **132**: 142–150.
96. Thomas P, Pinter J and Das S (2001). Upregulation of the maturation-inducing steroid membrane receptor in spotted seatrout ovaries by

- gonadotropin during oocyte maturation and its physiological significance. *Biol. Reprod.* **64**: 21–29.
97. Weber GM and Sullivan CV (2000). Effects of insulin-like growth factor-I on *in vitro* final oocyte maturation and ovarian steroidogenesis in striped bass, *Morone saxatilis*. *Biol. Reprod.* **63**: 1049–1057.
98. Patiño R and Kagawa H (1999). Regulation of gap junctions and oocyte maturational competence by gonadotropin and insulin-like growth factor-I in ovarian follicles of red seabream. *Gen. Comp. Endocrinol.* **115**: 454–462.
99. Kagawa H, Moriyama S and Kawauchi H (1995). Immunocytochemical localization of IGF-I in the ovary of the red seabream, *Pagrus major*. *Gen. Comp. Endocrinol.* **99**: 307–315.
100. Maestro MA, Planas JV, Moriyama S, Gutierrez J, Planas J and Swanson P (1997). Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. *Gen. Comp. Endocrinol.* **106**: 189–201.
101. Wood AW and Van Der Kraak G (2002). Inhibition of apoptosis in vitellogenic ovarian follicles of rainbow trout (*Oncorhynchus mykiss*) by salmon gonadotropin, epidermal growth factor, and 17 β -estradiol. *Mol. Reprod. Dev.* **61**: 511–518.
102. MacDougall TM and Van Der Kraak G (1998). Peptide growth factors modulate prostaglandin E and F production by goldfish ovarian follicles. *Gen. Comp. Endocrinol.* **110**: 46–57.
103. Pati D, Balshaw K, Grinwich DL, Hollenberg MD and Habibi HR (1996). Epidermal growth factor receptor binding and biological activity in the ovary of goldfish, *Carassius auratus*. *Am. J. Physiol.* **270**: R1065–1072.
104. Pang Y and Ge W (2002). Epidermal growth factor and TGF α promote zebrafish oocyte maturation *in vitro*: potential role of the ovarian activin regulatory system. *Endocrinology* **143**: 47–54.
105. Wang Y and Ge W (2003). Cloning of epidermal growth factor (EGF) and EGF receptor (EGFR) from the zebrafish ovary and its potential roles in the regulation of ovarian activin/follistatin system. *Biol. Reprod.*, in press.
106. Wong AOL, Li WS, Lee EKY, Leung MY, Tse LY, Chow BKC, Lin HR and Chang JP (2000). Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochem. Cell Biol.* **78**: 329–343.
107. Fradinger EA and Sherwood NM (2000). Characterization of the gene encoding both growth hormone-releasing hormone (GRF) and pituitary

- adenylylate cyclase-activating polypeptide (PACAP) in the zebrafish. *Mol. Cell. Endocrinol.* **165**: 211–219.
108. Wang Y, Wong AO-L and Ge W (2003). Cloning, regulation of mRNA expression and function of a new isoform of pituitary adenylylate cyclase-activating polypeptide (PACAP) in the zebrafish ovary. *Endocrinology* **144**: 4799–4810.
109. Wang Y and Ge W (2003). Gonadotropin regulation of follistatin expression in the cultured ovarian follicle cells of zebrafish, *Danio rerio*. *Gen. Comp. Endocrinol.* **134**: 308–315.
110. Knight PG and Glister C (2001). Potential local regulatory functions of inhibins, activins and follistatin in the ovary. *Reproduction* **121**: 503–512.
111. Nagahama Y (1994) Endocrine regulation of gametogenesis in fish. *Int. J. Dev. Biol.* **38**: 217–229.
112. Miura C, Miura T, Kudo N, Yamashita M and Yamauchi K (1999). cDNA cloning of a stage-specific gene expressed during HCG-induced spermatogenesis in the Japanese eel. *Dev. Growth Differ.* **41**: 463–471.
113. Miura T, Miura C, Yamauchi K and Nagahama Y (1995). Human recombinant activin induces proliferation of spermatogonia *in vitro* in the Japanese eel *Anguilla japonica*. *Fish. Sci.* **61**: 434–437.
114. Loir M (1999). Spermatogonia of rainbow trout: II. *In vitro* study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Mol. Reprod. Dev.* **53**: 434–442.

Chapter 6

Gonadal Steroidogenesis in Teleost Fish

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Abstract

Sex steroids are involved in all aspects of the regulation of reproductive processes in vertebrates. Teleost fish produce several types of bioactive gonadal steroids, including estrogens, progestogens, androgens and numerous other steroids. Bioactive steroids are produced in specialized cells within the ovarian follicle (theca, granulosa) and the testis (Leydig cells). Depending on species, sex, reproductive stage and cell identity,

these cells express an array of genes encoding steroidogenic enzymes that modify cholesterol and its derivatives. Steroidogenic cells may also express the gene encoding steroidogenic acute regulatory protein, which functions to transport cholesterol in mitochondria, the true rate-limiting step in steroidogenesis. In this review, we focus on the synthesis, function and excretion of gonadal steroids, emphasizing the diverse nature of steroid products in teleosts. We then examine how seasonal patterns of sex steroid production may be regulated at the molecular level. We review the numerous recent publications on the molecular characterization of the genes encoding steroidogenic proteins. Also, we describe recent studies aimed at understanding how differential expression of specific steroidogenic protein genes results in the production of appropriate sex steroid hormones at appropriate reproductive stages.

Introduction

Steroid hormones are small cholesterol-derived molecules universally found in chordates and arthropods. In both phyla, steroids are an absolute requirement for development, maintenance of homeostasis and/or reproduction. In adult vertebrates, three types of reproduction-related steroids (estrogens, androgens and progestogens) are produced at appropriate times in specialized steroid-producing cells in the gonads. These cells express an array of steroidogenic enzyme genes whose products modify cholesterol and its derivatives. Steroids direct the development of germ cells and accessory glands and organs, as well as the modification of behavior, to ensure that sexual reproduction can take place. Although many steroids are chemically identical in all major vertebrate classes, the role of these steroids may differ. Moreover, unique steroid hormones have evolved in some vertebrate classes, especially amongst fishes, to fulfill particular functions.

Kime¹ in his review of the reproductive steroids of fish, noted that in terms of sex steroids, teleosts are not ‘merely aquatic mammals.’ Not surprisingly, given the diverse nature and evolutionary history of the teleosts, there is enormous variation in the steroid biosynthetic pathways present in teleosts and a number of novel steroids have been identified over the past two decades since the major reviews of Idler²

and Fostier *et al.*³ Whereas many of these may simply reflect variation in catabolic pathways, in other cases potential bioactivity has not been assessed, and little is known about how their synthesis is regulated.

Our intention in this chapter is not to provide a comprehensive review on sex steroid biochemistry of teleosts but to give an overview of the numerous studies that have been conducted on steroid physiology at the tissue and organismal level. We will then focus in more depth on recent advances in understanding of the molecular and cellular processes underlying the seasonal profiles of sex steroids in sexually maturing teleosts, and the factors that potentially regulate the changes in the types of steroid produced. The major reviews of Idler and Safe,² Fostier *et al.*,³ Kime¹ and Devlin and Nagahama⁴ cover various aspects of sex steroid biosynthesis and action in teleosts.

Gonadal Steroids in Teleost Fishes: Synthesis, Function and Excretion

Steroid Synthesis

The production of the different classes of gonadal steroids hinges on the delivery of substrate, cholesterol, and its subsequent conversion, primarily by three enzymes belonging to the cytochrome P450 superfamily. The first of these P450 steroidogenic enzymes, cytochrome P450 side-chain cleavage (P450scc), is located at the inner mitochondrial membrane. Here, it hydroxylates carbons 20 and 22 (C_{20} , C_{22}) of cholesterol and then removes isocaproaldehyde,⁵ a six-carbon residue side-chain (C_{22-27}), to give rise to pregnenolone (Fig. 1). The production of pregnenolone by P450scc is rate-limited by cholesterol availability.⁶ Thus, whether produced *de novo* or obtained from the blood, cholesterol cannot reach P450scc from the cytoplasm, but instead a sterol transport protein is needed to deliver the substrate across the mitochondrial membrane. In mammals, this transport protein was first isolated in 1994 by Clark and others⁷ and termed steroidogenic acute regulatory protein, or StAR. Its involvement in cholesterol transport is evident from a number of experimental and clinical examples,^{6,8} but the precise mechanism of transport remains elusive.⁹ Possibilities include

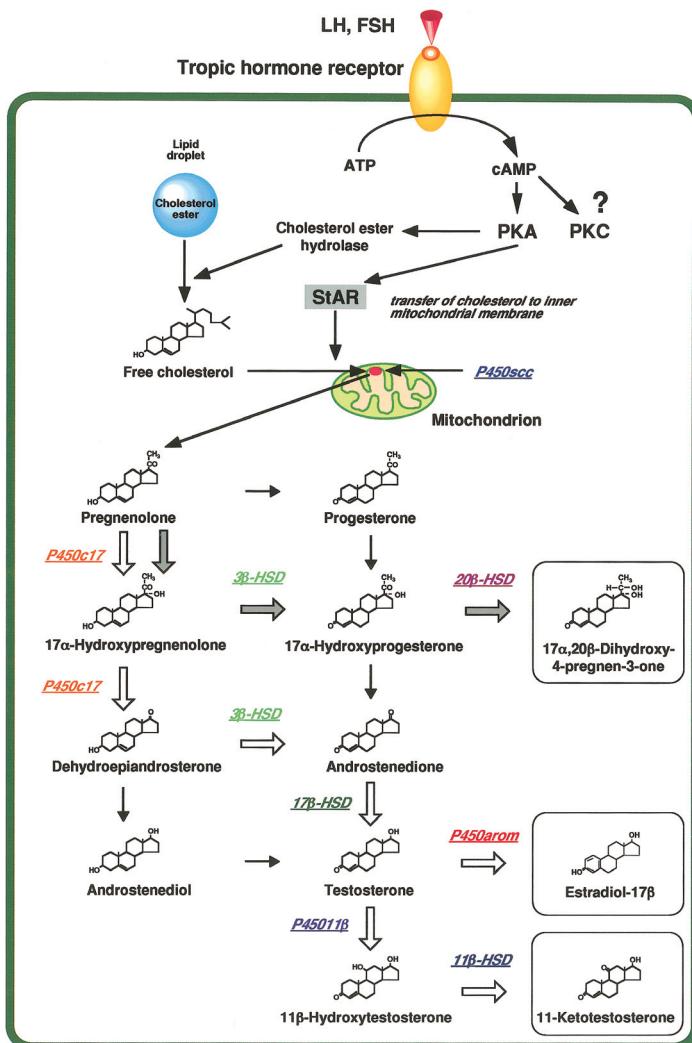


Fig. 1 Steroidogenic pathway in the gonads of teleost fish. Following binding of tropic hormones to their receptors, StAR protein is rapidly synthesized in the cytoplasm. The StAR protein becomes associated with cholesterol and quickly facilitates its transfer to the inner mitochondrion where P450scc is located. The pathways for E₂, 11-KT and 17,20 β -P biosynthesis are primarily based on studies concerning substrate preferences of 3 β -HSD and P450_{C17} in teleosts.^{24,123,127} White arrows indicate the proposed androgen synthesis pathway. Gray arrows indicate the proposed progestogen synthesis pathway.

StAR shuttling cholesterol across the mitochondrial membranes and forming a transport tunnel¹⁰ or cavity,¹¹ or StAR being a partially folded protein¹² or molten globule.¹¹ It is plausible that StAR interacts with contact sites where the inner and outer mitochondrial membranes are in close proximity.⁹

Delivery of cholesterol to the inner mitochondrial membrane is a prerequisite for gonadal and adrenal steroidogenesis and production of the basal steroid, pregnenolone. Pregnenolone, in turn, can serve as a substrate for cytochrome P450 17-hydroxylase/C₁₇₋₂₀lyase (P450_{C17}). P450_{C17} has dual enzymatic activity; it catalyses the hydroxylation of pregnenes at C₁₇ (17-hydroxylase) and it can promote the subsequent removal of the two-carbon C₂₀₋₂₁ acetic acid residues from 17-hydroxypregnanes by C₁₇₋₂₀lyase to yield the androgens dehydroepiandrosterone ($\Delta 5$ pathway: a double bond between C₅₋₆) or androstenedione ($\Delta 4$ pathway: a double bond between C₄₋₅). Lyase activity and 17-hydroxylase activity occur in the same enzymatic pocket (see citation¹³). However, lyase activity is regulated by its own set of modulators in a tissue-dependent fashion, thus explaining the much higher incidence of C₁₇₋₂₀ scission of 17-hydroxypregnanes in gonads than in adrenals. In mammals, modulators of C₁₇₋₂₀lyase activity include electron-donating redox partners, such as P450 reductase.^{14,15} Moreover, phosphorylation of serine and threonine residues on P450_{C17} is thought to increase the affinity of the enzyme for these redox partners.¹⁴ Cytochrome b5 has likewise been implicated in stimulating lyase activity, due to its action as an ‘allosteric facilitator’.¹³ It is noteworthy that cytochrome b5 did not affect C₁₇₋₂₀ scission by the sheep P450_{C17} ortholog.¹⁶

In fishes, regulation of lyase activity may be a key mechanism controlling the shift in the steroidogenic pathway from the production of androgens to progestogens in the prematurational phase. In both male and female fishes, this shift is associated with the luteinizing hormone (LH) surge, although it is often less clearly prominent in the circulating profiles of androgens and 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) of males. Studies on male cyprinids led Barry *et al.*¹⁷ to propose that the LH surge causes an increase in levels of 17-hydroxyprogesterone. C₁₇₋₂₀lyase activity is insufficiently high to

efficiently convert the higher levels of substrate to androgens and therefore 17-hydroxyprogesterone is made available as a substrate for both 20α - and 20β -hydroxysteroid dehydrogenase (20α -HSD; 20β -HSD). 20β -HSD in teleost testes drives the synthesis of $17,20\beta$ -P, the maturation-inducing steroid (MIS) in a large number of fishes. More specifically, 20β -HSD activity has been demonstrated in spermatozoa in a variety of fish species, including salmonids,¹⁸ cyprinids¹⁹ and grouper.²⁰ However, there is also evidence for the presence of 20β -HSD in non-flagellated sperm cells²¹ and testicular interstitial cells.^{22,23} In testes of carp, MIS was found to promote its own synthesis by inhibiting C₁₇₋₂₀lyase activity.¹⁷ The mechanism by which MIS inhibits C₁₇₋₂₀lyase activity may be based on modifying the availability of P450 reductase or cytochrome b5, or on changing the affinity for these factors by inhibiting phosphorylation of P450_{C17} serine or threonine residues. Observations on medaka ovarian follicles²⁴ support this notion, given the stimulatory actions of IBMX, a phosphodiesterase inhibitor, on androgen production *in vitro*. Interestingly, calcium ionophore A23187 and the protein kinase C activator, o-tetradecanoylphorbol 13-acetate (TPA), inhibited androgen production, prompting the authors to suggest that calcium affects P450_{C17} activity.²⁴

Irrespective of the finer biochemical details, observations on numerous fish species, both *in vivo* (amago salmon, *Oncorhynchus rhodurus*,^{25,26} carp, *Cyprinus carpio*,²⁷ chinook salmon, *Oncorhynchus tshawytscha*²⁸) and *in vitro* (amago salmon,^{18,29} yellowtail, *Seriola quinqueradiata*,³⁰ seabream, *Pagrus major* ;³¹ medaka, *Oryzias latipes*,²⁴ carp¹⁷), point to the LH-induced steroidogenic shift as a common mechanism involved in MIS synthesis in teleosts. However, it deserves mentioning here that temporal changes in steroid hormone levels are more difficult to interpret for teleosts with short cycles, such as Japanese whiting (*Sillago japonica*).³² In these fishes, different clutches of developing ovarian follicles contribute to circulating steroids concurrently.³² Hence, blood steroid levels do not tend to follow the pattern seen in fish with synchronously developing oocytes. A further confounding factor has been a tendency to use sampling strategies too coarse to detect cyclical

changes in steroid hormone levels in species with asynchronously developing oocytes.³³

Also, the importance of substrate levels on the steroidogenic pathway has been previously illustrated in female fishes. In goldfish for example, incubation of early vitellogenic follicles with excess 17-hydroxyprogesterone resulted in the untimely production of MIS.³⁴ In eel (*Anguilla* spp.), vitellogenic follicles were likewise found to produce 17,20 β -P when supplied with 17-hydroxyprogesterone *in vitro*.^{35,36} In salmon, too, substrate is a major limiting factor to MIS production, exemplified by the production of 17,20 β -P by vitellogenic follicles when supplied with 17-hydroxyprogesterone.³⁷ These data suggest that the mechanisms controlling the steroidogenic shift in males may also exist in females, in which 20 β -HSD is expressed in follicle cells (granulosa cells in the case of salmonids^{37,38}) surrounding the oocytes. Furthermore, these findings on vitellogenic females could indicate that the mechanisms controlling MIS production are in place very early in development and that 20 β -HSD may be constitutively expressed, at least in some fish species.

The third group of sex steroids, the estrogens, also depend on P450 enzymatic activity for their synthesis. Thus, cytochrome P450 aromatase (P450arom) uses Δ 4-androgens as substrates for conversion into estrogens by modifying the steroid A-ring into a benzene ring and removal of the C₁₉ methyl residue (Fig. 1). Estrogens have been mostly associated with female reproductive function, primarily as a central mediator of vitellogenesis in oviparous vertebrates. However, it has become evident that estrogens also play an important role in reproduction in males by stimulating proliferation of gonial stem cells (see Section on Germ Cell Development). In females, estrogen levels are generally elevated during the period of vitellogenic growth, but levels dramatically drop in the post-vitellogenic phase in a number of fish species, including salmonids,^{29,39} medaka⁴⁰ and goldfish.⁴¹ In salmonids, the decline in P450arom activity, responsible for the drop in estrogen levels, seems to be a key event preceding MIS production.^{39,42} Indeed, the resulting increase in androgens is believed to be important as a primer of LH synthesis in the pituitary, thus allowing the LH surge to occur and final maturation to proceed, at least in goldfish⁴³ (see Section on Steroid Feedback).

In spite of the numerous examples indicating a change in production of androgens to progestogens (males) or from estrogens to progestogens (females), a steroidogenic shift is not likely to be a universal mechanism explaining MIS production. In mummichog (*Fundulus heteroclitus*) for example, there is no evidence for a reduction in estrogen synthesis by maturing follicles.⁴⁴ Likewise, ovarian follicles from artificially matured Japanese eel have high P450arom activity at the migratory nucleus stage.⁴⁵ These examples clearly illustrate that MIS-inducing mechanisms other than gonadotropin-regulated decreases in P450arom may exist in teleost fishes.

Pregnene-, androstene- and estrene-derivatives can all be further modified into other steroids by a suite of hydroxylases, reductases, oxidoreductases and isomerases (Fig. 1). In fishes, 3 β -hydroxysteroid dehydrogenase (3 β -HSD), 17 β -HSD and 20 β -HSD deserve particular mention as important oxidoreductases in the synthesis of bioactive steroids. 3 β -HSD converts Δ 5- into Δ 4-steroids, whereas 17 β -HSD reduces 17-ketosteroids into 17-hydroxysteroids. Both conversions result in greatly increased bioactivity of the steroid product. 20 β -HSD too, has a pivotal role as a reductase, catalyzing the production of 20 β -hydroxysteroids that typically have maturation-inducing activity. Gonads of male and some female teleosts also produce a unique, very potent androgen, 11-ketotestosterone (11-KT). Synthesis of this compound requires cytochrome P450 11 β -hydroxylase (P450_{11 β}) to add a hydroxyl group to C₁₁, and the oxidoreductase 11 β -HSD to oxidize the 11-hydroxysteroid into an 11-ketosteroid.

As many as sixteen steroidogenic enzymes (Table 1) are possibly expressed in the gonads of teleost fish, allowing for the production, in theory, of an almost endless number of different steroid hormones. Many of these possible steroids have indeed been identified; most are considered intermediates in the pathway leading to bioactive end-products or are considered inactive metabolites that are being modified for excretion. Thus, only a handful of steroid metabolites from gonadal origin have been assigned biological function (see section on Steroid Action) in teleost fishes: the maturation-promoting progestogens 17,20 β -P and 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S); the androgens testosterone (T) and 11-KT; and the estrogen 17 β -estradiol

Table 1 Steroidogenic enzymes in gonads of teleost fish. Identification has either been based on DNA sequences (seq) or on steroid metabolites isolated from *in vitro* gonadal or gamete incubations (iv). Species and references for illustrative purposes only. Note that not all enzymes are necessarily found in all fish species.

Enzyme	Function	Species & Ref. No. []
P450 Side-chain cleavage	Pregnene synthesis after removal of C _{22–27} side-chain	Mummichog (iv [21]), trout (seq [26]), catfish (seq [29])
P450 17-Hydroxylase/C _{17–20} lyase	(1) Hydroxylation of C ₁₇ (2) Androgen synthesis after removal of C _{17–20} side-chain (3) Hydroxylation of C ₁₆ *	Trout (iv [1]), trout (seq [23]), eel (seq [8])
P450 Aromatase	Estrogen synthesis after removal of C ₁₉ and aromatization of A-ring	Goldfish (iv [7]), catfish (seq [28]), medaka (seq [5])
P450 C ₂₁ -Hydroxylase	Hydroxylation of C ₂₁	Perch (iv [27]), grouper (iv [15]), turbot (iv [19])
6α-Hydroxylase	Hydroxylation of C ₆	Carp (iv [10]), seabream (iv [3])
6β-Hydroxylase	Hydroxylation of C ₆	Seabream (iv [3])
7α-Hydroxylase	Hydroxylation of C ₇	Cyprinids (iv [11]), catfish (iv [20])
P450 11β-Hydroxylase	Hydroxylation of C ₁₁	Grouper (iv [15]), trout (seq [18]), trout (seq [12])
3β-Hydroxysteroid dehydrogenase/Δ5γ	(1) Oxidoreduction of 3-hydroxy/ketosteroids	Catfish (iv [31]), turbot (iv [19]), trout seq [24])
Δ4 isomerase	(2) Conversion of Δ5 into Δ4 steroids	
3α-Hydroxysteroid dehydrogenase	Oxidoreduction of 3-hydroxy/ketosteroids	Catfish (iv [31]), eel (iv [14]), grouper (iv [16])
11β-Hydroxysteroid dehydrogenase	Oxidoreduction of 11-hydroxy/ketosteroids	Betta (iv [17]), roach (iv [4]), trout (seq [13])

* No evidence for gonadal 16α-hydroxylase activity; this activity has been detected in mammalian P450_{c17}, and also in fish kidneys, but not in gonad.

Table 1 (Continued).

Enzyme	Function	Species & Ref. No. []
17 β -Hydroxysteroid dehydrogenase	Oxidoreduction of 17-hydroxy/ketosteroids	<i>Betta</i> (iv [16]), trout (iv [24]), eel (seq [9])
20 α -Hydroxysteroid dehydrogenase	Oxidoreduction of 20-hydroxy/ketosteroids	Perch (iv [26]), grouper (iv [15]), turbot (iv [18])
20 β -Hydroxysteroid dehydrogenase	Oxidoreduction of 20-hydroxy/ketosteroids	Brook trout (iv [26]), grouper (iv [15]), trout (seq [6])
5 α -Reductase	Reduction of the C ₄ –C ₅ double bond	Eel (iv [21]), perch (iv [26]), cyprinids (iv [11])
5 β -Reductase	Reduction of the C ₄ –C ₅ double bond	Grouper (iv [15]), brook trout (iv [26]), flounder (iv [2])

References

1. Andersson T and Rafter J (1990). Progesterone metabolism in the microsomal fraction of the testis, head kidney, and trunk kidney from the rainbow trout. *Gen. Comp. Endocrinol.* **79**: 130–135.
2. Canario AVM (1991). Sex steroids in marine flatfish. In: Scott AP, Sumpter JP, Kime DE and Rolfe MS (eds.), *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*. University of East Anglia Printing Unit, Norwich, UK, pp. 71–73.
3. Condeca JB and Canario AV (2001). Gonadal steroidogenesis in response to estradiol-17 β administration in the sea bream (*Sparus aurata* L.). *Gen. Comp. Endocrinol.* **124**: 82–96.
4. Ebrahimi M, Singh PB and Kime DE (1995). Biosynthesis of 17,20 α -dihydroxy-4-pregnen-3-one, 17,20 β -dihydroxy-4-pregnen-3-one, and 11-ketotestosterone by testicular fragments and sperm of the roach, *Rutilus rutilus*. *Gen. Comp. Endocrinol.* **100**: 375–384.
5. Fukada S, Tanaka M, Matsuyama M, Kobayashi D and Nagahama Y (1996). Isolation, characterization, and expression of cDNAs encoding the medaka (*Oryzias latipes*) ovarian follicle cytochrome P-450 aromatase. *Mol. Reprod. Dev.* **45**: 285–290.

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Table 1 (Continued).

6. Guan G, Tanaka M, Todo T, Young G, Yoshikuni M and Nagahama Y (1999). Cloning and expression of two carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). *Biochem. Biophys. Res. Commun.* **255**: 123–128.
7. Kagawa H, Young G and Nagahama Y (1984). *In vitro* estradiol-17 β and testosterone production by ovarian follicles of the goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* **54**: 139–143.
8. Kazeto Y, Ijiri S, Todo T, Adachi S and Yamauchi K (2000). Molecular cloning and characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. *Gen. Comp. Endocrinol.* **118**: 123–133.
9. Kazeto Y, Ijiri S, Matsubara H, Adachi S and Yamauchi K (2000). Cloning of 17 β -hydroxysteroid dehydrogenase-I cDNAs from Japanese eel ovary. *Biochem. Biophys. Res. Commun.* **279**: 451–456.
10. Kime DE (1990). *In vitro* metabolism of progesterone, 17-hydroxyprogesterone, and 17,20 β -dihydroxy-4-pregnen-3-one by ovaries of the common carp *Cyprinus carpio*: production rates of polar metabolites. *Gen. Comp. Endocrinol.* **79**: 406–414.
11. Kime DE (1991). Ovarian progestogens in cyprinid fish. In: Scott AP, Sumpter JP, Kime DE and Rolfe MS (eds.), *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*. University of East Anglia Printing Unit, Norwich, UK, pp. 77–79.
12. Kusakabe M, Kobayashi T, Todo T, Lokman PM, Nagahama Y and Young G (2002). Molecular cloning and expression during spermatogenesis of a cDNA encoding testicular 11 β -hydroxylase (P45011 β) in rainbow trout (*Oncorhynchus mykiss*). *Mol. Reprod. Dev.* **62**: 456–469.
13. Kusakabe M, Nakamura I and Young G (2003). 11 β -Hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in rainbow trout: cloning, sites of expression, and seasonal changes in gonads. *Endocrinology* **144**: 2534–2545.
14. Lambert JGD, Ouwend DM and Granneman JCM (1991). Steroidogenesis in the ovary of the European eel, *Anguilla anguilla*, at the silver stage. In: Scott AP, Sumpter JP, Kime DE and Rolfe MS (eds.), *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*. University of East Anglia Printing Unit, Norwich, UK, pp. 66–70.

(Continued)

Table 1 (Continued).

15. Lee ST, Lam TJ and Tan CH (2002). Increased 21-hydroxylase and shutdown of C(17,20) lyase activities in testicular tissues of the grouper (*Epinephelus coioides*) during 17 α -methyltestosterone-induced sex inversion. *Gen. Comp. Endocrinol.* **126**: 298–309.
16. Lee ST, Kime DE, Lam TJ and Tan CH (1998). Synthesis of 17,20 α/β -dihydroxy-4-pregnen-3-one and 5 β -pregnanes in spermatozoa of primary and 17 α -methyltestosterone-induced secondary male grouper (*Epinephelus coioides*). *Gen. Comp. Endocrinol.* **112**: 1–9.
17. Leitz T and Reinboth R (1985). *In vitro* bioconversion of [14C]androstenedione by testes of the Siamese fighting fish *Betta splendens* Regan (Anabantoidei, Belontiidae). *Gen. Comp. Endocrinol.* **58**: 471–477.
18. Liu S, Govoroun M, D'Cotta H, Ricordel MJ, Lareyre JJ, McMeel OM, Smith T, Nagahama Y and Guiguen Y (2000). Expression of cytochrome P450(11 β) (11 β -hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. *J. Steroid Biochem. Mol. Biol.* **75**: 291–298.
19. Mugnier C, Gaignon JL and Fostier A (1997). *In vitro* synthesis of 17,20 β ,21-trihydroxy-4-pregnen-3-one by ovaries of turbot (*Scophthalmus maximus* L.) during oocyte maturation. *Gen. Comp. Endocrinol.* **107**: 63–73.
20. Ponthier JL, Shackleton CH and Trant JM (1998). Seasonal changes in the production of two novel and abundant ovarian steroids in the channel catfish (*Ictalurus punctatus*). *Gen. Comp. Endocrinol.* **111**: 141–155.
21. Petrino TR, Lin YW and Wallace RA (1989). Steroidogenesis in *Fundulus heteroclitus*. I. Production of 17 α -hydroxy,20 β -dihydroprogesterone, testosterone, and 17 β -estradiol by prematurational follicles *in vitro*. *Gen. Comp. Endocrinol.* **73**: 147–156.
22. Querat B, Hardy A and Leloup-Hately J (1986). Ovarian metabolic pathways of steroid biosynthesis in the European eel (*Anguilla anguilla* L.) at the silver stage. *J. Steroid. Biochem.* **24**: 899–907.
23. Sakai N, Tanaka M, Adachi S, Miller WL and Nagahama Y (1992). Rainbow trout cytochrome P-450c17 (17 α -hydroxylase/17,20-lyase). cDNA cloning, enzymatic properties and temporal pattern of ovarian P-450c17 mRNA expression during oogenesis. *FEBS Lett.* **301**: 60–64.

(Continued)

Table 1 (Continued).

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24. Sakai N, Tanaka M, Takahashi M, Fukada S, Mason JI and Nagahama Y (1994). Ovarian 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ -4-isomerase of rainbow trout: its cDNA cloning and properties of the enzyme expressed in a mammalian cell. *FEBS Lett.* **350**: 309–313.
 25. Sire O and Depeche J (1981). *In vitro* effect of a fish gonadotropin on aromatase and 17β -hydroxysteroid dehydrogenase activities in the ovary of the rainbow trout (*Salmo gairdneri Rich.*). *Reprod. Nutr. Dev.* **21**: 715–726.
 26. Takahashi M, Tanaka M, Sakai N, Adachi S, Miller WL and Nagahama Y (1993). Rainbow trout ovarian cholesterol side-chain cleavage cytochrome P450 (P450scc). cDNA cloning and mRNA expression during oogenesis. *FEBS Lett.* **319**: 45–48.
 27. Theofan G and Goetz FW (1983). The *in vitro* synthesis of final maturational steroids by ovaries of brook trout (*Salvelinus fontinalis*) and Yellow perch (*Perca flavescens*). *Gen. Comp. Endocrinol.* **51**: 84–95.
 28. Trant JM (1994). Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom. *Gen. Comp. Endocrinol.* **95**: 155–168.
 29. Trant JM, Berard C and Byrne BJ (1998). *Isolation and Heterologous Expression of the cDNA Encoding the Cytochrome P450 Choleoestrol Side Chain Cleavage from the Channel Catfish (Ictalurus punctatus)*. GenBank Accession No. AF063836.
 30. Unger F and Gunville R (1997). Formation of 3α -hydroxy- 5β -pregnan-20-one in the ovaries of catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol.* **31**: 53–59.
 31. van den Hurk R and Richter CJ (1980). Histochemical evidence for granulosa steroids in follicle maturation in the African catfish, *Clarias lazera*. *Cell Tissue Res.* **211**: 345–348.
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(E₂). It has been emphasized, however, that other, “non-classical” steroids could well play an important role in the reproductive biology of teleost fish.¹

The presence of a large number of steroidogenic enzymes prompts an obvious question: *how is the production of steroids directed towards particular end-products?* To answer this key question, a thorough understanding of temporal changes in steroidogenic enzyme gene expression and the regulation of expression by gonadotropins and other hormones is required. The expression of genes encoding certain rate-limiting enzymes in particular may prove crucial for controlling steroid production, both quantitatively and qualitatively.

Steroid Release

Unlike peptide hormones, steroids are not normally stored in the cytoplasm following their synthesis, but they readily diffuse out of the cell and act by binding to nearby (paracrine, autocrine) or distant (endocrine) cytosolic or membrane receptors.

Steroid Action

Steroids exert their effects through receptors, either located in the cytosol (“classical” steroid receptors) or embedded in the membrane. Details on steroid receptors, their ligands, and their activation and signal transduction have been described.^{46–48}

Steroid Function

Both for the survival of a species and an individual animal, steroid hormones play a pivotal role. Briefly, in fishes, steroids have been implicated in sex determination and differentiation,⁴ in development and metamorphosis,^{49,50} in mineralocorticoid and glucocorticoid homeostasis,⁵¹ and in reproduction. Reproduction is geared toward the successful union of a male and a female gamete, and this process is heavily reliant on gonadotropins, i.e., follicle-stimulating hormone (FSH) and LH. Gonadotropin action generally results in the production of secondary mediators, the steroid hormones (e.g. Refs. 37 and 52). Thus, steroids

play a major role in germ cell development (see section on Germ Cell Development for review of recent progress on this subject), in the development of accessory organs and breeding coloration, in feedback on brain, pituitary and gonad (see section on Steroid Feedback), as pheromones for chemical communication (see section on Steroids and their Metabolites as Pheromones), and as modulators of behavior.

Germ cell development

Males

Developmental events in the testes of teleost fishes include spermatogonial renewal, spermatogonial proliferation, meiosis, spermiogenesis, and sperm maturation;⁵³ these processes and their control have recently been reviewed.⁵² Spermatogonial renewal is a process aimed at ensuring that stem cells are retained for future waves of spermatogenesis. A comprehensive study on Japanese eel (*Anguilla japonica*) has indicated that estrogens, notably E₂, control proliferation of stem cells in this species.⁵⁴ In contrast, spermatogonial proliferation, the generation of large numbers of germ cells needed to ensure a high chance of successfully fertilizing spawned eggs,⁵² is under androgen control. In eel, 11-KT can induce spermatogonial proliferation and subsequent stages of spermatogenesis, including spermiogenesis, *in vitro* (*Anguilla japonica*^{53,55}), provided insulin or insulin-like growth factor-I is present. The action of 11-KT is mediated, at least in part, by Sertoli cell-derived growth factors such as activin B that control development of spermatogenic cysts in eel.⁵⁶ In other teleost fishes, such as huchen, *Hucho perryi*, 11-KT has likewise been implicated in spermatogenesis as blood levels were elevated throughout germ cell development, starting from the spermatogonial proliferation phase.⁵⁷ However, spermatogonial proliferation in this species was not only induced in response to 11-KT, but E₂ and 17,20β-P also stimulated mitosis and meiosis, as evidenced by uptake of 5-bromo-2-deoxyuridine (BrdU) in germ cells *in vitro*.⁵⁷ In contrast, no such stimulatory effects of sex steroids were detected in rainbow trout, where only growth factors were effective.⁵⁸ Progress of germ cells through meiosis and spermiogenesis, which together dramatically change germ cell morphology, is also attributed to 11-KT in a wide range of teleost fishes as reviewed.⁵² The final developmental stage, sperm maturation, is associated with acquisition

of sperm motility, and again strongly hinges on gonadotropin-induced steroid action; thus, motile sperm can be obtained after exposure of sperm to 17,20 β -P, which increases the activity of carbonic anhydrase and, in turn, increases seminal plasma pH in eel.⁵⁹ In salmonids, 17,20 β -P is likewise the maturation-inducing hormone,⁶⁰ whereas 20 β -S is a likely candidate performing this function in other fishes, such as Atlantic croaker, in view of its maturational effects in females of this species.⁶¹

Females

Oogenesis in fishes involves an oogonial proliferation phase, followed by previtellogenic primary oocyte growth, vitellogenic growth, and final oocyte maturation preceding spawning. The reader is referred to several excellent reviews that have previously detailed the histological and ultrastructural changes occurring during these events.^{37,62-64}

Involvement of the endocrine system in both oogonial proliferation and previtellogenic growth has been little documented. Recent work on huchen⁶⁵ revealed that E₂ stimulates *in vitro* incorporation of BrdU into oogonia, suggesting a role for E₂ in oogonial proliferation. Furthermore, 17,20 β -P promoted entry of oogonia into meiosis,⁶⁵ giving rise to oocytes in prophase of the first meiotic division.

Growth of prophase-arrested oocytes of teleost fishes is reflected by a large number of morphological changes, such as increased numbers of mitochondria, increasing amounts of RNA and accumulation of lipid droplets, cortical alveoli and yolk granules.^{63,64} In hypophysectomized goldfish, *Carassius auratus*, Khoo⁶⁶ attributed formation of cortical alveoli to estrogens. More recently, our own observations point to an involvement of both growth factors and androgens, but not estrogens, in growth of previtellogenic eel ovarian follicles.^{49,67} No other studies have evaluated the possible importance of steroid hormones in early oogenesis in teleosts. This is in sharp contrast to the enormous number of publications on endocrine control and involvement of sex steroids in vitellogenesis and final oocyte maturation; the role of estrogens (E₂) in inducing vitellogenin synthesis in hepatocytes has received ample attention in a wide range of teleost fishes ever since the pioneering work by Wallace⁶⁸ and others in the 1960s and is not discussed in further detail in this review. Likewise,

the progestogens $17,20\beta$ -P and 20β -S have been widely implicated in induction of final maturation and ovulation in fishes and has been reviewed.^{37,63} It is noteworthy that these steroids may not be common to all teleosts, and that other mediators are occasionally proposed, such as in the case of the black sea bass.⁶⁹

Steroid feedback

Long-loop feedback

Steroid hormones do not only affect germ cell development by exerting effects on the gonads, but they are also important in regulating circulating levels of gonadotropins through feedback on the hypothalamus and pituitary. This steroid feedback is primarily mediated via estrogens and androgens in the brain or pituitary, and the direction (positive, negative) of the feedback can differ between seasons, between the sexes and between FSH and LH within the same species. Much remains to be learned, especially in view of the very few assay systems currently available for measurement of FSH.⁷⁰ A full discussion of steroid feedback control of gonadotropin synthesis and release is beyond the scope of this review, but several papers on this subject have recently appeared in print.⁷¹⁻⁷⁴

Short-loop feedback

The existence of short feedback loops in gonads of fishes has been demonstrated on several occasions. As illustrated earlier,¹⁷ a decrease in lyase activity in response to increasing levels of MIS in carp testes has been observed. As a result, MIS promotes its own synthesis. In the testis of the African catfish, *Clarias gariepinus*, steroids similarly affect steroidogenesis, with testosterone inhibiting androgen production by inhibiting the activity of P450_{C17}.⁷⁵ Accordingly, a short feedback loop seems to be operating.⁵²

Steroids and their metabolites as pheromones

Steroids and their metabolites have been recognized as pheromones, odorants that "improve an organism's chances for reproductive success

and survival⁷⁶ in males and/or females of a large number of fish species. They can do so by modifying behavior (aggression, courtship) and/or affect LH levels (e.g., goldfish⁷⁷). These steroidal pheromonal signaling molecules come in a variety of forms and, depending on the species, include progestogens, androgens and estrogens and their sulfated or glucuronidated derivatives. Examples include etiocholanolone in goby,⁷⁸ 17,20 β -P and 17, 20 β -P-sulfate in goldfish^{77,79} and tilapia⁸⁰ and 5 β -pregnane-3 α ,17 α -diol-20-one-glucuronide in catfish.⁸¹ Furthermore, sex-specific responsiveness to pheromones has been shown to be inducible by sex steroids in goby (*Neogobius melanostomus*).⁷⁸ In this species, the putative female pheromones estrone and estradiol glucuronide normally only produce a behavioral response in males, but treatment of females with androgens resulted in male-type responses following exposure to these pheromonal substances.⁷⁸ Cardwell *et al.*⁸² and Bhatt *et al.*⁸³ likewise argued for a stimulatory effect of androgens on olfactory pheromone receptors in the cyprinids *Puntius schwanenfeld* and *Barilius bendelisis*, respectively. For further details on the effects of steroid pheromones on physiology and behavior, the reader is referred to reviews by Van Weerd and Richter⁸⁴ and Kobayashi *et al.*⁴³

Steroid Metabolism and Excretion

In vertebrates, steroids circulating in blood are bound to carrier proteins with low (albumin) or high affinity (sex hormone binding proteins, SBPs). Classically, SBPs have been associated with controlling the delivery of steroids to target tissues, while excretion or inactivation is prevented. During the last decade, novel functions of SBPs as steroid signal transducers have been identified in mammals; thus, SBPs can bind to specific SBP membrane receptors (SBPR), and, on binding with a steroid, activate a G-protein, resulting in increased levels of intracellular cAMP.⁸⁵ The effects of steroids binding to the SBP-SBPR complex appear to be tissue-specific, resulting in either agonistic or antagonistic action. More recently, there has been speculation about a paracrine/autocrine regulatory function of SBPs, given indirect evidence of extra-hepatic, localized production of SBPs.⁸⁵

Studies directed towards understanding classical functions of SBPs in fishes have shown that, like other vertebrates, these proteins preferentially bind androgens and estrogens over progestogens. This has been demonstrated for a range of fish species, including flounder, seabream and Arctic char.^{86,87} It is further noteworthy that affinity of SBPs for the teleost-specific steroid 11-KT is also high, albeit markedly weaker, about one order of magnitude less in Arctic char,⁸⁷ than for E₂ and T. Like their mammalian counterparts, the two SBP cDNAs recently isolated from a carp liver cDNA library point to the liver as the main site of SBP synthesis.⁸⁸ Whether fish SBPs also perform the signal transducing functions recently reported for mammalian proteins remains to be investigated.

Only a fraction of steroids produced is likely to ever reach its membrane or intracellular receptor in target tissue. The fate of these steroids after receptor activation is not entirely clear; it is believed that ligand and receptor eventually dissociate, possibly due to structural changes following receptor activation. Thereafter, the steroid molecule is likely to be metabolized to an inactive form.⁸⁹ Steroid inactivation predominantly occurs in the kidney or liver by enzymatic conversion of bioactive steroids into inactive products, typically by steroid-metabolizing enzymes or glucuronosyl- or sulfotransferases. Both glucuronosyltransferases (separated into two families, with the UGT2B subfamily acting on sex steroids⁹⁰) and sulfotransferases (SULT cytosolic sulfotransferase superfamily) are groups of enzymes that may add glucuronide or sulfate groups to widely varying substrates.

Accordingly, substrate specificities differ for different enzymes: in mammals, for instance, estrogen sulphotransferase,⁹¹ and hydroxysteroid sulfotransferase SULT2B1⁹² have been described, both with their own substrate specificities. Comparable examples can be found for mammalian glucuronosyltransferases. In fishes, however, substrate-specificity or gene sequence data for steroid-metabolizing SULT and UGT2B gene products are essentially missing, with the exception of some SULT expressed sequence tags from catfish (Accession No. BM438784), flounder (Accession No. AW012984), and zebrafish (Accession No. CB354627).

The steroid-metabolizing enzymes include oxidases, reductases and members of the cytochrome P450 superfamily. Thus, cytochrome P450 3A (CYP3A) has been implicated in the metabolism of T to its 6β -, 2β -, and 16β -hydroxylated derivatives in liver and intestine of trout on the basis of functional expression of the enzyme in insect cells.⁹³ In medaka, CYP3A similarly converted T,⁹⁴ whereas the importance of hepatic CYP1A in modifying steroids was considered to be minor in a number of teleost species.⁹⁵ Nevertheless, glucuroconjugation of T by trout hepatocytes, thought to reflect CYP1A activity, has been reported.⁹⁶ Gill tissue, too, converted T, but into different products, i.e., mostly dihydrotestosterone and androstanediol.⁹⁶ Arguably, the enzyme carbonyl reductase, which has been implicated in the production of $17,20\beta$ -P in salmonids, may also play a role as a steroid inactivator, given its ability to reduce the carboxyl group of a number of steroid moieties, including 5α - and 5β -dihydrotestosterone.⁹⁷ The resulting steroid metabolite can then be excreted via the gills⁹⁸ or urine¹ as a waste product or, in the spawning season, as a pheromone. Interestingly, the form into which the steroid is modified appears to dictate the excretory routes. In a study by Vermeirssen and Scott,⁹⁹ the gills (free steroid), bile (glucuro-conjugated steroids) and urine (sulphated steroids) were identified as primary routes of steroid excretion in rainbow trout.

Molecular Cloning and Characterization of Steroidogenic Proteins

A number of studies aiming to clone and characterize cDNAs encoding steroidogenic proteins (StAR and the steroidogenic enzymes) of teleosts have been performed over the past decade. The main efforts by far have been directed towards cloning of P450arom cDNAs, a reflection of the importance of aromatase in understanding processes such as sexual differentiation and vitellogenesis, but information on other steroidogenic protein genes is emerging at an accelerating rate. In some cases, the catalytic activities of the encoded proteins have not been investigated and identification has been mainly based on high homology

with similar proteins in other animals. With some notable exceptions, information on promoter sequences is scarce.

StAR

Cloning and characterization of StAR cDNAs

Zebrafish StAR cDNA encoding a protein of 285 amino acids was the first teleost StAR cDNA to be cloned.¹⁰⁰ A primary transcript size of 1.5 kb was reported, along with minor transcripts of about 2.5 kb and 4.5 kb in testis. Since then, cDNAs containing full open reading frames have been cloned from rainbow trout and brook trout:¹⁰¹ both trout cDNAs encode proteins of 287 amino acids that are 97% identical. Trout StARs share approximately 80% overall homology with zebrafish and chicken StAR, and around 60–65% homology with mammalian StARs. However, a higher degree of homology is shared by the C-terminal portions of vertebrate StARs. The C-terminal is known to be essential for StAR function and over 85% homology is shared between hydrophobic residues in this region identified as important in forming a hydrophobic cavity structure thought to be involved in binding cholesterol.¹¹ Predicted motifs for protein kinase A (PKA) phosphorylation are close to 100% identical between species. More recently, StAR cDNA has been cloned from Japanese eel head kidney and cod ovaries, and structural analysis further confirms the high degree of conservation in known functional regions of StAR in these species.^{102,103}

Major StAR transcripts of about 2.3 kb and 1.8 kb are found in rainbow trout and brook trout, respectively, with larger minor transcripts of about 4.4 and 9.9 kb also reported for rainbow trout. A partial cDNA cloned from Arctic char (*Salvelinus alpinus*)¹⁰⁴ hybridized with a 1.8 kb transcript in Northern blots. Two StAR transcripts (1.1 and 1.4 kb), differing in length in the 3' untranslated region, occur in eel head kidney, with the expression of the two transcripts differing greatly between individual eels.¹⁰² In cod, two cDNAs of ~1.5 and 2.8 kb were isolated.¹⁰³ The smaller cDNA was similar in size and structure to cDNAs isolated from other fish, while the larger cDNA contained

introns. Further analysis of the cod StAR gene revealed that the larger cDNA was partially processed (as many as 5 of 6 introns were retained), yet this transcript was still present in the cDNA library since it contained a poly A tail. The StAR genes that have been characterized in mammals have 7 exons and 6 introns and are 6.5–7.5 kb in length^{105–107}. The same number of introns and exons are present in the cod and fugu StAR genes, though they are only half as large as the mammalian StAR gene.¹⁰³

In all species investigated to date, StAR mRNA has been detected at high levels in ovary, testis and head kidney. However, unlike mammals, where StAR gene expression is largely restricted to tissue known to be capable of *de novo* steroid synthesis, including brain, teleost StAR transcripts have been detected in a wider range of tissue, including rainbow trout spleen, pyloric caeca, intestine (Northern analysis,) and posterior kidney (RT-PCR).¹⁰¹ The Arctic char StAR transcripts have been detected in liver (RT-PCR),¹⁰⁴ and eel StAR transcripts have also been found in the brain (RT-PCR).¹⁰² In zebrafish, StAR transcripts were found in total kidney samples (head and posterior kidney were not separated because of the small size of the fish), gastrointestinal tract, gill, skin and muscle by RT-PCR.¹⁰⁸ The function of teleost StAR in these other tissues is unknown but may be related to the protein having other sterol carrier roles. In rainbow trout at least, these other tissues appear to be incapable of *de novo* steroid synthesis since they do not appear to express the gene encoding P450scc¹⁰¹ (Fig. 2). Intriguingly, RT-PCR using primers specific to the larger cod StAR cDNA revealed transcripts in tissues expressing the normal size cod StAR transcript. Thus, some of the larger transcripts observed in cod tissues highly expressing StAR could be a result of the expression of unprocessed or partially processed mRNA. Whether similar, unprocessed or partially processed StAR mRNAs exist in other species has not yet been determined. Finally, although there is high homology of teleost StARs with known functional domains of mammalian StARs, it is worth noting that it has not yet been demonstrated directly that teleost StARs function to transport cholesterol across the inner mitochondrial membrane.

Cloning of the upstream regulatory region of the brook trout StAR gene (GenBank accession # AY308064, F.W. Goetz), the first for

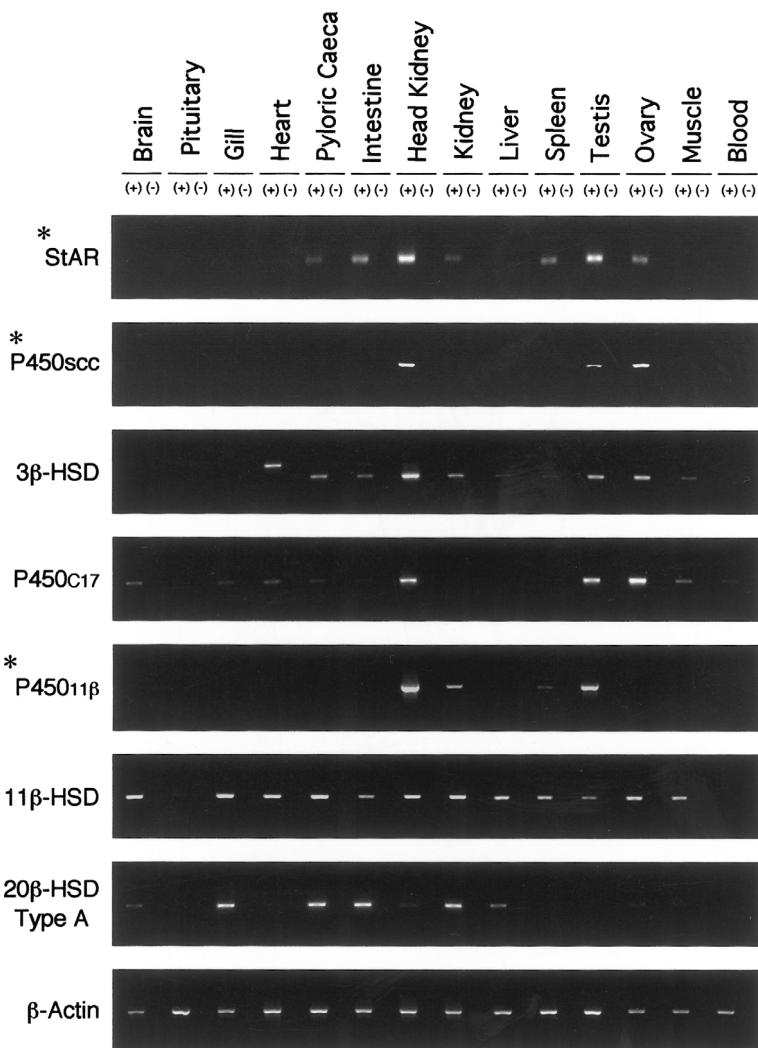


Fig. 2 Tissue distribution of rainbow trout steroidogenic protein mRNAs, analyzed by RT-PCR. First strand cDNAs were synthesized from 25 ng of poly(A)⁺ RNAs of two-year-old male rainbow trout. PCR amplification was carried out under the following conditions: denatured at 94°C for 20 sec, annealed at 60°C for 20 sec and extended at 72°C for 30 sec. Thirty cycles were performed for steroidogenic protein amplification and 20 cycles were performed for β -actin amplification. (+): reverse transcriptase added. (-): no reverse transcriptase added. *Results for StAR, P450scc and P45011 β mRNAs

are derived from data presented in Kusakabe *et al.*^{101,163} Two PCR products of different size were obtained for 3 β -HSD in some tissues. The smaller band is the one the PCR primers were designed to amplify and the larger band contains an extra 149 bp nucleotides compared to the original published sequence (sequence data not shown). The ratio of these two bands was tissue specific. Most tissues showed only the smaller transcript, but the larger transcript was also clearly found in intestine, spleen and ovary. Only the larger transcript was found in heart.

a non-mammal, has revealed several TATA boxes located within the first 200 bp of the ATG start site (Fig. 3), with the TATA site located more distally (-23-26) most likely being part of the transcriptional regulatory site. Typical TATA regions near the transcriptional start site are not present in mammalian StAR genes. Several other regulatory sites that are present in mammalian StAR promoters were present within 400 bp of the ATG start site. These include one Sp1 site (-82-87), two GATA sites (-63-68; -164-169), one CAAT enhancer binding protein (C/EBP) site (-33-41), and a site (TATCCTTGGC; -105 - 114) identical to a SF-1/Ad4BP site described in the bovine StAR promoter.¹⁰⁷ In addition, another C/EBP site (-629-636) and potential SF-1 (-638-644) site were located further upstream. Finally, a dinucleotide TG repeat region (-185-238) that resembles dinucleotide repeats in the mouse StAR promoter¹⁰⁹ exists approximately 400 bp upstream of the ATG start site. Thus, with the exception of SREBP (sterol regulatory element binding proteins), sites for transcriptional regulators that have been implicated in StAR expression in mammals, such as C/EBP, GATA, Sp1 and SF-1,¹¹⁰⁻¹¹² are present in the brook trout promoter.

A key feature of StAR expression in mammals is the strong induction by cAMP (see review by Stocco⁶). However, typical cAMP response elements (CREs) are not present in the upstream regulatory region of the mammalian and brook trout StAR genes. Steroidogenic factor 1 (SF-1) sites have been characterized in several mammalian StAR genes and suggested to be necessary for cAMP-mediated expression of StAR.^{109,113,114} However, this was not always observed.¹¹⁰ Although the SF-1 site in the brook trout promoter region is identical to that in the bovine gene, the involvement of the latter in StAR regulation has not been determined.¹⁰⁷

-1804 ATCACATCAATTGGGTGAGGTGGGGTGAATTGTGGGGTCAGGTCACTG
-1754 ATGCAGCAGTCACACTCTCTTCTGGTCAAATAGGCCCTAACAAAGCC
-1704 TGGTGTGGGTCAATTGTCCTGTGAAAACAAATGATAGTCCCCTAA
-1654 GCCCCAACAGATGGGATGCTGCGTGCAGAATGCTGTGGTAGCCAT
-1604 GCTGGTAAAGTGTGCTTGAAATTCTAAATAATCAGTCAGTGTCAACA
-1554 GCAAGGCACCCCCCATCCTCACACCTCTCCTCCATGCTCTGGGG
-1504 AACACACATGCGAGATCATCGTTACCTACCTAGTCTCACAAAGA
-1454 CACGGCGGTGGAACCAAAAACTCAAAATTGGACTCATCAGACAAAGG
-1404 ACAGATTCCTCGGTAAATGTCACATTCTCATGTTTCTTGGCCCAAGC
-1354 AAGTCTCTCTTATATTGATGTCTTAACTACGGTTCTTGCACCAA
-1304 TTCGACATGAAGGCCTGATTACACAGTCCTCTGAATAGTTGATGTT
-1254 GAGATGTTGTGTTACTGAACTGAAGCATTTATTGGGCTGCAAT
-1204 TTCTGAGGCTGGTAACCTAATGAACTTATCCTCTGCAGCAGATGTAAC
-1154 CTGGCTCTCCCTTCTCTGTGGGGTCTCATGAGGACAGTTTCACTATA
-1104 GCGCTGTGGTTTTCGCACTGCACTTGAAAGAAATTCAAAGTCTG
-1054 AAGTTCTGGATTGACTGATTGACTGACATGCTTTAGTAATGATGGA
-1004 CTGTCATTCTCTTCTGTCTTAACTGTTCTTGCCTTATATGGATT
-954 GGTATTTCACCAATAGGGGTATCTCTGTATACCCCCCTACCTTGTCACA
-904 ACACAACGTGGCTAAACGCTTAAAGAGGGAAAGAAATTCCACAAAT
-854 TAACCTTAAACATGCACACCTGTTAAATTGAAATGCAATTCCAGGTGACTA
-804 CCTCATGAATCTGGTTGAGAGAACTGCAAGAGTGTGCAAAGCTGTCA
-754 AGGGAAAGGGTTACTTTGGTTAAATCATGATTCCATATGTTGACTCTCA
-704 TAGTTGTGATGTTCTACATTATTCTACAATGTGGAAAATGTAAGGAA
SF-1 **C/EBP**
-654 AAAGAAAACCCTTGAAT **GACTAGGTGTGTC**AAACTGACTGGTACTAT
-604 ATATACAAAGTTGTTTACACAAAGTTACACTTCTCAAAAGGCCATA
-554 ATTGGAGGCATTACCATGCACTGTAAGAGGACAGAGTCACCTCTACCA
-504 AGCTTAAAGATGTCCTTACTCTTCTTAAAGATGGGATCATACTTACCAAAAGGTGGT
-454 GATCATATCATATTGTTAAAGATGGGATCATACTTACCAAAAGGTGGT
-404 TCAACACATACTGTTGACAGAATATCTGTCCAGCAACATCCAGTA
-354 ACTGTTTATTCTACATTCGGTGTGGGACCTTGGAGTGTGAGCGG
-304 ATGTCAGCTTACTGGTCTCTCTCTACTGCGCTGTGTGCA
-254 TGTCTGTGAGGGAAT**TGTGTGTGTGTGTGCGTGTGCGTGTGCGATGTG**
GATA
-204 **CGTGTGTGTGGTCAGTGTG**CCTGAGCACTGGATGTTATCTGACACAAATG
-154 TTTATTAGAGCTTGGAGACATTCTCAGCTTGTCAGGAGGTTACCTTGCCTG
SF-1/Ad4BP
-104 CACAGGGTCAATTAGAAC**GGCGGG**ATGATGTTGTT**TIA**TCTCCGAGGTG
SF-1 **C/EBP**
-54 ACAGCATCACGGC**CTTCTCC**ATGCTTATATAGACACACATGCAACT
+1
-4 CCCACAGACAGAATCTAAATCCTCTCGACAACACAAACCATCTGGCCT
+47 CTCTGTTTACAGTTCTAGGATTCTCTATTCTCTCTTGAGACACCAAG
+97 AACAAAATCGACAATCTAACGGGCTCTCG**TTT**TATATCT**TTT**TATTT
+147 TAGAAAATCTATTCACTCAGAAACTAAACACTAAATTGATACAAAATG

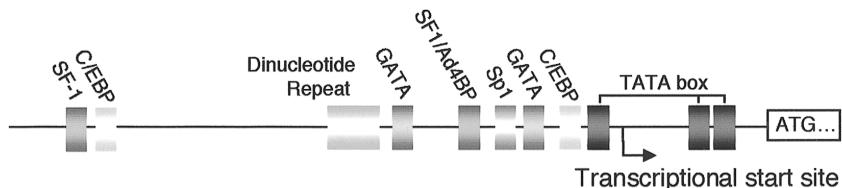


Fig. 3 Two thousand base pairs of the upstream regulatory region (Accession # AY308064) of the brook trout StAR gene. Putative transcriptional start site (arrow) upstream of the StAR open reading frame was determined using the Neural Network Promoter Prediction Program.¹⁹² Regions upstream of the transcription start site were

analyzed for putative transcriptional factor recognition sites using MatInspector (Genomatix Software)¹⁹³ and the Tfdsites.subseq. 7.0 program within MacVector 7.1 software. TATA sites are double underlined; all other sites are labeled and bolded/italicized. ATG site of cDNA is bolded and dinucleotide repeat region is underlined. The structure of the promoter region is presented schematically below the sequence.

Changes in and control of expression of StAR genes

Northern analysis of mRNA in rainbow trout ovarian follicles has revealed low, but detectable StAR mRNA levels during the greater part of vitellogenesis. StAR mRNA levels increased as vitellogenesis was completed and were about 30-fold higher in post-ovulatory follicles compared to levels in early vitellogenic follicles.¹¹⁵ However, the dynamics of StAR mRNA in rainbow trout follicles during the post-vitellogenic period have not yet been studied in detail in relation to changes in gonadotropin (GTH) secretion. Intensive sampling during final oocyte maturation showed that StAR mRNA levels were virtually non-detectable (assessed by Northern blotting) in full grown immature brook trout follicles but strongly and progressively increased during final oocyte maturation with highest expression around the time of ovulation. These studies on brook trout and rainbow trout suggest that increased StAR gene expression is required to increase steroid substrate production to support the pre-maturation surge in 17,20 β -P.¹⁰¹ StAR mRNA levels were reported to increase in Arctic char ovarian follicles at stages of development that appear to correspond to late vitellogenesis and pre-ovulation.¹⁰⁴ In male rainbow trout, a 40-fold increase in StAR mRNA in testes occurred over the course of spermatogenesis, with peak levels occurring during late spermatogenesis and spermiation.¹¹⁶ The same pattern seems to occur in Arctic char testes.¹⁰⁴

Thus far, aside from correlations between increases in StAR mRNA at times of acute increases in gonadal or interrenal steroid production,¹⁰¹ little is known about the regulation of StAR gene expression in teleosts. Compared to mammals, increases in StAR mRNA seem minor after incubation of salmonid gonadal or interrenal tissue with tropic hormones

or activators of the cAMP-PKA pathway^{102,117} (Hagen *et al.*, personal communication), although injection of ACTH led to a 2-fold increase in eel interrenal StAR mRNA.¹⁰² Similarly, acute stress led to a similar increase in trout interrenal StAR mRNA.¹⁰¹ These data suggest that post-transcriptional regulation of StAR may be important in controlling StAR activity.

P450scc

Cloning and characterization of P450scc cDNAs

A cDNA clone encoding a teleost P450scc was first isolated from a rainbow trout ovarian follicle cDNA library.¹¹⁸ The open reading frame encodes a protein of 514 amino acids with 46–48% homology with several mammalian P450scc. P450scc activity was confirmed using COS-1 cells transfected with a trout P450scc cDNA expression vector and estimation of conversion of 25-hydroxycholesterol to pregnenolone. The cDNA hybridized to a single 1.8 kb ovarian RNA transcript. Zebrafish P450scc cDNA encodes a protein of 509 amino acids that shares 78% homology with rainbow trout P450scc and 58% with human P450scc.¹¹⁹ A partial P450scc cDNA sequence that hybridized with 1.4 and 1.2 kb mRNAs in testis samples has been reported for Arctic char.¹⁰⁴

Changes in and control of expression of P450scc genes

Northern blotting did not detect P450scc transcripts in early vitellogenic ovarian follicles of rainbow trout, whereas the transcripts were barely detectable in post-vitellogenic follicles, and abundant in post-ovulatory follicles.¹¹⁸ *In situ* hybridization analysis of trout follicles showed that trends in thecal layer P450scc expression were generally similar to those for 3 β -HSD (see section on Changes in and Control of Expression of 3 β -HSD Genes; data not shown¹²⁰). In catfish ovarian follicles, P450scc transcript abundance increased progressively during vitellogenesis but decreased sharply with the completion of vitellogenesis.¹²¹ In Arctic char females, although details of stages of gametogenesis are not given, it appears that P450scc transcripts in ovarian follicles increased progressively to peak during

vitellogenesis, with a precipitous fall in fish displaying low E₂ levels (presumably post-vitellogenic). Observations on these latter two species are therefore contrary to the changes seen in rainbow trout follicles for increased expression of several steroidogenic protein genes, that likely supports increased 17,20 β -P production in the post-vitellogenic period.

P450scc transcripts in rainbow trout testes gradually increased from early to mid-spermatogenesis, rapidly increased to peak at late spermatogenesis and remained high in the spermating testis.¹¹⁶ In Arctic char testes, a pattern of increase and decline in P450scc transcripts was seen in testes, similar to that seen in ovaries.¹⁰⁴ Zebrafish P450scc is expressed temporally during development in two waves: during embryonic stages and when sexual differentiation begins. Transcripts (by RT-PCR) were found in adult testis, ovary, brain and head kidney. Within the adult ovary, transcripts were found within the ooplasm and in the thecal/granulosa follicle cells¹²² but changes with stage of oocyte development were not reported.

3 β -HSD

Cloning and characterization of 3 β -HSD cDNAs

The first cDNA clone encoding a fish 3 β -hydroxysteroid dehydrogenase $\Delta^{5,4}$ -isomerase (3 β -HSD) was isolated from a cDNA library of rainbow trout ovarian thecal cells. The cDNA hybridized to a 1.4 kb transcript isolated from rainbow trout ovaries. Recombinant trout 3 β -HSD showed a unique enzymatic 3 β -HSD activity, preferring dehydroepiandrosterone as substrate over 17 β -hydroxypregnенolone. Interestingly, recombinant trout 3 β -HSD exhibited minimal ability to convert pregnenolone to progesterone.¹²³ This observation suggests that conversion of pregnenolone to progesterone may be a minor pathway during salmonid ovarian steroidogenesis, even though progesterone production by salmonid ovarian follicles *in vitro* has been reported.¹²⁴

Recent work has identified an additional larger trout 3 β -HSD transcript in some tissues, with the heart only expressing the larger

form¹¹⁶ (see Fig. 2). Two cDNAs encoding 3 β -HSD, termed HSD 5 and HSD 17, have been isolated from zebrafish. The two forms may have resulted from alternative splicing.¹¹⁹ HSD 5 and HSD 17 encode proteins of 374 and 341 amino acids respectively. Both share 77% amino acid similarities with trout 3 β -HSD and 53% similarity with mouse 3 β -HSD. However, confirmation that the cDNAs encode functional proteins is lacking. A partial 3 β -HSD cDNA sequence has been reported for Arctic char.¹⁰⁴

Changes in and control of expression of 3 β -HSD genes

3 β -HSD transcripts were barely detectable by Northern blotting in vitellogenic rainbow trout ovarian follicles but were most abundant in fully-grown and post-ovulatory follicles.¹²³ *In situ* hybridization analysis (Fig. 4) showed that during early and mid-vitellogenesis, a relatively small number of cells within the thin thecal layer of follicles displayed weak to moderate hybridization signals for 3 β -HSD. These positive cells normally occurred singly or in pairs. However, in isolated thecal layer preparations in which thecal cells had lost their elongated profile, small nests of cells were seen, often associated with structures that appeared to be blood capillaries. Weak hybridization signals for 3 β -HSD mRNA were also sometimes found in granulosa cells. Numbers of positive thecal cells and intensity of signal increased in late-vitellogenic and post-vitellogenic follicles. Numbers and signal intensity were particularly elevated in post-ovulatory follicles, which reflects the high capacity of the salmonid post-ovulatory follicle for 17,20 β -P production. Weak hybridization signals for 3 β -HSD mRNA were also sometimes found in granulosa cells of post-ovulatory follicles. As with StAR, increased 3 β -HSD gene expression around the time of maturation and ovulation is correlated with the follicle acquiring the capacity to produce large amounts of 17,20 β -P over a relatively short time-frame. By contrast, 3 β -HSD mRNA levels were relatively stable in catfish ovarian follicles throughout ovarian recrudescence, vitellogenesis, and maturation.¹²¹ In rainbow trout testes, 3 β -HSD transcripts progressively

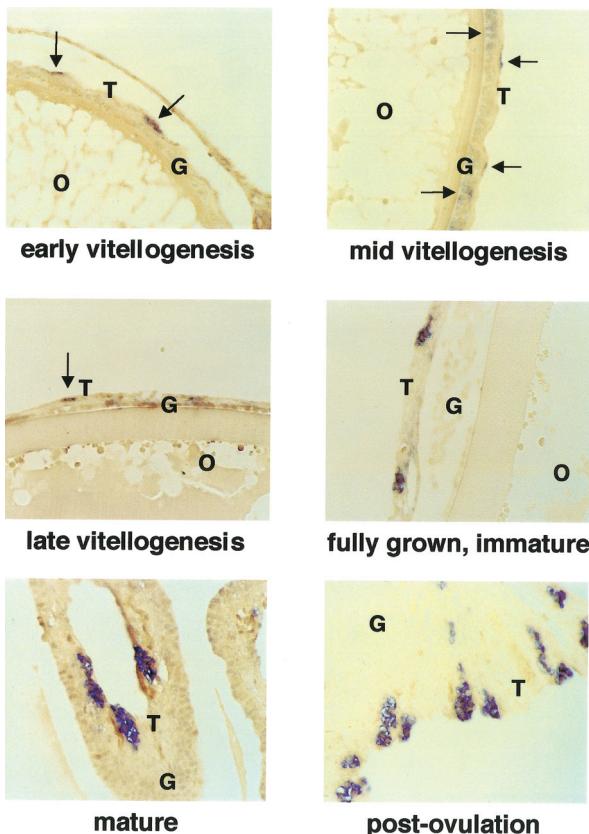


Fig. 4 *In situ* hybridization analysis of changes in 3β -HSD transcript abundance during growth and maturation of ovarian follicles of rainbow trout. Hybridization signals are indicated by the blue-purple color. T, thecal cell layer; G, granulosa cell layer; O; oocyte. Arrows indicate cells with relatively faint hybridization signals.

increased from early to late spermatogenesis and declined in the spermating testis.¹¹⁶

The trout 3β -HSD gene appears to be regulated by gonadotropins, at least partly via a cAMP-PKA-dependent pathway. Incubation of early vitellogenic trout ovarian follicles with either FSH, LH or forskolin (an activator of the cAMP-PKA pathway) resulted in striking increases in 3β -HSD mRNA and protein.^{125,126}

P450_{C17}

Cloning and characterization of P450_{C17} cDNAs

The first teleost cDNA encoding a P450_{C17} was isolated from a rainbow trout ovarian follicle cDNA library.¹²⁷ The 514 amino acid protein sequence of trout P450_{C17} displayed much greater homology with the chicken P450_{C17} sequence (64%) compared with those of human, bovine and rat proteins (46–48%). Recombinant trout P450_{C17} possesses both 17-hydroxylase and C₁₇₋₂₀lyase activities. The cDNA hybridized to a single species of ovarian mRNA (2.4 kb). Japanese eel ovarian P450_{C17} cDNA and protein have characteristics and activities similar to the trout forms.¹²⁸

Fathead minnow (*Pimephales promelas*) P450_{C17} cDNA encodes a 518 amino acid protein that shares high homology (up to 82%) with other teleost P450_{C17}, 62% with spiny dogfish P450_{C17}, 64% with chicken P450_{C17}, and up to 49% with mammalian forms.¹²⁹ Transcripts were found in the ovary and testis, and also in the brain but at levels at least 30 fold lower, at all stages of sexual development studied.

Changes in and control of expression of P450_{C17} genes

Rainbow trout P450_{C17} transcripts were not detected in early vitellogenic follicles, were barely detectable in mid-vitellogenic follicles but became increasingly abundant in follicles just prior to maturation and in post-ovulatory follicles.¹²⁷ Like other P450 steroidogenic enzymes (P450_{scc}, P450_{arom}), P450_{C17} transcript abundance increased progressively during vitellogenesis in catfish ovarian follicles but, unlike the trout follicle, decreased markedly with the completion of vitellogenesis.¹²¹ P450_{C17} transcripts in rainbow trout testes gradually increased from early to mid-spermatogenesis, rapidly increased to peak at late spermatogenesis and declined to low levels in the spermating testis.¹¹⁶

Eel P450_{C17} transcript levels significantly increased in ovarian follicles throughout artificially induced ovarian development. Since previous data showing that C₁₇₋₂₀lyase activity decreased from the vitellogenic to the maturational stage, whereas 17 α -hydroxylase activity increased, changes in C₁₇₋₂₀lyase activity (the production of androgens) may not depend

on changes in transcription of the P450_{C17} gene¹²⁸ (also see section on Steroid Synthesis).

P450_{C17} mRNA levels in the testis of the fathead minnow were negatively correlated with gonadal development, but there was no obvious association between P450_{C17} gene expression and sexual development in the ovary, or brain (in both males and females).¹²⁹

Nothing is currently reported on the endocrine regulation of P450_{C17} gene expression in fishes.

17 β -HSD

Cloning and characterization of 17 β -HSD cDNAs

Multiple forms of 17 β -HSD exist in mammals. Thus far, only complete cDNAs encoding eel 17 β -HSD type1 have been isolated from teleost fish. Eel 17 β -HSD type1 cDNAs were cloned from the ovary of the Japanese eel and the deduced amino acid sequences were approximately 50% identical to mammalian 17 β -HSD type1 proteins.¹³⁰ When expressed in HEK 293 cells, recombinant 17 β -HSD type 1 proteins showed high specificity for estrone, converting it to E₂, but neither androstenedione, nor testosterone, nor E₂ are substrates for this enzyme. The authors suggested that the substrate specificity of eel 17 β -HSD type1 indicates that a steroidogenic pathway for production of E₂ from androstenedione via estrone may exist in the Japanese eel ovary. 17 β -HSD type1 mRNA was only detected in ovary and testis. A partial tilapia 17 β -HSD type1 has been recently cloned.¹³¹

Changes in and control of expression of 17 β -HSD genes

17 β -HSD type1 mRNA was not detected in previtellogenic eel ovaries by Northern blotting.¹³⁰ However, transcript abundance increased in early vitellogenic ovaries obtained from fish artificially induced to mature using salmon pituitary homogenate injection, but thereafter did not appear to change further. Transcripts of different sizes were detected in ovarian follicles with marked variation in number and size between

follicles of individual eels.¹³⁰ Tilapia 17 β -HSD type 1 transcripts increased gradually during vitellogenesis and became faint or undetectable at the day of spawning, suggesting that the decline in E₂ at the time of maturation is partially due to decreased transcription of the tilapia 17 β -HSD type 1 gene.¹³¹

P450 Aromatase

Cloning and characterization of P450arom cDNAs

A substantial literature on P450arom is available for teleosts. A number of cDNAs encoding P450aroms have been isolated and the encoded proteins characterized to varying degrees. Distinct P450arom cDNAs derived from separate gene loci appear to be characteristic of teleosts. A recent review discusses the distinct gonadal and brain forms in detail.¹³² To avoid confusion, we will follow established terminology and refer to the predominant gonadal form as P450aromA (CYP19A gene product) and to the brain form as P450aromB (CYP19B gene product), although Kazeto *et al.*¹³³ have proposed a change in terminology to CYP19A1 and CYP19A2, respectively.

The first teleost P450arom cDNA (now known to encode a P450aromA type protein) was isolated from a rainbow trout ovarian cDNA library.¹³⁴ This cDNA is predicted to encode a protein of 522 amino acids, although like other teleost P450arom cDNAs, several potential initiation codons are present, and the true initiation codon is unknown. The trout protein shares 52–53% homology with chicken and mammalian P450arom. Recombinant trout P450arom efficiently catalyzed the conversion of testosterone to estradiol and the cDNA hybridized with 2.6 kb ovarian transcripts. Subsequently, P450arom cDNAs were cloned from catfish,¹³⁵ medaka¹³⁶ and tilapia ovary¹³⁷ and from a medaka genomic library.¹³⁸ Two distinct cDNAs encoding separate forms of P450arom were first reported in goldfish.^{139,140} P450aromA was expressed predominantly in goldfish ovary and P450aromB was expressed mainly in brain. Since then, one or two P450arom cDNAs have been cloned from a number of teleost species including: Japanese flounder

P450aromA,¹⁴¹ zebrafish P450aromA and P450aromB,¹⁴² which have been mapped to different chromosomes;^{143,144} tilapia P450aromA and P450aromB,¹⁴⁵ fathead minnow P450aromA and P450aromB,¹⁴⁶ rainbow trout P450aromB;^{147,148} European sea bass P450aromB;¹⁴⁹ and Japanese eel P450aromA.⁴⁵ The two forms of encoded proteins share only approximately 60% homology. The phylogenetic tree produced by Ijiri *et al.*,⁴⁵ based on published and unpublished teleost P450arom sequences, clearly segregates a P450aromA branch from a P450aromB branch. Within the P450aromA branch, ovarian P450arom of eel, a relatively primitive teleost, is located closest to the P450aromB branch. The P450aromB form was not identified in mRNA isolated from eel brains, and only P450aromA could be detected in this tissue. The authors speculated that the eel might exemplify a primitive/ancestral condition, possibly having only a single form of P450arom.⁴⁵ Recombinant goldfish P450aromA and B are able to catalyze the aromatization of androgens but with some differences in substrate preference and inhibition constants of a range of aromatase inhibitors.^{139,150} This suggests structural differences in the active sites of the enzymes, consistent with the differences in amino acid residues known to be functionally important.

Some species differences exist in sites of expression of the two P450arom genes in teleosts: P450aromB mRNA appears to be restricted to neural tissue (brain, pituitary, retina) of goldfish,¹³⁹ but is also found in gonads of zebrafish,¹⁴² tilapia,¹⁴⁵ fathead minnow¹⁴⁶ and rainbow trout.¹⁴⁷ Similarly, P450aromA transcripts have been found in neural tissue of goldfish,¹³⁹ zebrafish,¹⁴² rainbow trout¹⁴⁹ and eel.⁴⁵ Depending on species and P450arom type, transcripts have also been reported in various non-neural and non-gonadal tissues, such as rainbow trout gill (P450aromB),¹⁴⁹ and kidney (although it was unclear whether this sample included interrenal-containing head kidney tissue), eye (both P450aromB), and spleen (P450aromA) of tilapia.¹⁴⁵

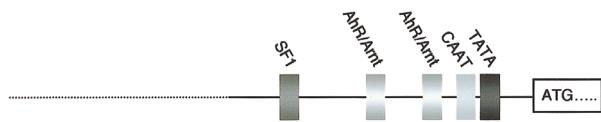
Promoter analysis has identified potential SF-1 sites and ERE half-sites in a medaka P450 gene (presumably encoding P450aromA).¹³⁸ The goldfish CYP19A gene promoter contains TATA and CAAT boxes, an SF-1 binding site, and two aryl hydrocarbon receptor nuclear translocator factor binding motifs. The latter observation is of

importance considering the potential direct effects of environmental pollutants on the steroidogenic pathway. The goldfish CYP19B gene promoter contains a TATA box, two EREs, a nerve growth factor inducible-B protein/Nur 77 responsive element containing an ERE half-site, and a sequence identical to the zebrafish GATA-2 gene neural specific enhancer. Nur 77, an orphan nuclear receptor, has been implicated in neuronal differentiation. Both genes contain numerous SRY/SOX binding sites.¹⁵¹

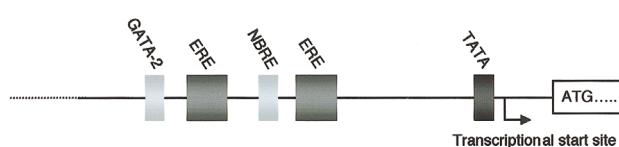
Promoter analyses of CYP19 genes of zebrafish have similarly revealed substantial differences in structure (see Fig. 5). The 5'-flanking region of CYP19A contains three CREs, an aryl hydrocarbon-responsive

Goldfish

CYP19A
(Ovary form)

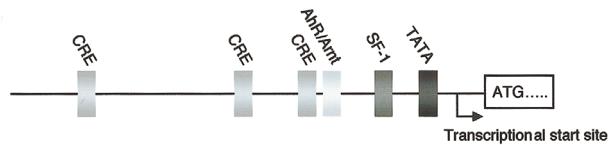


CYP19B
(Brain form)

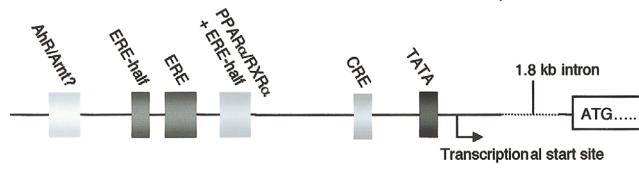


Zebrafish

CYP19A
(Ovary form)



CYP19B
(Brain form)



500 400 300 200 100 0 bp

Fig. 5 Diagrams illustrating the structure of the goldfish and zebrafish P450arom promoters, based on information in Tchoudokova *et al.*¹⁵¹ and Kazeto *et al.*¹⁷⁷ The transcription start site for goldfish CYP19A was not identified. See text for abbreviations.

element, an SF-1 site, and a TATA box. The 5'-flanking region of CYP19B contains a single CRE, an ERE, a peroxisome proliferator-activated receptor alpha/retinoid X receptor alpha heterodimer-responsive element (PPARalpha/RXRalpha), and a TATA box.¹³³

More recently, cloning and sequencing of the 5' flanking region of tilapia P450aromA has identified three CRE sites, two SF-1 binding motifs, a TATA box and five ERE half-sites. Comparison of the tilapia P450aromA promoter region with that of the medaka revealed a conserved region (218 to 230 bp) upstream from the TATA box. This conserved region included two putative SF-1 binding motifs at the same positions within both tilapia and medaka promoters.¹⁵²

Changes in and control of expression of P450arom genes

The functional significance of different types of P450arom during sexual differentiation has not been firmly established (see below). In rainbow trout, P450aromA mRNA is detectable in female gonads 3 weeks before the first sign of histological sex differentiation, with expression in male gonads being at least 100 times lower.¹⁵³ Two studies are available on the ontogeny of expression in zebrafish during sexual differentiation. In one study, P450aromA and B transcripts were first detected 3–4 days post-fertilization (dpf) and peak levels were found on 5 dpf. Although P450aromA transcripts were more abundant than P450aromB transcripts, the latter were segregated into two populations, presumably corresponding to males and females. This suggests that P450aromB transcript abundance and sexual differentiation are associated.¹⁴⁴ By contrast, P450aromB transcripts were much more abundant than P450aromA transcripts in another study on zebrafish. They both increased from 0.5–5 dpf, but P450aromB transcripts increased more rapidly and substantially.¹⁴² Although the relative abundance of the transcripts differed between these two studies, they both suggest the involvement of P450aromB in sexual differentiation of zebrafish. In both studies, exposure of developing embryos to estrogens resulted in an upregulation of P450aromB, but not P450aromA transcripts. Similarly, estrogen treatment increased P450aromB mRNA in brains of

adult goldfish.¹⁴⁰ Exposure to E₂ upregulated P450aromB mRNA in testes and ovaries of fathead minnow. No effect was seen on transcript levels in female brains, whereas an initial depression of transcript levels was followed by a significant increase in male brains.¹⁵⁴ A different scenario for the involvement of the CYP19 genes in sex differentiation comes from work on development in tilapia, using known genetic male and female stocks. Using whole body RNA extracts, P450aromB transcripts were found to increase gradually during the period of sexual differentiation, with no differences in expression pattern between the sexes. Conversely, a marked sexual dimorphism was observed in P450aromA transcript abundance. In females, P450aromA transcripts increased gradually during development. However, in males, P450aromA transcript levels decreased dramatically at 15–27 dpf. The authors interpret these data as indicating that P450aromA plays a dominant role in sexual differentiation, with a downregulation of expression needed for development of testes.¹⁴⁵ Two recent major reviews on sex determination and differentiation in fish discuss endocrine involvement in these processes.^{4,155}

Several studies have reported on changes in P450arom during oocyte growth and maturation. P450arom (the A form) mRNA was present at high levels in vitellogenic trout but in preparations from subsequent stages of ovarian development, no transcripts were detected.¹³⁴ This pattern is similar to the changes occurring in aromatase activity in salmonid follicles. Since P450arom transcripts were present only during the stage of E₂ production by the ovarian follicles, E₂ production may be regulated, in part, at the level of transcription of the P450aromA gene. *In situ* hybridization has confirmed this pattern of expression, and has also confirmed that only the granulosa cells of trout ovarian follicles express the P450aromA gene¹²⁰ (Fig. 6). Trout ovarian P450aromA expression appears to be regulated by both GTHs and IGF-1.¹²⁶

Like trout, P450aromA transcripts in tilapia ovarian follicles were low in early vitellogenic follicles, increased in mid-vitellogenic follicles and were non-detectable in post-vitellogenic follicles. Western blotting showed a similar pattern in P450aromA protein, and these changes correlated well with the ability of the follicle to convert testosterone to E₂.¹³⁷ Northern

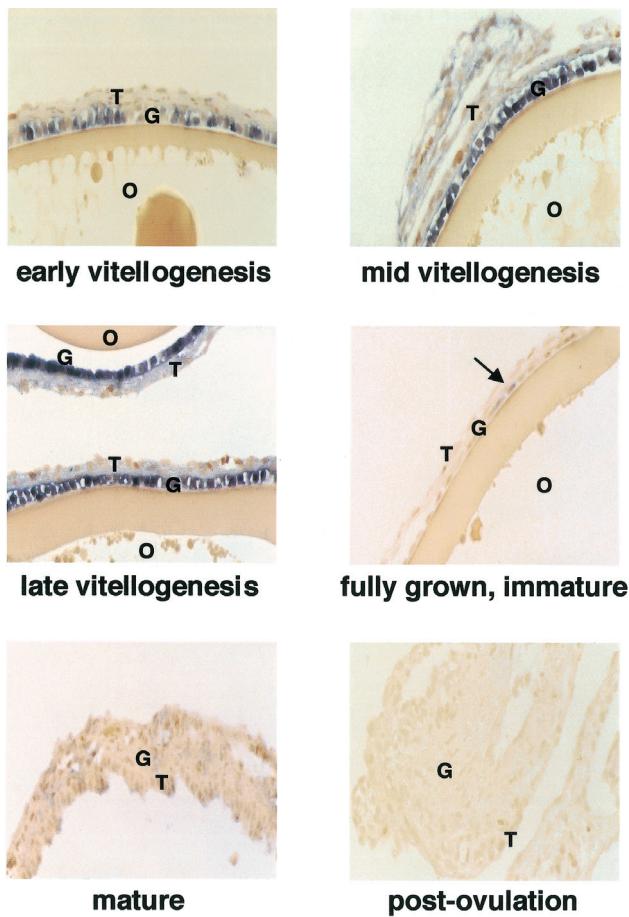


Fig. 6 *In situ* hybridization analysis of changes in P450arom transcript abundance during growth and maturation of ovarian follicles of rainbow trout. Hybridization signals are indicated by the blue-purple color. T, thecal cell layer; G, granulosa cell layer; O, oocyte. Arrows indicate cells with relatively faint hybridization signals.

blot analysis showed that the increase in P450aromA mRNA in tilapia follicles during vitellogenesis was paralleled by an increase in SF-1 mRNA, and both mRNA species declined in post-vitellogenic follicles, suggesting that SF-1 is involved in the transcriptional regulation of the tilapia P450aromA gene. Incubation of late vitellogenic follicles with hCG resulted in increased

abundance of P450aromA transcripts but little change in SF-1 transcripts, whereas incubation of post-vitellogenic follicles with hCG resulted in strong decreases in both P450aromA and SF-1 transcript levels.¹⁵² In mammals, the LH surge-induced reduction in P450arom gene expression in granulosa cells is similarly accompanied by a reduction in SF-1 transcripts.¹⁵⁶ The role of SF-1 in mediating potential FSH effects on P450aromA in the vitellogenic period remains to be established. SF-1 has also been implicated in regulating P450aromA in medaka ovarian follicles.¹⁵⁷

Similarly, increases in P450aromA transcripts in the ovary of red seabream correlated well with enzyme activity, with P450aromA mRNA increasing in abundance as vitellogenesis progressed in ovarian follicles and becoming undetectable in post-vitellogenic stages.¹⁵⁸ LH and IGF-1 both increased P450aromA mRNA levels in fragments of red seabream ovary.¹⁵⁹ In catfish and eel, ovarian P450aromA transcripts also correlated well with the production of E₂, levels being high during vitellogenesis in catfish¹²¹ and during the post-vitellogenic stage in eel.⁴⁵

Relatively less is known about the P450aromB form in relation to reproductive cycles. Goldfish P450aromB, which appears to be restricted to neural tissue, varied seasonally in the brain, with a peak in transcripts at the time of gonadal recrudescence, and higher transcript levels in reproductive fish.¹⁴⁰ In fathead minnow testes and, to a lesser extent, ovaries, P450aromB mRNA transcripts were negatively correlated with gonadal development, though with considerable variation between individuals, whereas a positive relationship was seen between female brain transcripts and ovarian development.¹⁵⁴ Thus, the contribution of expression of the P450aromB gene in ovarian follicles during vitellogenesis to circulating E₂ levels is unclear.

P450_{11β}

Cloning and characterization of P450_{11β} cDNAs

A cDNA encoding a teleost P450_{11β} was first isolated from the Japanese eel (*Anguilla japonica*) testis. The cDNA encodes a protein

of 511 amino acids that shares 38–48% homology with those of mammals and frog.¹⁶⁰ Northern blotting revealed a single 1.8 kb transcript expressed in testis and interrenal tissue. Expression of the cDNA in COS-1 cells confirmed that the protein had P450_{11 β} activity when testosterone was provided as substrate. Interestingly, the protein efficiently catalyzed the conversion of 11-deoxycorticosterone into corticosterone and 11-dehydrocorticosterone but no aldosterone was detected. Similarly, deoxycortisol was converted into cortisol and cortisone.¹⁶¹ These results show that unlike P450_{11 β} of frogs and several mammals, eel P450_{11 β} does not possess significant aldosterone synthase activity, reflecting the absence of significant quantities of aldosterone in teleosts.

Two cDNAs encoding rainbow trout P450_{11 β} have been isolated and characterized (P450_{11 β} type 2¹⁶²; P450_{11 β} ¹⁶³). The sequences of the encoded proteins differ by 15 amino acids and share 97% homology with one another, 65% homology with eel P450_{11 β} , and 33–45% homology with rat, human, and frog P450_{11 β} . Higher homology of 60–80% between the trout sequence and other sequences exists between the four different putative binding regions which are common in P450 enzymes: the steroid binding site; the oxygen binding site; the heme/steroid binding site; and the heme binding site.¹⁶³ The sequences of amino acids forming these sites are 100% identical between the two trout forms. Recombinant trout P450_{11 β} type 1 showed 11 β -hydroxylating activity when provided with exogenous testosterone.¹⁶³ Transcripts (1.8 kb) encoding trout P450_{11 β} have been found in testis and head kidney, with weaker signals in posterior kidney and spleen, but were absent in vitellogenic and post vitellogenic ovarian follicles. *In situ* hybridization revealed that transcripts were confined to testis Leydig cells (Fig. 7) and interrenal cells of the head kidney.¹⁶³ By contrast, vitellogenic ovarian follicles of Japanese eel contain relatively high levels of P450_{11 β} transcripts, consistent with the ability of this tissue to produce 11-KT *in vitro*.¹⁶⁴ Both P450_{11 β} activity and mRNA levels correlated with serum 11-KT levels, suggesting that the main source of high levels of 11-KT in female eels¹⁶⁵ is the ovary.¹⁶⁴

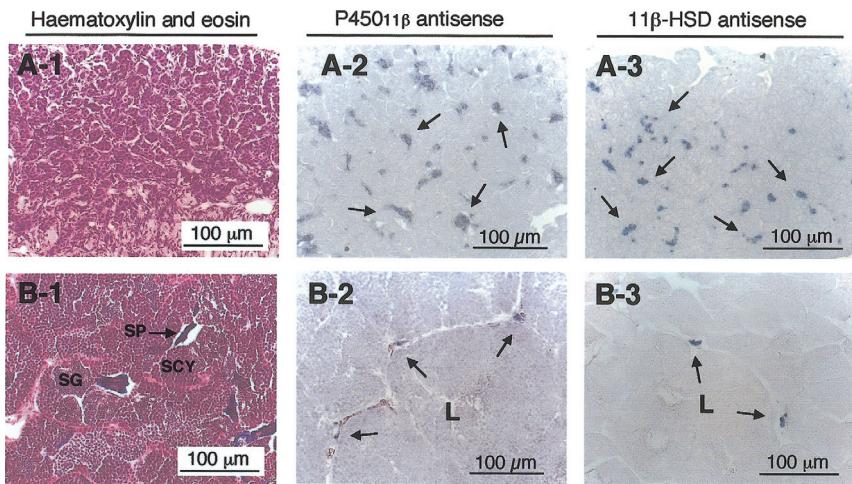


Fig. 7 Localization of $P450_{11\beta}$ and 11β -HSD type 2-like mRNA in juvenile (pre-spermatogenic, A2, A3) and maturing (B2, B3) rainbow trout testes. Hybridization signals (arrows) are indicated by the blue-purple color. Signals were confined to interstitial Leydig cells (L).

Changes in and control of expression of $P450_{11\beta}$ genes

During sexual differentiation of rainbow trout, $P450_{11\beta}$ mRNA was detectable in genetic males but not genetic females 3 weeks before morphological signs of gonadal sex differentiation were seen. Transcripts were abundant long after testis differentiation had occurred, suggesting $P450_{11\beta}$ gene expression plays a key role in testis differentiation.¹⁶² Signals were very weak (at least a 100 fold lower) in sexually differentiating females, assessed by RT-PCR.¹⁶² In adult rainbow trout males, two studies have reported increased abundance of $P450_{11\beta}$ transcripts in testes in early spermatogenesis, followed by declining levels thereafter, with no correlation with circulating androgens.^{162,163} However, these observations probably reflect ‘dilution’ of $P450_{11\beta}$ mRNA by the large amount of germ cell mRNAs being synthesized in later stages of spermatogenesis;^{162,163} when transcript abundance was expressed as total transcripts per testis, mRNA levels and plasma

androgens displayed a very similar pattern.¹⁶³ In eel, no expression of the P450_{11β} gene was seen in testes from immature eel but transcript abundance peaked 3 days after injection of eels with hCG, at a time when 11-KT levels increased.¹⁶⁰ Thus, in both species, regulation of 11-KT synthesis appears to depend in part on the regulation of transcription of P450_{11β} genes.

11β-HSD

Cloning and characterization of 11β-HSD cDNAs

Two distinct forms of 11β-HSD have been found in mammals. 11β-HSD type 1 is a low affinity, NADP(H)-dependent dehydrogenase/11-oxo-reductase found principally in glucocorticoid target tissues. In contrast, 11β-HSD type 2 is a high affinity, NAD-dependent 11β-dehydrogenase localized mainly in classical mineralocorticoid target tissues such as kidney and colon. In these tissues, 11β-HSD type 2 performs the key physiological role of protecting the non-selective mineralocorticoid receptor from overstimulation by glucocorticoids by converting the C₁₁-hydroxyl group of bioactive glucocorticoids (cortisol and corticosterone) to a keto group characteristic of inert glucocorticoids (cortisone and 11-dehydrocorticosterone) (see Kusakabe *et al.*¹⁶⁶ for references). In teleosts, only 11β-HSD type 2-like cDNAs have been cloned. Eel and tilapia 11β-HSD cDNAs cloned from testes¹⁶⁷ and rainbow trout 11β-HSD cloned from testis and head kidney¹⁶⁶ share 60–70% homology. The fish proteins exhibit 40–43% homology with mammalian 11β-HSD type 2, and about 20% homology with human and rat 11β-HSD type 1. Interestingly, the eel 11β-HSD is 19 amino acids shorter than other fish and mammalian type 2 enzymes in the N-terminus. Classification of the rainbow trout 11β-HSD as a type 2 enzyme is based on higher homology of the putative cofactor binding region with mammalian type 2 11β-HSDs, a hydropathy profile similar to those of mammalian 11β-HSD type 2, and demonstration that the recombinant protein possesses 11β-dehydrogenase activity but not 11-oxo-reductase activity, using corticosteroid and androgen substrates. Nonetheless, the

effects of co-factor abundance (reduced versus oxidized forms of NAD(P)) on the direction of the reaction have not been determined. Structurally, the eel and tilapia 11 β -HSDs also appear to be type 2 enzymes, and recombinant eel 11 β -HSD exhibits 11 β -dehydrogenase activity using corticosteroid substrates, although its enzymatic activity with 11-oxyandrogen substrates has not been determined.¹⁶⁷ A 3.5 kb transcript hybridized with the trout 11 β -HSD cDNA and was detectable in a wide variety of tissues by Northern blotting. However, positive *in situ* hybridization signals were seen only in testis (Fig. 7) and ovary (see below) and interrenal cells. Similarly, cDNA probes hybridize to 2.7 kb (eel) and 3.8 kb (tilapia) transcripts, and a widespread pattern of expression of 11 β -HSD type 2 cDNAs has been found in eel and tilapia tissues, with highest expression occurring in testes.

Changes in and control of expression of 11 β -HSD genes

In situ hybridization revealed strong signals in Leydig cells and weaker signals in head kidney interrenal cells from juvenile rainbow trout. Two different types of cell expressing 11 β -HSD type 2 were found in trout ovarian follicles. Strong signals were found in some theca cells of the early vitellogenic ovarian follicle, corresponding to steroid-producing cells identified in other studies, and in some theca and in all granulosa cells of the mid-late vitellogenic and post-ovulatory follicles. Seasonal changes in rainbow trout 11 β -HSD type 2 mRNA in testes and ovary, analyzed by Northern blot and *in situ* hybridization, showed a pattern that was more related to that of stress-induced plasma cortisol levels than to plasma 11-KT levels.¹⁶⁶ Thus, 11-KT production by the trout testis does not appear to be strongly regulated through transcription of the 11 β -HSD gene, although the reverse appears to be the case for P450_{11 β} . Conversely, increases in 11 β -HSD type 2 mRNA in eel testes occurred within one day of injection of hCG into immature eels, with a time-course similar to that of the increase in circulating 11-KT.¹⁶⁷ The presence of substantial 11 β -HSD transcripts in trout ovarian follicles (which do not express P450_{11 β} and therefore appear to be incapable of producing 11 β -hydroxyandrogens) raises the possibility of a role for the enzyme in the

protection of developing gonads from the inhibitory effects of stress-induced cortisol by converting it into the inactive metabolite, cortisone. By contrast, expression of the 11 β -HSD type 2 gene in tilapia ovary seems relatively low compared to that in the testis.¹⁶⁷

Although teleosts are considered to produce only one major bioactive corticosteroid, cortisol, that serves both glucocorticoid and mineralocorticoid roles, cDNAs encoding distinct glucocorticoid and mineralocorticoid receptors have been isolated from rainbow trout^{168–170}. It is not known whether these receptors have separate native ligands but it would be of considerable interest to determine whether, like in mammals, the fish mineralocorticoid receptor and 11 β -HSD type 2 are functionally linked.

20 β -HSD

Cloning and characterization of 20 β -HSD cDNAs

Two cDNAs (type A and type B) encoding carbonyl reductase-like 20 β -HSDs (CR/20 β -HSD) have been cloned from a rainbow trout ovarian cDNA library.⁹⁷ The encoded proteins differ by only three amino acids. Genomic DNA analysis showed that the two CR/20 β -HSD cDNAs are derived from different genes. Northern blot and RT-PCR analysis demonstrated that trout CR/20 β -HSDs are broadly expressed in various tissues. Studies to characterize the catalytic properties of recombinant proteins showed that the product of the type A cDNA possesses both 20 β -HSD and CR activity, but the type B protein was inactive in both assays.⁹⁷ Among the three different residues between the protein products encoded by the two cDNAs, two residues (positions 15 and 27) are located in the coenzyme binding site. Site-directed mutagenesis demonstrated that Ile-15 (present in type A, substituted with Thr-15 in type B) is critical for enzyme binding of the cofactor NADPH.¹⁷¹ cDNAs encoding CR-like 20 β -HSDs with high homology to the trout forms have subsequently been cloned from ovaries of tilapia,¹⁷² ayu¹⁷³ and zebrafish.¹⁷⁴ Like the trout forms, these cDNAs are expressed in a wide range of tissues. The tilapia and ayu forms

encode proteins that exhibit both 20β -HSD and CR activity, while the catalytic properties of the zebrafish protein have not been determined.

Changes in and control of expression of 20β -HSD genes

Earlier work clearly demonstrated that gonadotropins stimulate 20β -HSD activity in the granulosa cells of salmonid ovarian follicles, through a cAMP-dependent mechanism that is blocked by inhibitors of transcription and translation.¹⁷⁵ However, recent work on post-vitellogenetic rainbow trout follicles has reported that 20β -HSD transcript levels (primers apparently amplified both CR-like 20β -HSDs) do not change in association with the acquisition of maturation competence, nor do they increase as follicles undergo final maturation, even though $17,20\beta$ -P levels increased by about 30–40-fold during this time.¹⁷⁶ These authors suggest that this increase in $17,20\beta$ -P levels in rainbow trout is not mediated by increases in transcription of the 20β -HSD gene(s) but possibly by the activity of other steroidogenic enzymes or the availability of substrate. Whether the amplification of both the active type A form and the inactive type B form (which appears to be constitutively expressed) may partly explain the lack of observable change during a period when 20β -HSD activity is known to increase is not known. By contrast, the pattern of expression of the cDNAs for the tilapia and ayu CR/ 20β -HSDs in ovarian tissue increased in fully-grown, maturing ovarian follicles, consistent with the proposed role of CR-like 20β -HSDs in the production of $17,20\beta$ -P in response to GTHs. In addition, *in vivo* treatment of eels with salmon pituitary extract increased ovarian 20β -HSD activity¹⁷⁷ and incubation of tilapia ovarian follicles with hCG *in vitro* increased CR/ 20β -HSD mRNA levels.¹⁷² However, the zebrafish form appears to be constitutively expressed in ovarian follicles. Wang and Ge¹⁷⁴ suggest that unlike tilapia and ayu, gonadotropin-induced $17,20\beta$ -P production and final oocyte maturation in the zebrafish may either not involve significant change in expression of the CR/ 20β -HSD cDNA they isolated, or it may involve other isoforms whose expression is regulated by GTHs and other factors. 20β -HSD activity in testes has been associated both with germ cells

and with somatic cells (probably Leydig cells) but no information is currently available on changes in or control of CR/20 β -HSD gene expression during spermatogenesis.

Extragonadal Sources of Sex Steroids

Not surprisingly, study of the production of sex steroids has centered around gonadal physiology. However, a number of reports have indicated that tissues other than the gonads may play an important role in the production of steroids in teleost fish. In the section on Steroid Metabolism and Excretion, the ability of gills, kidney and liver to modify steroids, particularly as a means to excrete them as inactive metabolites or as steroid-conjugated pheromones, has been highlighted (also see Ref. 99). Skin may also play a pheromone-secreting function, at least in African catfish.¹⁷⁸

Extragonadal tissues have been implicated not only in the production of inactive metabolites or steroid conjugates, but also in the production of bioactive steroids. Thus, rainbow trout¹⁷⁹ and catfish interrenal tissue¹⁸⁰ secrete MIS, 17,20 β -P, *in vitro*. In the presence of substrate, rainbow trout milt and gill were similarly capable of producing small amounts of 17,20 β -P *in vitro*, whereas gill tissue had this ability in goldfish¹⁸¹ and carp.¹⁸² The presence of 20 β -HSD transcripts in a wide variety of trout and zebrafish tissues, most notably in gill, liver and kidney,^{97,174} is in general keeping with these *in vitro* observations, although enzymatic activity has only been demonstrated in gill and interrenal tissue. Ebrahimi *et al.*,¹⁸³ in discussing the presence of 20 α -HSD activity in several goldfish tissues, cautioned that their findings should be interpreted with care; they argued that substrate levels may often not be elevated sufficiently in extragonadal tissues for substantial MIS production to occur.

Does extragonadal synthesis of steroids ever substantially contribute to the total pool of bioactive steroids? In African catfish it clearly does, as illustrated by the findings of Cavaco *et al.*¹⁸⁴ Catfish testes do not appear to produce 11-KT *in vitro*, but instead, secrete large amounts of 11 β -hydroxyandrostenedione (11 β -OHA). *In vivo*, 11-KT is

prominently present, prompting the search for extragonadal sites of 11β -HSD and 17β -HSD activity by these authors. Several tissues, most notably the liver and seminal vesicles, had the ability to catalyze the conversion of 11β -OHA into 11-KT. This is perhaps not surprising given the widespread abundance of 11β -HSD transcripts amongst tissues in trout¹⁶⁶ and the seemingly common presence of 17β -HSD in blood (c.f. Refs. 185 and 186). Extragonadal conversion of testicular 11-oxyandrogen substrate to 11-KT has similarly been proposed for sticklebacks (*Gasterosteus aculeatus*)¹⁸⁶, whereas carp gills produced small amounts of 11-KT from tritiated 17-hydroxyprogesterone *in vitro*.¹⁸² In contrast, elevated levels of 11β -OHA (0.9 ng/ml), thought to reflect capture stress, did not result in measurable amounts of 11-KT in immature kingfish (*Seriola lalandi*). Likewise, only small amounts of 11-KT (<0.5 ng/ml) were found *in vivo* in stressed yellow-eyed mullet (*Aldrichetta forsteri*) in which 11β -OHA levels reached 3.0 ng/ml.¹⁶⁵

Extragonadal production of estrogens has long been known to occur, and is usually associated with brain-pituitary aromatase activity (see a study on turtles by Callard *et al.*,¹⁸⁷ also see section on P450 aromatase). However, there is some evidence for aromatase activity in other tissues, most notably fat. Thus, gonad-associated fat from black carp, *Mylopharyngodon piceus*, was capable of *de novo* secretion of E₂ *in vitro*, thought to reflect a common ancestry of these cells with ovarian steroidogenic cells.¹⁸⁸ The presence of aromatase transcripts in extragonadal tissues, such as gill, spleen and kidney (section on P450 aromatase) has also been documented, but activities have not been assessed.

Although the ability of the brain of teleosts to produce E₂ is well-established, the production of other steroids has not been documented, but several pieces of indirect evidence suggest that the teleost brain, like the mammalian brain, has the potential to produce other steroids, based both on the identification of transcripts for some of the steroidogenic proteins¹²⁹ (see section on Molecular Cloning and Characterization of Steroidogenic Proteins), and the immunohistochemical identification of 3β -HSD protein in the brain of zebrafish,¹⁸⁹ and in the brain of African lungfish, along with 5α -reductase.¹⁹⁰ Whether the teleost brain is capable

of *de novo* synthesis of sex steroid from cholesterol is less certain: although StAR mRNA has been identified in eel brain, for example,¹⁰² P450_{scc} transcripts have not been detected in rainbow trout brain¹¹⁶ (Fig. 2). The regulation of the various steroidogenic protein genes in sex-steroid producing extragonadal tissues has not been addressed. Are these genes constitutively expressed? Or, are some of the activities regulated by the endocrine system?

Summary and Conclusions

Considerable progress has been made over the last two decades in identifying bioactive steroids in teleosts and their sites of synthesis, and in determining some of their actions on reproductive processes. Understanding of the molecular processes underlying the temporal, quantitative and differential changes in sex steroids during sexual differentiation and reproduction is much less complete. Indeed, it is remarkable that our knowledge of the regulation of expression of many of the steroidogenic protein genes has not improved as much as might have been expected since the pioneering work of Nagahama and colleagues in the early-mid 1990s on the cloning of teleost cDNAs encoding a number of steroidogenic enzymes and transcription factors. Advances in methodology have made the task of reliably quantifying mRNA species routine for many laboratories. However, an underlying assumption in many of the recent studies is that an increase in a particular target mRNA indicates an increase in the final product of gene expression, a functional protein. While this might be largely accurate, this assumption is seldom tested, partly because of the lack of specific antisera suitable for protein quantification through Western blotting, and partly because the biochemical assessment of enzyme activity is not always straightforward. Studies on post-transcriptional regulation of expression of steroidogenic protein genes are sorely lacking. Although only a few comprehensive studies on steroidogenic protein expression (i.e., usually measurement of mRNAs and correlation with circulating steroid levels) are currently available, it is notable that mRNA

levels, levels of particular steroids, and steroidogenic capacity of gonadal tissue are sometimes not obviously correlated.

We also note that some reports on the cloning of steroidogenic protein cDNAs have based identification of encoded proteins largely on homology with related proteins. However, some caution should be exercised since substitution of a single isoleucine by threonine for rainbow trout CR/20 β -HSD results in a protein totally lacking CR or 20 β -HSD activity, despite the fact that this inactive protein is constitutively expressed at what appears to be relatively high levels in gonadal and non-gonadal tissue.

A shift in the steroidogenic pathway from androgens/estrogens during vitellogenesis or spermatogenesis to progestogens during final maturation appears to be a stereotypical feature of many, but not all teleosts studied to date, with a decline in aromatase activity and an increase in 17 α -hydroxylase activity apparently forming the basis for this shift. Information needed to understand this process more fully includes determining the role of GTHs and other factors in regulating P450aromA expression, and determining how 17 α -hydroxylase activity predominates over C₁₇₋₂₀lyase activity in the same enzyme at the time of gamete maturation. These other factors potentially include signalling molecules from the developing germ cells, and, based on the evidence for short loop feedback of steroids on steroidogenesis in the testes of goldfish and catfish, local effects of steroids. Despite the emphasis of research on the impacts of xenobiotics on reproductive processes in teleosts, we still have little understanding of the role of endogenous steroids in directly regulating steroidogenic protein gene expression, with the notable exception of estrogenic effects on P450aromB expression. Since StAR is the true rate-limiting step in steroid production, its potential as a target for xenobiotics deserves particular attention, as it has been shown to be a specific target of certain xenobiotics in mammalian studies (see Ref. 191). Given the impact of stress on reproductive processes, the effects of stress hormones on gonadal steroidogenic protein expression are similarly of importance.

In summary, researchers in the field of fish reproduction are now in a position, both methodologically and conceptually, to address many of

the questions that have arisen over the past two decades on the regulation of steroidogenic proteins in teleost ovaries and testes and the role of steroids in reproductive processes. Increased understanding of the molecular basis for the seasonal patterns in sex steroids, for example, should also lead to improvement in the ability both to understand the basis for and solutions to some of the reproductive problems prevalent in aquacultured species, and to document and predict the impact of endocrine-disrupting xenobiotics on reproductive processes.

References

1. Kime DE (1993). "Classical" and "non-classical" reproductive steroids in fish. *Rev. Fish. Biol. Fish.* **3**: 160–180.
2. Idler DR and Safe LM (1972). Separation of steryl acetates by silver ion chromatography. *Steroids* **19**: 315–324.
3. Fostier A, Jalabert B, Billard R, Breton B and Zohar Y (1983). The gonadal steroids. In: Hoar WS, Randall DJ and Donaldson EM (eds.), *Fish Physiology Vol IX A*, Academic Press, New York, pp. 277–372.
4. Devlin RH and Nagahama Y (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological and environmental influences. *Aquaculture* **208**: 191–364.
5. Lambeth JD (1990). Enzymology of mitochondrial side-chain cleavage by cytochrome P-450scc. In: Ruckpaul K and Rein H (eds.), *Frontiers in Biotransformation, Vol. 3: Molecular Mechanisms of Adrenal Steroidogenesis and Aspects of Regulation and Application*, Taylor & Francis, London, pp. 58–100.
6. Stocco DM (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annu. Rev. Physiol.* **63**: 193–213.
7. Clark BJ, Wells J, King SR and Stocco DM (1994). The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. *J. Biol. Chem.* **269**: 28314–28322.
8. Stocco DM (2002). Clinical disorders associated with abnormal cholesterol transport: mutations in the steroidogenic acute regulatory protein. *Mol. Cell. Endocrinol.* **191**: 19–25.
9. Thomson M (2003). Does cholesterol use the mitochondrial contact site as a conduit to the steroidogenic pathway? *Bioessays*. **25**: 252–258.

10. Tsujishita Y and Hurley JH (2000). Structure and lipid transport mechanism of a StARrelated protein. *Nat. Struct. Biol.* **7**: 40814.
11. Mathieu AP, Fleury A, Ducharme P and LeHoux J-G (2003). Insights into steroidogenic acute regulatory protein (StAR)-dependent cholesterol transfer in mitochondria: evidence from molecular modeling and structure-based thermodynamics supporting the existence of partly unfolded states of StAR. *J. Mol. Endo.* **29**: 327–345.
12. Bose HS, Whittal RM, Baldwin MA and Miller WL (1999). Related articles, links free in PMC: The active form of the steroidogenic acute regulatory protein, StAR, appears to be a molten globule. *Proc. Natl. Acad. Sci. USA* **96**: 7250–7255.
13. Auchus RJ and Miller WL (1999). Molecular modeling of human P450c17 (17 α -hydroxylase/17,20-lyase): insights into reaction mechanisms and effects of mutations. *Mol. Endocrinol.* **13**: 1169–1182.
14. Miller WL, Auchus RJ and Geller DH (1997). The regulation of 17,20 lyase activity. *Steroids* **62**: 133–142.
15. Soucy P, Lacoste L and Luu-The V (2003). Assessment of porcine and human 16-ene-synthase, a third activity of P450c17, in the formation of an androstenol precursor. Role of recombinant cytochrome b5 and P450 reductase. *Eur. J. Biochem.* **270**: 1349–1355.
16. Swart P, Lombard N, Swart AC, van der Merwe T, Murry BA, Nicol M and Ian Mason J (2003). Ovine steroid 17 α -hydroxylase cytochrome P450: characteristics of the hydroxylase and lyase activities of the adrenal cortex enzyme. *Arch. Biochem. Biophys.* **409**: 145–152.
17. Barry TP, Aida K, Okumura T and Hanyu I (1990). The shift from C-19 to C-21 steroid synthesis in spawning male common carp, *Cyprinus carpio*, is regulated by the inhibition of androgen production by progestogens produced by spermatozoa. *Biol. Reprod.* **43**: 105–112.
18. Sakai N, Ueda H, Suzuk N and Nagahama Y (1989). Steroid production by amago salmon (*Oncorhynchus rhodurus*) testes at different development stages. *Gen. Comp. Endocrinol.* **75**: 231–240.
19. Abdullah MA and Kime DE (1994). Increased substrate concentration causes a shift from production of 11-oxygenated androgens to 17,20-dihydroxy-progestogens during the *in vitro* metabolism of 17-hydroxyprogesterone by goldfish testes. *Gen. Comp. Endocrinol.* **96**: 129–139.

20. Lee ST, Kime DE, Lam TJ and Tan CH (1998). Synthesis of 17,20 α / β -dihydroxy-4-pregn-3-one and 5 β -pregnanes in spermatozoa of primary and 17 α -methyltestosterone-induced secondary male grouper (*Epinephelus coioides*). *Gen. Comp. Endocrinol.* **112**: 1–9.
21. Vizziano D, Fostier A, Le Gac F and Loir M (1996). 20 β -hydroxysteroid dehydrogenase activity in nonflagellated germ cells of rainbow trout testis. *Biol. Reprod.* **54**: 1–7.
22. Loir M (1990). Trout steroidogenic testicular cells in primary culture. II. Steroidogenic activity of interstitial cells, Sertoli cells, and spermatozoa. *Gen. Comp. Endocrinol.* **78**: 388–398.
23. Vizziano D, Le Gac F and Fostier A (1995). Synthesis and regulation of 17 α -hydroxy-20 β -dihydroprogesterone in immature males of *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **14**: 289.
24. Kobayashi D, Tanaka M, Fukada S and Nagahama Y (1996). Steroidogenesis in the ovarian follicles of the medaka (*Oryzias latipes*) during vitellogenesis and oocyte maturation. *Zool. Sci.* **13**: 921–927.
25. Ueda H, Young G, Crim LW, Kambegawa A and Nagahama Y (1983). 17 α ,20 β -dihydroxy-4-pregn-3-one: plasma levels during sexual maturation and *in vitro* production by the testes of amago salmon (*Oncorhynchus rhodurus*) and rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.* **51**: 106–112.
26. Young G, Crim LW, Kagawa H, Kambegawa A and Nagahama Y (1983). Plasma 17 α ,20 β -dihydroxy-4-pregn-3-one levels during sexual maturation of amago salmon (*Oncorhynchus rhodurus*): correlation with plasma gonadotropin and *in vitro* production by ovarian follicles. *Gen. Comp. Endocrinol.* **51**: 96–105.
27. Barry TP, Santos AJ, Furukawa K, Aida K and Hanyu I (1990). Steroid profiles during spawning in male common carp. *Gen. Comp. Endocrinol.* **80**: 223–231.
28. Slater CH, Schreck CB and Swanson P (1994). Plasma profiles of the sex steroids and gonadotropins in maturing female spring chinook salmon (*Oncorhynchus tshawytscha*). *Comp. Biochem. Physiol.* **109A**: 165–175.
29. Kanamori A, Adachi S and Nagahama Y (1988). Developmental changes in steroidogenic responses of ovarian follicles of amago salmon (*Oncorhynchus rhodurus*) to chum salmon gonadotropin during oogenesis. *Gen. Comp. Endocrinol.* **72**: 13–24.

30. Rahman MA, Ohta K, Chuda H, Nakano S, Maruyama K and Matsuyama M (2001). Gonadotropin-induced steroidogenic shift towards maturation-inducing hormone in Japanese yellowtail during final oocyte maturation. *J. Fish Biol.* **58**: 462–474.
31. Ohta K, Yamaguchi S, Yamaguchi A, Gen K, Okuzawa K, Kagawa H and Matsuyama M (2002). Biosynthesis of steroids in ovarian follicles of red seabream, *Pagrus major* (*Sparidae, Teleostei*) during final oocyte maturation and the relative effectiveness of steroid metabolites for germinal vesicle breakdown in vitro. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **133B**: 45–54.
32. Matsuyama M, Adachi S, Nagahama Y, Maruyama K and Matsura S (1990). Diurnal rhythm of serum steroid hormone levels in the Japanese whiting, *Sillago japonica*, a daily-spawning teleost. *Fish Physiol. Biochem.* **8**: 329–338.
33. Pankhurst NW and Carragher JF (1991). Seasonal endocrine cycles in marine teleosts. In: Scott AP, Sumpter JP, Kime DE and Rolfe MS (eds.), *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*, FishSymp 91, Sheffield, pp. 131–135.
34. Kime DE and Abdullah MA (1994). The *in vitro* metabolism of 17-hydroxyprogesterone by ovaries of the goldfish, *Carassius auratus*, is affected by substrate concentration. *Gen. Comp. Endocrinol.* **95**: 109–116.
35. Ijiri S, Kazeto Y, Takeda N, Chiba H, Adachi S and Yamauchi K (1995). Changes in serum steroid hormones and steroidogenic ability of ovarian follicles during artificial maturation of cultivated Japanese eel, *Anguilla japonica*. *Aquaculture* **135**: 3–16.
36. Lokman PM and Young G (1995). *In vitro* biosynthesis of oestradiol-17 β and 17 α , 20 β -dihydroxy-4-pregn-3-one by vitellogenic ovarian follicles from migrating New Zealand longfinned eels (*Anguilla dieffenbachii*). *Aquaculture* **135**: 17–26.
37. Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T and Katsu Y (1995). Regulation of oocyte growth and maturation in fish. *Curr. Top. Dev. Biol.* **30**: 103–145.
38. Young G, Adachi S and Nagahama Y (1986). Role of ovarian thecal and granulosa layers in gonadotropin-induced synthesis of a salmonid maturation-inducing substance (17 α , 20 β -dihydroxy-4-pregn-3-one). *Dev. Biol.* **118**: 1–8.

39. Young G, Kagawa H and Nagahama Y (1983). Evidence for a decrease in aromatase activity in the ovarian granulosa cells of amago salmon (*Oncorhynchus rhodurus*) associated with final oocyte maturation. *Biol. Reprod.* **29:** 310–315.
40. Sakai N, Iwamatsu T, Yamauchi K, Suzuki N and Nagahama Y (1988). Influence of follicular development on steroid production in the medaka (*Oryzias latipes*) ovarian follicle in response to exogenous substrates. *Gen. Comp. Endocrinol.* **71:** 516–523.
41. Kagawa H, Young G and Nagahama Y (1984). *In vitro* estradiol-17 β and testosterone production by ovarian follicles of the goldfish. *Gen. Comp. Endocrinol.* **54:** 139–143.
42. Afonso LO, Iwama GK, Smith J and Donaldson EM (1999). Effects of the aromatase inhibitor Fadrozole on plasma sex steroid secretion and ovulation rate in female coho salmon, *Oncorhynchus kisutch*, close to final maturation. *Gen. Comp. Endocrinol.* **113:** 221–229.
43. Kobayashi M, Sorensen PW and Stacey NE (2002). Hormonal and pheromonal control of spawning behavior in the goldfish. *Fish Physiol. Biochem.* **26:** 71–84.
44. Petrino TR, Hoch KL, Lin Y WP and Wallace RA (1990). Steroidogenesis in Fundulus heteroclitus III. Evidence for involvement of cAMP and protein synthesis in the gonadotropic modulation of ovarian steroid production and aromatase activity. *J. Exp. Zool.* **253:** 177–185.
45. Ijiri S, Kazeto Y, Lokman PM, Adachi S and Yamauchi K (2003). Characterization of a cDNA encoding P-450 aromatase (CYP19) from Japanese eel ovary and its expression in ovarian follicles during induced ovarian development. *Gen. Comp. Endocrinol.* **130:** 193–203.
46. Zhu Y, Rice CD, Pang Y, Pace M and Thomas P (2003). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc. Natl. Acad. Sci. USA* **100:** 2231–2236.
47. Lösel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haserot K and Wehling M (2003). Nongenomic steroid action: controversies, questions, and answers. *Physiol. Rev.* **83:** 965–1016.
48. Beato M, Chavez S and Truss M (1996). Transcriptional regulation by steroid hormones. *Steroids* **61:** 240–251.

49. Rohr DH, Lokman PM, Davie PS and Young G (2001). 11-Ketotestosterone induces silverying-related changes in immature female short-finned eels, *Anguilla australis*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **130**: 701.
50. Lokman PM, Rohr DH, Davie PS and Young G (2003). The physiology of silverying in anguillid eels — androgens and control of metamorphosis from the yellow to the silver stage. In: Aida K, Tsukamoto K and Yamauchi K (eds.), *Advances in Eel Biology*, Springer Verlag, Tokyo, pp. 331–350.
51. Wendelaar Bonga SE (1997). The stress response in fish. *Physiol. Rev.* **77**: 591–625.
52. Schulz RW and Miura T (2002). Spermatogenesis and its endocrine regulation. *Fish Physiol. Biochem.* **26**: 43–56.
53. Miura T (1998). Spermatogenetic cycle in fish. In: Knobil E and Neill JD (eds.), *Encyclopedia of Reproduction Vol 4*, Academic Press, San Diego, pp. 571–578.
54. Miura T, Miura C, Ohta T, Nader MR, Todo T and Yamuchi K (1999). Estradiol-17 β stimulates the renewal of spermatogonial stem cells in males. *Biochem. Biophys. Res. Commun.* **264**: 230–234.
55. Miura T, Yamauchi K, Takahashi H and Nagahama Y (1999). Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). *Proc. Natl. Acad. Sci. USA* **88**: 5774–5778.
56. Miura T, Miura C, Yamauchi K and Nagahama Y (1995). Human recombinant activin induces proliferation of spermatogonia *in vitro* in the Japanese eel *Anguilla-japonica*. *Fish. Sci.* **61**: 434–437.
57. Amer MA, Miura T, Miura C and Yamauchi K (2001). Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). *Biol. Reprod.* **65**: 1057–1066.
58. Loir M (1999). Spermatogonia of rainbow trout: II. *In vitro* study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Mol. Reprod. Dev.* **53**: 434–442.
59. Miura T and Miura C (2001). Japanese eel: A model for analysis of spermatogenesis. *Zool. Sci.* **18**: 1055–1063.
60. Miura T, Yamauchi K, Takahashi H and Nagahama Y (1992). The role of hormones in the acquisition of sperm motility in salmonid fish. *J. Exp. Zool.* **261**: 359–363.
61. Trant JM and Thomas P (1989). Isolation of a novel maturation-inducing steroid produced *in vitro* by ovaries of Atlantic croaker. *Gen. Comp. Endocrinol.* **75**: 397–404.

62. Patino R and Sullivan CV (2002). Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiol. Biochem.* **26**: 57–70.
63. Tyler CR and Sumpter JP (1996). Oocyte growth and development in teleosts. *Rev. Fish Biol. Fish.* **6**: 287–318.
64. Wallace RA and Selman K (1981). Cellular and dynamic aspects of oocyte growth in teleosts. *Am. Zool.* **21**: 325–343.
65. Higashino T, Miura T, Miura C and Yamauchi K (in press). Effects of several sex steroid hormones on early oogenesis in Japanese huchen (*Hucho perryi*). *Fish Physiol. Biochem.*
66. Khoo KH (1979). The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. *Can. J. Zool.* **57**: 617–626.
67. Lokman PM, George KAN and Young G (in press). Effects of steroid and peptide hormones on *in vitro* growth of previtellogenic oocytes from eel, *Anguilla australis*. *Fish Physiol. Biochem.*
68. Wallace RA and Jared DW (1968). Estrogen induces lipophosphoprotein in serum of male *Xenopus laevis*. *Science* **160**: 91–92.
69. Cerda J, Selman K and Wallace RA (1996). Observations on oocyte maturation and hydration *in vitro* in the black sea bass, *Centropristes striata* (*Serranidae*). *Aquat. Living Resour.* **9**: 325–335.
70. Okuzawa K (2002). Puberty in teleosts. *Fish Physiol. Biochem.* **26**: 31–41.
71. Borg B, Antonopoulou E, Mayer I, Andersson E and Swanson P (1998). Effects of gonadectomy and androgen treatments on pituitary and plasma levels of gonadotropins in Atlantic salmon, *Salmo salar*, mature male parr — physiological positive feedback control of both gonadotropins. *Biol. Reprod.* **58**: 814–820.
72. Khan IA, Hawkins MB and Thomas P (1999). Gonadal stage-dependent effects of gonadal steroids on gonadotropin II secretion in the Atlantic croaker (*Micropogonias undulatus*). *Biol. Reprod.* **61**: 834–841.
73. Antonopoulou E, Swanson P, Mayer I and Borg B (1999). Feedback control of gonadotropins in Atlantic salmon, *Salmo salar*, male parr.II. Aromatase inhibitor and androgen effects. *Gen. Comp. Endocrinol.* **114**: 142–150.
74. Mateos J, Mananos E, Carrillo M and Zanuy S (2002). Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotropin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **132**: 75–86.

75. Cavaco JE, van Blijswijk B, Leatherland JF, Goos HJ and Schulz RW (1999). Androgen-induced changes in Leydig cell ultrastructure and steroidogenesis in juvenile African catfish, *Clarias gariepinus*. *Cell Tissue Res.* **297**: 291–299.
76. Christensen TA and Sorensen PW (1996). Pheromones as tools for olfactory research. Introduction. *Chem. Senses.* **21**: 241–243.
77. Poling KR, Fraser EJ and Sorensen PW (2001). The three steroid components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129B**: 645–651.
78. Murphy CA and Stacey NE (2002). Methyl-testosterone induces male-typical ventilatory behavior in response to putative steroidal pheromones in female round gobies (*Neogobius melanostomus*). *Horm. Behav.* **42**: 109–115.
79. Sorensen PW, Scott AP, Stacey NE and Bowdin L (1995). Sulfated 17, 20 β -dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. *Gen. Comp. Endocrinol.* **100**: 128–142.
80. Rocha MJ and Reis-Henriques MA (1998). Steroid metabolism by ovarian follicles of the tilapia *Oreochromis mossambicus* (*Teleostei, Cichlidae*). *Comp. Biochem. Physiol. B* **121**: 85–90.
81. Van den Hurk R and Resink JW (1992). Reproductive-system as sex-pheromone producer in teleost fish. *J. Exp. Zool.* **261**: 204–213.
82. Cardwell JR, Stacey NE, Tan E SP, McAdam DSO and Lang SLC (1995). Androgen increases olfactory receptor response to a vertebrate sex-pheromone. *J. Comp. Physiol.* **176A**: 55–61.
83. Bhatt JP, Kandwal JS and Nautiyal R (2002). Water temperature and pH influence olfactory sensitivity to pre-ovulatory and post-ovulatory ovarian pheromones in male *Barilius bendelisis*. *J. Biosci.* **27**: 273–281.
84. Van Weerd JH and Richter CJ (1991). Sex pheromones and ovarian development in teleost fish. *Comp. Biochem. Physiol.* **100A**: 517–527.
85. Kahn SM, Hryb DJ, Nakhlia AM, Romas NA and Rosner W (2002). Sex hormone-binding globulin is synthesized in target cells. *J. Endocrinol.* **175**: 113–120.
86. Hobby AC, Pankhurst NW and Geraghty D (1999). Relative binding affinities of steroids for sex steroid binding protein (SBP) are similar despite differences between species in affinity and capacity for estradiol.

- In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (eds.), *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*, Bergern, Norway, p. 177.
87. Øvrevik J, Stenersen J, Nilssen K and Tollefsen KE (2001). Partial characterization of a sex steroid-binding protein in plasma from arctic charr (*Salvelinus alpinus* L.). *Gen. Comp. Endocrinol.* **122**: 31–39.
88. Nagae M, Okunaga M, Hidaka R, Ohkubo N and Matsubara T (in press). Molecular cloning of sex hormone-binding globulin cDNA in carp, *Cyprinus carpio*. *Fish Physiol. Biochem.*
89. Tsai M-J, Clark JH, Schrader WT and O’Malley BW (1998). Mechanisms of action of hormones that act as transcription-regulatory factors. In: Wilson JD, Foster DW, Kronenberg HM and Larsen PR (eds.), *Williams Textbook of Endocrinology* 9th edition, WB Saunders Co., Philadelphia, pp. 55–94.
90. de Leon J (2003). Glucuronidation enzymes, genes and psychiatry. *Int. J. Neuropsychopharmacol.* **6**: 57–72.
91. Falany JL, Lawing L and Falany CN (1993). Links Abstract Identification and characterization of cytosolic sulfotransferase activities in MCF-7 human breast carcinoma cells. *J. Steroid Biochem. Mol. Biol.* **46**: 481–487.
92. Meloche CA and Falany CN (2001). Expression and characterization of the human 3 β -hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b). *J. Steroid Biochem. Mol. Biol.* **77**: 261–269.
93. Lee SJ and Buhler DR (2002). Functional properties of a rainbow trout CYP3A27 expressed by recombinant baculovirus in insect cells. *Drug. Metab. Dispos.* **30**: 1406–1412.
94. Kullman SW, Hamm JT and Hinton DE (2000). Identification and characterization of a cDNA encoding cytochrome P450 3A from the fresh water teleost medaka (*Oryzias latipes*). *Arch. Biochem. Biophys.* **380**: 29–38.
95. Smeets JM, Wamsteker J, Roth B, Everaarts J and van den Berg M (2002). Cytochrome P4501A induction and testosterone hydroxylation in cultured hepatocytes of four fish species. *Chemosphere* **46**: 163–172.
96. Leguen II, Carlsson C, Perdu-Durand E, Prunet P, Part P and Cravedi JP (2000). Xenobiotic and steroid biotransformation activities in rainbow trout gill epithelial cells in culture. *Aquat. Toxicol.* **48**: 165–176.

97. Guan GJ, Tanaka M, Todo T, Young G and Nagahama Y (1999). Cloning and expression of two carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase cDNAs in ovarian follicles of rainbow trout (*Oncorhynchus mykiss*). *Biochem. Biophys. Res. Commun.* **255**: 123–128.
98. Cravedi JP, Delous G, Debrauwer L and Prome D (1993). Biotransformation and branchial excretion of 17 α -methyltestosterone in trout. *Drug. Metab. Dispos.* **21**: 377–385.
99. Vermeirssen ELM and Scott AP (1996). Excretion of free and conjugated steroids in rainbow trout (*Oncorhynchus mykiss*): Evidence for branchial excretion of the maturation-inducing steroid, 17,20 β -dihydroxy-4-pregnene-3-one. *Gen. Comp. Endocrinol.* **101**: 180–194.
100. Bauer MP, Bridgham JT, Langenau DM, Johnson AL and Goetz FW (2000). Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. *Mol. Cell. Endocrinol.* **168**: 119–125.
101. Kusakabe M, Todo T, McQuillan HJ, Goetz FW and Young G (2002). Characterization and expression of steroidogenic acute regulatory protein (StAR) and MLN64 cDNAs in trout. *Endocrinology* **143**: 2062–2070.
102. Li Y-Y, Inoue K and Takei Y (2003). Steroidogenic acute regulatory protein in eels: cDNA cloning and effects of ACTH and seawater transfer on its mRNA expression. *Zool. Sci.* **20**: 211–219.
103. Goetz FW, Norberg B, McCauley L and Iliev D (in review, 2003). Characterization of the cod (*Gadus morhua*) steroidogenic acute regulatory (StAR) protein sheds light on StAR gene structure in fish.
104. von Hofsten J, Karlsson J, Jones I and Olsson PE (2002). Expression and regulation of fushi tarazu factor-1 and steroidogenic genes during reproduction in Arctic char (*Salvelinus alpinus*). *Biol. Reprod.* **67**: 1297–1304.
105. Clark BJ, Soo SC, Caron KM, Ikeda Y, Parker KL and Stocco DM (1995). Hormonal and developmental regulation of the steroidogenic acute regulatory protein. *Mol. Endocrinol.* **9**: 1346–1355.
106. Sugawara T, Lin D, Holt JA, Martin KO, Javitt NB, Miller WL and Strauss JF (1995). Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. *Biochemistry* **34**: 12506–12512.
107. Rust W, Stedronsky K, Tillmann G, Morley S, Walther N and Ivell R (1998). The role of SF-1/Ad4BP in the control of the bovine gene for

- the steroidogenic acute regulatory (StAR) protein. *J. Mol. Endocrinol.* **21**: 189–200.
108. Bauer M (2001). Zebrafish mutagenesis: The isolation and characterization of reproductive mutations in the adult zebrafish, Ph.D. Thesis University of Notre Dame.
109. Caron KM, Ikeda Y, Soo SC, Stocco DM, Parker KL and Clark BJ (1997). Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Mol. Endocrinol.* **11**: 138–147.
110. Silverman E, Eimerl S and Orly J (1999). CCAAT enhancer-binding protein beta and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells. *J. Biol. Chem.* **274**: 17987–17996.
111. Christenson LK, Johnson PE, McAllister JM and Strauss JF 3rd (1999). CCAAT/enhancer-binding proteins regulate expression of the human steroidogenic acute regulatory protein (StAR) gene. *J. Biol. Chem.* **274**: 26591–26598.
112. Shea-Eaton WK, Trinidad MJ, Lopez D, Nackley A and McLean MP (2001). Sterol regulatory element binding protein-1a regulation of the steroidogenic acute regulatory protein gene. *Endocrinology* **142**: 1525–1533.
113. Sugawara T, Kiriakidou M, McAllister JM, Kallen CB and Strauss JF 3rd (1997). Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry* **36**: 7249–7255.
114. Sandhoff TW, Hales DB, Hales KH and McLean MP (1998). Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. *Endocrinology* **139**: 4820–4831.
115. Nakamura I and Young G (2002). Changes in steroidogenic enzyme and steroidogenic acute regulatory protein mRNAs during ovarian development of rainbow trout. In: *Proceedings of The Endocrine Society of Australia*, Vol. 45, p. 149.
116. Kusakabe M (2002). Steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes in rainbow trout (*Oncorhynchus mykiss*): cDNA cloning and expression during spermatogenesis, Ph.D. Thesis, University of Otago.
117. Hagen IJ (2002). Regulation of steroidogenesis and the Steroidogenic Acute Regulatory (StAR) protein messenger RNA in interrenal cells of rainbow trout, MSc thesis, University of Otago.

118. Takahashi M, Tanaka M, Sakai N, Adachi S, Miller WL and Nagahama Y (1993). Rainbow trout ovarian cholesterol side-chain cleavage cytochrome P450 (P450scc): cDNA cloning and mRNA expression during oogenesis. *FEBS Lett.* **319**: 45–48.
119. Lai WW, Hsiao PH, Guiguen Y and Chung BC (1998). Cloning of zebrafish cDNA for 3β -hydroxysteroid dehydrogenase and P450scc. *Endocr. Res.* **24**: 927–931.
120. Young G, Todo T, Kobayashi T, Guan G and Nagahama Y (1997). Steroidogenesis by the salmonid ovarian follicle: the two-cell type model revisited. In: Kawashima S and Kikuyama S (eds.), *Advances in Comparative Endocrinology*, Monduzzi Editore, Bologna, pp. 1443–1449.
121. Kumar RS, Ijiri S and Trant JM (2000). Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. *Biol. Reprod.* **63**: 1676–1682.
122. Hsu H-J, Hsiao P, Kuo M-W and Chung B-C (2002). Expression of zebrafish cyp11a1 as a maternal transcript and in yolk syncytial layer. *Gene Exp. Patt.* **2**: 219–222.
123. Sakai N, Tanaka M, Takahashi M, Fukada S, Mason JI and Nagahama Y (1994). Ovarian 3β -hydroxysteroid dehydrogenase Δ -isomerase of rainbow trout — its cDNA cloning and properties of the enzyme expressed in a mammalian cell. *FEBS Lett.* **350**: 309.
124. Young G, Kagawa H and Nagahama Y (1982). Oocyte maturation in the amago salmon (*Oncorhynchus rhodurus*): *in vitro* effects of salmon gonadotropin, steroids, and cyanoketone (an inhibitor of 3β -hydroxy- Δ^5 -steroid dehydrogenase). *J. Exp. Zool.* **224**: 365–375.
125. Young G, Todo T, Kusakabe M, Kobayashi T and Nagahama Y (1999). 3β -Hydroxysteroid dehydrogenase gene: sites of expression in trout gonads and cAMP-dependent regulation. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (eds.), *Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*, Bergern, Norway, p. 202.
126. Nakamura I, Kusakabe M and Young G (in press). Regulation of steroidogenic enzyme mRNAs in rainbow trout (*Oncorhynchus mykiss*) ovarian follicles *in vitro*. *Fish Physiol. Biochem.*
127. Sakai N, Tanaka M, Adachi S, Miller WL and Nagahama Y (1992). Rainbow trout cytochrome P450c17 (17 α -hydroxylase/17,20 lyase): cDNA

- cloning, enzymatic properties and temporal pattern of ovarian P450c17 mRNA expression during oogenesis. *FEBS Lett.* **301**: 60–64.
128. Kazeto Y, Ijiri S, Todo T, Adachi S and Yamauchi K (2000). Molecular cloning and characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. *Gen. Comp. Endocrinol.* **118**: 123–133.
129. Halm S, Kwon JY, Rand-Weaver M, Sumpter JP, Pounds N, Hutchinson TH and Tyler CR (2003). Cloning and gene expression of P450 17-hydroxylase,17,20-lyase cDNA in the gonads and brain of the fathead minnow *Pimephales promelas*. *Gen. Comp. Endocrinol.* **130**: 256–266.
130. Kazeto Y, Ijiri S, Matsubara H, Adachi S and Yamauchi K (2000). Cloning of 17 β -hydroxysteroid dehydrogenase-I cDNAs from Japanese eel ovary. *Biochem. Biophys. Res. Commun.* **279**: 451–456.
131. Zhou LY, Senthilkumaran B, Wang DS, Sudhakumari CC, Kobayashi T, Kajiura-Kobayashi H, Matsuda M, Yoshikuni M and Nagahama Y (in press). Partial cloning of 17 β -HSD-I from the Nile tilapia ovary and its expression pattern during spawning cycle. *Fish Physiol. Biochem.*
132. Callard GV, Tchoudakova AV, Kishida M and Wood E (2001). Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *J. Steroid. Biochem. Mol. Biol.* **79**: 305–314.
133. Kazeto Y, Ijiri S, Place AR, Zohar Y and Trant JM (2001). The 5'-flanking regions of CYP19A1 and CYP19A2 in zebrafish. *Biochem. Biophys. Res. Commun.* **288**: 503–508.
134. Tanaka M, Telecky TM, Fukada S, Adachi S, Chen S and Nagahama Y (1992). Cloning and sequence analysis of the cDNA encoding P-450 aromatase (P450arom) from a rainbow trout ovary: relationship between amount of P450 arom mRNA and the production of oestradiol 17 β in the ovary. *J. Mol. Endocrinol.* **8**: 53–61.
135. Trant JM (1994). Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom. *Gen. Comp. Endocrinol.* **95**: 155–168.
136. Fukada S, Tanaka M, Matsuyama M, Kobayashi D and Nagahama Y (1996). Isolation, characterization, and expression of cDNAs encoding the medaka (*Oryzias latipes*) ovarian follicle cytochrome P-450 aromatase. *Mol. Reprod. Dev.* **45**: 285–290.

137. Chang XT, Kobayashi T, Kajiura H, Nakamura M and Nagahama Y (1997). Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. *J. Mol. Endocrinol.* **18**: 57–66.
138. Tanaka M, Fukada S, Matsuyama M and Nagahama Y (1995). Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (*Oryzias latipes*). *J. Biochem. (Tokyo)* **117**: 19–25.
139. Tchoudakova A and Callard GV (1998). Identification of multiple CYP19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. *Endocrinology* **139**: 2179–2189.
140. Gelinas D, Pitoc GA and Callard GV (1998). Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. *Mol. Cell Endocrinol.* **138**: 81–93.
141. Kitano T, Takamune K, Kobayashi T, Nagahama Y and Abe SI (1999). Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). *J. Mol. Endocrinol.* **23**: 167–176.
142. Kishida M and Callard GV (2001). Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. *Endocrinology* **42**: 740–750.
143. Chiang EF, Yan YL, Guiguen Y, Postlethwait J and Chung BC (2001). Two Cyp19 (P450 aromatase) genes on duplicated zebrafish chromosomes are expressed in ovary or brain. *Mol. Biol. Evol.* **18**: 542–550.
144. Trant JM, Gavasso S, Ackers J, Chung BC and Place AR (2001). Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (*Danio rerio*). *J. Exp. Zool.* **290**: 475–483.
145. Kwon JY, McAndrew BJ and Penman DJ (2001). Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. *Mol. Biol. Cell* **12**: 2835–2845.
146. Halm S, Rand-Weaver M, Sumpter JP and Tyler CR (2001). Cloning and molecular characterization of an ovarian-derived (brain-like) P450 aromatase cDNA and development of a competitive RT-PCR assay to

- quantify its expression in the fathead minnow (*Pimephales promelas*). *Fish Physiol. Biochem.* **24:** 49–62.
147. Valle LD, Ramina A, Vianello S, Belvedere P and Colombo L (2002). Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum). *J. Steroid Biochem. Mol. Biol.* **82:** 19–32.
148. Menuet A, Anglade I, Le Guevel R, Pellegrini E, Pakdel F and Kah O (2003). Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: comparison with estrogen receptor α . *J. Comp. Neurol.* **462:** 180–193.
149. Valle LD, Lunardi L, Colombo L and Belvedere P (2002). European sea bass (*Dicentrarchus labrax* L.) cytochrome P450arom: cDNA cloning, expression and genomic organization. *J. Steroid. Biochem. Mol. Biol.* **80:** 25–34.
150. Zhao J, Mak P, Tchoudakova A, Callard G and Chen S (2001). Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. *Gen. Comp. Endocrinol.* **123:** 180–191.
151. Tchoudakova A, Kishida M, Wood E and Callard GV (2001). Promoter characteristics of two cyp19 genes differentially expressed in the brain and ovary of teleost fish. *J. Steroid Biochem. Mol. Biol.* **78:** 427–439.
152. Yoshiura Y, Senthilkumaran B, Watanabe M, Oba Y, Kobayashi T and Nagahama Y (2003). Synergistic expression of Ad4BP/SF-1 and cytochrome P-450 aromatase (ovarian type) in the ovary of Nile tilapia, *Oreochromis niloticus*, during vitellogenesis suggests transcriptional interaction. *Biol. Reprod.* **68:** 1545–1553.
153. Guiguen Y, Baroiller J-F, Ricordel M-J, Iseki K, McMeel OM and Fostier A (1999). Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). *Mol. Reprod. Develop.* **54:** 154–162.
154. Halm S, Pounds N, Maddix S, Weaver M, Sumpter JP, Hutchinson TH and Tyler CR (2002). Exposure to exogenous 17 β -oestradiol disrupts p450aromB mRNA expression in the brain and gonad of adult fathead minnows (*Pimephales promelas*). *Aquat. Toxicol.* **60:** 285–299.
155. Baroiller J-F, Guiguen Y and Fostier A (1999). Endocrine and environmental aspects of sex differentiation in fish. *Cell. Mol. Life Sci.* **55:** 910–931.

156. Carbone DL and Richards JS (1997). Evidence that functional interactions of CREB and SF-1 mediate hormone regulated expression of the aromatase gene in granulosa cells and constitutive expression in R2C cells. *J. Steroid. Biochem. Mol. Biol.* **61**: 223–231.
157. Watanabe M, Tanaka M, Kobayashi D, Yoshiura Y, Oba Y and Nagahama Y (1999). Medaka (*Oryzias latipes*) FTZ-F1 potentially regulates the transcription of P-450 aromatase in ovarian follicles: cDNA cloning and functional characterization. *Mol. Cell. Endocrinol.* **149**: 221–228.
158. Gen K, Okuzawa K, Kumakura N, Yamaguchi S and Kagawa H (2001). Correlation between messenger RNA expression of cytochrome P450 aromatase and its enzyme activity during oocyte development in the red seabream (*Pagrus major*). *Biol. Reprod.* **65**: 1186–1194.
159. Kagawa H, Gen K, Okuzawa K and Tanaka H (2003). Effects of luteinizing hormone and insulin-like growth factor-I on aromatase activity and P450 aromatase gene expression in the ovarian follicles of red seabream, *Pagrus major*. *Biol. Reprod.* **68**: 1562–1568.
160. Jiang JQ, Kobayashi T, Ge W, Kobayashi H, Tanaka M, Okamoto M, Nonaka Y and Nagahama Y (1996). Fish testicular 11 β -hydroxylase: cDNA cloning and mRNA expression during spermatogenesis. *FEBS Lett.* **397**: 250–252.
161. Jiang JQ, Young G, Kobayashi T and Nagahama Y (1998). Eel (*Anguilla japonica*) testis 11 β -hydroxylase gene is expressed in interrenal tissue and its product lacks aldosterone synthesizing activity. *Mol. Cell. Endocrinol.* **146**: 207–211.
162. Liu S, Govoroun M, D'Cotta H, Ricordel MJ, Lareyre JJ, McMeel OM, Smith T, Nagahama Y and Guiguen Y (2000). Expression of cytochrome P450(11 β) (11 β -hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. *J. Steroid. Biochem. Mol. Biol.* **75**: 291–298.
163. Kusakabe M, Kobayashi T, Todo T, Lokman PM, Nagahama Y and Young G (2002). Molecular cloning and expression during spermatogenesis of a cDNA encoding 11 β -hydroxylase (P45011 β) in rainbow trout. *Mol. Reprod. Develop.* **62**: 456–469.
164. Matsubara H, Lokman PM, Senaha A, Kazeto Y, Ijiri S, Kambegawa A, Hirai T, Young G, Todo T, Adachi S and Yamauchi K (in press). Synthesis and possible function of 11-ketotestosterone during oogenesis in eel (*Anguilla* spp.). *Fish Physiol. Biochem.*

165. Lokman PM, Harris B, Kusakabe M, Kime DE, Schulz RW, Adachi S and Young G (2002). 11-Oxygenated androgens in female teleosts: prevalence, abundance, and life history implications. *Gen. Comp. Endocrinol.* **129**: 1–12.
166. Kusakabe M, Nakamura I and Young G (2003). 11 β -hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in rainbow trout: cloning, sites of expression, and seasonal changes in gonads. *Endocrinology* **144**: 2534–2545.
167. Jiang JQ, Wang DS, Senthilkumaran, T Kobayashi, Kobayashi HK, Yamaguchi A, Ge W, Young G and Nagahama Y (2003). Isolation, characterization and expression of 11 β -hydroxysteroid dehydrogenase type 2 cDNAs from the testes of Japanese eel (*Anguilla japonica*) and Nile tilapia (*Oreochromis niloticus*). *J. Mol. Endocrinol.* **31**: 305–315.
168. Ducouret B, Tujague M, Ashraf J, Mouchel N, Servel N, Valotaire Y and Thompson EB (1995). Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. *Endocrinology* **136**: 3774–3783.
169. Takeo J, Hata J, Segawa C, Toyohara H and Yamashita S (1996). Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. *FEBS Lett.* **389**: 244–248.
170. Colombe L, Fostier A, Bury N, Pakdel F and Guiguen Y (2001). A mineralocorticoid-like receptor in the rainbow trout, *Oncorhynchus mykiss*: cloning and characterization of its steroid binding domain. *Steroids* **65**: 319–328.
171. Guan G, Todo T, Tanaka M, Young G and Nagahama Y (2000). Isoleucine-15 of rainbow trout carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase is critical for coenzyme (NADPH) binding. *Proc. Natl. Acad. Sci. USA* **97**: 3079–3083.
172. Senthilkumaran B, Sudhakumari CC, Chang XT, Kobayashi T, Oba Y, Guan G, Yoshiura Y, Yoshikuni M and Nagahama Y (2002). Ovarian carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase shows distinct surge in messenger RNA expression during natural and gonadotropin-induced meiotic maturation in nile tilapia. *Biol. Reprod.* **67**: 1080–1086.

173. Tanaka M, Nakajin S, Kobayashi D, Fukada S, Guan G, Todo T, Senthilkumaran B and Nagahama Y (2002). Teleost ovarian carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase: potential role in the production of maturation-inducing hormone during final oocyte maturation. *Biol. Reprod.* **66**: 1498–1504.
174. Wang Y and Ge W (2002). Cloning of zebrafish ovarian carbonyl reductase-like 20 β -hydroxysteroid dehydrogenase and characterization of its spatial and temporal expression. *Gen. Comp. Endocrinol.* **127**: 209–216.
175. Nagahama Y (1994). Endocrine regulation of gametogenesis in fish. *Int. J. Dev. Biol.* **38**: 217–229.
176. Bobe J, Maugars G, Nguyen T, Rime H and Jalabert B (2003). Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating hormone receptor and insulin-like growth factor 2 messenger RNAs. *Mol. Reprod. Dev.* **66**: 46–53.
177. Kazeto Y, Adachi S and Yamauchi K (2001). 20 β -hydroxysteroid dehydrogenase of the Japanese eel ovary: its cellular localization and changes in the enzymatic activity during sexual maturation. *Gen. Comp. Endocrinol.* **122**: 109–115.
178. Ali SA, Schoonen WG, Lambert JG, Van den Hurk R and Van Oordt PG (1987). The skin of the male African catfish, *Clarias gariepinus*: a source of steroid glucuronides. *Gen. Comp. Endocrinol.* **66**: 415–424.
179. Sangalang GB and Freeman HC (1988). *In vitro* biosynthesis of 17 α ,20 β -dihydroxy-4-pregnen-3-one by the ovaries, testes, and head kidneys of the Atlantic salmon *Salmo salar*. *Gen. Comp. Endocrinol.* **69**: 406–415.
180. Vermeulen GJ, Lambert JGD, Teitsma CA, Zandbergen MA and Goos HJT (1995). Adrenal tissue in the male African catfish, *Clarias gariepinus* — localization and steroid hormone secretion. *Cell Tissue Res.* **280**: 653–657.
181. Ebrahimi M and Kime DE (1998). Extragonadal steroidogenesis in teleost fish. *Ann. NY Acad. Sci.* **839**: 581–583.
182. Kime DE and Ebrahimi M (1997). Synthesis of 17,20 α - and 17,20 β -dihydroxy-4-pregnen-3-ones, 11-ketotestosterone and their conjugates by gills of teleost fish. *Fish Physiol. Biochem.* **17**: 117–121.

183. Ebrahimi M, Scott AP and Kime DE (1996). Extragonadal production of 17,20-dihydroxy-4-pregnen-3-ones in vitro in cyprinid fish. *Gen. Comp. Endocrinol.* **104**: 296–303.
184. Cavaco JEB, Vischer HE, Lambert JGD, Goos HJT and Schulz RW (1997). Mismatch between patterns of circulating and testicular androgens in African catfish, *Clarias gariepinus*. *Fish Physiol. Biochem.* **17**: 155–162.
185. Schulz R and Blum V (1991). Extragonadal 17 β -hydroxysteroid dehydrogenase activity in rainbow trout. *Gen. Comp. Endocrinol.* **82**: 197–205.
186. Mayer I, Borg B and Schulz R (1990). Conversion of 11-ketoandrostenedione to 11-ketotestosterone by blood cells of six fish species. *Gen. Comp. Endocrinol.* **77**: 70–74.
187. Callard GV, Petro Z and Ryan KJ (1997). Identification of aromatase in the reptilian brain. *Endocrinology* **100**: 1214–1218.
188. Yaron Z, Gur G, Melamed P, Levavi-Sivan B, Gissis A, Bayer D, Elizur A, Holland C, Zohar Y and Schreibman MP (1995). Blocks along the hypothalamo-hypophyseal-gonadal axis in immature black carp, *Mylopharyngodon piceus*. In: Goetz FW and Thomas P (eds.), *Proceedings of the 5th International Symposium on Reproductive Physiology of Fish*, Fish Symposium 95, Austin, Texas, pp. 22–24.
189. Sakamoto H, Ukena K and Tsutsui K (2001). Activity and localization of 3 β -hydroxysteroid dehydrogenase/ Δ^5 – Δ^4 isomerase in the zebrafish central nervous system. *J. Comp. Neuroendocrinol.* **439**: 291–305.
190. Mathieu M, Mensah-Nyagan AG, Vallarino M, Do-Rego JL, Beauljean D, Vaudry D, Luu-The V, Pelletier G and Vaudry H (2001). Immunohistochemical localization of 3 β -hydroxysteroid dehydrogenase and 5 α -reductase in the brain of the African lungfish *Protopterus annectens*. *J. Comp. Neuroendocrinol.* **438**: 123–135.
191. Stocco DM (2001). StAR protein and the regulation of steroid hormone biosynthesis. *Annu. Rev. Physiol.* **63**: 193–213.
192. Reese MG, Harris NL and Eeckman FH (1996). Large scale sequencing specific neural networks for promoter and splice site recognition.

- In: Hunter L and Klein TE (eds.), *Biocomputing: Proceedings of the 1996 Pacific Symposium*, World Scientific Publishing Co., Singapore.
193. Quandt K, Frech K, Karas H, Wingender E and Werner T (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**: 4878–4884.

Regulation and Function of Estrogen Receptors: Comparative Aspects

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Abstract

In vertebrates, estradiol (E_2) is involved in many physiological mechanisms starting in embryonic development and continuing throughout adult stages. In fish, its effects are best documented in the context of the hormonal regulations along the brain-pituitary-gonadal axis. Moreover, in oviparous species including fish, E_2 also controls the process of vitellogenesis at level of the liver. Genomic E_2 effects are mediated by estrogen receptors, namely ER α and ER β , acting as ligand-dependent transcription factors. Although, the teleost ERs share similar characteristics with their mammalian orthologs, studies on fish ERs have recently provided valuable and novel data on ER functions. First, in addition to the full length ER α , an N-terminal truncated ER α isoform was identified in teleost species. This novel isoform is mainly, if not exclusively, expressed in the liver and is characterized by constitutive ligand-independent activity. This short ER α isoform, which is also found in other oviparous species, may be relevant to hepatic vitellogenesis. Second, most fish are also characterized by the presence of two distinct forms of ER β , ER β 1 and ER β 2, arising from a gene duplication event early in the evolution of the fish lineage. The analysis of their transcriptional activities and their tissue distribution in zebrafish showed that these ER β forms are

functional and probably exert differential functions, as indicated by their different patterns of expression. This review focuses on the original characteristics of fish ERs and their involvement in the regulation of E₂-dependent genes.

Introduction

In teleost fish, as in other vertebrates, estradiol (E₂) is involved in the development and the maintenance of the reproductive system in both females and males. At the gonadal level, E₂ has been shown to play a crucial role in sexual differentiation¹ and is also involved in gonadal development and steroidogenesis during the reproductive cycle.² Through feedback mechanisms, E₂ also modulates the activity of the neuroendocrine pathways regulating the pituitary gonadotrope activity, which in turn controls the gonads.³ Moreover, E₂ is also able to affect directly the synthesis of gonadotropin subunits⁴ and is well known for exerting crucial effects in the control of vitellogenin production in the liver.⁵ Apart from the key role of E₂ in the development and functioning of the reproductive axis, research on estrogen receptors (ERs) in fish has been boosted by the fact that fish are primary targets for waterborne endocrine disrupters, many of which are well known estrogen-mimicking compounds.^{6,7} Consequently, the knowledge of the molecular mechanisms underlying the effects of E₂ is an important step to evaluate the impact of these chemicals on fish reproductive physiology.

E₂ genomic effects are mediated by specific nuclear receptors (ERs) acting as ligand dependent-transcription factors able to modulate target gene activity.⁸ In mammals, two ER subtypes have been identified ER α and ER β .^{9,10} Based on sequence homologies, these ER subtypes, share a common structural organization with the other members of the nuclear receptor superfamily.⁹⁻¹¹ Besides these genomic effects, there is accumulating evidence in mammals that E₂ can activate non-genomic pathways. Convincing studies suggest the existence of putative membrane-bound estrogen receptors

that could explain some of the rapid effects of E₂, but at the present time the molecular basis of such mechanisms remains to be established.¹²

ERs have been cloned and characterized in several teleost species. These fish ERs share a number of features with their mammalian counterparts, but present two main characteristics. First, in addition to the full-length ER α form, an isoform characterized by a constitutive ligand-independent transactivation function, has been isolated in fish^{13,14} and in other oviparous species.¹⁵⁻¹⁷ Second, our group has demonstrated,¹⁸ as have others,¹⁹⁻²¹ that most teleost fish possess two genes encoding two distinct ER β forms, ER β 1 and ER β 2. The objectives of the present review are to synthesize the most recent developments with respect to fish ERs.

ER α Structure-Function Relationships

Although the unliganded ERs are found mainly in the nucleus, they are in an inactive state and not tightly bound to nuclear components. Ligand binding triggers conformational changes in the receptor inducing receptor dimerization, receptor-DNA interaction, and recruitment of coactivators and the general transcription factors.²² This ER transcriptional activity is permitted by a complex modular organization of the receptor (Fig. 1). Because of the recent identification of ER β , most of the knowledge about the structure-function relationships of ERs comes from studies on the ER α subtype.

The N-terminal A/B domains are poorly conserved between members of the nuclear receptor family. These ER α domains contain a ligand-independent transactivation function AF1 (Fig. 1). Although little is known about this function, it was reported that a highly conserved helical structure and a phosphorylation site in the B domain are crucial elements for the AF1 activity. Consequently growth factors, such as IGF-1, EGF or TNF- α , can synergize with E₂ through activation of the MAP Kinase pathway which induces the phosphorylation of the

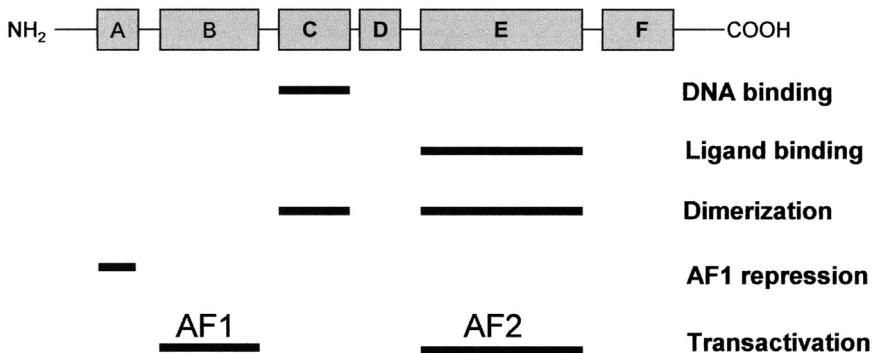


Fig. 1 Diagrammatic representation of the structural and functional domains of estrogen receptors.

serine 118 of the AF1 core. This phosphorylation permits recruitment of cofactors, such as p68 RNA helicase.^{23,24} More recently, structure analysis showed that, in absence of E₂, the A domain interacts with the C-terminal region inducing a repression of the AF1 function located in the B domain.^{25,26}

The C domain, also called DNA Binding Domain (DBD), is highly conserved between the members of the nuclear receptor superfamily¹¹ (40–50% of identity) and contains two zinc fingers permitting the recognition of a specific DNA hormone-responsive element. In the case of ERs, this element, known as estrogen-responsive element (ERE), is a palindromic sequence composed of two core 5'-AGGTCA-3' motifs with a 3 bp spacer (AGGTCA_{nnn}TGACCT).²⁷ The five residues of the D-box present in the second zinc finger are involved in the dimerization interface and the three residues of the P-box of the first zinc finger participate in the specific interaction with the DNA element.^{28,29} Several studies have indicated that regulatory sequences other than the ERE could also be targeted by ER suggesting that other mechanisms of transcriptional activation may also exist (Fig. 2). For example, analysis of the E₂-dependent regulation of the osteopontin gene showed that even if this gene is devoid of an ERE,

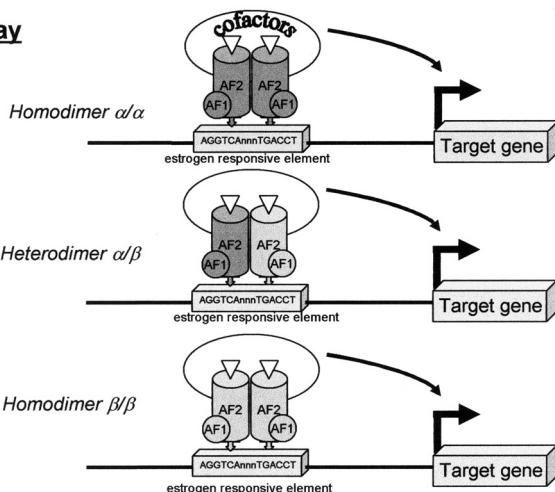
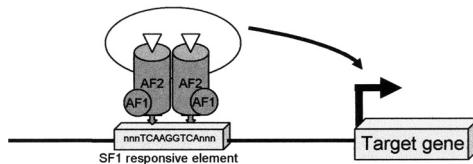
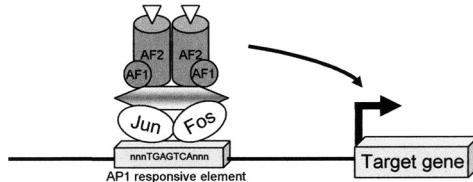
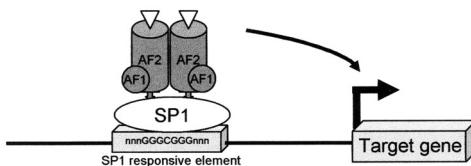
ERE pathway**SFRE pathway****AP1 pathway****SP1 pathway**

Fig. 2 ERs modulate the transcription of E₂-target genes by multiple pathways.

ER α was able to modulate its transcription by direct binding to the SF1 responsive element.³⁰ Alternatively, ER may modulate promoter activity, independently of the C domain, by interacting with different DNA-bound transcription factors. It appears to be the case of the AP1 pathway,³¹ since in the presence of E₂, ER α modulates the transcription of an AP1 responsive reporter gene. Mutation analysis of the P-box showed that this regulation is independent of a direct interaction of ER α with DNA,³² but requires the N-terminal and C-terminal regions of ER α to interact with coactivators of the Jun/Fos/AP1 complex. Additionally, ER action through pathways independent of direct DNA binding has been demonstrated with other transcription factors. For example, ER can also enhance gene activity by interacting directly with the SP1 transcription factor which specifically recognizes DNA GC-rich element.³³

Located in the C-terminal end of the C domain, the poorly conserved D domain is involved in the three-dimensional structure of the ERs and permits the stabilization of the DNA binding.²⁸ The function of this domain is still unclear, but recent studies showed that its lysine residues could be acetylation sites suggesting that this region is involved in regulation of the ER function.³⁴

The multifunctional E-domain, also called ligand binding domain (LBD), presents a globular organization which consists of a conserved arrangement of 12 helices (H1–H12) forming the hydrophobic ligand pocket closed by an antiparallel β -sheet and by helix H12.³⁵ The LBD recognizes a variety of compounds, exhibiting diversity in their size and in their chemical properties, which induce differential conformation of the ER protein. Mutation analysis has shown that residues G521, H524, L525 and M528 of helix H11 of ER α are clearly involved in E₂ recognition.³⁶ However, in the case of an antagonist such as tamoxifen, another set of residues, including R515, H516, G521 and L525, is involved in ligand recognition.³⁶ Consequently, agonist binding induces an intramolecular shift, which induces a repositioning of H12 known to be involved in the

transactivation function of AF2 and its ability to interact with cofactors. In contrast, the binding of an antagonist is accompanied by major structural reorganization disrupting the topography of the AF-2 and preventing the orientation of helix H12 in an agonist position.

Domain F is located at the C-terminal end of the protein. Its function is still unclear but recent mutation analysis showed that a helix in this domain modulates E₂ affinity, the interaction between the ER monomer and the antagonist effect. These data suggest that the F domain could be a modulator of the ER activity.³⁷

Estrogen Receptors in Teleost Fish

A Short Isoform of Teleost ER α in Oviparous Species

ER α was the first ER subtype identified in a fish, namely the rainbow trout.^{38,39} Compared with the mammalian ER α , this rainbow trout ER α (rtER α), of 65 kDa, is characterized in the N-terminal region by a deletion of 45 amino acid residues corresponding to the A domain. Following these pioneer studies, other groups described in different teleost species, the presence of an ER α form with the same N-terminal deletion,⁴⁰⁻⁴³ suggesting that this deletion could be specific to teleost ER α . However, the recent screening of a trout ovarian cDNA library permitted the cloning of a full length 71 kDa ER α , which was called rtER α -long.¹³ Consequently, the previously identified isoform was renamed rtER α -short. The sequence characterization of the rtER α gene using S1 nuclease protection assays demonstrated that these two isoforms were derived from two classes of mRNA generated by an alternative usage of two promoters. The first mRNA species, encoding the rtER α -short, was transcribed from exon1 and the second mRNA, encoding the rtER α -long, was transcribed from exon2a. Consequently, these mRNA species differ significantly in their 5' untranslated region, notably by the presence of an ATG in exon2a permitting the addition of 45 residues at the N-terminus of rtER α -

long.¹³ Analysis of the transcriptional activities of these isoforms in a yeast cell system demonstrated that, in contrast with rtER α -long, rtER α -short exhibits a ligand-independent transactivation capacity representing 15–25% of the full length receptor activity.^{13,44} Structural analysis of the AF1 function showed that as with mammalian ER α , in the absence of ligand, the A domain of the rtER α -long interacts with the C-terminal region,^{25,26} resulting in an inhibition of the AF1 activity located in the B domain. Thus, the deletion of A domain in rtER α -short explains the ligand independent AF1 activity of this truncated isoform.

Although in mammals an N-terminal truncated ER α isoform, devoid of the entire A/B domain, has been identified,⁴⁵ at the present time, no A domain truncated isoform has been isolated. However, two isoforms analogous to rtER α -long and rtER α -short have been identified in catfish,¹⁴ xenopus¹⁵ and chicken.^{16,17} These data suggest that the existence of the A-truncated ER α isoform could be a common feature of all oviparous species.

The organization of the rtER α gene, which generates two classes of transcripts significantly different in their 5' untranslated region, allowed us to generate specific probes to analyze precisely the tissue distribution of the two isoforms. S1 protection assays carried out in E₂ target tissues in trout demonstrated that the rtER α -short mRNAs were detectable only in liver samples.⁴⁶ In contrast, S1 nuclease assays using a probe specific for rtER α -long demonstrated that rtER α -long messengers could be detected in pituitary, brain, ovary, testis and liver samples. These data were confirmed by *in situ* hybridization experiments showing a clear expression of rtER α -long in the liver, the neuroendocrine regions of the brain, the proximal pars distalis of pituitary and in the follicular layers of the oocytes. In contrast, the rtER α -short specific riboprobe failed to detect any signal in all tissues except in hepatocytes. These results demonstrate a tissue-specific expression of the two rtER α isoforms, the rtER α -short being specifically expressed in the liver. As similar data have been obtained in other oviparous species,^{15–17} the short isoform, characterized by a constitutive ligand-independent

transactivation activity could be implicated in driving the liver towards its vitellogenic function not only during the reproductive cycle but also during development.⁴⁶

Interestingly, a recent study by Bouma and Nagler⁴⁷ in male rainbow trout showed that rtER α -long mRNAs are localized in the testicular interstitium at all stages of the annual reproductive cycle. RtER α -long was found particularly in the Leydig cell precursors suggesting that estrogens may participate in the differentiation process of those particular cells into mature Leydig cells.

Two ER β Forms from a Duplication of Genes in Teleosts

Recently, we and others have shown that, due to a duplication of the ER β gene within the teleost lineage, there are two ER β subtypes in most teleost species.^{18–21,48} Consequently, as found in Atlantic croaker (acER β and acER γ)¹⁹ and in goldfish (gfER β 1 and gfER β 2),²⁰ we have cloned and characterized two ER β forms in zebrafish, zfER β 1 and zfER β 2.¹⁸

Sequence analysis demonstrated that, as in mammals, the A/B domain is hypervariable between zfER α , zfER β 1 and zfER β 2 (Fig. 3). However, the serine residue corresponding to the Ser118 of the human ER α is

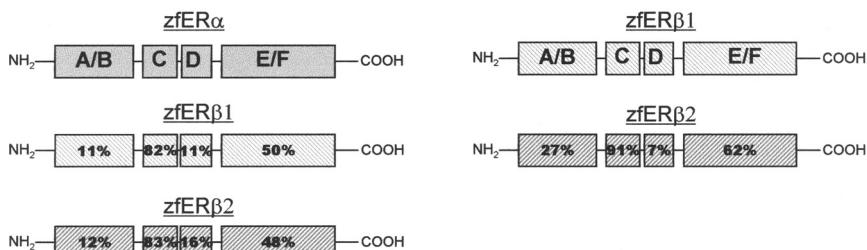


Fig. 3 Identity analysis of zfER α , zfER β 1 and zfER β 2 domains. The numbers within the ER receptor represent the degree of identity (%) between each receptor domain.

conserved. The phosphorylation of this residue by MAP kinase plays a key role in the potentiation of the AF1 activity of ER α , but also of ER β ^{49,50} and this point will require further investigation in teleost ERs.

The C-domain of zfER forms share more than 80% identity and possess a P-box identical to that of ER α suggesting that they could bind the same ERE (Fig. 3).¹⁸ However, in the case of mammalian ERs, clear differential affinities of ER α and ER β for various imperfect EREs (*c-fos*, *c-jun*, pS2, cathepsin D, choline acetyltransferase) have been demonstrated and could explain in part the differential transcriptional activity of the two ER subtypes⁵¹ on the expression of E₂ target gene.

The E-domain of zebrafish ER subtypes shares the same structural organization with an identity of approximately 55% (Fig. 3). This domain, known to contain the AF2 function involved in the recruitment of cofactors by helix H12 in an E₂-dependent manner, is present in both mammalian ERs. Examination of the zfER sequences suggests that this function exists in each zfER.¹⁸ The E domain is also involved in ligand binding. The tertiary structure of the hydrophobic cavity is conserved between mammalian ERs and explains the similar affinities of ER α and ER β for E₂. Our Scatchard analysis showed that each zfER protein binds E₂ with a high affinity close to that measured in mammals. However, the zfER β 2 showed a slightly higher affinity for E₂ ($K_d = 0.42$ nM) than zfER β 1 and zfER α ($K_d = 0.75$ nM).¹⁸ Despite the identity between ER α and ER β in mammalian species, it has been demonstrated, that ER α and ER β do not have the same affinity for various estrogenic components.⁵² In this respect, more investigation will be necessary to analyze the ligand binding affinities of each of the teleost ERs.

Analysis of the transcriptional activity of each zfER form has been investigated by co-transfection with a luciferase reporter gene under the control of a consensus ERE.¹⁸ These assays revealed that each form induced expression of the reporter gene in an E₂-dependent manner with approximately the same efficiency, suggesting that each form is able to specifically recognize the consensus ERE. However, probably

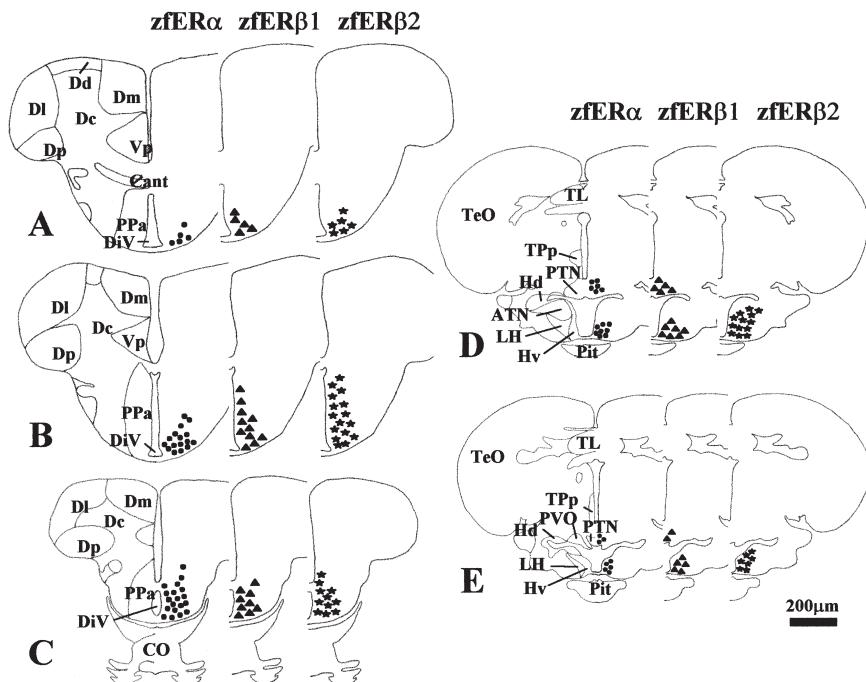


Fig. 4 Schematic drawings of transverse sections through the preoptic area (A,B,C) and the mediobasal hypothalamus (D–E) of zebrafish showing the distribution of the three estrogen receptor subtypes: α (solid circles), $\beta 1$ (triangles) and $\beta 2$ (stars). ANT — anterior tuberal nucleus; Cant — commissura anterior; CO — chiasma opticum; D — dorsal telencephalic area; Dc — central zone of D; Dd — dorsal zone of D; DiV — diencephalic ventricle; DL — lateral zone of D; Dm — medial zone of D; Dp — posterior zone of D; Hd — dorsal zone of the periventricular hypothalamus; Hv — ventral zone of the periventricular hypothalamus; LH — lateral hypothalamic nucleus; Pit — pituitary; PPa — parvocellular preoptic nucleus, anterior part; PTN — posterior tuberal nucleus; PVO — paraventricular organ; rl — lateral recess; TeO — tectum opticum; TL — torus longitudinalis; TPP — periventricular nucleus of posterior tuberculum; Vp — postcommisural nucleus of ventral telencephalic area.

reflecting its higher affinity for E_2 , zfER $\beta 2$ was transcriptionally active at doses ten times lower than zfER α and zfER $\beta 1$.¹⁸ These data are in contrast with those obtained in mammals showing that, in numerous cases, ER β has a lower transcriptional activity than ER α .⁵¹ Based on

this differential transcriptional activity between ER β in teleosts and in mammals, it is tempting to speculate that their functionality has diverged during evolution.

The first analyses of the respective sites of expression of teleost ERs by RT-PCR showed that like ER α , the two ER β forms were expressed in different E₂-target tissues with probably a higher expression in the ovary and the liver.^{18,53,54} At the level of the brain, and more specifically in the hypothalamus, previous results obtained in the Atlantic Croaker, showed that each ER messenger had a similar, but distinct distribution.¹⁹ By *in situ* hybridization, we analyzed precisely the distribution of each ER form in the adult zebrafish brain.¹⁸ Interestingly, the zfER mRNAs were found mainly in the neuroendocrine regions including the preoptic area and the mediobasal hypothalamus. However, their expression patterns were distinct, although partially overlapping (Fig. 4). Additionally, double *in situ* hybridization revealed that in the preoptic area and the mediobasal hypothalamus, zfERs were often colocalized in the same cells (unpublished data). The same situation was also found in rat brain where distinct patterns of expression were observed for ER α and ER β , although co-expression has been demonstrated in several regions.⁵⁵ *In vitro* analysis showed that when ER α and ER β are coexpressed, they preferentially form a heterodimer complex with a transcriptional activity similar to that of the ER α homodimer.^{49,56,57} Consequently, the ratio between the two subtypes in a given cell could be important for E₂-dependent induction of target genes. At the present time, the physiological role of the heterodimer complex is unclear.¹⁸

E₂-Target Genes in Fish

The cloning and characterization of specific promoters, together with the development of gene expression assays, are important steps in understanding how the specific gene might be regulated by steroid hormones and other signaling pathways. Although an important number of estrogen-regulated genes have been identified in mammals, to date little is known on estrogen target genes in fish species.

Hepatic E₂-sensitive Genes

In oviparous species, the process of vitellogenesis is tightly coupled to a clear E₂-dependent upregulation of ER α gene expression. In the trout, it was shown clearly that E₂ first induces ER α accumulation by its own induction of the ER α gene. This positive autoregulation, increasing the intracellular receptor concentration and therefore the E₂ sensitivity, is necessary for vitellogenin gene expression (Fig. 5). Consequently, the ER α gene autoregulation which is an important step of the vitellogenin production, was extensively studied in oviparous species such as chicken, xenopus and trout.⁵⁸⁻⁶⁰

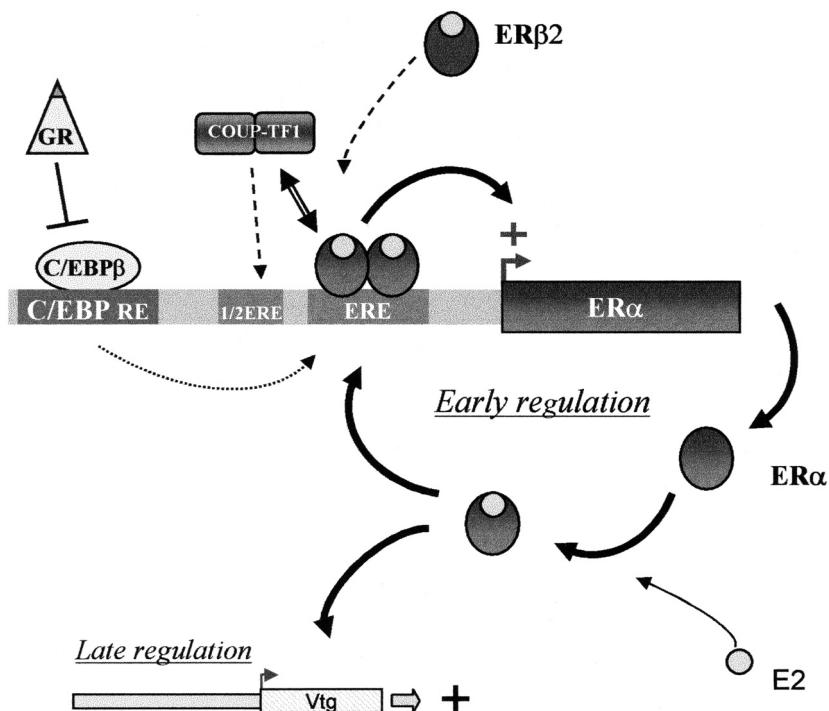


Fig. 5 Schematic representation of the ER α gene autoregulation: interaction with nuclear factors and permissive induction of late genes. Several studies showed that numerous nuclear factors modulate the autoregulation of the ER α gene process. Additionally, our preliminary experiments suggest that ER β 2 could be also involved (unpublished data).

Using a trout primary culture of hepatocytes, our earlier work showed that E₂-upregulation of the rtER α gene occurs at both the transcriptional and post-transcriptional levels.⁶¹ To analyze further the molecular basis of this autoregulation process, the promoter region (2 kb) has been isolated and analyzed by foot-printing, transfection and gel mobility shift assays.⁶²⁻⁶⁴ These experiments identified a fragment of 0.2 kb located at -40/-248 bp upstream of the transcription start site as being necessary and sufficient for rtER α autoregulation. This fragment contains an imperfect ERE (with one mutation on the 5' half site) and a consensus half-binding site ($\frac{1}{2}$ ERE). In addition, other upstream sequences in this 0.2 kb fragment were necessary to obtain maximal E₂ responsiveness, and were protected in footprint experiments with liver nuclear proteins, suggesting that other nuclear factors could also participate in the rtER α autoregulation process.⁶⁴ In fact, DNase protection assays with trout liver extracts showed that the orphan nuclear receptor COUP-TFI could maintain the rtER α gene promoter in an open state by binding to a direct repeat (DR24) consisting of the $\frac{1}{2}$ ERE and the 3' half site of the ERE in the 0.2 kb fragment.⁶⁴ This nucleosomal structure modification may facilitate ER α binding on the ERE. Additionally, COUP-TF1 also interacts directly with the N- and C-terminal region of ER α , inducing hyperphosphorylation of the rtER α protein, therefore increasing cofactor recruitment and its transcriptional activity (Fig. 5).⁶⁵

More recently, we showed that the transcription factor C/EBP β , corresponding to a leucine zipper protein of the CCAAT/enhancer binding protein family, was capable of binding to the 0.2 kb fragment at a specific site, and was also able to enhance the rtER α autoregulation process. It is interesting to note that *in vivo* studies showed that stress or cortisol treatment of maturing trout females caused a marked decrease of hepatic ER α and vitellogenin messenger levels.⁶⁶ Molecular analysis showed that, by direct interaction with C/EBP β , the liganded glucocorticoid receptor can repress its enhancer effect by preventing C/EBP β binding to the DNA, which interferes with the rtER α autoregulation process (Fig. 5).⁶⁷

To investigate the potential effects of ER β s on the ER α gene autoregulation, we isolated the 5' end of the ER α gene in zebrafish. The analysis of a series of mutations or deletions of the promoter region by transfection in CHO cells showed that this ER α gene was induced in an E₂-dependent manner by ER α itself and that the proximal region was characterized by a structural organization analogous, but not identical to that of the trout. In the ER α zebrafish gene, we also identified an imperfect ERE with three mutations in the 5' half site. Interestingly, 10 bp downstream from this ERE, another cis-element corresponding to an AP1-like site containing a half ERE was identified by computer analysis. Our preliminary experiments showed that this element could also enhance the autoinduction of the gene (unpublished data). Additionally, cotransfection experiments showed that zfER β 2, but not zfER β 1, was able to induce significantly zfER α gene expression. These data suggest that *in vivo* zfER β 2 could be implicated in the zfER α gene regulation during the vitellogenesis process (Fig. 5).

Another well known E₂-sensitive gene in fish is the egg yolk protein gene vitellogenin (VTG), which can be activated *de novo* from a totally silent state within hepatocytes by estrogens. The large amount of yolk stored during oocyte growth provides a nutrient source for the future developing embryo (see Chapter 8). Thus, proper vitellogenesis is a crucial mechanism of the reproductive process in all oviparous animals.⁶⁸ In fish, as in other oviparous species, liver-specific VTG synthesis is strictly regulated by E₂ at both the transcriptional and post-transcriptional levels and these effects are mediated by hepatic ER α .⁶¹ Moreover, transcription of the VTG gene requires the presence of high ER α levels and its transcriptional response is directly proportional to the amount of synthesized ER α . Indeed, a minimal basal expression of hepatic ER α is sufficient to highly (10–15 fold) induce the expression of its own gene during an estrogenic stimulation. However, the VTG gene exhibits a much lower sensitivity to ER α and therefore, under estrogenic treatment, stimulation of the VTG gene requires first an increase in the amount of hepatic ER α .⁵ Hence, VTG synthesized by the liver under estrogenic induction, is transported via the bloodstream to the ovary, where it is sequestered by growing oocytes in a cell-specific fashion by specific

membrane receptors, the VTG/VLDL receptors.⁶⁹ Our unpublished data suggest that genes encoding these receptors in the ovary might be potential targets for estrogens.

In fish, the egg envelopes are mainly constituted of the zona radiata (Zr), itself composed of three major proteins designated α -, β - and γ -Zr proteins possessing a molecular weight between 50–60 kDa in rainbow trout.⁷⁰ It is interesting to note that these Zr-proteins are also synthesized in the liver under estrogenic induction and are transported by the blood for deposition in ovarian follicles during oogenesis. It is also worth mentioning that the hepatic estrogen-dependent induction of VTG, Zr-proteins or ER- α expression were used to develop reliable and sensitive bioassays for the detection and assessment of estrogenic/antiestrogenic activity of environmental endocrine disruptors.^{71–73}

Another E₂-target is the albumin (Alb) gene encoding one of the most abundant serum protein synthesized in the liver. However, unlike the other mentioned E₂-target genes, the expression of hepatic Alb gene is decreased by estrogens in several oviparous species including fish,^{74–76} suggesting that E₂-downregulation of serum protein Alb during vitellogenesis may be widespread among oviparous vertebrates. The serum protein Alb binds and transports into various tissues a wide variety of lipophilic compounds including steroids, other lipophilic hormones and various phytochemicals and xenobiotics. Although the significance of the E₂-downregulation of Alb gene expression is unclear, a high level of serum Alb during vitellogenesis may have adverse effects on the hormonal or lipid profiles since estrogen is also known to increase hepatic triglyceride production.

Brain and Pituitary E₂ Sensitive Genes

In vertebrates, the local synthesis of estrogens in the brain is thought to play a critical role in the growth, differentiation and also protection of specific brain regions involved in the reproductive function. These effects are largely mediated by the central expression of the cytochrome P450 aromatase which converts androgens into estrogens and is well conserved during evolution among vertebrates.⁷⁷ Mammalian aromatase

is generated from a single gene and its hormonal regulation appears to be multifactorial, implying tissue-specific multiple promoters and alternative splicing.⁷⁸ By contrast, two distinct aromatase genes have been identified in fish: aromatase A (cyp19a), which is specifically expressed in the gonads, and aromatase B (cyp19b), which is mainly expressed in the pituitary and in the anterior ventral brain.^{79–82} Interestingly, in teleosts, the level of aromatase in the brain is about 100- to 1000-fold higher than in the brain of mammals.^{83,84} Studies in goldfish and zebrafish have shown that the expression of the brain aromatase (aromatase B) is markedly upregulated by estrogens, whereas the gonad isoform (aromatase A) remains unchanged or even repressed after E₂-treatment.^{85,86} Moreover, this estrogen-upregulation of aromatase B is functional as early as 24 hours postfertilization (hpf), since E₂ increases more than 10 times the level of aromatase B mRNA found very early (24–48 hpf) during zebrafish development. This E₂-stimulation of aromatase B is antagonized by the pure antiestrogens ICI 164,384 indicating that ERs mediate the effect of E₂.⁸⁷ In addition, in goldfish and zebrafish the promoter region of the aromatase B gene, in contrast with that of the aromatase A gene, exhibits an ERE-like element,^{88–90} which was shown to be functional *in vitro* (unpublished observations). Taken together, these data suggest that E₂-dependent regulation of the aromatase B gene involves a direct interaction with ERs. To investigate this pathway, we analyzed the cell distributions of aromatase B and ER α in trout.⁸² *In situ* hybridization and immunohistochemistry revealed that aromatase B mRNAs were highly expressed in the ER α positive area, but in contrast with the neuronal expression of ER α , aromatase was mainly detected in radial glial cells, as also shown in the plainfin midshipman.⁸¹ However, RT-PCR experiments on trout brain cell primary culture enriched in glial cells suggest that ER α could be weakly expressed in aromatase positive glial cells.⁸² More investigation will be necessary to understand the functions of brain aromatase during development or adulthood, and the molecular mechanisms involved in the regulation of its gene. Indeed, because of its early expression during development, it is tempting to suggest that this gene could represent a key marker to test potential effects of endocrine disrupters.

Estrogens are also important signaling molecules that modulate specific target genes directly involved in reproductive functions at the brain and pituitary levels. In the pituitary, gonadotropin (GTH) I and II also called, follicle stimulating hormone (FSH) and luteinizing hormone (LH) respectively, are involved in reproductive events such as steroidogenesis, vitellogenesis and ovulation. The production and release of these pituitary hormones is controlled by neuroendocrine systems including the positive regulator GnRH and the negative regulator dopamine.³ Although several reports indicate that estrogens and androgens affect GnRH synthesis and release,⁹¹ the mechanisms underlying these effects are unknown. Until now, ER α has not been detected in GnRH neurons of teleosts,⁹² however the possibility exists for a low expression of ER β as recently observed in mammals.⁹³ In contrast, a strong expression of ER α has been reported in the dopaminergic neurons of the preoptic area responsible for the dopamine inhibition of GTH secretion.⁹⁴ Accordingly, the dopaminergic inhibition has been shown to be dependent upon the steroid environment and several mechanisms can account for this regulation. In goldfish and trout, estrogens and androgens are known for increasing the pituitary dopaminergic turnover,^{95,96} therefore reinforcing the dopaminergic inhibitory tone. However, estrogen can also modulate tyrosine hydroxylase expression⁹⁷ and/or that of brain and pituitary D2 receptors.⁹⁸

Pituitary gonadotropins (GTH I or FSH, and GTH II or LH) are synthesized in separate gonadotrope cells whose activity is also modulated by sexual steroids. The two GTHs are composed of two noncovalently associated subunits: a common α -subunit, and a specific β -subunit which confers hormonal specificity.⁹⁹ Studies in salmonids have shown that both E₂ and testosterone specifically stimulate the coho salmon LH β gene expression in cultured pituitary cells.⁴ Transfection assays with the 5'-flanking regions of the LH β gene into juvenile rainbow trout pituitary cells, clearly showed that LH β is positively regulated by estradiol through a direct action of ER α . The promoter region of the LH β gene possesses an ERE that allows direct ER binding and confers estrogenic sensibility on the gene. However, transfection studies revealed that in combination with ER, other cell-specific transcription factors are needed to mediate E₂-induction. Indeed, E₂-activation of LH β requires a synergistic effect

between ER and the orphan nuclear receptor SF-1 (steroidogenic factor). The interaction seems to play a crucial role in the cell-type specificity and control of expression of the α - and β -subunit genes.¹⁰⁰ The SF-1 DNA-binding site, also called gonadotrope-specific element (GSE), was actually found in the promoter of the α - and β -subunit genes of all vertebrate species characterized. In addition, knock-out experiments in mouse showed that SF-1 is essential for the development of gonadotrope cells in the pituitary.¹⁰¹ Recently, it was shown that the pituitary homeobox factor-1 (Pitx-1) also plays a crucial role in the regulation of salmon LH β acting synergically with SF-1 alone or in combination with ER α .¹⁰² Moreover, Pitx-1 facilitates the effect of GnRH on the salmon LH β (see Chapter 3).

Conclusion and Perspectives

Over the last few years, our views on the estrogen receptors in fish have changed dramatically with the discovery of new forms and isoforms: (1) two ER α isoforms, including ER α -short, which lacks the A domain and exhibits a constitutive transcriptional activity, and (2) two ER β functional forms, ER β 1 and ER β 2, which have differential transcriptional activities on the ER α gene. The specific physiological functions sustained by these novel teleost ERs are far from being understood. At the present time, it is clear that ER α plays a key role in the E₂-dependent regulation of the liver vitellogenesis process. However, in the liver, we demonstrated that the ER α gene generated two functionally distinct isoforms, ER α -long and ER α -short, and that two ER β forms can be also expressed in hepatocytes. Therefore, it would be interesting to investigate the complex interplay that probably occurs between these new teleost ERs in the process of vitellogenesis. Moreover, our results in zebrafish showed that zfER α , zfER β 1 and zfER β 2 are differentially distributed in discrete areas of the preoptic region and hypothalamus, suggesting that they could be involved in different functions in the neuroendocrine system. However, the fact that they are often co-expressed within the same cells also suggests that they can interact in a complex manner to regulate

target genes. The mouse knock-out technology revealed that mammalian ERs could be implicated in several distinct physiological processes, but the results are still unclear and controversial. The zebrafish is a small and hardy fish, with a relatively short generation time, currently used in developmental biology and toxicology studies. Additionally, the transparent embryos permit one to observe the organogenesis processes without disturbing development. Moreover, multiple techniques are currently being developed to specifically decrease or even block the expression of a given gene in a transitory or permanent manner. Therefore, the zebrafish could be proposed as an alternative model to dissect the physiological functions of each ER form not only in adults, but also during development.

In mammals, numerous studies have reported the neurotrophic and neuroprotective effects of estrogens in the developing embryo and the adult. In particular, it is known that radial glial cells, which disappear in adult mammals, are involved in neurogenesis and that there is a peak of aromatase activity at the time of neurogenesis. Such mechanisms have not been studied in the brain of fish. Curiously, adult teleost fish are characterized by a high aromatase activity in the radial glial cells, which could be correlated with the capacity of the fish brain to grow during adulthood. Consequently, it is tempting to suggest that developing and adult fish could be good models to study the role of E₂ in neurogenesis and the respective role of each ER form in this process.

It has been known for a long time that a wide range of endocrine disrupting components (EDC) sharing structural similarities with steroid hormones, can act as estrogens and may have the potential to modify sexual development in exposed fish. For example, bisexual gonads have been observed in several fish species after exposure to EDCs. Consequently, the characterization of three distinct ER forms in zebrafish will be interesting tools to investigate the potential binding of these EDCs. Additionally, the use of E₂ dependent genes such as ER α , VTG and aromatase as biomarkers is highly interesting to evaluate the potential impacts of EDCs on the reproductive axis in adults and also during development.

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References

1. Guiguen Y, Baroiller JF, Ricordel MJ, Iseki K, Mcmeel OM, Martin SA and Fostier A (1999). Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*). *Mol. Reprod. Dev.* **54:** 154–162.
2. Dorrington JH, Bendell JJ and Khan SA (1993). Interactions between FSH, estradiol-17 beta and transforming growth factor-beta regulate growth and differentiation in the rat gonad. *J Steroid Biochem. Mol. Biol.* **44:** 441–447.
3. Kah O, Anglade I, Leprêtre E, Dubourg P and De Monbrison D (1993). The reproductive brain in fish. *Fish Physiol. Biochem.* **11:** 85–98.
4. Xiong F, Liu D, Le Drean Y, Elsholtz HP and Hew CL (1994). Differential recruitment of steroid hormone response elements may dictate the expression of the pituitary gonadotropin II beta subunit gene during salmon maturation. *Mol. Endocrinol.* **8:** 782–793.
5. Flouriot G, Pakdel F, Ducouret B, Le Drean Y and Valotaire Y (1997). Differential regulation of two genes implicated in fish reproduction: vitellogenin and estrogen receptor genes. *Mol. Reprod. Dev.* **48:** 317–323.
6. Arukwe A (2001). Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction. *Mar. Pollut. Bull.* **42:** 643–655.
7. McLachlan JA (2001). Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocr. Rev.* **22:** 319–341.
8. Couse JF and Korach KS (1999). Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr. Rev.* **20:** 358–417.
9. Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P and Chambon P (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320:** 134–139.

10. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S and Gustafsson JA (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* **93**: 5925–5930.
11. Evans RM (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240**: 889–895.
12. Coleman KM and Smith CL (2001). Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front. Biosci.* **6**: D1379–D1391.
13. Pakdel F, Metivier R, Flouriot G and Valotaire Y (2000). Two estrogen receptor (ER) isoforms with different estrogen dependencies are generated from the trout ER gene. *Endocrinology* **141**: 571–580.
14. Patino R, Xia Z, Gale W, Wu C, Maule AG and Chang X (2002). Novel transcripts of the estrogen receptor alpha gene in channel catfish. *Gen. Comp. Endocrinol.* **120**: 314–325.
15. Claret FX, Chapel S, Garces J, Tsai-Pflugfelder M, Bertholet C, Shapiro DJ, Wittek R and Wahli W (1994). Two functional forms of the *Xenopus laevis* estrogen receptor translated from a single mRNA species. *J. Biol. Chem.* **269**: 14047–14055.
16. Griffin C, Flouriot G, Sonntag-Buck V, Nestor P and Gannon F (1998). Identification of novel chicken estrogen receptor-alpha messenger ribonucleic acid isoforms generated by alternative splicing and promoter usage. *Endocrinology* **139**: 4614–4625.
17. Griffin C, Flouriot G, Sonntag-Buck V and Gannon F (1999). Two functionally different protein isoforms are produced from the chicken estrogen receptor-alpha gene. *Mol. Endocrinol.* **13**: 1571–1587.
18. Menuet A, Pellegrini E, Anglade I, Blaise O, Laudet V, Kah O and Pakdel F (2002). Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. *Biol. Reprod.* **66**: 1881–1892.
19. Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A and Thomas P (2000). Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proc. Natl. Acad. Sci. USA* **97**: 10751–10756.
20. Ma CH, Dong KW and Yu KL (2000). cDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (*Carassius auratus*). *Biochim. Biophys. Acta* **1490**: 145–152.

21. Bardet PL, Horard B, Robinson-Rechavi M, Laudet V and Vanacker JM (2002). Characterization of oestrogen receptors in zebrafish (*Danio rerio*). *J. Mol. Endocrinol.* **28**: 153–163.
22. Klinge CM (2001). Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res.* **29**: 2905–2919.
23. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D and Chambon P (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**: 1491–1494.
24. Endoh H, Maruyama K, Masuhiro Y, Kobayashi Y, Goto M, Tai H, Yanagisawa J, Metzger D, Hashimoto S and Kato S (1999). Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. *Mol. Cell. Biol.* **19**: 5363–5372.
25. Metivier R, Petit FG, Valotaire Y and Pakdel F (2000). Function of N-terminal transactivation domain of the estrogen receptor requires a potential alpha-helical structure and is negatively regulated by the A domain. *Mol. Endocrinol.* **14**: 1849–1871.
26. Metivier R, Stark A, Flouriot G, Hubner MR, Brand H, Penot G, Manu D, Denger S, Reid G, Kos M, Russell RB, Kah O, Pakdel F and Gannon F (2002). A dynamic structural model for estrogen receptor-alpha activation by ligands, emphasizing the role of interactions between distant A and E domains. *Mol. Cell.* **10**: 1019–1032.
27. Martinez E and Wahli W (1989). Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. *EMBO J.* **8**: 3781–3791.
28. Zilliacus J, Wright AP, Carlstedt-Duke J and Gustafsson JA (1995). Structural determinants of DNA-binding specificity by steroid receptors. *Mol. Endocrinol.* **9**: 389–400.
29. Ruff M, Gangloff M, Wurtz JM and Moras D (2000). Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand-binding domains of estrogen receptors. *Breast Cancer Res.* **2**: 353–359.
30. Vanacker JM, Pettersson K, Gustafsson JA and Laudet V (1999). Transcriptional targets shared by estrogen receptor-related receptors (ERRs) and estrogen receptor (ER) alpha, but not by ERbeta. *EMBO J.* **18**: 4270–4279.

31. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ and Scanlan TS (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277**: 1508–1510.
32. Jakacka M, Ito M, Weiss J, Chien PY, Gehm BD and Jameson JL (2001). Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. *J. Biol. Chem.* **276**: 13615–13621.
33. Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA and Safe S (2000). Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. *J. Biol. Chem.* **275**: 5379–5387.
34. Wang C, Fu M, Angeletti RH, Siconolfi-Baez L, Reutens AT, Albanese C, Lisanti MP, Katzenellenbogen BS, Kato S, Hopp T, Fuqua SA, Lopez GN, Kushner PJ and Pestell RG (2001). Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. *J. Biol. Chem.* **276**: 18375–18383.
35. Shiu AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA and Greene GL (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**: 927–937.
36. Ekena K, Weis KE, Katzenellenbogen JA and Katzenellenbogen BS (1997). Different residues of the human estrogen receptor are involved in the recognition of structurally diverse estrogens and antiestrogens. *J. Biol. Chem.* **272**: 5069–5075.
37. Schwartz JA, Zhong L, Deighton-Collins S, Zhao C and Skafar DF (2002). Mutations targeted to a predicted helix in the extreme carboxyl-terminal region of the human estrogen receptor-alpha alter its response to estradiol and 4-hydroxytamoxifen. *J. Biol. Chem.* **277**: 13202–13209.
38. Pakdel F, Le Guellec C, Vaillant C, Le Roux MG and Valotaire Y (1989). Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. *Mol. Endocrinol.* **3**: 44–51.
39. Pakdel F, Le Gac F, Le Goff P and Valotaire Y (1990). Full-length sequence and *in vitro* expression of rainbow trout estrogen receptor cDNA. *Mol. Cell. Endocrinol.* **71**: 195–204.
40. Tan NS, Lam TJ and Ding JL (1995). Molecular cloning and sequencing of the hormone-binding domain of *Oreochromis aureus* estrogen receptor gene. *DNA Seq.* **5**: 359–370.

41. Xia Z, Gale WL, Chang X, Langenau D, Patino R, Maule AG and Densmore LD (2000). Phylogenetic sequence analysis, recombinant expression, and tissue distribution of a channel catfish estrogen receptor beta. *Gen. Comp. Endocrinol.* **118**: 139–149.
42. Munoz-Cueto JA, Burzawa-Gerard E, Kah O, Valotaire Y and Pakdel F (1999). Cloning and sequencing of the gilthead sea bream estrogen receptor cDNA. *DNA Seq.* **10**: 75–84.
43. Touhata K, Kinoshita M, Toyohara H and Sakaguchi M (1998). Sequence and expression of a cDNA encoding the red seabream estrogen receptor. *Fish Sci.* **64**: 131–135.
44. Petit F, Valotaire Y and Pakdel F (1995). Differential functional activities of rainbow trout and human estrogen receptors expressed in the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **233**: 584–592.
45. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, Sonntag-Buck V and Gannon F (2000). Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. *EMBO J.* **19**: 4688–4700.
46. Menuet A, Anglade I, Flouriot G, Pakdel F and Kah O (2001). Tissue-specific expression of two structurally different estrogen receptor alpha isoforms along the female reproductive axis of an oviparous species, the rainbow trout. *Biol. Reprod.* **65**: 1548–1557.
47. Bouma J and Nagler JJ (2001). Estrogen receptor-alpha protein localization in the testis of the rainbow trout (*Oncorhynchus mykiss*) during different stages of the reproductive cycle. *Biol. Reprod.* **65**: 60–65.
48. Robinson-Rechavi M, Marchand O, Escriva H, Bardet PL, Zelus D, Hughes S and Laudet V (2001). Euteleost fish genomes are characterized by expansion of gene families. *Genome Res.* **11**: 781–788.
49. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F and Giguere V (1997). Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol. Endocrinol.* **11**: 353–365.
50. Tremblay GB, Tremblay A, Labrie F and Giguere V (1999). Dominant activity of activation function 1 (AF-1) and differential stoichiometric requirements for AF-1 and -2 in the estrogen receptor alpha-beta heterodimeric complex. *Mol. Cell. Biol.* **19**: 1919–1927.

51. Klinge CM (2000). Estrogen receptor interaction with co-activators and co-repressors. *Steroids* **65**: 227–251.
52. Kuiper GG, Carlsson B, Grandien K, Enmark E, Hagglad J, Nilsson S and Gustafsson JA (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**: 863–870.
53. Xia Z, Patino R, Gale WL, Maule AG and Densmore LD (1999). Cloning, *in vitro* expression, and novel phylogenetic classification of a channel catfish estrogen receptor. *Gen. Comp. Endocrinol.* **113**: 360–368.
54. Socorro S, Power DM, Olsson PE and Canario AV (2000). Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution. *J. Endocrinol.* **166**: 293–306.
55. Shughrue PJ, Scrimo PJ and Merchenthaler I (1998). Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. *Endocrinology* **139**: 5267–5270.
56. Cowley SM, Hoare S, Mosselman S and Parker MG (1997). Estrogen receptors alpha and beta form heterodimers on DNA. *J. Biol. Chem.* **272**: 19858–19862.
57. Pettersson K, Grandien K, Kuiper GG and Gustafsson JA (1997). Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. *Mol. Endocrinol.* **11**: 1486–1496.
58. Pakdel F, Feon S, Le Gac F, Le Menn F and Valotaire Y (1991). *In vivo* estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. *Mol. Cell. Endocrinol.* **75**: 205–212.
59. Lee JH, Kim J and Shapiro DJ (1995). Regulation of *Xenopus laevis* estrogen receptor gene expression is mediated by an estrogen response element in the protein coding region. *DNA Cell Biol.* **14**: 419–430.
60. Ninomiya Y, Mochii M, Eguchi G, Hasegawa T, Masushige S and Kato S (1992). Tissue-specific response of estrogen receptor gene expression to estrogen in chick. *Biochem. Biophys. Res. Commun.* **187**: 1374–1380.
61. Flouriot G, Pakdel F and Valotaire Y (1996). Transcriptional and post-transcriptional regulation of rainbow trout estrogen receptor and vitellogenin gene expression. *Mol. Cell. Endocrinol.* **124**: 173–183.
62. Le Roux MG, Theze N, Wolff J and Le Pennec JP (1993). Organization of a rainbow trout estrogen receptor gene. *Biochim. Biophys. Acta* **1172**: 226–230.

63. Le Drean Y, Lazennec G, Kern L, Saligaut D, Pakdel F and Valotaire Y (1995). Characterization of an estrogen-responsive element implicated in regulation of the rainbow trout estrogen receptor gene. *J. Mol. Endocrinol.* **15**: 37–47.
64. Lazennec G, Kern L, Valotaire Y and Salbert G (1997). Cooperation between the human estrogen receptor (ER) and MCF-7 cell-specific transcription factors elicits high activity of an estrogen-inducible enhancer from the trout ER gene promoter. *Mol. Cell. Biol.* **17**: 5053–5066.
65. Metivier R, Gay FA, Hubner MR, Flouriot G, Salbert G, Gannon F, Kah O and Pakdel F (2002). Formation of an hER alpha-COUP-TFI complex enhances hER alpha AF-1 through Ser118 phosphorylation by MAPK. *EMBO J.* **21**: 3443–3453.
66. Lethimonier C, Flouriot G, Valotaire Y, Kah O and Ducouret B (2000). Transcriptional interference between glucocorticoid receptor and estradiol receptor mediates the inhibitory effect of cortisol on fish vitellogenesis. *Biol. Reprod.* **62**: 1763–1771.
67. Lethimonier C, Flouriot G, Kah O and Ducouret B (2002). The glucocorticoid receptor represses the positive autoregulation of the trout estrogen receptor gene by preventing the enhancer effect of a C/EBPbeta-like protein. *Endocrinology* **143**: 2961–2974.
68. Tyler CR, Sumpter JP and Withthames PR (1990). The dynamics of oocyte growth during vitellogenesis in the rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod.* **43**: 202–209.
69. Davail B, Pakdel F, Bujo H, Perazzolo LM, Waclawek M, Schneider WJ and Le Menn F (1998). Evolution of oogenesis: the receptor for vitellogenin from the rainbow trout. *J. Lipid Res.* **39**: 1929–1937.
70. Oppen-Berntsen DO, Gram-Jensen E and Walther BT (1992). Zona radiata proteins are synthesized by rainbow trout (*Oncorhynchus mykiss*) hepatocytes in response to oestradiol-17 beta. *J. Endocrinol.* **135**: 293–302.
71. Sumpter JP and Jobling S (1995). Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* **7**: 173–178.
72. Flouriot G, Pakdel F, Ducouret B and Valotaire Y (1995). Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. *J. Mol. Endocrinol.* **15**: 143–151.
73. Arukwe A, Celius T, Walther BT and Goksøyr A (2000). Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). *Aquat. Toxicol.* **49**: 159–170.

74. Riegel AT, Aitken SC, Martin MB and Schoenberg DR (1987). Posttranscriptional regulation of albumin gene expression in *Xenopus* liver: evidence for an estrogen receptor-dependent mechanism. *Mol. Endocrinol.* **1**: 160–167.
75. Selcer KW and Palmer BD (1995). Estrogen downregulation of albumin and a 170-kDa serum protein in the turtle, *Trachemys scripta*. *Gen. Comp. Endocrinol.* **97**: 340–352.
76. Flouriot G, Ducouret B, Byrnes L and Valotaire Y (1998). Transcriptional regulation of expression of the rainbow trout albumin gene by estrogen. *J. Mol. Endocrinol.* **20**: 355–362.
77. Balthazart J and Ball GF (1998). New insights into the regulation and function of brain estrogen synthase (aromatase). *Trends Neurosci.* **21**: 243–249.
78. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorenz S, Amarneh B, Ito Y, Fisher CR and Michael MD (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.* **15**: 342–355.
79. Tchoudakova A, Kishida M, Wood E and Callard GV (2001). Promoter characteristics of two *cyp19* genes differentially expressed in the brain and ovary of teleost fish. *J. Steroid Biochem. Mol. Biol.* **78**: 427–439.
80. Timmers RJ, Lambert JG, Peute J, Vullings HG and van Oordt PG (1987). Localization of aromatase in the brain of the male African catfish, *Clarias gariepinus* (Burchell), by microdissection and biochemical identification. *J. Comp. Neurol.* **258**: 368–377.
81. Forlano PM, Deitcher DL, Myers DA and Bass AH (2001). Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. *J. Neurosci.* **21**: 8943–8955.
82. Menuet A, Anglade I, Le Guevel R, Pellegrini E, Pakdel F and Kah O (2003). Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. *J. Comp. Neurol.* **462**: 180–193.
83. Pasmanik M and Callard GV (1985). Aromatase and 5 alpha-reductase in the teleost brain, spinal cord, and pituitary gland. *Gen. Comp. Endocrinol.* **60**: 244–251.
84. Pasmanik M and Callard GV (1988). Changes in brain aromatase and 5 alpha-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius auratus*). *Endocrinology* **123**: 1162–1171.

85. Gelinas D, Pitoc G and Callard G (1998). Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. *Mol. Cell. Endocrinol.* **138**: 81–93.
86. Kishida M, McLellan M, Miranda JA and Callard GV (2001). Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129**: 261–268.
87. Kishida M and Callard GV (2001). Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development. *Endocrinology* **142**: 740–750.
88. Kazeto Y, Ijiri S, Place AR, Zohar Y and Trant JM (2001). The 5'-flanking regions of CYP19A1 and CYP19A2 in zebrafish. *Biochem. Biophys. Res. Commun.* **288**: 503–508.
89. Callard GV, Tchoudakova AV, Kishida M and Wood E (2001). Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. *J. Steroid Biochem. Mol. Biol.* **79**: 305–314.
90. Tchoudakova A and Callard GV (1998). Identification of multiple CYP19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. *Endocrinology* **139**: 2179–2189.
91. Breton B, Motin A, Billard R, Kah O, Geoffre S and Precigoux G (1986). Immunoreactive gonadotropin-releasing hormone-like material in the brain and the pituitary gland during the periovulatory period in the brown trout (*Salmo trutta* L.): relationships with the plasma and pituitary gonadotropin. *Gen. Comp. Endocrinol.* **61**: 109–119.
92. Navas JM, Anglade I, Bailhache T, Pakdel F, Breton B, Jego P and Kah O (1995). Do gonadotrophin-releasing hormone neurons express estrogen receptors in the rainbow trout? A double immunohistochemical study. *J. Comp. Neurol.* **363**: 461–474.
93. Herbison AE and Pape JR (2001). New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. *Front. Neuroendocrinol.* **22**: 292–308.
94. Linard B, Anglade I, Corio M, Navas JM, Pakdel F, Saligaut C and Kah O (1996). Estrogen receptors are expressed in a subset of tyrosine hydroxylase-positive neurons of the anterior preoptic region in the rainbow trout. *Neuroendocrinology* **63**: 156–165.

95. Saligaut C, Linard B, Mananos E, Kah O, Breton B and Govoroun M (1998). Release of pituitary gonadotrophins GtH I and GtH II in the rainbow trout (*Oncorhynchus mykiss*): modulation by estradiol and catecholamines. *Gen. Comp. Endocrinol.* **109**: 302–309.
96. Trudeau VL, Soley BD, Wong AO and Peter RE (1993). Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. *Gen. Comp. Endocrinol.* **89**: 39–50.
97. Vetillard A, Atteke C, Saligaut C, Jego P and Bailhache T (2003). Differential regulation of tyrosine hydroxylase and estradiol receptor expression in the rainbow trout brain. *Mol. Cell. Endocrinol.* **199**: 37–47.
98. Vacher C, Pellegrini E, Anglade I, Ferriere F, Saligaut C and Kah O (2003). Distribution of dopamine D2 receptor mRNAs in the brain and the pituitary of female rainbow trout: An *in situ* hybridization study. *J. Comp. Neurol.* **458**: 32–45.
99. Swanson P, Suzuki K, Kawauchi H and Dickhoff WW (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. *Biol. Reprod.* **44**: 29–38.
100. Le Drean Y, Liu D, Wong AO, Xiong F and Hew CL (1996). Steroidogenic factor 1 and estradiol receptor act in synergism to regulate the expression of the salmon gonadotropin II beta subunit gene. *Mol. Endocrinol.* **10**: 217–229.
101. Ingraham HA, Lala DS, Ikeda Y, Luo X, Shen WH, Nachtigal MW, Abbud R, Nilson JH and Parker KL (1994). The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev.* **8**: 2302–2312.
102. Melamed P, Koh M, Preklathan P, Bei L and Hew C (2002). Multiple mechanisms for Pitx-1 transactivation of a luteinizing hormone beta subunit gene. *J. Biol. Chem.* **277**: 26200–26207.

Vitellogenesis and Vitellogenin Uptake into Oocytes

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Abstract

The yolk proteins found in the oocyte of oviparous animals are synthesized by the liver as a large lipophosphoglycoprotein precursor, vitellogenin (Vtg), which represents a vital, sole source of nutrient for the developing larvae. Vitellogenesis underlies early development of oviparous animals. The Vtg gene is extremely sensitive to estrogen and other steroid hormones. Thus, vitellogenesis is a useful model for studying hormonal regulation of eukaryotic gene expression. The *Oreochromis aureus* vitellogenin, OaVtg gene spans 9 kb and contains 34 exons. Although the OaVtg promoter has a nonconsensus TATA, this promoter is capable of driving basal transcription. Two imperfect estrogen response elements, ERE_p (proximal) and ERE_d (distal), located in the promoter at -532 and -1352 elicit a significant increase in estrogen-dependent reporter gene activity. Competition gel mobility shift assays have demonstrated that ERE_p and a novel ERE_{exon2} (located within exon 2) exhibit specific binding of estrogen receptor. Thus, nonconsensus OaVtg EREs contribute to the estrogen-dependent regulation of the OaVtg gene *in vivo*. During estrogen-induced vitellogenesis, the massive rate of transcription and translation of Vtg followed by its secretion into the blood makes this a major circulating protein that is taken up by membrane-bound vitellogenin receptors (VtgR) found on oocytes. The VtgR, which belongs to the low density lipoprotein receptor family, interacts with the N-terminal region of Vtg

via electrostatic attraction. Once in the oocytes, Vtg is cleaved into yolk proteins (lipovitellin and phosvitin), which are stored as nutrients for the developing embryo.

Introduction

One of the major challenges to the survival of the developing fish larva and fry is the level of nutrition supplied in the form of its yolk protein which is the ultimate nutrient source. Vitellogenin (Vtg) is a principal precursor of egg-yolk proteins, and is thus crucial for successful embryonic and larval growth. Vitellogenesis presents a versatile model for the study of hormone-inducible gene expression. Estradiol (E_2)-induced vitellogenin mRNA synthesis in oviparous vertebrates has been well-documented in amphibians,¹ avians,² reptiles³ and fish.⁴ In numerous oviparous animals, Vtgs are large phospholipoglycoproteins, varying in size from 200 to 700 kDa. The Vtg protein is secreted into the bloodstream by the intestines of nematodes, fat bodies of insects, and livers of vertebrates. In vertebrates, plasma Vtg dimers are sequestered via membrane-bound Vtg receptor-mediated endocytosis into the growing oocytes where further site-specific cleavages occur, yielding smaller yolk proteins.⁵

This review will encompass a survey of the Vtg proteins from various oviparous species and will examine the Vtg gene and hormonal⁶ regulation of its expression. Finally, the molecular biology of the VtgR:Vtg interaction during uptake of the Vtg protein into the oocyte will be considered.

Hormone-Induced Vitellogenesis

The classical role of estradiol (E_2) in the activation of vitellogenesis in oviparous vertebrates has been widely accepted for teleosts, amphibians, reptiles, and avians.^{1,4,5,7-11} E_2 -induced vitellogenesis in male animals or hepatocyte cultures typically ceased when the hormone was withdrawn.^{7,9} However, later investigations in other species suggest that

the Vtg gene can be modulated by factors other than estrogens. Vtg gene expression is more complicated than originally thought. The effects of ambient temperature,¹² androgens,¹³ a pituitary factor,^{9,14,15} growth hormone,¹⁴ pituitary homogenate,¹⁵ prolactin,¹⁶⁻¹⁸ thyroid hormone,^{19,20} and glucocorticoids such as dexamethasone²¹ and cortisol,²² on vitellogenesis have called for a reinterpretation of hormone-induced Vtg gene expression.

In situ hybridization studies²³ showed that E₂-stimulation of male *Oreochromis aureus* (tilapia, a teleost fish from the Cichlidae family) gave rise to uneven distribution of the Vtg mRNA in the liver. E₂ was also found to induce the synthesis of low levels of Vtg mRNA in the testis, indicating the presence of functional estrogen receptor (ER) in the testis. Interestingly, when cortisol was implanted 72 h after E₂-stimulation, there was a potentiation 24 h later of Vtg mRNA synthesis in both the hepatic and gonadal tissues. Such observations have similarly been made with catfish.²² When administered alone, cortisol suppressed the catfish plasma Vtg protein.²⁴ Since cortisol is a hormone produced in excess during stress, its seemingly deleterious effects in teleost reproduction *in vivo*^{25,26} and its inability to induce Vtg protein *in vitro*²⁷ appear convincing. However, the increase of cortisol during ovarian recrudescence,^{22,28-30} preovulation,³¹ postovulation,^{32,33} or spawning^{28,34} led to speculations on the biological significance of cortisol in teleost reproduction apart from its other well-established roles in osmoregulation, ionic balance, metabolism of proteins and carbohydrates, and stress responses. It has been suggested that cortisol is indirectly involved in oocyte maturation.³⁵ Thus, Ding *et al.*³⁶ studied the effects of cortisol on vitellogenin gene expression in tilapia, whose biology is not complicated by problems of osmoregulation, migration, or senescence. This study reported that cortisol triggers a rapid (within 1 h) but transient surge (30-fold) in transcription of the silent Vtg gene in the male, producing short-lived Vtg mRNA, which was probably not efficiently translated into Vtg proteins.

Vtg mRNA of ~6 kNt has been characterized from the livers of female and E₂-treated male *O. aureus*.¹⁰ By hybridization with a specific *O. aureus* cDNA probe, the Vtg mRNA transcript was detected as early

as 1 h following primary and secondary E₂-stimulation, although for the latter, the rate of accumulation of Vtg-specific mRNA was 20-fold higher.⁴ Under the influence of E₂, the *O. aureus* Vtg mRNA peaked at 72 h and 48 h, respectively, for primary and secondary stimulation. At the translational level, the increase in plasma Vtg was further enhanced during the secondary stimulation. There was a distinct shift in the peak of plasma Vtg from day 14 in the primary induction to day 3 in the secondary stimulation.⁴ This rapid and massive rise in E₂-stimulated vitellogenesis is more recently explained by the discovery of strong interplay between liver-specific transcription factors such as ER, Vtg-binding protein (VBP) and GATA on the *O. aureus* Vtg promoter, which culminates in synergistic effects of these nuclear factors in potentiating vitellogenin gene transcription.³⁷

Vitellogenin Proteins — Interspecific Diversity in Molecular Size and Domain Structure

Studies on the physiology and biochemistry of vitellogenesis showed that under denaturing conditions of SDS-PAGE, the monomeric size of Vtg precursors is between 180–250 kDa in insects,³⁸ *Xenopus*,³⁹ and chicken.^{40,41} Investigations of the *Caenorhabditis elegans*⁴² have revealed two monomeric precursor Vtgs of different molecular weights, at 170 and 180 kDa. The study of vitellogenesis has also been extended to a few fish species to give more disparate observations. By gel filtration under native conditions, Plack *et al.*⁴³ identified cod Vtg to be 400 kDa. The killifish, *Fundulus heteroclitus* has a monomeric Vtg of 200 kDa.⁴⁴ By denaturing conditions of SDS-PAGE, the monomeric form of *Salmo gairdneri* Vtg was found to range from 170 kDa⁴⁵ to 200 kDa.⁴⁶ Ding *et al.*⁴⁷ reported two forms of Vtg of monomeric sizes, 130 and 180 kDa in the evolutionarily more advanced teleost fish, *O. aureus*. Vtg variation studied in 11 species of Perciformes, encompassing the families Cichlidae, Serranidae, Lutjanidae, Centropomidae, and Eleotrididae confirmed that in this order of fish the main features of Vtg protein diversity are interfamily variability in size and subunit number.¹¹

Invertebrate Vtg characteristically consists of two modules, the N-terminal lipovitellin I (LVI) and the C-terminal lipovitellin II (LVII). Vertebrate Vtg contains an extra serine-rich domain, phosvitin (PV), found between LVI and LVII. Invertebrate Vtgs lack the PV domain, although some possess similar intervening serine repeats.⁴⁸ These insect polyserines are much shorter and share no homology with vertebrate PV.⁴⁹ Typically, PV consists of long chains of serine residues interrupted by short stretches of basic amino acids. It was predicted that PV size expands in more recently evolved species, as fish have smaller PV than *Xenopus* and chicken.⁵⁰ However, contrary to this prediction, Lim *et al.*⁵¹ reported that the Vtg cDNA of *O. aureus* has a prominent PV domain, comparable in size to that of *Xenopus* and chicken Vtgs.

The Vtg Gene and Its Molecular Evolution

Vtg belongs to an ancient family of genes conserved from protostomes to deuterostomes. Most studies to date show Vtg to be encoded by a small family of genes, with the exception of the single gene in sea urchin.⁵ The *Xenopus* Vtg genes form two linkage groups, the A1, A2 and B1 genes constituting the first, whereas the B2 gene segregates independently.⁵² The expression of these A and B group genes can be non-coordinated in primary stimulation and coordinated in secondary estrogen stimulation.⁵³

In chicken, the gene encoding the major protein (VtgII) has been characterized.⁵⁴ In *Drosophila*, three Vtg genes located on the X chromosome have been isolated.⁵⁵ The *C. elegans* has been reported to possess six Vtg genes, the first five (vit-1 to vit-5) are linked to the X-chromosome, while the vit-6 gene is autosomal.⁵⁶ Analysis of cDNA libraries constructed from the livers of male and female *O. aureus* that were stimulated with E₂ indicated variant forms of Vtg genes.¹⁰ Further characterization of these Vtg cDNAs suggested the existence of 2 major groups of tilapia Vtg genes.⁵⁷

In recent years, contiguous conserved sequence motifs have been identified between Vtg orthologues and some non-Vtg proteins.

Phylogenetic analyses indicate that LVI and lipoproteins such as insect apolipoporphin I/II precursor, human apolipoprotein B (Apo B100) and the large subunit of mammalian microsomal triglyceride transfer proteins were derived from a common ancestral functional unit.⁵⁸ LVII contains conserved sequences related to the von Villebrand factor (VWF) D. Like other storage proteins, Vtggs are also prone to accumulating mutations along the evolutionary pathway. However, there is sufficient conservation to recognize homology among Vtggs of different animal classes. Being structurally and functionally conserved but divergent in primary sequences, this ancient storage protein of more than 500 million years^{42,54} may be useful in providing insights into the radiation of multicellular animals. In recent years, sequence data have accumulated on insect Vtggs in addition to those from nematode, *Xenopus*, chicken, lamprey, and chondrostean fish. Although the rainbow trout Vtg gene has been sequenced,⁵⁹ comparative analysis of the relationship between fish Vtg genes and those of other oviparous vertebrates needs to be extended to other groups of teleost for sufficient characterisation of their similarity to the vertebrate Vtg gene family. In view of the great extent of teleost diversification, the number of teleost Vtg orthologues is far under-represented. Lim *et al.*⁵¹ reported the cloning and analysis of the cDNA of *Oreochromis aureus* Vtg (*pOaVtg*). Phylogenetic analyses of the *pOaVtg* indicated a close relationship between nematodes and arthropods. Furthermore, Lim *et al.*⁵¹ showed evidence for a closer interspecific relationship between *O. aureus* Vtg I and Fundulus VtgII than the intraspecific relationship between Fundulus VtgI and II isoforms, suggesting that teleost ancestors had at least two Vtg isoforms.

The Vtg Promoter: Regulation of the Tilapia Vtg Estrogen Responsive Elements

Vtg transcription is effected by binding of estrogen to estrogen receptor (ER) which dimerises and binds to a target DNA sequence known as estrogen response element (ERE). Such regulatory enhancers have been localized to the 5'-flanking region of Vtg genes of *X. laevis* and

chicken.^{60,61} Single copies of these elements matching the palindromic consensus sequence, GGTCAnnnTGACC, are able to confer significant estrogen inducibility on reporter genes. Various non-consensus EREs have also been identified upstream of the *Xenopus* and chicken Vtg genes. On their own, imperfect EREs of the *Xenopus* Vtg B1 and B2 genes lack transcriptional enhancer activity. However, by synergistic action of two imperfect EREs, *cis*-linked genes acquire high estrogen inducibility.⁶² In addition, an imperfect ERE at -350 of the chicken VtgII gene lies within an estrogen response unit (ERU) that requires the synergy of a GATA factor and ER to function as an estrogen-dependent enhancer.⁶³ Although the *Xenopus* and chicken Vtg gene promoters have been well studied, such information had been lacking for the piscine Vtg gene. Earlier studies on *O. aureus* have shown that the OaVtg mRNA was detectable within 1 h of E₂-stimulation,²³ and peaked at 72 and 48 h upon primary and secondary stimulation, respectively, resulting in a 20-fold increase in Vtg mRNA accumulation.⁴ Thus, it was pertinent that Teo *et al.*⁶⁴ identified the structural features in this piscine Vtg gene that are responsible for such high and rapid expression of the Vtg gene, and characterized the molecular interactions of estrogen-responsive regulatory elements in the OaVtg promoter.³⁷

A survey of all Vtg promoters studied to date, including the OaVtg, revealed with a single exception of the sea urchin Vtg gene,⁶⁵ that most Vtg genes display neither a well-positioned consensus TATA box, nor a CAAT box sequence.^{42,66,67} A consensus TATA is found in the Vtg genes of *C. elegans*, sea urchin and *Drosophila*. The chicken and *Xenopus* Vtg (XIVg) genes also contain a TATA-like element, ATAAA.⁶⁵ In trout, the sequence TTAAAA found 27 bp upstream of the transcription initiation site was identified as the TATA signal. The OaVtg promoter has a similar AT-rich region, TTAAAAA, between 23 and 29 bp upstream of the transcription initiation site, which may function *in vivo* as a basal promoter.⁶⁴

Detailed analyses of the cloned OaVtg genomic sequence⁶⁴ showed that it spans 9 kb, containing 34 exons and 33 introns. Its transcription start site is located 15 bp upstream of the translational start codon. Transient transfection of the nonconsensus OaVtg TATA showed that

this promoter is capable of driving basal transcription. Two imperfect estrogen response elements: ERE_p (proximal) and ERE_d (distal) are located in the promoter at -532 and -1352, respectively. Conservation of an ERE, at a similar position as ERE_p across taxonomic lines (*Xenopus* Vg1 and chicken VTGII⁶⁵) strongly indicates its importance in the regulation of Vtg gene expression. Furthermore, in OaVtg, only ERE_p exhibits specific binding of the homologous recombinant estrogen receptor, OaER.⁶⁸ An imperfect ERE (ERE_{exon2}) found within exon 2 of the OaVtg gene also binds recombinant OaER at Kd of 1.4 nM, an affinity comparable to that of OaER binding to consensus ERE.⁶⁹ Hence, ERE_{exon2} may be strategically located internally/downstream of the transcription initiation site to confer correct folding of the DNA upon binding of ER. This conformational change is crucial to facilitate interaction with basal promoter complex and to cooperate with ERE_p by inducing DNA bending to activate rapid transcription of the OaVtg gene. The position-dependent location of functional internal ERE has also been alluded to by Nardulli *et al.*⁷⁰ Although ERE_p and ERE_d are nonconsensus in sequence, they are partially responsible for the estrogen-dependent activation (14-fold) of the OaVtg gene *in vitro*.⁶⁴ Furthermore, in addition to ERE_p, ERE_d and ERE_{exon2}, the OaVtg also contains four half-EREs at -927, -587, -579 and -179. Findings of functional half-EREs in the homologous OaER gene⁷¹ as well as reports on co-operation of half-EREs to confer inducibility of a gene^{72,73} indicate that the putative imperfect full and half EREs in the OaVtg gene are functional, either individually and/or synergistically.

To elucidate the full physiological potential of the Vtg promoter activity where > 100-fold increase in yolk production is observed *in vivo*, Teo *et al.*³⁷ further examined other liver tissue-specific transcription factor binding sites within -625 bp of the OaVtg promoter. Multiple GATA binding sites (WGATAR) are mapped within the OaVtg promoter. Since GATA-6 is reported to synergise with ER in an ERU in the chicken VTGII gene,⁶³ it was envisaged that GATA plays a crucial role in regulating the OaVtg gene, perhaps by nucleosome re-positioning/chromatin remodeling. The Vtg-binding protein (VBP) plays a pivotal role in E₂-dependent regulation of the *Xenopus* and

chicken Vtg genes⁷⁴ and the tilapia Vtg gene also contains a VBP binding site, albeit imperfect. Teo *et al.*³⁷ postulated that VBP plays a crucial role in regulating the OaVtg gene. It was found that in the presence of E₂, GATA and VBP synergise with ER, and the interplay of the corresponding promoter elements mediates proper hormone-dependent expression of the OaVtg gene, regardless of non-consensus sequence context of EREs and VBP. The interaction of GATA, VBP and ER was pronounced at $\geq 10^{-7}$ M E₂. In salmon, the gonadotropin II β subunit gene is transactivated through synergism between its ER and SF-1 only at high concentration of E₂, close to 10^{-7} M.⁷⁵ In tilapia and trout, the level of E₂ observed in the plasma during vitellogenesis is $\sim 2 \times 10^{-8}$ to 10^{-7} M.^{76,77} These observations indicate that a high E₂ environment similar to that occurring during vitellogenesis *in vivo* may be necessary for synergism between GATA, VBP and ER.

Transfection of the OaVtg promoter constructs into a liver cell line suggests that in addition to GATA and VBP, liver-specific transcription factors such as C/EBP and HNF5, as well as the ubiquitous factor, CTF/NF-1, also play an important role in potentiating the OaVtg gene. This accounts for the massive surge in Vtg gene expression that occurs *in vivo*.³⁷ Thus, the Vtg promoter may be maintained in the silent state by interaction with nucleosomes. The liganded ERs, assisted by liver-specific transcription factors, would then primarily act to make the promoter region accessible for the RNA polymerase complex.⁷⁸ Therefore, it is likely that ER alone cannot overcome tissue-specific barriers and that one or several additional liver components participate in mediating tissue-specific expression of the Vtg genes.⁷⁹

VtgR:Vtg — The Receptor: Ligand Interaction

Plasma Vtg binds to the vitellogenin receptor (VtgR) on the surface of oocytes and is taken up by receptor-mediated endocytosis.⁸⁰ A large amount of Vtg accumulates in the oocytes within a relatively short time. Once in the oocytes, Vtg is cleaved into yolk proteins, namely, lipovitellin (LV) and phosvitin (PV), which are stored as nutrients for

the developing embryo.⁸¹ Although many Vtg and VtgR genes have been cloned and characterized in recent years,⁸² knowledge on the molecular interaction between VtgR and Vtg remains limited. Thus, using full length Vtg⁵¹ and different domains of Vtg and VtgR from tilapia, Li *et al.*⁸³ have examined in detail, the interaction between VtgR and Vtg.

VtgR belongs to the low density lipoprotein receptor (LDLR) gene family. The members of this family bind to various ligands and are involved in lipid metabolism in both vertebrates and invertebrates. These receptors have common structural features^{84,85} including (i) cysteine-rich ligand-binding repeats, LBRs, (ii) cysteine-rich epidermal growth factor precursor (EGFP)-like repeats spaced by cysteine-poor spacer regions, (iii) a single transmembrane domain, and (iv) a short carboxyl-terminal cytoplasmic tail. In addition, a short region highly enriched in serine and threonine residues may exist in some receptors. The number of LBRs varies among different receptors. The LDLR contains seven LBRs, whereas very low density lipoprotein receptor (VLDLR) and VtgR in vertebrates have eight LBRs. Larger receptors such as LDLR-related protein and megalin have more than 30 LBRs in several clusters.^{86,87} Each LBR consists of about 40 amino acids including six cysteine residues, participating in the formation of three disulfide bonds, which are crucial for its proper folding.⁸⁸ At the carboxyl terminus of each LBR, there is a consensus acidic tripeptide, ser-asn-glu (SDE). Recent structural studies of LBRs 1, 2, 5 and 6 from LDLR have revealed that the side chains of many of the asp and glu residues in the consensus peptides are involved in coordinating the calcium ion into a folded calcium cage.⁸⁹⁻⁹⁴

The binding sites of Vtg for VtgR were presumed to be located on the LV1 domain.⁹⁵ Residue modification studies showed that lys and arg residues were important for binding with the acidic clusters in LBR of VtgR through ionic interactions.⁹⁶ However, new structural studies of LBR⁸⁹⁻⁹⁴ indicate that those acidic residues might not be accessible to Vtg. Thus, Li *et al.*⁸³ reassessed the current models for the binding of VtgR to Vtg. Because the sequences of LBRs in different receptors are highly homologous, their backbone structures are very likely to be identical.⁸⁸ The distinct affinity to different ligands may result from

differential participation of individual LBRs; for example, LBR 5 is essential for binding of apoE; and LBRs 2–7 cooperatively bind apoB.⁹⁷ Thus, Vtg binding may require the involvement of different LBRs of VtgR.

Li *et al.*⁸³ identified two forms of tilapia VtgR cDNAs of 2500 and 2560 bp, encoding 800 and 820 residues, respectively. One of these contains the O-linked sugar domain which is a thr- and ser-rich region. The existence of 2 forms of VtgR, one with and one without the O-linked sugar domain, was also reported in other species and in other tissues.^{98–100} The O-linked form of VtgR may be responsible for controlling receptor recycling and degradation.^{101,102} The alignment of the amino acid sequences of VtgR to the VLDLR and VtgR from other species showed high homology. By RT-PCR, all the tilapia tissues consistently exhibited 2 forms of VtgR mRNAs. But, Northern analysis revealed only one form of VtgR (3.5 kNt) in the ovarian tissue. Thus, VtgR is transcribed in both ovarian and non-ovarian tissues. However, only the ovarian VtgR mRNA is sufficiently abundant to be detectable by Northern analysis. The major form of VtgR in the ovary lacks the O-linked sugar domain. In chicken, the VtgR was reported to function as VLDLR/VtgR in different tissues.¹⁰³ The existence of VtgR mRNA in non-ovarian tissues suggests that this receptor may function as VLDLR/LDLR or VtgR in different piscine tissues.

Using a yeast two-hybrid system, Li *et al.*⁸³ showed that the ligand-binding domain is sufficient to bind Vtg. The deletions of different LBRs provided evidence that LBRs 1, 2 and 3 constitute the major ligand-binding subdomain in VtgR for binding of Vtg. The binding sites within the Vtg are localized to 84 amino acids at the N-terminal of LV1, between amino acids 162 and 248. Sequence analysis of the binding sites in Vtg and VtgR together with other members of LDLR and apolipoprotein suggests that VtgR may interact with Vtg via electrostatic attraction. LBR3 is the only repeat that contains glu-asp-glu (EDE) instead of the consensus sequence, SDE. In chicken VtgR/VLDLR, this acidic region has been proposed to bind the basic residues on RAP because it has the highest negative charge density.¹⁰⁴ However, according to the known structures of LBRs 1,2,5 and 6, many residues in these regions are involved in the formation of a calcium cage and are most likely not accessible to the ligand. By site-directed mutagenesis

of LBR3, Li *et al.*⁸³ affirmed that the change of EDE sequence to SDE did not affect the binding of Vtg. In addition, mutagenesis of SDE to EDE in LBR6 failed to gain the function of binding Vtg. Thus, EDE sequence alone is insufficient for Vtg binding. The structural determinants in different LBRs, viz, three disulfide bonds and calcium cage-forming amino acids are highly conserved, and these features form the fundamental 3-D structure of ligand binding domain to constitute the correct surface patch which recognizes Vtg.

Vtg and apolipoprotein belong to the large lipid transfer protein, and they were found to be evolutionarily related not only in function but also in sequence.⁵⁸ Li *et al.*⁸³ showed that the receptor binding region in tilapia Vtg lies within 84 amino acids, between 162 and 248 residues in the N-terminal of LV1 (VtgSE domain). In particular, lys¹⁸⁵ which is highly conserved in Vtg, plays a crucial role in receptor binding. In a single deviant case in chicken Vtg, the lys is substituted with another basic residue, arg, thus, strongly suggesting the importance of electrostatic interaction between VtgR and Vtg.

In nature, Vtg exists as a dimer containing symmetric binding sites.¹⁰⁵ Steyrer *et al.*¹⁰⁶ suggested a 1:1 stoichiometry of interaction between VtgR:Vtg. Therefore, to bind the Vtg dimer, VtgR must contain 2 Vtg-binding sites. LBRs 1–3 may contain more than one binding site for Vtg. Two molecules of Vtg dimerize through the dimerization domain in LV1.¹⁰⁷ The symmetric receptor binding sites in Vtg bind to two sites in the LBRs 1–3 of the VtgR. The carboxyl terminal of Vtg will form the lipid-binding cavity to transport lipid into the oocytes. Thus, transportation of a complex of 2 Vtg:1VtgR into the oocytes presents an efficient mechanism to meet the temporal demands of oogenesis. Figure 1 illustrates a proposed model of VtgR-Vtg interaction.

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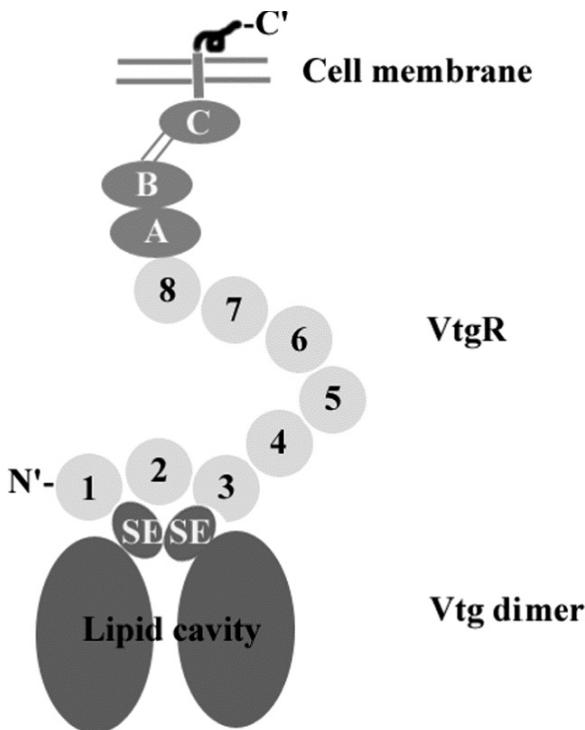


Fig. 1 A model of VtgR-Vtg interaction. The ligand binding repeats (LBRs) are labeled 1–8. The epidermal growth factor precursor (EGFP)-like repeats A, B and C are annotated in VtgR. In a stoichiometric ratio of interaction between 2Vtgs:1VtgR, the Vtg dimer facilitates the binding of the LBRs 1–3 via the SE fragment (amino acids 162–248) in each amino-terminal of the Vtgs. There is more than one ligand-binding site within LBRs 1–3, and binding of VtgR to Vtg requires the combination of these 3 LBRs, which make electrostatic contacts with the VtgSE regions of the Vtg dimer. Adapted from Li *et al.*⁸³

References

1. Tata JR (1978). Induction and regulation of vitellogenin synthesis by estrogen. In: Litwack G (ed.), *Biochemical Actions of Hormones*. Vol. 5. Academic Press, New York, pp. 397–431.
2. Lazier CB and Haggerty AJ (1979). A high-affinity estrogen-binding protein in cockerel liver cytosol. *Biochem. J.* **180:** 347–353.

3. Riley D, Haisermann GJ, MacPherson R and Callard IP (1987). Hepatic estrogen receptor in the turtle, *Chrysemys picta*: partial characterization, seasonal changes and pituitary dependence. *J. Steroid Biochem.* **26**: 41–47.
4. Lim EH, Ding JL and Lam TJ (1991). Estradiol-induced vitellogenin gene expression in a teleost fish *Oreochromis aureus*. *Gen. Comp. Endocrinol.* **82**: 206–214.
5. Wahli W (1988). Evolution and expression of vitellogenin genes. *Trends Genet.* **4**: 227–232.
6. Shapiro D (1982). Steroid hormone regulation of vitellogenin gene expression. *CRC Crit. Rev. Biochem.* **12**: 187–203.
7. Tata JR, Ng WC, Perlman AJ and Wolffe AP (1987). Activation and regulation of the vitellogenin gene family. In: Roy AK and Clark JH (eds.), *Gene Regulation by Steroid Hormones III*. Springer-Verlag, New York, pp. 205–233.
8. Vaillant C, Le Guellec K, Pakdel F and Valotaire Y (1988). Vitellogenin gene expression in primary culture of male rainbow trout hepatocytes. *Gen. Comp. Endocrinol.* **70**: 284–290.
9. Callard IP, Riley D and Perez L (1990). Vertebrate vitellogenesis: molecular model for multi-hormonal control of gene regulation. *Progr. Clin. Biol. Res.* **342**: 343–348.
10. Ding JL, Ho B, Valotaire Y, Le Guellec K, Lim EH, Tay SP and Lam TJ (1990). Cloning, characterization and expression of vitellogenin gene of *Oreochromis aureus* (Teleostei, Cichlidae). *Biochem. Int.* **20**: 843–852.
11. Lee KBH, Lim EH, Lam TJ and Ding JL (1992). Vitellogenin diversity in the Perciformes. *J. Exp. Zool.* **264**: 100–106.
12. Mackay ME and Lazier CB (1993). Estrogen responsiveness of vitellogenin gene expression in rainbow trout (*Oncorhynchus mykiss*) kept at different temperatures. *Gen. Comp. Endocrinol.* **89**: 255–266.
13. Hori SH, Kodama T and Tanahashi K (1979). Induction of vitellogenin synthesis in goldfish by massive doses of androgens. *Gen. Comp. Endocrinol.* **37**: 306–320.
14. Ho SM, Wangh LJ and Callard IP (1985). Sexual differences in the *in vitro* induction of vitellogenesis in the turtle: Role of pituitary and growth hormone. *Comp. Biochem. Physiol.* **B81**: 467–472.

15. Vaisius A, Fletcher GL and So YP (1991). Vitellogenin gene transcription is not under strict estrogen control in winter flounder. *Cell. Mol. Biol.* **37**: 617–622.
16. Carnevali O and Mosconi G (1992). *In vitro* induction of vitellogenin synthesis in *Rana esculenta*: role of pituitary. *Gen. Comp. Endocrinol.* **86**: 352–358.
17. Carnevali O, Mosconi G, Yamamoto K, Kobayashi T, Kikuyama S and Polzonetti-Magni AM (1992). Hormonal control of *in vitro* vitellogenin synthesis in *Rana esculenta* liver: effects of mammalian and amphibian growth hormone. *Gen. Comp. Endocrinol.* **88**: 406–414.
18. Carnevali O, Mosconi G, Yamamoto K, Kobayashi T, Kikuyama S and Polzonetti-Magni AM (1993). *In vitro* effects of mammalian and amphibian prolactins on hepatic vitellogenin synthesis in *Rana esculenta*. *J. Endocrinol.* **137**: 383–389.
19. Wangh LJ (1982). Glucocorticoids act together with estrogens and thyroid hormones in regulating the synthesis and secretion of *Xenopus* vitellogenin, serum albumin, and fibrinogen. *Dev. Biol.* **89**: 294–298.
20. Rabelo EM and Tata JR (1993). Thyroid hormone potentiates estrogen activation of vitellogenin genes and autoinduction of estrogen receptor in adult *Xenopus* hepatocytes. *Mol. Cell. Endocrinol.* **96**: 37–44.
21. Wangh LJ and Schneider W (1982). Thyroid hormones are corequisites for estradiol-17 β *in vitro* induction of *Xenopus* vitellogenin synthesis and secretion. *Dev. Biol.* **89**: 287–293.
22. Sundararaj BI, Goswami SV and Lamba VJ (1982). Role of testosterone, estradiol-17 β and cortisol during vitellogenin synthesis in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol.* **48**: 390–397.
23. Ding JL, Ng WK, Lim EH and Lam TJ (1993). *In situ* hybridization shows the tissue distribution of vitellogenin gene expression in *Oreochromis aureus*. *Cytobios* **73**: 197–208.
24. Sundararaj BI and Nath P (1981). Steroid-induced synthesis of vitellogenin in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Comp. Endocrinol.* **43**: 201–210.
25. Carragher JF, Sumpter JP, Pottinger TG and Pickering AD (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. *Gen. Comp. Endocrinol.* **76**: 310–321.

26. Carragher JF and Sumpter JP (1990). The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. *Gen. Comp. Endocrinol.* **77**: 403–407.
27. Pelissero C, Flouriot G, Foucher JL, Bennetau B, Dunogues J, Le Gac F and Sumpter JP (1993). Vitellogenin synthesis in cultured hepatocytes: An *in vitro* test for the estrogenic potency of chemicals. *J. Steroid Biochem. Mol. Biol.* **44**: 263–272.
28. Fuller JD, Scott DBC and Fraser R (1976). The reproductive cycle of *Coregonus lavaretus* (L) in Loch Lomond, Scotland, in relation to seasonal changes in plasma cortisol concentration. *J. Fish Biol.* **9**: 105–117.
29. Wingfield JC and Grimm AS (1977). Seasonal changes in plasma cortisol, testosterone and oestradiol-17 β in the plaice, *Pleuronectes platessa* L. *Gen. Comp. Endocrinol.* **31**: 1–11.
30. Lamba VJ, Goswami SW and Sundararaj BI (1982). Circannual and circadian variations in plasma levels of steroids (cortisol, estradiol-17 β , estrone, and testosterone) correlated with the annual gonadal cycle in the catfish, *Heteropneustes fossilis* (Bloch). *Gen. Com. Endocrinol.* **50**: 205–225.
31. Cook CE, Smith ML, Telford MJ, Bastianello A and Akam M (1980). Hox genes and the phylogeny of the arthropods. *Curr. Biol.* **11**: 759–763.
32. Bry C (1985). Plasma cortisol levels of female rainbow trout (*Salmo gairdneri*) at the end of the reproductive cycle: relationship with oocyte stages. *Gen. Comp. Endocrinol.* **57**: 47–52.
33. Pickering AD and Christie P (1981). Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **44**: 487–496.
34. Katz Y and Eckstein B (1974). Changes in steroid concentration in blood of female *Tilapia aurea* (Teleostei, Chichlidae) during initiation of spawning. *Endocrinology* **95**: 963–967.
35. Jalabert B (1976). *In vitro* oocyte maturation and ovulation in the rainbow trout (*Salmo gairdneri*), northern pike (*Esox lucius*), and goldfish (*Carassius auratus*). *Bull. Fish Res. Board Can.* **33**: 974–988.
36. Ding JL, Lim EH and Lam TJ (1994). Cortisol-induced hepatic vitellogenin mRNA in *Oreochromis aureus* (Steindachner). *Gen. Comp. Endocrinol.* **96**: 276–287.

37. Teo BY, Tan NS, Lam TJ and Ding JL (1999). Synergistic effects of nuclear factors — GATA, VBP and ER in potentiating vitellogenin gene transcription. *FEBS Lett.* **459**: 57–63.
38. Bownes M (1986). Expression of the genes coding for vitellogenin (yolk protein). *Annu. Rev. Entomol.* **31**: 507–531.
39. Wahli W, Dawid IB, Ryffel GU and Weber R (1981). Vitellogenesis and the vitellogenin gene family. *Science* **212**: 298–304.
40. Beuving G and Gruber M (1971). Isolation of phosvitin from plasma of estrogenised roosters. *Biochim. et Biophys. Acta* **232**: 524–528.
41. Bergink EW, Kloosterboer JJ, Gruber M, and Ab E (1973). Estrogen-induced phosphoprotein synthesis in roosters. *Biochim. et Biophys. Acta* **294**: 497–506.
42. Spieth J and Blumenthal T (1985). The *Caenorhabditis elegans* vitellogenin gene family includes a gene encoding distantly related protein. *Mol. Cell. Biol.* **5**: 2495–2501.
43. Plack PA, Pritchard DJ and Fraser NW (1971). Egg proteins in cod serum. *Biochem. J.* **121**: 847–856.
44. Selman K and Wallace RA (1983). Oogenesis in *Fundulus heteroclitus*. *J. Exp. Zool.* **226**: 441–457.
45. Chen TT (1982). Identification and characterization of estrogen-responsive gene products in the liver of rainbow trout. *Can. J. Biochem.* **61**: 802–810.
46. Valotaire Y, Tenniswood M, LeGuellec K and Tata JR (1984). The preparation and characterization of vitellogenin messenger RNA from rainbow trout (*Salmo gairdneri*). *Biochem. J.* **217**: 73–77.
47. Ding JL, Hee PL and Lam TJ (1989). Two forms of vitellogenin in the plasma and gonads of male *Oreochromis aureus*. *Comp. Biochem. Physiol.* **93**: 363–370.
48. Martin D, Piulachs MD, Comas D and Belles X (1998). Isolation and sequence of a partial vitellogenin cDNA from the cockroach, *Blattella germanica* (L.) (Dictyoptera, Blattellidae), characterization of the vitellogenin gene expression. *Arch. Insect Biochem. Physiol.* **38**: 137–146.
49. Chen JS, Sappington TW and Raikhel AS (1997). Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *J. Mol. Evol.* **44**: 440–451.
50. LaFleur Jr. GJ, Byrne BM, Kanungo J, Nelson LD, Greenberg RM and Wallace RA (1995). *Fundulus heteroclitus* vitellogenin: the deduced primary

- structure of a piscine precursor to noncrystalline, liquid-phase yolk protein. *J. Mol. Evol.* **41**: 505–521.
51. Lim EH, Teo BY, Lam TJ and Ding JL (2001). Sequence analysis of a fish vitellogenin cDNA with a large phosvitin domain. *Gene* **277**: 175–186.
 52. Schubiger JL and Wahli W (1986). Linkage arrangement in the vitellogenin gene family of *Xenopus laevis* as revealed by gene segregation analysis. *Nucleic Acids Res.* **14**: 8723–8734.
 53. Williams JL and Tata JR (1983). Simultaneous analysis of conformation and transcription of A and B groups of vitellogenin genes in male and female *Xenopus* during primary and secondary activation by estrogen. *Nucleic Acids Res.* **11**: 1151–1165.
 54. Van het Schip FD, Samallo J, Ophius JBJ, Mojet M, Gruber M and Ab G (1987). Nucleotide sequence of a chicken vitellogenin gene and derived amino acid sequence of the encoded yolk precursor protein. *J. Mol. Biol.* **196**: 245–260.
 55. Hung MOC, Barnett T, Woolford C and Wensink PC (1982). Transcript maps of *Drosophila* yolk protein genes. *J. Mol. Biol.* **154**: 581–602.
 56. Heine U and Blumenthal T (1986). Characterization of regions of the *Caenorhabditis elegans* X chromosome containing vitellogenin genes. *J. Mol. Biol.* **188**: 301–312.
 57. Lee BH, Lim EH, Lam TJ and Ding JL (1994). Two major groups of vitellogenin cDNA in *Oreochromis aureus* (Steindachner). *Biochem. Mol. Biol. Int.* **34**: 75–83.
 58. Babin PJ, Bogerd J, Kooiman FP, Van Marrewijk WJA and Van der Horst DJ (1999). Apolipoporphin I/II, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. *J. Mol. Evol.* **49**: 150–160.
 59. Mouchel N, Trichet V, Naimi BY, Pennec JPL and Wolff J (1997). Structure of a fish (*Onchorynchus mykiss*) vitellogenin gene and its evolutionary implication. *Gene* **197**: 147–152.
 60. Klein-Hitpass L, Schorpp M, Wagner U and Ryffel GU (1986). An estrogen-responsive element derived from the 5'flanking region of the *Xenopus* vitellogenin A2 gene functions in transfected human cells. *Cell* **46**: 1053–1061.
 61. Seiler-Tuyns A, Walker P, Martinez E, Merillat AM, Givel G and Wahli W (1986). Identification of estrogen-responsive DNA sequences

- by transient expression experiments in a human breast cancer cell line. *Nucleic Acids Res.* **14:** 8755–8770.
- 62. Chang TC, Nardulli AM, Lew D and Shapiro JD (1992). The role of estrogen response element in expression of the *Xenopus laevis* vitellogenin B1 gene. *Mol. Endocrinol.* **6:** 346–354.
 - 63. Davies DL and Burch JBE (1996). The chicken vitellogenin II gene is flanked by a GATA factor-dependent estrogen response unit. *Mol. Endocrinol.* **10:** 937–944.
 - 64. Teo BY, Tan NS, Lim EH, Lam TJ and Ding JL (1998). A novel piscine vitellogenin gene: structural and functional analyses of estrogen-inducible promoter. *Mol. Cell. Endocrinol.* **146:** 103–120.
 - 65. Shyu A-B, Blumenthal T and Raff RA (1987). A single gene encoding vitellogenin in the sea urchin *Strongylocentrotus purpuratus*: sequence at the 5' end. *Nucleic Acids Res.* **15:** 10405–10417.
 - 66. Walker P, Brown-Luedi M, Germond J-E, Wahli W, Meijlink FCPW, van het Schip AD, Roelink H, Gruber M and Ab G (1983). Sequence homologies within the 5' end region of the estrogen-controlled vitellogenin gene in *Xenopus* and chicken. *EMBO J.* **2:** 2271–2279.
 - 67. Rina M and Savakis C (1991). A cluster of vitellogenein genes in the Mediterranean fruit fly, *Ceratitis capitata*: sequence and structural conservation in Diptera proteins and their genes. *Genetics* **127:** 769–780.
 - 68. Tan NS, Lam TJ, and Ding JL (1996a). The first contiguous estrogen receptor gene from a fish, *Oreochromis aureus*: evidence for multiple transcripts. *Mol. Cell. Endocrinol.* **120:** 177–192.
 - 69. Gray WG and Gorski J (1996). Identification and characterization of an estrogen-responsive element binding protein repressed by estradiol. *Biochemistry* **35:** 11685–11692.
 - 70. Nardulli AM, Romine LE, Carpo C, Greene GL and Rainish B (1996). Estrogen receptor affinity and location of consensus and imperfect estrogen response elements influence transcription activation of simplified promoters. *Mol. Endocrinol.* **10:** 694–704.
 - 71. Tan NS, Lam TJ and Ding JL (1996b). Transcription regulatory signals in the 5' and 3' regions of *Oreochromis aureus* ER gene. *Mol. Cell. Endocrinol.* **123:** 149–161.
 - 72. Tora L, Gaub MP, Mader S, Dierich A, Bellard M and Chambon P (1988). Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive

- element in the chicken ovalbumin gene promoter. *EMBO J.* **7**: 3771–3778.
73. Kato S, Tora L, Yamauchi J, Masushige S, Bellard M and Chambon P (1992). A far upstream response element of the ovalbumin gene contains several half-palindromic 5'-TGACC-3' motifs acting synergistically. *Cell* **68**: 721–731.
 74. Iyer SV, Davis DL, Seal SN and Burch JBE (1991). Chicken vitellogenin gene-binding protein, a leucine zipper transcription factor that binds to an important control element in the chicken vitellogenin II promoter, is related to rat DBP. *Mol. Cell. Biol.* **11**: 4863–4875.
 75. Dréan YL, Liu D, Wong AO, Xiong F and Hew CL (1996). Steroidogenic factor 1 and estradiol receptor act in synergism to regulate the expression of the salmon gonadotropin II beta subunit gene. *Mol. Endocrinol.* **10**: 217–229.
 76. Scott AP, Bye VJ and Baynes SM (1980). Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri*). *J. Fish Biol.* **17**: 587–592.
 77. Smith CJ and Haley SR (1988). Steroid profiles of the female tilapia, *Oreochromis mossambicus*, and correlation with oocyte growth and mouthbrooding behavior. *Gen. Comp. Endocrinol.* **69**: 88–98.
 78. Pina B, Bruggemeier U and Beato M (1990). Nucleosome positioning modulates accessibility of regulatory proteins to the mouse mammary tumor virus promoter. *Cell* **60**: 719–731.
 79. Corthesy B, Corthesy-Theulaz I, Cardinaux J-R and Wahli W (1991). A liver protein fraction regulating hormone-dependent *in vitro* transcription from the vitellogenin genes induces their expression in *Xenopus* oocytes. *Mol. Endocrinol.* **5**: 159–169.
 80. Opresko LK and Wiley HS (1987). Receptor-mediated endocytosis in *Xenopus* oocytes — characterization of the vitellogenin receptor system. *J. Biol. Chem.* **262**: 4109–4115.
 81. Deeley RG, Mullinix DP, Wetekam W, Kronenberg HM, Meyers M, Eldridge JD and Goldberger RF (1975). Vitellogenin synthesis in the avian liver — vitellogenin is the precursor of the egg yolk phosphoproteins. *J. Biol. Chem.* **250**: 9060–9066.
 82. Schneider WJ (1996). Vitellogenin receptors: oocyte-specific members of the low-density lipoprotein receptor supergene family. *Int. Rev. Cytol.* **166**: 103–137.
 83. Li AK, Murali S and Ding JL (2003). Receptor-ligand interaction: vitellogenin receptor and vitellogenin, implications on LDLR and

- Apo B/E. The first three ligand binding repeats of vitellogenin receptor interact with the N-terminal region of vitellogenin. *J. Biol. Chem.* **278**: 2799–2806.
- 84. Willnow TE, Nykjaer A and Herz J (1999). Lipoprotein receptors: new roles for ancient proteins. *Nat. Cell Biol.* **1**: E157–E162.
 - 85. Herz J (2001). Deconstructing the LDL receptor — a rhapsody in pieces. *Nat. Struct. Biol.* **8**: 476–478.
 - 86. Willnow TE, Orth K and Herz J (1994). Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *J. Biol. Chem.* **269**: 15827–15832.
 - 87. Saito A, Pietromonaco S, Loo AKC and Farquhar MG (1994). Complete cloning and sequencing of rat gp330/“megalin,” a distinctive member of the low density lipoprotein receptor gene family. *Proc. Natl. Acad. Sci. USA* **91**: 9725–9729.
 - 88. Koduri V and Blacklow SC (2001). Folding determinants of LDL receptor type A modules. *Biochemistry* **40**: 12801–12807.
 - 89. Bieri S, Atkins AR, Lee HT, Winzor DJ, Smith R and Kroon PA (1988). Folding, calcium binding, and structural characterization of a concatemer of the first and second ligand-binding modules of the low-density lipoprotein receptor. *Biochemistry* **37**: 10994–11002.
 - 90. Daly NL, Djordjevic JT, Kroon PA and Smith R (1995). Three-dimensional structure of the second cysteine-rich repeat from the human low-density lipoprotein receptor. *Biochemistry* **34**: 14474–14481.
 - 91. North CL and Blacklow SC (1999). Structure independence of ligand-binding modules five and six of the LDL receptor. *Biochemistry* **38**: 3926–3935.
 - 92. North CL and Blacklow SC (2000). Solution structure of the sixth LDL-A module of the LDL receptor. *Biochemistry* **39**: 2564–2571.
 - 93. Beglova N, North CL and Blacklow SC (2001). Backbone dynamics of a module pair from the ligand-binding domain of the LDL receptor. *Biochemistry* **40**: 2808–2825.
 - 94. Clayton D, Brereton IM, Kroon PA and Smith R (2000). Three-dimensional NMR structure of the sixth ligand-binding module of the human LDL receptor: comparison of two adjacent modules with different ligand binding specificities. *FEBS Lett.* **479**: 118–122.
 - 95. Stifani S, Nimpf J and Schneider WJ (1990). Vitellogenesis in *Xenopus laevis* and chicken: cognate ligands and oocyte receptors. *J. Biol. Chem.* **265**: 882–888.

96. Roehrkasten A and Ferenz HJ (1992). Role of the lysine and arginine residues of vitellogenin in high affinity binding to vitellogenin receptors in locust oocyte membranes. *Biochim. et Biophys. Acta* **1133**: 160–166.
97. Russell DW, Brown MS and Goldstein JL (1989). Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *J. Biol. Chem.* **264**: 21682–21688.
98. Bujo H, Lindstedt KA, Hermann M, Dalmau LM, Nimpf J and Schneider WJ (1995). Chicken oocytes and somatic cells express different splice variants of a multifunctional receptor. *J. Biol. Chem.* **270**: 23546–23551.
99. Nakamura Y, Yamamoto M and Kumamaru E (1998). A variant very low density lipoprotein receptor lacking 84 base pairs of O-linked sugar domain in the human brain myelin. *Brain Res.* **793**: 47–53.
100. Margrane J, Reina M, Pagan R, Luna A, Casaroli-Marano RP, Angelin B, Gafvels M and Vilaro S (1998). Bovine aortic endothelial cells express a variant of the very low density lipoprotein receptor that lacks the O-linked sugar domain. *J. Lipid Res.* **39**: 2172–2181.
101. Margrane J, Casaroli-Marano RP, Reina M, Gafvels M and Vilaro S (1999). The role of O-linked sugars in determining the very low density lipoprotein receptor stability or release from the cell. *FEBS Lett.* **451**: 56–62.
102. Iijima H, Miyazawa M, Sakai J, Magoori K, Ito MR, Suzuki H, Nose M, Kawarabayasi Y and Yamamoto TT (1998). Expression and characterization of a very low density lipoprotein receptor variant lacking the O-linked sugar region generated by alternative splicing. *J. Biochem.* **124**: 747–755.
103. Bujo H, Hermann M, Kaderli MO, Hacobsen L, Sugawara S, Nimpf J, Yamamoto T and Schneider WJ (1994). Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. *EMBO J.* **13**: 5165–5175.
104. Bajari TM, Lindstedt KA, Reipl M, Mirsky VM, Nimpf J, Wolfbeis OS, Dresel HA, Bautz EK and Schneider WJ (1998). A minimal binding domain of the low density lipoprotein receptor family. *Biol. Chem.* **379**: 1953–1962.
105. Baert JL, Britel M, Slomianny MC, Delbart C, Fournet B, Sautier P and Malecha J (1991). Yolk protein in leech — identification, purification and characterization of vitellin and vitellogenin. *Eur. J. Biochem.* **201**: 191–198.

106. Steyrer E, Kostner GM and Schneider WJ (1995). A double labeling procedure for lipoproteins: independent visualization of dual ligand-receptor interaction with colloidal gold- and ^{125}I -labeled ligands. *Anal. Biochem.* **226**: 44–50.
107. Anderson TA, Levitt DG and Banaszak LJ (1998). The structure basis of lipid interactions in lipovitellin, a soluble lipoprotein. *Structure* **6**: 895–909.

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