Structural and functional evolution of GnRH and its receptors in three chordate models: *Branchiostoma floridae*, *Ciona intestinalis* and *Danio rerio*

by

Javier Ananda Tello
B.Sc., University of Victoria, 2002

A dissertation submitted in partial fulfillment
of the requirements for the degree of

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Abstract

Neural control of reproduction in vertebrates and invertebrates has generated considerable interest due to the presence of common neuropeptides. Gonadotropin-releasing hormone (GnRH), a neuropeptide, is the final integrator of neural regulation governing reproduction in vertebrates by controlling the release of gonadotropins. Little is known about GnRH before the origin of vertebrates or about the biological significance of multiple GnRH forms in a single species. To understand the role of GnRH in invertebrates, I selected a tunicate, *Ciona intestinalis*, the sister group to vertebrates and amphioxus, *Branchiostoma floridae*, a group more basal than tunicates. Neural control of reproduction in these chordates was compared with that in the zebrafish, *Danio rerio*. From the zebrafish, I isolated four GnRH receptor cDNAs that each map to a distinct chromosome and are expressed in a variety of tissues. Each receptor was functional, as shown by its response to physiological doses of native GnRH peptides. Also, two receptors showed selectivity between GnRH1 and GnRH2. Protein localization of each zebrafish GnRH receptor with specific antisera showed that all four receptors are present in the pituitary. However, the most striking localization revealed the presence of GnRH networks in a major motor control centre and fibre tract

system in the hindbrain and spinal cord. Both structures are major components in the control of motor movements, such as swimming. Phylogenetic and synteny analysis segregates the four zebrafish GnRH receptors into two distinct phylogenetic groups that are separate gene lineages conserved throughout vertebrate evolution.

In *Ciona intestinalis*, we found two GnRH genes that each encode three GnRH decapeptides in tandem, for six unique GnRH forms from this species. These genes are expressed throughout development. With an immunocytochemical approach, at least one peptide was found in the dorsal strand nerve plexus adjacent to the gonads in adults. Injection near the gonads of gravid *Ciona* quickly induced spawning, suggesting a novel action for control of reproduction by GnRH. My further studies identified four novel GnRH receptors encoded within the genome of this protochordate, and showed that three receptors responded to *Ciona* GnRHs by stimulating intracellular accumulation of cAMP. In contrast, only one receptor activated inositol phosphate turnover in response to one of the *Ciona* GnRHs.

My final study involved identifying the GnRH signalling components in amphioxus. I found four novel GnRH receptors, with three displaying sensitivity to the highly conserved vertebrate GnRH2 and one of these showing selectivity for GnRH1. My pharmacological testing showed that the capacity to respond to GnRH1 and GnRH2 is evolutionarily conserved between amphioxus and vertebrates, and that key motifs found to be important in GnRH binding, signalling and activation are present in the amphioxus receptors. Phylogenetic analysis showed that two receptors cluster with the recently identified octopus GnRHR-like sequence; the other two receptors group at the base of the vertebrate GnRHR clade and may represent the proto-vertebrate condition, after which gene duplication and

sequence divergence resulted in the four contemporary vertebrate GnRHRs. This work reveals novel and important features of the GnRH signalling axis throughout chordate evolution.

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Dedication

I dedicate this thesis to my late grandparents, Gladys and John Houghton.

Chapter 1

General Introduction

The diversity of life on earth has been facilitated by gene duplication events that supply novel genes to enable organisms to evolve and adapt to new environments. Multigene families are formed after gene duplication enables one gene copy to escape purifying selection. Although a redundant copy often becomes a non-functional pseudogene, occasionally the gene develops a novel function. There are a number of theories that describe potential mechanisms through which novel functions have evolved. A method first proposed by Ohno describes the evolution of novel functions as a result of the accumulation of chance mutations (Ohno, 1970). Others argue that gene copies divide functions that were once performed by the ancestral gene (Force *et al.*, 1999; Lynch and Force, 2000). In addition to duplication of single genes, whole genome duplications have also occurred, allowing large-scale proliferation of multigene families. Two such rounds of genome duplication are thought to have occurred between ancestral protochordates and the lineage that led to jawed vertebrates, with an additional round occurring in a lineage of teleost fishes (Ohno, 1970; Ohno, 1996, 1999; Taylor *et al.*, 2001; Furlong and Holland, 2002; Panopoulou *et al.*, 2003). Others argue that the complexity of vertebrates is likely due to the accumulation of tandem duplication events (Hughes, 1999; Hughes and Friedman, 2003; Friedman and Hughes, 2004). In whatever manner these duplications occur, diverse multigene families have become integral to the reproductive fitness of many organisms and are essential for the survival of all vertebrates.

The capacity of a metazoan organism to respond to various environmental stimuli requires communication between specialized cells and tissues. For example, the endocrine system utilizes small molecules to coordinate the function of these specialized cells and tissues. Traditionally, the endocrine system has included a network of ductless

glandular structures that secrete specific chemical messengers, called hormones, into the general circulation to communicate with non-adjacent cells. Now, the study of endocrinology encompasses all aspects of intercellular communication, where a hormone is more broadly defined as a non-metabolic chemical, regardless of whether it is produced by a gland. The hormone can act on distant or local target tissues by binding to a specific receptor. For example, a cell can release a hormone that acts on the cell that released it (autocrine), a nearby cell in the same tissue (paracrine), or a distant cell target (endocrine).

Protein hormones are a distinct class of endocrine hormones that can be utilized immediately or stored for later use. They make excellent models for the investigation of molecular evolution because they can be compared for structural changes between divergent animals and tested for function by a vast array of scientific methods. Such techniques include: characterizing isolated proteins, testing protein function at the cellular level, or measuring the involvement/absence of a protein in the physiology of a whole organism. The recent unification of molecular biology and genetic manipulation techniques has sparked a number of novel developments in our understanding of various aspects of endocrinology in general, and specifically regarding the reproductive axis. At the center of many discoveries is the use of genetic information being compiled into exponentially expanding DNA and protein databases. Molecular comparison of sequence elements between distantly related organisms, including previously unknown genes, helps decipher conserved or novel gene functions during evolution. My interest includes the structural and functional evolution of a protein hormone called gonadotropin-releasing hormone (GnRH) and its cognate receptors (GnRHRs) which are classically

known as the main regulators of reproduction in vertebrates. I have chosen three chordate models to delineate differences in the GnRH system from basal chordates to evolved vertebrates. My aim is to understand how functional changes may be the result of expanded gene content and development of a more complex chordate body plan.

1.1 Gonadotropin-Releasing Hormone

1.1.1 Discovery

Several critical discoveries were made in the beginning of the 20th century that have shaped our current understanding of reproduction. In 1910, the work of Crowe and colleagues established the foundation for the endocrine control of reproductive biology with the anterior pituitary acquiring prominence as a "master gland" (Crowe *et al.*, 1910). The idea of a reciprocal relationship between the pituitary and the gonads was pioneered by the work of Moore & Price (Moore and Price, 1932), following a report of the vascular connection between the hypothalamus and the anterior pituitary by Popa & Fielding (Popa and Fielding, 1930). The concept of neural control by the central nervous system (CNS) on the pituitary became prevalent after the publication of a small book titled "Neural control of the pituitary gland" by Geoffrey Harris (Harris, 1955). The identification of reproductive hormones began with estradiol in 1931 (Thayer *et al.*, 1931), followed by testosterone in 1935 (David *et al.*, 1935), gonadotropin-releasing hormone in 1971, and the gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in 1971-1974 (Papkoff *et al.*, 1971; Liu *et al.*, 1972; Liu *et al.*, 1972; Shome and Parlow, 1973, 1974); knowledge of these hormone structures revolutionized the field of reproductive biology.

Normal reproductive function involves complex coordination between the hypothalamus, pituitary and gonads during sexual differentiation, puberty and gametogenesis. Higher brain centers integrate endogenous and exogenous environmental cues (i.e. developmental benchmarks, hormonal signals, light cycles, nutrient status, etc.) that initiate or augment reproductive functions acting through the hypothalamus at the base of the brain. Positioned as the final integrator of neural regulation governing reproduction is the hypothalamic peptide hormone called GnRH. Two competing groups (Matsuo et al., 1971; Burgus et al., 1972) first elucidated the primary structure of this small decapeptide hormone, initially termed luteinizing hormone - releasing hormone. This form was later shown to also stimulate the release of FSH, leading to a new name, gonadotropin-releasing hormone. Both of these groups purified the hormone from over 150,000 ovine or porcine hypothalamic extracts and obtained the same peptide sequence, a form that has now been found in most mammals investigated, commonly called mammalian GnRH or mGnRH. In 1976, Roger Guillemin and Andrew Schally shared the Nobel Prize in medicine for establishing the primary structure of GnRH (Schally) and thyrotropin releasing hormone (TRH) (Guillemin).

1.1.2 Structure

In the early 1980s, novel forms of GnRH were identified by protein isolation and sequencing methods in chicken (cGnRH-I) (Miyamoto *et al.*, 1982), fish (salmon GnRH, sGnRH) (Sherwood *et al.*, 1983), then a second chicken form called cGnRH-II (GnRH2) (Miyamoto *et al.*, 1984) and lamprey GnRH in 1986 (Sherwood *et al.*, 1986). The first GnRH gene transcript (mGnRH) was cloned and characterized from human placental

tissue by Seeburg and Adelman (Seeburg and Adelman, 1984). The human mGnRH gene (~5.1 kb) is composed of three exons and two intervening introns and has been mapped to chromosome 8p21. The gene transcript (1,512 bp) has a long 5' untranslated region (UTR) (1,074 bp), a short 3' UTR (159 bp) and a precursor peptide (prepro-GnRH) encoded by three of the exons. All GnRH peptides are derived from precursors with similar overall structure, including a signal peptide of around 20-25 amino acids, the GnRH peptide sequence, a dibasic cleavage site, and a GnRH-associated peptide (GAP) of approximately 40-60 residues. The highly conserved GnRH decapeptide usually consists of a pyroglutamic acid N-terminus, followed by His^2, Trp^3, Ser^4 and an amidated Pro^9-Gly^{10} at the C-terminus. These conserved residues play an important role in binding and/or activation of the GnRH receptor. There is considerable variation seen in positions 5, 7, 8 of the peptide structure, which affects ligand selectivity (Millar *et al.*, 2004). Arg^8, a residue present in mGnRH, has been shown to be critical for high affinity binding to its cognate pituitary receptor (Millar *et al.*, 2004). At position 6, a glycine is present in all forms of GnRH found in jawed vertebrates. This small achiral amino acid introduces flexibility in the center of the molecule and facilitates a tight type II β-turn that brings the N- and C-termini in close proximity necessary for efficient receptor binding (Karten and Rivier, 1986; Millar *et al.*, 2004). However, this prerequisite is not seen in protochordates because multiple tunicate GnRHs possess chiral residues at position 6 and are extremely potent at their receptors (Adams *et al.*, 2003; Tello *et al.*, 2005). In addition, a 12 amino acid form of a GnRH-like peptide has been isolated from the cephalopod octopus with LH-releasing properties after application to quail pituitary explants (Iwakoshi *et al.*, 2002). When I began my research, 14 distinct decapeptide forms of GnRH had been

identified in vertebrates (8 were identified in our lab) and two in the protochordate tunicate (also from our lab) (Powell *et al.*, 1996). All 16 GnRH forms maintain these highly conserved peptide features with the exception of two tyrosine substitutions (Figure 1.1). Included in this dissertation is the identification and characterization of seven GnRH forms from two sister tunicate species, *Ciona intestinalis* and *Ciona savignyi*, bringing the total number of unique decapeptide GnRH forms to 23.

1.1.3 Evolution and Phylogeny

Comparative studies have demonstrated that most jawed vertebrates (gnathostomes) possess at least two GnRH forms, one always corresponding to cGnRH-II (now renamed GnRH2) (White *et al.*, 1998; Sherwood and Adams, 2005; Kah *et al.*, 2007) and a more variable second form analogous to mGnRH (see Figure 1.2 for each species' endogenous GnRH forms). Two phylogenetically distinct GnRH genes are thought to have been present as early as 450 million years ago (mya) in the cartilaginous fish, one being GnRH2 (Lovejoy *et al.*, 1992). GnRH2 is the most widely distributed GnRH form found in over 90 different organisms from multiple taxonomic classes, including most jawed vertebrates. However, in some mammalian species (cow, sheep and chimpanzee) the GnRH2 gene has been functionally inactivated (Morgan *et al.*, 2006) or is missing completely (rat and mouse) (Morgan and Millar, 2004). Mammalian GnRH is detected later in early-derived bony fish (Lescheid *et al.*, 1995) with an origin dating back

Gonadotropin-Releasing Hormones

	1	2	3	4	5	6	7	8	9	10
Mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Guinea Pig	pGlu	**Tyr**	Trp	Ser	Tyr	Gly	**Val**	Arg	Pro	Gly-NH_2
Chicken-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Gln**	Pro	Gly-NH_2
Chicken-II	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Tyr**	Pro	Gly-NH_2
Frog	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Trp**	Pro	Gly-NH_2
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	**Trp**	**Leu**	Pro	Gly-NH_2
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	**Ser**	Pro	Gly-NH_2
Pejerrey	pGlu	His	Trp	Ser	**Phe**	Gly	Leu	**Ser**	Pro	Gly-NH_2
Herring	pGlu	His	Trp	Ser	**His**	Gly	Leu	**Ser**	Pro	Gly-NH_2
Catfish	pGlu	His	Trp	Ser	**His**	Gly	Leu	**Asn**	Pro	Gly-NH_2
Whitefish	pGlu	His	Trp	Ser	Tyr	Gly	**Met**	**Asn**	Pro	Gly-NH_2
Dogfish	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Leu**	Pro	Gly-NH_2
Lamprey-I	pGlu	His	**Tyr**	Ser	**Leu**	**Glu**	**Trp**	**Lys**	Pro	Gly-NH_2
Lamprey-III	pGlu	His	Trp	Ser	**His**	**Asp**	**Trp**	**Lys**	Pro	Gly-NH_2
Tunicate-1	pGlu	His	Trp	Ser	**Asp**	**Tyr**	**Phe**	**Lys**	Pro	Gly-NH_2
Tunicate-2	pGlu	His	Trp	Ser	**Leu**	**Cys**	**His**	**Ala**	Pro	Gly-NH_2
Octopus	pGlu	His	**Phe**	Ser	**Asn**	Gly	**Trp**	**His**	Pro	Gly-NH_2

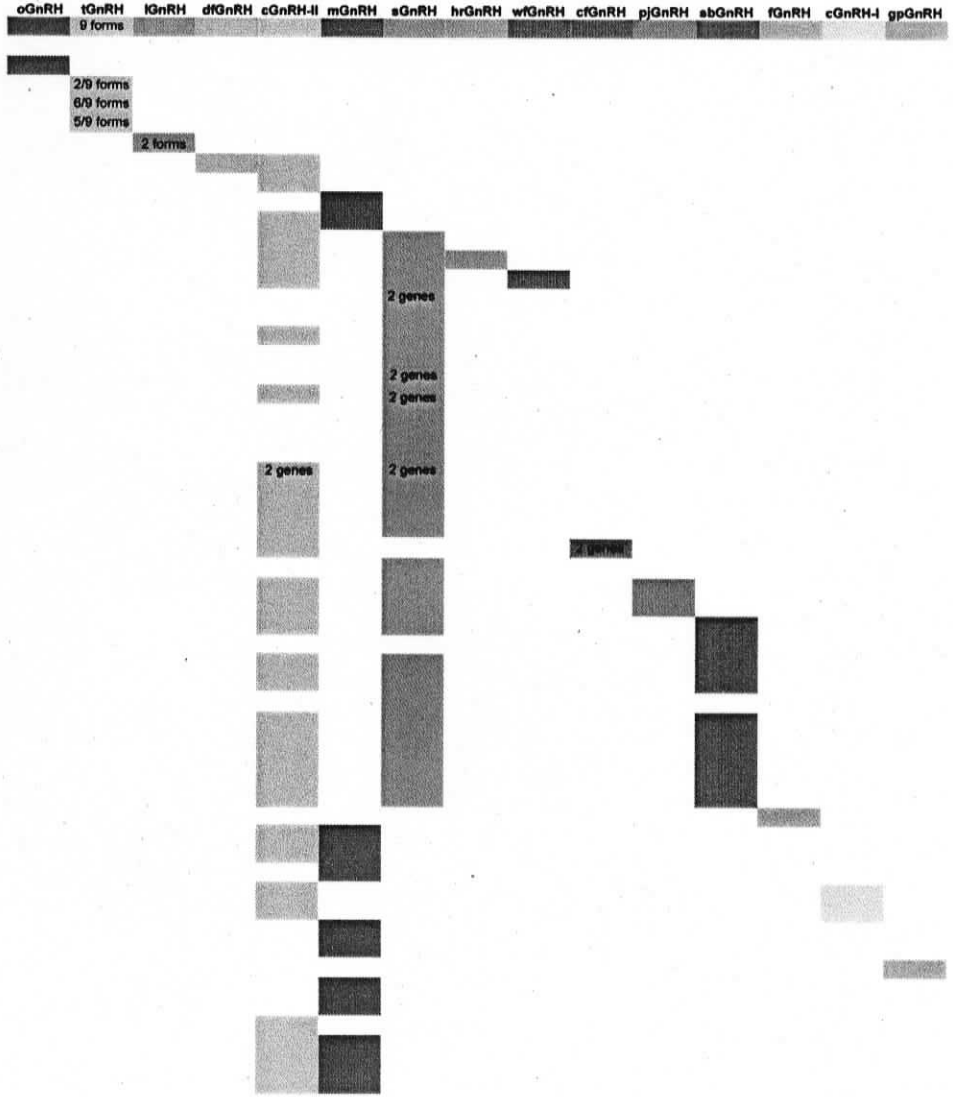
Asn **Tyr**

Figure 1.1. Primary amino acid sequences of naturally occurring GnRH forms in 2004. The boxed regions represent conserved N- and C-terminal residues that play important functional roles. Non-conserved residues (bolded), shown in the intervening regions, can convey ligand selectivity for a particular GnRH receptor. Variants are named according to the animal in which they were first discovered. The octopus GnRH-like decapeptide has two additional amino acids shown as an insert after the first residue for alignment purposes.

Figure 1.2. Species in which GnRH structure is determined by protein, cDNA or gene. A coloured box indicates the form identified in each species and an asterisk (*) indicates if a cDNA or gene has been isolated. (modified from Sherwood and Adams, 2005).

Species in which GnRH structure is determined by protein, cDNA or gene

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

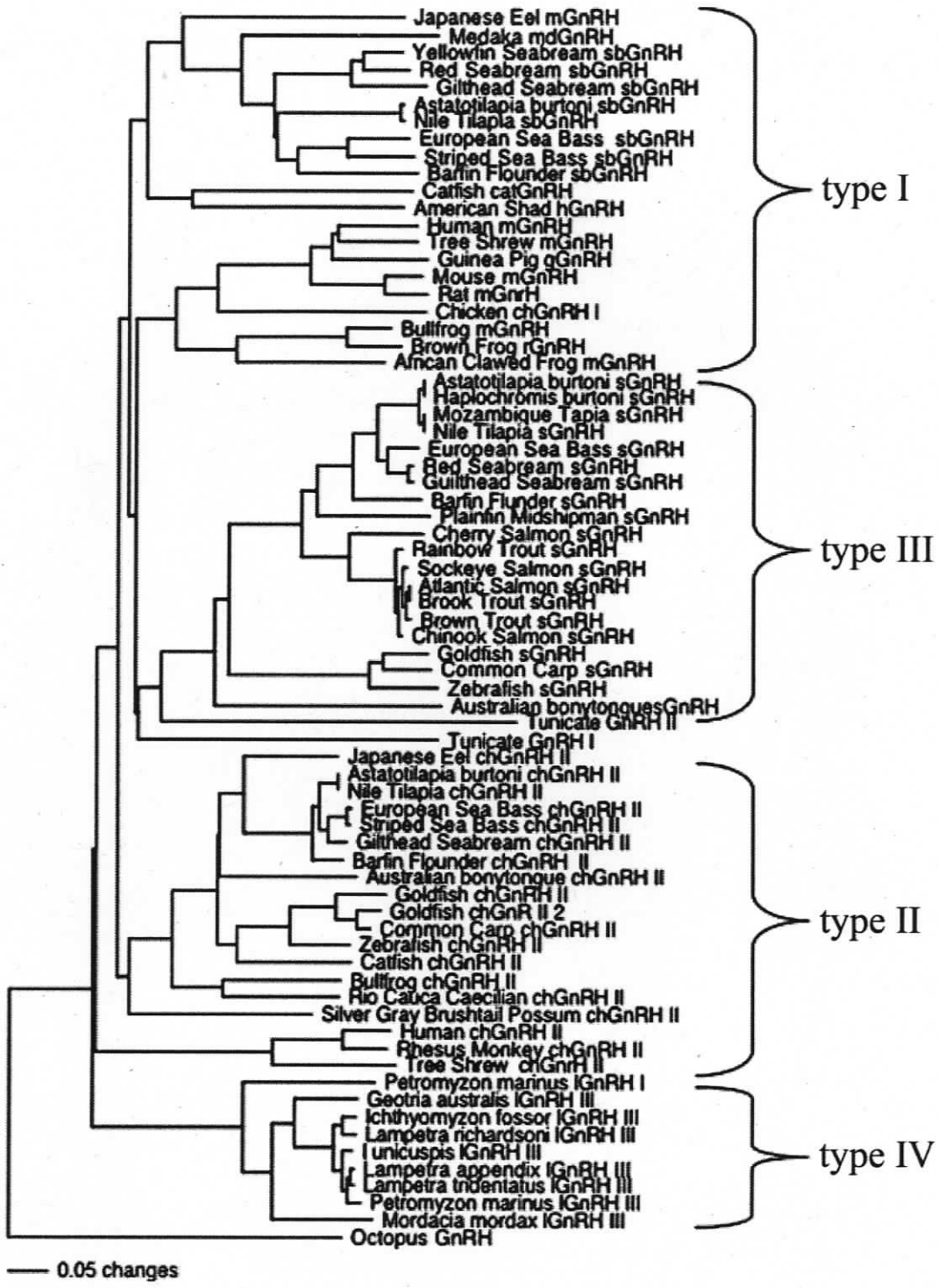


* - gnrh1 cDNA, * * = gnrh1,2 * * * = gnrh 1,2,3; blank space = protein only: gnrh2 = cGnRH-II, gnrh3 = sGnRH, gnrh1 = all other forms

~410 mya. It is conserved in the amphibian and mammalian lineage, but is missing from the reptilian and avian lines. A third form, first isolated from salmon, is present among the Osteichthyes superclass of fish (bony fish) (Sherwood *et al.*, 1983; Sherwood and Adams, 2005). This gene is proposed to have arisen from a genome duplication specific to the teleost lineage about 320-350 mya (Kuo *et al.*, 2005). Other forms of GnRH precede both mammalian and GnRH2 in chordate history. Evidence for this is found in the lamprey, a type of jawless fish, which possesses two distinct forms of GnRH (Sherwood *et al.*, 1986; Sower *et al.*, 1993), and in the protochordate *Chelyosoma productum* which has two unique GnRH peptides (Powell *et al.*, 1996).

Phylogenetic comparison of the amino acid sequences of the preproGnRH precursors can be used to classify GnRH forms from jawed vertebrates into three types (White *et al.*, 1998). When the sequences from lamprey are included in the analysis, all vertebrate GnRHs segregate into four distinct groups where the lamprey sequences are monophyletic (Figure 1.3) (Silver *et al.*, 2004). Type I includes the variants that are all mGnRH orthologs, including pejerrey, seabream, catfish, herring, guinea pig, frog, and chicken-I forms; type II includes the sequences that encode GnRH2 from mammals and fish; type III encodes sGnRH forms found only in teleost fish; and type IV includes the lamprey forms. Syntenic mapping of chromosomal regions surrounding GnRH genes in medaka and humans confirm the relationships among pejerrey and mGnRH in group I as well as GnRH2 orthologs between fish and man (Okubo *et al.*, 2002).

Figure 1.3. Phylogenetic tree of all GnRH precursors. Neighbour-joining tree was generated using the amino acid sequences of GnRH precursors, including the signal peptide, GnRH decapeptide, dibasic cleavage site and GAP protein, adapted from Silver *et al.*, 2004.


Japanese Eel mGnRH
Medaka mdGnRH
Yellowfin Seabream sbGnRH
Red Seabream sbGnRH
Gilthead Seabream sbGnRH
Astatotilapia burtoni sbGnRH
Nile Tilapia sbGnRH
European Sea Bass sbGnRH
Striped Sea Bass sbGnRH
Barfin Flounder sbGnRH
Catfish catGnRH
American Shad hGnRH
Human mGnRH
Tree Shrew mGnRH
Guinea Pig gGnRH
Mouse mGnRH
Rat mGnrH
Chicken chGnRH I
Bullfrog mGnRH
Brown Frog rGnRH
African Clawed Frog mGnRH
type I
Astatotilapia burtoni sGnRH
Haplochromis burtoni sGnRH
Mozambique Tapia sGnRH
Nile Tilapia sGnRH
European Sea Bass sGnRH
Red Seabream sGnRH
Gulthead Seabream sGnRH
Barfin Flunder sGnRH
Plainfin Midshipman sGnRH
Cherry Salmon sGnRH
Rainbow Trout sGnRH
Sockeye Salmon sGnRH
Atlantic Salmon sGnRH
Brook Trout sGnRH
Brown Trout sGnRH
Chinook Salmon sGnRH
Goldfish sGnRH
Common Carp sGnRH
Zebrafish sGnRH
Australian bonytongueGnRH
Tunicate GnRH II
type III
Tunicate GnRH I
Japanese Eel chGnRH II
Astatotilapia burtoni chGnRH II
Nile Tilapia chGnRH II
European Sea Bass chGnRH II
Striped Sea Bass chGnRH II
Gilthead Seabream chGnRH II
Barfin Flounder chGnRH II
Australian bonytongue chGnRH II
Goldfish chGnRH II
Goldfish chGnR II 2
Common Carp chGnRH II
Zebrafish chGnRH II
Catfish chGnRH II
Bullfrog chGnRH II
Rio Cauca Caecilian chGnRH II
Silver Gray Brushtail Possum chGnRH II
Human chGnRH II
Rhesus Monkey chGnRH II
Tree Shrew chGnrH II
type II
Petromyzon marinus lGnRH I
Geotria australis lGnRH III
Ichthyomyzon fossor lGnRH III
Lampetra richardsoni lGnRH III
Lunicuspis lGnRH III
Lampetra appendix lGnRH III
Lampetra tridentatus lGnRH III
Petromyzon marinus lGnRH III
Mordacia mordax lGnRH III
type IV
Octopus GnRH
0.05 changes

1.1.4 Distribution and Physiology

GnRH was first classified based on the anatomical location of the neuronal cell bodies in fish (Zandbergen *et al.*, 1995) and then in mammals (Kasten *et al.*, 1996). In species that express two GnRH variants, the type I GnRH neurons, which are implicated in gonadotropin release, are located in the preoptic area, whereas type II are in the midbrain. Type I neurons originate from outside of the brain in the region of the olfactory/nasal placode and migrate along the olfactory nerves to reside in the medial preoptic area and the hypothalamus during prenatal development (Wray, 2002). Small cell clusters remain along this migratory route, occupying distinct populations in the olfactory bulbs/terminal nerves, ventral telencephalon and preoptic area, but all of these neurons project fibres to the medium eminence (tetrapods) or directly to the anterior pituitary (many teleost fish). In species with three GnRH forms (some teleosts), type II neurons remain in the mesencephalon, whereas type I and type III GnRH neurons may be in separate locations, or overlap in the same region in the ventral forebrain (Gonzalez-Martinez et al. 2002; Lethimonier *et al.*, 2004; Kah *et al.*, 2007). In some fish species, notably zebrafish, goldfish, trout and salmon, mGnRH orthologs (type I) are missing and it appears that sGnRH fills the role as the gonadotropin secretagogue (Sherwood and Adams, 2005; Kah *et al.*, 2007).

In humans at the time of puberty, mGnRH is released intermittently from nerve terminals in the region of the median eminence at the base of the hypothalamus (Ganong, 2005). This region is rich in nerve endings from the preoptic and hypothalamic nuclei and is in close proximity to the fenestrated capillaries from which the portal vessels originate. GnRH is secreted into the hypophysial circulation to reach the gonadotropes present in

the anterior pituitary. Under exposure to pulsatile GnRH, the gonadotropes synthesize and secrete the gonadotropins, LH and FSH. Gonadotropins enter the general circulation to reach their gonadal targets, where they stimulate steroidogenesis and development of the gonads. FSH stimulates follicular maturation and estrogen secretion in females and spermatogenesis in males, whereas LH triggers ovulation and corpus luteum formation (luteinisation) in females and testosterone secretion in males. Neural input from the CNS and feedback of gonadal steroids modulate GnRH and the gonadotropins at the level of both the hypothalamus and pituitary, completing the cycle.

GnRH is maintained at very high levels (~10^9 molecules/cell) and its secretion can vary widely over developmental stages and the female menstrual cycle (Sarkar *et al.*, 1976; Wray *et al.*, 1989). The anterior pituitary is therefore exposed to high levels of GnRH in the portal blood for brief periods of time. Studies in sheep have investigated the generation of the GnRH pulse from GnRH neurons and have shown that it begins suddenly, plateaus for 5-6 minutes and then falls rapidly (Moenter *et al.*, 1991). The frequency of pulses *in vivo* has been found to range between 30-120 minutes in primates (Gearing and Terasawa, 1988; Millar *et al.*, 2004). A high GnRH amplitude and pulse frequency with a 30 minute interval preferentially stimulates the biosynthesis and secretion of LHβ and the common glycoprotein subunit α, leading to ovulation, whereas a lower pulse frequency (every 120 minutes) preferentially stimulates FSHβ biosynthesis, leading to ovarian follicle development (Dalkin *et al.*, 1989; Burger *et al.*, 2002; Ferris and Shupnik, 2006). Continuous or long-term exposure to mGnRH leads to the suppression of the gonadotropins, but can be reversed once pulsatile administration is returned (Belchetz *et al.*, 1978).

A discrete population of GnRH neurons residing in an unusual location in the tegmentum of the midbrain was first identified in platyfish in 1981 (Munz *et al.*, 1981). Over the subsequent years this population was found in all fish and in many other vertebrates, and was identified as expressing GnRH2 (Amano *et al.*, 1991). This research led to the discovery of the second GnRH gene in humans (White *et al.*, 1998). The type II GnRH genes are now believed to have a distinct developmental origin in the mesencephalic primordium, a derivative of the neural crest (Gonzalez-Martinez *et al.*, 2002; Whitlock, 2005). The potential role of GnRH2 in the neural control of fertility has garnered considerable attention in the past. Efforts to locate GnRH2-expressing cell bodies within the midbrain revealed that nerve fibres are even more widely distributed. GnRH2 projections have been located in the forebrain, hypothalamus and midbrain. As well, its transcript has been detected in many tissues outside of the brain, particularly in the kidney, bone marrow and prostate (White *et al.*, 1998). GnRH2 has also been implicated in reproductive control via the portal system, as a few immunoreactive fibres have been localized to the region of the median eminence in monkeys and musk shrews (Dellovade *et al.*, 1993; Lescheid *et al.*, 1997; Chen *et al.*, 1998; Urbanski *et al.*, 1999). The evidence supporting LH and FSH-releasing roles is less clear, with mixed effects seen after exogenous administration of GnRH2 to monkeys, rats, sheep and shrews. Adding to the complexity of this system, the effects of GnRH2 in human, monkey and sheep gonadotropes are thought to act via the mGnRH receptor. As well, the presence of GnRH2 in portal blood has not been established (Neill, 2006). Furthermore, the widespread expression of GnRH2 throughout the CNS makes it plausible that it is involved in a large number of different neuronal circuits.

Some researchers have attributed the roles of neuromodulator and/or neurotransmitter to GnRH (mainly GnRH2) and this is supported by studies in amphibians, fish and some mammals. Studies in frogs have shown that GnRH can act directly on sympathetic neurons to produce a depolarizing response lasting minutes, affect neuron excitability and act as a neurotrophic factor to regulate the expression of Ca^{2+} channels (Jan and Jan, 1981; Bosma *et al.*, 1990; Ford *et al.*, 2003). In addition, the terminal nerve of fish contains sGnRH, which has been proposed to modulate chemosensory function through the olfactory mucosa (Wirsig-Wiechmann and Oka, 2002) and may play a role in the perception of sex pheromones (Demski and Northcutt, 1983). This theory may be extended to mammals as shown by a study where selective lesions of the terminal nerve in male hamsters severely impaired mating behaviour (Wirsig and Leonard, 1987). Recent studies conducted in the musk shrew have shown that GnRH may serve a permissive role by regulating reproductive behaviour according to a female's energy status. Female shrews that are food restricted for 48 hours display decreased mating behaviours, which can be reversed by a central administration of GnRH2 (Temple *et al.*, 2003). These authors posit that GnRH2 may have evolved to help coordinate the interaction between food intake, energy balance and reproduction. However, the lack of a functional GnRH2 system in some mammals without an apparent detriment warrants further investigation into its role.

In fishes, various forms of GnRH have been shown to induce the release of other pituitary hormones. In goldfish, sGnRH has been shown to have a growth hormone-releasing effect (Marchant *et al.*, 1989) and GnRH2 is very potent at stimulating the release of prolactin in tilapia (Weber *et al.*, 1997). Additionally, GnRH was shown to be

produced in the gonads, where it is presumed to act as an autocrine/paracrine factor (Pati and Habibi, 2000). In zebrafish, sGnRH has been detected in the interstitial cells of the testis using immunoaffinity labelling (Kuo *et al.*, 2005). Studies in rat and human testes show that GnRH messenger RNA (mRNA) is expressed in Sertoli cells (Bahk *et al.*, 1995; Botte *et al.*, 1998) and in cultured rat testes, and that GnRH agonists can block steroidogenesis (Dufau *et al.*, 1984; Botte *et al.*, 1999). In the mature goldfish testes, GnRH-induced apoptosis occurs during the late stage of spermatogenesis (Andreu-Vieyra *et al.*, 2005). In the ovary, *in situ* expression studies have shown mGnRH mRNA in granulosa cells at many stages of follicular development, with the changes in ovarian receptor expression shown to correlate with the degree of development across the human estrous cycle (Kang *et al.*, 2003). GnRH has been shown to have many effects in the ovary, such as inhibition of DNA synthesis, induction of apoptosis and activation of genes encoding factors important for follicular rupture and oocyte maturation (Ramakrishnappa *et al.*, 2005).

1.2 GnRH Receptors

The capacity of an animal to respond to a wide variety of sensory and chemical stimuli such as light, neurotransmitters, pheromones and hormones depends upon the presence of cellular receptors. Receptors are the link between the detection of a stimulus and the internalization of the signal to evoke a cellular or systematic response. The effects of GnRH on target tissues depend on specific membrane-embedded receptors called GnRH receptors (GnRHRs). GnRHRs belong to the seven-transmembrane guanosine triphosphate (GTP)-binding (G) protein-coupled receptors (GPCRs) which are

members of family A or the rhodopsin family (Tsutsumi *et al.*, 1992). These receptors share a similar overall structure, which includes an extracellular N-terminal region, seven hydrophobic transmembrane spanning domains (TMDs) connected by alternating intracellular loops (ICLs) and extracellular loops (ECLs) (three of each), and an intracellular C-terminal tail.

1.2.1 Discovery

In the early 1990s, the cDNA encoding the GnRH receptor was first isolated and characterized from the mouse αT3-1 gonadotropic cell line, revealing the absence of a C-terminal tail, a feature unique from all other GPCRs (Tsutsumi *et al.*, 1992). Subsequently, additional GnRH receptors were cloned from other mammalian species, including rat (Eidne *et al.*, 1992; Kaiser *et al.*, 1992), human (Kakar *et al.*, 1992), sheep (Brooks *et al.*, 1993), cow (Kakar *et al.*, 1993) and pig (Weesner and Matteri, 1994), which all lacked an intracellular tail, now referred to as type I GnRH receptors (see below). Additional GnRHRs were cloned from non-mammalian vertebrates such as the African catfish (Tensen *et al.*, 1997) and goldfish (Illing *et al.*, 1999). The existence of multiple GnRH and GnRHR genes in some vertebrates prompted investigation into finding concordant receptor gene duplications in mammalian species. A partial second GnRHR gene sequence was isolated from human tissue (Troskie *et al.*, 1998) and a human expressed sequence tag (EST) library confirmed its presence as a distinct GnRHR gene (now referred to as a type II receptor) (Millar *et al.*, 1999). The first full-length type II mammalian GnRHR gene was identified in the macaque and green monkey (Neill *et al.*, 2001), followed by the marmoset monkey (Millar *et al.*, 2001). This newly identified

mammalian GnRHR type showed higher sequence similarity to the non-mammalian receptors (~55 %) than to the other mammalian GnRHRs (~40 %). With the aid of the growing number of genome sequencing projects, large numbers of GnRH receptors have been identified, with most vertebrates possessing at least two GnRHR homologs, and up to five present in some fish species (salmon, pufferfish and sea bass) (Jodo *et al.*, 2003; Ikemoto and Park, 2005; Kah *et al.*, 2007). Recently, a GnRHR-like sequence was cloned from the common octopus and shown to be functional with octopus GnRH peptide (Kanda *et al.*, 2006), suggesting a common origin for GnRH receptors before the protostome and deuterostome split. Phylogenetic analysis using both maximum likelihood and neighbour-joining algorithms, and using the classification scheme first proposed by Millar et al. (2004), groups vertebrate GnRHRs into four main types: mammalian type I, non-mammalian type I, type II, and type III GnRHRs (see Figure 1.4).

1.2.2 Structure

In humans, the type I GnRHR gene is composed of three exons interrupted by two large introns and spans approximately 19 kb on the long arm of chromosome four. This gene encodes a 328 amino acid protein with features common to all GPCRs, with the exception that it lacks an intracellular C-terminal tail, making type I GnRHRs the smallest of all GPCRs. A putative human type II receptor gene is located on chromosome 1q12 and is also composed of three exons. This gene is approximately 7.5 kb and is expressed in many extrapituitary tissues (Millar *et al.*, 1999). However, this human gene contains a frame shift mutation in addition to a C-to-T substitution that introduces a premature stop codon (see Entrez Gene GeneID: 114814). Some investigators propose

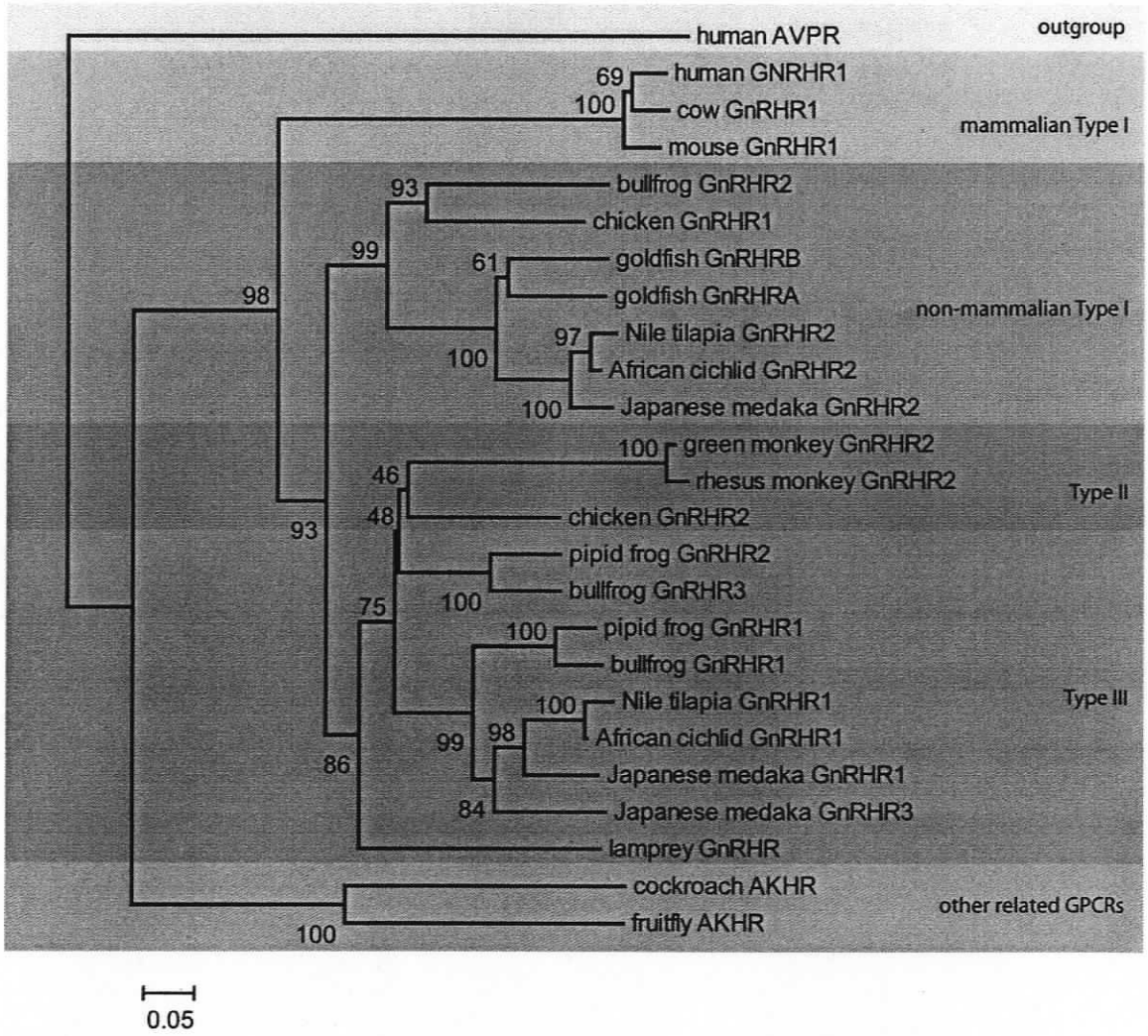


Figure 1.4. Phylogenetic tree of GnRH receptors. Consensus tree of GnRH receptor sequence relationships was generated using a neighbour-joining algorithm with Mega software suite (Version 4). Bootstrap proportions for each hypothesized group are identified above or below the branch leading to the group. The human vasopressin receptor is used an outgroup. The classification of each GnRH type is according to Millar *et al.* (2004).

that this gene has become silenced and is only a gene remnant (Morgan and Millar, 2004). Analogous to the lost or inactive GnRH2 peptide gene in some mammals, the type II receptor is also missing in the mouse and rat, and has been functionally inactivated in the cow and sheep (Gault *et al.*, 2004; Morgan *et al.*, 2006). Some mammals do possess a functional type II GnRHR with a long intracellular C-terminal tail, a feature found in all non-mammalian GnRH receptors identified to date.

The current three-dimensional structure of the human GnRH receptor is based on X-ray analysis of the ground state of bovine rhodopsin. The GnRHR amino acids have been threaded onto the rhodopsin atomic map and then refined by incorporating the angles, kinks and side chain orientations of the specific amino acids in each of the seven TMDs. The arrangement of the seven TMDs forms a tight bundle, enclosing a hydrophilic pocket surrounded by the hydrophobic cellular membrane. In many chordate GnRHRs there is a high conservation of key residues along the hydrophilic face of the TMDs. To validate the GnRHR model, the involvement of certain amino acids is tested using *in vitro* site-directed mutagenesis studies in various heterologous expression systems (Millar *et al.*, 2004). Integrating many of these mutational studies has produced a generalized model where the ECLs and superficial regions of the TMDs form a hydrophilic pocket that facilitates the binding of GnRH, whereas the ICLs and C-terminal tail are involved in coupling to G proteins. The C-terminal tail is also the target for GPCR kinase phosphorylation and is responsible for ligand-induced receptor internalization and desensitization.

After mutating over 30 % of mammalian type I GnRHR positions, researchers have identified many residues that are critical for functions such as maintenance of

receptor structure, formation of the binding pocket, interaction with GnRH ligands, coupling to G proteins, receptor activation and changing the receptor from inactive to active conformations. Two conserved cysteine residues have been found to form a disulfide bridge between ECL1 and ECL2 (Cys^{114} and Cys^{195} in the rat), which stabilizes the GnRHR structure (Cook and Eidne, 1997). Substitution of either cysteine residue to alanine abolished ligand binding and ligand-induced second messenger activation. Another Cys found in the N-terminal extracellular domain along with Asp^{98}, Trp^{101}, Asn^{102} (TMD2), Lys^{121} (TMD3), Asn^{212} (TMD5), Tyr^{290} (TMD6), Asp^{302} (Glu^{301} in rat, ECL3) and Trp^{280} (TMD6) are thought to be involved with formation of the ligand binding pocket (Flanagan *et al.*, 1994; Zhou *et al.*, 1995; Davidson *et al.*, 1996; Flanagan *et al.*, 2000; Hoffmann *et al.*, 2000; Chauvin *et al.*, 2001; Fromme *et al.*, 2001; Hovelmann *et al.*, 2002; Millar *et al.*, 2004). Arg^{139}, Ser^{140} (ICL2), Thr^{239} (ICL3), Leu^{300} (ECL3) and Phe^{326} (TMD7) have been shown to be important for agonist binding affinity. Residues in ICL2 (Asp^{138}, Pro^{146} and Leu^{147}), in ICL3 (Leu^{238}, Arg^{240}, Val^{241}, Leu^{242}, Arg^{260}, Ala^{261} and Arg^{262}), in TMD6 (Trp^{280}) and in TMD7 (Phe^{311}, Asp^{319}, Tyr^{323}, Ser^{327}) have been shown to be involved with $G_{q/11\alpha}$ protein-coupling and/or agonist-induced inositol phosphate (IP) production (Millar *et al.*, 2004). Alternatively, several residues within TMD1 (Leu^{58}) or ICL1 (Leu^{73}, Ser^{74}, Arg^{75} and Leu^{80}) have been implicated in coupling to another G protein ($G_{s\alpha}$) (Millar *et al.*, 2004). In addition, post-translational modifications contribute to GnRHR structure and expression. For example, N-glycosylation sites present in the N-terminal extracellular extension are common in many GnRHRs. Mutation of two glycosylation sites in the mouse GnRHR caused a decrease in membrane expression, but had no affect on ligand binding or activation of intracellular IP

accumulation. This suggests that glycosylation has a role in maintaining receptor expression levels, presumably by improving receptor trafficking or stability (Davidson *et al.*, 1995). Additional residues that have been implicated in receptor expression are Asp^{138} (ICL2), Leu^{238}, Thr^{265} (ICL3), and Phe^{272} (TMD6) (Neill, 2006).

Few mutational studies have been performed on other GnRHRs beside the mammalian type I. However, some structural commonalities and distinctions have been made with other GnRHR types. In the marmoset type II receptor, mutations of Asp^{98}, Asn^{102} and Lys^{121} demonstrated that these residues display functions similar to mammalian type I receptors. In addition, the absence of Asp^{302} in other vertebrate GnRHR types, including the marmoset type II, is not surprising, as this residue is proposed to interact with Arg^{8} in mGnRH and is not required for high affinity binding of these receptor's cognate ligand, GnRH2 (Millar *et al.*, 2004).

GnRH-mediated gonadotropic responses correlate directly with the number of GnRHRs present within the cell membranes of the pituitary gonadotropes. GnRH has a bimodal effect on receptor expression. On the one hand, low concentrations can stimulate receptor gene expression in pituitary cell cultures and immortalized murine gonadotrope cell lines by up to 50 % in less than 20 minutes (Tsutsumi *et al.*, 1993). On the other hand, high GnRH concentrations, which can occur during the proestrus phase in rodents, can reduce receptor expression through internalization and desensitization of the receptor (Pawson and McNeilly, 2005).

Like many GPCRs, GnRH receptors are down-regulated by their ligands through internalization. Ligand-bound receptors are endocytosed in small vesicles (sometimes coated with clathrin) via an association with the GTPase dynamin (Urrutia *et al.*, 1997).

Endocytosed GnRH-bound receptors are subject to one of two fates: either the GnRHR is uncoupled from its ligand in Golgi complexes and is recycled to the cell surface via secretory granules (Cornea *et al.*, 1999), or the complexes are dissociated and the proteins are degraded in lysosomes (Schvartz and Hazum, 1987). The exact mechanism of internalization is highly dependent on the receptor subtype as well as the cell type. For GnRHRs that contain intracellular C-terminal tails (all receptors except mammalian type I), a method that involves β-arrestin, clathrin-coated pits and the GTPase dynamin is proposed. This model suggests that once the receptor is activated by a ligand, G protein-coupled receptor kinases (GRKs) are targeted to the receptor by the generation of free βγ-subunits. GRKs phosphorylate specific serine or threonine residues, often within the C-terminal tail, which enhances β-arrestin binding (McArdle *et al.*, 1999). The β-arrestin interaction is rapid and serves to target the ligand-coupled receptor to clathrin-coated pits for internalization, where the invaginated pits are excised through a dynamin-dependent process. Other methods have been proposed for tailed GnRHRs, where internalization is dynamin-independent, but the schemes can vary, as some are β-arrestin and/or clathrin independent. In the mammalian type I GnRHRs, on the other hand, internalization is not as rapid, presumably due to the absence of the C-terminal tail with its associated phosphorylation sites and/or β-arrestin binding sites. A recent study showed that the tail-less human GnRHR underwent slow agonist-independent constitutive internalization, which did not increase after agonist-stimulation (Pawson *et al.*, 2007).

Sustained exposure to high GnRH concentrations reduces the response of the gonadotropes to subsequent stimulation, a process called homologous desensitization. The mechanism of desensitization tends to vary depending on the specific receptor profile

(Willars *et al.*, 1998). Early desensitization appears to interfere with G protein-coupling and loss of downstream signalling through the phosphorylation of specific sites within ICL3 or the C-terminal tail. Overall, desensitization is likely the result of reduced receptor expression at the cell membrane and is maintained by loss of receptor-effector coupling, low rates of second messenger turnover or loss of a functional Ca^{2+} channel (Neill, 2006).

1.2.3 Physiology

In mammals, the type I "tail-less" GnRHRs are the hypophysiotropic targets of mGnRH because they show the highest sensitivity to mGnRH in functional assays and are present on the gonadotropes of the anterior pituitary. However, in some animals with multiple GnRH and receptor forms, it is a complex task to discern which form is responsible for gonadotropin release. Ligand binding and receptor activation studies often do not distinguish clear ligand/receptor pair relationships because various GnRH ligands have the ability to bind and stimulate multiple GnRH receptors. Instead, in addition to binding and activation data, hypophysiotropic function is attributed to specific GnRH/receptor pairs after a receptor is located on the pituitary gonadotropes concurrent with enhanced reproductive status. The accumulation of functional data from multiple GnRHR studies has revealed a trend within vertebrates; all mammalian type I GnRHRs show the highest affinity and sensitivity to mGnRH; all other GnRHRs are most sensitive to GnRH2, although in two cases the highest sensitivity is shared with sGnRH (Kah *et al.*, 2007). The retention of a receptor with high binding affinity for GnRH2 indicates that is has an important function that has been conserved in many animals, but remains to be

elucidated. In addition, GnRH function maybe important outside the pituitary system, as GnRH hormone expression occurs outside the hypothalamus and many receptors have also been localized to extrapituitary tissues. In primates, type II GnRH receptors are widely expressed throughout the CNS, adrenal gland, thyroid gland, heart, skeletal muscle and many reproductive tissues such as the ovary, testis, prostate and mammary glands (Millar *et al.*, 2001; Neill *et al.*, 2004; Ramakrishnappa *et al.*, 2005). This trend is also apparent in fishes, where GnRH receptors are expressed in diverse tissues, including the eye, olfactory epithelium, cerebellum, skeletal muscle, ovaries and testes (Lethimonier *et al.*, 2004). The presence of multiple receptors (which are highly sensitive to GnRH2) suggests the existence of distinct role(s) for GnRHs, which reside outside the pituitary, possibly in local autocrine/paracrine networks.

1.2.4 Signalling

Ligand binding induces a rapid conformational change within the GnRHR that leads to the propagation of an intracellular signalling event. GnRHRs can activate multiple second messenger pathways (IP_3, cyclic adenosine monophosphate [cAMP], Ca^{2+} and others) through association with different classes of heterotrimeric G proteins ($G_{q/11\alpha}$, $G_{s\alpha}$ and $G_{i\alpha}$). Receptor activation acts to exchange guanosine diphosphate (GDP) with GTP on the coupled $G\alpha$ subunit, which then dissociates and is free to activate downstream cascades (Figure 1.5).

In the pituitary gonadotropes, GnRHRs have been shown to predominantly activate phospholipase C (PLC) after coupling to $G_{q/11\alpha}$. There are three types of PLC isoenzymes (β, γ and δ); two members of the β isoform are responsible for IP production

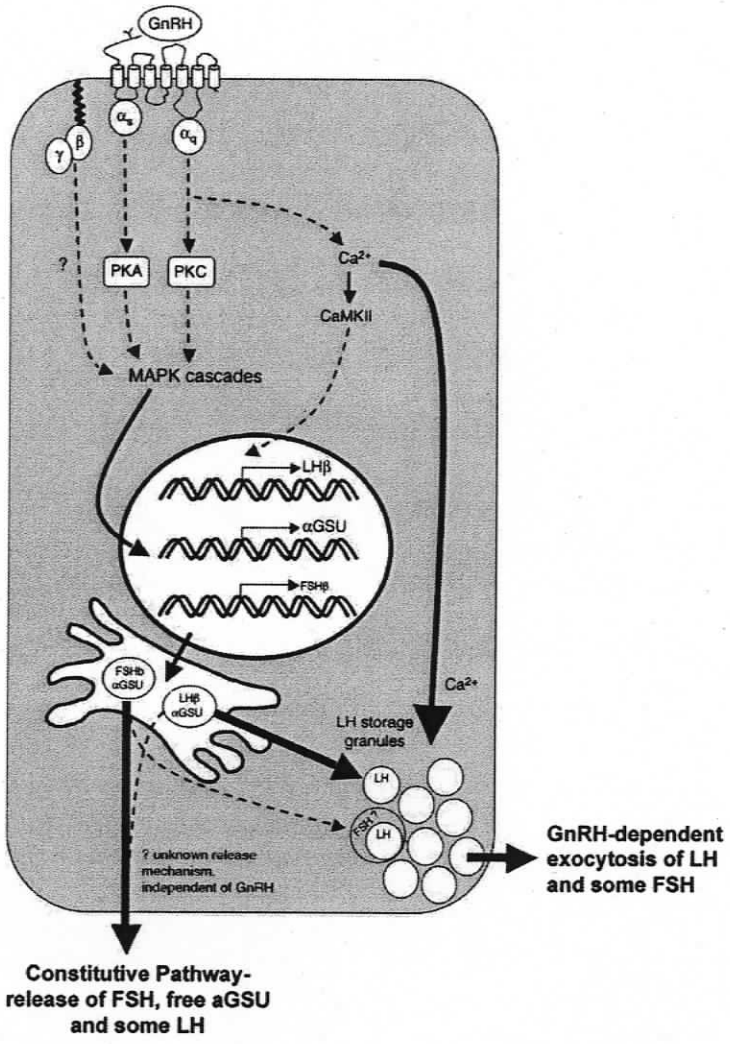


Figure 1.5. Summary of gonadotrope signalling and trafficking pathways. Agonist-induced GnRH receptor activation leads to LH and FSH secretion. Signalling is initiated via predominantly $G_{q/11\alpha}$ and a minor contribution from Gsα towards the MAPK cascades, which drive the synthesis of LHβ, FSHβ and αGSU gonadotropin subunits. LH is stored in storage granules which are released in response to increases in Ca^{2+} induced by GnRH (adapted from Pawson and McNeilly, 2005).

regulated by GPCRs (Spiegel *et al.*, 1992). Once activated, PLCβ1 catalyzes the rapid hydrolysis and turnover of 4,5-bisphosphate (PIP_2) to generate the transient metabolites 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). In many GnRHR activation experiments, it is the accumulation of radioactively-labelled inositol phosphates ($[^3H]IP_x$) in the presence of Li^+, which is used to block degradation by inositol monophosphatase, that provides the index of total PLCβ1 activity (McArdle *et al.*, 1995). IP_3 acts through specific receptors to trigger the release of Ca^{2+} from intracellular stores and to enhance Ca^{2+} uptake through voltage sensitive calcium channels (VSCC) from the extracellular pool. This mobilized calcium triggers an initial burst of LH secretion within 10 seconds, which lasts for up to 100 seconds (Leong and Thorner, 1991; Neill, 2006). DAG, in concert with Ca^{2+} and phosphatidylserine, has been shown to activate protein kinase C (PKC) isoforms by inducing their translocation from the cytosol to the plasma membrane. PKC, in turn, controls Ca^{2+} release and influx by modulation of IP_3 receptors and VSCCs (Stojilkovic *et al.*, 1994). PKC participates in gonadotropin gene transcription (LH and FSH) as well as cell growth and development through phosphorylation of four key MAPK subfamilies: c-Jun N-terminal protein kinase (JNK), p38 MAPK, the extracellular signal regulated protein kinase (ERK) and the big MAPK (BMK) (Pawson and McNeilly, 2005; Dobkin-Bekman *et al.*, 2006). These MAPK effectors ultimately regulate target gene expression, including immediate-early genes such as *jun*, *fos* and *egr-1* (Neill, 2006). FSH secretion is predominantly regulated by gonadal peptides producing both stimulatory (activins) and inhibitory (inhibins, follistatin) effects (Bilezikjian *et al.*, 1998).

Although the biological actions of GnRH are largely dependent on $G_{q/11\alpha}$ coupling, modulation of intracellular cAMP levels, presumably through $G_{s\alpha}$, and $G_{i\alpha}$, have been demonstrated in pituitary cultures/cell lines (Naor *et al.*, 1979; Bourne and Baldwin, 1987, 1987; Liu *et al.*, 2002), in heterologous cell lines (Kuphal *et al.*, 1994; Arora *et al.*, 1998) and in human reproductive tract tumours (Imai *et al.*, 1996; Gether, 2000). Modulation of intracellular cAMP, either up by $G_{s\alpha}$, or down by $G_{i\alpha}$, regulates protein kinase A (PKA), which can either attenuate or activate various MAPK signalling cascades (Cheng and Leung, 2000). A recent study also implicated calcium/calmodulin kinase type II in regulating LHβ expression by acting on GnRH-sensitive transcription factors following modulation by intracellular calcium (Haisenleder *et al.*, 2003).

1.2.5 Clinical implications

If during development, type I GnRH neurons fail to migrate into the hypothalamus, then GnRH fails to make a connection with the pituitary and animals will remain prepubertal and infertile (Hardelin *et al.*, 2000). A rare X-linked disease caused by a mutation in the KAL1gene highlights the crucial integration between olfactory cortex development and the migration of GnRH neurons. Alteration of the *KAL1* gene product (anosmin-1), which normally has branch-promoting and neuronal guidance functions (Cariboni *et al.*, 2004), disrupts the normal development of the olfactory cortex. As a by-product, the migration of GnRH neurons is prevented, which results in infertility (hypogonadism) and the inability to smell (anosmia).

Another disease, called isolated hypogonadotropic hypogonadism (IHH), is due to spontaneous mutations in the gene for GnRH or its receptor. It results in a dramatic

decrease in circulating gonadotropins, leading to pubertal failure and infertility (Cattanach *et al.*, 1977; Tao, 2006; Bedecarrats and Kaiser, 2007). Over the last ten years, 21 mutations in the GnRH receptor gene have been reported to cause partial or complete impairment of gonadotropin secretion resulting in IHH (Tao, 2006; Bedecarrats and Kaiser, 2007). Males generally present with small testes and infertility, whereas females present with primary amenorrhea, often with a lack of pubarche and thelarche (Tao, 2006). Most of the GnRHR mutations are missense mutations (see Figure 1.6). One mutation is in a splice acceptor site that leads to receptor truncation (Silveira *et al.*, 2002). It appears that most patients are compound heterozygotes and only a few are homozygous for a single mutation (Bhagavath *et al.*, 2005). Functional studies of these defective GnRHRs in cell culture show that all of the missense mutations lead to absent or decreased ligand binding and/or intracellular signalling. This is likely due to decreased receptor expression at the plasma membrane as a result of misrouting of the mutated GnRHRs and retention in the ER. This theory was validated by experiments where partial function of 11 missense mutations were recovered by the use of either non-specific antagonists or pharmacological chaperones, which are presumed to act as a folding template to improve trafficking and expression at the cellular membrane (Leanos-Miranda *et al.*, 2002).

1.3 Objectives

The focus of this dissertation research is the investigation of naturally-occurring GnRH signalling components throughout chordates. At the onset of my graduate research in May, 2003, the genetic and biochemical characteristics of the GnRH receptor had not

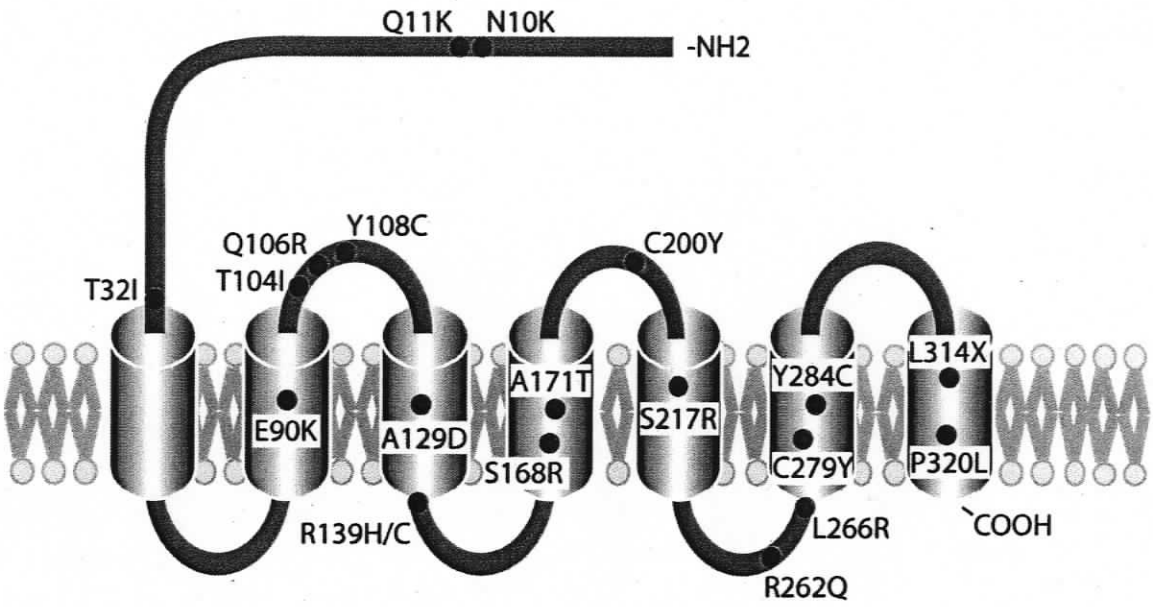


Figure 1.6. Inactivating mutations of the GNRHR (red circles) identified in patients with isolated hypogonadotropic hypogonadism (IHH). Missense mutations are noted with amino acid changes and positions from the wildtype residues in the human GnRHR. Single amino acid nomenclature is used and X indicates a receptor truncation at that position. (modified from de Roux, 2006).

yet been characterized outside of vertebrates. Therefore, I set out to expand our understanding of the structural and functional evolution of GnRH and its receptors in chordates through the identification, characterization and comparison of the GnRH axis in key chordates. Basal chordates provide essential models for the comparison of structural and functional relationships with vertebrates by helping to delineate which characteristics are conserved in the chordate lineage and which are innovations specific to vertebrates. This work is directed towards three models positioned at key junctions throughout chordate evolution: zebrafish (*Danio rerio*), tunicate (*Ciona intestinalis*) and amphioxus (*Branchiostoma floridae*).

The phylogeny and diversity of teleost fish represent the genetic, structural and functional diversity of the vertebrate GnRH system. The zebrafish is an excellent model, among a group of evolved teleost fishes, which is believed to have undergone large gene duplications, possibly as the result of a whole genome duplication event. Zebrafish maintain some of these duplicated genes, including GnRHRs, which may confer novel functional innovations when compared to their tetrapod and basal chordate counterparts. To compare vertebrate traits with more ancient species, I also analyzed two invertebrate chordates, the urochordate tunicate and the cephalochordate amphioxus, as they are believed to contain a basic set of genes with little genomic duplication, allowing the delineation of the prevertebrate condition. Recently, the phylogenetic position of amphioxus, thought to represent the closest extant relative to vertebrates, has been called into question. Phylogenetic comparisons of 146 protein-coding sequences present in the amphioxus genome have led to a decision to reverse the evolutionary positions of urochordates and cephalochordates (Bourlat *et al.*, 2006; Delsuc *et al.*, 2006). The

placement of amphioxus at the base of the chordate lineage provides an important touchstone for predicting the nature of the GnRHR gene family in the shared chordate ancestor. The simple body plan of the tunicate, as an immediate sister group of the vertebrates, represents a unique experimental model toward the understanding of functions and evolution of the GnRH neuronal system of chordates. In this dissertation, I present my research findings working back through evolution, starting with the vertebrate zebrafish, followed by the tunicate and ending with the amphioxus.

In Chapter 2, I describe the characterization of the GnRH system in the evolved teleost zebrafish. I used this model to address questions concerning the functional evolution of the vertebrate GnRH system. First, I addressed the question of whether duplicated genes are functional. I cloned and characterized four novel receptors and found that each displayed differential stimulation profiles with their two endogenous ligands and an additional mammalian GnRH variant (mGnRH). Second, I addressed the question of whether GnRH has functions outside of the pituitary. To do this, I generated antibodies to each zebrafish GnRH receptor for the first time and found that in addition to all of the receptors being expressed on the gonadotropes, one was also expressed in the motor control regions of the hindbrain. Third, to conclude this study, I identified GnRH neurites in the vicinity of these receptors, indicating an immunoreactive network outside of the pituitary.

In Chapter 3, I describe my contributions to the discovery of new members of the GnRH peptide family from the tunicate, *Ciona intestinalis*. I helped to identify and clone two GnRH genes that encode six novel GnRH forms. As well, we also identified an additional form in a sister species, *Ciona savignyi*, revealing a total of seven unique

GnRH forms. I also describe our studies showing that synthetic *Ciona* GnRHs are highly potent at inducing gamete release after injection into gravid animals.

In Chapter 4, I reveal the functional targets of these *Ciona* GnRH forms by isolating and characterizing the GnRH receptor complement from *Ciona intestinalis*. I found that three of the four cloned receptors were functional with each of the six native *Ciona* GnRH peptides, as well as the highly conserved vertebrate form, GnRH2. This was the first study to reveal that *Ciona* GnRH receptors predominantly signal through the cAMP pathway, with only a single GnRH/receptor pair able to induce intracellular IP accumulation.

To elucidate the nature of GnRH receptors at the base of chordates, I describe the amphioxus GnRH receptor complement in Chapter 5. I cloned four receptors from *Branchiostoma floridae* and found that two show high phylogenetic association with the vertebrate GnRHRs; even more so than the *Ciona* GnRHRs. The other two GnRHR genes showed phylogenetic grouping with the octopus GnRHR-like sequence. This is the first discovery of functional GnRH receptors in amphioxus and reveals that two distinct classes of GnRH receptors were present at the base of chordate evolution. Physiological testing of these receptors demonstrates that two receptors have sensitivity to two vertebrate GnRH forms (mGnRH and GnRH2) and one receptor is functional with GnRH2 as well as another neuropeptide, adipokinetic hormone.

1.4 References

Adams, B. A., J. A. Tello, J. Erchegyi, C. Warby, D. J. Hong, K. O. Akinsanya, G. O. Mackie, W. Vale, J. E. Rivier and N. M. Sherwood (2003). "Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, Ciona intestinalis." Endocrinology **144**(5): 1907-19.

Amano, M., Y. Oka, K. Aida, N. Okumoto, S. Kawashima and Y. Hasegawa (1991). "Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, Oncorhynchus masou." J Comp Neurol **314**(3): 587-97.

Andreu-Vieyra, C. V., A. G. Buret and H. R. Habibi (2005). "Gonadotropin-releasing hormone induction of apoptosis in the testes of goldfish (Carassius auratus)." Endocrinology **146**(3): 1588-96.

Arora, K. K., L. Z. Krsmanovic, N. Mores, H. O'Farrell and K. J. Catt (1998). "Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor." J Biol Chem **273**(40): 25581-6.

Bahk, J. Y., J. S. Hyun, S. H. Chung, H. Lee, M. O. Kim, B. H. Lee and W. S. Choi (1995). "Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis." J Urol **154**(5): 1958-61.

Bedecarrats, G. Y. and U. B. Kaiser (2007). "Mutations in the human gonadotropin-releasing hormone receptor: insights into receptor biology and function." Semin Reprod Med **25**(5): 368-78.

Belchetz, P. E., T. M. Plant, Y. Nakai, E. J. Keogh and E. Knobil (1978). "Hypophysial responses to continuous and intermittent delivery of hypopthalamic gonadotropin-releasing hormone." Science **202**(4368): 631-3.

Bhagavath, B., M. Ozata, I. C. Ozdemir, E. Bolu, D. P. Bick, R. J. Sherins and L. C. Layman (2005). "The prevalence of gonadotropin-releasing hormone receptor mutations in a large cohort of patients with hypogonadotropic hypogonadism." Fertil Steril **84**(4): 951-7.

Bilezikjian, L. M., A. V. Turnbull, A. Z. Corrigan, A. L. Blount, C. L. Rivier and W. W. Vale (1998). "Interleukin-1beta regulates pituitary follistatin and inhibin/activin betaB mRNA levels and attenuates FSH secretion in response to activin-A." Endocrinology **139**(7): 3361-4.

Bosma, M. M., L. Bernheim, M. D. Leibowitz, P. J. Pfaffinger and B. Hille (1990). "Modulation of M current in frog sympathetic ganglion cells." Soc Gen Physiol Ser **45**: 43-59.

Botte, M. C., A. M. Chamagne, M. C. Carre, R. Counis and M. L. Kottler (1998). "Fetal expression of GnRH and GnRH receptor genes in rat testis and ovary." J Endocrinol **159**(1): 179-89.

Botte, M. C., Y. Lerrant, A. Lozach, A. Berault, R. Counis and M. L. Kottler (1999). "LH down-regulates gonadotropin-releasing hormone (GnRH) receptor, but not GnRH, mRNA levels in the rat testis." J Endocrinol **162**(3): 409-15.

Bourlat, S. J., T. Juliusdottir, C. J. Lowe, R. Freeman, J. Aronowicz, M. Kirschner, E. S. Lander, M. Thorndyke, H. Nakano, A. B. Kohn, A. Heyland, L. L. Moroz, R. R. Copley and M. J. Telford (2006). "Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida." Nature **444**(7115): 85-8.

Bourne, G. A. and D. M. Baldwin (1987). "Evidence for cAMP as a mediator of gonadotropin secretion from female pituitaries." Am J Physiol **253**(3 Pt 1): E290-5.

Bourne, G. A. and D. M. Baldwin (1987). "Evidence for cAMP as a mediator of gonadotropin secretion from male pituitaries." Am J Physiol **253**(3 Pt 1): E296-9.

Brooks, J., P. L. Taylor, P. T. Saunders, K. A. Eidne, W. J. Struthers and A. S. McNeilly (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**(2): R23-7.

Burger, L. L., A. C. Dalkin, K. W. Aylor, D. J. Haisenleder and J. C. Marshall (2002). "GnRH pulse frequency modulation of gonadotropin subunit gene transcription in normal gonadotropes-assessment by primary transcript assay provides evidence for roles of GnRH and follistatin." Endocrinology **143**(9): 3243-9.

Burgus, R., M. Butcher, M. Amoss, N. Ling, M. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale and R. Guillemin (1972). "Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH-hypothalamus-LRF-gas chromatography-mass spectrometry-decapeptide-Edman degradation)." Proc Natl Acad Sci U S A **69**(1): 278-82.

Cariboni, A., F. Pimpinelli, S. Colamarino, R. Zaninetti, M. Piccolella, C. Rumio, F. Piva, E. I. Rugarli and R. Maggi (2004). "The product of X-linked Kallmann's syndrome gene (KAL1) affects the migratory activity of gonadotropin-releasing hormone (GnRH)-producing neurons." Hum Mol Genet **13**(22): 2781-91.

Cattanach, B. M., C. A. Iddon, H. M. Charlton, S. A. Chiappa and G. Fink (1977). "Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism." Nature **269**(5626): 338-40.

Chauvin, S., M. Hibert, A. Berault and R. Counis (2001). "Critical implication of transmembrane Phe310, possibly in conjunction with Trp279, in the rat gonadotropin-releasing hormone receptor activation." Biochem Pharmacol **62**(3): 329-34.

Chen, A., D. Yahalom, N. Ben-Aroya, E. Kaganovsky, E. Okon and Y. Koch (1998). "A second isoform of gonadotropin-releasing hormone is present in the brain of human and rodents." FEBS Lett **435**(2-3): 199-203.

Cheng, K. W. and P. C. Leung (2000). "The expression, regulation and signal transduction pathways of the mammalian gonadotropin-releasing hormone receptor." Can J Physiol Pharmacol **78**(12): 1029-52.

Cook, J. V. and K. A. Eidne (1997). "An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation." Endocrinology **138**(7): 2800-6.

Cornea, A., J. A. Janovick, X. Lin and P. M. Conn (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**(9): 4272-80.

Crowe, S., H. Cushing and J. Homans (1910). "Experimental hypophysectomy." Bull. Johns Hopkins Hosp. **21**: 127-169.

Dalkin, A. C., D. J. Haisenleder, G. A. Ortolano, T. R. Ellis and J. C. Marshall (1989). "The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression." Endocrinology **125**(2): 917-24.

David, K., E. Dingemanse, J. Freud and E. Laqueur (1935). "Ueber krystallinisches männliches Hormon Hoden (Testosteron), wirksamer als aus Harn oder aus Cholesterin bereitetes Androsteron." Ztschr. f. physiol. chem **233**: 281.

Davidson, J. S., C. A. Flanagan, W. Zhou, Becker, II, R. Elario, W. Emeran, S. C. Sealfon and R. P. Millar (1995). "Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: role in receptor expression but not ligand binding." Mol Cell Endocrinol **107**(2): 241-5.

Davidson, J. S., C. A. McArdle, P. Davies, R. Elario, C. A. Flanagan and R. P. Millar (1996). "Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide." J Biol Chem **271**(26): 15510-4.

de Roux, N. (2006). "GnRH receptor and GPR54 inactivation in isolated gonadotropic deficiency." Best Pract Res Clin Endocrinol Metab **20**(4): 515-28.

Dellovade, T. L., J. A. King, R. P. Millar and E. F. Rissman (1993). "Presence and differential distribution of distinct forms of immunoreactive gonadotropin-releasing hormone in the musk shrew brain." Neuroendocrinology **58**(2): 166-77.

Delsuc, F., H. Brinkmann, D. Chourrout and H. Philippe (2006). "Tunicates and not cephalochordates are the closest living relatives of vertebrates." Nature **439**(7079): 965-8.

Demski, L. S. and R. G. Northcutt (1983). "The terminal nerve: a new chemosensory system in vertebrates?" Science **220**(4595): 435-7.

Dobkin-Bekman, M., M. Naidich, A. J. Pawson, R. P. Millar, R. Seger and Z. Naor (2006). "Activation of mitogen-activated protein kinase (MAPK) by GnRH is cell-context dependent." Mol Cell Endocrinol **252**(1-2): 184-90.

Dufau, M. L., D. W. Warren, G. F. Knox, E. Loumaye, M. L. Castellon, S. Luna and K. J. Catt (1984). "Receptors and inhibitory actions of gonadotropin-releasing hormone in the fetal Leydig cell." J Biol Chem **259**(5): 2896-9.

Eidne, K. A., R. E. Sellar, G. Couper, L. Anderson and P. L. Taylor (1992). "Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor." Mol Cell Endocrinol **90**(1): R5-9.

Ferris, H. A. and M. A. Shupnik (2006). "Mechanisms for pulsatile regulation of the gonadotropin subunit genes by GNRH1." Biol Reprod **74**(6): 993-8.

Flanagan, C. A., Becker, II, J. S. Davidson, I. K. Wakefield, W. Zhou, S. C. Sealfon and R. P. Millar (1994). "Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone." J Biol Chem **269**(36): 22636-41.

Flanagan, C. A., V. Rodic, K. Konvicka, T. Yuen, L. Chi, J. E. Rivier, R. P. Millar, H. Weinstein and S. C. Sealfon (2000). "Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction." Biochemistry **39**(28): 8133-41.

Force, A., M. Lynch, F. B. Pickett, A. Amores, Y. L. Yan and J. Postlethwait (1999). "Preservation of duplicate genes by complementary, degenerative mutations." Genetics **151**(4): 1531-45.

Ford, C. P., W. F. Dryden and P. A. Smith (2003). "Neurotrophic regulation of calcium channels by the peptide neurotransmitter luteinizing hormone releasing hormone." J Neurosci **23**(18): 7169-75.

Friedman, R. and A. L. Hughes (2004). "Two patterns of genome organization in mammals: the chromosomal distribution of duplicate genes in human and mouse." Mol Biol Evol **21**(6): 1008-13.

Fromme, B. J., A. A. Katz, R. W. Roeske, R. P. Millar and C. A. Flanagan (2001). "Role of aspartate7.32(302) of the human gonadotropin-releasing hormone receptor in stabilizing a high-affinity ligand conformation." Mol Pharmacol **60**(6): 1280-7.

Furlong, R. F. and P. W. Holland (2002). "Were vertebrates octoploid?" Philos Trans R Soc Lond B Biol Sci **357**(1420): 531-44.

Ganong, W. F. (2005). Review of medical physiology. Los Altos, Calif., Lange Medical Publications.

Gault, P. M., K. Morgan, A. J. Pawson, R. P. Millar and G. A. Lincoln (2004). "Sheep exhibit novel variations in the organization of the mammalian type II gonadotropin-releasing hormone receptor gene." Endocrinology **145**(5): 2362-74.

Gearing, M. and E. Terasawa (1988). "Luteinizing hormone releasing hormone (LHRH) neuroterminals mapped using the push-pull perfusion method in the rhesus monkey." Brain Res Bull **21**(1): 117-21.

Gether, U. (2000). "Uncovering molecular mechanisms involved in activation of G protein-coupled receptors." Endocr Rev **21**(1): 90-113.

Gonzalez-Martinez, D., N. Zmora, S. Zanuy, C. Sarasquete, A. Elizur, O. Kah and J. A. Munoz-Cueto (2002). "Developmental expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (Dicentrarchus labrax)." J Chem Neuroanat **23**(4): 255-67.

Haisenleder, D. J., H. A. Ferris and M. A. Shupnik (2003). "The calcium component of gonadotropin-releasing hormone-stimulated luteinizing hormone subunit gene transcription is mediated by calcium/calmodulin-dependent protein kinase type II." Endocrinology **144**(6): 2409-16.

Hardelin, J. P., N. Soussi-Yanicostas, O. Ardouin, J. Levilliers and C. Petit (2000). "Kallmann syndrome." Adv Otorhinolaryngol **56**: 268-74.

Harris, G. (1955). Neural control of the pituitary gland. Monographs of the Physiological Society. London, Edward Arnold Ltd.

Hoffmann, S. H., T. ter Laak, R. Kuhne, H. Reilander and T. Beckers (2000). "Residues within transmembrane helices 2 and 5 of the human gonadotropin-releasing hormone receptor contribute to agonist and antagonist binding." Mol Endocrinol **14**(7): 1099-115.

Hovelmann, S., S. H. Hoffmann, R. Kuhne, T. ter Laak, H. Reilander and T. Beckers (2002). "Impact of aromatic residues within transmembrane helix 6 of the human gonadotropin-releasing hormone receptor upon agonist and antagonist binding." Biochemistry **41**(4): 1129-36.

Hughes, A. L. (1999). "Phylogenies of developmentally important proteins do not support the hypothesis of two rounds of genome duplication early in vertebrate history." J Mol Evol **48**(5): 565-76.

Hughes, A. L. and R. Friedman (2003). "2R or not 2R: testing hypotheses of genome duplication in early vertebrates." J Struct Funct Genomics **3**(1-4): 85-93.

Ikemoto, T. and M. K. Park (2005). "Identification and molecular characterization of three GnRH ligands and five GnRH receptors in the spotted green pufferfish." Mol Cell Endocrinol **242**(1-2): 67-79.

Illing, N., B. E. Troskie, C. S. Nahorniak, J. P. Hapgood, R. E. Peter and R. P. Millar (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 U S A **96**(5): 2526-31.

Imai, A., H. Takagi, S. Horibe, T. Fuseya and T. Tamaya (1996). "Coupling of gonadotropin-releasing hormone receptor to Gi protein in human reproductive tract tumors." J Clin Endocrinol Metab **81**(9): 3249-53.

Iwakoshi, E., K. Takuwa-Kuroda, Y. Fujisawa, M. Hisada, K. Ukena, K. Tsutsui and H. Minakata (2002). "Isolation and characterization of a GnRH-like peptide from Octopus vulgaris." Biochem Biophys Res Commun **291**(5): 1187-93.

Jan, L. Y. and Y. N. Jan (1981). "Role of an LHRH-like peptide as a neurotransmitter is sympathetic ganglia of the frog." Fed Proc **40**(11): 2560-4.

Jodo, A., H. Ando and A. Urano (2003). "Five different types of putative GnRH receptor gene are expressed in the brain of masu salmon (Oncorhynchus masou)." Zoolog Sci **20**(9): 1117-25.

Kah, O., C. Lethimonier, G. Somoza, L. G. Guilgur, C. Vaillant and J. J. Lareyre (2007). "GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective." Gen Comp Endocrinol **153**(1-3): 346-64.

Kaiser, U. B., D. Zhao, G. R. Cardona and W. W. Chin (1992). "Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor." Biochem Biophys Res Commun **189**(3): 1645-52.

Kakar, S. S., L. C. Musgrove, D. C. Devor, J. C. Sellers and J. D. Neill (1992). "Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor." Biochem Biophys Res Commun **189**(1): 289-95.

Kakar, S. S., C. H. Rahe and J. D. Neill (1993). "Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH)." Domest Anim Endocrinol **10**(4): 335-42.

Kanda, A., T. Takahashi, H. Satake and H. Minakata (2006). "Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor isolated from the common octopus (Octopus vulgaris)." Biochem J **395**(1): 125-35.

Kang, S. K., K. C. Choi, H. S. Yang and P. C. Leung (2003). "Potential role of gonadotrophin-releasing hormone (GnRH)-I and GnRH-II in the ovary and ovarian cancer." Endocr Relat Cancer **10**(2): 169-77.

Karten, M. J. and J. E. Rivier (1986). "Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective." Endocr Rev **7**(1): 44-66.

Kasten, T. L., S. A. White, T. T. Norton, C. T. Bond, J. P. Adelman and R. D. Fernald (1996). "Characterization of two new preproGnRH mRNAs in the tree shrew: first direct evidence for mesencephalic GnRH gene expression in a placental mammal." Gen Comp Endocrinol **104**(1): 7-19.

Kuo, M. W., S. W. Lou, J. Postlethwait and B. C. Chung (2005). "Chromosomal organization, evolutionary relationship, and expression of zebrafish GnRH family members." J Biomed Sci **12**(4): 629-39.

Kuphal, D., J. A. Janovick, U. B. Kaiser, W. W. Chin and P. M. Conn (1994). "Stable transfection of GH3 cells with rat gonadotropin-releasing hormone receptor complementary deoxyribonucleic acid results in expression of a receptor coupled to cyclic adenosine 3',5'-monophosphate-dependent prolactin release via a G-protein." Endocrinology **135**(1): 315-20.

Leanos-Miranda, A., J. A. Janovick and P. M. Conn (2002). "Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism." J Clin Endocrinol Metab **87**(10): 4825-8.

Leong, D. A. and M. O. Thorner (1991). "A potential code of luteinizing hormone-releasing hormone-induced calcium ion responses in the regulation of luteinizing hormone secretion among individual gonadotropes." J Biol Chem **266**(14): 9016-22.

Lescheid, D. W., J. F. Powell, W. H. Fischer, M. Park, A. Craig, O. Bukovskaya, I. A. Barannikova and N. M. Sherwood (1995). "Mammalian gonadotropin-releasing hormone (GnRH) identified by primary structure in Russian sturgeon, Acipenser gueldenstaedti." Regul Pept **55**(3): 299-309.

Lescheid, D. W., E. Terasawa, L. A. Abler, H. F. Urbanski, C. M. Warby, R. P. Millar and N. M. Sherwood (1997). "A second form of gonadotropin-releasing hormone (GnRH) with characteristics of chicken GnRH-II is present in the primate brain." Endocrinology **138**(12): 5618-29.

Lethimonier, C., T. Madigou, J. A. Munoz-Cueto, J. J. Lareyre and O. Kah (2004). "Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish." Gen Comp Endocrinol **135**(1): 1-16.

Liu, F., I. Usui, L. G. Evans, D. A. Austin, P. L. Mellon, J. M. Olefsky and N. J. Webster (2002). "Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L beta T2 cells." J Biol Chem **277**(35): 32099-108.

Liu, W. K., H. S. Nahm, C. M. Sweeney, G. N. Holcomb and D. N. Ward (1972). "The primary structure of ovine luteinizing hormone. II. The amino acid sequence of the reduced, S-carboxymethylated A-subunit (LH-)." J Biol Chem **247**(13): 4365-81.

Liu, W. K., H. S. Nahm, C. M. Sweeney, W. M. Lamkin, H. N. Baker and D. N. Ward (1972). "The primary structure of ovine luteinizing hormone. I. The amino acid sequence of the reduced and S-aminoethylated S-subunit (LH-)." J Biol Chem **247**(13): 4351-64.

Lovejoy, D. A., W. H. Fischer, S. Ngamvongchon, A. G. Craig, C. S. Nahorniak, R. E. Peter, J. E. Rivier and N. M. Sherwood (1992). "Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution." Proc Natl Acad Sci U S A **89**(14): 6373-7.

Lynch, M. and A. Force (2000). "The probability of duplicate gene preservation by subfunctionalization." Genetics **154**(1): 459-73.

Marchant, T. A., J. P. Chang, C. S. Nahorniak and R. E. Peter (1989). "Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish." Endocrinology **124**(5): 2509-18.

Matsuo, H., Y. Baba, R. M. Nair, A. Arimura and A. V. Schally (1971). "Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence." Biochem Biophys Res Commun **43**(6): 1334-9.

McArdle, C. A., J. S. Davidson and G. B. Willars (1999). "The tail of the gonadotrophin-releasing hormone receptor: desensitization at, and distal to, G protein-coupled receptors." Mol Cell Endocrinol **151**(1-2): 129-36.

McArdle, C. A., W. Forrest-Owen, G. Willars, J. Davidson, A. Poch and M. Kratzmeier (1995). "Desensitization of gonadotropin-releasing hormone action in the gonadotrope-derived alpha T3-1 cell line." Endocrinology **136**(11): 4864-71.

Millar, R., D. Conklin, C. Lofton-Day, E. Hutchinson, B. Troskie, N. Illing, S. C. Sealfon and J. Hapgood (1999). "A novel human GnRH receptor homolog gene: abundant and wide tissue distribution of the antisense transcript." J Endocrinol **162**(1): 117-26.

Millar, R., S. Lowe, D. Conklin, A. Pawson, S. Maudsley, B. Troskie, T. Ott, M. Millar, G. Lincoln, R. Sellar, B. Faurholm, G. Scobie, R. Kuestner, E. Terasawa and A. Katz (2001). "A novel mammalian receptor for the evolutionarily conserved type II GnRH." Proc Natl Acad Sci U S A **98**(17): 9636-41.

Millar, R. P., Z. L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan and S. R. Maudsley (2004). "Gonadotropin-releasing hormone receptors." Endocr Rev **25**(2): 235-75.

Miyamoto, K., Y. Hasegawa, T. Minegishi, M. Nomura, Y. Takahashi, M. Igarashi, K. Kangawa and H. Matsuo (1982). "Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone." Biochem Biophys Res Commun **107**(3): 820-7.

Miyamoto, K., Y. Hasegawa, M. Nomura, M. Igarashi, K. Kangawa and H. Matsuo (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 U S A **81**(12): 3874-8.

Moenter, S. M., A. Caraty, A. Locatelli and F. J. Karsch (1991). "Pattern of gonadotropin-releasing hormone (GnRH) secretion leading up to ovulation in the ewe: existence of a preovulatory GnRH surge." Endocrinology **129**(3): 1175-82.

Moore CR and D. Price (1932). "Gonad hormone functions and the reciprocal influence between gonads and hypophysis with its bearing on the problem of sex hormone antagonism." Am J Anat **50**: 13-67.

Morgan, K. and R. P. Millar (2004). "Evolution of GnRH ligand precursors and GnRH receptors in protochordate and vertebrate species." Gen Comp Endocrinol **139**(3): 191-7.

Morgan, K., R. Sellar, A. J. Pawson, Z. L. Lu and R. P. Millar (2006). "Bovine and ovine gonadotropin-releasing hormone (GnRH)-II ligand precursors and type II GnRH receptor genes are functionally inactivated." Endocrinology **147**(11): 5041-51.

Munz, H., W. E. Stumpf and L. Jennes (1981). "LHRH systems in brain of platyfish." Brain Res **221**(1): 1-13.

Naor, Z., C. P. Fawcett and S. M. McCann (1979). "Differential effects of castration and testosterone replacement on basal and LHRH-stimulated cAMP and cGMP accumulation and on gonadotropin release from the pituitary of the male rat." Mol Cell Endocrinol **14**(3): 191-8.

Neill, J. D. (2006). Knobil and Neill's The Physiology of reproduction. New York, Academic Press.

Neill, J. D., L. W. Duck, J. C. Sellers and L. C. Musgrove (2001). "A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates." Biochem Biophys Res Commun **282**(4): 1012-8.

Neill, J. D., L. C. Musgrove and L. W. Duck (2004). "Newly recognized GnRH receptors: function and relative role." Trends Endocrinol Metab **15**(8): 383-92.

Ohno, S. (1970). Evolution by gene duplication. Berlin, Springer-Verlag.

Ohno, S. (1996). "The notion of the Cambrian pananimalia genome." Proc Natl Acad Sci U S A **93**(16): 8475-8.

Ohno, S. (1999). "The one-to-four rule and paralogues of sex-determining genes." Cell Mol Life Sci **55**(6-7): 824-30.

Okubo, K., H. Mitani, K. Naruse, M. Kondo, A. Shima, M. Tanaka, S. Asakawa, N. Shimizu, Y. Yoshiura and K. Aida (2002). "Structural characterization of GnRH loci in the medaka genome." Gene **293**(1-2): 181-9.

Panopoulou, G., S. Hennig, D. Groth, A. Krause, A. J. Poustka, R. Herwig, M. Vingron and H. Lehrach (2003). "New evidence for genome-wide duplications at the origin of vertebrates using an amphioxus gene set and completed animal genomes." Genome Res **13**(6A): 1056-66.

Papkoff, H., M. R. Sairam and C. H. Li (1971). "Amino acid sequence of the subunits of ovine pituitary interstitial cell-stimulating hormone." J Am Chem Soc **93**(6): 1531-2.

Pati, D. and H. R. Habibi (2000). "Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis." Mol Cell Endocrinol **160**(1-2): 75-88.

Pawson, A. J., E. Faccenda, S. Maudsley, Z. L. Lu, Z. Naor and R. P. Millar (2007). "Mammalian Type I GnRH Receptors Undergo Slow, Constitutive, Agonist-Independent Internalization." Endocrinology.

Pawson, A. J. and A. S. McNeilly (2005). "The pituitary effects of GnRH." Anim Reprod Sci **88**(1-2): 75-94.

Popa, G. T. and U. Fielding (1930). "A portal circulation of the pituitary to the hypothalamic region." J Anat **65**(88).

Powell, J. F., S. M. Reska-Skinner, M. O. Prakash, W. H. Fischer, M. Park, J. E. Rivier, A. G. Craig, G. O. Mackie and N. M. Sherwood (1996). "Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications." Proc Natl Acad Sci U S A **93**(19): 10461-4.

Ramakrishnappa, N., R. Rajamahendran, Y. M. Lin and P. C. Leung (2005). "GnRH in non-hypothalamic reproductive tissues." Anim Reprod Sci **88**(1-2): 95-113.

Sarkar, D. K., S. A. Chiappa, G. Fink and N. M. Sherwood (1976). "Gonadotropin-releasing hormone surge in pro-oestrous rats." Nature **264**(5585): 461-3.

Schvartz, I. and E. Hazum (1987). "Internalization and recycling of receptor-bound gonadotropin-releasing hormone agonist in pituitary gonadotropes." J Biol Chem **262**(35): 17046-50.

Seeburg, P. H. and J. P. Adelman (1984). "Characterization of cDNA for precursor of human luteinizing hormone releasing hormone." Nature **311**(5987): 666-8.

Sherwood, N., L. Eiden, M. Brownstein, J. Spiess, J. Rivier and W. Vale (1983). "Characterization of a teleost gonadotropin-releasing hormone." Proc Natl Acad Sci U S A **80**(9): 2794-8.

Sherwood, N. M. and B. A. Adams (2005). Gonadotropin-releasing Hormone in Fish: Evolution, Expression and Regulation of the GnRH gene. Hormones and their receptors in fish reproduction. P. Melamed and N. M. Sherwood. Singapore, World Scientific Publishing Co. Pte. Ltd. **4:** 1-39.

Sherwood, N. M., S. A. Sower, D. R. Marshak, B. A. Fraser and M. J. Brownstein (1986). "Primary structure of gonadotropin-releasing hormone from lamprey brain." J Biol Chem **261**(11): 4812-9.

Shome, B. and A. F. Parlow (1973). "The primary structure of the hormone-specific, beta subunit of human pituitary luteinizing hormone (hLH)." J Clin Endocrinol Metab **36**(3): 618-21.

Shome, B. and A. F. Parlow (1974). "Human follicle stimulating hormone: first proposal for the amino acid sequence of the hormone-specific, beta subunit (hFSHb)." J Clin Endocrinol Metab **39**(1): 203-5.

Silveira, L. F., P. M. Stewart, M. Thomas, D. A. Clark, P. M. Bouloux and G. S. MacColl (2002). "Novel homozygous splice acceptor site GnRH receptor (GnRHR) mutation: human GnRHR "knockout"." J Clin Endocrinol Metab **87**(6): 2973-7.

Silver, M. R., H. Kawauchi, M. Nozaki and S. A. Sower (2004). "Cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey representing the three families of Petromyzoniformes." Gen Comp Endocrinol **139**(1): 85-94.

Sower, S. A., Y. C. Chiang, S. Lovas and J. M. Conlon (1993). "Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain." Endocrinology **132**(3): 1125-31.

Spiegel, A. M., A. Shenker and L. S. Weinstein (1992). "Receptor-effector coupling by G proteins: implications for normal and abnormal signal transduction." Endocr Rev **13**(3): 536-65.

Stojilkovic, S. S., J. Reinhart and K. J. Catt (1994). "Gonadotropin-releasing hormone receptors: structure and signal transduction pathways." Endocr Rev **15**(4): 462-99.

Tao, Y. X. (2006). "Inactivating mutations of G protein-coupled receptors and diseases: structure-function insights and therapeutic implications." Pharmacol Ther **111**(3): 949-73.

Taylor, J. S., Y. Van de Peer, I. Braasch and A. Meyer (2001). "Comparative genomics provides evidence for an ancient genome duplication event in fish." Philos Trans R Soc Lond B Biol Sci **356**(1414): 1661-79.

Tello, J. A., J. E. Rivier and N. M. Sherwood (2005). "Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate Ciona intestinalis GnRH receptors and the green monkey type II GnRH receptor." Endocrinology **146**(9): 4061-73.

Temple, J. L., R. P. Millar and E. F. Rissman (2003). "An evolutionarily conserved form of gonadotropin-releasing hormone coordinates energy and reproductive behavior." Endocrinology **144**(1): 13-9.

Tensen, C., K. Okuzawa, M. Blomenrohr, F. Rebers, R. Leurs, J. Bogerd, R. Schulz and H. Goos (1997). "Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor." Eur J Biochem **243**(1-2): 134-40.

Thayer, S., L. Levin and E. Doisy (1931). "Characteriztion of Theelol." J Biol Chem **91**: 655-665.

Troskie, B., N. Illing, E. Rumbak, Y. M. Sun, J. Hapgood, S. Sealfon, D. Conklin and R. Millar (1998). "Identification of three putative GnRH receptor subtypes in vertebrates." Gen Comp Endocrinol **112**(3): 296-302.

Tsutsumi, M., S. C. Laws and S. C. Sealfon (1993). "Homologous up-regulation of the gonadotropin-releasing hormone receptor in alpha T3-1 cells is associated with unchanged receptor messenger RNA (mRNA) levels and altered mRNA activity." Mol Endocrinol **7**(12): 1625-33.

Tsutsumi, M., W. Zhou, R. P. Millar, P. L. Mellon, J. L. Roberts, C. A. Flanagan, K. Dong, B. Gillo and S. C. Sealfon (1992). "Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor." Mol Endocrinol **6**(7): 1163-9.

Urbanski, H. F., R. B. White, R. D. Fernald, S. G. Kohama, V. T. Garyfallou and V. S. Densmore (1999). "Regional expression of mRNA encoding a second form of gonadotropin-releasing hormone in the macaque brain." Endocrinology **140**(4): 1945-1948.

Urrutia, R., J. R. Henley, T. Cook and M. A. McNiven (1997). "The dynamins: redundant or distinct functions for an expanding family of related GTPases?" Proc Natl Acad Sci U S A **94**(2): 377-84.

Weber, G. M., J. F. Powell, M. Park, W. H. Fischer, A. G. Craig, J. E. Rivier, U. Nanakorn, I. S. Parhar, S. Ngamvongchon, E. G. Grau and N. M. Sherwood (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**(1): 121-32.

Weesner, G. D. and R. L. Matteri (1994). "Rapid communication: nucleotide sequence of luteinizing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary." J Anim Sci **72**(7): 1911.

White, R. B., J. A. Eisen, T. L. Kasten and R. D. Fernald (1998). "Second gene for gonadotropin-releasing hormone in humans." Proc Natl Acad Sci U S A **95**(1): 305-9.

Whitlock, K. E. (2005). "Origin and development of GnRH neurons." Trends Endocrinol Metab **16**(4): 145-51.

Willars, G. B., C. A. McArdle and S. R. Nahorski (1998). "Acute desensitization of phospholipase C-coupled muscarinic M3 receptors but not gonadotropin-releasing hormone receptors co-expressed in alphaT3-1 cells: implications for mechanisms of rapid desensitization." Biochem J **333 (Pt 2)**: 301-8.

Wirsig-Wiechmann, C. R. and Y. Oka (2002). "The terminal nerve ganglion cells project to the olfactory mucosa in the dwarf gourami." Neurosci Res **44**(3): 337-41.

Wirsig, C. R. and C. M. Leonard (1987). "Terminal nerve damage impairs the mating behavior of the male hamster." Brain Res **417**(2): 293-303.

Wray, S. (2002). "Development of gonadotropin-releasing hormone-1 neurons." Front Neuroendocrinol **23**(3): 292-316.

Wray, S., A. Nieburgs and S. Elkabes (1989). "Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode." Brain Res Dev Brain Res **46**(2): 309-18.

Zandbergen, M. A., O. Kah, J. Bogerd, J. Peute and H. J. Goos (1995). "Expression and distribution of two gonadotropin-releasing hormones in the catfish brain." Neuroendocrinology **62**(6): 571-8.

Zhou, W., V. Rodic, S. Kitanovic, C. A. Flanagan, L. Chi, H. Weinstein, S. Maayani, R. P. Millar and S. C. Sealfon (1995). "A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites." J Biol Chem **270**(32): 18853-7.

Chapter 2

Four functional GnRH receptors that are expressed in a motor control centre and/or reproductive tissues in zebrafish: analysis of structure, signalling, synteny and evolutionary relationships

Sheng Wu assisted in identifying the 5' sequence of zfGnRHR-3, after which I completed the analysis of this receptor.

All peptides used in this study were synthesized by Jean Rivier. Conjugation of receptor peptides to their carrier proteins was done by Joan Vaughan.

2.1 Introduction

Gonadotropin-releasing hormone has been tightly linked to the control of reproduction in vertebrates through the hypothalamic-pituitary-gonadal axis (Schally *et al.*, 1971). However, there are additional forms of GnRH found in most chordate species that are expressed in different brain and peripheral locations. Accumulating evidence suggests that GnRH has functions outside of pituitary regulation. The key to understanding novel GnRH functions depends on locating other GnRH target tissues.

The hypophysiotropic function of GnRH in the mammalian brain is activated by mGnRH (aka GnRH1), which is released by neurons in the preoptic-hypothalamic area (Wray, 2002). In the zebrafish, mGnRH has not been found; instead, salmon GnRH (sGnRH or GnRH3), present mainly in the area of the terminal nerve and ventral telencephalon, is thought to be recruited for control of pituitary function (Torgersen *et al.*, 2002; Steven *et al.*, 2003). This arrangement is closer to that in mammals, where mGnRH is found both in the preoptico-hypothalamic and terminal nerve areas (Schwanzel-Fukuda, 1999). In addition, zebrafish possess GnRH2 (aka chicken GnRH-II), a highly conserved GnRH form that has been localized in the midbrain of most jawed vertebrates, although its role remains unclear.

Preservation of multiple GnRH forms, each localized to anatomically distinct neuronal populations in the brain and peripheral tissues, suggests roles outside of the stimulation of gonadotropes in the pituitary. For example, GnRH has been shown to increase circulating levels of growth hormone in both goldfish and tilapia (Marchant *et al.*, 1989; Melamed *et al.*, 1995), increase serum prolactin in tilapia (Weber *et al.*, 1997) and stimulate spawning behaviour in female goldfish (Volkoff and Peter, 1999). In

addition, GnRH has been shown to have neuromodulatory effects in the salamander by increasing the excitability of olfactory receptor neurons (Eisthen *et al.*, 2000).

The obvious clues to novel GnRH functions are the location, signalling pathways and effectors of GnRH reception. The effects of GnRH on target tissues depend on specific GnRHRs, which are members of the GPCR family. Vertebrate GnRHRs share a common overall structure; they have an N-terminal extracellular region with seven TMDs connected by alternating hydrophilic intracellular and extracellular loops terminating in a cytoplasmic C-terminal tail, although the tail is missing in the type I mammalian GnRHRs (Millar *et al.*, 2004). GnRHRs are widely conserved in evolution, as demonstrated recently with the cloning of a functional GnRHR from the octopus (Kanda *et al.*, 2006) and three functional receptors in the invertebrate tunicate (Kusakabe *et al.*, 2003; Tello *et al.*, 2005). Since the cloning of the first GnRHR from the mouse αT3 gonadotrope cell line (Reinhart *et al.*, 1992; Tsutsumi *et al.*, 1992) and the first teleost GnRHR from the African catfish (Tensen *et al.*, 1997), many teleost GnRHRs have been identified. Some species have been shown to encode up to five GnRHRs in their genome, as is the case for each of two pufferfish (*Fugu rupripes* and *Tetradon nigroviridis*), the cherry salmon (*Oncorhynchus masou*) and the European sea bass (*Dicentrachus labrax*), although the functional status of only a few is known (Jodo *et al.*, 2003; Ikemoto and Park, 2005; Moncaut *et al.*, 2005).

The zebrafish, *Danio rerio*, is an excellent model to elucidate novel functions of the GnRH ligand-receptor partnership during both development and in the adult. With sequencing of the genome nearly complete, three GnRHRs and one partial GnRHR have been annotated, but not cloned. The present study lays the foundation for delineating the

roles of GnRH outside of the direct regulation of pituitary gonadotropes. In this study, I characterize the zebrafish GnRH receptor system at the molecular level by cloning the four complete zebrafish GnRHRs. Zebrafish GnRH receptor functions are assessed by measuring ligand-induced intracellular accumulation of IP and cAMP in transfected COS7 cells. After generating polyclonal antisera specific to each GnRH receptor, I locate the expression of each zebrafish receptor protein in the pituitary, olfactory epithelium and within the central nervous system, including a motor control centre and associated fibres. Using antisera that recognize both native GnRH peptides (GnRH2, sGnRH) I show that GnRH axons project to the cerebellum and are present in the zebrafish hindbrain. Expression of the four GnRH receptor transcripts in peripheral tissues is detected by RT-PCR. Finally, each zebrafish GnRHR sequence is mapped to its chromosomal location and placed into its evolutionary context using both synteny mapping and phylogenetic methods.

2.2 Materials and Methods

2.2.1 Animals

All experiments were approved by the Animal Care Committee at the University of Victoria. Adult zebrafish (*Danio rerio*) were obtained from a local dealer (Safari Pets, Victoria, BC). Zebrafish were anaesthetised with tricaine methanesulfonate (MS222; Argent Chemical Laboratories, Inc., Redmond, WA) and tissues were dissected under a microscope. Tissues used for RNA extraction were immediately frozen in liquid nitrogen. Twenty zebrafish were used for immunocytochemistry; whole heads and dissected brains were immediately processed as described below.

2.2.2 Gene organization

To identify the complete GnRH receptor gene complement in the zebrafish, genomic sequences from the Sanger Institute's *Danio rerio* Sequencing Project (http://www.sanger.ac.uk/Projects/D_rerio/) were screened. The entire open reading frame (ORF) nucleotide sequences of all reported GnRHRs were used with default parameters. Each search generated closely matched fragments. The DNA regions encoding four putative GnRHRs were compiled, examined for exon/intron boundaries and analyzed for an in-frame ORF. Primers were designed and the complete receptor ORFs were amplified from brain tissue using PCR and sequenced (see below). Exon/intron boundaries were established by comparing the cDNA to the genome project sequence or to amplified genomic DNA. To complete any missing regions in the coding sequences, 5'- and 3'-rapid amplification of cDNA ends (RACE) was implemented.

2.2.3 Isolation of zebrafish mRNA for cDNA synthesis and RACE

The mRNA was isolated from brain tissue using a Micro Poly(A) Pure mRNA isolation kit according to the manufacturer's protocol (Ambion Inc., Austin, TX). The mRNA was reverse-transcribed in a 50 μl reaction that contained mRNA, 2 µM oligo dT, 2 mM deoxynucleoside triphosphates, 1x first strand reaction buffer, 0.01 M dithiothreitol, 40 U RNase inhibitor, and 100 U Superscript II reverse transcriptase (Invitrogen, Burlington, ON). The reaction was incubated at 42 °C for 90 min, and the enzyme was heat-inactivated at 70 °C for 15 min.

For RACE-PCR, 250 ng of mRNA were used to prepare RACE-ready cDNA using the RLM-RACE kit (Ambion) according to the manufacturer's instructions, except that the DNA was dissolved in DNase/RNase-free distilled water (Invitrogen).

2.2.4 PCR and sequencing of cDNA

Oligonucleotide primers were designed to regions encoding candidate GnRHRs based on the compiled sequences for zebrafish GnRHR genes 1, 2, 3 and 4. Each 50 μl reaction contained 2.5 U Platinum *Taq* polymerase High Fidelity (Invitrogen), 1X High Fidelity PCR buffer, 2.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates (Invitrogen), and 0.4 μM of each Koz forward (f) and Stop reverse (r) primer (Table 2.1). PCRs were performed under the following conditions: 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, and a 5 min final extension. The PCR amplicons were separated by electrophoresis on a 1.3 % (w/v) agarose gel and visualized with ethidium bromide staining using an Eagle Eye II still video system (Stratagene, La Jolla, CA). Bands were selected, isolated (Qiagen, Valencia, CA), and cloned or cloned directly as amplicons into pGEM Vector-T (Promega Corp., Madison, WI) and sequenced. The SequiTherm EXCEL II DNA sequencing kit was used by the University of Victoria Sequencing Centre. Each gene was amplified using M13 forward and reverse priming sites present on pGEM-T and sequenced on a LI-COR 4200 – Global IR^2.

Table 2.1. Primers used to amplify target cDNA for GnRH, GnRHR and housekeeping genes in the zebrafish, *Danio rerio*.

Primer	Sequence (5'-3')	Target	Target size (bp)
zfR1Koz f	TCTAGAGCCACCATGGCAGGTAACGTGT	*zfGnRHR1*	1,165
zfR1Stop r	GCGGCCGCGGATTGACCACCTATGCTGTT TG		
zfR2Koz f	TCTAGAGCCACCATGGACACAACTCAATT GATCGAGGAT	*zfGnRHR2*	1,266
zfR2Stop r	GCGGCCGCTGGCTATTCATTCCACTGTGG CGA		
zfR3Koz f	ATGCTAGCGCCACCATGGCTGGTAACTGG TCTCA	*zfGnRHR3*	1,052
zfR3Stop r	ATCTCGAGCTCCAGTGTTCCTCCTTTGT		
zfR4Koz f	TCTAGAGCCACCATGGATGACAGCTCTC	*zfGnRHR4*	1,256
zfR4Stop r	GCGGCCGCTGCTTTCTTTCCCCACTATTCT		
zfR1 TE f	GCGATGCCATGTGTAAACTTCTCTG	*zfGnRHR1*	582
zfR1 TE r	GGGCGTATACCGGCGTGACCT		
zfR2 TE f	TGATTAGTCTGGATCGACAGG	*zfGnRHR2*	765
zfR2 TE r	GCACAAACTCAGCATCCCATTCTAGC		
zfR3 TE f	CGCCGGGGACGTGGTGTGTA	*zfGnRHR3*	421
zfR3 TE r	GCGCCTCAGAGAATCACCTTTGTTG		
zfR4 TE f	TGGCCTGGGCGATGAGTGTTGTTCT	*zfGnRHR4*	298
zfR4 TE r	CGGGCCTTGGGGATGTTGCTGT		
GnRH2 TE f	ATTAGACTGAAGTGATGGTG	*zfGnRH2*	493
GnRH2 TE r	AGCCTTTATTGTAGGAACTG		
GnRH3 TE f	AAGGTTGTTGGTCCAGTTGTTGCT	*zfGnRH3*	226
GnRH3 TE r	CAAACCTTCAGCATCCACCTCATTCA		
TBP TE f	ACACCACTTTATACCACACC	*zfTBP*	100
TBP TE r	ACAATATTCTGTAACTGCGGG		

Abbreviations: bp= base pair; f = forward; Koz = Kozak; R= receptor; r = reverse; TBP= TATA-box binding protein; TE= tissue expression; zf= zebrafish.

2.2.5 Isolation of total RNA for receptor tissue expression profiling

Total RNA was isolated from male and female tissues separately using TRIzol reagent according to the manufacturer's protocol (Invitrogen). Total RNA samples were run on the Eukaryote Total RNA Nano chip to assess RNA quality using the Agilent 2100 Bioanalyzer with resulting RNA integrity numbers (RIN) ranging between 5.6 and 9.2 (Agilent Technologies Canada Inc., Mississauga, ON). Total RNA of 1.5 μg from each tissue was DNased with the DNA-free kit according to the manufacturer's directions (Ambion). DNased total RNA (800 μg) from male and female tissues were pooled to create an equal mix of male and female RNA for each tissue. The RNA was reverse-transcribed in a 20-μl reaction that contained, 2.5 μM oligo dT, 0.5 mM deoxynucleoside triphosphates, 1x first strand reaction buffer, 5 mM dithiothreitol, 40 U RNaseOUT, and 200 U Superscript III reverse transcriptase (Invitrogen). The reaction was incubated at 50 °C for 60 min, and the enzyme was heat inactivated at 70 °C for 15 min.

2.2.6 PCR for tissue distribution

Primer pairs used in each tissue expression PCR reaction are listed in Table 2.1. Each 50 μl reaction contained 2 U Platinum Taq polymerase High Fidelity (Invitrogen), 1X High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates (Invitrogen), and 0.2 μM of each Koz forward (f) and Stop reverse (r) primer. PCRs were performed under the following conditions: 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a 10 min final extension. The PCR amplicons were separated by electrophoresis on a 3 % (w/v) agarose gel.

2.2.7 Inositol phosphate (IP) accumulation assay

The cDNA encoding each GnRHR ORF was altered to include a strong context Kozak sequence (GCCACCATGG) (start codon is underlined) (Kozak, 1996) using the same PCR conditions as mentioned previously. The altered cDNA was then cloned into pcDNA3.1(-) (Invitrogen). COS7 cells (Invitrogen) were seeded and grown into monolayer cultures in T-175 cm^2 flasks in growth medium consisting of Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 4 mM L-glutamine, 0.1 mM non-essential amino acids (Invitrogen) and 10 % fetal bovine serum (Invitrogen) at 37 °C in 5 % CO_2. After 3 days, the monolayers were trypsinized with TrypLE (Invitrogen) and seeded in 24-well tissue culture-treated plates (Corning-Costar Corp., Cambridge, MA) at a density of 55,000 cells per well and grown overnight in growth medium. At 85-95 % confluence, approximately 24 h post seeding, the cells were washed and incubated with serum-free medium (VP-SFM, Invitrogen), then transfected with 0.8 µg/well of receptor-encoded plasmid DNA using Lipofectamine according to the manufacturer's protocol (Invitrogen). After another 24 h, the cells were washed with labelling medium: Medium 199 (Invitrogen) containing 0.3 % bovine albumin (Sigma-Aldrich, Oakville, ON) and subsequently labelled with 0.9 μCi/well of *myo*-[2-^{3}H]-Inositol (Amersham, Piscataway, NJ) in labelling medium for 24 h. The cells were washed and pre-incubated for 30 min at 37 °C in labelling medium containing 10 mM Li Cl. The cells were stimulated with various concentrations of GnRH (Table 2.2) for 1h at 37 °C with gentle agitation, and then lysed using 200 μl of 0.1 M formic acid after the medium in each well was removed. Measurement of total inositol phosphates (IPs) in cell extracts was performed by the multi-well filtration method (Chengalvala *et al.*, 1999).

Table 2.2. Amino acid sequences of ligands used to test zebrafish GnRHR activation.

Peptide	Sequence
GnRH1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH2	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
GnRH3	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2

Abbreviations: GnRH1= mammalian GnRH; GnRH2= chicken GnRH-II; GnRH3= salmon GnRH.

2.2.8 cAMP accumulation assay

COS7 cells were grown, seeded and transfected in 24-well plates as described above. The cells were allowed to grow in VP-SFM for 24 h post-transfection after which the media was switched to Medium 199 (Invitrogen) containing 0.3 % bovine albumin (Sigma-Aldrich). Following another 24 h, these cells were washed with Hank's Buffered Salt Solution (HBSS) (Invitrogen) supplemented with 20 mM HEPES and 300 μM IBMX (Sigma), pH 7.4 and pre-incubated for 15 min at 37 °C. These cells were stimulated with various concentrations of ligands for 1 h at 37 °C with gentle agitation. Intracellular cAMP concentrations were measured using a cAMP Direct Enzyme Immunoassay according to the manufacturer's protocol (Amersham).

2.2.9 Data analysis

All IP and cAMP samples were measured at least in duplicate within each assay. All assays were repeated on three independent occasions. Data analysis was performed using nonlinear regression; GnRH concentrations inducing half-maximal stimulation (EC_{50}) were calculated using PRISM5 software (GraphPAD Software, Inc., San Diego CA). The Log EC_{50} values were generated from the mean ± SEM of three independent experiments and differences between Log EC_{50} values were analyzed by one-way ANOVA followed by Tukey's multiple comparison post-test, where $P < 0.05$ was considered statistically significant.

2.2.10 Development of GnRHR-specific antisera

Polyclonal antisera were raised in rabbits against an extracellular portion of the zebrafish GnRHR predicted from each coding sequence. An amino acid peptide corresponding to a short sequence (19-25 residues) at the N-terminus of each putative zebrafish GnRHR was chosen. This region was unique to each receptor with little sequence similarity to other proteins identified in the zebrafish genome. The fragments were synthesized as described (Rivier *et al.*, 1984) and were then conjugated to either human α-globulin using bisdiazotized benzidine coupled through an added carboxy terminal tyrosine residue (GnRHR1, GnRHR2 and GnRHR4), or to *Megathura crenulate* keyhole limpet hemocyanin (GnRHR3) via a terminal cysteine residue. A separate rabbit was immunized for each of the following:

zfGnRHR1 against peptide MSGNVSLSLISLLENSSLASY-NH_2;

zfGnRHR2 against peptide MNTTQLIEDLLQNSSSKHEAY-NH_2;

zfGnRHR3 against peptide MSGNWSQYNASLLPVWTAC-NH_2;

and zfGnRHR4 against peptide MNDSSPTSENIMFHQLTADTLNGSY-NH_2. The peptide fragments coupled to their carrier proteins were diluted with phosphate buffered saline (PBS) to a final concentration of 1 mg of total protein per millilitre. The immunogens for the initial injection were prepared by mixing Freund's complete adjuvant (Sigma) with an equal volume of PBS containing 1 mg conjugate/ml. The boosters were generated by mixing equal volumes of Freund's incomplete adjuvant and conjugate (0.5 mg/ml). Emulsification of the adjuvant and conjugate was performed using two 3 ml syringes connected with a luer-lock to generate a homogenous mixture. For each immunization, a rabbit received a total of 1 ml of emulsion in 20-30 sites

(intradermal for the initial injection and subcutaneous for the boosters). Animals were injected every two weeks and bled through an ear vein seven days after each booster. Blood was allowed to clot and serum was separated from cells by centrifugation. Twenty-four hours before use, each antiserum was preadsorbed with its respective carrier proteins, which were diluted 1:100 into 5 mg/ml of either human globulins Cohn fraction IV-1 (predominantly α-globulins) (Sigma-Aldrich) or *Megathura crenulate* keyhole limpet hemocyanin (Sigma), and placed on a nutator overnight at 4 °C. The antiserum from each bleed was characterized with respect to titre and cross-reactivity using the indirect enzyme-linked immunosorbent assay (data not shown) and immunoblot spot assay. The uncoupled receptor peptides and lysates from 293T cells expressing whole receptor proteins were used as antigens (see below).

2.2.11 Immunoblots to determine polyclonal zfGnRHR antisera specificity

Zebrafish GnRH receptor proteins were prepared from cultured 293T cells. In summary, cells were seeded in 10 cm plates (VWR, Edmonton, AB) at a density of 2.5 million viable cells/plate and grown overnight in growth medium (Dulbecco's Modified Eagle Medium with 4 mM L-glutamine supplemented with 0.1 mM MEM non-essential amino acids and 10 % fetal bovine serum) in a 37 °C incubator containing a humidified atmosphere of 5 % CO_2 in air. At 85-95 % confluence, 24 h after seeding, the cells were washed and incubated with serum-free medium (VP-SFM, Invitrogen) and then transfected with 12 μg/plate of receptor-encoded plasmid DNA using Lipofectamine according to the manufacturer's protocol (Invitrogen). After 24 h, the transfection medium was replaced with regular growth medium. At forty-eight hours post-transfection

the cells were washed in cold PBS on ice and then lysed with 1 ml of lysis buffer (1 % Igepal CA-630, 50 mM Tris-Cl, 150 mM NaCl, 2 mM EDTA, 10 % glycerol, 1 Complete Mini protease inhibitor tablet [Roche, Mississauga, ON], 1 mM PMSF). Cell homogenates were poured into microfuge tubes and then placed on a nutator for 30 min at 4 ˚C to allow for membrane disruption. The homogenates were clarified by centrifugation at 16,000 x g (4 ˚C) and the total protein content of each supernatant was measured using the detergent compatible (Dc) Bio-Rad Protein Microplate Assay (Bio-Rad, Hercules, CA) following the manufacturer's directions.

Aliquots from 293T cell lysates were spotted onto Odyssey nitrocellulose membrane (LI-COR Biosciences, Licoln, NB) (5 μg/spot) and allowed to dry at room temperature (RT). The membrane was blocked in 5 % Blotto (5 % non-fat skim milk powder in TBST, 150 mM NaCl, 0.1 % Triton X-100, 0.1 M Tris-HCl, pH 7.4) for 1.5 h with gentle shaking. The blot was cut into strips and each strip was incubated with the corresponding polyclonal rabbit anti-zfGnRHR antibody (α-zfGnRHR1 1:900, α-zfGnRHR2 1:3,000, α-zfGnRHR3 1:600, and α-zfGnRHR4 1:3,000) in 5 % Blotto for 1.5 h at RT with shaking. The strips were then washed 3x (5 min/10 min/15 min) in TBST. All strips were then incubated with a 1:10,000 dilution of goat anti-rabbit HRPO secondary antibody (Cedarlane Laboratories Ltd., Burlington, ON) in TBST for 1h at RT. The strips were washed 3x (5 min/10 min/15 min) in TBST and finally rinsed in TBS for 5 min. Chemiluminescence detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and the blot was exposed to Clonex Bioflex ECL film (Cedarlane). The film was developed using an X-ray developer (Kodak, New Haven, CT).

2.2.12 Western blots of zebrafish GnRHR expressing HEK cells

Aliquots from human embryonic kidney (HEK, 293T) cell lysates (10 μg/well for GnRHR1-3; 20 μg/well for R4) were mixed with Red Loading buffer reagent pack (New England Biolabs, Ipswich, MA) according to the manufacturer's directions and electrophoresed on 10 % acrylamide SDS-PAGE gels by the method of Laemmli (Laemmli, 1970). After electrophoresis, proteins were transferred to an Odyssey nitrocelluse membrane (LI-COR Biosciences, Licoln, NB) at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3). Blotted membranes were rinsed in TBST and then blocked in blocking buffer (5 % Blotto and 2 % normal goat serum) for 1.5 h with gentle agitation. Strips were cut from membranes and then incubated with each corresponding rabbit zfGnRHR antibody (α-zfGnRHR1 1:1,000, α-zfGnRHR2 1:3,000, α-zfGnRHR3 1:500, and α-zfGnRHR4 1:1,000) in blocking buffer. Preabsorption controls were run to determine the specificity of each antibody, where an aliquot of each rabbit zfGnRHR antiserum dilution was mixed with the corresponding immunizing peptide (10 μM) and then incubated on a nutator at RT for 1 h before addition to membranes. Primary antibodies and preabsorption mixes were incubated at RT for 1.5 h with gentle agitation and membranes were washed 3x in TBST (5 min/10 min/15 min). All strips were then incubated with a 1:10,000 dilution of goat anti-rabbit HRPO secondary antibody (Cedarlane) in TBST at RT for 1 h. The strips were washed 3x (5 min/10 min/15 min) in TBST and finally rinsed in TBS for 5 min. Chemiluminescence detection was performed as described above.

2.2.13 Immunocytochemistry

Whole zebrafish heads were fixed overnight in 4 % paraformaldehyde at 4 °C and decalcified with 3 changes of 0.5 M EDTA (pH 7) over one week at 4 °C. The decalcified whole heads were paraffin-embedded and cut into sagittal sections (5 μm thick). The slides were deparaffinized by heating them for 30 min at 58 °C followed by two rinses in xylene for 2 min each, one rinse in 1:1 xylene and 100 % ethanol for 2 min, one rinse in 100 % ethanol for 2 min, one rinse in 90 % ethanol for 2 min, one rinse in 80 % ethanol for 2 min, and one rinse in 70 % ethanol for 2 min. Immunocytochemistry was performed using the automated Ventana Discovery XT platform with a DAB Map XT protocol and heat-induced epitope retrieval (HIER) (Ventana, Tucson, AZ). Cells were initially conditioned using the following HIER protocol, with all wash steps using Ventana reaction buffer: slides were incubated in Ventana citrate buffer for 2 x 4 min at 95 °C with a single wash in between cycles, incubated again for 4 min in citrate buffer at 100 °C, washed, then cooled by turning off the heat for 8 min, with a wash after 4 and 8 min. The slides were treated with a 4 min hydrogen peroxidase blocking step to quench endogenous tissue peroxidases. In addition to newly generated GnRH receptor antisera, we used polyclonal GnRH antisera previously raised to locate GnRH immunoreactive neurons throughout the CNS. GF-4 antisera were raised against sGnRH, but it also recognizes GnRH2 as shown previously in our laboratory (Yu *et al.*, 1988). Both GnRH and GnRHR primary antisera (100 μl) at 1:600 dilutions were added and incubated for 1 h at 37 °C. As a negative control, preimmune rabbit serum (collected from each of the four rabbits before immunization with GnRHR peptide immunogen) was used instead of the primary antiserum at the same dilution. Slides were washed with washing buffer and

incubated with 1:500 goat anti-rabbit biotinylated IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at 37 °C for 30 min. Slides were washed according to the Ventana protocol and antibody binding was detected with a peroxidase-labeled streptavidin-biotin technique with diaminobenzidine (DABMap kit, Ventana). All slides were counterstained with hematoxylin.

In addition, immunocytochemistry was performed on freshly cryosectioned decalcified zebrafish heads using a simplified immunofluorescence method. The sections (12-14 μM) were adhered to Super Frost Plus slides (Fisher Scientific, Ottawa, ON) and allowed to dry at 37 °C for 3 h. A HIER protocol was then performed by immersing the slides in 10 mM Sodium Citrate (pH 6) for 20 min at 90 °C. The slide vessel was then removed from the heat and the slides were allowed to cool to RT. The slides were rinsed in PBS and sections were then blocked with 2 % NGS/BSA in PBS for 1 h at 37 °C. Following this blocking step, primary antibodies were applied to each corresponding section (α-zfGnRHR1 1:200, α-zfGnRHR2 1:400, α-zfGnRHR3 1:150, and α-zfGnRHR4 1:300) in blocking buffer for 1 h at 37 °C. The slides were then washed 3x in PBST (PBS containing 0.05% Tween-20). Localization of primary antibody binding was detected using a goat anti-rabbit IgG secondary antibody (Alexa Fluor 488, Invitrogen) diluted to 1:300 in PBST and incubated for 1 h at 37 °C. The slides were washed 3x in PBST and then mounted in aqueous mounting media (Vector Labs, Burlingame, CA). Slides were visualized immediately using a fluorescein isothiocyanate (FITC) filter set on a Leica inverted microscope.

To ensure detection of specific signals, controls for antisera specificity to GnRHRs were carried out by: i) substitution of pre-immune serum in the same dilution as

the primary antiserum; ii) phosphate buffered saline alone; and iii) preabsorption with 10 μM of corresponding antigen incubated with the same dilution of the primary antiserum for 24 h or 1 h before use. Preabsorption of anti-zfGnRHR4 with corresponding immunizing peptide blocked all positive immunoreactivity in the cerebellum and in the medial longitudinal fasciculus. Preabsorption with their corresponding antigen blocked all positive immunoreactivity with the putative receptor protein in western blots.

2.2.14 Phylogenetic analysis

The deduced amino acid sequences for the four zebrafish GnRHRs and all full-length teleost GnRHRs that have been published or reported in GenBank were aligned using the ClustalW program (a component of the Mega software suite, Version 3.1). Other select GnRHRs were included in the alignment to help distinguish each GnRHR group: mammalian type I, non-mammalian type I, type II, and type III; the human oxytocin (OXTR, GenBank accession no. NP_000907) and arginine vasopressin type 1A (AVPR1A, NP_000697) receptors were added as outgroups. Receptor names are as reported: human (*Homo sapiens*) GnRHR1 (NP_000397); green monkey (*Cercopithecus aethiops*) GnRHR2 (AAK52746); rhesus monkey (*Macaca mulatta*) GnRHR2 (NP_001028014); house mouse (*Mus musculus*) GnRHR1 (AAA37716); cow (*Bos taurus*) GnRHR1 (NP_803480); two chicken (*Gallus gallus*) GnRHRs (GnRHR1: NP_989984, GnRHR2: NP_001012627); three bullfrog (*Rana catesbeiana*) GnRHRs (GnRHR1: AAG42575, GnRHR2: AAG42949, GnRHR3: AAG42574); two pipid frog (*Xenopus tropicalis*) GnRHRs (GnRHR1: compiled from the Ensembl *X. tropicalis* genome project (version JGI 4.1) using peptide ID ENSXETP00000038462 and scaffold

22, GnRHR2: compiled from Ensembl peptide ID ENSXETP00000036235 and scaffold 3972); silverfish (*Odontesthes bonariensis*) GnRHR2A; Japanese flounder (*Paralichthys olivaceus*) GnRHR (AAY28982), Japanese eel (*Anguilla japonica*) GnRHR (BAB11961); two Nile tilapia (*Oreochromis niloticus*) GnRHRs (type 1: BAC77240, type 2: BAC77241); three dwarf gourami (*Colisa lalia*) GnRHRs (GnRHR1-1: BAE87048, GnRHR1-2: BAE87049, GnRHR2-1: BAE87050), black sea bream (*Acanthopagrus schlegelii*) GnRHR1 (AAV71128); African catfish (*Clarias gariepinus*) GnRHRs (GnRH-II-R: O42329, GnRHR2: AAM95605); striped sea bass (*Morone saxatilis*) GnRHR (AAF28464); three Japanese medaka (*Oryzias latipes*) GnRHRs (GnRHR1: BAB70506, GnRHR2: BAB70505, GnRHR3: BAC97833); two tilapia hybrid (*Oreochromis aureus* x *Oreochromis niloticus*) GnRHRs (type 1: AAQ88391, type 3: AAQ88392); rainbow trout (*Oncorhynchus mykiss*) GnRHR (CAB93351); amberjack (*Seriola dumerili*) GnRHR (CAB65407); five European sea bass GnRHRs (*Dicentrarchus labrax*) GnRHRs (GnRHR1A: CAE54804, GnRHR1B: CAE54806, GnRHR2A: CAD11992, GnRHR2B:CAE54807, GnRHR2C from (Moncaut *et al.*, 2005); orange-spotted grouper (*Epinephelus coioides*) GnRHR1 (ABF93210); two goldfish (*Carassius auratus*) GnRHRs (type A: AAD20001, type B: AAD20002); Atlantic croaker (*Micropogonias undulatus*) GnRHR2A (ABB97085); two African cichlid (*Astatotilapia burtoni*) GnRHRs (GnRHR1: AAU89433, GnRHR2: AAK29745); five spotted green pufferfish (*Tetraodon nigroviridis*) (type 1/III-1: BAE45694, type 1/III-2: BAE45697, type 1/III-3: BAE45699, type 2/nmI-1: BAE45701, type 2/nmI-2: BAE45702); lamprey (*Petromyzon marinus*) GnRHR (AAQ04564), four sea squirt (*Ciona intestinalis*) GnRHRs (GnRHR1: NP_001028997, GnRHR2: NP_001028996,

GnRHR3: NP_001028995, GnRHR4: NP_001028994); and octopus (*Octopus vulgaris*) GnRHR (BAE66648). The phylogenetic tree was constructed using the maximum likelihood method with 500 bootstrap pseudoreplicates using default parameters (available online at http://atgc.lirmm.fr/phyml/).

2.2.15 Chromosomal synteny

The chromosomal locations and organization of neighbouring gene clusters surrounding each GnRHR locus in select model genomes were determined using the following genome assemblies available in ensembl at http://www.ensembl.org/index.html (zebrafish assembly Version 7, Zv7; *Xenopus tropicalis* assembly version 4.1; chicken genome assembly Version 2.1; cow preliminary genome assembly 3.1; and human genome assembly NCBI 36).

2.3 Results

2.3.1 Zebrafish encode four full-length GnRH receptors

I cloned each zebrafish GnRHR cDNA to functionally characterize the individual receptors. The four zebrafish GnRHRs were amplified by PCR from reverse-transcribed poly(A)$^+$ mRNA extracted from brain tissue. An *in silico* study performed previously by Lethimonier *et al.* (2004) identified the putative amino acid sequence of three full-length zebrafish GnRHRs and one partial receptor sequence from the Zebrafish Draft Genome assembly. To avoid unnecessary confusion, we have maintained the same naming convention. Our complete ORFs were deposited in GenBank under the following

accession numbers: zfgnrhr1, EF571592; zfgnrhr2, EF571593; zfgnrhr3, EF571594; zfgnrhr4, EF571595.

The four zebrafish GnRHR cDNAs encode proteins with the profile characteristics of family A GPCRs. The isolated *zfgnrhr1* cDNA clone encoded a putative GnRHR similar to the zfGnRHR1 previously predicted (Lethimonier *et al.*, 2004) with the exception of 8 amino acid differences. The 1,134 bp ORF (including the stop codon) spans 12.596 kb on chromosome 19 and encodes a putative protein of 377 amino acids. The ORF contains three exons of 519 bp, 208 bp and 407 bp, separated by two introns of 9,312 bp and 2,150 bp. The ORF encoding zfGnRHR2 is 1,239 bp, spans 4.096 kb on chromosome 7, and encodes a putative protein of 412 amino acids. The ORF has three exons of 531 bp, 205 bp and 503 bp interrupted by two introns of 2,451 bp and 406 bp. The translated protein matches the previously identified zfGnRHR2, except for 2 amino acid differences. After performing 5'-RACE, we isolated a complete 1,011 bp ORF for *zfgnrhr3* encoding a putative protein of 336 amino acids. The ORF spans approximately 6.2 kb when compared to isolated genomic DNA. The zfGnRHR3 ORF is comprised of three exons of 474 bp, 205 bp and 332 bp separated by a first intron of approximately 3,700 bp and a second intron of 1,518 bp. When the zebrafish genome was searched with this cDNA sequence, only a partial match resulted without significant hits for the first exon. Inspection of exon 2 and 3 revealed that this receptor maps to chromosome 16, which is embedded in ambiguous sequence, probably the result of genome assembly problems. Finally, the ORF encoding zfGnRHR4 was shown to be 1,221 bp, which spans 9.832 kb on chromosome 18 and encodes a putative protein of 406 amino acids. The ORF contains three exons of 543 bp, 205 bp and 473 bp with two large

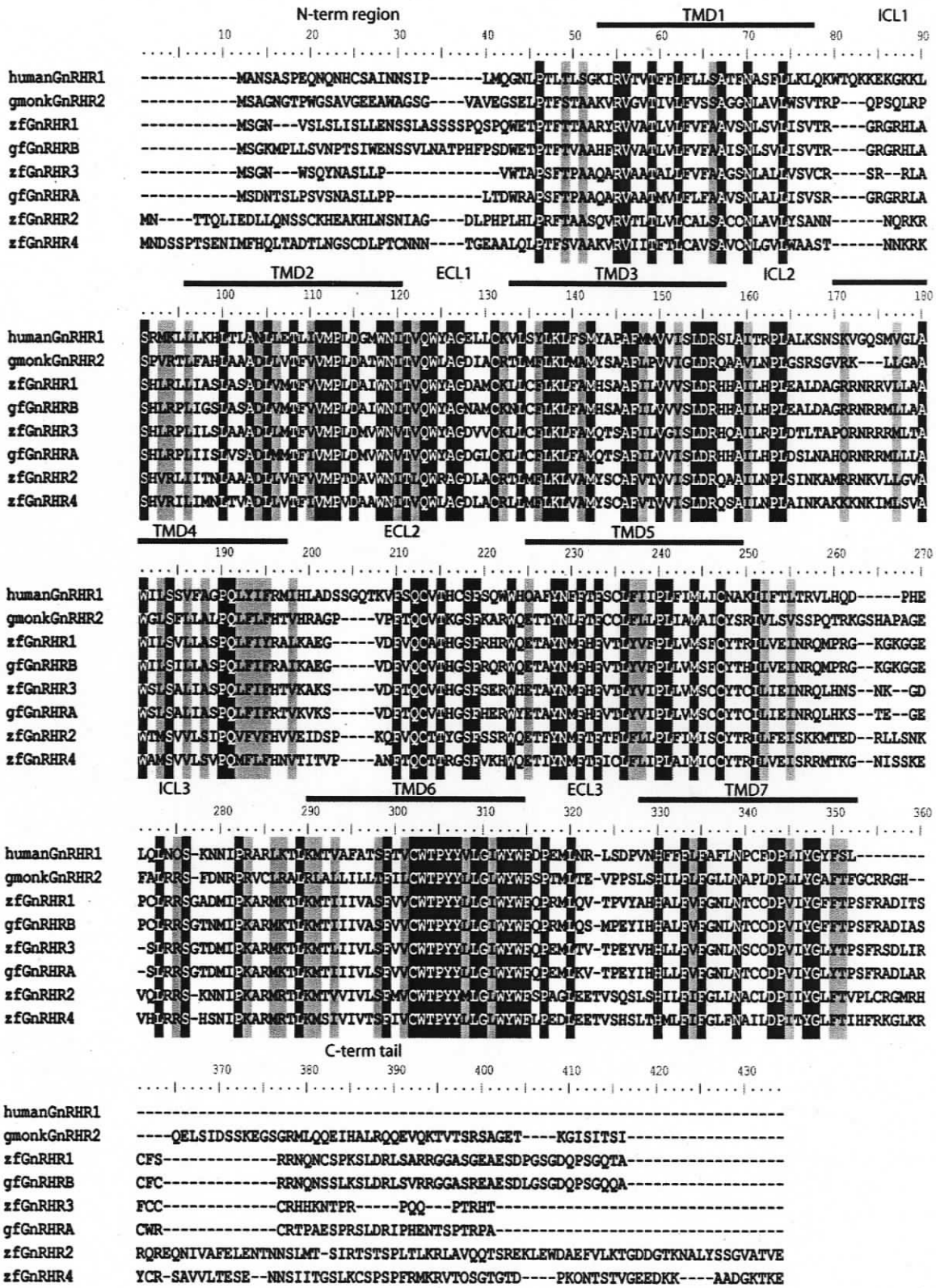
introns (4,559 bp and 4,052 bp). Interestingly, our translated zfGnRHR4 had one amino acid substitution and included an additional five amino acids encoded at the junction of exons 2 and 3 when compared to the previously annotated sequence.

After querying the National Center for Biotechnology Information's (NCBI) non-redundant protein database with the four translated zebrafish GnRHRs, two zebrafish receptors displayed the highest sequence similarity to the two goldfish GnRH receptors; zfGnRHR1 had 93 % similarity to the goldfish type B receptor and zfGnRHR3 had 89 % similarity to goldfish type A receptor. ZfGnRHR2 generated the closest match to the spotted green pufferfish GnRHR type1/III-3 (65 % identity and 76 % similarity), whereas zfGnRHR4's closest match was to the European sea bass (*Dicentrarchus labrax*) GnRHR-2B (76 % identity and 84 % similarity).

2.3.2 GnRHRs have conserved domains

In the seven TMDs, the four zebrafish receptor proteins show high conservation with other vertebrate GnRHRs, with some conservation in intracellular and extracellular loops. All zfGnRHRs possess potential N-glycosylation sites in their N-terminal domains (at positions 4 and 15 for zfGnRHR1; 2 and 13 for zfGnRHR2; 4 and 9 for zfGnRHR3; 2, 22 and 32 for zfGnRHR4). The N-terminal extracellular regions show little conservation with other vertebrate GnRHRs. The zfGnRHR1 and R4 have intracellular tails with high sequence similarity to those of other teleost GnRHRs, whereas the tails for zfGnRHR2 and R3 show no conservation. All zebrafish GnRHRs share many conserved residues (Figure 2.1) and motifs with other vertebrate GnRHRs, including human; the conserved residues and their putative functions for zfGnRHR3 are highlighted in Figure 2.2. Several

Figure 2.1. Sequence alignment of zebrafish GnRHRs with other vertebrate GnRHRs. Deduced amino acid sequences were aligned using ClustalW. Dashes indicate gaps introduced in the sequence to optimize the alignment. The putative TMDs are indicated by horizontal bars and ICLs and ECLs are noted above the alignment. Sequences are shaded according to the Blosum62 matrix.


N-term region
TMD1
ICL1
TMD2
ECL1
TMD3
ICL2
TMD4
ECL2
TMD5
ICL3
TMD6
ECL3
TMD7
C-term tail
humanGnRHR1
gmonkGnRHR2
zfGnRHR1
gfGnRHRB
zfGnRHR3
gfGnRHRA
zfGnRHR2
zfGnRHR4

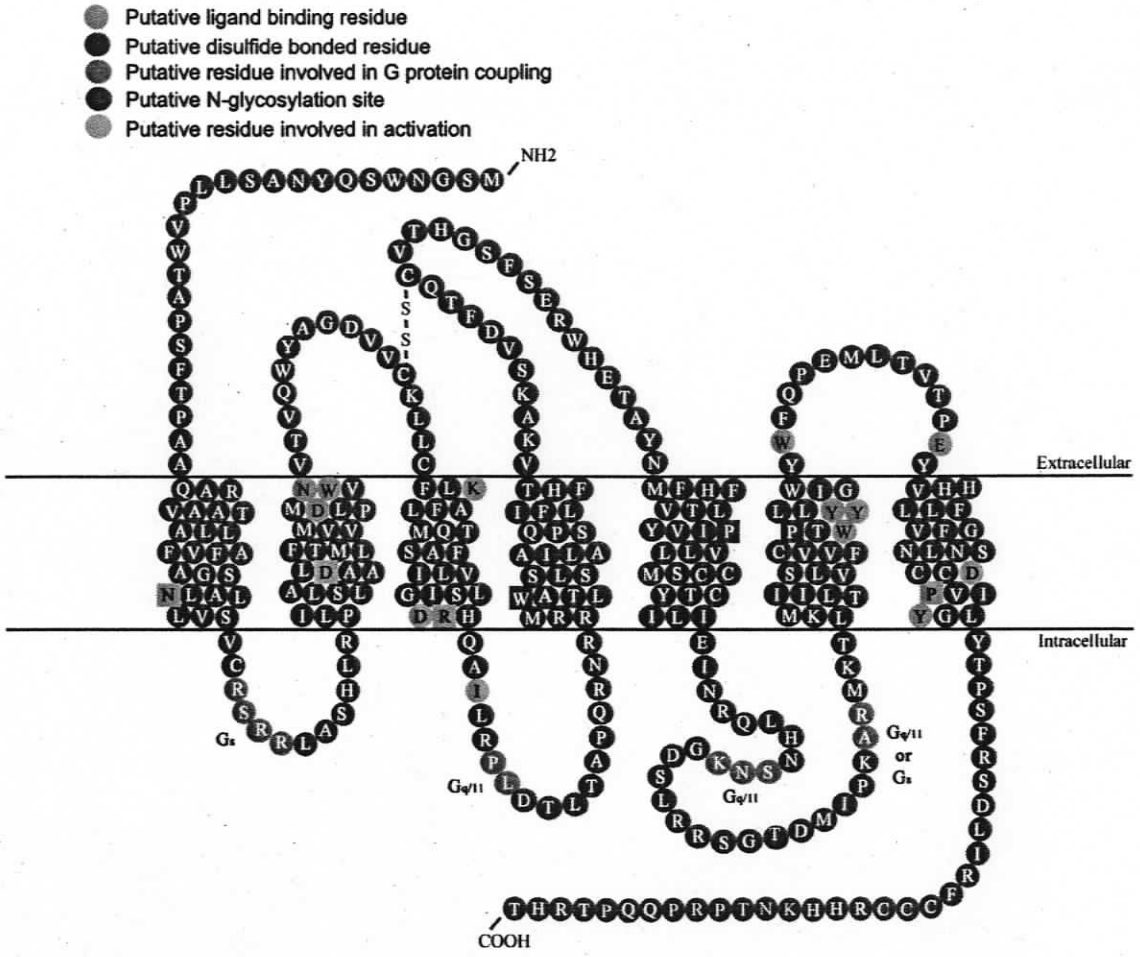


Figure 2.2. Helical net representation of the zebrafish GnRHR3. Key functional residues that are conserved with other GnRHRs are highlighted (see key). The reference residue in each helix is indicated by a square. For the GnRHR numbering convention, see Sealfon *et al.*, 1997.

residues previously characterized to be involved in ligand binding or formation of binding pockets are conserved in the four zebrafish GnRHRs: $Asp^{2.61(98)}$ (D), $Trp^{2.64(101)}$ (W), and $Asn^{2.65(102)}$ (N) in TMD2; $Lys^{3.32(121)}$ (K) in TMD3; $Trp^{6.48(280)}$ (W), $Tyr^{6.51(283)}$ (Y), and $Tyr^{6.52(284)}$ (Y) in TMD6; and $Trp^{6.59(291)}$ (W) in ECL3. Conserved microdomains that are involved in receptor activation are also present, with $Asn^{1.50}$ (N) in TMD1, $Asp^{2.50}$ (D) in TMD2, the DRxxxI/V motif within TMD3 and N/DPxxY in TMD7 (DPxxY in all zfGnRHRs) (Millar *et al.*, 2004).

2.3.3 Dot and western blots confirm specificity of rabbit polyclonal antibodies

Immunoblotting (Figure 2.3) confirmed that each polyclonal zebrafish GnRHR antiserum was specific for the targeted receptor protein heterologously expressed in 293T cells with no detectable cross-reactivity observed between the four antisera. Western blots (Figure 2.4) confirmed that each antiserum recognized the expected sized proteins, which could be abrogated with 10 μM of the corresponding peptide antigen. In addition, we used an indirect ELISA and were unable to detect any cross-reactivity between our four GnRHR antisera (data not shown).

2.3.4 Immunocytochemistry shows localization of four GnRH receptors in the pituitary

The expression of GnRH receptors in the adult zebrafish was detected with polyclonal antisera raised against an N-terminal portion unique to each receptor. Immunocytochemistry was performed on sagittal sections of decalcified whole heads.

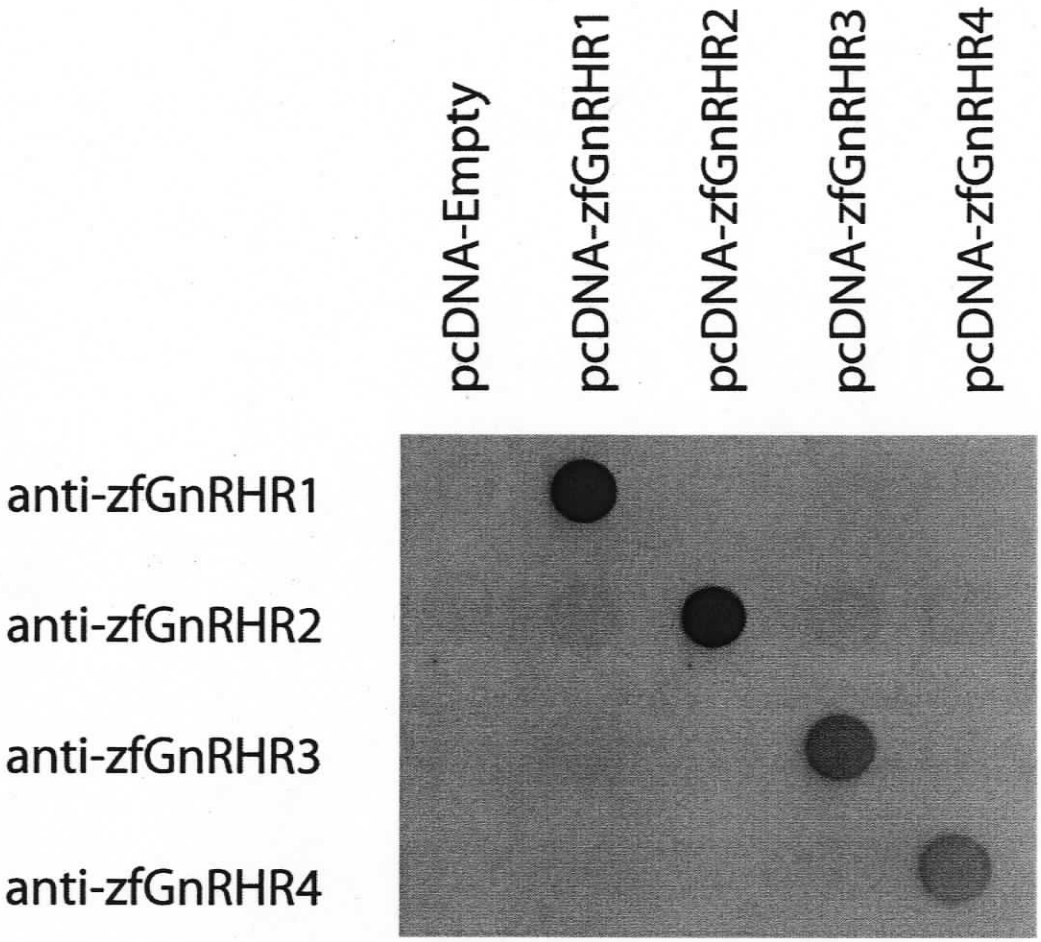


Figure 2.3. Dot blot assay using zebrafish GnRH receptor antisera. Heterologously expressed zebrafish GnRHR HEK cell lysates and the empty vector control were spotted in corresponding columns. Each row was incubated with the corresponding zebrafish GnRH receptor antisera.

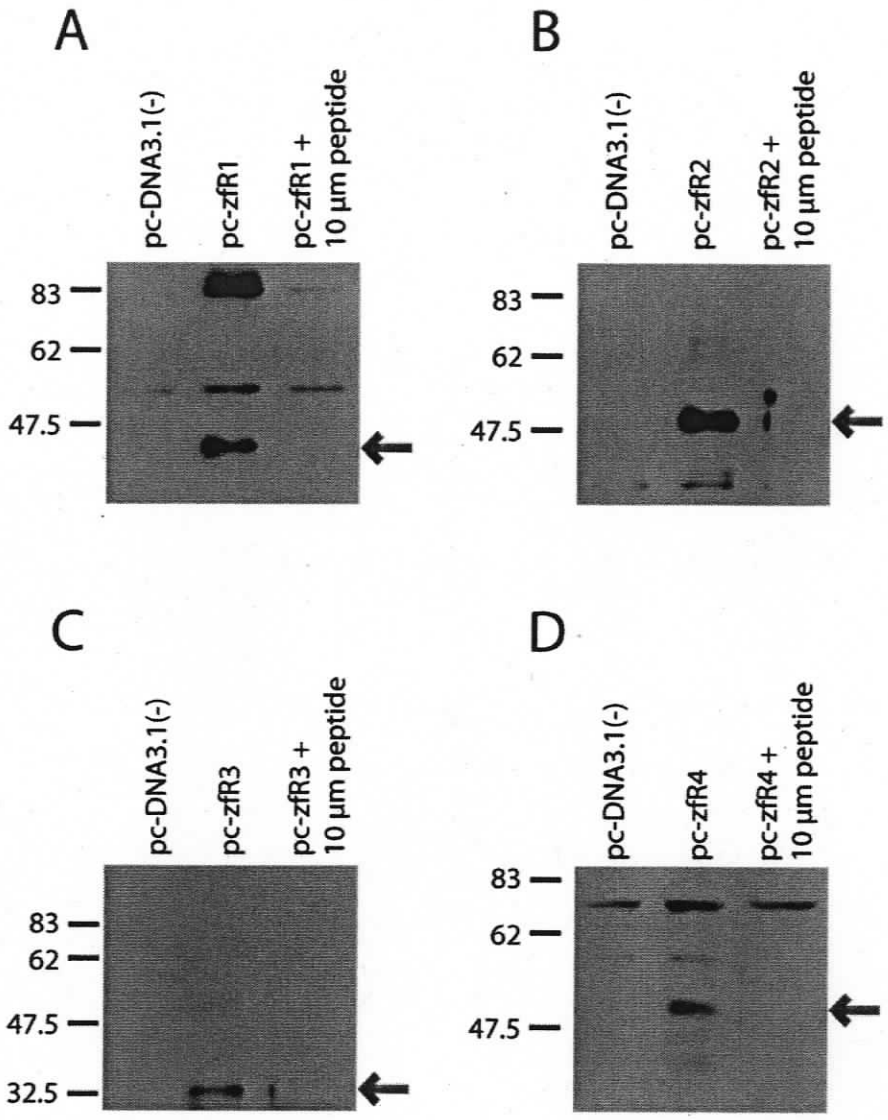


Figure 2.4. Immunoblots of zebrafish GnRH receptor proteins expressed in 293T cells. Empty vector transfected 293T cells were run to detect cross-reactive host cell proteins (left lanes). The middle lanes correspond to the heterologously expressed zebrafish GnRHR proteins as indicated by the red arrows. The preabsorption controls were run on the right lane of each blot. The two left lanes were incubated with corresponding antibody dilutions, whereas preabsorption controls were spiked with immunizing peptide. The corresponding positions of the respective molecular mass markers run simultaneously are indicated to the left of each blot (kDa).

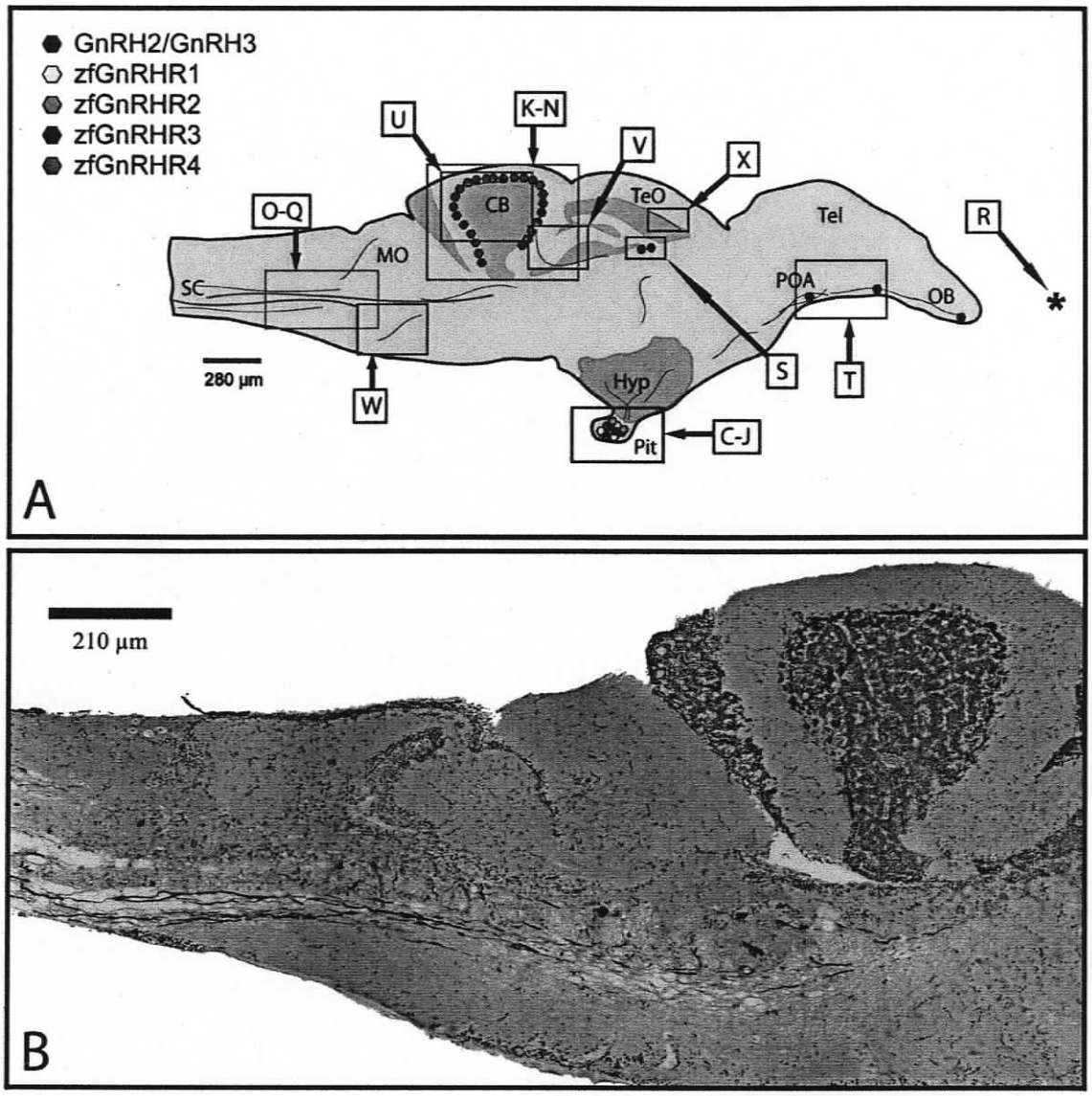
A diagram showing an overview of the immunocytochemistry results (Figure 2.5.A) is accompanied by the immunolabelling results for the hindbrain and spinal cord showing an overview of a motor control centre and associated fibres labelled for zfGnRHR4 (Figure 2.5.B). Each GnRHR is detected in the pituitary (Figures 2.5 C-J) with cell clusters visualized predominantly in the proximal pars distalis; the most intense staining was for zfGnRHR3 and R4.

2.3.5 Zebrafish GnRH receptors show novel localizations

ZfGnRHR4 was localized to perikarya along the periphery of the granular layer in the Purkinje layer with little staining in the molecular layer (Figures 2.5 K-M). Intense zfGnRHR4 immunoreactive fibre tracts were observed throughout the medial longitudinal fasciculus clearly detected just below the cerebellum and proceeding back into the spinal cord (Figures 2.5 B, O, Q). Negative controls for the cerebellum and medial longitudinal fasciculus (MLF) displayed no immunoreactivity in these regions (Figures 2.5 N, P). In addition, zfGnRHR2 was localized inside the nasal cavity within the olfactory epithelium (Figure 2.5 R).

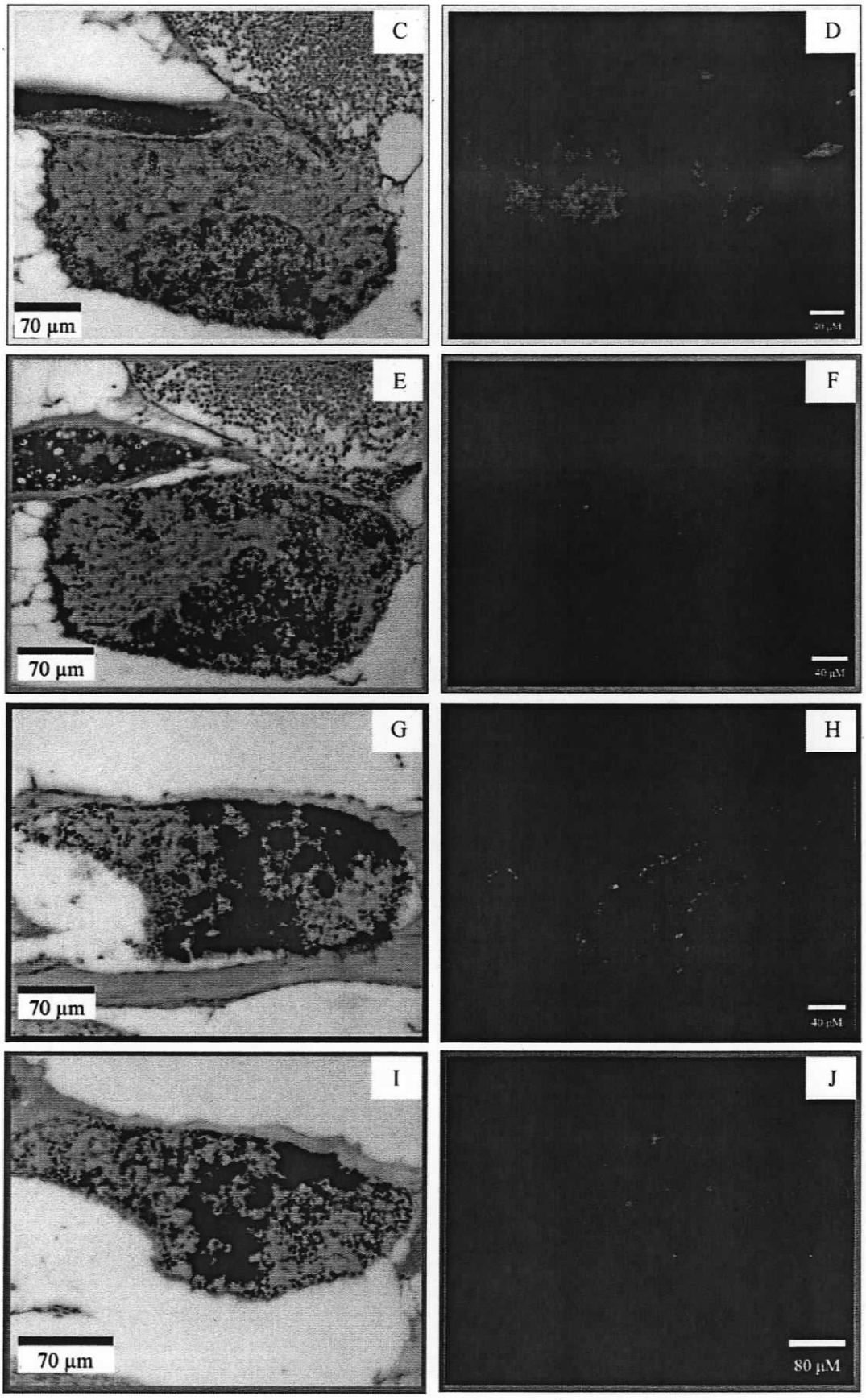
2.3.6 GnRH axons project to the cerebellum and the hindbrain

Previously generated GnRH antiserum (GF-4) raised against sGnRH, but which also cross-reacts with GnRH2, was used to localize novel GnRH projections. Cell bodies in conventional locations were visualized: in the area of the nucleus of the medial longitudinal fasciculus (nMLF) (Figure 2.5 S); in the olfactory bulb (not shown); in the

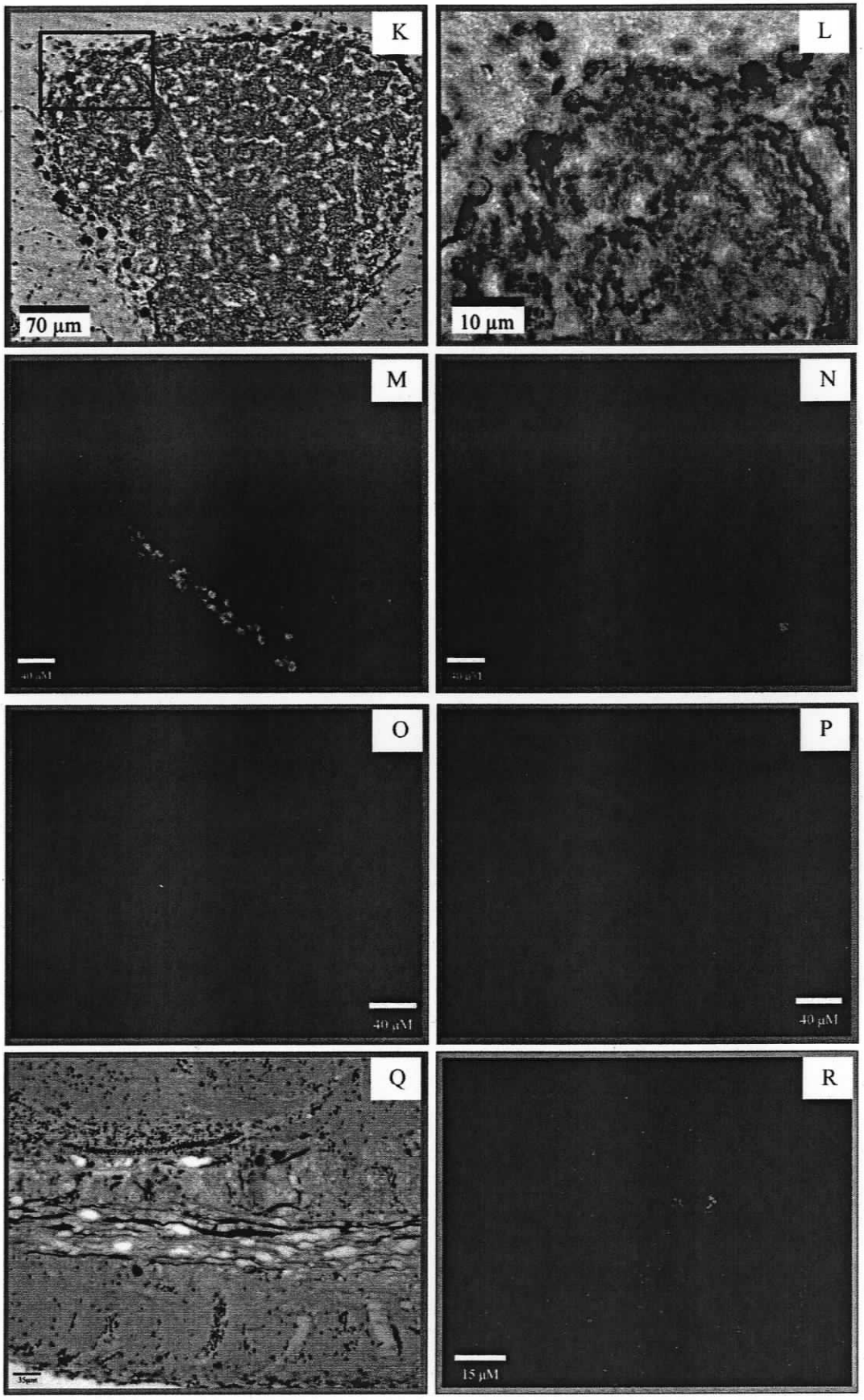


Figures 2.5 Schematic representation and micrograph displaying the immunocytochemical location of zebrafish GnRHRs in the CNS of the adult zebrafish. (A) Schematic of GnRH2/3 and the four zfGnRHRs in the central nervous system of the adult zebrafish. CB, cerebellum; Hyp, hypothalamus; MO, medulla oblongata; OB, olfactory bulb; Pit, pituitary; POA, preoptic area; SC, spinal cord; TeO, optic tectum; Tel, telencephalon, * olfactory epithelium (B) Light micrograph of 5 µm, sagittal section of an adult zebrafish hindbrain displaying the expression of zfGnRHR4 immunoreactive soma and fibre tracts (dark brown staining) with hematoxylin counterstain (dark blue).

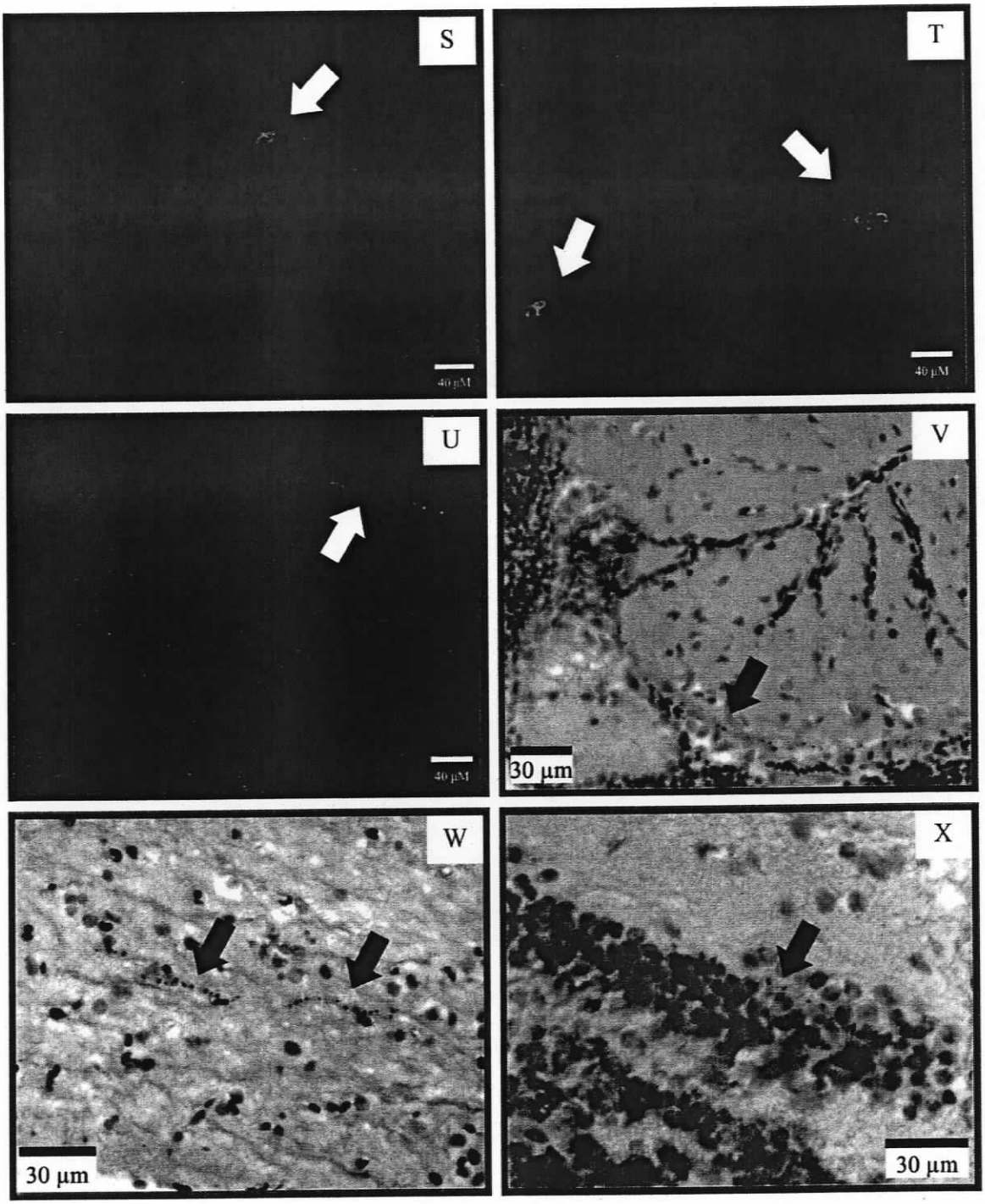
Figures 2.5. C-J. Immunoreactivity of zebrafish pituitary to anti-GnRHR antisera. Antisera were generated against: anti-zfGnRHR1 (C, D); anti-zfGnRHR2 (E, F); anti-zfGnRHR3 (G, H) and with anti-zfGnRHR4 (I, J).


C
70 µm
D
E
70 µm
F
G
70 µm
H
I
70 µm
J
80 µM

Figures 2.5 K-R. Immunoreactivity of the zebrafish brain to anti-GnRHR antisera. Immunoreactivity in the Purkinje cell layer of the zebrafish cerebellum localized with anti-zfGnRHR4 antiserum (K-M). (L) is an enlargement of the inset shown in (K). Lack of anti-zfGnRHR4 immunoreactivity in an adjacent section after preabsorption with 10 μM immunizing peptide (N). Immunoreactivity in the medial longitudinal fasciculus (O, Q) with anti-GnRHR4 antisera with no detectable immunoreactivity in the preabsorption control in an adjacent section (P). Immunofluorescence localized inside the nasal cavity within the olfactory epithelium shown using anti-zfGnRHR2 (R).


K
70 µm
L
10 µm
M
N
O
40 µM
P
40 µM
Q
R
15 µM

Figures 2.5. (S-X) Immunoreactivity of the zebrafish brain to anti-GnRH antisera. Immunoreactivity in the zebrafish brain to GF-4 antiserum, which detects both endogenous GnRH forms (GnRH2 and sGnRH). Immunoreactive cell bodies in the midbrain tegmentum (S) and in the preoptic area and in the region of the terminal nerve (T). Immunoreactive neurites were localized in the cerebellum (U); at the periphery of the cerebellum (V); adjacent to the medial longitudinal fasciculus (W) and in the optic tectum (X). Arrows identify GnRH varicosities or perikarya.


S
40 µM
T
40 µM
U
40 µM
V
30 µm
W
30 µm
X
30 µm

preoptic area and region of the terminal nerve (Figure 2.5 T). Novel immunopositive axons were visualized in the cerebellum (Figure 2.5 U) and entering the cerebellum (Figure 2.5 V) as well as GnRH axons located either crossing or adjacent to the medial longitudinal fasciculus (Figure 2.5 W). In addition, GnRH projections were visualized throughout the optic tectum (Figure 2.5 X).

2.3.7 GnRH receptors show distinct distribution in peripheral adult tissues

Expression of each GnRH and GnRHR gene was analyzed by RT-PCR using total RNA isolated from four male and four female adult zebrafish. Tissues examined included intestine, skeletal muscle, gill, eye, brain, ovary, testes, heart and dorsal skin (not shown). Amplicons were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized using a UV transilluminator. A control without template was included as a negative control for each primer pair and TATA-box binding protein (TBP) was used as a housekeeping gene for each tissue. Representative data regarding the GnRH2, sGnRH (GnRH3), zfGnRHR1, zfGnRHR2, zfGnRHR3, zfGnRHR4, and TBP mRNAs from each tissue are shown in Figure 2.6. As to the hormone, the GnRH2 primer pair generated PCR amplicons of the expected size (493 bp) predominantly in brain, eye, testis, ovary and skeletal muscle, and produced a faint band in the gill and skin (not shown). The sGnRH target sequence (226 bp) was prominent in brain, ovary and testis and showed a faint band in the rest of the tissues examined. In the receptor set, the zfGnRHR1 primer pair amplified the expected target sequence (582 bp) in the brain, eye, gill, ovary and testis, and a faint band was seen in both the heart and intestine. The zfGnRHR2 PCR product

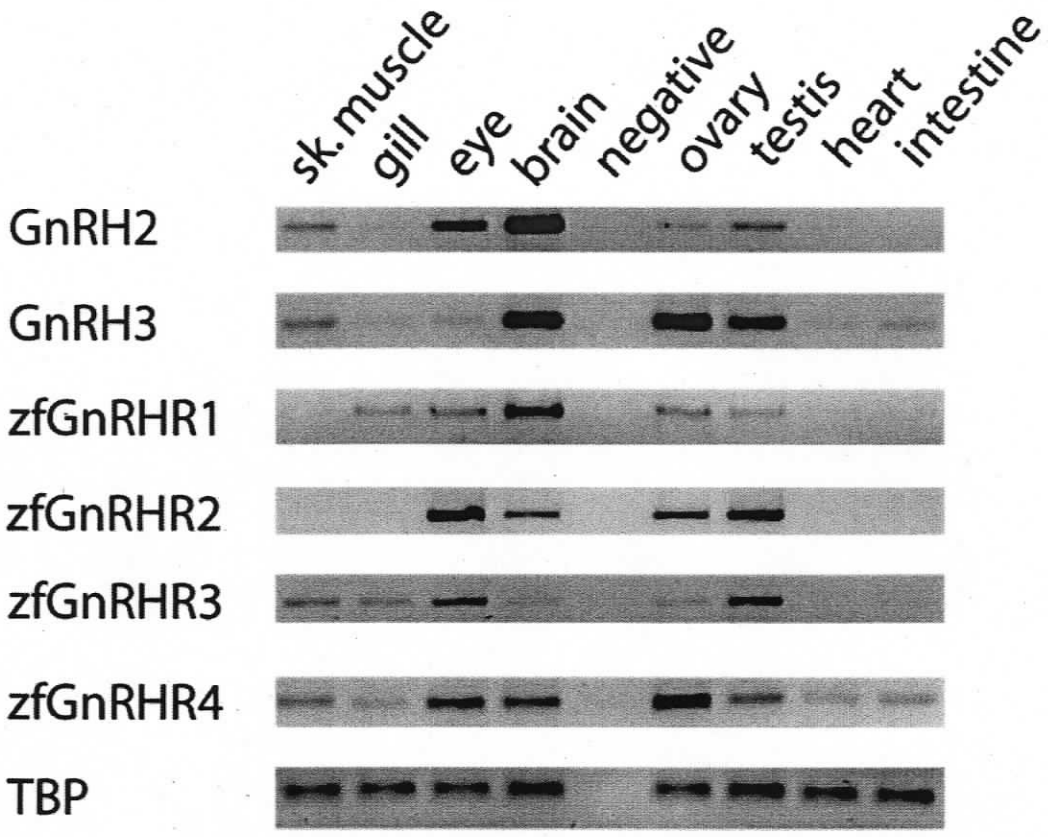


Figure 2.6. Tissure expression of the zebrafish GnRH and receptor cDNAs. Two control reactions were run; one negative (no template DNA) for each primer set and one positive control TATA-box binding protein (TBP) for each tissue examined. A representative amplicon from each primer pair was cloned to confirm the specificity of each primer pair.

(765 bp) was visualized in the eye, brain, ovary and testis. The zfGnRHR3 target sequence (421 bp) was observed in all tissues except for the heart, whereas the zfGnRHR4 target sequence (298 bp) was visualized in all tissues examined.

2.3.8 The four GnRH receptors signal through the IP pathway

To confirm the functional identity of the four zfGnRHR cDNAs, each receptor cDNA was expressed in COS7 cells and exposed to graded concentrations of mGnRH, GnRH2 and sGnRH. Each native ligand (GnRH2 and sGnRH) stimulated inositol phosphate accumulation in zfGnRHR-expressing cells (see Figure 2.7), indicating that all four receptors couple to $G_{q/11\alpha}$. Both zfGnRHR1 and R3 showed a greater preference for GnRH2 compared with sGnRH, whereas zfGnRHR2 and R4 showed relatively equal potency with both native ligands (Table 2.3). GnRH2 was the most potent with zfGnRHR3, inducing a dose-dependent stimulation with EC_{50} values of 0.10 nM. The least potent endogenous ligand-receptor pair was sGnRH with zfGnRHR1, stimulating intracellular IP accumulation dose dependently with EC_{50} values of 308 nM. Another distinct characteristic of the receptors is that zfGnRHR2 and R4 showed a distinct response to the non-native mGnRH (EC_{50} -7.75 and -7.97), whereas zfGnRHR1 and R3 did not respond to the peptide in a physiological range. As a control, inositol phosphate levels were measured in COS7 cells transfected with empty vector and incubated with either GnRH2 or sGnRH; IP did not rise above basal levels (data not shown).

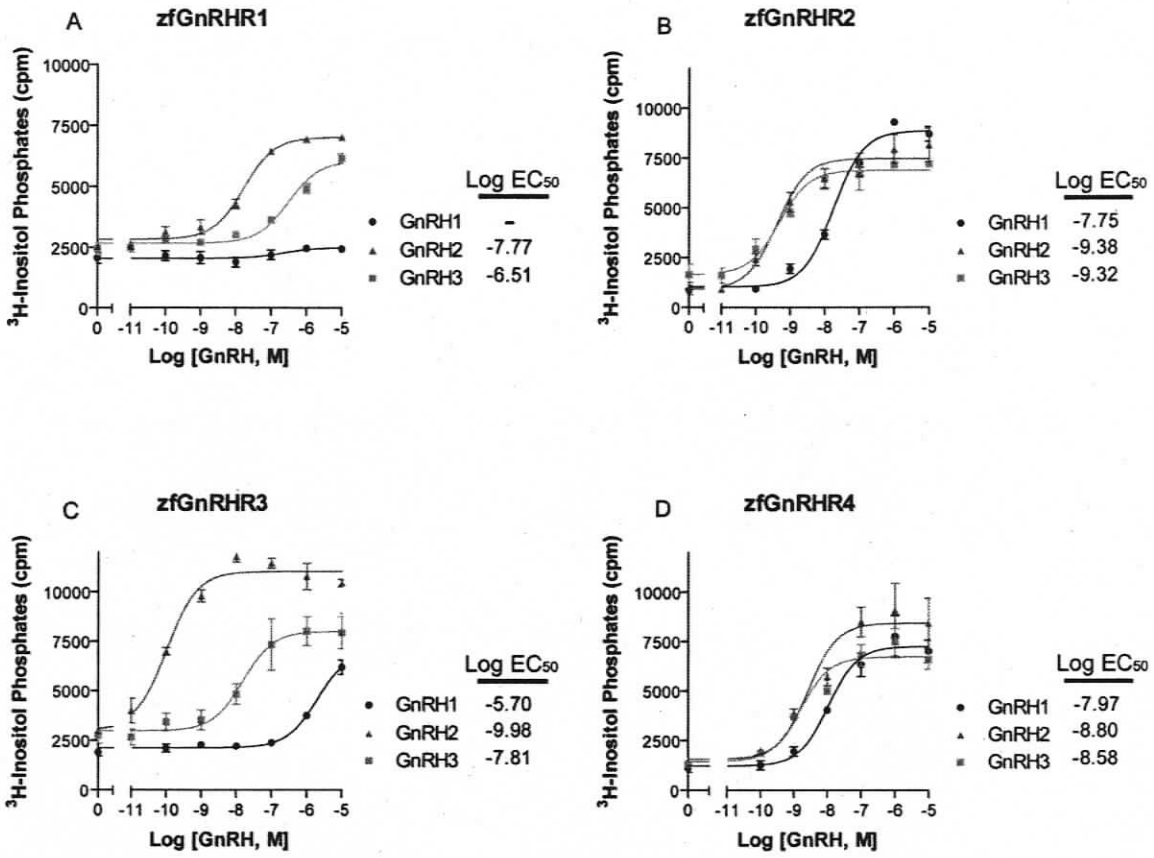


Figure 2.7. Inositol phosphate accumulaton in COS7 cells expressing zebrafish GnRH receptors. *myo*-[2-^{3}H]IP accumulation in cells expressing either zfGnRHR1 (A), zfGnRHR2 (B), zfGnRHR3 (C), or zfGnRHR4 (D) was induced with graded concentrations of indicated peptide for 1 h. Cells were transfected with 0.8 μg of vector containing GnRHR cDNA. At 24 h after transfection, labelled inositol was added and 48 h post-transfection the cells were washed and then stimulated. The intracellular ^{3}H-IP concentrations are shown as scintillation counts per minute (cpm) and *error bars* represent mean ± SEM of a minimum of three independent experiments performed in triplicate. GnRH1=mGnRH, GnRH3=sGnRH.

Table 2.3. Inositol phosphate accumulation after incubation of mGnRH, GnRH2 and sGnRH ligands with each zebrafish GnRHR (zfGnRHR).*

	Inositol Phosphate Accumulation **EC_{50} (log_{10} M)			
Ligand	**zfGnRHR-1**	**zfGnRHR-2**	**zfGnRHR-3**	**zfGnRHR-4**
GnRH1	-	-7.75±0.08^{d}	-5.70±0.12^{f}	-7.97±0.14^{d}
GnRH2	-7.77±0.11^{d}	-9.38±0.13^{b}	-9.98±0.10^{a}	-8.80±0.13^{c}
GnRH3	-6.51±0.14^{e}	-9.32±0.18^{b}	-7.81±0.23^{d}	-8.58±0.18^{c}

* IP measurements in COS7 cells, which were transfected with receptor cDNA 48 h earlier. Also added to the cells was 0.9 µCi/well *myo*-[2-^{3}H]-inositol at 24 h and GnRH peptides at 1 h before measurements.

** Dose of peptide stimulating half-maximal IP response (EC_{50}). Data were derived from the means of triplicate samples from three or more independent experiments.

$^{a\text{-}f}$ Means that are significantly different are indicated by different superscript letters. For each zfGnRHR, GnRH concentrations that induce dose-dependent responses and are not significantly different from one another share the *same superscript letter* ($P < 0.05$).

A dash (-) denotes no response detected.
GnRH1=mGnRH, GnRH3=sGnRH

2.3.9 One zebrafish GnRHR activates the cAMP signaling pathway

The ability of GnRH2 and sGnRH to induce cAMP accumulation in zfGnRHR transfected COS7 cells was analyzed. Only treatment with increasing concentrations of GnRH2 elicited a continuous rise in cAMP accumulation in zfGnRHR3-expressing cells. The maximal response was at least two-fold over basal levels with an estimated EC_{50} of 56 nM (Figure 2.8). We were unable to detect any significant responses with the other combinations of GnRHs and zfGnRHRs (Table 2.4).

2.3.10 Analysis of zebrafish GnRH receptors show conserved relationships

The chromosome locations and neighbouring gene clusters surrounding GnRHR loci are shown for human, cow, chicken, pipid frog and zebrafish (Figure 2.9). A molecular phylogenetic tree highlighting teleost GnRHRs was constructed for each putative amino acid sequence using the maximum likelihood algorithms (available at http://atgc.lirmm.fr/phyml/). Figure 2.10 shows a maximum likelihood tree based on the degapped regions encoding transmembranes 1-7. Human oxytocin and vasopressin receptors were included as outgroups. The robustness of the internal branches was estimated by 500 bootstrap resamplings. Vertebrate GnRHRs separated into four groups. GnRHR classifications were based upon a previous classification scheme (Millar *et al.*, 2004). The zebrafish GnRHRs cloned in the present study grouped into two types: non-mammalian type I and type III.

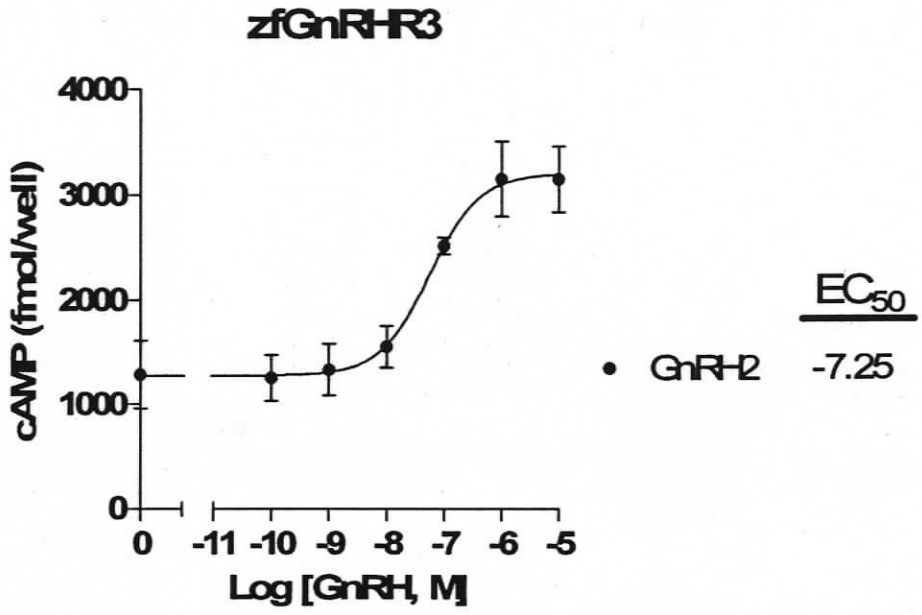


Figure 2.8. cAMP accumulation in COS7 cells expressing zfGnRHR3. Cells were transiently transfected with 0.8 μg of vector containing zfGnRHR3 cDNA. At 48 h post-transfection, cells were washed and stimulated with GnRH2 for 1 h. The intracellular concentrations are shown as femtomoles per well and error bars represent mean ± SEM of a minimum of three independent experiments performed in duplicate.

Table 2.4. cAMP accumulation after incubation of GnRH2 and GnRH3 ligands with each zebrafish GnRHR (zfGnRHR).*

	cAMP Accumulation **EC_{50} (log_{10} M)			
Ligand	**zfGnRHR-1**	**zfGnRHR-2**	**zfGnRHR-3**	**zfGnRHR-4**
GnRH2	-	-	-7.25±0.13	-
GnRH3	-	-	-	-

* cAMP measurements in COS7 cells, which had been transfected with receptor cDNA 48 h earlier.

** Dose of peptide stimulating half-maximal cAMP response (EC_{50}).

Data were derived from the means of triplicate samples from three or more independent experiments.

For each GnRH peptide, doses resulting in responses that are not different from one another are identified by the same subscript letter ($P < 0.05$).

A dash (-) denotes no response detected.

GnRH3=sGnRH

Figure 2.9. Zebrafish GnRH receptor gene synteny. Chromosomal arrangement of conserved neighboring genes surrounding each GnRHR gene locus in human, cow, chicken, pipid frog, and zebrafish (locations in megabases). Gene abbreviations are: ARNT2, aryl-hydrocarbon receptor nuclear translocator 2; CHD2, chromodomain helicase DNA binding protein 2; CHRNB2, neuronal acetylcholine receptor subunit beta-2 precursor; CREB3L4, cAMP responsive element binding protein 3-like 4; DCST1, DC-STAMP domain-containing protein 1; FLAD1, FAD1 flavin adenine dinucleotide synthetase homolog; GNRHR/GnRHR, gonadotropin-releasing hormone receptor; GNRHR pseudo, gonadotropin-releasing hormone receptor non-functional pseudogene; HISPPD2A, histidine acid phosphatase domain containing 2A; HFE2, hemochromatosis type 2; IDH2, isocitrate dehydrogenase 2; ITGA10, integrin, alpha 10; IQGAP1, Q motif containing GTPase activating protein 1; KIF23, kinesin family member 23; MAPIP, mitogen-activated protein-binding protein-interacting protein; MTFMT, mitochondrial methionyl-tRNA formyltransferase; NTRK1, neurotrophic tyrosine kinase, receptor, type 1; NUDT17, nudix (nucleoside diphosphate linked moiety X)-type motif 17; PEX11A, peroxisomal biogenesis factor 11A; PLIN, perilipin; RBM8A, RNA binding motif protein 8A; RGMA, RGM domain family, member A; SEMA4B, semaphorin 4B; SLCO3A1, solute carrier organic anion transporter family, member 3A1; STAP1, signal-transducing adaptor protein 1; ST8SIA2, ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2; TCF12, transcription factor 12 (HTF4, helix-loop-helix transcription factors 4); TMPRSS11A, transmembrane protease, serine 11A; TMPRSS11D, transmembrane protease, serine 11D; UBE1L2, ubiquitin-activating enzyme E1-like 2; VPS33B, vacuolar protein sorting 33 homolog B; ZBTB7B, zinc finger and BTB domain containing 7B.

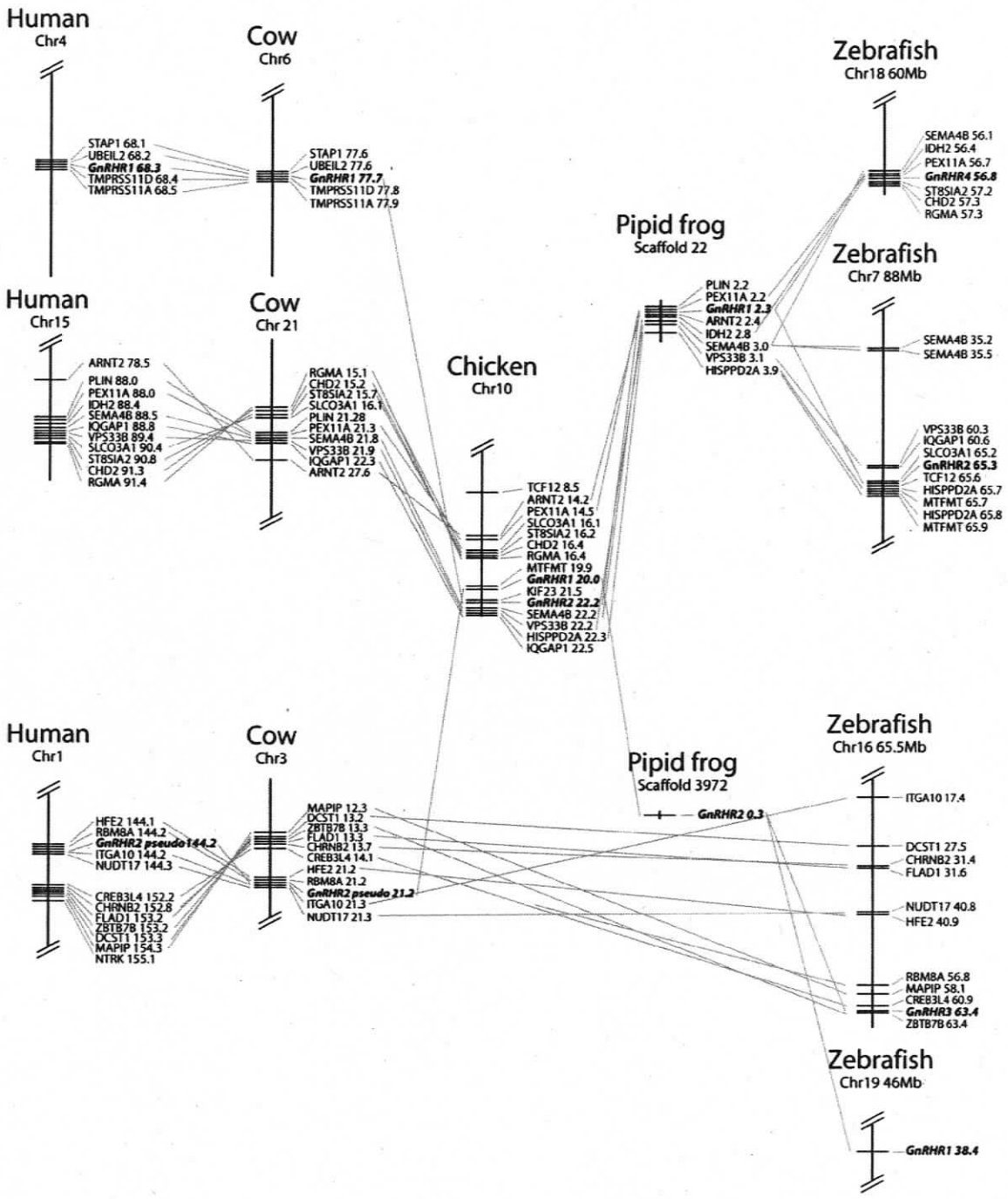
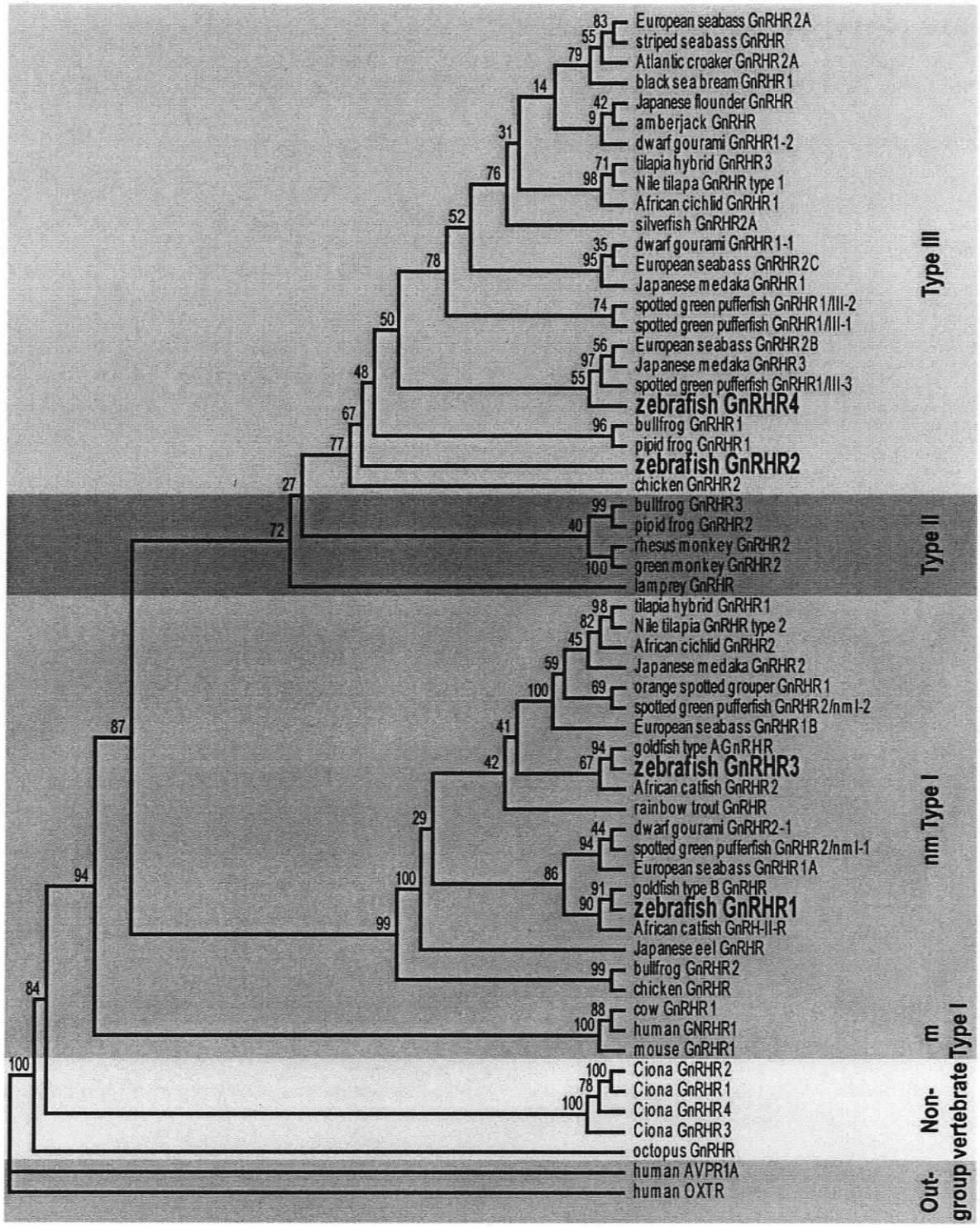

Human
Chr4
STAP1 68.1
UBEIL2 68.2
GnRHR1 68.3
TMPRSS11D 68.4
TMPRSS11A 68.5
Cow
Chr6
STAP1 77.6
UBEIL2 77.6
GnRHR1 77.7
TMPRSS11D 77.8
TMPRSS11A 77.9
Zebrafish
Chr18 60Mb
SEMA4B 56.1
IDH2 56.4
PEX11A 56.7
GnRHR4 56.8
ST8SIA2 57.2
CHD2 57.3
RGMA 57.3
Pipid frog
Scaffold 22
PLIN 2.2
PEX11A 2.2
GnRHR1 2.3
ARNT2 2.4
IDH2 2.8
SEMA4B 3.0
VPS33B 3.1
HISPPD2A 3.9
Zebrafish
Chr7 88Mb
SEMA4B 35.2
SEMA4B 35.5
VPS33B 60.3
IQGAP1 60.6
SLCO3A1 65.2
GnRHR2 65.3
TCF12 65.6
HISPPD2A 65.7
MTFMT 65.7
HISPPD2A 65.8
MTFMT 65.9
Human
Chr15
ARNT2 78.5
PLIN 88.0
PEX11A 88.0
IDH2 88.4
SEMA4B 88.5
IQGAP1 88.8
VPS33B 89.4
SLCO3A1 90.4
ST8SIA2 90.8
CHD2 91.3
RGMA 91.4
Cow
Chr 21
RGMA 15.1
CHD2 15.2
ST8SIA2 15.7
SLCO3A1 16.1
PLIN 21.28
PEX11A 21.3
SEMA4B 21.8
VPS33B 21.9
IQGAP1 22.3
ARNT2 27.6
Chicken
Chr10
TCF12 8.5
ARNT2 14.2
PEX11A 14.5
SLCO3A1 16.1
ST8SIA2 16.2
CHD2 16.4
RGMA 16.4
MTFMT 19.9
GnRHR1 20.0
KIF23 21.5
GnRHR2 22.2
SEMA4B 22.2
VPS33B 22.2
HISPPD2A 22.3
IQGAP1 22.5
Human
Chr1
HFE2 144.1
RBM8A 144.2
GnRHR2 pseudo144.2
ITGA10 144.2
NUDT17 144.3
CREB3L4 152.2
CHRNB2 152.8
FLAD1 153.2
ZBTB7B 153.2
DCST1 153.3
MAPIP 154.3
NTRK 155.1
Cow
Chr3
MAPIP 12.3
DCST1 13.2
ZBTB7B 13.3
FLAD1 13.3
CHRNB2 13.7
CREB3L4 14.1
HFE2 21.2
RBM8A 21.2
GnRHR2 pseudo 21.2
ITGA10 21.3
NUDT17 21.3
Pipid frog
Scaffold 3972
GnRHR2 0.3
Zebrafish
Chr16 65.5Mb
ITGA10 17.4
DCST1 27.5
CHRNB2 31.4
FLAD1 31.6
NUDT17 40.8
HFE2 40.9
RBM8A 56.8
MAPIP 58.1
CREB3L4 60.9
GnRHR3 63.4
ZBTB7B 63.4
Zebrafish
Chr19 46Mb
GnRHR1 38.4

Figure 2.10. Phylogenetic analysis of zebrafish GnRH receptors. All complete teleost GnRHRs available in GenBank are used with other select GnRHRs to resolve vertebrate groupings. Human vasopressin and oxytocin receptors are used as outgroups. Receptors characterized in this report are bolded. GnRHR classifications are according to Millar *et al*. (2004).


European seabass GnRHR2A
striped seabass GnRHR
Atlantic croaker GnRHR2A
black sea bream GnRHR1
Japanese flounder GnRHR
amberjack GnRHR
dwarf gourami GnRHR1-2
tilapia hybrid GnRHR3
Nile tilapa GnRHR type 1
African cichlid GnRHR1
silverfish GnRHR2A
dwarf gourami GnRHR1-1
European seabass GnRHR2C
Japanese medaka GnRHR1
spotted green pufferfish GnRHR1/III-2
spotted green pufferfish GnRHR1/III-1
European seabass GnRHR2B
Japanese medaka GnRHR3
spotted green pufferfish GnRHR1/III-3
zebrafish GnRHR4
bullfrog GnRHR1
pipid frog GnRHR1
zebrafish GnRHR2
chicken GnRHR2
bullfrog GnRHR3
pipid frog GnRHR2
rhesus monkey GnRHR2
green monkey GnRHR2
lamprey GnRHR
tilapia hybrid GnRHR1
Nile tilapia GnRHR type 2
African cichlid GnRHR2
Japanese medaka GnRHR2
orange spotted grouper GnRHR1
spotted green pufferfish GnRHR2/nmI-2
European seabass GnRHR1B
goldfish type AGnRHR
zebrafish GnRHR3
African catfish GnRHR2
rainbow trout GnRHR
dwarf gourami GnRHR2-1
spotted green pufferfish GnRHR2/nmI-1
European seabass GnRHR1A
goldfish type B GnRHR
zebrafish GnRHR1
African catfish GnRH-II-R
Japanese eel GnRHR
bullfrog GnRHR2
chicken GnRHR
cow GnRHR1
human GNRHR1
mouse GnRHR1
Ciona GnRHR2
Ciona GnRHR1
Ciona GnRHR4
Ciona GnRHR3
octopus GnRHR
human AVPR1A
human OXTR
Type III
Type II
nm Type I
m Type I
Non-vertebrate
Out-group

2.4 Discussion

Zebrafish offer an important model system in which to examine the physiological actions of GnRH in vertebrates. Our laboratory has shown previously that both GnRH2 and sGnRH contribute to early brain development in zebrafish in regard to the anterior and posterior midbrain boundaries as well as development of the eye cup and stalk (Wu *et al.*, 2006). Recent reports have elucidated the ontogeny of both GnRH2 and sGnRH in the developing zebrafish brain and placed salmon GnRH (GnRH3) in context with the well-accepted view of GnRH migration from the region of the nasal placode/anterior neural plate into the hypothalamus (Schwanzel-Fukuda, 1999; Wray, 2002; Whitlock, 2005; Whitlock *et al.*, 2006; Palevitch *et al.*, 2007).

In addition to the conventional mid-brain GnRH2 population, a new promoter-reporter study identified novel forebrain and hindbrain GnRH2 populations (Palevitch *et al.*, 2007). Despite these recent findings, there has been a paucity of investigation into the receptors that mediate GnRH signalling in the zebrafish. The complete characterization of the zebrafish GnRH receptors, especially their locations, is a key part of understanding the functional significance of two GnRH populations during development and in the adult.

This study reports the presence of four full-length zebrafish GnRHRs with conserved structural characteristics. Each receptor is physiologically active with two native GnRH forms in IP intracellular signalling assays. The spatial expression of each receptor analyzed by RT-PCR is widespread, with high expression in the brain, eye and gonads. The most striking and novel localization with antibodies is in the major motor control centre (cerebellum) and the motor tract of the MLF from the midbrain to the

spinal cord. Finally, the mapping of each zebrafish GnRHR gene to its respective chromosome and the identification of conserved gene neighbours helps to elucidate each receptor's gene history in context with those of other vertebrates, including humans.

2.4.1 Motifs for receptor activation, signalling and internalization are conserved

The cloning of four complete GnRH receptors from the zebrafish brain confirms that each transcript encodes a putative protein characteristic of non-mammalian GnRH receptors. Unlike the tail-less human type I GnRH receptor, the zebrafish receptors have tails ranging from 28-81 amino acids. The C-terminal tail has been implicated in controlling expression, receptor desensitization, membrane cycling and G protein activation (Sealfon *et al.*, 1997; Blomenrohr *et al.*, 2002; Millar *et al.*, 2004). On the N-terminal extracellular extension, zfGnRHR1, R2, and R3 have two consensus glycosylation sites (N-x-S/T), whereas zfGnRHR4 has three.

Zebrafish GnRHRs contain key residues and motifs for activation, G protein-coupling, and internalization. All zebrafish GnRHRs contain the arginine-cage motif (DRxxxI/V) at the intracellular junction of TMD3 and a DPxxY domain in TMD7. The Arg in the cage motif has been shown to be necessary for the transition of the receptor into an active confirmation by coordinating interactions with the conserved N/DPxxY micro-domain in TMD7 (Ballesteros *et al.*, 1998; Oliveira *et al.*, 1999). The N/DPxxY motif has been shown to be important for internalization, agonist-induced receptor activation and G protein signal transduction (Arora *et al.*, 1996). Like other GPCRs in family A, the zfGnRHRs possess conserved cysteine residues, two of which form a disulfide bond between the first two ECLs necessary for correct receptor folding (Karnik

et al., 1988; Gether, 2000). ZfGnRHR2 and R4 retain an additional cysteine residue in the N-terminus that may form a second disulfide bond, as in the human type I GnRH receptor (Davidson *et al.*, 1997). Each receptor has at least one cysteine residue in its C-terminal tail in context with neighbouring basic and/or hydrophobic residues that may facilitate tail palmitoylation. This may create an additional fourth ICL by anchoring the tail into the plasma membrane. Palmitoylation of other GPCRs in family A has been shown to contribute to the accessibility of regulatory phosphorylation sites located down-stream of the palmitolyated cysteine (Moffett *et al.*, 1996). Each zfGnRHR has one or more consensus phosphorylation motifs (S/T)x(R/K) thought to be targets of protein kinase C (Hanyaloglu *et al.*, 2001). Only zfGnRHR3 has a putative Src homology 3 (SH3) binding motif (PxxP) in its intracellular tail, which could facilitate the coupling to MAP kinases (Millar *et al.*, 2004).

2.4.2 The four GnRH receptors activate the inositol phosphate pathway

Both GnRH2 and sGnRH stimulated dose-dependent inositol phosphate accumulation after heterologous expression of the four zebrafish GnRH receptors in COS7 cells, indicating that each was functional and able to activate the IP_3 signalling pathway. The sensitivity of the receptors showed that GnRH2 was more potent than sGnRH with zfGnRHR3 and R1, but was equally potent with zfGnRHR2 and R4. Mammalian GnRH has been identified in bony fish that evolved before teleosts, but was lost early in the teleost lineage (Sherwood *et al.*, 1991). As shown in Figures. 2.7 (B and D), two zebrafish GnRHRs retained the ability to respond to mGnRH at physiological concentrations.

ZfGnRHR1 had ~18-fold higher response to GnRH2 than to sGnRH and zfGnRHR3 had ~149-fold higher potency to GnRH2 than to sGnRH. ZfGnRHR1 and R3 displayed a similar pattern of inositol phosphate accumulation in response to GnRH2 and sGnRH as the two goldfish GnRHRs, type B and A, respectively (Illing *et al.*, 1999). There was a difference in absolute EC_{50} values between the two zebrafish and goldfish GnRHRs, with the goldfish receptors displaying ~4-fold higher sensitivity to each of the endogenous ligands tested. To date, the goldfish lacks equivalents to zfGnRHR2 and zfGnRHR4.

In addition to activation of the intracellular inositol phosphate accumulation, zfGnRHR3 elicited the intracellular accumulation of cAMP in response to graded concentrations of GnRH2, presumably by coupling to an alternate G protein ($G_{s\alpha}$) pathway. Previous studies have shown that some mammal, bullfrog, teleost and protochordate GnRHRs show a ligand-induced cAMP turnover via activation of adenylyl cyclase (Levavi-Sivan and Yaron, 1992; Blomenrohr *et al.*, 2002; Oh *et al.*, 2003; Tello *et al.*, 2005). A more recent study demonstrated that only sustained stimulation of the GnRH receptor in LBetaT2 gonadotrope cells led to a concomitant increase in cAMP. These authors suggest that *in vivo* the cAMP signalling pathway may be selectively recruited under intense GnRH release, such as during the preovulatory surge (Lariviere *et al.*, 2007). The ability of zfGnRHR3 to activate this alternate pathway may indicate that this receptor has additional functions *in vivo* unique from the other receptors.

ZfGnRHR3 was the most sensitive of the four receptors (EC_{50} 0.10 nM) and the most selective; the response to GnRH2 was ~149-fold higher than to sGnRH. GnRHR1 was similar but less sensitive and showed higher selectivity for GnRH2 over sGnRH. In

contrast, both zfGnRHR2 and R4 were equally sensitive to GnRH2 and sGnRH, which was also demonstrated with the Japanese medaka GnRHR3 and the European sea bass GnRHR2A (Okubo *et al.*, 2003; Kah *et al.*, 2007). In addition, zfGnRHR1 and R3 segregate to the same group (non-mammalian type I) in the phylogram, whereas zfGnRHR2 and R4 are in type III, indicating that these receptors may also share a conserved function.

2.4.3 All four receptors are pituitary targets for GnRH

As in mammals, the population of endocrine GnRH cells responsible for pituitary functions resides in the hypothalamic/preoptic area of teleost fish. In zebrafish, sGnRH neurons arise from the region of the nasal placode and follow the olfactory/vomeronasal tracts migrating through the olfactory bulbs and along the ventral telencephalon to their final position in the hypothalamus (Steven *et al.*, 2003; Palevitch *et al.*, 2007). Fish do not have a median eminence and instead, the axons of GnRH perikarya located in the preoptic/hypothalamic regions directly innervate the pituitary. It has been proposed that sGnRH is the stimulating factor in the zebrafish pituitary due to its presence in hypothalamic neurons and its prominence in the adult pituitary (Steven *et al.*, 2003). We showed that sGnRH had the highest potency with zfGnRHR2 (EC_{50} of 0.40 nM). Our antibodies detected expression of zfGnRHR1 and R2 in the proximal pars distalis, with weak labelling throughout the rest of the pituitary. In contrast, both zfGnRHR3 and R4 showed prominent expression within the pituitary with sharp labelling of cells in the proximal pars distalis and some labelling throughout the rostral pars distalis and the pars intermedia. Conversely to mammals, FSHβ and LHβ reside in separate cell populations

within the zebrafish pituitary. The *fshβ*-positive cells are scattered as small clusters or single cells, whereas the *lhβ*-positive cells are arranged in the same area in large clusters (So *et al.*, 2005). Future studies are needed to discriminate whether different GnRH receptors are responsible for the release of the two gonadotropins under separate afferent stimulation. All four GnRHR antisera displayed some labelling in the cytoplasm and cell nuclei of pituitary cells, which has also been seen using another GnRHR antibody in the cichlid brain (Soga *et al.*, 2005). These non-conventional locations for traditionally membrane-bound receptors, although high affinity GnRH receptors have been localized to the nuclei of some tumor cells, suggest unknown functions (Szende *et al.*, 1991).

2.4.4 GnRH targets include a major motor control system

The immunocytochemical data indicate the presence of complete GnRH networks within the hindbrain, including the cerebellum. Our labelling of perikarya mainly within the Purkinje cell layer of the cerebellar corpus showed distinct cell-membrane expression of zfGnRHR4. The long fibre tracts of the MLF, which run just below the cerebellum, were also intensely immunopositive for zfGnRHR4. The nucleus of the medial longitudinal fasciculus is an efferent target of the cerebellum with neurons that project to the spinal cord in salmon (Oka *et al.*, 1986; Ikenaga *et al.*, 2002). Uematsu and Todo (1997) suggested that these neurons have a role in the spinal cord to initiate swimming and regulate activities of individual central pattern generators (Uematsu and Todo, 1997).

As to the peptides, labelling of neurons and fibres containing GnRH in the same mid- and hindbrain regions suggests that there are afferents for activation of GnRH receptors in these motor control areas. GF-4 antiserum localized GnRH varicosities

projecting from the midbrain tegmentum into the cerebellum and diffuse GnRH ramifications could be visualized within the cerebellar granular layer. Our labeling of GnRH neurons and fibres in the vicinity of these receptors in the mid- and hindbrain suggests involvement of GnRH in this motor control area. In the adult zebrafish, our two GnRH antisera confirmed the expression of GnRH perikarya (presumably GnRH2) in the midbrain tegmentum, specifically within the nucleus of the MLF. The target of this GnRH2 population may be the adjacent MLF fibre tracts. With the expression of GnRH2 in the midbrain tegmentum in teleosts and other developmental populations found in zebrafish, whitefish and gilthead sea bream, it has been suggested that GnRH2 may act both as a neurotransmitter and neuromodulator in some fish (Vickers *et al.*, 2004; Wong *et al.*, 2004; Palevitch *et al.*, 2007). These data indicate the presence of complete GnRH networks within the hindbrain, which may be necessary for modification of fine muscle control and to integrate complex swimming behaviours.

2.4.5 GnRH targets in the olfactory epithelium may relate to reproduction

The anti-zfGnRHR2 antiserum labeled cells in the olfactory epithelium (Figure 2.5 R). This result is supported by previous studies reporting GnRHR proteins in the olfactory epithelium using an antibody (ISPR3) raised against the extracellular loop of the medaka GnRHRs to study zebrafish (Whitlock *et al.*, 2006) and cichlid fish (Soga *et al.*, 2005). Studies in aquatic salamanders demonstrated that GnRH can increase the excitability of olfactory receptor neurons in mudpuppies and can modulate odorant responses from the main olfactory epithelium in another salamander (Eisthen *et al.*, 2000; Park and Eisthen, 2003). Studies in rodents suggest that GnRH is released from the

terminal nerve into the olfactory and vomeronasal epithelium where it can modulate odorant processing and chemoreception (Wirsig-Wiechmann, 2001). Wirsig-Wiechmann suggests that the intranasal GnRH system modifies olfactory information at reproductively auspicious times, leading to appropriate reproductive behaviours.

Recent reports have found that GnRHRs in the goldfish and cichlid are expressed throughout the forebrain and midbrain, suggesting multiple roles for GnRH in the brain of teleosts (Peter *et al.*, 2003; Chen and Fernald, 2006). Our immunocytochemistry studies focused on the midsagittal sections, enabling us to include the pituitary as a positive control within most sections.

2.4.6 Functional targets are widespread in peripheral organs

Past studies have focused on delineating possible autocrine/paracrine GnRH mechanisms within unconventional organs mainly in gonadal tissues due to the presence of both GnRH and receptors (Harrison *et al.*, 2004; Ramakrishnappa *et al.*, 2005). We amplified transcripts of both forms of GnRH and all four receptors from the testes and ovaries of zebrafish, indicating that local networks may be present. In zebrafish, sGnRH has been detected previously in the interstitial cells of the testes using immunoaffinity assays (Kuo *et al.*, 2005). Studies in rat and human testes showed that GnRH mRNA is expressed in Sertoli cells, whereas the receptor is expressed in Leydig cells (Bahk *et al.*, 1995; Botte *et al.*, 1998). In cultured rat testes, GnRH was shown to increase the expression of GnRH receptors and incubation with GnRH agonists blocked steroidogenesis (Dufau *et al.*, 1984; Botte *et al.*, 1999). GnRH-induced apoptosis occurs only during the late stage of spermatogenesis in the mature goldfish testes (Andreu-

Vieyra *et al.*, 2005). Detailed studies with zfGnRHR antibodies will be essential to establish specific testicular targets.

The presence of GnRH networks within the gonads of vertebrates may have been conserved during evolution. Our earlier studies using protochordate tunicates identified two genes expressing a total of six unique GnRH forms, and showed that injection of these peptides near the gonads *in vivo* resulted in spawning (Powell *et al.*, 1996; Adams *et al.*, 2003; Tello *et al.*, 2005). The absence of classical pituitary hormones in these animals suggests that pituitary involvement is an evolved characteristic.

Expression of all four GnRH receptors and both native GnRHs were found in the zebrafish eye. Studies in the goldfish found GnRH axons from the terminal nerve projecting to the eye and a more recent study in the cichlid, *Astatotilapia burtoni*, has localized GnRHR1 mRNA to the amacrine cell layer in the retina and GnRHR2 mRNA to the ganglion cells, which convey visual information to the brain (Demski and Northcutt, 1983; Grens *et al.*, 2005). Fernald's group suggests that GnRH from the terminal nerve could broadly influence sensory processing of retinal signals both in the lateral and vertical processing circuits via these two receptors, respectively. A more recent study found that application of both GnRH2 and sGnRH increased excitatory postsynaptic currents from retinal fibres to the periventricular neurons in rainbow trout. One speculation is that these neuromodulatory effects on the brain regulate both homing behaviour in salmonids, in addition to reproductive behaviours in other animals (Kinoshita *et al.*, 2007).

I found expression of receptors in various other tissues, notably the skin, muscle and gill, and I confirmed that zfGnRHR3 was expressed as protein in the skin and gill

(data not shown). A recent study found GnRH receptors in canine skin using both real-time PCR and immunocytochemical labelling with a human GnRHR antibody (Welle *et al.*, 2006).

2.4.7 Molecular evolution of GnRH receptor loci in vertebrates

To delineate vertebrate GnRHR gene history we analyzed the neighbouring chromosomal regions surrounding each receptor locus from model genomes at important evolutionary positions. A combined synteny mapping and phylogenetic approach was used to analyze the GnRH receptor gene history in this study.

Our phylogram shows that the four zebrafish GnRHRs segregate into two distinct GnRHR types (non-mammalian type I and type III), with sister relationships shared between zfGnRHR1 and R3 and zfGnRHR2 and R4, respectively. The presence of four GnRH receptors in this teleost species relative to two in most tetrapods may be explained by genome duplication in the Actinopterygii lineage, in a common ancestor of teleost fish (Amores *et al.*, 1998). The doubling of the genome (and the resultant GnRHR repertoire) is thought to have occurred before the divergence of zebrafish, medaka and pufferfish. In support of this theory, two homologous genes are often found in zebrafish for each single copy found in the chicken, mouse and human genomes. Many zebrafish paralogs are unlinked and are estimated to have been formed around the same time between 300 and 450 mya (Taylor *et al.*, 2001). We found that neighbouring genes clustered around zfGnRHR2 and R4 are present on a single locus in the pipid frog and chicken. Of interest in our two mammalian models is that many of the conserved GnRHR neighbours are seen on chromosomes separate from the GnRHR1 gene locus. It appears that early in the

mammalian lineage, the GnRHR type I gene was mobilized (to chr 4 in human) without its neighbouring genes. The selective loss of the C-terminal tail in all type I mammalian GnRHRs supports the possibility of an interchromosomal rearrangement.

Our synteny analysis is less revealing regarding the history of the GnRHR2 locus. ZfGnRHR3 appears to be the ortholog of the type II GnRHR gene, sharing many of the neighbouring genes present in the GnRHR2 locus from the mammalian lineage. However, we did not find many of these shared genes surrounding the zfGnRHR1 locus. Phylogenetic comparisons as well as high amino acid similarity with zfGnRHR3 show that zfGnRHR1 is the paralog of zfGnRHR3. The incomplete genome sequencing project of the pipid frog (*Xenopus tropicalis*) is unable to clarify GnRHR2 relationships, as the GnRHR2 gene is located on a small scaffold without available neighbouring gene data. Adding to the complexity, the two chicken GnRHR gene loci are present on the same chromosome (chr 11) within three megabases of each other. Our phylogenetic analysis indicates that the receptors segregate into two distinct groups, implying that these genes were not the result of recent tandem duplication. This organization in the chicken may have been the result of an interchromosomal rearrangement, possibly by a chromosomal fusion event present in the avian lineage. Surprisingly, the GnRHR2 gene locus in select mammals has been the target of mutational events acting to silence this gene (Morgan *et al.*, 2006). Our gene synteny and phylogeny indicate that mammalian type I and vertebrate type III GnRHRs evolved from the same gene locus, whereas the non-mammalian type I and vertebrate type II GnRHRs evolved from a separate GnRHR gene. Taken together, these analyses highlight vertebrate GnRHR evolution suggesting that two GnRHR loci were present in a vertebrate ancestor over 420 mya.

The maintenance of four functional receptors in the zebrafish after large-scale duplication events indicate that each of the two duplicates may have partitioned functions that were previously covered by an ancestral ortholog (subfunctionalization). Alternatively, one of two gene paralogs may have undergone critical structural changes that impart a novel function (neofunctionalization), which allows them to be maintained within the genome by a method first proposed by Ohno (Ohno, 1970). Finally, the distribution of two distinct types of GnRH ligands, each segregated to specific areas in the brain with distinct potencies at each receptor, suggests that different physiological pathways may be served by each receptor in this model.

2.5 References

Adams, B. A., J. A. Tello, J. Erchegyi, C. Warby, D. J. Hong, K. O. Akinsanya, G. O. Mackie, W. Vale, J. E. Rivier and N. M. Sherwood (2003). "Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, Ciona intestinalis." Endocrinology **144**(5): 1907-19.

Amores, A., A. Force, Y. L. Yan, L. Joly, C. Amemiya, A. Fritz, R. K. Ho, J. Langeland, V. Prince, Y. L. Wang, M. Westerfield, M. Ekker and J. H. Postlethwait (1998). "Zebrafish hox clusters and vertebrate genome evolution." Science **282**(5394): 1711-4.

Andreu-Vieyra, C. V., A. G. Buret and H. R. Habibi (2005). "Gonadotropin-releasing hormone induction of apoptosis in the testes of goldfish (Carassius auratus)." Endocrinology **146**(3): 1588-96.

Arora, K. K., Z. Cheng and K. J. Catt (1996). "Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor." Mol Endocrinol **10**(8): 979-86.

Bahk, J. Y., J. S. Hyun, S. H. Chung, H. Lee, M. O. Kim, B. H. Lee and W. S. Choi (1995). "Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis." J Urol **154**(5): 1958-61.

Ballesteros, J., S. Kitanovic, F. Guarnieri, P. Davies, B. J. Fromme, K. Konvicka, L. Chi, R. P. Millar, J. S. Davidson, H. Weinstein and S. C. Sealfon (1998). "Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor." J Biol Chem **273**(17): 10445-53.

Blomenrohr, M., J. Bogerd, R. Leurs and H. Goos (2002). "Differences in structure-function relations between nonmammalian and mammalian GnRH receptors: what we have learnt from the African catfish GnRH receptor." Prog Brain Res **141**: 87-93.

Botte, M. C., A. M. Chamagne, M. C. Carre, R. Counis and M. L. Kottler (1998). "Fetal expression of GnRH and GnRH receptor genes in rat testis and ovary." J Endocrinol **159**(1): 179-89.

Botte, M. C., Y. Lerrant, A. Lozach, A. Berault, R. Counis and M. L. Kottler (1999). "LH down-regulates gonadotropin-releasing hormone (GnRH) receptor, but not GnRH, mRNA levels in the rat testis." J Endocrinol **162**(3): 409-15.

Chen, C. C. and R. D. Fernald (2006). "Distributions of two gonadotropin-releasing hormone receptor types in a cichlid fish suggest functional specialization." J Comp Neurol **495**(3): 314-23.

Chengalvala, M., B. Kostek and D. E. Frail (1999). "A multi-well filtration assay for quantitation of inositol phosphates in biological samples." J Biochem Biophys Methods **38**(3): 163-70.

Davidson, J. S., D. Assefa, A. Pawson, P. Davies, J. Hapgood, I. Becker, C. Flanagan, R. Roeske and R. Millar (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**(42): 12881-9.

Demski, L. S. and R. G. Northcutt (1983). "The terminal nerve: a new chemosensory system in vertebrates?" Science **220**(4595): 435-7.

Dufau, M. L., D. W. Warren, G. F. Knox, E. Loumaye, M. L. Castellon, S. Luna and K. J. Catt (1984). "Receptors and inhibitory actions of gonadotropin-releasing hormone in the fetal Leydig cell." J Biol Chem **259**(5): 2896-9.

Eisthen, H. L., R. J. Delay, C. R. Wirsig-Wiechmann and V. E. Dionne (2000). "Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons." J Neurosci **20**(11): 3947-55.

Gether, U. (2000). "Uncovering molecular mechanisms involved in activation of G protein-coupled receptors." Endocr Rev **21**(1): 90-113.

Grens, K. E., A. K. Greenwood and R. D. Fernald (2005). "Two visual processing pathways are targeted by gonadotropin-releasing hormone in the retina." Brain Behav Evol **66**(1): 1-9.

Hanyaloglu, A. C., M. Vrecl, K. M. Kroeger, L. E. Miles, H. Qian, W. G. Thomas and K. A. Eidne (2001). "Casein kinase II sites in the intracellular C-terminal domain of the thyrotropin-releasing hormone receptor and chimeric gonadotropin-releasing hormone receptors contribute to beta-arrestin-dependent internalization." J Biol Chem **276**(21): 18066-74.

Harrison, G. S., M. E. Wierman, T. M. Nett and L. M. Glode (2004). "Gonadotropin-releasing hormone and its receptor in normal and malignant cells." Endocr Relat Cancer **11**(4): 725-48.

Ikemoto, T. and M. K. Park (2005). "Identification and molecular characterization of three GnRH ligands and five GnRH receptors in the spotted green pufferfish." Mol Cell Endocrinol **242**(1-2): 67-79.

Ikenaga, T., M. Yoshida and K. Uematsu (2002). "Efferent connections of the cerebellum of the goldfish, Carassius auratus." Brain Behav Evol **60**(1): 36-51.

Illing, N., B. E. Troskie, C. S. Nahorniak, J. P. Hapgood, R. E. Peter and R. P. Millar (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 U S A **96**(5): 2526-31.

Jodo, A., H. Ando and A. Urano (2003). "Five different types of putative GnRH receptor gene are expressed in the brain of masu salmon (Oncorhynchus masou)." Zoolog Sci **20**(9): 1117-25.

Kah, O., C. Lethimonier, G. Somoza, L. G. Guilgur, C. Vaillant and J. J. Lareyre (2007). "GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective." Gen Comp Endocrinol **153**(1-3): 346-64.

Kanda, A., T. Takahashi, H. Satake and H. Minakata (2006). "Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor isolated from the common octopus (Octopus vulgaris)." Biochem J **395**(1): 125-35.

Karnik, S. S., T. P. Sakmar, H. B. Chen and H. G. Khorana (1988). "Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin." Proc Natl Acad Sci U S A **85**(22): 8459-63.

Kinoshita, M., S. Kobayashi, A. Urano and E. Ito (2007). "Neuromodulatory effects of gonadotropin-releasing hormone on retinotectal synaptic transmission in the optic tectum of rainbow trout." Eur J Neurosci **25**(2): 480-4.

Kozak, M. (1996). "Interpreting cDNA sequences: some insights from studies on translation." Mamm Genome **7**(8): 563-74.

Kuo, M. W., S. W. Lou, J. Postlethwait and B. C. Chung (2005). "Chromosomal organization, evolutionary relationship, and expression of zebrafish GnRH family members." J Biomed Sci **12**(4): 629-39.

Kusakabe, T., S. Mishima, I. Shimada, Y. Kitajima and M. Tsuda (2003). "Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian Ciona intestinalis." Gene **322**: 77-84.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-5.

Lariviere, S., G. Garrel, V. Simon, J. W. Soh, J. N. Laverriere, R. Counis and J. Cohen-Tannoudji (2007). "Gonadotropin-releasing hormone couples to 3',5'-cyclic adenosine-5'-monophosphate pathway through novel protein kinase Cdelta and -epsilon in LbetaT2 gonadotrope cells." Endocrinology **148**(3): 1099-107.

Lethimonier, C., T. Madigou, J. A. Munoz-Cueto, J. J. Lareyre and O. Kah (2004). "Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish." Gen Comp Endocrinol **135**(1): 1-16.

Levavi-Sivan, B. and Z. Yaron (1992). "Involvement of cyclic adenosine monophosphate in the stimulation of gonadotropin secretion from the pituitary of the teleost fish, tilapia." Mol Cell Endocrinol **85**(3): 175-82.

Marchant, T. A., J. P. Chang, C. S. Nahorniak and R. E. Peter (1989). "Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish." Endocrinology **124**(5): 2509-18.

Melamed, P., N. Eliahu, B. Levavi-Sivan, M. Ofir, O. Farchi-Pisanty, F. Rentier-Delrue, J. Smal, Z. Yaron and Z. Naor (1995). "Hypothalamic and thyroidal regulation of growth hormone in tilapia." Gen Comp Endocrinol **97**(1): 13-30.

Millar, R. P., Z. L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan and S. R. Maudsley (2004). "Gonadotropin-releasing hormone receptors." Endocr Rev **25**(2): 235-75.

Moffett, S., L. Adam, H. Bonin, T. P. Loisel, M. Bouvier and B. Mouillac (1996). "Palmitoylated cysteine 341 modulates phosphorylation of the beta2-adrenergic receptor by the cAMP-dependent protein kinase." J Biol Chem **271**(35): 21490-7.

Moncaut, N., G. Somoza, D. M. Power and A. V. Canario (2005). "Five gonadotrophin-releasing hormone receptors in a teleost fish: isolation, tissue distribution and phylogenetic relationships." J Mol Endocrinol **34**(3): 767-79.

Morgan, K., R. Sellar, A. J. Pawson, Z. L. Lu and R. P. Millar (2006). "Bovine and ovine gonadotropin-releasing hormone (GnRH)-II ligand precursors and type II GnRH receptor genes are functionally inactivated." Endocrinology **147**(11): 5041-51.

Oh, D. Y., L. Wang, R. S. Ahn, J. Y. Park, J. Y. Seong and H. B. Kwon (2003). "Differential G protein coupling preference of mammalian and nonmammalian gonadotropin-releasing hormone receptors." Mol Cell Endocrinol **205**(1-2): 89-98.

Ohno, S. (1970). Evolution by gene duplication. Berlin, Springer-Verlag.

Oka, Y., M. Satou and K. Ueda (1986). "Descending pathways to the spinal cord in the hime salmon (landlocked red salmon, Oncorhynchus nerka)." J Comp Neurol **254**(1): 91-103.

Okubo, K., S. Ishii, J. Ishida, H. Mitani, K. Naruse, M. Kondo, A. Shima, M. Tanaka, S. Asakawa, N. Shimizu and K. Aida (2003). "A novel third gonadotropin-releasing hormone receptor in the medaka Oryzias latipes: evolutionary and functional implications." Gene **314**: 121-31.

Oliveira, L., A. C. Paiva and G. Vriend (1999). "A low resolution model for the interaction of G proteins with G protein-coupled receptors." Protein Eng **12**(12): 1087-95.

Palevitch, O., K. Kight, E. Abraham, S. Wray, Y. Zohar and Y. Gothilf (2007). "Ontogeny of the GnRH systems in zebrafish brain: in situ hybridization and promoter-reporter expression analyses in intact animals." Cell Tissue Res **327**(2): 313-22.

Park, D. and H. L. Eisthen (2003). "Gonadotropin releasing hormone (GnRH) modulates odorant responses in the peripheral olfactory system of axolotls." J Neurophysiol **90**(2): 731-8.

Peter, R. E., P. D. Prasada Rao, S. M. Baby, N. Illing and R. P. Millar (2003). "Differential brain distribution of gonadotropin-releasing hormone receptors in the goldfish." Gen Comp Endocrinol **132**(3): 399-408.

Powell, J. F., S. M. Reska-Skinner, M. O. Prakash, W. H. Fischer, M. Park, J. E. Rivier, A. G. Craig, G. O. Mackie and N. M. Sherwood (1996). "Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications." Proc Natl Acad Sci U S A **93**(19): 10461-4.

Ramakrishnappa, N., R. Rajamahendran, Y. M. Lin and P. C. Leung (2005). "GnRH in non-hypothalamic reproductive tissues." Anim Reprod Sci **88**(1-2): 95-113.

Reinhart, J., L. M. Mertz and K. J. Catt (1992). "Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor." J Biol Chem **267**(30): 21281-4.

Rivier, J., C. Rivier and W. Vale (1984). "Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat." Science **224**(4651): 889-91.

Schally, A. V., A. Arimura, A. J. Kastin, H. Matsuo, Y. Baba, T. W. Redding, R. M. Nair, L. Debeljuk and W. F. White (1971). "Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones." Science **173**(4001): 1036-8.

Schwanzel-Fukuda, M. (1999). "Origin and migration of luteinizing hormone-releasing hormone neurons in mammals." Microsc Res Tech **44**(1): 2-10.

Sealfon, S. C., H. Weinstein and R. P. Millar (1997). "Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor." Endocr Rev **18**(2): 180-205.

Sherwood, N. M., S. Doroshov and V. Lance (1991). "Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: reedfish (Calamoichthys calabaricus), sturgeon (Acipenser transmontanus), and alligator gar (Lepisosteus spatula)." Gen Comp Endocrinol **84**(1): 44-57.

So, W. K., H. F. Kwok and W. Ge (2005). "Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits--their spatial-temporal expression patterns and receptor specificity." Biol Reprod **72**(6): 1382-96.

Soga, T., S. Ogawa, R. P. Millar, Y. Sakuma and I. S. Parhar (2005). "Localization of the three GnRH types and GnRH receptors in the brain of a cichlid fish: Insights into their neuroendocrine and neuromodulator functions." J Comp Neurol **487**(1): 28-41.

Steven, C., N. Lehnen, K. Kight, S. Ijiri, U. Klenke, W. A. Harris and Y. Zohar (2003). "Molecular characterization of the GnRH system in zebrafish (Danio rerio): cloning of

chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II." Gen Comp Endocrinol **133**(1): 27-37.

Szende, B., G. Srkalovic, J. Timar, J. J. Mulchahey, J. D. Neill, K. Lapis, A. Csikos, K. Szepeshazi and A. V. Schally (1991). "Localization of receptors for luteinizing hormone-releasing hormone in pancreatic and mammary cancer cells." Proc Natl Acad Sci U S A **88**(10): 4153-6.

Taylor, J. S., Y. Van de Peer, I. Braasch and A. Meyer (2001). "Comparative genomics provides evidence for an ancient genome duplication event in fish." Philos Trans R Soc Lond B Biol Sci **356**(1414): 1661-79.

Tello, J. A., J. E. Rivier and N. M. Sherwood (2005). "Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate Ciona intestinalis GnRH receptors and the green monkey type II GnRH receptor." Endocrinology **146**(9): 4061-73.

Tensen, C., K. Okuzawa, M. Blomenrohr, F. Rebers, R. Leurs, J. Bogerd, R. Schulz and H. Goos (1997). "Distinct efficacies for two endogenous ligands on a single cognate gonadoliberin receptor." Eur J Biochem **243**(1-2): 134-40.

Torgersen, J., R. Nourizadeh-Lillabadi, H. Husebye and P. Alestrom (2002). "In silico and in situ characterization of the zebrafish (Danio rerio) gnrh3 (sGnRH) gene." BMC Genomics **3**(1): 25.

Tsutsumi, M., W. Zhou, R. P. Millar, P. L. Mellon, J. L. Roberts, C. A. Flanagan, K. Dong, B. Gillo and S. C. Sealfon (1992). "Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor." Mol Endocrinol **6**(7): 1163-9.

Uematsu, K. and T. Todo (1997). "Identification of the midbrain locomotor nuclei and their descending pathways in the teleost carp, Cyprinus carpio." Brain Res **773**(1-2): 1-7.

Vickers, E. D., F. Laberge, B. A. Adams, T. J. Hara and N. M. Sherwood (2004). "Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, Coregonus clupeaformis." Biol Reprod **70**(4): 1136-46.

Volkoff, H. and R. E. Peter (1999). "Actions of two forms of gonadotropin releasing hormone and a GnRH antagonist on spawning behavior of the goldfish Carassius auratus." Gen Comp Endocrinol **116**(3): 347-55.

Weber, G. M., J. F. Powell, M. Park, W. H. Fischer, A. G. Craig, J. E. Rivier, U. Nanakorn, I. S. Parhar, S. Ngamvongchon, E. G. Grau and N. M. Sherwood (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**(1): 121-32.

Welle, M. M., I. M. Reichler, A. Barth, U. Forster, U. Sattler and S. Arnold (2006). "Immunohistochemical localization and quantitative assessment of GnRH-, FSH-, and

LH-receptor mRNA Expression in canine skin: a powerful tool to study the pathogenesis of side effects after spaying." Histochem Cell Biol **126**(5): 527-35.

Whitlock, K. E. (2005). "Origin and development of GnRH neurons." Trends Endocrinol Metab **16**(4): 145-51.

Whitlock, K. E., N. Illing, N. J. Brideau, K. M. Smith and S. Twomey (2006). "Development of GnRH cells: Setting the stage for puberty." Mol Cell Endocrinol.

Wirsig-Wiechmann, C. R. (2001). "Function of gonadotropin-releasing hormone in olfaction." Keio J Med **50**(2): 81-5.

Wong, T. T., Y. Gothilf, N. Zmora, K. E. Kight, I. Meiri, A. Elizur and Y. Zohar (2004). "Developmental expression of three forms of gonadotropin-releasing hormone and ontogeny of the hypothalamic-pituitary-gonadal axis in gilthead seabream (Sparus aurata)." Biol Reprod **71**(3): 1026-35.

Wray, S. (2002). "Development of gonadotropin-releasing hormone-1 neurons." Front Neuroendocrinol **23**(3): 292-316.

Wu, S., L. Page and N. M. Sherwood (2006). "A role for GnRH in early brain regionalization and eye development in zebrafish." Mol Cell Endocrinol **257-258**: 47-64.

Yu, K. L., N. M. Sherwood and R. E. Peter (1988). "Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (Carassius auratus)." Peptides **9**(3): 625-30.

Chapter 3

Six novel GnRH hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*.

A modified version of this chapter has been published:
Adams BA, **Tello JA**, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE, Sherwood NM. 2003 Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. **Endocrinology 144:1907-1919.**

(Note: Data from DJH, KOA and WV are not included in the results; JE and JER prepared the synthetic peptides)

I assisted in all bioinformatic techniques used in this study, including identification of each GnRH gene, promoter analysis and identification of the nearest neighbouring genes. I was involved with the experimental design and assisted in cloning each GnRH gene and nearest neighbouring gene, culturing *Ciona* embryos, and the *in vivo* gamete release assay. I also was involved in writing portions of the manuscript and assisted in proofing and editing the final document.

3.1 Introduction

Duplications of the complete genome have been proposed to explain the presence of multigene families in vertebrates compared to a single copy of each gene found in invertebrates. One theory suggests that two complete genomic duplications have occurred between ancestral protochordates and jawed vertebrates, although the timing of the duplication varies between theories (Ohno, 1970; Ohno, 1996; Furlong and Holland, 2002; Dehal and Boore, 2005). Thus, the protochordates are a focal point in comparing genes with those in the vertebrate lineage (Dehal *et al.*, 2002). The genes that encode GnRH are of particular interest because GnRH controls the cascade of events that lead to reproduction throughout the vertebrates.

A GnRH structure of ten amino acids was first isolated in mammals (Matsuo *et al.*, 1971; Burgus *et al.*, 1972). In vertebrates, each form of GnRH is encoded on a separate gene. Mammals usually express two forms of GnRH, mGnRH (aka GnRH1) and GnRH2, although a few species express guinea pig (gp)GnRH (Jimenez-Linan *et al.*, 1997). In evolution, mGnRH is first detected in early-derived bony fish and GnRH2 is detected even earlier in cartilaginous fish. In the teleosts (later-evolving bony fish), most species studied to date express three different forms of GnRH: GnRH2, salmon (s)GnRH (aka GnRH3) and other forms known as GnRH1 that can vary in structure among species.

The multiple forms of GnRH are distinguished not only by their structure, but also by their anatomical location in the brain of mammals (Kasten *et al.*, 1996) and fish (Zandbergen *et al.*, 1995). Neuronal cell bodies containing GnRH2 are mainly in the midbrain region for all species, whereas the other GnRH forms are in neurons in the anterior brain.

Our current understanding of GnRH in invertebrates is derived primarily from three GnRH structures. Two of those GnRH peptides were identified in our laboratory by primary structure as tunicate GnRH-1 (tGnRH-1) and -2 (tGnRH-2) from the protochordate, *Chelyosoma productum* (Powell *et al.*, 1996). More recently, a GnRH of 12 amino acids has been identified by protein sequence and cDNA isolation in octopus (Iwakoshi *et al.*, 2002). These studies establish that GnRH is present in protochordates and in animals that evolved earlier, but all of the GnRH forms may not be identified due to lack of specific antisera.

Identification of an ancestral GnRH gene with subsequent duplications or mutations has been approached in several ways. First, the distribution of different GnRH forms with known peptide structures was mapped among animals with an established place in evolution based on fossil records and morphological comparisons. Second, phylogenetic analysis was used based on the cDNAs for the coding part of the precursors. Third, a linkage method was used to map each GnRH gene and the nearest upstream gene to determine GnRH orthologs in humans and medaka (Okubo *et al.*, 2002). GnRH-coding genes with the same upstream gene were considered to be orthologs. The nearest upstream gene was identified for each of the three GnRH forms in medaka and two forms in human. A highly conserved protein (FLJ20038) preceded the medaka form of GnRH (mdGnRH) and human mGnRH; protein tyrosine phosphatase alpha (PTPα) was the upstream neighbor for GnRH2 in both species; and PTPϵ preceded sGnRH in medaka. This type of linkage analysis for GnRH has not been used to date in tunicates.

In the present study, we began by searching the complete genome for GnRH genes in two tunicate species, *Ciona intestinalis* and *Ciona savignyi*. We used molecular

biological techniques to isolate and sequence the gene and cDNA structures from *C. intestinalis*. In addition, the alternative splicing and expression pattern of both genes in *C. intestinalis* were examined during early development and in adults. Tunicate GnRH was localized using immunocytochemical techniques. All six novel GnRH peptides from *C. intestinalis* and a seventh from *C. savignyi* were synthesized and tested for biological activity by two methods: *in vivo* activity in releasing gametes from a tunicate and activation of the human GnRH-I receptor using a gene reporter assay. We also examined if the nearest upstream gene was the same as in medaka and human GnRH genes.

3.2 Materials and Methods

3.2.1 Analysis of gene organization

C. intestinalis gene arrangements were discovered initially using the Department of Energy Joint Genome Institute *C. intestinalis* (tunicate or sea squirt) genome project database (http://www.jgi.doe.gov/programs/ciona.htm). Tunicate GnRH-1 and tGnRH–2, as well as mammalian and frog GnRH amino acid sequences, were used to search the available TBLASTN input form. We used the PAM30 matrix to optimize small matching fragments. Each search generated closely matched fragments. These DNA regions for the matching fragments were translated and examined for elements that might suggest peptide cleavage.

Identification of the *C. savignyi* GnRH genes utilized sequence data from the Whitehead Institute *C. savignyi* sequencing project (http://www.genome.wi.mit.edu/annotation/ciona/). This database was limited to nucleotide similarity searches using

MEGABLAST. To generate an amino acid-searchable database, the genome-read sequences were downloaded and compiled into a local blast database. These sequences were then compared using TBLASTN analysis with the same parameters described above. Each of the *C. intestinalis* GnRH forms was used to search the *C. savignyi* genome and resulted in similar blast matches, allowing for the characterization of two *C. savignyi* gene arrangements.

To determine the transcription start site for each *C. intestinalis* GnRH gene, a 1,000 bp upstream sequence to the GnRH peptide coding region was entered into the neural network promoter prediction site (http://www.fruitfly.org/seq_tools/promoter/html) using the default minimum promoter score of 0.80.

3.2.2 Animals

Adult *C. intestinalis* (Subphylum Tunicata, Class Ascidiacea) were obtained from Woods Hole Biological Station (Woods Hole, MA) and treated under the guidelines of the Animal Care Committee at the University of Victoria. The cerebral ganglion (brain), neural gland, gonads and intestine, were dissected and frozen in liquid nitrogen. *Ciona* are hermaphrodites, but eggs and sperm were collected from the terminal ends of gonoducts from different animals and mixed for fertilization. *C. intestinalis* embryos were collected at four stages (4-cell, gastrulation, tail-release and tail-resorption) and frozen.

3.2.3 Isolation of mRNA and synthesis of cDNA

Messenger RNA was isolated from tissues and embryos using a Micro Poly(A) Pure mRNA isolation kit (Ambion). Messenger RNA was reverse transcribed in a 50 µl reaction that contained 2 mM oligo dT, 2 mM dNTPs, 1x first strand reaction buffer, 0.01 M DTT, 5 U RNase inhibitor and 100 U Superscript II reverse transcriptase (Invitrogen). The reaction was incubated at 42 °C for 90 min and the enzyme was heat inactivated at 90 °C for 10 min.

For RACE-PCR, approximately 200 ng of mRNA was used to prepare RACE-ready cDNA using the RLM-RACE kit (Ambion) according to the manufacturer's instructions.

3.2.4 Isolation of genomic DNA

Genomic DNA was isolated using TRIzol reagent (Invitrogen). DNA was precipitated from the non-aqueous phase of the first phenol-chloroform phase separation following the manufacturer's instructions, except the DNA was dissolved in water.

3.2.5 PCR of cDNA and genomic DNA

Oligonucleotide primers were designed against regions encoding candidate GnRH prepropeptides based on the compiled genomic sequences for *C. intestinalis* GnRH genes 1 and 2 (Table 3.1). Each 50 µl reaction contained 2.5 U Taq polymerase, 1x Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen) and 20 pmol of each 5' and 3' primer. PCRs were carried out under the following conditions: denaturation at 94 °C for 30 s; annealing at 55 °C for 30 s; extension at 72 °C for 30 s for 35 cycles and a 7-minute extension.

Table 3.1. Primers used to amplify cDNA and genomic sequences for GnRH genes and upstream neighbouring genes in *Ciona intestinalis*. All primers were used at an annealing temperature of 55 °C and the direction of the primer, either forward (f) or reverse (r) is indicated.

Primer	Sequence (5'-3')	Direction	Target
G1	GGAACAGATACAAGCAAGCCAAC	f	5' *Ci-gnrh1* cDNA
G2	GACGAATTGCCCGCCGAGTC	f	5' *Ci-gnrh2* cDNA
G3	CAACGAGGAGCGGCGTCAGC	f	5' *Ci-gnrh2* cDNA
G4	CTTGTACCTATTCGCGTCT	f	5' *Ci-gnrh1* gene
G5	AACGGCTCTTCCGGCATTCC	r	3' *Ci-gnrh1* cDNA
G6	GGTTGTTCAACTTTGAACGGCTC	r	3' *Ci-gnrh1* cDNA
G7	TTCCCTTGTAGCGACCGAAG	r	3' *Ci-gnrh2* cDNA
G8	GCATAAAGCGTGCACACAAGAC	r	5' *Ci-gnrh1* gene
G9	ACACGCGAATAGGTACAAG	r	5' *Ci-gnrh1* gene
G10	GCAGATCACTAATGACGTCA	r	5' *Ci-gnrh2* gene
G11	ATGAGCGATAGCGGGAAATTCG	f	5' *Ci-gnrh1* gene
F1	TCGCCGCTTATTCTTCTACGC	f	FLJ20038 gene
F2	ACTGTGGGGTAAGACGGGACAC	f	FLJ20038 gene
P1	TGGCCTGAGACTGGACCTCC	f	PTPα gene
P2	TCATCAGGCAACCATCCTATTAC	f	PTPα gene

The PCR products were separated by electrophoresis on a 1.3 % (w/v) agarose gel and visualized with ethidium bromide staining using an Eagle Eye II still video system (Stratagene). Amplicons were selected, isolated (Qiagen) and cloned or cloned directly as PCR products into pGEM Vector-T (Promega) and sequenced.

3.2.6 Synthesis and testing of tunicate GnRH peptides

Jean Rivier (JER) synthesized and purified the GnRH peptides as described in Chapter 2. Judit Erchegyi (JE) completed the LHα subunit promoter assay to test for activation of the human GnRHR1 by tunicate GnRH peptides via the described protocol (Adams *et al.*, 2003).

3.2.7 Screening tunicate peptides with available GnRH antisera

Carol Warby performed the screening of tunicate peptides in our laboratory. Sixteen different antisera that were raised in rabbits against different forms of GnRH were initially screened for binding to tunicate peptides. The antisera included: Jas-2 through -11 (anti-tunicate GnRH-1); Bla-5, Her-4, Jul-5 (anti-lamprey GnRH-1); Emily and Gertie (anti-lamprey GnRH-III); 7CR-10 (anti-dogfish GnRH); GF-6, FP-5, PBL-45, PBL-49 and Aida (anti-salmon GnRH); 8CR-6 and –10, 9CR-6 (anti-catfish GnRH); Adams-100 (anti cGnRH-II); and B-7 (anti-mammalian GnRH). Ten antisera were prepared in the Sherwood laboratory; the others included Emily and Gertie (a gift of Dr. Stacia Sower), PBL-45 and –49 (a gift of Dr. Wylie Vale), Aida (a gift of Dr. Katsumi Aida) and Adams-100 (a gift of Dr. Tom Adams). Each antiserum was prepared at a dilution of 1:1,000 except for B-7 (1:2,000), GF-6 and Aida (1:5,000), 7CR-10 (1:7,500),

Gertie (1:8,000), Adams-100 (1:10,000), PBL-49 (1:30,000) and PBL-45 (1:50,000). The tGnRH-3, -5, -6, and -7 peptides were iodinated with ^{125}I. The percent binding (maximum binding) of the four iodinated tunicate GnRH peptides with each antiserum was determined and compared with the total counts. Only six of the antisera (Jas-2 through -6, Bla-5, FP-5, 8CR-6, PBL-45 and PBL-49) had binding of greater than 5 %; most antisera had binding of less than 1 %. Two antisera were selected for further studies. Bla-5 had 30-32 % binding with tGnRH-3 and –5; FP-5 had 7-13 % binding with tGnRH-5 and –6. Jas-2 had 38 % binding with the tGnRH-3 trace, but did not show an immunocytochemical reaction, so it was not tested further. Each of the nine tunicate peptides, plus mGnRH and GnRH2, were tested at 10, 100, 500, 1,000, 10,000 and 50,000 pgs in four assays: with antibody Bla-5 and trace tGnRH-3 or tGnRH-5 or with antibody FP-5 and trace tGnRH-5 or tGnRH-6. The percent cross-reactivity was calculated as the reference peptide in picomoles at 50 % B/B_0 divided by the test peptide in picomoles at 50 % B/B_0 times 100. Thus the reference peptide had 100 % cross-reactivity.

3.2.8 Immunolocalization

George Mackie performed the immunocytochemistry experiment on *Ciona intestinalis* using our specimens and antibodies. Specimens of *C. intestinalis* were relaxed in 0.01 % MS222 for 30 min. Portions of the dorsal fold were pinned out in Petri dishes lined with Sylgard 184 (Dow-Corning) using cactus spines. The dorsal blood sinus was opened to allow access of reagents to the dorsal strand and nerves lying within the sinus. The dorsal strand was fixed for 1 hour in 4 % paraformaldehyde in 0.1 M PBS at pH 7.3

at room temperature, followed by washing and storage in 0.1 M PBS containing 0.35 % Triton X-100 and 0.03 % sodium azide (PTA). Preparations were treated with one of three primary antibodies, Jas-2, Bla-5 or FP-5, in 1:100, 1:1,000 or 1:2,000 (diluted with PTA), whereas controls were incubated in solutions omitting the antibody. All preparations had 1.5 % goat serum added and were incubated for 12-24 hours. After a PTA wash, preparations were incubated in fluorescein-isothiocyanate-goat anti rabbit gamma globulin (Sigma #F-0382) for 12-24 hours. Following a PTA wash, preparations were mounted in 50 % glycerol containing 1.5 % N-propyl pyrogallate and examined by laser-scanning confocal microscopy using a Zeiss LSM 410.

3.2.9 Assay for gamete release induced by tunicate GnRH peptides

Seven novel tGnRH peptides (tGnRH-3 through –9) and tGnRH-2 (Figure 3.1) were tested to determine if they induce gamete release in mature adults. *C. intestinalis* were selected by the presence of a white sperm duct and/or a pink oviduct. GnRH peptides were dissolved in a saline solution (3 g NaCl/100 ml) and diluted at a final injection dose of 10 ng/g tunicate. The injection volume was based on the body mass of representative individuals (6.0 to 8.0 g). Peptides were injected with a 25-gauge needle beside the gonoducts. Then, each individual was placed in a 300 ml glass stacking dish filled with fresh, filtered seawater and monitored for response. A positive response was the release of a visible plume of either eggs or sperm. A preliminary test injection of only saline solution was conducted on ten tunicates, none of which released gametes during a 30 min period.

	1	2	3	4	5	6	7	8	9	10
mGnRH	**pGlu**	**-**	**His**	**-**	**Trp**	**-**	**Ser**	**-**	**Tyr**	**-Gly-Leu-Arg-Pro-Gly-NH2**
tGnRH-1	pGlu	-	His	-	Trp	-	**Ser**	-	**Asp**	-**Tyr**-**Phe**-**Lys**-Pro-Gly-NH2
tGnRH-2	pGlu	-	His	-	Trp	-	**Ser**	-	**Leu**	-**Cys**-**His**-**Ala**-Pro-Gly-NH2
tGnRH-3	pGlu	-	His	-	Trp	-	Ser	-	Tyr	-**Glu**-**Phe**-**Met**-Pro-Gly-NH2
tGnRH-4	pGlu	-	His	-	Trp	-	Ser	-	**Asn**	-**Gln**-Leu-**Thr**-Pro-Gly-NH2
tGnRH-5	pGlu	-	His	-	Trp	-	Ser	-	Tyr	-**Glu**-**Tyr**-**Met**-Pro-Gly-NH2
tGnRH-6	pGlu	-	His	-	Trp	-	Ser	-	**Lys**	-Gly-**Tyr**-**Ser**-Pro-Gly-NH2
tGnRH-7	pGlu	-	His	-	Trp	-	Ser	-	Tyr	-**Ala**-Leu-**Ser**-Pro-Gly-NH2
tGnRH-8	pGlu	-	His	-	Trp	-	Ser	-	**Leu**	-**Ala**-Leu-**Ser**-Pro-Gly-NH2
tGnRH-9	pGlu	-	His	-	Trp	-	Ser	-	**Asn**	-**Lys**-Leu-**Ala**-Pro-Gly-NH2
cGnRH-II	**pGlu**	**-**	**His**	**-**	**Trp**	**-**	**Ser**	**-**	**His**	**-Gly-Trp-Tyr-Pro-Gly-NH2**

Figure 3.1. Amino acid sequences of tunicate and two vertebrate GnRH peptides. GnRH peptides, including the seven novel forms identified in the tunicates (tGnRH) *C. intestinalis* and *C. savignyi* were compared to mammalian (m)GnRH and GnRH2 = cGnRH-II. Amino acids that are different from mGnRH in the tunicate GnRH peptides are underlined and bold, whereas differences in GnRH2 from mGnRH are in bold.

3.2.10 *In silico* analysis of GnRH promoters

Upstream promoter regions of 1,000 nucleotides preceding the predicted transcription start site for each of the two GnRH genes in *C. intestinalis* and *C. savignyi* were generated with BLASTN similarity searches. To build the upstream promoter region for *C. intestinalis*, we used data from a different sequencing project that allowed for an increased number of overlapping fragments (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). Matching fragments were aligned using Bioedit Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A technique to walk 1,000 bp upstream from each predicted transcription start site by overlapping matching fragments was utilized to generate a consensus sequence with a minimum of four matching fragments covering any given region. The promoter sequences were confirmed using the Department of Energy Joint Genome Institute *Ciona intestinalis* v1.0 (http://genome.jgi-psf.org/ciona4/ciona4.home.html) on scaffolds 1051 (*Ci-gnrh1*) and 410 (*Ci-gnrh2*).

To predict the transcription factor binding sites on the various promoter regions, each 1,000 bp upstream region was entered into the MatInspector input form using Matrix Family Library Version 2.4 (http://www.genomatix.de/cgi-bin/mat_fam.pl) with default settings (cutoff of 0.75 and offset of –1000). This bioinformatics tool recognized transcription factor binding site matrix information and resolved a greater number of GnRH-specific transcription binding sites than other methods such as IUPAC consensus (TESS filtered) and context (Alibaba 2.1).

3.2.11 *In silico* and experimental analysis of nearest genes

To identify the nearest upstream gene to GnRH, we first searched the *C. intestinalis* database using TBLASTN for the last exon of the medaka FLJ20038 prepropolypeptide gene (medaka, *Oryzias latipes*; gi: 21955956). This gene has been identified as the nearest upstream gene to mdGnRH in medaka and to mGnRH gene in human. This search resulted in a very high match (70 % amino acid similarity) to the *C. intestinalis* FLJ20038 gene. A similar procedure of using overlapping fragments as described above was used to determine if a gene similar to FLJ20038 was located within 3 kb upstream of the GnRH genes. We imported the FLJ20038 matched sequence fragments into Bioedit and walked downstream past the 3' UTR of the peptide. Using the Whole-Genome Shotgun approach (WGS) and Bacterial Artificial Chromosome (BAC) ends in the database we constructed overlapping fragments until the database returned fewer than two similar fragments. We also used the Department of Energy Joint Genome Institute *Ciona intestinalis* v1.0 to identify the scaffolds containing the two *Ciona* GnRH genes as well as the *Ciona* FLJ20038 and PTPα genes. Primers F1 and F2 (see Table 3.1) were designed for the region coding for FLJ20038 and were used in a PCR reaction with reverse primers G9 for *Ci-gnrh1* and G10 for *Ci*-gnrh2 to amplify possible products. PCR was performed using genomic DNA, Platinum Taq DNA polymerase (Invitrogen), and primers F1 or F2 and G9 or G10 under the following conditions: 94 °C for 3 min, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min 45s, and finally a 7 min extension at 72 °C.

A similar procedure, both *in silico* and PCR using primers P1 or P2 and G9 or G10, was used to determine if a PTPα gene is upstream of the two *Ciona* GnRH genes.

The final exon of the medaka PTPα peptide (gi: 21955958) was found to have highly matching fragments within the *C. intestinalis* database. These matching nucleotide fragments were then used to construct a 1,800 bp downstream region.

3.3 Results

3.3.1 Six novel peptides are distributed on two genes in *C. intestinalis*

In silico analysis revealed six candidate GnRH peptides in *C. intestinalis*. Each peptide has a unique 10-amino-acid sequence and does not match any known GnRH peptides identified to date. The peptides were assigned numbers (Figure 3.1) as to whether they are present in *Chelyosoma productum* only (tGnRH-1 and –2), in *C. intestinalis* only (tGnRH-3 and –4), in both *Ciona* species (tGnRH-5 through –8), or in *C. savignyi* only (tGnRH-9). Analysis of the genomic regions for these candidates showed that three of the peptides, tGnRH-3, tGnRH-5 and tGnRH-6, were found as a triplet on one gene (*Ci-gnrh1*). These peptides are each bordered by basic amino acids (R or KR before the N-terminus, and GRR or GKR after the C-terminus) that are potential peptide cleavage sites separating the peptides. The GnRHs are separated by intervening peptides of 13 or eight amino acids (Figure 3.2A).

Three more of these candidate peptides, tGnRH-7, tGnRH-8, and tGnRH-4, were found in tandem on a second gene, *C. intestinalis gnrh2* (*Ci-gnrh2*) (Figure 3.2B). The peptides in *Ci-gnrh2* are each bordered by basic amino acids (RR or GKR) but lack intervening peptides.

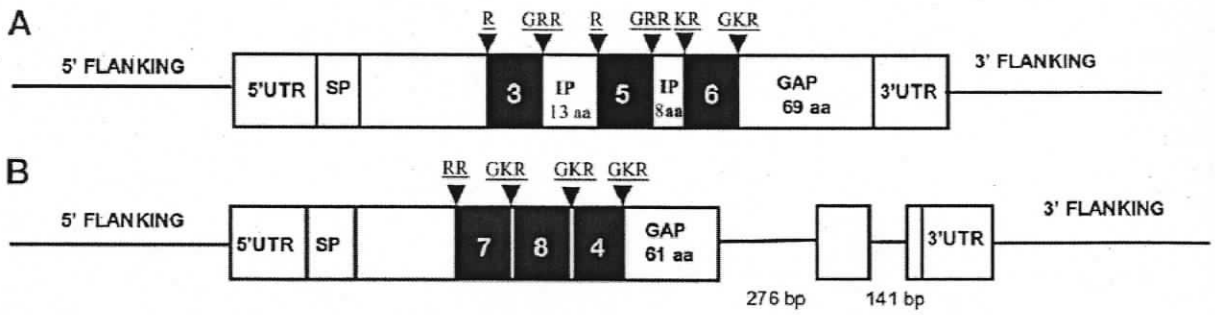


Figure 3.2. Schematic arrangement of two GnRH genes in *Ciona intestinalis*. The figure contains GnRH genes for *Ci-gnrh1* (A) and *Ci-gnrh2* (B). Tunicate GnRH peptides are indicated by black boxes with the appropriate tGnRH peptide number in white. Single letter codes are used to indicate the bordering basic amino acids. 5' flanking region (5' flanking), 5'untranslated region (5' UTR), signal peptide (SP), GnRH-associated peptide (GAP), 3' untranslated region (3' UTR), 3' flanking region (3' flanking), amino acids (aa), base pairs (bp) in introns.

3.3.2 PCR of mRNA transcripts from both GnRH genes in adult *C. intestinalis*

Using gene-specific primers, we amplified a single product from cDNA prepared from adult *C. intestinalis* brain tissue for each GnRH gene 1 and 2. Initial *Ci-gnrh1* products were amplified with primers G1 and G5 resulting in a 327 bp product and G1 and G6 resulted in a 343 bp product. 5' RACE-PCR resulted in a number of products for the 5' end of *Ci-gnrh1*, but only the longest 346 bp product made with G5 and the adapter inner primer matched the expected sequence. This product was used to compare to products in public EST and genomic databases. 3' RACE-PCR using G1 and the appropriate adapter primer resulted in a 708 bp cDNA fragment. This product was used to overlap and construct a full-length profile cDNA (Figure 3.3) for comparison to public EST and genomic databases.

Initial *Ci-gnrh2* products were amplified with primers G2 and G7 resulting in a 600 bp product, and G3 and G7 resulting in a 559 bp product. 3'RACE-PCR using G2 and the appropriate adapter primer resulted in a 609 bp cDNA fragment. This product was used to overlap and construct a full-length cDNA profile (Figure 3.4) for comparison with public EST and genomic databases. We were not able to isolate products for the 5' end of *Ci-gnrh2* cDNA by RACE-PCR. However, we were able to amplify genomic DNA that codes for this region. The final cDNA and genomic sequences for each gene has been submitted to the DDBJ/EMBL/Genbank databases under accession numbers AY204706 (*Ci-gnrh1* cDNA), AY204707 (*Ci-gnrh1* gene), AY204708 (*Ci-gnrh2* cDNA) and AY204709 (*Ci-gnrh2* gene).

```
cttgtacctattcgcgtgtataagttattctgcaatcgcttatccaacttcacaaatgtt      60
                                                        M  L       2
ggatatcgaaaaagacgaacttgcggctttgttgcagcgagaaaactcagctttccgtga     120
  D  I  E  K  D  E  L  A  A  L  L  Q  R  E  N  S  A  F  R  D      22
cctgttataccacaagaacgcagggaacttcgaaaagagcgatagcgggaagttcggctc     180
  L  L  Y  H  K  N  A  G  N  F  E  K  S  D  S  G  K  F  G  S      42
cctgaagccgcagaataatttccctcatctcgatcttggtttaggagtcgatttggacgc     240
  L  K  P  Q  N  N  F  P  H  L  D  L  G  L  G  V  D  L  D  A      62
agtggatcaatggaacagatacaagcaagccaacgcacagcggatgcaagatctcggggt     300
  V  D  Q  W  N  R  Y  K  Q  A  N  A  Q  R  M  Q  D  L  G  V      82
cccagtgaacgcacgccaacattggagctacgagtttatgccgggtggacgcagagctgc     360
  P  V  N  A  R  Q  H  W  S  Y  E  F  M  P  G  G  R  R  A  A     102
ttgggaaaacgcaaacgtcggtgttccagtttcaagacaacactggagttatgaatacat     420
  W  E  N  A  N  V  G  V  P  V  S  R  Q  H  W  S  Y  E  Y  M     122
gccgggtggtagaagatcggctggtcgacatgctatgaccaaacgtcaacattggagcaa     480
  P  G  G  R  R  S  A  G  R  H  A  M  T  K  R  Q  H  W  S  K     142
aggttattctcccggtggtaagcgaagtgtggatctgtccgaattcgatgaccaaggccg     540
  G  Y  S  P  G  G  K  R  S  V  D  L  S  E  F  D  D  Q  G  R     162
cagaatcacaaaacacgaaggaatgccggaagagccgttcaaggttgaacaaccaagacc     600
  R  I  T  K  H  E  G  M  P  E  E  P  F  K  V  E  Q  P  R  P     182
gaggaatggaatccatggaccggctggtttggaccagaacgaaccggattggaaaaactg     660
  R  N  G  I  H  G  P  A  G  L  D  Q  N  E  P  D  W  K  N  W     242
gatgaacgaacaaccagcagtcagcagcgacgacaaaggatctgacgtagaataattccc     720
  M  N  E  Q  P  A  V  S  S  D  D  K  G  S  D  V  E  -           259
gccgagtttgtcacgtgatcactagtggcattctgtttgttttaaaaatttgtgcacgta     780
agaaaattttaatctttaacattaaaccggatataccagatcaaaacttaaaccagtgtt     840
aaatagcgatagaaatgctcaaaactgttcaataacttgtcgtgtaactaaaatgtgaaa     900
gggcgctctcccataatataaagtagaccagatgccttaccaaaacagattcaaacagtt     960
tttctctgtttttggaatgtttttatgcgggttcaattcaaatgcatgtcttgtgtgcac    1020
gctttatgc                                                       1029
```

Figure 3.3. Complementary DNA sequence of *Ci-gnrh1*. *Ci-gnrh1* encodes three tunicate GnRH peptides in tandem, tGnRH-3, -5, and -6, which are bordered by basic amino acid residues (indicated by underlines), but are separated by intervening peptides. cDNA was prepared from mRNA isolated from nervous system tissue of adult *C. intestinalis*.

```
atgacgtcattagtgatctgcttattgtctgtgtttgtttttcttcacgttgctcagtgc     60
 M  T  S  L  V  I  C  L  L  S  V  F  V  F  L  H  V  A  Q  C      20
cacgtccttcgcaaccaggaagcgcttggtaacttcgattggaacgaggataattccgaa    120
 H  V  L  R  N  Q  E  A  L  G  N  F  D  W  N  E  D  N  S  E      40
actcgaccggacttcgaggacgaattgcccgccgagtcattccaaaatctcccttctaac    180
 T  R  P  D  F  E  D  E  L  P  A  E  S  F  Q  N  L  P  S  N      60
aacgaggagcgacgacagcattggtcttatgctttatcaccaggaggaaagcggcaacac    240
 N  E  E  R  R  Q  H  W  S  Y  A  L  S  P  G  G  K  R  Q  H      80
tggtctcttgcgttgtctccgggcggtaaaaggcaacactggtcgaaccaacttacccca    300
 W  S  L  A  L  S  P  G  G  K  R  Q  H  W  S  N  Q  L  T  P     100
ggtggcaagagggtgattccccgaatgcgagagcagaagaaagtggatttcgatgaaata    360
 G  G  K  R  V  I  P  R  M  R  E  Q  K  K  V  D  F  D  E  I     120
acttacaacaagatctataatctattacgacaatatttagaggcggcggccgaatacgaa    420
 T  Y  N  K  I  Y  N  L  L  R  Q  Y  L  E↑A  A  A  E  Y  E     140
gaaggagacttcggtcgcaacaagggaaatcaacgcaatcaacttgaaacaataaaggac    480
 E  G  D  F  G  R  N  K  G  N  Q  R  N  Q  L  E  T  I  K↑D     160
gacattataaccgaatgattctaattgtgacgtcatattcaaaccatggacacaccattt    540
 D  I  I  T  E  -                                              165
gtatttttgttggaaattcttcacaaacttcaacatttatcaaagttatgtttcaattgt    600
cgttgtatattatgagagtattgtatgatactgtggggtaagatggataccgttagcaca    660
taatgtcccatatttcctaatcgtgtttttatcaattaacaacgctttttagagtcgtg    720
agaaaacggttatataaatctgtagtatgctttgtttaataccaaacgaaacaataaatt    780
aaaataaaaacatgaaccatcttcccccaacctactttatgccatgttaaacaaactaca    840
aaatgacgtaacagtgtgtcatgacg                                     866
```

Figure 3.4. Complementary DNA sequence of *Ci-gnrh2*. *Ci-gnrh2* encodes three tunicate GnRH peptides in tandem, tGnRH-7, -8, and -4, which are bordered by basic amino acid residues (indicated by underlines). Arrow heads indicate the position of two introns, the first of which is sometimes retained in mRNA.cDNA was prepared from mRNA isolated from nervous system tissue of adult *C. intestinalis*.

A.

```
atgttggataatgaaaaggatcaattggccgcttttcttcaacgtgaaagttcagctttccgagatattctgtac
 M  L  D  N  E  K  D  Q  L  A  A  F  L  Q  R  E  S  S  A  F  R  D  I  L  Y
caccacgcggcaagcggctttgagaagagcgacaatggcatactaggaccacaaagcccggaaaatatattcaat
 H  H  A  A  S  G  F  E  K  S  D  N  G  I  L  G  P  Q  S  P  Q  N  I  F  N
Caacaaggagatgggtggagcaggtacgcccaaggcggaaagcaaacacatgcgatacaagaaggaggaattcca
 Q  Q  G  D  G  W  S  R  Y  A  Q  G  G  K  Q  T  H  A  I  Q  E  G  G  I  P
gtgaacgctcgtcaacattggagctacgagtacatgccaggaggtagaaggtcgtcatgggaaaacattggcgcg
 V  N  A  R  Q  H  W  S  Y  E  Y  M  P  G  G  R  R  S  S  W  E  N  I  G  A
cctgtttcaaggcaacattggagttatgagtacatgccaggcggacgccgatccgtacctacacatgcattgttc
 P  V  S  R  Q  H  W  S  Y  E  Y  M  P  G  G  R  R  S  V  P  T  H  A  L  F
aagcgacaacattggagcaagggatattctcctggtggaaaacgaagcgtggacctggcagaatttgacgaccaa
 K  R  Q  H  W  S  K  G  Y  S  P  G  G  K  R  S  V  D  L  A  E  F  D  D  Q
ggacatcgtattatggaacacaatggtatgaaagagaaagcctttgatattcagcaacctcaaccaagtaatggc
 G  H  R  I  M  E  H  N  G  M  K  E  K  A  F  D  I  Q  Q  P  Q  P  S  N  G
atccacggccctggcatgaatcagaaaaaaacggattggaaaaactggctgagcaacaatccagcaactgaagac
 I  H  G  P  G  M  N  Q  K  K  T  D  W  K  N  W  L  S  N  N  P  A  T  E  D
accatcgatcaaaaccaaaacgacgaggcataa
 T  I  D  Q  N  Q  N  D  E  A  -
```

B.

```
                  ******* *** **** *********  *  ***** *       ** *
Ci-gnrh1          MLDIEKDELAALLQRENSAFRDILYHKNAGNFEKSDSGKFDSLKLQNNFP
Cs-gnrh1          MLDIEKDQLAAFLQRESSAFRDILYHHAASGFEKSDNGILGPQSPQNIF-

                              * * ** *   *    *  * *********** ****
Ci-gnrh1          HLDLGLGVDLDAVDQWNRYKQANAQ--RMQNLGVPVNARQHWSYEFMPGG
Cs-gnrh1          --------NQQG-DGWSRYAQGGKQTHAIQEGGIPVNARQHWSYEYMPGG

                  **  **  * * ******************   **  *************
Ci-gnrh1          RRAVWENANVGVPVSRQHWSYEYMPGGRRSAGQHAMTKRQHWSKGYSPGG
Cs-gnrh1          RRSSWE--NIGAPVSRQHWSYEYMPGGRRSVPTHALFKRQHWSKGYSPGG

                  ****** ****** **    ** *  *   ** * ****** *  *   *
Ci-gnrh1          KRSVDLSEFDDQGRRITKHEGMPEEPFKVEQPRPRNGIHGPAGLDQNEPD
Cs-gnrh1          KRSVDLAEFDDQGHRIMEHNGMKEKAFDIQQPQPSNGIHGP-GMNQKKTD

                  ****    **     *    *
Ci-gnrh1          WKNWMNEQPAA-SS-DDKGSDVE
Cs-gnrh1          WKNWLSNNPATEDTIDQNQNDEA
```

Figure 3.5. Complementary DNA sequence of *Cs-gnrh1* and comparison to *Ci-gnrh1*. (A) Nucleotide sequence from *C. savignyi* genome and derived amino acid sequence of gene *Cs-gnrh1*. Basic amino acids bordering the tGnRH peptides are underlined. (B) Alignment of the derived amino acids of *Ci-gnrh1* and *Cs-gnrh1*. Identical amino acids are indicated by an asterisk (*). tGnRH-5 (two) and -6 (*C. savignyi*) and -3, -5 and -6 (*C. intestinalis*) are indicated by a black background.

3.3.3 Triplet peptides are also found on two genes in a second tunicate species

Search results of the *C. savignyi* genome resulted in identification of fragments that group into similar gene structures as those in *C. intestinalis*. In *C. savignyi*, *Cs-gnrh1* encodes two copies of tGnRH-5 and one copy of tGnRH-6 (Figure 3.5A), whereas *Cs-gnrh2* encodes tGnRH-7, tGnRH-8 and tGnRH-9 with the appropriate cleavage sites (Figure 3.6A). Hence, the two *Ciona* species share the same general gene structures for *gnrh1* and *gnrh2*.

3.3.4 Determination of transcription start sites for *C. intestinalis* GnRH genes

Ci-gnrh1 and *Ci-gnrh2* have predicted transcription start sites at 649 bp (score 0.99) and 282 bp (score 0.92) upstream from the GnRH peptide coding regions, respectively. Transcription start sites for GnRH gene 1 and gene 2 in *C. savignyi* were located at 467 bp (score 0.99) and 237 bp (score 0.98) upstream from the GnRH coding regions, respectively.

3.3.5 Early developmental expression of mRNA of both genes

Throughout development, both *Ci-gnrh1* and *Ci-gnrh2* are expressed by *C. intestinalis*. A single 327 bp product, amplified by PCR for *Ci-gnrh1,* is expressed at each stage: 4-cell, gastrulation, tail-release and tail-resorption (Figure 3.7). This is the same transcript that was amplified from adult *Ciona* tissue. In contrast, two transcripts, 310 bp and 602 bp, were amplified for *Ci-gnrh2* at the 4-cell stage and gastrulation, although only the shorter transcript was detected at the tail release and tail resorption

A.

```
atgaagtcaggtatttcattactgtctcttttagttataatcaatgtcgtgtattgccatgtcttg
 M  K  S  G  I  S  L  L  S  L  L  V  I  I  N  V  V  Y  C  H  V  L
cccagtagaggcggacaagctaatttcgattgggccaggcagaaatctgatatctcgcctgatttc
 P  S  R  G  G  Q  A  N  F  D  W  A  R  Q  K  S  D  I  S  P  D  F
ggggctgattatccgtcagattcgtctgaggaggttcccccgttgggtgcaaacagacgacagcat
 G  A  D  Y  P  S  D  S  S  E  E  V  P  P  L  G  A  N  R  R  Q  H
tggtcgtacgcactttcacccgggggaaagcgacagcactggtcactcgcgttatccccaggcggg
 W  S  Y  A  L  S  P  G  G  K  R  Q  H  W  S  L  A  L  S  P  G  G
aagcgacaacattggtcaaacaagcttgctccaggcggaaaaagggtaaacccctattcgtcagca
 K  R  Q  H  W  S  N  K  L  A  P  G  G  K  R  V  N  P  Y  S  S  A
aaagcgaaccctgttacagatgaagaaaattacaacaaactgtacaatcttctcagacagtat...
 K  A  N  P  V  T  D  E  E  N  Y  N  K  L  Y  N  L  L  R  Q  Y  ...
```

B.

```
                * *  *  *** *   *  ****        ***        ***    *
Ci-gnrh2        MTSLVICILSLFVFLHVAQCHVLRNQEALGSFDWDEENPEVRPDFEDELP
Cs-gnrh2        MKS-GISLLSLLVIINVVYCHVLPSRGGQANFDWARQKSDISPDFGADYP

                  *    *     ********************************** * *
Ci-gnrh2        AESFQNLPSNNEERRQHWSYALSPGGKRQHWSLALSPGGKRQHWSNQLTP
Cs-gnrh2        SDSSEEVPPLGANRRQHWSYALSPGGKRQHWSLALSPGGKRQHWSNKLAP

                ***** *          ** ** * *******
Ci-gnrh2        GGKRVIPRMREQKKADFDEINYTKIYNLLRQY...
Cs-gnrh2        GGKRVNPYSSAKANPVTDEENYNKLYNLLRQY...
```

Figure 3.6. Complementary DNA sequence of *Cs-gnrh2* and comparison to *Ci-gnrh2*. (A) Nucleotide sequence from the *C. savignyi* genome and derived amino acid sequence of gene *Cs-gnrh2*. Basic amino acids bordering the tGnRH peptides are underlined. (B) Alignment of the derived amino acids of *Ci-gnrh2* and *Cs-gnrh2*. Identical amino acids are indicated by an asterisk (*). tGnRH-7, -8 and -9 (*C. savignyi*) and -7, -8 and -4 (*C. intestinalis*) are indicated by a black background.

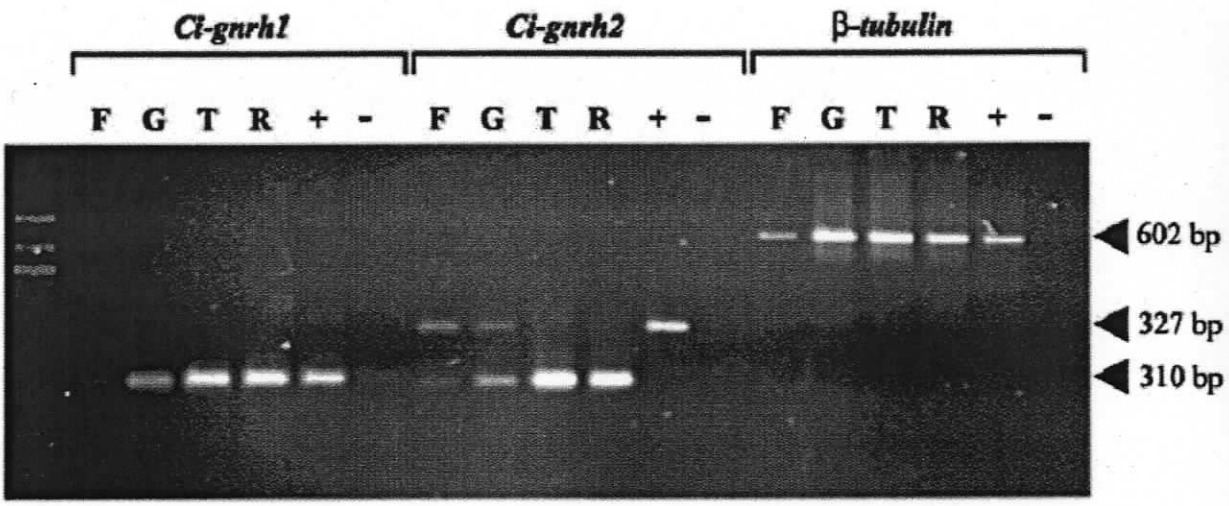


Figure 3.7. Developmental expression of both *Ci-gnrh1* and *Ci-gnrh2* at four stages. These four stages are: 4-cell (F), gastrulation (G), tail-release (T), and tail-resorption (R). Amplification of β-tubulin cDNA was used to ensure quality control for cDNA synthesis. Two control reactions were also included for each set of primers: one negative without DNA (-) and one positive control using adult *C. intestinalis* intestine cDNA (+).

stages. Sequencing of these products revealed that one transcript has introns removed, whereas the second transcript retains an intron. Adult tissue in the same study expressed one transcript for gene 1, but only the longer transcript for gene 2.

3.3.6 Cross-reactivity of tGnRH peptides

The nine tunicate GnRH peptides synthesized for this study had a purity of greater than 95 % after purification by capillary zone electrophoresis and an observed monoisotropic mass $(M + H)^+$ within 0.02 to 0.24 compared with the calculated mass (Table 3.2). Two out of nine tunicate peptides (tGnRH-3 and –5) showed the highest cross-reactivity with antiserum Bla-5, which was raised against lamprey GnRH-I (Table 3.3). Both peptides had between 77-117 % cross-reactivity. The other tunicate peptides had less than 2.5 % cross-reactivity; mGnRH (GnRH1) had $\leq$3.5 % cross-reactivity; and GnRH2 did not cross-react (< 0.1 %). All of the peptides were tested with antiserum FP-5 and iodinated tGnRH-5 or tGnRH-6, but the cross-reactivity was so weak that only GnRH2 and tGnRH-5 reached 50 % B/B_0 binding at a concentration below 50 nM.

3.3.7 Immunolabeling of GnRH containing neurons in the dorsal strand plexus

Both Bla-5 and FP-5 antisera labelled neurons in the dorsal strand plexus along with isolated neurites that run within branches of the visceral nerve (Figure 3.8). This confirms previous reports for *C. intestinalis* using antisera raised against GnRH2 (Mackie, 1995), lamprey GnRH (Bollner *et al.*, 1997) and salmon GnRH (Tsutsui *et al.*, 1998). These antisera cross-react with several forms of GnRH. There was no labelling in the preparations incubated with the Jas-2 antibody or in controls.

Table 3.2. Structure and physico-chemical characteristics of synthetic tunicate GnRHs.

Peptide	Structure	% purity HPLC	% purity CZE	MH+(mono) calculated	MH+(mono) observed
tGnRH-1	<EHWSDYFKPG-NH_2	99	99	1246.56	1246.73
tGnRH-2	[<EHWSLCHAPG-NH_2]$_2$	96	95	2231.98	2232
tGnRH-3	<EHWSYEFMPG-NH_2	93	96	1263.53	1263.32
tGnRH-4	<EHWSNQLTPG-NH_2	98	99	1149.55	1149.46
tGnRH-5	<EHWSYEYMPG-NH_2	93	97	1279.52	1279.76
tGnRH-6	<EHWSKGYSPG-NH_2	98	98	1128.53	1128.45
tGnRH-7	<EHWSYALSPG-NH_2	94	96	1127.53	1127.76
tGnRH-8	<EHWSLALSPG-NH_2	95	96	1077.54	1077.49
tGnRH-9	<EHWSNKLAPG-NH_2	99	97	1119.57	1119.54

Abbreviations: <E , pyroglutamic acid; CZE, capillary zone electrophoresis; MH+ (mono), monoisotopic mass of [M + H]+

Table 3.3. Cross-reactivity of the BLA-5 antibody with each of the nine tunicate GnRH forms, mammalian GnRH and GnRH2. The percent cross-reactivity (%) of antibody BLA-5 was standardized against tGnRH-3 or tGnRH-5.

Antibody	**BLA-5**	**BLA-5**
Trace	**tGnRH-3**	**tGnRH-5**
Reference	**tGnRH-3**	**tGnRH-5**
Peptides	**%**	**%**
tGnRH-1	< 0.1	< 0.1
tGnRH-2	0.5	0.4
tGnRH-3	**100.0**	76.8
tGnRH-4	< 0.1	< 0.1
tGnRH-5	117.0	**100.0**
tGnRH-6	< 0.1	< 0.1
tGnRH-7	1.5	1.3
tGnRH-8	2.2	1.8
tGnRH-9	< 0.1	< 0.1
mGnRH	3.5	2.9
GnRH2	< 0.1	< 0.1

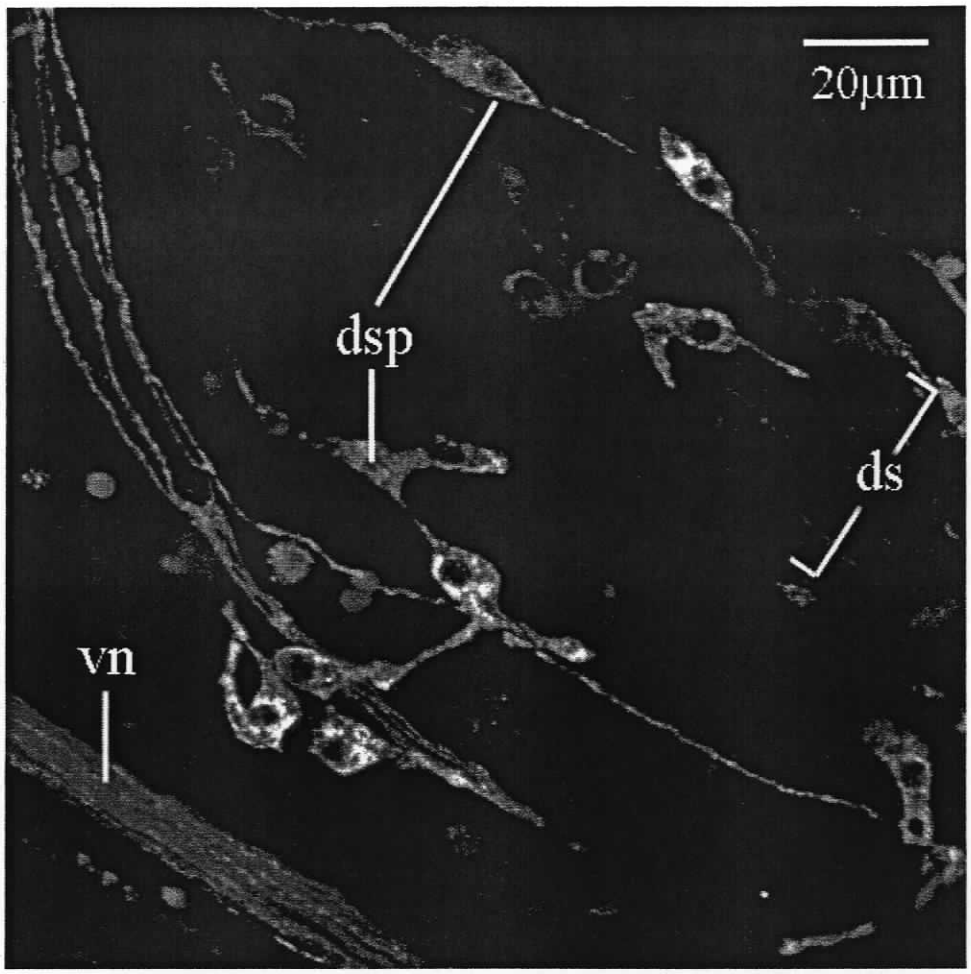


Figure 3.8. Whole-mount preparation of the dorsal wall of the blood sinus of *Ciona*. GnRH-immunoreactive neurons were visualized in the dorsal strand nerve plexus (dsp) lying near the dorsal strand (ds). The latter (an epithelial structure) did not label, but was faintly visible due to background illumination. GnRH-immunoreactive neurites also run within branches of the visceral nerve (vn).

3.3.8 Novel tunicate GnRH peptides are bioactive, resulting in the release of gametes

The novel tunicate peptides identified in this study were biologically active and caused the release of eggs and/or sperm from mature adult *C. intestinalis* (Table 3.4). Administration of the peptide initially caused an increase in water flow by bodily contraction. At least two tunicates from each group injected with GnRH peptide released gametes. The most effective peptides in this study were tGnRH-5, which caused 63 % of tunicates to spawn, and tGnRH-3, which caused 50 % of tunicates to spawn. The time it took for tunicates to spawn ranged from 1 min 33 s to 36 min. There was also a great range in the intensity and number of eggs or sperm released, though this was not associated with a particular peptide. Three animals injected with GnRH peptide underwent increased water flow and large bodily contractions, but they did not release eggs or sperm. This observation suggests that the GnRH may have been effective, but the gametes were not ripe for release at the time of our experiment.

3.3.9 Promoter consensus sites for transcription factors

We identified a number of potential binding sites for transcription factors in the *Ciona* GnRH genes (Figure 3.9); these DNA sequences are involved in the regulation of GnRH gene expression in promoter studies of other species. The relevant binding sites in the *Ciona* promoter regions are predicted to have binding affinity to POU factors (Brn-2, Oct-1, Pit-1, and Tst-1), GATA factors (GATA), androgen receptors (AR), glucocorticoid receptors (GR), progesterone receptors (PR), cyclic AMP response element binding protein (CREB) and its variants, CREB-1 and CREB-1/c-Jun

Table 3.4. Release of eggs or sperm from gravid *Ciona intestinalis* after injections of tunicate GnRH peptides or saline.

Treatment	Number injected	Number spawned	Percent spawned	Time to spawn (min)
Control	19	1	5	27:32
tGnRH-2	8	2	25	7:39-29:11
tGnRH-3	8	4	50	5:00-19:15
tGnRH-4	8	2	25	5:02-13:55
tGnRH-5	8	5	63	2:00-8:40
tGnRH-6	10	2	20	1:33-3:51
tGnRH-7	8	3	38	2:30-14:32
tGnRH-8	9	3	33	2:49-18:30
tGnRH-9	8	2	25	14:22-36:00

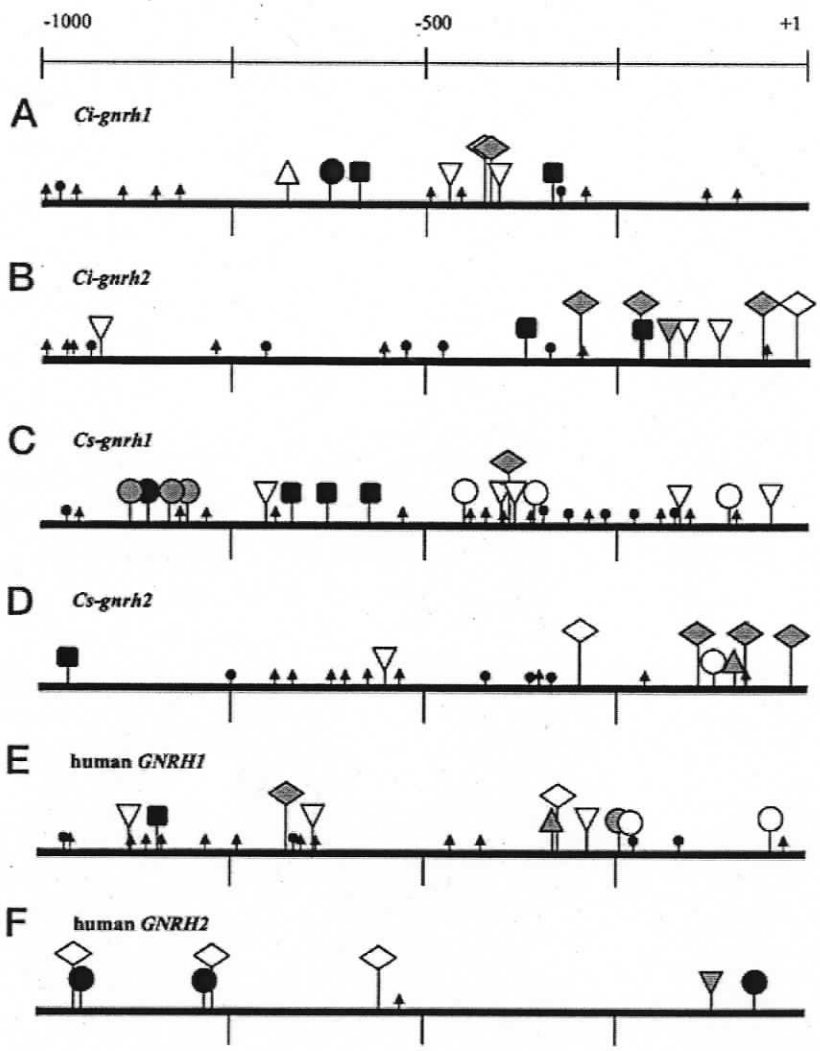


Figure 3.9. Promoter elements identified *in silico* using 1,000 bp of gene sequence compiled upstream of the transcription start site for (A) *Ci-gnrh1*, (B) *Ci-gnrh2*, (C) *Cs-gnrh1*, (D) *Cs-gnrh2*, (E) human *GNRH1*, and (F) human *GNRH2*. △ ARE, androgen response element; ◇ AP-1 response element; ■ Brn-2 binding; ● CRE, cAMP response element for CRE binding protein (CREB); ○ CREB-1, cAMP response element for CRE binding protein-1; ● CREB-1/cJUN, cAMP response element for CREB-1/cJun heterodimer; ▮ GATA response element; ▲ GRE, glucocorticoid response element; ▼ Oct-1 binding site; ▽ Pit-1 binding site; ◆ PRE, progesterone response element; ▮ Tst-1 binding site.

heterodimer, and activator protein -1(AP-1). No obvious pattern was seen with respect to the location of the binding sites of the above transcription factors, but the frequencies of many transcription-binding sites are similar between the two *Ciona* species (Figure 3.9).

We compared the 1,000 bp upstream promoter regions in *C. intestinalis* and *C. savignyi* against the 1,000 bp upstream promoter regions from human *GNRH1* (gi:19923125) and *GNRH2* (gi:2833652). The human *GNRH1* upstream region was closest in transcription binding sites to *Cs-gnrh2*, as each had the same number of AP-1, Brn-2, GATA and GR sites, and each had the same binding sites but different numbers for Pit-1, Tst-1, CREBP-1 and Oct-1. The only difference in types of response elements was that the human *GNRH1* promoter had a CREB-1/cJun heterodimer site and *Cs-gnrh2* did not. Human *GNRH2* was closest to the *Ci-gnrh1* promoter as they both share CREB, AP-1 and Oct-1 binding sites.

3.3.10 Nearest upstream gene for GnRHs

DNA fragments were identified in *C. intestinalis* EST databases that had 73 % amino acid identity to the last exon of human FLJ20038 and 75 % identity to the last exon of human PTPα. However, these two genes were not detected upstream of *Ci-gnrh1* or *Ci-gnrh2*. To prove this, a region of approximately 4,000 bp 3' to the FLJ20038 coding regions in *C. intestinalis* was compiled, but neither *Ci-gnrh1* nor *Ci-gnrh2* was within this distance. We also walked 4,000 bp upstream of *Ci-gnrh*1 and 2,500 bp upstream of *Ci-gnrh2* in an attempt to locate FLJ20038, but none of these fragments matched the fragments used in constructing the FLJ20038 downstream region. Areas amplified by PCR did not contain the expected products. In addition, *in silico* analysis

showed that the two genes neither were within this downstream region of the PTPα peptide nor matched any fragments used to construct the upstream regions of either *Ci-gnrh1* or *Ci-gnrh2*. Products were not amplified by PCR using the PTPα forward primer and the *Ci-gnrh1* or *Ci-gnrh2* reverse primer. Furthermore, using the Department of Energy JGI database, we identified our four genes of interest on different scaffolds: *Ci-gnrh1* on scaffold 1,051, *Ci-gnrh2* on scaffold 410, the FLJ20038 gene on scaffold 91 and the PTPα gene on scaffold 104. The distance between the genes was even greater than stated above.

3.4 Discussion

3.4.1 GnRH gene structure suggests that exon duplication preceded gene duplication in *Ciona* stem line

We show here for the first time that there are two genes for GnRH in *Ciona*. This is unusual in that vertebrates are thought to have evolved from an ancestral protochordate in which two complete genome duplications occurred. Therefore, the prediction would be for a single gene encoding a single GnRH. In contrast, each *Ciona* species has two genes, each encoding three GnRH peptides. One possible explanation is that a single GnRH gene encoding one peptide may have been present in the stem line of ancestral tunicates (at least for *Ciona*), but that exon duplication producing three peptides occurred first, followed by gene duplication.

3.4.2 The organization of GnRH mRNA is distinct in tunicates and vertebrates

The general gene structure of the *Ciona* genes is different from vertebrate GnRH genes. In most vertebrates, the first exon is non-coding and contains the 5'-untranslated region; exon two encodes the signal peptide, the GnRH decapeptide and the first portion of the GnRH-associated peptide (GAP); the third exon encodes the bulk of GAP; and the fourth and final exon encodes the last few amino acids of GAP and the 3'-untranslated region.

Here we have shown that the gene for *Ci-gnrh1* contains only one exon, which encodes a 5'UTR, possibly a signal peptide, and three GnRH peptides that are separated by intervening peptides of 13 and 8 amino acids. The GnRH peptides are followed by a candidate GAP of 69 amino acids and finally a 3'UTR. In contrast, *Ci-gnrh2* contains three exons, with a large first exon containing the 5'UTR as well as most of the coding region for the three GnRH peptides and their cut sites (RR or GKR) but no intervening peptides, as well as most of a candidate GAP. Exon 2 is 276 bp downstream and has no predictable function. The third exon is 141 bp further downstream and contains a stop codon followed by the 3'UTR.

3.4.3 Regulation of GnRH triplet peptides may enhance tunicate peptide output

In vertebrates, each GnRH peptide is coded on its own gene, allowing for separate regulation of GnRH production for each peptide. However, in *Ciona* sp. three GnRH peptides are encoded on each of two genes, suggesting that the regulation of all three of the peptides coded on one gene is the result of common gene regulation. The multiplication of exons encoding GnRH may simply increase the output of peptides. The

large number and type of promoter binding sites (identified *in silico*) shared with the human *GNRH1* gene suggests that some aspects of regulation have been conserved.

3.4.4 Analysis of the *Ciona* genome for other forms of GnRH

Our laboratory has previously identified two novel forms of GnRH, tGnRH-1 and tGnRH-2, in the protochordate tunicate *Chelyosoma productum* (Powell *et al.*, 1996). We did not find any evidence for either of these two peptides in *C. intestinalis*. Thus, the peptides appear to be genus-specific.

Two GnRH peptides, cGnRH-I and mGnRH, were reported previously to be in the gonads of *C. intestinalis* using HPLC, radioimmunoassay and mass spectrometry (Di Fiore *et al.*, 2000). We have not found evidence for these two peptides in the genome of *C. intestinalis*, in EST databases, nor using molecular techniques.

3.4.5 Tunicate GnRH genes are expressed early in development

We found that the two GnRH genes in *C. intestinalis* are expressed as early as the four-cell stage in development. Both genes are expressed, but *Ci-gnrh2* has one transcript that retains an intron. It is not clear if this is a functional mRNA, a stored mRNA or a transcript that was not completely processed at the time the tissue was collected for PCR analysis. However, we amplified the transcript with the intron retained in adult tunicate tissue as well, suggesting this is a common phenomenon. Intron retention in the salmon GnRH cDNA occurs in adult rainbow trout (Von Schalburg and Sherwood, 1999; Gray *et al.*, 2002) and mGnRH cDNA from human reproductive tissues (Dong *et al.*, 1993). However, the impact of this, if any, on regulation is not clear. GnRH is expressed early in

fish development (von Schalburg *et al.*, 1999), in human placenta (Tan and Rousseau, 1982) and in mouse embryos (Raga *et al.*, 1999), suggesting that the function of GnRH in early development is conserved in tunicates and vertebrates.

3.4.6 Significance of two genes for GnRH in protochordates

The tunicates are invertebrate chordates belonging to the subphylum Urochordata or Tunicata. Tunicates represent a branch of the phylum Chordata after the emergence of Cephalochordates (*Amphioxus*) but before vertebrates. Tunicates may represent a body plan with a minimum set of mostly single-copy genes that are needed for chordate development. We do not think our finding of two genes for GnRH refutes the concept that an ancestral tunicate had mainly single-copy genes. Instead, we propose there was one GnRH gene in ancestral tunicates and that a second GnRH gene resulted from duplication after expansion of the peptide-coding region. The two genes we have identified are different from each other in many regards, including the encoded peptides and presence or lack of introns. However, even with these differences, the similarity in number and type of promoter elements suggests conserved regulatory strategies between these two genes and between these genes and human *GNRH1* or *GNRH2*.

3.4.7 Tunicate GnRH promoters have consensus binding sites that are conserved in humans

A comparison of the tunicate and human GnRH gene promoters includes 1,000 bp for each of the genes. This area was selected because the human *GNRH1* gene has both a downstream transcription start site at +1 and the upstream start site at -579 within this 1

kb region (Dong *et al.*, 1993). In addition, *in vivo* studies have shown that transgenic mice with a human *GNRH1* construct require the promoter region from -992 to -763 bp to regulate the expression of a luciferase reporter gene in a cell-specific manner so that expression is restricted to GnRH cells mainly in the hypothalamus (Wolfe *et al.*, 2002). The rat *Gnrhl* promoter is not used for upstream comparison, as a neuron-specific enhancer is located further upstream at –1,571 to –1,863 (Whyte *et al.*, 1995), and the human and rat have marked differences in the structural organization of these promoters with the exception of the proximal region at –343 to -1 (Kepa *et al.*, 1996). Therefore, our discussion is restricted primarily to 1,000 bp of the human promoter.

Each of the tunicate or human promoter regions had an abundance of POU-family binding sites, except human *GNRH2.* POU-domain transcription factors such as Brn-2, Oct-1, Pit-1 and Tst-1 may bind to these promoter regions as they are highly expressed in the developing neural tube and adult brain (He *et al.*, 1989). In the human *GNRH1* promoter, Brn-2 binds to a site within the region essential for cell-specific expression (Wolfe *et al.*, 2002). The tunicate promoters each contain one to three Brn-2 binding sites, but it is not known if they are functional or within a cell-specific region. Oct-1, another POU factor, binds within or near to the same cell-specific region, but does not stimulate human *GNRH1* transcription of the reporter gene (Dong *et al.*, 2001; Wolfe *et al.*, 2002). For the Tst-1 transcription factor, one to three binding sites were identified for each of the four tunicate promoters, and one Tst-1 site for the human *GNRH1* promoter. In the rat *Gnrh-l* promoter, Tst-1 (referred to as SCIP/Oct-6/Tst-1) bound three sites within the proximal 333 nucleotides from the transcription start site, where rat and human *GnRH1* promoters are similar, and repressed GnRH transcription (Wierman *et al.*, 1997).

There is controversy about steroid receptor binding to the GnRH promoter for human or rat. In the tunicate GnRH genes, there are consensus sites for steroid receptors, but the *C. intestinalis* genome appears not to have genes that encode steroid receptors or genes for enzymes that synthesize steroids (Dehal *et al.*, 2002).

The CRE (cAMP response element) is interesting because this is one of only a few binding sites identified for the human *GNRH2* promoter; the human *GNRH1* promoter does not have sites for binding CREB, although there are CREBP-1 and CREBP-1/cJun sites. The transcription factor (CREB), which binds to the CRE site, is downstream from a cAMP phosphorylation cascade. The CRE site in the human *GNRH2* promoter appears to be functional as a cAMP analog upregulated expression of *GNRH2* mRNA and GnRH2 peptide (Chen *et al.*, 2001). Mutation within the CRE site at –67 to –60 reduced both the basal activity and the cAMP analog response of the *GNRH2* promoter.

3.4.8 Biological activity of GnRH in tunicates

GnRH has direct effects on gonads in some invertebrates. Five GnRH forms (mGnRH, cGnRH-I, GnRH2, sGnRH and lGnRH-I) increased mitogenic activity in gonial cells of oysters, *Crassostrea gigas* (Pazos and Mathieu, 1999). Injection of tGnRH-1 and -2 induced spawning in *C. intestinalis* generally within minutes, though tGnRH-2 was less effective than tGnRH-1 (Terakado, 2001). Each of the tGnRH peptides we tested induced gamete release, although some peptides appeared to be more effective than others, as the percent effectiveness ranged from 20 to 63 %. Each gene produces peptides that can induce the release of eggs and/or sperm. It is possible that the time of

year of our experiment did not coincide with all the animals being fully mature. Animals were selected based on visual assessment of maturity – a white sperm duct and/or a pink oviduct. GnRH may act in concert with other biological or environmental factors to induce spawning, but may not be sufficient on its own. Only one of 19 saline-injected *C. intestinalis* released eggs. The bioactivity of the peptides, including tGnRH-2 found in *Chelyosoma productum* and tGnRH-9 that we have identified in the *C. savignyi* genome, suggests that the receptor(s) in *C. intestinalis* are able to bind and activate many forms of tunicate GnRH (See Chapter 4).

3.4.9 Tunicate GnRH peptides are inactive at rat and human GnRH-I receptors

The novel tunicate GnRH-3, -4, -5, -6, -7, -8 and -9 are inactive at the human GnRH-I receptor. This was expected on the basis of the amino acid substitutions at positions 5, 6, 7 and 8. All but tunicate GnRH-2 and -7 (with 4 and 2 substitutions, respectively) have three substitutions when compared to mammalian GnRH at positions 5-8. From known studies on structure-activity relationships in mammalian systems, an L-amino acid substitution for Gly at position 6 is conformationally unfavorable. Of the nine tunicate GnRHs, only tGnRH-6 could have been predicted to have affinity for the mammalian GnRH-I receptor because of an achiral Gly residue present at that position. Whether it is the lack of a basic residue at position 8 (Ser), or the presence of a basic residue at position 5 (Lys) that determines the loss of potency at the human GnRH-I receptor is uncertain. Because GnRH2 is biologically active in mammalian systems even though it has substitutions at positions 5 (His for Tyr), 7 (Trp for Leu) and 8 (Tyr for

Arg) compared with mGnRH, it cannot be excluded that tGnRH analogs with a D-residue at position 6 would have some binding affinity for the mammalian GnRH-I receptor.

3.5 Conclusion

Tunicates of the genus *Ciona* are favourable models for study of the function and regulation of genes important in development (Corbo *et al.*, 2001). The same characteristics make *Ciona* excellent for hormone studies. Our *in silico* identification of genes encoding the hormone GnRH was confirmed by our sequencing of genomic DNA. We have shown that both of the GnRH genes are expressed as mRNA early in development and in adult *Ciona* tissue. The approach of identifying GnRH orthologs in protochordates by the nearest upstream gene does not appear to be suitable because the marker genes that are present in medaka and human are not upstream of *C. intestinalis* GnRH genes, although the genes are present in tunicate. The novel peptides do not represent potential analogs for rat or human GnRH studies because the peptides do not activate the human GnRH-I receptor. This is most likely due to the presence of an L-amino acid (in place of glycine) at position 6 in the tunicate peptides. It is accepted from structure-activity relationship studies in mammalian systems that non-glycine residues (except possibly for proline) are detrimental to biological activity (*in vitro* and *in vivo*) (Monahan *et al.*, 1973). However, this explanation of structure-activity relationships from the structural perspective cannot ignore the fact that each residue at positions 5-8 (Tyr-Gly-Leu-Arg) in mGnRH has been selected for optimal interaction with the mammalian receptor type I and that any deviation from that sequence results in significant loss of potency.

3.6 References

Adams, B. A., J. A. Tello, J. Erchegyi, C. Warby, D. J. Hong, K. O. Akinsanya, G. O. Mackie, W. Vale, J. E. Rivier and N. M. Sherwood (2003). "Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, Ciona intestinalis." Endocrinology **144**(5): 1907-19.

Burgus, R., M. Butcher, M. Amoss, N. Ling, M. Monahan, J. Rivier, R. Fellows, R. Blackwell, W. Vale and R. Guillemin (1972). "Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH-hypothalamus-LRF-gas chromatography-mass spectrometry-decapeptide-Edman degradation)." Proc Natl Acad Sci U S A **69**(1): 278-82.

Chen, A., O. Laskar-Levy, N. Ben-Aroya and Y. Koch (2001). "Transcriptional regulation of the human GnRH II gene is mediated by a putative cAMP response element." Endocrinology **142**(8): 3483-92.

Corbo, J. C., A. Di Gregorio and M. Levine (2001). "The ascidian as a model organism in developmental and evolutionary biology." Cell **106**(5): 535-8.

Dehal, P. and J. L. Boore (2005). "Two rounds of whole genome duplication in the ancestral vertebrate." PLoS Biol **3**(10): e314.

Dehal, P., Y. Satou, R. K. Campbell, J. Chapman, B. Degnan, A. De Tomaso, B. Davidson, A. Di Gregorio, M. Gelpke, D. M. Goodstein, N. Harafuji, K. E. Hastings, I. Ho, K. Hotta, W. Huang, T. Kawashima, P. Lemaire, D. Martinez, I. A. Meinertzhagen, S. Necula, M. Nonaka, N. Putnam, S. Rash, H. Saiga, M. Satake, A. Terry, L. Yamada, H. G. Wang, S. Awazu, K. Azumi, J. Boore, M. Branno, S. Chin-Bow, R. DeSantis, S. Doyle, P. Francino, D. N. Keys, S. Haga, H. Hayashi, K. Hino, K. S. Imai, K. Inaba, S. Kano, K. Kobayashi, M. Kobayashi, B. I. Lee, K. W. Makabe, C. Manohar, G. Matassi, M. Medina, Y. Mochizuki, S. Mount, T. Morishita, S. Miura, A. Nakayama, S. Nishizaka, H. Nomoto, F. Ohta, K. Oishi, I. Rigoutsos, M. Sano, A. Sasaki, Y. Sasakura, E. Shoguchi, T. Shin-i, A. Spagnuolo, D. Stainier, M. M. Suzuki, O. Tassy, N. Takatori, M. Tokuoka, K. Yagi, F. Yoshizaki, S. Wada, C. Zhang, P. D. Hyatt, F. Larimer, C. Detter, N. Doggett, T. Glavina, T. Hawkins, P. Richardson, S. Lucas, Y. Kohara, M. Levine, N. Satoh and D. S. Rokhsar (2002). "The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins." Science **298**(5601): 2157-67.

Di Fiore, M. M., R. K. Rastogi, F. Ceciliani, E. Messi, V. Botte, L. Botte, C. Pinelli, B. D'Aniello and A. D'Aniello (2000). "Mammalian and chicken I forms of gonadotropin-releasing hormone in the gonads of a protochordate, Ciona intestinalis." Proc Natl Acad Sci U S A **97**(5): 2343-8.

Dong, K. W., K. L. Yu and J. L. Roberts (1993). "Identification of a major up-stream transcription start site for the human progonadotropin-releasing hormone gene used in reproductive tissues and cell lines." Mol Endocrinol **7**(12): 1654-66.

Dong, K. W., H. M. Zheng, Z. Y. Wen and Z. G. Chen (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**(6): 2838-44.

Furlong, R. F. and P. W. Holland (2002). "Were vertebrates octoploid?" Philos Trans R Soc Lond B Biol Sci **357**(1420): 531-44.

Gray, S. L., B. A. Adams, C. M. Warby, K. R. Von Schalburg and N. M. Sherwood (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**(5): 1621-7.

He, X., M. N. Treacy, D. M. Simmons, H. A. Ingraham, L. W. Swanson and M. G. Rosenfeld (1989). "Expression of a large family of POU-domain regulatory genes in mammalian brain development." Nature **340**(6228): 35-41.

Iwakoshi, E., K. Takuwa-Kuroda, Y. Fujisawa, M. Hisada, K. Ukena, K. Tsutsui and H. Minakata (2002). "Isolation and characterization of a GnRH-like peptide from Octopus vulgaris." Biochem Biophys Res Commun **291**(5): 1187-93.

Jimenez-Linan, M., B. S. Rubin and J. C. King (1997). "Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain." Endocrinology **138**(10): 4123-30.

Kasten, T. L., S. A. White, T. T. Norton, C. T. Bond, J. P. Adelman and R. D. Fernald (1996). "Characterization of two new preproGnRH mRNAs in the tree shrew: first direct evidence for mesencephalic GnRH gene expression in a placental mammal." Gen Comp Endocrinol **104**(1): 7-19.

Kepa, J. K., A. J. Spaulding, B. M. Jacobsen, Z. Fang, X. Xiong, S. Radovick and M. E. Wierman (1996). "Structure of the distal human gonadotropin releasing hormone (hGnrh) gene promoter and functional analysis in Gt1-7 neuronal cells." Nucleic Acids Res **24**(18): 3614-20.

Matsuo, H., Y. Baba, R. M. Nair, A. Arimura and A. V. Schally (1971). "Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence." Biochem Biophys Res Commun **43**(6): 1334-9.

Monahan, M. W., M. S. Amoss, H. A. Anderson and W. Vale (1973). "Synthetic analogs of the hypothalamic luteinizing hormone releasing factor with increased agonist or antagonist properties." Biochemistry **12**(23): 4616-20.

Ohno, S. (1970). Evolution by gene duplication. Berlin, Springer-Verlag.

Ohno, S. (1996). "The notion of the Cambrian pananimalia genome." Proc Natl Acad Sci U S A **93**(16): 8475-8.

Okubo, K., H. Mitani, K. Naruse, M. Kondo, A. Shima, M. Tanaka, S. Asakawa, N. Shimizu, Y. Yoshiura and K. Aida (2002). "Structural characterization of GnRH loci in the medaka genome." Gene **293**(1-2): 181-9.

Pazos, A. J. and M. Mathieu (1999). "Effects of five natural gonadotropin-releasing hormones on cell suspensions of marine bivalve gonad: stimulation of gonial DNA synthesis." Gen Comp Endocrinol **113**(1): 112-20.

Powell, J. F., S. M. Reska-Skinner, M. O. Prakash, W. H. Fischer, M. Park, J. E. Rivier, A. G. Craig, G. O. Mackie and N. M. Sherwood (1996). "Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications." Proc Natl Acad Sci U S A **93**(19): 10461-4.

Raga, F., E. M. Casan, J. Kruessel, Y. Wen, F. Bonilla-Musoles and M. L. Polan (1999). "The role of gonadotropin-releasing hormone in murine preimplantation embryonic development." Endocrinology **140**(8): 3705-12.

Tan, L. and P. Rousseau (1982). "The chemical identity of the immunoreactive LHRH-like peptide biosynthesized in the human placenta." Biochem Biophys Res Commun **109**(3): 1061-71.

Terakado, K. (2001). "Induction of gamete release by gonadotropin-releasing hormone in a protochordate, Ciona intestinalis." Gen Comp Endocrinol **124**(3): 277-84.

von Schalburg, K. R., W. L. Harrower and N. M. Sherwood (1999). "Regulation and expression of gonadotropin-releasing hormone in salmon embryo and gonad." Mol Cell Endocrinol **157**(1-2): 41-54.

Von Schalburg, K. R. and N. M. Sherwood (1999). "Regulation and expression of gonadotropin-releasing hormone gene differs in brain and gonads in rainbow trout." Endocrinology **140**(7): 3012-24.

Whyte, D. B., M. A. Lawson, D. D. Belsham, S. A. Eraly, C. T. Bond, J. P. Adelman and P. L. Mellon (1995). "A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons." Mol Endocrinol **9**(4): 467-77.

Wierman, M. E., X. Xiong, J. K. Kepa, A. J. Spaulding, B. M. Jacobsen, Z. Fang, G. Nilaver and S. R. Ojeda (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**(3): 1652-65.

Wolfe, A., H. H. Kim, S. Tobet, D. E. Stafford and S. Radovick (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**(3): 435-49.

Zandbergen, M. A., O. Kah, J. Bogerd, J. Peute and H. J. Goos (1995). "Expression and distribution of two gonadotropin-releasing hormones in the catfish brain." Neuroendocrinology **62**(6): 571-8.

Chapter 4

Tunicate GnRH peptides selectively activate *Ciona intestinalis* GnRH receptors and the green monkey type II GnRH receptor

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JER prepared the synthetic peptides

4.1 Introduction

Gonadotropin-releasing hormone is an ancient peptide that mediates the release of luteinizing hormone and follicle-stimulating hormone from the pituitary gland, which in turn, induce gametogenesis and steriodogenesis in the vertebrate gonads. A highly conserved molecule, GnRH is 10 amino acids in length, with 24 family members identified to date (Adams *et al.*, 2003). The GnRH receptor was first cloned from a murine gonadotrope cell line, αT3-1 (Reinhart *et al.*, 1992; Tsutsumi *et al.*, 1992) and was shown to be a member of the seven transmembrane G protein-coupled receptors. It is now clear that most vertebrates each have more than one GnRHR subtype that coincides with expression of up to three GnRH ligands in each species. One human GnRH ligand is GnRH1 (aka mammalian GnRH or mGnRH); it was identified in bony fish (sturgeon) that evolved before teleosts, in lungfish, amphibians and most mammals examined (Lescheid *et al.*, 1997). The other human GnRH form is GnRH2 (aka chicken GnRH-II or cGnRH-II), which was first isolated from chicken (Miyamoto *et al.*, 1984) and is found in most jawed vertebrates from sharks to humans. Identification and characterization of each GnRHR is crucial for determining the GnRH target sites and signalling pathways. The several GnRH ligands and receptors found within *Ciona intestinalis* (a sea squirt) may be involved in a number of distinct functions.

Genes in protochordates are of interest because they represent the foundation from which vertebrates evolved. The origin of vertebrates is thought to be an ancestral protochordate such as amphioxus or a tunicate. One hypothesis is that the vertebrates evolved as a result of more than one duplication of the genome, which is supported by the presence of one cluster of Hox genes in tunicates but four clusters in mouse and human

(Dehal *et al.*, 2002). The recent sequencing of two tunicate genomes, *Ciona intestinalis* and *Ciona savignyi* (whole-genome shotgun databases located at http://www.jgi.doe.gov/ciona and http://www.broad.mit.edu/annotation/ciona, respectively) (Dehal *et al.*, 2002), has added to the debate regarding the evolutionary origin of higher chordates in general, and of GnRH and GnRHR molecules in particular. One can now examine the possible origin of a number of molecules, including GnRH and its cognate receptor that may contain conserved characteristics ancestral to those in vertebrates.

In Chapter 3, I described characterization of two genes from a tunicate, *C. intestinalis*, in which each gene encoded three distinct GnRH peptides for a total of six deduced peptides (Adams *et al.*, 2003). In addition, we characterized three other distinct tunicate GnRH peptides: one was deduced from the *Ciona savignyi* genomic sequence (Adams *et al.*, 2003) and two were isolated as peptides from *Chelyosoma productum* (Powell *et al.*, 1996). In addition, a GnRH-like peptide of 16 amino acids, which we refer to as tunicate-X, was annotated in the *C. intestinalis* genome as the homolog of a gonadoliberin-II precursor. All of the tunicate GnRH peptides are closely related to vertebrate GnRH peptides with high conservation of key amino acids. The apparent lack of steroid receptors and key enzymes essential for sex steroid synthesis in the *Ciona* genome (Dehal *et al.*, 2002) precludes GnRH from an involvement in stimulation and release of reproductive steroids. Our laboratory previously detected estradiol in tunicate gonads by radioimmunoassay (RIA) (Craig *et al.*, 1997), but tunicate extracts may have interfered with the assay designed for human steroids. In tunicates, GnRH is evidently involved in reproductive functions and may act directly on the gonads and/or gonoducts instead of stimulating the hypophyseal release of LH and FSH, as in higher chordates.

Previous studies by our lab are consistent with findings by Terakado that have shown that injection with physiological concentrations of tunicate GnRHs into gravid *C. intestinalis* induces the release of gametes from the atrial siphon (Terakado, 2001; Adams *et al.*, 2003). These injections may mimic the release of tunicate GnRH located in nerves that terminate in a blood sinus near both the testis and ovary (Mackie, 1995).

Given that native GnRH peptides are bioactive in *C. intestinalis*, I was interested in establishing if these peptides transmit their signal through a GnRH receptor similar to those possessed by vertebrates. I found four putative GnRHR homologs encoded in the *C. intestinalis* genome. These *Ciona* receptors possess a feature common to non-mammalian and type II mammalian GnRHRs, which is a long carboxyl-terminal tail required for membrane expression, rapid desensitization and receptor internalization. In contrast, the type I mammalian GnRHR lacks a tail. Two of the GnRH receptors were previously identified by cDNA structure for *C. intestinalis* (Kusakabe *et al.*, 2003), but the receptors were only tested with two GnRHs from *Chelyosoma productum*. It is vital to determine if *Ciona* receptors stimulate inositol phosphate turnover similar to the type I mammalian pituitary GnRHRs. It is also important to determine whether the receptors are activated by GnRH, as a recent report showed that a receptor isolated from Drosophila appeared to be structurally related to a GnRHR, but was activated by adipokinetic hormone (AKH) rather than by GnRH (Staubli *et al.*, 2002).

In this study, I examined whether tunicates have functional GnRH receptors. I provide the cDNA sequences for four putative GnRH receptors from *Ciona intestinalis* and compare these sequences to the genomic structure to establish the gene organization and exon boundaries. Two of the receptor sequences are novel and the other two

sequences confirm the structures of a previous report (Kusakabe *et al.*, 2003). To determine receptor function, I expressed each receptor in COS7 cells, incubated them with each of nine native tunicate GnRHs, one novel GnRH (tunicate-X) and one related peptide (AKH). I tested receptor activation by assaying the levels of GnRH-induced second messengers: inositol phosphates and cAMP. I also examined whether critical motifs are conserved for ligand binding, G protein-coupling and receptor activation. Hereafter, I use the abbreviated name *Ciona* for the species *Ciona intestinalis*; if other species of *Ciona* are mentioned, the full genus and species are given.

4.2 Materials and Methods

4.2.1 Gene organization

Gene arrangements were discovered initially using the database from the Department of Energy Joint Genome Institute's *C. intestinalis* genome project (http://www.jgi.doe.gov/programs/ciona.htm) and N. Satoh and Y. Satou's Ghost *C. intestinalis* cDNA database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). Human, rat and horse GnRHR amino acid sequences were used to search the available TBLASTN input form. Using the default parameters (BLOSUM 60 matrix), each search generated closely matched fragments. The DNA regions for the matching fragments of four putative receptors were compiled, examined for exon/intron boundaries and analyzed for a complete open reading frame. Primers were designed and the complete receptor cDNAs were amplified using PCR and sequenced as stated below. To complete any missing regions in the receptor open-reading frames (ORF), 5' or 3' RACE was used.

4.2.2 Animals

Adult *C. intestinalis* (subphylum Tunicata, class Ascidiacea) were obtained from Woods Hole Biological Station and treated under the guidelines of the Animal Care Committee at the University of Victoria. The tissues were dissected and frozen in liquid nitrogen.

4.2.3 Isolation of mRNA and synthesis of cDNA

The mRNA was isolated from tissues and embryos using a Micro Poly(A) Pure mRNA isolation kit (Ambion). Then mRNA was reverse-transcribed in a 50 μl reaction that contained mRNA, 2mM oligo dT, 2mM deoxynucleoside triphosphates, 1x first strand reaction buffer, 0.01 M dithiothreitol, 5 U RNase inhibitor, and 100 U Superscript II reverse transcriptase (Invitrogen). The reaction was incubated at 42 °C for 90 min, and the enzyme was heat-inactivated at 90 °C for 10 min. To check for genomic DNA contamination, a negative reverse transcription reaction was run for each tissue sample with no reverse transcriptase.

For RACE-PCR, 250 ng of mRNA was used to prepare RACE-ready cDNA using the RLM-RACE kit (Ambion) according to the manufacturer's instructions, except that the DNA was dissolved in distilled water.

4.2.4 PCR and sequencing of cDNA

Oligonucleotide primers were designed to regions encoding candidate GnRHRs based on the compiled sequences for *Ciona* GnRHR genes 1-4. Each 50-μl reaction contained 2.5 U Platinum *Taq* polymerase High Fidelity (Invitrogen), 1x High Fidelity

PCR buffer, 2.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates (Invitrogen), and 0.4 μM of each Koz forward (f) and Stop reverse (r) primer (Table 4. 1). PCRs were performed under the following conditions: initial denaturation at 94 °C for 2 min, 33 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 2 min, and a 5-min final extension. The PCR amplicons were separated by electrophoresis on a 1.3 % (w/v) agarose gel and visualized with ethidium bromide staining using an Eagle Eye II still video system (Stratagene). Bands were selected, isolated (Qiagen), and cloned or cloned directly as amplicons into pGEM Vector-T (Promega) and sequenced. The SequiTherm EXCEL II DNA sequencing Kit utilizing the Sanger sequencing method was performed by the University of Victoria Sequencing Centre. Each gene was amplified using M13 forward and reverse priming sites present on pGEM-T and sequenced on a LI-COR 4200 – Global IR^2.

4.2.5 PCR for tissue distribution

The tissue expression primer pairs (Table 4.1) were used in each tissue expression PCR reaction. Each 50-μl reaction contained 1 U Platinum *Pfx* DNA polymerase (Invitrogen), 1X *Pfx* Amplification buffer, 1 mM $MgSO_4$, 0.3 mM deoxynucleoside triphosphates (Invitrogen), and 0.4 μM of each forward (f) and reverse (r) primer. PCRs were performed under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 68 °C for 2 min 30 s, and a 3-min final extension. The PCR amplicons were separated by electrophoresis on a 1.3 % (w/v) agarose gel as stated above.

Table 4.1. Primers used to identify GnRHR genes in *Ciona intestinalis.*

Primer	Sequence (5'-3')	Direction	Target
ciGnRHR1Koz	ATAATGGTGACGTCAGACATCTCCCAA	f	*ciGnRHR-I*
ciGnRHR1Stop	TCACAGGGCCGACTCGGAGCTCA	r	*ciGnRHR-I*
ciGnRHR2Koz	CGTATAGAAATGGCTACGGCA	f	*ciGnRHR-II*
ciGnRHR2Stop	GTTATAATTGAGCAATTACAGAGACGAGT	r	*ciGnRHR-II*
ciGnRHR3Koz	AGCTGCGTCATGGCGACAGTTA	f	*ciGnRHR-III*
ciGnRHR3Stop	CACGCACGGAGGGAAGTCTGT	r	*ciGnRHR-III*
ciGnRHR4Koz	GTCATGGTGACGTCACCAACTA	f	*ciGnRHR-IV*
ciGnRHR4Stop	ATTAAACCAATGCATCAACCC	r	*ciGnRHR-IV*
ciGnRHR1 f TE	ATAATGGTGACGTCAGACATCTCCCAA	f	*ciGnRHR-I*
ciGnRHR1 r TE	TCACAGGGCCGACTCGGAGCTCA	r	*ciGnRHR-I*
ciGnRHR2 f TE	TTTTATTAAGTTGAACGATCTGAGT	f	*ciGnRHR-II*
ciGnRHR2 r TE	GCCGGTTTGTTGTTGTGATTATTTTTAC	r	*ciGnRHR-II*
ciGnRHR3 f TE	TGCTTCATGGCGACAGTTAGTTC	f	*ciGnRHR-III*
ciGnRHR3 r TE	TGGAATAAGGAAGCTCACACACA	r	*ciGnRHR-III*
ciGnRHR4 f TE	TGGCTGGAAATCTAACCGTGCT	f	*ciGnRHR-IV*
ciGnRHR4 r TE	TAAGGTCCCCAACAAATAACGA	r	*ciGnRHR-IV*

Abbreviations: f = forward; r = reverse; Koz = Kozak; TE= tissue expression.

4.2.6 Receptor and green fluorescent protein mRNA vector design

A clone for green monkey type II GnRHR (gm-GnRHR) was a gift from Dr. Jimmy Neill, University of Alabama. The cDNAs containing full-length open reading frames of each *Ci-gnrhr* and *gm-gnrhr* were altered to include a Kozak sequence (A/G)NNATGG) (where N is any amino acid) using the same PCR conditions as mentioned above (Kozak, 1996) and then cloned into pcDNA3.1(-) (Invitrogen) in front of an internal ribosome entry site and an enhanced green fluorescent protein (eGFP) coding region. This system generates co-expression of the receptor protein and eGFP allowing for live selection of COS7 cells with high transfection efficiency. In addition, we fused the eGFP coding sequence directly to the 3' end of the ORFs for *Ci-gnrhr-1* and *-4* to verify that the receptors were expressed as a protein. After I determined that Ci-GnRHR-4 was not bioactive in our assays, I used an alternate Kozak sequence (GCCACCatgG) for the translation start site for *Ci-gnrhr-4* and for the cDNA fused directly to eGFP to verify that the receptor was translated into protein.

4.2.7 Inositol phosphate accumulation assay

No *Ciona* cell lines were available to facilitate our receptor signalling assays instead I used COS7 cells, which have been well utilized in previous GnRH receptor assays (Okubo *et al.*, 2001; Wang *et al.*, 2001; Fujii *et al.*, 2004). COS7 cells (Invitrogen) were seeded and grown into monolayer cultures in T-175 cm^2 flasks in growth medium containing Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 0.1 mM non-essential amino acids (Invitrogen) and 10 % fetal bovine serum (Invitrogen) at 37 °C in 5 % CO_2. After 3 days, the monolayers were trypsinized and seeded in 24-well tissue

culture treated plates (Corning-Costar Corp., Cambridge, MA) at a density of 65,000 cells per well and grown overnight in growth medium. At 85-95 % confluence, usually 24 h post seeding, the cells were washed and incubated with serum-free medium (VP-SFM, Invitrogen), then transfected with 0.8 μg/well of receptor encoded plasmid DNA using lipofectamine according to the manufacturer's protocol. After another 24 h, the cells were washed with labelling medium (Medium 199, Invitrogen) containing 0.3 % bovine albumin (Sigma) and subsequently labelled with 0.9 μCi/well of *myo*-[2-^{3}H]-Inositol (Amersham) in labelling medium for approximately 24 h. The wells were screened for high transfection efficiency by comparing cellular levels of eGFP using an inverted microscope with a FITC filter set. The cells were washed and pre-incubated for 30 min at 37 °C in labelling medium containing 20 mM LiCl and then incubated with various concentrations of ligands (Table 4.2) for 1 h at 37 °C with gentle agitation. The medium in each well was then removed and 200 μl of 0.1 M formic acid was added to lyse the cells. Quantification of inositol phosphates (IPs) was performed in cell extracts by the multi-well filtration method (Chengalvala *et al.*, 1999).

4.2.8 cAMP accumulation assay

COS7 cells were grown, seeded and transfected in 24-well plates as described above. The cells were allowed to grow in VP-SFM for 48 h post-transfection, after which wells with high transfection efficiency were selected. These cells were washed with Hank's Buffered Salt Solution (HBSS) supplemented with 20 mM HEPES and 300 μM IBMX. The cells were pre-incubated for 15 min at 37 °C and then incubated with various concentrations of ligands (Table 4.2) for 1 h at 37 °C with gentle agitation. Intracellular

Table 4.2. Amino acid sequences for peptides tested in *Ciona* GnRHR activation assay.

Peptide	Sequence
Tunicate-1	pQHWSDYFKPG-NH_2
Tunicate-2	pQHWSLCHAPG-NH_2
Tunicate-3	pQHWSYEFMPG-NH_2
Tunicate-4	pQHWSNQLTPG-NH_2
Tunicate-5	pQHWSYEYMPG-NH_2
Tunicate-6	pQHWSKGYSPG-NH_2
Tunicate-7	pQHWSYALSPG-NH_2
Tunicate-8	pQHWSLALSPG-NH_2
Tunicate-9	pQHWSNKLAPG-NH_2
Tunicate-X	pQHWSNWWIPGAPGYNG-NH_2
GnRH2	pQHWSHGWYPG-NH_2
BomAKH	pQLTFTSSWG-NH_2

Two tunicate GnRH peptides were identified in *C. productum* (Tunicate-1 and -2), seven forms were identified in *C. intestinalis* and *C. savignyi* (Tunicate-3 thru Tunicate-9), mammalian GnRH2, and *Bombyx mori* Adipokinetic Hormone (BomAKH).

cAMP concentrations were measured using a cAMP direct enzyme immunoassay following the manufacturer's protocol (Amersham).

4.2.9 Data Analysis

All IP samples were measured in triplicate and cAMP samples in duplicate within each assay. All assays were repeated in at least three independent experiments. Data analysis was performed using nonlinear regression. The GnRH concentrations inducing half-maximal stimulation (EC_{50}) were calculated using PRISM3 software (GraphPAD). The GnRH concentrations inducing half maximal stimulation (EC_{50}) presented in Tables 4.3 and 4.4 were calculated from the mean $\pm$ SEM of at least three independent experiments. The data were analyzed by one-way ANOVA followed by Newman-Keuls test. $P < 0.05$ was considered statistically significant.

4.2.10 Phylogenetic Analysis

The deduced amino acid sequences of the four *Ciona* GnRHRs were aligned with GnRHR and GnRHR-like proteins from other species using the ClustalW program. The sequences were: two human *Homo sapiens* GnRHRs type I (GenBank Accession Number NP000397) and type II (Q96P88); rubber eel *Typhlonectes natans* GnRHR (NF174481); African clawed frog *Xenopus laevis* type II GnRHR (AF257320); bullfrog *Rana catesbeiana* GnRHR-3 (AF144062); house mouse *Mus musculus* type I GnRHR (NP034453); African green monkey *Cercopithecus aethiops* type II GnRHR (Q95MH6); striped sea bass *Marone saxitilis* GnRHR (AAF28464); fruitfly *Drosophila melanogaster* adipokinetic hormone receptor (AF077299); and the human *Homo sapiens* alpha-1

Table 4.3. Inositol phosphate accumulation after incubation of various ligands with *Ciona* GnRHRs and the green monkey GnRHR (Gm-GnRHR).[1]

	Inositol Phosphate Accumulation EC_{50} (logM)[2]				
Ligand	Ci-GnRHR1	Ci-GnRHR2	Ci-GnRHR3	Ci-GnRHR4	Gm-GnRHR
tGnRH-1	-	-	-	-	-
tGnRH-2	-	-	-	-	-
tGnRH-3	-	-	-	-	$-5.41 \pm 0.18_a$
tGnRH-4	-	-	-	-	-
tGnRH-5	-	-	-	-	$-5.55 \pm 0.26_a$
tGnRH-6	-6.90 ± 0.17	-	-	-	-
tGnRH-7	-	-	-	-	-
tGnRH-8	-	-	-	-	-
tGnRH-9	-	-	-	-	-
tGnRH-X	-	-	-	-	-
GnRH2	-	-	-	-	$-8.91 \pm 0.21_a$
BomAKH	-	-	-	-	-

[1] IP measurements in COS7 cells, which had been transfected with receptor cDNA 48 h earlier. Also added to the cells was 0.9 µCi/well *myo*-[2-^{3}H]-inositol at 24 h and GnRH peptides at 1 h before measurements.

[2] Dose of peptide stimulating half-maximal IP response (EC_{50})

Data were derived from the means of triplicate samples from three independent experiments.

For each GnRH peptide, doses resulting in responses that are not different from one another are identified by the same subscript letter ($P < 0.05$)

Table 4.4. cAMP accumulation after incubation of various ligands with *Ciona* GnRHRs.[1]

	cAMP Accumulation EC_{50} (logM)[2]			
Ligand	Ci-GnRHR1	Ci-GnRHR2	Ci-GnRHR3	Ci-GnRHR4
tGnRH-3	$-6.02 \pm 0.05_{bc}$	$-4.77 \pm 0.42_{a}$	$-8.84 \pm 0.21_{ab}$	-
tGnRH-4	$-5.34 \pm 0.39_{c}$	$-5.93 \pm 0.10_{a}$	$-5.34 \pm 0.34_{c}$	-
tGnRH-5	$-5.95 \pm 0.17_{bc}$	$-5.09 \pm 0.38_{a}$	$-9.51 \pm 0.19_{a}$	-
tGnRH-6	$-9.01 \pm 0.12_{a}$	$-6.95 \pm 0.11_{a}$	$-6.06 \pm 0.27_{c}$	-
tGnRH-7	$-7.12 \pm 0.13_{bc}$	$-7.41 \pm 0.12_{a}$	$-5.77 \pm 0.11_{c}$	-
tGnRH-8	$-6.97 \pm 0.07_{bc}$	$-7.44 \pm 0.09_{a}$	$-5.22 \pm 0.27_{c}$	-
tGnRH-X	-	-	-	-
GnRH2	$-7.68 \pm 0.11_{b}$	$-5.85 \pm 0.09_{a}$	$-7.34 \pm 0.13_{bc}$	
BomAKH	-	-	-	-

[1] cAMP measurement of cell content in COS7 cells, which had been transfected with *Ciona* receptor cDNA 48 h earlier and treated with GnRH peptides for 1 h before measurements.

[2] Dose of GnRH stimulating half-maximal cAMP response (EC_{50}).

Data were derived from the means $\pm$ SEM of duplicate samples, from three independent experiments.

Means in the same column that are significantly different are indicated by different subscript letters. For each GnRH peptide, doses resulting in responses that are not different from one another are identified by the same subscript letter ($P < 0.05$)

adrenergic receptor (AAQ91331) as an outgroup. The phylogenetic tree was generated based on the ClustalW alignment using a topological algorithm with PHYLIP software available at the EMBnet Node: http://www.genebee.msu.su/emb.html. Branch lengths are to scale.

4.2.11 GnRH peptide synthesis

Ten tunicate GnRH peptides (Table 4.2) were synthesized automatically on a CS-Bio Peptide Synthesizer (Model CS536, CS Bio Co., Inc. San Carlos, CA) on a methyl benzhydrylamine resin using the Boc-strategy at the Salk Institute. The peptides were cleaved with hydrofluoric acid, concomitantly deprotected and then purified as described by Adams et al. (2003).

4.3 Results

4.3.1 Two novel *Ciona* cDNAs encode putative seven transmembrane GnRH receptors

In silico analysis revealed four candidate GnRHR genes in *Ciona*. Each of four cDNAs, encoding distinct GnRHR-like receptors, was amplified from poly(A)$^+$ mRNA extracted from the neural complex by RT-PCR. Two GnRHRs were unique sequences that did not match any known GnRHR proteins to date. The other two GnRHR cDNAs were similar to previously identified *Ciona* GnRHRs (Kusakabe *et al.*, 2003) in which one had 98 % amino acid identity with Ci-GnRHR1 (AB103333) and the other had 96 % amino acid identity with Ci-GnRHR2 (AB103334). Our two novel GnRHRs were designated Ci-GnRHR3 and Ci-GnRHR4. The isolated *Ci-gnrhr1* confirmed a 2,043 bp

cDNA encoding a 450 amino acid protein, whereas the cDNA for *Ci-gnrhr2* was 1,675 bp encoding a protein of 401 amino acids. The novel full-length *Ci-gnrhr3* cDNA consisted of 1,752 bp encoding a 454 amino acid protein, and the novel *Ci-gnrhr4* cDNA consisted of 1,666 bp encoding a protein of 366 amino acids. The four *Ciona* GnRHR cDNAs encoded proteins with the hydrophobicity profile characteristic of GPCRs: seven TMDs connected by alternating intracellular and extracellular loops, and extracellular N-terminal and intracellular C-terminal domains (Figure 4.1).

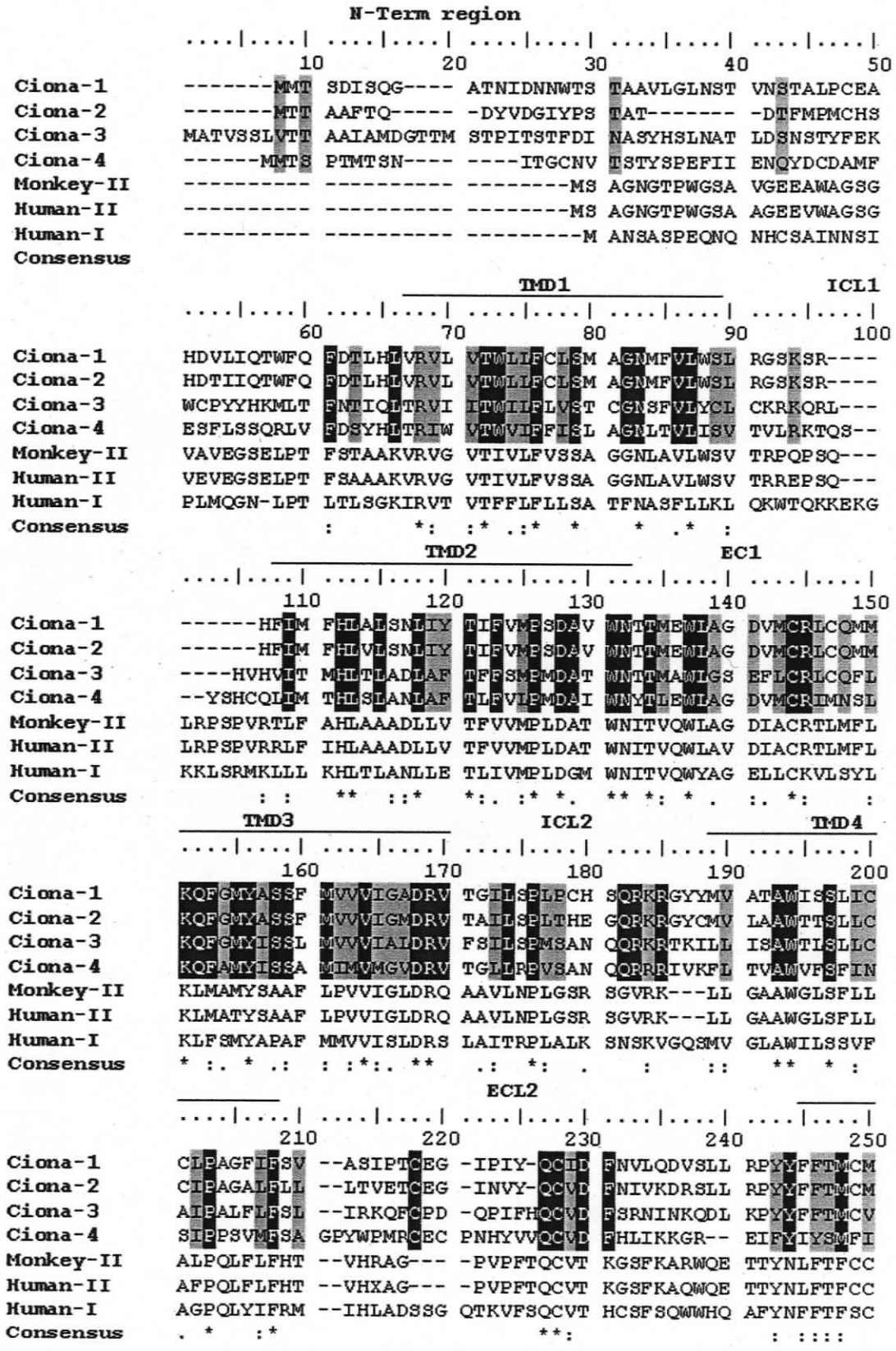
To establish the identity of the receptors, the four *Ciona* GnRHR-like amino acid sequences were entered into the TBLASTN input form and searched against the NCBI databases. The sequence identity of each *Ciona* GnRHR was compared to each other (Table 4.5). The highest matches of the novel receptors to other GnRHRs were to the rubber eel GnRHR (AAD49750) in which Ci-GnRHR3 and Ci-GnRHR4 exhibited 36 % and 52 % amino acid identity, respectively.

The nucleotide sequences for the two novel *Ciona* GnRHR-like cDNAs, *Ci-gnrhr3* and *Ci-gnrhr4,* are in the GenBank Nucleotide Database under GenBank Accession Numbers AY742890 and AY742891. The open reading frames for *Ci-gnrhr1* and Ci-gnrhr2 that we identified are submitted under the GenBank Accession Numbers AY742888 and AY742889, respectively.

4.3.2 *Ciona* GnRH receptor genes each maintain consistent exon arrangement but not intron size

The receptor cDNAs were aligned with the *C. intestinalis* genome to determine the intron/exon boundaries. This technique revealed gene sizes of 8378 bp, 7419 bp,

Figure 4.1. Comparison and consensus of the deduced amino acid sequence of *Ciona* GnRHR with representative vertebrate GnRH receptors. Amino acid sequences were aligned using the ClustalW program. Dashes indicate gaps introduced in the sequence to optimize the alignment. The putative transmembrane domains (TMD1-TMD7) are indicated by horizontal bars above the alignment. Identity among the four Ci-GnRHRs is shown by black; similarity is shown by gray shading. Within the consensus sequence, an asterisk indicates the position in which all receptor sequences exhibit the same amino acid, a colon indicates the position in which all residues share a similar amino acid and a dot indicates a position that is shared between two distinct amino acids.


N-Term region
TMD1
ICL1
TMD2
EC1
TMD3
ICL2
TMD4
ECL2
Ciona-1
Ciona-2
Ciona-3
Ciona-4
Monkey-II
Human-II
Human-I
Consensus

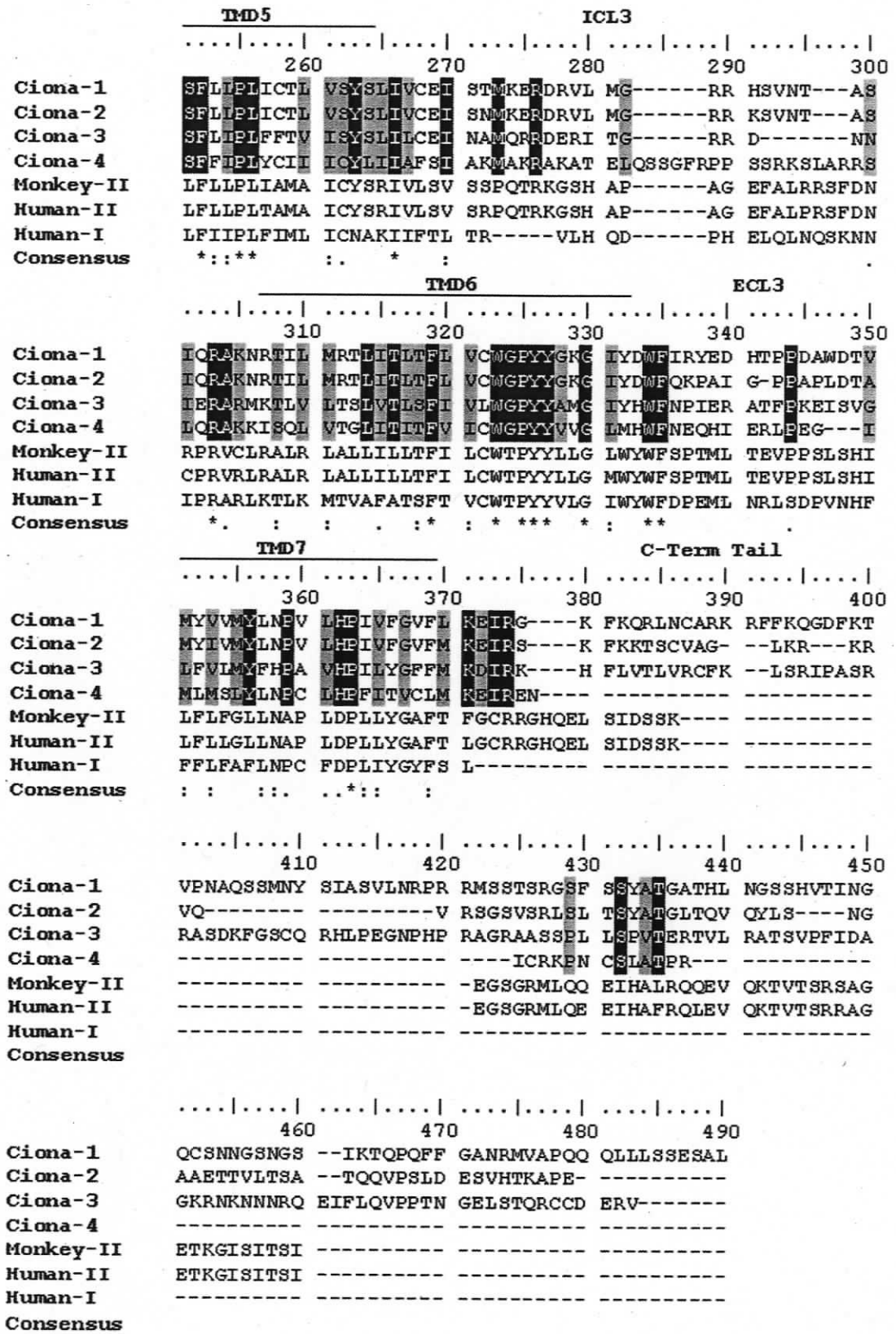

TMD5
ICL3
260 270 280 290 300
Ciona-1 SFLIPLICTL VSYSLIVCEI STMKERDRVL MG------RR HSVNT---AS
Ciona-2 SFLIPLICTL VSYSLIVCEI SNMKERDRVL MG------RR KSVNT---AS
Ciona-3 SFLIPLFFTV ISYSLILCEI NAMQRRDERI TG------RR D-------NN
Ciona-4 SFFIPLYCII ICYLIIAFSI AKMAKRAKAT ELQSSGFRPP SSRKSLARRS
Monkey-II LFLLPLIAMA ICYSRIVLSV SSPQTRKGSH AP------AG EFALRRSFDN
Human-II LFLLPLTAMA ICYSRIVLSV SRPQTRKGSH AP------AG EFALPRSFDN
Human-I LFIIPLFIML ICNAKIIFTL TR-----VLH QD------PH ELQLNQSKNN
Consensus
TMD6
ECL3
310 320 330 340 350
Ciona-1 IQRAKNRTIL MRTLITLTFL VCWGPYYGKG IYDWFIRYED HTPPDAWDTV
Ciona-2 IQRAKNRTIL MRTLITLTFL VCWGPYYGKG IYDWFQKPAI G-PPAPLDTA
Ciona-3 IERARMKTLV LTSLVTLSFI VLWGPYYAMG IYHWFNPIER ATFPKEISVG
Ciona-4 LQRAKKISQL VTGLITITFV ICWGPYYVVG LMHWFNEQHI ERLPEG---I
Monkey-II RPRVCLRALR LALLILLTFI LCWTPYYLLG LWYWFSPTML TEVPPSLSHI
Human-II CPRVRLRALR LALLILLTFI LCWTPYYLLG MWYWFSPTML TEVPPSLSHI
Human-I IPRARLKTLK MTVAFATSFT VCWTPYYVLG IWYWFDPEML NRLSDPVNHF
Consensus
TMD7
C-Term Tail
360 370 380 390 400
Ciona-1 MYVVMYLNPV LHPIVFGVFL KEIPG----K FKQRLNCARK RFFKQGDFKT
Ciona-2 MYIVMYLNPV LHPIVFGVFM KEIPS----K FKKTSCVAG- --LKR---KR
Ciona-3 LFVLMYFHPA VHPILYGFFM KDIPK----H FLVTLVRCFK --LSRIPASR
Ciona-4 MLMSLYLNPC LHPFITVCLM KEIPEN---- ---------- ----------
Monkey-II LFLFGLLNAP LDPLLYGAFT FGCRRGHQEL SIDSSK---- ----------
Human-II LFLLGLLNAP LDPLLYGAFT LGCRRGHQEL SIDSSK---- ----------
Human-I FFLFAFLNPC FDPLIYGYFS L--------- ---------- ----------
Consensus
410 420 430 440 450
Ciona-1 VPNAQSSMNY SIASVLNRPR RMSSTSRGSF SSYATGATHL NGSSHVTING
Ciona-2 VQ-------- ---------V RSGSVSRLSL TSYATGLTQV QYLS----NG
Ciona-3 RASDKFGSCQ RHLPEGNPHP RAGRAASSPL LSPVTERTVL RATSVPFIDA
Ciona-4 ---------- ---------- ----ICRKPN CSLATPR--- ----------
Monkey-II ---------- ---------- -EGSGRMLQQ EIHALRQQEV QKTVTSRSAG
Human-II ---------- ---------- -EGSGRMLQE EIHAFRQLEV QKTVTSRRAG
Human-I ---------- ---------- ---------- ---------- ----------
Consensus
460 470 480 490
Ciona-1 QCSNNGSNGS --IKTQPQFF GANRMVAPQQ QLLLSSESAL
Ciona-2 AAETTVLTSA --TQQVPSLD ESVHTKAPE- ----------
Ciona-3 GKRNKNNNRQ EIFLQVPPTN GELSTQRCCD ERV-------
Ciona-4 ---------- ---------- ---------- ----------
Monkey-II ETKGISITSI ---------- ---------- ----------
Human-II ETKGISITSI ---------- ---------- ----------
Human-I ---------- ---------- ---------- ----------
Consensus

Table 4.5. Amino acid identity among *C. intestinalis* GnRHRs

1	*2*	*3*	*4*		
100	70	38	36	1	Ci-GnRHR1
	100	41	37	2	Ci-GnRHR2
		100	33	3	Ci-GnRHR3
			100	4	Ci-GnRHR4

Amino acid identity was determined using the CLUSTAL W alignment program.

15795 bp, and 5960 bp for *Ci-gnrhr1*, *Ci-gnrhr2*, *Ci-gnrhr3* and C*i-gnrhr4*, respectively. Each gene for *Ci-gnrhr1*, *Ci-gnrhr2*, and *Ci-gnrhr3* consists of eight exons separated by seven introns, whereas the *Ci-gnrhr4* gene contains seven exons separated by six introns (Figure 4.2). The corresponding sizes of exons three, four and five are similar for all *Ciona.*GnRHRs. The 3' exon deviates most in size among the four *Ci-gnrh* receptor transcripts.

4.3.3 *Ciona* GnRH receptor proteins have conserved domains like vertebrate GnRHRs

The four amino acid sequences of *Ciona* GnRHRs were aligned with type I human, type II human and type II monkey GnRHRs (Figure 4.1). There is high conservation in all seven putative TMD regions with some conservation in intracellular loop 2 and extracellular loops (ECL) 1 and 2. Three of the *Ciona* GnRHRs possess two potential glycosylation sites in the N- terminal domain (at positions 16 and 27 for Ci-GnRHR1; 31 and 44 for Ci-GnRHR3; 10 and 15 for Ci-GnRHR4). The N-terminal extracellular region and C-terminal tail show no conservation with the vertebrate receptors. Ci-GnRHR1, Ci-GnRHR2 and Ci-GnRHR3 each possess a C-terminal tail longer than the monkey type II receptor. Ci-GnRHR4 has a short 18 amino acid C-terminal tail more analogous in length to type I mammalian GnRHRs.

Ci-GnRHRs share conserved residues and motifs with vertebrate GnRHRs; the conserved residues for Ci-GnRHR1 are highlighted in Figure 4.3. Several previously characterized ligand binding residues are conserved in all tunicate GnRHRs: Asp (D) in TMD2, Trp (W) and Arg (R) positioned in ECL1, and Lys (K) in TMD3. Also, Ci-

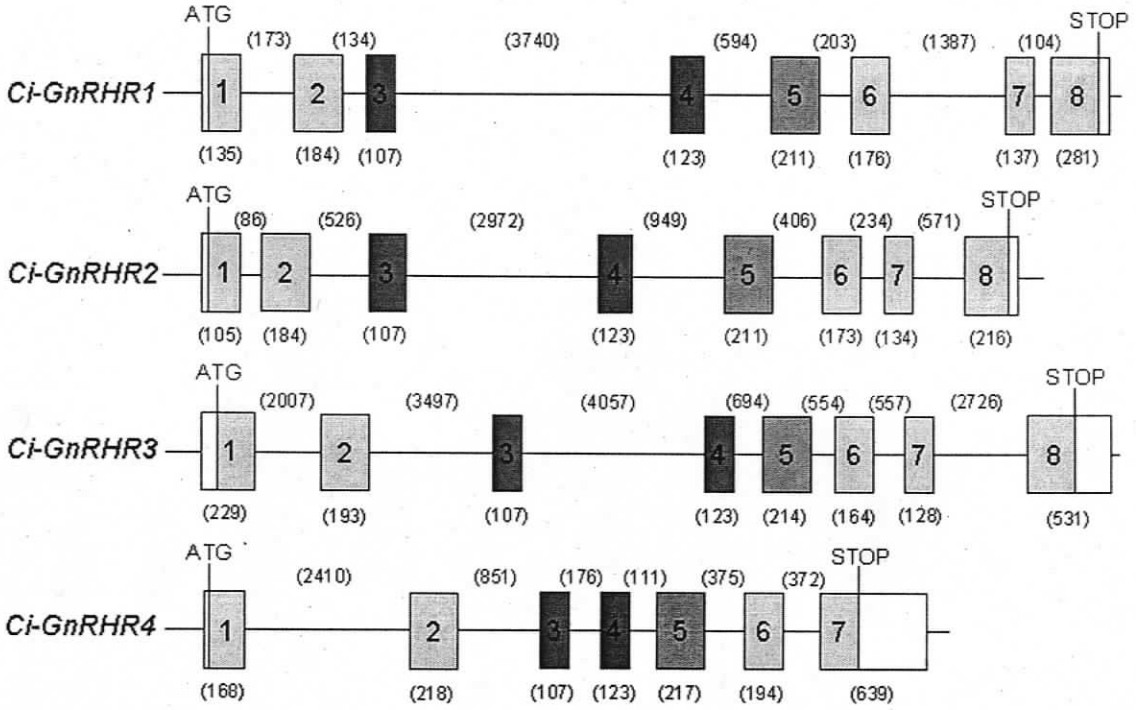


Figure 4.2. Schematic of four *Ci-gnrhr* genes present in the *Ciona* genome. Shading of exons 3 and 4 indicates completely conserved exon size between each gene. Shading of exon 5 indicates highly conserved exon size between each gene. Exons are boxed with size shown below (in base pairs) and intron size shown above. Scale is approximate.

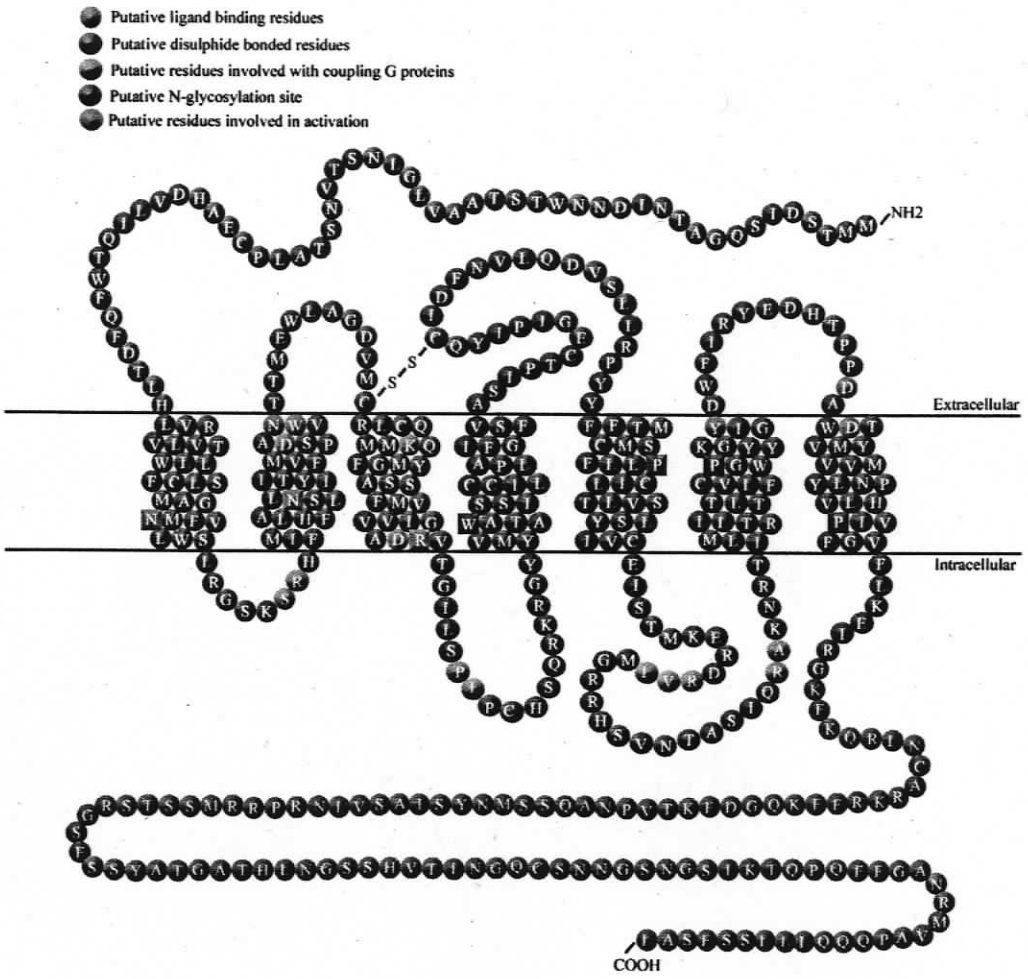


Figure 4.3. Two-dimensional representation of the *Ciona* GnRH receptor-1. The schematic shows the transmembrane domains within the extracellular and intracellular environments. Squared residues are used as reference points for the consensus numbering of the rhodopsin family of GPCRs. Conserved residues with other characterized GnRHRs are highlighted (see key).

GnRHRs have two highly conserved Cys (C) residues in ECL1 and ECL2. Highly conserved microdomains that may be involved with G protein activation are present, with Asn (N) in TMD1, Asp (D) or Asn (N) in TMD2, and the DRxxxI/V motif within and adjacent to TMD3. This motif is modified to DRxxxL in Ci-GnRHR4.

4.3.4 The four GnRH receptor transcripts are widely distributed in adult *Ciona*

Transcripts for each *Ciona* GnRHR were found in the neural complex, gonad, heart, intestine, endostyle and branchial sac (Figure 4.4). The control reaction that lacked template was negative for all four receptor transcripts. Cloning the *Ci-GnRHR3* revealed a splice variant present in the neural complex of mature specimens. The splice variant molecule named C*i-gnrhrΔ3* contains a 271 bp deletion in exons 1 and 2 (from nucleotide positions 213 to 484). Comparison of C*i-gnrhrΔ3* coding regions with the full-length *Ciona* GnRHRs reveals that this deletion is predicted to cause a shift in the open reading frame, leading to protein truncation eight amino acids downstream from the splice site, resulting in an incomplete receptor protein.

4.3.5 Only one *Ciona* GnRH receptor activates the IP signalling pathway

To determine whether each C*i-gnrhr* cDNA encoded a functional GnRHR, we expressed each receptor cDNA in COS7 cells. The control and transfected COS7 cells were exposed to 10^{-5} to 10^{-11} M concentrations for each of the ten *Ciona* GnRH peptides and the related peptides (Table 4.2). Untransfected COS7 cells were unable to elicit a dose response with either IP or cAMP with any of the peptides tested. Tunicate (t) GnRH-6 was able to stimulate IP accumulation in Ci-GnRHR1 (Figure 4.5A). None of

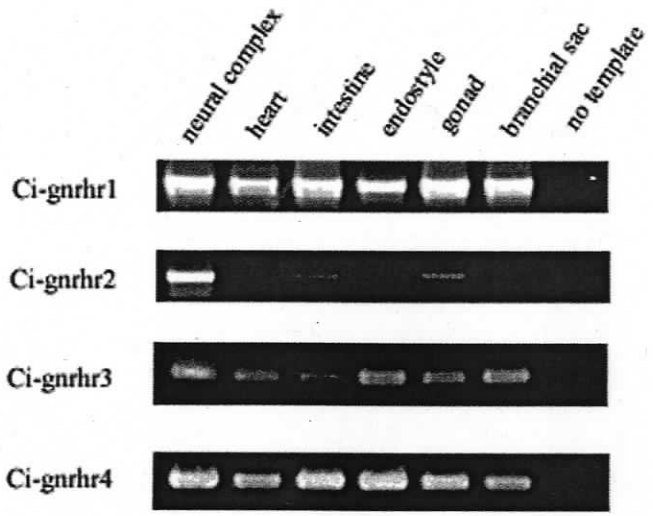


Figure 4.4. Tissue expression of *Ciona* GnRHR cDNAs using RT-PCR. Two control reactions were also run: one negative (no template DNA) for each primer set and one positive control (α-tubulin) for each tissue examined (results not shown). A faint second band can be seen in the *Ci-gnrhr3* lanes and has been sequenced and shown to be the *Ci-gnrhr3* splice variant *Ci-gnrhr3*Δ.

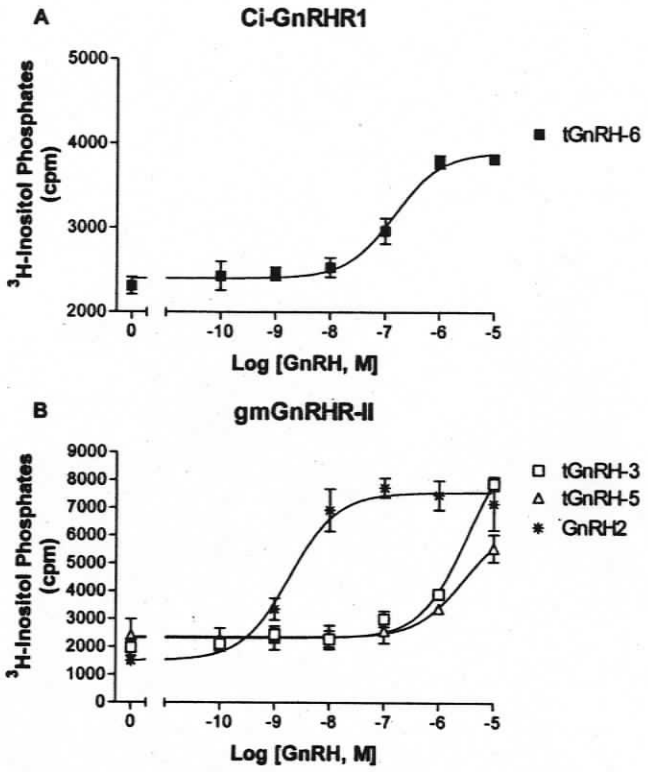


Figure 4.5. Inositol phosphate accumulation in COS7 cells expressing either Ciona or green monkey GnRHR induced with GnRHs. *myo*-[2-^{3}H]-inositol phosphate accumulation in cells expressing either Ci-GnRHR (A) or gm-GnRHR (B) induced with various concentrations of indicated peptide for one hour. Cells were transiently transfected with 0.8 μg of indicated GnRHR cDNA. Labelled inositol was added 24 h post-transfection, and cells were stimulated 48 h post-transfection. The results are from a single experiment representative of a total of three or more.

the ligands tested were able to stimulate ^{3}H-IP accumulation with the other Ci-GnRHRs (Table 4.4).

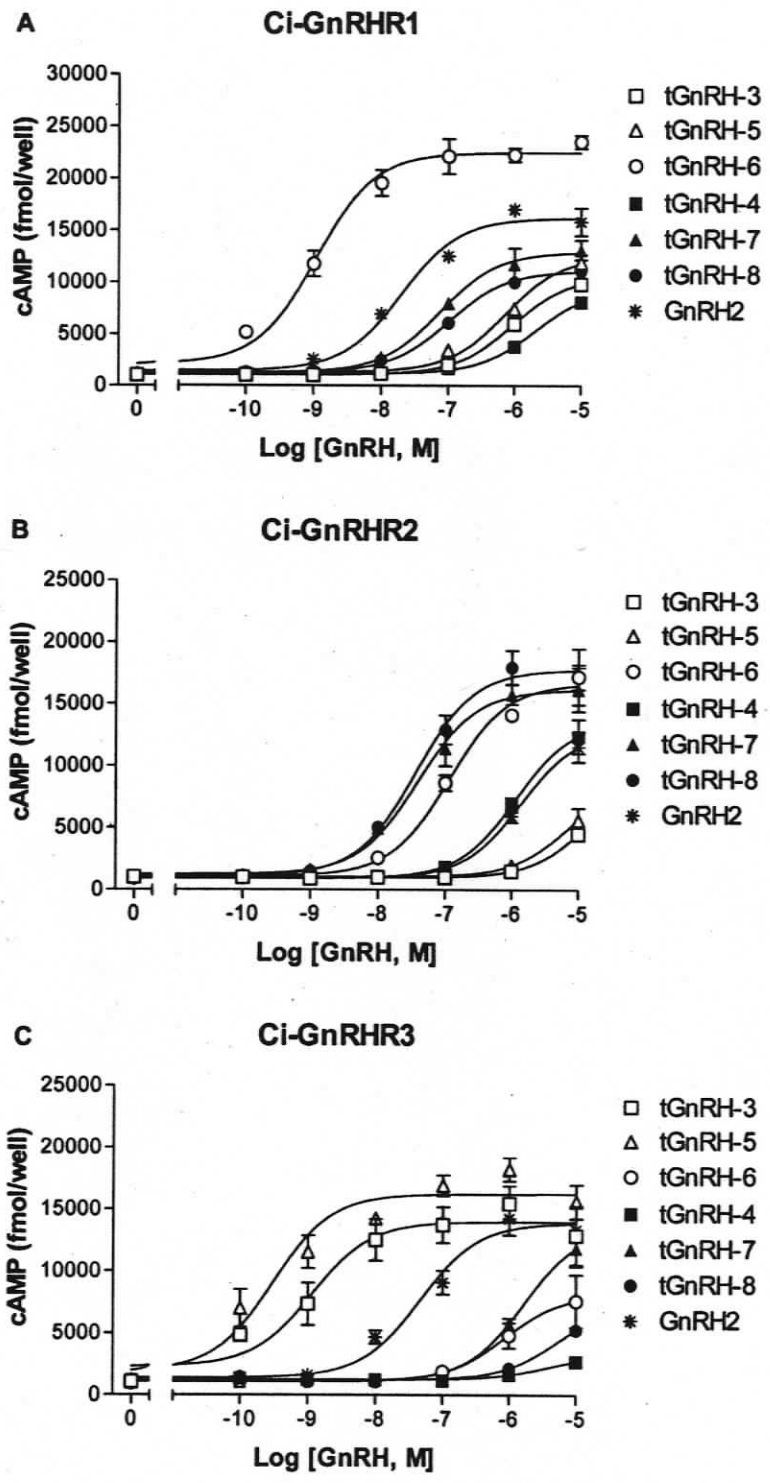
4.3.6 Two *Ciona* GnRH ligands stimulate the IP pathway through the green monkey type II GnRHR

To compare IP stimulation profiles with a prototypical mammalian type II GnRHR, the green monkey type II GnRHR was assayed and shown to be activated with GnRH2 > tGnRH-5 > tGnRH-3 (Figure 4.5B).

4.3.7 Three *Ciona* GnRHRs signal through the cAMP pathway

The limited IP activation response led us to examine whether the *Ciona* receptors would couple to $G_{\alpha s}$ and elicit a cAMP response after stimulation. COS7 cells were transfected with each *Ciona* GnRH receptor cDNA and cAMP production was measured in response to native *Ciona* GnRHs and vertebrate GnRH2. For Ci-GnRHR1, the potency was tGnRH-6 > GnRH2 > tGnRH-7 > tGnRH-8 > tGnRH-3 > tGnRH-5 > tGnRH-4; statistical analysis showed that tGnRH-6 was the most potent (see Figure 4.6A and Table 4.5). For Ci-GnRHR2, the order of effectiveness was tGnRH-8 > tGnRH-7 > tGnRH-6 > tGnRH-4 > GnRH2 > tGnRH-5 > tGnRH-3; statistical analysis showed that there was no significant difference between the GnRH peptides (see Figure 4.6B and Table 4.5). Ci-GnRHR3 was active with: tGnRH-5 > tGnRH-3 > GnRH2 > tGnRH-6 > tGnRH-7 > tGnRH-4 > tGnRH-8; statistical analysis showed that tGnRH-5 and tGnRH-3 were the most potent of the tunicate GnRHs (see Figure 4.6C and Table 4.5). Ci-GnRHR4 did not

Figure 4.6. cAMP accumulation in COS7 cells expressing three Ciona GnRHRs after incubation with GnRH ligands. The accumulation of cAMP was measured for Ci-GnRHR1 (A), Ci-GnRHR2 (B) and Ci-GnRHR3 (C) incubated with various concentrations of native *Ciona* GnRHs for one hour. Cells were transiently transfected with 800 ng of indicated GnRHR cDNA. At 48 h post-transfection, cells were washed and then stimulated with the indicated ligand. The intracellular concentrations are shown as fmol/well and error bars represent the mean $\pm$ SEM of a minimum of three independent experiments performed in duplicate.


A
Ci-GnRHR1
cAMP (fmol/well)
30000
25000
20000
15000
10000
5000
0
0
-10
-9
-8
-7
-6
-5
Log [GnRH, M]
tGnRH-3
tGnRH-5
tGnRH-6
tGnRH-4
tGnRH-7
tGnRH-8
GnRH2
B
Ci-GnRHR2
cAMP (fmol/well)
25000
20000
15000
10000
5000
0
Log [GnRH, M]
C
Ci-GnRHR3
cAMP (fmol/well)
25000
20000
15000
10000
5000
0
Log [GnRH, M]

demonstrate cAMP accumulation with any of the tunicate GnRHs nor did tGnRH-X or AKH activate any of the Ci-GnRHRs.

4.3.8 *Ciona* GnRH receptors form an independent phylogenetic branch

The primary amino acid sequences of the four *Ciona* GnRHRs were aligned with selected type I, type II and type III vertebrate GnRHRs, as previously classified (Millar *et al.*, 2004), to construct a phylogenetic tree (Figure 4.7). The four *Ciona* receptors form their own monophyletic group compared with the vertebrate GnRHR types. Ci-GnRHR1 and Ci-GnRHR2 are direct descendants from the same node with Ci-GnRHR3 closely associated. Ci-GnRHR4 groups basally to the other *Ciona* receptors.

4.4 Discussion

4.4.1 *Ciona* encodes four full-length GnRH receptors

In the present study, we found four full-length GnRHR transcripts encoded within the tunicate *C. intestinalis* genome. Each *Ciona* GnRHR transcript predicts a protein with an extracellular N-terminus, seven hydrophobic stretches that correspond to putative alpha-helical transmembrane domains linked by alternating intracellular and extracellular loops, and an intracellular C-terminal tail. The *Ciona* receptors have a number of features common to many GnRHRs (Figure 4.3). At the proximal end of the second intracellular loop is a region thought to be involved in G protein coupling (Ballesteros *et al.*, 1998). Three *Ciona* GnRHRs have the motif DRxxxI but CiGnRHR4 has DRxxxL compared to DRxxxI/V in vertebrates. Also, three *Ciona* GnRHRs but not Ci-GnRHR2 have consensus glycosylation sites (N-X-S/T) on their N-terminal extracellular extension. In

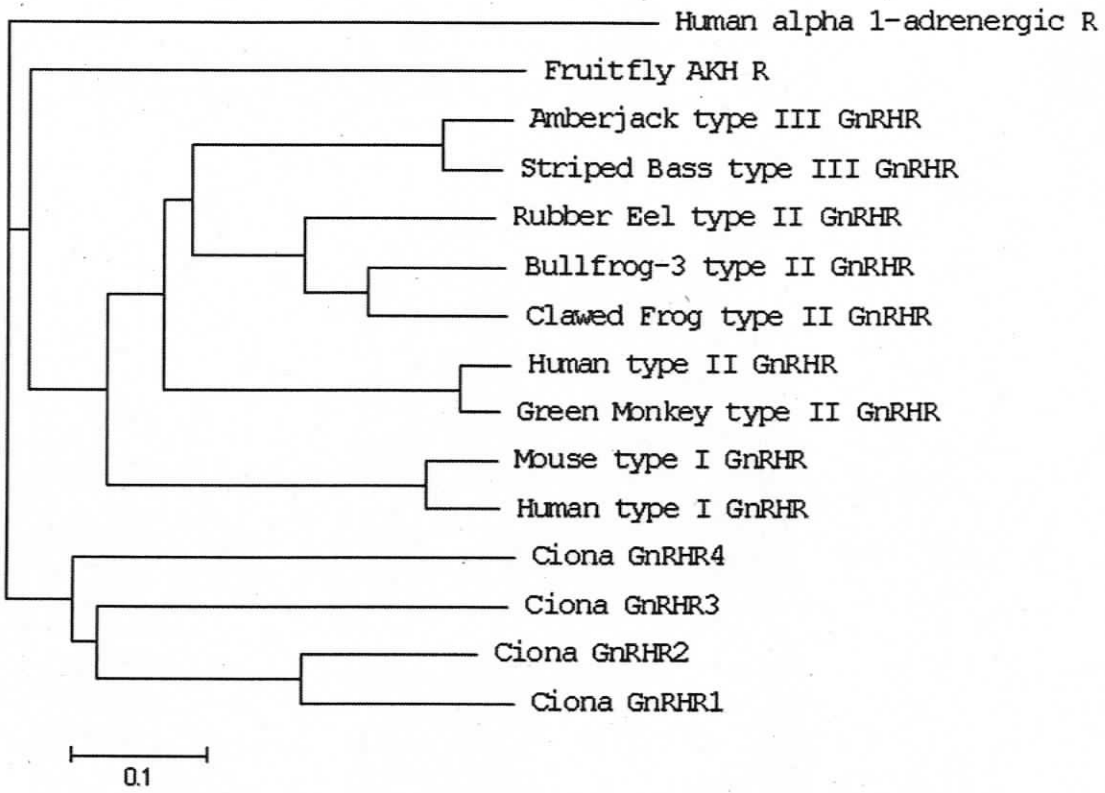


Figure 4.7. A phylogenetic diagram showing the evolutionary relationship of the four *Ciona intestinalis* GnRH receptors. These receptors are compared to other non-mammalian and mammalian GnRH receptors, the structurally related fruitfly adipokinetic hormone receptor and the human alpha 1-adrenergic receptor (as an outgroup).

the murine GnRHR, interference with glycosylation decreases receptor expression possibly by an increase in degradation (Davidson *et al.*, 1995). The alteration of receptor glycosylation did not affect receptor affinity or transport of the mouse GnRHR to the cell surface (Keinan and Hazum, 1985; Schvartz and Hazum, 1985; Davidson *et al.*, 1995). Like the rhodopsin family of GPCRs, all *Ciona* GnRHRs possess conserved cysteine residues predicted to form a disulfide bond between the first two extracellular loops, as in mammalian GnRHRs (Tsutsumi *et al.*, 1992). All *Ciona* GnRHRs possess additional cysteine residues in the N-terminal extension and extracellular loops, which may form a second disulfide bond, as in the mouse and human type I GnRH receptors (Sealfon *et al.*, 1997).

All four *Ciona* receptors appear to be true GnRH receptors based on conservation of critical motifs. All *Ciona* GnRHRs possess either $Asn^{2.50}$ or $Asp^{2.50}$ in TMD2. In the mammalian GnRHRs, $Asn^{2.50}$ is thought to interact with $Asp^{7.49}$ through a water molecule helping to stabilize the receptor structure (Zhou *et al.*, 1994; Okada *et al.*, 2002). *C. intestinalis* GnRHRs possess $His^{7.49}$ as a replacement for $Asp^{7.49}$ in TMD7, a feature unique to the *Ciona* GnRHRs.

4.4.2 *Ciona* GnRH receptors have key amino acids for binding to GnRH

Residues $Asp^{2.61(98)}$, $Asn^{2.65(102)}$ and $Lys^{3.32(121)}$ in the primate GnRHR are present in homologous positions in *Ciona* GnRHRs (Figure 4.1). $Asp^{2.61(98)}$ is proposed to interact with His^2, Trp^3 and Ser^4 of GnRH (Zhou *et al.*, 1995; Flanagan *et al.*, 2000), whereas $Asn^{2.65(102)}$ and $Lys^{3.32(121)}$ are thought to be important in the ligand binding pocket for mGnRH (Davidson *et al.*, 1996; Hoffmann *et al.*, 2000; Millar *et al.*, 2004). Aromatic

residues $Trp^{2.64(101)}$ and $Trp^{6.48(280)}$ in the mammalian GnRHR are in homologous positions in the *Ciona* GnRHRs. $Trp^{2.64(101)}$ affects the formation of the ligand binding pocket and its role has been reviewed (Wang *et al.*, 2004). Mutation of $Trp^{6.48(280)}$ to Ser or Arg abolished inositol phosphate production and was shown to decrease ligand binding (Chauvin *et al.*, 2000). In many GPCRs, this residue is involved with receptor activation, but not ligand binding (Millar *et al.*, 2004).

The CWTPYYLLGL/IWYWF motif in the extracellular end of TMD6 ($Cys^{6.47}$-$Phe^{6.60}$) is partially conserved in all *Ciona* GnRHRs. This motif is thought to form an aromatic cage around the conserved $Pro^{6.50}$ and is thought to be important for receptor expression, ligand recognition and G protein-coupling efficiency (Seong *et al.*, 2003). Ala mutants of aromatic residues $Tyr^{6.51(283)}$, $Tyr^{6.52(284)}$ and $Trp^{6.59(291)}$ in the human GnRHR abolished both ligand binding and signal transduction (Hovelmann *et al.*, 2002). Perturbation of these residues may impair integration of the human receptor into the cellular membrane, which would target the misfolded protein for destruction by the proteosome. $Trp^{6.57}$ is conservatively replaced by Tyr in Ci-GnRHR1, Ci-GnRHR2 and Ci-GnRHR3, and is altered to Met in Ci-GnRHR4. $Tyr^{6.58}$ is changed to Asp in Ci-GnRHR1 and Ci-GnRHR2, and to His in Ci-GnRHR3 and Ci-GnRHR4. $Tyr^{6.58(290)}$ is thought to interact with Tyr^{5} in GnRH1 (Hovelmann *et al.*, 2002). Hence, the absence of $Tyr^{6.58}$ is not unexpected in *Ciona* GnRHRs because tunicate GnRH peptides lack this Tyr^{5} residue.

The type I mammalian GnRHRs favour binding mGnRH (GnRH1) due to a positively charged Arg residue in position 8 of the ligand. It seems unnecessary for *Ciona* GnRHRs to contain this $Asp^{7.32}$ because they all lack Arg^{8}. Marmoset, green monkey,

rhesus monkey and human type II GnRH receptors all lack $Asp^{7.32}$, which indicates a plasticity for another residue in this position for type II GnRHRs. *Ciona* GnRHR1, GnRHR2 and GnRHR3 each have a long tail (78-116 amino acids) that is characteristic of mammalian type II and other non-mammalian GnRHRs, whereas ciGnRHR4 has a short C-terminal tail (21 amino acids), which is more analogous to the mammalian type I receptor tail (3 amino acids).

4.4.3 Four GnRHR mRNAs are found ubiquitously in *Ciona*

Transcripts of each *Ciona* GnRHR were found in all tissues sampled, including the neural complex, gonad, heart, intestine, endostyle and branchial sac. We previously found that each GnRH gene is co-expressed in the above tissues (data not shown). These results suggest that GnRHRs may have local paracrine or autocrine functions in the tunicate. A screen of cDNA produced from 4-cell and gastrulation stages of the developing *Ciona* embryo showed early expression of *ci-GnRHR3* and *ci-GnRHR4* but not *ci-GnRHR1* and *ci-GnRHR2* (results not shown), implying that these receptors may have a role during development and metamorphosis.

4.4.4 One *Ciona* GnRH activates the IP pathway through Ci-GnRHR1

Results of the receptor activation assay showed that only tGnRH-6 was able to induce inositol phosphate accumulation in one of the *Ciona* GnRH receptors. Ci-GnRHR1 was shown to be the only *Ciona* GnRHR that was able to couple to the IP signalling pathway and elicit a dose response within the transfected COS7 cells.

4.4.5 cAMP signalling response is dominant in the *Ciona* GnRHRs

Ci-GnRHR1, Ci-GnRHR2, and Ci-GnRHR3 were able to activate a cAMP dose response with all of the tGnRHs tested. However, for Ci-GnRHR1, tGnRH-6 was 1,000-fold more potent than tGnRH-3,-4, and -5. Ci-GnRHR2 did not show significant difference in potency between the GnRH peptides. In contrast, Ci-GnRHR3 had two highly potent GnRH peptides (tGnRH-5 and tGnRH-3) that were 1,000-fold more effective than tGnRH-4, -6, -7 and -8.

A $G_{s\alpha}$ protein recognition sequence in the ICL1 of the mouse GnRHR was identified as a BBxB or BBxxB motif, where B was a basic residue and x was any amino acid. Any substitutions to this region drastically impaired $G_{s\alpha}$ coupling and resulted in a sharp decrease in cAMP accumulation (Arora *et al.*, 1998). Ci-GnRHR1 and Ci-GnRHR2 each have a BxxBxB sequence and Ci-GnRHR3 has a BBBxB sequence in ICL1. These slightly modified but heavily basic sequences may be responsible for $G_{s\alpha}$ coupling and the GnRH-induced cAMP responses observed. Ci-GnRHR4 contained only two adjacent basic residues within ICL1, which may explain the negative cAMP response observed.

To date, Ci-GnRHR1 is the only known invertebrate GnRHR that activates both the IP and cAMP pathway. Previously, a GnRHR-like protein was identified in *Drosophila melanogaster* and was initially thought to be activated by GnRH (Hauser *et al.*, 1998). Later studies revealed that adipokinetic hormone (AKH), a peptide structurally similar to GnRH, activated this receptor (Staubli *et al.*, 2002). As a precaution, we tested silkworm AKH against all of the *Ciona* GnRHRs and found it unable to induce either inositol phosphate production or cAMP accumulation, even at 10 μM concentrations.

The different stimulation profiles displayed by the functional *Ciona* GnRHRs suggest distinct roles for each receptor. tGnRH-3, tGnRH-5 and tGnRH-7 were shown to have the highest potency for inducing gamete release in live animals and were also the most effective at activating Ci-GnRHR3. It is plausible that Ci-GnRHR3 is responsible for the induction of gamete release within the gravid animal. However, because it is expressed ubiquitously, other functions are plausible, therefore, further investigation is needed.

Kusakabe *et al.* (2003) showed similar expression results with *Ci-gnrhr1* and *Ci-gnrhr2* detected in the neural complex, gonad, stomach, intestine, endostyle and branchial sac. In our studies, we also identified *Ci-gnrhr2* transcripts in the heart and ovary (data not shown); two areas in which they failed to detect them. Kusakabe *et al.* identified a partial gene encoding *Ci-gnrhr3*, but were unable to predict the full-length coding sequence or isolate a cDNA clone representing this third GnRHR transcript. We show that *Ci-gnrhr3* is a processed transcript in all of the tissues we examined. Kusakabe *et al.* were able to detect inward currents using *Xenopus* oocytes injected with *Ci-gnrhr1* mRNA after stimulation with tGnRH-1 isolated from *Chelyosoma productum* (Kusakabe *et al.*, 2003). However, we were unable to elicit a response to tGnRH-1 from Ci-GnRHR1 expressed in COS7 cells through the IP pathway.

Our previous study showed that the nine tGnRHs were unable to activate either the human or the rat type I GnRHRs via the IP pathway (endpoint was activation of LHα promoter) (Adams *et al.*, 2003). However, we found that the *Ciona* GnRHRs share features common to type II receptors, as evidenced by the high matches to African clawed frog and the rubber eel GnRHRs. Therefore, we tested the tunicate GnRH and

related peptides on a type II receptor. Inositol phosphate accumulation assays showed that tGnRH-5 and tGnRH-3 were able to stimulate the green monkey GnRHR at high concentrations (see Table 4.3). In addition, the structurally related peptide, α_1-mating factor, was able to activate the green monkey GnRHR at very high concentrations ($10^{-4.5}$ M) (data not shown).

4.4.6 Phylogeny of the *Ciona* GnRH receptors and polymorphism

The sequences for Ci-GnRHR1 and Ci-GnRHR2 submitted by Kusakabe *et al.* (2003) showed higher sequence similarity to the published genome than our two sequences. However, a high level of allelic polymorphism was found in *Ciona* individuals in the sequencing of their genome (Dehal *et al.*, 2002). Our animals were obtained from a different population compared with Kusakabe and this likely accounts for the observed differences.

Our *Ciona* receptor proteins were compared with other type I, type II, and type III GnRH receptors, and were found to cluster to their own branch of the cladogram. This indicates that the *Ciona* GnRHRs are more similar among themselves than to any previously identified GnRHR types. When the NCBI non-redundant protein database was searched using the *Ciona* receptor proteins, the closest external species match was either the rubber eel or the African clawed frog type II GnRHR, both proposed by Millar *et al.* (2004) to cluster with type II GnRHRs. Like many non-mammalian GnRHRs, the *Ciona* receptors possess a C-terminal tail, which has been implicated in rapid agonist-induced receptor internalization, coupling to G protein-signalling paths and increased membrane expression (Blomenrohr *et al.*, 1999). The high sequence identity between Ci-GnRHR1

and Ci-GnRHR2 suggest that these two receptors are the result of a more recent duplication event. *Ci-gnrhr3* and *Ci-gnrhr4* appear to have been generated from much earlier duplications, perhaps in an ancestral tunicate due to their low sequence conservation with the other *Ciona* receptors.

The functional data suggest that Ci-GnRHR2, Ci-GnRHR3 and Ci-GnRHR4 have lost the ability to couple to $G_{q/11\alpha}$ in COS7 cells possibly through the loss of $G_{q/11\alpha}$ coupling motif contained in ICL3. It appears that cAMP is the dominant pathway utilized by three *Ciona* GnRHRs, which suggests that the preference for cAMP may be a specific for the urochordate lineage as amphioxus GnRHRs retain the ability to activate IP accumulation (See Chapter 5). Like Ci-GnRHR1, the bullfrog, mouse and the type II green monkey GnRHRs were also shown to couple to both the inositol phosphate and the adenylyl cyclase-signalling pathways, suggesting that Ci-GnRHR1 may be an ancient ortholog (Arora *et al.*, 1998; Oh *et al.*, 2003; Wang *et al.*, 2003).

Ci-GnRHR4 does not have the ability to activate either inositol phosphate or cAMP accumulation, at least in response to the tested peptides, and is distinctly different from all of the other *Ciona* receptors. *Ci-gnrhr4* possesses only seven exons, whereas the others each have eight. It has a much shorter C-terminal tail, with only 18 amino acid residues, whereas the rest have longer tails (more than 70 residues). Ci-GnRHR4 is also the only receptor with DRxxxL in TMD3 and $Met^{6.57}$ instead of $Trp^{6.57}$. To be certain that GnRHR4 is expressed as a protein, we fused the eGFP coding sequence to the 3' end of the receptor cDNA and found that the protein product was expressed. It is possible that Ci-GnRHR4 is able to be activated by another ligand, has improper post-translational processing or has the ability to activate an alternate signalling pathway.

Tunicates are classified as both invertebrates and protochordates, which makes them an important group for understanding the transition from invertebrates to vertebrates (Dehal *et al.*, 2002). This study reveals that functional GnRHRs exist in animals without a pituitary gland or sex steroids. Because the tunicate larvae share many chordate features with higher chordates, including humans, delineation of the function of each *Ciona* GnRHR reveals clues to the function and origin of all GnRHR subtypes, and may uncover novel functions that are masked in the vertebrates.

4.5 References

Adams, B. A., J. A. Tello, J. Erchegyi, C. Warby, D. J. Hong, K. O. Akinsanya, G. O. Mackie, W. Vale, J. E. Rivier and N. M. Sherwood (2003). "Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, Ciona intestinalis." Endocrinology **144**(5): 1907-19.

Arora, K. K., L. Z. Krsmanovic, N. Mores, H. O'Farrell and K. J. Catt (1998). "Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor." J Biol Chem **273**(40): 25581-6.

Ballesteros, J., S. Kitanovic, F. Guarnieri, P. Davies, B. J. Fromme, K. Konvicka, L. Chi, R. P. Millar, J. S. Davidson, H. Weinstein and S. C. Sealfon (1998). "Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor." J Biol Chem **273**(17): 10445-53.

Blomenrohr, M., A. Heding, R. Sellar, R. Leurs, J. Bogerd, K. A. Eidne and G. B. Willars (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**(6): 1229-37.

Chauvin, S., A. Berault, Y. Lerrant, M. Hibert and R. Counis (2000). "Functional importance of transmembrane helix 6 Trp(279) and exoloop 3 Val(299) of rat gonadotropin-releasing hormone receptor." Mol Pharmacol **57**(3): 625-33.

Chengalvala, M., B. Kostek and D. E. Frail (1999). "A multi-well filtration assay for quantitation of inositol phosphates in biological samples." J Biochem Biophys Methods **38**(3): 163-70.

Craig, A. G., W. H. Fischer, M. Park, J. E. Rivier, B. D. Musselman, J. F. Powell, S. M. Reska-Skinner, M. O. Prakash, G. O. Mackie and N. M. Sherwood (1997). "Sequence of two gonadotropin releasing hormones from tunicate suggest an important role of conformation in receptor activation." FEBS Lett **413**(2): 215-25.

Davidson, J. S., C. A. Flanagan, W. Zhou, Becker, II, R. Elario, W. Emeran, S. C. Sealfon and R. P. Millar (1995). "Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: role in receptor expression but not ligand binding." Mol Cell Endocrinol **107**(2): 241-5.

Davidson, J. S., C. A. McArdle, P. Davies, R. Elario, C. A. Flanagan and R. P. Millar (1996). "Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide." J Biol Chem **271**(26): 15510-4.

Dehal, P., Y. Satou, R. K. Campbell, J. Chapman, B. Degnan, A. De Tomaso, B. Davidson, A. Di Gregorio, M. Gelpke, D. M. Goodstein, N. Harafuji, K. E. Hastings, I. Ho, K. Hotta, W. Huang, T. Kawashima, P. Lemaire, D. Martinez, I. A. Meinertzhagen,

S. Necula, M. Nonaka, N. Putnam, S. Rash, H. Saiga, M. Satake, A. Terry, L. Yamada, H. G. Wang, S. Awazu, K. Azumi, J. Boore, M. Branno, S. Chin-Bow, R. DeSantis, S. Doyle, P. Francino, D. N. Keys, S. Haga, H. Hayashi, K. Hino, K. S. Imai, K. Inaba, S. Kano, K. Kobayashi, M. Kobayashi, B. I. Lee, K. W. Makabe, C. Manohar, G. Matassi, M. Medina, Y. Mochizuki, S. Mount, T. Morishita, S. Miura, A. Nakayama, S. Nishizaka, H. Nomoto, F. Ohta, K. Oishi, I. Rigoutsos, M. Sano, A. Sasaki, Y. Sasakura, E. Shoguchi, T. Shin-i, A. Spagnuolo, D. Stainier, M. M. Suzuki, O. Tassy, N. Takatori, M. Tokuoka, K. Yagi, F. Yoshizaki, S. Wada, C. Zhang, P. D. Hyatt, F. Larimer, C. Detter, N. Doggett, T. Glavina, T. Hawkins, P. Richardson, S. Lucas, Y. Kohara, M. Levine, N. Satoh and D. S. Rokhsar (2002). "The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins." Science **298**(5601): 2157-67.

Flanagan, C. A., V. Rodic, K. Konvicka, T. Yuen, L. Chi, J. E. Rivier, R. P. Millar, H. Weinstein and S. C. Sealfon (2000). "Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction." Biochemistry **39**(28): 8133-41.

Fujii, Y., M. Enomoto, T. Ikemoto, D. Endo, K. Okubo, K. Aida and M. K. Park (2004). "Molecular cloning and characterization of a gonadotropin-releasing hormone receptor in the guinea pig, Cavia porcellus." Gen Comp Endocrinol **136**(2): 208-16.

Hauser, F., L. Sondergaard and C. J. Grimmelikhuijzen (1998). "Molecular cloning, genomic organization and developmental regulation of a novel receptor from Drosophila melanogaster structurally related to gonadotropin-releasing hormone receptors for vertebrates." Biochem Biophys Res Commun **249**(3): 822-8.

Hoffmann, S. H., T. ter Laak, R. Kuhne, H. Reilander and T. Beckers (2000). "Residues within transmembrane helices 2 and 5 of the human gonadotropin-releasing hormone receptor contribute to agonist and antagonist binding." Mol Endocrinol **14**(7): 1099-115.

Hovelmann, S., S. H. Hoffmann, R. Kuhne, T. ter Laak, H. Reilander and T. Beckers (2002). "Impact of aromatic residues within transmembrane helix 6 of the human gonadotropin-releasing hormone receptor upon agonist and antagonist binding." Biochemistry **41**(4): 1129-36.

Keinan, D. and E. Hazum (1985). "Mapping of gonadotropin-releasing hormone receptor binding site." Biochemistry **24**(26): 7728-32.

Kozak, M. (1996). "Interpreting cDNA sequences: some insights from studies on translation." Mamm Genome **7**(8): 563-74.

Kusakabe, T., S. Mishima, I. Shimada, Y. Kitajima and M. Tsuda (2003). "Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian Ciona intestinalis." Gene **322**: 77-84.

Lescheid, D. W., E. Terasawa, L. A. Abler, H. F. Urbanski, C. M. Warby, R. P. Millar and N. M. Sherwood (1997). "A second form of gonadotropin-releasing hormone

(GnRH) with characteristics of chicken GnRH-II is present in the primate brain." Endocrinology **138**(12): 5618-29.

Mackie, G. O. (1995). "On the 'visceral nervous system' of Ciona." J Mar Biol Assoc UK **75**: 141–151.

Millar, R. P., Z. L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan and S. R. Maudsley (2004). "Gonadotropin-releasing hormone receptors." Endocr Rev **25**(2): 235-75.

Miyamoto, K., Y. Hasegawa, M. Nomura, M. Igarashi, K. Kangawa and H. Matsuo (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 U S A **81**(12): 3874-8.

Oh, D. Y., L. Wang, R. S. Ahn, J. Y. Park, J. Y. Seong and H. B. Kwon (2003). "Differential G protein coupling preference of mammalian and nonmammalian gonadotropin-releasing hormone receptors." Mol Cell Endocrinol **205**(1-2): 89-98.

Okada, T., Y. Fujiyoshi, M. Silow, J. Navarro, E. M. Landau and Y. Shichida (2002). "Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography." Proc Natl Acad Sci U S A **99**(9): 5982-7.

Okubo, K., S. Nagata, R. Ko, H. Kataoka, Y. Yoshiura, H. Mitani, M. Kondo, K. Naruse, A. Shima and K. Aida (2001). "Identification and characterization of two distinct GnRH receptor subtypes in a teleost, the medaka Oryzias latipes." Endocrinology **142**(11): 4729-39.

Powell, J. F., S. M. Reska-Skinner, M. O. Prakash, W. H. Fischer, M. Park, J. E. Rivier, A. G. Craig, G. O. Mackie and N. M. Sherwood (1996). "Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications." Proc Natl Acad Sci U S A **93**(19): 10461-4.

Reinhart, J., L. M. Mertz and K. J. Catt (1992). "Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor." J Biol Chem **267**(30): 21281-4.

Schvartz, I. and E. Hazum (1985). "Tunicamycin and neuraminidase effects on luteinizing hormone (LH)-releasing hormone binding and LH release from rat pituitary cells in culture." Endocrinology **116**(6): 2341-6.

Sealfon, S. C., H. Weinstein and R. P. Millar (1997). "Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor." Endocr Rev **18**(2): 180-205.

Seong, J. Y., L. Wang, D. Y. Oh, O. Yun, K. Maiti, J. H. Li, J. M. Soh, H. S. Choi, K. Kim, H. Vaudry and H. B. Kwon (2003). "Ala/Thr(201) in extracellular loop 2 and Leu/Phe(290) in transmembrane domain 6 of type 1 frog gonadotropin-releasing hormone

receptor confer differential ligand sensitivity and signal transduction." Endocrinology **144**(2): 454-66.

Staubli, F., T. J. Jorgensen, G. Cazzamali, M. Williamson, C. Lenz, L. Sondergaard, P. Roepstorff and C. J. Grimmelikhuijzen (2002). "Molecular identification of the insect adipokinetic hormone receptors." Proc Natl Acad Sci U S A **99**(6): 3446-51.

Terakado, K. (2001). "Induction of gamete release by gonadotropin-releasing hormone in a protochordate, Ciona intestinalis." Gen Comp Endocrinol **124**(3): 277-84.

Tsutsumi, M., W. Zhou, R. P. Millar, P. L. Mellon, J. L. Roberts, C. A. Flanagan, K. Dong, B. Gillo and S. C. Sealfon (1992). "Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor." Mol Endocrinol **6**(7): 1163-9.

Wang, A. F., J. H. Li, K. Maiti, W. P. Kim, H. M. Kang, J. Y. Seong and H. B. Kwon (2003). "Preferential ligand selectivity of the monkey type-II gonadotropin-releasing hormone (GnRH) receptor for GnRH-2 and its analogs." Mol Cell Endocrinol **209**(1-2): 33-42.

Wang, C., O. Yun, K. Maiti, D. Y. Oh, K. K. Kim, C. H. Chae, C. J. Lee, J. Y. Seong and H. B. Kwon (2004). "Position of Pro and Ser near Glu7.32 in the extracellular loop 3 of mammalian and nonmammalian gonadotropin-releasing hormone (GnRH) receptors is a critical determinant for differential ligand selectivity for mammalian GnRH and chicken GnRH-II." Mol Endocrinol **18**(1): 105-16.

Wang, L., J. Bogerd, H. S. Choi, J. Y. Seong, J. M. Soh, S. Y. Chun, M. Blomenrohr, B. E. Troskie, R. P. Millar, W. H. Yu, S. M. McCann and H. B. Kwon (2001). "Three distinct types of GnRH receptor characterized in the bullfrog." Proc Natl Acad Sci U S A **98**(1): 361-6.

Zhou, W., C. Flanagan, J. A. Ballesteros, K. Konvicka, J. S. Davidson, H. Weinstein, R. P. Millar and S. C. Sealfon (1994). "A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor." Mol Pharmacol **45**(2): 165-70.

Zhou, W., V. Rodic, S. Kitanovic, C. A. Flanagan, L. Chi, H. Weinstein, S. Maayani, R. P. Millar and S. C. Sealfon (1995). "A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites." J Biol Chem **270**(32): 18853-7.

Chapter 5

GnRH peptides found in humans and other vertebrates selectively activate three amphioxus GnRH receptors

5.1 Introduction

Amphioxus (lancelet) is a key chordate model which shares with vertebrates many genetic programs that pattern the organization of the developing embryo (Minguillon *et al.*, 2005). As a result, many features of the basic body plan are common between amphioxus and vertebrates, including a notochord, a dorsal tubular nervous system, segmented muscles, gill slits, an endostyle and a post-anal tail. Recently, the phylogenetic position of amphioxus as the closest living relative to vertebrates has been called into question. Phylogenetic comparisons of 146 protein coding sequences present in the amphioxus genome have led to the reversal of positions between the urochordates and cephalochordates (Bourlat *et al.*, 2006; Delsuc *et al.*, 2006). The placement of amphioxus at the base of the phylum chordata provides an important touchstone for predicting the nature of the GnRHR gene family in the shared chordate ancestor, and the GnRH receptor condition from which all vertebrates evolved.

The presence of GnRH immunoreactivity has been reported in the brain and Hatschek's pit of amphioxus using antiserum raised against mammalian GnRH (Chang *et al.*, 1984; Fang *et al.*, 1999). However, the labelling of Hatschek's pit was not accomplished with antiserum raised against lamprey GnRH-I in another study (Castro *et al.*, 2006). Instead, this antiserum labelled rhabdomeric photoreceptor cells, helping to reveal the organization of the Hesse cell axonal system in the amphioxus CNS. These data predict the presence of a GnRH-like peptide in amphioxus, but our genomic searches to date have been unable to locate a putative GnRH gene.

In our previous studies, we reported that *C. intestinalis* has a complex GnRH system, with six native GnRH peptides and three functional receptors. In the present study, I investigated the presence of functional GnRH receptors in the cephalochordate amphioxus. I identified and cloned four candidate GnRH receptor sequences from the North American amphioxus, *Branchiostoma floridae*. Three GnRHR genes map to the same genomic region, whereas the fourth is located on a separate scaffold. The four amphioxus GnRHRs cluster into two paralogous phylogenetic relationships, with two receptors showing high sequence similarity with vertebrate GnRHRs and two grouping closely with the recently cloned octopus GnRHR. Lacking native amphioxus GnRH candidates, I evaluated the functional conservation of each amphioxus GnRH receptor by testing it with vertebrate GnRH peptides. In addition, I also tested another protostome neuropeptide, adipokinetic hormone (AKH), as its receptor shows very high similarity to the octopus GnRHR. Our results provide insight into the functional evolution of the GnRH receptor gene family within the chordates and show a conserved relationship with the GnRHR from the molluscan lineage.

5.2 Methods and Materials

5.2.1 Gene organization

Gene arrangements were found using the Department of Energy Joint Genome Institute's *Branchiostoma floridae* genome project (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html). Human, zebrafish, *C. intestinalis* and octopus GnRHR protein sequences were used to search the available TBLASTN input form. Using default parameters (BLOSUM 62 matrix), each search generated closely matched fragments. The

DNA regions encoding the matching fragments of putative receptors were compiled, examined for exon/intron boundaries, and analyzed for complete open reading frames (ORFs). Primers were designed and the receptor cDNAs encompassing the complete ORFs were amplified using PCR and sequenced as stated below. To complete any missing regions in the protein coding regions, 5' and/or 3' RACE was utilized.

5.2.2 Animals

Sexually mature wild-caught *Branchiostoma floridae* (subphylum Cephalochordata), approximately 4 cm in length, were purchased from Gulf Specimen Marine Laboratory (Panacea, FL). For RNA preparation, animals were rinsed in DEPC-treated PBS, snap frozen by immersion in liquid nitrogen and stored whole at -80 °C.

5.2.3 Isolation of mRNA and synthesis of cDNA

Total RNA was isolated from male and female animals separately using Trizol reagent (Invitrogen) according to the manufacturer's protocol. In summary, whole animals were homogenized using tungsten carbide beads in 1ml Trizol reagent in microfuge tubes with vigorous shaking in a mixer mill (Retsch, Inc. Newton, PA) at 20 Hz for 2x 3 min (rotating the tubes 180 ° between cycles). Total RNA samples were treated with RNase-free DNase (Ambion Inc., TX) to eliminate genomic DNA contamination and total RNA was quantified in each sample according to absorbance at 260 nm. A mix of equal male and female RNA was pooled and used in subsequent reactions. Total RNA (2.5 μg) was reverse transcribed in a 50 µl reaction that contained 2 mM oligo dT, 2 mM dNTPs, 1x first strand reaction buffer, 0.01 DTT, 5 U RNase

inhibitor and 100 U Superscript III reverse transcriptase (Invitrogen). The reaction was incubated at 50 °C for 60 min and the enzyme was heat inactivated at 70 °C for 15 min. To check for genomic contamination, a negative control was run with all the reaction components, but lacking the reverse transcriptase enzyme.

5.2.4 5'- and 3'- RACE-PCR, RT-PCR and cloning into expression vector

For 5' RACE-PCR, mRNA was purified from the total RNA using a Micro Poly(A) Pure mRNA isolation kit (Ambion). Messenger RNA (300 ng) was used to prepare 5' RACE-ready cDNA using the RLM-RACE kit (Ambion) according to the manufacturer's instructions. For 3' RACE, 1 μg of total RNA was used to prepare 3' RACE-ready cDNA using the same kit.

Oligonucleotide primers were designed to regions encoding candidate GnRHRs based on the compiled sequences for amphioxus GnRHR genes. The full-length ORFs of each *Branchiostoma floridae* gnrhr were altered to include a strong context Kozak sequence (GCCACCATGG) (where the start codon is underlined) using a modified primer (Table 5.1). Each 50-μl reaction contained 2 U Platinum® Taq DNA Polymerase High Fidelity (Invitrogen), 1x High Fidelity PCR buffer, 2 mM $MgSO_4$, 0.2 mM dNTP mixture (Invitrogen), and 0.4 mM of each Kozak forward (f) and stop reverse (r) primer (Table 5.1). PCRs were performed under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 68 °C for 2 min, and a 5-min final extension. The PCR amplicons were separated by electrophoresis on a 1.3 % (w/v) agarose gel and visualized with ethidium bromide staining using an Eagle EyeII still Video system (Stratagene). Bands were

Table 5.1. Primers used to amplify *Branchiostoma floridae* GnRHR cDNAs.

Primer name	Sequence (5'-3')	Direction	Target
R1 F	ACGGACGAACTGAACGATGTGGAC	F	*GnRHR1*
R1 R	CGGTCTAGACGTCTCAGGTGGGTG	R	*GnRHR1*
R1 NheI KozF	GCTAGCGCCACCATGGTGAACGCTTCAGAAACTCA	F	*GnRHR1*
R1 StopR XhoI	CTCGAGTCAGGTGGGTGTGCTGATGAACT	R	*GnRHR1*
R2 NheI KozF	GCTAGCGCCACCATGGATTGCCCTGGCCAAGGA	F	*GnRHR2*
R2 StopR XhoI	CTCGAGTCAGGTGGTTGATGTTCCGTTGC	R	*GnRHR2*
R2 5'O	CCAGGATGAGGAGGTAACAC	R	*GnRHR2*
R2 5'I	ATGACCATGATCTTGCATCTC	R	*GnRHR2*
R3 ApaI KozF	GGGCCCGCCACCATGGCTGATGCTAGCAGCAA	F	*GnRHR3*
R3 StopR AflII	CTTAAGGATTCTCAAGGTAACGTGGACGATGT	R	*GnRHR3*
R4 NheI KozF	GCTAGCGCCACCATGGCGCCTTCACAGTCACCACG	F	*GnRHR4*
R4 StopR XbaI	TCTAGATTACTATACCCTACTCCCAGAT	R	*GnRHR4*

Koz=Kozak; F=forward; R=reverse.

excised and isolated using a QIAquick® Gel extraction kit (Qiagen), cloned into pGEM-T (Promega) and sequenced. The SequiTherm EXCELII DNA sequencing kit using the Sanger sequencing method was performed by the University of Victoria Sequencing Centre. Each gene was amplified using M13 forward and reverse priming sites present on pGEM-T and sequenced on a LI-COR 4200-Global IR^2. To generate expression clones, each receptor open-reading frame was excised from pGEM-T with restriction enzymes and then ligated into pcDNA 3.1(-) (Invitrogen) and resequenced to confirm sequence integrity.

5.2.5 Peptide synthesis

GnRH peptides (Table 5.2) were synthesized automatically on a CS-biopeptide synthesizer (model CS536, CS BioCo) on methylbenzhydrylamine resin using the Boc strategy by Jean Rivier at the Salk Institute. The peptides were cleaved with hydrofluoric acid, concomitantly deprotected, and then purified as described by Adams *et al.* (2003).

Octopus GnRH was a gift from Dr. Minakata (Suntory Institute for Bioorganic Research, Osaka, Japan). *Bombyx mori* AKH (H-5792) was purchased from BaChem.

5.2.6 IP accumulation assay

Each GnRHR was heterologously expressed in COS7 cells and assayed for ligand-induced inositiol phosphate accumulation by the method described previously.

5.2.7 Phylogenetic analysis

The deduced amino acid sequences of the four *Branchiostoma floridae* GnRHRs

Table 5.2. Sequences of ligands used to test amphioxus GnRHR activation.

Peptide	Sequence
GnRH1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH2	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2
OctGnRH	pGlu-Asn-Tyr-His-Phe-Ser-Asn-Gly-Trp-His-Pro-Gly-NH_2
AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH_2

mammalian GnRH (GnRH1), chicken GnRH-II (GnRH2) and silkworm adipokinetic hormone (AKH).

were aligned with GnRHRs and structurally related receptors (GnRHR-like) from other species using the ClustalW program. Protein sequences were trimmed to include the regions encompassing transmembrane domains 1-7 then manually degapped. The phylogenetic tree was constructed by the maximum likelihood method using the online PhyML portal available at (http://atgc.lirmm.fr/phyml/). The parameters were: WAG model; Gamma distribution parameter= estimated; starting tree bionj; 100 bootstrap replicates; optimized tree topology, branch lengths, and rate parameters (Guindon *et al.*, 2005). Other related receptors were included in the alignment to help distinguish each GnRHR group: mammalian type I, non-mammalian type I, type II and type III; the human arginine vasopressin type 1A (AVPR1A, GenBank accession no. NP_000697) and oxytocin (OXTR, NP_000907) receptors were added as outgroups to root the tree. Receptor names are as reported: human (*Homo sapiens*) GnRHR1 (NP_000397); green monkey (*Cercopithecus aethiops*) GnRHR2 (AAK52746); rhesus monkey (*Macaca mulatta*) GnRHR2 (NP_001028014); house mouse (*Mus musculus*) GnRHR1 (AAA37716); two chicken (*Gallus gallus*) GnRHRs (GnRHR1: NP_989984, GnRHR2: NP_001012627); three bull frog (*Rana catesbeiana*) GnRHRs (GnRHR1: AAG42575, GnRHR2: AAG42949, GnRHR3: AAG42574); two pipid frog (*Xenopus tropicalis*) GnRHRs (GnRHR1: compiled from the Ensembl *X. tropicalis* genome project (version JGI 4.1) using peptide ID ENSXETP00000038462 and scaffold 22, GnRHR2: compiled from Ensembl peptide ID ENSXETP00000036235 and scaffold 3972); two Nile tilapia (*Oreochromis niloticus*) GnRHRs (type 1: BAC77240, type 2: BAC77241); three Japanese medaka (*Oryzias latipes*) GnRHRs (GnRHR1: BAB70506, GnRHR2: BAB70505, GnRHR3: BAC97833; two goldfish (*Carassius auratus*) GnRHRs (type A:

AAD20001, type B: AAD20002); two African cichlid (*Astatotilapia burtoni*) GnRHRs (GnRHR1: AAU89433, GnRHR2: AAK29745); lamprey (*Petromyzon marinus*) GnRHR (AAQ04564), four sea squirt (*Ciona intestinalis*) GnRHRs (GnRHR1: NP_001028997, GnRHR2: NP_001028996, GnRHR3: NP_001028995, GnRHR4: NP_001028994); octopus (*Octopus vulgaris*) GnRHR (BAE66647); fruitfly (*Drosophila melanogaster*) adipkinetic hormone receptor (AKHR, AAC61523); and the cockroach (*Periplaneta Americana*) AKHR (AAQ17230).

5.2.8 Data analysis

All IP samples were measured in duplicate within each assay and all assays were repeated in at least three independent experiments. Dose response curves were plotted using non-linear regression, a feature in the PRISM5 software suite (GraphPad). The peptide concentrations inducing half-maximal stimulation (log EC_{50}) were calculated and presented in Table 5.3. The data were calculated from the mean ± SEM of three independent experiments and differences between log EC_{50} values were compared using a paired t-test with a two-tailed P-value. $P<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Cloning and structure of four Amphiouxs GnRHR candidates

Our *in silico* analysis identified gene fragments encoding four candidate GnRH receptors from the *Branchiostoma floridae* draft genome assembly. Four complete open-reading frames encoding a putative GnRHRs were cloned. 5'- and 3'-RACE were used to complete any missing regions and irregularities in the genomic sequence. The four

putative GnRHR coding sequences, as well as an amphioxus GnRHR2 variant that has a deletion in the region coding for ICL3 (referred to as GnRHRΔICL3), were deposited in GenBank under the following accession numbers: amphioxus gnrhr1, EU433377; amphioxus gnrhr2, EU433378; amphioxus gnrhrΔICL3, EU433379; amphioxus gnrhr3, EU433380; amphioxus gnrhr4 (to be obtained).

The molecular organizations of each GnRHR ORF, as well as their scaffold arrangements, are shown in Figure 5.1. The isolated amphioxus GnRHR1 ORF was 1,212 bp (including the stop codon), encoding a putative protein of 403 amino acids. This gene contains three exons (567 bp, 211 bp and 434 bp) separated by two introns (553 bp and 588 bp). It spans 2.35 kb on scaffold 15. The ORF for amphioxus GnRHR2 consisted of 1,194 bp, encoding a putative protein of 397 amino acids. It has three exons (564 bp, 199 bp and 428 bp) that are interrupted by two introns (604 bp and 572 bp) and spans 2.37 kb on the same scaffold. In the process of cloning GnRHR2, I isolated a variant of GnRHR2 (GnRHRΔICL3) in which the proximal region of intracellular loop 3 contained a 21 bp deletion (Figure 5.2). After performing both 5'- and 3'-RACE, we isolated a complete 2,496 bp ORF for GnRHR3, encoding the largest putative GnRHR protein identified to date (831 amino acids). This gene is encoded by four exons (426 bp, 156 bp, 196 bp and 1,718 bp), is separated by three introns (617 bp, 1,175 bp and 1,284 bp) and spans 5.57 kb on scaffold 629. Finally, the ORF (1,266 bp) for amphioxus GnRHR4 was determined using 5'- and 3'- RACE, and consists of 5 exons (237 bp, 174 bp, 159 bp, 202 bp and 494 bp), with four introns (~4,200 bp, 512 bp, 1,322 bp, and 641 bp). It encodes a protein of 421 amino acids. This gene is located 883 kb downstream of GnRHR1 on scaffold 15.

Table 5.3. Inositol phosphate measurements after incubation of mGnRH, GnRH2 and AKH with amphioxus GnRHR1-3.*

	Inositol Phosphate Accumulation **EC_{50} (logM)			
Ligand	GnRHR1	GnRHR2	GnRHR2ΔICL3	GnRHR3
mGnRH	$-5.56\pm0.03_a$	$-8.35\pm0.04_c$	$-7.13\pm0.05_e$	-
GnRH2	$-6.23\pm0.05_b$	$-7.30\pm0.09_d$	$-6.27\pm0.05_f$	$-7.07\pm0.03_g$
AKH	-	-	-	$-6.43\pm0.02_h$

* IP measurements in COS7 cells, which had been transfected with receptor cDNA 48 h earlier. 0.9 µCi/well *myo*-[2-^{3}H]-inositol was added to the cells after 24 h post-transfection and ligands were added for 1 h at 48 h post-transfection.

** Dose of peptide stimulating half-maximal IP response (EC_{50}). Data were derived from the means of triplicate samples from three or more independent experiments.

a-h EC_{50} within each column that are significantly different are indicated by *different subscript letters* ($P < 0.05$)

A dash (-) denotes no response detected.
GnRH1=mGnRH

5.3.2 Amphioxus GnRHRs share conserved motifs with vertebrate GnRHRs

The four amphioxus GnRHR cDNAs encode proteins with the hydrophobicity profiles characteristic of seven transmembrane GPCRs. When compared to other chordate GPCRs, the four amphioxus receptors showed high conservation in the TMDs, with some conservation seen in the intracellular and extracellular loops. The N-terminal extracellular region and the C- terminal intracellular tails showed little conservation with GnRHRs from any other species. Sequence similarity searches querying the NCBI non-redundant protein database with the translated amphioxus GnRHRs all returned GnRHRs as the top BLAST hits. Amphioxus GnRHR1 and R2 displayed the highest sequence conservation to the rubber eel (*Typhlonectes natans*, AF174481) GnRH receptor, both with 46 % identity and 63 % similarity. GnRHR3 generated the closest match to the octopus GnRHR (with 38 % identity and 57 % similarity in the aligned region), whereas GnRHR4 showed the highest sequence match to the European sea bass (*Dicentrarchus labrax*) GnRHR2B (35 % identity and 56 % similarity).

Motifs that are common among GPCRs generally, and GnRHRs specifically, include potential N-glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid residue except Pro) in their N-terminal domains. Amphioxus GnRHR1 has five potential N-glycosylation sites at positions 3, 16, 19, 27 and 39; GnRHR2 also has five sites at positions 13, 16, 32, 35 and 40; GnRHR3 has four sites at positions 7, 25, 38 and 42; GnRHR4 has four sites at positions 11, 27, 36, and 42. The intracellular loops and C-terminal tail of amphioxus GnRHRs possess many putative serine or threonine residues that may be targets for both protein kinase A and protein kinase C. These residues were characterized based on their placement near basic residues in context with a consensus

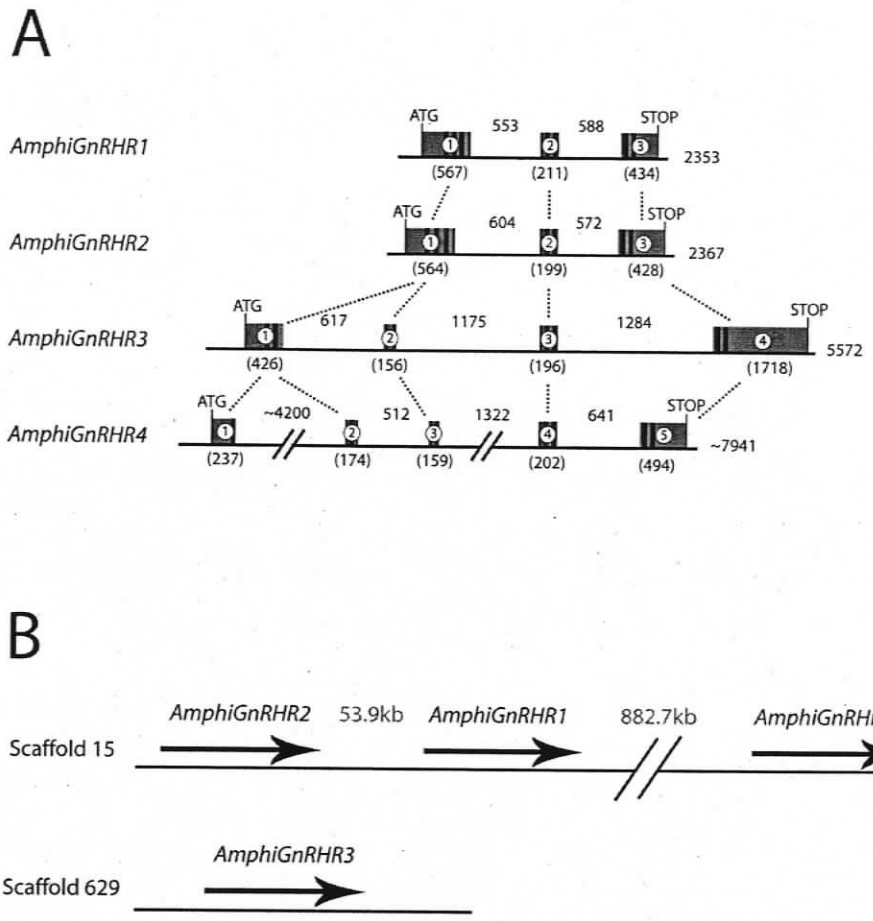


Figure 5.1. Genomic structure and location of amphioxus GnRH receptor open reading frames. (A) Detailed structure of the amphioxus GnRH receptor ORFs. The exons are boxed and numbered. The seven TMDs are indicated by black bars. Numbers in the parentheses below each exon indicate its size in base pairs (bp); the numbers above each ORF indicate the size (bp) of corresponding introns. Homologous exons between the ORFs are indicated by a dashed line. In GnRHR3 and GnRHR4, two exons can be joined to represent one corresponding homologous exon, as indicated by the dashed lines. (B) Genomic structures of the amphioxus GnRH receptor protein coding regions. Three GnRH receptors are encoded by a single gene cluster in the *B. floridae* genome. The 950 kb genomic region on scaffold 15 contains three genes, each encoding a GnRH receptor homolog. Arrows indicate the location and orientation of the ORFs.

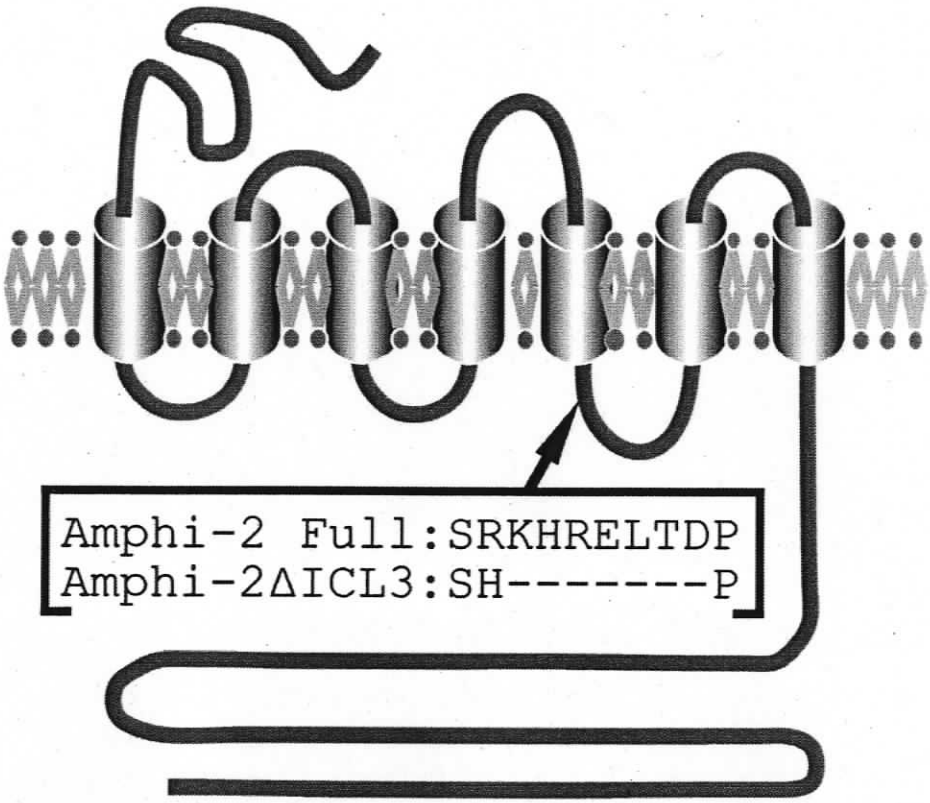


Figure 5.2. Schematic comparing the full-length and ΔICL3 variants of amphioxus GnRHR2. The heptahelical structure shows the site of nucleotide deletion and the corresponding amino differences between the full-length and ΔICL3 variants.

sequence as described (Blom *et al.*, 2004). The potential sites for phosphorylation in GnRHR-1 include serine$^{(89, 163, 272, 274, 391)}$ and threonine$^{(252, 375, 403)}$; for GnRHR2, they include serine$^{(88, 268, 366, 386, 395)}$ and threonine$^{(79, 155, 254, 389, 390, 396)}$. As a result of the deletion in in ICL3, the GnRHR2 variant, GnRHR2ΔICL3, was missing the putative PKA phosphorylation site at threonine$^{(254)}$. As well, each downstream putative phosphorylation site from ICL3 was also shifted by 7 residues towards to the N-terminus. Amphioxus GnRHR3 had many potential phosphorylation sites due to its long C-terminal tail. These include serine$^{(85, 99, 339, 351, 369, 375, 379, 384, 411, 426, 433, 449, 471, 489, 554, 558, 586, 595, 621, 648, 674, 683, 688, 695, 724, 735, 739,747, 754, 765, 781, 811, 827, 828)}$ and threonine$^{(172, 248, 261, 280, 283, 344, 345, 377, 385, 464, 466, 476, 609, 799, 804, 823, 826, 829)}$, whereas GnRHR4 has serine$^{(90, 274, 340,370, 380, 381, 393)}$ and threonine$^{(83, 257, 262, 343, 371, 397)}$. In addition, all amphioxus GnRHRs, except GnRHR4, possess a conserved G/S-X-X-X-G/S motif in TMD4, which is found in some GPCRs that can function as a dimer interface (Dawson *et al.*, 2002).

Amphioxus GnRHR1 and R2 share many conserved residues (Figure 5.3) and motifs with other vertebrate GnRHRs, including human, more so than GnRHR3 and R4. Several residues previously characterized to be involved in ligand binding or formation of binding pockets are conserved only in GnRHR1 and R2: Asp$^{2.61(98)}$ (D), Asn$^{2.65(102)}$ (N) in TMD2; Lys$^{3.32(121)}$ (K) in TMD3; Tyr$^{6.51(283)}$ (Y), Tyr$^{6.52(284)}$ (Y) in TMD6. Other key residues are present in all amphioxus receptors: Trp$^{2.64(101)}$ (W) in TMD2; Asn$^{5.39(212)}$ in TMD5; Trp$^{6.48(280)}$ (W) and Trp$^{6.57(289)}$ (W) in TMD6. Conserved residues and microdomains that are involved in receptor activation are also present in the four amphioxus GnRHRs, with Asn$^{1.50(53)}$ (N) in TMD1, Asp$^{2.50(86)}$ (D) in TMD2, the

DRxxxI/V motif within TMD3 and N/DPxxY in TMD7 (DPxxY in GnRHR1 and R2, and NPxxY in GnRHR3 and R4).

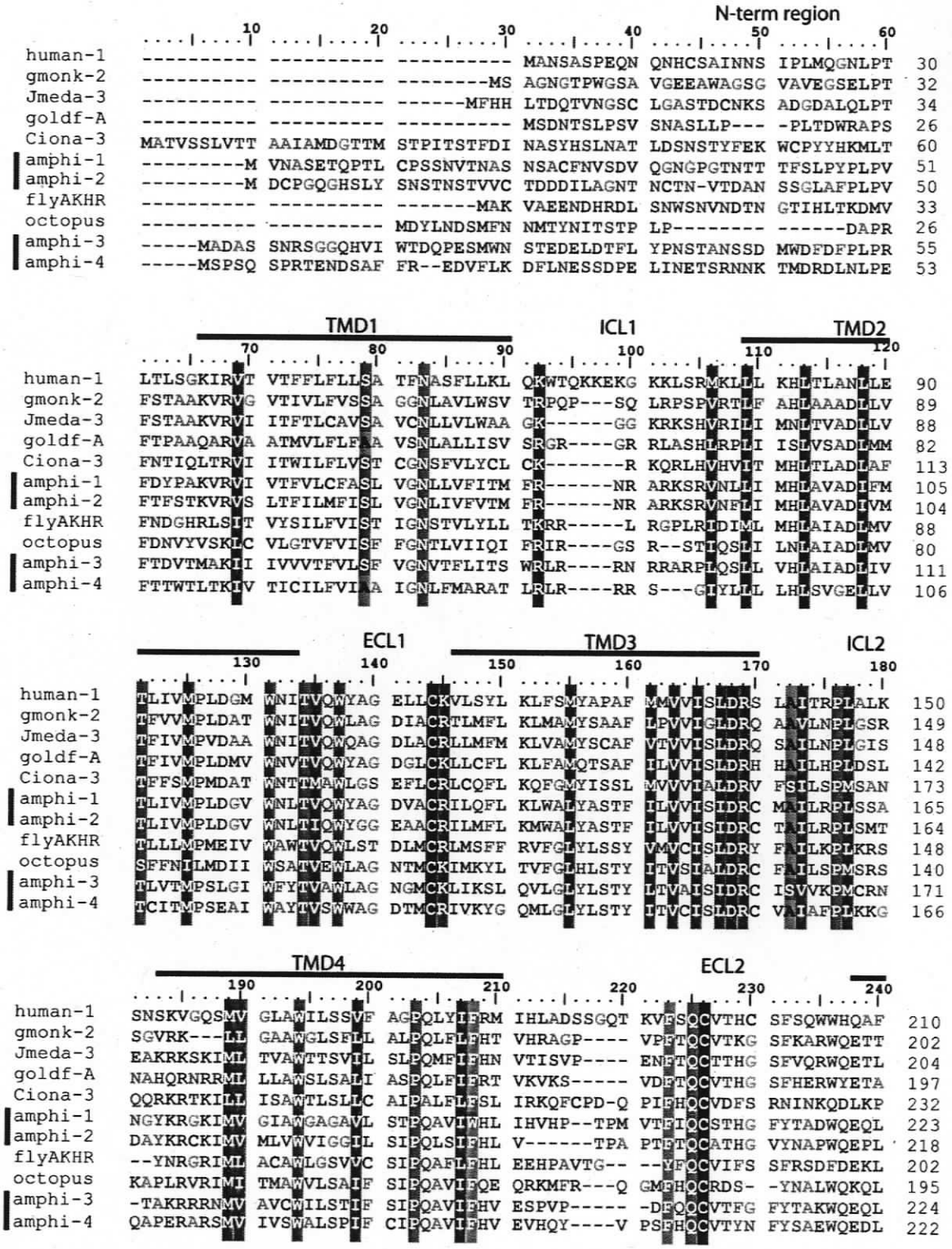
5.3.3 Phylogenetic relationships

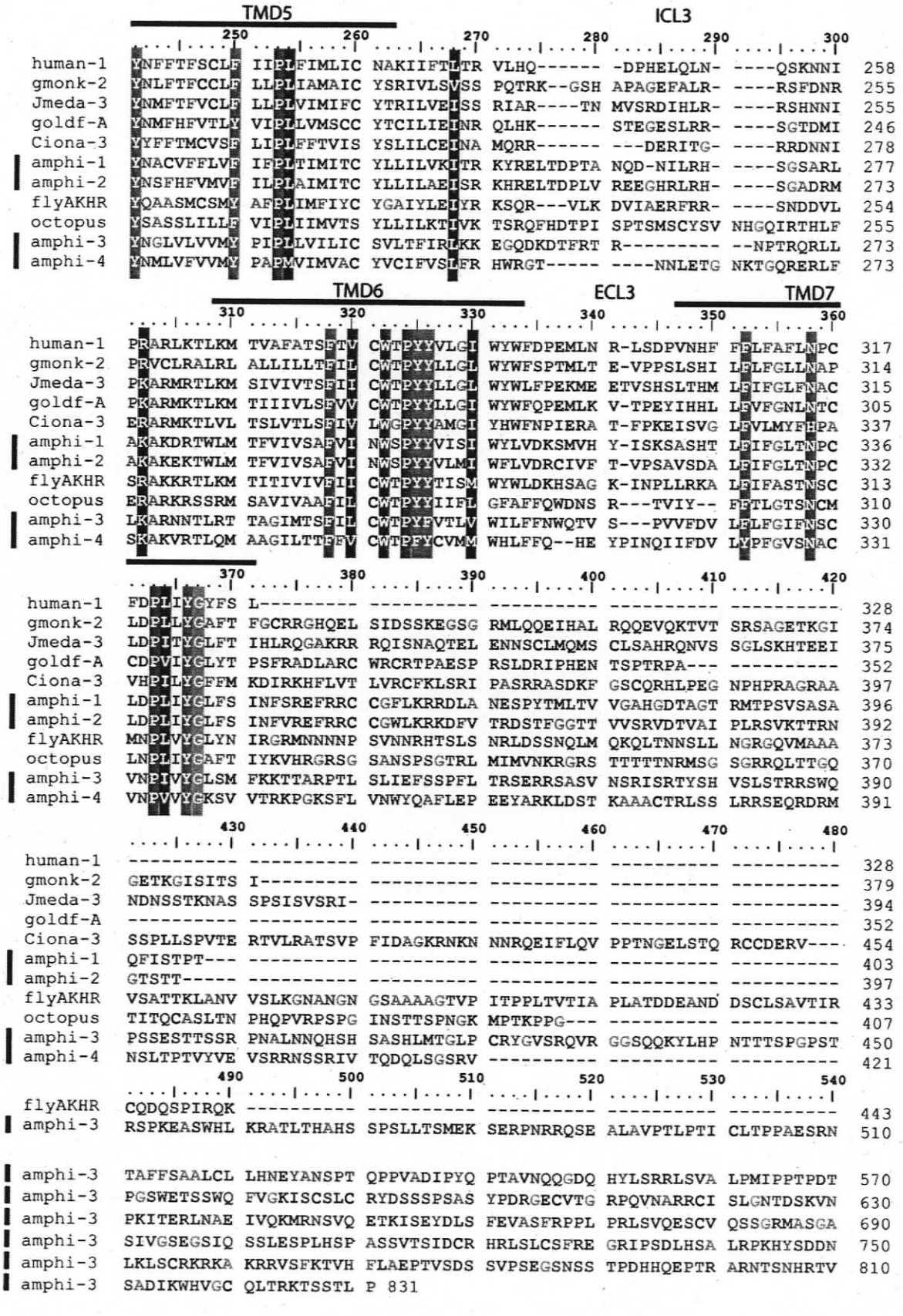
Maximum likelihood phylogenetic analysis of the protein coding region spanning the seven transmembrane domains of the amphioxus GnRH receptor candidates with GnRHR representatives from all vertebrate types, the tunicate GnRHRs, the octopus GnRHR-like sequence, as well as the related adipokinetic hormone receptors, yielded a consensus tree that separates the amphioxus receptors into two groupings (Figure 5.4). In one group, amphioxus GnRHR1 and R2 are placed basally in a monophyletic group that includes all of the vertebrate types, achieving robust support (85 % bootstrap proportion). Amphioxus GnRHR3 and R4 showed the closest association with the octopus GnRHR-like sequence (91 % bootstrap proportion), which formed a monophyletic group with the related AKHRs.

5.3.4 Two vertebrate GnRHs stimulate two amphioxus GnRH receptors through the IP pathway

To determine whether each amphioxus cDNA encoded a functional GnRHR, COS7 cells were transfected with each amphioxus GnRH receptor cDNA, and intracellular IP production was measured with two vertebrate GnRHs (mGnRH and GnRH2) and insect adipokinetic hormone (AKH) (peptide structures are shown in Table. 5.2). The ability of each ligand to induce an IP dose response is shown in Table 5.3 (log EC_{50} values). COS7 cells transfected with an empty vector did not induce an IP dose

Figure 5.3. Sequence alignment of amphioxus GnRHRs with representative vertebrate GnRHRs and invertebrate GnRHR-like sequences. Putative amino acid sequences were aligned using ClustalW. Dashes indicate gaps introduced in the sequence to optimize the alignment. The putative transmembrane domains (TMDs) are indicated by horizontal bars and intracellular and extracellular loops (ICLs and ECLs) are noted above the alignment. Sequences are shaded according to the Blosum62 matrix where 100% identity or conservative replacement is seen in all sequences.


N-term region
TMD1
ICL1
TMD2
ECL1
TMD3
ICL2
TMD4
ECL2
human-1
gmonk-2
Jmeda-3
goldf-A
Ciona-3
amphi-1
amphi-2
flyAKHR
octopus
amphi-3
amphi-4


TMD5
ICL3
TMD6
ECL3
TMD7
human-1
gmonk-2
Jmeda-3
goldf-A
Ciona-3
amphi-1
amphi-2
flyAKHR
octopus
amphi-3
amphi-4

response with any of the peptides tested.

The two vertebrate forms of GnRH were able to elicit robust intracellular inositol phosphate accumulation in amphioxus GnRHR1, R2, and R2ΔICL3 (Figure 5.5). Statistical analysis showed that GnRH2 was much more potent than mGnRH (GnRH1) at GnRHR1 (EC_{50} of 580 nM compared to 2,750 nM). This trend was reversed for receptor 2, which was more selective for mGnRH (EC_{50} of 4.5 nM) compared to GnRH2 (50 nM). GnRHR2ΔICL3 showed a pronounced reduction in sensitivity to both GnRH2 and mGnRH when compared to the full-length clone (GnRHR2), where increased concentrations of ligand were necessary to induce a dose response (11-fold and 17-fold reduction was seen for GnRH2 and mGnRH, respectively). GnRHR3 was activated by GnRH2 and AKH with EC_{50} values of 85.1 nM and 372 nM, respectively. Mammalian GnRH-induced inositol phosphate accumulation did not reach 2-fold above basal levels and was considered ineffective with GnRHR3, whereas GnRHR4 remains to be tested.

5.4 Discussion

Repositioned at the base of the chordates, amphioxus is an important model in which to examine the pre-vertebrate condition of GnRH signalling components. Various immunocytochemical studies using antibodies raised against vertebrate GnRH forms report the presence of GnRH-like molecules within this cephalochordate. However, the exact structure or characteristics of any component of the GnRH system in amphioxus is still unknown. Here, we show for the first time the presence of four GnRH receptors that cluster into two distinct phylogenetic groups in amphioxus. Cloned are four GnRHRs, two that show high conservation with the GnRHRs found in vertebrates, and two that

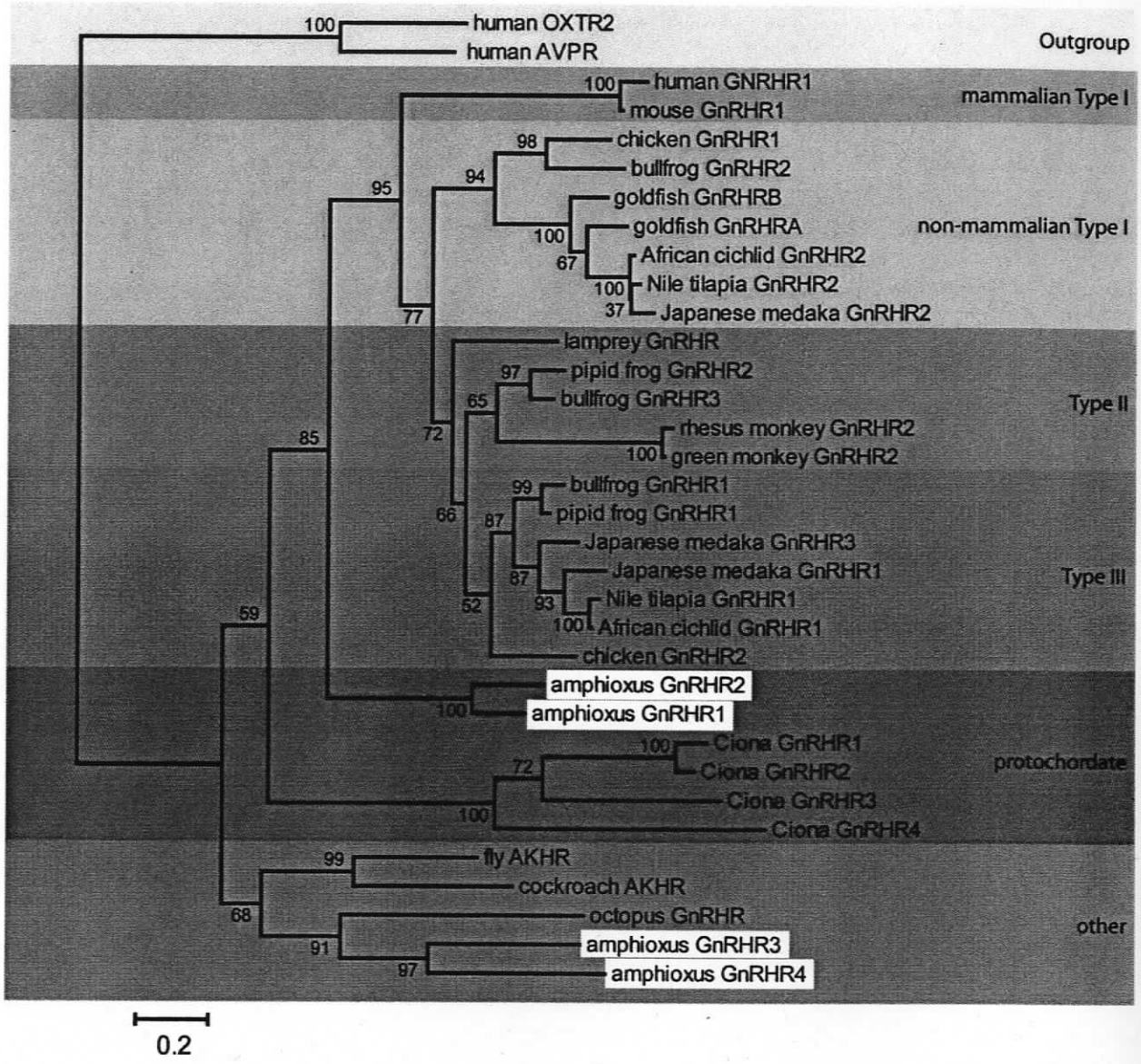
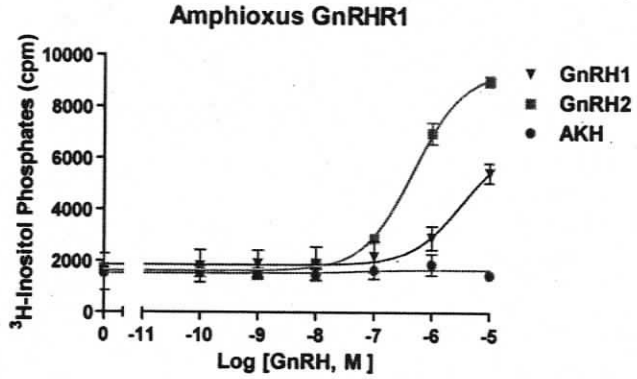


Figure 5.4. Consensus maximum likelihood phylogenetic tree of vertebrate GnRH receptors and invertebrate GnRHR-like sequences. Bootstrap proportions for each hypothesized receptor group are indicated above or below the branch leading to the group (100 bootstrap replicates). The human oxytocin and vasopressin receptors are used as outgroups to root the tree. Receptors identified in this report are boxed in white.

Figure 5.5. Inositol phosphate accumulation in COS7 cells expressing amphioxus GnRHRs induced with various peptides. *myo*-[2-^{3}H]IP accumulation is measured in cells expressing either amphioxus GnRHR1 (A), GnRHR2 (B), GnRHR2ΔICL3 (C), or GnRHR3 (D) induced with graded concentrations of indicated peptide for 1h. Cells were transfected with 0.8 μg of vector containing GnRHR cDNA. At 24 h after transfection, labeled inositol was added and 48 h post-transfection the cells were washed and then stimulated. The intracellular ^{3}H-IP concentrations are shown as scintillation counts per minute (cpm). Each dose response curve is from a single experiment representative of a total of three or more and *error bars* signify the mean ± SEM of the replicates. GnRH1=mGnRH


Amphioxus GnRHR1
^{3}H-Inositol Phosphates (cpm)
10000
8000
6000
4000
2000
0
0 -11 -10 -9 -8 -7 -6 -5
Log [GnRH, M]
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GnRH2
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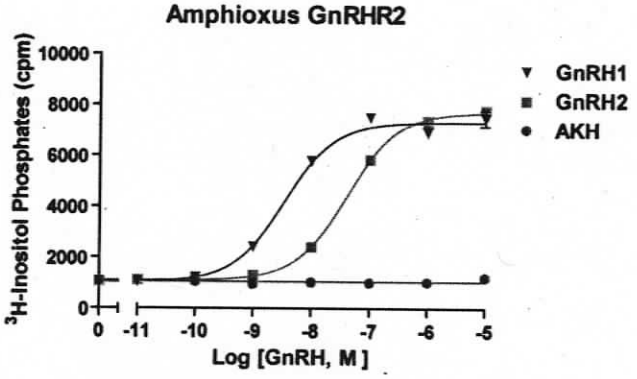
Amphioxus GnRHR2
^{3}H-Inositol Phosphates (cpm)
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GnRH1
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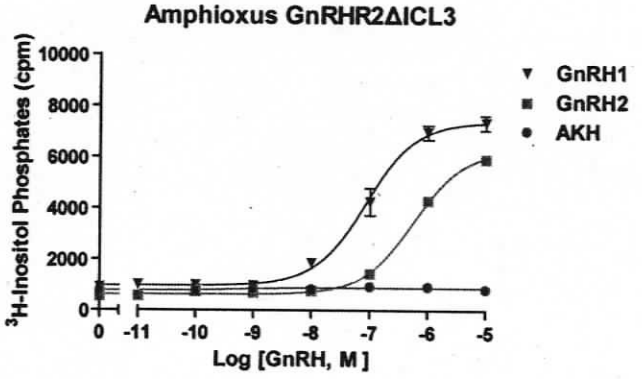
Amphioxus GnRHR2ΔICL3
^{3}H-Inositol Phosphates (cpm)
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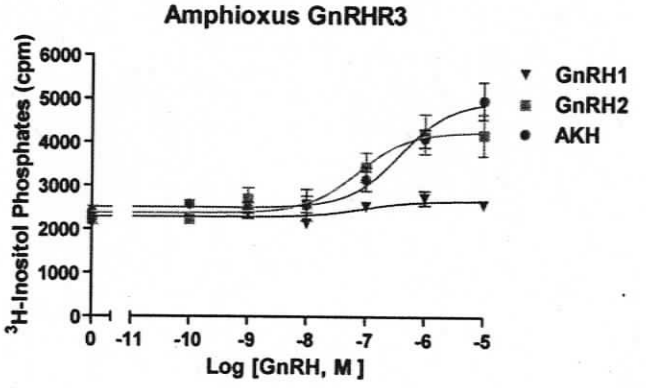
Amphioxus GnRHR3
^{3}H-Inositol Phosphates (cpm)
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Log [GnRH, M]
GnRH1
GnRH2
AKH

demonstrate close phylogenetic relationship with the recently cloned octopus GnRH receptor. To identify evolutionarily conserved functions between vertebrate and basal chordate GnRH receptors, two vertebrate GnRH forms were investigated for their ability to activate the amphioxus receptors, as measured by intracellular accumulation of inositol phosphates. Two receptors were physiologically active with both vertebrate GnRH forms. A unique characteristic among non-mammalian type I GnRHRs is selectivity for GnRH2 (Millar *et al.*, 2004; Kah *et al.*, 2007). However, amphioxus GnRHR2 displayed a higher sensitivity for mGnRH compared with GnRH2, and had ligand potency in the nM range. One of the amphioxus GnRHR-type receptors possessed sensitivity to GnRH2, as well as to insect AKH, which has been shown to stimulate a receptor with high sequence similarity to that of the octopus GnRHR.

5.4.1 The amphioxus genome encodes four GnRH receptors

Searches of the amphioxus draft genome revealed the presence of four candidate GnRH receptors, which were subsequently cloned and characterized. Amphioxus GnRHR1 and R2 are encoded by very similar ORFs, containing three exons located in tandem on scaffold 15. The close proximity of amphioxus GnRHR1 and R2 to each other, in addition to their high sequence similarity, indicate that one of these genes likely resulted from a tandem duplication event. Whether the same theory can be applied to GnRHR3 and R4 is less clear because each gene is on a separate genomic scaffold. Mapping of these scaffolds to their respective chromosomes is needed to clarify their locations with respect to each other. We found that GnRHR3 encodes the largest putative GnRH receptor identified to date, with a C-terminal tail of 490 amino acids (Kakar *et al.*,

1992). GnRHR3 also contains many putative serine and threonine residues, which may be targets for phosphorylation by protein kinase A and protein kinase C (Blom *et al.*, 2004). The putative phosphorylation sites within the four amphioxus GnRHRs are characteristic of many GPCRs, where phosphorylation of intracellular serine or threonine sites by PKC can lead to uncoupling of the receptor from signalling pathways, resulting in receptor desensitization (Pitcher *et al.*, 1998).

The availability of increasing numbers of genome sequencing projects has allowed us to compare the amphioxus GnRH receptors with a diverse sample of vertebrate receptors. Perusal of the GnRHR protein coding sequences, available in the Ensembl genomic databases (http://www.ensembl.org/), in addition to those sequences identified in our laboratory, showed that amphioxus GnRHR1 and R2 are like most vertebrate GnRH receptors; they are encoded by open-reading frames consisting of three exons, with the same intron phase and positions of intronic insertion. The corresponding three exons encode the same regions of the GnRH receptor protein; exon 1 encodes the N-terminal extension to the middle of TMD4; exon two encodes the protein region from TMD4 to ICL3; and exon three encodes the protein region from the middle of the ICL3 to the end of the C-terminal tail. When differences in the length of exon 2 are accounted for, which can vary at its 3' end to accommodate for various lengths of ICL3, a homologous exon can be found in most vertebrate GnRHRs, as well as those of *Ciona* and octopus (Kanda *et al.*, 2006; Fridmanis *et al.*, 2007). This homologous exon is shifted to exon 3 in amphioxus GnRHR3, and to exon 4 in GnRHR4 (Figure 5.1). The conservation of this exon from mollusks to vertebrates indicates a truly ancient gene history, possibly present in the bilaterian ancestor before the deuterostome/protostome split over 600 million years

ago (Tello *et al.*, 2005; Kanda *et al.*, 2006). A recent study comparing rhodopsin GPCR intron density suggests that ancestral GPCRs were largely intronless, or with a limited number of introns, and that invertebrate GPCRs accumulated a larger proportion of introns during their evolution than mammals (Fridmanis *et al.*, 2007). In addition, several groups have suggested that a proto-splice site may contribute to positional intron insertion, which may explain why some specific lineages have larger numbers of introns. If this proposal is correct, an ancestral chordate GnRHR gene may have been composed of three exons, much like those of amphioxus GnRHR1 and R2.

5.4.2 Amphioxus receptors possess many conserved GnRHR structural motifs

Mutation of over 30 % of the positions in the human GnRH receptor has revealed many key residues and motifs that are important for expression, structural integrity, ligand binding, receptor activation and coupling to various intracellular signalling pathways. Although the context is different in each amphioxus GnRHR, many of the sites present at homologous positions in the human GnRHR are well conserved in amphioxus GnRHR1 and R2, but less so in receptors R3 and R4. All four amphioxus receptors possess two invariant cysteine residues in ECL1 and ECL2 that have been shown in many GPCRs to provide stability to the tertiary structure and maintain the receptor in a ready conformation (Strader *et al.*, 1994). As well, each receptor has multiple putative N-glycosylation sites in their N-terminal regions that have been shown to be important for cell surface expression, but not ligand binding (Davidson *et al.*, 1995). A conserved $Asp^{2.61(98)}$ residue at the extracellular end of TMD2 of the human GnRHR, proposed to be involved in multiple GnRH interactions (Flanagan *et al.*, 2000; Millar *et al.*, 2004), is

present in amphioxus receptor R1 and R2. In amphioxus GnRHR4, Glu is present at the homologous position of $Asp^{2.61(98)}$. Mutation of the human receptor from $Asp^{2.61(98)}$ to $Glu^{2.61(98)}$ displayed decreased binding, expression and G protein-coupling efficiency (Flanagan *et al.*, 2000). As well, structural modeling has shown that $Asp^{2.61(98)}$ promotes an intramolecular salt bridge with $Lys^{3.32(121)}$ in TMD3 (Flanagan *et al.*, 2000). However, $Lys^{3.32(121)}$ is only present in amphioxus receptors R1 and R2, and is replaced by glutamine in the other two receptors. Interestingly, mutation of the human GnRHR from $Lys^{3.32(121)}$ to $Gln^{3.32(121)}$ decreased receptor sensitivity to mGnRH, but not to antagonists (Zhou *et al.*, 1995), suggesting that this residue in amphioxus GnRHR3 and R4 may contribute to the low sensitivity to vertebrate GnRHs. The conserved $Asn^{2.65(102)}$ is present in GnRHR1 and R2, but is substituted for Phe in GnRHR3 and for Ala in GnRHR4. Mutation of the human GnRHR $Asn^{2.65(102)}$ to $Ala^{2.65(102)}$ showed a decrease in GnRH agonist binding, likely due to an interaction with Gly^{10} of GnRH (Davidson *et al.*, 1996; Hoffmann *et al.*, 2000). The hydrophobic residues at the extracellular end of TMD6 ($Tyr^{6.51(283)}$, $Tyr^{6.52(284)}$ $Trp^{6.57(289)}$) that have been shown to be critical for GnRH receptor function (Millar *et al.*, 2004) are retained in both GnRHR1 and R2 but not in R3 and R4. The low conservation of many key residues in GnRHR3 and R4 with those of vertebrate GnRHRs suggests that these receptors may possess different functional arrangements that contribute to the low sensitivity to vertebrate GnRHs. The high structural conservation of both amphioxus receptor R1 and R2 with vertebrate GnRH receptors supports the pharmacological data with mGnRH and GnRH2, showing potent IP turnover. The selectivity for mGnRH by amphioxus receptor R2 is not seen in any

other non-mammalian receptor, and represents an important model to investigate which residues confer this selectivity.

5.4.3 Two amphioxus GnRHRs possess a proto-vertebrate condition

Our phylogenetic analysis of the four amphioxus GnRHRs places two receptors at the base of the clade comprising the four extant vertebrate GnRH types; the other two receptors position in close association with the octopus GnRHR-like sequence. The grouping of the four amphioxus GnRH receptors into two monophyletic pairs suggests that a lineage-specific duplication event (possibly specific to the amphioxus line) formed these paralogous relationships. The position of amphioxus GnRHR1 and R2 at the base of vertebrates, without the inclusion of the *Ciona* GnRHRs, indicates that a high rate of lineage specific sequence divergence occurred in the urochordate lineage after its split from an amphioxus-like ancestor. This is supported by analysis of the *Ciona* genome, which found many derived gene features, possibly due to specific adaptations made by the urochordate lineage (Hughes and Friedman, 2005).

It was suggested that the octopus GnRH receptor was the counterpart to that of chordates (Kanda *et al.*, 2006). However, the presence of two distinct GnRH receptor types in amphioxus, one being proto-vertebrate-like and the other more like the octopus GnRHR, suggests that the latter type was eventually lost in the chordate lineage. Whether the bilaterian ancestor possessed a GnRH receptor gene with characteristics similar to that of protochordates or that of the octopus GnRHR-like receptor remains to be resolved. Analysis of additional classes of invertebrates may help to clarify this question.

5.4.4 Two amphioxus GnRHRs show selectivity with two vertebrate GnRHs

To determine whether amphioxus possesses functional GnRH receptors, I tested the ability of graded concentrations of two vertebrate GnRH forms to induce intracellular accumulation of inositol phosphates in amphioxus GnRHR-expressing COS7 cells. Our pharmacological testing of the three amphioxus GnRHRs suggests that each is functional with GnRH2 (see Table 5.3). Amphioxus GnRHR2 displayed high sensitivity to mGnRH with an EC_{50} value of 4.5 nM, and less so to GnRH2 (EC_{50} = 50 nM). Interestingly, this preference for mGnRH over GnRH2 has only been seen in mammalian type I GnRHRs (Flanagan *et al.*, 2007; Kah *et al.*, 2007). This selectivity was reversed for GnRHR1, although overall, GnRH2 and mGnRH had significantly reduced potencies compared to GnRHR2. Future mutagenesis studies will be important to resolve which specific residues confer the selectivity differences between these two receptors. This may also help to uncover novel GnRH/receptor residue interactions in higher vertebrates.

The isolated GnRHR2ΔICL3 displayed over a 10-fold reduction in ligand potency to both mGnRH and GnRH2, indicating that this deletion may act to disrupt $G_{q/11\alpha}$ coupling sites present within the neighbouring ICL3. We are currently unsure whether this variant is a splicing artifact or alternate allele possessed by the specific amphioxus population used in our study.

GnRH2, as well as the insect metabolic hormone AKH, albeit at high ligand concentrations (in the μM range), induced intracellular accumulation of inositol phosphates in GnRHR3- expressing COS7 cells. Adipokinetic hormone is the ligand for an insect GPCR, which shows very high structural similarity to that of the octopus GnRH receptors and are thought to be evolutionarily related to the vertebrate GnRHRs (Staubli

et al., 2002). The low potency of GnRH2 and the sensitivity to AKH for amphioxus GnRHR3 may indicate a different endogenous ligand for R3 from that of amphioxus GnRHR1 and R2. The recent cloning of two mollusk GnRH genes, one from the common octopus and one from the sea hare (*Aplysia califonica*), indicates a deviation from the decapeptide architecture found in tunicates and vertebrates, and suggests that a structurally distinct cognate ligand may be present in amphioxus as well.

Our pharmacological testing shows that the capacity to respond to mGnRH and GnRH2 is evolutionarily conserved between amphioxus and vertebrates, and that key motifs found to be important in GnRH binding, signalling and activation are present in amphioxus receptors (primarily in GnRHR1 and R2). The higher sensitivity of GnRHR1 and R2 to two vertebrate GnRH forms indicates that this paralogous pair may represent the proto-vertebrate condition, after which gene duplication and sequence divergence acted to elaborate into the four contemporary GnRHRs found in vertebrates.

5.4.5 Amphioxus GnRHRs may resolve the role of Hatschek's pit

Earlier studies have proposed that amphioxus contains a rudimentary pituitary homolog in the form of a ciliated organ called Hatschek's pit. To support this theory, several groups have found immunoreactivity in Hatschek's pit to GnRH (Chang *et al.*, 1984; Fang *et al.*, 1999), luteinizing hormone (Chang *et al.*, 1982; Nozaki and Gorbman, 1992), follicle-stimulating hormone (Chang *et al.*, 1984) and human chorionic gonadotropin (Nozaki and Gorbman, 1992). However, many of these vertebrate antibodies were raised against antigens that may differ significantly in structure from their amphioxus counterparts, or may cross-react with non-target proteins, as has been

shown to be the case with LH antibodies (Kubokawa, 2001). In addition, homologous genes of the vertebrate reproductive glycoproteins and their receptors are lacking in the genomes of both amphioxus (Holland *et al.*, 2008) and *C. intestinalis*, suggesting that these genes are vertebrate innovations (Hsu *et al.*, 2002). Although GnRH immunoreactivity in Hatschek's pit could not be confirmed (Nozaki and Gorbman, 1992; Castro *et al.*, 2006), the presence of GnRH receptors (this paper) suggests that amphioxus does contain a GnRH peptide, which may be found once analysis of the genome is optimized for detecting these small peptide sequences. Our ongoing studies aim to identify the locations of GnRH receptor expression and may clarify if Hatschek's pit is involved with amphioxus reproductive function. However, reproductive function at the level of the gonad appears to be the most ancient of all modes of GnRH receptor control (Sherwood *et al.*, 2005; Tsai, 2006; Kah *et al.*, 2007).

In summary, we demonstrated that four GnRH receptors are encoded in the genome of the Florida lancelet, *Branchiostoma floridae*. The four receptors group into two paralogous pairs, with one GnRH receptor pair grouping basally to the vertebrate GnRH receptors, and the other two GnRH receptors grouping with the newly identified octopus GnRHR-like sequence. Our pharmalogical studies indicate that the capacity to respond to mGnRH and GnRH2 is evolutionarily conserved in two amphioxus GnRH receptors. This study is an example of how comparison of evolutionarily distant animal species may reveal similarities and differences in molecular forms of hormone receptors, and allow us to speculate on the ancestral system from which all vertebrate GnRHRs evolved.

5.5 References

Adams, B. A., J. A. Tello, J. Erchegyi, C. Warby, D. J. Hong, K. O. Akinsanya, G. O. Mackie, W. Vale, J. E. Rivier and N. M. Sherwood (2003). "Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, Ciona intestinalis." Endocrinology **144**(5): 1907-19.

Barran, P. E., R. W. Roeske, A. J. Pawson, R. Sellar, M. T. Bowers, K. Morgan, Z. L. Lu, M. Tsuda, T. Kusakabe and R. P. Millar (2005). "Evolution of constrained gonadotropin-releasing hormone ligand conformation and receptor selectivity." J Biol Chem **280**(46): 38569-75.

Blom, N., T. Sicheritz-Ponten, R. Gupta, S. Gammeltoft and S. Brunak (2004). "Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence." Proteomics **4**(6): 1633-49.

Bourlat, S. J., T. Juliusdottir, C. J. Lowe, R. Freeman, J. Aronowicz, M. Kirschner, E. S. Lander, M. Thorndyke, H. Nakano, A. B. Kohn, A. Heyland, L. L. Moroz, R. R. Copley and M. J. Telford (2006). "Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida." Nature **444**(7115): 85-8.

Cameron, C. B., G. O. Mackie, J. F. Powell, D. W. Lescheid and N. M. Sherwood (1999). "Gonadotropin-releasing hormone in mulberry cells of Saccoglossus and Ptychodera (Hemichordata: Enteropneusta)." Gen Comp Endocrinol **114**(1): 2-10.

Castro, A., M. Becerra, M. J. Manso, N. M. Sherwood and R. Anadon (2006). "Anatomy of the Hesse photoreceptor cell axonal system in the central nervous system of amphioxus." J Comp Neurol **494**(1): 54-62.

Chang, C., Y. Chu and D. Chen (1982). "Immunocytochemical demonstration of luteinizing hormone (LH) in Hatschek's pit of amphioxus (*Branchiostoma belcheri*)." Kexue Tongbao **27**: 1233-1234.

Chang, C., Y.-X. Liu, Y. Zhu and H. Zhu (1984). The reproductive endocrinology of amphioxus. . Cambridge, Cambridge University Press.

Davidson, J. S., C. A. Flanagan, W. Zhou, Becker, II, R. Elario, W. Emeran, S. C. Sealfon and R. P. Millar (1995). "Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: role in receptor expression but not ligand binding." Mol Cell Endocrinol **107**(2): 241-5.

Davidson, J. S., C. A. McArdle, P. Davies, R. Elario, C. A. Flanagan and R. P. Millar (1996). "Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide." J Biol Chem **271**(26): 15510-4.

Dawson, J. P., J. S. Weinger and D. M. Engelman (2002). "Motifs of serine and threonine can drive association of transmembrane helices." J Mol Biol **316**(3): 799-805.

Delsuc, F., H. Brinkmann, D. Chourrout and H. Philippe (2006). "Tunicates and not cephalochordates are the closest living relatives of vertebrates." Nature **439**(7079): 965-8.

Fang, Y. Q., W. Huang, A. Chen and J. Zhang (1999). "Distribution of gonadotropin-releasing hormone in the brain and Hatschek's pit of amphioxus (*Branchiostoma belcheri*)." Acta Zoologica Sinica **45**: 106-111.

Flanagan, C. A., C. C. Chen, M. Coetsee, S. Mamputha, K. E. Whitlock, N. Bredenkamp, L. Grosenick, R. D. Fernald and N. Illing (2007). "Expression, structure, function, and evolution of gonadotropin-releasing hormone (GnRH) receptors GnRH-R1SHS and GnRH-R2PEY in the teleost, Astatotilapia burtoni." Endocrinology **148**(10): 5060-71.

Flanagan, C. A., V. Rodic, K. Konvicka, T. Yuen, L. Chi, J. E. Rivier, R. P. Millar, H. Weinstein and S. C. Sealfon (2000). "Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction." Biochemistry **39**(28): 8133-41.

Fridmanis, D., R. Fredriksson, I. Kapa, H. B. Schioth and J. Klovins (2007). "Formation of new genes explains lower intron density in mammalian Rhodopsin G protein-coupled receptors." Mol Phylogenet Evol **43**(3): 864-80.

Gorbman, A., A. Whiteley and S. Kavanaugh (2003). "Pheromonal stimulation of spawning release of gametes by gonadotropin releasing hormone in the chiton, Mopalia sp." Gen Comp Endocrinol **131**(1): 62-5.

Guindon, S., F. Lethiec, P. Duroux and O. Gascuel (2005). "PHYML Online--a web server for fast maximum likelihood-based phylogenetic inference." Nucleic Acids Res **33**(Web Server issue): W557-9.

Hoffmann, S. H., T. ter Laak, R. Kuhne, H. Reilander and T. Beckers (2000). "Residues within transmembrane helices 2 and 5 of the human gonadotropin-releasing hormone receptor contribute to agonist and antagonist binding." Mol Endocrinol **14**(7): 1099-115.

Holland, L. Z., R. Albalat, K. Azumi, E. Benito-Gutiérrez, M. J. Blow, M. Bronner-Fraser, F. Brunet, T. Butts, S. Candiani, L. J. Dishaw, D. E. K. Ferrier, J. Garcia-Fernàndez, J. J. Gibson-Brown, C. Gissi, A. Godzik, F. Hallböök, D. Hirose, K. Hosomichi, T. Ikuta, H. Inoko, M. Kasahara, J. Kasamatsu, T. Kawashima, A. Kimura, M. Kobayashi, Z. Kozmik, K. Kubokawa, V. Laudet, G. W. Litman, A. C. McHardy, D. Meulemans, M. Nonaka, R. P. Olinski, Z. Pancer, L. A. Pennacchio, M. Pestarino, J. P. Rast, I. Rigoutsos, M. Robinson-Rechavi, G. Roch, H. Saiga, Y. Sasakura, M. Satake, Y. Satou, M. Schubert, N. Sherwood, T. Shiina, N. Takatori, J. Tello, P. Vopalensky, S. Wada, A. Xu, Y. Ye, K. Yoshida, F. Yoshizaki, J. K. Yu, Q. Zhang, C. M. Zmasek, N. Putnam, D. S. Rokhsar, N. Satoh and P. W. Holland (2008). "The amphioxus genome illuminates vertebrate origins and cephalochordate biology." Genome Res Accepted 02/25/2008

Hsu, S. Y., K. Nakabayashi and A. Bhalla (2002). "Evolution of glycoprotein hormone subunit genes in bilateral metazoa: identification of two novel human glycoprotein hormone subunit family genes, GPA2 and GPB5." Mol Endocrinol **16**(7): 1538-51.

Hughes, A. L. and R. Friedman (2005). "Loss of ancestral genes in the genomic evolution of Ciona intestinalis." Evol Dev **7**(3): 196-200.

Iwakoshi-Ukena, E., K. Ukena, K. Takuwa-Kuroda, A. Kanda, K. Tsutsui and H. Minakata (2004). "Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (Octopus vulgaris) by in situ hybridization and immunohistochemistry." J Comp Neurol **477**(3): 310-23.

Iwakoshi, E., K. Takuwa-Kuroda, Y. Fujisawa, M. Hisada, K. Ukena, K. Tsutsui and H. Minakata (2002). "Isolation and characterization of a GnRH-like peptide from Octopus vulgaris." Biochem Biophys Res Commun **291**(5): 1187-93.

Kah, O., C. Lethimonier, G. Somoza, L. G. Guilgur, C. Vaillant and J. J. Lareyre (2007). "GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective." Gen Comp Endocrinol **153**(1-3): 346-64.

Kakar, S. S., L. C. Musgrove, D. C. Devor, J. C. Sellers and J. D. Neill (1992). "Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor." Biochem Biophys Res Commun **189**(1): 289-95.

Kanda, A., T. Takahashi, H. Satake and H. Minakata (2006). "Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor isolated from the common octopus (Octopus vulgaris)." Biochem J **395**(1): 125-35.

Karten, M. J. and J. E. Rivier (1986). "Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective." Endocr Rev **7**(1): 44-66.

Kubokawa, K. (2001). "Hatschek's pit: its endocrinological significance." Zoolog Sci **18**: 462-463.

Kusakabe, T., S. Mishima, I. Shimada, Y. Kitajima and M. Tsuda (2003). "Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian Ciona intestinalis." Gene **322**: 77-84.

Millar, R. P., Z. L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan and S. R. Maudsley (2004). "Gonadotropin-releasing hormone receptors." Endocr Rev **25**(2): 235-75.

Minguillon, C., J. Gardenyes, E. Serra, L. F. Castro, A. Hill-Force, P. W. Holland, C. T. Amemiya and J. Garcia-Fernandez (2005). "No more than 14: the end of the amphioxus Hox cluster." Int J Biol Sci **1**(1): 19-23.

Nozaki, M. and A. Gorbman (1992). "The Question of Functional Homology of Hatscheks Pit of Amphioxus (Branchiostoma-Belcheri) and the Vertebrate Adenohypophysis." Zoological Science **9**(2): 387-395.

Pitcher, J. A., N. J. Freedman and R. J. Lefkowitz (1998). "G protein-coupled receptor kinases." Annu Rev Biochem **67**: 653-92.

Powell, J. F., S. M. Reska-Skinner, M. O. Prakash, W. H. Fischer, M. Park, J. E. Rivier, A. G. Craig, G. O. Mackie and N. M. Sherwood (1996). "Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications." Proc Natl Acad Sci U S A **93**(19): 10461-4.

Reinhart, J., L. M. Mertz and K. J. Catt (1992). "Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor." J Biol Chem **267**(30): 21281-4.

Sherwood, N. M. and B. A. Adams (2005). Gonadotropin-releasing Hormone in Fish: Evolution, Expression and Regulation of the GnRH gene. Hormones and their receptors in fish reproduction. P. Melamed and N. M. Sherwood. Singapore, World Scientific Publishing Co. Pte. Ltd. **4:** 1-39.

Sherwood, N. M., B. A. Adams and J. A. Tello (2005). "Endocrinology of protochordates." Canadian Journal of Zoology-Revue Canadienne De Zoologie **83**(1): 225-255.

Silver, M. R., H. Kawauchi, M. Nozaki and S. A. Sower (2004). "Cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey representing the three families of Petromyzoniformes." Gen Comp Endocrinol **139**(1): 85-94.

Staubli, F., T. J. Jorgensen, G. Cazzamali, M. Williamson, C. Lenz, L. Sondergaard, P. Roepstorff and C. J. Grimmelikhuijzen (2002). "Molecular identification of the insect adipokinetic hormone receptors." Proc Natl Acad Sci U S A **99**(6): 3446-51.

Strader, C. D., T. M. Fong, M. R. Tota, D. Underwood and R. A. Dixon (1994). "Structure and function of G protein-coupled receptors." Annu Rev Biochem **63**: 101-32.

Tello, J. A., J. E. Rivier and N. M. Sherwood (2005). "Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate Ciona intestinalis GnRH receptors and the green monkey type II GnRH receptor." Endocrinology **146**(9): 4061-73.

Terakado, K. (2001). "Induction of gamete release by gonadotropin-releasing hormone in a protochordate, Ciona intestinalis." Gen Comp Endocrinol **124**(3): 277-84.

Tsai, P. S. (2006). "Gonadotropin-releasing hormone in invertebrates: structure, function, and evolution." Gen Comp Endocrinol **148**(1): 48-53.

Tsutsumi, M., W. Zhou, R. P. Millar, P. L. Mellon, J. L. Roberts, C. A. Flanagan, K. Dong, B. Gillo and S. C. Sealfon (1992). "Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor." Mol Endocrinol **6**(7): 1163-9.

Zhang, L., J. A. Tello, W. Zhang and P. S. Tsai (2007). "Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, Aplysia californica." Gen Comp Endocrinol.

Zhou, W., V. Rodic, S. Kitanovic, C. A. Flanagan, L. Chi, H. Weinstein, S. Maayani, R. P. Millar and S. C. Sealfon (1995). "A locus of the gonadotropin-releasing hormone receptor that differentiates agonist and antagonist binding sites." J Biol Chem **270**(32): 18853-7.

Chapter 6

Summary

Many different forms of gonadotropin-releasing hormone have been found in a wide diversity of species. To date, twenty-five unique forms of GnRH have been identified in vertebrate and invertebrate species, including the newly identified second protostome form (Zhang *et al.*, 2007). The presence of GnRH and its receptor in different animal lineages provides an opportunity to learn how the structure and function of a highly conserved hormone system has evolved in chordates. GnRH is the final integrator of neuronal regulatory control of the reproductive axis in vertebrates via the pituitary gonadotropes. It is unclear if a true pituitary homolog exists outside of the vertebrates, although Hatschek's pit in amphioxus or the neural gland in tunicates have been suggested as homologs. The gonadotropins and their receptors are lacking in basal chordates and invertebrates, indicating that the ancestral condition of the reproductive axis is quite different from that in vertebrates. This dissertation describes my investigation of GnRH systems in three chordate models: the vertebrate zebrafish, *Danio rerio*, the protochordate tunicate, *Ciona intestinalis* and the cephalochordate amphioxus, *Branchiostoma floridae*; the latter two are invertebrates.

Chapter 2 described the comprehensive analysis of the GnRH system of the tetraploid teleost zebrafish. Four unique GnRH receptors were cloned and then mapped to separate chromosomes. My phylogenetic, syntenic and functional studies found that the zebrafish receptors group into two paralogous pairs. One group, classified as non-mammalian type I, shows selectivity for GnRH2 over sGnRH. The other two receptors (type III) demonstrate equal potency to both endogenous GnRH forms. The dominant signaling pathway ($G_{q/11\alpha}$) was shown by the induction of inositol phosphates, although one receptor was effective at inducing intracellular accumulation of cAMP with GnRH2.

Localization of these receptors at the level of both mRNA and protein demonstrates receptor expression concurrent with GnRH ligands in many extra-pituitary tissues. As well, the generation of antibodies specific to each zebrafish receptor protein localized the expression of all four receptors to the pituitary of both male and female adult zebrafish, with overlapping regions in the gonadotropes. One of the most striking results with our antisera is the localization of GnRH receptors in the Purkinje cell layer of the cerebellum and the motor fibres of the medial longitudinal fasciculus in the vicinity of GnRH expressing neurites. These areas are responsible for integration of fine motor control, a plausible target for GnRH in the zebrafish. The zebrafish genome appears to have undergone duplications in many of its genes allowing for new gene copies to occasionally partition functions or gain a new function. The GnRH receptor complement also appears to have been duplicated in this model vertebrate, which may allow for the gain of novel GnRH functions. My study is a novel approach in that GnRH is suggested to have functions outside of the brain-pituitary-gonadal axis where these functions may be related or independent of reproduction.

In Chapter 3, I described the identification and characterization of multiple GnRH forms from the tunicate *Ciona intestinalis*. Two GnRH genes were cloned from this *Ciona* species, each encoding three GnRH peptides for a total of six novel peptides. An additional GnRH form was identified after mining the genetic data from a sister tunicate species, *Ciona savignyi*. Immunocytochemistry with GnRH antisera found labelling of the dorsal strand nerve plexus close to the gonads of *C. intestinalis*. To investigate if GnRH can affect the gonads, injection of tunicate GnRH forms near the gonads of gravid *Ciona* was performed; results indicated that synthetic GnRH had a potent effect on the

release of gametes, usually within minutes. A comparison of the GnRH gene structure with that of vertebrates not only detected conserved elements within the open-reading frame, but also found similar promoter elements that may bind orthologous transcription factors. The discovery that a GnRH gene can encode more than one functional peptide is novel but opens the question of regulation of a gene that presumably releases three distinct GnRH peptides. It is not clear if the purpose of three peptides is to enhance the tunicate peptide output or to activate/inhibit distinct functions.

Chapter 4 described the identification, cloning and physiological testing of the *Ciona* GnRH receptors. The ability of all six endogenous GnRH forms to induce two intracellular signalling pathways in the four *Ciona* GnRH receptors were tested. Only a single ligand/receptor pair (tGnRH-6 with Ci-GnRHR1) activated intracellular accumulation of radioactively-labelled inositol phosphates. Unexpected, three *Ciona* GnRH receptors displayed differential stimulation profiles when assayed for induction of cAMP after incubation with *Ciona* GnRH forms. This study shows that *Ciona* GnRHRs predominantly activate an alternate signaling pathway compared with the pituitary gonadotropes of vertebrates. Genetic analysis found that the *Ciona* GnRH receptor genes share a similar overall structure, with analogous positions of intron insertion. One notable exception is GnRHR4, which has one less exon. Phylogenetic analysis also shows that the four tunicate GnRH receptors form a monophyletic group, indicating a higher relationship among each other than to any vertebrate GnRH type. The present research establishes that a chordate has GnRH peptides and receptors remarkably similar to those in vertebrates and that the reproductive system can operate without a pituitary that contains gonadotropins.

In chapter 5, I described the genetic organisation and functional analysis of four amphioxus GnRH receptors. The phylogenetic relationships of the amphioxus GnRH receptors were analysed with select invertebrate and vertebrate types. Two paralogous relationships were observed, where two amphioxus receptors grouped basally to those of vertebrates, and two clustered with the recently identified octopus GnRHR-like receptor. These data reveal that two distinct GnRH receptor types were present in this cephalochordate; one receptor type was subsequently lost during the evolution to higher chordates. Pharmacological testing of two vertebrate GnRH forms showed that two amphioxus GnRHRs were sensitive to both forms, but displayed different selectivity to the two peptides. In addition, an insect neuropeptide was able to induce the activation of one amphioxus GnRHR, albeit at high concentrations. The presence of these receptors suggests that amphioxus possesses GnRH peptides, and although we have searched for them, we have not been successful in identifying them to date. Whether the amphioxus GnRH is more like that of octopus GnRH or those of *Ciona* remains to be seen, but it is also possible that one of each type will be present. This research presents the first hypothesis concerning the transition from an ancestral receptor to a GnRH receptor, which appears to have occurred in an invertebrate.

My studies have shown that the GnRH receptor system has changed dramatically during the evolution of chordates, as well as in the urochordate lineage after its split from the evolutionary path that led to vertebrates (Figure 6.1). One pair of amphioxus GnRHRs represents a proto-vertebrate state that led to the four existing vertebrate GnRHR types, whereas the *Ciona* GnRHRs appear to have undergone lineage specific adaptations, which may be attributed to the changes observed in their genome (Hughes

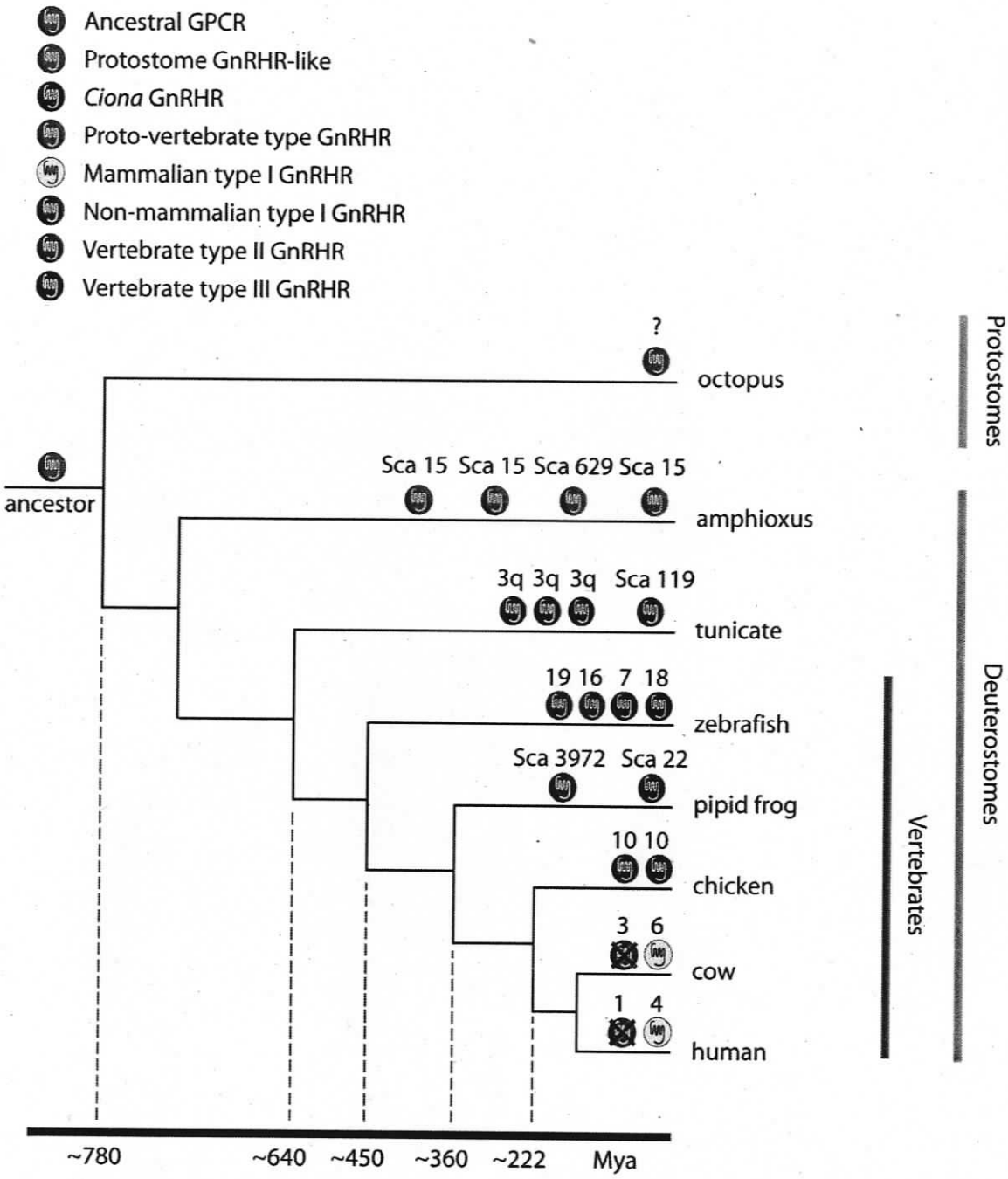

Figure 6.1 Chordate GnRH receptor phylogeny. Types of GnRH receptors are indicated in the key and scaffold (Sca) or chromosome locations are indicated above each receptor.

and Friedman, 2005). Evidence that supports the derived nature of *Ciona* includes their disruption of the linked HOX genes, which are present as a single gene cluster in amphioxus (Dehal *et al.*, 2002). Many invertebrates, including *Ciona*, possess GnRH-containing neurons, suggesting an evolutionarily ancient role for GnRH in neuromodulation and regulation of reproduction. GnRH is capable of inducing spawning in hemichordates, amphioxus and ascidians (Schlosser, 2005), and modulates ovarian functions in many chordates, including humans (Leung *et al.*, 2003). A generalized evolutionary scenario has been proposed, where GnRH-directed reproductive functions (such as regulating gamete maturation/release in response to environmental cues) before the gonadotropins evolved in the lineage that led to vertebrates after its separation from the urochordate-like ancestor (Sherwood *et al.*, 2005; Sherwood *et al.*, 2006; Tsai, 2006; Kah *et al.*, 2007). The GnRH system has continued to evolve in vertebrates, where duplication of the GnRH receptors occurred in teleost fishes, such as the zebrafish. Even in mammals, notable changes in the GnRH receptor system are seen, where the type II receptor gene has become silenced or lost in some species, including human and mouse. Evidence from octopus demonstrates a related GnRH system is present outside the deuterostome lineage and suggests a GnRH-like system may have been present in the last common ancestor of the deuterostome and protostome taxa over 600 million years ago.

A multitude of future directions are suggested by this research. There are many novel GnRH peptides and receptors to be cloned from diverse species. Each structure elucidates the evolutionary process further. A detailed understanding of the origin of the pituitary is needed. Mapping of glycoproteins in early vertebrates would be helpful in understanding the origin of the pituitary in regard to reproduction. However, I find the

determination of alternative GnRH receptor signaling pathways including elucidating novel mitogen-activated protein kinase actions and probing the mechanism for the activation of second messengers to be interesting. New methods like FRET are available for elucidating the possible roles for receptor heterodimerization and the molecular movements of the receptor during activation. Finally, one question continues to plague us, which is the origin of GnRH and its receptors along with the evolution of their structure and function thereafter.

6.1 References

Dehal, P., Y. Satou, R. K. Campbell, J. Chapman, B. Degnan, A. De Tomaso, B. Davidson, A. Di Gregorio, M. Gelpke, D. M. Goodstein, N. Harafuji, K. E. Hastings, I. Ho, K. Hotta, W. Huang, T. Kawashima, P. Lemaire, D. Martinez, I. A. Meinertzhagen, S. Necula, M. Nonaka, N. Putnam, S. Rash, H. Saiga, M. Satake, A. Terry, L. Yamada, H. G. Wang, S. Awazu, K. Azumi, J. Boore, M. Branno, S. Chin-Bow, R. DeSantis, S. Doyle, P. Francino, D. N. Keys, S. Haga, H. Hayashi, K. Hino, K. S. Imai, K. Inaba, S. Kano, K. Kobayashi, M. Kobayashi, B. I. Lee, K. W. Makabe, C. Manohar, G. Matassi, M. Medina, Y. Mochizuki, S. Mount, T. Morishita, S. Miura, A. Nakayama, S. Nishizaka, H. Nomoto, F. Ohta, K. Oishi, I. Rigoutsos, M. Sano, A. Sasaki, Y. Sasakura, E. Shoguchi, T. Shin-i, A. Spagnuolo, D. Stainier, M. M. Suzuki, O. Tassy, N. Takatori, M. Tokuoka, K. Yagi, F. Yoshizaki, S. Wada, C. Zhang, P. D. Hyatt, F. Larimer, C. Detter, N. Doggett, T. Glavina, T. Hawkins, P. Richardson, S. Lucas, Y. Kohara, M. Levine, N. Satoh and D. S. Rokhsar (2002). "The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins." Science **298**(5601): 2157-67.

Hughes, A. L. and R. Friedman (2005). "Loss of ancestral genes in the genomic evolution of Ciona intestinalis." Evol Dev **7**(3): 196-200.

Kah, O., C. Lethimonier, G. Somoza, L. G. Guilgur, C. Vaillant and J. J. Lareyre (2007). "GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective." Gen Comp Endocrinol **153**(1-3): 346-64.

Leung, P. C., C. K. Cheng and X. M. Zhu (2003). "Multi-factorial role of GnRH-I and GnRH-II in the human ovary." Mol Cell Endocrinol **202**(1-2): 145-53.

Schlosser, G. (2005). "Evolutionary origins of vertebrate placodes: insights from developmental studies and from comparisons with other deuterostomes." J Exp Zoolog B Mol Dev Evol **304**(4): 347-99.

Sherwood, N. M., B. A. Adams and J. A. Tello (2005). "Endocrinology of protochordates." Canadian Journal of Zoology-Revue Canadienne De Zoologie **83**(1): 225-255.

Sherwood, N. M., J. A. Tello and G. J. Roch (2006). "Neuroendocrinology of protochordates: insights from Ciona genomics." Comp Biochem Physiol A Mol Integr Physiol **144**(3): 254-71.

Tsai, P. S. (2006). "Gonadotropin-releasing hormone in invertebrates: structure, function, and evolution." Gen Comp Endocrinol **148**(1): 48-53.

Zhang, L., J. A. Tello, W. Zhang and P. S. Tsai (2007). "Molecular cloning, expression pattern, and immunocytochemical localization of a gonadotropin-releasing hormone-like molecule in the gastropod mollusk, Aplysia californica." Gen Comp Endocrinol.