The Role of Two Neuropeptide Families and their Receptors in Developing and Adult Zebrafish and Mice

by

Sheng Wu
B.Sc, Ocean University of China, 1991
M.Sc, Wageningen University, 2000

A Dissertation Submitted in Partial Fulfillment
of the Requirements for the Degree of

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in the Department of Biology

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ABSTRACT

Two groups of neuropeptides (secretin and gonadotropin-releasing hormone groups, GnRH) and their receptors are of considerable interest because they are highly conserved in structure during evolution and have important physiological effects. To study the role of these neuropeptides during development, the zebrafish was selected initially as a model because the embryo is transparent and accessible for genetic manipulation. Later, a mouse model was added to evaluate the effects of permanent gene loss.

The purpose of this thesis was to 1) characterize the structure and function of several peptides and receptors within the secretin group that had not been previously identified for zebrafish, 2) examine brain development in zebrafish after gene knock down of an important peptide in each of the secretin and GnRH groups, 3) identify the pattern and location of expression of the GnRH receptors in zebrafish embryos, and 4) study the
functional importance of the GnRH receptor in a mouse line deficient in the receptor. The secretin superfamily of hormones includes 10 structure-related polypeptides in mammals, but fewer in zebrafish. In this thesis it was discovered that within the secretin group, zebrafish have two peptides, peptide histidine-isoleucine (PHI) and vasoactive intestinal polypeptide (VIP), in addition to two receptors, PHI receptor and growth hormone-releasing hormone-like peptide (GHRH-LP) receptor that were not previously identified. After isolation of the cDNA for each, the signaling pathways were characterized in vitro by transfection of the receptors into COS cells. The PHI-R was activated by PHI but not by VIP or pituitary adenylate cyclase-activating polypeptide (PACAP). The GHRH-LP receptor was activated by GHRH-LP1 and GHRH but not by GHRH-LP2. A novel observation was that the PAC₁ receptor is activated by both PACAP and VIP, whereas the PAC₁ receptor is usually specific to PACAP. Also novel was evidence that a VPAC₂ receptor in zebrafish was structurally and functionally a PHI receptor. In examining the role of PACAP in early brain development in zebrafish, morpholinos were used to knockdown PACAP1 and PACAP2 in the zygote. The study revealed that loss of PACAP leads to change in the expression of brain markers.

GnRH and its receptors (GnRH-R) are key regulators of reproduction and sexual behaviour. In zebrafish, morpholino-induced knock down of GnRH revealed that GnRH is critical in eye and brain development by affecting transcription factors and/or secreted factors: pax2.1, fgf8, pax6.1 and mab. Further, the GnRH receptor was expressed at 24h post fertilization showing that GnRH and its receptor could influence early development.

To determine whether the GnRH receptor is critical at all stages of reproduction, a mouse line was created in which the GnRH receptor was disrupted using the gene trap
method. This model has a similar phenotype to the clinical syndrome of hypogonadotropic hypogonadism. In mice, GnRH receptor disruption results in small sexual organs, low gonadotropin and steroid hormone levels, failure of sexual maturation, and lack of reproduction. Also, the GnRH receptor may be important in the fetal testis.

This thesis provides evidence that 1) in evolution the PAC$_1$ receptor may have responded to both PACAP and VIP in fish, 2) zebrafish have a PHI receptor rather than a VPAC$_2$ receptor, 3) PACAP and GnRH peptides have a fundamental role in early brain development, 4) the GnRH receptor is not only important in reproduction but unexpectedly, in zebrafish embryos is localized in hindbrain motor neurons and fibers, which are involved normally in movement and escape, and 5) GnRH receptor-disrupted mice should be a valuable model for understanding reproduction and clinical approaches to hypogonadotropic hypogonadism.
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Dedication

I would like to dedicate this thesis to my parents

who give me support all the time.
Chapter 1

General Introduction:

Two groups of neuropeptides and their receptors
I. Secretin superfamily and receptors

A. Hormones: discovery, structure, evolution and function

Discovery

The first hormone to be discovered was named secretin in 1902 (Bayliss and Starling, 1902). Now, the secretin superfamily of hormones includes 10 structure-related polypeptides: pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), peptide histidine-isoleucine (PHI or PHM in human), secretin, glucagon, glucagon-like peptide (GLP-1 and GLP-2), glucose-dependent insulinoergic polypeptide (GIP), growth hormone-releasing hormone (GHRH) and GHRH-like peptide (GHRH-LP), which is also known as PACAP-related peptide (PRP) (Sherwood et al., 2000). This thesis is primarily concerned with PACAP, VIP, PHI, GHRH and GHRH-LP. PACAP, originally isolated from the ovine hypothalamus, is 38 amino acids and results in a remarkable increase in adenyl cyclase activity (cAMP) in rat pituitary cell culture (Miyata et al., 1989). VIP was originally isolated from porcine duodenum and is characterized as a factor of vasodilation of blood vessels (Said and Mutt, 1980). GHRH was isolated and sequenced from human pancreatic tumors in 1982 (Rivier et al., 1982). PRP (or GHRH-LP in non-mammals) was found encoded in the same gene as PACAP (Kimura et al., 1990).
Figure 1.1. The human secretin superfamily genes. The exons are shown as boxes and the introns as lines. The lengths of the exons and introns are not proportional so that the exons can be aligned between genes. Exon 1 is an open box at the 5'-end of the gene and contains the 5'-UTR except for the secretin gene. The open box at the 3'-end of the gene is the 3'-UTR. Abbreviations are: GIP, glucose-dependent insulino tropic polypeptide; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; GLUC, glucagon; GHRH, growth hormone releasing hormone; PACAP, pituitary adenylate cyclase-activating polypeptide; PHM, peptide histidine-methionine; PRP, PACAP-related peptide; SECR, secretin; SP, signal peptide; VIP, vasoactive intestinal polypeptide (modified from Sherwood et al., 2000).
Structure

In humans, ten members of this superfamily are encoded by six structurally related genes (Fig. 1.1). Each gene encodes a signal peptide, one to three bioactive peptides, and a C-terminal peptide; in mammals, only PACAP-related peptide (PRP) has not been shown to be bioactive. Also in mammals, the PACAP precursor exhibits strong similarities with that of the VIP precursor (Vaudry et al., 2000). And only VIP and PACAP have an additional exon (cryptic peptide) between the signal peptide and the bioactive peptide. The PACAP precursor has mono or dibasic residues that can be cleaved by prohormone convertases (PC) to produce PRP47 and PACAP38. The structure of the PACAP precursor and post-translational processing is illustrated in Fig. 1.2. Cleavage at all Arg (R), Arg Arg (RR) or Lys Arg (KR) residues generates PRP29 and PACAP27. PRP is hydrolyzed at the C-terminal by carboxypeptidases (Rouillé et al., 1995) and PACAP is amidated by the glycine residue through the α-amidating monoxygenase (Eipper et al., 1992 a,b).

Evolution

Recently, GHRH was discovered as a separate gene in not only mammals but also chicken and fish (Lee et al., 2007; Wang et al., 2007). I have updated the models of PACAP, GHRH, PHI/VIP evolution in Fig. 1.3. First, an exon encoding a superfamily hormone in an ancestral invertebrate is hypothesized to undergo exon duplication, followed by gene duplication to form the ancestral PACAPs. Possibly in the second round of duplication in an early vertebrate, one of the ancestral GHRH/PACAP genes duplicated into GHRH-LP/PACAP and PHI/VIP, and in the other copy, GHRH was
preserved while PACAP sequences were lost by exon deletion. This second round of
gene duplication also occurred in an early vertebrate. In mammals, only one copy of PHI
Fig. 1.2. Schematic representation of the post-translational processing of the rat PACAP
precursor. The nature and allocation of each cleavage and amidation site is specified.
PACAP, pituitary adenylate cyclase-activating polypeptide; PAM, peptidyl glycine α-
amidating monooxygenase; PC, prohormone convertase; PRP, PACAP related peptide;
SP, signal peptide (adapted from Vaudry et al., 2000).
Figure 1.3 Hypothetical model for the evolution of the PACAP, GHRH and PHI/VIP. Boxes represent exons that encode hormones. Abbreviations are: GHRH, growth hormone releasing hormone; GHRH-LP, growth hormone releasing hormone-like peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; PHI, peptide histidine-isoleucine; PHM, peptide histidine-methionine; PRP, PACAP related peptide; VIP, vasoactive intestinal polypeptide.
(PHM)/VIP, PRP-PACAP and GHRH were kept. After the third round of gene duplication (mainly in fish), GHRH-LP-PACAP duplicated to two genes (GHRH-LP/PACAP1 and GHRH-LP/PACAP2). Also, PHI/VIP and GHRH are predicted to have two copies, although there is no other copy isolated so far.

**Function**

Secretin superfamily members are classified as neuropeptides since nine of ten of the superfamily members are found in the brain except GIP. All of the members are found in the gut. The functions of this superfamily are very diverse but I will focus mainly on PACAP, VIP, PHI, GHRH and GHRH-LP. PACAP and VIP, which are widely expressed in the central and peripheral nervous system, and are tightly conserved for 700 million years suggesting their function may be essential for survival. PACAP and its related peptides act as neurotransmitters, neurotrophic factors and neuromodulators.

PACAP stimulates the release of growth hormone (GH) from somatotrophs in fish (Parker *et al.*, 1997; Wong *et al.*, 2000; Sawisky and Chang, 2005; Mitchell *et al.*, 2007), amphibians (Yon *et al.*, 2001) and some mammals (Montero *et al.*, 2000). In the lower vertebrates, GHRH-LP and PACAP plus the newly discovered GHRH are important for GH secretion (Sherwood *et al.*, 2000; Lee *et al.*, 2007), whereas in mammals GHRH is more potent for stimulating GH than PACAP (Montero *et al.*, 2000). PACAP also stimulates Gonadotropin-II (LH) secretion in goldfish pituitary (Chang *et al.*, 2001).

PACAP, VIP and PHI are neuroprotective. PACAP was first reported to have neuroprotective action when low concentrations were used to protect cerebral cortical neurons from neuronal loss (Brenneman *et al.*, 1986). PACAP, VIP and PHI can protect cells from neurotoxic effects caused by ethanol, hydrogen peroxide ($\text{H}_2\text{O}_2$), glutamate, $\beta$-
amyloid (Vaudry et al., 2002, 2005; Onoue et al., 2002; Morio et al., 1996; Brenneman et al., 2007). These peptides or their derivatives, which have a neuroprotective function, are promising therapeutic agents in several psychoneurological disorders like Alzheimer’s disease and Parkinson’s disease. Also these peptides have been implicated in modulating pain in chronic nerve injury (Mabuchi et al., 2004).

In general, PACAP and GIP are involved in glucose-dependent insulin release and lipid and carbohydrate metabolism. Also PACAP influences smooth muscle contraction in blood vessels and lung, and germ cell maturation in reproduction (Sherwood et al., 2000). Finally, PACAP has an important role in early development (Wu et al., 2006).

B. Receptors: discovery, structure, and signaling

Discovery

The peptides of the secretin superfamily bind to their specific G-protein coupled receptors (GPCR), which are included in the secretin receptor family (family B or family 2). VIP receptors are also members of the secretin receptor family; they were found in the 1970s in liver and adipose tissue (Desbuquois et al., 1974; Bataille et al., 1974). In 1992, a VIP receptor cDNA was cloned from rat and is now referred to as VPAC₁R. Soon thereafter, VPAC₂R was cloned from rat pituitary gland (Lutz et al., 1993). VPAC receptors are known to share VIP and PACAP as ligands with similar potency (Sherwood et al., 2000). These VPAC receptors also respond to other neuropeptides such as secretin, PHI, helodermin and GHRH with a lower potency (Laburthe et al., 2002). Also in 1993, a specific PACAP receptor (PAC₁) was cloned by a different laboratory (Laburthe et al., 2002). This receptor has very low affinity for VIP (Vaudry et al., 2000).
Figure 1.4. Schematic representation of the 7 transmembrane (TM) G-protein coupled receptor hPACAP-R. Amino acids for the 7 TM region are in a gray box; potential sites for N-linked glycosylation are shown as tridents; the signal peptide cleavage site is indicated by an arrow; seven highly conserved cysteine residues are shown by triangles; intracellular loop 3 for PAC1R, where cassette insertions occur, is enclosed in a dashed open box (Modified from Pisegna et al., 1996).
**Structure**

Secretin family receptors have seven transmembrane domains, three extracellular loops (EC1, EC2, EC3), three intracellular loops (IC1, IC2 and IC3), an extracellular amino-terminus and an intracellular carboxyl terminus just like other GPCRs (Fig. 1.4). The major features of family B are: conserved cysteine residues, an amino-terminal signal peptide and N-terminal glycosylation sites. The extracellular domains are essential for ligand binding.

**Signaling**

All the secretin receptors mediate their action by activating a heterotrimeric G-protein. The major signaling pathway for secretin receptor family members is coupling to adenylyl cyclase via Gs to increase intracellular cAMP production. However, further studies revealed that the secretin-type receptors can activate additional signaling pathways like phospholipase C (PLC) and phospholipase D (PLD). The most intensively studied receptors in this family are PAC₁R and VPAC₁R. PAC₁R has multiple isoforms with deletions in the N-terminal sequence and inclusions of short amino acid cassettes in the intracellular loop 3 (Fig 1.4 dashed open box). In humans, more than 14 variants of PAC₁R have been identified (Lutz et al., 2006). These splicing variants greatly influence the interactions of a receptor with different heterotrimeric and small G protein ADP ribosylation factors (ARF) to couple to PLC (McCulloch et al., 2001). For example, the PAC₁ receptor could be a moderately strong activator of phospholipase C (PLC) via Gq/11 in a pertussis toxin (PTx)-insensitive way (Spengler et al., 1993). However, VPAC₂R activates PLC through G proteins that include both PTx-insensitive Gq/11 and
sensitive Gi/Go types (Luo et al., 1999) (Table. 1.1). Two major signaling pathways (cAMP and IP₃) are described in Fig. 1.5.
<table>
<thead>
<tr>
<th>Effector</th>
<th>Coupling mechanism</th>
<th>Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>$G_s$</td>
<td>All</td>
</tr>
<tr>
<td>PLC</td>
<td>$G_{q/11}$</td>
<td>$\text{PAC}<em>{1\text{-null}} &gt; \text{PAC}</em>{1\text{-hop1}} &gt; \text{VPAC}_{2/1}$</td>
</tr>
<tr>
<td></td>
<td>$G_{i/o}$</td>
<td>$\text{VPAC}<em>{2}$ (not $\text{PAC}</em>{1}$)</td>
</tr>
<tr>
<td>PLD</td>
<td>ARF</td>
<td>$\text{VPAC}<em>{2/1} = \text{PAC}</em>{1\text{-hop1}} &gt; \text{PAC}_{1\text{-null}}$</td>
</tr>
<tr>
<td></td>
<td>Downstream of PLC</td>
<td>$\text{PAC}<em>{1\text{-null}} &gt; \text{PAC}</em>{1\text{-hop1}}$</td>
</tr>
</tbody>
</table>

AC, adenylyl cyclase; PLC, phospholipase C; PLD, phospholipase D; ARF, ADP ribosylation factors. Modified from McCulloch et al., 2002.
Fig. 1.5. Schematic diagram of the secretin receptor family with signal transduction pathways: cAMP (left) and IP₃ (right). cAMP pathway: ligand and receptor binding trigger the GTP to couple with the Gₛ protein. Dissociation of the α-subunit from Gβ and Gγ protein stimulates AC. AC catalyzes the synthesis of cAMP from ATP, and phosphorylates a number of cellular proteins via cAMP-dependent protein kinase (PKA).

IP pathway: ligand and receptor binding enhance receptor coupling to the GTP-Gₒ protein. Dissociation of the α-subunit activates phospholipase Cβ, which cleaves PIP₂ into DAG and IP₃. DAG activates PKC, which, in turn, phosphorylates cellular proteins.

IP₃ causes the release of Ca²⁺ from endoplasmic reticulum. The released Ca²⁺ leads to exocytosis and various other cellular responses. Abbreviations: cAMP: cyclic adenosine 5'-monophosphate; GDP: guanosine 5'-biphosphate; GTP: guanosine 5'-triphosphate; AC: adenylyl cyclase; DAG: diacylglycerol; IP₃: inositol-1,4,5-triphosphate; PIP₂: phosphatidylinositol-4,5-bisphosphate; PLC-β: phospholipase C-β; PKC: protein kinase C; ER: endoplasmic reticulum (Modified from Siu et al., 2006).
II. GnRH and its receptors in fish and mammals

A. GnRH: discovery, structure, evolution and function

Discovery

Gonadotropin-releasing hormone (GnRH) is a decapeptide that plays a fundamental role in reproduction. It was first isolated from the mammalian hypothalamus during the early 1970s by two competing groups (Matsuo et al., 1971; Burgus et al., 1972). Currently there are 24 structural variants that have been identified across various species from invertebrates and vertebrates (Adams et al., 2003). These GnRHs were divided into three types in vertebrates. The type 1 hypothalamic forms of GnRH (mammalian GnRH or GnRH-I) with variable structures (Sealfon et al., 1997) are synthesized in the hypothalamus and released in a pulsatile manner through axons to the external zone of median eminence in mammals (Seeburg et al., 1987). The released GnRH enters the hypophysial portal circulation and then exits from the blood in the pituitary to stimulate the biosynthesis and secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fink et al., 1988). GnRH-I neurons originate from the olfactory region and migrate into the forebrain and hypothalamus (Wray et al., 1989; Schwanzel-Fukuda et al., 1990).

The second type of GnRH (chicken GnRH or GnRH2) was first identified from chicken brain (Miyamoto et al., 1984). Its structure and midbrain location have been conserved in species from jawed fish to human (Lovejoy et al., 1992; White et al., 1998a), which suggests it has critical and specific functions. GnRH-II neurons appear
first in a mesencephalic primordium, which is derived from the neural crest (White and Fernald, 1998b; Muske et al., 1994; Gonzalez-Martinez et al., 2004; Whitlock et al., 2005; Wu et al., 2006). The third type of GnRH (salmon GnRH or GnRH3), which is preferentially expressed in the olfactory terminal region, was first identified in 1983 in chum salmon (Sherwood et al., 1983). It is clear that each type of the GnRH has its unique distribution within the brain (Fig. 1.6) (Gothilf et al., 1996; Okubo et al., 2000; Sherwood and Adams, 2005).

**Structure**

The 24 forms of GnRH are decapeptides with a pyroglutamyl amino terminus and amidated carboxy terminus (Fig. 1.7). The N-terminus (pGlu-His-Trp-Ser) and C-terminus (Pro-Gly NH$_2$) sequences are highly conserved for more than 500 million years. These conserved amino acids are critical for receptor binding and activation. The amino acid in position eight is the most variable residue and this residue seems to play an important role in selection of a receptor by ligands (Lu et al., 2007) (Fig. 1.8). The first GnRH gene to be cloned showed that GnRH is encoded with a GnRH-associated peptide (GAP), which does not have a hormone function (Seeburg et al., 1987)

**Evolution**

GnRH peptides are only 10 amino acids, which is too short for phylogenetic analysis. Therefore their phylogeny was established on the basis of their cDNA sequences. The phylogenetic trees agree that GnRH cDNAs segregate into three branches, which are types I, II and III (White et al., 1998a, Vickers et al., 2004) (Fig 1.9) and these three
Fig. 1.6. Sagittal drawing of the fish brain (sea bass) summarizing the distribution of GnRH. GnRH-I (green dots), cGnRH-II (black dots), and sGnRH (GnRH-III) (gray dots) immunoreactive cells and the main projection pathways. The dashed circle, open box, and dashed square box represent the major distribution of the three types of GnRH (modified from Kah et al., 2007).
Fig. 1.7. Amino acid sequences of GnRH found in Phylum Chordata. Tunicate-II-GnRH exists as a homodimer; the underlined cysteine residue is the site of a disulfide bond. Shaded amino acids are conserved residues. Peptides identified by Dr. Sherwood’s lab are shown with red open boxes (modified from Sherwood and Adams et al., 2005).
Fig. 1.8 Structure of human GnRH. The N-terminus and C-terminus are involved in receptor binding and the residue in position 8 is involved in ligand-receptor selectivity (Modified from Lu et al., 2007).
Fig. 1.9. Unrooted neighbor-joining phylogenetic tree of GnRH peptides. The tree was generated from an alignment of GnRH preprohormones by using the neighbor-joining algorithm and simplified to three groups (modified from White et al., 1998).
types match their three major distribution patterns in the brain. Branch 1 includes the classical hypophysiotropic system in the preoptic-hypothalamic region (GnRH-I); branch 2 includes GnRH-II expressed in the midbrain in various tetrapods or fish; and branch 3 only contains GnRH-III expressed mainly in the forebrain of teleost fish. That there were three rounds (1R, 2R, 3R) of genome duplication was proposed for vertebrate evolution (Fig. 1.10) (Panopoulu et al., 2003; Steinke et al., 2006). The theory assumes that invertebrates have at least one GnRH gene and that 1R, 2R duplications occurred after the divergence of the urochordates from the line leading to vertebrates (Steinke et al., 2006). The 3R duplication happened in teleost fish about 320-350 million years ago (Kah et al., 2007).

Functions

GnRH functions have been intensively investigated in vertebrates (Chang et al., 1996; Pawson et al., 2003; Millar et al., 2004; Ferris et al., 2006). In addition to reproductive functions, neuroendocrine (GH release in fish) (Uretsky et al., 2003), paracrine (in gonads), autocrine (in breast cancer cells and GnRH neurons) and neurotransmitter/neuromodulatory roles have been studied (Millar et al., 2005). GnRHs are also expressed in non-reproductive tissues like the adrenal gland, extrahypothalamic brain, immune system, retina and pancreas (Millar, 2005), which suggests GnRH peptides are possible regulators in these tissues. GnRH-I (also called LHRH in human) plays a key regulatory role in the control of reproduction through the hypothalamic-pituitary-gonadal axis by stimulating the release of the pituitary gonadotropins LH and FSH. GnRH is secreted in pulses, which vary widely over the female menstrual cycle and the reproductive developmental stages of both males and females (Wray et al., 1989). The
Fig. 1.10. Kah et al., (2007) explain the evolution of different forms of GnRH in the following way: "Schematic representation of a hypothesis on the evolution of GnRH genes in vertebrates based on the existence of a third full genome duplication, specific to teleost fish (3R). This hypothesis postulates that invertebrates have at least one GnRH gene, which would make it expected that the 1R and 2R duplications gave birth to four potential GnRH genes as is the case for GnRH-R. However, the current information suggests that one copy of these ancestral duplicated genes was rapidly lost. Thus, the current available information suggests that two GnRH genes type 1 (green) and type 2 (purple) were present in basal representatives of both early actinopterygians and sarcopterygians. Following the teleost specific genome duplication, a second copy of the type 1 GnRH gene emerged (type 3, red). With respect to the type 2 gene, it is probable that the second copy of the type 2 gene was rapidly lost. In teleosts, more recent tetraploidization events also explain the presence of two copies of cfGnRH in catfish, sGnRH in salmonids or cGnRH-II in cyprinids. GnRH genes in dogfish and lampreys appear in grey as their relationships to the other genes are currently unknown".
pulse frequency and or amplitude have critical effects on gonadotropin release. Low
frequency pulses appear to favor increased FSHβ mRNA levels, leading to ovarian
follicle development, whereas higher frequency and amplitude pulses stimulate LHβ and
α subunit mRNA, leading to ovulation of ovarian eggs (Dalkin et al., 1989; Burger et al.,
2002; Ferris et al., 2006). However, constant exposure to GnRH-I in vivo leads to a
suppression of FSH and LH. Administration of GnRH-I in regular pulses rescues FSH
and LH pulsatile secretion (Belchetz et al., 1978). Current clinical studies also show that
long-term administration of GnRH agonists induces depletion of LH and FSH (Lahlou et
al., 1987). A natural genetic defect in GnRH-I in rodents and humans results in
hypogonadotropic hypogonadism, with a dramatic decrease of gonadotropins in serum
together with failure of puberty and infertility phenotype (Mason et al., 1986; Legouis et
al., 1991).

GnRH-II is ubiquitous in jawed vertebrates and is conserved over 500 million years.
The wide distribution of GnRH-II in central and peripheral systems suggests a
neurotransmitter/neuromodulatory role. Evidence also suggests that GnRH-II is involved
in reproductive behaviour. For example, GnRH-II stimulated female marmoset sexual
behaviour (Millar, 2005). In our lab, we investigated GnRH function in zebrafish early
brain development. We showed that two native GnRHs and their receptors are expressed
early (24h post fertilization). Both peptides influenced brain and eye development after
knock down of the translation of GnRH-I and/or GnRH-II (Wu et al., 2006). Moreover,
GnRH is also involved in modulation of cell functions such as inhibition of proliferation
and stimulation of apoptosis in tumour cells (Ramakrishnappa et al., 2005).
B. GnRH receptors: discovery, structure, function, signaling and disease

Discovery

GnRH receptors belong to the G-protein coupled receptors. The first cloned GnRH receptor was the mouse receptor from the gonadotrope-derived α T3-1 tumor cell line (Tsutsumi et al., 1992, Reinhart et al., 1992). Based on this sequence, subsequent cloning has identified the GnRH-R in the rat (Kaiser et al., 1992), human (Kakar et al., 1992; Chi et al., 1993), cow (Karkar et al., 1993), sheep (Illing et al., 1994), and pig (Weesner and Matteri, 1994). These mammalian GnRH receptors share over 80% amino acid identity. Following the identification of mammalian GnRH-Rs, more GnRH-Rs were cloned from fish, frog and chicken (Millar et al., 2005). The presence of three forms of GnRH in many fish suggested three cognate GnRH receptors would also be present in vertebrates (Troskie et al., 1998). However, according to functional assays, except for the mammalian type-I receptors, all GnRH receptors from tetrapods are “type-II” receptors, as they have a higher affinity for the GnRH-II ligand. The mammalian type-I GnRH receptor shows higher efficacy when stimulated by GnRH-I ligand (Neill et al., 2001; Millar et al., 2005). However, the type-II GnRH receptor has been silenced in the mouse, rat, sheep, cow, chimp and human; in human there is a shift in the reading frame that produces a premature stop codon (Neill et al., 2004). Also, the GnRH-II peptide has been silenced in the mouse, rat, sheep, cow and chimp but not in human (Millar et al., 2005).

Structure

All GnRH receptors are like the secretin receptor family in having seven transmembrane domains, which are involved in conformational changes associated with
receptor activation. The extracellular domains and superficial regions of the TM5s contribute to form a hydrophilic pocket to facilitate the ligand binding for GnRH. The intracellular domains and C-terminal tail are involved in interacting with G-proteins for intracellular signal transduction. The C-terminal tail is the target for GPCR kinase phosphorylation. Phosphorylated GPCR enhances β-arrestin binding, which separates G-proteins from the receptor. Thereafter, the receptor is desensitized and internalized. Once the receptor is internalized, either it is degraded by lysosomes or recycled back to the plasma membrane (Rispoli et al., 2005). However, the mammalian GnRH receptor does not have a carboxyl terminal tail that is present in all other GPCRs, so mGnRH is desensitized and internalized slower and more poorly than type II receptor (Counis et al., 2005). Also GPCRs have conserved cysteines in the first and second extracellular loops that form disulfide bonds for stabilizing their 7TM structure. Mutation of these cysteines greatly reduced the ability of the receptor to bind GnRH (Keinan and Hazum, 1985). Like other GPCRs, GnRH-Rs have two potential N-linked glycosylation sites (Sealfon and Millar, 1995). Mutation of these two sites does not influence receptor binding for ligand or receptor activity but decreases the number of receptors on the cell surface (Davidson et al., 1995). The glycosylation sites of the GnRH-R are required for receptor stability rather than for receptor transport to the cell surface like the β-adrenergic receptor (Rands et al., 1990).

**Functions**

GnRH-R, expressed on the membranes of pituitary gonadotropes, controls reproductive functions (Clayton and Catt, 1981; Childs and Unabia, 1997) as the receptor is crucial in regulating the gonadotropin subunits. Also, the GnRH receptor is present in other tissues
such as gonads, central nervous system, placenta, somatotropes and various pancreatic tumours (Rispoli et al., 2005). Further, GnRH-Rs inhibit proliferation of human tumour cells and increase apoptosis (Neill et al., 2004).

**Signaling**

It has been established that GnRH-Rs activate several distinct signaling pathways by interacting with multiple G-proteins (Gq/11, Gs and Gi) in different cells. The classical coupling in the gonadotrope with Gαq/Gα11 activates phospholipase C (PLC) to produce inositol-triphosphate (IP₃) and diacylglycerol (DAG) (Grosse et al., 2000). These two second messengers (IP₃ and DAG) are responsible for the activation of protein kinase C (PKC) isoforms and the intracellular Ca²⁺ mobilization. PKC, in turn, leads to the activation of the four key mitogen-activated protein kinase (MAPK) subfamilies and calcium/calmodulin-dependent kinase type-II (Ca/CaMKII), which acts to stimulate the biosynthesis and secretion of LH and FSH. In a minor pathway, GnRH-R activates Gs resulting in an increase in cAMP, which in turn stimulates PKA and then the MAPK cascades (Fig. 1.11) (Pawson and McNeilly, 2005).

**Disease**

So far, 19 mutations in the GnRH receptor gene have been reported from patients with idiopathic hypogonadotropic hypogonadism (Tao, 2006). In this syndrome, the GnRH-R does not bind or misroutes, as reproduction could be rescued by pharmacological or chemical chaperones (Janovick et al., 2002; Leanos-Miranda et al., 2002). In our GnRH receptor gene trap mice (see chapter 6), we found the homozygous mice were overweight after 6 months of age compared to the heterozygous or wild type mice.
Fig. 1.11. Schematic summary of mammalian type I GnRH receptor signaling pathway.

GnRH binding to its receptor initiates two signaling pathways with PKA and PKC, which can activate the MAPK cascade. These pathways eventually drive the biosynthesis of LHβ, FSHβ and αGSU gonadotropin subunits. After transcription in the nucleus and transport through the Golgi, FSH, some LH, and free αGSU are released from the gonadotrope. In contrast, most LH associates with secretogranin II (SgII) into granules, which are released in response to local increases in Ca²⁺ at the cell membrane, induced by GnRH (modified from Pawson and McNeilly, 2005).
Constitutive Pathway - release of FSH, free alphaGSU, and some LH

GnRH-dependent exocytosis of LH, Sigma, and some FSH
Thesis

My thesis is a study of the hormones and receptors in the secretin superfamily and in the GnRH family. Chapter 2 is an examination of the structure and function in vitro of two hormones and two receptors within the zebrafish secretin superfamily. This study provides a fundamental framework for a more accurate and completes superfamily in zebrafish. Chapter 3 then examines the in vivo function of PACAP, as one of the most important hormones in the superfamily. Knockdown of PACAP and localization are used to understand the function of PACAP during early development in zebrafish.

Chapter 4 is an examination of the function of two types of GnRH during early brain and eye development of zebrafish. Knockdown technology is also used here. Chapter 5 determines the location of GnRH receptors during early development. The receptor data explain why zebrafish show defects in specific brain areas after knockdown of GnRH peptides. Chapter 6 uses gene targeting to disrupt the GnRH receptor in mice. The study provides insight into GnRH function regarding reproduction during early development but mainly in adults.
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Chapter 2

Zebrafish as a model to analyze structure and function of the secretin superfamily of peptides and receptors
Introduction

In vertebrates, the secretin family of hormones contains peptides with related structures (see Chapter 1). Humans have ten hormones in this family (Fig. 1.1) but fish lack some members.

PACAP is the most conserved member of the secretin superfamily in terms of length and sequence identity of both nucleotides and amino acids (Sherwood et al., 2000; Vaudry et al., 2000). Two biologically active forms of PACAP, PACAP27 and PACAP38, have been identified in vertebrates, and they are derived from the same precursor in which PACAP-38 is a C-terminal extension of PACAP27; both PACAPs are amidated at the C-terminus (Miyata et al., 1989 and Miyata et al., 1990). PACAP has a wide range of functions as a neuromodulator, neurotrophin, smooth muscle relaxant, and releasing factor in the pituitary (Sherwood et al., 2000; Chow et al., 2003).

VIP was first discovered in the porcine gastrointestinal tract (Said and Mutt, 1970) as was PHI (Tatemoto et al., 1980). PHI and VIP are transcribed from the same precursor in which VIP is encoded immediately following PHI. They are also colocalized in the central and peripheral nervous system (Cauvin et al., 1989). In humans, PHI is also called peptide histidine methionine (PHM) as its last amino acid is methionine. It was generally accepted that PHI functions were mediated through VIP receptors; PHI had lower potency than VIP (Moriarty et al., 1984; Palle et al., 1989; Suzuki et al., 1991). However, there is one specific PHI receptor in goldfish (Tse et al., 2002), the only one identified so far in vertebrates. Hence, PHI could be mediated by its own receptor in addition to VIP receptors. Some functions of VIP and PACAP are close to each other as they share two specific receptors: VPAC₁ and VPAC₂ with equal high affinity (Kimura et al., 1987;
Nurko et al., 1989). A third receptor, PAC₁ preferentially binds PACAP with a much lower affinity for VIP (Lelièvre et al., 1998; Sherwood et al., 2000). In addition, at least 14 splice variants have been identified for the PAC₁ receptor (Spengler et al., 1993; Lutz et al., 2006). These variant receptors have been reported to have a different binding ability for PACAP analogs or VIP and have different coupling to signaling pathways (Lutz et al., 2006).

GHRH, also known as growth hormone-releasing factor (GRF), was first isolated from human pancreatic tumors by its ability to stimulate growth hormone (GH) synthesis and release (Guillemi et al., 1982; Rivier et al., 1982). GHRH interacts with its receptor (GHRH-R) to stimulate cell proliferation, differentiation, and growth of somatotrophs in the anterior pituitary (Billestrup et al., 1987; Mayo et al., 1988; Lin et al., 1992). In mammals, GHRH and PACAP are encoded by two separate genes, but PACAP and PACAP-related peptide (PRP) (aka GHRH-like peptide or GHRH-LP) are located in the same gene (Mayo et al., 1985; Hosoya et al., 1992; Sherwood et al., 2000). In 2007, the same pattern in which GHRH and PACAP are encoded by separate genes was found in nonmammalian vertebrates such as chicken, frog, zebrafish, and goldfish (Wang et al., 2007; Lee et al., 2007). However, GHRH-LP and PACAP are encoded and transcribed from the same gene (Sherwood et al., 2000). Further evidence suggested that GHRH and PACAP are two major peptides that stimulate growth hormone release in teleost fish and that GHRH and GHRH-LP each act on a specific receptor, GHRH-R and GHRH-LP-R, respectively (Porter et al., 2006; Lee et al., 2007; Wang et al., 2007).

The physiological actions of all members of the family are mediated by their interactions with cell-surface receptors known as G-protein coupled receptors (GPCRs).
Within this superfamily of GPCRs, the hormones considered here act on the secretin family (family B or family2) of receptors (Ulloa-Aguirre et al., 2000; Laburther et al., 2003; Fradinger et al., 2005). GPCRs have common features (Cardoso et al., 2005 and see Chapter 1). The receptors in the secretin superfamily have been given various names, but the present study will use the ones suggested by The International Union of Pharmacology (Foord et al., 2005).

To date, the isolation and characterization of two VIP/PACAP receptors (VPAC₁R and VPAC₂R), PACAP-specific receptor (PAC₁R), GHRH-LP receptor (GHRH-LP-R) and GHRH receptor (GHRH-R) have been reported in a number of vertebrate species (Sherwood et al., 2000; Hoo et al., 2001; Fradinger et al., 2005; Cardoso et al., 2005; Lutz et al., 2006; Lee et al., 2007). However, for fish the partial coding sequence for VPAC₂R has been recently predicted only by data mining from fugu and zebrafish genome (Cardoso et al., 2004, 2005). GHRH-LP-R cDNA has only been reported as a partial sequence from TMD5 to TMD7 in zebrafish (Fradinger et al., 2005). In the present study, I selected zebrafish as a model to investigate the expression and isolation of the full length cDNA sequence of the peptide of PHI/VIP, and the receptors of GHRH-LP-R and VPAC₂R. I show that the latter is structurally and functionally the PHI receptor. Also, functional analysis of PAC₁R, VPAC₁R, PHI-R and GHRH-LP-R was performed by stimulating with zebrafish peptides PACAP27 isoform1 and isoform2, VIP, PHI, GHRH-LP, and GHRH. I also compare these zebrafish receptors with human VPAC₂R and GHRH-R in two signaling pathways: cAMP and IP.
Materials and Methods

Animals and peptides

Wild-type zebrafish (*Danio rerio*) were purchased from a local pet store and maintained in our lab with a 14h light and 10 h dark cycle at 28.5 °C. Procedures were approved by the Animal Care Committee at the University of Victoria. The tissues of adult zebrafish were dissected and frozen in liquid nitrogen.

Peptides (Table 2.1) of zfVIP (isolated in this project), zfPACAP-27 isoform 1 (Fradinger et al., 2000), zfPACAP-27 isoform 2 (Wang et al., 2003), zfGHRH-like peptide 1 (1-28) (zfGHRH-LP-1) (Fradinger et al., 2000) which is identical to carpGHRH-like peptide (Vaughan et al., 1992), zfGHRH-LP-2 (1-29) (Wang et al., 2003) and zfGHRH (Lee et al., 2007; Wang et al., 2007) were synthesized at the Salk Institute, San Diego, CA and were a gift of Dr. Jean Rivier. The purity of the peptides was characterized by capillary zone electrophoresis performed on a Beckman P/ACE System 2050 connected to a ChromJet integrator (Spectra Physics, San Jose, CA). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of the peptides was measured on an ABI Perseptive DE-STR instrument (PE Applied Biosystems, Foster City, CA).
Table 2.1 Amino acid sequences of the zebrafish peptides tested in the receptor activation assay

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PACAP&lt;sub&gt;1-27&lt;/sub&gt; isoform1</td>
<td>HSDGVPTDSYSRYRKQMAVKKYLATVL-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PACAP&lt;sub&gt;1-27&lt;/sub&gt; isoform2</td>
<td>HSDGIFTDIYSRYRKQMAVKKYLAAVL-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>VIP</td>
<td>HSDAIPTDNYSFRKQMAVKKYNLSVT-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PHI</td>
<td>HADGIFTSGYKLLLGLSARRYLESLI-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>GHRH-LP-1&lt;sub&gt;1-28&lt;/sub&gt;</td>
<td>HADGMFNKAYRKGQLSARKYLHTLMA</td>
</tr>
<tr>
<td>GHRH-LP-2&lt;sub&gt;1-29&lt;/sub&gt;</td>
<td>HADGLLDRLRDILVQLSARKYLHSLMAV</td>
</tr>
<tr>
<td>GHRH&lt;sub&gt;1-27&lt;/sub&gt;</td>
<td>HADAIFTNSYRKVLGQISARKFLQTVM-NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>
Total RNA isolation and cDNA synthesis

Total RNA was extracted from 11 tissues of adult zebrafish: brain, eye, heart, testis, ovary, spleen, kidney, gill, skin, gut and swimbladder using a kit according to the manufacturer’s protocol (RNeasy Mini Kit, Qiagen, Inc., Mississauga, ON). The concentration of total RNA was measured and 900ng total RNA from each sample were reverse transcribed with oligo (dT) and Superscript II (Invitrogen, Burlington, ON) according to the manufacturer’s instructions.

RACE (rapid amplification of cDNA ends) for zebrafish PHI-R, GHRH-LP-R, and PHI/VIP

Total RNA (500-600ng) from adult zebrafish brains was used for synthesis of cDNA with the First Choice™ RLM-RACE kit (Ambion, Austin, TX) following the manufacturer’s instructions. The 5’ and 3’ RACE primers for PHI-R and GHRH-LP-R were designed from published partial sequences (Wang et al., 2003; Fradinger et al., 2005). A partial predicted zebrafish VIP peptide sequence was found by searching with sequences of VIP of rat/ mouse, guinea pig, chick, frog, trout/bowfin, cod, dogfish in the Vega Multi Blast View program (http://vega.sanger.ac.uk/Multi/blastview?species=Danio_rerio); this partial predicted zebrafish VIP peptide was identical to trout VIP. Primers for 3’ RACE of PHI/VIP were designed from this predicted nucleotide sequence. Later, 5’ RACE primers were designed from our 3’RACE PCR sequence. RACE-PCR reactions were carried out for 35 cycles at 94°C for 30 seconds, 55°C annealing temperature for 30 seconds and 72°C for 1 minute with a 7-min extension at 72°C on the last cycle. All primers are listed in Table 2.2. The
PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Bands were selected and isolated with QIAquick Gel Extraction Kit (QIAGEN), and ligated into PGEM-T vector (Promega Corp., Madison, WI).

**Tissue distribution of zfPHI-R, PAC₃R, VPAC₁R, GHRH-LP-R and PHI/VIP mRNA**

Tissue specific distribution of each mRNA was performed by RT-PCR on eleven tissues of zebrafish. Primers for PAC₃R-short isoform and VPAC₁R were designed according to the NCBI Gene Bank database (accession No.: AAW65134.1; NP_001013371.1). PHI-R, GHRH-LP-R and PHI/VIP primers were designed according to the 5' and 3' RACE sequences. A control reaction was prepared by using 1μl of cDNA amplified with zebrafish actin primers. All PCR reactions were carried out on 1μl cDNA for 35 cycles at different annealing temperatures: 55°C (PHI-R), 58°C (actin), 60°C (PAC₃R), 62°C (VIP, VPAC₁R, GHRH-LP-R, GHRH-R). All PCR products spanned at least two exons as a control check for potential genomic contamination. PCR products of the right size were sequenced and confirmed.

**Transfection of zebrafish PHIR, PAC₃R short, VPAC₁R, GHRH-LP-R and human VPAC₂R and GHRHR**

Constructs of cDNA full length coding sequences for receptors PAC₃R short and VPAC₁R were a gift from Javier Tello and have been previously reported (Fradinger et al., 2005). The cDNAs for PHI-R and GHRH-LP-R containing full length open reading frames with flanking restriction enzymes sites and a Kozak sequence were subcloned into pcDNA3.1 (-) (Invitrogen). Two rounds of PCR reactions were conducted with platinum
Taq polymerase high fidelity (Invitrogen) as outlined by the manufacturer. Primers were listed on Table 2.2. Human vasoactive intestinal peptide receptor 2 (VPAC₂R) and GHRH-R cDNA clones were obtained from University of Missouri-Rolla (UMR) cDNA Resource Center (Clone ID: VIPR200000, GHRHR00000; GB Acc. No.: NM_003382, AY557192). All six receptors were separately transfected into COS-7 cells (American Type Culture Collection, Manassas, VA) according to the procedure described by Tello et al. 2005 with a few modifications: cells were plated at a density of 60,000 cells/well instead of 65,000; after 14 hr of transfection, medium was replaced with fresh VP-SFM (Invitrogen) for later cAMP assay or replaced with labeling medium (Medium 199 (Invitrogen)) plus 0.3% bovine albumin (Sigma-Aldrich, St. Louis, MO) for the inositol phosphate (IP) assay; and labeling medium with IBMX and 10 mM LiCl was used to incubate cells during ligand stimulation. Measurement of IPs and cAMP were described by Tello et al., 2005.

**Data analysis**

All IP and cAMP samples were measured in triplicate within each assay and each experiment was independently repeated at least three times. Data analysis was performed using PRISM3 software (GraphPad Software, Inc., San Diego, CA) with nonlinear regression (curve fit) and sigmoidal dose-response curves. The EC₅₀ values (dose of peptide stimulating half-maximal IP or cAMP response) of each peptide were calculated from the means of at least three independent experiments. The data were analyzed by one-way ANOVA followed by Tukey’s Multiple Comparison Test. \( P < 0.05 \) was considered as statistically significant.
Table 2.2 Primers used to amplify cDNA in zebrafish

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
<th>Target</th>
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<tbody>
<tr>
<td>RACE</td>
<td></td>
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<tr>
<td>PHI-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R274a</td>
<td>CAGGTTCAAGATGGATGTAGT</td>
<td>5' cDNA</td>
</tr>
<tr>
<td>R267b</td>
<td>AGATGGATGTTGCTCCTGGTG</td>
<td>5' cDNA</td>
</tr>
<tr>
<td>F3b</td>
<td>GTCCTGTGACCCCTCAAAA</td>
<td>3' cDNA</td>
</tr>
<tr>
<td>F191</td>
<td>CTCATCGCCCTCATCACC</td>
<td>3' cDNA</td>
</tr>
<tr>
<td>GHRH-LP-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-615</td>
<td>TCCAGAACGCCCAGTCTGTCATCAA</td>
<td>5' cDNA</td>
</tr>
<tr>
<td>R-190</td>
<td>CCCCAGCTCCAGGACTTTTC</td>
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<td>PHI/VIP</td>
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<td>AATAAAGATTTTCAATGCCCACCC</td>
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<td>R584</td>
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<td>5' cDNA</td>
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<tr>
<td>F260</td>
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Clones for functional study

- PHI-R
  - F1: AAAGTACTGCAAAACCGCACATGAT
  - R2096: CTTACAATCCGGCTTCACA
  - F172Nhel: TGCTAGCGCCAACCATGGACATGAGCCTCGTCAACT
  - R1551XbaI: ATTCTAGAAGGGCAGTGAAGAGCGAGCG

- GHRH-LP-R
  - F5: AAGTGAATCTGACTGTCG
  - R1675: ATCATATATATTTTAAATCATTTCTTATTTCC
  - F120Nhel: TTGCTAGCGCCAACCATGGCGTTGTACATCTGTGCT
  - R1436XhoI: TTCTCGAGTCACAATCCGAGTGCGGCGAGTTCA

Tissue expression primers

- PHI-R
  - F19: GATTTTTTGGCAGCTGACAG
  - R618: GATGGTGCTGCGGGTGATGA

- GHRH-LP-R
  - F79: GTGTGACTGAGAAGAGCCGGCTGT
  - R701: GAAGAAACATCTTCAAGACAATACTG

- GHRH-R
  - F30: CCGTGATGAGAAGAGCCGGCTGT
  - R625: CAGGCAAGTGAGACAGCAGACAG

- VIP
  - F41: CAGGGTGTGGCGCTG
  - R596: AGGGCTCCATCGAAGCGAG

- VPAC1R
  - F66: AACTTGCTATGTTTTACTCGTCTG
  - R537: ATCGTCAACAGTGGCCATTCA
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<td>β-actin</td>
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<sup>a</sup> F: forward primer. <sup>b</sup> R: reverse primer. The enzyme restriction sites are underlined.
Data mining and phylogenetic analysis

The NCBI database was used for searching VPAC₂R, GHRH-LP-R, PAC₁R, VPAC₁R and GHRH-R in human, chicken, frog, goldfish, fugu and zebrafish. These deduced amino acid sequences were aligned with each other using the Clustal W alignment program with BLOSUM62. The phylogenetic tree was generated based on the ClustalW alignment (amino acid from transmembrane domain I to VII) by MEGA3.0 to produce neighbor-joining trees with the Dayhoff matrix. The Gene Bank accession numbers of these sequences are: human (Homo Sapiens) GHRH-R (NP000814); chicken GHRH-R (ABB84385); goldfish GHRH-R (ABJ55978); zebrafish GHRH-R (ABJ55981); chicken PACAP-related peptide receptor (PRP-R) (CAC82589); goldfish GHRH-LP-R (AAC15698); fugu GHRH-LP-R (CAC82589); zebrafish GHRH-LP-R (isolated in the present project); human VPAC₂R (P41587); chicken VPAC₂R (NP_001014970); zebrafish PHI-R (isolated in present project); fugu VPAC₂RA (AJ408877); fugu VPAC₂RB (AJ296143); human VPAC₁R (P32241); marsh frog VPAC₁R (Q9YHC6); goldfish VPAC₁R (AAB05459); zebrafish VPAC₁R (NP_001013371.1); fugu VPAC₁RA (CAC82588); fugu VPAC₁BR (CAC82587); human PAC₁R (P41586); goldfish PAC₁R (O73769); zebrafish PAC₁R short (AAW65134.1); fugu PAC₁RA (CAD35690); and fugu PAC₁RB (CAD33842). The zebrafish glucagon receptor (GluR) clone (XM_685886) was used as the out group for tree construction. Goldfish PHI-R was reported earlier but doesn’t have an accession number (Tse et al, 2002).
Results

Isolation and structural analysis of the full-length cDNAs of zebrafish PHI-R, GHRH-LP-R and PHI/VIP

Full length cDNAs obtained from total RNA of zebrafish brain were determined in both the forward and reverse direction by RACE. The zebrafish PHI-R cDNA is 2088bp in length (submitted to Gene Bank, accession No. EU150381) with an open reading frame of 1323bp (from 169-1491) encoding a 441 amino-acid protein (Fig. 2.1 A, B). A Kyte-Doolittle hydrophobicity plot (data not shown) of the deduced protein revealed it has seven transmembrane domains. Comparison of the zebrafish PHI-R deduced amino acid sequence revealed it shares sequence identity with chicken VPAC₂R (59%), human VPAC₂R (56%), fugu VPAC₂RA-partial (55%) and VPAC₂RB-partial (62%). However, the highest identity is 80% with goldfish PHI-R. For other members of the secretin receptor family, zebrafish PHI-R shares sequence identity with zebrafish PAC₁R-short (42%), VPAC₁R (42%), GHRH-LP-R (37%) and GHRH-R (35%) (Table 2.3). The percent identity plus the functional study leads me to argue that the cDNA I isolated is a receptor that only responds to PHI (not to PACAP or VIP) and thus is not a VPAC₂R. Therefore, I refer to the receptor as PHI-R hereafter.

The full length cDNA of the PHI-R blasted against the zebrafish genome database (Vega Multi Blast View) shows that the zebrafish PHI-R gene spans more than 79kb on chromosome 24 and consists of 14 exons (Fig. 2.1A). Sequence alignment of PHI-Rs and VPAC₂Rs from various species (Fig. 2.1B) indicates that zebrafish PHI-R has signature motifs for VIP binding (PDV) and has RLAK between TMD5 and TMD6 for the coupling to Gsa (Chow et al. 1997; Cardoso et al. 2004). Also, other motifs such as the
Fig. 2.1 (A) Predicted structure of zebrafish PHI-R gene from zebrafish genome database in Vega. The gene size (>79kb) is indicated on the right of the gene. Exons are numbered and are shown as closed boxes; a solid horizontal black line indicates introns. The distance between the first five exons is indicated by a number above the intron line.

(B) ClustalW alignment of amino acids of zebrafish PHI-R with other known PHI-R and VPAC₂Rs. The transmembrane domains are annotated above the sequence. Thirteen conserved cysteines are marked with a black dot (except fugu VPAC₂RB). Two potential conserved N-glycosylation sites (Asn-X-The/Ser) in the N-terminal extracellular domain for zebrafish are labeled with a black triangle. Opened circles or ovals show where Asn is not conserved. All signature motifs for both PHI-Rs and VPAC₂Rs are in open boxes.

Zebrafish PHI-R (zfPHI-R); goldfish PHI-R (gfPHI-R); human VPAC₂R (hVPAC₂R).
### Signal peptide

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| hVPAC2R    | SWVG-------- --CITKSL VILNYFIMAN FYWLLVGEGL YHTLMLMVLFS |

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Table 2.3. Percentage of amino acid identity of aligned zebrafish PHIR with others in the same gene family

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Amino acid identity was determined by using the ClustalW alignment program with BLOSUM62. * fugu VPAC2R sequences, lack approximately 20 amino acid in the N-terminus.
highly conserved region for the mammalian class II family members at TMD7, FQGBBVXXXBYCFXNXEXQ (Lok et al., 1994), were identified where X represents any amino acid residue and B represents any hydrophobic amino acid residue (Fig. 2.1B). In addition to these motifs, two conserved residues, R and L, localized within TMD2 and associated with VPAC1R activation (Solano et al., 2001) were identified. Thirteen conserved cysteines, which are involved in tertiary structure and ligand binding, were also found in the zebrafish PHI-R. There are only two conserved putative glycosylation sites in PHI-Rs in zebrafish at N-terminal extracellular sites in contrast to three conserved sites in those of chicken and mammals. Interestingly, goldfish PHI receptor is the same as zebrafish and fugu VPAC2RA and VPAC2RB with only two putative glycosylation sites (Asn-X-Ser/Thr, where X is any amino acid except proline) in the N-terminal extracellular domain.

The zebrafish GHRH-LP-R cDNA is 1747bp in length (submitted to Gene Bank, accession No.:EU150382) with an open reading frame of 1296bp (from 181-1476) encoding a 432 amino-acid protein (Fig. 2.2). The amino acid sequence of the zebrafish GHRH-LP-R shares the highest level of identity with goldfish GHRH-LP-R (88%), followed by fugu (64%) and chicken (43%) (Table 2. 4). On the other hand, zebrafish GHRH-LP-R only shares 40% sequence identity with GHRH-R of zebrafish, goldfish, chicken and human. The intron and exon organization was not revealed by BLAST searching the full length cDNA of GHRH-LP-R in the zebrafish genome database (Vega Multi Blast View) as the genome data is not complete. Sequence alignment of GHRH-LP-R (PRP-Rs) from various species (Fig. 2.2) revealed that zebrafish GHRH-LP-R is very similar and structurally related to GHRH-LP receptors of other fish.
Fig. 2.2 ClustalW alignment of the zebrafish GHRH-LP-R amino acids with goldfish (gf), fugu (fu) and chicken (ch) GHRH-LP-Rs (a.k.a PACAP-related peptide receptor or PRP-R). The transmembrane domains are annotated above the sequence. Thirteen conserved cysteines in the fish receptors are marked with black dots and potential conserved N-glycosylation sites in the N-terminal extracellular domain in fish are labeled with a black triangle. Signature motif “RLAK” is shown by an open box. As noted, chicken GHRH-LP-R has only three conserved cysteines and one glycosylation site in the N-terminus compared with the GHRH-LP-R of fish.
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<td>chGHRH-LP-R</td>
<td>IPRFGLMTL TFLPENTGE IIRFYIELGL GSFQFVAVLV LACFLNGEVQ</td>
<td>chGHRH-LP-R</td>
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<td>Clustal Cons</td>
<td>Clustal Cons</td>
<td>Clustal Cons</td>
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Table 2.4. Percentage of amino acid identity of aligned zebrafish GHRH-LP-R with others in the same gene family

<table>
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<tr>
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<th>zebrafish GHRH-LP-R</th>
<th>goldfish GHRH-LP-R</th>
<th>fugu GHRH-LP-R</th>
<th>chicken GHRH-LP-R</th>
<th>zebrafish GHRH-R</th>
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<tr>
<td>zebrafish GHRH-LP-R</td>
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<td>88</td>
<td>64</td>
<td>43</td>
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<td>goldfish GHRH-LP-R</td>
<td>88</td>
<td>88</td>
<td>64</td>
<td>43</td>
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<tr>
<td>fugu GHRH-LP-R</td>
<td>64</td>
<td>64</td>
<td>44</td>
<td>39</td>
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<tr>
<td>chicken GHRH-LP-R</td>
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<td>44</td>
<td>31</td>
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<tr>
<td>zebrafish GHRH-R</td>
<td>40</td>
<td>39</td>
<td>39</td>
<td>31</td>
<td>78</td>
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<tr>
<td>goldfish GHRH-R</td>
<td>40</td>
<td>40</td>
<td>39</td>
<td>33</td>
<td>78</td>
<td>54</td>
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<tr>
<td>chicken GHRH-R</td>
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<td>34</td>
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<tr>
<td>human GHRH-R</td>
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<td>33</td>
<td>49</td>
<td>51</td>
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Amino acid identity was determined by using the ClustalW alignment program with BLOSUM62.
Thirteen conserved cysteines and three putative glycosylation sites, and signature motifs like RLAK, FQGBBVXXBYCFXNXEXQ, and SQR were observed in zebrafish, fugu and goldfish receptors.

The zebrafish PHI/VIP cDNA is 1092bp in length (submitted to Gene Bank, accession no.: EU150383) with an open reading frame of 459bp (from 117-576) encoding a 153 amino-acid polypeptide. The genomic database in Vega revealed that the PHI/VIP gene spans approximately 3584bp on chromosome 13 and contains six exons (Fig. 2.3A, B). The zebrafish mature PHI peptide between residues 40 and 66 shares high sequence identity with goldfish A (85%) and B (89%), chicken (67%) and human (78%) PHIs (Tse et al, 2002) (Fig. 2.3C). The zebrafish amino acid residues between 84 and 112 are identical with the mature VIP peptide of trout. Both PHI and VIP in zebrafish are flanked at the C-terminus by a GKR, indicating the peptides are amidated and have potential proteolytic cleavage sites (Fig. 2.3B).

**Functional analysis of zebrafish PHIR compared with related zebrafish and human receptors**

To evaluate whether the identified PHIR and PHI peptide are able to transduce a physiological signal, zebrafish PHIR, VPAC₁R, PAC₁R-short and human VPAC₂R were transfected separately into COS7L cells. The ability of zebrafish peptides to activate adenylyl cyclase (AC) and phospholipase C (PLC) pathways was assessed by the cAMP and [³H] inositol phosphate assay (Fig. 2.4). In COS7L cells expressing the PAC₁R, three peptides were able to activate the cAMP and inositol phosphate (IP) accumulation in a dose-dependent manner with different half maximal response (EC₅₀) values (Fig. 2.4A, B). The EC₅₀ values of PACAP isoform1 were 1.23X10⁻⁹ M (cAMP) and 4.62X10⁻⁸ M
Fig. 2.3 (A) Predicted structure of zebrafish PHI/VIP gene from the zebrafish genome data base in Vega blasted with our cDNA sequence. The gene size (3584bp) is indicated on the right of the gene. Exons are numbered and are presented by closed boxes and a solid horizontal gray line indicates introns. The distance between the first five exons is indicated by numbers above the intron line. (B) cDNA and predicted amino acid sequences of zebrafish PHI/VIP. The numbers on the right and left are the positions of the nucleotide sequences. The putative sequence of mature PHI and VIP are underlined and the putative PHM (C-terminal extension of PHI to the last residue (Met) before "KR") is labeled with a black dot (●). The potential polyadenylation signals (AATAAA) are double underlined. (C) Amino acid sequence alignment of mature PHI peptides by ClustalW in vertebrates. Shaded areas represent identical amino acid residues. The sequences of goldfish A and B, chicken, mouse and human are from Tse et al, 2002.
### PHI

<table>
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<tr>
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<th>Sequence 3</th>
<th>Identity</th>
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<td>SKLIGGSAK</td>
<td>SAR RYLESLI-NH$_2$</td>
<td>100%</td>
</tr>
<tr>
<td>goldfish-A</td>
<td>HADGLFTSGY</td>
<td>SKLIGGSAK</td>
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<tr>
<td>goldfish-B</td>
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<td>SKLIGGSAK</td>
<td>EYLESLIL</td>
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<td>SOLLAKLAVK</td>
<td>RYHLHSLI</td>
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<td>mouse</td>
<td>HADGVFTSDY</td>
<td>SRLIGQISAK</td>
<td>KYLESLI-NH$_2$</td>
<td>78%</td>
</tr>
<tr>
<td>human</td>
<td>HADGVFTSDF</td>
<td>SKLIGQISAK</td>
<td>KYLESLM-NH$_2$</td>
<td>78%</td>
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### VIP

<table>
<thead>
<tr>
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<th>Sequence 3</th>
<th>Identity</th>
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</thead>
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<td>SRERKQMAVK</td>
<td>KYLNSVL-T-NH$_2$</td>
<td>100%</td>
</tr>
<tr>
<td>cod</td>
<td>HSDAVFTDNY</td>
<td>SRERKQMA AK</td>
<td>KYLNSVLA-NH$_2$</td>
<td>89%</td>
</tr>
<tr>
<td>chicken</td>
<td>HSDAVFTDNY</td>
<td>SRERKQMAVK</td>
<td>KYLNSVL-T-NH$_2$</td>
<td>96%</td>
</tr>
<tr>
<td>human</td>
<td>HSDAVFTDNY</td>
<td>TLRKQMAVK</td>
<td>KYLNSLI-IN-NH$_2$</td>
<td>82%</td>
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<tr>
<td>Clustal Co</td>
<td>****:*****</td>
<td><em>:</em>:*****:* ****<em>:</em></td>
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Fig. 2.4 Accumulation of total cAMP and total inositol phosphates (IPs) in COS7L cells transfected with different receptors and stimulated with graded concentrations of zebrafish (zf) peptides. (A) cAMP and (B) IP of zebrafish PAC1R; (C) cAMP and (D) IP of zebrafish VPAC1R; (E) cAMP and (F) IP of zebrafish PHI-R; (G) cAMP and (H) IP of human VPAC2R. Values represent means ± SEM from a minimum of three independent experiments each in triplicate. VIP, vasoactive intestinal polypeptide; VPAC1R, VIP/PACAP shared receptor 1; VPAC2R, VIP/PACAP shared receptor 2.
isoform2 were 1.73 \times 10^{-9} M (cAMP) and 6.66 \times 10^{-9} M (IP); and VIP were 3.44 
\times 10^{-8} M (cAMP) and 6.68 \times 10^{-7} M (IP). There was no significance difference (P<0.05) in 
EC_{50} values of PAC_{1}R stimulation by the two different PACAP isoforms in either the 
cAMP or IP pathway. But the EC_{50} values of the PAC_{1}R response to VIP were 
significantly higher (P<0.05) than the EC_{50} values of PACAPs in either the cAMP or IP 
pathway. Hence zebrafish PACAP was more potent than zfVIP on the zf PAC_{1}R.

In cells expressing the VPAC_{1}R, the same three peptides were able to activate the 
cAMP pathway but none of the peptides were able to stimulate VPAC_{1}R in the IP 
pathway in the physiological range (<10^{-6} M) (Fig. 2.4C, D). The EC_{50} values of PACAP 
isoform-1 were 2.93 \times 10^{-7} M (cAMP); isoform2 were 1.54 \times 10^{-7} M (cAMP) and VIP 
were 1.96 \times 10^{-7} M (cAMP). Hence PACAP and VIP are nearly equal in potency for 
stimulation the zfVPAC_{1}R.

PHI-R had a different response pattern to PACAP and VIP compared with zf VPAC_{1}R 
or VPAC_{2}R from other species in that the PHI-R did not respond to VIP and PACAP 
(Fig. 2.4E, F). In contrast to VPAC_{1}R or VPAC_{2}Rs, there is no significant dose response 
of PHI-R to VIP stimulation (P>0.05) although PHI-R has high identity to VPAC_{2}Rs of 
other species. However, PHI-R had a high potency to response to PHI in cAMP 
accumulation and the EC_{50} value is 1.77 \times 10^{-9} M (Fig. 2.4E).

The physiological characteristics of the zebrafish receptors were compared with 
human VPAC_{2}R using our isolated zebrafish VIP (82% identical to human VIP in amino 
acids). Surprisingly, zebrafish VIP was able to activate PAC_{1}R-short (Fig 2.4 A, B), 
VPAC_{1}R (Fig. 2.4 C) and human VPAC_{2}R in both the cAMP and IP signaling pathway in 
a dose-dependent manner (Fig. 2.4G, H). PAC_{1}R is thought to have a more specific
response to PACAP stimulation except for the variant described by Lutz et al., 2006. However, in the present study, I found zfVIP was able to stimulate PAC1R-short in both the cAMP and IP paths. As expected, VIP did not stimulate the zfVPAC1R in the IP path. Unexpectedly, human VPAC2R has a strong response to zfVIP stimulation in the IP signaling pathway (Fig. 2.4 H). The EC50 values of human VPAC2R are 5.41X10^{-9} M (cAMP) and 5.07X10^{-8} M (IP) in response to zebrafish VIP. EC50 values are listed in Table. 2.5.

**Functional analysis of zebrafish GHRH-LP-R and human GHRH-R activated by zebrafish GHRH and two GHRH-like peptides**

To confirm the functional identity of zebrafish GHRH-LP-R, we used three peptides: zebrafish GHRH-LP-1, GHRH-LP-2 and GHRH. Each peptide was tested on the zebrafish GHRH-LP-R transfected into COS7L cells. For comparison of the physiological profiles, human GHRH-R was also expressed in COS7L cells. Both cAMP and IP production were measured after stimulation with different doses of peptides. Zebrafish GHRH-LP-R responded to zfGHRH-LP-1 and zfGHRH in a similar dose-dependent manner in the cAMP signaling pathway (Fig. 2.5); the response was similar to that in a chicken or goldfish system (Wang et al, 2007 and Lee et al., 2007). However, there was no response of zfGHRH-LP-R to GHRH-LP-2 stimulation, which is the same lack of response in goldfish (Chan et al, 1998). Human GHRH-R had no response to either GHRH-LP-1 or GHRH-LP-2. The EC50 values of GHRH-LP-1 and GHRH are 3.26X10^{-8} and 1.15X10^{-7} M respectively with the zebrafish GHRH-LP-R in the cAMP
### Table 2.5. EC50 values of receptor responses to peptides

<table>
<thead>
<tr>
<th></th>
<th>cAMP response EC50 (nM)</th>
<th>IP response EC50 (nM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>zfPACAP isoform 1</td>
<td>zfPACAP isoform 2</td>
</tr>
<tr>
<td>zfPAC1R-short</td>
<td>1.23 ± 0.22</td>
<td>1.73 ± 0.54</td>
</tr>
<tr>
<td>zfVPAC1R</td>
<td>293 ± 90</td>
<td>154 ± 85</td>
</tr>
<tr>
<td>zfPHIR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>hVPAC2R</td>
<td></td>
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</tr>
<tr>
<td>zfGHRH-LP-R</td>
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<td></td>
</tr>
<tr>
<td>hGHRHR</td>
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</table>

* NR, no response; blank space, not tested.
Fig. 2.5 Functional assay using graded concentrations of zebrafish GHRH-LP-1, GHRH-LP-2 and GHRH on (A) cAMP and (B) IP accumulation in COS7L cells transfected with zebrafish growth hormone-releasing hormone-like peptide receptor (zfGHRH-LP-R); (C) cAMP and (D) IP accumulation in COS7L cells transfected with human GHRH-R. Values in cAMP assay represent means ± SEM from three independent experiments each in triplicate. Values in IP assay represent means ± SE from at least two independent experiments each in triplicate.
A  

zfGHRH-LP-R

![Graph A: cAMP (fmol/well) vs Log [peptides, M]]

- zfGHRH-LP1
- zfGHRH-LP-2
- zfGHRH

B  

zfGHRH-LP-R

![Graph B: 3H-Inositol Phosphates (cpm) vs Log [peptides, M]]

- zfGHRH-LP1
- zfGHRH-LP2
path. There is no significant difference (P>0.05) between these two EC₅₀ values. EC50 values are listed in Table 2.5.


To investigate the expression of zebrafish PHI-R, GHRH-LP-R and PHI/VIP mRNA distribution in comparison with zfPAC₁R, VPAC₁R and GHRH-R, I used RT-PCR in eleven tissues (Fig. 2.6). Zebrafish β-actin (1014bp) was used as a control to verify the quality of the first-strand cDNA synthesized. The most widely distributed mRNA is PHI-R, which is expressed strongly in all tissues except ovary where only a faint band is present. The PAC₁R-short mRNA (arrow in Fig. 2.6) was also widely expressed in adult zebrafish with stronger expression in the eye, brain, testis, gill, skin and ovary; lower levels were detected in the heart, kidney, swimbladder, skin and gut. The longer bands (arrow head in Fig. 2.6) are PAC₁R-hop, which has a hop cassette inserted in ICL3. PAC₁R-short is more widely distributed than other forms of PAC₁R in zebrafish (Fradinger et al, 2005), PAC₁R-short is also the predominant isoform in goldfish (Kwok et al, 2006). Also, VPAC₁R was expressed in all eleven tissues with a lower level of expression in the gill, kidney, skin and ovary. PHI/VIP mRNA was more strongly expressed in the eye, heart, brain, testis, gill, gut and skin than in the swimbladder or ovary. There was no detection in the kidney and spleen. GHRH-LP-R and GHRH-R are distributed to only a few tissues. GHRH-LP-R was only strongly expressed in eye, brain, testis and kidney with very weak expression in swimbladder and heart. There was no expression observed in gill, gut, skin, ovary and spleen. The GHRH-R is mainly
Fig. 2.6 Tissue distribution of the zebrafish (zf) PAC₁R-short, VPAC₁R, PHI-R, PHI/VIP, GHRH-LP-R and GHRH-R by RT-PCR. Zebrafish β-actin is a positive control for each tissue examined. In the negative lane, no template was added. All PCR products spanned at least two exons. The arrow points to the PAC₁R-short PCR product and the arrow head represents the PAC₁R-hop product in different tissues. PCR was repeated three times. Abbreviations: PAC₁R, PACAP-specific receptor; VPAC₁R, VIP/PACAP shared receptor1; PHI, peptide histamine isoleucine; VIP, vasoactive intestinal polypeptide; GHRH-LP, growth hormone-releasing hormone-like peptide.
distributed in the brain, testis, and gut with very faint bands in the eye, kidney, swimbladder. β-actin was expressed in a relatively similar manner in the eleven tissues.

**Phylogenetic analysis**

Phylogenetic analysis was carried out on zebrafish PHI-R and GHRH-LP-R with other members of the secretin receptor superfamily. Analysis of all ClustalW multiple aligned receptors was conducted by the Neighbour Joining method with the zebrafish glucagon receptor (GLUR) as the out group (Fig. 2.7). The analysis indicates that the zebrafish PHI-R was grouped with goldfish PHI-R, and in a subgroup with fugu, human and chicken VPAC₂Rs. The identified zebrafish GHRH-LP-R was grouped together with all other previously cloned or predicted GHRH-LP-R (PRP-Rs).

**Discussion**

**Proof that a novel zebrafish receptor is PHI-R and not VPAC₂R**

Initially we thought our newly isolated receptor of 2088 bp was a VPAC₂R because it encoded a small section that was identical to a published fragment of VPAC₂R (274bp) (Wang *et al*, 2003). Also, alignment of our deduced amino acid sequence with human and chicken VPAC₂R showed a relatively high sequence identity of 56% and 59%, respectively. Signature motifs characteristic of human and chicken VPAC₂R were present in the zebrafish sequence. For example, in the zebrafish sequence, there are 13 conserved cysteines including seven in the N-terminal extracellular domain, two potential N-glycosylation sites, PDV and RLAK binding motifs and other highly conserved amino
Fig. 2.7 Phylogenetic analysis of the zebrafish PHIR and GHRH-LP-R amino acid sequences with other known related receptors. Receptors identified in this report are underlined. Zebrafish glucagon receptor (GluR) was used as an out group. PHI-R, peptide histamine isoleucine-receptor; GHRH-LP, growth hormone-releasing hormone-like peptide.
acid residues for VIP binding (Couvineau et al., 1995). Phylogenetic analysis also grouped this sequence with other VPAC₂Rs to form a subbranch. However, this receptor was not significantly stimulated by VIP even with a $10^{-5}$ M concentration analyzed in both cAMP and IP signaling pathways. Other evidence that our receptor might not be a VPAC₂R was in the N-terminal extracellular site, that is fundamental for VIP binding (Laburthe and Couvineau, 2002), where the zebrafish sequence is missing a potential glycosylation site, which is expressed in the human, chicken and frog VPAC₂R (Hoo et al, 2001). N-glycosylation sites are critical for ligand binding and correct delivery to the plasma membrane of the human VPAC₁R (Couvineu et al, 1995, 1996). Although asparagine in position 58 and 69 is crucial in human VPAC₁R, a single mutation of asparagine (one of three) in the N-terminal extracellular domain did not influence receptor function (Couvineu et al, 1996). Finally, we discovered that our receptor has 80% amino acid sequence identity to goldfish PHI-R; the latter has been published (Tse et al., 2002) but the sequence has not been entered into the NCBI genome database. The goldfish PHI-R is the only one characterized in vertebrates so far as we know. The sequence identity of our receptor with goldfish PHI receptor is higher than for all reported VPAC₂Rs (up to 62% in fugu). Phylogenetic analysis also grouped the two fish PHI-Rs together. Moreover, in the N-terminal glycosylation site, goldfish has isoleucine in the same position as zebrafish (Fig. 1B, position 91, opened circle) instead of asparagine. Based on functional studies (specific response to PHI and no response to VIP), sequence identity and phylogenetic analysis, we conclude that this receptor is zebrafish PHI-R. Whereas our PHI-R is not VPAC₂R, further analysis of fugu VPAC₂RA and VPAC₂RB for PHI-R characteristics is essential. First, fugu VPAC₂Rs also only have
two glycosylation sites each (Fig. 1B, positions 61 and 95 for RB and RA, respectively, open circles). Second, fugu VPAC$_2$Rs share relatively high sequence identity with zebrafish PHI-R: 63% and 56% of VPAC$_2$RB and VPAC$_2$RA, respectively. Third, in a phylogenetic tree, fugu VPAC$_2$RB and fugu VPAC$_2$RA grouped more closely with zebrafish PHI-R than with chicken and human VPAC$_2$R. However, the sequence similarity may not represent functional equivalence as addressed by Cardoso et al., 2005. The more exact relationship of fugu VPAC$_2$RA and VPAC$_2$RB with PHI-R will be defined by functional assays.

**zebrafish PAC$_1$R-short has unconventional response to VIP**

It was generally accepted that PAC$_1$R specifically binds and responds to PACAP with a very low affinity to VIP and that only VPACRs have similar affinity to both PACAP and VIP (Vaudry et al., 2000). However, we show here that PAC$_1$R-short isoform in zebrafish has a significant dose-dependent response to PACAPs and VIP in both cAMP and IP assays. Although VIP is not as potent as PACAP, the EC$_{50}$ values of VIP are still all below the pharmacological concentration (10$^{-6}$M) and there is no significant difference (P<0.05) between PAC$_1$R and VPAC$_1$R in response to VIP in cAMP pathway. There are no data available in other teleost fish as to whether PAC$_1$R-short isoform responds to VIP. However, there are at least 14 variants of the PAC$_1$R in human neuroblastoma cells due to alternative splicing and some of these variants also respond to VIP stimulation in a dose-dependent manner in the cAMP and IP signaling pathways (Lutz et al., 2006). It is possible that certain variant PAC$_1$ receptors retained the function of responding to VIP from fish to human. Moreover, it is interesting to note that human
VPAC₂R also responds well to zebrafish VIP and there is no significant difference (P>0.05) compared with zebrafish PAC₁R in both the cAMP and IP signaling pathways. The zebrafish VIP peptide shares 82% amino acid identity with human.

*zf GHRH-LP-R responds to both zebrafish GHRH-LP-1 and GHRH*

Recently published data suggests that fish (Lee et al., 2007; Wang et al., 2007) and mammals have GHRH in addition to GHRH-LP on separate genes. Each of these two peptides has a specific receptor. In the present study, we identified a new receptor in zebrafish, which we argue is GHRH-LP-R because it has 88% sequence identity with goldfish GHRH-LP-R but only 40% identity with zebrafish and human GHRH-R. Sequence alignment with goldfish and fugu GHRH-LP-Rs reveals closely related structures: 13 conserved cysteines, three N-glycosylation sites, and signature motifs like “SQR” for protein kinase C binding, RLAK for the coupling to Gsα and FQBBVZXBYCFXNXEXQ. Also phylogenetic analysis grouped zebrafish and goldfish GHRH-LP-Rs along with other GHRH-LP-Rs. The analysis revealed that GHRH-LP-Rs grouped near to GHRH-Rs among the family B members.

Functional assays revealed that the GHRH-LP receptors in both zebrafish and goldfish are most sensitive to GHRH-LP-1 but both respond also to GHRH at about a 3 fold less potency in zebrafish and 30 fold less in goldfish. The activation of the zebrafish GHRH-LP-R was concentration-dependent in the cAMP pathway and the potency of zfGHRH-LP-1 and zfGHRH had no significant difference (P>0.05). Similarly, goldfish GHRH-LP-R (PRP-R) was stimulated by carp GHRH-LP-1 (identical to zfGHRH-LP-1) and goldfish GHRH (Lee et al, 2007). Although the goldfish GHRH-LP-R was transfected
into a CHO cell line and our zebrafish receptor was in a COS7 line, the EC$_{50}$ values between goldfish (1.8x10$^{-7}$ M) and zebrafish (1.15X10$^{-7}$M) response to fish GHRH are nearly the same. However, in response to GHRH-LP-1, the EC$_{50}$ values are 5.8X10$^{-9}$ M and 3.41X10$^{-8}$ M for goldfish and zebrafish, respectively. Moreover, neither zebrafish nor goldfish GHRH-LP-R responded to the second form of GHRH-LP. We did not detect any response of human GHRH-R to zebrafish GHRH-LP-1 or GHRH-LP-2. We conclude that zfGHRH-LP-R (PRP) has at least two specific ligands: zebrafish GHRH-LP-1 and zebrafish GHRH in the cAMP and IP path. However, zfGHRH-R only responds to zfGHRH not the carpGHRH-LP stimulation. In chicken, further work is needed, as one report states that chicken GHRH-R has no response to chicken GHRH-LP (Toogood et al., 2006), whereas another report shows that chicken GHRH-R has a dose dependent response to chicken GHRH-LP (Wang et al., 2007).

**Cleavage of zebrafish prepro-PHI/VIP is predicted to produce two amidated peptides or extended forms without amidation**

PHI and VIP are both transcribed from the same gene. PHI has a cleavage site of GKR, which implies that PHI is amidated at position 27. Another zebrafish peptide is PHM, which is a C-terminal extension of PHI to a methionine at position 42; PHM would not be amidated as it is followed by only a KR cleavage site. VIP also has a GKR cleavage site after position 28. The zebrafish VIP C-terminus can be extended, which is similar to human VIP. Both PHI and VIP are amidated at the C-terminus but goldfish and chicken PHIs are not amidated because of the lack of glycine residue (Talbot et al., 1995; Tse et al., 2002). Also, a VIP extension is absent in goldfish because VIP-28 is directly followed by a stop codon. The primary structure of zebrafish PHI has been conserved
with 6 amino acid substitutions compared with the human peptide. Zebrafish mature PHI peptide shares high sequence identity with human PHM (78%) and goldfish PHI (85-89%). In general, PHI activates all known VIP receptors with moderate to weak activation (Palle et al., 1989; Moriarty et al., 1984; Lelièvre et al., 1998) and PHI is a weak agonist of VIP. However, Lelièvre and his coauthors (1998) suggested that PHI had its own specific receptor that preferentially binds PHI over VIP. In their study, PHI had a 100 fold more potent anti-proliferative action than VIP using radio-labeled PHI as a tracer for binding studies on neuroblastoma cells. The study also revealed there are high affinity receptors that selectively bound PHI over VIP. The existence of zebrafish and goldfish PHI-R and the high conservation of PHI from fish to human suggest that not only VIP but also PHI plays an important role in fish and possibly other vertebrates.

*Location of receptors indicates diverse target tissues for peptides*

To compare the location of zebrafish PHI-R and GHRH-LP-R with other related peptide receptors, we performed an RT-PCR study. PHI-R is one of the most widely distributed receptors and is highly expressed in 10 different tissues with weak expression in the ovary. The coexpression of PHI-R with PHI in the eye, heart, brain, testis, gill, gut, swimbladder, skin and ovary indicates that PHI may act in an autocrine/paracrine manner. Goldfish PHI-R expression was quantified by real time PCR (Tse et al., 2002). High expression levels were found in the brain, heart, testis and gut, which is similar to zebrafish. In ovary, neither goldfish PHI mRNA nor fugu VPAC2-R mRNA were detected (Cardoso et al., 2005). The low or undetectable expression of PHI-R and PHI in the ovary of zebrafish and goldfish contrasts with previous data that a fragment of the zebrafish
VPAC₂R (274bp, now identified as PHI-R) was highly expressed in follicle cells in cultured ovary (Wang et al., 2003). In rat, VPAC₂Rs were found by RNA blot analysis in brain, stomach, colon, lung, heart, pancreatic islets and an insulin-secreting cell line (MiN6) (Inagaki et al., 1994).

Zebrfish PAC₁R-short is expressed widely except in the spleen. This result is similar to the observation of Fradinger et al., 2005, except we found additional weak expression in the heart and swim bladder. Goldfish PAC₁R-short was not detected in the spleen either but has strong expression in the heart conducted by real time PCR (Kwok et al., 2006). PAC₁R-short isoform is one of the most widely distributed receptors tested here, along with PHI-R in goldfish and zebrafish. In frog and mammal, the distribution was largely investigated by RT-PCR and in situ hybridization in the brain and pituitary (Hashimoto et al., 1996; Shioda et al., 1997; Hu et al., 2000) where PAC₁Rs are abundantly expressed.

In contrast, compared with other receptors studied here, the distribution of zebrafish GHRH-LP-R and GHRH-R are quite limited. This probably relates to tissue specific functions. Zebrafish GHRH-R was only strongly expressed in the brain, testis and gut with very weak signals in the eye, kidney, swimbladder, skin and ovary. GHRH-LP-R was only detected in the eye, brain, testis and kidney with very weak signal in the heart. But GHRH-LP-R was expressed strongly in the eye compared with GHRH-R. This indicates that the role of zebrafish GHRH-LP-R in the eye should be studied further. The distribution of zebrafish GHRH-LP-R is comparable to a previous study in our laboratory (Fradinger et al., 2005).
The newly isolated zebrafish PHI-R and GHRH-LP-R will contribute to a better understanding of the family B receptors from an evolutionary perspective. That PAC₁R is activated by both PACAP and VIP in both zebrafish and humans offers an insight into functional conservation and evolution. Two critical questions that arise from this study are whether both PACAP and VIP are ligands for certain variant PAC₁Rs and whether there is an evolutionary transformation from the PHI-R to VPAC₂R or whether tetrapods also have a PHI-R.
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Chapter 3

Role of two genes encoding PACAP in early brain development in zebrafish

Introduction

In Chapter 2, the superfamily members of hormones PHI and VIP were established, as were the receptors for PHI and GHRH-LP in zebrafish by cloning the cDNAs and testing in vitro functions. As a result, the zebrafish VPAC$_2$ receptor was shown to be a PHI receptor and the GHRH-LP receptor was shown to be activated with both the GHRH-like peptide, and GHRH. In the present Chapter 3, the in vivo expression, localization and function of PACAP, GHRH-LP and their receptors are examined.

PACAP and its PAC$_1$ receptor are expressed in the neural tube of a mouse embryo at E10 suggesting that PACAP might be important in development (Sherwood et al., 2000). Although our group (Gray et al., 2001) and others (Hashimoto et al., 2001; Hamelink et al., 2002; Colwell et al., 2004) have created lines of mice with the PACAP gene disrupted, the zebrafish offers advantages in the study of development. In zebrafish it is possible to avoid the influence of maternal PACAP and to use a nearly transparent embryo for constant observation. The method of gene knockdown offers only a temporary blocking of the PACAP translation, but is sufficient for developmental studies in zebrafish where hatching occurs at 72 hours. Although PACAP is highly conserved between mammals and fish, there are some interesting differences between them. First, PACAP is encoded on two separate genes in zebrafish (Fradinger et al., 2000; Wang and Wong 2003), unlike the single copy gene in mice (Cummings et al., 2002). In zebrafish, the two forms of PACAP (PACAP1 and PACAP2) are each 27 or 38 amino acids in length and the two forms are 82% identical, but we do not know if the peptides have distinct functions. Therefore, each of the two PACAPs would have to be eliminated to examine their separate effects. Second, there is alternative splicing in the zebrafish
PACAP2 transcript in which exon 4 is cut out of the mRNA. Third, the PACAP genes of all fish studied to date encode a growth hormone-releasing hormone (GHRH)-like peptide (LP) on exon 4, which would be blocked also if the mRNA is knocked down. However, GHRH-LP in fish appears to have minor functional effects for release of GH compared with PACAP (Parker et al., 1997).

To date, the temporal expression of PACAP1 and PACAP2 in zebrafish is known through expression studies using RT-PCR (Fradinger et al., 2000; Wang et al., 2003). More specific localization in the brain and eye was provided by an immunocytochemistry study in zebrafish from the 24-hour embryo through to the adult, but the antibody could not distinguish the two PACAP forms (Mathieu et al., 2004). The functions of the two zebrafish PACAPs are not known, but in mice and other mammals PACAP has a role in the regulation of other endocrine systems (Vaudry and Arimura, 2003), in carbohydrate and protein metabolism (Gray et al., 2000), in the stress response (Gray et al., 2000; Hamelink et al., 2002), in the immune system (Vaudry and Arimura, 2003), as a neuromodulator in the sympathetic and parasympathetic nervous system (May et al., 2000; Parsons et al., 2000) and in the brain as a neurotrophic, neuroprotective or proliferative factor (Vaudry and Arimura, 2003).

The structures of zebrafish receptors that bind PACAP and VIP, PAC1 receptor and VPAC1 receptor have been fully identified (chapter 2; Wang et al., 2003; Fradinger et al., 2005). Also, Chapter 2 provided the complete structure and proof that "VPAC2R" is not a VIP/PACAP receptor, but a PHI receptor. When the zebrafish PAC1 receptor was expressed in monkey COS cells, it responded equally to zebrafish PACAP1 or PACAP2 as measured by an increase in cAMP or inositol phosphate (Fradinger et al., 2005). In
contrast, only cAMP signaling was activated when the zebrafish VPAC₁ receptor was tested with PACAP1 or PACAP2 (Fradinger et al., 2005). In conclusion, zebrafish have two genes encoding PACAP1 and PACAP2, which can be distinguished as to structure, but not yet as to exact brain location and function in development or in adults. Evidence to date suggests that the two zebrafish PACAPs activate PAC₁ and VPAC₁ receptors in the same way.

Here, we examine early brain development and the role of PACAP using zebrafish (Danio rerio), where rapid development occurs outside of the mother in contrast to mammals. The first appearance of mRNA for PACAP1, PACAP2, and receptors, PAC₁, VPAC₁, PHI and GHRH-LP, is determined with RT-PCR and for PACAP2 using in situ hybridization. The effects of morpholino (MO)-induced knockdown of PACAP1 or PACAP2 is determined initially by screening morphological defects in the embryos at 27 hours post fertilization (hpf) and subsequently by detailed examination of the expression pattern of brain genes that can be used as molecular markers for early stages of development.

Materials and Methods

Zebrafish

All zebrafish (Danio rerio) were kept in dechlorinated water at 28°C with 14 hours of light and 10 hours of dark. Embryos were collected in the morning after natural spawning of adult fish. Times of development are referred to as hours (h) after
fertilization at 28°C. All procedures were approved by the Animal Care Committee at the University of Victoria.

**RT-PCR of pacap and its receptors**

To examine the onset of *pacap* expression, mRNA from 30 embryos at developmental stages ranging from 30 min to 25 hours after fertilization was extracted with the RNeasy mini-kit (Qiagen, Mississauga, ON). The samples were treated with Turbo RNase-free DNase (Ambion). Single stranded cDNA was synthesized using oligo (dT) and Superscript II RT (Invitrogen, Burlington, ON) according to the manufacturer’s instructions. The primers for PCR of *pacap1* and *pacap2* mRNA are shown in Table 3.1. A control reaction was prepared using 1 µl of cDNA amplified with primers for RNA polymerase II (Table3.1). PCR reactions were carried out for 35 cycles at 55°C annealing temperature with a 10 min extension at 72°C on the last cycle. The PCR products were normalized to the expression of RNA polymerase II. Reaction products were separated by 1.5% agarose gel electrophoresis and visualized using ethidium bromide staining. The differences were evaluated visually. To examine the onset of receptor expression, mRNA from five embryos at developmental stages ranging from 1-18 hours after fertilization was extracted with the Poly (A) Pure kit (Ambion, Austin, TX). Single stranded cDNA was synthesized using oligo (dT) and Superscript II RT (Invitrogen) according to the manufacturer’s instructions. The transcripts encoding the PAC₁, VPAC₁ and GHRH-LP receptors, and tubulin control were amplified from 2 µl of cDNA by PCR and detected by Southern blot analysis with $^{32}$P-dCTP-labeled receptor specific probes as previously described (Fradinger *et al.*, 2005).
Table 3.1. Primer sequences for PCR and probes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Direction-Use</th>
<th>Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PACAP1</td>
<td>F</td>
<td>CGCCTCTGAGTTACCCCGAAA</td>
</tr>
<tr>
<td>PACAP1</td>
<td>R</td>
<td>TAGCGAGCCCGCTCCTTTTG</td>
</tr>
<tr>
<td>PACAP2</td>
<td>F</td>
<td>TCAGGGGAAGGCTGTGTGAGGA</td>
</tr>
<tr>
<td>PACAP2</td>
<td>R</td>
<td>CATCTGTGGTTTGAGTCCGTGT</td>
</tr>
<tr>
<td>VPAC&lt;sub&gt;2&lt;/sub&gt; receptor</td>
<td>F</td>
<td>GTCTCTGCTGCCCCTCAA AAA</td>
</tr>
<tr>
<td>VPAC&lt;sub&gt;2&lt;/sub&gt; receptor</td>
<td>R</td>
<td>AGATTGGGATGAGTTCTGCGT</td>
</tr>
<tr>
<td>RNA polymerase II</td>
<td>F</td>
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<td>CACAGCAATAACCTGAGAAA</td>
</tr>
<tr>
<td>tubulin</td>
<td>F</td>
<td>CAGGAGCGACGCGGCTGCTTG</td>
</tr>
<tr>
<td>tubulin</td>
<td>R</td>
<td>AGTTGGTCCACAGGCTGCTTG</td>
</tr>
<tr>
<td><strong>Probes</strong></td>
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<td></td>
</tr>
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<td>PACAP2</td>
<td>F</td>
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<tr>
<td>PACAP2</td>
<td>R</td>
<td>CAAAGCGCAACTGCTGCTTAAC</td>
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<tr>
<td>mab</td>
<td>F</td>
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</tr>
<tr>
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<tr>
<td>fgf8</td>
<td>R</td>
<td>GTCGCTACCTTTACTTTGCTCAC</td>
</tr>
<tr>
<td>meis1.1</td>
<td>F</td>
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</tr>
<tr>
<td>meis1.1</td>
<td>R</td>
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<tr>
<td>eng2</td>
<td>F</td>
<td>GACGCAGCAATCGTTTGG</td>
</tr>
<tr>
<td>eng2</td>
<td>R</td>
<td>GTCGCGCTTCATCCCCCTTT</td>
</tr>
<tr>
<td>ephA4</td>
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<td>CAAGCGATAAGGATGAGC</td>
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<tr>
<td>mbx</td>
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<td>CAGCGAGACGAGGAGTA</td>
</tr>
</tbody>
</table>
The sequence of the PHI receptor (formerly thought to be a VPAC2 receptor) was not identified until after the other three receptors had been studied. To examine expression of PHI receptor in embryos, the same procedure was followed as for RT-PCR of pacap1 and pacap2 mRNA, described above. The primers are listed (Table 3.1).

**Microinjection of morpholino antisense oligonucleotides**

Morpholinos (MOs) are antisense oligonucleotides that are designed to block a specific mRNA from being translated into a protein (Nasevicius and Ekker 2000). The morpholine ring modifies the nucleotides so that degradation is prevented (Summerton and Welller, 1997), although the original quantity injected is diluted at each cell division. In developmental studies of zebrafish, MOs are effective blockers of translation, but are mainly restricted to the period before hatching. The methods for knockdown in zebrafish have been described by others (Ekker and Larson, 2001).

MOs, including those for control injections, were designed and synthesized by Gene Tools (Eugene, OR). The design of the MOs against the RNA region around the AUG translation start site (ATG) and the 5’ untranslated region (UTR) ensured that the two MOs did not overlap (Table 3.2). Also, this design resulted in blockage of translation of both the full length and splice variants lacking exon 4 of PACAP. In addition, a PACAP splicing MO (Ekker and Larson, 2001) was used to confirm the specificity of the PACAP1-UTR-MO. MOs were dissolved in filtered ddH₂O to a working concentration of 2 - 12µg/µl. Zebrafish embryos were injected with 1-1.5nl of the MO solution into the yolk at the 1-cell stage (Xu, 1999; Nasevicius and Ekker, 2000). Pictures of morphants were captured by an Olympus (SZX9) dissection microscope with a Sony color video
Table 3.2. Morpholino sequences and rescue primers

<table>
<thead>
<tr>
<th>Morpholino name</th>
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<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>pacap1 UTR</td>
<td>5' UTR</td>
<td>CGGACGGATGCTGTCCAATGGAGGC</td>
</tr>
<tr>
<td>pacap1 ATG</td>
<td>Start codon</td>
<td>GAGTCGTTTTGCTGCTCGTAATCAT</td>
</tr>
<tr>
<td>pacap2 UTR</td>
<td>5' UTR</td>
<td>GAAATGCTGTTGGAAATGCAGCTCGGG</td>
</tr>
<tr>
<td>pacap2 ATG</td>
<td>Start codon</td>
<td>GCCATGCTATTGCAGAGTAGGTAGA</td>
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</tbody>
</table>

**Rescue primers**

<table>
<thead>
<tr>
<th>pacap1</th>
<th>F</th>
<th>AGAATGATTGCGAGGCAGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pacap1</td>
<td>R</td>
<td>TGGAGCAAGCATGATAAAC</td>
</tr>
<tr>
<td>pacap2</td>
<td>F</td>
<td>TATCTACTACTCTGCAATAGCATG</td>
</tr>
<tr>
<td>pacap2</td>
<td>R</td>
<td>ATATCGGCAGCATAACGT</td>
</tr>
</tbody>
</table>
camera (DXC-950P). Images were imported into Northern Eclipse software (Empix Imaging Inc, Mississauga, ON).

**Rescue of morpholino-induced effects by injection of pacap mRNA**

To rescue morpholino-treated embryos, mRNA was prepared by amplification using PCR of the corresponding pacap2 cDNA containing part of the 5' UTR plus the coding sequence and 3' UTR. The rescue mRNA did not overlap with the morpholino pacap2-UTR sequence. The primers for PCR amplification of rescue mRNA are shown (Table 3.2). The PCR products were ligated into pGEM-T vector and sequenced. The vector with insert was linearized with NdeI, and synthetic mRNA was transcribed from the T7 promoter using the T7 mMESSAGE mMACHINE kit (Ambion) following the manufacturer’s directions. After synthesis, all capped RNAs were purified by MEGAclear kit (Ambion) and precipitated by ammonium acetate and ethanol. Capped mRNA was mixed with the MO solution just before microinjection and co-injected into the 1-cell stage embryos. The co-injection concentrations for rescue were 2ng pacap2-UTR-MO + 50pg pacap2 mRNA of either long (5 exons) mRNA or short (lacking exon 4) mRNA. Embryos were scored for defects 27h after fertilization.

**Whole mount in situ hybridization**

RNA probes were prepared from a linearized template using the Digoxigenin-RNA labeling kit (Roche, Germany) according to the manufacturer’s instructions. *In situ* hybridization and signal detection with anti-digoxigenin antibody coupled to alkaline phosphatase (Roche) were performed as described (Krueckl et al., 2003) with a few
modifications. Anti-dig-alkaline phosphatase antibody was diluted 1:3000 in blocking solution. After a blue product was visible, the reaction was stopped by washing twice in PBS. Embryos were dehydrated in a series of solutions: 70% PBS-30% methanol, 50% PBS-50% methanol, 30% PBS-70% methanol, and 100% methanol. Expression patterns were photographed after clearing in benzyl benzoate:benzyl alcohol (1:2). The primers used to prepare probes for the brain gene markers eng2, fgf8, pax2.1, meis1.1, eph4A, mab21l2, and mbx are listed in Table 3.1. To analyze expression patterns for pacap2, the probe was prepared using primers listed in Table 3.1 and the procedure was modified from the one listed above by storing embryos in a 10% glycerol in PBS solution, instead of dehydrating in methanol, before photographing them.

Results

Expression of pacap1 and pacap2 transcripts in early embryos

To determine the earliest expression of pacap mRNA in the embryo, we used RT-PCR. Our analysis showed the presence of both pacap1 and pacap2 cDNA beginning at 0.5 hours after fertilization and continuing until the last measurement at 25 hours (Fig. 3.1A). However, the pattern of expression was quite different for the two mRNAs. The pacap1 cDNA was always expressed as a single band of the same length with the strongest expression at 0.5-1.5 h and 18-25h; expression was least intense at 4 and 14h compared to the control expression of RNA polymerase II. In contrast, pacap2 cDNA always appears as a double band with the strongest expression at 14-25h compared to the control cDNA.
**First expression of transcripts encoding PAC₁, VPAC₁, PHI and GHRH-LP receptors**

The cDNAs encoding the PAC₁ or GHRH-LP receptors were expressed beginning at 1-2 hours after fertilization, whereas the cDNA encoding VPAC₁R was detected at 5-6h after fertilization (Fig. 3.1B). Once the expression began, it was continuous throughout the early period measured until 18h. The expression pattern showed double bands at 1-8h for the PAC₁ receptor and single bands of either the long or short form thereafter. Sequence analysis demonstrated that the shorter PAC₁ receptor was a full length receptor without inserts, whereas the longer PAC₁ receptor had a hop1 cassette inserted into its third intracellular loop. The other receptors were expressed as single-size transcripts only. The PHI receptor was analyzed at a later time using regular RT-PCR rather than the Southern blot method used for the other three receptors. The PHI receptor is detected as a single band beginning at 0.5h after fertilization and is detected thereafter with varying intensity using RNA polymerase II as the control for comparison (Fig.3.1A).

**In situ hybridization of pacap mRNA in 24-hour embryos**

Previously our lab showed that pacap1 mRNA is expressed in 24h embryos in the eye, midbrain, midbrain-hindbrain boundary (MHB), and hindbrain (Krueckl et al., 2003). In the present study, pacap2 mRNA at 24h is expressed extensively in the forebrain, in the diencephalon near the eyes, and in the hindbrain as shown from both the lateral and dorsal views (Fig. 3.2).
Figure 3.1. Expression of zebrafish PACAP and receptors at different stages of development. **A.** PACAP1, PACAP2 and PHI receptor cDNA are shown in comparison to control cDNA amplified from RNA polymerase II in zebrafish embryos at times from 0.5 to 25 hours post fertilization. RT-PCR was used to amplify the mRNA. PACAP1 is not spliced, thus the 440bp form represents the long form. PACAP2 is alternatively spliced and shows both the short form (484bp) and long form (544bp).

**B.** PAC1 receptor (R), VPAC1-R and GHRH-R cDNA converted to Southern blots are shown in comparison to control cDNA amplified from tubulin in zebrafish embryos at times from 1-18 hours after fertilization. The length of each band is shown at the right in base pairs (bp). The developmental stage represented by hours after fertilization is shown at the bottom. PCR was repeated three times.
Figure 3.2. In situ hybridization of PACAP2 in a zebrafish embryo at 24 hours after fertilization. A. lateral view. B. dorsal view.
Brain and eye morphology after knockdown of PACAP1 or PACAP2

A series of MO injections at 2, 4 and 8 ng were tested to determine the threshold dose that hybridizes to pacap mRNA resulting in blockage of translation of PACAP1 or PACAP2 as determined by altered brain morphology, especially in the midbrain-hindbrain boundary (MHB) where the fold can be easily viewed in whole embryos. For pacap1 mRNA, the threshold was 4 ng MOs whereas for pacap2 mRNA hybridization, the threshold was 2 ng MOs to alter brain morphology. These are low doses and far below any reported to cause toxic effects. In this study, we only used 2 or 4 ng of MOs. The effects caused by using either UTR- or ATG-directed MOs were similar and suggested that the MOs were not misdirected. Injections of unrelated MOs into control embryos did not result in any developmental delays with 2-6% showing some altered morphology at 24 h.

The screening of embryos (n= 80-90/ injection group) at 27 hours after injection of MOs is shown in representative embryos in Fig. 3.3. The knockdown or blocking of PACAP1 peptide resulted in morphological changes in which the most obvious were the distortion of the forebrain and mid-hindbrain boundary. The knockdown of PACAP2 at only 2 ng MO always produced more severe changes than knockdown of PACAP1 with 4 ng MO. The lateral view shows an aberrant MHB and the dorsal view shows distortion of the forebrain in the embryos in which PACAP2 was knocked down (Fig. 3.3). Eye size was measured at its greatest diameter in ten embryos in each of three groups to verify our initial observations: wildtype (156 μm), PACAP1-UTR MO of 4 ng (124 μm) and PACAP2-UTR MO of 2 ng (110 μm). The two knockdown groups had eyes that were
Figure 3.3. Morphological changes in 27-hour zebrafish embryos, which are wild type or injected with 4ng of morpholinos to knockdown (KD) PACAP1 or PACAP2 in the one-celled stage. Lateral view is above; dorsal view is below. Arrows point to mid-hind brain boundary (MHB).
significantly smaller than wild type embryos. Statistical analysis (P<0.05) was performed by Prism using one way ANOVA and Tukey’s Multiple Comparison test.

To further demonstrate that the MOs were hybridizing to the correct mRNA, rescue experiments with exogenous mRNA were done. In 82 embryos, 2ng MO directed against pacap2 mRNA resulted in 8.7% normal embryos; in 100 embryos treated in the same amount and type of MO along with 50pg of the long form of pacap2 mRNA including all five exons, 22.1% of the embryos were normal; and in 83 embryos treated with the same MO along with 50pg of the short form (lacking exon 4) of pacap2, 34.8% of the embryos were normal. The results in both rescued groups were both significantly changed compared to the group without mRNA rescue. Statistical analysis (P<0.05) was Tukey’s Multiple Comparison test as above.

The screening process showing morphological defects after knockdown of either PACAP1 or PACAP2 with low doses of morpholinos and the partial rescue of the morphants provided evidence that further testing after PACAP knockdown was warranted. Therefore, marker gene expression for specific brain areas was evaluated after PACAP knockdown as described below.

*Change in expression of markers for brain and eye after knockdown of PACAP1 or PACAP2*

Seven different brain markers were selected for their expression in different brain regions including the midbrain-hindbrain boundary (MHB), the hindbrain, midbrain, diencephalon-midbrain boundary (DMB), diencephalon, forebrain and eyes. All markers were examined in the 24 hpf embryos, which were wild type or treated with 2-4ng of MOs against pacap1 or pacap2 mRNA.
The midbrain-hindbrain boundary is an important organizing center for this region of the brain and hence three markers were selected to study the region. Marker *eng2* is expressed in the MHB in a similar pattern for both the wild type embryo and PACAP1-blocked embryo (Fig. 3.4). In contrast, *eng 2* is not only overexpressed in the MHB as judged by its intensity of labeling, but the expression shows a striking increase in the posterior midbrain and rostral hindbrain (presumptive cerebellum) in embryos in which PACAP2 is knocked down compared with the wild type. The *fgf8* gene marker also has more intense labeling of the MHB, especially after PACAP2 knockdown, than the wild type embryo (Fig. 3.4). The third MHB marker, *pax2.1*, had similar labeling intensity in the MHB to that for the wild type embryo (Fig. 3.4).

To analyze alterations in the hindbrain after PACAP knockdown, two markers, *ephA4* and *meis1.1* were used. The expression of *ephA4* in specific rhombomeres appears to be almost identical in the wild type and in PACAP-blocked embryos (Fig. 3.4). Changes of expression of *ephA4* in other parts of the brain are discussed below. Marker *meis1.1* expression in hindbrain is decreased after PACAP1 or PACAP2 are blocked.

Three midbrain markers include *mbx, mab21l2* and *meis1.1*. There is a severe reduction in the midbrain for expression of marker *mbx* but only a modest reduction in *mab21l2 and meis1.1* in a PACAP1-blocked embryo (Fig. 3.4). In PACAP2-blocked embryos, expression of the same gene markers is not reduced but the shape of the midbrain is altered in the embryo (Fig. 3.4).

In the diencephalic-midbrain boundary, *eph4A* was used as a marker. There is a marginal increase in *eph4A* expression in embryos with both types of PACAP knockdowns compared to wild type embryos, but other markers are needed to confirm
Figure 3.4. Zebrafish embryos at 24 hours after fertilization in which in situ hybridization has been used to show gene expression of specific factors listed on the left. The vertical column on the left has wild type embryos; the column in the middle has embryos injected with 4ng of morpholinos directed against PACAP1 to knockdown translation of the mRNA; and the column on the right is the same as the middle column except that PACAP2 was knocked down. White arrow points to the mid-hindbrain boundary, red arrow points to the eye stalk, black arrow points to the hindbrain, and black arrowhead points to the midbrain. The scale bar in each picture represents 250μm. All embryos are dorsal view.
this result (Fig. 3.4). In the diencephalon, *eph4A* expression shows an increase after PACAP2 loss, and *fgf8* shows a substantial increase in expression in the diencephalon in PACAP1- and PACAP2-blocked embryos.

Forebrain development is visualized with markers *pax2.1*, *fgf8* and *meis1.1*. Marker *fgf8* showed an increased expression in the absence of either PACAP1 or PACAP2 compared to wild type zebrafish. Gene *meis1.1* expression is decreased with lack of PACAP1, but expression is lower with lack of PACAP2. A striking change in the pattern of expression is seen with marker *pax2.1* in which overexpression in cells of the eye cup and optic stalk in the forebrain occurs after knockdown of either form of PACAP.

Further, a decrease in eye size and a decrease in gene marker expression in the eye occurred for *mbx* after loss of PACAP1 or PACAP2 and for *mab21l2* after loss of PACAP2 compared to wild type embryos (Fig. 3.4).

**Discussion**

*Early expression of transcripts encoding PACAP peptides and their receptors*

The early expression of *pacap1* and *pacap2* mRNA at only 0.5 hours after fertilization suggests that PACAP could potentially influence the early development of the brain. The transcripts at 0.5 and 1.5 hours are likely to be maternal transcripts, as zebrafish embryos do not begin to make their own mRNA until 3-4 hours post fertilization (hpf) (Kane and Kimmel, 1993). The earlier expression of PACAP1 in the present study compared to our previous study (Krueckl et al., 2003) is thought to be due to the use of 30 rather than 5
embryos at each time point and to the use of different primers. However, PACAP1 and PACAP2 may not have the same role, as their patterns of expression differ during the first 25 hpf. Transcripts for pacap1 are constantly expressed except for a decrease at 4-14 hpf, whereas transcripts of two sizes for pacap2 are always present except for lower levels at 0.5-10 hpf. The shorter form is the result of alternative splicing of the full length form, which removes exon 4 encoding GHRH-LP1-32, the biologically active portion (Guillemín, 1986). The functional implication is that a higher ratio of PACAP to GHRH-LP is produced in any tissue with both forms of the pacap2 transcript. Also, GH release in fish depends primarily on PACAP (Paker et al., 1997) and not on GHRH-LP, although GHRH, recently discovered on a separate gene, may be important for GH release (Lee et al., 2007).

The early expression of receptors activated by PACAP is the other essential component along with ligand expression that indicates PACAP is involved in development. In zebrafish, only one gene copy of each receptor that is activated by PACAP or GHRH is present. The expression of PAC1, PHI and GHRH-LP receptors occurs at 1-2 hpf, which implies they are maternal transcripts, whereas the expression of VPAC1 receptor begins at 5-6 hpf implying that it is an embryonic transcript. PAC1 receptor binding is most sensitive to PACAP but also binds VIP with lower potency (Chapter 2). I found that zebrafish PACAP1 (1-27) and PACAP2 (1-27) activate zebrafish PAC1 receptor expressed in monkey COS cells with similar profiles as measured by cAMP and inositol phosphate (IP) accumulation (Fradinger et al., 2005 and Chapter 2). VPAC1 receptor, which binds both PACAP and VIP equally, is not expressed until 5-6 hpf. Thereafter, VPAC1 receptor continues to be expressed as a single form for at least 25 hpf. The
zebrafish VPAC₁ receptor was expressed also in COS cells and found to be activated by PACAP₁, PACAP₂ and VIP in an almost identical manner with an increase in cAMP but not IP accumulation (Chapter 2). In regard to early brain development, all of the components, two ligands and four receptors, were expressed no later than 5-6 hpf indicating that PACAP may be involved in early development.

**Location of pacap1 and pacap2 mRNA expression in 24-hour embryo**

Previously we showed that pacap₁ mRNA is expressed in the eye, midbrain, midbrain-hindbrain boundary, and hindbrain (Krueckl et al., 2003). Clearly, the location of the two pacap mRNAs is different in that pacap₂ is shown here to be widely expressed in the forebrain, diencephalon, and hindbrain, but not in the eye. With two PACAPs, large areas of the brain and eye could be affected. *In situ* hybridization shows only the location of cell bodies, so the influence via axonal extensions is even greater. Although we only tested *in situ* hybridization at 24 hpf, the mRNA is present much earlier as shown by the RT-PCR.

The translation of the mRNA to protein is important as developmental effects depend on the protein expression of the PACAP peptides. A new study has examined the presence of PACAP peptides by immunocytochemistry from 24 hpf to adulthood (Mathieu et al., 2004). The antisera used in the study did not distinguish between PACAP₁ and PACAP₂. Nonetheless, PACAP is present in the telencephalon, diencephalon, eye, and rhombencephalon in the 24 hpf embryo. The protein may be present even earlier, but was not checked and, indeed, may be present even though it can not be detected by immunocytochemistry. The receptors have not been studied for
protein expression because antibodies are not available. In comparing zebrafish to a mammal such as mouse, the single copy gene in mouse is expressed early in development at embryonic day 10.5 and the protein is detected at embryonic day 14; the PAC1 receptor mRNA is expressed on embryonic day 10 (Sherwood et al., 2000). Thus, the zebrafish embryo, which has no maternal protection, appears to express PACAP at an earlier stage of development than mammals suggesting PACAP is essential whether obtained from embryonic translation in zebrafish or the placenta in mice.

Morphological changes in brain and eye after knockdown of PACAP1 or PACAP2

The MO doses of 2-4ng are very low for threshold doses that are effective in terms of morphological changes in brain and eye morphology. A number of controls, including the injection of an unrelated MO, the injection of MOs directed against the UTR or ATG regions for comparison of effects, the injection of a PACAP1 splicing MO, and the injection of exogenous mRNA along with morpholinos to partially rescue embryos provide evidence that the MOs were targeting the correct mRNA and that the morphological defects were not due to the injection process or toxicity of the MO. That rescue is partial and not complete is known from the literature (Cui et al., 2001; Hashiguchi and Asashima, 2004; Kanzler et al., 2003) and is thought to reflect the difficulty of injecting mRNA at exactly the needed dose or location Kennedy et al., 2004).

The observation of morphological changes in the 27h brain and eye provides a screening method to determine if knockdown of a factor expressed early in development has a critical role that needs to be examined in detail. Both PACAP1 and PACAP2
produced observable changes in the size and shape of the brain and eye. PACAP 2 knockdown at a lower threshold dose (2ng) is more effective than PACAP1 with a threshold dose of 4ng. However, both produced similar morphological effects in reduction of brain size and distortion of the midbrain-hindbrain boundary, which usually results from changes in the midbrain, hindbrain or both. The size of the eye was significantly reduced by both forms of PACAP. Although we tested higher doses of morpholinos (8ng), which produced the same type of defects with greater severity, these doses were not used further to ensure that toxic effects were not a factor in the observed neural changes.

**Selective changes in expression of gene markers in brain and eye after PACAP knockdown**

Development involves cascades of factors, both transcription factors and secreted factors, in which sequential events must occur in a strict spatial and temporal pattern to produce a normal brain. Furthermore, some steps involve feedback loops so that it is more difficult to determine the exact sequence of events. Brain marker genes can be used to visualize the developmental events as they are expressed at a specific time and in a specific pattern within the three dimensional space of the brain during development. We selected seven marker genes that have distinct expression patterns in brain regions of interest. Any change in marker expression suggests PACAP is important at some point in the cascade, although not necessarily immediately before marker gene expression. Lack of PACAP can reduce or enhance marker gene expression. The markers we chose, *eng2*, *fgf8*, *pax2.1*, *meis1.1*, *ephA4*, *mab21l2*, and *mbx* are all zebrafish homologs of similar proteins in mice and humans.
The *pax 2.1* marker is one of the earliest acting genes needed for the development of the midbrain, MHB (isthmus) and cerebellum from gastrulation onward. The loss of *pax2.1* protein leads to loss of the midbrain tectum, MHB, and cerebellum in zebrafish (Brand et al., 1996). In our embryos, *pax2.1* is expressed in the MHB regardless of PACAP knockdown demonstrating that PACAP is unlikely to act in the midbrain-hindbrain region before *pax2.1* expression. Our *pax2.1* in situ hybridization images show that the region has formed normally. The expression of *pax2.1* in eyes and forebrain is discussed below.

PACAP may act after the expression of *pax2.1*. Downstream genes of *pax2.1* include *eng2* and *eng3* with a feedback loop among the three (Scholpp and Brand, 2003). Expression of *eng2* is strongly dependent on *pax2.1* (Rhinn and Brand, 2001). Others have shown that a MO knockdown of *eng2* and *eng3* results in an absence of *pax 2.1* and that the MHB does not develop (Scholpp and Brand, 2001). In our experiments, the absence of PACAP1 had little effect on the expression of *eng2*, but the knockdown of PACAP2 had a striking effect in that *eng2* expression was substantially increased in the MHB. This suggests that PACAP2 acts in the cascade involving *eng2* and normally suppresses the expression. PACAP2 is a secreted protein and hence acts in a different way from *pax2.1*, which is thought to act as an activating transcription factor that directly binds to the promoter region of *eng2*. In contrast, PACAP2, which activates 7-transmembrane receptors in the outer membrane, has been shown to increase both cAMP and IP accumulation in zebrafish (Fradinger et al., 2005). The PAC1, VPAC1 receptors may be available for PACAP activations, as shown here the mRNA is expressed by 1-6 hpf, although their expression as protein has not yet been studied.
Further support for an early role of PACAP as a suppressor in the cascade involving formation of the MHB, is the striking overexpression of *fgf8* in embryos that have MO-induced knockdown of PACAP2. There may be an effect of PACAP1 knockdown, but the greater effect depends on lack of PACAP2. The *fgf8* gene is expressed as early as *pax2.1* and its expression is independent of *pax2.1* (Rhinn and Brand, 2001). Fgf8 is important in the mid-hindbrain boundary organizer (Rhinn and Brand, 2001) and if *fgf8* is mutated, the zebrafish lack a cerebellum (Scholpp and Brand, 2003; Reifers et al., 1998). After PACAP knockdown, the cerebellum is present and *fgf8* expression is increased. One hypothesis is that PACAP is a target gene for *fgf8* (and *eng2*) and expression of PACAP feeds back to inhibit *fgf8* and *eng2*. Rhinn and Brand have clearly stated the problem: “Given the potency as a signaling molecule, the activity of Fgf8 must be carefully controlled in the embryo. An emerging theme for several signaling pathways is that extracellular or intracellular inhibitors control their activity.” PACAP is a candidate as an extracellular inhibitor of this pathway.

In hindbrain formation, there is some indication that PACAP1 and PACAP2 may normally influence the pathway regulating the expression of *meis1.1* in the hindbrain. The expression of the *meis1.1* gene in wild type zebrafish has been described (Waskiewicz et al., 2001). Another gene marker for the hindbrain is *epha4*, which is an ephrin receptor; *epha4* is clearly expressed in specific rhombomeres in the wild type embryo (Scholpp and Brand, 2003). However, the expression of *epha4* and the morphology of the hindbrain are not altered by knockdown of either PACAP1 or PACAP2 suggesting that PACAP does not act in this cascade of factors, although PACAP1 and PACAP2 may act in a minor way through *meis1.1*. 
PACAP1 and PACAP2 both influence the formation of the midbrain. The expression of the *mab21l2* gene (Kennedy et al., 2004; Kudoh and Dawid, 2001) was affected, although to a lesser extent than *mbx* expression. The reduction of *mbx* expression in the midbrain was even more evident than *mab21l2* after PACAP1 was blocked. Both the intensity of expression and morphology of tissue were altered after knockdown. Thus PACAP affects the midbrain by activating *mbx, mab21l2* and *meis1.1* expression; the three genes appear later in development than *pax2.1, fgf8* or *eng2*.

Our data on the diencephalon and the diencephalon-midbrain boundary are both derived from our in situ hybridization study of *eph4A* and *fgf8* genes. Both *eph4A* and *fgf8* show an increase in expression if PACAP1 or PACAP2 are absent, but the greatest increase is in *fgf8* after PACAP2 knockdown. Again, the suggestion is that PACAP normally acts to suppress these genes. *Fgf8* is thought to be one of several factors that are involved in setting the boundary between the diencephalon and midbrain. *Fgf8, pax2.1* and *eng* genes are all thought to be involved in repressing forebrain fate (Scholpp and Brand, 2003), so it is of interest that PACAP normally acts to inhibit the expression of *fgf8* and *eng2*. There are other studies in mice that show an interaction of PACAP with other genes expressed in the diencephalon, such as sonic hedgehog (*shh*), and these genes will be of interest in future studies.

Our evidence suggests that PACAP peptides affect the formation of the forebrain. Patterning of the forebrain in zebrafish begins in gastrulation, and as in the midbrain-hindbrain region, *fgf8* and *pax2.1* are known to play a role (Walshe and Mason, 2003; Macdonald et al., 1995). Gene *fgf8* showed a substantial increase in expression, after blockage of either form of PACAP.
In forebrain and eye, either PACAP1 or PACAP2 appears to activate the cascade that includes the \textit{pax2.1} gene. After knockdown of either form of PACAP, expression of \textit{pax2.1} in the anterior forebrain and eye cup is greatly increased. Therefore, PACAP appears to be an inhibitor of \textit{pax2.1}.

The \textit{mab21l2} gene is expressed in eye primordia and midbrain at 11 hpf; others showed that knockdown of this gene resulted in a 20\% decrease in eye size at 24 hpf in zebrafish, and in mice was embryonically lethal (Kudoh and Dawid, 2001; Wong and Chow, 2002; Yamada et al., 2003; Kennedy et al., 2004). Although our in situ hybridization studies showed \textit{pacap1} location in the eye and midbrain, \textit{pacap2} fibers may be distributed in the midbrain so that the knockdown of either peptide could explain the results. Other evidence that PACAP affects development of the eyes is the decreased expression of \textit{mab21l2} in the absence of PACAP and our measurement of eye diameter which was significantly reduced after knockdown of either form of PACAP.

The strategy of examining the role of a hormone by eliminating it in a living organism is a long established method to elucidate function and identify novel actions. Knockdown of either PACAP1 or PACAP2 has shown that both secreted proteins have a role in early development. The most dramatic results are the increase of \textit{pax2.1} in the eye and forebrain in the absence of both PACAPs, the increase of \textit{eng2} and \textit{fgf8} in the absence of PACAP2, and the decrease in \textit{mbx} in the absence of PACAP1. The result of the combined gene markers is that PACAP is shown to have an effect in early development in the midbrain, hindbrain and their boundary and on the diencephalon and forebrain. The changes in marker gene expression and in brain morphology imply that PACAP is normally translated into a protein early in development to act on the brain and
eye. A study by others (Mathieu et al., 2004) substantiates this claim by immunocytochemistry. The authors do not test for protein detection until the 24 hpf embryo, but already PACAP is detected in the telencephalon, diencephalon, retina and rhombencephalon.

The zebrafish offers a model for the study of PACAP on the expression of specific genes during early development. Currently, microarray projects to identify target genes of PACAP are likely to produce long lists of genes. The zebrafish offers a method to study the effects of PACAP on these genes in a living organism in which the spatial and temporal aspects of gene expression can be observed after the absence of PACAP. We examined seven marker genes in the absence or PACAP1 or PACAP2, but other genes known to be interrelated in the formation of a brain region or pituitary could be examined.
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Chapter 4

A role for GnRH in early brain regionalization and eye development in zebrafish

Introduction

In this thesis two neuropeptide families and their receptors are examined. The gonadotropin-releasing hormone (GnRH) family has many similarities to the secretin superfamily in that both groups of hormones are synthesized in nerve cells, have a wide distribution of the hormone and receptors, and have multiple functions. It is interesting that during evolution, GnRH has been conserved as a single family without evolving into a superfamily like the secretin hormones. Again, zebrafish are an excellent model to study function of GnRH during early development.

In mammals, gonadotropin-releasing hormone (GnRH) is first expressed in the embryo pre-implantation. Immunoreactive GnRH1 was present in the morula and blastocyst stages in vitro in humans (Casañ et al., 1999), rhesus monkey (Seshagiri et al., 1994) and mice (Raga et al., 1999) and was shown to be secreted in the rhesus monkey study. Both GnRH1 and GnRH receptor mRNA were detected in embryos at the same stages (Casañ et al., 1999; Raga et al., 1999). Later in development, GnRH1 was detected in the mouse at embryonic day 10.75-11.5 in neurons anterior to the brain (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Livne et al., 1993; Wu et al., 1997). Within a day the neurons began to migrate into the brain and assume their adult locations in the forebrain and diencephalon. A second population of neurons expresses a different form of GnRH (GnRH2) in most mammals but not in mice as they lack the gene encoding GnRH2 (Pawson et al., 2003). In rhesus monkey, GnRH1 was expressed at embryonic day 30 (Quanbeck et al., 1997), whereas the midbrain neurons expressed GnRH2 at embryonic day 34 (Terasawa et al., 2000). Both groups of neurons continued to express GnRH throughout life. These studies were based on the detection of GnRH as a peptide using
immunocytochemistry, whereas GnRH mRNA can be detected much earlier across several species (see Witkin et al., 2003). The function of GnRH in the forebrain and diencephalon is known to be the control of reproduction via the pituitary gland. However, the function of GnRH in the midbrain of the adult has not been clearly determined and the role of GnRH expression in the brain in early development is not fully understood.

The highly conserved structure of GnRH, ten amino acids in length, is thought to be related to its crucial role in the initiation and maintenance of reproduction in all vertebrates and some invertebrates. However, the conserved structure would also be important if GnRH has a role in development. At present, 24 distinct GnRH structures have been determined from brains of various species from octopus to human (Sherwood and Adams, 2005, see Chapter 1). Almost all vertebrate species have at least two GnRH forms coexisting in the central nervous system. Most mammals, including humans, have GnRH1 and GnRH2, whereas zebrafish have GnRH2 and GnRH3 (Powell et al., 1996; Torgersen et al., 2002; Steven et al., 2003; Kuo et al., 2005). Each of these three forms of GnRH are widely shared by vertebrates: GnRH1 (mammalian GnRH or LHRH) is present in all mammals and some non-mammals; in some classification schemes, GnRH1 also refers to several GnRH isoforms that substitute for mammalian GnRH in other species to control pituitary secretion. GnRH2 (chicken GnRH-II) is a form shared among most jawed vertebrates; and GnRH 3 (salmon GnRH) is a form shared among most teleost fish. One form of GnRH, GnRH1 in mammals and GnRH3 in zebrafish, is found in neurons in several locations in the forebrain and diencephalon, which includes the hypothalamus, whereas GnRH2 is present in midbrain neurons (Sherwood and Adams,
2005). Recent evidence in zebrafish suggests that GnRH 2 cells in the midbrain and
GnRH3 cells in the forebrain and diencephalon are derived from cranial neural crest cells
(Whitlock et al., 2003, 2005). Also, the GnRH2 and GnRH3 genes in zebrafish were
shown not to be derived from a GnRH1 gene by analysis of their neighboring
chromosomal genes (Kuo et al., 2005). Reproduction is initiated in both mammals and
fish by release of GnRH from nerve cells in the brain, leading to a cascade of events and
culminating in gametogenesis. In addition to the brain, GnRH peptides are present in
several other tissues and are known to interact with their specific, membrane-bound, G
protein-coupled receptors to influence gene expression in pituitary gonadotropin cells and
other organs (Lethimonier et al., 2004; Millar et al., 2004). Even though GnRH is present
in all chordates, the question of GnRH's function in early development needs to be
examined.

To study the role of GnRH in the earliest stages of development, we selected zebrafish
(Danio rerio) as a model because the embryo develops both rapidly and free from
maternal influences, such as GnRH peptides. The functions of GnRH2 and GnRH3 were
examined by injecting morpholino (MO) antisense oligonucleotides at the one-cell stage
to suppress translation of the gnrh transcripts that normally produce GnRH2 and GnRH3
protein. After the gross morphological changes and their rescue were examined
following various doses of MOs, markers for the brain and eye were used to identify
more specific effects of GnRH knockdown after low doses of MOs. Gene markers for
the forebrain and/or diencephalon (fgf8, pax6.1, meis1.1), the diencephalon-midbrain
boundary (fgf8, pax6.1), midbrain (mab2112), midbrain-hindbrain boundary (fgf8, pax2.1)
and hindbrain (meis1.1, ephA4) were examined at 24 hours post fertilization after
knockdown of GnRH2 or GnRH3 in the one-celled embryo. Apoptosis was examined by the TUNEL method and the caspase activity assay to determine if GnRH loss-of-function involves apoptosis.

Materials and Methods

Animals

Wild type zebrafish (*Danio rerio*) were kept at 28°C in dechlorinated water on a 14 hour light/10 hour dark cycle (Westerfield, 1995). Embryos were collected each morning after natural spawning of adult fish. Times of development are expressed as hours post fertilization at 28°C. All procedures were approved by the Animal Care Committee at the University of Victoria.

Rapid amplification of cDNA ends (RACE) for *gnrh2* and *gnrh3*

Adult zebrafish brains were used to isolate mRNA with the Micropoly (A) pure mRNA isolation kit (Ampion, Austin, Texas). For preparation of cDNA reactions, we used 100 to 250ng of mRNA in the First Choice™ RLM-RACE kit (Ambion), according to the manufacturer's instructions. All primers are shown in Table 4.1. The 3’ RACE reactions were performed using s-3’ F1 and c-3’ F1 as first round primers, and s-3’ F2 and c-3’ F2 as second round primers for 3’ partial *gnrh3* and *gnrh2*. 5’ RACE reactions were performed using s-5’ R1 and c-5’ R1 as first round primers, and s-5’ R2 and c-5’ R2
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<td>meis1.1</td>
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<tr>
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<tr>
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<td>AGCCGCAATGGGTGTCG</td>
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<tr>
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<tr>
<td><strong>Morpholinos</strong></td>
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<td>Location</td>
<td>Target</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>grnrh3- ATG</td>
<td>CCAACAAACTCCTTCCCTCCTCCTCCTCCTCCTC</td>
<td>I to 25</td>
<td>grnrh3 mRNA</td>
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<tr>
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<td>-10 to -15</td>
<td>grnrh2 mRNA</td>
</tr>
<tr>
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<td>grnrh2 mRNA</td>
</tr>
<tr>
<td>control MO</td>
<td>CCGTCTTTACCTCAGTTTTAATTAT</td>
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</tr>
</tbody>
</table>

$^a$The direction of the primer, either forward (F) or reverse (R) is indicated.
as second round primers for 5’ partial gnrh3 and gnrh2. PCR reactions were carried out for 35 cycles at 55°C annealing temperature with a 7-min extension at 72°C on the last cycle.

**RT-PCR**

Zebrafish embryos at different stages of development were used to extract mRNA with the MicroPoly (A) Purist kit (Ambion). The samples were treated with Turbo RNase-free DNase (Ambion). Single cDNA was synthesized with Oligo (dT) and Superscript II RT (Invitrogen, Burlington, ON) according to the manufacturer’s instructions. The primers for additional PCR of gnrh3 were s-3’ F1 and s-5’ R1 (spanning three exons) and for gnrh2 were C-F and C-R (spanning two exons) (Table 4.1). A control reaction was prepared using 1 μl of cDNA amplified with tubulin primers (Table 4.1). PCR was performed for 35 cycles as above; the PCR products were normalized to the expression of β-tubulin. Products of the reaction were separated by 1.5% agarose gel electrophoresis and visualized using ethidium bromide staining. To determine if other forms of GnRH were present in the zebrafish genome, all 24 known sequences for GnRH were blasted against the genome.

**Microinjection of morpholino antisense oligonucleotides**

Morpholino oligonucleotides (MOs) were designed and synthesized by Gene Tools (Eugene, OR) against the RNA region around the AUG translation start site (ATG) and the 5’ untranslated region (UTR). These “translational-RNA” MOs were selected as they are effective in blocking both maternal and zygotic transcripts. The design ensured that
the two morpholinos did not overlap. The sequence of each morpholino was searched against the zebrafish genome and the maximum match was three nucleotides or less except for the desired target sequence. The sequences and hybridization locations of morpholinos relative to the start site are shown in Fig. 4.1A, B. The morpholino sequence for control injections is supplied by Gene Tools and is listed in Table 4.1. This control MO is designed against a mutant site in human β-globin and has been shown not to target the zebrafish genome in many papers. MOs were solubilized in ddH₂O to a stock concentration of 25µg/µl. The stock solution was diluted to a working concentration of 2 - 12µg/µl in filtered ddH₂O. Zebrafish embryos were injected with 1-1.5nl of the MO solution into the yolk at the 1-cell stage. The procedure for the microinjection has been described (Xu, 1999; Nasevicius and Ekker, 2000). Pictures of morphants were captured by an Olympus (SZX9) dissection microscope with a Sony color video camera (DXC-950P). Images were imported into Northern Eclipse software (Empix Imaging Inc, Mississauga, ON). Additional pictures were taken with a Leica DMIRE2 microscope with a Retica 2000R FAST camera. Images were imported into Openlab Software.

*Rescue of morpholino-induced effects by injection of gnrh2 or gnrh3 mRNA*

To rescue morpholino-treated embryos, the mRNA of *gnrh2* was prepared by amplification using PCR of the corresponding *gnrh2* cDNA containing part of the 5’ UTR plus the coding sequence and 3’ UTR. The rescue mRNA did not overlap with the morpholino *gnrh2*-UTR sequence. Amplification was done by PCR with the forward primer (C-T-F): 5’ TATTAGACTGAAGTGATGGTTGCTGG 3’ (start codon is
Fig. 4.1. (A) Nucleotide and deduced amino acid sequences of the cDNA encoding the *gnrh2* precursor. The regions against which the morpholinos (MO) were designed are highlighted in gray. The nucleotide difference compared to genbank (gi: 40254663) is marked by an asterisk below the nucleotide. The stop signal is marked by ^^^. The poly A signal peptide is marked by a double line. (B) Nucleotide and deduced amino acid sequences of the cDNA encoding the *gnrh3* precursor. The regions against which the morpholinos (MO) were designed are highlighted in gray. The nucleotide difference compared to genbank (gi: 33504552) is marked by an asterisk. Stop signal is marked by ^^^. Poly A signal peptide is marked by a double line.
| A | 164 | aga atg gtt gga aga ggt tca gag -136 |
|   | 135 | gct tgg tga ttt tac tca acc gcc cac ttc agg aaa aag aga aca -91 |
|   | 90  | ttt cag gat tac cca cac cag gac tgc agt aga gga gct aca gca -46 |
| MO-gmrh2-UTR | MO-gmrh2-ATG |
|   | -45 | gaa gat acc tca aga gaa gac gty cca aaa tat tag acc gaa gta -1 |
| 1  | ATG GTC CTG GTC TGC AGG CTG CTG TTG GTC ATG GGG CTG ATG CTG 45 |
|    | M V L V C R L L L V *M G L M L |
|    | Signal peptide |
| 46 | TGT CTG AGT GGT CAG TGG AGC AGC GCT CAG CAC TGG TCT CAC GGC 90 |
|    | C L S A Q L S S A Q H W S H G 30 |
|    | GnrH2 |
| 91 | TGG TAT CCT GGA GGA AAG AGA GAG ATA GAC CTC TAC GAC ACC TCA 135 |
|    | W Y P G K R E I D L Y D T S 45 |
|    | cut site GnrH-associated peptide |
| 136 | GAG GTT TCA GAG GAA GTG AAG CTC TGG GAG GCA GGA AAA TGC AGT 180 |
|    | E V S E V K L C E A G K C S 60 |
| 181 | TAC CTG AGA CCG CAG GGA AGA AAC ATC CTC AAG ACA ATA CTG CTG 225 |
|    | Y L R P Q G R N I L K T I L 75 |
| 226 | GAT GCC CTC ATT CGT GAT TTC CAA AAG AGA AAG tga cac csa gct 270 |
|    | D A L I R D F Q K R K ** 86 |
| 271 | gat gtt tca gcc tgt gtc cca aag agar act ttt tcc acc gaa cac 315 |
| 316 | att tgg cct gcc ttt tca ttc cca acc gta tat att gtt tta ttc 360 |
| 361 | ctc tga ctt ctt ttc ttt ttt gta tgt gta tgt gta tga ctt cgc 405 |
| 406 | ttc tca tat tca tgg tga aat cga ctt cca cag cca cgc tgt acc 450 |
| 451 | cat tgg cag cag cag cta cca aat agg ctt tat ttt ggt - PolyA 489 |

| B | 27 | atc tgg acc aac cca cac agc agt ttt agc -1 |
| MO-gmrh3-UTR |
| 1  | ATG GAG TGG AAA GGA AGG TTG TTC GAT TCA CAC TGG TTG CTG TTA GTT 45 |
|    | M E W K G R L L L V Q L L L L V 15 |
|    | Signal peptide |
| 46 | TGG GTC TTG GAG GTC AGT CTT TGC CAG CAC TGG TCA TAT GGT TGG 90 |
|    | C V L E V S L C Q M W S Y G W 30 |
|    | GnrH3 |
| 91 | CTT CCC GGT GGA AAA AGA AGC GTC GTT GAA ATG GAG GCA ACA TTC 135 |
| 31 | L P G K R S V G E M E A T F 45 |
|    | cut site GnrH-associated peptide |
| 136 | AGG ATG TTG GAT CCA GGT GAC ACA ATG CTG ATT CTC GCT GAT 180 |
| 46 | R M L D P G D T V L S I P A D 60 |
| 181 | TCT CCA ATG GAG CAG CTT TCA CCA ATA CAC ATA GAT AGA GGT 225 |
| 61 | S P M E Q L S P I H I V N E V 75 |
| 226 | GAT GCT GAA GGT TTG CTT CCA AAA GGA AAA GAA AGA TAT TCC GAC AGA 270 |
| 76 | D A E G L P L K G Q R Y S D R 90 |
| 271 | CCG GGA AGA GTG taa aat tat aat tca tga agc tga tat gag tat 315 |
| 316 | gaa ttc tgt tgc cca gga gac cat gtt ttt gga ctc cta atg aag 360 |
| 361 | tat cct cca aat tat gga ttt aat aat tac act tca cgt aag gaa 405 |
| 406 | cagatggggctgt -Poly A 489 |
underlined) and reverse primer (C-R): 5’ CACAATGAATATGAGAACAGAACTAA 3’.
The mRNA of gnrh3 was prepared by the same method except that the PCR primer starts
from the start codon and alters 5 nucleotides in the proximal initiation codon without
altering the amino acids in the coding region (Cui et al., 2001). The forward primer (S-ATG-M) was: 5’ ATG GAA TGC AAG GGT AGA TTG TTG GTC CAG T 3’ (5
mutated bases are underlined) and reverse primer (S-prob-R): 5’ TTG GAG GAT ATT
TCA TTA GGA GTC 3’. The PCR products were ligated into PGEM-T vector and
sequenced. The vector with insert was linearized with Sal1; synthetic mRNA was
transcribed from the T7 promoter using the T7 mMESSAGE mMACHINE kit (Ambion)
following the manufacturer’s directions. After synthesis, all capped RNAs were purified
by MEGAclean kit (Ambion) and precipitated by ammonium acetate and ethanol.
Capped mRNA was mixed with the morpholino solution just before microinjection and
co-injected into the 1-cell stage embryos. The co-injection concentrations for gnrh2
rescue were 5ng gnrh2-UTR-MO + 100pg gnrh2 mRNA or 5ng + 200pg, or 10ng +
400pg. The co-injection concentrations for gnrh3 rescue were 10ng gnrh3-ATG-MO +
100pg gnrh3 mRNA or 12ng gnrh3-ATG-MO + 120pg gnrh3 mRNA. Embryos were
scored for defects 27h after fertilization.

**Whole-mount immunocytochemistry**

Microinjected embryos at different stages were dechorionated and fixed in 4%
paraformaldehyde in phosphate buffered saline (PBS) (0.1 M, pH 7.3) for 5h at room
temperature or 4°C overnight. Embryos were washed two times in PBS, one time in
100% methanol, and then stored in 100% methanol at −20°C. Embryos were rehydrated
and treated for 3-5h with blocking solution (5% normal goat serum, and 0.5% Tween-20 in PBS). After blocking, embryos were incubated overnight at 4°C with primary antiserum GF-6 at a dilution of 1:200 (5% normal goat serum, and 0.5% Triton-100 in PBS). Antiserum GF-6, raised against salmon GnRH in rabbits in our laboratory, cross-reacts with both GnRH2 and GnRH3 (Lescheid et al., 1997). After washing with PBST (0.5% Tween-20 in PBS), embryos were incubated with a secondary antibody for 3-5h at room temperature with gentle rocking. The secondary antibody was horse radish peroxidase (HRPO) goat anti-rabbit IgG at a dilution of 1:1000. The color reaction for the HRPO antibody was DAB (DAB Substrate Kit for Peroxidase, Vector Laboratories Inc., Burlingame, CA). The kit was used according to the manufacturer's instructions. Signals were captured by a microscope (Leica DMIRE2) with a Retica 2000R FAST camera. Images were imported into Openlab Software.

**Whole mount in situ hybridization**

RNA probes were prepared from a linearized template using the Digoxigen-RNA labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. *In situ* hybridization and signal detection with anti-digoxigenin antibody coupled to alkaline phosphatase (Roche) was performed as described (Krueckl et al., 2003) with a few modifications. Anti-dig-alkaline phosphatase antibody was diluted 1:3000 in blocking solution. After a blue product was visible, the reaction was stopped by washing twice in PBS. Embryos were dehydrated in a series of solutions: 70% PBS/30% methanol, 50% PBS/50% methanol, 30% PBS/70% methanol, and 100%
methanol. Expression patterns were photographed after clearing in benzyl benzoate/benzyl alcohol (2:1).

Probes were used to analyze expression patterns for *gnrh2*, *gnrh3*, and six gene markers for specific brain areas (Table 4.1). For hindbrain markers, genes *meis1.1* and *ephA4* were used (Xu et al., 1994, 1995; Waskiewicz et al., 2001, 2002; Scholpp and Brand, 2003). For the MHB markers, two genes were used: *fgf8* and *pax2.1* (Krauss et al., 1991b; Ekker et al. 1995; Brand et al., 1996; Reifers et al. 1998; Rhinn and Brand, 2001; Scholpp and Brand, 2003). For the midbrain tectum, gene markers *mab2112* and *meis1.1* were used (Kudoh and Dawid, 2001; Waskiewicz et al., 2001, 2002; Kennedy et al., 2004). Genes *fgf8* and *pax6.1* marked the diencephalon-midbrain boundary, whereas *fgf8*, *pax6.1* and *meis1.1* were expressed in the diencephalon and *fgf8* and *meis1.1* marked the forebrain (Krauss et al., 1991a, 1991c; Macdonald et al. 1995; Reifers et al. 1998; Waskiewicz et al., 2001; Scholpp and Brand, 2003). The genes *pax6.1*, *mab2112* and *meis1.1* are expressed in the eye cup whereas genes *pax2.1* and *fgf8* are in the stalk (Krauss et al., 1991a, 1991b, 1991c; Macdonald et al., 1995; Reifers et al. 1998; Waskiewicz et al., 2001, 2002; Walshe and Mason, 2003). For each probe, in situ hybridization was conducted for three groups of embryos (wildtype, *gnrh2*-MO and *gnrh3*-MO) at the same time with the same conditions. In situ hybridization was repeated three times for each probe using three independent collections of embryos.

**TUNEL assay**

Apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using the In Situ Cell Death Detection kit,
Fluorescein (Roche). Dechorionated embryos at 12h, 19h and 24h post fertilization for three different groups (control, gn rh2-MO, gn rh3-MO) were fixed in 4% paraformaldehyde and stored in methanol at -20°C. Embryos were rehydrated, permeabilized by proteinase K (10μg/ml) and washed by PBS. The yolk was removed at this step. Embryos were incubated 2h at 37°C under dark conditions with terminal deoxynucleotidyl transferase and fluorescein dUTP. The labeling reaction was stopped by washing with PBS. Embryos were immersed in the mounting medium Vectorshield (Vector Laboratories Inc.), positioned on a slide and cover slipped. Labeled cells were imaged with a confocal microscope (Nikon, C1-T-SM, Japan) using 10μm scans.

**Assay for caspase-3 and caspase-9 activity**

Caspase-3 and -9 activity in 24 hpf zebrafish embryos was examined in control and gn rh-MO groups by using Caspase Fluorometric Assay kits (Biovision, Mountain View, CA). Embryos were collected so that control, gn rh2-MO and gn rh3-MO groups could be tested at one time under the same conditions. The collection and testing was repeated a total of three times. Embryos were homogenized in ice-cold lysis buffer and centrifuged for 20 min at 12000 rpm at 4°C in a microcentrifuge. Supernatant was collected and protein concentrations were measured by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Each well in the 96-well plate was loaded with 150 μg of protein. For the caspase-9 assay, samples from each treatment were loaded into three individual wells and the assay was conducted according to the manufacturer’s instructions, although a technical support person from Bio-Rad Lab suggested that incubation be overnight instead of 2h because tissue was being assayed. The assay was carried out as three
independent experiments. For the caspase-3 assay, samples were loaded into three
individual wells and assays were conducted in two independent experiments. The
fluorescence of free AFC (after cleavage of the substrate from LEHD-AFC for caspase 9
or DEVO-AFC for caspase-3) was quantified using a fluorescence microtiter plate reader
(Perkin Elmer, VICTOR³ V) with an excitation filter 360 nm and 535 nm emission filter.
Changes in caspase activity in gnrh2-MO or gnrh3-MO injected embryos was calculated
against the mean value of caspase activity in the control group and expressed as a fold
increase compared to the control group.

**Statistical Analysis**

Statistical significance (P<0.05) was determined by Prism using one way ANOVA and
Tukey’s Multiple Comparison Test. This test was used to compare eye diameter, the
percentage of morphants after MO treatments, the percentage of embryos rescued after
injection of mRNA (see legend for Fig. 4.5), and the caspase-3 and caspase-9 activity in
control and gnrh-MO groups.

**Results**

*Expression of gnrh2 and gnrh3 transcripts and peptides in early embryos*

The nucleotide sequences of zebrafish gnrh2 (AY 657018) and gnrh3 (AY 657019)
(Fig. 4.1A, B) were consistent with previously reported sequences (gi: 33504552 and gi:
40254663) except for a few nucleotide differences that did not change the protein
sequence (Torgersen et al., 2002; Steven et al., 2003).
To determine the earliest expression of GnRH in the embryo, we used three methods: RT-PCR, *in situ* hybridization, and immunocytochemistry. Our RT-PCR analysis showed the presence of *gnrh2* and *gnrh3* mRNA at an early stage of development in embryos (Fig. 4.2). The mRNA for *gnrh2* was detected beginning at 1.5 hours post fertilization (hpf), and *gnrh3* mRNA could be detected at 0.5 hpf. RT-PCR for gnrh2 at 0.5 hpf was repeated three times with three different sets of probes. Each test used 30 embryos per group but transcripts were not detected. Both mRNA transcripts were present additionally in five later stages: 4 hpf (sphere), 6 hpf (shield), 8 hpf (75% epiboly), 10 hpf (bud) and 20 hpf (22-somites).

Our *in situ* hybridization for *gnrh* mRNA detected the *gnrh2* transcripts in brain (dorsal view) at 22, 24 and 32 hpf (Fig. 4.3A-C) and the gnrh3 transcripts (ventral view) at 24, 28 and 48 hpf (Fig. 4.3D-F). Each sequential time point shows an increase in the number of cells expressing *gnrh* mRNA.

Our immunocytochemistry results (Figs. 4.3G-L) showed that GnRH was expressed in cells at 30-31 hpf in the same brain positions as those detected by *in situ* hybridization (Figs. 4.3M-N). GnRH2-immunoreactive cells were in the midbrain (lateral view) in Figs. 4.3G (with inset) and 3K. Similar cells, also in the lateral view, are shown with *in situ* hybridization in Fig. 4.3M. GnRH3-immunoreactive cells are near to or at the edge of the olfactory placode (ventral view) in Figs. 4.3H (with inset) and 3J, whereas similar cells are shown by *in situ* hybridization in Fig. 4.3N. Immunoreactive GnRH-containing cells in the hypothalamus could not be detected at 30-31 hpf.
Fig. 4.2. RT-PCR analysis of *gnrh2* and *gnrh3* mRNA expression, compared with tubulin mRNA. Equal aliquots of first strand cDNA were used as templates in PCR reactions using specific primers to amplify *gnrh2*, *gnrh3*, and tubulin at 7 stages of development: 30', 1h 30', 4h, 6h, 8h, 10h, and 20h post fertilization. The negative control represents a PCR reaction done without template.
Fig. 4.3. *In situ* hybridization and immunocytochemistry of zebrafish embryos. (A-C) *in situ* hybridization of gonadotropin-releasing hormone2 (*gnrh2*) mRNA located in midbrain at 22h (arrows), 24h and 32h post fertilization, dorsal view, (D-F) *in situ* hybridization of *gnrh3* mRNA in forebrain at 24h, 28h and 48h, ventral view, (G-L) immunocytochemistry (ICC) of brains at 30-31h with GnRH cells shown by black arrows, (G) wild type GnRH2 protein in embryonic midbrain (lateral view) with enlargement of cells as inset, (H) GnRH3 cells in the wild type forebrain (ventral view) with enlargement of cells as inset, (I) lack of GnRH2 cells in midbrain (lateral view) after knockdown of GnRH2 and (J) forebrain (ventral view) after knockdown of GnRH2 leaving the GnRH3 protein intact, (K) midbrain (lateral view) after knockdown of GnRH3 leaving GnRH2 protein intact, (L) lack of GnRH3 cells in forebrain (ventral view) after knockdown of GnRH3. (M) *in situ* hybridization of *gnrh2* mRNA (lateral view) and (N) *in situ* hybridization of *gnrh3* mRNA (ventral view) at 30-31h for both M and N. The scale bars are: A, 20μm; B-N, 15μm.
Morphant brain phenotype after knockdown of GnRH2 and/or GnRH3

To determine the functional roles of GnRH2 and GnRH3 in zebrafish early development, morpholinos (MO) against gnrh2 and gnrh3 at both the 5' untranslated region (UTR) and start codon region (ATG) were injected separately into 1-cell stage embryos. Hence, two different non-overlapping morpholinos for each transcript were tested. In a systematic screen, the brain of each living zebrafish embryo was examined after knockdown at 27 and 48 hpf using a Nikon SMZ-2T dissecting microscope. The size and shape of the forebrain, diencephalon, midbrain, hindbrain, midbrain-hindbrain boundary and ventricles were observed; the MHB and eyes were scored as the defects were most clear in these regions. Typical defects in the brain and eyes of the embryo (morphant) at 27 hpf are shown in Figs. 4.4A-C from the lateral view and in Figs. 4.4D-F from the dorsal view. After knockdown of GnRH2 or GnRH3, the boundary between the midbrain and hindbrain was disturbed (Figs. 4.4B, C, E, F), the midbrain shape was altered and the eyes were smaller (Figs. 4.4E-F). Embryos that were not injected or injected only with control morpholinos (Figs. 4.4A, D) had less than 1% abnormalities observed at 26-27hr provided that only egg batches of good quality were used.

To assess a change in the size of the eyes, we measured eye diameter at the greatest length and found that the average for control eyes (n=10) was 168.2 ± 2.3μm; for GnRH2 (4ng) knockdown (n=10) was 97.7 ± 13.0μm; and for GnRH3 (13ng) knockdown (n=10) was 108.9 ± 10.3μm. The reduced eye diameter in GnRH2 and GnRH3 knockdown groups was statistically significant (p<0.001) from the control group and diameter in the GnRH 2 group was statistically different (p<0.05) from the GnRH 3 group. Thus, the
Fig. 4.4. Zebrafish embryos at 27 h after morpholino knockdown of GnRH (MO-gnrh2 or MO-gnrh3) and rescue. Inserts above A, B, C are group pictures. (A) embryo after injection with control morpholino in lateral view with anterior to left and black arrow showing location of midbrain-hindbrain boundary (MHB), (B) morphant with disrupted midbrain-hindbrain boundary (MHB) and eye reduced in size after knockdown of GnRH2 or (C) GnRH3, (D-F) embryo head in dorsal view at same stage and treatments as A-C. MHB is indicated by black arrow. (G-I) embryo in lateral view for injected control or rescue with gnrh mRNA. Scale bar for all photos is shown in A and is 250μm.
diameter of the eye size was only 58% in embryos with blocked GnRH2 and 65% in embryos with blocked GnRH3 compared with MO-controls.

To establish that the morphant phenotype was not due to toxic effects or mistargeting of morpholinos to unintended nucleotides, a series of morpholino doses was initially tested showing that 2.5ng gnrh2-MO was the lowest dose to consistently result in MHB defects in at least 50% of the embryos. Hence, low doses of 2.5, 3.2 and 5ng of morpholinos were selected and shown to block translation of gnrh2 (Table 4.2). At 2.5ng, the midbrain-hindbrain boundary was reduced or disrupted in 72% of the morphants and the eyes were small in 71% of the morphants. There were significant differences in the percentage of morphants between the gnrh-MO-treated embryos and injected control group (P< 0.001) at each dose. To further avoid mistargeting, low doses with two different non-overlapping morpholinos were used so that any non-specific hybridization would be different for each morpholino. Two morpholinos (ATG and UTR) against gnrh2 were combined at only 2 ng each (Table 4.2). The same types of defects resulted as for a single morpholino: midbrain-hindbrain boundary reduction or disruption (89%) and small eyes (88%). The combination of 2.5ng each or 5ng each of ATG + UTR morpholinos resulted in a further increase in the percentage of morphants and the same types of defects (Table 4.2) showing that mistargeting was not responsible for unexpected morphological defects. In these studies on mistargeting, there was no significant difference in the percentage of morphants between those injected with the same dose of ATG or UTR morpholinos against gnrh2 or gnrh3. Morpholinos against gnrh3 at a dose of 10ng gnrh3-MO induced defects in the MHB greater than 50% (Table 4.2). There were significant differences (P< 0.001) in the percentage of morphants
Table 4.2. Defects in embryos with blocked GnRH2 or GnRH3 after injection with threshold doses of morpholinos

<table>
<thead>
<tr>
<th>Morpholinos</th>
<th>N(^a)</th>
<th>27h</th>
<th>48h</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Disrupted midbrain-hindbrain boundary (%)(^b)</td>
<td>Defective and/or small eyes (%)</td>
</tr>
<tr>
<td>2.5ng UTR (gnrh2)</td>
<td>78</td>
<td>72</td>
<td>71</td>
</tr>
<tr>
<td>3.2ng UTR (gnrh2)</td>
<td>100</td>
<td>86</td>
<td>84</td>
</tr>
<tr>
<td>5ng UTR (gnrh2)</td>
<td>174</td>
<td>94</td>
<td>87</td>
</tr>
<tr>
<td>2ng each ATG + UTR (gnrh2)</td>
<td>89</td>
<td>89</td>
<td>88</td>
</tr>
<tr>
<td>2.5ng each ATG + UTR (gnrh2)</td>
<td>82</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>5ng each ATG + UTR (gnrh2)</td>
<td>108</td>
<td>97</td>
<td>98</td>
</tr>
<tr>
<td>10ng ATG (gnrh3)</td>
<td>97</td>
<td>68</td>
<td>66</td>
</tr>
<tr>
<td>13ng ATG (gnrh3)</td>
<td>92</td>
<td>71</td>
<td>70</td>
</tr>
<tr>
<td>10ng UTR (gnrh3)</td>
<td>141</td>
<td>57</td>
<td>62</td>
</tr>
<tr>
<td>13ng UTR (gnrh3)</td>
<td>147</td>
<td>61</td>
<td>63</td>
</tr>
</tbody>
</table>

\(^a\) N is the number of injected live embryos at 27h pooled from three independent experiments.

\(^b\) The percentages are the number of morphants with a specific defect compared to the number of injected live embryos. Uninjected embryos and Controls were examined but had less than 1% morphants.
between the gnrh-MO-treated embryos and injected control group. The defects were not statistically different whether the ATG or UTR MO was used.

After determining that the ATG and UTR sites were equivalent for morpholino knockdown, we examined the effect of combining two types of morpholinos (gnrh2-UTR and gnrh3-ATG). There was no significant difference in percentage of morphants after a single dose of 8 ng of gnrh2-MO compared with a dose of 4ng each for gnrh2- and gnrh3-MOs.

*Rescue of morphants after knockdown of GnRH2 or GnRH3*

To determine if morphants could be rescued with injected gnrh mRNA, embryos were injected simultaneously with a morpholino against gnrh2-UTR and gnrh2 mRNA that did not include any part of the UTR region that hybridizes with the morpholino. In the present experiments, a morpholino against gnrh2-UTR was injected at 5 or 10 ng each. The rescue for individual defects is detailed in Table 4.3. For example, a deformed MHB occurred in 94% of embryos after injection with 5ng of gnrh2 morpholino, but this value was reduced to 41% with rescue by 100 pg of gnrh2 mRNA. Eye defects (92%) were rescued with a reduction to 8%.

The results for injection of gnrh3-ATG morpholino and its rescue mRNA are detailed in Table 4.3. For example, a dose of 10ng of the gnrh3 morpholino with rescue mRNA of 100pg resulted in a reduction of embryos with disrupted midbrain-hindbrain boundary from 56% to 42% and small eyes from 72% to 42%. We found that the percentage of normal embryos was significantly greater in the rescued group (p<0.05) than that of the knockdown group injected only with morpholinos for GnRH2 or GnRH3 (Fig. 4.5).
Table 4.3. Rescue from morpholinos

<table>
<thead>
<tr>
<th>Morpholinos+mRNA</th>
<th>N(^a)</th>
<th>Disrupted Mid-hind brain boundary (%)(^b)</th>
<th>Defective and/or small eyes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10ng UTR (gnrh2)</td>
<td>60</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>10ng UTR+400pg mRNA (gnrh2)</td>
<td>60</td>
<td>74</td>
<td>30</td>
</tr>
<tr>
<td>5ng UTR (gnrh2)</td>
<td>174</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>5ng UTR+200pg mRNA (gnrh2)</td>
<td>100</td>
<td>54</td>
<td>20</td>
</tr>
<tr>
<td>5ng UTR+100pg mRNA (gnrh2)</td>
<td>142</td>
<td>41</td>
<td>8</td>
</tr>
<tr>
<td>10ng ATG(gnrh3)</td>
<td>55</td>
<td>56</td>
<td>72</td>
</tr>
<tr>
<td>10ng ATG+100pg mRNA (gnrh3)</td>
<td>76</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>12ng ATG(gnrh3)</td>
<td>64</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>12ng ATG+120pg mRNA (gnrh3)</td>
<td>72</td>
<td>42</td>
<td>42</td>
</tr>
</tbody>
</table>

\(^a\) N is the number of injected live embryos at 27h pooled from at least two independent experiments.

\(^b\) The percentages are the number of morphants with a specific defect compared to the number of injected live embryos.
Fig. 4.5. Comparison of percentage of normal embryos among control, morpholino knockdown and rescue groups. A. The percentage of embryos in the gnrh2-MO (5ng UTR) group is significantly lower than the control group or rescued group (5ng UTR gnrh2-MO + 100pg mRNA). B. The percentage of embryos in gnrh3-MO (12ng ATG) group is significantly lower than the control group or rescued group (12ng ATG gnrh3-MO + 120pg mRNA). Both rescued groups are significantly lower than the control group. P<0.05.
Examples of the phenotype of rescued embryos compared to control embryos are shown in Figs.4.4H and 4.4I. In addition, in situ hybridization with brain markers pax6.1 and mab2112 showed recovery of the patterns and intensity of expression as discussed below for the two markers.

*Inhibition of the fgf8 gene by GnRH2 and GnRH3 in early brain regionalization*

Fgf8 is a secreted factor that is expressed very early in brain formation. Our wild type embryos showed a distinct band expressing fgf8 at the boundary between the midbrain and hindbrain and another weaker band between the diencephalon and midbrain (Fig. 4.6A). These regions, known as the midbrain-hindbrain boundary (MHB) and the diencephalon-midbrain boundary (DMB), are important landmarks in the anterior-posterior regionalization of the brain (Kiecker and Lumsden, 2005). In our study, the overexpression of fgf8 was striking in the MHB and noticeable in the DMB after knockdown of GnRH2 (Fig. 4.6B) or GnRH3 (Fig. 4.6C), showing that these neuropeptides normally reduce the expression of fgf8 at its boundary locations. Overexpression of fgf8 in the eye stalk was strong after knockdown of GnRH3 but was modest after knockdown of GnRH2.

*Inhibition of pax2.1 gene by GnRH2 or GnRH3 in early mid-hindbrain boundary and eye*

A transcription factor, pax2.1, is expressed in the developing brain at the same early stage as fgf8 in zebrafish, although the initial expression of the two genes is independent (Rhinn and Brand, 2001). Our wild type embryo showed expression of pax2.1 in the
Fig. 4.6. *In situ* hybridization for specific gene markers at 24h in wild type (WT) zebrafish embryos and embryos after GnRH2 or GnRH3 knockdown with morpholinos (gnrh2-MO and gnrh3-MO). All photos are dorsal view. (A-C) embryos after *in situ* with *fgf8* probe; black arrow shows the MHB, (D-F) embryos after *pax2.1* probe; arrow shows the eye stalk; an arrow also shows the pronephros in the trunk, (G-I) embryos after *pax6.1* probe; arrow shows the diencephalon, (J-L) embryos after *mab21l2* probe; arrow shows midbrain, (M-O) embryos after *meis1.1* probe; arrow shows rhombomere, (P-R) embryos after *ephA4* probe; arrows show rhombomeres. Scale bar for all photos is shown in A and is 250μm.
MHB and in the eye stalk (Fig. 4.6D) as expected (Brand et al., 1996). Overexpression of \textit{pax2.1} in the MHB was modest after knockdown of GnRH2 (Fig. 4.6E) but strong after knockdown of GnRH3 (Fig. 4.6F), although it was not as dramatic as overexpression of \textit{fgf8} gene (Fig. 4.6C). Also, overexpression of \textit{pax2.1} in the eye cup was clearly visible in both GnRH knockdowns but was modest in the eye stalk (Figs. 4.6E, 4.6F). Thus, GnRH peptides would normally reduce the early expression of \textit{fgf8} and \textit{pax2.1} at least by 24 hpf in zebrafish embryos. Also, \textit{pax2.1} is expressed in the otic vesicles (Püschel et al., 1992) and is shown in Figs 4.6 D-F; labeling was similar in the control and knockdown embryos.

\textbf{Role of GnRH2 in development of diencephalon and eye as shown by \textit{pax6.1}}

\textit{Pax6.1} is a marker for the eye and more dorsal brain regions; it is involved in regionalization of the diencephalon (Krauss et al., 1991a, 1991c). The expression pattern of \textit{pax6.1} was shown in our wild type embryo in the diencephalon with a delineated boundary at the midbrain. Also, \textit{pax6.1} was expressed throughout the eye (Fig. 4.6G). Expression was severely reduced in the diencephalon and eye after GnRH2 was blocked (Fig. 4.6H). In contrast, little change was observed in \textit{pax6.1} expression after knockdown of GnRH3 (Fig. 4.6I). Thus, GnRH2 would be necessary for full expression of \textit{pax6.1} in the eye and diencephalon. In addition, brain marker \textit{pax6.1} was used to show that its expression was rescued with gnrh mRNA in knockdown fish (Fig. 4.7A-C).
Fig. 4.7. In situ hybridization showing expression of two brain markers in rescued embryos. The expression pattern and intensity of *pax6.1* and *mab2112* is shown in rescued embryos (B, C, E and F) compared to wildtype (A and D) and knocked down embryos in Fig. 4.6H, I, K and L. All embryos are shown in the dorsal view. The scale bar for all photos is shown in A and is 250μm.
Role of GnRH2 in development of midbrain and eye as shown by mab21l2

The Mab21l2 gene is expressed during midbrain and eye morphogenesis (Kudoh and Dawid, 2001; Wong and Chow, 2002; Kennedy et al., 2004). We confirmed its expression in our wild type 24 hpf-embryos (Fig. 4.6J). We found that mab21l2 expression was markedly reduced as were the shape and development of the eye and midbrain after GnRH2 knockdown (Fig. 4.6K) compared with the control tissue at 24 hpf. The midbrain after GnRH3 knockdown was altered in shape. Also, brain marker mab21l2 was used to show that its expression was rescued with gnrh mRNA in knockdown fish (Fig. 4.7D-F).

Differing roles of GnRH2 and GnRH3 emphasized by meis1.1, pax6.1 and mab21l2

Marker meis1.1, which is part of a transcription factor complex, was found in the forebrain, diencephalon, eye, midbrain and hindbrain in our wild type embryos (Fig. 4.6M) as expected (Waskiewicz et al., 2001). A striking reduction in the expression of meis1.1 is seen only after knockdown of GnRH2, similar to the effect on expression of pax6.1. We observed that GnRH2 loss-of-function had more severe effects on the expression of pax6.1, mab21l2 and meis1.1 than GnRH3 loss, demonstrating that GnRH2 is more important in regulating these transcription factors during normal development. Likewise, the shape and growth of the midbrain are more dependent on GnRH2 than GnRH3 as seen in Figs 4.6B, E, H, K and N.
Expression of EphA4 not changed in hindbrain or diencephalon after knockdown of GnRHs

The expression of ephA4, a tyrosine kinase receptor, normally occurs in the diencephalon and in specific rhombomeres (numbers 1, 3 and 5) in the hindbrain as shown in the wild type embryo (Fig. 4.6P); little change in expression occurred after knockdown of the GnRHs (Figs. 4.6Q, 4.6R). As noted above, meisl/1 expression in the hindbrain was reduced after GnRH2 knockdown.

GnRH knockdown confirmed by immunocytochemistry

GnRH was detected in the forebrain, diencephalon and midbrain at 30-31hpf in wild type embryos (Figs. 4.3G, H). After injection of only the gnrh2 morpholino, immunocytochemistry was used to show that GnRH2 protein expression was blocked (Fig. 4.3I), but that of GnRH3 protein was present in the olfactory region (Fig. 4.3J). Likewise, knockdown of GnRH3 translation resulted in immunoreactivity only for GnRH2 (Fig. 4.3K) and not GnRH3 in the brain (Fig. 4.3L).

To additionally determine the length of time of blockade, embryos at 26h–120h were examined after immunocytochemistry with primary antiserum 7CR-10, which detects both GnRH2 and GnRH3 (Lescheid et al., 1997). Even at 99hr, GnRH protein was detected primarily in the mild morphants and at a lesser level than the control (data not shown).

Little effect on apoptosis after GnRH knockdown
In order to understand whether changes resulting from loss of GnRH were due to apoptotic death in early development of zebrafish, we compared the TUNEL assay in control, gnrh2-MO injected and gnrh3-MO injected embryos at three different stages in the dorsal view (Fig.4.8). There wasn’t a dramatic change among the three groups or compared to an earlier study of apoptosis in normal developing zebrafish (Cole and Ross, 2001). The gnrh3-MO group did show more apoptotic cells in the hindbrain at 19 and 24 hpf. The caspase-3 and caspase-9 assay results for 24 hpf embryos in the three groups was consistent with the TUNEL assay. There was no significant difference among the groups. The fold increase of caspase-3 activity as a ratio to the mean value of the control group was 1.027 ± 0.024 for gnrh2-MO group and 1.088 ± 0.049 for the gnrh3-MO group. For caspase-9, the ratio was 1.039 ± 0.097 for the gnrh2-MO group and 0.989 ± 0.072 for the gnrh3-MO group (Fig. 4.9).

Discussion

**GnRH peptides affect early brain and eye development in zebrafish**

In this study we isolated two GnRH cDNAs (gnrh2 and gnrh3) from zebrafish and showed they are expressed early in development. The transcripts encoding both GnRH peptides were detected in embryos within two hours of fertilization by RT-PCR suggesting that they are initially maternal transcripts but later are maintained as embryonic transcripts. By 22-24 hours the transcripts were detected by in situ hybridization. The GnRH proteins were detected in the forebrain (GnRH3) and in the midbrain (GnRH2) at 30-31 hours after fertilization by immunocytochemistry. However,
Fig. 4.8. Apoptotic cell labeling by the TUNEL method at different hours and among different groups. All embryos are show in the dorsal view. The left column is the control group; the middle column is the gnrh2-MO group; and the right column is the gnrh3-MO group. Embryos at 12h, 19h and 24h post fertilization in the three groups are compared.
Fig. 4.9. Assay of caspase-3 and caspase-9 activity for apoptosis in zebrafish embryos at 24h post fertilization among different groups. (A) Caspase-3 activity and (B) caspase-9 activity are shown as fold increases compared to the mean value of the control group. There were no significant differences among the control, gnrh2-MO and gnrh3-MO groups. Caspase activity was determined by a fluorescence kit.
immunocytochemistry is not a sensitive method to measure protein so that GnRH was undoubtedly secreted in small amounts long before its detection at 30-31 hours. We show that knockdown of translation for GnRH mRNA, beginning in the fertilized egg, results in defects in the brain and eye at a low dose of morpholinos.

The mechanism of action of GnRH in early development was examined using the expression of early brain genes after loss-of-function for GnRH. These experiments reveal three major functions for GnRH in early brain development. The first is that both GnRHs affect early genes that are important in defining the boundaries of the midbrain in its posterior extent with the hindbrain and in its anterior extent with the diencephalon. The second function is that both GnRH peptides contribute to the development of the eye cup and stalk. The third function involves primarily GnRH2 in the enhancement of growth and development of the midbrain before 24 hpf and in the regulation of several transcription factors in the midbrain.

*Early transcripts encoding GnRH2 and GnRH3 are likely maternal*

The transcripts that we detected by RT-PCR in embryos less than 2 hpf are likely to be maternal, as zygotic transcription begins in the zebrafish only at 3-4 hpf (Kane and Kimmel, 1993). The maternal transcripts are likely to be rare as they were detected only by the more sensitive PCR assay and not by *in situ* hybridization. Also, gnrh2 mRNA was not detected at 30 min after fertilization despite repeated attempts. It is possible that gnrh2 mRNA expression was inhibited briefly because it was clearly expressed at 1h 30min. Further proof that gnrh transcripts may be maternal in fish comes from Okubo et
al. (2006) who showed that green fluorescent protein fused to the gnrh3 promoter was expressed in eggs inside the transgenic mother before fertilization.

Later embryonic transcripts are detected in three brain locations

In situ hybridization was reported to detect mRNA for gnrh2 and gnrh3 at 24-26 h (Gopinath et al., 2004; Kuo et al., 2005); we found that we could detect gnrh2 mRNA at 22 hpf and gnrh3 by 24 hpf. The location of gnrh2 mRNA expression, when viewed dorsally, was in the midbrain between the posterior part of the eyes. Expression of gnrh3 mRNA was in two locations with one group of cells first detected near the olfactory placode at 24h and the other cells in the hypothalamic region at 28h and 48h; these are similar positions to cells with immunoreactive GnRH at 52h, as reported by Gopinath et al. (2004). Also, GnRH peptides are expressed during early embryogenesis as we have detected them by 30-31h with immunocytochemistry (Fig. 4.3). Other methods such as radioimmunoassay combined with high performance liquid chromatography (RIA-HPLC) or mass spectrometry are more sensitive than immunocytochemistry for detection of GnRH peptides but require more tissue for initial purification steps than is available. GnRH peptides are undoubtedly produced earlier than 30-31 hours and only small amounts would be necessary in the early embryo.

Morpholino antisense oligos specifically blocked translation of GnRH proteins

The morpholino technology is based on a chemical modification of antisense oligonucleotides to prevent their degradation by nucleases (Summerton and Weller, 1997). Morpholinos are useful to study early development as they block protein
translation, but are effective only in the early stages of development as the morpholinos become diluted during repeated cell division. Nonetheless, morpholinos function by blocking translation initiation in both maternal and zygotic transcripts (Nasevicius and Ekker, 2000). In the present experiments, a number of procedures have been carried out to determine if the results are specific (Ekker and Larson, 2001; Heasman, 2002). First, we selected a control morpholino for injection to evaluate toxic side effects from morpholinos in general or from residues of the synthesis process. Second, for each gnrh transcript, we designed two distinct morpholinos targeting two independent regions, the 5' UTR and the start ATG codon. Third, to confirm that GnRH peptides were efficiently blocked, we used immunocytochemistry to detect the GnRH peptide. Fourth, to confirm that the morpholino blockade of GnRH was specific, we did rescue experiments with exogenous gnrh mRNA. We found rescue effects in regard to the brain and eye for both gnrh2 and gnrh3 mRNA. Not all embryos were rescued but the percent of normal embryos in the rescued group was significantly higher than in the knockdown groups. Also, the in situ hybridization pattern and intensity of expression showed recovery in rescued embryos. Our partial recovery results were as expected from the literature on zebrafish (Cui et al., 2001), Xenopus (Hashiguchi et al., 2004) and mouse (Kanzler et al., 2003). It is known that incorrect temporal and spatial expression is inherent to RNA injection experiments, and may not reproduce full rescue of function for the gene of interest (Kennedy et al., 2004).

Both GnRHs have a role in anterior-posterior brain regionalization
Brain development depends on the sequential expression of factors, some of which are secreted proteins, whereas others are transcription factors. Here we selected six factors that are present early in the brain; Fgf8 is a secreted protein; EphA4 is a tyrosine kinase receptor; Pax2.1, Pax6.1 and Mab21l2 are transcription factors, whereas Meis1.1 is part of a transcription complex. Each protein is a zebrafish homologs of similar proteins in mice and humans: Fgf8 (Reifers et al., 1998), Pax2.1 (Krauss et al., 1991a, 1991b; Puschel et al., 1992b; Pfeffer et al., 1998), Pax6.1 (Krauss et al., 1991c; Puschel et al., 1992a), Mab21l2 (Kudoh and Dawid, 2001), Meis1.1 (Waskiewicz et al., 2001), EphA4 (Xu et al., 1994, 1995).

The boundary between the midbrain and hindbrain (MHB) is an important organizing center in the developing brain for induction and patterning of the midbrain and cerebellum (Reifers et al., 1998; Kiecker and Lumsden, 2005). We examined the expression pattern of fgf8 and pax2.1, which are required for proper formation of this boundary. Each is expressed early in the MHB of the embryo at 8-9 hpf (70-80% epiboly)(Krauss et al., 1991b; Rhinn and Brand, 2001). If the fgf8 gene is disrupted, the MHB and cerebellum are absent along with other defects (Brand et al., 1996; Reifers et al., 1998; Rhinn and Brand, 2001). If the pax2.1 gene is disrupted, the MHB and cerebellum are absent and the midbrain tectum degenerates later (Brand et al., 1996; Rhinn and Brand, 2001; Scholpp and Brand, 2003). This boundary is crucial for defining the anterior limit of the hindbrain and posterior limit of the midbrain.

The knockdown of GnRH2 or GnRH3 resulted in overexpression of both fgf8 and pax2.1 at the MHB region, which implies that if the GnRH peptides were present, they would partially suppress these early genes. Our evidence suggests that GnRH peptides
are not necessary for induction of $fgf8$ or $pax2.1$, but are downstream acting back to regulate $fgf8$ and $pax2.1$. Rhinn and Brand (2001) have proposed that Fgf8 as a protein is a potent signaling molecule that must be carefully controlled in the embryo by inhibitors that are intracellular or extracellular. The same proposal could be applied to Pax2.1. The evidence presented here suggests that GnRH2 and GnRH3 are examples of secreted inhibitors. Other neuropeptides such as PACAP may play a similar role (Wu et al., 2006). We suggest that regulation of $fgf8$ and $pax2.1$ by GnRH peptides is part of a molecular network defining the midbrain-hindbrain boundary, which is an important component of brain regionalization.

A second boundary affected by GnRH peptides is the one between the diencephalon and midbrain (DMB). In wild type embryos, $fgf8$ and $pax6.1$ are expressed in the diencephalon with a clearly defined boundary at the midbrain region, which is the position of the posterior commissure in the!brain. Fgf8 is thought to be one of the signals that maintains forebrain identity. In the zebrafish $fgf8$ mutant, there is some posterior expansion of the forebrain region into the midbrain region, implying that Fgf8 normally represses the forebrain (Scholpp and Brand, 2003). A modest overexpression of $fgf8$ and a more anterior site for the DMB (reduction of diencephalon) was seen in our experiments after knockdown of GnRH, especially GnRH3. The implication is that if GnRH were present, $fgf8$ would be reduced in the region, which would shift the diencephalic border to a more posterior position. Likewise, $pax6.1$ expression in the diencephalon, mainly in the thalamus and pretectum, formed a clear cut boundary with the midbrain (Krauss et al., 1991a; 1991c). However, loss of GnRH2 resulted in loss of $pax6.1$ expression, which is different from the $fgf8$ pattern. The implication is that
GnRH2 normally regulates \textit{pax6.1} by increasing its expression in the diencephalon. This is logical as mutant \textit{pax6} mice show an expansion of the midbrain into the diencephalon (Scholpp and Brand, 2003), which implies that \textit{pax6} stimulates the diencephalon. Thus, GnRH peptides appear to reduce a diencephalic inhibitor (\textit{fgf8}) and stimulate a diencephalic putative growth factor (\textit{pax6.1}).

In conclusion, GnRH peptides appear to have a role in the anterior-posterior regionalization of the brain by acting back to regulate \textit{fgf8}, \textit{pax2.1} and \textit{pax6.1}. GnRHs are likely to be downstream of these three early genes in defining the MHB and DMB. Certainly the location of GnRH3 neurons in the diencephalon and GnRH2 neurons in the midbrain are appropriate for the proposed functions and the axons from GnRH neurons extend the sphere of influence for GnRH. It is crucial to show that GnRH receptors are present in the target tissues. To date, four zebrafish GnRH receptors have been sequenced and their location includes areas in the midbrain and hindbrain (Tello, Wu, Cervini, Vaughan, Rivier and Sherwood, submitted).

\textbf{Both GnRHs affect development of the eye cup and/or stalk}

Our results indicate that knockdown of GnRH affects early genes involved in eye cup or eye stalk formation. In the eye cup, a striking reduction in expression occurs in genes \textit{pax6.1}, \textit{mab21l2} and \textit{meis1.1} after GnRH2 loss. In contrast, expression of \textit{pax2.1} in the eye cup is not present in the wild type embryo but is observed after knockdown of either GnRH peptide. In the eye stalk, the most dramatic increase in expression is for \textit{fgf8} and \textit{pax2.1} after GnRH3 knockdown, but there are smaller increases after GnRH2 loss. The size of the eye is reduced more after loss of GnRH2 than GnRH3.
Gene *pax6.1* is expressed in the eye beginning at 12 hpf in the zebrafish and is expressed throughout the eye (retina and lens) by 24 hpf (Krauss et al., 1991a, 1991c; Puschel et al., 1992a; Ekker et al., 1995) as seen in our wildtype embryos. In mouse, the pax6 gene is required for eye formation as shown by the mutant known as *small eye* (Hill et al., 1991). In our experiments loss of GnRH2 resulted in an embryo with almost no *pax6.1* expression in the eye cup implying the GnRH2 normally upregulates this transcription factor during development.

A similar reduction in *meis1.1* expression in the eye cups occurs at 24 hpf after loss of GnRH2. Meis1.1, which is a member of the Meis family of transcription factors, is expressed early in developing eye fields. At 24 hpf, *meis1.1* is widely expressed with high levels of expression within the retina, midbrain and hindbrain (Waskiewicz et al., 2001). Therefore, the dramatic reduction in *meis1.1* not only in the eye cups but throughout the brain is of interest and parallels that seen with *pax6.1*.

In addition, a noticeable reduction in *mab21l2* expression was observed after loss of GnRH2. The gene *mab21l2* is one of the earliest genes expressed in the zebrafish eye primordia prior to complete separation of the two eyes (Kudoh and Dawid, 2001) and is critical in eye formation (Wong and Chow, 2002; Kennedy et al., 2004). In wild type zebrafish embryos, *mab21l2* was expressed at 11h after fertilization as detected by RT-PCR; the level continued to increase through 18h (Wong and Chow, 2002). At 24 hpf, eye expression is concentrated in the retina and lens (Kudoh and Dawid, 2001). In mouse, knockout of the *mab21l2* gene was lethal to the embryo (Yamada et al., 2003; Kennedy et al., 2004). In contrast, knockdown of *mab21l2* by morpholinos in zebrafish was not lethal, but loss of mab21l2 prevented survival of progenitor cells during eye
formation and resulted in a 20% decrease in eye size at 24h (Kennedy et al., 2004). Our data show that knockdown of GnRH2 or GnRH3 resulted in a significant decrease in eye size but secondary apoptotic cell death did not play a major role.

Knockdown of either GnRH2 or GnRH3 resulted in unexpected expression of pax2.1 in the eye cup, which implies that GnRH, if present, would partially suppress pax2.1 in the eye.

GnRH, especially GnRH3 was involved in feedback inhibition of fgf8 in the eyestalks. Retinal ganglion cell axons form the eye stalks, which are detected as they grow to form the optic chiasm beneath the hypothalamus. Because GnRH3 is expressed in the hypothalamic region (Figs. 4.3E, F; Steven et al., 2003; Gopinath et al., 2004), it is reasonable that this peptide has an effect on the eye stalks. The loss of GnRH3 results in overexpression of fgf8 in the stalks and to some extent of pax2.1. Earlier reports on the zebrafish fgf8 mutant showed that "projection of the retinal ganglion cell axons to the midbrain and the retinotectal map were disturbed" (Rhinn and Brand, 2001). Our evidence suggests that GnRH normally restrains fgf8 expression in the eye stalks.

The role of GnRH in eye development is a new concept, but a number of researchers have reported that GnRH and/or its receptor exist in the eye or retina in different species of adult fish (Stell et al., 1984; Okubo et al., 2000a, 2000b; Madigou et al., 2000; Robison et al., 2001). The present evidence shows that knockdown of GnRH affects several genes in the formation of the eye cup (pax6.1, meis1.1, mab21l2, and pax2.1) and eye stalk (fgf8 and pax2.1). The action of GnRHs as secreted peptides could be direct or indirect on the gene markers but would occur before 24h after fertilization.
GnRH2 affects the growth and development of the midbrain

Two of the gene markers, \textit{mab21l2} and \textit{meis1.1}, were expressed in the midbrain. Expression of each of these markers decreased compared to wild type embryos after loss of GnRH2. Because the midbrain tectum receives an input from the retina via the optic nerves, both \textit{mab21l2} and \textit{meis1.1} are useful markers for tracing the morphological development of the eyes and midbrain tectum (Kudoh and Dawid, 2001; Waskiewicz et al., 2001). In zebrafish, \textit{mab21l2} is expressed in the midbrain beginning at 11 hpf and is critical for midbrain formation (Wong and Chow, 2002; Kennedy et al., 2004). After GnRH3 knockdown, the shape of the midbrain was extended (Fig. 4.6C, F, O, R).

In the midbrain, it was obvious that knockdown of GnRH2 also resulted in a substantial decrease in growth of the midbrain at 24 hours. The inhibition of growth, presumably cell proliferation, can be observed in several of the embryos after GnRH2 loss in which the shape of the midbrain was altered. The lack of normal midbrain growth implies that GnRH2 normally feedbacks, directly or indirectly, to enhance the effect of transcription factors such as \textit{mab21l2} and \textit{meis1.1}. Because GnRH2 is exclusively in the midbrain, it has an appropriate location to upregulate these presumptive growth factors. It remains to be shown that at least one of the four types of zebrafish GnRH receptors is present in the midbrain.

\textit{Meis1.1} and \textit{ephA4} are markers for the zebrafish hindbrain. Gene \textit{meis1.1} is thought to be the DNA-binding protein that forms a complex of Pbx-Hox-Meis for specifying the rhombomeres in hindbrain segmentation; \textit{meis1.1} is highly expressed at 24h in several regions including the hindbrain (Waskiewicz et al., 2001). Our GnRH2 knockdown results showed considerable reduction in \textit{meis1.1} expression in all the regions where it is
normally expressed. In contrast, ephA4 is known to have weak expression in rhombomere 1 with strong expression in rhombomeres 3 and 5 (Waskiewicz et al., 2002); our wild type embryos showed this pattern and knockdown of either GnRH did not alter the pattern. From this evidence we conclude that loss of GnRH does not affect ephA4 in the hindbrain.

**GnRH may have a role in mammalian fetal development**

GnRH was shown to be expressed in mammalian embryos using cultured rhesus monkey embryos in which immunoreactive GnRH was detected in vitro from the pre-implantation morula to the attached blastocyst stages. Furthermore, GnRH was secreted into the medium at the same stages with a considerable increase when the blastula hatched and attached (Seshagiri et al., 1994). Similar results were found in human and mouse embryos where GnRH and its receptor were detected as mRNA in pre- and peri-implantation embryos and immunoreactive GnRH was present at the 8-cell, morula, and blastocyst stages (Casan et al., 1999; Raga et al., 1999). Also, GnRH mRNA and protein were detected in the human uterine endometrium at all stages of the menstrual cycle (Raga et al., 1998) and in the fallopian tube in the luteal phase (Casan et al., 2000). The GnRH receptor, like GnRH, was found as mRNA in the uterus at all stages (Raga et al., 1998). Additionally, there is evidence that pre-implantation embryonic development is significantly improved by incubation with increasing concentrations of GnRH agonist, as determined by the number of mouse embryos reaching the hatching blastocyst stage (Raga et al., 1999). Raga et al. (1999) and Casan et al. (2000) suggest that GnRH has a role in mammalian pre-implantation development, which occurs before differentiation of
There may be differences in role of GnRH in development of mammals and fish

Mice and zebrafish do not always have the same phenotype after loss of function in genes that are homologous. As noted above, the loss of \textit{mab21l2} was lethal to the mouse embryo, but mainly affected the eye in zebrafish (Kennedy et al., 2004). In the present study, loss of function for GnRH2 and GnRH3 resulted in defects in regionalization of the brain, and in development of the eye and midbrain in zebrafish. However, it is not yet clear if mammals would have the same phenotype. All mammals develop in utero and thus are exposed to GnRH from the placenta (Tan and Rousseau, 1982; Seeburg and Adelman, 1984) because the mother, whether natural or foster, will produce GnRH in the brain and placenta. Both type I and type II GnRH receptors in the fetus would have to be inactivated from the time of fertilization to prevent GnRH effects during development, as each type can bind both ligands. In the hypogonadal mouse (Cattanach et al., 1977; Mason et al., 1986), GnRH1 is thought to be nonfunctional due to a genetic defect and mice as a species lack GnRH2 and its receptor (Millar et al., 2004) but the type I receptor could still respond to the mother’s GnRH in utero. A knockout of GnRH receptor type I would test the hypothesis that GnRH is necessary in early development in mice (see chapter 6). In humans, the type II GnRH receptor may not be functional because it has an early stop codon in exon 2 (Pawson et al., 2003). In addition, the human type I GnRH receptor can have a number of mutations that have been associated with clinical idiopathic hypogonadotropic hypogonadism (IHH) (Bedecarrats, 2003). Some patients did not develop sexually. It still remains to be shown whether both type I and type II
GnRH receptors are completely nonfunctional in the IHF fetus. GnRH2 normally does not reach the pituitary, but would be released in midbrain and other brain areas that contain GnRH2 axon terminals and could act on type II receptors within the brain during development, but not for reproduction after birth. Until we are certain that both GnRH receptors are nonfunctional, we do not know if GnRH affects early development in mammals.

**Conclusion**

This report shows that GnRH has a critical role in the early development of the brain and eye in a non-mammalian embryo. GnRHS affect the expression of markers in the MHB, which organizes the spatial development of the mesencephalon, as shown by the strong expression of *fgf8* and *pax2.1* after GnRH knockdown. GnRHS also affect the position of the DMB. The loss of GnRH2 results in a decrease in the growth, presumably in proliferation, in the midbrain, as shown by reduction of several marker genes. Although GnRH is known to be present in the adult retina, this is the first demonstration that GnRHS affect the development of the eye cup and stalk. We propose that certain neuropeptides that are expressed early in the brain and eye during development may not be the inducers of brain structures, but may be part of downstream networks that regulate early factors controlling brain and eye. The exact location of GnRH in the cascade of factors controlling growth and patterning of the brain and eye is not certain but apoptosis after GnRH loss appears not to play a major role. Zebrafish will provide an excellent model to study the interaction of GnRH with different signaling pathways in development.
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Chapter 5

GnRH receptor localization in early development of zebrafish
Introduction

The decapptide gonadotropin-releasing hormone (GnRH) plays a key role in reproduction by controlling luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH has been divided into three groups: GnRH1, GnRH2 and GnRH3; these three groups are distributed in three distinct areas of the brain (Fernald and White, 1999). During development, GnRH1 originates from the olfactory placode and migrates through the ventral telecephalon to enter the preoptico-hypothalamic area (Wray et al., 1989, 2002; Schwanzel-Fukuda and Pfaff, 1990). GnRH2 (cGnRH-II) cell bodies are found in the midbrain. The location and peptide sequence of GnRH2 have been conserved from jawed fish to humans (Millar et al., 2003). GnRH3 (salmon GnRH) also originates in the olfactory placode, but migrates to the terminal nerve/ventral telencephalon and is exclusively found in teleost fish.

In species that have two forms of GnRH (GnRH2 and GnRH3), like zebrafish, the terminal nerve GnRH3 is also expressed in preoptic-hypothalamic cells (Palevitch et al., 2007). The diversity of GnRH locations in brain and extrapituitary tissues such as eye, gonad, liver, kidney, placenta, and prostate (Pati and Habibi, 1993; Leung et al., 1996; Maruska and Tricas, 2007) suggests GnRH also acts as a neuromodulator in an autocrine and paracrine pattern. GnRH action occurs by binding to its seven transmembrane domain G-protein coupled receptor to activate a variety of intracellular signaling pathways. The major signaling pathway is the IP₃ in which GnRH binds its specific receptor on the cell membrane, which triggers the Gq/11 G protein to couple with the GnRH-R. The coupling activates phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4, 5- bisphosphate and produces inositol 1, 4, 5-triphosphate (IP₃).
and diacylglycerol (DAG). These products further activate transcription factors that regulate mRNA transcription (Millar, 2005). However, other signaling pathways like cAMP are involved also with certain receptors (Tello et al., 2005).

GnRH mRNA and peptide have been found expressed at 24h post fertilization in zebrafish (Gopinath et al., 2004; Wu et al., 2006; Palevitch et al., 2007). If GnRH2 and GnRH3 genes are knocked down at the zygote stage, then changes occur in eye and brain development (Sherwood and Wu, 2005; Wu et al., 2006). To better understand how GnRH plays a role in early development and its site of action, it is critical to study GnRH receptor expression and localization.

In the present study, I used in situ hybridization and immunocytochemistry for localization of the putative zebrafish GnRH receptors in embryos. In the zebrafish genome, data mining revealed four putative GnRH receptors in zebrafish but GnRH receptor 3 was suspected to be a truncated gene (Lethimonier et al., 2004). The open reading frames (ORFs) of GnRH receptor 1, 2 and 4 were originally cloned by Javier Tello in our laboratory. For in situ hybridization, I performed 5' and 3' RACE to sequence the untranslated regions (UTRs) of each receptor as these are the most unique areas in a receptor cDNA for designing specific probes against each receptor. Also, I completed the ORF for receptor 3. I then sequenced the four complete receptor cDNAs, which confirmed the ORFs above. For early developmental stages of zebrafish, I used in situ hybridization to study mRNA expression and immunocytochemistry to study protein expression of the four GnRH receptors. The results revealed the in vivo location of the GnRH receptors, which are the target sites for GnRH.
Materials and Methods

**Animals and antibodies**

Wild-Type zebrafish (*Danio rerio*) were purchased from a local pet store and maintained in our lab under a 14h light and 10h dark cycle at 28.5°C. Procedures were approved by the Animal Care committee at the University of Victoria. The tissues of adult zebrafish brains and different stages of embryos were frozen in liquid nitrogen. Antibodies raised against the N-terminal coding sequence of the four zebrafish receptors were a gift from Javier Tello at University of Victoria.

**Total RNA isolation and cDNA synthesis**

Total RNA was extracted and DNase digested from zebrafish brain and embryos at different stages of early development: 30', 1h30', 4h, 6h, 8h, 10h, 14h, 18h, 25h30' post fertilization (hpf) according to the manufacturer’s protocol (RNeasy Mini Kit, Qiagen, Inc., Mississauga, ON). The concentration of total RNA was measured and 900ng total RNA from different samples was reverse transcribed with Oligo dT and Superscript II (Invitrogen, Burlington, ON) according to the manufacturer’s instructions. Meanwhile, total RNA from different samples was reverse transcribed using the same conditions except without Superscript II as a negative control.

**RACE (rapid amplification of cDNA ends) for zebrafish GnRH receptors**
Total RNA (500-600ng) from adult zebrafish brains was used for synthesis of cDNA with the First Choice™ RLM-RACE kit (Ambion, Austin, TX) following the manufacturer’s instructions. The 5’ and 3’ RACE primers for GnRHs were designed by using published in silico amino acid sequences (Lethimonier et al., 2004) to search the cDNA sequences in the zebrafish genomic database in Vega Multi Blast View Programme. RACE-PCR reactions were carried out for 35 cycles at 94°C for 30 s, 55°C annealing temperature for 30 s, 72°C for 1 min with a 7-min extension at 72°C on the last cycle. All primers were listed in Table 5.1. The PCR products were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Bands were selected and isolated with QIAquick Gel Extraction Kit (QIAGEN) and ligated into PGEM-T vector (Promega, Corp., Madison, WI).

**RT-PCR**

The temporal expression pattern of GnRH receptors was performed by RT-PCR. One μl of cDNA from different stages of embryos was amplified with specific primers. The positive control reaction was conducted with zebrafish polymerase II primers to verify the cDNA quality. Negative controls were carried out by two methods: the cDNA sample was replaced by 1μl H2O or the cDNA was made without reverse transcriptase. All PCR reactions were performed for 35 cycles at 95°C for 30 s, 55°C annealing temperature for 30 s, 72°C for 1 min with a 7-min extension at 72°C on the last cycle. All primers were designed either to the exon-intron junction position or to a site that was two exons apart. PCR products were sequenced and confirmed. Primers are listed on Table. 5.1.
TABLE 5.1. Primers used to amplify cDNA of GnRH receptors in zebrafish

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Target</th>
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<td><strong>RACE</strong></td>
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<td>GnRH-R1</td>
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<td>R² 543</td>
<td>CGCCCTGTAAATAAATGAACTGT</td>
<td>5’ cDNA</td>
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<td>R322</td>
<td>ATGGCGTGCTACACCCGCTACAC</td>
<td>5’ cDNA</td>
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<td>F² 105</td>
<td>TGCTGGGACACCGTATTATACTGCTG</td>
<td>3’ cDNA</td>
</tr>
<tr>
<td>F126</td>
<td>CTGGGCTCTGGGATTGG</td>
<td>3’ cDNA</td>
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<tr>
<td><strong>GnRH-R2</strong></td>
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<tr>
<td>R680</td>
<td>GAACAGTGGTGAAGAGGAAGA</td>
<td>5’ cDNA</td>
</tr>
<tr>
<td>R573</td>
<td>TGGGGAATCAATCTCCACAAAC</td>
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<tr>
<td>F525</td>
<td>ACCGCAGTATTGATTATTGCTG</td>
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<tr>
<td>F734</td>
<td>GATTGTGAACAAATGAGC</td>
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<td><strong>GnRH-R3</strong></td>
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<td>R431</td>
<td>ATCACAGGGTGACACGAGGAGTT</td>
<td>5’ cDNA</td>
</tr>
<tr>
<td>R279</td>
<td>ATCTTCACTGTCTTCTCATCC</td>
<td>5’ cDNA</td>
</tr>
<tr>
<td>F105</td>
<td>TGCTGGGACACGATTTATCTGCTG</td>
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<td>F126</td>
<td>CTGGGCTCTGGGATTGG</td>
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<td><strong>GnRH-R4</strong></td>
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<td>F267</td>
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<td>F809</td>
<td>GCATAGTTATCCTGACCTCCTT</td>
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<td><strong>RT-PCR</strong></td>
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<td><strong>GnRHR1</strong></td>
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<tr>
<td>F390</td>
<td>TTCAGGCAGCATTTATCTTGG</td>
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<td>TACAGGCTGACACAAAGTGAAA</td>
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<td><strong>GnRH-R2</strong></td>
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<td>F420</td>
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<td>R680</td>
<td>GAACAGTGGTAAGAGGAAGA</td>
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<td>F1321</td>
<td>TGGAGGGACATTGAAGGAACACT</td>
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</tr>
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<td>TTCTTACGCTGCTTCTACTGCTTTCT</td>
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<td><strong>GnRH-R4</strong></td>
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<td>F670</td>
<td>ATATGCTCTTTCTGTATGCTCC</td>
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<td>set 4F</td>
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<tr>
<td>set 3R</td>
<td>CACAGCAATAACCTGAGAAA</td>
<td>(from Javier Tello)</td>
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<td>Primers</td>
<td>Sequence (5’-3’)</td>
<td>Target</td>
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<td><strong>Probes</strong></td>
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<td><strong>GnRHR1</strong></td>
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<tr>
<td>F1445</td>
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<td>R1746</td>
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<td>F3</td>
<td>AACTGTAGTGTTTGATGCCGTTGCT</td>
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<td>R558</td>
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<td>F43</td>
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<tr>
<td>R331</td>
<td>GAAGATGAAGAGGATGAGGAAG</td>
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<td><strong>GnRHR3</strong></td>
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<td>F1321</td>
<td>TGGAGGGGACATTGAAGGAAACC</td>
<td>3’ cDNA</td>
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<td>R2045</td>
<td>TCTCTCAGCGTGCCCTCTACTGTTTCT</td>
<td>3’ cDNA</td>
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<td>F3</td>
<td>AAGATGGAAGTAGTGGATGCTTG</td>
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<td>R420</td>
<td>CTGCCGCCGCCCAAACACG</td>
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<td><strong>GnRHR4</strong></td>
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<td>TCGAAAGGAAGCTGAAGCGCTACT</td>
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<tr>
<td>F27</td>
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<tr>
<td>R614</td>
<td>GCCAATGCCGCTTCACCTGTG</td>
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<td><strong>Clones for functional study</strong></td>
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<td>cDNA</td>
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<tr>
<td><strong>GnRH-R3</strong></td>
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<td>F260</td>
<td>ATCTGACATCTTGCAACCAAC</td>
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<tr>
<td>R1352</td>
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<tr>
<td>F297NheI</td>
<td>A<strong>T</strong>GCTAGG<strong>C</strong>G<strong>C</strong>C<strong>A</strong>C<strong>A</strong>T<strong>G</strong>G<strong>G</strong>T<strong>G</strong>A<strong>C</strong>T<strong>G</strong>G<strong>T</strong>G<strong>T</strong>C<strong>T</strong>C<strong>T</strong>C<strong>T</strong>T<strong>G</strong>T**</td>
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<tr>
<td>R1326Xhol</td>
<td>A<strong>T</strong>C<strong>T</strong>C<strong>G</strong>A<strong>G</strong>C<strong>T</strong>C<strong>C</strong>A<strong>G</strong>T<strong>G</strong>T<strong>C</strong>C<strong>T</strong>C<strong>C</strong>T<strong>T</strong>T<strong>G</strong>T**</td>
<td></td>
</tr>
</tbody>
</table>

a F: forward primer.  b R: reverse primer. The enzyme restriction sites are underlined. The red color is the Kozak sequence.
Transfection of zebrafish GnRH-R3

The predicted GnRH-R3 had a stop codon in its N-terminal part and was suspected to be a truncated gene (Lethimonier et al., 2004). After I isolated the complete cDNA sequence of GnRH-R3, I tested its activation. The cDNA construct with a full length open reading frame, flanked restriction enzymes, and a Kozak sequence (A/G CCATGG) (Kozak, 1996), was subcloned into PCDNA 3.1(-) (Invitrogen). Two rounds of PCR reactions were conducted with plantinum Taq polymerase high fidelity (Invitrogen) as outlined by the manufacturer. Primers are listed on Table 5.1. I amplified the GnRH-R3 clone with Maxi Prep (QIAGEN) and transfected it into COS-7L cells (Invitrogen). COS-7L cells were seeded at 1-2X10^4 cells /cm^2 in flasks with growth medium of Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen) supplemented with 10mM nonessential amino acids (NEAA, Invitrogen) and 10% fetal bovine serum (Invitrogen). Cells were cultured at 37°C in 5% CO₂. After 3 days, the monolayers were approximately 90% confluent and cells were trypsinized by Tryple (Invitrogen) for 5 minutes and seeded in 24-well tissue culture-treated plates (Corning Costar Corp., Cambridge, MA) at a density of 60,000 cells/well in growth medium. Twenty four hours after seeding, the cells were washed and incubated with serum-free medium (VP-SFM, invitrogen); cells were transfected with 0.8 μg/well of the GnRH-R3 cDNA clone and lipofectamine according to the manufacturer’s protocol (Invitrogen). After 14h of transfection, the cells were washed and incubated with labeling medium (Medium 199, Invitrogen) containing 0.3% bovine albumin (Sigma-Aldrich, St. Louis, MO). Twenty four hours after transfection, cells were labelled with 0.9 μCi/well of Myo-[2-³H] Inositol (Amersham, PiscataWay, NJ) in labeling medium. After a 20h labeling, cells were washed and preincubated more
than 30 min at 37°C in labeling medium containing 10 mM LiCl. Cells were stimulated with various concentrations of ligands for 1h at 37°C with gentle agitation. After stimulation, the medium was removed and 200 μl of 0.1 M formic acid were added to each well to lyse the cells. IP quantitation was measured in cell extracts by the multiwell filtration method as described by Chengalvala et al., 1999.

Whole mount in situ hybridization

The method for in situ hybridization was described by Wu et al., 2006. Briefly, two probes were synthesized (about 400bp) targeting the 5’ untranslated region or 3’ untranslated region of each receptor. The sense probe as a negative control was synthesized at the same time. Embryos at 24hpf, 36hpf, 48hpf, and 72hpf were collected, dechorionated and preserved in 4% freshly made paraformaldehyde for 2h at room temperature or overnight at 4°C. After a wash with 1XPBS two times, embryos were transferred to cold 100% methanol for 10 min and stored in 100% methanol at -20°C until use. Embryos were rehydrated at 70% methanol/PBS, 50% methanol/PBS, 30% methanol/PBS and PBS twice with 5 min for each step. Then embryos were treated with proteinase K (10μg/ml) for different periods (5-12 min) and fixed again with 4% paraformaldehyde. After a 2h prehybridization, probes were added in a final concentration of approximately 0.5 ng/μl and embryos were incubated overnight at 65°C. After a series of washings at 65°C, embryos were incubated with antidig-alkaline phosphatases (Roche) in blocking solution (1:3000). Blue signals were developed in NBT/BCIP (Roche) coloration buffer, then stopped by washing twice in PBS. Embryos were dehydrated in a series of solutions until 100% methanol was reached. Embryos were
cleared in benzyl benzoate / benzyl alcohol (2:1). Signals were captured by a microscope (Leica DMIRE2) with a Retica 2000R fast camera. Images were imported into OpenLab software.

**Whole-mount immunocytochemistry**

Embryos preserved in methanol at -20°C were rehydrated, treated with 1% triton in PBS for 30’, washed in PBS twice and incubated overnight with blocking solution (10% normal goat serum, 2% BSA, 0.1% tween-20 in PBS). Embryos were incubated 48h-72h at 4°C with primary antiserum (polyclonal) raised in rabbits against the four different GnRH receptors. The antisera were a gift from Javier Tello who raised them in our laboratory against the N-terminal peptides (19-25 residues) of the receptors. I used the antisera at a dilution of 1:200 or 1:500 (5% normal goat serum, 0.1% tween-20 and sodium azide in PBS). After four washes with PBS at 15’ intervals each, embryos were incubated with a secondary antibody for 24h-48h at 4°C. The secondary antibody was Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen, Eugene, Oregon, USA) at a dilution of 1:500. The reaction was stopped by washing with PBS. Embryos were immersed in the mounting medium Vectorshield (Vector Laboratories Inc.), positioned on a slide and cover slipped. Signals were imaged with a confocal microscope (Nikon, C1-T-SM, Japan) using 10μm scans.

**Results**

*Isolation of 5’ and 3’ UTRs for four GnRH receptor cDNAs in zebrafish*
The ORFs of GnRH-R1, R2 and R4 were first isolated by Javier Tello. However, to detect specific signals by in situ hybridization, I used RACE to obtain full length cDNAs from total RNA of zebrafish brains; the sequences were determined in both the forward and reverse direction. These data showed that the zebrafish GnRH-R1 cDNA is 1757bp in length and encodes a 337 amino-acid protein (Fig. 5.1A). The full length cDNA of the GnRH-R1 blasted against the zebrafish genome database (Ensemble Multi BlastView, http://www.ensembl.org/multi/blastview) shows that the zebrafish GnRH-R1 gene spans approximately 21.7 kb on chromosome 14 and consists of 4 exons (Fig. 5.2A).

The zebrafish GnRH-R2 cDNA is 1159bp in length and encodes a 412 amino-acid protein (Fig. 5.1B). The full length cDNA of GnRH-R2 blasted against the zebrafish genome database (Ensemble Multi BlastView) shows that the zebrafish GnRH-R2 gene spans 4435bp on chromosome 7 and consists of 3 exons (Fig. 5.2B).

The zebrafish GnRH-R3 is 2336bp in length with an open reading frame of 1008bp (from 293-1300) encoding a 336 amino-acid protein (Fig. 5.1C). The full length cDNA of GnRH-R3 blasted against the zebrafish genome database (Ensemble Multi BlastView) failed to reveal the intron-exon structure due to the incompletely genome but the majority of the GnRH-R3 sequence localized on chromosome 16.

The zebrafish GnRH-R4 is 1861bp in length and encodes a 401 amino-acid protein (Fig. 5.1D). The full length cDNA of GnRH-R4 blasted against the zebrafish genome database (Ensemble MultiBlast view) shows that the zebrafish GnRH-R4 gene spans 11.6kb on chromosome 18 and consists of 4 exons (Fig. 5.2C).
Fig. 5.1. Nucleotide and amino acid sequences of four full length GnRH receptors. (A) GnRH-R1; (B) GnRH-R2; (C) GnRH-R3; (D) GnRH-R4. Coding sequences of amino acids are shown by Single letters in bold and listed below the nucleotides.

* represents stop codon. The regions that were used for design of the hybridization probes are shown with an underline.
GGCTGTACACACCCCTCCTTCCGCTCCGACCTGATACGCTTCTGCCTGGCCGACCAAC 1262
GLYTPSFRRDLSLLRFCCCRHH
AAGAACCCCGGCCGCCCTACGCAACCCACGACGACATAAACAAGGAGGAACACTGGA 1322
KNTPRPQQOPTHRH*
GAGACACTGGAAGGAACACTGGAAGGAGACACTGAAGAGATATACGAGGAACACTGGAAGG 1382
ACACTGAAAGATATACTAGAGGAACACTGCAGAAACACTGGAGGACACTGGAGGAACA 1442
CTGCAGAAACACTTCTAGGCAACTGGAAGGGACATTGAAAGGAACACTGCAGAAACTTCA 1502
TGAACACTGGAAGGAACACTGGAAGGAACACTGCAAGAAAGCAGTAGGACACTGGAAGGAC 1562
ACTGAAGGAATACACTGGGAGGACACTGCAAGAAACACTGGAGGACACTGGAAGGACACT 1622
GCAGAACACTGGAAGGAACACTGGAAGGAGATGTCGACTGGAGGGGACACTGCAGAAACACTGGA 1682
GGCAGAAGGAAACTTTACATGAAACACTGGGAGGAAACACTGGCAGAAACACTGGAAGGAC 1742
ACTGGAAGGAACACTGGAAGGAGATGTCGACTGGAGGGGACACTGGAAGGAAACACTGCACT 1802
GGGTAATACACTGCAAGAAACAAAGGGGACACTGGAAGGAAACTGGAAGGAGATGCACCTGGA 1862
GGGACACTGGGAAGGAAACTTTACATGAAACACTGGGAGGAAACACTGCAAGGAACACTGGAAC 1922
ACTGGAAGGAACACTGCAAGAAACACTGGAAGGAGTGCACGCTGGAAACACTGAAGGAAC 1982
AGGAAACACTGGAAGGAGATGTCGACTGGGAGGAAACACTGCAGAAACAGTAGGGGACGCTGAAAG 2042
AACGCTTCATGAAACACTGGAAGGAAACCTGCTGGAACACTGGGAGGAAACTGGAAGGAAAC 2102
TTCATGAAACACTGGAAGGAAACACTTCTGAAACACTGGAAGGAAACCTGCTGGAACACTGGGAG 2162
GAACACTGGAAGGAAACACTGCAGAAACACTGGAAGGAGCTCGGGAAGAAACACTTTCATGAACA 2222
CTGGAAGGAGCACTGGAAGGAGATGCACGTTGGAAGGACACTGCAAGAAACACTAAGGGGACGCTG 2282
AAGAAACACTTTCATGAAACACTGGAAGGAAACACTGCAAGGACACTGGGGAAGAAAC 2336
D

ACTCTGCAACTGAGGGAGAAACACCCAGCTAAGGATGCGGTGAAAGGCAACATATCGTGTAAT 62
ATCACCCGAAAATTACTTTCTGCAAAACTATTTAGTCTATTAAAGGTACTTTTCCTGAA 122
AAAAGCATAGATGGTGCTTGCAAAACTCGTACTAATCTTGGATGAGACGAATGTCACC 182
TTTCGAAAAGCTTGTGTTGTTGATAGGGAACCTCGTTTCTTTCATCACATCTCTGTGCA 242
TTTCGAACTTTGAAGATCGGGTTTGAAGCCACATGAAGAGATGGAATATTAATCAAGTTT 302
TTTTATGCTTGGAGATGTCTCAAAACAATAAATTTTCAAGGTCTATTTGATTTCTTCTAACC 362
CATCCCAATTGCATCAGCGCTGTAAATATATAAGGAGCTTTTTCATCTGAAATCTGAA 422
TCCTCTGCTGATCCCTACTGCAGCAGGCTGAGCAATATGCGACACACACACAGCTTTC 482
GCTTTCCGAGTGAATGAAATGAGCGATCCTGCTCCAACACTGAGAAACATCTAGATGTTTCCACAGCTG 542
M N D S S P T S E N I M F H Q L

ACTGCAGATATCTCTGAATGTGCGAGTTGATTTGCCACGCTGAACAAACAAATCAAGCTGAA 602
T A D T L N G S C D L P T C N N N T G E

GGCGGTCATTGGAGCTGCCACATTTCATAGGCAGCAAAGAGTGAGGGATGATAATACCTTC 662
A A L Q L P T S M A A K V R V I I T F

ACTCTTTGCGACAGGTGCTAGCTGTGCTGAACCTCGGCGTGGTGGGCTGCAGACCAACAC 722
T L C A V S A V C N L G V L W A A S T N

AACAAGCGCAAGTCCCAGATGCAGATCTCAAGTAATTTACATTAGACACCTCACTCGGCGACCTACTG 782
N K R K S H V R I L I M N L T V A D L L

GTGACTTTCTCATGTCGATGCGAGATGCGCAGGCTGGAACATCACAGTTCTAGTGCTGCCGQ 842
V T F I V M P V D A W N I T V Q W L A

GGAGATCTGCGCTTGACAGATGTCTTTAGTCTGAAAGCTCTGCTGGCATGCTACTCTCTGTGCA 902
G D L A C R L L M F L K L V A M Y S C A

TTTGTGACCCTGTCCTGCTGTTGTAACGCTAGCCATAGCAGACTCGGCCATTCACCCTCCTGCGCATT 962
F V T V V I S L D R Q S A I L N P L A I

AATAAGGCTAAAGAAAGAAGAAACAAAATAAGCTGAGCAGCTGGCCTGGCAGTGGAGGTGTGTTT 1022
N K A K K K K N K I M L S V A W A M S V V

CTCTCGGTCCCTACAGTGATTCTTGCTTTCCACATGTCAGCATGCTGGCAGCAGCAATTTTC 1082
L S V P Q M F L F H N V T I T V P A N F

ACTAGTGTACCTCGAGGAAGCTTCTGCTAAACACACTGCGAGAACCACATCTACAATATG 1142
T Q C T T R S F V K H W Q E T I Y N M

TTCACATTATCGCTCTTTCTGAGTCTCTCCTGGCCATCAGTCTGCTACACAGGG 1202
F T F I C L F L P L A I M I C C Y T R

ATTCTAGTGGAAAATCTTCGAGGAGGTGACCCAAAGGAACACAGTCTACTCAGCCGCTTCGAC 1262
I L V E I S R R M T K G N S H L R R S H
AGCAACATCCCCAAAGCCCGTATGAGGACTTTAAAATGAGCATAAGTTATCGTGACCTCC 1322
SNIPKARMRTLKMSSIVIVTS
TTTATAGTTTGGCTGGACTCCGTACTACCTGCTGGTCTCTGACTGGTTCTTCCTCCAGAG 1382
FIVCWTPYYLLGLWYWFLPE
GATTAGAGAGAGCCGTTTCTCACTCTCTGACTCCATGCGTTTTATTTTTGACTGTTT 1442
dleeETVSHSLTHTMLIFGGLF
AATGCAATCTGGATCCCCATCACTTTACGGCCTCTTACCCATCCACCTTTCCCAGAGAC 1502
NAILDPITYGLFTIFHRKGGL
AAGCGCTACTGTCGAGGCGGTCGTGCTCACCAGTCAGAGAATAACTCCATCCTACG 1562
KRYCRSAVVTESENNSTIT
GGCTCATGAAATGCTACCACTCCCATTTTCGATGAAAGAGTGACCCAGTCGGGCACA 1622
gsLKCSPSPFRMKRVTQGSCT
GGAACTGACCCAAAGCAACACAAAGACACGCTGAGGAGGAGATAAGAGGAGCTGAC 1682
GTDPKQNTSTVGEEDKKAAD
GGTTAAAACATAAAGAAATGTTGGGAAAGAAGACATCCACATTTGAGCAGCAGCTTATTGATT 1742
GKTKE*
AGAGATACGGATGACATGGCCAGCTTTCTGCTACAGCAGTCTAGATTACATACAA 1802
TGAGACAAAGAACACAAACACTGCTGTTTGCTCGACGAAACCATCTAAAAAAAAAA 1861
Fig. 5.2. Predicted structure of three zebrafish GnRH-R genes from the zebrafish genome database in Ensemble. (A) GnRH-R1; (B) GnRH-R2; (C) GnRH-4. The gene size is indicated on the right of the gene. Exons are shown as closed boxes and exon length is numbered above the box. A solid horizontal black line indicates introns and the distance between two exons is numbered above the intron line in A and C, but below the line in (B). The dashed line in (C) indicated the extent of the data not found in the genomic database.
Detection of GnRH-R mRNA by RT-PCR in early development of zebrafish

GnRH receptors were investigated for their expression pattern in early stages of development. To determine the earliest expression of GnRH-Rs in the embryo, we used three methods: RT-PCR, in situ hybridization (ISH) and immunocytochemistry (ICC). Our RT-PCR analysis showed the presence of mRNA for three GnRH-R (GnRH-R1, R2 and R4) at an early stage of 0.5hpf. But GnRH-R3 was not detected until 8hpf. The mRNA transcripts of GnRH-R1, R2, R4 were present additionally in seven later stages: 4hpf, 6hpf, 8hpf, 10hpf, 14hpf, 18hpf and 25.5hpf, whereas GnRH-R3 mRNA was only detected at 8hpf, 14hpf, 18hpf and 25.5hpf. Polymerase II (1150bp) was used as a control to verify the quality of the first-strand cDNA synthesized (Fig. 5.3).

Localization of GnRH-R4 mRNA and protein by ISH and ICC

The ISH and ICC results revealed that GnRH-R4 had the most distinct signals among all four receptors. By ISH, embryos at 24hpf showed two groups of GnRH-R4 mRNA signals (Fig. 5. 4A). One group is just below the olfactory placode near the eye (white arrow). Another group of cells are located in the cerebellar area (white arrowhead). At 36hpf (Fig. 5. 4B), a new group of cells was detected in the lateral telencephalon/diencephalon between the eyes (black arrow). Some of these cells were more ventral and close to the midline (black arrow Fig. 5. 4B inset). A single cell was detected also in the preoptic area (black arrowhead, Fig. 5. 4B inset). The number of cells
Fig. 5. 3. RT-PCR analysis of GnRH receptor (R) mRNA expression, compared with polymerase- II mRNA. Equal aliquots of first strand cDNA were used as templates in PCR reactions using specific primers to amplify GnRH-Rs, and polymerase-II at nine stages of development: 30 min, 1h 30 min, 4, 6, 8, 10, 14, 18 and 20h post fertilization. The negative control represents a PCR reaction done without template.
Fig. 5.4. Localization of GnRH receptor 4 (GnRH-R4) mRNA by *in situ* hybridization. (A) 24hpf, dorsal view with anterior at the top. Signals were detected just caudal to the olfactory placode, between the eyes (white arrow). The inset is a blow up of white dashed box. White arrowheads show signals in the cerebellum. (B) 36hpf, dorsal view with anterior at the top. Cells were labeled at the lateral telencephalon/diencephalon between the eyes. Top inset with black arrow is a blow up of the black dashed box. Some cells migrated toward the ventral brain. Bottom inset is a blow up of the dashed gray box with a black arrowhead pointing to a single cell in the preoptic area. The white arrowheads point to signals in the hindbrain. (C) 48hpf, dorsal view with anterior at the top. Inset shows signal near olfactory placode in the white dashed box. Black arrows show signals in the lateral midbrain and white arrow points to signals in the caudal midbrain/cerebellum. (D) Negative control of 24hpf embryos labeled with GnRH-R4 sense probe, dorsal view. No specific signals were detected. (E) 48hpf, lateral view with anterior to the right. Black arrow points to signals in the midbrain. (F) Negative control of 48hpf embryos labeled with GnRH-R4 sense probe, lateral view with anterior to the left. No specific signals were detected. Experiment was repeated at least three times from different embryos at different times of collection.
36hpf. The inset of Fig. 5. 4C is an enlargement of these two sites. Signals in the lateral midbrain (black arrow, Fig. 5. 4E) shown in the lateral view were compared with the negative control of sense probe (Fig. 5. 4F).

With ICC in embryos, at 24hpf (Fig. 5. 5A), GnRH-R4 neurons were faintly detected in the hindbrain (white arrowhead). The axons of GnRH-R4 projected toward midbrain, eye and hindbrain. At 36hpf (Fig. 5. 5B, C), GnRH-R4 neurons in the cerebellar area had long axons projecting toward the midbrain, inner eye and hindbrain along the notochord and spinal cord (white arrowhead). By 48hpf, a striking distribution of neurons and fibers was observed in the spinal cord with some fibers crossing to the opposite side and others remaining ipsilateral (white arrowhead, Fig. 5. 5D, E). Many punctuate areas are labeled throughout the brain and may represent small cell bodies, axons or dendrites containing GnRH receptors. The GnRH-R4 labeled neurons match the seven clusters of reticulospinal neurons described by Kimmel’s group by morphological criteria only (Metcalf et al., 1986). The large Mauthner cell is identified by the GnRH-R4 labeling; the third cluster shows the giant cells with axons crossing the midline. The other neurons have ipsilateral axons that are labelled but do not cross. In addition, a group of cells is labeled just caudal to the eye; the axons enter the lateral spinal cord. These neurons are in the area of the trigeminal ganglion (Mueller and Wullimann, 2005). The axons form a path that is lateral to the MLF (Medial Longitudinal Fasciculus), which is the route for the descending trigeminal axons (Vth cranial nerve) (Wullimann et al., 1996).
Fig. 5.5. Localization of GnRH receptor 4 (GnRH-R4) protein by immunocytochemistry. (A) 24hpf, lateral view with anterior to the top. A neuron is detected at the base of cerebellum (midbrain/hindbrain boundary) with axon projections toward the midbrain, eye and hindbrain (white arrowhead). (B) 36hpf, lateral view with anterior to the top. Cells are labeled at the base of the cerebellum and along the spinal cord (white arrowhead). (C) 36hpf, dorsal view with anterior toward top. Cells are labeled at the base of cerebellum and along the spinal cord and notochord (white arrowhead). (D) 48hpf, dorso-lateral view with anterior toward top. Nerve fibers surround the eye, and are in the spinal cord. White arrowhead show neurons with GnRH-R4. (E) 48hpf, dorsal view with anterior to the top. Nerve fibers are in spinal cord where some cross to the opposite side. White arrows show neurons with GnRH-R4. (F) 48hpf, dorsal view with anterior up. White arrows show signal near olfactory placode. (G) 72hpf, lateral view with anterior to the top. GnRH-R4 protein was detected in the midbrain (purple arrow). (H) 48hpf, negative control in which embryos were exposed to prebleed serum. No signal was detected. Experiment was repeated three times from different embryos of different collecting time.
Meanwhile, a single cell body was also detected below the olfactory placode near the eyes (white arrow, Fig. 5. 5F). At 72hpf, more neurons were detected in the midbrain (purple arrow, Fig. 5. 5G). The ISH and ICC matched each other as they both detected signals in the midbrain, cerebellum and near the olfactory placode. The negative controls using sense probes and prebleeding serum had no specific signals at 36hpf (data not shown) and 48hpf (Fig. 5. 5H).

**Localization of GnRH-R3 mRNA and and GnRH-R2 by ISH**

With ISH, GnRH-R3 mRNA was first detected at 48hpf (Fig. 5. 6A, B). Signals were located at the border of the olfactory placode and olfactory bulb (white arrow, Fig. 5.6A). Also, strong signals were detected (Fig. 5.6 A, B) in the ventral preoptic area and hypothalamus (white arrowhead), cranial ganglia (black arrow) and midbrain/cerebellum (black arrowhead). Todate, labeling has not been detected for GnRH-R2

**Localization of GnRH-R1 mRNA and protein by ISH and ICC**

With ISH, GnRH-R1 mRNA was first detected at 24hpf in the preoptico/hypothalamic area (white arrow, Fig. 5. 7A). At 48hpf (Fig. 5. 7B, C), a larger cluster of cells was detected. One population was observed in the forebrain between the olfactory placodes (white arrow). Another population was along the ventral preoptic area around the eye (white arrow head). A third population were detected in the telencephalon (black arrow) and lateral midbrain and hindbrain (black arrow head). The fourth population was strongly expressed in the heart (white double arrow, Fig. 5. 7D). At 72hpf, more cells expressed GnRH-R1 mRNA around the dorsal and ventral eye (Fig. 5. 7E). However,
Fig. 5.6. Localization of GnRH receptor 3 (GnRH-R3) mRNA by *in situ* hybridization (A-B). (A) 48 hours post fertilization (hpf), ventral view, anterior on the top. Signals were detected at the border of olfactory placode and olfactory bulb (white arrow); at preoptico/hypothalamic area (white arrowhead); and at a cranial ganglia (black arrow). (B) 72hpf, lateral view with anterior to the right. Signals were detected at preoptico/hypothalamic area (white arrowhead), in a cranial ganglia (black arrow), and in the midbrain, midbrain/hindbrain boundary (black arrowhead).
Fig. 5.7. Localization of GnRH receptor 1 (GnRH-R1) mRNA and protein by *in situ* hybridization (ISH) and immunocytochemistry (ICC). ISH (A-E) and ICC (F-G). (A) 24 hours post fertilization (hpf), ventral view with anterior on top. Signals were detected in the hypothalamic region (white arrows). (B) 48hpf, ventral view with anterior on top. Signals were detected in the forebrain near the olfactory placode (white arrows). (C) 48hpf, lateral view with anterior toward the right. Black arrow points to a signal in the telencephalon; black arrowhead points to signals on the midbrain/hindbrain boundary; white arrowheads show signals in the preoptico/hypothalamic area. Caudal arrow head shows putative trigeminal ganglion (D) 48hpf, lateral view. Signals of GnRH-R1 in the heart (white double arrow). (E) 72hpf, lateral view, signals were detected in the preoptic/hypothalamus area (white arrowhead); midbrain/hindbrain (black arrow head); telencephalon (black arrow). (F) 48hpf, lateral view with anterior to the right. GnRH-R1 protein was detected in the heart (white double arrow). (G) Negative control. Embryo was exposed to prebleed serum.
ICC signals were only detected in the heart at 48h (white double arrow, Fig. 5. 7F). There is no signal in the negative control at 48hpf (Fig. 5. 7G).

**Functional analysis of zebrafish GnRH-R3**

To evaluate whether the identified GnRH-R3 has physiological activity, GnRH-R3 was transfected into COS7L cells. The function of GnRH-R1, R2 and R4 were tested by Javier Tello after he isolated the ORFs. The major signaling pathway of GnRH-R in all vertebrates is Phospholipase C, this path was tested with [³H] inositol phosphate assasy (IP₃). In COS7L cells expressing the GnRH-R3, both native peptides GnRH2 and GnRH3 were able to activate IP₃ accumulation in a dose dependent manner with different half maximal response (EC₅₀) values (Fig. 5.8). The EC₅₀ values of GnRH2 is 1.74X10⁻¹⁰ M and GnRH3 is 1.16X10⁻⁶ M. Thus, GnRH2 has much higher potency (6667 fold) than GnRH3 to stimulate GnRH-R3. The EC₅₀ value of GnRH2 is in the physiological range (10⁻⁹ M to 10⁻¹² M) and the EC₅₀ value of GnRH3 is near the pharmacological doses (10⁻⁶ M) (Hadley and Levine, 2006).

**Discussion**

**cDNAs of four GnRH receptors have distinct 5’ and 3’ UTRs**

A comparison of the organization of the three available gene structures of GnRH-R1, GnRH-R2 and GnRH-R4 (Fig. 5.2), shows that the GnRH receptor in zebrafish vary in length from 4.4kb to 21.7kb and have 3 or 4 exons. Each of the four receptors is present
Fig. 5.8. Accumulation of inositol phosphate (IP) in COS 7L cells transfected with GnRH receptor 3 (GnRH-R3) and stimulated with graded concentrations of zebrafish GnRH2 and GnRH3 peptides. Values represent means ± SEM from three independent experiments each in triplicate.
on a different chromosome suggesting they are the result of large scale genomic duplications. GnRH-R1 has four exons and has the longest intron; the gene spans approximately 21.7kb in length. GnRH-R2 has the shortest structure and only contains 3 exons; this gene is only about 4.4kb in length. GnRH-R4 also has four exons but the gene is shorter than GnRH-R1 and spans 11.6kb in length. The GnRH-R3 gene structure couldn’t be predicted due to the incomplete GeneBank database. However, the zebrafish GnRH-R3 can activate the inositol phosphate signaling pathway and is highly selective for GnRH2. The activities of the four GnRH-Rs are described in Tello, Wu et al., (submitted).

When comparing the cDNA sequence of each of the four receptors, I found they exhibit distinct structures in the 5’ and 3’ untranslated region. The length of 5’ untranslated sequences are 5’R4 (≈ 490bp) > 5’R1 (≈ 430bp) > 5’R3 (≈ 290 bp) > 5’R2 (≈ 200bp). The length of 3’ untranslated sequences are 3’R3 (≈ 1000bp) > 3’R1 (≈ 180bp) >3’R4 (≈ 135bp) > 3’R2 (≈ 125bp). The most unusual result is that GnRH-R3 has a long 3’ untranslated region with many repeated sequences: 32 repeats of CACTGGA, 9 repeats of CACTGAA and 6 repeats of CACTGCA. The function of these repeats remains unclear. The structures of the coding sequence of the four receptors are not discussed here, but are described in detail in another paper (Tello, Wu et al., submitted).

In brief, each receptor has all the components of a typical 7 transmembrane receptor including a C-terminal tail.

*Four GnRH receptors are expressed early in specific brain regions*
All the GnRH-Rs except GnRH-R3 were detected at 30 min post fertilization by RT-PCR, which means they are maternal transcripts, as zygotic transcription begins in the zebrafish only at 3–4 hpf (Kane and Kimmel, 1993). This result differs from Whitlock’s paper, as they detected mRNA of GnRH receptors at 36hpf (Whitlock et al., 2006), which is much later than our results. There are probably a few reasons: first, they used different numbers of embryos for isolation of mRNA or total RNA; second, their primers were less sensitive; third, their techniques were different. However, there are no other data for comparison of expression of GnRH receptors in early development of zebrafish.

With in situ hybridization (ISH) in the present study, zebrafish GnRH-R4 and GnRH-R1 signals were detected at 24hpf in different positions. GnRH-R4 signals were detected just caudal to the olfactory placode and at the midbrain/hindbrain boundary. GnRH-R1 signals were detected at the preoptico/hypothalamic area. At 24hpf using immunocytochemistry (ICC), I detected GnRH-R4 protein in the hindbrain with the neuronal axons projecting toward the midbrain, eye and hindbrain. These results again differ from the Whitlock’s study (2006), in which they did not detect signals until 56hpf for GnRH-R4 and GnRH-R3. However, these early expressions of GnRH-R mRNA and protein in our study and the location of expression match our previous observations. We found that if GnRH2 and / or GnRH3 are knocked down, the gene expression pattern of fgf8, pax2.1, pax6.1 and mab is altered in the area of midbrain, midbrain/hindbrain boundary (near the cerebellum), forebrain and eye (Wu et al., 2006). More cells expressing GnRH-Rs were detected as zebrafish embryo development. A group of GnRH-R4 labeled cells (36hpf, 48hpf) had mRNA that was detected lateral of the
midbrain and some of them toward the midline; also the midbrain expressed the GnRH2 mRNA (Gopinath, *et al.*, 2004; Palevitch, *et al.*, 2006; Wu *et al.*, 2006).

At 48hpf, GnRH-R1 mRNA signals were detected in the eye stalk of the forebrain near to the olfactory placode. GnRH-R3 was detected at the border of the olfactory placode and olfactory bulb. Interestingly, the signals of GnRH-R1, GnRH-R3 and GnRH-R4 mRNA were all expressed in the preoptico/ hypothalamic area and in cranial ganglia (trigeminal, V), lateral midbrain and midbrain/ hindbrain boundary area. However, in the heart, the GnRH-R1 proteins were only detected at 48hpf and in the preoptic/hypothalamic area GnRH-R3 proteins were not detected until 56hpf. The delay in protein detection or the reduced abundance compared to mRNA probably is due to the embryos being too thick for better scanning of signals. In Whitlock’s paper, GnRH-R4 was expressed in the lateral midbrain at 56hpf and GnRH-R3 mRNA was found near the ganglia cells, which is the same location as we detected at 36hpf and 48hpf. Additionally, other locations for GnRH-R1, R3 and R4 were detected with my 5’ probe (targeted to the 5’ UTR of cDNA) and 3’ probe (targeted to the 3’ UTR of cDNA). The negative control of the prebleeding serum and sense probe didn’t label any specific signals. In rat, the GnRH-R was first detected in pituitary primordia of 13-day-old fetuses by autoradiography (Jennes, 1990). However, in human GnRH-R was detected at 8-cell embryo by RT-PCR (Casanã et al., 1999).

**GnRH receptors in embryos are expressed in classical and unconventional sites**

GnRH-R localization has recently been studied in our lab in adult fish brains and all four GnRH-Rs were localized in the pituitary (Tello, Wu, *et al.*, submitted). Similar to
expression in zebrafish embryos, GnRH-R4 was also expressed in the adult zebrafish midbrain and hindbrain, in areas that are associated with control of muscle movement and swimming behavior (Uematsu and Todo, 1997; Wullimann, 1998). In the present study, beginning at 36hpf, the Purkinje cells expressed GnRH-R4 proteins and the axons extended along the spinal cord, which is similar with the zebrafish adult brain (Tello, Wu, et al., submitted). In adult seabass, GnRH-R4 mRNA was detected in the forebrain, ventral telecephalon, preoptic area and hypothalamus (Gonzalez-Martinez et al., 2004), and in our zebrafish, GnRH-R2, R3 and R4 also were expressed in the embryonic brain. In adult cichlid fish, the GnRH-R positive neurons and fibers were detected in the optic tectum and cerebellum, and hindbrain, in addition to the pituitary, olfactory bulb and/or telencephalic regions (Chen and Fernald, 2006). Our observations in early development also matched this expression pattern. In the retina, GnRH and GnRH-R were also detected in four coral fishes and other teleost fish (Maruska and Tricas, 2007). Our GnRH-R4 labeled neurons also project axons into the eye of the zebrafish. However, our most novel result was the presence of GnRH-Rs in motor neurons and fibers including the Mauthner neurons. The GnRH-R4 labeled nerves showed that seven clusters of neurons with axons in the MLF are present. This location for GnRH receptors in Mauthner cells suggests GnRH is involved in this escape motor system that is fundamental to survival (Zottoli and Donald, 2000).

The wide expression of GnRH-Rs in different fish and in different stages of development suggests that GnRH has multiple functions in addition to reproduction. The multiple areas related to motor control and sensory systems including the lateral line where we detected GnRH-Rs in zebrafish and evidence from other fish suggest GnRH
modulates movement, influences visual processing, and acts as a neuromodulator not only in adult fish but also in developing fish. These results further confirm that GnRH has effects on nonreproductive functions.
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Chapter 6

Phenotype of adult and developing mice with GnRH receptor disruption
Introduction

Evidence presented in Chapters 4 and 5 suggests that the GnRH-Rs in zebrafish are expressed early in development in a variety of tissues, especially nerve cells. Also, temporary loss of GnRH peptides in zebrafish during early development results in changes in the brain. To further investigate the role of GnRH in early development, before birth, we selected a mouse model in addition to zebrafish so that the GnRH-R gene could be permanently disrupted. The mouse is the only vertebrate in which a specific gene can be targeted for preparation of a transgenic animal.

In humans, mutations in the GnRH receptor gene have resulted in hypogonadotropic hypogonadism (HH), which is defined by low plasma levels of gonadal sexual hormone and low or normal gonadotropins (LH and FSH) (Iovane et al., 2004). There are two types of gonadotropic deficiency in human: one is combined with anosmia called Kallmann’s syndrome (KS) (Kallmann et al., 1944); another one is a normal sense of smell called normosmic idiopathic hypogonadotropic hypogonadism (nIHH) (Boyar et al., 1976). Patients with these syndromes show no evidence of puberty and male patients have small testes and female patients have primary amenorrhea. The mutation genes related to KS and IHH are: *Kallmann syndrome1 sequence (KAL1), FGF receptor1 (FGFR1), prokineticin receptor2 (PROKR2), Prokineticin 2 (PROK2), gonadotropin-releasing hormone (GNRH) and its receptor (GNRHR), G-protein coupled receptor 54 (GPR54), KISS-1, nasal embryonic LHRH factor (NELF)* (Pitteloud et al., 2007). However, these genes influence reproductive function through the brain-pituitary-gonadal axis. In the brain, gonadotropin-releasing hormone (GnRH) is one of the key neuropeptides that control reproduction by acting on its G-protein coupled receptor
(GnRHR) to stimulate the synthesis and release of gonadotropins. So far 19 mutations in the GnRHR gene have been reported from patients with HH (Tao et al., 2006) and many of them could be rescued by pharmacological chaperones (Janovick et al., 2002; Leanos-Miranda et al., 2002). Numerous medical therapies such as targeted gene therapy also have been intensively investigated because more than 80% of ovarian and endometrial cancers and more than 50% of breast cancers express the GnRH receptor (Gründker et al., 2002).

To investigate the neural control of reproduction, the GPR54 and Kiss-1 genes were recently knocked out in mice using gene targeting strategy (De Roux et al., 2003; Funes et al., 2003; Kaiser UB and Kuohung W, 2005; Smith et al., 2006; Smith et al., 2007; Pitteloud et al., 2007; Luque et al., 2007). In the present study, we generated a disrupted GnRH receptor (Gnhrhr) mouse line, with a HH phenotype. This mouse line is different from the line containing an ENU-induced point mutation in the GnRH receptor (Pask et al., 2005). In our mouse line, exon2 and exon3 of the Gnhrhr gene were deleted by using the gene trap method. Also, a lacZ gene in the gene trap which encodes β-galactosidase facilitated the observation of GnRHR expression. The gene trap phenotype was more severe than the ENU-induced mutant. Our targeted mouse line together with the ENU-induced mutant mouse line offer unique models for studying the HH syndrome.

**Materials and Methods**

**Gene targeting and generation of gnhrhr mutant mice**
Embryonic stem (ES) cells containing Gnrhr gene trap clone 181A6 were obtained from the Centre for Modelling Human Disease at the Toronto Center for Phenogenomics. This gene trap clone was created by using the gene trap vector Gen-SD5 and R1 ES cells (129 S1/SV). The strategy for disrupting the Gnrhr gene was to insert a vector into an intron; the vector disrupted transcription beyond its point of insertion. The Gen-SD5 vector has a splice acceptor site upstream of the β-galactosidase gene followed by a three-frame stop codon. The insertion of Gen-SD5 into an intron produces a β-gal fusion protein and creates a null allele by truncating the host transcript (Fig. 6.1A). Furthermore, the Gen-SD5 vector carries a PGK promoter-driven neomycin gene with a three-frame stop codon followed by a splice donor site. In theory, this neomycin transcript could splice into downstream exons and create a chimeric mRNA that would be a candidate for nonsense-mediated RNA decay (Fig. 6.1A). The Gnrhr trapped gene sequence was obtained from Dr. Stanford’s lab (University of Toronto) where it was sequenced by 3’RACE and inversed PCR.

At our request, chimera mice were generated in the Centre for Molecular Medicine and Therapeutics (CMMT) at the University of British Columbia. Briefly, ES cells were injected into C57Bl/6J blastocysts, and then implanted into pseudopregnant foster mice. Seven-week-old chimeric males were cross bred with 6-week-old C57Bl/6J female mice (Jackson lab) for germline transmission of the mutated allele. The heterozygotes were bred to generate homozygous animals for Gnrhr targeted disruption.

**Animal housing**
All mice were kept in cages of 1-4 animals with food and water *ad libitum*. Mice were housed at 21°C with a 12h light cycle. All procedures were approved by the Animal Care Committee of University of Victoria.

**ES cell culture and genotyping design**

ES cells with the disrupted *Gnrhr* gene were cultured in our lab for isolation of genomic DNA. The procedure for culturing ES cells followed the protocol of Mouse Embryonic Stem (ES) Cell Culture manual (Invitrogen). ES cells were collected after reaching 70% confluency and ES cell genomic DNA was isolated with a DNAeasy kit (QIAGEN). For genotyping, two primers: *gnrhr*-5pF4 and *gnrhr*-int1-3pR1 were designed to a region outside of the insertion vector at the 5' and 3' flanking position and one primer 3p-trap-F2 was located inside of the vector at a 3' position (Fig. 6.1B). These 3 primers together give two bands of PCR product: Wild-type (260bp) by *gnrhr*-5pF4 and *gnrhr*-int1-3pR1 and mutant (744bp) by 3p-trap-F2 and *gnrhr*-int1-3pR1. Primer sequences are: *gnrhr*-5pF4 TCTAGGCATTTCCCCAATCCA; 3p-trap-F2 GAGCCCCCAATGAAAGAC; *gnrhr*-int1-3pR1 acaatgaaacgtgacgacg. The PCR reaction was carried out as follows: 94°C for 2 min, 35 cycles with 94 °C for 30 s, 54 °C for 30 s, 72°C for 1 min and last cycle at 72°C for 7 min. Genotyping mice of a different age was conducted with the same PCR conditions but the genomic DNA was extracted in a more economical way. A clipping from the ear or tail was collected from the mice and put into 10% chelex-100 resin (Bio-Rad, Hercules, CA) with 0.1% tween-20 and 0.1mg/ml proteinase K. Samples were incubated at 50°C for 45 min; proteinase K was inactivated at 95°C for 15 min; and the solution was cooled down at 4°C. One μl of supernatant was removed for PCR
Fig. 6.1 Characterization of the GnRH receptor (Gnrhr) gene trap clone and genotyping of transgenic mice. (A) and (C) are contributed by Michael Wilson.

(A) Overview of the gene trap insertion into the Gnrhr gene. Probe location and enzyme restriction sites were labeled accordingly. (B) Primers for genotyping are labeled on the targeted Gnrhr gene. (C) Southern blot screening of offspring from Gnrhr^{gt/wt} mice intercrosses using NcoI, EcoRI and the Gnrhr intron1 probe; and EcoRI with neo probe. NcoI digestion produced a wild-type band of 3.9kb and a mutant band of 4.6kb; EcoRI digestion produced a wild-type band of 6.7bp and a mutant band of 4.8bp; and EcoRI and the neomycin probe gave a 4.8kb band only in null (-/-) and heterozygous (+/-) mice. (D) RT-PCR for genotyping of genomic DNA confirmed that the wild-type band was 260bp, and the mutant band was 744bp.
南方印迹

南方印迹是一种用于验证单个插入的工具。使用尾部剪切的野生型、杂合型和纯合型小鼠的尾部组织，南方印迹分析被进行，正如之前所述（Hames and Higgins, 1995）。基因组DNA被用EcoRI或NcoI消化，并设计了两个探针以靶向内含子1和载体，分别。内含子探针1的引物是：3p-probe F1 5′CAGTCACGAAACATCGCCTTA3′和3p-probe R1 5′GCAGAGGTCTTTTCAGGATG-3。新霉素-盒探针，它只靶向载体的新霉素序列，是一个来自Michael Wilson博士的礼物。

表型

野生型、杂合型和无小鼠被每10天称重一次。性成熟度的雌性是由阴道开口的有无来判断的。阴道涂片用0.9% NaCl溶液获取。生殖脏器（睾丸和卵巢）的重量，脑和垂体的重量被测量在2个月大的小鼠。

对于生育能力的评估，5只同型基因诱捕小鼠的每一种性别被放在一个笼子里和一个野生型的一只相反性别的小鼠。怀孕被用作生育能力的证明。

整体制备β-半乳糖苷酶染色

为了检测GHRH-R表达模式在同型小鼠中，从同窝的同一胎的9.5 dpc（受精后天数）到18.5 dpc小鼠都被基因型鉴定并固定在冰上。
type band was 6.7kb in length and the mutant band was 4.8kb in length. Thus, wild-type genomic DNA had one band (6.7kb); heterozygous genomic DNA had two bands (6.7kb and 4.8kb); homozygous genomic DNA had one band (4.8kb) (Fig. 6. 1C). Genomic DNA was also separately digested with NcoI with intron1 probe, which produced the same pattern except the sizes of bands were different (mutant 4.6kb and wild-type 3.9kb) (Fig. 6. 1C). Genotyping of wild-type, heterozygous and homozygous mice with primers shown in Fig. 6.1B resulted in a RT-PCR mutant band of 744bp and a wild-type band of 260bp (Fig. 6.1D).

Crossing germline chimeras with C57 BL/6J wild type mice produced heterozygous mice for the gene trap allele 129 S1/Sv/C57 BL6 Jax-Gnrhr<sup>gt1NMS</sup> (designated here after as GT-Gnrhr).

**Characterization of gene trap Gnrhr-null mice**

*GT-Gnrhr* mice were viable and born with expected frequency. The ratio of offspring of heterozygous crosses was: 85/187/87 (WT/heterozygous/homozygous). The major gross anatomical or developmental abnormalities were external and internal sex organs. The null males were distinguishable from wild type or heterozygous males by the extremely small size of their genital papilla and reduced anal-genital distance (Fig. 6.2A). The testes of 2-month-old mutant mice were pale and weighted only 1/32 of wild-type testes (Fig. 6.2B and Table 6.1). The size of the secondary sex glands such as the androgen-dependent seminal vesicle and epididymis were dramatically reduced in null mice compared with wild-type littermates (Fig. 6.2 C).
Fig. 6.2 Gross analysis of the male reproductive organs of 2-month-old wild-type (+/+) and GT-Gnrhr null mice (−/−).

(A) Two external views of wild-type and null mice, showing dramatically different size of penis (arrows). (B) Testes (white arrow) and seminal vesicles (white arrowhead) of null mice were extremely small and pale compared with the organs in wild-type mice. (C) Epididymis is small and pale in null mice compared with wild-type mice (white arrow).

scale bar: 1cm
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<td>1.08±0.10</td>
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<td>463.1±10.94</td>
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P is a statistic analysis value; space without data means no significant difference. N is the number of samples.
Null females are only distinguishable from wild type littermates by having a delayed opening of the vagina and a pin point vaginal opening. In about 25% of null mice the vagina was not open even by 6 months. There is a significant delay in vagina opening of null mice compared with the wild-type and heterozygous mice even though data are not included for null mice in which the vagina never opens (Fig. 6.3). The vaginal smears where physically possible showed that null females did not have estrous cycles (data not shown). The uterus was thread-like and the ovaries of 2-month-old null mice (−/−) weighted only 1/4 of sex-matched wild-type littermates (Fig. 6.4; Table 6.1).

The null mice were infertile as shown by absence of pregnancy when wild-type females were paired with null males or null females were paired with wild-type males.

The body weight of male and female mice at different days after birth were compared among wild-type (+/+), heterozygous (+/−) and null (−/−) mice. There was no significant difference at any age among male mice or female mice (P>0.05), except at 40 and 60 days; null male mice were significantly lighter than heterozygous mice (P< 0.05) (Fig. 6.5 A, B).

The weights of the brain and pituitary were not significantly different between wild-type and null mice (Table 6.1). Also, pituitary structure and shape appeared similar in null and wild-type 2-month-old mice (Fig. 6.6).

**Histological analysis of gene trap Gnrhr - null mice**

Histological sections and photographs of adult gonads were done by Emma Isaac. The testis of a wild-type male mouse showed different stages of spermatogenesis and
Fig. 6.3 Day of vaginal opening in female mice.

The day of vaginal opening in null female mice is significant delayed compared with heterozygous (P<0.001) and wild-type (P<0.01) mice. Data do not include unopened vaginas. Values are mean ± SEM.
Fig. 6.4 Female reproductive organs of 2-month-old wild-type and GT-Gnhr null mice.

Uterine horns (arrowhead) and ovaries (arrow) of null mice are dramatically reduced in size compared with wild-type mice. scale bar: 1cm
Fig. 6.5 Body weight in mice from birth to 60 days.

(A) The body weight of null male mice was significantly (P<0.05) smaller only at days 40 and 60 compared with heterozygous mice, but was not significantly different from wild-type mice. (B) There was no significant difference among female wild-type, heterozygous and null mice. Also there was no significant difference among the three genotypes at birth (data not shown). * represents statistical difference. Data are means ± SEM.
Fig. 6.6 Pituitaries of 2-month-old wild-type and GT-Gnrhr null mice. There was no difference in size or gross structure.
relatively large numbers of Leydig cells (Fig. 6.7A, C, E). However, the spermatogenesis in null mice only proceeded to an early stage as spermatogonia, primary spermatocytes, and meiotic division were not observed; Leydig cells were rarely seen in the interstitial tissue (Fig. 6.7. B, D, F).

In the wild-type female ovary, there are different stages of follicles (Fig. 6.8A, C) but in the null female ovary, follicles only proceeded to the early antral stage (Fig. 6.8 B, D). Also, in null mice corpora lutea were not found and many atretic follicles were observed.

**Hormone assays**

The most dramatic hormonal reduction was FSH in null male mice (3.39 ± 0.86 ng/ml, N=15), which was significantly lower (P<0.0001) than wild-type mice (33.47 ± 2.83 ng/ml, N=11) (Fig. 6.9A). Also the testosterone of null male mice (0.06 ± 0.00 ng/ml, N=15) was significantly lower (P= 0.0041) than in the wild-type male mice (0.72 ± 0.24 ng/ml, N=11) (Fig. 6.9B).

The progesterone of null female mice (3.20 ± 0.37 ng/ml, N=13) was significantly lower (P= 0.039) than the level in wild-type female mice ((9.35 ± 0.3.5 ng/ml, N=8) (Fig. 6.9C). The FSH of null female mice (2.34 ± 0.53 ng/ml, N=11) was significantly lower (P=0.041) than the wild-type female mice (8.02 ± 3.39 ng/ml, N=6) (Fig.6.9A). In null female mice, LH (0.34 ± 0.03 ng/ml, N=13) was significantly (P=0.0017) lower than the level in the wild-type female mice (0.6 ± 0.08 ng/ml, N=15) but LH in null and wild-type male mice was not significantly different (Fig. 6. 9D). However, the LH data is not accurate due to the limited serum volume and low concentration of LH, which fell at the “low end” of the standard curve, where quantitative discrimination is poor (personal
Fig. 6.7 Histological Analysis of testes (2-month old mice).

Histological photographs show wild-type (+/+ ) testis (A, C, E) and null (−/−) testis (B, D, F). Null mice have relatively small seminiferous tubules (black circle, B) compared with wild-type testis (black circle, A). (C) and (D) magnification with 20X objective, scale bar: 30 μm. (E) and (F) 63X objective. Wild-type tubules have different stages of spermatogenesis with early spermatids (green arrow, E) and late spermatids (red arrow, E). Spermatogonium (yellow arrow) and primary spermatocyte (blue arrow, E) are presented also in a wild-type testis. Leydig cells are present in the interstitial area of a wild-type testis (white arrow, C and E). However, null mice expressed more elongated cells (black arrows, F) and myoid cells (white arrow, F) instead of Leydig cells in the interstitial tissue.

Inside of seminiferous tubules of null male mice, most cells are spermatogonia (yellow arrow, F) with a few primary spermatocyte (blue arrow, F). Early spermatids were not detected in null mice. However, many small elongated shaped cells existed in the seminiferous tubules (red arrow, F)
Fig. 6.8 Histological analysis of ovaries (2-month-old mice)

Histological photographs show wild-type (+/+) (A, C) and null (-/-) ovary (B, D). (C) and (D) are blow ups of wild-type and null ovary (A) and (B). The follicle of the wild-type ovary has an antrum (white arrow) close to the egg (A) and (C); the null ovary only has an early stage antral follicle with an antrum that is only partly around the egg and more distant (white arrow, D). The null ovary has no corpus luteum and more atretic follicles (black circle). Black arrow points to the corpus luteum in (A). Scale bar: 15 μm in (C) and (D). Sections and photographs were prepared by Emma Isaac.
Fig. 6.9. Circulating hormone levels of 2-month-old mice.

(A) FSH; (B) testosterone (male); (C) progesterone (female); (D) LH. *, represents statistical difference (P<0.05) by an unpaired t-test. Wild-type (+/+); null (-/-). Data are means ± SEM
communication with Dr. A.F. Parlow).

Immunohistochemistry labeling of LH and prolactin is shown in Fig. 6.10 (A). The number of LH expressing cells in the pituitary between wild-type and null mice were not significantly different (Fig. 6.10B). A large number of PRL-expressing cells were detected, but were difficult to count accurately in either wild-type or null mice.

**GnRH receptor protein expression detected with β-galactosidase**

As the null mice and heterozygous mice have an insertion of a reporter gene, the lacZ gene, the expected expression of GnRH receptor proteins encoded by exon1 can be detected by the enzymatic activity of the gene product β-galactosidase. In the present experiment, GnRH receptor (β-gal) proteins were first detected at 14.5 dpc in the olfactory bulb (data not shown) and at 15.5 days post coitus (dpc) in wide spread tissues (arrow, Fig. 6.11 A, B). At 16.5dpc, strong signals were found in the lateral nose cavity area (arrow, Fig. 6.11 D). At 15.5 dpc, GnRH receptor (β-gal) protein (product of exon1) is detectable at the anterior pituitary (Fig. 6.11C) and is expressed more strongly at 17.5 dpc (arrow, Fig. 6.11E). As expected, the wild-type embryos have no signal (Fig. 6.11 F, G, H, I).
Fig. 6.10 Pituitary LH and prolactin expression in 2-month-old mice.

(A) Immunohistochemistry of LH and PRL in pituitaries. Antibody labeling is shown in brown. Tissue is counterstained with Eosin. Scale bar: 15 μm. Middle insert is a blow up of a cell labelled with antibody.

(B) Statistical analysis of immunopositive cell counts. The number represents the positive cells per field of view under the 40X objective (8 fields per pituitary were counted). Wild-type (+/+); null (-/-). Pictures and cell counts were conducted by Emma Issac.
Fig. 6.11 β-gal staining of targeted GnRH receptor protein in null mice.

As the lacZ gene was driven by the Gnrhr gene promoter (see Fig. 1A), the GnRH receptor expression pattern could be detected by β-galactosidase activity in null mice. (A) and (B) A few single cells were detected (arrows) in the olfactory placode of the 15.5 dpc embryo. (B) A blow up of (A) is shown. (C) Signals were present in the anterior pituitary (arrow) of 15.5 dpc embryo. (D) At 16.5 dpc, β-gal is detected in the lateral nasal cavity area (arrow). (E) At 17.5 dpc, signals were detected at the anterior pituitary. (F-I) Wild type embryos at the same age as the null mice do not express the same signals as wild-type embryos, as they do not have the lacZ gene insertion or its product, β-galactosidase (β-gal).
Discussion

The present study demonstrates that a gene trap disruption of the GnRH-R in mice results in loss of fertility in both sexes. Sexual development does not occur and key reproductive hormones remains low including FSH, testosterone and progesterone. We compare our GT-Gnhr null mice with a spontaneous GnRH gene deletion mouse (aka hypogonadotropic hypogonadal or hpg mouse) (Cattanach et al., 1977), an ENU-induced point mutation Gnhr mutant mice (ENU-Gnhr) (Pask et al., 2005), a null GPR 54 mouse (de Roux et al., 2003; Seminara et al., 2003; Funes et al., 2003) and null Kiss-l mouse (d’Anglenont de Tassigny et al., 2007; Lapatto et al., 2007). The GnRH null mouse lacks exons 3 and 4 in the gene, which results in failure to process GnRH properly. The ENU-induced point mutation in the GnRH receptor causes a one amino-acid change from leucine to proline at position 117 (L117P) in the third helix, which prevents binding of GnRH and transduction of the signals. Kiss-1 and its GPR54 receptor are upstream of GnRH and thought to regulate the synthesis and/or release of GnRH. Although the mice strains are not identical, our GT-Gnhr null mice show a more severe phenotype in males compared with ENU-Gnhr mutant mice but are affected to a similar degree as hpg male mice. First, the testes weight of 2-month-old GT-Gnhr null mice were 1/32 of wild-type mice; hpg testes weight was about 1/31 of wild-type at 65 days; but ENU-Gnhr mutant testes weight was about 1:10 of wild-type mice. Second, in our GT-Gnhr mice, testicular interstitial tissues were either degraded or full of elongated cells or myeloid cells; Leydig cells were barely visible. Also, there were no Leydig cells in hpg mice but the ENU-Gnhr mutant mice appeared to have normal interstitial tissue. Third, GT-Gnhr null mice had dramatically decreased FSH, about 1/10 of wild-type,
which is similar to hpg males (1/11 of wild-type at 51-65 days). The FSH of ENU-Gnrhr mutant male mouse was about 1/5 of wild-type levels. However, the testosterone is not comparable as there is no data on hpg mice and the testosterone level of ENU-Gnrhr mutant mice is measured from mixed sex samples. The spermatogenesis of ENU-Gnrhr mutant mice was arrested at the pachytene stage of meiosis and hpg mice rarely advanced beyond the diplotene stage. However, the magnification is not sufficient for either mouse model to identify the different stage of spermatogenesis for comparison with our line. Rarely does spermatogenesis go through meiosis in our GT-Gnrh males. In both Kiss-1 and GPR54−/− null mice, spermatogenesis reached at least the early haploid spermatid (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007) and Leydig cells were not overly different from wild-type mice. Leydig cells secrete testosterone to facilitate the last stage of spermatid maturation. FSH plays a major role in regulating spermatogonia development and testosterone is essential for the completion of spermiogenesis (Donnell et al., 2006). Because hpg and GT-Gnrhr mice have much lower FSH and testosterone compared to wild-type mice, the GT-Gnrh spermatogenesis arrest at spermatogonia or primary spermatogenesis stages is expected. An important clue is provided by the finding that the GPR54−/− and Kiss-1 null mice are less severely affected than GT-Gnrhr, hpg and ENU-Gnrhr mutant mice, as GPR54 and Kiss-1 genes influence the brain-pituitary-gonad axis by regulating GnRH. Our evidence suggests that other factors in addition to Kisspeptin influence the release of GnRH; the evidence includes more severe decrease in testes weight, no Ledig cells and no maturation of male gametes in mice lines with disrupted GnRH or receptor genes, compared with knockout of kisspeptin or its receptor. However, the female mutant mice did not show a dramatic difference among GT-Gnrhr,
ENU-Gnrhr, GPR54<sup>−/−</sup> and Kiss-1<sup>−/−</sup> null mice by histological analysis as they all have early stage follicles and no corpora lutea which secrete progesterone. However, the weight of the ovary for GT-Gnrhr mice is 1/4 that of wild-type mice and the ovary of GPR54<sup>−/−</sup> and ENU-Gnrhr mutant mice is 1/2 of wild-type (Funes et al., 2003; Pask et al., 2005). The hpg female mice have more severe defects, as the weight of the ovary is 1/11 to 1/38.5 of wild-type at age 31-71 days (Cattanach et al., 1977). Also, an antrum was not detected in follicles and interstitial tissue seemed to be atrophic in hpg female mice.

During the first 30 postnatal-days, there is no significant difference in body weight between GT-Gnrhr and wild-type mice, although at 40 days and 60 days, male- Gnrhr mice are significantly lighter than the heterozygous male mice. In hpg mice, the weight of 6-week-old (42 days) males was significantly smaller than wild-type male mice. However, in Kiss-1<sup>−/−</sup> mice, both male and female mutant mice were significantly smaller than sex-matched littermates at 2-months of age (d’Anglemont deTassigny et al., 2007). However, another group (Lapatto et al., 2007) reported that there is no significant difference in the weight of Kiss-1<sup>−/−</sup> and GPR54<sup>−/−</sup> mice from wild-type mice at 9-12 weeks. This may be due to weight differences in mice of different ages. In our mouse line, the weight of null mice exceeded the wild-type mice after 6-months of age (data not shown).

In the GT-Gnrhr null mice, the expression pattern of truncated GnRH receptor protein was observed using a reporter gene. Expression begins at 14.5 dpc and increases at 15.5 dpc in the olfactory placode, anterior pituitary (Fig. 6.12), and hippocampus (data not shown). At 16.5 dpc, further expression was detected at the lateral side of the nose cavity. Others have reported that GnRH peptide fibers were expressed in the nose cavity area and
olfactory bulb at 15.5 dpc (Daikokus and Koide, 1998). In the adult rat brain, GnRH receptor was express also in the olfactory bulb, hippocampus and anterior pituitary (Jennes, 1994).

In most patients with IHH syndrome, the defects are caused by mutation of the Gnrh receptor although other genes are also involved (Kottler et al., 1999; Themmen et al., 2000; Beranova et al., 2001). Our GT-Gnhr model is similar to the abnormalities found in human IHH. It will be a valuable tool to use this model to understand further GnRH receptor signaling and function and for better clinical treatment of human IHH.
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Chapter 7

Conclusions
For my Ph.D research, I carried out 5 projects involving two different neuropeptide families: the secretin superfamily of peptides and receptors (PACAP, PHI/VIP, GHRH-LP and their receptors) and the rhodopsin superfamily of peptides and receptors (GnRH and its receptor). In this research, I used two animal models (zebrafish and mouse) and two cell lines (COS7L and ES). Below are the general conclusions from these projects.

I. Expansion of the secretin superfamily of peptides and receptors

In our laboratory, we have isolated the cDNAs of peptides and receptors of the secretin family of zebrafish and tested their biological activities by cAMP and IP assays. My research revealed a novel function in that the PAC1R is not only specific to PACAP but is regulated also by VIP (Table 7.1). Further, I showed that the previously described VPAC2 receptor in zebrafish is structurally and functionally a PHI receptor. To date, only zebrafish and goldfish have an identified PHI-R, although all vertebrates have the PHI/PHM peptide.

II. Role of neuropeptides in early development

A. Effect of PACAP in early development

To study the role of PACAP in early brain development, I examined the expression of PACAP and its receptors at different stages and then examined the effects of separate knock down of the two forms of PACAP in zebrafish where development is rapid and
Table 7.1 Comparison of identified peptides and receptors of secretin family

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<tr>
<th>Peptides</th>
<th>human</th>
<th>zebrafish</th>
<th>peptides activation of receptors in zebrafish</th>
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<tbody>
<tr>
<td>PACAP</td>
<td></td>
<td>PACAP1, PACAP2</td>
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<td>VIP</td>
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<td>VIP</td>
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<td>PHM</td>
<td></td>
<td>PHI</td>
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<td>GHRH-LP?</td>
<td></td>
<td>GHRH-LP-1, GHRH-LP2</td>
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<td>GHRH</td>
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<td>GHRH</td>
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<td>GLUC-GLP1-GLP2</td>
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<td>GLUC-GLP1-GLP2</td>
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<td>GIP</td>
<td></td>
<td>GIP</td>
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<td>secretin</td>
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<td>secretin?</td>
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<table>
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<tr>
<th>Receptors</th>
<th>human</th>
<th>zebrafish</th>
<th>peptides activation of receptors in zebrafish</th>
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<tr>
<td>PAC1R</td>
<td></td>
<td>PAC1R</td>
<td>PACAP1,2; VIP</td>
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<td>VPAC1R</td>
<td></td>
<td>VPAC1R</td>
<td>PACAP1,2; VIP</td>
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<tr>
<td>PHMR?</td>
<td></td>
<td>PHI-R</td>
<td>PHI?</td>
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<td>GHRH-LP-R (PRP-R)?</td>
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<td>GHRH-LP-1; GHRH</td>
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<td>GLUC-R</td>
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<td>Secretin-R</td>
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<td>Secretin-R</td>
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Names highlighted in green were isolated by our lab, Names highlighted in yellow were isolated by me. Receptor activation by specific peptides tested by me or our lab uses the same color scheme.
observable. I injected morpholinos (antisense oligonucleotides) into fertilized eggs to block translation of PACAP into protein. Morphological changes in the brain were observed in 27-hour embryos. Using in situ hybridization of early brain marker genes, I found that the most striking effects were an increase in pax2.1 expression in eyes and forebrain associated with absence of either form of PACAP or an increase in eng2 and fgf8 in the midbrain-hindbrain boundary after loss of PACAP2. These marker genes are among the earliest factors in the formation of the midbrain-hindbrain boundary, an early organizing center. We suggest that PACAP is a target gene with feedback inhibition on pax2.1, eng2 or fgf8 in specific brain areas. In both the diencephalon and/or forebrain, lack of PACAP1 or PACAP2 led to a substantial increase in fgf8, again suggesting a suppressive effect of PACAP during development on these important genes that help to define cells in the forebrain. The early expression of transcripts for PACAP and its receptors by 0.5-6 hours after fertilization makes both PACAP1 and PACAP2 candidates for factors that influence brain development.

B. Effect of GnRH in early development

Gonadotropin-releasing hormone (GnRH) is a highly conserved peptide that is expressed early in brain development in vertebrates even though it is normally associated with puberty. In zebrafish, we detected GnRH mRNA within two hours post fertilization by RT-PCR and 24hpf by in situ hybridization (Wu et al., 2006). Their receptors are expressed in a similar pattern. To determine if GnRH is involved in development, we used gene knockdown techniques to block translation of gnrh2 or gnrh3 mRNA after which the expression patterns for gene markers were examined at 24 hours post fertilization with in situ hybridization. First, loss of either GnRH2 or GnRH3 affected
regionalization of the brain as shown by a change in expression of \textit{fgf8} or \textit{pax2.1} genes in the midbrain-hindbrain boundary or diencephalon-midbrain boundary. Second, lack of GnRH2 and/or GnRH3 altered gene markers expressed in the formation of the eye cup (\textit{pax2.1, pax6.1, mab21l2} and \textit{meis1.1}) or eye stalk (\textit{fgf8} and \textit{pax2.1}) (Fig. 7.1). Third, knockdown of GnRH2 affected the size and shape of the midbrain and expression of gene markers therein. Results from assays with the TUNEL method and caspase-3 and -9 activities showed the brain and eye changes were unlikely to result from secondary apoptotic cell death before 24hpf. These experiments suggest the novel concept that GnRH loss-of-function affects early brain and eye formation during development.

III. Role of neuropeptide receptors in early development

A. GHRH-LP-R and PHI-R are activated by specific peptides

We investigated that zebrafish GHRH-LP-R is regulated by both GHRH-LP and GHRH through cAMP signaling pathway. PHI-R is a very specific receptor, which was only stimulated by PHI peptide.

B. Novel location of GnRH-R in larval motoneurons

Zebrafish has four GnRH receptors and they are distributed in different locations during early development. The most novel location is in specific neurons the hindbrain and in axons along the spinalcord (Fig. 7.2). GnRH-R antiserum labeled Mauthner cells and other reticulospinal neurons in the hindbrain. These motor neurons are known to be
involved in movement, swimming and in rapid escape. This is a newly identified function that GnRH is involved in motor control in addition to reproduction.

**IV. GnRH-R is essential for reproductive maturation and function**

We found that GnRH is involved in the early development of zebrafish and we predict this is the same in mice. We created a mouse line in which the GnRH receptor is disrupted preventing GnRH from acting on its receptor to regulate gonadotropin secretion and release. The adult mice showed the hypogonadotropic and hypogonadism phenotype in which gonad development and fertility were blocked. However, we did not detect any specific defects in embryonic development so far. Testicular structure and spermatogenesis of testis at 16.5dpc is similar between wild-type and GT-Gnrhr null mice (Fig. 7.1). However, the testis of null mouse appears to have more seminiferous tubules than in the wild-type mouse. Also, the spermatogenesis cells are less dense per seminiferous tubule than in the wild-type mouse. These results will be requested at several stages of development. This is supported by the observation that expression of GnRH and GnRH receptor begins at this stage and could, in theory, influence testis development.
Fig. 7.1 16.5 dpc testis of wild-type (+/+, A, C) and Gnrhr^{G1NMS} null mice (-/-, B, D).

(Sections and pictures were carried out by Emma Isaac).
**Future work**

GnRH has been conserved from octopus to human for more than 650 million years. It is well documented that gonadotropin-releasing hormone (GnRH), also known as LHRH in human, is a key regulator of reproduction and sexual behaviour. GnRH stimulates the synthesis and release of gonadotropins (LH, FSH) from the pituitary, which act on the gonads to regulate steroid hormones. However, GnRH and its receptor are expressed long before puberty in mammals. We showed that GnRH acts in early development to affect brain regionalization and eye formation in zebrafish.

In the future, I am interested in working with both zebrafish and mice. For zebrafish, I would like to continue the following experiments: 1. find the function of PHI in zebrafish by gene targeting techniques using morpholinos. 2. determine if VPAC2R is present in fish species other than zebrafish and goldfish by isolating the cDNA and performing 5' and 3'RACE. 3. trace the evolutionary pattern in which PHI-R becomes a VPAC2R by phylogenetic analysis. 4. do electrophysiological experiments in zebrafish to determine the influence of GnRH in larvae on the Mauthner cells.

For mice, I am interested in studying how the endocrine neuropeptides and their receptors control the reproductive brain-pituitary-gonadal axis and the interaction with other related peptides or proteins in developing and adult mice.

I would like to address the following questions:

1. Do GnRH and its receptor have feedback influence on the GnRH neuron during embryogenensis and in adult mice?

2. At what stage does the GnRH receptor mediate effects on gonadal development?
3. Is there feedback of GnRH and its receptor on the kisspeptin receptor GPR54, recently confirmed as upstream of the GnRH signaling pathway?

4. Why do GnRH receptor-disrupted mice become obese after 6 months of age?

5. Do mice in which both GnRH and its receptor are knocked out have a more severe or different effects than single-knockout mice?

First, I will use the mouse line, in which the GnRH receptor is disrupted by the gene trap method (GT-GnRH-R) that I helped to create during my Ph.D study. I will cross GT-GnRH-R mice with mice in which GnRH neurons are labelled with GFP (Green GnRH mice commercially available) to generate heterozygous Green GnRH/GT-GnRH-R mice. The heterozygous mice will be used as breeders to make homozygous mice. Brain sections or whole mounts will be prepared at different stages and neurons that express GnRH with fluorescence will be examined to compare receptor KO and wild type mice in which both groups have green GnRH neurons. From the spatial and temporal expression pattern, we can conclude if the GnRH/GnRH receptor complex feeds back to influence GnRH expression in early development. Second, I will inbreed the heterozygous GT-GnRH-R mice to generate homozygous KO GT-GnRH-R mice from 18.5 days post conception (fetal) to 20-day old pups (postnatal). I will examine if there is a stage of testis that shows structural differences between KO and wild type by histology. Third, I will examine the relationship between the GPR54 and GnRH receptors. I will collect the brain and pituitary tissue from the KO and wild type mice for analyzing the mRNA level of GPR54 by real time PCR and in situ hybridization and the protein level of GPR 54 by immunocytochemistry and western blot. Fourth, I will investigate why GT-GnRH-R KO mice become obese after 6 months of age and what the mechanism is involved by using
the microarray method to detect genes with mRNA levels that are changed by more than two fold in KO mice compared to wild-type mice. I will select known regulated genes that are related to diabetes to conduct further investigation by real time PCR and in situ hybridization for confirming the RNA levels. **Fifth,** I will create double KO mice in which both GnRH and its receptor are non functional by crossing the heterozygous hpg-GnRH with our GT-GnRH-R mice. I will identify the double KO mice by genotyping. The double KO offspring will be used to study the loss of GnRH and its receptor in early brain regionalization and eye formation, and sexual organ development. For comparison, both GnRH and its receptors will be knocked down in zebrafish and the results examined with multiple methods involving genetic, molecular and cellular biology along with physiology.

We do not understand the exact interactions of GnRH, GnRH-R and GPR54 in vivo and how GnRH and its receptor together influence development. Understanding the mechanism of action of GnRH and its receptor in early development is fundamental in all vertebrates and in some invertebrates. Transgenic methods offer a window to investigate why GnRH is so tightly conserved during evolution and GnRH-R gene trap mouse is a valuable model for studying hypogonadotropic hypophysial disease in human.
References
