Structural and Functional Evolution of Gonadotropin-Releasing Hormone (GnRH) and Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) in Chordates

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ABSTRACT

Neuropeptide hormones arose early in evolution. Multigene families in vertebrates are proposed to have arisen initially in early vertebrates by genome duplication events. In its simplest form, the theory suggests that the copy of a duplicated, ancestral single gene diverged in sequence, and possibly function, from its original match. My goal was to understand the structural and functional evolution of two neuropeptides, gonadotropin-releasing hormone (GnRH), a member of a single gene family, and pituitary adenylate cyclase-activating polypeptide (PACAP), a member of a multigene superfamily of hormones.

GnRH is the primary regulator of reproduction in vertebrates, but the evolutionary origin of GnRH is not clear. In the protochordate tunicate *Ciona intestinalis*, I found there are two genes that encode GnRH peptides, however each gene encodes different GnRH peptides. Furthermore, these peptides are novel structures for GnRH and quickly induce spawning in *Ciona*, suggesting a novel and direct action for GnRH in the control of reproduction. In studies of the novel form of GnRH in lake whitefish, wfGnRH, I provide proof wfGnRH is a gonadotropin-releasing form in whitefish by showing it to be an activator of pituitary gonadotropin and growth hormone gene expression, and is co-localized in the forebrain region.

PACAP is a hormone structurally related to glucagon and has been tightly conserved in structure during evolution. PACAP is produced as either a 27 or a 38 amino acid form in vertebrates, whereas in one tunicate studied to date, PACAP is produced from each of two genes as a 27 amino acid form. PACAP regulates several endocrine systems and has direct and indirect actions on metabolism, growth, and reproduction, and is well-known for its ability to potently secrete insulin in laboratory testing.

I studied a number of species to increase our understanding of PACAP gene diversity in evolution. I was unable to identify a PACAP-like gene in the tunicate species, *C. intestinalis*. However, I identified a number of novel PACAP peptide structures in nine fish species by molecular biological and bioinformatic approaches. I was able to identify a second copy of a PACAP gene in five of the nine species. Using these data, I constructed a phylogenetic relationship for prohormones for PACAP in chordates and

propose a updated explanation for the evolution of the PACAP/glucagon superfamily of genes in vertebrates. Using morpholino-based knockdown of the PACAP peptides in zebrafish early development, I showed that each copy of these two genes is functional and important in normal development in zebrafish, suggesting that divergence in function of the two different PACAP genes coincided with divergence in sequence.

I also studied mice to determine the proposed role for PACAP in thermogulation. Recently, the pups born to a new model of mouse with a targeted disruption of the PACAP gene (PACAP-null) have been found to have disruption of normal lipid and carbohydrate metabolism and die early in the second postnatal week. Furthermore it has been determined this phenotype is temperature sensitive. I hypothesized that there is a disruption of the thyroid axis in these mice that contributes to their problems with thermogenesis, and because these mice have a compromised adrenergic response, they are more sensitive to obesity.

I showed that there is twice as much of the active form of thyroid hormone (TH), 3,5,3'-triiodothyronine (T3), in PACAP-null mice compared to their wildtype littermates. Mice reared at different temperatures (21, 24 and 28 °C) had increased survival with increasing temperature from 14% surviving at 21°C to 79 % at 28 °C, and mice held at 28°C had lower levels of THs compared to 21°C. Treatment of PACAP-null mice with methimizole decreased their level of T3 and increases their survival suggesting the levels of T3 in mice at 21 °C are toxic.

Mice raised at 28 °C on one of two diets, regular chow (low fat) and high fat were studied for difference in appetite and in tolerance to obesity. There were no differences in either appetite or many obesity-related parameters such as mass, fed and fasted glucose levels, fat distribution or plasma levels of leptin in PACAP-null mice compared to their sex- and diet-matched wildtype comparison groups. However, there was an increase in insulin sensitivity in PACAP-null mice fed a high fat diet.

Examiners:

Dr. N. M. Sherwood, Supervisor (Biology)

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LIST OF ABBREVIATIONS

BAT: brown adipose tissue

bp: base pairs

cAMP: cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

CRH: corticotropin-releasing hormone

GH: growth hormone

GHRH: growth hormone-releasing hormone

GIP: glucose-dependent insulinotropic polypeptide

GnRH: gonadotropin-releasing hormone

HPLC: high performance liquid chromatography

mRNA: messenger ribonucleic acid

PAC1: PACAP specific receptor

PACAP: pituitary adenylate cyclase-activating polypeptide

PHI: peptide histidine isoleucine

PHM: peptide histidine methionine

PRP: PACAP-related peptide

RT-PCR: reverse transcription-polymerase chain reaction

T3: 3,5,3'-triiodothyrnine

T4: L-thyroxine

TH: thyroid hormone

TRH: thyrotropin-releasing hormone

TSH: thyroid stimulating hormone; thyrotropin

UCP: uncoupling protein

UTR: untranslated region

VIP: vasoactive intestinal polypeptide

VPAC1: VIP and PACAP shared receptor 1

VPAC2: VIP and PACAP shared receptor 2

RIA: radioimmunoassay

WAT: white adipose tissue

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To my parents

CHAPTER 1: INTRODUCTION

A hormone multigene family and a superfamily: Case studies for molecular evolution.

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Introduction

Multigene families in vertebrates are proposed to have arisen initially in early vertebrates by genome duplication events (Ohno, 1970). In its simplest form, the theory suggests that a copy of a duplicated, ancestral single gene diverged in sequence, and possibly function, from its original match. One theory suggests that two complete genomic duplications occurred between ancestral protochordates and jawed vertebrates, although the timing of the duplication varies (Ohno, 1970; Ohno, 1998; Furlong and Holland, 2002).

Coordination of function among specialized cells and tissues in an organism requires extracellular communication regulated typically by one of three systems: the nervous system, the immune system, or the endocrine system. The endocrine system has been traditionally thought to be a network of glandular structures that release chemical messengers called hormones into the blood as a means to communicate been non-adjacent cells and tissues. A modern, inclusive definition of a hormone is a non-metabolic chemical that is produced by any cell and released into circulation for a distant target tissue, but also that can be released and act on the cell that secreted it (autocrine action), or on nearby cells in the same tissue (paracrine action) without moving into general circulation, and that will bind to a specific receptor.

Many hormones are proteins, produced as gene products, that can be released or stored for use at a later time. Hormones that are gene products are excellent models for studies of molecular evolution not only because they can be identified by an array of methods, but also because it is possible to test for conservation or change in function of the hormones using physiological tests in isolated cells or whole organisms. My interest is in the structural and functional evolution of hormones, and in particular understanding the expanded repertoire of peptide hormones and functions as a consequence of expanded gene content. The first of two hormones that I selected to study is the gene family for gonadotropin-releasing hormone (GnRH), the main hormone regulating reproduction in vertebrates. The second hormone I selected to study is pituitary adenylate cyclase-activating polypeptide (PACAP), a highly conserved member of the

PACAP/glucagon superfamily of hormones that is expressed mainly in the nervous system and gonads.

Gonadotropin-releasing hormone: discovery and structure

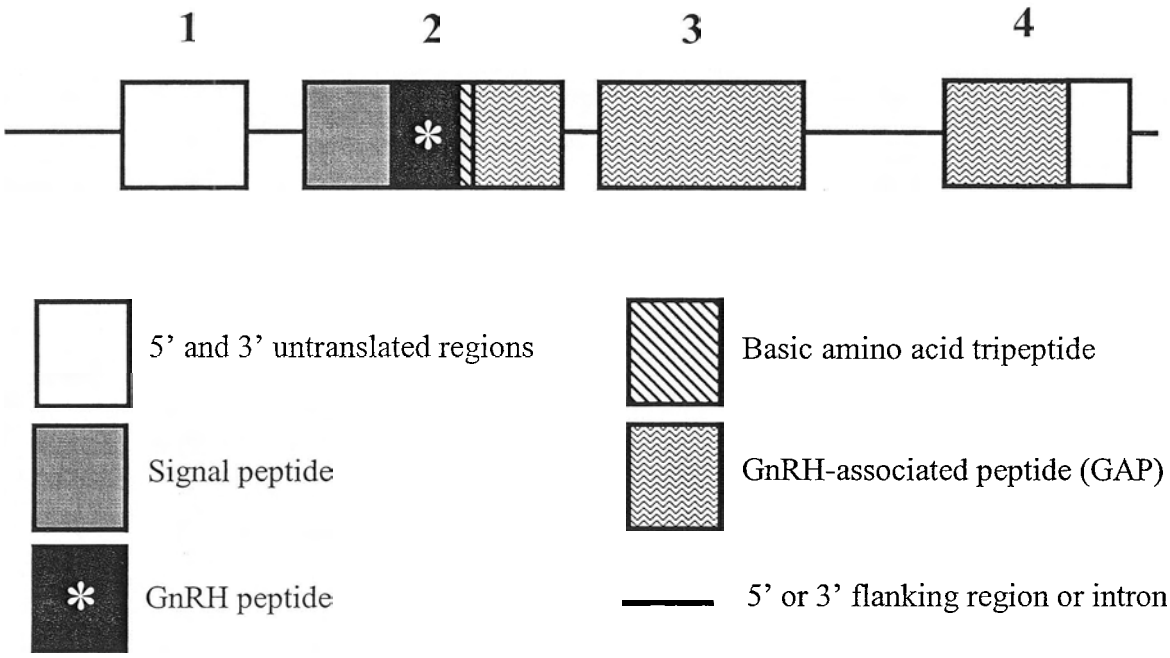
GnRH is best known for its physiological role in the regulation of the synthesis and release of the pituitary gonadotropin hormone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). A GnRH structure of ten amino acids was first isolated from mammals (Matsuo et al., 1971; Burgus et al., 1972), followed by the identification of a unique form in chicken, cGnRH-I (Miyamoto et al., 1982) and fish (Sherwood et al., 1983). By the beginning of my research, twelve GnRH forms of ten amino acids each had been identified, mainly by protein isolation and sequencing techniques (Carolsfeld et al., 2000). Since that time, a novel GnRH form was identified in pejerrey by protein isolation and sequencing (Montaner et al., 2000) and a cDNA coding for this form was reported the same year in medaka (Okubo et al., 2000). Also, a cDNA for a novel form was identified in frog (Yoo et al., 2000) plus another of 12 amino acids in octopus was reported in 2002 (Iwakoshi et al., 2002) (Fig. 1.1). Each of these forms of GnRH is encoded by a separate precursor. The structure of the genes for GnRH peptides that have been isolated to date are very similar, including a signal peptide of around 20-25 amino acids, the GnRH sequence, three basic amino acids, and the GnRH-associated peptide (GAP) of approximately 40-60 residues (Adelman et al., 1986; Klungland et al., 1992; White et al., 1994) (Fig. 1.2).

Mammals, including humans, usually express two forms of GnRH, mammalian (m)GnRH (also known as GnRH1) and chicken (c)GnRH-II (also known as GnRH2), although a few species additionally express guinea pig (gp)GnRH. In evolution mGnRH is first detected in early-derived bony fish (Sherwood et al., 1991; Lescheid et al., 1995) and cGnRH-II is detected even earlier in cartilaginous fish (Lovejoy et al., 1991). Other forms of GnRH preceded mGnRH and cGnRH-II in evolution. Jawless fish have lamprey-I and –III GnRH (Sherwood et al., 1986; Sower et al., 1993), whereas limited evidence in

Figure 1.1. Amino acid sequences of the GnRH peptides.

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

Figure 1.2. Schematic arrangement of vertebrate GnRH gene.


1
2
3
4
5' and 3' untranslated regions
Signal peptide
GnRH peptide
Basic amino acid tripeptide
GnRH-associated peptide (GAP)
5' or 3' flanking region or intron

invertebrates indicates the existence of two ten-amino acid forms in the tunicate *Chelyosoma productum* (Powell et al., 1996) and a 12 amino acid form in octopus (Iwakoshi et al., 2002). In the teleosts (later-evolving bony fish), most species studied to date express three different forms of GnRH: a GnRH-I form that can vary in structure among species (see Sherwood and Adams, 2004 for review); cGnRH-II, and salmon (s)GnRH (also known as GnRH3).

GnRH distribution and physiology

The multiple forms of GnRH are distinguished by their anatomical location in the brain of mammals (Kasten et al., 1996) and fish (Zandbergen et al., 1995). Neurons containing cGnRH-II are mainly in the midbrain region for all species, whereas the other GnRH forms are in neurons in the anterior brain. In species with only two forms of GnRH (e.g. most tetrapods and some fish), cGnRH-II is mainly in the midbrain and the second form is in both the olfactory region/terminal nerve (OLF/TN) and ventral telencephalic/preoptic (VT/PO) region. In species with three GnRH forms, cGnRH-II is in the midbrain, but the two GnRH forms in the anterior brain may be in separate anterior locations or overlap within the same region (Gonzalez-Martinez et al., 2002).

GnRH is generally considered the master regulator governing the reproductive axis because it is understood to be the main brain hormone that initiates a cascade of other reproductive hormones and events in the body, beginning with release and synthesis of the pituitary hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In fish, GnRH also releases growth hormone (Marchant et al., 1989) and prolactin (Weber et al., 1997) from the pituitary. Gonadotropins LH and FSH released from the pituitary travel to their main target tissues, ovary or testes, to regulate local steroid production. GnRH is increasingly being studied for its expression and direct function in peripheral tissues outside of the pituitary. The gonads are another site of GnRH synthesis, where for example, in fish it acts in a paracrine manner to influence cell cycling events and steroid production in the ovary (Pati and Habibi, 2000; see Sherwood and Adams, 2004 for review).

GnRH mechanism of action

GnRH is released from the brain and binds to a seven transmembrane, G-protein coupled receptor (GPCR) found on the surface of target cells of the pituitary and a number of extra-pituitary tissues (Hsieh and Martin, 1992; see Millar et al., 2004 for review). The subsequent events follow the De Lean model for GPCRs as first described for the β-adrenergic receptor (De Lean et al., 1980). The binding of GnRH causes the receptor to undergo a change in conformation such that there is a change to an activated state of the complex including the associated G protein. The GnRH receptors identified in vertebrates to date have been grouped into three GnRH receptor types (Type I, Type II and Type III) that differ in structure and selectivity for different GnRH forms (see Millar et al., 2004 for review). All of the GnRH receptors identified to date are believed to act mainly through the $G_{q/11}$ pathway to cause changes in intracellular calcium concentrations via generation of inositol triphosphate (IP_3), whereas the diacylglycerol (DAG) that is also formed activates protein kinase C. Furthermore, there is evidence for some non-$G_{q/11}$ protein specific intracellular messenger activation that is best explained at this time by interaction of GnRH receptors with other G proteins, including Gs (Janovick and Conn, 1993; Stanislaus et al., 1998), Gi, and Go (Hawes et al., 1993).

PACAP: discovery, structure and superfamily members

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated from the hypothalamus of sheep as a peptide of either 27 or 38 amino acids (Miyata et al., 1989; Miyata et al., 1990). The PACAP/glucagon superfamily contains nine hormones in humans including PACAP, growth hormone releasing hormone (GHRH), glucagon, glucagon-like peptide (GLP)-1, GLP-2, glucose-dependant insulinotropic polypeptide (GIP), secretin, vasoactive intestinal polypeptide (VIP), and peptide histidine methionine (PHM) (peptide histidine isoleucine (PHI) in other species) that are clustered together in a superfamily because of their related protein structures particularly in the first 27 amino acids (Sherwood et al., 2000) (Fig. 1.3). The gene organization of the

superfamily members shows a common pattern. The bioactive core (27 amino acids) is encoded always on one exon within the gene, although a second exon may encode the C-terminal extension of the peptides, as in GHRH and GIP. Some genes encode more than one peptide so that glucagon, GLP-1 and GLP-2 are in one gene and PHM and VIP are in another gene (Fig.1.4). GIP and secretin are each encoded on a separate gene.

Our understanding of the evolution of the PACAP/glucagon superfamily of peptides has resulted from structural studies in a relatively small number of different classes of animals. An ancestral PACAP peptide is thought to be the precursor of the entire PACAP/glucagon superfamily because PACAP is tightly conserved in structure from protochordates to humans (McRory and Sherwood, 1997). GHRH is encoded on the same gene with PACAP in protochordates (McRory and Sherwood, 1997), fish (Fradinger and Sherwood, 2000; McRory et al., 1995; Parker et al., 1993) amphibians (Alexandre et al., 2000), and birds (McRory et al., 1997). The generally accepted hypothesis (Sherwood et al., 2000) is that the first ancestral peptide was encoded on one exon within a gene. During evolution a series of exon and gene duplications followed by DNA base substitutions have resulted in nine separate bioactive hormones encoded on six genes in humans (Fig. 1.4 and 1.5). At some point before or during the emergence of mammals, PACAP and GHRH became encoded on separate genes. However, the amino acid sequence of PACAP is identical in the mammalian species studied to date (Kimura et al., 1990; Miyata et al., 1989; Ogi et al., 1990). In mammals the PACAP mRNA precursor also encodes PACAP-related peptide (PRP), though no function is yet associated with this peptide.

Generally speaking members of the PACAP/glucagon superfamily members have both distinct and overlapping functions. The superfamily hormones (with the exception of GIP) are found in the brain, so can be considered to be neuropeptides. However, the brain distribution of each is distinct, as is the central control of various functions. Also, all family members are found in the

Figure 1.3. Bioactive human PACAP/glucagon superfamily members arranged by length .

Peptide	Sequence	# amino acids
$PACAP_{27}$	HSDGIFTDSYSRYRKQMAVKKYLAAVL-NH_2	27
SECRETIN	....T..SEL..L.EGARLQRL.QGLV-NH_2	27
PHM	.A..V..SDF.KLLG.LSA....ESLM-NH_2	27
VIP	...AV...N.T.L.....NSI.N-NH_2	28
GLUCAGON	..Q.T..SD..K.LDSRRAQDFVQWLMNT-OH	29
GLP-1	.AE.T..SDV.S.LEGQ.A.EFI.WLVKGR-NH_2	29
GLP-2	.A..S.S.EMNTILDNL.ARDFINWLIQTKITD-OH	33
$PACAP_{38}$	.....GKRYKQRVKNK-NH_2	38
GIP	YAE.T.ISD..IAMDKIHQQDFVNWL.AQKGKKNDWKHNITQ-OH	42
GHRH	YA.A...N..RKVLG.LSAR.L.QDIMSRQQGESNQERGARARL-NH_2	44

Figure 1.4. Organization of the genes encoding the PACAP/glucagon superfamily members. White boxes represent 5'- and 3'-untranslated regions; crosshatched boxes represent signal peptides; black boxes represent bioactive superfamily members, and shaded boxes represent N- and C-terminal peptides and intervening domains. Introns are depicted as lines, but are not drawn to scale.

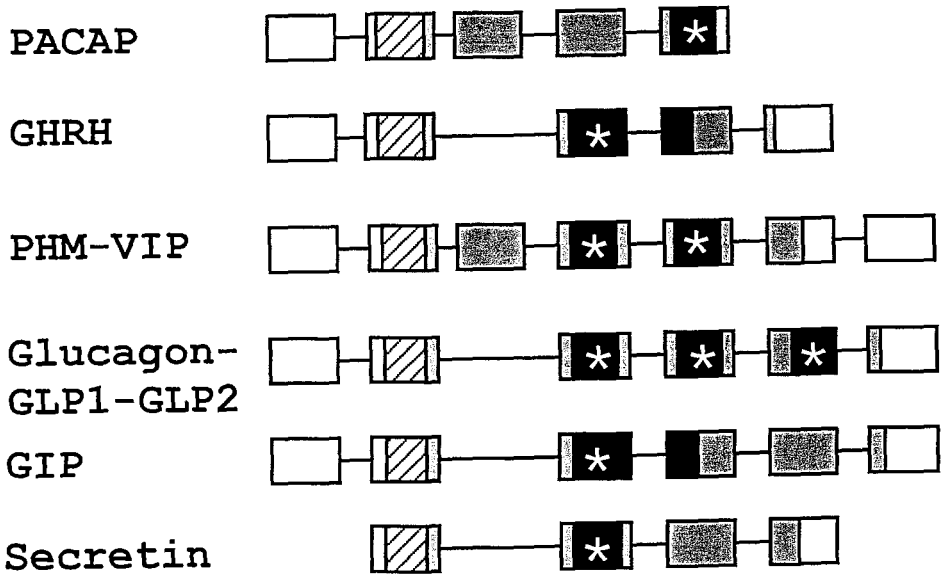
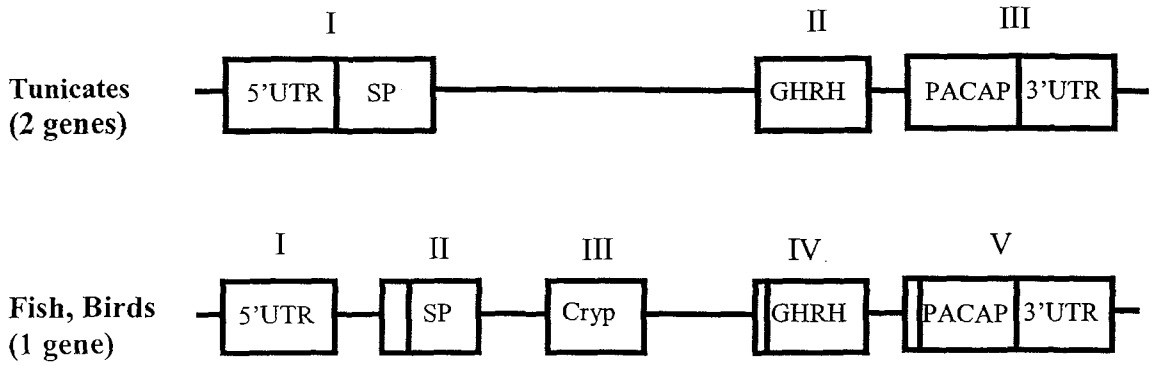

PACAP
GHRH
PHM-VIP
Glucagon-
GLP1-GLP2
GIP
Secretin

Figure 1.5. Framework for the evolution of the PACAP/glucagon superfamily of hormones. 5'UTR, 5'-untranslated region; 3'UTR, 3'-untranslated region; SP, signal peptide; Cryp, cryptic peptide.



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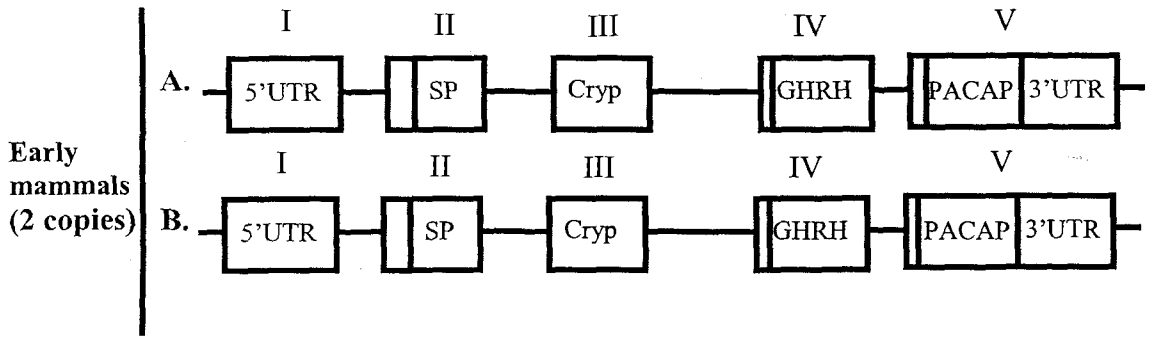

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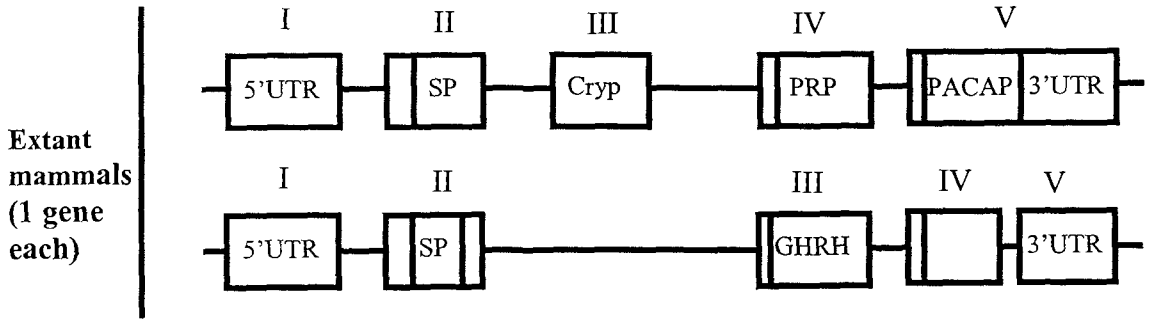

gastrointestinal tract, but GIP, GLP-1 and GLP-2 are the primary gut peptides that act within the GI tract. A few of the hormones (PACAP, VIP, PHM) can release pituitary hormones (Miyata et al., 1989), but only GHRH has a primary role to affect pituitary hormone release in mammals (Rivier et al., 1982). Finally, several of the peptides act as growth factors, and PACAP and VIP are associated with brain development (Mercer et al., 2004; Moody et al., 2003).

Mechanism of PACAP action

Each hormone, with the exception of VIP and possibly PHM/PHI, has a specific receptor (Tse et al., 2002). VIP shares two receptors with PACAP. The collection of receptors for individual members of the superfamily is most interesting. All of the superfamily receptors are seven-transmembrane, G protein-coupled receptors, which describes more than 1000 different receptors in the body. However, the superfamily receptors as a whole, along with several other hormone receptors, form a subset called family B (Kolakowski, 1994). Structural differences separate family B from other receptors suggesting the B receptors evolved in parallel with the PACAP/glucagon superfamily (Sherwood et al., 2000).

There are three types of PACAP receptors, each encoded on a separate gene. One of these receptors, labeled PAC_1 receptor, is specific for PACAP. The other two receptors, $VPAC_1$ and $VPAC_2$, bind both PACAP and VIP (Harmar et al., 1998). The most interesting of these three receptors is PAC_1 because it is not only specific for PACAP, but has nine forms (the normal receptor plus eight variant forms) (Table 1.1) (Chatterjee et al., 1996; Daniel et al., 2001; Dautzenberg et al., 1999; McCulloch et al., 2000; Pantaloni et al., 1996; Pisegna et al., 1996; Spengler et al., 1993). These variants have insertions in the third intracellular loop or deletions in the extracellular domain or substitutions in the second and fourth transmembrane region. PAC_1 variants are different in their abilities to increase cAMP via adenylyl cyclase activation, or activate calcium channels, often because of the alteration in intracellular signaling pathways that are

Table 1.1. Splice variants of PAC_1 receptors (adapted from Laburthe et al., 2003).

Variant	Structural modification	Phenotype		Reference
		Binding	Signaling	
PAC_1	None			(a)
Hip	Insertion (28 aa) in IL3	No data	Decreased potency for cAMP response	(a,b)
Hop 1	Insertion (28 aa) in IL3	No change	Small changes in response and facilitation of Phospholipase D activation	(a,b,c)
Hop 2	Insertion (28 aa) in IL3	No data	Small changes in response	(a,b)
Hip Hop 1	Insertion (56 aa) in IL3	No data	Small changes in response	(a,b)
TM4	Modification of TMII and TMIV	No data	1. Abolition of IP-PLC and AC responses 2. PACAP stimulates L-type calcium channels	(d)
S	Deletion (21 aa; 89-109) in N-terminal domain	Loss of selectivity towards VIP	No change (cAMP), increased potency of $PACAP_{1-27}$ for IP production	(e, f)
VS	Deletion (57 aa; 53-109)	Loss of PACAP affinity	Loss of potency of PACAPs in stimulating cAMP	(f)
3a	Insertion (24 aa) in N-terminal domain	Selective increase of affinity for $PACAP_{1-38}$	Reduced sensitivity (cAMP, IP)	(g)

(a) Spengler et al., 1993,; (b) Pisegna et al., 1996; (c) McCulloch et al., 2000; (d) Chatterjee et al., 1996; (e) Pantaloni et al., 1996; (f) Dautzenberg et al., 1999; (g) Daniel et al., 2001.

activated, including a shift to activation of phospholipase effectors (Dautzenburg et al., 1999; McCulloch et al.. 2000). In contrast, the receptors shared with VIP do not have variant forms; they induce post-receptor effects primarily through cAMP (see Vaudry et al., 2000, for review).

The sum of the expression patterns of all three receptors for PACAP implies that many tissues and cell types are targets for PACAP (see Sherwood et al., 2000; Vaudry et al., 2000, for reviews). The PAC_1 receptors are widely distributed in the brain. $VPAC_1$ and $VPAC_2$ receptors are in the hippocampus and hypothalamus, but are far less abundant than PAC_1 receptors. PAC_1 is also found in the pituitary whereas in peripheral tissues PAC_1 receptors are found in the eye, pituitary, adrenal medulla, pancreas, liver, ovary, lung, gut and lymphoid tissue. The $VPAC_1$ receptors are in the eye, adrenal gland, pancreas, liver, testis, lung, gut and lymphoid tissue. The $VPAC_2$ receptors are in similar locations plus in the pituitary and ovary. There is less overlap among receptor location than the list suggests as the receptors may be expressed only in specific cells within the organs and at specific times.

PACAP physiology

The functions of PACAP are diverse, but to date it is not clear as to the physiological stimulus that releases PACAP from nerve endings or cells. Regardless of the control of PACAP, this hormone has a number of functions (see Sherwood et al., 2000 for review) many of which have been identified in mammalian systems or using mammalian cell-based systems. PACAP stimulates the release of other hormones; it releases insulin and glucagon from the pancreas (see below), catecholamines from the adrenal medulla (Hamelink et al., 2003), glucocorticoids from the adrenal cortex in some species (Nussdorfer and Malendowicz, 1998, for review), and growth hormone from the pituitary gland (Murakami et al., 2001). PACAP acts on blood vessels to cause vasorelaxation (Absood et al., 1992) and can inhibit platelet formation in blood (Freson et al., 2004). In the developing nervous system, PACAP acts to alter proliferation and differentiation. Recently, PACAP's actions have been examined in mice in which

either the PACAP gene or the receptor (PAC_1) gene has been knocked out by targeted disruption of the gene (see Sherwood et al., 2003). These studies show that PACAP plays a role in behavior (Hashimoto et al., 2001; Otto et al. 2001), and in lipid and carbohydrate metabolism (Gray et al., 2001), including the response to a metabolic stress such as an insulin challenge (Gray et al., 2001; Hamelink et al., 2002). One hypothesis to explain the many actions of PACAP is that it is used for responses to environmental or metabolic stress (Hamelink et al, 2001).

With regards to disease, PACAP is over-expressed in a number of tumors including gliomas (Robberecht et al., 1994), neuroblastomas (Suzuki et al., 1993), pheochromocytomas (Fahrenkrug et al., 1995) and in tumors of pancreas (Douziech et al., 1998), ovary (Odum and Fahrenkrug, 1998), and breast (Garcia-Fernandez et al., 2004). However, solid evidence is lacking as to symptoms associated with alterations in the human genes encoding PACAP or its receptors. Nearly every type of tumor expresses the receptors for PACAP, so there is a great interest in the actions of PACAP in the proliferation or survival of tumor cells (see Lelievre et al, 2003).

A major site of PACAP action is the pancreas (For review see Filipsson et al., 2001). PACAP has been identified in nerve fibers that innervate the ganglia of the pancreas as well as the islets of Langerhans (Fridolf et al., 1992; Yada et al., 1994) and PACAP and the PAC_1 receptor are expressed and synthesized in islet cells. A number of studies support this, including pancreas infusion with PACAP that results in significant release of insulin, and when given simultaneously with glucose results in enhanced glucose-evoked insulin secretion (Yamaguchi, 2001). Furthermore, transgenic mice over-expressing PACAP in the pancreas have enhanced glucose-induced insulin secretion with normal glucose tolerance, and amelioration of streptozotocin-induced diabetes (Yamamoto et al., 2003), and improve the insulin profile when crossed with obesity- and diabetes-prone Agouti yellow mice (Tomimoto et al., 2004). PACAP-null mice exhibit significantly impaired glucose-induced insulin secretion but still have normal glucose tolerance (Shintani et al., 2002). Finally, PACAP has also been implicated in β-cell

proliferation based on studies in adult mice over-expressing PACAP in β-cells (Yamamoto et al., 2003; Tomimoto et al., 2004).

There is also evidence for a role in reproduction. Whereas the role of PACAP in reproduction stems mostly from localization of mRNA and protein for both the ligand and receptors, increasingly this type of data is being integrated to understand the role of PACAP in sexual maturation. For example, PACAP is now being demonstrated to have a role in the progression of puberty, presumably in part through involvement of the GnRH axis (Choi et al., 2000).

In mammals, PACAP is highly abundant in testes (Shioda et al., 1994) and ovary (Arimura et al., 1991). Most of the evidence in mammals to date suggests PACAP is an auto- or paracrine regulator of spermatogenesis. PACAP expression is restricted to specific stages, mostly during early spermatogenesis. Also, this increase in expression coincides with the expression of receptors for PACAP, which are PAC_1 receptors (Hannibal and Fahrenkrug 1995). Sertoli cells contain PACAP mRNA and cell functions can be modulated by PACAP (Heindel et al., 1992). Leydig cells have PACAP receptors and cell function is also influenced by PACAP. Finally, *in vivo* studies show that PACAP affects the blood flow in the testis and caput epididymis (Lissbrant et al., 1999). It is still unknown whether PACAP has the same functions in the fish testis.

In the mammalian ovary, both PACAP and PACAP receptor mRNAs are present (Koh et al., 2000; Park et al., 2000). PACAP causes a stimulation of cAMP accumulation, estradiol secretion and progesterone secretion *in vitro* (Hiendel et al., 1996). Regulatory control of ovarian PACAP may be under the influence of both pituitary gonadotropins as well as local progesterone receptor activation (Ha et al., 2000). PACAP may be a survival factor against follicular atresia (Lee et al., 1999). In total, the expression and functional data to date suggest PACAP may have autocrine and paracrine actions in ovarian function.

Models for study in comparative physiology and evolution

There are several theories about the timing of genome duplication events in chordate evolution (Dehal et al., 2002). Nonetheless, the protochordates provide a

reference group for comparison of genes with those in the vertebrate lineage. Nonetheless, the genes that encode GnRH are of particular interest because GnRH controls the cascade of events that lead to reproduction throughout the vertebrates. The PACAP/glucagon superfamily of hormones is suspected of having benefited dramatically in terms of number of extant superfamily members as a consequence of genome and gene duplications that were experienced in the lineage that led to vertebrates since the split from chordates. Therefore, I have selected model species, including tunicates, several teleost fish species, and mouse, to represent different lineages in the chordate phylogeny in order to further our understanding of the structural and functional evolution of these hormones. The understanding of the effects of PACAP until 2001 was largely the result of experimental addition of the peptide, so the physiological role had yet to be clearly established. As of 2001, there are now five laboratories that have generated mice that have targeted disruptions of the PACAP gene (see Sherwood et al., 2003, for review).

Objectives

The general objectives of my research are to expand our understanding of the structural and functional evolution of GnRH and PACAP in chordates. At the outset of my research in 1999, the complete sequence of the genome was available for a small number of organisms and none was known for a chordate. GnRH and PACAP provide interesting markers in gene evolution, as we know they are both conserved in these chordates studied to date.

In the first two chapters, I describe the identification of new members of the GnRH peptide family. I found that in the invertebrate chordate tunicate, *Ciona intestinalis*, there is a very different gene structure for GnRH that codes for three GnRH peptides on each of two genes. In the next chapter, I describe my part in isolating and characterizing a novel GnRH form from a tetraploid salmonid species, lake whitefish, and show that the peptide form is active biologically, and is structurally similar to the vertebrate GnRH1 grouping of these peptides.

The diversity and phylogeny of fish species provides an excellent group of animals to track gene structural and functional diversity. I selected a number of

fish species that represented different orders of fish and identified cDNAs generated from mRNA that potentially code for GHRH and PACAP to gain some sense of the diversity of the structures of the mature polypeptides. Interestingly, this study revealed that a number of fish species that are model genomic species in fact have two copies of the genes that code for PACAP, and I used this information to provide an updated framework for the PACAP/glucagon superfamily of hormones in vertebrates.

I then used fish models to address questions concerning the functional evolution of PACAP. First, I addressed the question of whether both duplicate PACAP genes are functional. To answer this question, I used gene knockdown in which modified DNA oligonucleotides that are complementary to each of the zebrafish PACAP genes blocked the translation of the genes in early zebrafish development and studied zebrafish for anatomical defects. Next I wanted to address the question of whether PACAP has a conserved function in gonadal function in fish. More simply, I was interested if PACAP is present in the gonads of mature fish, and if so, what functions it may have. To do this, I studied the brain and gonad of first-time sexually maturing adult rainbow trout for gene and protein expression of PACAP.

In the final two chapters, I expanded our current understanding of the physiological role of PACAP in mammals. To accomplish this, I used a mouse model for PACAP that was recently generated in our laboratory in which the gene coding for PACAP, *Adcyap1*, was knocked out by targeted disruption (Gray et al., 2001). Mice homozygous for the targeted disruption (PACAP-null) are born from heterozygous parents in the expected Mendelian ratio, suggesting that PACAP is not essential for development *in utero* (Gray et al., 2001). The most profound alteration of the phenotype of the mouse is seen by the second week after birth when most of the PACAP-null pups undergo reduced growth as part of a general wasting followed by death by two-weeks of age. Further analysis revealed a more complex phenotype, including a mobilization of subcutaneous fat stores and massive fat deposition in liver, skeletal muscle and heart muscle (Gray et al., 2001). This phenotype is to some degree temperature sensitive: mice that are

raised in housing conditions with a room temperature of 21°C die, whereas the majority of mice raised at 24°C survive to weaning and adulthood (Gray et al., 2002). These results and those of others suggest a problem with the catecholamine response and the sympathetic nervous system in PACAP-null mice (Hamelink et al., 2002). Indeed short-term hyperthermia has been noted in rodents treated with PACAP (Pataki et al., 2000). The temperature sensitive phenotype and the possible involvement with the sympathetic nervous system led me to design studies investigating two main questions. First, is there a problem with the thyroid system in the newborn mouse pups that results in the reduced growth, problems with lipid metabolism and temperature sensitivity? Secondly, given the possible impact of PACAP on the sympathetic nervous system, I asked whether adult mice lacking PACAP would have a different response to a high fat diet than mice with a gene encoding PACAP. It was possible that weight control in the PACAP-null mouse might be aggravated by the fact PACAP has a role in normal insulin release and glucose balance.

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CHAPTER 2: Six novel GnRH hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*.

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

Introduction

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

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

In the present study, I began by searching the complete genome for GnRH genes in two tunicate species, *Ciona intestinalis* and *C. savignyi*. I then used molecular biological techniques to isolate and sequence the gene and cDNA structures from *C. intestinalis*. In addition, the alternative splicing and expression pattern of mRNA for both genes in *C. intestinalis* were examined during early development and in adults. All six novel GnRH peptides from *C. intestinalis* and a seventh from *C. savignyi* were synthesized and tested for biological activity.

We determined the cross-reactivity of the new peptides with a suite of GnRH-specific antisera and used three of these to test for the presence of mature GnRH peptide in a dorsal strand preparation. Finally, we examined if the nearest upstream gene was the same as in medaka fish and human GnRH genes.

Materials and methods

Analysis of gene organization

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

Identification of the *Ciona savignyi* GnRH genes utilized sequence data from the Whitehead Institute *C. savignyi* sequencing project (http://www-genome.wi.mit.edu/annotation/ciona/). This database was limited to nucleotide similarity searches using MEGABLAST. To generate an amino-acid-searchable database, the genome-read sequences were downloaded and compiled into a local blast database. These sequences were then compared using TBLASTN analysis with the same parameters described above. Each of the *C. intestinalis* GnRH forms was used to search and resulted in similar blast matches allowing the characterization of two *C. savignyi* gene arrangements.

To determine the transcription start site for each gene, a sequence of 1000 bp that was 5' to the GnRH peptide coding region was entered into the neural network promoter prediction site (http://www.fruitfly.org/seq_tools/promoter/html) using the default minimum promoter score of 0.80.

I also searched the *C. intestinalis* genome with known forms of PACAP (both 27 and 38 amino acid forms) from a number of species including the tunicate *Chelyosoma productum*, mouse, human, and fish. I performed a search using full-

length and N-terminal segments ($PACAP_{1-12}$) of other PACAP/glucagon superfamily of peptides as well as full-length and transmembrane domain coding regions of PAC_1 and the two VPAC receptors.

Animals

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

Isolation of mRNA and synthesis of cDNA

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

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

Isolation of genomic DNA

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

PCR of cDNA and genomic DNA

Oligonucleotides were designed against regions encoding candidate GnRH prepropeptides based on the compiled genomic sequences for *C. intestinalis* GnRH genes 1 and 2 (Table 2.1). Each 50 µl reaction contained 2.5 U Taq polymerase, 1x Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen) and 20 pmol of each 5' and 3' primer. PCRs were carried out under the following conditions: denaturation at 94 °C for 30 sec; annealing at 52 °C for 30 sec; extension at 72 °C for 30 sec for 35 cycles and a 7 minute extension. The PCR products were separated by electrophoresis on a 1.3% agarose gel and visualized with ethidium bromide staining using an Eagle Eye II still video system (Stratagene, La Jolla, CA). Bands were selected and isolated (Qiagen) and cloned or cloned directly as PCR products into pGEM Vector-T (Promega, Madison, WI) and sequenced.

Peptide synthesis

All peptides were synthesized using an automated method by Jean Rivier and his colleagues at the Salk Institute as outlined in Adams *et al.*, 2003.

Screening tunicate peptides with available GnRH antisera

Sixteen different antisera that were raised in rabbits against different forms of GnRH were initially screened for binding to tunicate peptides. The antisera included: Jas-2 through -11 (anti-tunicate GnRH-1); Bla-5, Her-4, Jul-5 (anti-lamprey GnRH-1); Emily and Gertie (anti-lamprey GnRH-III); 7CR-10 (anti-dogfish GnRH); GF-6, FP-5, PBL-45, PBL-49 and Aida (anti-salmon GnRH); 8CR-6 and –10, 9CR-6 (anti-catfish GnRH); Adams-100 (anti cGnRH-II); and B-7 (anti-mammalian GnRH). Ten antisera were prepared in the Sherwood laboratory; the others included Emily and Gertie (a gift of Dr. Stacia Sower), PBL-45 and –49 (a gift of Dr. Wylie Vale), Aida (a gift of Dr. Katsumi Aida) and Adams-100 (a gift of Dr. Tom Adams). Each antiserum was used at a dilution of 1:1000 except for B-7 (1:2000), GF-6 and Aida

Table 2.1. Primer names and sequences used to amplify cDNA and genomic sequences for GnRH genes and to identify the upstream neighbor gene in *C. intestinalis*. All primers were used at an annealing temperature of 55 °C. The direction of the primer, either forward (f) or reverse (r) is indicated.

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

(1:5,000), 7CR-10 (1:7500), Gertie (1:8000), Adams-100 (1:10,000), PBL-49 (1:30,000) and PBL-45 (1:50,000). The tGnRH-3, -5, -6, and -7 peptides were iodinated with I^{125}. The percent binding (maximum binding) of the four iodinated tunicate GnRH peptides with each antiserum was determined compared with the total counts.

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

Immunolocalization

Immunolocalization was performed by Dr. George Mackie using antisera from our laboratory. Specimens of *C. intestinalis* were relaxed in 0.01% MS222 for 30 min. Portions of the dorsal fold were pinned out in Petri dishes lined with Sylgard 184 (Dow-Corning) using cactus spines. The dorsal blood sinus was opened to allow access of reagents to the dorsal strand and to nerves lying within the sinus. The dorsal strand was fixed for 1 hour in 4% paraformaldehyde in 0.1 M PBS at pH 7.3 at room temperature followed by washing and storage in 0.1 M PBS containing 0.35 % Triton X-100 and 0.03 % sodium azide (PTA).

Preparations were treated with one of three primary antibodies, Jas-2, Bla-5 or FP-5 in 1:100, 1:1000 or 1:2000 (diluted with PTA), whereas controls were incubated in solutions omitting the antibody. All preparations had 1.5 % goat serum added and were incubated for 12-24 hours. After a PTA wash,

preparations were incubated in fluorescein isothiocyanate-labelled goat anti rabbit gamma globulin (Sigma #F-0382) for 12-24 hours. Following a PTA wash, preparations were mounted in 50 % glycerol containing 1.5 % N-propyl pyrogallate and examined by laser-scanning confocal microscopy using a Zeiss LSM 410 microscope.

Assay for gamete release induced by tunicate GnRH peptides

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

In silico analysis of promoter

Upstream promoter regions of 1000 nucleotides preceding the predicted transcription start site for each of the two GnRH genes in *C. intestinalis* and *C. savignyi* were generated with BLASTN similarity searches. To build the upstream promoter region for *C. intestinalis*, we used http://ghost.zool.kyoto-u.ac.jp/indexr1.html. Matching fragments were aligned using Bioedit Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A technique to walk 1000 bp upstream from each predicted transcription start site by overlapping matching fragments was utilized to generate a consensus sequence with a minimum of four agreeing fragments covering any given region. The promoter sequences were confirmed using the Department of Energy Joint Genome Institute *C. intestinalis*

v1.0 (http://genome.jgi-psf.org/ciona4/ciona4.home.html) on scaffolds 1051 (*Ci-gnrh1*) and 410 (*Ci-gnrh2*).

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

In silico and experimental analysis of nearest gene

To determine if any tunicate GnRH is an ortholog to a vertebrate GnRH, we identified the nearest upstream gene to GnRH. We searched the *C. intestinalis* databases using TBLASTN with the last exon in medaka FLJ20038 prepropolypeptide (medaka, *Oryzias latipes*; gi: 21955956). This gene has been identified as the closest upstream gene to mdGnRH in medaka and to mGnRH gene in human. This search resulted in a very high match (70% amino acid similarity) to a *C. intestinalis* FLJ20038 gene. A similar procedure of using overlapping fragments as described above was used to determine if a gene similar to FLJ20038 was located upstream of the GnRH genes. We also used the Department of Energy Joint Genome Institute *C. intestinalis* v1.0 to identify the scaffolds containing the two *C. intestinalis* GnRH genes as well as the *Ciona* FLJ20038 and PTPα genes. Primers F1 and F2 (see Table 1) were designed for the region coding for FLJ20038 and used in a PCR reaction with reverse primers G9 for *Ci-gnrh1* and G10 for *Ci*-gnrh2 to amplify possible products. PCR was performed using genomic DNA (see below), Platinum Taq DNA polymerase (Invitrogen), and the primers F1 or F2 and G9 or G10 under the following conditions: 94°C for 3 minutes, followed by 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1m 45s for 32 cycles, followed by a 7 min extension at 72 °C.

A similar procedure, both *in silico* and PCR using primers P1 or P2 and G9 or G10, was used to determine if a PTPα gene is upstream of the two *C. intestinalis* GnRH genes. The final exon of the medaka PTPα peptide (gi: 21955958) was found to have highly matching fragments within the *C. intestinalis* database. These matching nucleotide fragments were then used to construct an 1800 bp downstream region.

Results

GnRH peptides and gene arrangement

In silico analysis revealed six candidate GnRH peptides in *C. intestinalis*. The six peptides are each unique 10-amino-acid sequences and do not match any known GnRH peptides identified to date. The peptides were assigned numbers (Fig. 2.1) indicating whether they are present in *C. productum* only (tGnRH-1 and –2), in *C. intestinalis* only (tGnRH-3 and –4), in both *Ciona* species (tGnRH-5 through –8) or in *C. savignyi* only (tGnRH-9). Analysis of the genomic regions for these candidates showed that three of the peptides, tGnRH-3, tGnRH-5, and tGnRH-6, were found in close proximity as a triplet on one gene (*Ci-gnrh1*). These peptides are each bordered by basic amino acids (R or KR before the N-terminus, and GRR or GKR after the C-terminus) that are potential peptide cleavage sites separating the peptides. The GnRHs are separated by intervening peptides of 13 or eight amino acids (Fig. 2.2A).

Three more of these candidate peptides, tGnRH-7, tGnRH-8, and tGnRH-4, were found in tandem on a second gene, *C. intestinalis gnrh2* (*Ci-gnrh2*) (Fig. 2.2B). The peptides in *Ci-gnrh2* are each bordered by basic amino acids (RR or GKR) but lack intervening peptides.

I was unable to identify any sequence in the *Ciona* genome that would indicate a highly conserved or even generally similar candidate PACAP or PACAP superfamily peptide or receptors. However, annotated sequences for the GPCR family B member PTH-receptor were noted.

Sequences of GnRH genes in adult C. intestinalis

Using gene-specific primers, we amplified a single product from cDNA prepared from *adult C. intestinalis* brain tissue for each GnRH gene 1 and 2. Initial *Ci-gnrh1* products were amplified with primers G1 and G5 resulting in a 327 bp product and G1 and G6 resulted in a 343 bp product. 5'RACE-PCR resulted in a number of products for the 5' end of *Ci-gnrh1*, but only the longest 346 bp product made with G5 and the adapter inner primer matched the expected sequence. This product was used to compare to products in public EST and genomic databases. 3'RACE-PCR using G1 and the appropriate adapter primer resulted in a 708 bp cDNA fragment. This product was used to overlap and construct a full-length profile cDNA (Fig. 2.3) for comparison to public EST and genomic databases.

Initial *Ci-gnrh2* products were amplified with primers G2 and G7 resulting in a 600 bp product and G3 and G7resulting in a 559 bp product. 3'RACE-PCR using G2 and the appropriate adapter primer resulted in a 609 bp cDNA fragment. This product was used to overlap and construct a full-length profile cDNA (Fig. 2.4) for comparison with public EST and genomic databases. We were not able to isolate products for the 5' end of *Ci-gnrh2* cDNA by RACE-PCR. However, we were able to amplify genomic DNA that codes for this region.

A final cDNA and genomic sequence for each gene have been submitted to the DDBJ/EMBL/Genbank databases under accession numbers AY204706 (*Ci-gnrh1* cDNA), AY204707 (*Ci-gnrh1* gene), AY204708 (*Ci-gnrh2* cDNA), and AY204709 (*Ci-gnrh2* gene).

GnRH peptides in C. savignyi

Search results of the *C. savignyi* genome were used to identify fragments that group into similar gene structures as those in *C. intestinalis*. In *C. savignyi, Cs-gnrh1* encodes two copies of tGnRH-5 and one copy of tGnRH-6 (Fig. 2.5A), whereas *Cs-gnrh2* encodes tGnRH-7, tGnRH-8 and tGnRH-9 with the appropriate cleavage sites (Fig. 2.6A). Hence, the two *Ciona* species share the

Figure 2.1. Amino acid sequences of the tunicate (t) GnRH peptides, including the seven novel forms (tGnRH-3 to –9) identified in *C. intestinalis* and *C. savignyi*, and two previously identified forms in *Chelyosoma productum* (tGnRH-1 and –2) compared to mammalian (m)GnRH and chicken (c)GnRH-II. Amino acids different from mGnRH in tunicate GnRH peptides are underlined and bold, whereas differences in cGnRH-II from mGnRH are shown only in bold.

	1	2	3	4	5	6	7	8	9	10
mGnRH	**pGlu**	**His**	**Trp**	**Ser**	**Tyr**	**Gly**	**Leu**	**Arg**	**Pro**	**Gly-NH_2**
tGnRH-1	pGlu	His	Trp	Ser	**Asp**	**Tyr**	**Phe**	**Lys**	Pro	Gly-NH_2
tGnRH-2	pGlu	His	Trp	Ser	**Leu**	**Cys**	**His**	**Ala**	Pro	Gly-NH_2
tGnRH-3	pGlu	His	Trp	Ser	Tyr	**Glu**	**Phe**	**Met**	Pro	Gly-NH_2
tGnRH-4	pGlu	His	Trp	Ser	**Asn**	**Gln**	Leu	**Thr**	Pro	Gly-NH_2
tGnRH-5	pGlu	His	Trp	Ser	Tyr	**Glu**	**Tyr**	**Met**	Pro	Gly-NH_2
tGnRH-6	pGlu	His	Trp	Ser	**Lys**	Gly	**Tyr**	**Ser**	Pro	Gly-NH_2
tGnRH-7	pGlu	His	Trp	Ser	Tyr	**Ala**	Leu	**Ser**	Pro	Gly-NH_2
tGnRH-8	pGlu	His	Trp	Ser	**Leu**	**Ala**	Leu	**Ser**	Pro	Gly-NH_2
tGnRH-9	pGlu	His	Trp	Ser	**Asn**	**Lys**	Leu	**Ala**	Pro	Gly-NH_2
cGnRH-II	pGlu	His	Trp	Ser	**His**	Gly	**Trp**	**Tyr**	Pro	Gly-NH_2

Figure 2.2. Schematic arrangement of the genes for *Ci-gnrh1* (A) and *Ci-gnrh2* (B) in *C. intestinalis*. tGnRH peptides are indicated by black boxes with the appropriate tGnRH peptide number in white as well as single letter codes for bordering basic amino acids. 5' flanking region (5' flanking), 5'untranslated region (5'UTR), signal peptide (SP), GnRH-associated peptide (GAP), 3' untranslated region (3'UTR), 3' flanking region (3' flanking), amino acids (aa), base pairs (bp) in introns.

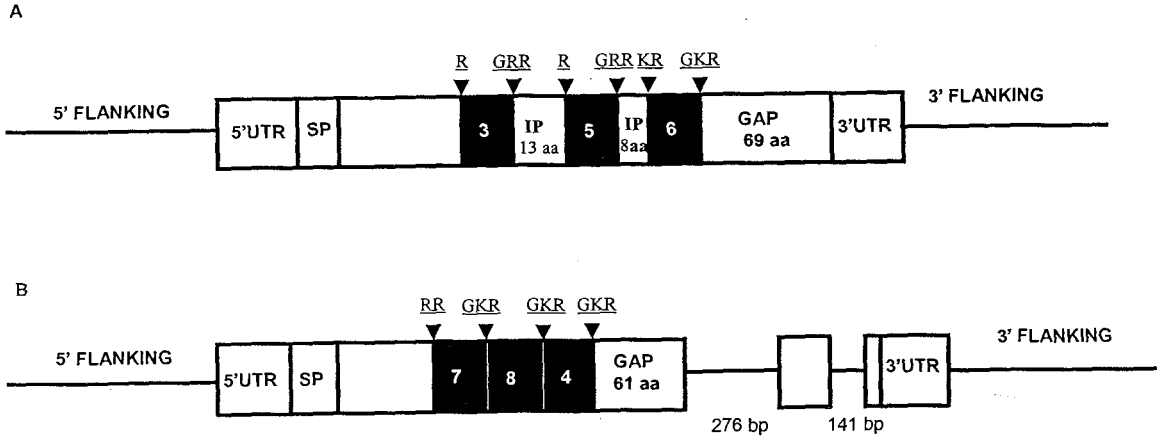

A
R
GRR
R
GRR
KR
GKR
5' FLANKING
5'UTR
SP
3
IP
13 aa
5
IP
8aa
6
GAP
69 aa
3'UTR
3' FLANKING
B
RR
GKR
GKR
GKR
5' FLANKING
5'UTR
SP
7
8
4
GAP
61 aa
3'UTR
3' FLANKING
276 bp
141 bp

Figure 2.3. Nucleotide sequence of cDNA, including derived amino acids, for *Ci-gnrh1*. *Ci-gnrh1* encodes three tunicate GnRH peptides in tandem, tGnRH-3, -5, and -6, which are bordered by basic amino acid residues (indicated by underlines), but are separated by intervening peptides. cDNA was prepared from mRNA isolated from nervous system tissue of adult *C. intestinalis*.

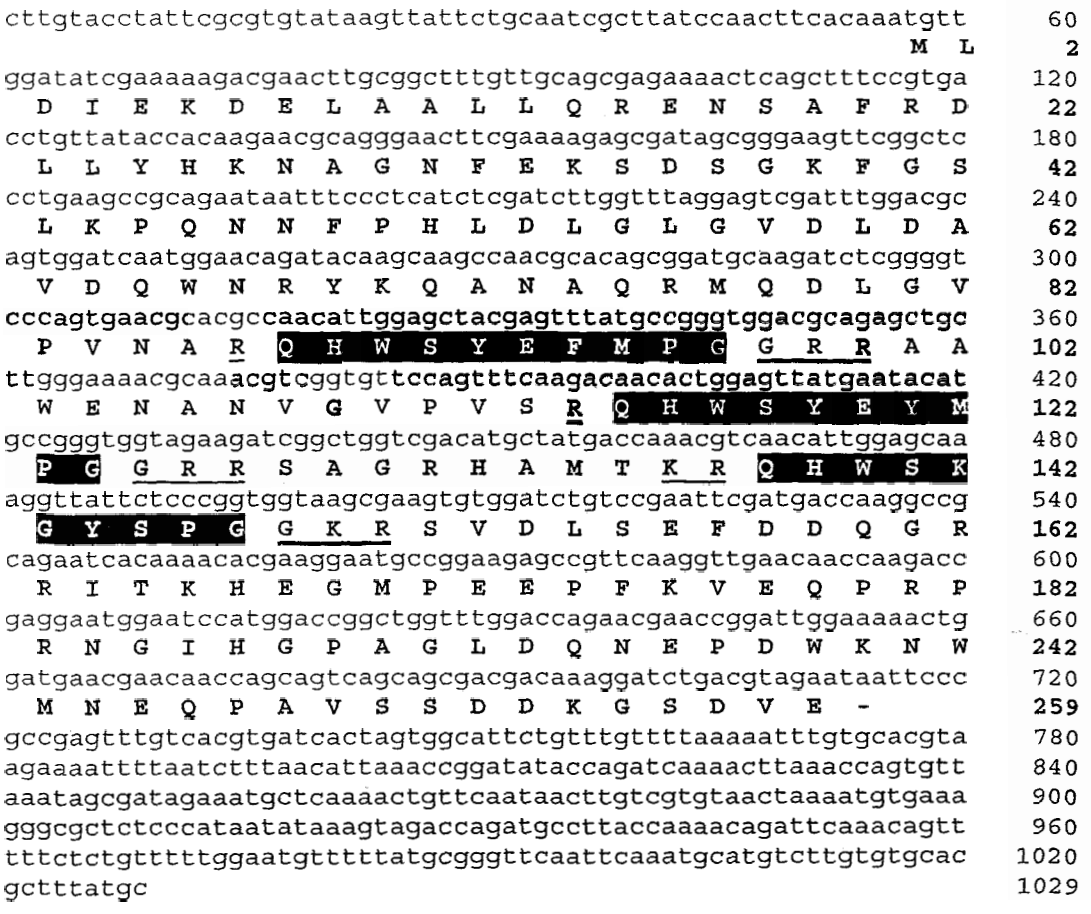

cttgtacctattcgcgtgtataagttattctgcaatcgcttatccaacttcacaaatgtt 60
M L 2
ggatatcgaaaaagacgaacttgcggctttgttgcagcgagaaaactcagctttccgtga 120
D I E K D E L A A L L Q R E N S A F R D 22
cctgttataccacaagaacgcagggaacttcgaaaagagcgatagcgggaagttcggctc 180
L L Y H K N A G N F E K S D S G K F G S 42
cctgaagccgcagaataatttccctcatctcgatcttggtttaggagtcgatttggacgc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

Figure 2.4. Nucleotide sequence of cDNA, including derived amino acids, for *Ci-gnrh2*. *Ci-gnrh2* encodes three tunicate GnRH peptides in tandem, tGnRH-7, -8, and -4, which are bordered by basic amino acid residues (underlined). Arrow heads indicate positions of two introns, the first of which is sometimes retained in mRNA. cDNA was prepared from mRNA isolated from nervous system tissue of adult *C. intestinalis*.

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

Figure 2.5. (A) Nucleotide sequence from the *Ciona savignyi* genome and derived amino acid sequence of gene *Cs-gnrh1*. Basic amino acids bordering the tGnRH peptides are underlined. (B) Alignment of the derived amino acids of *Ci-gnrh1* and *Cs-gnrh1*. Identical amino acids are indicated by an asterisk (*). tGnRH-5 (two) and -6 (*C. savignyi*) and -3, -5 and -6 (*C. intestinalis*) are indicated by a black background.

A.

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

B.

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

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

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

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

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

Figure 2.6. (A) Nucleotide sequence from *Ciona savignyi* genome and derived amino acid sequence of gene *Cs-gnrh2*. Basic amino acids bordering the tGnRH peptides are underlined. (B) Alignment of the derived amino acids of *Ci-gnrh2* and *Cs-gnrh2*. Identical amino acids are indicated by an asterisk (*). tGnRH-7, -8 and -9 (*C. savignyi*) and -7, -8 and -4 (*C. intestinalis*) are indicated by a black background.

A.

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

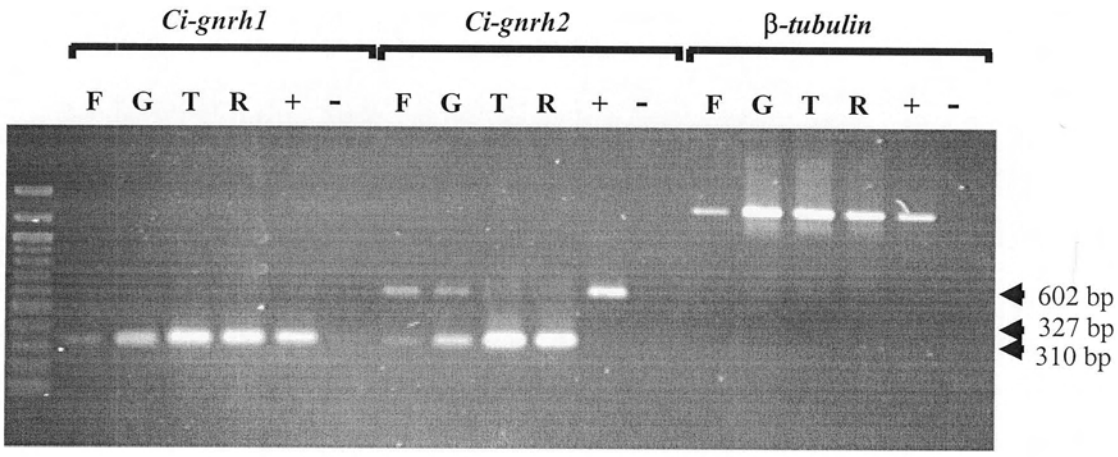
B.

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

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

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

Figure 2.7. PCR products showing developmental expression of both *Ci-gnrh1* and *Ci-gnrh2* in *C. intestinalis* at four stages: 4-cell (F), gastrulation (G), tail release (T), and tail resorption (R). Primers to amplify β-tubulin cDNA were used as a quality control for cDNA synthesis. Two control reactions were also included for each set of primers: one negative without DNA (-) and one positive control using adult *C. intestinalis* intestine cDNA (+).


Ci-gnrh1
Ci-gnrh2
β-tubulin
F G T R + - F G T R + - F G T R + -
602 bp
327 bp
310 bp

same general gene structures for *gnrh1* and *gnrh2* (Figs. 2.5B, 2.6B).

Determination of transcription start site

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

Developmental expression

Throughout development both *Ci-gnrh1* and *Ci-gnrh2* are expressed by *C. intestinalis*. A single 327 bp product, amplified by PCR for *Ci-gnrh1,* is expressed at each stage: 4-cell, gastrulation, tail release, and tail resorption (Fig. 2.7). This is the same transcript that was amplified from adult *Ciona* tissue. In contrast, two transcripts, 310 bp and 602 bp, were amplified for *Ci-gnrh2* at the 4-cell stage and gastrulation, although only the shorter transcript was detected at the tail release and tail resorption stages. Sequencing of these products revealed that one transcript had introns removed, whereas the second transcript retained an intron. Adult tissue in the same study expressed one transcript for gene 1, but only the longer transcript for gene 2.

Cross-reactivity of tGnRH peptides

The nine tunicate peptides synthesized for this study had a purity >95% after purification by capillary zone electrophoresis. The observed monoisotopic mass $[M + H]^+$ values of each peptide corresponded with the calculated values (Table 2.2). Two out of nine tunicate peptides (tGnRH-3 and –5) showed the highest cross-reactivity with antiserum Bla-5, which was raised against lamprey GnRH-I (Table 2.3). Both peptides had between 77-117 % cross-reactivity. The other tunicate peptides had less than 2.5 % cross-reactivity; mGnRH had 3.5% or less cross-reactivity; and cGnRH-II did not cross-react (<0.1 %). All the peptides were tested with antiserum FP-5 and iodinated tGnRH-5 or tGnRH-6, but the

cross-reactivity was so weak that only cGnRH-II and tGnRH-5 reached 50% B/Bo binding at a concentration below 50 nM.

Immunolabeling of GnRH containing neurons in dorsal strand plexus

Both Bla-5 and FP-5 antisera labeled neurons in the dorsal strand plexus along with isolated neurites running within branches of the visceral nerve (Fig. 2.8), as previously reported for *C. intestinalis* using antisera raised against cGnRH-II (Mackie, 1995), lamprey GnRH (Bollner et al., 1997) and salmon GnRH (Tsutsui et al., 1998). These antisera cross-react with several forms of GnRH. There was no labeling in the preparations incubated with the anti-Jas-2 antibody or in controls.

Bioactivity of tunicate peptides

The novel tunicate peptides identified in this study were biologically active and caused release of eggs and/or sperm from mature adult *C. intestinalis* (Table 2.4). Administration of the peptide initially caused an increase in the water flow by bodily contraction. At least two tunicates from each group injected with GnRH peptide released gametes. The most effective peptides in this study were tGnRH-5, which caused 63 percent of tunicates to spawn, and tGnRH-3, in which 50 percent of tunicates spawned. The time for the tunicates to spawn ranged from 1 min 33 sec to 36 min. There was also a great range in the intensity and number of eggs or sperm released, though this was not associated with a particular peptide. Three animals injected with GnRH peptide underwent increased water flow and large bodily contractions, but they did not release eggs or sperm. This observation suggests that the GnRH may have been effective, but the gametes were not ripe for release at the time of our experiment.

Promoter consensus sites for transcription factors

We identified a number of potential binding sites for transcription factors in the *Ciona* GnRH genes (Fig. 2.9); these DNA sequences are involved in the regulation of GnRH gene expression in promoter studies of other species. The

relevant binding sites in the *Ciona* promoter regions are predicted to have binding affinity to POU factors (Brn-2, Oct-1, Pit-1, and Tst-1), GATA motif binding factor (GATA), androgen receptors (AR), glucocorticoid receptors (GR), progesterone receptors (PR), cyclic AMP response element binding protein (CREB), and its variants, CREB-1 and CREB-1/c-Jun heterodimer, and activator protein-1 (AP-1). No obvious pattern was seen with respect to the location of the binding sites of the above transcription factors but the frequencies of many transcription-binding sites are similar between the two *Ciona* species (Fig. 2.9).

We compared the 1000 bp upstream promoter regions in *C. intestinalis* and *C. savignyi* against the 1000 bp upstream promoter regions from human *GNRH1* (gi:19923125) and *GNRH2* (gi:2833652). The human *GNRH1* upstream region was closest in transcription binding sites to *Cs-gnrh2*, as each had the same number of AP-1, Brn-2, GATA, and GR sites, and each had the same sites but different number for Pit-1, Tst-1, CREBP-1, and Oct-1 binding sites. The only difference in types of response elements was that the human *GNRH1* promoter had a CREB-1/cJun heterodimer site and *Cs-gnrh2* did not. Human *GNRH2* was closest to the *Ci-gnrh1* promoter as they both shared CREB, AP-1 and Oct-1 binding sites.

Nearest upstream gene for GnRHs

DNA fragments were identified in *C. intestinalis* EST databases that had 73 % amino acid identity to the last exon of human FLJ20038 and 75 % identity to the last exon of human PTPα. However, these two genes were not detected upstream of *Ci-gnrh1* or *Ci-gnrh2*. To prove this, a region of approximately 4000 bp 3' to the FLJ20038 coding regions in *C. intestinalis* was compiled but neither *Ci-gnrh1* nor *Ci-gnrh2* was within that distance. We searched 4000 bp upstream of *Ci-gnrh*1 and 2500 bp upstream of *Ci-gnrh2* in an attempt to locate FLJ20038 but none of these matched the fragments used in constructing the FLJ20038 downstream region. Areas amplified by PCR did not contain the expected products. Also, *in silico* analysis showed that the two genes were neither within this downstream region of the PTPα peptide nor matched any

Table 2.2. Structure and physico-chemical characteristics of synthetic tunicate GnRHs

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

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

Table 2.3. The percent cross-reactivity (%) of antibody BLA-5 with each of the nine tunicate (t)GnRH forms, mammalian (m)GnRH and chicken (c)GnRH-II when standardized against tGnRH-3 or tGnRH-5.

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

Table 2.4. Release of eggs or sperm from mature adult *Ciona intestinalis* after injections of tunicate (t)GnRH peptides 2-9 or saline.

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

Figure 2.8. Whole-mount preparation of the dorsal wall of the dorsal blood sinus of *Ciona intestinalis* with GnRH-immunoreactive neurons of the dorsal strand nerve plexus (dsp) lying in the vicinity of the dorsal strand (ds). The latter (an epithelial structure) did not label but was faintly visible due to background illumination. GnRH-immunoreactive neurites also run within branches of the visceral nerve (vn).

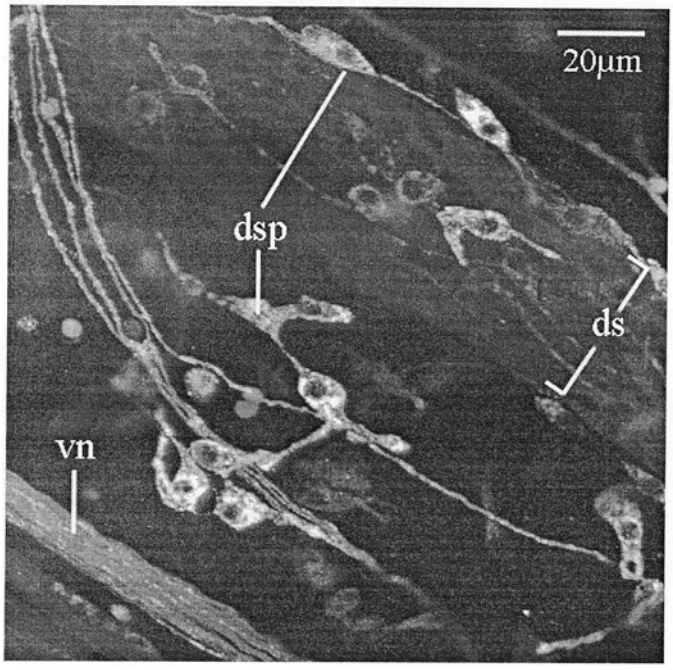
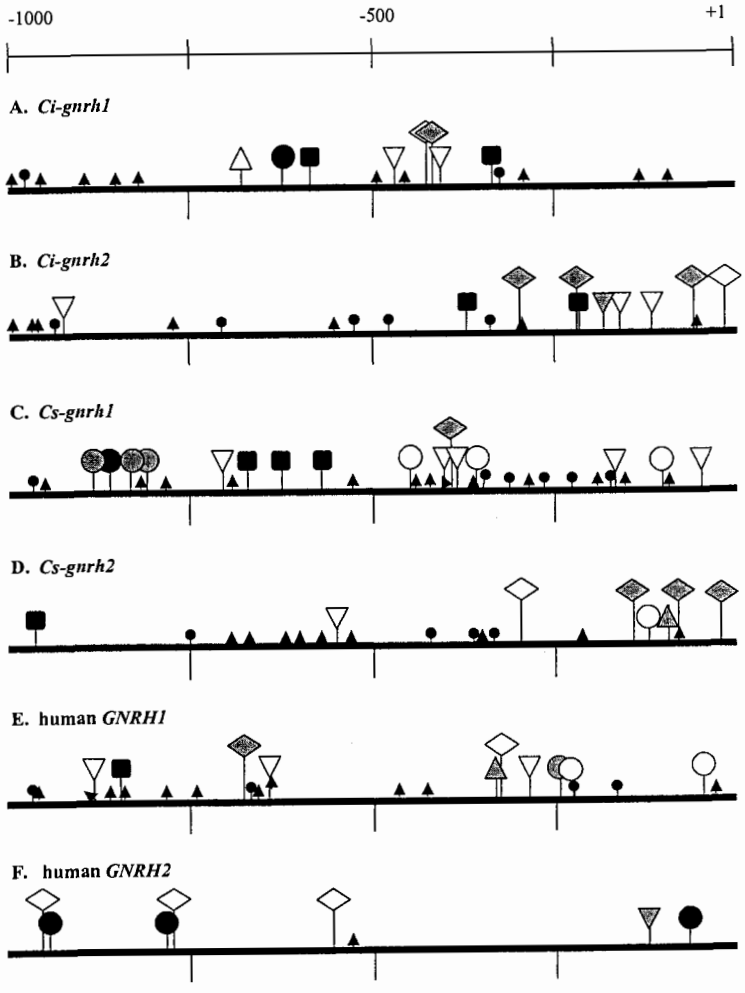

20µm
dsp
ds
vn

Figure 2.9. Promoter elements identified *in silico* using 1000 bp of gene sequence compiled upstream of the transcription start site for (A) *Ci-gnrh1*, (B) *Ci-gnrh2*, (C) *Cs-gnrh1*, (D) *Cs-gnrh2*, (E) human *GNRH1*, and (F) human *GNRH2*. △ ARE, androgen response element; ◇ AP-1 response element; ■ Brn-2 binding site; ● CRE, cAMP response element for CRE binding protein (CREB); ○ CREBP-1, cAMP response element for CRE binding protein-1; ● CREBP-1/cJUN, cAMP response element for CREBP-1/cJun heterodimer; ▮ GATA response element; ▲ GRE, glucocorticoid response element; ▲ Oct-1 binding site; ▽ Pit-1 binding site; ▼ PRE, progesterone response element; ◆ Tst-1 binding site.


-1000
-500
+1
A. Ci-gnrh1
B. Ci-gnrh2
C. Cs-gnrh1
D. Cs-gnrh2
E. human GNRH1
F. human GNRH2

fragments used to construct the upstream regions of either *Ci-gnrh1* or *Ci-gnrh2*. Products were not amplified by PCR using the PTPα forward primer and the *Ci-gnrh1* or *Ci-gnrh2* reverse primer. Furthermore, using the Department of Energy JGI database, we identified the four genes of interest on different scaffolds: *Ci-gnrh1* on scaffold 1051, *Ci-gnrh2* on scaffold 410, the FLJ20038 gene on scaffold 91, and the PTPα gene on scaffold 104. The distance between the genes was even greater than stated above.

Discussion

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

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

In my study, the entire gene *Ci-gnrh1* contains only one exon that encodes a 5'UTR, a putative signal peptide, then three GnRH peptides separated by intervening peptides of 13 and 8 amino acids. These are followed by a candidate GAP of 69 amino acids and finally a 3'UTR. In contrast, *Ci-gnrh2* contains three exons, with a large first exon containing the 5'UTR as well as most of the coding region for the three GnRH peptides and their cut sites (RR or GKR) but no intervening peptides, as well as most of a candidate GAP. Exon 2 is 276 bp

downstream and has no predictable function. The third exon is 141 bp further downstream and contains a stop codon followed by the 3'UTR.

In vertebrates each GnRH peptide is coded by its own gene allowing separate regulation of GnRH production for each peptide. However, in *Ciona* sp. three GnRH peptides are encoded on each of two genes, suggesting that the regulation of all three of the peptides coded on one gene is the result of common gene regulation. The multiplication of exons encoding GnRH may simply increase the output of peptides. The large number and type of promoter binding sites (identified *in silico*) that are shared with the human *GNRH1* gene suggest that some aspects of regulation have been conserved.

Two novel forms of GnRH, tGnRH-1 and tGnRH-2, were identified in our laboratory in the protochordate tunicate *Chelyosoma productum* (Powell et al., 1996). I did not find any evidence of DNA sequences in either of these two peptides in *C. intestinalis*. Thus, the peptides appear to be genus-specific.

Two GnRH peptides, cGnRH-I and mGnRH, were reported previously to be in the gonads of *C. intestinalis* using HPLC, radioimmunoassay, and mass spectrometry (Di Fiore et al., 2000). I did not find evidence of DNA sequences in either the genome or EST databases or by PCR for these two peptides in *C. intestinalis*.

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

and Rousseau, 1982) and mouse embryo (Raga et al., 1999), suggesting that a function for GnRH in early development is conserved in tunicates and vertebrates.

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

A comparison of the tunicate and human GnRH gene promoters includes 1000 bp for each of the genes. Within this 1 kb region the human *GNRH1* gene has both a downstream transcription start site at +1 and an upstream start site at -579 (Dong et al., 1993). Also, the proximal 1 kb of promoter is the region that restricts expression of GnRH to cells mainly in the hypothalamus; the proof is that transgenic mice with a human *GNRH1* construct require the promoter region from -992 to -763 bp to restrict the expression of a luciferase reporter gene (Wolfe et al., 2002). The rat *Gnrhl* promoter is not used for upstream comparison, as a neuron-specific enhancer is located further upstream at –1571 to –1863 (Whyte et al., 1995) and the human and rat promoters have marked differences in the structural organization of the promoters except in the proximal region at –343 to -1 (Kepa et al., 1996).

Each of the tunicate or human promoter regions had an abundance of POU-family binding sites, except human *GNRH2*. In the human *GNRH1* promoter, Brn-2 (a POU factor) binds to a site within the region essential for cell-specific expression (Wolfe et al., 2002). The tunicate promoters each contain one to three

Brn-2 binding sites, but it is not known if the sites are functional. Oct-1, another POU factor, binds within or near to the same cell-specific region, but did not stimulate human *GNRH1* transcription of the reporter gene (Wolfe et al., 2002; Dong et al., 2001). For the Tst-1 transcription factor, one to three binding sites were identified for each of the four tunicate promoters and one Tst-1 site for the human *GNRH1* promoter. In the rat *Gnrh-1* promoter, Tst-1 (referred to as SCIP/Oct-6/Tst-1) bound three sites within the proximal 333 nucleotides from the transcription start site and repressed GnRH transcription (Wierman et al., 1997). These data may be relevant to human *GNRH1* as the proximal promoter is similar to that of the rat.

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

The *Ciona* genes *Ci-gnrh1* and *Cs-gnrh1* both contain a single cAMP response element (CRE) site, whereas none were detected in *Ci-gnrh2* and *Cs-gnrh2* promoters. CRE is interesting because this is one of only a few binding sites identified for the human *GNRH2* promoter; the human *GNRH1* promoter does not have sites for binding CREB, although there are CREBP-1 and CREBP-1/cJun sites. The CRE site in the human *GNRH2* promoter appears to be functional as a cAMP analog upregulated expression of *GNRH2* mRNA and GnRH-II peptide (Chen et al., 2001). Mutation within the CRE site at –67 to –60 reduced both the basal activity and the cAMP analog response of the *GNRH2* promoter.

GnRH has direct effects on gonads in some invertebrates. Five GnRH forms (mGnRH, cGnRH-I, cGnRH-II, sGnRH and lGnRH-I) increased mitogenic activity in gonial cells of oysters, *Crassostrea gigas* (Pazos and Mathieu, 1999). Injection of tGnRH-1 and – 2 induced spawning in *C. intestinalis* generally within minutes, though tGnRH-2 was less effective than tGnRH-1 (Terakado, 2001). Each of the tGnRH peptides we tested induced gamete release, although some peptides appeared to be more effective than others, as the percent effectiveness

ranged from 20 to 63 percent. Each gene produces peptides that can induce the release of eggs and/or sperm. It is possible that the time of year of our experiment did not coincide with all the animals being fully mature. Animals were selected based on visual assessment of maturity – a white sperm duct and/or a pink oviduct. GnRH may act in concert with other biological or environmental factors to induce spawning, but may not be sufficient on its own. Only one of 19 saline-injected *C. intestinalis* released eggs. The bioactivity of the peptides, including tGnRH-2 found in *Chelyosoma productum* and tGnRH-9 that we have identified in the *C. savignyi* genome, suggests that the receptor(s) in *C. intestinalis* are able to bind many forms of tunicate GnRH. The GnRH receptor may initiate a direct or indirect effect on the gonoducts, but in either case, the isolation and localization of GnRH receptors are needed to demonstrate the target organs of GnRH in tunicates.

Subsequent studies using the nine tunicate GnRH peptides by our collaborators investigated the therapeutic potential and the nature of interaction between the ligands and mammalian GnRH receptors (Adams et al., 2003). Cells expressing the human GnRH-I receptor and a luciferase reporter gene driven by the LH α subunit were tested for receptor activation. Only three (tGnRH-3, tGnRH-5, and tGnRH-7) of the nine tunicate peptides tested activated the human GnRH-I receptor, and only slightly at the highest concentration tested, 1 μM (Adams et al., 2003). Also, the tunicate GnRH peptides were not able to stimulate LH from rat anterior pituitary cells in culture, suggesting no binding to either the type I or II GnRH receptor (Adams et al., 2003).

One surprise from this study was the lack of evidence for either a peptide of similar sequence to PACAP or to one of the PACAP/glucagon superfamily members or one of their receptors. Two cDNAs have been described previously in *C. productum* that each code for a PACAP peptide of 27 amino acids (McRory and Sherwood, 1997). The basis for the theory of PACAP evolution has its roots in the high conservation of this peptide in *C. productum*. The data suggest further efforts are warranted to gain insight into the extent of PACAP genes in tunicate species and to identify their receptors. It is possible there are still regions of the

genome which are not yet interpretable, and PACAP or PACAP-like genes could be contained within these DNA reads.

Tunicates of the genus *Ciona* are favourable models for study of the function and regulation of genes important in development (Corbo et al., 2001). Our *in silico* identification of genes encoding the hormone GnRH was confirmed by our sequencing of genomic DNA. We have shown that both of the GnRH genes are expressed as mRNA early in development and in adult *Ciona* tissue. The approach of identifying GnRH orthologs in protochordates by the nearest upstream gene does not appear to be suitable because the marker genes that are present in medaka and human are not upstream of *C. intestinalis* GnRH genes, although the genes are present in tunicate. The novel peptides do not represent potential analogs for rat or human GnRH studies because the peptides do not activate the human GnRH-I receptor or cause LH release in rat pituitary cell cultures. This is most likely due to the presence of an L-amino acid (in place of glycine) at position 6 in the tunicate peptides. It is accepted from structure-activity relationship studies in mammalian systems that non-glycine residues (except possibly for proline) are detrimental to biological activity (*in vitro* and *in vivo*) (Monahan et al., 1973). However, this explanation of structure activity relationships from the structural perspective cannot ignore the fact that each residue at positions 5-8 (Tyr-Gly-Leu-Arg) in mGnRH has been selected for optimal interaction with the mammalian receptor type 1 and that any deviation from that sequence results in significant loss of potency.

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CHAPTER 3: Protein characterization and cloning of three forms of GnRH, including a novel form, in a basal salmonid, *Coregonus clupeaformis*.

Parts of this chapter are included in the following publications:

Adams BA, Vickers ED, Warby CM, Park M, Fisher WH, Craig AG, Rivier JE, Sherwood NM 2002 Three forms of gonadotropin-releasing hormone (GnRH), including a novel form, in a basal salmonid, *Coregonus clupeaformis*. Biology of Reproduction 67: 232-239.

Vickers ED, Laberge F, Adams BA, Hara TJ, Sherwood NM 2004 Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, *Coregonus clupeaformis*. Biology of Reproduction 70: 1136-1146.

Introduction

The salmonids provide an interesting opportunity to study gene duplicates and how the genes are lost or undergo changes to produce novel peptide sequences and possibly specialization of function. Three subfamilies of the salmonids have species that still exist today, and therefore provide the opportunity to study the evolution of GnRH and PACAP genes in tetraploid species.

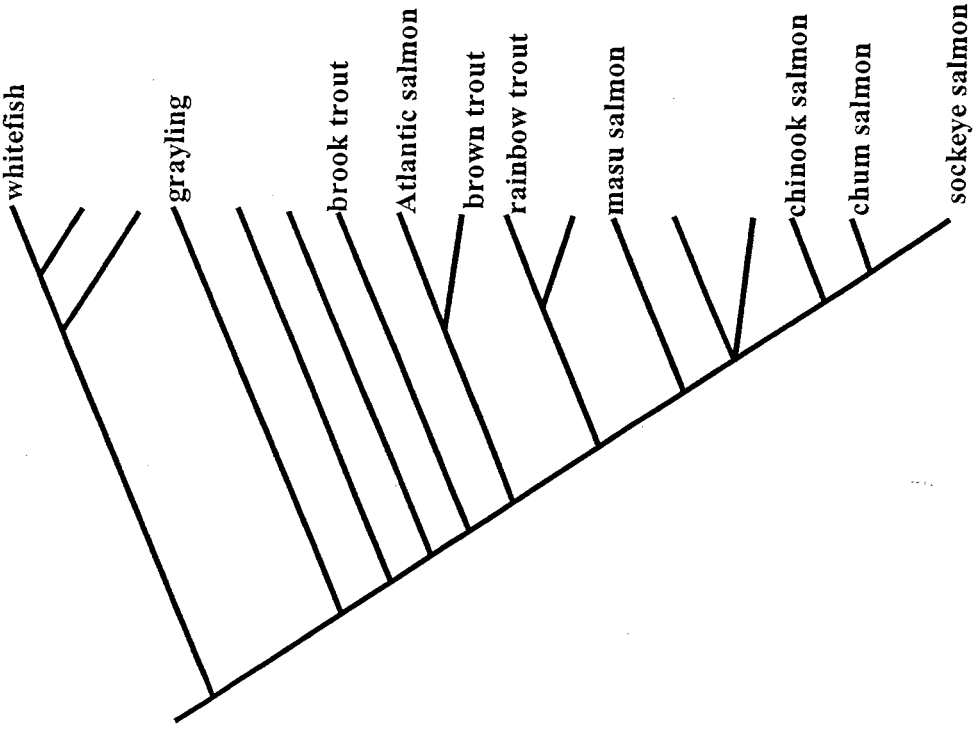
In this chapter, my objective was to determine whether a representative of the earliest subfamily of salmonids (Coregoninae) had more than two forms of GnRH in the brain. The presence of a third form implies that the loss of one form of GnRH occurred much later than the initial tetraploidization of the ancestral salmonid. Lake whitefish (*Coregonus clupeaformis*) were selected as a representative because of their phylogenetic position (Fig. 3.1), but also because they are available in a laboratory setting as well as in sufficient numbers in the wild for peptide purification. I was able to obtain more than a thousand lake whitefish brains that underwent HPLC purification by colleagues in the laboratory and subsequent identification by protein sequencing and mass spectrometry by Jean Rivier and colleagues at the Salk Institute. The primary structure of the third form of GnRH in whitefish was identified (Adams et al., 2002). Following the determination of the sequence, we wanted to determine whether the peptide was biologically active and to localize the different GnRH peptides using immunocytochemistry. Finally, we determined the cDNA structure of each form of GnRH in lake whitefish, including the cDNA for wfGnRH. This is the first time a third cDNA has been isolated from a tetraploid species. Finally, using these sequences, I performed a phylogenetic analysis to understand the relationship of the three whitefish cDNAs to those from other vertebrates.

Materials and methods

Collection of specimens

Lake whitefish were collected from two sources. Twelve fish (six males, six females) were netted from the Freshwater Research Institute, Winnipeg, Manitoba. Eleven wild lake whitefish were collected by gill netting at Exeter Lake near 100 Mile House, British Columbia, Canada. In each case, fish were deeply anesthetized with MS-222 (0.1 mg/ml) and killed. For isolation of mRNA, brains were dissected with

Figure 3.1. The relationship of salmonid species and their three subfamilies. Lake whitefish are members of the basal group of salmonids, the Coregoninae. Salmonid species such as sockeye salmon and rainbow trout are examples of Pacific salmon in the later-evolving Salmoninae subfamily.



Order: Salmoniformes

pituitaries attached if possible and frozen in liquid nitrogen or stored in RNAlater (Ambion Inc., Austin, TX). All brains were transported to the University of Victoria where the tissues were frozen at –80 °C.

Bioactivity of wfGnRH peptide

Synthetic wfGnRH was tested for its effect on pituitary hormone expression in dispersed rainbow trout (*Oncorhyncus mykiss*) pituitary cells. Procedures were approved by the University of Victoria Animal Care Committee. cGnRH-II was used for a comparison. The pituitary hormones for whitefish have not been isolated as proteins or cDNA. Instead, we developed an assay for the rainbow trout, a closely related salmonid, based on modification of the procedures in goldfish (Chang et al., 1994; Klausen et al., 2001). Briefly, wfGnRH and cGnRH-II were solubilized in water and stored at –20 °C. Rainbow trout growth hormone (GH) and the α-subunit that serves for both gonadotropins and thyroid-stimulating hormone (GtH/TSH) were cloned in our laboratory using rainbow trout pituitary total RNA and primers based on reported sequences (Agellon and Chen, 1998; Kitihara et al., 1988). Primers used to clone GH were 5'-AACGGCTCTTCAACATCG-3' and 5'-GACGGTCAGGTAGGTCTCG-3', forward and reverse, respectively. A nested primer strategy was used to generate the partial glycoprotein α-subunit cDNA. First round products were generated by the forward 5'-CAACTGGACTATTCCTCATCC-3' and reverse 5'-GCCCATACAGACAGTTTAT-3' primers. The nested primers were forward 5'GTCCGCACTTCTAGTCAT-3' and reverse 5'-AATAGCAGGTGCTGCAGT-3'. Rainbow trout were anesthetized in 50 mg/l Eugenol and killed by decapitation. Pituitaries were removed, washed and treated using a combination of physical fragmentation and a trypsin/DNase treatment. Fragments were dispersed in calcium-free media (Dulbecco's PBS, 25 mM Hepes, 2.2 g/L sodium bicarbonate, 0.3% bovine serum albumin, 100 000 U/L penicillin and 100 mg/L streptomycin, pH 7.2). Cells were harvested and cell yield and viability were determined. Cells were resuspended in culture medium (Medium 199 with Earle salts, 25 mM Hepes, 2.2 g/L $NaHCO_3$, 100 000 U/L penicillin and 100 mg/L streptomycin, pH 7.2; Life Technologies, Burlington, ON) and 140 000 to 180 000 cells per well were added to a 96 well plate for 2 hours at 18 °C, 5% CO_2 and saturated humidity. Then, horse serum (Life Technologies, Burlington, ON) was added to each well to a final concentration of 1% and incubated overnight at 18 °C, 5% CO_2 and saturated

humidity. The next morning cells were treated with 10^{-9}, 10^{-8} or 10^{-7} M wfGnRH or 10^{-7} M cGnRH-II for 12 hours. After the incubation cells, were harvested and total RNA was extracted.

Determination of mRNA levels in trout pituitary primary cell culture

Total RNA was extracted using TRIzol (Life Technologies, Burlington, ON) based on the guanidium thiocyanate-phenol-chloroform method of extraction (Chomzynski and Sacchi, 1987). The ratio of the absorbance wavelength (nm) at 260/280 for the samples ranged between 1.7 and 2.0. Subsequently, total RNA (approximately 5 ug) was loaded onto a 1.5% formaldehyde/agarose gel at 30 Volts for 4-5 hours. The RNA was transferred using the capillary transfer method with 0.1 N NaOH to a GeneScreen plus nylon membrane (New England Nuclear Life Science Products, Boston, MA), rinsed and fixed on the membrane by baking for 1 hour. Purified cDNA fragments for rainbow trout GH and GtH/TSH α-subunit were labeled using the Random Primers DNA Labeling System (Life Technologies, Burlington, ON) with [α-^{32}P]-deoxycytidine 5'-triphosphate (dCTP) 3000 Ci/mmol (New England Nuclear, Boston, MA). A probe of 18S rRNA was made using an end-labeling kit (Amersham Pharmacia Biotech, Baie d'Urfé, QC) incorporating [γ-^{32}P]-deoxycytidine 5'-triphosphate (dATP) 3000 Ci/mmol (New England Nuclear, Boston, MA). The membrane was prehybridized for 1.5-2 hours at 55 °C in 30 ml of ULTRAhyb (Ambion Inc., Austin, TX). The specific probe of interest was added and left to hybridize overnight at 55 °C. We used the 18S rRNA probe to control for possible variation in loading amounts. The membrane was washed following hybridization with a series of washes: 2 X 15 min with 2 % SSC and 0.2 % SDS; 1X 60 min with 0.2 % SSC and 0.2 % SDS at 55 °C. The membrane was exposed for 24-48 hours in a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Signals were analyzed with ImageQuant software. Between hybridizations the membrane was stripped of probe with 2 X 10 min washes containing 0.2 % SSC and 0.2 % SDS. mRNA levels were expressed with respect to the 18S rRNA signal obtained for each lane and reported as a percentage with respect to the control (where control is 0%). Statistical analyses were performed using ANOVA followed by a Student t Test. Differences between groups were considered significant when $P < 0.05$.

Lake whitefish RNA isolation and cDNA synthesis

Brain tissue was ground to a powder in liquid nitrogen using a cold mortar and pestle. Total RNA was isolated using TRIzol RNA isolation reagent (Invitrogen, Burlington, ON), based on an acid guanidinium-thiocyanate extraction method (Chomzynski and Sacchi, 1987). The mRNA was extracted from total RNA using the Micro Poly(A)$^+$ Pure kit (Ambion Inc.) as per manufacturer's directions. The concentration of mRNA was quantified by a spot test on an ethidium bromide agar plate. The mRNA was stored at –80 °C.

For sGnRH and cGnRH-II cDNA isolation, both 5' and 3' rapid amplification of cDNA ends (RACE) were done using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA) by following the manufacturer's instructions. For wfGnRH cDNA isolation, 5' and 3' RACE products were made using the First Choice RNA Ligase Mediated (RLM)-RACE kit (Ambion Inc.) as per the manufacturer's directions. 3'RACE was performed using two rounds of PCR. First round used forward (F) primers for salmon (s)F1, chicken (c)F1, or whitefish (w)F1 (Table 3.1) with reagents provided in the kit, including first round anchor primer, and DNA Taq Polymerase (Invitrogen). The second round used 1 µl of first round PCR product and forward primers sF2, cF2, or wF2 with the nested adapter primer. Also, 5'RACE reactions involved two rounds of PCR. The first round used reverse (R) primers sR1, cR1, or wR1 with the appropriate first round primer, and second round used 1 µl of first round product and the reverse primers sR2, cR2 or wR2 with the appropriate nested adapter primer. All cDNAs were amplified at annealing temperatures indicated in Table 3.1, for 35 cycles with a 7-minute extension at 72°C. All second round PCR products were separated on a 1.5% agarose gel using electrophoresis and visualized by ethidium bromide stain on the Eagle-Eye II still video system (Stratagene, La Jolla, CA).

PCR products that were considered to be candidate GnRH cDNAs (200-400 bp) were ligated into the pGEM T-Vector plasmid (Promega, Madison, WI). Plasmid DNA was incorporated into *E. coli* XL-1 blue competent cells (Stratagene, Cedar

Table 3.1. 5' and 3' PCR primer sequences for amplifying sGnRH, wfGnRH, and cGnRH-II from lake whitefish.

Primer Name	Primer Sequence*	Annealing Temperature (°C)
sF1	5'-cagcactggtcgtatggvtgg-3'	45
sF2	5'-ggctacctggaggraagagaa-3'	50
sR1	5'-cttcacctcctgtgtccatcatc-3'	50
sR2	5'-atcctgatggtggcctccagctc-3'	56
wF1	5'-cagcactggtcgtatggvatg-3'	50
wF2	5'-atgaaycctggaggvaagagr-3'	55
wR1	5'-gcaagtaatgcctcagcct-3'	55
wR2	5'-gcctgtacattttggcatgag-3'	57
cF1	5'-cagcactggtcncayggntggta-3'	50
cF2	5'-tggtcncayggntggtayccngg-3'	55
cR1	5'-cttcgctgtggtctcaggtagct-3'	52
cR2	5'-ctgcattctcctgcctcacaga-3'	58
sF3	5'-gtctcagaaagactgagacca-3'	55
sR3	5'-aatcactctttattacaattt-3'	55
wF3	5'-atgaaycctggaggvaagagr-3'	55
wR3	5'-gacccccaacaaagaccacc-3'	55
cF3	5'-acgcgggaagcactggtatca-3'	55
cR3	5'-ctagcccactgaccatcact-3'	55

*Exact nucleotide sequence of each primer is included where r = a + g, y = c + t, v = a + c + g, and n = a + c + g + t.

Creek, TX) by electrotransformation. Colonies were grown on plates treated with 100 mg/ml ampicillin, 0.1M IPTG, and 20 mg/ml X-Gal. White colonies, assumed to be recombinant colonies, were picked and grown overnight in 3 ml LB broth supplemented with 100 mg/ml ampicillin. All plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's instructions. A 3-µl sample of DNA was digested using the restriction enzymes Pst-1 and Sph-1 (New England Biolabs, Mississauga, Ontario, Canada), followed by separation on a 1.5% agarose gel. Gels were stained in ethidium bromide and plasmids containing candidate GnRH cDNA inserts were sequenced in both directions.

Phylogenetic analysis

Gene and cDNA sequences encoding 71 different GnRH precursors were obtained from Genbank (http://www.ncbi.nlm.nih.gov) and the literature. The GnRH precursors (signal peptide, GnRH, processing site (GKR), and GnRH-associated peptide, GAP) were entered into a common file using BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999). Phylogenetic analyses were carried out with Clustal X using the Neighbor-Joining method. The data were re-sampled by 1000 bootstrap replicates, and the proportion of Neighbor-Joining trees possessing each particular internal branch is indicated to express its level of support. Phylogenetic trees were generated using the TreeView software package, version 1.5.2 for Microsoft Windows 32 bit (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html), and formatted using Microsoft Word. Full species names and GenBank accession numbers for the sequences of the GnRH precursors used are listed in the legend of the tree. Only sequences that included a full coding DNA sequence were used in analyses.

Immunocytochemistry

The antisera GF-6 and 7CR-10 were raised in rabbits in our laboratory against sGnRH or dogfish GnRH, respectively. Cross-reactivity studies showed that GF-6 strongly cross-reacted with wfGnRH and sGnRH, whereas 7CR-10 cross-reacted only with cGnRH-II and sGnRH (Table 3.2). Cross-reactivity was determined as described earlier (Lescheid et al., 1997).

Four female and seven male fish were deeply anesthetized with MS-222, then perfused through the heart with 30 ml of PBFS followed by 250 ml of 4%

paraformaldehyde in PBFS. The brain was removed from the skull, left in the fixative solution overnight, and then cryoprotected in 30% sucrose PBFS for a day. Sagittal sections 40 μm thick were cut on a cryostat (American Optical Corp.). Consecutive sections were distributed among the different reactions (antibody or antibody plus blocking peptide). Immunocytochemistry was performed on free-floating sections.

The sections were incubated in PBFS containing 4% normal donkey serum, 0.4% TRITON-X and 1% bovine serum albumin (BSA) for 20 minutes (blocking step), followed by incubation with the first antiserum (rabbit anti-GnRH) diluted 1:2500 or 1:5000 in PBFS with 0.4% TRITON-X, 1% normal serum and 1% BSA at 4°C overnight. The next day the sections were rinsed 10 × 10 minutes in PBFS with 0.02% TRITON-X and 0.25% BSA, followed by incubation with the secondary antibody (biotinylated donkey anti-rabbit) diluted 1:1000 in PBFS with 0.02% TRITON-X and 1% BSA for 60 minutes. The antigen of the secondary antibody was whole rabbit IgG. The sections were rinsed again 2 × 15 minutes in PBFS with 0.25% BSA, followed by incubation with avidin-biotin: peroxidase with 1%BSA and 0.4% TRITON-X for 120 minutes. After 3 × 10 minute rinses in PBFS, the chromagen solution (100 mM $NiSO_4$, 125 mM acetate, 10 mM imidazole, 0.03% diaminobenzidine, 0.003% H_2O_2) was applied to the sections for 2-15 min. The sections were finally mounted on gelatinized glass slides, dried, dehydrated in graded alcohols and cleared in toluene. Coverslips were applied. The secondary antibodies and normal serum were from Jackson ImmunoResearch Laboratories (West Grove, PA) and the ABC kit was from Vector Laboratories (Burlingame, CA). Additional chemical supplies were from Sigma Chemical Co. (St Louis, MO).

Controls were performed by adding 100 μg of wfGnRH or sGnRH peptide to the primary antisera solutions at 30 minutes prior to application to the sections.

Statistical analysis

A two-way ANOVA with unweighted means was used to determine if the number of neurons labeled by the two antisera was different in the four brain regions that showed labeling with both antisera.

Results

Physiological effects

Both wfGnRH and cGnRH-II peptides significantly increased GH mRNA expression in dispersed rainbow trout pituitary cells by 42% and 80%, respectively (Fig. 3.2). wfGnRH stimulated GtH/TSH-α subunit mRNA expression 90% (a significant increase) compared to controls in dispersed rainbow trout pituitary cells. However, the 30% increase by cGnRH-II was not statistically significant. *cDNA sequences*

We isolated three cDNA sequences that encode sGnRH, wfGnRH, and cGnRH-II separately from lake whitefish by overlapping the 5' and 3' ends. The lake whitefish sGnRH cDNA is 491 bp in length, and consists of a 44 bp 5'UTR, a 249 bp open reading frame, a stop codon (TAA), and a 195 bp 3'UTR (Fig. 3.3). The cDNA for wfGnRH is 511 bp long, which includes a 67 bp 5'UTR, a 279 bp open reading frame (encoding the signal peptide, wfGnRH, cut site, and GAP), a stop codon (TAA), and a 162 bp 3'UTR (Fig. 3.4). The lake whitefish cGnRH-II cDNA is 636 bp long, which includes a 202 bp 5'UTR, a 258 bp open reading frame (encoding the signal peptide, cGnRH-II, cut site, and GAP), a stop codon (TAA), and a 173 bp 3'UTR (Fig. 3.5).

Phylogenetic relationships

An unrooted phylogenetic tree based on the amino acids of the GnRH precursors revealed three major groupings of vertebrate GnRH (GnRH1, GnRH2 and GnRH3) (Fig. 3.6). The lake whitefish wfGnRH precursor fits with the GnRH1 group; whitefish cGnRH-II and sGnRH each formed a group, GnRH 2 and GnRH 3, with the same molecules from other species.

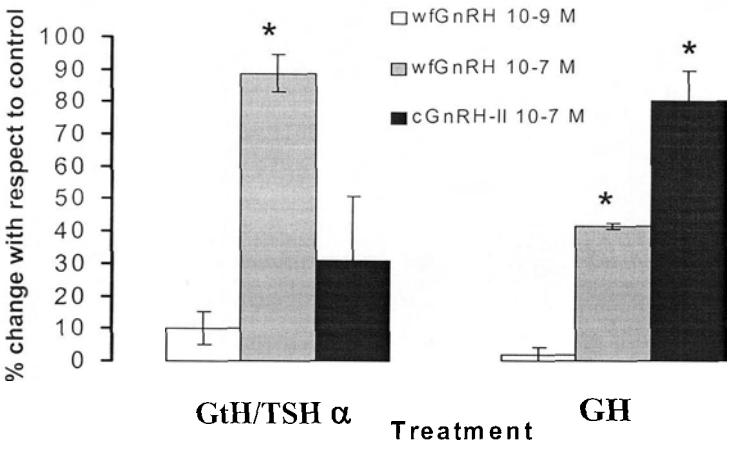
GnRH I includes the precursors of ten vertebrate GnRH forms that have been sequenced and are expressed mainly in the preoptic-hypothalamic areas, the main location for neurons that control reproduction. However, within GnRH1, fish precursors group together, whereas tetrapod GnRH1 precursors form a separate group of two main branches: one branch includes three amphibian precursors and the second includes precursors found in birds and mammals. The precursors for a specific form, for example mammalian (m)GnRH, do not group based on the GnRH peptide produced, but group instead with precursors from organisms that are more closely related phylogenetically. For example, the mGnRH precursor from Japanese eel is more closely related to other fish GnRH precursors that code for different forms of

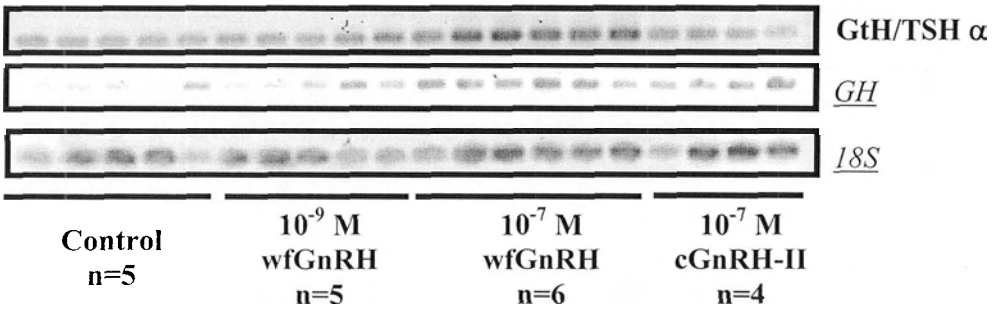
TABLE 3.1. Gonadotropin-releasing hormones.

Peptide	Species	Group*	Amino acids 1 2 3 4 5 6 7 8 9 10	Cross-reactivity antisera GF-6	7CR-10
mGnRH	m= mammal	GnRH 1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	100%	**<0.03%**
gpGnRH	gp=guinea pig	GnRH 1	pGlu-**Tyr**-Trp-Ser-Tyr-Gly-**Val**-Arg-Pro-Gly-NH_2	nt	<0.04%
cGnRH-I	c=chicken	GnRH 1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-**Gln**-Pro-Gly-NH_2	nt	<0.04%
fGnRH	f=frog	GnRH 1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-**Trp**-Pro-Gly-NH_2	nt	<0.04%
wfGnRH	**wf=whitefish**	**GnRH 1**	pGlu-His-Trp-Ser-Tyr-Gly-**Met**-**Asn**-Pro-Gly-NH_2	124%	<0.04%
sGnRH	s=salmon	**GnRH 3**	pGlu-His-Trp-Ser-Tyr-Gly-**Trp**-**Leu**-Pro-Gly-NH_2	69%	85%
sbGnRH	sb=seabream	GnRH 1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-**Ser**-Pro-Gly-NH_2	nt	<0.04%
pjGnRH	pj=pejerrey	GnRH 1	pGlu-His-Trp-Ser-**Phe**-Gly-Leu-**Ser**-Pro-Gly-NH_2	nt	<0.04%
hrGnRH	hr=herring	GnRH 1	pGlu-His-Trp-Ser-**His**-Gly-Leu-**Ser**-Pro-Gly-NH_2	nt	<0.04%
cfGnRH	cf=catfish	GnRH 1	pGlu-His-Trp-Ser-**His**-Gly-Leu-**Asn**-Pro-Gly-NH_2	nt	<0.04%
cGnRH-II	c=chicken	**GnRH 2**	pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Tyr**-Pro-Gly-NH_2	4%	100%
lGnRH-I	l=lamprey	GnRH 1	pGlu-His-**Tyr**-Ser-**Leu**-**Glu**-**Trp**-**Lys**-Pro-Gly-NH_2	nt	6%
dfGnRH	df=dogfish	-	pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Leu**-Pro-Gly-NH_2	nt	25%
lGnRH-III	l=lamprey	-	pGlu-His-Trp-Ser-**His**-**Asp**-**Trp**-**Lys**-Pro-Gly-NH_2	nt	13%

*The cDNA for dfGnRH and lGnRH-I have not been determined. nt (not tested)

Figure 3.2. Northern blot analysis of relative mRNA expression for GtH/TSH glycoprotein α-subunit and GH in dispersed rainbow trout pituitary cells 12 hours after treatment with wfGnRH or cGnRH-II. a) Values (+/- SEM) represent the percent change compared to control values, $P \leq 0.05$. b) Representative imaging of bound and labeled probes for GtH/TSH glycoprotein α-subunit, GH and ribosomal subunit 18S, to correct for RNA loading.


% change with respect to control
100
90
80
70
60
50
40
30
20
10
0
wfGnRH 10-9 M
wfGnRH 10-7 M
cGnRH-II 10-7 M
*
*
*
GtH/TSH α
GH
Treatment


GtH/TSH α
GH
18S
Control
n=5
10-9 M
wfGnRH
n=5
10-7 M
wfGnRH
n=6
10-7 M
cGnRH-II
n=4

GnRH than it is to mGnRH-encoding precursors from tetrapods. The second group, GnRH 2, is composed solely of cGnRH-II-encoding precursors. These precursors also group according to our current understanding of phylogenetic relationships for these species. The third group, GnRH 3, is composed solely of precursors encoding sGnRH, which are expressed in neurons in the olfactory and other regions. The GnRH 3 precursors are the most different from those in GnRH 1 and GnRH 2.

Immunocytochemistry

Immunocytochemistry revealed the existence of neurons positive for GnRH in several regions of the lake whitefish brain. Figure 3.7 shows representative examples of GnRH-positive neurons in the olfactory nerve, olfactory bulb, ventral telencephalon, preoptic area and midbrain. Both 7CR-10 and GF-6 antisera labeled a similar number of neurons in anterior brain regions (P=0.608) except the midbrain, where only 7CR-10 labeled neurons (Table 3.3). In the forebrain, 7CR-10 antiserum shows the distribution of sGnRH neurons as it does not cross-react with wfGnRH. The neurons labeled with 7CR-10 were present from the olfactory nerve-bulb junction to the preoptic area with the highest number of neurons in the ventral telencephalon (Table 3.3).

The GnRH neurons of lake whitefish were of three types: 1) large darkly stained neurons with extensive arborization in the ventral telencephalon and preoptic area; 2) small fusiform neurons found around the olfactory bulb and sometimes in the preoptic area; and 3) large lightly stained neurons with a limited arborization in the midbrain. Some GnRH-positive neurons in the ventral telencephalon appeared to be in contact with each other (Fig. 3.7C inset).

The distribution of labeled fibers was similar with both antisera. The fibers were widely distributed throughout the brain although few were in the cerebellum and spinal cord. However, fibers stained with GF-6 were less abundant around the midbrain. Few pituitaries in this study were attached to the brain preventing the study of GnRH neuron projections to the pituitary.

The addition of sGnRH peptide to the 7CR-10 primary antibody solution abolished all cell labeling, whereas the addition of whitefish GnRH peptide had no effect. This confirms the specificity of sGnRH for antiserum 7CR-10. The addition of whitefish GnRH peptide to the GF-6 primary antibody solution abolished all cell labeling, as expected.

Figure 3.3. Lake whitefish sGnRH cDNA. The nucleotide and derived amino acid sequence that encodes the sGnRH precursor of lake whitefish is shown. Nucleotides are numbered 5' to 3' and amino acids N-terminal to C-terminal. Signal peptide, cut site and GnRH-associated peptide are all underlined. Salmon GnRH and the nucleotides corresponding to the polyadenylation signal (aataaa) are in bold. The dash (-) indicates the stop codon.

```
acgcggggactgtaggaaggaatatacagaacggagacactcca ATG GAT CTT AGC AGC AGA   62
                                               M   D   L   S   S   R     6
                                                   Signal Peptide

ACG GTC GTG CAG GTG GTG GTG CTG GTA TTG GTA GCT CAG GTC ACT CTC TCT  113
 T   V   V   Q   V   V   V   L   V   L   V   A   Q   V   T   L   S   23

CAG CAT TGG TCA TAT GGG TGG CTA CCT GGA  GGG AAG AGA  AGT GTT GGG     164
 Q   H   W   S   Y   G   W   L   P   G   G   K   R   S   V   G      40
             salmon GnRH                    cut site

GAG CTG GAG GCC ACC ATC AGG ATG ATG GAC ACA GGA GGT GAA GTG GCT CTT  215
 E   L   E   A   T   I   R   M   M   D   T   G   G   E   V   A   L  57
                     GnRH-associated peptide

CCC GAG GAG ACA AGC GCC CAT GTC TCA GAA AGA CTG AGA CCA TAT GAT GTA  266
 P   E   E   T   S   A   H   V   S   E   R   L   R   P   Y   D   V  74
     •

ATA TCG AAG AAA TGG ATG CCC CAT AAA TAA agaactgagaccattattcacaaaag  322
 I   S   K   K   W   M   P   H   K   -                              83

aagcaagaagacaacatcaagcagacattcagcatcactatcaacatcaatgatggagcattcaac  388

tacagttctgtccatttacaactaacatcacatttagatttatgttatttacttgaagtatgaaac  454

tttaacctcctgtcaaattgtaataaagagtgattg                                491
```

Figure 3.4. Lake whitefish wfGnRH cDNA and derived amino acids. Signal peptide, cut site and GnRH-associated peptide are underlined. Whitefish GnRH and the nucleotides corresponding to the polyadenylation signal (aataaa) are in bold. The dash (-) indicates the stop codon.

```
cgcggatccgaacactgcgtttgctggctttgatgaaaagctagagtaataaggctgactttgcaga  67

ATG GAA GAG AAA AAG GTC CTG TTG TTG CTG CTG CTT TTG GTG GTG GCT CTA  118
M   E   E   K   K   V   L   L   L   L   L   L   L   V   V   A   L   17
                                Signal Peptide

GTG TCA CAG GGT TGC TGT  CAA CAT TGG TCC TAT GGC ATG AAC CCA GGG     166
V   S   Q   G   C   C    Q   H   W   S   Y   G   M   N   P   G      33
                              whitefish GnRH

GGG AAA AGA  GCG ACT GGC AGC CTG TCT GAC ACC CAG GAC AAT ATG GCT GAA 217
G   K   R    A   T   G   S   L   S   D   T   Q   D   N   M   A   E  50
cut site     GnRH-associated peptide

GAC CTT CTG AAG ATA GAC CCT TCT TGC AGT TTG TTT GGC TGT GCT GAT GTC  268
D   L   L   K   I   D   P   S   C   S   L   F   G   C   A   D   V   67

TCA CCT CAT GCC AAA ATG TAC AGG CTG AGG GCA TTA CTT GCA AGC CTC GCT  319
S   P   H   A   K   M   Y   R   L   R   A   L   L   A   S   L   A   84

GAC AGA CAA AGT GGA CTC AAT AAT ATA TAG caaatgtatgctagctaactcaatgaaa 377
D   R   Q   S   G   L   N   N   I   -                               93

cattgcagtgccatcattgtgttttatggtggtctttgttgggggtcccatttttgttggtttgcata 445

ttacatagtatggctttaaaacagtggaacacgatgtcaaataaaagtgccaacacatgattgat   511
```

Figure 3.5. Lake whitefish cGnRH-II cDNA and derived amino acids. Signal peptide, cut site and GnRH-associated peptide are underlined. Chicken GnRH-II and the nucleotides corresponding to the polyadenylation signal (aataa) are in bold. The dash (-) indicates the stop codon.

```
                                                    gagagatatcatcagaggccagctctcta   29

caacactcatcgtcgcctcaggaaaagtgatttgtcactgagttgctggataccaacaggttaata   95

ataggcagaagtgaacagcgggcacacagacgtacctggcggacatcaacagtgataaccgattcc   161

ataccacagtgattatcagactaagggtctaagctccagtg ATG GTC AGT GTG GCT AGA   220
                                             M   V   S   V   A   R     6

CTG GTG TTT ATG CTG GGG CTG CTG CTG TGT CTG GGA GCC CAG CTG TCC TCC   271
 L   V   F   M   L   G   L   L   L   C   L   G   A   Q   L   S   S    23
                                 signal peptide

TCC  CAG CAC TGG TCC CAT GGC TGG TAC CCA GGA  GGC AAG AGG  GAG CTG   319
 S    Q   H   W   S   H   G   W   Y   P   G    G   K   R    E   L    39
                  cGnRH-II                     cut site

GAC TCA TTT ACC ACC TCT GAG ATT TCA GAG GAG ATT AAA CTC TGT GAG GCA   370
 D   S   F   T   T   S   E   I   S   E   E   I   K   L   C   E   A    56
                         GnRH-associated peptide

GGA GAA TGC AGC TAC CTG AGA CCA CAG CGA AGG AAC ATC CTT AAG AAC ATT   421
 G   E   C   S   Y   L   R   P   Q   R   R   N   I   L   K   N   I    73

CTT TTG GAT GCT CTG GCC AGA GAA TTC GAG AAG AGA AAG TAA cctatcaactg   474
 L   L   D   A   L   A   R   E   F   E   K   R   K   -                86

tggttgacttaaatccttccttgtctatctgtttattccatatgaccactattttaatcttatgtcc   541

tttgatccaattttgtggtttatccattttgataataatgtagcatcactcctttacccttgcaccc   608

tctttattgtccctttttccgctgtag                                          636
```

Table 3.3. Number of labeled neurons in different brain regions in lake whitefish*

Antiserum	ON-OB	OB-Tel.	VT	POA	Midbrain
7CR-10 (n=6)	1.17±0.98 (5)	3.67±4.23 (3)	13.17±6.49 (6)	3.83±4.71 (5)	8.17±8.82 (4)
7CR-10 + 100 µg wfGnRH (n=2)	1.5±2.12 (1)	4±4.24 (2)	6±0 (2)	-	3±4.24 (1)
7CR-10 + 100 µg sGnRH (n=2)	-	-	-	-	-
GF-6 (n=4)	2±2.83 (2)	4±3.37 (3)	9.75±3.1 (4)	1±1.15 (2)	-
GF-6 + 100 µg wfGnRH (n=2)	-	-	-	-	-

*the numbers shown are for one quarter of a brain.
The symbol - indicates the absence of labeled neurons. The numbers in parentheses indicate the number of fish in which the presence of GnRH neurons was observed in that brain region. Other symbols: n: sample size; wfGnRH: whitefish form of gonadotropin-releasing hormone; sGnRH: salmon form of gonadotropin-releasing hormone; ON-OB: olfactory nerve-olfactory bulb region; OB-Tel.: olfactory bulb-telencephalon transition region; VT: ventral telencephalon; POA: preoptic area.

Discussion

We have characterized the novel GnRH peptide, wfGnRH (pGlu-His-Trp-Ser-Tyr-Gly-Met-Asn-Pro-Gly-NH_2), which was isolated from lake whitefish brain. I showed that the ability of wfGnRH to increase the expression of mRNA encoding GtH/TSH-α subunit and GH in dispersed fish pituitary cells, suggesting that the novel peptide is biologically active. The cDNA sequence for each of the genes coding for the GnRH peptides matches those determined by protein sequencing. This proof of three GnRH forms provides a mechanism to explain why tetraploid salmon have only two forms of GnRH, ie. ancestral salmon retained all three forms (presumably with duplicate copies), but later-evolving salmon had chromosomal rearrangements that resulted in a loss of the third GnRH form.

The specialized nature and importance of GnRH in vertebrates may be inferred from the presence of the peptide in extant protochordate tunicate species that diverged as early as 600 million years ago from the ancestral line that led to vertebrates (Powell et al., 1996; Adams et al., 2003). The novel wfGnRH has the basic structure that is common to most GnRH peptides isolated to date: a length of 10 amino acids and conserved amino acids in positions 1-4, 9 and 10. The substitutions in wfGnRH involve the part of the GnRH peptide that is believed to be important for receptor binding, but not for any known functional effects.

Synthetic wfGnRH at a concentration of 10^{-7} M stimulated a significant increase in mRNA expression of GtH/TSH-α subunit and GH in dispersed rainbow trout pituitary cells. wfGnRH had a greater effect on GtH/TSH-α subunit and a similar effect on GH compared with cGnRH-II. Proof of biological activity is important because characterization of a GnRH form is determined in part by its function, mainly pituitary activity involving gonadotropic hormone (GtH) and, in fish, growth hormone (GH). Past reports show that the endogenous forms of GnRH in different fish species can release GtH and GH from pituitary cells *in vitro*. Each of sGnRH, cGnRH-II, hrGnRH and pjGnRH is able to act *in vitro* in goldfish pituitary cells to release GtH and GH (Carolsfeld et al., 2000; Montaner et al., 2001; Chang et al., 1990; Marchant et al., 1989). GnRH increases the gene expression of the GtHs, FSH and LH, in mammals (Dalkin et al., 2001). In fish, sGnRH elevated mRNA expression of all three GtH subunits (α, β_1, β_2) in cultured hybrid tilapia pituitary cells (Yaron et al., 2001), and

sGnRH and cGnRH-II each increased mRNA expression of all three GtH subunits and GH in goldfish pituitary cells *in vitro* (Klausen et al., 2001).

The origin of wfGnRH could be due to substitutions in one of the GnRH genes, possibly a duplicated gene, known to exist in early teleosts before whitefish evolved. Early teleost GnRH forms include mGnRH, cGnRH-II, sGnRH and hrGnRH (Carolsfeld et al., 2000; O'Neill et al., 1998; Okubo et al., 1999). One clue to the origin of wfGnRH may lie in the distinct embryological origins of GnRH neurons. cGnRH-II neuroblasts originate in the midbrain in contrast to all other GnRH neuroblasts, which are born in or near the olfactory placode outside the brain and then migrate into the forebrain and diencephalon (Sherwood et al., 1997; Whitlock et al., 2003). cGnRH-II can probably be eliminated as the source of wfGnRH because no immunoreactive wfGnRH neurons were found in the midbrain. Another possibility is that wfGnRH resulted from nucleotide substitutions in either herring GnRH or another ancestral GnRH, possibly mGnRH or sGnRH. The cloning of GnRH cDNAs in early teleosts and the bony fishes that preceded them should give more information as to the GnRH lineage. However, limited sequence from species such as the ancient teleost arowana offer our only insight into the sGnRH and cGnRH-II paralog relationships (Okubo and Aida, 2001).

GnRH was originally thought to be present in the brain as a single form that controlled both FSH and LH. It is now clear that most, if not all, vertebrates have at least two forms of GnRH in the brain and most advanced bony fish (teleosts) have three forms of GnRH. However, only the GnRH that is primarily in neurons of the preoptic area of the brain (GnRH 1 group; Fig. 3.6) regulates the synthesis and release of LH and FSH. Neither the function of the GnRH in the midbrain neurons (GnRH 2) nor the GnRH in cells of the terminal nerve or the olfactory bulb (GnRH 3) is clearly known, but the location of their axons suggests that very little GnRH is delivered to the pituitary (Oka, 2002; Gonzalez-Martinez et al., 2002). The highly conserved structure of the GnRH family of peptides (i.e. human, sturgeon and eel have identical forms of both GnRH 1 and GnRH 2), means that many forms of GnRH are effective in releasing pituitary hormones, but their location in the brain prevents them from reaching the pituitary.

The distribution of a third form of GnRH in teleost fish is puzzling. In orders of fish that are thought to have had a complete duplication of the genome, including orders that contain salmon, trout, zebrafish, goldfish or catfish, there are only two

forms of GnRH detected in the brain. One hypothesis is that one form of GnRH is no longer expressed or is lost due to rearrangements within the chromosome or deletions/mutations within the gene or its promoter after the duplication of the genome (Phillips and Rab, 2001). The other hypothesis is that the third form has not yet been detected. Our evidence is the first to show that at least one species of fish with a doubled genome still retains three forms of GnRH, each encoded on a separate gene.

The three full-length cDNAs isolated from whitefish brain all have a similar structural organization to other vertebrate GnRH cDNA sequences in vertebrates. Each sequence consists of 5'UTR, signal peptide, GnRH, GnRH-associated peptide, and 3'UTR; the region that is most conserved encodes the GnRH peptide with its 3' cut site. These data support the idea that each GnRH form is encoded on a different gene in vertebrates.

The peptide structure deduced from the cDNAs for the three forms of GnRH in the whitefish matched our earlier results from peptide isolation and showed that the GnRH 2 form (cGnRH-II) and the GnRH 3 form (sGnRH) are identical to those in other vertebrates. The cGnRH-II form has been identified from sharks through humans in all jawed vertebrates. The sGnRH form is present in almost all teleost fish. In contrast, the GnRH I form (wfGnRH) is a novel form and a new member of the GnRH peptide family. Comparing the whitefish and other GnRH peptides over ten amino acid differences limits any deductions about their evolutionary relationship.

The full-length cDNA sequences for all three GnRH forms found in lake whitefish are useful for understanding the evolution of the peptide by phylogenetic analysis. Grober et al. (1995) first constructed a phylogenetic tree based on 18 prepro-GnRH cDNA sequences from vertebrate species. Later, other trees were constructed based on prepro-GnRH amino acid sequences (Gothilf et al., 1996; White et al., 1998). The phylogenetic analyses from these earlier studies showed three major precursor groups: group one (GnRH 1), which contained forms of GnRH that were located in neurons of the preoptic region; group two (GnRH 2), which were mainly in neurons of the midbrain; and group three (GnRH 3), which were located in neurons of the olfactory region and ventral telencephalon. The new tree that we have generated is based on 71 vertebrate GnRH precursors. Lake whitefish wfGnRH fits with the GnRH1 group that includes ten vertebrate GnRH forms (Fig. 3.6). The phylogenetic analysis of the precursors in this group suggests they shared a common ancestral gene that underwent changes, including mutations in the GnRH-coding part of the gene. In analysis of

Figure 3.6. Phylogenetic relationship of precursors derived from known DNA sequences encoding gonadotropin-releasing hormone (GnRH). The relationship was generated with CLUSTAL W and the unrooted tree was generated using Treeview version 1.5.2. The scale bar represents the estimated evolutionary distance as 0.1 amino acid substitutions per site. In alphabetical order the precursors, species and accession numbers are listed on the next page.

chicken (c)GnRH-I

chicken (*Gallus gallus*)	X69491

chicken (c)GnRH-II

arowana (*Scleropages jardinii*)	AB047326
caecilian (*Typhlonectes natans*)	AF167558
carp (*Cyrpinus carpio*)	AY147400
catfish (*Clarias gariepinus*)	X78047
cichlid (*Haplochromis burtoni*)	AF076962
eel (*Anguilla japonica*)	AB026990
European bass (*Dicentrarchus labrax*)	AF224281
flounder (*Verasper moseri*)	AB066359
frog (*Rana catesbeiana*)	AF186096
gilthead seabream (*Sparus aurata*)	U30325
goldfish 1 (*Carassius auratus*)	U40665
goldfish 2	U40568
human (*Homo sapiens*)	NM001501
medaka (*Oryzias latipes*)	AB041334
musk/house shrew (*Suncus murinus*)	AF107315
possum (*Trichosurus vulpecula*)	AF193516
rainbow trout (*Oncorhynchus mykiss*)	AF125973
rhesus monkey (*Macaca mulatta*)	AF097356
roach (*Rutilus rutilus*) cGnRH-II	U60668
striped bass (*Morone saxatilis*)	AF056313
tree shrew (*Tupaia glis*)	U63327
whitefish (*Coregonus clupeaformis*)	AY245102
zebrafish 1(*Danio rerio*)	AF511531
zebrafish 2	AY094357

catfish (cf)GnRH

catfish 1	X78049
catfish 2	X78048

guinea pig (gp)GnRH

guinea pig (*Cavia porcellus*)	AF033346

herring (hr)GnRH

shad (*Alosa sapidissima*)	AF536381

lamprey (l)GnRH-I

lamprey (*Petromyzon marinus*)	AF144481

mammalian (m)GnRH

eel	AB026989
frog (*R. catesbeiana*)	AF188754
human	NM000825
mouse (*Mus musculus*)	M14872
rat (*Rattus norvegicus*)	NM012767
rhesus monkey	Dong et al., 1996
tree shrew	U63326
Xenopus laevis	L28040

octopus (oc)GnRH

octopus (*Octopus vulgaris*)	AB037165

pejerrey (pj)GnRH

medaka	AB041336

***Rana* (r)GnRH**

frog (*Rana dybowskii*)	AF139911

salmon (s)GnRH

arowana	AB047325
Atlantic salmon (*Salmo salar*)	X79709
brook trout (*Salvelinus fontinalis*)	X79712
brown trout (*Salmo trutta*)	X79713
carp	AF521130
cichlid	AF076961
European seabass	AF224280
flounder	AB066358
gilthead seabream	U30311
goldfish sGnRH1	U30301
goldfish sGnRH2	AB017271
masu salmon (*Oncorhynchus masou*)	S44614
midshipman (*Porichthys notatus*)	S79620
medaka	AB041335
Mozambique tilapia (*O. mossambicus*)	AY167989
Nile tilapia (*Oreochromis niloticus*)	AF467291
rainbow trout 1	AF232212
rainbow trout 2	AF232213
red seabream (*Pagrus major*)	D26108
roach	U60667
sockeye salmon 1 (*Oncorhynchus nerka*)	D31868
sockeye salmon 2	D31869
whitefish	AY245103
zebrafish	AJ304429

seabream (sb)GnRH

cichlid	F076961
European bass	AF224279
flounder	AB066360
gilthead seabream	U30320
Nile tilapia	AF467291
red seabream	D86582
striped bass	AF056314
yellowfin seabream (*Acanthopagrus latus*)	AB089312

whitefish (wf)GnRH

whitefish	AY245104

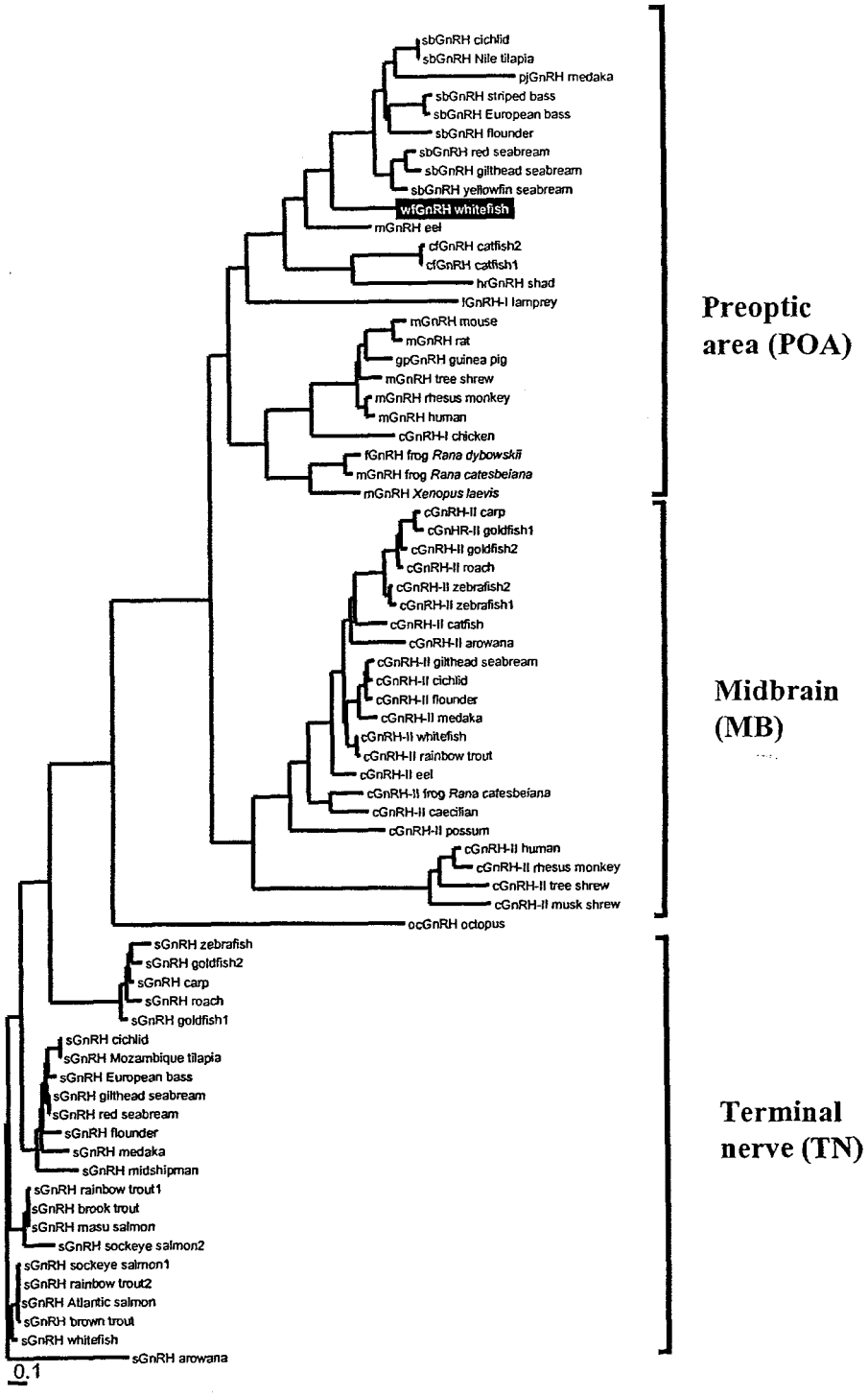
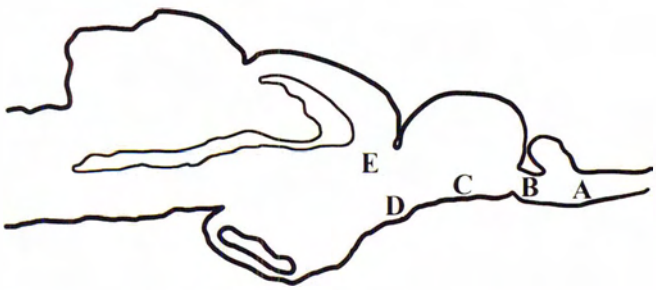

sbGnRH cichlid
sbGnRH Nile tilapia
pjGnRH medaka
sbGnRH striped bass
sbGnRH European bass
sbGnRH flounder
sbGnRH red seabream
sbGnRH gilthead seabream
sbGnRH yellowfin seabream
wfGnRH whitefish
mGnRH eel
cfGnRH catfish2
cfGnRH catfish1
hrGnRH shad
lGnRH-I lamprey
mGnRH mouse
mGnRH rat
gpGnRH guinea pig
mGnRH tree shrew
mGnRH rhesus monkey
mGnRH human
cGnRH-I chicken
fGnRH frog Rana dybowskii
mGnRH frog Rana catesbeiana
mGnRH Xenopus laevis
cGnRH-II carp
cGnHR-II goldfish1
cGnRH-II goldfish2
cGnRH-II roach
cGnRH-II zebrafish2
cGnRH-II zebrafish1
cGnRH-II catfish
cGnRH-II arowana
cGnRH-II gilthead seabream
cGnRH-II cichlid
cGnRH-II flounder
cGnRH-II medaka
cGnRH-II whitefish
cGnRH-II rainbow trout
cGnRH-II eel
cGnRH-II frog Rana catesbeiana
cGnRH-II caecilian
cGnRH-II possum
cGnRH-II human
cGnRH-II rhesus monkey
cGnRH-II tree shrew
cGnRH-II musk shrew
ocGnRH octopus
sGnRH zebrafish
sGnRH goldfish2
sGnRH carp
sGnRH roach
sGnRH goldfish1
sGnRH cichlid
sGnRH Mozambique tilapia
sGnRH European bass
sGnRH gilthead seabream
sGnRH red seabream
sGnRH flounder
sGnRH medaka
sGnRH midshipman
sGnRH rainbow trout1
sGnRH brook trout
sGnRH masu salmon
sGnRH sockeye salmon2
sGnRH sockeye salmon1
sGnRH rainbow trout2
sGnRH Atlantic salmon
sGnRH brown trout
sGnRH whitefish
sGnRH arowana
0.1
Preoptic area (POA)
Midbrain (MB)
Terminal nerve (TN)

Figure 3.7. Gonadotropin-releasing hormone (GnRH)-positive neuron populations found in the lake whitefish brain. A drawing of a complete medial parasagittal section of the lake whitefish brain is shown to help localize the micrographs (rostral to the right and dorsal on top). A) olfactory nerve-olfactory bulb junction (antiserum GF-6), B) olfactory bulb-telencephalon transition region (GF-6), C) ventral telencephalon (antiserum 7CR-10), D) preoptic area (GF-6), E) midbrain tegmentum (7CR-10). Note that the micrographs shown in B and E are at a level more medial than that illustrated by the drawing. Scale bars are 100 μm.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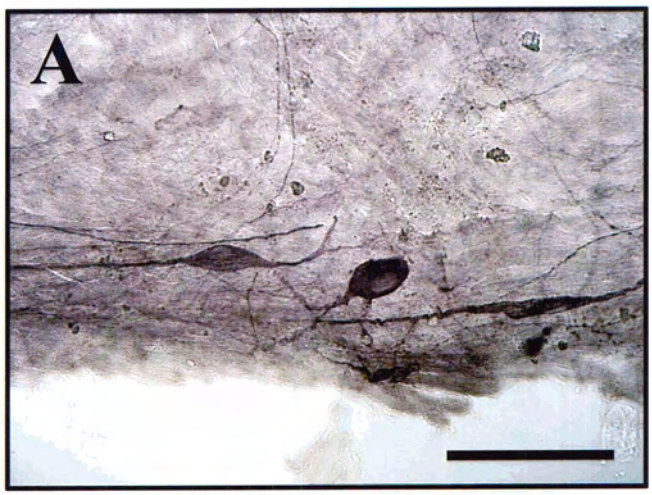
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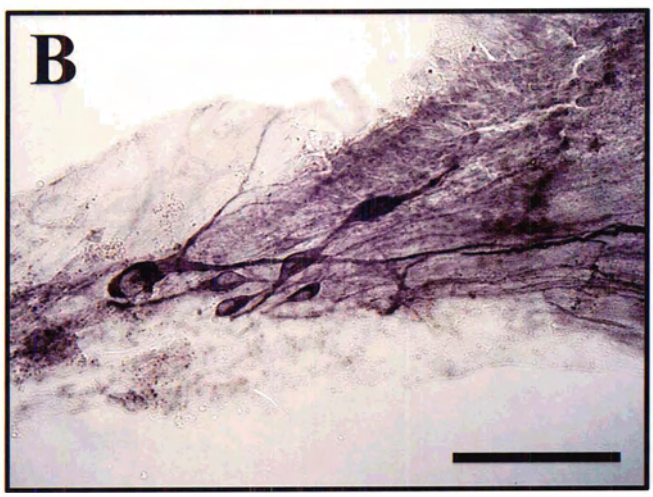
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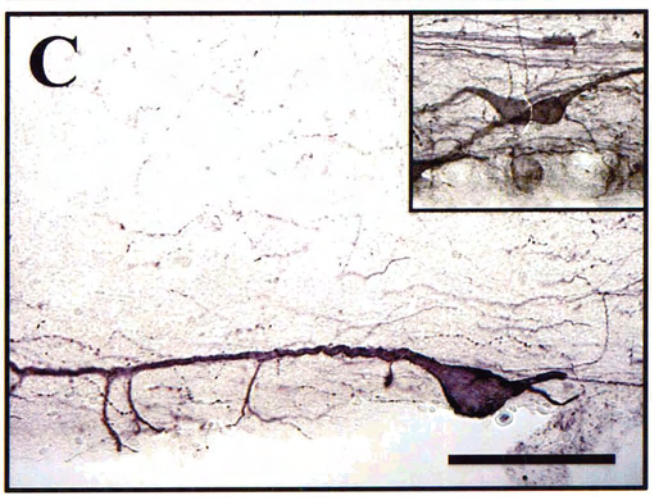
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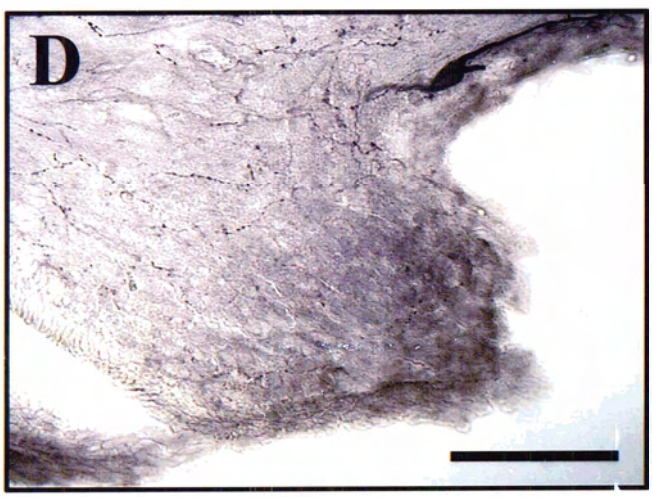
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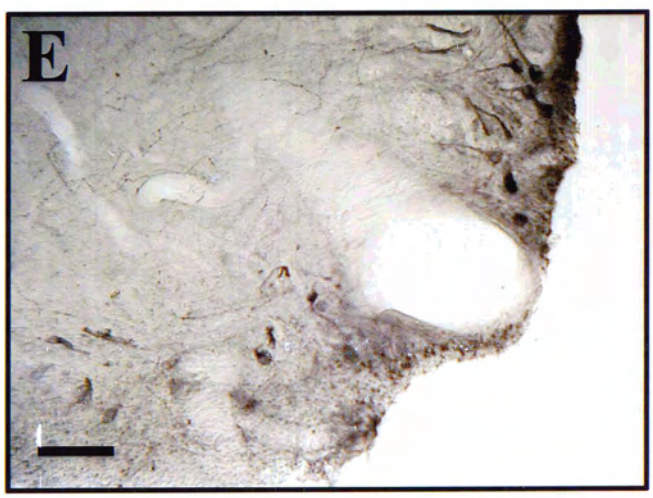
E

whitefish cGnRH-II and sGnRH precursors, each grouped with the same molecules from other species. The precursors for the GnRH2 group all share the same peptide structure (cGnRH-II), which suggests a common ancestral gene, one common to both the fish and tetrapod lineages, and also a gene that has been tightly conserved. Also, neurons containing cGnRH-II occupy a unique location in the brain compared to neurons producing other GnRH forms, suggesting a specialized and conserved function for this gene. The presence of group 3 (sGnRH) only in fish species suggests that this GnRH form may be derived from gene duplication early in the bony fish lineage. In teleosts, the sGnRH coding region has resisted mutation pressure.

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CHAPTER 4: Structural and functional fates of genes encoding PACAP in teleost fish.

Part of this chapter has been published in the following form:

Adams BA, Lescheid DW, Vickers ED, Crim LW, Sherwood NM 2002 Pituitary adenylate cyclase-activating polypeptide and growth hormone-releasing hormone-like peptide in sturgeon, whitefish, grayling, flounder and halibut: cDNA sequence, exon-skipping and evolution. Regulatory Peptides 109: 27-37.

Introduction

The PACAP/glucagon superfamily of hormones consists of nine members in humans encoded on six genes. However, in fish only two types of genes (one encoding both GHRH and PACAP (Fradinger and Sherwood, 2000; Parker et al., 1993, 1997) and a second encoding glucagon, GLP-1 and GLP-2 (Irwin and Wong, 1995) have been described to date. Increasingly, there is an understanding of the functions of PACAP/glucagon superfamily members in fish. However, there is much less data available for interpretation than in mammalian systems. Given the framework of PACAP evolution (see Fig. 1.5 in Chapter 1), an increased understanding of PACAP structure and function in fish will help to complete the PACAP/glucagon superfamily evolution story. Furthermore, considering the hypothesis that fish genomes have undergone extra genome duplication events (Vandepoele et al., 2004; see Phillips and Rab, 2001, for review of salmon polyploidy), the possibility of extra copies of PACAP/glucagon superfamily genes is higher and provides an opportunity to study their subsequent evolutionary fate (e.g. gene loss or new functions).

In fish PACAP potently stimulates the secretion of growth hormone (GH) (Montero et al., 1998; Parker et al., 1997; Rousseau et al., 2001; Wirachowsky et al., 2000; Wong et al., 1998, 2000) and gonadotropin (Chang et al., 2001). In contrast, GHRH is less consistent in releasing GH in fish in vitro (Luo et al., 1991; Montero et al., 1998; Parker et al., 1997; Peter et al., 1984; Rousseau et al., 2001; Vaughan et al., 1992;). Also PACAP in fish can control contractions in the intestine (Matsuda et al., 2000; Olsson and Holmgren, 2000) and can induce catecholamine secretion from chromaffin tissue (Montpetit et al., 2000). However, the separate genome duplication event that has been proposed in the teleost lineage (Vandepoele et al., 2004) could have resulted in the retention of second copies of genes. Three possible fates for one copy of each gene duplicate would have included: 1) gene loss, 2) mutation to a new gene product or a pseudogene, or 3) retention and subsequent change in function or new function (Force et al., 1999; see Taylor and Raes, 2005 for review). At the outset of my research program, the only known duplicates for any of the PACAP/glucagon superfamily genes were the two genes that code for GHRH and PACAP in the tunicate, *C. productum* (McRory and Sherwood, 1997, although, there was some

indication, for example in rainbow trout (Krueckl and Sherwood, 2001), that a second gene may exist.

My hypothesis is that duplicate genes for the PACAP/glucagon superfamily would have been lost in many of the extant species of teleost, although there could be duplicates that still exist in teleost species that have undergone further genome or large scale gene duplication such as the tetraploid salmonids.

To gain insight into the evolution of PACAP and its related superfamily members, I identified DNA sequences encoding PACAP and GHRH from nine species of teleost fish, and compared these to known sequences. Arctic grayling and lake whitefish represent the two subfamilies (Thymallineae and Coregoninae, respectively) that were the earliest of the extant salmonids to evolve (see Fig. 3.1 in Chapter 3). The third subfamily in the salmonid lineage includes rainbow trout and Atlantic salmon for which one gene encoding PACAP and GHRH is known. Next, I studied the structure of PACAP and GHRH in yellowtail flounder and Atlantic halibut, two flatfish from one of the most recently derived fish lineages, the Pleuronectiformes, and grass rockfish. Finally, I searched the genomes of two pufferfish species, *Fugu rubripes* and *Tetraodon nigroviridis*, to identify sequence with the potential to code for PACAP and GHRH.

Force and co-authors (1999) proposed the duplication-degeneration-complementation (DDC) model. This model predicts that "(1) degenerative mutations in regulatory elements can increase rather than reduce the probability of duplicate gene preservation and (2) the usual mechanism of duplicate gene preservation is the partitioning of ancestral functions rather than the evolution of new functions " (Force et al., 1999). The identification of a second gene encoding PACAP in zebrafish by Wang and co-workers (2003) during the course of my research provided me the opportunity to determine the 'functional fate' of the two gene copies of a PACAP/glucagon superfamily member using the developmental model system of zebrafish. Zebrafish *ghrh-pacap1* mRNA is expressed in early development and adult zebrafish (Fradinger and Sherwood, 2000; Krueckl et al., 2003). Zebrafish *ghrh-pacap2* mRNA is expressed in a number of tissues in adult zebrafish, and the synthetic $PACAP_{1-38}$ deduced from *ghrh-pacap2* mRNA was able to stimulate oocyte maturation

in vitro (Wang et al., 2003). In order to determine that both genes have a functional role, I injected 1-2 cell stage zebrafish embryos with morpholinos designed against each of the two *ghrh-pacap* gene sequences in zebrafish to test specifically if each gene has a functional role in development, and if so, if the two genes are able to compensate for one another, or if they have non-overlapping roles.

Materials and methods

cDNAs in grayling, whitefish, rainbow trout, grass rockfish, flounder and halibut: 5' and 3'-Rapid Amplification of cDNA Ends (RACE)

Lake whitefish (*Coregonus clupeaformis*) were collected at the Fresh Water Institute (Winnipeg, MB, Canada). The fish were anesthetized and brains were dissected into RNA*later* (Ambion Inc., Austin, TX) and stored on ice for shipping. Arctic grayling (*Thymallus arcticus*) were caught in the Kakisa River (Northwest Territories, Canada). Grayling were killed and their brains were dissected into RNA*later* and shipped on ice to the University of Victoria. Grass rockfish (*Sebastes rastrelliger*) were killed and their brains were quick frozen at the University of California at Santa Barbara. Yellowtail flounder (*Pleuronectes ferrugineus*) and Atlantic halibut (*Hippoglossus hippoglossus*) were killed and their brains were quick frozen in liquid nitrogen at the Marine Sciences Research Laboratory, Memorial University of Newfoundland. Rockfish, flounder and halibut brains were shipped on dry ice to the University of Victoria. Rainbow trout from the aquatic facilities at the University of Victoria were anesthetized in clove oil and subsequent killed by cutting of the spinal cord. The brains were dissected and quick frozen in liquid nitrogen and transferred to storage at –80°C for RNA isolation.

Total RNA was extracted using TRIzol (Invitrogen/Life Technologies, Burlington, ON) based on the guanidium thiocyanate-phenol-chloroform method of extraction (Chomczynski and Sacchi, 1987). mRNA was then isolated from total RNA using the Ambion MicroPoly(A) Pure mRNA isolation kit (Ambion Inc.). Separate cDNA reactions were prepared for each species using 100 to 300

Table 4.1. Sequences of primers and annealing temperatures used in the amplification of cDNAs identified in this study. Forward primers are indentified by numbers, whereas reverse primers are indicated by letters.

Primer	Sequence	Annealing temperature (°C)
1	5'-CGACTTTAGCCTTACTCATC-3'	50-54
2	5'-CATGCAGACGGAATGTTTAA-3'	50-54
3	5'-CA[TC]TCGGA[TC]GGGATCTTCACGGA[TC]AG-3'	50-54
A	5'-CTCTGTCTATACCTTTTCCC-3'	50-54
B	5'-GCCATTTGCTTTCGGTAGCG-3'	50-54
C	5'-ACACGCTTTGCCATCAGAGA-3'	50-54

ng of mRNA in the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. 3'RACE reactions were performed using forward primers 1, 2, or 3 (Table 4.1) in conjunction with the kit reagents and first reaction round primer as well as DNA Taq Polymerase (Invitrogen/Life Technologies). Only one round of 3'RACE was performed. 5'RACE reactions were done over two rounds. The first round
reaction used reverse primers A, B, or C as well as the first round primer from the kit. Second round reactions included 2 µl of the first round PCR reaction as well as B and/or C and the nested primer included in the kit. All PCR reactions ran for 35 cycles with a seven-minute extension at 72°C on the last cycle. Annealing temperatures used for each primer are indicated in Table 1. RACE PCR reactions were separated on 1.5% agarose gels using electrophoresis. RACE PCR products were visualized by staining the gels in ethidium bromide and viewed with the Eagle-Eye II still video system (Stratagene, La Jolla, CA). Ligation into pGEM-T vector (Promega, Madison, WI) was performed using the PCR reaction mixture and/or DNA from bands isolated from the agarose gels using phenol-chloroform extraction. DNA was transformed and processed as described above except that DNA was isolated using the QIAGEN miniprep kit (QIAGEN Inc., Mississauga, ON), according to the manufacturer's instructions. After enzyme digestion with Sph-1 and Pst-1 (New England Biolabs Inc., Beverly, MA), preparations with inserts of expected size were sequenced.

DNA sequences coding for PACAP in rainbow trout and Atlantic salmon

Using the two rainbow trout cDNAs that I constructed from overlapping 5' and 3'RACE sequences, I searched the sequence data available from the Genomic Research on Atlantic Salmon Project (http://web.uvic.ca/cbr/grasp/) that includes expressed sequence tags (ESTs) from salmon species, including Atlantic salmon and rainbow trout. Matches from the database (two for salmon and two for rainbow trout) were selected and a new 3 ml culture was inoculated with the original plasmid, followed by DNA purification and sequencing.

DNA sequence coding for PACAP in pufferfish species, F. rubripes and T. nigroviridis

To identify PACAP and GHRH coding segments in *F. rubripes*, I searched the data set available to the public at the United Kingdom *Fugu* Genome Project website (http://fugu.hgmp.mrc.ac.uk/) using the amino acid sequences for PACAP and glucagon family members already known in other vertebrates (Sherwood et al., 2000 for compilation of sequences). Regions with high indentity to the exons of other fish *ghrh-pacap* prohormone sequences were linked together to reconstruct a full prohormone sequence. For candidate sequences in *T. nigroviridis*, I used the coding DNA-sequence for the GHRH and PACAP I generated from *F. rubripes* for comparison to the NCBI protein database, as well as the genome site for the *T. nigroviridis* sequencing project (http://www.genoscope.cns.fr/externe/tetraodon/).

Sequence analysis

DNA sequences were submitted to the BLASTn program available at the NCBI internet website (http://www.ncbi.nlm.nih.gov) for analysis. Significant similarities were assumed when the P value was less than 0.0001. Protein translations were done using the Expasy biology tools internet website (http://expasy.ch/tools). The subsequent alignment of the prohormone sequences was done using the GeneBee internet-based alignment and phylogenetic tree generation program (http://www.genebee.msu.su/services/phtree_reduced.html). The alignment was inspected visually to ensure the expected corresponding hormone-coding regions were aligned.

Zebrafish morpholino studies

Zebrafish (*Danio rerio*) were kept at 28 ^{0}C in dechlorinated water on a 14 hour light / 10 hour dark cycle. Embryos were collected each morning after natural spawning of adult fish. Times of development are expressed as hours after fertilization at 28 ^{0}C. All the studies using zebrafish were approved by the University of Victoria Animal Care Committee.

Microinjection of antisense morpholino oligonucleotides

Two morpholino oligonucleotides (MOs) were designed and synthesized for each of the two GHRH-PACAP-encoding genes by Gene Tools (Eugene, OR). For each gene, a MO was designed against the RNA region for the 5'UTR and a second against the translation start site (ATG). There was no overlap in the regions against which the four MOs were made. The sequences and the hybridization locations of MOs relative to the start site are indicated in Table 4.2. MOs were solubilized in ddH_2O to make a stock concentration. Next, the stock solution was further diluted to a working concentration of 2 – 16 μg/μl. Zebrafish embryos were injected with 1-1.5 nl of the MOs solution at the 1-2 cell stage into the yolk cell. The procedure for the microinjection followed that described by Nasevicius and Ekker (2000). Zebrafish were monitored through development by dissecting microscope. At 24 hours, I recorded the number and description of morphants. 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) and edited in Adobe Photoshop 6.0 and Microsoft PowerPoint.

Results

Whitefish, Arctic grayling and rainbow trout ghrh-pacap cDNAs

Full-length cDNAs encoding PACAP and GHRH from the brains of lake whitefish, Arctic grayling and rainbow trout were constructed from overlapping 5' and 3'RACE products; the cDNAs were 774 bp, 672 bp (Fig. 4.1), and 647 bp respectively. A second 3'-RACE product was obtained for each of rainbow trout and whitefish using a forward primer that sits in the region of the signal peptide, and resulted in products with very different sequence after what normally codes for the $PACAP_{1\text{-}27}$ N-terminal processing site. However, I was not successful at identifying a 5'-RACE product to construct a full-length cDNA sequence. The full-length cDNAs that I constructed have the same arrangement of domains as that found in other non-mammalian vertebrates. Each of the three species had a smaller PCR product that was shown to lack 105 bp compared to longer sequences. This region encodes 35 amino

Table 4.2. Sequences and the hybridization locations for morpholinos to zebrafish GHRH-PACAP genes used in knockdown experiments.

Morpholino Name	Sequence	Location
GHRH-PACAP GENE 1		
ghrh-pacap1 UTR	cggacggatgctgtccaatggaggc	5'UTR
ghrh-pacap1 ATG	gagtcgttttgctgctcgtaatcat	Start codon
GHRH-PACAP GENE 2		
ghrh-pacap1 UTR	gaaatgctgttggaatgcgactcggg	5'UTR
ghrh-pacap1 ATG	gccatgctattgcagagtaggtaga	Start codon

acids, including the first 32 amino acids for the GHRH peptide. For the translation start site, the grayling cDNA appears to lack an upstream triplet coding for a methionine as in whitefish. Rather, a methionine that is in a comparable position to the 16^{th} amino acid position in whitefish is used as the start site in grayling. Apart from the shorter 5'end of the cDNA in grayling, the two cDNAs encode for the same number of amino acids in the hormone encoding regions. They differ in the GHRH coding region by two amino acids: asparagine (position 74) in grayling is a lysine (position 89) in whitefish and asparagine (position 101) in grayling is a serine (position 116) in whitefish (Fig. 4.1).

The coding DNA regions of the whitefish and grayling are closely related to two other salmonids for which sequences are available, sockeye salmon and rainbow trout (Fig. 4.2). Whitefish PACAP is only three amino acids different from human $PACAP_{38}$ (92 % identical) and one different from grayling and other closely related salmonids (Fig.4.2). The grayling cDNA codes for a $PACAP_{38}$ peptide that is identical to that found in five species of salmon (Fig. 4.2). Whitefish GHRH is one amino acid different from grayling. Compared to human GHRH, whitefish is only 41 % and grayling is 43 % identical in amino acids (Fig. 4.3).

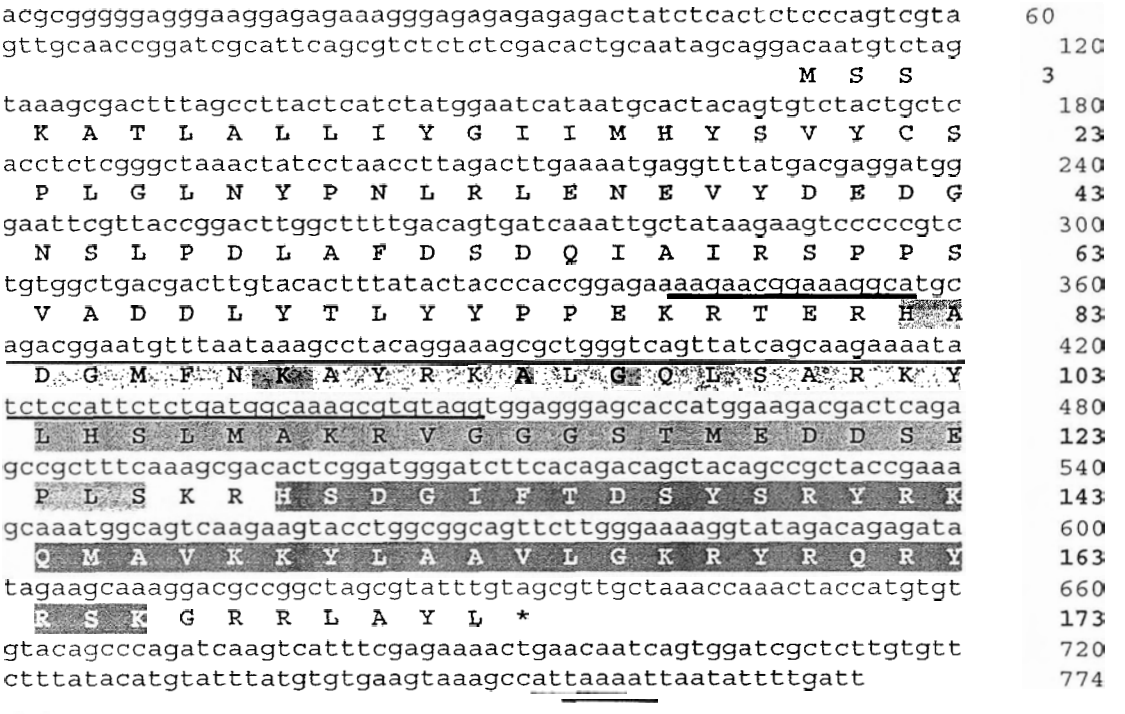
The overlapping, full-length sequence that I determined for rainbow trout matched one already in the NCBI database (accession number AF343976) that was reported by Krueckl and Sherwood (2001). Database searching using the second 3'RACE fragment revealed a match to the $PACAP_{1-27}$ processing site to rainbow trout *ghrh-pacap*, whereas the later 3' end of this sequence had four strong matches: a 100 of 155 bp region in the 3'UTR of the human PACAP gene; 86 of 98 bp region of the 3'UTR of the mouse PACAP gene; 102 of 122 bp region of the 3'UTR of the zebrafish *ghrh-pacap1* gene; and two regions in the scaffold 4 (92 of 104 bp and 61 of 68 bp) and one region of scaffold 836 (45 of 51 bp) of the *Fugu* genome sequence.

Rockfish, flounder and halibut ghrh-pacap cDNAs

Full length cDNAs encoding PACAP and GHRH from the brains of rockfish, flounder and halibut were constructed from overlapping 5' and 3'RACE products,

Figure 4.1. Nucleotide and deduced amino acid sequences for growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) in whitefish (A) and grayling (B). GHRH amino acid sequence is highlighted in light gray whereas PACAP is dark gray. The underline indicates the section of the transcript this is alternatively spliced. The nucleotides highlighted in black show differences from the whitefish GHRH peptide. The double underline at the 3'end indicates a possible polyadenylation signal.

A.



B.

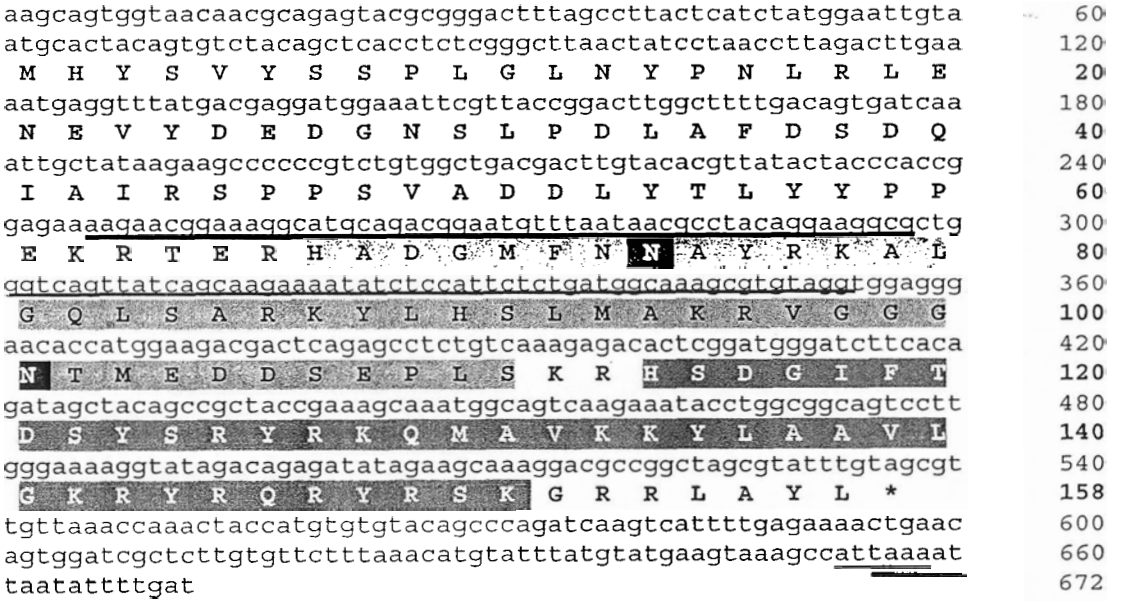


Figure 4.2. Comparison of pituitary adenylate cyclase-activating polypeptide (PACAP) amino acid sequences to the human sequence. a) Ogi et al., 1990, b) Okazaki et al., 1995, c) Kimura et al., 1990, d) McRory et al., 1997, e) Yoo et al., 2000, f) Hu et al., 2000, g) Alexandre et al., 2000, h) Matsuda et al., 1997; i) Jiang et al., 2003, j) McRory et al., 1995, k) Small and Nonneman, 2001, l) Fradinger and Sherwood, 2000, m) Wong et al., 2003, n) Parker et al., 1997, o) Adams et al., 2002, p) Matsuda et al, 1998, q) McRory and Sherwood, 1997. An asterisk in the reference column is a sequence identified in this study. A black highlight identifies an amino acid that is not identical to the human sequence.

Species (number)	PACAP	%Identity	Ref.
Human/mammal (4)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	100	a-c
Bird (2)	HTDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	97	d,e
Frog (2)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRIKNK	97	f,g
Flounder,halibut	HSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNK	89	*
Stargazer,grouper	HSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNK	89	h,i
Catfish (2)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGRRYRQRFRNK	89	j,k
Rockfish	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRIRNK	95	*
Fugu-1	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRIRNK	95	*
Tetraodon-1	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRIRNK	95	*
Fugu-2	HSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNK	95	*
Tetraodon-2	HSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNK	95	*
Zebrafish-1	HSDGVFTDSYSRYRKQMAVKKYLATVLGKRYRQRYRSK	84	l
Zebrafish-2	HSDGIFTDIYSRYRKQMAVKKYLAAVLGRRYRQRVKNK	92	m
Salmon-1 (5 sp)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRSK	89	n
Grayling	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRSK	89	*
Whitefish	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRYRNK	92	*
Rainbow trout-2	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKSPEDLGFHH...	79	*
Salmon-2a(asplnba)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKSPEDLGFHI...	79	*
Salmon-2b(asplnbb)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKSPDDLGFHH...	79	*
Sturgeon	HSDGIFTDSYSRYREQMAVKKYLAAVLGKRYRQRVRNK	92	o
Stingray	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKPKVKNS	87	p
Tunicate-1	HSDGIFTDSYSRYRNQMAVKKYLAAVL	96	q
Tunicate-2	HSDGIFTDSYSRYRNQMAVKKYINALL	85	q

Figure 4.3. Comparison of growth hormone-releasing hormone (GHRH) amino acid sequences. a) Rivier et al, 1982, b) Guillemin et al., 1982, c) Bohlen et al., 1983, d) Esch et al., 1983, e) Brazeau et al., 1984, f) Ono et al., 1994 g) Yamamoto et al., 1998, h) Spiess et al., 1983; i) Bohlen et al., 1984, j) McRory et al., 1997, k) Yoo et al., 2000, l) Alexandre et al., 2000, m) Hu et al., 2000, n) Fradinger and Sherwood, 2000, o) Wang et al., 2003 p) Jiang et al., 2003, q) Vaughan et al., 1992, r) McRory et al, 1995, s) Small and Nonneman, 2001, t) Parker et al., 1997, u) Adams et al., 2002, v) McRory and Sherwood, 1997. An asterisk in the reference column is a sequence identified in this study. A black highlight identifies an amino acid that is not identical to the human sequence.

Species	GHRH	% Identity	Ref.
Human	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL	100	a,b
Pig	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGARVRL	93	c
Cattle/goat	YADAIFTNSYRKVLGQLSARKLLQDIMNRQQGERNQEQGAKVRL	87	d,e
Sheep	YADAIFTNSYRKILGQLSARKLLQDIMNRQQGERNQEQGAKVRL	86	d
Hamster	YADAIFTSSYRKVLGQLSARKLLQDIMSRQQGERNQEQGPRVRL	87	f
Mouse	HVDAIFTTNYRKLLSQLYARKVIQDIMNKQ-GERIQEQRARLS	59	g
Rat	HADAIFTSSYRRILGQLYARKLLHEIMNRQQGERNQEQRSRFN	67	h,i
Chicken1	HADGIFSKAYRKLLGQLSARNYLHSLMAKRVG---SGLGDEAEPLS	43	j
Chicken2	HADGIFSKAYRKLLGQLSARNYLHSLMAKRVGGASSGLGDEAEPLS	43	j
Turkey	HADGIFSKAYRKVLGQLSARKYLHSLMAKRVGGVSSGLGDEAEPLS	47	k
Rana	HADDLLNKAYRNLLGQLSARKYLHTLMAKHLGAVSSSLEDDSEPLS	34	l
Xenopus	HADELLNKVYRNVLGHLSARKYLHTLMAQRLGTVSSSLEDESEPLS	34	m
Flounder/hal.	HADGMFNKAYRKALGQISARKYLHSLMAKRVGGGSTMEDD-SEPLS	39	*
Fugu-1	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGNTLEDS-SEPLS	36	*
Fugu-2	HAEEELDRALREILGQLTARHYRHFLMTIR-GGGSSAEED-SEPLS	25	*
Tetraodon-1	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGK-TLEDS-SEPLS	41	*
Tetraodon-2	HAEGELDRALREILGQLTARHYRHFLMTVR-G----SAEE-SEPLS	27	*
Zebrafish-1	HADGMFNKAYRKALGQLSARKYLHTLMAKRVGGGSTTEDD-NEPLS	41	n
Zebrafish-2	HADGLLDRALRDILVQLSARKYLHSLMAVRVGGGSSEEDE-SEPLS	34	o
Grouper	HAEEELDRALREILGQLTARHYLHSLMTIRAGEDNSMEEE-SEPLS	30	p
Rockfish	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGKTLDDS-SEPLS	41	*
Carp	HADGMFNKAYRKALGQLSARKYLHTLMAKRVGGGS-MIEDDNEPLS	41	q
Catfish (2)	HADGLLDRALRDILVQLSARKYLHSLTAVRVGEEEE-DEEDSEPLS	32	r,s
Salmon (4)	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGST-MEDDSEPLS	41	t
Chinook Salm.	HADGMLNKAYRKALGQLSARKYLHSLMAKRVGGGST-MEDDSEPLS	39	t
Atl salmon2b	HADGMFNKAYRKALGQLSARQYLHSLMAKRVGGGST-MEDDSEPLS	39	*
Atl salmon2a	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGST-MEDDSEPLS	41	*
Whitefish	HADGMFNKAYRKALGQLSARKYLHSLMAKRVGGGST-MEDDSEPLS	41	*
Grayling	HADGMFNNAYRKALGQLSARKYLHSLMAKRVGGGNT-MEDDSEPLS	43	*
Sturgeon	HADGIFNKTYRKVLGQLSARKYLHSVMAKRVGGVSS-MEEDSEPLS	45	u
Tunicate-1	HSDGIFTKDYRKYLGQLRAQKFLQWLM	59	v
Tunicate-2	HSDGIFTSDYRRYLGQLSAQKFLQWLM	59	v

resulting in cDNAs that are 928 (Fig. 4.4), 1044, and 932 bp (Fig. 4.5), respectively. The cDNAs had the expected arrangement of domains. Rockfish, flounder and halibut had additional PCR products that lacked 105 bp of sequence compared to longer sequences. This missing region in the short transcript is present in the long transcript as the one described above for rainbow trout, whitefish and grayling that codes for 35 amino acids, including the first 32 for the GHRH peptide. Overall, the flounder and halibut cDNAs are very similar. They are identical in the coding region except for one nucleotide, which results in an amino acid difference in the cryptic peptide at position 48 with phenylalanine in flounder and serine in halibut. Whereas the 5'UTRs have only a small region that can be aligned, the 3'UTR has significant alignment.

The coding DNA regions of the flounder and halibut cDNAs are more closely related phylogenetically to each other than to any other species, and as a pair they are more closely related to the rockfish than to other fish species (Fig. 4.6). Flounder and halibut PACAP amino acids are identical to stargazer, but four amino acids different from human and salmonid $PACAP_{1-38}$ (89 % identical) (Fig. 4.2). Flounder and halibut GHRH are identical and are only one amino acid different from GHRH common to four salmon species. In contrast, the flounder and halibut peptides are only 39 % identical to the human GHRH peptide (Fig.4.3).

Rainbow trout and Atlantic salmonid EST identification

Using the rainbow trout 3'RACE to search the Atlantic salmon and rainbow EST database from the GRASP project, I identified two sequences, rga1 and rga2 rainbow trout ESTs. Sequencing and construction revealed a 1101 bp product in rainbow trout that could code for a PACAP1-27 and then continues to code for up to a total of 72 amino acids before a stop codon (Fig. 4.7). This sequence lacked the region that codes for $GHRH_{1-32}$ but encodes $GHRH_{33-45}$ and a dibasic cleavage site (KR). This appears to represent a clone in which the original mRNA had undergone alternative splicing of this gene, splicing out the exon coding for $GHRH_{1-32}$. The dibasic cleavage site at the N-terminal of $PACAP_{1-27}$ is intact as KR, but only a single K is present for cleavage after $PACAP_{1-27}$ amidation site (G). It is possible that the $PACAP_{1-27}$-NH_2 peptide is released.

Figure 4.4. Nucleotide and deduced amino acid sequences for pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH) in grass rockfish. GHRH amino acid sequences is highlighted in light gray whereas PACAP is dark gray. The underline indicates the section of the transcript this is alternatively spliced.

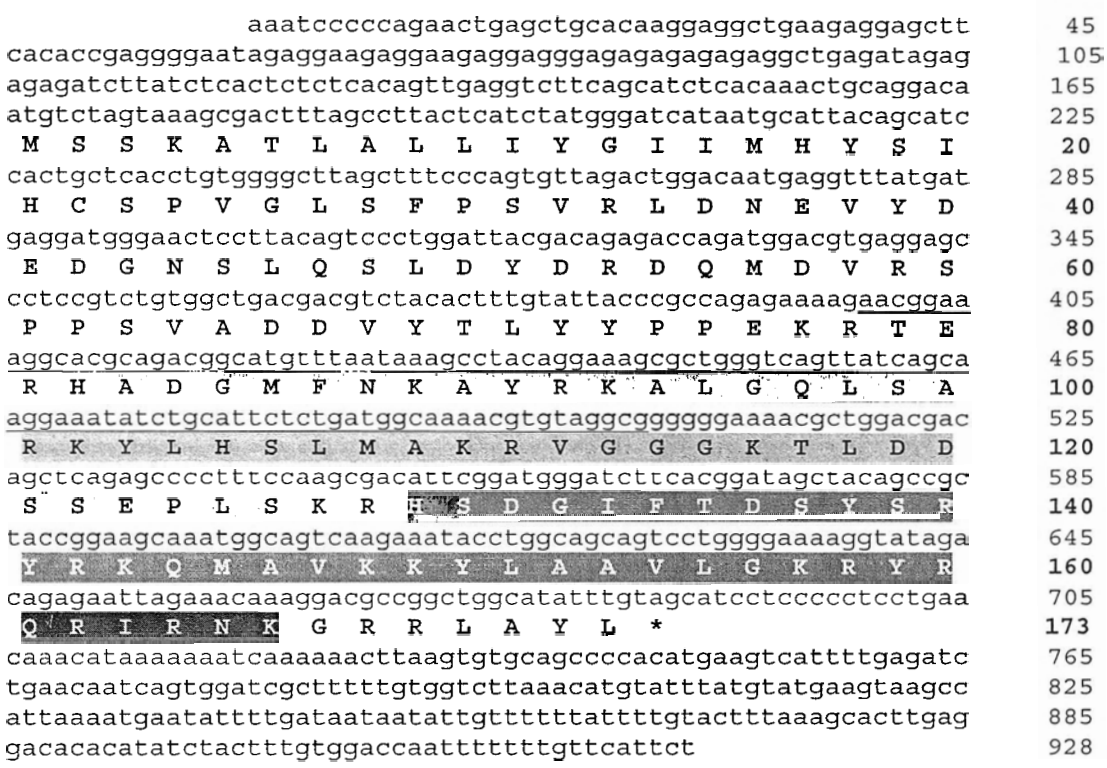

aaatcccccagaactgagctgcacaaggaggctgaagaggagctt 45
cacaccgaggggaatagaggaagaggaagaggagggagagagagagaggctgagatagag 105
agagatcttatctcactctctcacagttgaggtcttcagcatctcacaaactgcaggaca 165
atgtctagtaaagcgactttagccttactcatctatgggatcataatgcattacagcatc 225
M S S K A T L A L L I Y G I I M H Y S I 20
cactgctcacctgtggggcttagctttcccagtgttagactggacaatgaggtttatgat 285
H C S P V G L S F P S V R L D N E V Y D 40
gaggatgggaactccttacagtccctggattacgacagagaccagatggacgtgaggagc 345
E D G N S L Q S L D Y D R D Q M D V R S 60
cctccgtctgtggctgacgacgtctacactttgtattacccgccagagaaaagaacggaa 405
P P S V A D D V Y T L Y Y P P E K R T E 80
aggcacgcagacggcatgtttaataaagcctacaggaaagcgctgggtcagttatcagca 465
R H A D G M F N K A Y R K A L G Q L S A 100
aggaaatatctgcattctctgatggcaaaacgtgtaggcgggggggaaaacgctggacgac 525
R K Y L H S L M A K R V G G G K T L D D 120
agctcagagccccttccaagcgacattcggatgggatcttcacggatagctacagccgc 585
S S E P L S K R H S D G I F T D S Y S R 140
taccggaagcaaatggcagtcaagaaatacctggcagcagtcctggggaaaaggtataga 645
Y R K Q M A V K K Y L A A V L G K R Y R 160
cagagaattagaaacaaaggacgccggctggcatatttgtagcatcctccccctcctgaa 705
Q R I R N K G R R L A Y L * 173
caaacataaaaaaatcaaaaaacttaagtgtgcagccccacatgaagtcattttgagatc 765
tgaacaatcagtggatcgctttttgtggtcttaaacatgtatttatgtatgaagtaagcc 825
attaaaatgaatattttgataataatattgtttttttattttgtactttaaagcacttgag 885
gacacacatatctactttgtggaccaatttttttgttcattct 928

Figure 4.5. Nucleotide and deduced amino acid sequences for pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH) in flounder (A) and halibut (B). GHRH amino acid sequences is highlighted in light gray whereas PACAP is dark gray. The underline indicates the section of the transcript this is alternatively spliced.

A.

```
                                                    acgcggggaggga      13
aaaggtatagacagagaagctgcgcgtaaaaccccccgagaagtcagctgcgggcgcacg          73
gagcgctgctgcactctccgctaacaagagaccggacaggcgcttcatcgagggaacaga         133
ggaagaggaggggaagaggagatagagagagagagagagaggggctgagatagagatc           193
ctatctcactctcccc agttgaggtcttcagcggatcgttcagcggatcgcaactgg aca       253
atgtctagcaaagcgactttagccttactcatctatggaatcataatgcactacagcgtc         313
 M  S  S  K  A  T  L  A  L  L  I  Y  G  I  I  M  H  Y  S  V         20
agctgctcacctgtggggcttagcttccccactgttagacttgacagtgaggtttatgat        373
 S  C  S  P  V  G  L  S  F  P  T  V  R  L  D  S  E  V  Y  D         40
gaggatggaaattccttaccgccctggattatgacagagaccaaatggatgtgagaaac         433
 E  D  G  N  S  L  P  P  L  D  Y  D  R  D  Q  M  D  V  R  N         60
cctccgcctgtcgatggcgacgtctacgctttgtattaccctccagacaaaagaacggaa        493
 P  P  P  V  D  G  D  V  Y  A  L  Y  Y  P  P  D  K  R  T  E         80
aggcacgcagacggcatgtttaataaagcctacaggaaagcgctgggtcagatatcagca        553
 R  H  A  D  G  M  F  N  K  A  Y  R  K  A  L  G  Q  I  S  A        100
aggaaatatctgcattctctgatggcaaagcgtgtaggtggagggagcaccatggaagac        613
 R  K  Y  L  H  S  L  M  A  K  R  V  G  G  G  S  T  M  E  D        120
gactcagagcctctgtcaaagagacattcggatgggatcttcacggatagctacagtcgc        673
 D  S  E  P  L  S  K  R  H  S  D  G  I  F  T  D  S  Y  S  R        140
tatagaaagcagatggccgtgcagaagtacctggcagcggttctgggaagaaggtacaga        733
 Y  R  K  Q  M  A  V  Q  K  Y  L  A  A  V  L  G  R  R  Y  R        160
cagagagttaggaacaaaggacgccgacttgcctatttgtagcgttgctaaagcgcccca        793
 Q  R  V  R  N  K  G  R  R  L  A  Y  L  *                        173
gctgccctcctgtgtatatacatccagtcgttaaatcaaagtcattcagatatatctgac        853
caaccagtggattgcgcctgtgttctttcaacatgtatttatgtatgaagtaaagccatt        913
aaaatgaatattttaataataatatcgttttttttctttttgtacaaaagcacttgatac        973
cgcacagttatgccctgtggaccaatattttattttcatgttgagatgttgaaaacaaaa       1033
caaaatgccct                                                        1044
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B.

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                                                    aaaagttgaggtcttcagcggatc      24
gttcagcggatcgcaactggtaagaggaggcacttttacgctgcgtaaaggcgcggagc           84
gtgtgaggggaaactcaggtgtggctgttttttttttccttagaaactcacagcaggaca         144
atgtctagcaaagcgactttagccttactcatctatggaatcataatgcactacagcgtc         204
 M  S  S  K  A  T  L  A  L  L  I  Y  G  I  I  M  H  Y  S  V         20
agctgctcacctgtggggcttagcttccccactgttagacttgacagtgaggtttatgat        264
 S  C  S  P  V  G  L  S  F  P  T  V  R  L  D  S  E  V  Y  D         40
gaggatggaaattccttaccgtccctggattatgacagagaccaaatggatgtgagaaac         324
 E  D  G  N  S  L  P  S  L  D  Y  D  R  D  Q  M  D  V  R  N         60
cctccgcctgtcgatggcgacgtctacgctttgtattaccctccagacaaaagaacggaa        384
 P  P  P  V  D  G  D  V  Y  A  L  Y  Y  P  P  D  K  R  T  E         80
aggcacgcagacggcatgtttaataaagcctacaggaaagcgctgggtcagatatcagca        444
 R  H  A  D  G  M  F  N  K  A  Y  R  K  A  L  G  Q  I  S  A        100
aggaaatatctgcattctctgatggcaaagcgtgtaggtggagggagcaccatggaagac        504
 R  K  Y  L  H  S  L  M  A  K  R  V  G  G  G  S  T  M  E  D        120
gactcagagcctctgtcaaagagacattcggatgggatcttcacggatagctacagtcgc        564
 D  S  E  P  L  S  K  R  H  S  D  G  I  F  T  D  S  Y  S  R        140
tatagaaagcagatggccgtgcagaagtacctggcagcggttctgggaagaaggtacaga        624
 Y  R  K  Q  M  A  V  Q  K  Y  L  A  A  V  L  G  R  R  Y  R        160
cagagagttaggaacaaaggacgccgacttgcctatttgtagcgttgctaaagcgcccca        684
 Q  R  V  R  N  K  G  R  R  L  A  Y  L  *                        173
gcgctcctgtgtatatacatccagtcgttaaatcaaagtcattcagatatatctgaccaa        744
ccagtggattgcgcctgtgttctttcaacatgtatttatgtatgaagtaaagccattaaa        804
atgaatattttaataataatatcgttttttttctttttgtacaaaagcacttgataccgc        864
acagttatgccctgtggaccaatattttattttcatgttgagatgttgaaaacaaaacaa        924
aacgcccg                                                           932
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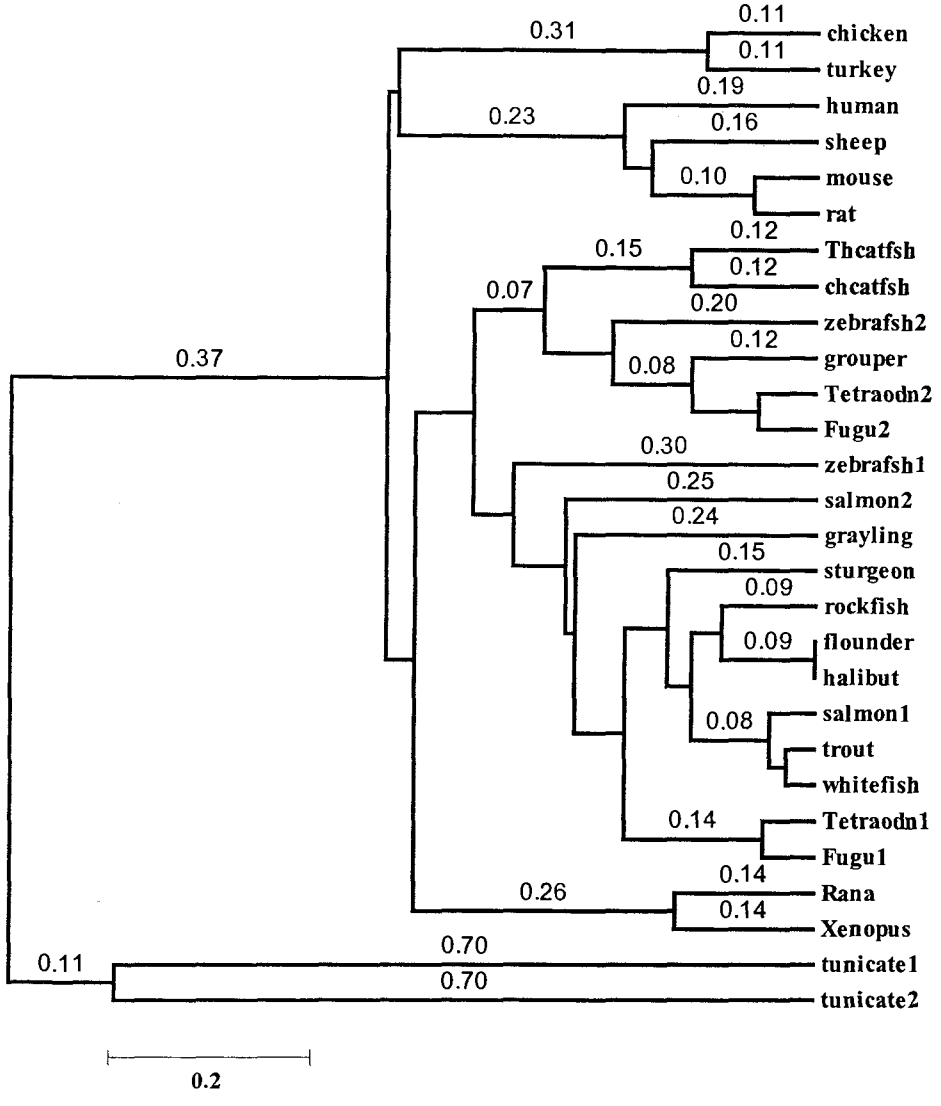
Figure 4.6. Phylogenetic relationship of known pituitary adenylate cyclase-activating polypeptide (PACAP)-encoding prohormones in chordates. A distrance matrix was generated using Protdist, part of the PHYLIP phylogenetic comparison software.


0.11
0.31
chicken
0.11
turkey
0.19
human
0.23
0.16
sheep
0.10
mouse
rat
0.12
0.15
Thcatfsh
0.12
chcatfsh
0.07
0.20
zebrafsh2
0.12
0.37
0.08
grouper
Tetraodn2
Fugu2
0.30
zebrafsh1
0.25
salmon2
0.24
grayling
0.15
sturgeon
0.09
rockfish
0.09
flounder
halibut
0.08
salmon1
trout
whitefish
0.14
Tetraodn1
Fugu1
0.14
0.26
Rana
0.14
Xenopus
0.70
0.11
tunicate1
0.70
tunicate2
0.2

A search using the same rainbow trout 3'RACE product I generated revealed two sequences, plnb-A and plnb-B, with high match in GRASP Atlantic salmon EST database. Sequencing of these two clones resulted in two highly similar sequences with that included the expected predicted coding region up to the end of $PACAP_{1-27}$ at which point the sequence closely matches that of my rainbow trout 3'RACE product and has high sequence homology with the 3'UTR of PACAP-encoding genes in human, zebrafish and *Fugu* (Fig. 4.8). Furthermore, the high similarity in plnb-A and plnb-B, suggests a degree of polymorphism in the two alleles for this gene in Atlantic salmon.

Identification of two genes encoding PACAP and GHRH in Fugu and Tetraodon

Searching the public database of the *Fugu* genome project with PACAP and GHRH amino acid sequences resulted in matches to a small set of sequences. On closer analysis, I determined that two of the sequences coded for PACAP and GHRH, whereas the other candidate sequences were high matches because they are the *Fugu* genes that code for other PACAP/glucagon superfamily members including VIP, glucagon, and GLP-1 (data not shown). Using the two fragments that more closely match GHRH and PACAP sequences (*Fugu* scaffolds 4 and 836) I reconstructed the pututative coding for the two PACAP and two GHRH polypeptides (Fig. 4.2 and 4.3) and their prohormones (Fig. 4.9). Using these protein sequences, I matched two unidentified protein sequences in the NCBI protein database: the scaffold 4 construct had a 94% identity with accession number CAG10213, an unidentified protein product from *Tetraodon*, whereas the scaffold 836 construct highly matched accession number CAG12289, another unidentified protein product from *Tetraodon*. I subsequently confirmed these results (Fig. 4.9) by searching the *Tetraodon* genome database.

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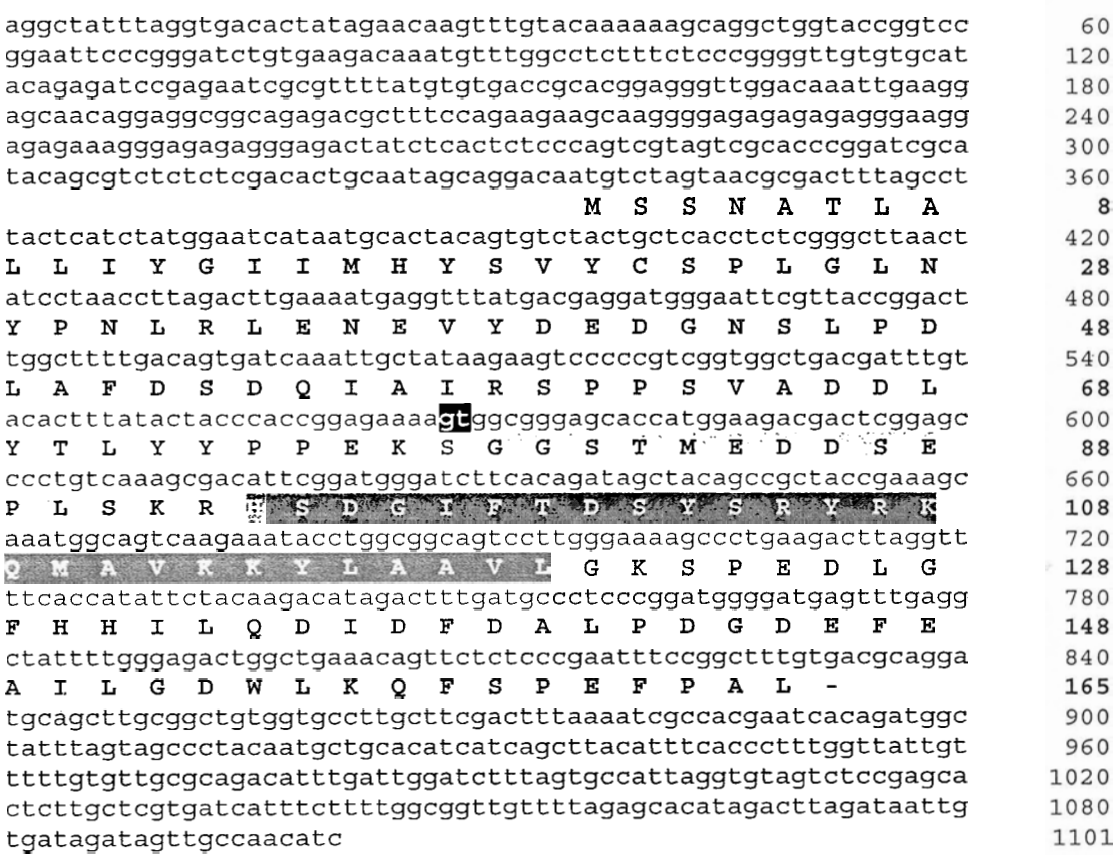
Figure 4.7. Nucleotide and deduced amino acid sequence of clone identified and sequence from rainbow trout expressed-sequence tag project. $GHRH_{33-45}$ is highlighted in light gray whereas $PACAP_{1-27}$ is dark gray.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M S S N A T L A 8
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L L I Y G I I M H Y S V Y C S P L G L N 28
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L A F D S D Q I A I R S P P S V A D D L 68
acactttatactacccaccggagaaaagtggcgggagcaccatggaagacgactcggagc 600
Y T L Y Y P P E K S G G S T M E D D S E 88
ccctgtcaaagcgacattcggatgggatcttcacagatagctacagccgctaccgaaagc 660
P L S K R S D G I F T D S Y S R Y R K 108
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Q M A V K K Y L A A V L G K S P E D L G 128
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F H H I L Q D I D F D A L P D G D E F E 148
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A I L G D W L K Q F S P E F P A L - 165
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ttttgtgttgcgcagacatttgattggatctttagtgccattaggtgtagtctccgagca 1020
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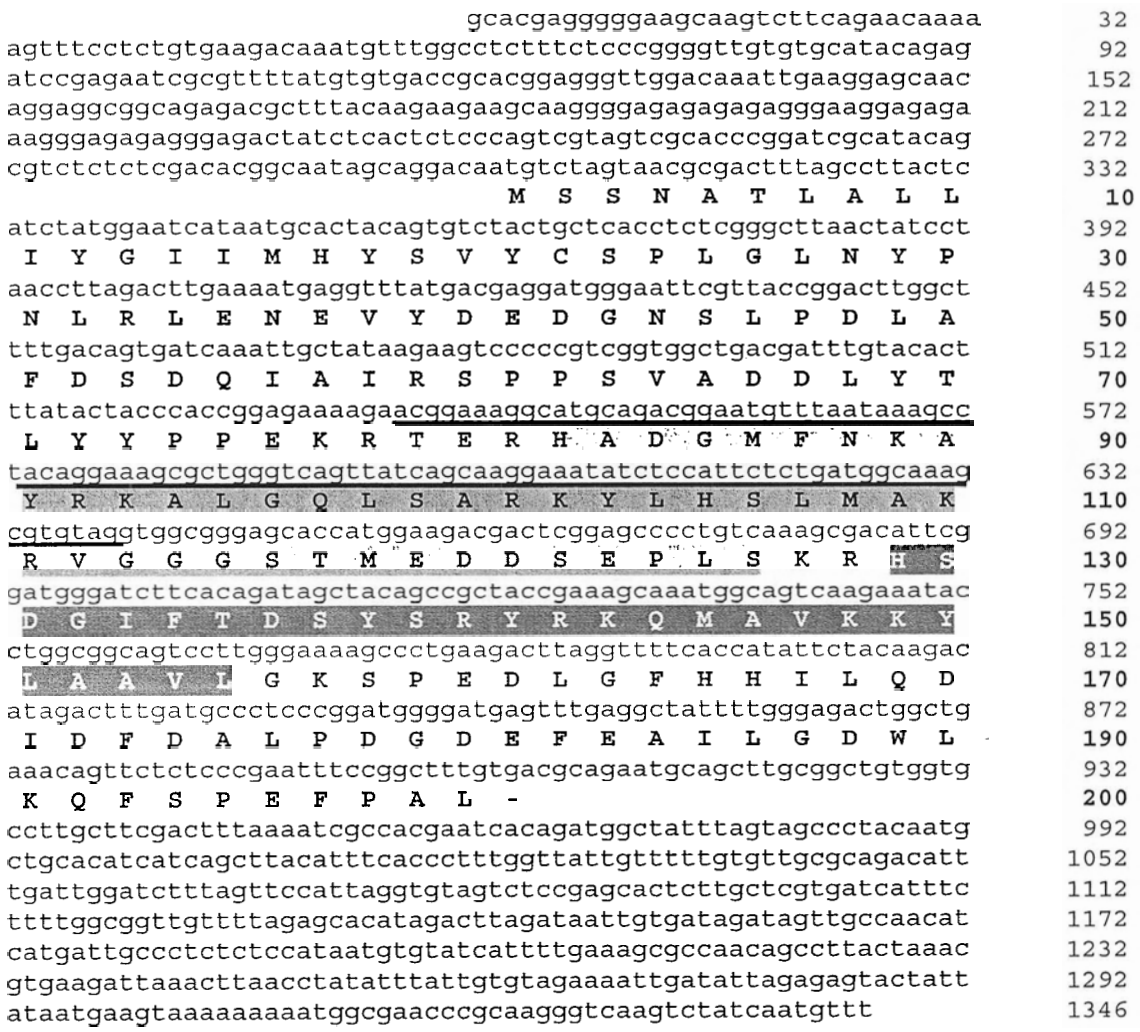
tgatagatagttgccaacatc 1101

Figure 4.8. Nucleotide and deduced amino acid sequences for two cDNAs, asplna-A (A) and asplnb-B (B) determined for a second gene for pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH) in Atlantic salmon. GHRH amino acid sequence is highlighted in light gray whereas PACAP is dark gray. The underline indicates the section of the transcript this is alternatively spliced.

A.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R V G G G S T M E D D S E P L S K R H S 130
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D G I F T D S Y S R Y R K Q M A V K K Y 150
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L A A V I G K S P E D L G F H H I L Q D 170
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I D F D A L P D G D E F E A I L G D W L 190
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K Q F S P E F P A L - 200
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B.

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gaaaattgatattagagagtactattataatgagaaaaaaatggcgatccacaagggtta                 1343
agtctataaatgt                                                               1356
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genes that code for other PACAP/glucagon superfamily members including VIP, glucagon, and GLP-1 (data not shown). Using the two fragments that more closely match GHRH and PACAP sequences (*Fugu* scaffolds 4 and 836) I reconstructed the pututative coding for the two PACAP and two GHRH polypeptides (Fig. 4.2 and 4.3) and their prohormones (Fig. 4.9). Using these protein sequences, I matched two unidentified protein sequences in the NCBI protein database: the scaffold 4 construct had a 94% identity with accession number CAG10213, an unidentified protein product from *Tetraodon*, whereas the scaffold 836 construct highly matched accession number CAG12289, another unidentified protein product from *Tetraodon*. I subsequently confirmed these results (Fig. 4.9) by searching the *Tetraodon* genome database.

Knockdown of zebrafish ghrh-pacap1 and ghrh-pacap2 translation

Inspection at 24 hours revealed a number of embryos with general defects in some of the injected groups. To take into account the variability in egg quality, I have reported the number of morphants in relation to the number of embryos that appeared to be living at 24 hours (Table 4.3). I decreased the initial dosage of 16 ng to lower doses until I determined the lowest effective dose to be 4 ng. I found that the low dose of 2 ng did not cause morphants (ie. non-toxic). However, upon addition of two different MOs (UTR and ATG) at this low dose of only 2 ng each, I saw a similar number of morphants at 24 hours that I saw with the lowest effective dose of 4 ng (Table 4.3). I did not find an increase in morphants with increasing dose of any of the four MOs as you might expect with a chemical that was toxic.

Morphants were identified because of their typically smaller size, mainly a result of a curved or bent tail and reduced eye or head size (Fig. 4.10). In some instances the fish only had one of the three main charactertics (ie. a curved tail or small head only), and these were scored equally with morphants that had more than one defect. In some instances whole body pigmentation was reduced at 48 hours, though there was no correlation of this observation with the particular MO. Batches of eggs on different days seem to vary in both their quality as determined by the amount of embryos that survive to 24 hours, and I have noted variance as well in the degree of whole body

Figure 4.9. Prohormone sequences for ghrh-pacap1 and ghrh-pacap2 in *Fugu* and *Tetraodon*. GHRH amino acid sequence is highlighted in light gray whereas PACAP is dark gray.

Fugu ghrh-pacap1

MSSKATLAFLICGVIMHYSVICSPVGLSFPSVRLDSEVYDDGGNSLQSLDYDRDMEVR
SPPSVADDLYSFYYAPEKRTERHADGMFNKAYRKALGQLSARKYLHSLMAKRVGGNTL
EDSSEPLSKRHSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYRQRIRNKGRRMAYL

Fugu ghrh-pacap2

MASSSKATLILLIYGILMHYSVFCTPIGLGYPKIRLDNDAFDEDGNSLSNMGFDSDQI
AIRSPPSVDDDAYSLYYPQEQRRPERHAEEELDRALREILGQLTARHYRHFLMTIRGS
AEEDSEPLSKRHSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNKGRRLAYL

Tetraodon ghrh-pacap1

MSSKATLAFLICGVLMRHSVICSPVGLSFPSVRLGSEVYDDGGNSLQSLDYDRDMEVR
TPASVADDLYSYYYAPEKRTERHADGMFNKAYRKALGQLSARKYLHSLMAKRVGGKTL
EDSSEPLSKRHSDGIFTDSYSR

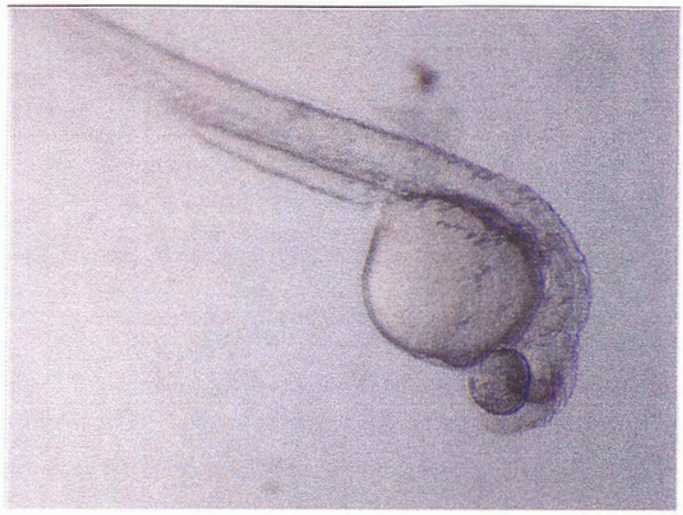
Tetraodon ghrh-pacap2

MASSSKATLILLIYGIIMHYSAFCTPIGLGYPKIRLDNDAFDEDGNSLSNMGFDSDQI
AIRSPPSVDDDAYSLYYPQEQRPERHAEGELDRALREILGQLTARHYRHFLMTVRGSA
EESEPLSKRHSDGIFTDSYSRYRKQMAVQKYLAAVLGRRYRQRVRNKGRRLAYL

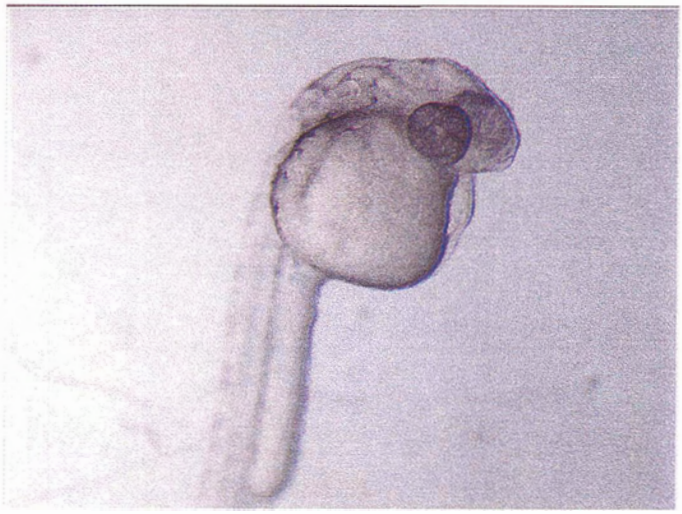
Table 4.3. Effect of morpholino-based knockdown of GHRH-PACAP genes in zebrafish at 24 hours.

Morpholino (dose)	Dose	N	Morphant %
CONTROLS			
Not injected	-	127	**10.0**
Control morpholino	16 ng	193	**6.3**
ghrh-pacap1			
ghrh-pacap1 UTR	2 ng	118	**1.7**
ghrh-pacap1 UTR	4 ng	108	**41.0**
ghrh-pacap1 UTR	8 ng	113	**49.0**
ghrh-pacap1 UTR	16 ng	100	**58.0**
ghrh-pacap1 ATG	2 ng	93	**4.7**
ghrh-pacap1 ATG	4 ng	86	**29.0**
ghrh-pacap1 ATG+UTR	2 ng each	90	**33.0**
ghrh-pacap2			
ghrh-pacap2 UTR	2 ng	150	**6.7**
ghrh-pacap2 UTR	4 ng	139	**52.0**
ghrh-pacap2 UTR	8 ng	139	**51.0**
ghrh-pacap2 UTR	16 ng	139	**42.0**
ghrh-pacap2 ATG	2 ng	129	**7.0**
ghrh-pacap2 ATG	4 ng	99	**31.0**
ghrh-pacap2 ATG+UTR	2 ng each	200	**60.0**

Figure 4.10. Morphological changes in zebrafish embryos at 24 and 48 hours after injection with morpholinos designed to knockdown each of the two genes encoding GHRH and PACAP.

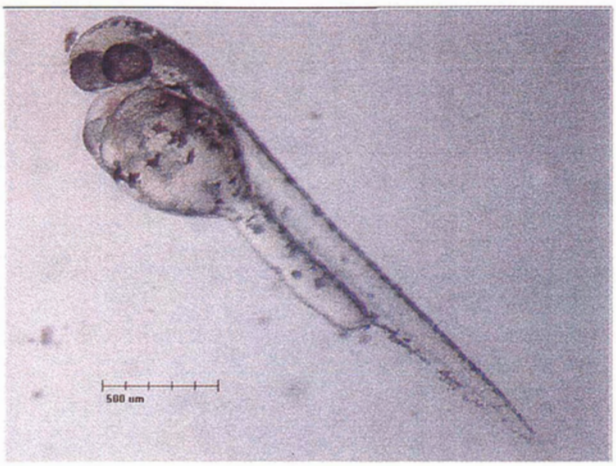


Control-injected

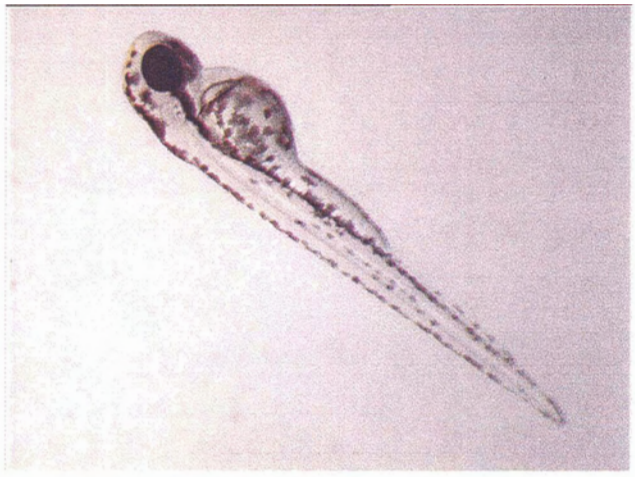


***ghrh-pacap2* (2 ng UTR)**

***ghrh-pacap2* (2 ng ATG + 2 ng UTR)**



Control-injected



***ghrh-pacap2* (2 ng UTR)**

***ghrh-pacap2* (2 ng ATG + 2 ng UTR)**

pigmentation that occurs at 48 hours. If there is some impact on pigmentation by the four MOs I have injected, I have lost it in the 'natural background' in subtle developmental variation between batches of eggs.

Discussion

I have identified DNA sequences encoding GHRH and PACAP in nine species of teleost fish (rainbow trout, grayling, whitefish, flounder, halibut, rockfish, Atlantic salmon, and two pufferfish species, *Fugu* and *Tetraodon*) using a combination of molecular biological and bioinformatic techniques. Interestingly, sequences indicating a second gene coding for PACAP were identified in five of the nine species. Using morpholino injection, I was able to show in zebrafish that both copies of the gene are functional, but neither is critical alone, in early development, and cannot compensate totally for the second *ghrh-pacap* gene suggesting specialization in function.

Most fish species for which cDNAs encoding PACAP have been isolated to date are teleost species. An older fish lineage that includes sturgeons, which are basal bony fishes that are estimated to have diverged from teleosts about 400 million years ago, has recently had a cDNA encoding GHRH and PACAP identified (Adams et al., 2002). The organization of the precursor is the same for all the fish sequences identified, and the regions are the same as that described in other non-mammals: 5'UTR, signal peptide, cryptic peptide, GHRH-like peptide, PACAP, and 3'UTR, except in the case where alternative splicing has removed the exon that primarily codes for GHRH. The identification of new DNA sequences encoding GHRH and PACAP in nine teleost species provides an opportunity to compare the evolution of GHRH and PACAP over a shorter time interval, but also in relation to evolutionary events at the level of DNA that are hypothesized to occur since the split of these two lineages. Indeed, the identification of a number of GHRH-PACAP sequences suggests that duplicate genes coding for PACAP in the teleost species in my study corresponds with the timing of the genome duplication event that is hypothesized to have occurred in the teleost lineage (Vandepoele et al., 2004). However, three of the duplicate sequences are found in salmonid species that are proposed to have had a unique genome duplication event (Phillips and Rab, 2001).

The DNA sequences identified would encode a number of novel PACAP peptide forms. A number of previously identified sequences were also found in these species. Grayling PACAP is identical to the one already described in several salmon and rainbow trout, whereas flounder and halibut PACAP is the same as that in stargazer, which was isolated in peptide form (Matsuda et al., 1997). Compared with human $PACAP_{38}$, fish have sequence identities of 79 – 95 %, whereas frogs and birds each have 97 % identity. This may reflect the diversity within the large number of fish species or the amount of time since fish diverged from the lineage that led to mammals.

The high conservation of the PACAP peptide sequence among fish species reaffirms the notion that this peptide is functionally important in fish (Sherwood et al., 2000). The actions of PACAP in fish are widespread. PACAP acts as a hypophysiotropic agent (Wong et al., 2001); it functions in the gut to influence contractions in the intestine (Olsson and Holmgren, 2000; Matsuda et al., 2000); and PACAP can induce catecholamine secretion from chromaffin tissue (Montpetit and Perry, 2000). The diversity of structure for PACAP in fish may suggest that there is also variation of the function of PACAP in fish, with possible specialization of function of PACAP in different groups of fish.

Although the 25 – 45 % conservation of fish GHRH structure compared to human GHRH may seem low, it is higher than frog (34%) and as high as bird (43 – 47 %). The lower conservation of the GHRH peptide compared to PACAP suggests that the GHRH is functionally less important in fish. This may be supported by studies in fish that suggest GHRH has less ability to stimulate secretion of GH and gonadotropins than PACAP.

The phylogenetic tree was constructed from the deduced amino acid sequences for the prohormones. The prohormone sequences grouped nearly as predicted based on what we know about the evolution of the different groups of animals. Tunicate cDNAs for GHRH-PACAP are most different from the vertebrate sequences because the nucleotides encoding the signal peptide and cryptic peptide are variable. Within the vertebrates mammalian cDNA sequences are more similar to each other than to other groups but the second highest similarity is with bird sequences. The two sequences

from frogs, *Xenopus* and *Rana*, are also more similar to each other than to other vertebrates. Flounder and halibut are nearly identical at the preprohormone level, and their sequences are most similar to rockfish. A second major grouping in fish includes the sequences that have a GHRH sequence that is less conserved. This grouping includes one of the paralogs from each of the two pufferfish species and zebrafish, as well as the catfish and grouper sequences.

The species we studied represent various degrees of polyploidy. As salmonids grayling and whitefish are tetraploid (Phillips and Rab, 2001); and flounder and halibut are thought to be diploid based on cellular DNA content (Hinegarder and Rosen, 1972). Although there is typically extensive reorganization of chromosomes following a polyploidy event, many of the genes are unaffected. For example, the fraction of genes retained in duplicate after a polyploidy event varies with each species, but is considered to be about 70 % in salmonids over 25-100 million years (Phillips and Rab, 2001).

Changes that occur in two copies of a ghrh-pacap gene in one species probably undergo changes first in introns, second in the untranslated regions of the mRNA and finally in the coding region. Studies of the PACAP gene in sturgeon suggest the two copies of a gene are different in the 5'UTR, but not in the 3'UTR or coding region (Adams et al., 2002). The two cDNAs encoding GHRH and PACAP in zebrafish and pufferfish species may represent an extreme example of these changes in duplicate copies of a gene. For example, the gene duplication event in zebrafish is estimated to have occurred between 300 and 450 million years ago (Taylor et al., 2001) and the large difference between the two zebrafish and pufferfish coding regions supports such an ancient duplication event. The second sequence for salmon suggests that it is more similar to the already known salmon sequences, suggesting that either there was less pressure on DNA sequences in the salmonid lineage, or the second salmon gene is the result of a more recent salmonid-specific genome or large scale gene duplicaton event. The region that normally codes for a GHRH-like peptide in the second gene of pufferfish species has undergone a dramatic change, suggesting the mutation pressures on the gene, or at least that region of the gene, have been relaxed. Indeed there is evidence for the duplication of other components of the PACAP/glucagon superfamily

in these species. Two glucagon genes have been identified in the genomes of Fugu and zebrafish (Zhou and Irwin, 2004), and a second GHRH receptor has been found in Fugu (Cordoso et al., 2003). I also identified putative duplicates for a PHI-VIP in the Fugu genome during my current attempts to identify new PACAP genes (date not shown). The genes encoding the PACAP/superfamily of hormones are an excellent case study for the hypothesis and observation by Jordan et al. (2004), which suggests that although there is an initial acceleration of substitution immediately following the duplication event as a result of the relaxing of purifying selection, there is a subsequently slower rate of mutation/substitution on these paralogs because of their functional importance (reviewed in Taylor and Raes, 2005). I was not able to identify candidates for other superfamily members such as GIP and secretin in the genomes of pufferfish, suggesting that the number of members increased after the split of fish and tetrapods.

The shorter PCR products are the result of exon skipping, a phenomenon that has been shown to occur in fish (Parker et al., 1992; McRory et al., 1995; Parker et al., 1997; Krueckl and Sherwood, 2001), frog (Alexandre et al., 2000) and bird (McRory et al., 1997; Yoo et al., 2000). Exon skipping is a specific form of alternative splicing in which an exon is deleted during the formation of mRNA. In the fish presented here, it is exon four containing $GHRH_{1-32}$ that is deleted and allows for the expression of PACAP independently of GHRH. This mechanism can be used as early as embryonic day four in fish (Krueckl and Sherwood, 2001) and results in different ratios of two *ghrh-pacap* transcripts during development in turkeys (Yoo et al., 2000). To date there is no evidence for skipping of exon 5, which encodes PACAP. Exon skipping allows a higher ratio of PACAP peptide to GHRH peptide.

Alternative processing, including exon skipping, is a major method of regulating the ratio of peptides from multi-peptide coding genes in the PACAP/glucagon superfamily. In fish and birds, the proglucagon gene is alternatively spliced in a tissue-specific way to produce either glucagon or glucagon-like peptide-1 and –2 peptides (Irwin and Wong, 1995). In birds, the vasoactive intestinal polypeptide can be produced without peptide histidine isoleucine (PHI), when the exon encoding the latter is skipped (Talbot et al, 1995). However, there is no evidence for exon skipping in the

mammalian vasoactive intestinal polypeptide transcript (Linder et al., 1987). These examples imply that tissue-specific factors activate the exon skipping action. However, the factors regulating exon skipping of the *ghrh-pacap* transcripts are not well understood. Exon skipping in the *ghrh-pacap* mRNA likely represents the ancestral version of regulating GHRH and PACAP production. Mammals have achieved control over the production of GHRH and PACAP by having these two peptides encoded on separate genes.

Gene duplicates are also candidates for new gene functions, or genes can simply divide the same function. To test this possibility I took advantage of the fact that two cDNA sequences are available for the zebrafish *ghrh-pacap* gene paralogs, as well as the fact that zebrafish are such excellent model of early development. I was interested to know whether each of the two genes is functional, as the data for zebrafish *ghrh-pacap1* and *2* mRNA (although some function for zebrafish PACAP2 has been shown in vitro (Wang et al., 2003)) is limited to mRNA expression (Fradinger and Sherwood, 2000; Krueckl et al., 2003; Wang et al., 2003), which is a possible fate of a pseudogene. Indeed at least one of these genes is producing mature peptide, as immunofluorescence studies show PACAP-like immunoreactivity as early as 24 hours post fertilization in the brain, spinal cord and retina of zebrafish. My results imply that each gene has a function in development, and that neither gene is able to compensate fully for the second (if at all). Studies that would complement these MO knockdown studies would include colocalization studies of the two mRNA populations by *in situ* hybrization in order to determine tissue-specific expression patterns, as well as quantitative PCR studies that would give an idea as the relative amounts of expression of each gene at different time points in development. Lastly, localization of receptors may be critical if it is determined the two peptides activate different signal transduction pathways.

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CHAPTER 5: PACAP and GHRH expression in brain and gonads of sexually maturing rainbow trout.

A version of this chapter will be submitted for publication in the following form:

Adams BA, Gray SL, Warby CM, Rivier JE, Sherwood NM Growth hormone releasing hormone (GHRH)-pituitary adenylate cyclase-activating polypeptide (PACAP) expression in brain and gonads of sexually maturing rainbow trout. Molecular Reproduction and Development.

Introduction

As described in Chapter 1, the actions of PACAP are widespread and include the gonads. One approach to understanding whether PACAP has a local, perhaps autocrine or paracrine action in gonads is to determine the expression patterns of mRNA encoding PACAP and its receptor and PACAP peptide in mammalian gonadal tissue (see Arimura and Shioda, 1995; Moretti *et al.*, 2002 for reviews).

In mammals, PACAP is widely expressed in testes (Shioda et al., 1994) and has been detected in ovary using RIA (Arimura et al., 1991). In ovary, PACAP expression has been identified for rat (Arimura, 1991; Scaldaferri et al., 1996), as well as in chicken (Peeters et al., 1999) and zebrafish (Wang et al., 2003). PACAP rat ovarian cell expression over the course of the estrous cycle has been identified for both *adcyap* (PACAP) mRNA and PACAP immunoreactivity in nerve cells, most granulosa and cumulus cells from large preovulatory follicles, most cells of the interstitial glandular tissue and in thecal cells of both growing and mature follicles (Gras et al., 1996).

There is little known about the expression and possible function(s) of PACAP in non-mammalian gonads. The gene coding for GHRH and PACAP is expressed starting early in development in the teleost rainbow trout (Krueckl and Sherwood, 2001) and zebrafish (Krueckl et al, 2003) and is expressed in adult salmon (Parker et al., 1997), catfish (McRory et al., 1995; Small and Nonneman, 2001) and zebrafish tissue (Fradinger and Sherwood, 2000). There is, however, little evidence to date for a role of GHRH or PACAP in the development and function of the germ cell line and mature gonadal structures in non-mammalian male and female reproductive systems. Recently a second gene for PACAP was identified and studied in the ovary of maturing zebrafish (Wang et al., 2003). This second form of PACAP in zebrafish is proposed to be regulated in the ovarian follicle by gonadotropin and to act via a Gs, cAMP-inducing, pathway, with the capability of stimulating oocyte maturation in vitro (Wang et al., 2003). The authors concluded that PACAP has a paracrine/autocrine role in these tissues. A similar paracrine/autocrine action has been proposed for other hormones that have their genes expressed in the gonads of fish, including GnRH (Madigou et al. 2002; von

genes expressed in the gonads of fish, including GnRH (Madigou et al. 2002; von Schalburg and Sherwood, 1999). We subsequently determined that in rainbow trout there is no or very little mature GnRH peptide produced in the gonads even though expression of mRNA occurs (Gray et al., 2002). Consequently, before a paracrine/autocrine action for PACAP in the gonads of fish is considered further, it would be useful to consider if there is a local supply of protein available.

My first objective was to identify whether *ghrh-pacap* mRNA is expressed in the brain and gonads of adult rainbow trout and then to determine the expression of this gene during the final 13 months in first time sexually maturing male and female rainbow trout. Finally, we developed a highly specific RIA based on $PACAP_{1-12}$ to detect if mature PACAP peptide is present in the brains and gonads of adult rainbow trout.

Materials and methods

Animal care and tissue collection

For mRNA analysis, rainbow trout were maintained for research according to the guidelines outlined by the Animal Care Committee of the University of Victoria. On November 1, 1998, two-year-old rainbow trout (~400-500 g average) were obtained from Mountain Trout Sales (Sooke, BC, Canada). Male and female rainbow trout were randomly selected on or about the 12^{th} day of each month from December 1998 to December 1999. For protein studies, gravid four-year-old trout were netted. Fish were housed in a 4000 litre tank and fed commercial trout pellets. Trout were killed by submersion in a lethal concentration of tricaine methanosulfate (MS-222) (Argent Chemical Laboratories, Redmond, WA) and followed by severing of the spinal column. Brains, testes or ovaries, and eggs (if present) were quickly dissected and immediately frozen in liquid nitrogen or kept on dry ice until transferred to a –80°C freezer.

RNA isolation

For each month of the 13-month study, total RNA was isolated from separate pools of brain, ovary or testis tissue using the TRIzol Reagent (Life Technologies,

Burlington, ON) according to the manufacturer's instructions. Quantification of total RNA was made by measuring the optical density at 260 nm and quality was determined by the 260nm/280nm optical density ratio. Next, mRNA was isolated from total RNA using the Poly-A Pure mRNA isolation kit (Ambion Inc., Austin, TX) and quantified with a dot assay using dilutions of a known RNA standard to compare samples.

RT-PCR analysis

cDNA was synthesized using 10 µl of total RNA for each sample, using 200 U avian reverse transcriptase (Superscript II RT, Life Technologies), 10 mM diothiothreitol, 2 mM each dNTP, 160 U RNA guard, 2 mM oligo dT16-20, and 1 X RT buffer, to a total reaction volume of 50 µl. The reaction proceeded for 90 min at 42°C, followed by 5 min at 90°C. DNA amplifications were done in a 50-µl vol that contained 1-3 µl cDNA, 1 X Taq buffer, 0.5 µM dNTPs, 2.5 mM $MgCl_2$ and 20 pmol of each primer. PCR was performed with 1 cycle at 94°C followed by 33 cycles at 94°C for 30 sec, 57°C for 30 sec, and 74°C for 30 sec.

Primers were designed based on conserved regions of the *ghrh-pacap* cDNA sequence from salmon (Parker et al, 1993). The first primer (P1) is a forward primer that is housed in the second exon, the signal peptide of the salmon cDNA. The second primer (P2) is a reverse primer that is found within exon 5, the exon that codes for PACAP. PCR with P1 and P2 would be expected to yield a product of slightly greater than 600 base pairs (bp) based on the known sequence for salmon GHRH-PACAP cDNA. A third primer (P3) is a reverse primer from within the fourth exon, the exon coding for GHRH. PCR with P1 and P3 would result in a smaller product of about 300 bp.

Products were separated by electrophoresis on a 1.2 or 1.5 % agarose gel, and the gel was photographed after staining in ethidium bromide solution. To confirm the PCR products as *ghrh-pacap* transcripts, PCR products were ligated, cloned and sequenced as described above.

Bands of the products of interest were cut from the agarose gel in preparation for DNA isolation. The bands were minced with a blade and DNA-pH-equilibrated phenol was added before freezing. Subsequent steps resulted in a small pellet of DNA, which was taken up in 10 µl of sterile water and 2 µl of this product was used in ligation reaction using the Vector T cloning kit (Promega, Madison, WI). Plasmids were then introduced into competent bacteria cells by electroporation. After a short incubation 200 µl of bacterial culture was spread onto antibiotic-treated plates coated with X-gal, which allowed for blue-white selection for colonies with plasmids containing the insert of interest. Plates were incubated overnight at 37°C. Up to 10 white colonies were selected and grown up overnight in a 3 ml culture to increase the amount of insert DNA. The plasmid DNA was then isolated using the MiniPrep kit (Qiagen, Mississauga, ON). A sample of each plasmid DNA was subjected to an enzyme digest using the appropriate enzymes for the plasmid insert, Sph1 and Pst1. Products of the digest were electrophoresed on a 1.5 % agarose gel to confirm the presence of the insert of size of interest. Candidate preparations of DNA were sent for sequencing.

Sequencing

At least two plasmids for each of the reactions were sequenced with M13 forward and reverse primers using LI-COR model 4200 DNA sequencer (LI-COR Inc, Lincoln, NE). Sequencing results were analyzed using the BLAST similarity searching technique offered by the National Institute of Health (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

Protein Extraction

Brains and gonads of 14 rainbow trout were individually extracted. Frozen tissues were powdered with a chilled pestle and mortar and liquid nitrogen. Peptides were extracted with cold acetone/HCl and soluble lipids removed with petroleum ether as previously described (Gray et al., 2002). Acetone was removed and the volume reduced to approximately 2 ml on a vacuum centrifuge. The extract was filtered thru a µStar-LB 45 µm filter (Costar Corning, NY).

HPLC

Each filtered extract was analyzed by HPLC chromatography (Beckman System Gold) using a Vydac C-18 column for proteins and peptides (catalogue #218TP54; Hesperia, CA) 25 cm x 4.5 mm, 5μm particle size with guard column (catalogue #VY218GK54) of the same material. Loading occurred thru a 1 ml injection loop in a series of three injections (approximately 600 μl each) at 0, 2.5 and 5 minutes of the first 10 minute isocratic period of 10% acetonitrile (ACN) with 0.1% trifluroacetic acid (TFA). After 10 minutes, the % ACN with 0.1% TFA was increased on a gradient at a rate of 1% per minute until reaching 60% ACN at 60 minutes. During this time, 60 x 1 ml/minute fractions were collected into polyallomer tubes.

Prior to each extract being loaded onto the HPLC, a blank was analyzed in which 700 μl of deionized water was injected onto the column and eluted under conditions identical to those described above. Fractions were collected so that strategic blank samples could be assayed to determine if the columns was clean prior to the injection of a tissue extract. Only one set of blank samples was assayed, as the results as the extract fractions had little protein. The other blank fractions were saved.

Synthetic standards of salmon PACAP $(sPACAP)_{1-27}$ (40 μg) and $sPACAP_{1-38}$ (15 μg) (a gift from J. Rivier, Salk Institute) were previously run with identical column and conditions, except without a guard column. The optical density peak for $sPACAP_{1-27}$ was at 36 mins and for $sPACAP_{1-38}$ was 32 mins.

Radioimmunoassay

Samples were taken from each HPLC fraction, dried on a vacuum centrifuge and rehydrated in buffer to assay for salmon PACAP. Initially, samples from fractions of brains and gonads from two fish (one male and one female) were assayed for $sPACAP_{1-27}$ using a Peninsula RIA kit for mammalian $PACAP_{1-27}$ (Cat # S2165) (which is the same amino acid sequence as $sPACAP_{1-27}$) using 500 μl per from each of fractions 30 to 50.

(Cat # S2165) (which is the same amino acid sequence as $sPACAP_{1-27}$) using 500 µl per from each of fractions 30 to 50.

Next, an assay was developed using antibody 15CR-5 made against sPACAP 38 in rabbit in our laboratory (Parker, 1992). Other RIA materials used were the same as previously described for GnRH RIA (Gray et al., 2002) but the method was slightly adapted as follows. First, synthetic $sPACAP_{1-12}$ (J. Rivier, Salk Institute) was iodinated as a tracer using chloramine T method followed by Sep Pak separation (Waters C-18). $sPACAP_{1-12}$ was chosen because sPACAP38 has more than one tyrosine residue that can iodinated. Second, dried samples were reconstituted in 200 µl PBS-gelatin solution. Third, 100 µl of antibody 15Cr-5 at a 1:5000 dilution were added on day one, followed by addition of 100 µl of I-125 tracer $sPACAP_{1-12}$ on day two. Finally on day three, 1 ml of charcoal-dextran-PBS was added to bring down the unbound trace by centrifuging and the supernatant was counted on a gamma counter.

Eighteen point standard curves (1 pg – 128 ngs) were assayed for each $sPACAP_{1-12}$, $sPACAP_{1-27}$ and $sPACAP_{1-38}$. HPLCs of all 14 fish brains and gonads were assayed with this method using 100 µl per fraction of the samples, assaying all 60 fractions of each run and measuring the results against the standard curve for $sPACAP_{1-38}$.

Results

ghrh-pacap gene expression

Testis and ovary began to increase in size rapidly beginning in June (Fig. 5.1). RT-PCR indicated expression of *pacap-ghrh* in brain and gonads in each of the 13 months of this study. Two transcripts of 540 and 645 bp were detected in the brain RT-PCR. However, only the shorter 540 bp transcript is found in the ovary and testis (Fig. 5.2A). Sequencing results identified the long transcript that appears in brain, but not gonads, as five exons that have been shown to exist previously in rainbow trout and other fish species (Fig. 5.2). On sequencing, the shorter product of 540 bp in gonads and also brain was missing exon 4, the segment coding for $GHRH_{1-32}$ (Fig. 5.2B). The lack of a longer transcript in trout gonads was

confirmed by the lack of product after RT-PCR of gonad cDNA with primers p1 and p3 (specific to exon 4 sequence) (data not shown).

Radioimmunoassay for PACAP

Using the RIA we developed based on $PACAP_{1-12}$, we found that only one of the five male fish we studied had significant amount of PACAP in the brain (Fig. 5.3) and gonads (Fig. 5.4), whereas three other male fish had lower but significant levels of PACAP in the brain, but very low or undetectable levels of PACAP in the gonads (Table 5.1). Only one of the 14 female fish studied had significant levels of PACAP in the brain (Fig. 5.3) and ovary (Fig. 5.4), whereas the other females had low or undetectable levels (Table 5.1).

Discussion

This study extends earlier studies showing expression of *ghrh-pacap* in rainbow trout during early development in whole embryos 4-19 days after fertilization (Krueckl and Sherwood, 2001). In the present study specific tissues were analyzed at 24-37 months of age to show that *ghrh-pacap* is also expressed in the central nervous system and gonads
of sexually mature adult rainbow trout. The presence of two *ghrh-pacap* transcripts in brain tissue suggests that the brain produces mature PACAP and GHRH peptide. It is unclear why the rainbow trout gonad only expresses an exon-4 spliced transcript in gonadal tissues. However, in another salmonid, lake whitefish, I have detected the same short transcript expression pattern in brain (chapter 4; Adams et al., 2002) and ovary (unpublished observation). Deletion of an exon as a form of alternative splicing may allow for differential regulation of downstream peptide products that are found on the same gene. At the time of the experiment the presence of a second gene for PACAP in rainbow trout had not been reported.

I confirmed the finding by Fradinger and Sherwood (2000) of no alternative splicing of the *ghrh-pacap1* gene in zebrafish gonads, and those of a recent study of zebrafish *ghrh-pacap2*, in which two transcripts, one of which is alternatively

spliced, were expressed in ovary (Wang et al., 2003) (data not shown). In earlier studies of fish *ghrh-pacap* expression, no alternative splicing was noted in either brain or gonads of the gene for PACAP and GHRH of catfish (McRory *et al.*, 1995). The two-transcript expression occurs in the brain of other salmonids such as salmon (Parker *et al.*, 1997) and lake whitefish (Adams *et al.*, 2002) as well as for zebrafish *pacap-ghrh2* (Wang *et al.*, 2003). The presence of a precursor that contains only information for translating PACAP but not GHRH suggests that PACAP production by the testes and ovaries may be the result of tissue-specific differences in prohormone cleavage by different prohormone converting enzymes as is the case in mammals (Li et al., 1998). In mammals, the gene for PACAP in the testis is under control of a different upstream promoter than in nervous tissue (Daniel and Habener, 2000), although it is unknown if a similar phenomenon occurs in fish.

In order to gain a more reliable measurement for the amount of PACAP protein that is found in brain and gonad, we were required to develop a RIA that accounts for the nature of the polypeptide being studied. RIA includes the peptide of interest that is iodinated on its tyrosine residues. PACAP is a peptide that includes more than one tyrosine residue and requires a special approach to detecting an accurate amount in a sample. Commercially available PACAP RIA kits use full length PACAP peptide as tracer so that up to three tyrosine residues may be iodinated. Consequently there is a population of tracers with variability in the degree of iodination of peptides that compromises the ability to interpret the results from the standard curve that is generated. The assay that we developed was less sensitive for detecting PACAP than the commercially available kit, namely because the $PACAP_{1-12}$ trace has such a high affinity for the binding sites on the antibody that it was difficult for the tracer to displace PACAP in the extract. Therefore the $PACAP_{1-12}$ RIA is less sensitive, but more accurate. One interesting side effect of the $PACAP_{1-12}$ RIA is that it does present the possibility that

Figure 5.1. Average monthly diameter of ovaries and testes as an indicator of growth over 13 months in sexually maturing rainbow trout.

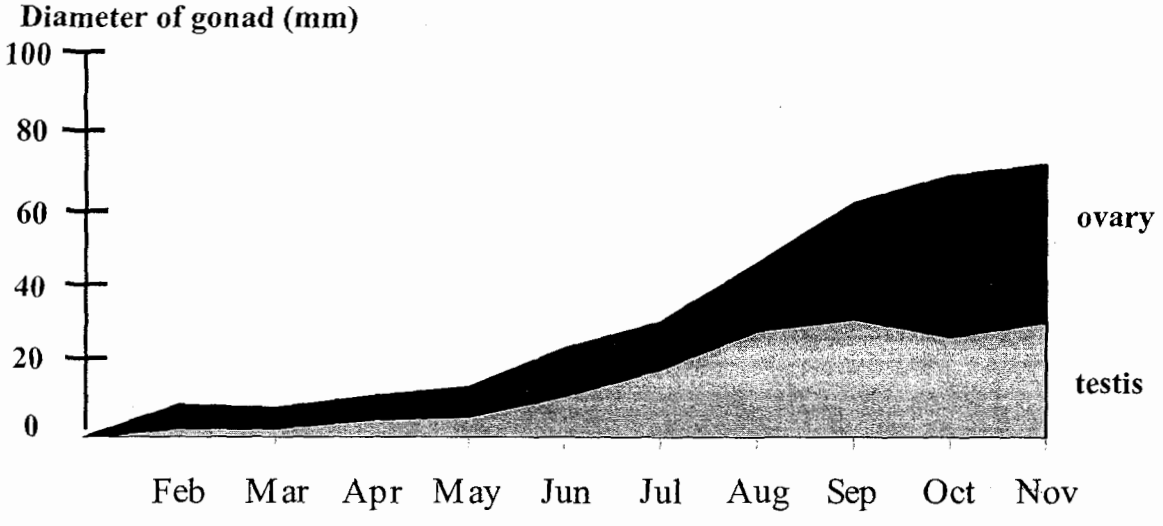
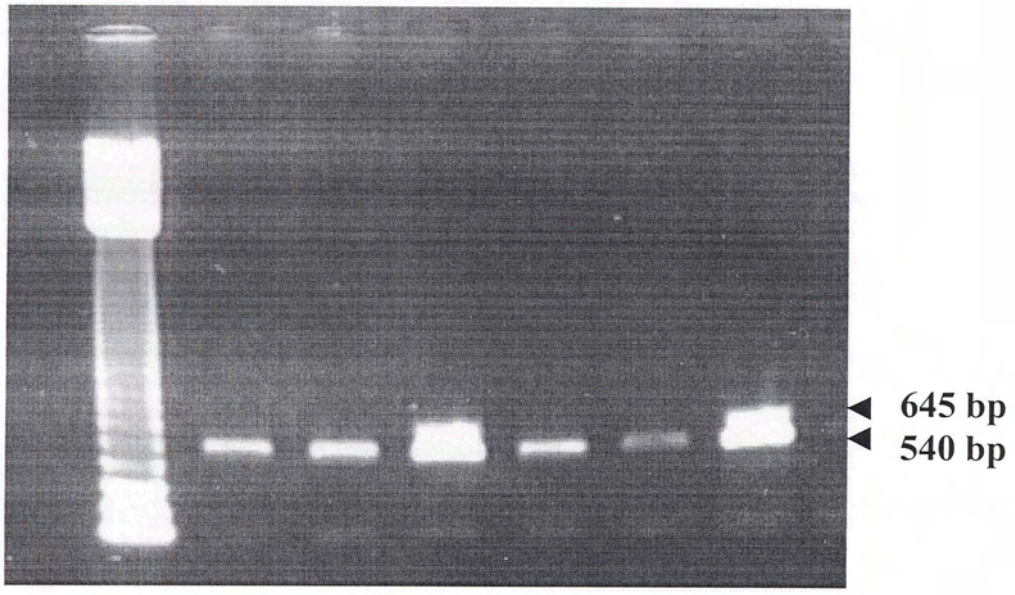

Diameter of gonad (mm)
100
80
60
40
20
0
ovary
testis
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov

Figure 5.2. A. Gel of RT-PCR results from ovary (O), testis (T) and brain (B) tissue collected from rainbow trout in August and September. **B.** Organization of the rainbow trout PACAP gene indicating the regions that code for the signal peptide (SP), cryptic peptide (cryptic), growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP). The transcript that results from alternative splicing lacks exon 4, the one that codes for $GHRH_{1\text{-}32}$. The schematic is not drawn to scale.

A.


645 bp
540 bp

B.

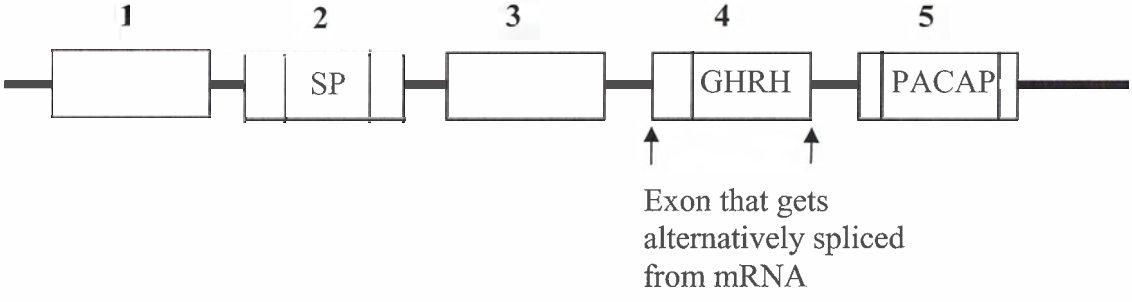

1
2
3
4
5
SP
GHRH
PACAP
Exon that gets
alternatively spliced
from mRNA

Figure 5.3. Immunoreactive PACAP content by fraction in from male #4 brain (a), female #9 brain (b), and blank run (c) as determined by RIA.

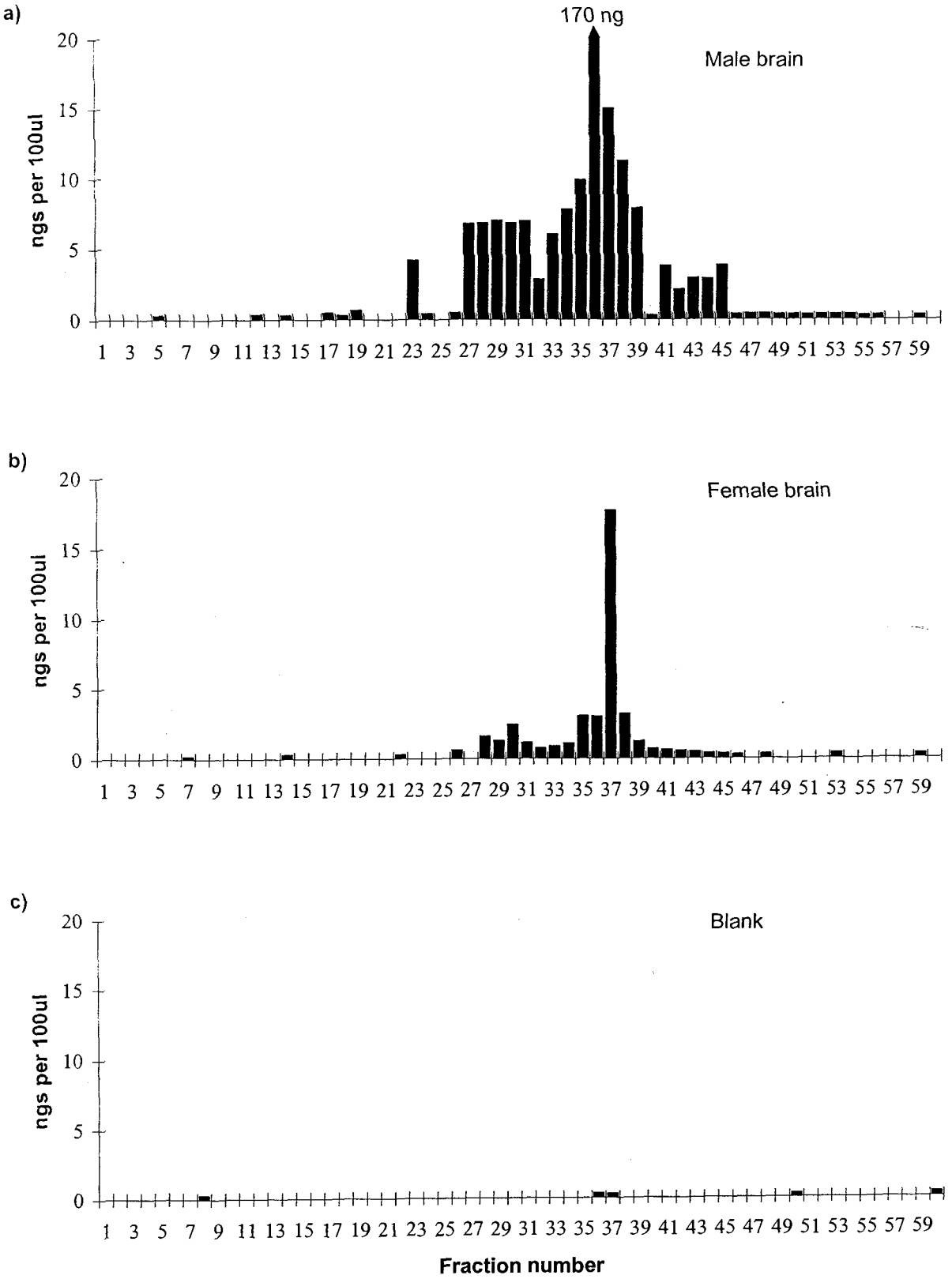

a)
170 ng
Male brain
ngs per 100ul
20
15
10
5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
b)
Female brain
ngs per 100ul
20
15
10
5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
c)
Blank
ngs per 100ul
20
15
10
5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Fraction number

Figure 5.4. Immunoreactive PACAP content by fraction in from male #4 testis (a), and female #9 ovary (b) as determined by RIA.

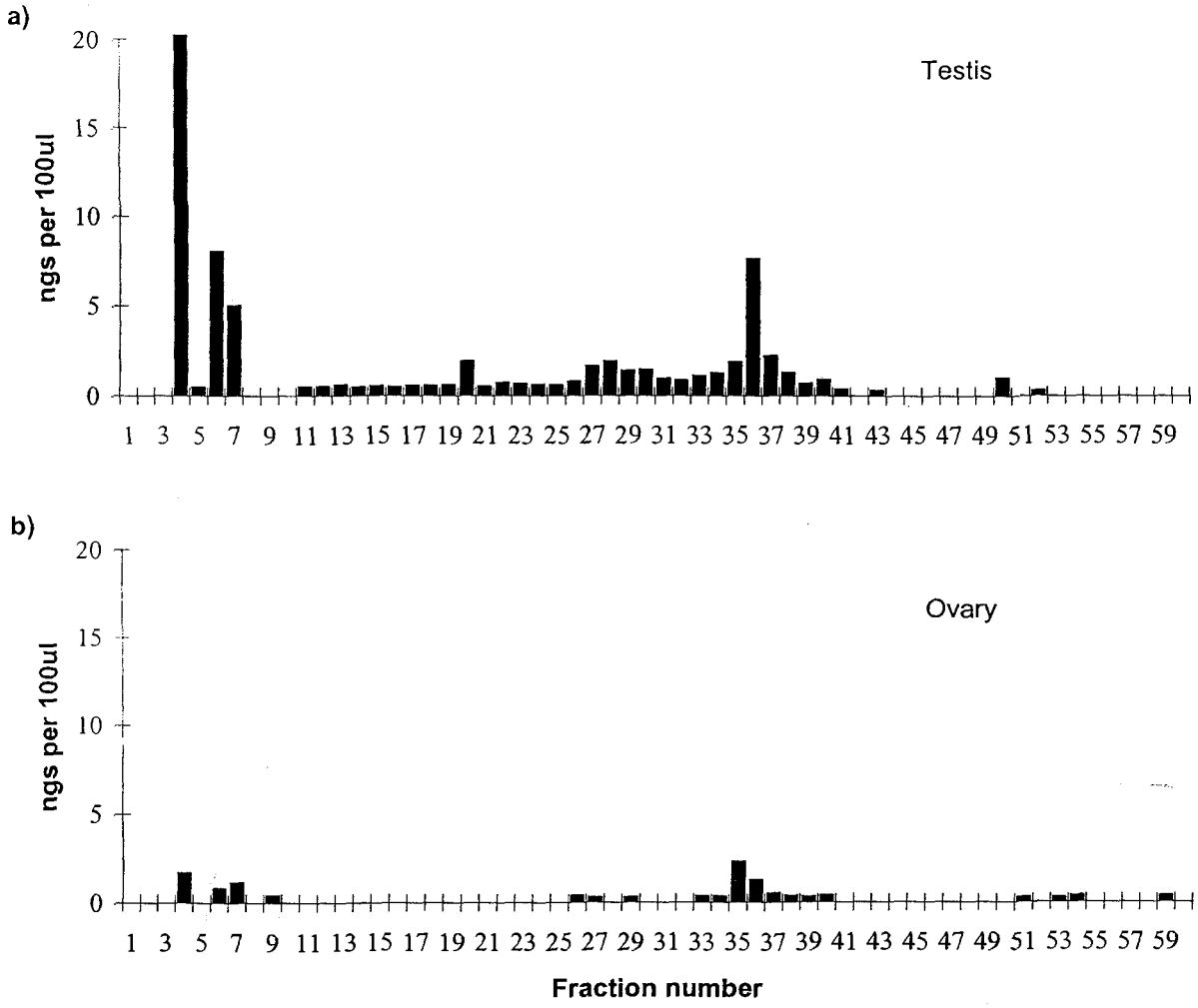

a)
Testis
ngs per 100ul
20
15
10
5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
b)
Ovary
ngs per 100ul
20
15
10
5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Fraction number

Table 5.1. Quantities of pituitary adenylate cyclase-activating polypeptide (PACAP) in brain and gonads of 14 rainbow trout determined by radioimmunoassay.

Mass (g)	Gonadosomatic index	Tissue	Quantity of PACAP (ng/100 µl)
Males			
414.3	6.97	Brain	nd
		Testes	<1
701.9	2.05	Brain	170
		Testes	8
472.3	2.78	Brain	<1
		Testes	<1
436.9	1.16	Brain	2
		Testes	nd
712.4	2.28	Brain	4
		Testes	<1
Females			
838	6.49	Brain	<1
		Ovary	<1
976.5	0.04	Brain	<1
		Ovary	<1
831	0.01	Brain	<1
		Ovary	<1
605.2	0.08	Brain	<1
		Ovary	nd
636.5	4.5	Brain	17.5
		Ovary	3
881	0.07	Brain	<1
		Ovary	nd
810.4	0.08	Brain	nd
		Ovary	<1
377	7.01	Brain	<1*
		Ovary	nd
448.5	0.34	Brain	<1*
		Ovary	nd

*Brains from these two fish were pooled and extracted.

$PACAP_{1-12}$ could be useful as a pharmacological agent because it would have a high affinity for native PACAP receptors and not be out-competed by endogenous compounds.

We found that only 2 of 14 (5 males and 9 females) fish used in analysis had significant levels of PACAP present, suggesting that high levels of PACAP polypeptide are not critical for gonadal function in gravid rainbow trout. We recently completed a study of the gonads of sexually maturing rainbow trout for the presence of gonadotropin-releasing hormone (GnRH) (Gray et al., 2002), another factor thought to have important peripheral actions. As mentioned above, a number of studies including our own show the expression of mRNA in the mature gonads. However, we were unable to detect GnRH peptide. It is not clear why the mature trout gonad expresses so many of the neuropeptide hormones, but fails to produce or accumulate any significant quantity of peptide, which would be necessary for autocrine or paracrine actions of the peptides in gonadal tissues.

Another finding was that the only significant amount of material was eluted at or about 36 minutes, suggesting only one of two forms of PACAP peptide, $PACAP_{1-27}$ or $PACAP_{1-38}$ is being produced at detectable levels. In a recent report in which a newly developed enzyme-immunoassay was developed and tested on fish tissue extracts only $PACAP_{1-38}$, but no radioimmunoreactive $PACAP_{1-27}$, was detected in tissues of two species, the stargazer, a teleost, or the stingray, an elasmobranch (Matsuda et al., 2002). However, given the identification of a candidate second gene in rainbow trout described in chapter 4, I would speculate that a prohormone from that mRNA would preferentially produce a 27 amino acid PACAP, as the deduced amino acid sequence after the dibasic cut site would not produce an expect 38 amino acid products followed shortly thereafter by a stop codon. Furthermore, it is possible the much longer 72 amino acid product is generated, but was not recognized by our antibody.

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CHAPTER 6: Thyroid homeostasis is disrupted in PACAP-null mice and contributes to the early death phenotype.

Introduction

The recent development by four independent laboratories of mice with targeted-disruption of the gene encoding PACAP (PACAP-null mice) (Colwell et al., 2004; Gray et al., 2001; Hamelink et al., 2002; Hashimoto et al., 2001), as well as one lab which has generated a mouse that over-expresses PACAP (Yamamoto et al., 2003), present new models for deciphering PACAP function. A number of physiological and behavioural phenotypes in these genetically modified mice are being studied (Colwell et al., 2004; Sherwood et al., 2003; Yamamoto et al., 2003). The PACAP-null mouse produced a phenotype that is consistent with altered thermogenesis and lipid metabolism in neonatal mice. Specifically, the mice underwent a wasting syndrome that includes mobilization and redistribution of subcutaneous fat, followed by death of most of the mice in the second post-natal week (Gray et al., 2001). Furthermore, PACAP-null mouse pups lost body temperature faster than their wild type littermates upon removal from their nest and mother, and had increased survival only when housed at 24°C (Gray et al., 2002). This phenotype was associated with a compromised catecholamine response (Gray et al., 2002; Hamelink et al., 2002).

Many of the symptoms of catecholamine insufficiency or hyper-stimulation mimic symptoms of disruption of thyroid hormone (TH) homeostasis, including cardiac or thermoregulatory disorders. Hormonal control of cold-induced thermogenesis is modulated by energy production, with catecholamines and thyroid hormones acting in a closely related and complementary manner (Blouquit et al, 1990; Herpin et al., 1995; Lanni et al., 2003). Catecholamines and THs are major factors that interact at the level of brown adipose tissue (BAT) to regulate uncoupling protein (UCP)1, the protein responsible for uncoupling of the mitochondrial respiration pathway to increase heat production. Catecholamine activation by the sympathetic nervous system is associated with increased thermogenesis via the β-adrenergic stimulation of BAT. Furthermore, catecholamine release in BAT activates local TH conversion of L-thyroxine (T4) to 3,5,3'-triiodothyronine (T3), the more biologically active form of TH, by

increasing the transcription and activity of the enzyme responsible, type II 5'-deiodinase (Hofer et al., 2000).

THs are known to have a wide range of functions in development and the physiology of vertebrates (see Yen, 2001 for review). THs are synthesized in the thyroid gland that is under regulatory and feedback control of the pituitary and circulating TH levels. The brain releases thyrotropin-releasing hormone (TRH) to cause release of thyroid stimulating hormone (thyrotropin; TSH) from the pituitary, which in turn stimulates the release of THs from the thyroid. THs circulate in the blood to peripheral tissues where they are taken up and managed by activation and deactivation schemes (e.g. deiodination, sulfation, or conjugation). Circulating levels of THs can provide feedback at each of the central axis control centers (Porterfield and Hendrich, 1993). During embryogenesis, THs acts primarily to promote differentiation and to slow proliferation. Therefore, either insufficient levels of T3 or the premature exposure of the embryo to adult T3 concentrations can be detrimental and can result in abnormal development. In mammals, THs are involved in heat generation and fat metabolism (Bianco and Silva, 1987; see Silva, 2001 for review).

Considering the temperature-sensitive phenotype of the PACAP-null pups, I proposed that the thyroid axis in the pups might also be disrupted. There are four reasons for focusing on the interaction of PACAP and THs at this time: 1) PACAP affects cAMP in peripheral targets and lack of PACAP leads to impaired thermogenesis (Gray et al., 2001); 2) Noradrenalin, adrenalin, and THs are known to affect heat production in BAT, but the effects of PACAP are unknown; 3) T3 can directly and independently stimulate mitochondrial uncoupling proteins in BAT and skeletal muscles (Lombardi et al, 2002; Silva, 2001); and 4) TH status affects the expression of β-adrenergic receptor mRNA in BAT and white adipose tissue (WAT). Studies in rats indicate that TH has differential effects on the expression of β3-adrenergic receptor in BAT and WAT, based on an increase in β3-adrenergic receptors in hypothyroid BAT, but a decrease in WAT (Rubio et al., 1995).

UCPs are also expressed in skeletal muscle, and are activated in response to a number of similar factors as UCP1 in BAT. For example, cold and TH are among the activators of UCP3 in skeletal muscle and heart (Larkin et al., 1997; Gong et al., 1997), although the contribution of UCP3 to the cold response and general metabolic efficiency, at least via mitochondrial uncoupling, is considered less likely due to studies in UCP3-null mice that are not cold sensitive (Gong et al., 2000).

PACAP has been demonstrated as having a role in heat generation in rodents. Mice can have drug-induced hypothermia reversed by administration of $PACAP_{1-38}$ (Masuo et al., 1995). This effect could not be achieved with VIP, suggesting a PAC_1 receptor-mediated event. The hyperthermic effect of PACAP is hypothesized to act by dopaminergic mediation (Pataki et al., 2003) and involves prostaglandin products (Pataki et al., 2000).

A small body of evidence supports an influence of PACAP on the brain-pituitary-thyroid axis, as well as altered thyroid economy in a PACAP-receptor transgenic mouse model. In early studies, PACAP was shown to stimulate cAMP production in pig thyroid cells *in vitro*, and increase T4-release from mouse thyroid *in vivo* (Chen et al., 1993). However, since then, evidence shows little activation of thyrotrophs by PACAP (Alarcon et al., 2000; Vigh et al., 1993). In the hypothalamus, synapses have been found between PACAP- immunoreactive nerve terminals and TRH-immunoreactive perikarya and various dendritic profiles (Legradi et al., 1997). More recently, mRNA for the PACAP receptor VPAC2 has been identified in thyroid follicles (Harmar et al., 2004), and VPAC2-null mice have decreased levels of free T3 in adults along with the more general phenotype of growth retardation and increased metabolic rate (Asnicar et al., 2002).

To address my hypothesis that TH homeostasis is disrupted in PACAP-null mice, I studied 7-day old wild type and PACAP-null pups for indicators of thyroidal status, namely systemic levels of TSH, T4 and T3, as well as to measure deiodinase activity in brain, liver and BAT. I also tested plasma TH levels in mice held at different housing temperatures. Next, I treated PACAP-null pups with T3 to induce hyperthyroidism, and with a drug, methimizole (MMI), to

induce hypothyroidism and monitored them for changes in phenotype, including survival. Finally, I exposed two-month old adult wild type and PACAP-null mice to a 4°C cold challenge and monitored their ability to retain core body temperature.

Materials and methods

Animals

Mice used for initial TH and deiodinase activity assessment were generated from 129SvJ/C57BL/6 mixed-strain breeding pairs housed at 21°C. PACAP-null and wild type mice used in studies for the second part of the TH assessment and for the adult 4 °C cold challenge were generated from mixed-strain breeding pairs that were housed at 28 °C. Pups born at the different temperatures were monitored and mortality was recorded. The University of Victoria Animal Care Committee approved the procedures used in this study.

Mice were genotyped according to the protocol outline in Gray et al., 2002. Briefly, genomic DNA was extracted from the ear clipping that is also used for identification purposes. Extraction was done using a solution of 5% chelex (Bio-Rad Laboratories, Inc., Hercules, CA) with 2 mM proteinase K (Invitrogen, Carlsbad, CA) and 0.1% Tween 20, incubated at 50 °C for 45 min, followed by a 15-min incubation at 94 °C to inactivate the proteinase K. Then, genomic DNA (1 µl) was added to a 50-µl PCR reaction containing 2.5 U Taq polymerase (Invitrogen), 1x Taq buffer, 2.5 mM MgCl2, 0.2 mM deoxynucleotide triphosphates (dNTPs), and 20 pmol of three primers: 5'MP1 (5' ATGTGTAGCGGAGCAAGGCTGG 3'), PA1 (5'CACTCGGACGGCA-TCTTCACAGATAG 3'), and 3'UTR-1 (5'GGCCATTATTGGTATCTTCAAGACGG 3'). The reaction conditions were: denaturation at 94 °C for 5 min, then for 32 cycles 94 °C for 30 sec; annealing at 67 °C for 30 sec; extension at 72 C for 30 sec and a long extension at 72 °C for 7 min. Products were run on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The wild-type allele produced a band of approximately 550 bp, whereas the knockout allele produced a band of

approximately 950 bp. Thus, the three genotypes produce distinct banding patterns. A PCR that contained 1 µl of ddH_2O instead of genomic DNA served as a negative control for each litter.

Assessment of thyroid-axis in 7-day-old pups and adult mice

All mice were anesthetized for blood collection by cardiac puncture and immediately euthanized by isofluorane or carbon dioxide overdose and cervical dislocation. Plasma total T4, free T4, and total T3 were measured by commercial RIA kits (Diagnostics Products, Los Angeles, CA). Plasma TSH was measured by RIA in the laboratory of A. F. Parlow of the National Hormone & Peptide Program, Harbor-UCLA Medical Center, Torrance, California.

Induction of hyperthyroidism and hypothyroidism

To induce hyperthyroidism, mouse pups were given an i.p. injection of T3 (Sigma, St. Louis, MO, USA) once per day from birth until postnatal day ten. To induce hypothyroidism, the dams of pregnant mice were presented with 0.05 % methimizole (MMI) via their drinking water. Mice pups were counted daily to record survival. To assess if MMI was affecting TH levels, a subset of wild type pups was used as control and pups exposed to MMI were sacrificed for blood collection on day seven. All mice were anesthetized for blood collection by cardiac puncture and immediately euthanized by carbon dioxide overdose and cervical dislocation.

Deiodinase activity assays

Deiodination assays were performed in the laboratory of Dr. Geoff Eales, Department of Zoology, University of Manitoba, according to protocol described previously (Adams et al., 2000). Supplies of I^{125}-labeled T4 (*T4; specific activity = 1250 µCi/µg) were obtained from New England Nuclear (NEN) Life Science Products. Unlabelled T4 and diothiothreitol (DTT) were obtained from Sigma. Brain, liver, and BAT were dissected, frozen in liquid nitrogen and subsequently

stored at –80°C. Tissues were packed on dry ice and sent to the University of Manitoba where they were stored at – 76 °C.

Preparation of microsomes, deiodinase assays, and HPLC analyses were carried out as described previously (Adams et al., 2000) with minor changes. Tissue microsomal fractions were thawed on ice and diluted with buffer (pH 7.2) containing cofactor DTT (10 mM for mouse liver and BAT, 50 mM for mouse brain) and 1 mM EDTA to an estimated final protein concentration of 0.2 to 0.9 mg/ml. A volume of 0.5 ml-1.0 ml of diluted microsomal fraction was added to siliconized test tubes and equilibrated in darkness in a water bath for 30 minutes (37°C; 140 rpm). Adding a mixture of cold and I^{125}-labeled T4 (mouse liver substrate = 5.38 nM; mouse brain substrate = 0.55 nM) started the reaction. After 60 minutes the reaction was stopped with 0.5 ml of methylmercaptomethizmadol-methanol (MMI-methanol), vortexed and centrifuged at 15 600 g for 4 to 5 minutes. A 420-ul aliquot of the supernatant was transferred to 700-ul amber vials for HPLC analysis.

Cold challenge of adult mice

Two- to three-month-old adult mixed strain male and female wild type and PACAP-null mice were transferred to a 4 °C cold room for up to 24 hours. Rectal temperatures were taken using a rectal probe for mice (Harvard Instruments) at the start of the exposure, after the first half hour, and at every hour thereafter. Following the Animal Care Committee protocol, I removed and euthanized the mouse at any sampling time point in which the rectal temperature of that mouse had dropped ten Celsius-degrees. Furthermore, the animals were monitored for signs of behavioural responses to cold and for consciousness. Mice were anesthetized for blood collection by cardiac puncture, and then euthanized with carbon dioxide and cervical dislocation. Brain, liver, and BAT were collected by dissection, quick frozen in liquid nitrogen and stored at –80 °C.

Statistics

The means in most cases were compared using a Student's t-test after testing for equality of variance. Abilities of mice of each genotype to maintain thermoregulation for cold challenges of adult mice were compared using the log rank test. Differences were considered significant if $p < 0.05$.

Results

Effect of ambient temperature on survival of mixed-strain PACAP-null mice

There was an increase in survival of PACAP-null mice with increasing temperature, from 14% for mice housed at 21°C to 79% percent of mice surviving to weaning in the 28°C housing conditions (Table 6.1).

Assessment of thyroid-axis in 7-day-old pups and adult mice

Plasma levels of total T4 were reduced in PACAP-null 7-day-old pups housed at 21°C compared to wild type mice (Table 6.2). However, there were no differences between the two genotypes in the amount of free T4, the pool available for uptake into target tissues (data not shown). Surprisingly, plasma levels of T3 in the PACAP-null mice were nearly twice that of wild type mice (Table 6.2, Fig. 6.1). TH levels decreased in mouse pups of both genotypes housed at 28°C (Table 6.2, Fig. 6.1A).

Adult PACAP-null mice raised at 28°C had no difference from wild type littermates in their levels of T4 or T3. However, in the few pups that survived at 21°C the values suggest increased plasma levels of T3, although this was not significant. There was no difference in plasma levels of T4 in wild type adults housed at 21°C or 28°C, however, plasma T4 levels in PACAP-null adults at 28°C were reduced compared to mice housed at 21°C.

Induction of hyperthyroidism and hypothyroidism

Daily i.p. injections of T3 did not improve the number of mice that survive to weaning at 21°C (Table 6.3). However, treating the drinking water of females housed at 21°C with MMI dramatically improved the survival of PACAP-null

mice (Table 6.3). MMI treatment reduced the amount of plasma T4 in the blood of both wild type and PACAP-null mice by 60%. The plasma level of T3 was reduced in both genotypes to the same degree when pups are treated with MMI. However, T3 is reduced in PACAP-null mice to the level of wild type mice at 21°C that have had no MMI treatment, but remains elevated compared to wild type mice housed at the same temperature (Fig. 6.1).

Preliminary assessment of thyroid hormone deiodination capacity

Deiodinase activity was measured in small set of samples to determine the capacity for T4 metabolism (T3 and rT3 generation) in brain, liver and BAT of 7-day-old PACAP-null and wild type mice, as well as in 14-day-old and adult tissues for a comparison (Table 6.4). Activity was found in at least one tissue sample studied of each representative tissue from each age class except for the four samples of BAT from 7-day-old PACAP-null mice in which case no activity was detected. However, it is important to note that there was no microsomal protein content detected in any of those four samples at the end of the assay, so it is not clear if this is a technical problem or an indication of a problem with BAT protein content. PACAP-null mice had high levels of T4 outer-ring deiodinase (T4ORD) activity in liver, responsible for the conversion of T4 to T3. However, these values were not significant, possibly due to the variability of the small sample sizes used in the analysis.

Cold challenge of adult mice

A 4°C cold challenge caused all of the mice in the experiment, both wild type and PACAP-null, to drop at least 10 °C in body temperature from their initial temperature by the end of the 24-hour experiment. By five hours the average body temperature in PACAP-null mice tended to be lower than in wild type mice (Fig. 6.2A), though this difference was not statistically significant. However, PACAP-null mice did lose their ability to thermoregulate (as indicated by a 10 degree or greater drop in rectal temperature) faster than their wild type counterparts (Fig. 6.2B) ($p < 0.05$). Blood taken from mice at the end of the cold

challenge indicated plasma T4 dropped to 1.03 μg/dl, a drop of about 75% from wild type mice held at 28°C, and PACAP-null mice dropped to 1.45 μg/dl, about 57% of PACAP-null mice held at 28°C. Plasma levels of T3 were maintained in mice that underwent the 4°C cold challenge at levels of adult mice at 28°C, suggesting both genotypes of mice were able to induce T4 metabolism, possibly to maintain plasma T3 levels. These data also suggest that the T4 available, but not T3, was a factor in their loss of ability to thermoregulate.

Discussion

The most severe phenotype of the PACAP-null mice is early death in the second postnatal week (Gray et al., 2001). Previously, Gray et al. (2002) reported that at 24°C 76% PACAP-/- mice were alive at postnatal day 14 compared with only 11% mice at 21°C (Gray et al., 2002). I found that fewer mice die when raised at 28°C, a temperature that is in the thermoneutral range of mice, than at 21°C, but not fewer than that found by Gray et al. when housed at 24°C. A difference in my studies is that mice have been housed in a vented racking system, in which air in the cage is constantly replaced by room temperature air. In the non-vented cages in which the mice reported by Gray et al. were housed, the air temperature in the cage can rise as much as 1 to 2°C above the regulated ambient temperature has a dramatic effect on the percent of mice that survive to weaning. In my experiments, I only attained the higher rate of survival by housing mice in higher room temperature. Mice strains vary in their sensitivity to cold and obesity. Obligatory thermogenesis sustains a core temperature of approximately 37°C only over a narrow range of ambient temperatures (26–28°C). Therefore, room temperature (21–22°C) is a significant cold stress for small mammals, including newborn humans and rodents, creating the need for additional heat, a process known as adaptive thermogenesis, to allow effective thermoregulation (Bianco et al., 2002; Lowell and Spiegelman, 2000). THs are part of the adaptive thermogenesis process. Therefore assessment of TH status is an important part in determining the lack of adaptive thermogenesis PACAP-null mice.

Table 6.1. Survival of 129SvJ/C57BL/6 mixed-strain PACAP-null mice at different housing temperatures.

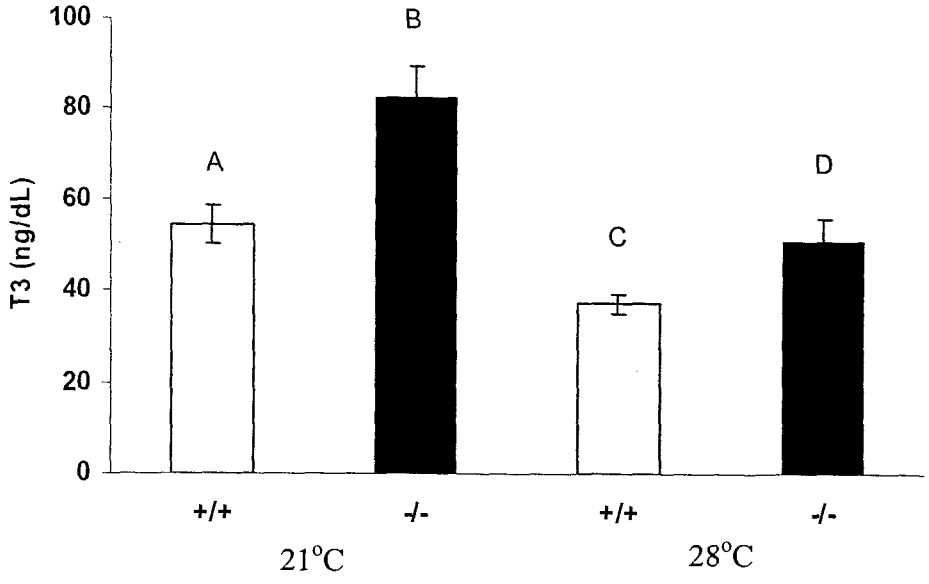
Number born	Temperature	Number survived to weaning (%)
14	21 °C	2 (14 %)
29	24 °C	14 (48 %)
56	28 °C	44 (79 %)

Table 6.2. Plasma levels (standard error of the mean; N) of TSH in 7-day-old mice and total L-thyroxine (TT4) and total 3,5,3'-tri-iodothyronine (TT3) in mixed-strain PACAP-null and wild type mice at different ages and housing temperature. #, significantly different from same genotype and age at lower temperature; *, significantly different from wild type mice at same temperature and age.

Age	Genotype	Ambient temperature (°C)	TSH (ng/ml)	TT4 (μg/dl)	TT3 (ng/dl)
7 d	-/-	21	152.25 (11.25; 12)	1.34 (0.13; 14)*	107.5 (13; 14)*
7 d	-/-	28	-	1.88 (0.1; 10)#	50.9 (5.0; 10)#,*
7 d	+/+	21	148.33 (5.14; 12)	2.14 (0.13; 14)	61.8 (4.2; 14)
7 d	+/+	28	-	1.93 (0.16; 10)	37.1 (2.3; 10)*
14 d	+/+	21	-	5.39 (0.34; 5)	95.5 (3.9; 5)
Adult	-/-	21	-	3.98 (0.18; 4)	82.4 (11.2; 4)
Adult	-/-	28	-	2.71 (0.26; 5)#,*	58.0 (6.7; 5)
Adult	+/+	21	-	4.01 (0.53; 4)	57.7 (5.63; 3)
Adult	+/+	28	-	4.01 (0.23; 5)	58.3 (5.1; 5)

Figure 6.1. Plasma levels of 3,5,3'-triiodothyronine (T3) (ng/dl) in 7-day-old wild type and PACAP-null mice raised at 21°C or 21°C (A) or raised at 21°C and treated with or without methimizole (MMI).

A.


100
80
60
40
20
0
T3 (ng/dL)
A
B
C
D
+/+
-/-
+/+
-/-
21°C
28°C

B.

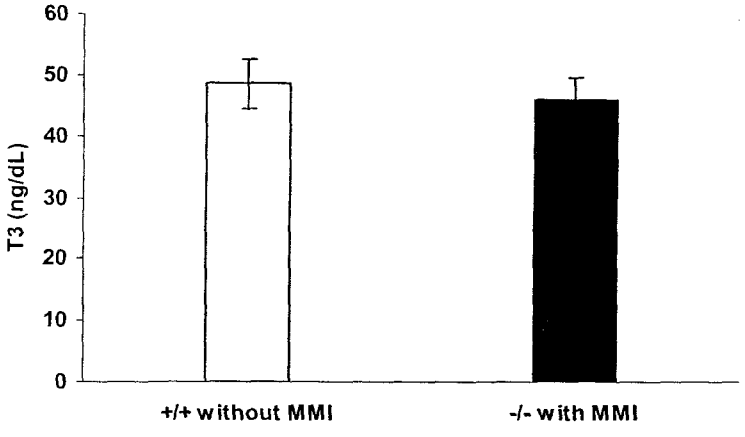

60
50
40
30
20
10
0
T3 (ng/dL)
+/+ without MMI
-/- with MMI
21°C

Table 6.3. Survival of 129SvJ/C57BL/6 mixed-strain PACAP-null mice housed at 21°C with treatment to induce hypothyroidism (MMI) or hyperthyroidism (T3 injection).

Number of pups	Treatment	Number survived to weaning (%)
14	control	2 (14 %)
21	MMI	15 (71 %)
13	saline injection	2 (15 %)
19	T3 injection	1 (5%)

The requirement for adaptive thermogenesis afforded by TH and adrenergic responsiveness is decreased as ambient temperature approaches thermoneutrality. Consequently, the factors involved in the heat-generating machinery of cells, such as UCP1, are also less required. The decrease in T3 by neonates of both genotypes of mice in my study when raised at 28°C suggests neonatal mice adjust their metabolic rate according to ambient temperature. These reduced levels of THs probably coincide with a reduction in sympathetic nervous system activation and UCP1 expression in BAT.

The reason for the lack of reduced levels of TSH in response to high plasma levels of T3 in the PACAP-null neonate is not known. It is possible that the neonate does not yet have a fully integrated or maximally responsive brain-pituitary-thyroid pathway since normally at one week of age circulating levels of TH are low. It is possible that TSH levels may be sustained as another way to increase activation of BAT in the compromised heat-producing state of the PACAP-null mouse, as TSH can act on BAT cells to increase 5'-deodinase activity (Murakami et al., 2001).

The only tissue without detectable 5'deiodinase activity was PACAP-null BAT tissue. However, there was no quantifiable protein in the extract used in the assay, suggesting that there may have been a technical problem with the isolation of the protein, or alternatively, there is greatly reduced protein expression in PACAP-null BAT, but this is unlikely, as UCP1 and hormone sensitive lipase have already been detected in PACAP- null mice (Gray et al., 2002).

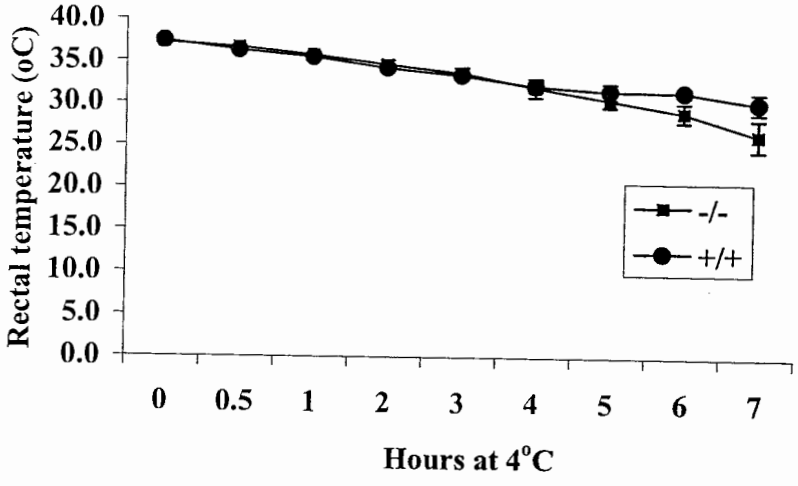
Although PACAP-null mice have reduced adrenergic responsiveness, they manage to increase BAT expression of UCP1 (Gray et al., 2002), although the regulation of this has not been determined in the PACAP-null mice specifically. The general understanding of UCP regulation suggests there is variation not only in the methods of regulation of the different UCPs, but also tissue-specific regulation for each of the UCPs. For example, one β-adrenergic receptor agonist can cause a large increase in expression of UCP1, UCP2 and UCP3 in BAT, whereas the same agonist increased expression of only UCP1 and UCP3, but not UCP2 in white adipose tissue (Yoshitomi et al, 1998). That same study showed

Table 6.4. Levels of deiodinase activity in liver, brain and BAT of PACAP-null mice compared to wild type littermates, juveniles, and adults.

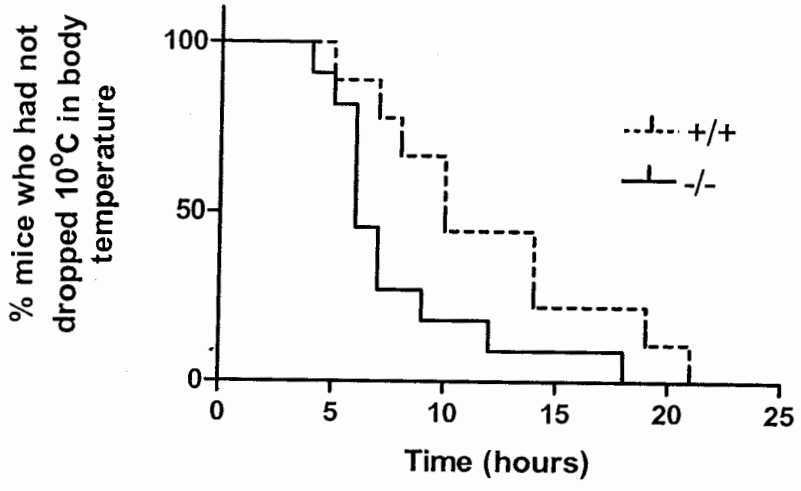
Tissue	Age	Genotype	T4ORD (pmol T4 deiodinated/hr/mg protein)	T4IRD (pmol T4 deiodinated/hr/mg protein)	N
Liver	7 d	-/-	0.084	0.032	4
	7 d	+/+	0.011	0.005	4
	14 d	+/+	0.033	0.117	6
	adult	+/+	0.056	0	3
Brain	7 d	-/-	0.005	0.018	4
	7 d	+/+	0.003	0.012	4
	14 d	+/+	-	-	-
	adult	+/+	0.003	0.007	3
BAT	7 d	-/-	0	0	4
	7 d	+/+	0.006	0	4
	14 d	+/+	0.005	0.008	7
	adult	+/+	0.035	0	1

Figure 6.2. Average rectal temperature in adult wild type and PACAP-null mice during the first seven hours at 4°C.

A.


Rectal temperature (oC)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
0.5
1
2
3
4
5
6
7
Hours at 4°C
-/-
+/+

B.


% mice who had not dropped 10°C in body temperature
100
50
0
0
5
10
15
20
25
Time (hours)
+/+
-/-

agonist-induced reduction of UCP2 and UCP3 expression in skeletal and heart muscle, a phenomenon that coincided with decrease plasma insulin and circulating free fatty acids (FFA). This led to the speculation that UCP1 and UCP3 gene expression in the BAT and WAT are mainly controlled by the hypothalamus via the sympathetic nervous system. However, insulin, FFA or both may play important roles in the control of UCP2 and UCP3 gene expression in skeletal muscle and heart (Yoshitomi et al., 1998). THs are also regulators of UCP expression based on the evidence that *in vitro* brown adipocyte T3 is required to sensitize UCP mRNA to adrenergic stimulation (Hernandez and Obregon, 2000). The increased levels of plasma T3 in the PACAP-null mice may have been in response to decreased adrenergic stimulation, as part of a compensating pathway for heat production. This proposed T3-compensating pathway may be available normally as a temporary backup system, one in which an animal never has to rely on chronically, so the consequences of prolonged, unnatural lipid metabolism are never realized.

The general physiological effects that result from excess catecholamines results in symptoms similar to the hyperthyroid state. However, recent studies in mice lacking all three β-adrenergic receptors indicate that the metabolic and cardiovascular effects of hyperthyroidism are largely independent of β - adrenergic stimulation (Bachman et al., 2004). It appears that two distinct thyroid-dependent pathways, stimulation of UCP1 and augmentation of adrenergic responsiveness, are mediated by different TH receptor isoforms in the same tissue (Ribeiro et al, 2001). In the PACAP-null mouse, the increased levels of plasma T3 could support the increase in BAT UCP1 expression reported by Gray et al. (2002) by one TH receptor subtype, whereas reducing the β-adrenergic capacity of BAT cells via another subtype. It is still possible that even though the two states act via different mechanisms, they can still compensate for one another. Furthermore, an increase in BAT UCP1on its own does not indicate increased BAT lipogenesis or an improved ability to induce adaptive thermogenesis (Christoffolete et al., 2004).

There are a number of physiological implications of hyperthyroidism. In humans, hyperthyroidism increases adipose-tissue lipolysis that results in fatty acid delivery from adipose tissue (Beylot et al., 1991; Wahrenburg et al., 1994). Furthermore hyperthyroid patients have increased concentrations of triglycerides contributed by enhanced liver lipogenesis (Cachefo et al., 2001).

There are similar responses to hyperthyroidism and thyrotoxicosis in studies using rodents. Hyperthyroidism and thyrotoxicosis can be induced artificially by daily injection of T3 (Rubio et al., 1995). Induction of thyrotoxicosis in rats nearly eliminated any expression of the β3-adrenergic receptor mRNA in BAT, but increased nearly five-fold its expression in WAT (Rubio et al., 1995). The high levels of plasma T3 in PACAP-null pups may be having a similar effect, and consequently a reduced adrenergic response in BAT. The reduction in plasma T3 levels induced by MMI treatment may allow for increased expression of β3-adrenergic receptor mRNA in BAT and its reduction in WAT, and allow for a more euthyroid state of BAT-generated-heat production. Hyperthyroidism also enhances the expression of proteins involved in lipogenesis, including fatty acid synthase and acetyl-CoA-carboxylase, involved in lipogenesis (Blennemann et al., 1995). Hyperthyroidism has been shown in rat to cause liver dysfunction by inducing apoptosis via a mitochondria-dependent pathway (Upadhyay et al., 2004). The PACAP-null mouse has a phenotype that can be explained in part by disrupted lipid metabolism. It is unclear if the different state of lipid metabolism is in response to an altered adrenergic pathway. However, treatment with MMI seems to act by putting T3 levels within the 'tolerable' range of neonatal thyroid economy, but whether this causes changes in liver and BAT metabolism remains to be determined. The brain can also be affected. Exposure of the neonatal rat to excessive TH causes accelerated morphogenesis of pyramidal neurons and their dendritic spines in the cerebrum as well as a reduction in the total neuronal cell number (Pasquini and Adamo, 1994). As mentioned above, PACAP-containing neurons synapse onto TRH neurons (Legradi et al, 1997). It is not clear if the PACAP-null mouse has defective TRH neuron response, although this could account for the lack of response in levels of TSH we detected. There was no

change in serum TSH levels in mice that have a targeted disruption of the TRH-receptor gene, although there were reduced levels of circulating T3 and T4 (Rabeler et al., 2004).

In conclusion, the PACAP-null mice may induce increased production or decreased elimination of T3 in order to increase the sensitivity of BAT to reduced adrenergic stimulation. However, systemic levels of T3 are in the range that causes thyrotoxicosis and the PACAP-null mice do not appear to respond with an appropriate decrease in TSH. It is unclear if the increased liver deiodinase activity is a response to an increase in plasma T3 as a result of production in other tissues, or if the high levels of activity are generating the high plasma T3 levels. This seems to be a developmentally-associated change in requirements and capacity to manage the thyroid axis effectively in neonatal mice compared with adult mice, which may explain why mice that survive to two-weeks can tolerate and manage the system for survival, but still have problems compared with wild type littermates to cold challenge. The altered adrenergic system of the PACAP-null mouse pup provides the opportunity to consider the impact of such an altered adrenergic system in the adult mouse, including any impact on more general questions of energetics and metabolism in relation to food intake and susceptibility to obesity, and forms the basis of the next chapter.

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CHAPTER 7: Appetite and high fat diet tolerance in PACAP-null mice.

Introduction

The current interest in understanding the physiological regulation of appetite and energy balance stems from the high incidence in the Western world, and rapid increase in parts of the East, of obesity (and obesity-related disorders such as Type 2 diabetes) that is now considered to be an epidemic by many public health officials, physicians and researchers (Flier, 2004). There is, therefore, a great interest in understanding the hormonal regulation of energy balance and appetite regulation with the goal of identifying a neuropeptide(s) or neuropeptide agonist and antagonist that would be candidates for obesity treatment.

Increasingly, there is a focus on determining all of the hormones that play a role in coordinating appetite regulation and energy balance as well as their interplay (see Zigman and Elmquist, 2003 for review). Traditionally, the hypothalamus was considered to be the master regulator of energy balance. In the past decade, peripheral factors such as ghrelin, derived from the endocrine cells of the gastrointestinal tract, and leptin, secreted by white adipose tissue, have been identified and found to serve as communicators to the hypothalamus on the state of energy reserves. Leptin and ghrelin act mainly at the level of the arcuate nucleus to affect two classes of neurons; one that is appetite-inducing (orexigenic) and the second is appetite suppressing (anorexigenic). The first class of neurons expresses the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP). NPY and AgRP-expressing neurons are activated by ghrelin and inactivated by leptin. The second class of anorexigenic neurons express proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) and are activated by leptin, but blocked by ghrelin. So, in the case of high levels of energy stores (or reduced energy use), leptin acts to decrease the signal for energy input by activating a suite of hypothalamic neurons the products of which (e.g. norepinephrine and thyrotropin hormone-releasing hormone (TRH)), lead to increased energy output. Humans and rodents that are obese are often resistant to the weight reducing effects of leptin (Kershaw and Flier, 2004). Conversely, decreased energy reserves stimulate ghrelin release from gastrointestinal tract to act on the hypothalamus to decrease energy expenditure and stimulate energy stores.

The widespread actions of PACAP including influences on glucose homeostasis, circadian rhythms and reproduction would suggest PACAP is not a prime candidate for obesity treatment because of the effects that could be imparted on systems outside the central energy balance axis (Christophe, 1998). There is no association between polymorphisms in the human gene that codes for PACAP, *ADCYAP1*, and clinical symptoms in Type 2 diabetic patients (Gu, 2002). However, the VPAC2-receptor-null, PAC1-receptor-null and PACAP-null mice are models that present a complex phenotype of different aspects of obesity including disrupted glucose and insulin sensitivity and altered metabolic states (see Brabet et al., 2003, and Sherwood et al., 2003, for reviews), and therefore the candidates for studies of obesity and energy balance. Features such as a defective sympathetic nervous system would suggest that PACAP-null mice are prone to obesity. Injection of PACAP into rodent models of Type 2 diabetes does reduce blood glucose levels (Yada et al., 2000b), presumably by aiding in release of insulin and uptake of glucose in peripheral tissues such as white adipose tissue (Yada et al., 2000a).

PACAP has also been shown to have a role in appetite regulation. Intracerebroventricular injection of PACAP has been shown to reduce short-term food intake in mice (Morley et al., 1992), rats (Chance et al., 1995; Mizuno et al., 1998) and chicks (Tachibana et al., 2003a,b). The mechanism for this action of PACAP is not clear. In rats, there is no effect of PACAP on expression of NPY or corticotrophin-releasing hormone (CRH) (Mizuno et al., 1998). However, in the chick, evidence suggests the anorexigenic action of PACAP is mediated by CRH neurons (Tachibana et al., 2004). PACAP has a role in reducing appetite, suggesting that appetite suppression could be compromised in the PACAP-null mouse. In order to test these two hypotheses, I fed wild type and PACAP-null mice regular chow or a high-fat chow until 30 weeks of age and assessed them for differences in mass, amount of food eaten, glucose and insulin responses, and indicators of fat distribution.

Materials and methods

Animals and feeding

Adult wild type and PACAP-null mice were raised at 28°C and were genotyped and identified by ear-clipping during the first post-natal week. Genomic DNA was generated

and PCR performed as described in Gray et al., 2001. Upon weaning, mice were fed either a regular chow consisting of 10% kcal from fat (referred hereafter as the low fat diet) or a high fat diet in which 45% kcal were from fat (Research Diets, New Brunswick, New Jersey, USA) until they were 30 weeks of age. Weekly recordings were made of the mass of each mouse and the amount of food eaten. Upon completion of testing, animals were euthanized for dissection of brain, BAT, liver, skeletal muscle and WAT that were quick-frozen in liquid nitrogen and later transferred to –80°C for long-term storage.

Blood glucose levels

Blood was collected from the femoral vein of mice fed *ad libitum* and from the same mice after 5 hours of fasting. Glucose was measured using a Glucometer Elite (Bayer) and recorded.

Glucose tolerance test

Adult wild type and PACAP-null mice were fasted at least five hours and had a blood sample taken from the femoral vein in the leg prior to glucose adminstration. D-glucose (200 mg in 500 µl) was given by gavage tube directly into the stomach. Blood samples were taken 10, 30, 60, 90 and 120 minutes after gavage and glucose was measured using a glucometer.

Insulin tolerance test

The same mice that were tested for glucose tolerance were tested one week later for insulin tolerance. Mice were again fasted for at least 5 hours, and had a blood sample taken before i.p. injection of insulin (0.75 U human insulin/kg). Blood samples were collected at 10, 30, 60, 90 and 120 minutes after the injection, and measured as above.

Insulin and leptin RIAs

Mice were deeply anesthetized with isofluorane (flow rate = 5 l/min), and blood was collected by cardiac puncture. The blood was kept at 4°C for up to 1 hour and centrifuged in a microcentrifuge for 5 minutes at 5 000 g, then the serum was removed and aliquoted to fresh tubes and stored at –80°C. Serum insulin and leptin levels were measured using

commercially available RIA kits (Rat Sensitive Insulin RIA Kit and Mouse Leptin RIA Kit, respectively, Linco Research Inc.) according to the manufacturer's directions.

Fat pads

To make an assessment of fat distribution, the weight of dissected perirenal fat pads from male and female mice and epididimal fat pads from male mice was recorded and the tissues were subsequently quick-frozen in liquid nitrogen and stored at –80°C.

Statistics

Significant differences in the means between treatment groups were determined by ANOVA using the Instat statistical software program.

Results

Body weight and food intake

I measured the mass of the mice and the amount of food each mouse ate weekly to gain insight into differences in growth and feeding behaviour in PACAP-null mice and any difference they may have compared to wild type mice in their tolerance to a high fat diet. There were no differences between wild type mice and PACAP-null groups on the same diet in growth as determined by mass over the 30 weeks of the study (Fig. 7.1). However, mice on high fat diets weighed more than their low fat-fed counterparts, and female mice grew less than males.

Food consumption relative to size was the same between genotype comparison groups (Fig.7.1A, B). However, mice on a low fat diet ate about two percent more food/average weight/day than mice on a high fat diet.

Blood glucose and insulin

Because PACAP potently induces insulin release, and because a high fat diet can lead to insulin insensitivity I was interested in the levels and tolerance of glucose and insulin in PACAP-null mice on the two different diet regimes. Blood glucose levels were the same within a given feed group provided fasted or fed groups were compared (Fig. 7.2). There was a trend for an increase in the fasted glucose level in each of the high fat diet

groups compared with their regular diet comparison group but it was not significant. Furthermore, there were no significant differences between wild type mice and the PACAP-null group in response to a glucose challenge by oral gavage (Fig. 7.3). However, mice fed a high fat diet had more glucose (area under curve) in their bloodstream than did their low-fat diet comparison groups over 90 minutes, suggesting insulin insensitivity in the high-fat diet mice.

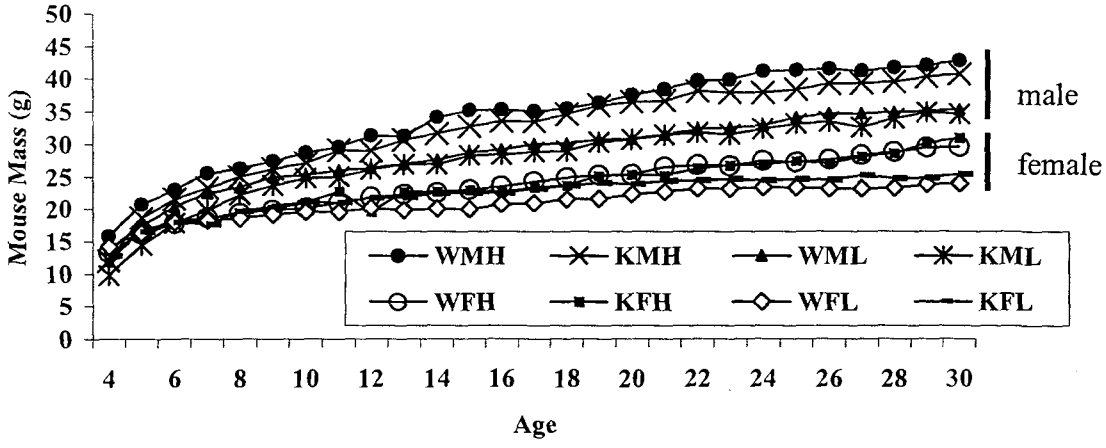
There was no trend in insulin levels between regular chow-fed mice verses their high-fat diet counterparts. There were no differences among the female groups in non-fasted serum insulin levels (Fig 7.4A). Wild type males fed regular chow had insulin levels that were more than twice that of their PACAP-null counterparts, although there was no other differences among the groups (Fig.7.4B). When given an insulin challenge by i.p. injection, PACAP-null mice of both sexes that were fed high fat diets had greatly reduced glucose profiles over the course of the experiment, suggesting they are more insulin-sensitive than wild type mice. There were no differences in PACAP-null mice fed regular chow compared to their wild type comparison group (Fig. 7.5).

Fat distribution and serum leptin

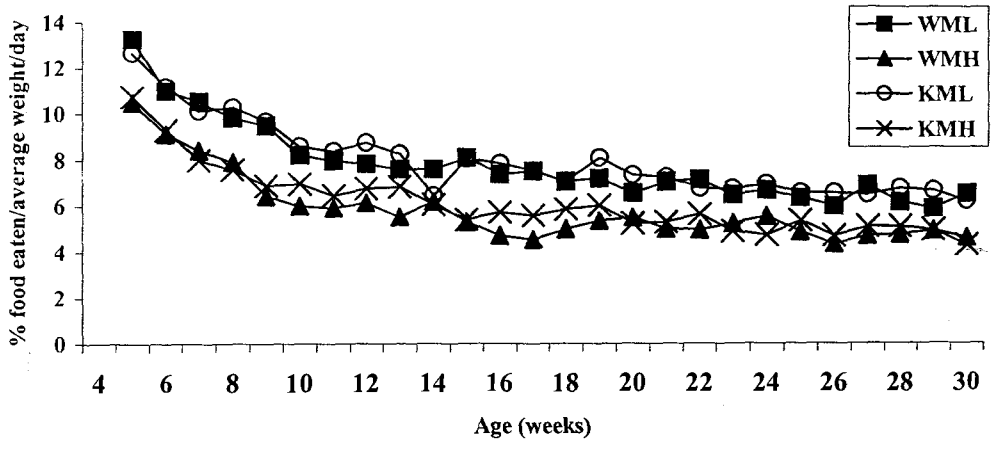
Many animal models of altered energy metabolism or feeding behaviour have differences in parameters such as fat pad mass and serum leptin levels. I found no significant difference between the size of fat pads between diet and sex-matched wild type mice and PACAP-null counterparts (Fig. 7.6). Nor were there differences in perirenal fat pad sizes among the female mice groups. However, male mice fed a low fat diet had significantly smaller perirenal fat pads than males fed the high-fat chow (Fig. 7.6A). Furthermore, PACAP-null male mice fed regular chow had significantly smaller epididymal fat pads than PACAP-null mice fed high-fat chow (Fig.7.6B). However there were no differences in the serum levels of leptin among either the female or male groups if comparing wild type and null within a feed group (Fig.7.7).

Figure 7.1. The mass of male and female C57Bl/6-129SvJ mixed-strain PACAP-null and control mice on high or low fat diet up to 30 weeks of age (A) and the food consumption of the males (B) and female (C) expressed as % of food eaten/per average weight/day of weight. WFL, wild-type female low fat diet; WFH, wild-type female high fat diet; KFL, knockout female regular chow diet; KFH, knockout female high fat diet; WML, wild-type male regular chow diet; WMH, wild-type male high fat diet; KML, knockout male regular chow diet; KMH, knockout male high fat diet.

A.



B. Males



C. Females

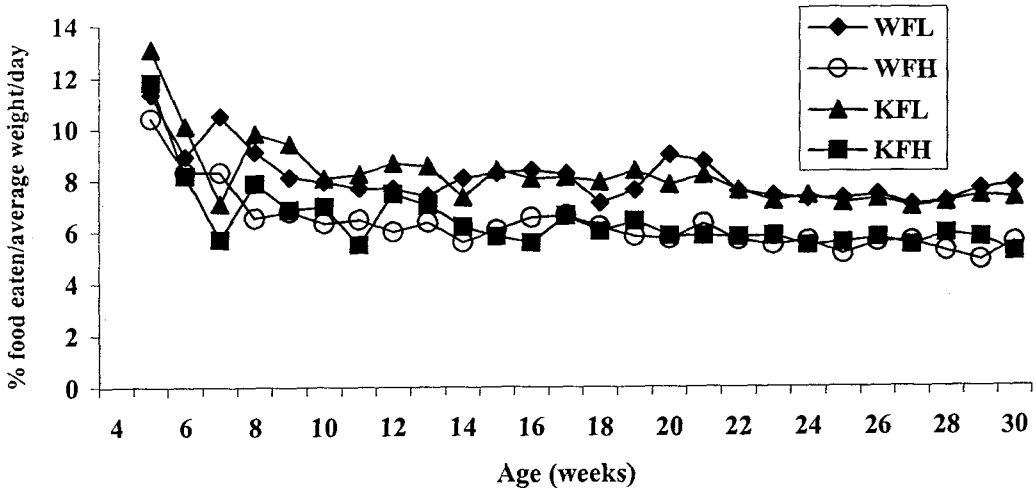
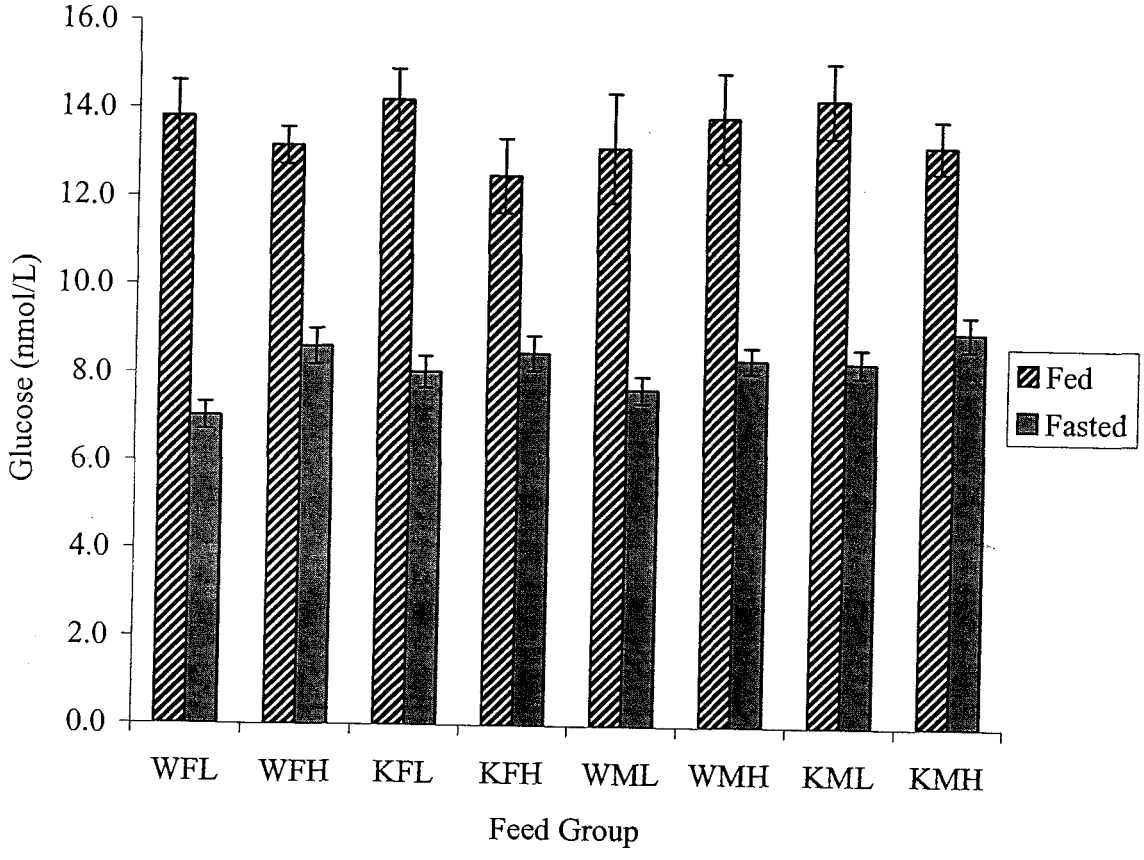


Figure 7.2. Blood concentrations of glucose in low or high fat diet male and female wild-type or PACAP-null mice that were fed or fasted. WFL, wild-type female low fat diet; WFH, wild-type female high fat diet; KFL, knockout female regular chow diet; KFH, knockout female high fat diet; WML, wild-type male regular chow diet; WMH, wild-type male high fat diet; KML, knockout male regular chow diet; KMH, knockout male high fat diet.


16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Glucose (nmol/L)
WFL
WFH
KFL
KFH
WML
WMH
KML
KMH
Feed Group
Fed
Fasted

Discussion

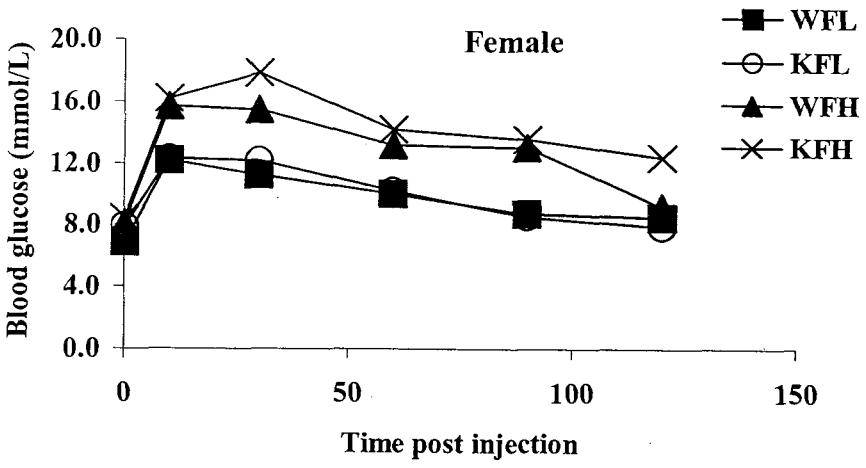
There is evidence that PACAP has a role in insulin secretion and glucose regulation, but there has been little study to date on what, if any, action PACAP may play in general energy homeostasis. Furthermore, a role for PACAP in inhibiting feeding has been suggested, but the degree of effect and the mechanism have still not been determined. The distribution of PACAP in the region of the hypothalamus that is involved in energy balance suggests PACAP has an influence in these pathways as well.

Rodents and chicks given exogenous PACAP have reduced short-term food intake, however, I found no difference in appetite of PACAP-null mice over the course of the 30-week study. It is possible that over the long-term, there are over-riding or compensating pathways for the lack of PACAP. Alternatively, treatment with exogenous PACAP is a pharmacologically-induced effect, and PACAP has no endogenous role in appetite regulation. I did, however, note that there was a decrease in the amount of high fat chow ate compared to regular chow for both males and females, even though there was more growth in the high fat diet feed groups. The difference in growth of mice on the different diets suggests that PACAP-null mice adapt in the same way as wild type mice over the long term to energy intake requirements depending on food quality.

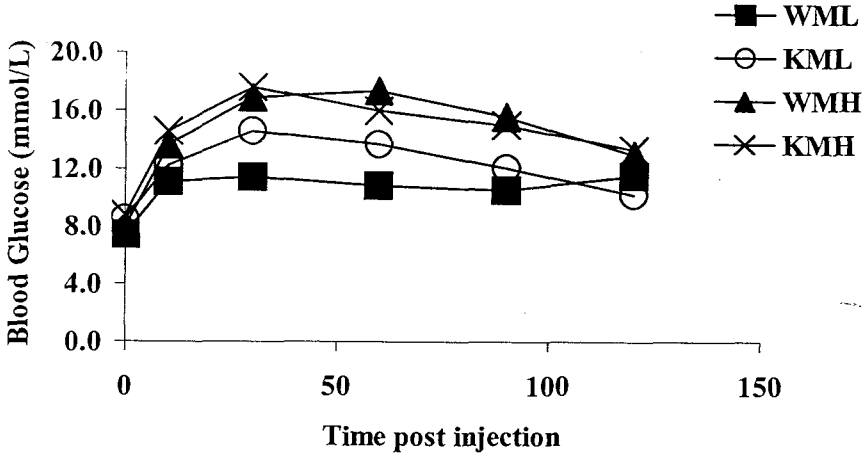
In the previous chapter, I determined that there is a disrupted thyroid homeostasis in neonatal PACAP-null mice housed at 21°C compared to mice at 28°C. Since the adult mice in this study have been raised at 28°C, it is unclear if they would have disrupted TH hormone homeostasis if housed at 21°C. If that was the case, feeding and energy balance pathways could be disrupted. Changes in eating induce changes in a number of endocrine and metabolic pathways that include decreases in THs (Ahima et al., 1996; Blake et al., 1991). This involves a complex interplay of energy play factors, including hormones mentioned above. For example, AgRP have been studied for their role in acting on the hypothalamic-pituitary-thyroid axis (Fekete et al., 2002; 2004). However, not all of the interactions and pathways are understood. Leptin is also known to play a role in activation of the thyroid axis when fat stores are high. Leptin is secreted by white adipocytes in proportion to adipose tissue mass serves as an indicator of sufficient energy stores. Leptin has direct and indirect regulation over thyrotropin hormone-releasing (TRH) hormone (Harris et al., 2001; Nillni et al., 2000). PACAP-expressing neurons also

Figure 7.3. Glucose tolerance test (2 g D-glucose/kg body mass) in PACAP knockout and wild type male and female mice 31 to 32 weeks old fed a regular chow or high fat chow. (A) Percent fasted glucose in (A) male mice and (B) female mice, and (C) area under the curve analyses for the gavage response as an indicator of glucose uptake into tissues. WFL, wild-type female low fat diet; WFH, wild-type female high fat diet; KFL, knockout female low fat diet; KFH, knockout female high fat diet; WML, wild-type male low fat diet; WMH, wild-type male high fat diet; KML, knockout male low fat diet; KMH, knockout male high fat diet.

A.


Female
WFL
KFL
WFH
KFH
Blood glucose (mmol/L)
20.0
16.0
12.0
8.0
4.0
0.0
0
50
100
150
Time post injection

B.


WML
KML
WMH
KMH
Blood Glucose (mmol/L)
20.0
16.0
12.0
8.0
4.0
0.0
0
50
100
150
Time post injection

C.

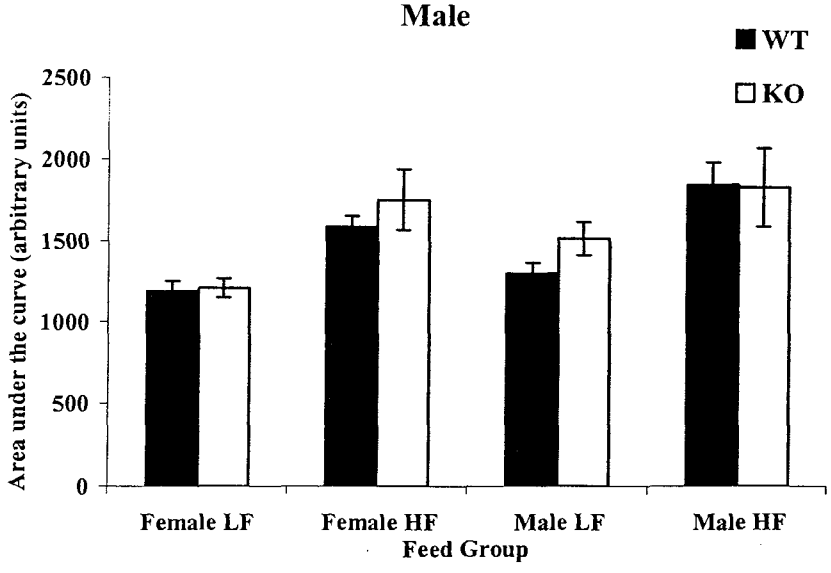
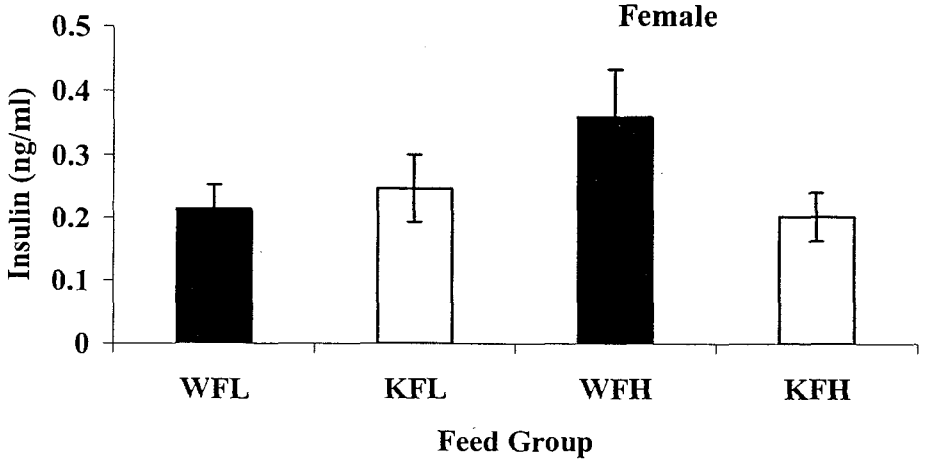

Male
WT
KO
Area under the curve (arbitrary units)
2500
2000
1500
1000
500
0
Female LF
Female HF
Male LF
Male HF
Feed Group

Figure 7.4. Serum concentrations of insulin (ng/ml) in low or high fat diet female (A) and male (B) wild-type or PACAP-null mice that were fed *ad libitum*. WFL, wild-type female regular chow diet; WFH, wild-type female high fat diet; KFL, knockout female regular chow diet; KFH, knockout female high fat diet; WML, wild-type male regular chow diet; WMH, wild-type male high fat diet; KML, knockout male regular chow diet; KMH, knockout male high fat diet.

A.


Female
0.5
0.4
0.3
0.2
0.1
0
Insulin (ng/ml)
WFL
KFL
WFH
KFH
Feed Group

B.

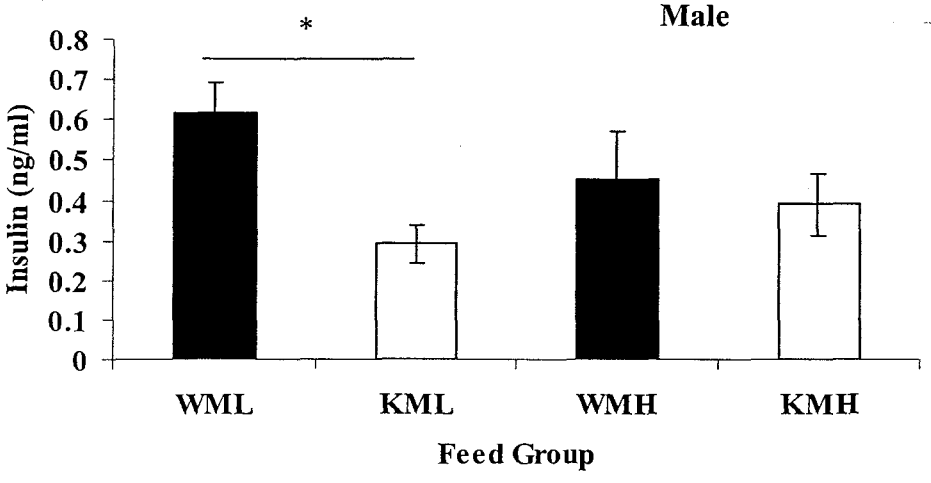
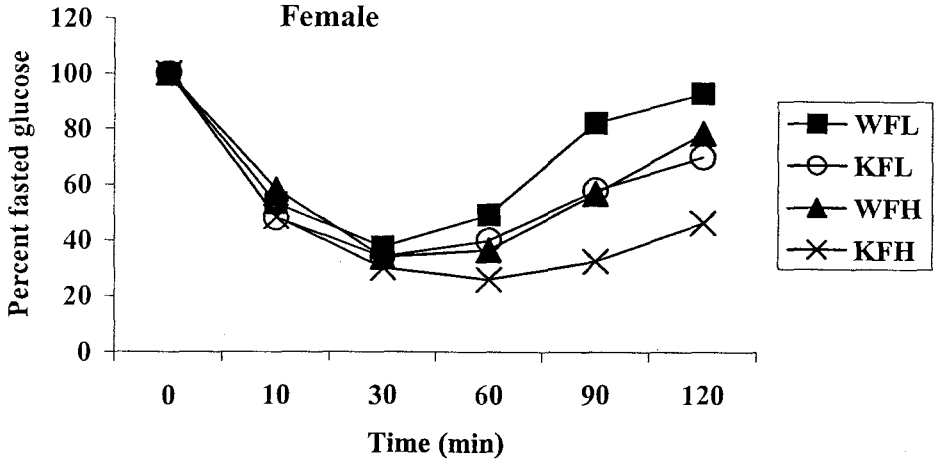
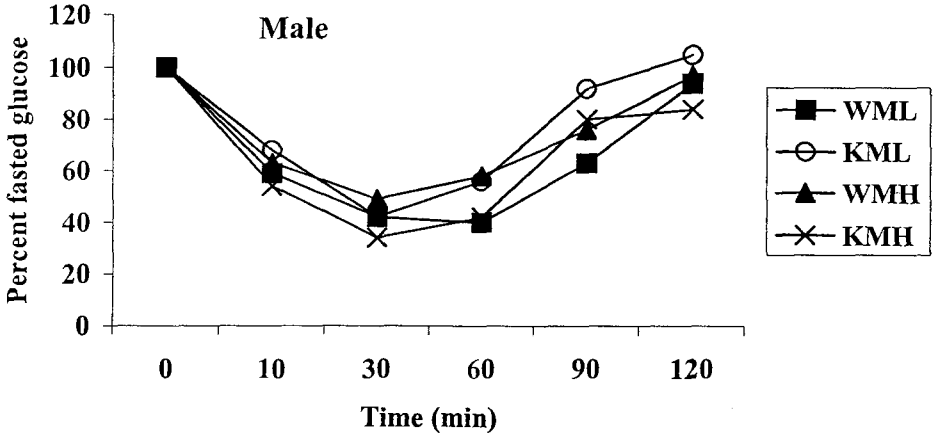

Male
*
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Insulin (ng/ml)
WML
KML
WMH
KMH
Feed Group

Figure 7.5. Insulin tolerance test (human insulin/kg body mass) in PACAP knockout and wild type male and female mice 31 to 32 weeks old fed a regular chow or high fat chow. (A) Blood glucose concentration (mM) in (A) male mice and (B) female mice, and (C) area under the curve analyses for the gavage response as an indicator of insulin sensitivity. WFL, wild-type female low fat diet; WFH, wild-type female high fat diet; KFL, knockout female low fat diet; KFH, knockout female high fat diet; WML, wild-type male low fat diet; WMH, wild-type male high fat diet; KML, knockout male low fat diet; KMH, knockout male high fat diet.

A.


Female
Percent fasted glucose
120
100
80
60
40
20
0
0
10
30
60
90
120
Time (min)
WFL
KFL
WFH
KFH

B.


Male
Percent fasted glucose
120
100
80
60
40
20
0
0
10
30
60
90
120
Time (min)
WML
KML
WMH
KMH

C.

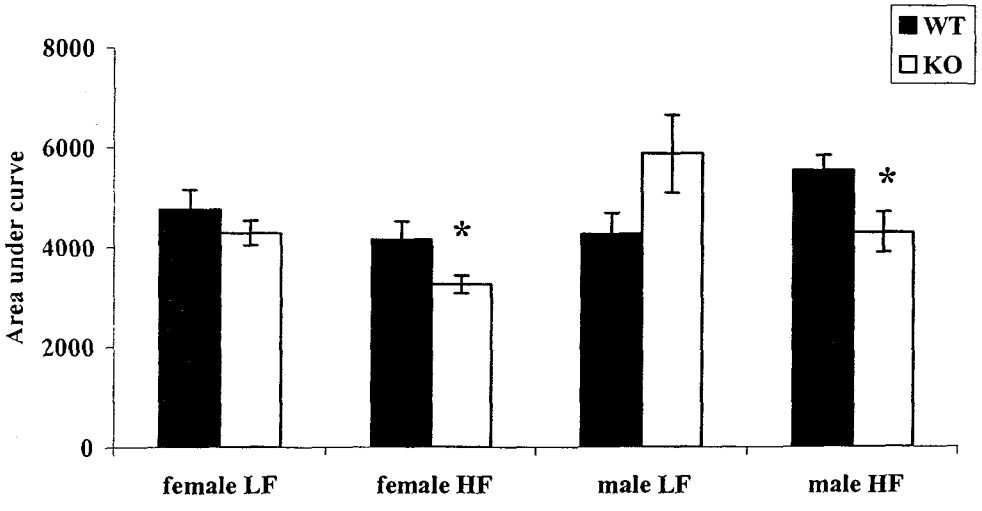
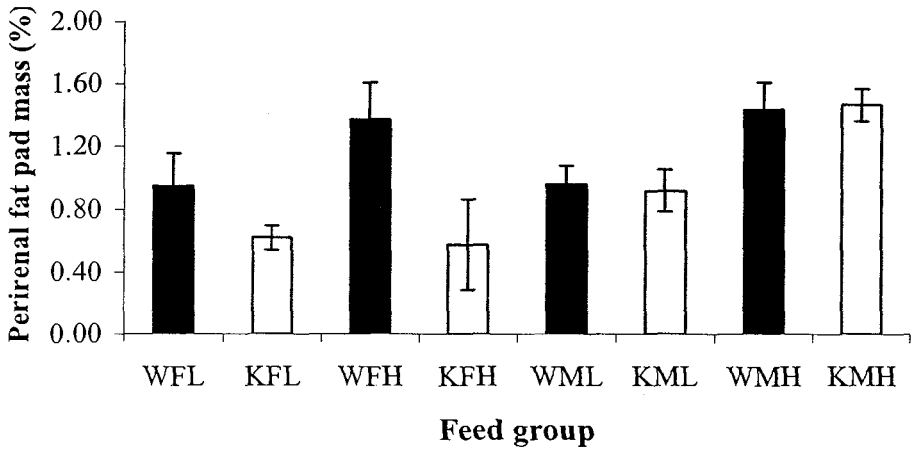

WT
KO
Area under curve
8000
6000
4000
2000
0
*
*
female LF
female HF
male LF
male HF

Figure 7.6. Perirenal (A) and epididymal (B) fat pad mass as a percent of whole body mass of wild type and PACAP-null mice fed a regular or high-fat chow diet for 30 weeks. WFL, wild-type female regular chow diet; WFH, wild-type female high fat diet; KFL, knockout female regular chow diet; KFH, knockout female high fat diet; WML, wild-type male regular chow diet; WMH, wild-type male high fat diet; KML, knockout male regular chow diet; KMH, knockout male high fat diet.

A.


Perirenal fat pad mass (%)
2.00
1.60
1.20
0.80
0.40
0.00
WFL
KFL
WFH
KFH
WML
KML
WMH
KMH
Feed group

B.

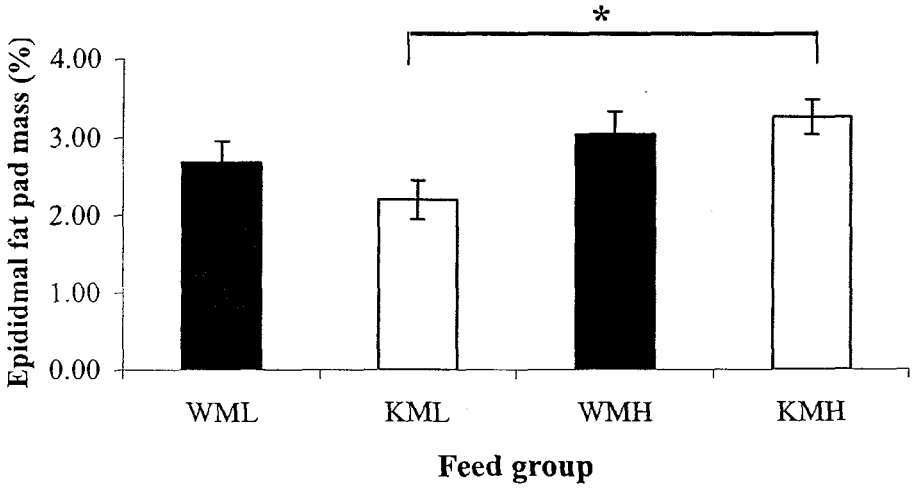
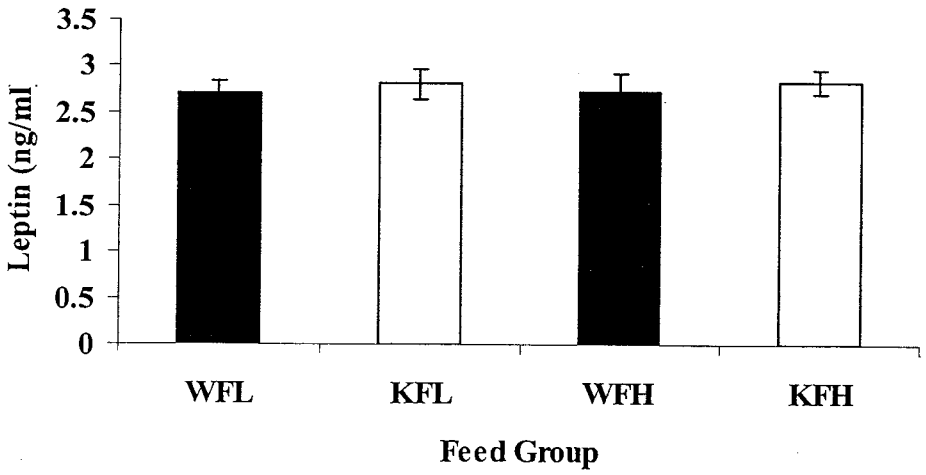
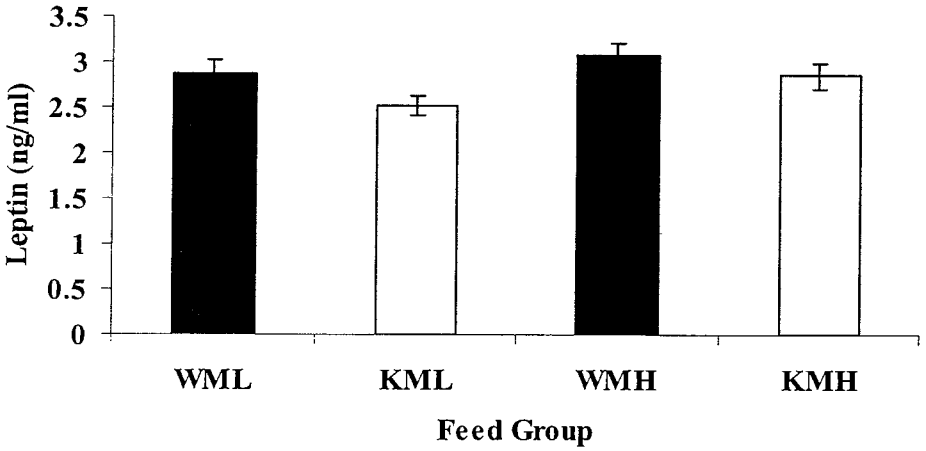

*
Epididmal fat pad mass (%)
4.00
3.00
2.00
1.00
0.00
WML
KML
WMH
KMH
Feed group

Figure 7.7. Serum concentrations of leptin in regular chow-fed or high fat diet male and female wild-type or PACAP-null mice. WFL, wild-type female regular chow diet; WFH, wild-type female high fat diet; KFL, knockout female regular chow diet; KFH, knockout female high fat diet; WML, wild-type male regular chow diet; WMH, wild-type male high fat diet; KML, knockout male regular chow diet; KMH, knockout male high fat diet.

A.


3.5
3
2.5
2
1.5
1
0.5
0
Leptin (ng/ml)
WFL
KFL
WFH
KFH
Feed Group

B.


3.5
3
2.5
2
1.5
1
0.5
0
Leptin (ng/ml)
WML
KML
WMH
KMH
Feed Group

associate with TRH neurons, suggesting TRH neurons as a site of overlap for these two peptides in energy regulation. PACAP-null mice had no difference in either fat accumulation as determined by fat pad mass or levels of serum leptin, which is consistent with fat pad data. VPAC2-receptor-deficient mice had increased lean mass and decreased fat mass with reduced serum leptin levels. However, the only indicator of thyroid status was a measure of free T3, which was elevated (Asnicar et al., 2002).

If the sympathetic nervous system must be activated for the actions of PACAP to be revealed, then it is possible that there would be no effects on either the feeding behaviour or the tendency to become obese in mice that are housed near thermoneutrality at 28°C. Another version of this study should consider the same low and high fat-fed mice held at 28°C in comparison with a group under the same conditions except for environmental temperature, which should be set at 21°C, the temperature at which the PACAP-null phenotype, at least in neonate and juvenile mice, is fully expressed. Mice housed at or near thermoneutrality have very different energy requirements and energy-consuming behaviour than mice held at typical housing conditions (21-23°C) (Overton and Williams, 2004). Mice with ablation of BAT do not become obese when raised at thermoneutrality as they do when housed under standard room temperature (Melnyk et al., 1997). However, mice with a targeted disruption of UCP1 in BAT are cold-sensitive but do not get obese even at when raised at 24°C (Enerback et al., 1997). The difference in the two models (one with intact BAT but not UCP1 and the second with no BAT), have led to the suggestion that a second satiety factor other than leptin originates in the BAT, specifically in UCP1-expressing cells, that would explain the differences influencing obesity based on the activation of the sympathetic nervous system and activation of BAT (Melnyk and Himms-Hagen, 1998). This factor would adjust food intake in response to altered ambient temperature as an indicator of energetic requirements. Based on the data from my study, PACAP is not a strong candidate for such a satiety factor, but it is possible that the BAT-mediated actions of PACAP could be a mediator of such a factor. A similar study to mine conducted at the normal ambient housing temperature of mice, 21-23°C, would allow testing of that hypothesis.

PACAP acts on the pancreas mainly via PAC1 receptors (Borboni et al., 1999) and is a potent releaser of glucose-induced insulin secretion (see Filipsson et al., 2001, and

Shintani et al., 2003, for review). However, a complete understanding of the total glucohomeostatic action by PACAP is still not known. Mouse models with targeted disruption of either the PAC1 or VPAC2 receptor have problems with insulin physiology (see Brabet et al., 2003 for review). In this study, PACAP-null mice were able to dispose of glucose by oral gavage as well as wild type mice, however when fed a high fat diet, they are more insulin sensitive. About 10% of the PACAP-null mice given an i.p. insulin injection in this study became hypoglycemic, a trait that was noted by another group that studied PACAP-null mice (Hamelink et al., 2002). Another model of PAC1-receptor null mice had decreased secretion in response to PACAP, although the mice had normal pancreas production based on pancreatic insulin content and fasting serum levels of insulin (Jamen et al., 2000). VPAC2-receptor deficient mice are more sensitive to insulin combined with increased lean mass and decreased fat mass with reduced serum leptin levels (Asnicar et al., 2002). This hypoglycemia is most likely the result of abnormal glucagon response to hypoglycemia (Persson and Ahren, 2002). However, these data may also reflect extra-pancreatic functions of PACAP as PACAP receptors are highly expressed in tissues heavily involved in glucose uptake and release, such as liver, muscle and heart. Exogenous PACAP treatment of mice with an intact PACAP gene has reduced insulin sensitivity (Filipsson et al., 1998), therefore, lack of PACAP may allow an increased rate of glucose uptake into tissues. This, in some ways, is consistent with the phenotype of abnormal fat uptake into liver, heart and liver of the PACAP-null mice when housed at 21°C (Gray et al., 2001) due to increased sensitivy to insulin's action on fatty acids, amino acid and lucose uptake into cells. The PACAP-null mouse has more glucose (area under curve) after the glucose challenge, suggesting they are not insulin insensitive they just are not releasing enough insulin because of the lack of PACAP. Back-up proof is that when PACAP-null mice are given an injection of insulin (insulin challenge test), they are very good at moving glucose into cells (hypoglycemia). The full suite of PACAP-mediated action in the regulation of glucose homeostasis remains to be elucidated.

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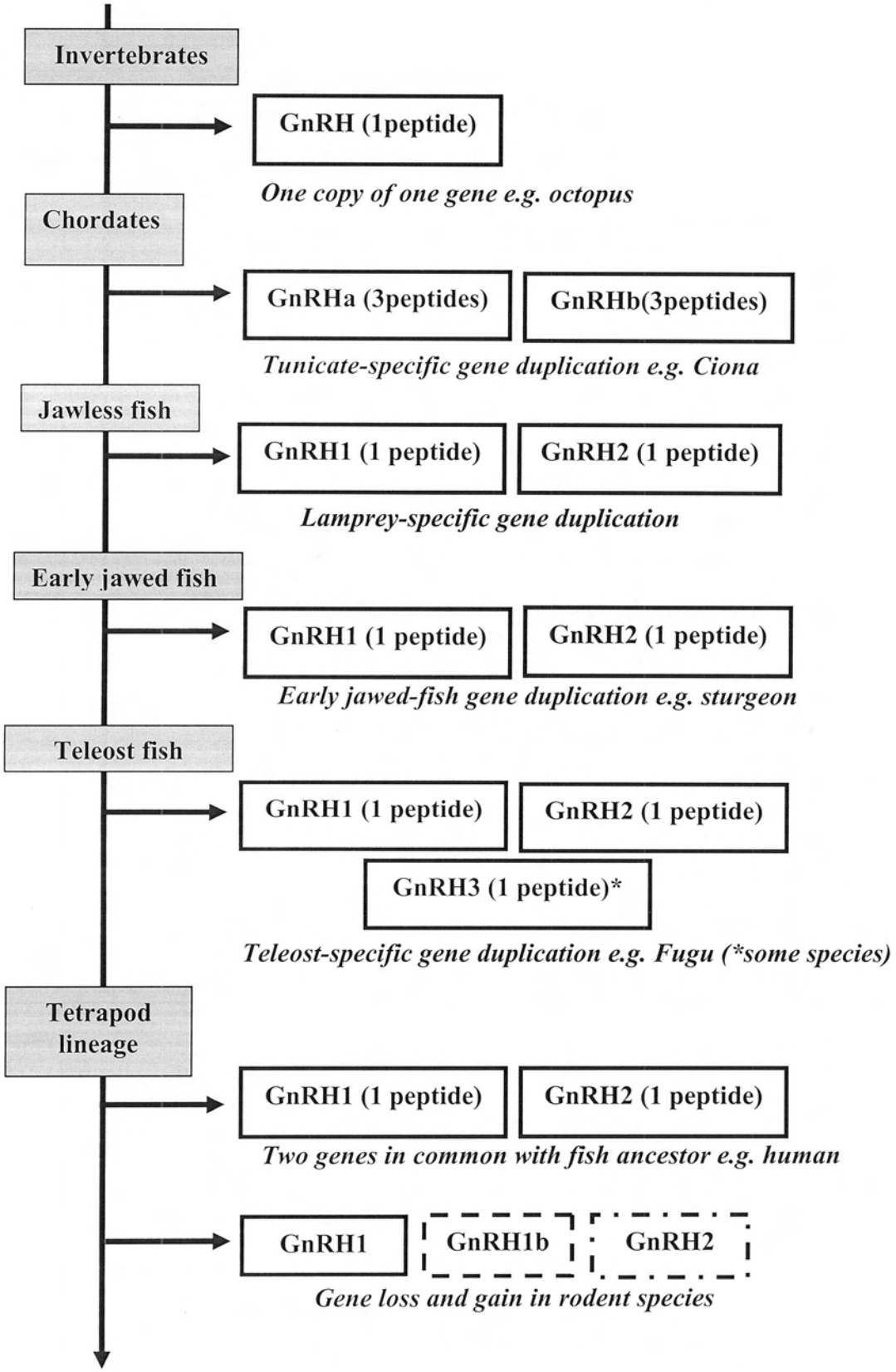
Chapter 8: Conclusions

The presence of GnRH and PACAP in different lineages of animals provides the opportunity to learn how two highly conserved peptides have developed in evolution in terms of structure as well as functionally. My studies have provided new information that radically changes our ideas of the molecular evolution of these two peptides, and gives some insight into the array of functional divergence that coincided with the structural divergence over time.

GnRH is considered the master regulator of the reproductive axis in vertebrates. My data show clearly the nature of the gene for GnRH changed in the evolution of protochordates after the split from the evolutionary path that led to vertebrates. The two genes in *Ciona* each encode three GnRH peptides for a total of six novel peptides. Evidence from octopus suggests the GnRH gene structure is generally similar to that of vertebrates (Iwakoshi et al., 2002), although it is not yet confirmed if there is more than one GnRH gene in octopus. A GnRH gene remains to be identified in amphioxus, representative of the second protochordate lineage considered to be the sister group of vertebrates in evolution. I propose there is a single copy of a GnRH gene in pre-chordate invertebrates that encodes one GnRH peptide, which is similar to the current vertebrate gene template (Fig. 8.1). A gene that codes for a single GnRH peptide is the form that probably existed in the last common ancestor in the chordate lineage before the split of protochordates and vertebrates. The GnRH gene in an ancestral tunicate underwent dramatic change that led to the form we see in extant tunicate species. A role for GnRH in reproduction is conserved, based on my studies that show GnRH can release eggs in *Ciona*. However, the ability for GnRH to act directly on spawning suggests a major alteration in the system-level pathway compared to vertebrates. It remains to be seen if there are other functions of GnRH in tunicates. Clearly, although functional significant, GnRH genes are not exempt from the forces of gene loss in evolution as evidenced by our ability to uncover a novel third form of GnRH in lake whitefish, from a lineage whose later-derived species, the salmon, only have two forms of GnRH. This has also occurred in mammals, as some species such as the guinea pig have three forms of GnRH, but we can only find one form in the genome of the mouse. These examples suggest

Figure 8.1. Evolutionary scheme for GnRH.


Invertebrates
GnRH (1peptide)
One copy of one gene e.g. octopus
Chordates
GnRHa (3peptides)
GnRHb(3peptides)
Tunicate-specific gene duplication e.g. Ciona
Jawless fish
GnRH1 (1 peptide)
GnRH2 (1 peptide)
Lamprey-specific gene duplication
Early jawed fish
GnRH1 (1 peptide)
GnRH2 (1 peptide)
Early jawed-fish gene duplication e.g. sturgeon
Teleost fish
GnRH1 (1 peptide)
GnRH2 (1 peptide)
GnRH3 (1 peptide)*
Teleost-specific gene duplication e.g. Fugu (*some species)
Tetrapod lineage
GnRH1 (1 peptide)
GnRH2 (1 peptide)
Two genes in common with fish ancestor e.g. human
GnRH1
GnRH1b
GnRH2
Gene loss and gain in rodent species

there is a significant specialization in duplicate gene function that has taken place in some species.

The theory of the evolution of the PACAP/glucagon superfamily has provided a framework for the development and expansion of superfamily members based on the identification of a set of genes in a single tunicate species, *C. productum* and the number of genes that have been found in mammals (see Sherwood et al., 2000 for review). However the explanations to date have not accounted for the issue of massive gene and genome duplication events within the vertebrates that could have contributed to the structural diversity that we see in different groups of vertebrates today. In fish, I have identified duplicates for PACAP genes in five species of teleost. These finding have allowed me to propose a revised sheme for the evolution of the PACAP/glucagon superfamily in vertebrates (Fig. 8.2). My research findings suggest that in fish there are only three of the six PACAP/glucagon gene family members that are found in mammals, a GHRH and PACAP-coding gene (see Chapter 4 for review), a glucagon-, GLP-1- and GLP-2-coding gene (Zhou and Irwin, 2004; unpublished results) and VIP (Wang and Conlon, 1995; unpublished results). In my scheme, the last common ancestor of tetrapods (the line that led to amphibians, birds, reptiles and mammals) and fish had one copy of each of these three genes. Early in fish evolution, probably in teleosts, there was a genome duplication that led to the duplication of these genes. In some species the duplicate gene would have been subsequently lost, but clearly many have been maintained and specialized in function. I further propose that the lineage that led to the salmon species had only one copy, but the salmonid-specific genome duplication created a duplication of these genes, for which I have presented evidence that at least one, *ghrh-pacap*, has retained its duplicate. It is possible that in the tetrapod lineage there was a duplication of each of the three genes (PACAP-GHRH, VIP, and glucagon) and second copies of these genes changed to become the three genes that today code for the balance of PACAP/glucagon superfamily peptides we find in mammals including GIP and secretin (Fig. 1.4).

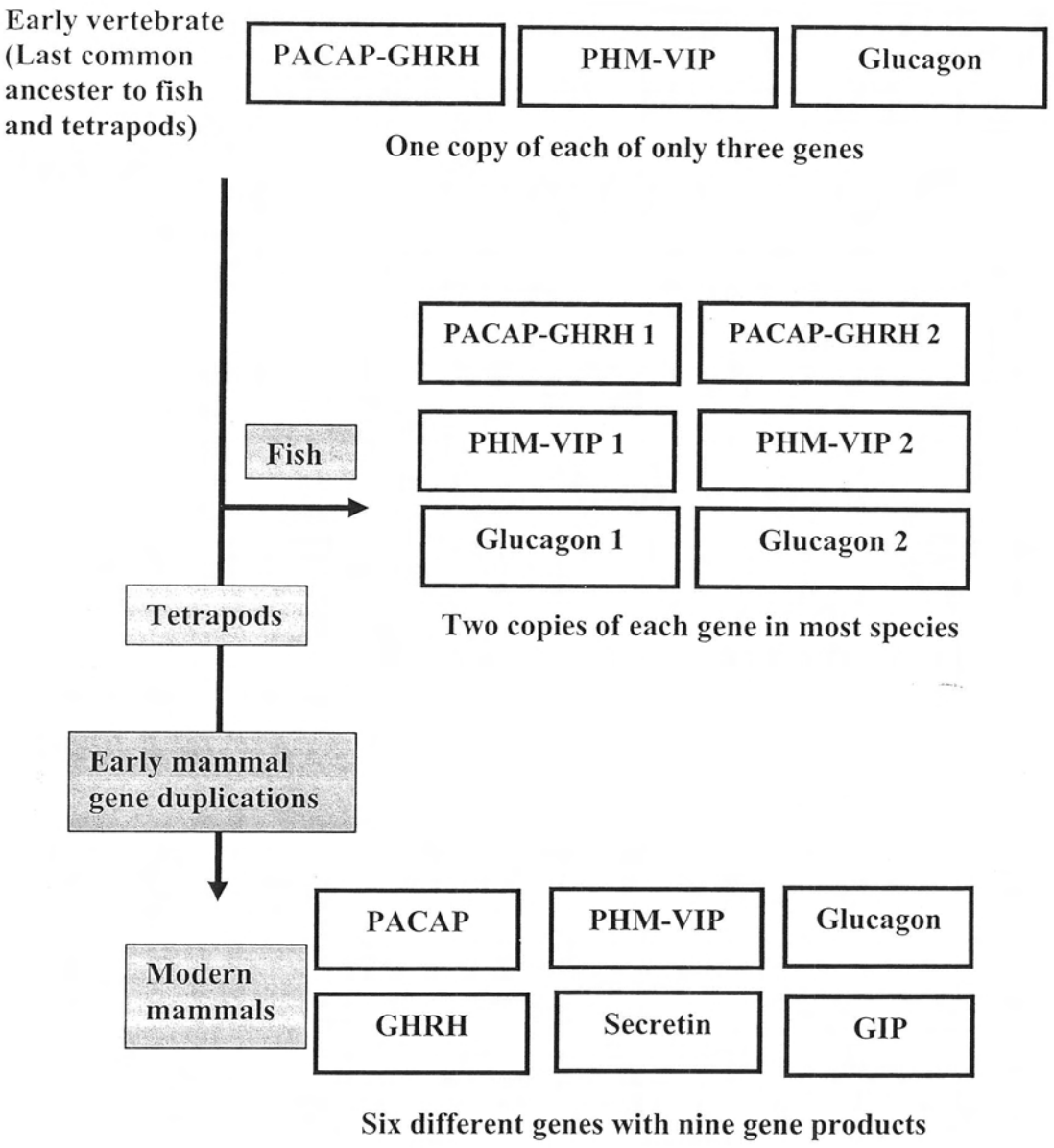
Based on my results it appears that both duplicates of the PACAP gene in fish remain functional. Using zebrafish I showed that both PACAP-encoding mRNAs have a functional role in early development, and that one gene alone cannot compensate for the other gene. It is not yet clear from these results if there are completely distinct functions for these two genes or if there is partial overlap in function. A detailed study of the expression pattern of the two genes in early zebrafish development may help to answer that question. Furthermore, assessment by quantitative PCR may further provide an idea as to the degree of expression of each gene at particular developmental stages.

Do these fish provide another model to study peripheral actions by PACAP that are implied based on studies in mammals? The role PACAP may have in peripheral tissues such as the gonads of fish remains to be determined. Although I showed that the gene for PACAP is expressed in both ovary and testis of maturing rainbow trout, we developed a specific RIA to detect PACAP and we found that the mature PACAP polypeptide is present in brain, ovary and testis of some, but not all of the maturing fish, suggesting that PACAP is not a constitutive component of the mature trout gonadal proteome. These data raise the issue of the nature of the transcriptome in relation to the proteome of mature gonadal tissue.

In further studies on the functional role of PACAP, the phenotype of the PACAP-null mouse developed by Gray and colleagues in 2001 was unexpected. In retrospect the timely development of a model with disorders of lipid and carbohydrate metabolism, sympathetic response, and, as my studies show, thyroid homeostasis and insulin response in a time when there are so many unexplained connections between central and peripheral system in regulating energy balance and explaining diabetes type 2 and obesity, provides the opportunity to study what role PACAP has in regulating or acting as a communicator in these systems.

GnRH and PACAP are peptides conserved over millions of years of evolution, yet provide unique aspects of physiology and behaviour that we find in species today. The sequencing of new genomes of both vertebrates and invertebrate model species will provide new opportunity to gain insight into the evolution of GnRH and PACAP.

Figure 8.2. Proposed new evolutionary scheme of the PACAP/glucagon superfamily of hormones in vertebrates.


Early vertebrate (Last common ancester to fish and tetrapods)
PACAP-GHRH
PHM-VIP
Glucagon
One copy of each of only three genes
Fish
PACAP-GHRH 1
PACAP-GHRH 2
PHM-VIP 1
PHM-VIP 2
Glucagon 1
Glucagon 2
Tetrapods
Two copies of each gene in most species
Early mammal gene duplications
Modern mammals
PACAP
PHM-VIP
Glucagon
GHRH
Secretin
GIP
Six different genes with nine gene products

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