Characterization of gonadotropin-releasing hormone (GnRH) from lake whitefish (*Coregonus clupeaformis*): structure, function, and location.

by

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We accept this thesis as conforming to the required standard.

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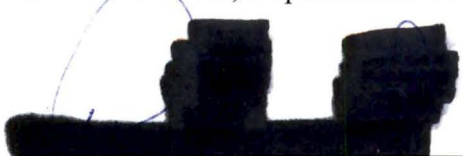

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) is a small peptide hormone that is essential for reproduction in vertebrates and some invertebrates. GnRH is synthesized in the brain and acts on the pituitary gland stimulating the synthesis and release of gonadotropins into the blood. These gonadotropins circulate to the gonads where they stimulate gametogenesis and the synthesis and release of reproductive steroid hormones such as estrogen and testosterone. Seventeen GnRH forms have been isolated and named after the species in which they were first identified. The structure of this ten-amino-acid hormone is highly conserved among vertebrates, except for the four amino acids located in positions five to eight. To date, species contain either two or three GnRH forms.

Lake whitefish was chosen as the study animal because it is a representative of the earliest subfamily of salmonids (*Coregoninae*). All salmonids studied so far express only two GnRH forms: salmon (s)GnRH and chicken (c)GnRH-II. However, our previous protein work done on lake whitefish identified three GnRH forms: sGnRH, cGnRH-II, and the third form called whitefish (wf)GnRH. There are three objectives in this study: (1) to obtain the cDNA sequences of each form of GnRH in lake whitefish, (2) to determine the releasing potential of wfGnRH on pituitary cells, and (3) to determine the location of each GnRH form in the whitefish brain using *in situ* hybridization. An examination of the structure (both protein and DNA), specific brain function, and location of each GnRH form within a species provides clues to the evolution of the hormone.

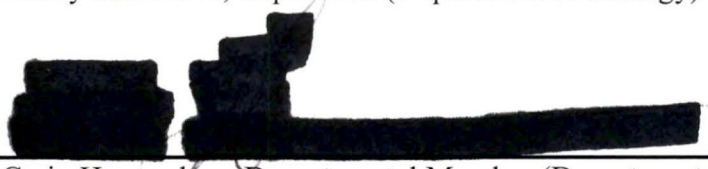
The cDNA sequence of each form of GnRH in lake whitefish was isolated using 5' and 3' rapid amplification of cDNA ends (RACE) and reverse transcription polymerase chain reaction (RT- PCR). Each sequence consists of a 5' untranslated region (UTR), signal peptide, GnRH-encoding region, cut site, GnRH-associated peptide (GAP), and 3'UTR. The lake whitefish cGnRH-II precursor has 88% nucleotide and 79% amino acid identity with rainbow trout cGnRH-II. The lake whitefish sGnRH precursor shows 93% nucleotide and 79% amino acid identity when compared to Atlantic salmon sGnRH. As seen in other vertebrate GnRH sequences, the regions that are most conserved encode the GnRH peptide and the cut site. The novel wfGnRH cDNA reveals little nucleotide sequence identity to any known GnRH sequences but shows 60% amino acid identity with *Haplochromis burtoni* seabream (sb)GnRH. This suggests the ancestral forms of wfGnRH and sbGnRH were closely related.

Whitefish GnRH is biologically active, as it stimulated cultured pituitary cells to increase the expression of the α-subunit of gonadotropin mRNA. This suggests that wfGnRH has a role in gonadotropin-release from the pituitary in lake whitefish. Also, cGnRH-II had some biological activity, but is unlikely to cause gonadotropin-release *in vivo* due to its location in the midbrain.

Localization of brain GnRH by *in situ* hybridization revealed an overlap of regions expressing sGnRH or wfGnRH in the anterior brain from olfactory bulb to preoptic area (POA). The only form located in the hypothalamus was wfGnRH and in the midbrain was cGnRH-II. It is concluded that wfGnRH plays the main role in gonadotropin release in lake whitefish and that sGnRH may have a minor role. Also, both sGnRH and cGnRH-II probably have neuromodulatory roles as well.

Examiners:

Dr. Nancy Sherwood, Supervisor (Department of Biology)



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I figure this part of the thesis is probably one of the hardest to write. So many people have helped me through the last two years. Of course how is anyone going to know that this actually belongs to me unless I put my signature on it? So here goes...

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things that can not be forgotten, I have them now I go:
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The experience and the memories of my masters will last awhile,
to all of you above, please know when I think of you I smile!

Chapter 1

Introduction

Gonadotropin releasing hormone (GnRH) is a small peptide hormone that is essential for reproduction in vertebrates and some invertebrates. It is synthesized in the hypothalamus and acts on the anterior pituitary causing the synthesis and release of gonadotropins into the blood. These gonadotropins (follicle stimulating hormone, FSH, and luteinizing hormone, LH, in mammals and gonadotropin-I and –II in fish) circulate to the gonads where they stimulate gametogenesis and the synthesis and release of reproductive steroid hormones such as estrogen and testosterone.

GnRH Structure

GnRH was first discovered three decades ago in ovine (Burgus *et* al., 1972) and porcine (Matsuo *et al.*, 1971) hypothalami. It was first named luteinizing hormone-releasing hormone (LHRH) and was later called mammalian GnRH (mGnRH). Since then 16 other GnRH forms have been identified and named after the species from which they were first isolated (Table 1.1). The GnRH forms include chicken (c)GnRH-I (Miyamoto *et al.*, 1983) and cGnRH-II (Miyamoto *et al.*, 1984), salmon (s)GnRH (Sherwood *et al.*, 1983), lamprey (l)GnRH-I (Sherwood *et al.*, 1986) and lGnRH-III (Sower *et al.*, 1993), catfish (cf)GnRH (Ngamvongchon *et al.*, 1992a; Bogerd *et al.*, 1992), dogfish (df)GnRH (Lovejoy *et al.*, 1992b), sea bream (sb)GnRH (Powell *et al.*, 1994), tunicate (t)GnRH-I and tGnRH-II (Powell *et al.*, 1996), guinea-pig (gp)GnRH (Jimenez-Linan *et al.*, 1997), herring (hr)GnRH (Carolsfeld *et al.*, 2000), frog (f)GnRH (Yoo *et al.*, 2000), pejerrey (pj)GnRH (Montaner *et al.*, 2001) also named medaka (md)GnRH (Okubo *et al.*, 2000), and whitefish (wf)GnRH (Adams *et al.*, 2002). An

Table 1.1. Amino acid structure of GnRH.

The amino acid sequences of all 17 GnRH forms known to date are presented. Only amino acids that differ from whitefish GnRH are shown.

FORM* (abbreviation)	1	2	3	4	5	6	7	8	9	10	Reference
WHITEFISH (wf)	pGlu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	Gly-NH_2	Adams et al., 2002
MAMMAL (m)	-	-	-	-	-	-	Leu	Arg	-	-	Matsuo et al., 1971; Burgus et al., 1972
GUINEA PIG (gp)	-	Tyr	-	-	-	-	Val	Arg	-	-	Jimenz-Linan et al., 1997
CHICKEN-I (c-I)	-	-	-	-	-	-	Leu	Gln	-	-	Miyamoto et al., 1983
FROG (f)	-	-	-	-	-	-	Tyr	Arg	-	-	Yoo et al., 2000
SEABREAM (sb)	-	-	-	-	-	-	Leu	Ser	-	-	Powell et al., 1994
PEJERREY (pj)/MEDAKA (md)	-	-	-	-	Phe	-	Leu	Ser	-	-	Okubo et al., 2000; Montaner et al., 2001
HERRING (hr)	-	-	-	-	His	-	Leu	Ser	-	-	Carolsfeld et al., 2000
CATFISH (cf)	-	-	-	-	His	-	Leu	-	-	-	Ngamvongchon et al., 1992; Bogerd et al., 1992
SALMON (s)	-	-	-	-	-	-	Trp	Leu	-	-	Sherwood et al., 1983
DOGFISH (df)	-	-	-	-	His	-	Trp	Leu	-	-	Lovejoy et al., 1992
CHICKEN-II (c-II)	-	-	-	-	His	-	Trp	Tyr	-	-	Miyamoto et al., 1984
LAMPREY-I (l-I)	-	-	-	-	His	Asp	Trp	Lys	-	-	Sherwood et al., 1986
LAMPREY-III (l-III)	-	-	Tyr	-	Leu	Glu	Trp	Lys	-	-	Sower et al., 1993
TUNICATE-I (t-I)	-	-	-	-	Asp	Tyr	Phe	Lys	-	-	Powell et al., 1996
TUNICATE-II (t-II)	-	-	-	-	Leu	Cys	His	Ala	-	-	Powell et al., 1996
OCTOPUS (oct)	- Asn-Tyr	-	Phe	-	Asn	-	Trp	His	-	-	Iwakoshi et al., 2002

*Only amino acids that differ from whitefish are indicated.

additional GnRH is found in octopus, although it is 12 amino acids in length (Iwakoshi *et al.*, 2002).

GnRH is ten amino acids long with the overall structure conserved in the end groups. That is, amino acids in positions 1-4 and 9-10 are conserved, whereas amino acids in positions 5-8 tend to vary among forms. GnRH is usually recognized by its pyroglutamic acid N-terminus, followed by histidine, tryptophan, and serine, and ending with a proline and glycine C-terminus. The four varying amino acids that are not conserved, and the C-terminal end are part of the hormone that is important for receptor binding. Amino acids 1-3 are important for the functional release of gonadotropins from the pituitary gland (see review by Sherwood, 1986). The overall conservation of GnRH is not surprising as it is essential for reproduction to occur. If the GnRH gene is altered genetically or if GnRH is blocked by immunoneutralization, then reproduction does not occur (see review by Sherwood, 1986).

GnRH Structure in Fish

One of the major GnRH forms found in fish is similar in structure to that of mammalian GnRH except for 2 amino acid substitutions in positions 7 and 8. This form was characterized and named salmon (s)GnRH by Sherwood *et al.* (1983). Shortly after the discovery of sGnRH a second form was found in cartilaginous fish (Lovejoy *et al.*, 1991; 1992b). This GnRH form had the same primary structure as chicken (c)GnRH-II. Subsequently, cGnRH-II was found as a second form in all jawed vertebrate species (see Sherwood *et al.*, 1993), from cartilaginous fish to humans (White *et al.*, 1998). Salmon GnRH and cGnRH-II are not the only GnRH forms found in fish; lamprey, dogfish,

catfish, pejerrey, herring, sea bream and lake whitefish all have distinct GnRH molecules that are found in specific groups of fish (see Figure 1.1, pg 12).

GnRH Function

GnRH is secreted in the median eminence of mammals and acts on the anterior pituitary where it stimulates the synthesis and release of LH and FSH (Schally *et al.*, 1973). In many vertebrates GnRH is released from the brain into the brain-pituitary portal vessels shortly before gonadotropins are released (Fink and Jamieson, 1976). However, there are two exceptions. The first is apparent in more primitive species, like the lamprey, where there is no portal system between the brain and the pituitary gland. Here the two organs are apposed and neurosecretions diffuse between them (Gorbman, 1980) or GnRH reaches the pituitary via the general circulation (see Muske, 1993). The second exception is evident in teleosts, where neurons containing GnRH have axons that extend from the brain into the pituitary gland and GnRH is released directly into the pituitary without use of a portal link (see Sherwood *et al.*, 1991).

In the pituitary of all vertebrates, GnRH interacts with membrane-associated high affinity receptors on gonadotropin secreting cells. The binding of GnRH to its receptor triggers downstream events (see section marked GnRH receptor).

Research done with the pituitary supports the evidence that GnRH is released in pulses to act on the pituitary. For example, Duan *et al.* (2002) found that GnRH pulses maintain LH and FSH gene expression: LHβ by high-frequency pulses and FSHβ by lower frequency pulses. Also, they determined that GnRH specifically stimulates early

growth response protein-1 (Egr-1) expression, which is an essential transcription factor for LHβ gene expression in the pituitary.

GnRH has other proposed functions that include acting as a stimulator of sexual behaviour, as a neuromodulator and/or neurotransmitter in the central and sympathetic nervous systems, and as a paracrine/autocrine regulator in the pituitary, gonads, placenta, and in tumour cells (see Millar and King, 1988; Sherwood *et al.*, 1993; Rastogi and Iela, 1994). It is also known that GnRH can stimulate the release of other pituitary hormones in fish including growth hormone (Marchant *et al.*, 1989), prolactin (Weber *et al.*, 1997), and possibly somatolactin (Parhar, 1997).

Within the CNS

Contrasting with the septo-preoptico-infundibular GnRH system, where neurons extend fibers to the pituitary for gonadotropin release, GnRH neurons in the terminal nerve send fibers throughout the brain but not near the pituitary (Yamamoto *et al.*, 1995). Abe and Oka (2000) suggest that GnRH found in the terminal nerve acts as a neuromodulator with endogenous pacemaker activity and is involved in the regulation of many long lasting changes in animal behaviour, such as motivational and arousal states. Salmon (s)GnRH made in the terminal nerve acts on its own or neighboring neurons to change the discharge patterns of terminal nerve neurons in dwarf gourami (Abe and Oka, 2000).

GnRH found in the olfactory tract (olfactory epithelium, nerve and bulb) could mediate responses to sex pheromones (see reviews by Sherwood *et al.*, 1993, and Muske, 1993). In concordance, Wirsig-Wiechmann (2001) found that GnRH altered responses of chemosensory neurons and speculated that the purpose of altered olfactory sense is to

increase detectability of pheromones. Okubo and coworkers (1999) concluded that GnRH in the olfactory epithelium of the eel is involved in the pathway through which environmental and behavioral inputs influence the neuroendocrine systems. They suggest that the presence of GnRH here has an important role for the reproductive migration of the eel.

Although specific function is unknown, GnRH has been located in the amygdala and neighboring areas of mammals (Silverman *et al.*, 1987; Stopa *et al.*, 1991; Quanbeck *et al.*, 1997), retina of fish (Stell *et al.*, 1984), sympathetic ganglia of bullfrog (Jan *et al.*, 1979; Jan *et al.*, 1983), and GnRH binding sites have been located in the limbic region (Millan *et al.*, 1986). For a more detailed review see Muske (1993).

GnRH in the midbrain, hindbrain and spinal cord suggests GnRH may regulate sexual behavior as it has a role in increasing rat sexual receptivity (Moss and McCann, 1973), and a role in mating behavior in the musk shrew (Rissman *et al.*, 1997) and in newts (Muske *et al.*, 1993). Other speculative functions of GnRH include: gonadal duct contractility in fish (GnRH terminals on neurosecretory cells located in the caudal part of the spinal cord in fish) (Miller and Kriebel, 1986), sperm release in fish (Demski *et al.*, 1975), and clasper control in cartilaginous fish (GnRH terminals in the spinal cord overlap areas for clasper control) (see Wright and Demski, 1991).

Outside the CNS

Other locations for GnRH secretion include: (1) human placenta, where GnRH causes the release of chorionic gonadotropin (CG); (2) mammary gland tumors; (3) tumor cell lines; (4) pancreas, although the function remains unknown, and (5) pancreatic tumor cells (for review see Sherwood *et al.*, 1993). GnRH has also been reported to act in the

immune system (both hormone and receptor) where it stimulates LH production in lymphocytes (Blalock and Costa, 1989).

Gonadal GnRH function

GnRH has functions within the gonads of mammals, fish, and tunicates. It is reported to affect gonadal steroids, act as a meiosis-stimulating factor and help in the regulation of ovarian function. GnRH mRNA was isolated locally from gonads in fish (von Schalburg and Sherwood, 1999; Gray *et al.*, 2002), although only rarely is the protein found (von Schalburg and Sherwood, 1999). GnRH induces an increase in tunicate gonadal estradiol (Craig *et al.*, 1997; Di Fiore *et al.*, 2000). GnRH has been suggested to play a role in the regulation of ovarian function in both the goldfish (Pati and Habibi, 1998) and in the seabream (Nabissi *et al.*, 1997). Hillensjo and LeMaire (1980) found GnRH stimulated oocyte meiosis in rat and the same effect was found in goldfish (Pati and Habibi, 2000). Pazos and Mathieu (1999) found that mGnRH, sGnRH, cGnRH-I and –II, and lGnRH-I significantly increased DNA synthesis in gonial cells of a marine bivalve and suggest that GnRH might promote the G1-S phase transition in the gonial cell cycle. Parhar (1997) speculates that preoptic sea bream (sb)GnRH has an indirect role (via the pituitary) in gonadal sex differentiation in the sea bream because the development of both GnRH and gonads are synchronized, although serum concentration of pituitary gonadotropins has not been measured. Also, GnRH can affect sex steroids. Van der Kraak *et al.* (1984) found that injected LHRH (mGnRH) caused an increase in 17 alpha, 20 beta dihydroxy-4-pregnen-3-one plasma levels in ovulating coho salmon (*Oncorhynchus kisutch*). Similarly, Joss and coworkers (1994) reported that injected, synthetic mGnRH caused male and female lungfish to elevate their circulating steroid

hormones at the beginning of spawning. Testosterone increased in males; estradiol and testosterone increased in females. However, steroid levels at the end of spawning were lower than at the beginning of spawning and were abolished in early autumn.

GnRH Evolution

The phylogenetic pattern of GnRH in different species is determined in part by structural changes in the peptide, cDNA or gene. The presence of GnRH in a jawless vertebrate, the lamprey, whose ancestors arose about 500 million years ago, suggests the early appearance of the peptide in vertebrate evolution (Sherwood *et al.*, 1986). However, the exact time GnRH arose still remains to be determined. The peptide is now widely studied to determine if different forms common to different vertebrates will establish the evolutionary thread of GnRH in vertebrates (Sherwood *et al.*, 1997). GnRH is not only found in vertebrates; more recently the peptide was detected by immunoreactivity in neurons of invertebrates such as the gastropod mollusc *Helisoma* (Goldberg *et al.*, 1993), the acorn worm *Saccoglossus* (Cameron *et al.*, 1999) and the ancestral chordate *Ciona intestinalis* (Tsutsui *et al.*, 1998). In addition, the primary structure of two distinct GnRH forms was determined for the tunicate *Chelyosoma productum* (Powell *et al.*, 1996) and one form of 12 amino acids for octopus, *Octopus vulgaris* (Iwakoshi *et al.*, 2002).

There are some GnRH structural patterns among species studied to date. Chicken GnRH-II is first detected in cartilaginous fish, such as ratfish and dogfish, and is conserved in all vertebrates thereafter including bony fish, amphibians, reptiles, birds and mammals (see Sherwood *et al.*, 1997). Salmon GnRH is confined to teleosts whereas

cGnRH-I is present in reptiles and birds only (Sherwood *et al.*, 1991). A few forms, such as cGnRH-I, pjGnRH, and sbGnRH are confined to closely related orders (Sherwood *et al.*, 1993). So far, the remaining forms of cfGnRH, dfGnRH, tGnRH-I, tGnRH-II, lGnRH-I, lGnRH-III, hrGnRH, and wfGnRH are located only in their respective species or in closely related species. However, future work may find the above forms in other related species as well. The pattern of mammalian (m)GnRH is interesting as it first appears in older bony fish and their descendants (early-teleosts), then disappears in teleosts that evolve thereafter. Also, mGnRH is present in amphibians and mammals (Sherwood *et al.*, 1991; Sherwood *et al.*, 1993). Because mGnRH appeared in ancient ancestral bony fish, but not in cartilaginous fish, it is thought to have evolved before the actinopterygian and sarcopterygian split leading to bony fish and tetrapods, respectively (Sherwood *et al.*, 1991).

The peptide structures, determined by peptide or DNA sequencing, of all 17 GnRHs published to date can be used to provide clues on the evolution of the peptide. One proposed scheme of GnRH evolution is shown in Figure 1.1. So far, studies show the pattern that most tetrapods have two forms of GnRH, whereas most teleosts have three forms. In humans, for example, only two forms of GnRH are shown to be encoded (mGnRH and cGnRH-II) in an analysis of the genome (Neill, 2002). Chicken GnRH-II is found in all other tetrapods along with mGnRH or an alternate form (guinea pig, frog, or chicken-I) that arose from mutations of mGnRH. Chicken GnRH-II is an apparent GnRH lineage beginning in cartilaginous fish; and mGnRH represents another lineage first seen in ancient bony fish. However, O'Neill *et al.* (1998) found that mGnRH disappears in

Figure 1.1. General GnRH evolution.

A hypothetical scheme for the evolution of the 17 known GnRHs from an ancestral GnRH (A) is shown. Numbers indicate the amino acid differences between GnRH forms. Forms shown are: A, ancestral; T-I, tunicate GnRH-I; T-II, tunicate GnRH-II; OCT, octopus GnRH; L-I, lamprey GnRH-I; L-III, lamprey GnRH-III; C-II, chicken GnRH-II; DF, dogfish GnRH; M, mammalian GnRH; S, salmon GnRH; H, herring GnRH; CF, catfish GnRH; SB, seabream GnRH; WF, whitefish GnRH; PJ, pejerrey GnRH; F, frog GnRH; C-I, chicken GnRH-I (adapted from Sherwood *et al.*, 1997; and Montaner *et al.*, 2001).

PRE-CHORDATES

PROTOCHORDATES

Octopus

Tunicates

JAWLESS FISH

Lamprey

CARTILAGINOUS FISH

Ratfish

Dogfish

BONY FISH-PRE-TELEOSTS

Sturgeon

BONY FISH-EARLY TELEOSTS

Eel

Herring

BONY FISH-EUTELEOSTS

Whitefish

Salmon

Catfish

Pacu

BONY FISH-NEOTELEOSTS

Pejerrey, Medaka

Sea bream, Cichlid

AMPHIBIANS

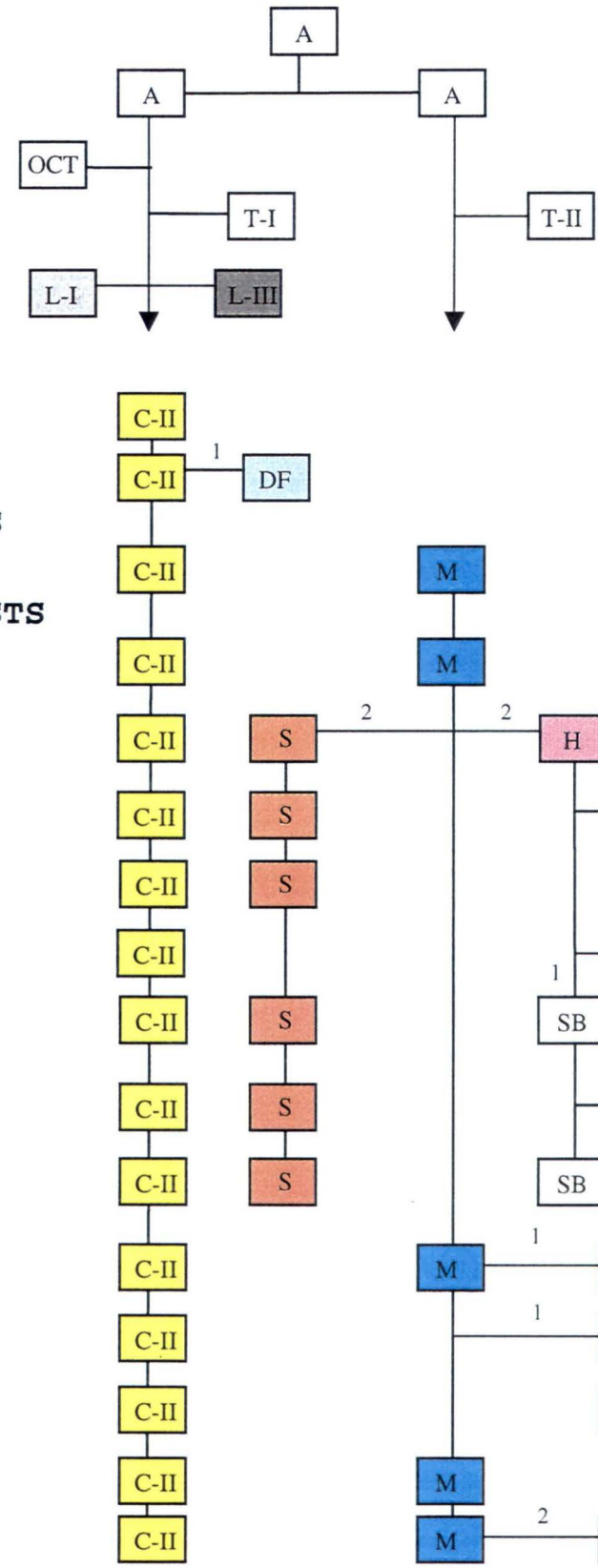
Frog

REPTILES

BIRDS

MAMMALS

Guinea Pig



bony tongue fish and sGnRH appears. Also, a third from of GnRH appears for the first time in teleost species in herring (Carolsfeld *et al.*, 2000). Okubo and Aida (2001) have suggested that the three GnRH lineages in teleosts began in bony tongue fish, but they have only been able to identify two forms of GnRH in any bony tongue species. The rest of the GnRH forms do not fall into lineages so easily, although sGnRH could represent another lineage starting in herring and appearing in all other teleosts except catfish. Also, the origin of the third form of GnRH found in teleosts still needs to be explained, and the relationship (orthologous or paralogous) among the teleost GnRH forms remains to be identified. It is clear that a different kind of analysis is required to find the orthologs for sGnRH and the others.

Another problem that the evolutionary scheme in Fig 1.1 does not account for is that it has not been possible to identify the orthologs for cGnRH-II and mGnRH in jawed fish. The short 10 amino acid peptide does not reveal the ancestral relationship of octopus (oct)GnRH, tGnRH-I, tGnRH-III, lGnRH-I, and lGnRH-II, with mGnRH and cGnRH-II or whether mGnRH is a result of a cGnRH-II duplication. The line of descent for orthologous or paralogous forms of GnRH would not necessarily become clear even if all forms between octopus to jawless fish were known. The primary structure of GnRH peptides in ancient, but living animals may be far removed from the ancestral form at its stem. For example, lamprey GnRH-I and -III are not found in other vertebrates to date and these forms have been evolving for 500 million years from the ancestral lamprey stem form. The same is true for the octopus and tunicate GnRHs. The GnRH form in living animals may be distinct from the ancestral animals.

An alternate approach to determine GnRH evolution is phylogenetic tree analysis based on the GnRH preprohormones. This method uses the nucleotide sequence of not only the GnRH encoding region, but also the nucleotide sequence of the signal peptide, the cut site, and the GAP region. It is a valuable method for grouping related GnRH peptides among all the different forms found in teleosts; however, it does not show the ortholog for any of the forms and does not connect these forms in jawed vertebrates to lamprey, protochordate or octopus forms. Other problems are that alternative splicing could produce altered forms and expression is not constant so a form could be missed.

The form of analysis I would suggest for further GnRH phylogeny information is linkage analysis. In this method, GnRH genes would be identified with other genes on the same chromosome. These linked genes could then be compared. It is likely in evolution that genes on the same chromosome will remain on that chromosome, although not necessarily in the same order. The next step would be to establish GnRH in relation to marker genes on specific chromosomes. This would help to account for the GnRH lineage during genomic duplications that are proposed: (1) between protochordates and vertebrates, (2) leading up to cartilaginous fish, and (3) in early teleosts.

GnRH Origin, Precursor and Expression

GnRH origin

GnRH neurons in the terminal nerve ganglion (TN) and septo-preoptic areas originate embryonically in the olfactory placode of vertebrates and migrate to adult destinations (TN and preoptic area) in the forebrain (Muske and Moore, 1987). In addition, Quanbeck and coworkers (1997) found two different GnRH cell types migrate

from the olfactory placode in the rhesus macaque. They identified them as early neurons and late neurons. Early neurons appear first, are smaller than late neurons, and migrate to the preoptic region before settling in the extrahypothalamic regions. In contrast, late neurons, which contain mGnRH, migrate to the basal hypothalamus and regulate the pituitary-gonadal axis (Quanbeck *et al.*, 1997).

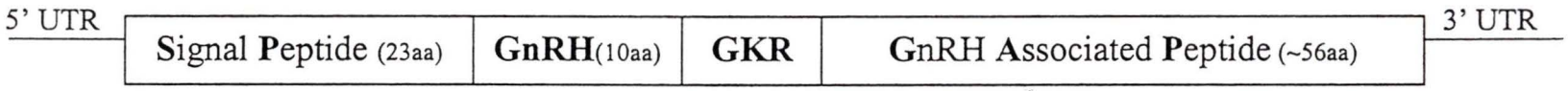
GnRH neurons in the midbrain were later found to originate from neuroblasts in the midbrain region; they send fibers that extend throughout the ventral brainstem and into the spinal cord (Muske and Moore, 1990; Witkin, 1990). To further this observation, Parhar (1997) reported midbrain-GnRH neurons originated from the ventricular ependyma, and Lescheid *et al.* (1997) suggested cGnRH-II neurons originate in the ventricular wall of the posterior hypothalamus and the midbrain.

GnRH precursor

The general prepro-GnRH precursor structure is similar among vertebrates (Figure 1.2). The cDNA of GnRH contains a 5' untranslated region (UTR), signal peptide, GnRH, a cut site (containing an amide-donating glycine and a lysine, arginine processing site), a GnRH associated-peptide (GAP), and a 3' UTR (see Sherwood *et al.*, 1993). For example, Coe and coworkers (1995) sequenced the gene for sGnRH in sockeye salmon. They identified the cDNA to be 475 base pairs (bp) in length with a 40bp 5'UTR, an open reading frame (or coding region) of 240bp (including the stop signal), and a 195bp 3'UTR. Similarly, the cDNA for cGnRH-II in rainbow trout has also been identified (Penlington *et al.*, 1998). It was found to be 544bp in length with a 27bp 5'UTR, a 258bp open reading frame, and a 256bp 3'UTR.

Figure 1.2. GnRH precursor.

A diagram showing the general GnRH precursor molecule including the signal peptide, 23 amino acids in length, the GnRH region, 10 amino acids in length, the cut site (GKR), 3 amino acids in length, and the GAP region, approximately 56 amino acids in length.


5' UTR
Signal Peptide (23aa)
GnRH(10aa)
GKR
GnRH Associated Peptide (~56aa)
3' UTR

The GnRH portion of the precursor is highly conserved among species, whereas the signal and GAP portions are not (see Coe *et al.*, 1995). The GAP region was initially thought to inhibit secretion of PRL but now is thought to be involved in the correct processing and packaging of GnRH as in other associated peptides (see Sherwood *et al.*, 1993).

Even though many species express more that one form of GnRH, to date each form is encoded by separate genes in vertebrates (see Sherwood *et al.*, 1997; Chow *et al.*, 1998). Generally, the GnRH gene contains four exons (Figure 1.3). The first encodes the 5' UTR; the second encodes the signal peptide, GnRH, the cut site, and the first 11 amino acids of the GAP region; the third exon encodes GAP amino acids 12-43; and the last exon codes for the rest of the GAP region and the 3' UTR (Sherwood *et al.*, 1993).

GnRH Expression

In general, GnRH expression patterns are similar among fish, with sGnRH being expressed in the forebrain, cGnRH-II in the midbrain, and the third form in the preoptic area (Figure 1.4). Table 1.2 summarizes all expression studies done on fish and the brain location of different GnRH forms. Localization of GnRH can be done using the following techniques: high-pressure liquid chromatography (HPLC) in combination with radioimmunoassay (RIA), Northern blot and reverse-transcription PCR (RT-PCR), *in situ* hybridization, and immunocytochemistry (similar to immunohistochemistry). The most precise localization studies use a combination of *in situ* hybridization, which is very specific to each GnRH form, and immunocytochemistry, which locates both cells and fibers containing GnRH. For example, in the European sea bass Zmora *et al.* (2002) used both *in situ* hybridization and immunocytochemistry techniques. *In situ*

Figure 1.3. GnRH gene structure.

The general structure of the GnRH gene has four exons (numbered 1-4). 5' and 3' UTRs are shown by diagonal marks (/), the signal peptide is shown by dots (▒), GnRH is shown by circles (••), the cut site is shown by chevrons (<), and the GAP region has been left white.

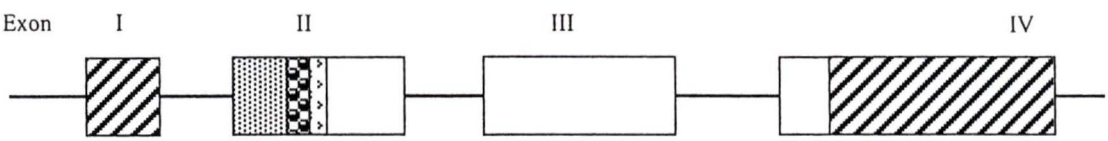

Exon
I
II
III
IV

Figure 1.4. GnRH brain location.

This drawing of a saggital section from a salmonid brain shows the three brain locations for GnRH. Triangles (▲) represent salmon GnRH, diamonds (♦) represent chicken GnRH-II, and circles (●) represent third GnRH form.

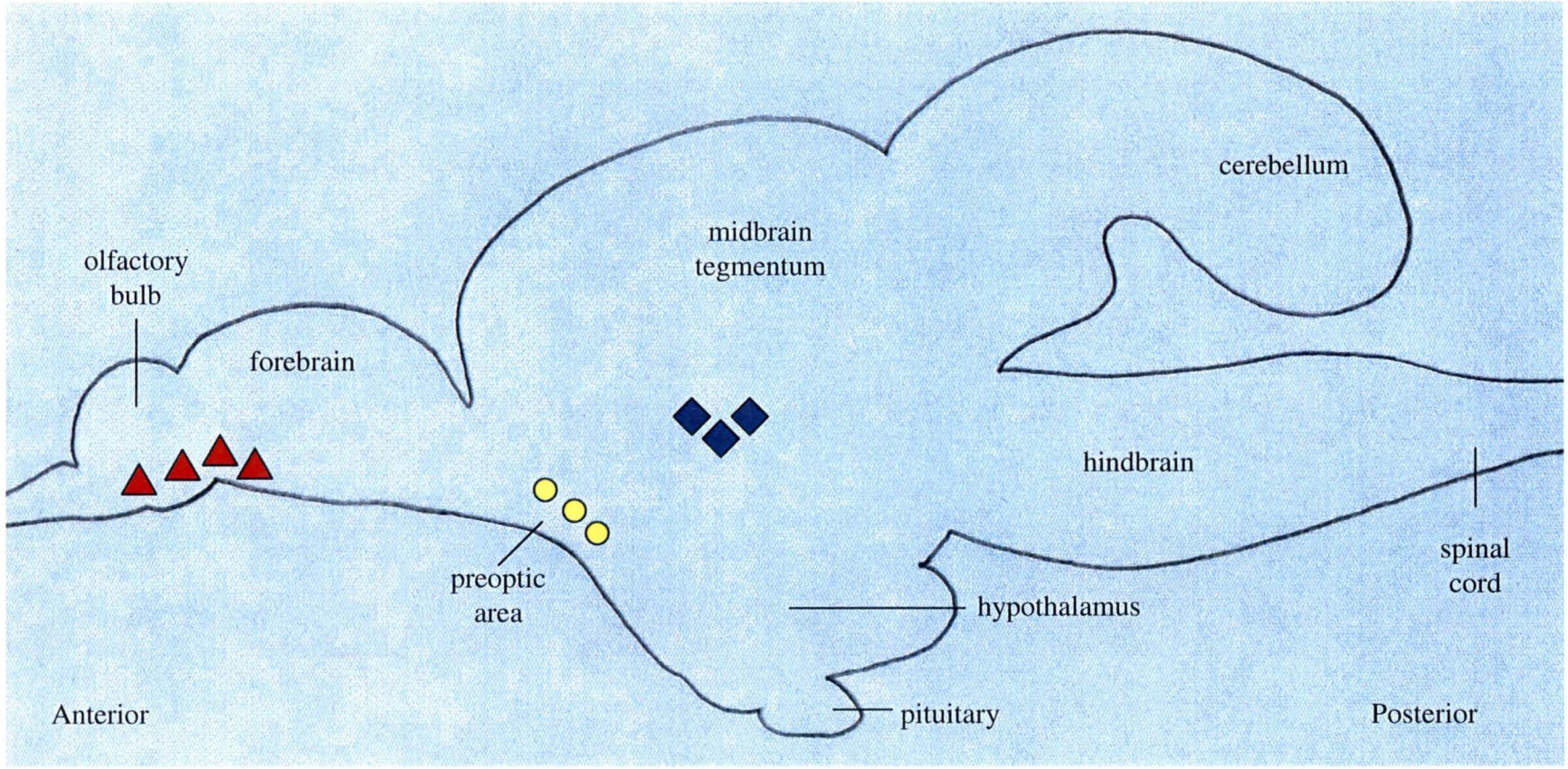

cerebellum
midbrain
tegmentum
olfactory
bulb
forebrain
hindbrain
spinal
cord
preoptic
area
hypothalamus
pituitary
Anterior
Posterior

Table 1.2. GnRH expression in fish.

A comparison of all GnRH expression studies done to date states the species used, the method of detection, the specific brain location of each GnRH, whether or not cross-reactivity of GnRH forms was considered, and references. Method abbreviations: IL – immunolabeling, which includes immunocytochemistry, immunohistochemistry, radioimmunoassay, and double antibody immunofluoresence; ISH – *in situ* hybridization. Brain abbreviations: NOR – nucleus olfactoretinalis, NPP – nucleus preopticus periventricularis, NLT – nucleus lateralis tuberis, NP – nucleus preopticus, NPBL – nucleus preopticus basalis lateralis, POA – preoptic area, NMLF – nucleus of medial longitudinal fasciculus (in midbrain), NT – nervus terminalis, TN – terminal nerve, PM – pars magnocellularis, and PP – pars parvicellularis. GnRH abbreviations: mGnRH – mammalian GnRH, sGnRH – salmon GnRH, cGnRH-II – chicken-II GnRH, cfGnRH – catfish GnRH, mdGnRH – medaka GnRH, and lGnRH – lamprey GnRH.

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
1. barfin flounder	ISH	sbGnRH – POA sGnRH – olfactory bulb, NT cGnRH-II – midbrain	Probes specific for each GnRH form.	Amano *et al.*, 2002
2. sole	IL	sGnRH – cells - in olfactory nerve and bulb, POA – fibers - in retina, telencephalon, hypothalamus, pituitary, optic tectum	Cross-reactivity is referenced in the paper.	Nunez-Rodriguez *et al.*, 1985
3. Atlantic croaker	IL	GnRH – cells - in olfactory bulb, POA, hypothalamus – fibers - in pituitary	Not tested.	Khan and Thomas, 1993
4. dwarf gourami	IL	sGnRH – cells - in olfactory nerve and bulb, POA, pituitary, and few in midbrain cGnRH-II – cells - few in telencephalon, in midbrain	sGnRH antibody – cross-reacts 1.58% with cGnRH-II. cGnRH-II antibody – cross-reacts 16% with sGnRH.	Yamamoto *et al.*, 1995
5. sea bass	IL	sGnRH – cells - olfactory bulb, POA, telencephalon, hypothalamus – fibers - from olfactory bulb into pituitary, in optic tectum, cerebellum, vagal lobe, medulla oblongata, spinal cord	sGnRH antibody - 2.5% cross-reactivity with cGnRH-II, and 1.2% with mGnRH	Kah *et al.*, 1991

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
6. European sea bass	ISH	sbGnRH – cells - olfactory bulb, POA, telencephalon sGnRH – cells - same as above, and hypothalamus cGnRH-II – cells - NMLF	Probes specific to each GnRH form.	Gonzalez-Martinez *et al.*, 2001
7. European sea bass	ISH and IL	sbGnRH – cells - in POA – fibers - in POA, hypothalamus, pituitary sGnRH – cells - in olfactory bulb – fibers - in forebrain, some into pituitary cGnRH-II – cells - in midbrain – fibers - in forebrain	ISH – Probes specific to the GAP region of each GnRH form. IL – Not tested. Antibodies made against the GAP region of the different GnRH forms.	Zmora *et al.*, 2002
8. Gilthead seabream	ISH	sGnRH – olfactory bulb, NT sbGnRH – POA, thalamus cGnRH-II – midbrain	Probes specific to each GnRH form.	Gothilf *et al.*, 1996
9. red seabream	ISH and IL	sGnRH – both ISH and IL – cells - NT sbGnRH – both ISH and IL – cells - POA, – fibers - into pituitary	ISH – Probes specific to sGnRH and sbGnRH. IL – Cross-reactivity tested but no results discussed.	Okuzawa *et al.*, 1997
10. tilapia	ISH	sGnRH – NOR cGnRH-II – midbrain	Probes specific to sGnRH and cGnRH-II.	Parhar *et al.*, 1996a

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
11.tilapia	IL	– sGnRH – cells - in NOR, telencephalon, POA, midbrain – mGnRH – cells - as above minus midbrain – fibers (both forms) - olfactory bulb, telencephalon, hypothalamus, pituitary, optic tectum, spinal cord	Referenced in paper.	Parhar *et al.*, 1996b
	ISH	– sbGnRH – NOR – cGnRH-II – midbrain	Probes specific to sbGnRH and cGnRH-II.	
12. H. burtoni	ISH	sGnRH – NT sbGnRH – POA cGnRH-II – midbrain	Probes specific to each GnRH form.	White *et al.*, 1994; 1995; White and Fernald, 1998
13. labrid fish	IL	GnRH (LHRH) – cells - in POA, midbrain – fibers - in olfactory nerve and bulb, POA, hypothalamus, midbrain	Not tested. Antibody made against either mGnRH or sGnRH.	Grober and Bass, 1991
14. guppy	IL	GnRH (LHRH) – telencephalon, POA, midbrain	Not tested.	Zentel *et al.*, 1987
15. platyfish	IL	GnRH (LHRH) – cells - in telencephalon, NLT – fibers - into pituitary	Not tested.	Schreibman *et al.*, 1979

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
16. platyfish	IL	GnRH (LHRH) – cells - in telencephalon, POA, midbrain	Not tested.	Munz *et al.*, 1981
17. platyfish	IL	GnRH (LHRH) – cells - in NOR, NPP, NLT	Not tested. Antibody made against mGnRH.	Halpern-Sebold and Schreibman, 1983
18. green molly	IL	GnRH – cells - in NOR, telencephalon, POA, midbrain – fibers - in NT	Table with cross-reactivity is included in the paper.	Batten *et al.*, 1990
19. medaka	ISH	sGnRH – NOR mdGnRH – POA cGnRH-II – midbrain	Probes specific to each GnRH.	Okubo *et al.*, 2000
20. pejerrey	IL	GnRH – cells - in NT, POA, midbrain – fibers - in olfactory nerve and bulb, hypothalamus, pituitary	Table with cross-reactivity is included in the paper.	Stefano *et al.*, 2000
21. rainbow trout	IL	GnRH – cells - in telencephalon – fibers - toward pituitary	Not tested. Antibody made against mGnRH.	Goos and Murathanoglu, 1977

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
22. rainbow trout	IL	GnRH – cells - in POA, PP, PM, and pituitary – fibers - in PP, PM, into hypothalamus and pituitary	Not tested. Antibody made against mGnRH.	Schafer *et al.*, 1989
23. rainbow trout	ISH	sGnRH – olfactory bulb, telencephalon, and POA	Probe specific to sGnRH.	Bailhache *et al.*, 1994
24. masu salmon	IL	sGnRH – cells - in olfactory bulb, telencephalon, and POA – fibers - in olfactory bulb, POA, hypothalamus, and pituitary cGnRH-II – cells - in midbrain – fibers - in olfactory bulb, POA, and hypothalamus	sGnRH antibody – cross-reacts 1.58% with cGnRH-II and 0.63% with lGnRH. cGnRH-II antibody – cross-reacts 16% with sGnRH.	Amano *et al.*, 1991
25. masu salmon	ISH	sGnRH – olfactory nerve and bulb, telencephalon, POA	Probe specific to sGnRH.	Suzuki *et al.*, 1992
26. Atlantic salmon	ISH	sGnRH – olfactory bulb, telencephalon, and POA	Probe specific to sGnRH.	Bailhache *et al.*, 1994
27. Atlantic salmon	IL	GnRH – cells - in olfactory nerve and bulb, POA	Not tested.	Nevitt *et al.*, 1995

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
28. sockeye salmon	IL	– sGnRH – cells - in NT, telencephalon, midbrain – cGnRH-II – cells - in NT – fibers (all forms) – in olfactory bulb, telencephalon, hypothalamus, pituitary, optic tectum, spinal cord	Referenced in paper.	Parhar *et al.*, 1996b
	ISH	– sGnRH – NT	Probe specific to sGnRH.	
29. chum salmon	ISH	– GnRH – nasal epithilium, olfactory bulb, NT	Probe specific to sGnRH.	Parhar *et al.*, 1994
	IL	– GnRH – same as above and midbrain	Not tested. Antibody made against sGnRH.	
30. African catfish	IL	GnRH (LHRH) – cells - in POA – fibers - in pituitary	Not tested. Antibody made against mGnRH	Goos *et al.*, 1985
31. catfish	IL	GnRH (LHRH) – cells - in NPBL, NPP, NP, NLT – fibers - in retina, cerebellum, medulla oblongata – cells and fibers - in olfactory nerve and bulb, pituitary, midbrain	Not tested. Antibody made against mGnRH.	Subheader and Krishna, 1988

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
32. catfish	ISH	cfGnRH – ventral forebrain cGnRH-II – midbrain	Probes were specific to each GnRH form.	Zandbergen *et al.*, 1995
33. goldfish	IL	GnRH (LHRH) – cells - NPP, NLT – fibers - from hypothalamus into pituitary, in olfactory bulb and optic tectum	Not tested. Antibody made against mGnRH	Kah *et al.*, 1984
34. goldfish	IL	LHRH – cells - olfactory nerve – fibers - olfactory bulb, telencephalon, optic tectum, retina, optic nerve	Not tested. Antibody made against mGnRH.	Stell *et al.*, 1984
35. goldfish	IL	GnRH – cells - olfactory nerve and bulb, telencephalon, POA, midbrain, hypothalamus, pituitary, medulla oblongata, spinal cord	Not tested. Antibody made against sGnRH.	Kah *et al.*, 1986
36. goldfish	IL	sGnRH – cells - olfactory nerve and bulb, POA (telencephalon), hypothalamus cGnRH-II – cells - same as above and midbrain – fibers (both) – olfactory bulb to spinal cord, hypothalamus into pituitary	sGnRH antibody - 0.8% cross-reactivity with cGnRH-II, 69.7% with mGnRH, 82.1% with cGnRH-I, and 1.0% with lGnRH. cGnRH-II antibody - 0.3% cross-reactivity with sGnRH, and 0.5% with lGnRH.	Kim *et al.*, 1995

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
37. goldfish	IL	sGnRH – cells - telencephalon, POA, and diencephalon cGnRH-II – cells - cerebellum and brain stem	Same antisera as above.	Rosenblum *et al.*, 1994
38. goldfish	IL	sGnRH and cGnRH-II – olfactory bulb, telencephalon, hypothalamus, pituitary, optic tectum, cerebellum, medulla, and spinal cord	Table with cross-reactivity is included in the paper.	Yu *et al.*, 1988
39. white sucker	IL	lGnRH-like and sGnRH – cells - in POA, hypothalamus – fibers - in telencephalon, diencephalon, pituitary cGnRH-II – fibers - in telencephalon, diencephalon	Tables with cross-reactivity is included in the paper.	Robinson *et al.*, 2000
40. American eel	IL	GnRH (LHRH) – cells - in junction of olfactory bulb and telencephalon – fibers - in olfactory epithelium, retina	Not tested.	Grober *et al.*, 1987
41. European silver eel	IL	mGnRH – cells - in olfactory bulb, NOR, telencephalon, POA, hypothalamus – fibers - as above, mesencephalon, pituitary cGnRH-II – cells - in NMLF (midbrain) – fibers - throughout brain, in pituitary	mGnRH antibody - no cross-reactivity with cGnRH-II. cGnRH-II antibody - cross reacts 90% with mGnRH.	Montero *et al.*, 1994

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
42. Japanese eel	IL	GnRH (LHRH) – cells - in olfactory bulb, NT, telencephalon – fibers - in pituitary	Not tested. Antibody made against mGnRH.	Nozaki *et al.*, 1984
43. Siberian sturgeon	IL	mGnRH – cells - in olfactory nerve and bulb, POA, hypothalamus – fibers - in olfactory nerve and bulb, hypothalamus, pituitary	Tested for cross-reactivity but no results discussed.	Lepretre *et al.*, 1993
44. black skate	IL	GnRH – fibers - in telencephalon, hypothalamus, midbrain, cerebellum, brain stem	Table with cross-reactivity is referenced in paper.	Lovejoy *et al.*, 1992a
45. leopard shark	IL	GnRH – fibers - in NT, POA	Not tested. Antibody made against mGnRH.	Nozaki *et al.*, 1984
46. leopard shark	IL	GnRH – cells - in midbrain – fibers - throughout the mesencephalon, in optic tectum, medulla, spinal cord	Not tested. Antibodies made against sGnRH and mGnRH.	Wright and Demski, 1991
47. spiny dogfish	IL	GnRH – fibers - in NT, telencephalon, midbrain, cerebellum, brain stem	Table with cross-reactivity is referenced in paper.	Lovejoy *et al.*, 1992a
48. sea lamprey	IL	GnRH (LHRH) – cells - in POA – fibers - in pituitary	Not tested. Antibody made against mGnRH.	Nozaki *et al.*, 1984

Species	Method Used	GnRH Brain Location	Antibody Cross Reactivity or Probe Specificity	Reference
48. hagfish	IL	GnRH – not found	Not tested. Antibody made against mGnRH.	Nozaki *et al.*, 1984
49. Pacific hagfish	IL	GnRH-like – fibers - in POA, hypothalamus	Table with cross-reactivity is included in the paper.	Braun *et al.*, 1995

hybridization located sGnRH mRNA in the olfactory bulb, sbGnRH in the preoptic area and cGnRH-II in the syencephalon (transition area between diencephalon and mesencephalon); and immunocytochemistry detected sGnRH fibers throughout the forebrain (with a few into the pituitary), sbGnRH fibers in the preoptic area, mediobasal hypothalamus and pars distalis of the pituitary, and cGnRH-II fibers widely in the posterior brain.

For those fish species that only express two forms of GnRH, the gonadotropin-releasing form is present in both the forebrain and the POA and cGnRH-II remains in the midbrain. For example, catfish express only cGnRH-II and catfish (cf)GnRH. Zandbergen and coworkers (1995) used both *in situ* hybridization and immunohistochemical techniques to locate brain GnRH in the catfish. *In situ* hybridization detected cfGnRH in the ventral forebrain and cGnRH-II in the midbrain, and immunohistochemistry located cfGnRH also in the medial olfactory tract, and the ventral forebrain with fibers extending into the pituitary, and cGnRH-II cells and fibers in the midbrain (Zandbergen *et al.*, 1995).

There is one fish model that does not seem to fit the usual pattern. In goldfish, which has two forms of GnRH, Kim and coworkers (1995) used immunocytochemical techniques to visualize GnRH neurons in the brain. They found sGnRH cell bodies in the olfactory nerve and bulb, the ventral telencephalon, the preoptic area and in the hypothalamus. However, instead of finding cGnRH-II in the midbrain only, they also found cGnRH-II in all the same areas as sGnRH. They found both sGnRH and cGnRH-II fibers innervated the pituitary and suggested both GnRH forms have a gonadotropin-releasing role. Unfortunately, they did not use *in situ* hybridization.

GnRH is expressed at different times depending on its location. In catfish, it was found that cfGnRH GAP mRNA expression increased in the pituitary during puberty (Dubois *et al.*, 2000); and in salmon, GnRH is expressed at smoltification and not earlier (Parhar *et al.*, 1994). GnRH mRNA is expressed in the brain continuously from before hatching through adult life in rainbow trout (von Schalburg *et al.*, 1999). However, GnRH mRNA expressed in the gonads appears during the first year, in an intermittent pattern during the juvenile stage, and up to the first spawning at 3 years of age (von Schalburg and Sherwood, 1999; Gray *et al.*, 2002). It was shown that the gonads and brain used downstream promoters; but gonadal mRNA only used an upstream promoter until the year before spawning when upstream and downstream promoters were used. The 5'UTR in the upstream (gonadal) precursor was longer than the downstream (brain) precursor (von Schalburg and Sherwood, 1999). In frogs, Yuanyou and Haoran (2000) used RIA to determine GnRH peptide expression in *Rana rugulosa* tadpoles and found that cGnRH-II was expressed in the brain before metamorphosis, whereas mGnRH was not detected until the stage of metamorphosis. However, both forms were present in the pituitary at each stage of maturity.

GnRH Regulation

In fish, a number of studies report consensus sites for transcription factors binding to the GnRH gene (Klungland *et al.*, 1992; Chow *et al.*, 1998; von Schalburg and Sherwood, 1999; Dubois *et al.*, 2000). In striped bass, Chow and coworkers (1998) analyzed the sbGnRH gene sequence and found a glucocorticoid receptor element present. Chow and coworkers (1998) found other potential GnRH regulators in both sbGnRH and cGnRH-II in the striped bass. They suggest that the glucocorticoid receptor

(GR) influences GnRH synthesis as GRs were localized to GnRH neurons in the rainbow trout brain. This study also found AP-1 binding sites in both sbGnRH and cGnRH-II, and SF-1 DNA binding domain in sbGnRH. AP-1 binding sites have previously been found in sGnRH gene sequence in Atlantic salmon (Klungland *et al.*, 1992) and Oct-1, Brn-2 and AP-1 sites are reported for Pacific salmon (von Schalburg and Sherwood, 1999). In Atlantic salmon (*Salmo salar*) human estrogen receptor was shown to bind to two estrogen receptor element (ERE)-like motifs in the 5' flanking region of the GnRH gene (Klungland *et al.*, 1993). In rainbow trout, a potential ERE in the sGnRH gene sequence was reported (von Schalburg and Sherwood, 1999). Also, an ERE was found within the sbGnRH promoter (Chow *et al.*, 1998) and testosterone and estradiol both increased pituitary GnRH levels (Dubois *et al.*, 2000). However, more detailed studies in mammals found no estrogen receptors in GnRH-producing neurons so it is unlikely that estrogen has a direct effect on GnRH expression in the hypothalamus (Shivers *et al.*, 1983; Herbison *et al.*, 1993; Sullivan *et al.*, 1995).

GnRH Receptor

The GnRH receptor was first identified and characterized from mammalian species in the early 1990's, and it was found to trigger G-protein-modulated release of hormones, cytokines or growth factors (Alok *et al.*, 2000). The GnRH receptor is the smallest member of the G-protein-coupled receptor (GPCR) superfamily (Cornea *et al.*, 2001). In most vertebrates, two forms of the GnRH receptor are known, although a third type has been found in frogs, *Rana rana* (Neill, 2002). The first GnRH receptor is referred to as type I or mammalian receptor; the second is type II, which is both a

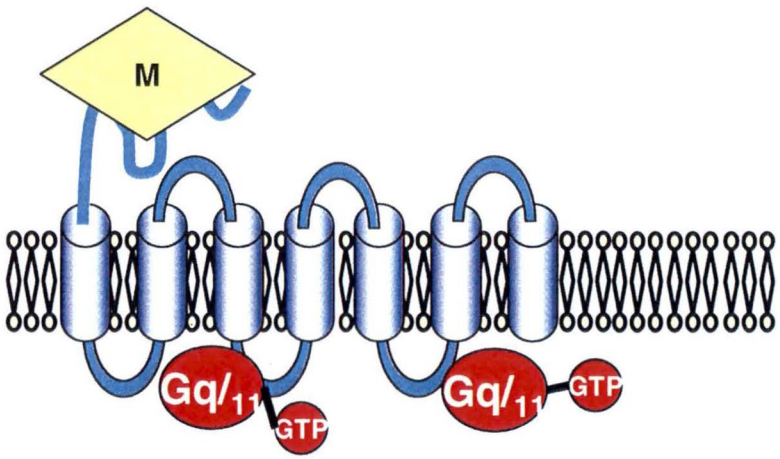
mammalian and non-mammalian receptor. Mammals studied thus far have both type I and II receptors (Millar *et al.*, 2001; Neill *et al.*, 2001; Neill, 2002), whereas other vertebrates usually only express type II receptors (Alok *et* al., 2000; Madigou *et al.*, 2000). Both type I and II receptors are 7 transmembrane with an extracellular N-terminal region (Figure 1.5). The two common receptors differ in C-terminal tail length and ligand binding. Type I receptors have virtually no C-terminal tail (Cornea *et al.,* 2001) and preferentially bind mGnRH (Neill *et al.,* 2001) whereas, type II receptors are functional with cGnRH-II (and some other GnRH forms, such as sGnRH) and have a cytoplasmic tail (Neill, 2002). The tail appears to be phosphorylated when GnRH binds, which results in receptor desensitization and internalization (Neill *et al.,* 2001). It is thought that the intracellular tail (C-terminal) is important for receptor functioning as truncation of this portion results in loss of ligand binding and GnRH-stimulated cAMP production (Blomenrorh *et al.*, 1997).

When GnRH binds to the receptor, it activates multiple signal transduction pathways such as protein kinase C (PKC), protein kinase A (PKA), arachidonic acid, and calmodulin (Alok *et al.,* 2000). It has been suggested that in mammals and goldfish the PKC pathway is used to cause synthesis and release of LH (in mammals) and gonadotropin II (in goldfish) from the pituitary (Alok *et al.,* 2000). In perciform fish, such as striped bass, cross talk between cAMP-PKA and PKC pathways are probably used (Alok *et al.,* 2000). Primates have both type I and II receptors; it is thought that the function of type II receptor is synergistic with type I as both are coupled to Gqα protein, which stimulates inositol phosphate production (Neill *et al.,* 2001). The binding of different GnRH forms to the receptor causes different receptor potencies. Weber and

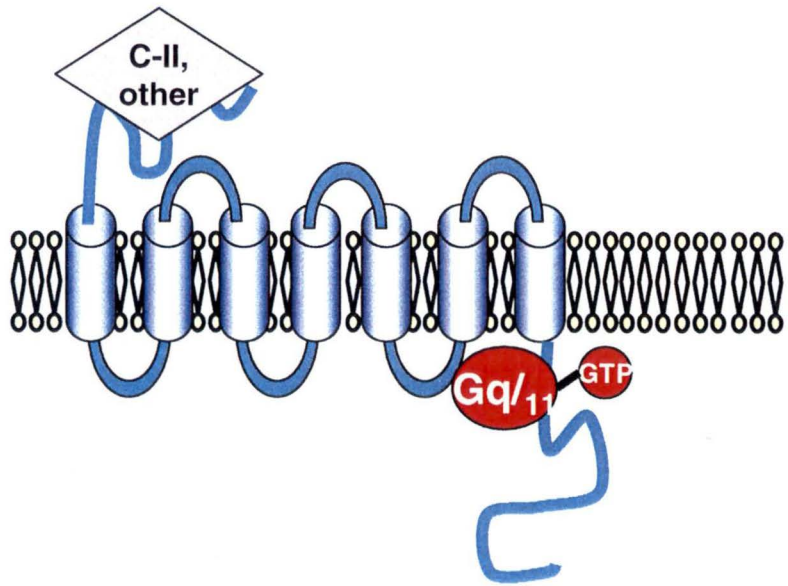
Figure 1.5. GnRH receptor.

A drawing that shows both Type 1 and Type 2 GnRH receptors. Both are 7 transmembrane G-protein-coupled receptors with an extracellular N-terminal tail. Type 1 binds only mammalian GnRH (M) and has no intracellular C-terminal tail. Type 2 binds chicken-II GnRH (C-II) and other GnRH forms and has an intracellular C-terminal tail (adapted from a drawing by Erica Fradinger).

TYPE 1



TYPE 2



coworkers (1997) suggested that the reasons for different releasing potential of prolactin by GnRH include: receptor affinity, signal transduction pathway and enzymatic degradation rates of GnRH at the pituitary. Also, the binding of different GnRH forms to the receptor may lead to distinct receptor conformational changes and alternative G-protein linkages (Chang *et al.*, 1993). Possible roles for type II receptor include induction of mating behaviour, suppression of cell growth in tumor cell lines (Neill *et al.,* 2001), and a regulatory role in hCG secretion (Cheng *et al.,* 2001).

Expression of the GnRH receptor is known in only a few species. Northern blot experiments in rainbow trout found GnRH type II receptors in the brain, pituitary and liver, whereas RT-PCR techniques revealed the receptor in ovary and retina also (Madigou *et al.*, 2000). In striped bass, the GnRH type II receptor is expressed in pituitary and in extrapituitary tissues (Alok *et al.,* 2000), but in humans type II receptor mRNA is expressed ubiquitously (Neill *et al.*, 2001). Cheng and coworkers (2001) mapped the promoters for GnRH receptor expression from human placenta and found that placental receptor expression is controlled by a distal promoter and pituitary receptor expression is controlled by a proximal promoter. They also identified a cAMP response element and AP-1 binding motif in the placental promoter that means receptor gene expression could be regulated by either the PKC or PKA pathway.

Lake Whitefish

Lake whitefish belong to the order Salmoniformes, family Salmonidae (Salmonids), subfamily Coregoninae, genus *Coregonus,* species *clupeaformis* (Nelson, 1994). The fossil record and morphological characteristics place lake whitefish as an

ancient (early-evolving) salmonid. It has an overall silver colour with darker dorsal scales and light brown fins (Figure 1.6).

Lake whitefish are widely distributed throughout North American fresh waters. Within Canada, lake whitefish are found in Newfoundland, New Brunswick, Quebec, Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia, and throughout the territories in most large lakes and larger rivers (Scott and Crossman, 1973). Lake whitefish were introduced into British Columbia, Newfoundland, and Alberta as a forage fish and as an attempt to establish commercial fisheries (Scott and Crossman, 1973).

Lake whitefish spawn in the fall, around November, or earlier the farther north the fish are located. Eggs are randomly deposited, usually in shallow water over a stony bottom. Males usually mature faster but die sooner that females (Scott and Crossman, 1973). They are a cool water species and so move to deeper, cooler waters in the summer in southern lakes.

Coregonus clupeaformis can grow up to 80 cm (Page and Burr, 1991) but average around 40 to 50 cm (Scott and Crossman, 1973). They weigh anywhere from 908g to 3.63kg but the commercial size for lake whitefish around 1kg; this weight is usually reached at age 3 or 4 years in eastern lakes and at age 9 or 10 in the west. It has been documented that lake whitefish from the Great Lakes can be either normal or dwarf. A normal lake whitefish at age 5 would be around 24 to 30 cm, whereas the dwarf lake whitefish measures 13-19 cm at the same age (Scott and Crossman, 1973).

Lake whitefish feed at the bottom of lakes during the summer and on plankton around spawning (Nelson, 1994). When at the bottom of lakes, they will eat mainly invertebrates such as insect larvae, molluscs and amphipods. When top feeding their diet

Figure 1.6. Lake whitefish.

A photograph of lake whitefish, *Coregonus clupeaformis*, that was caught at Exeter Lake in 100 Mile House, B.C. November, 2001.

consists of copepods, cladocerans, and terrestrial insects. Lake whitefish occasionally eat small fish like ninespine stickleback, Johnny darters and alewives (Scott and Crossman, 1973).

Also belonging to the genus *Coregonus* are ciscoes, which differ from lake whitefish by gill raker numbers (Nelson, 1994). Whitefish share the subfamily Coregoninae with *Stenodus* (Inconnu) and *Prosopium* (Round whitefishes). Salmonids include three related subfamilies: Coregoninae, Thymallinae, and Salmoninae (Figure 1.7).

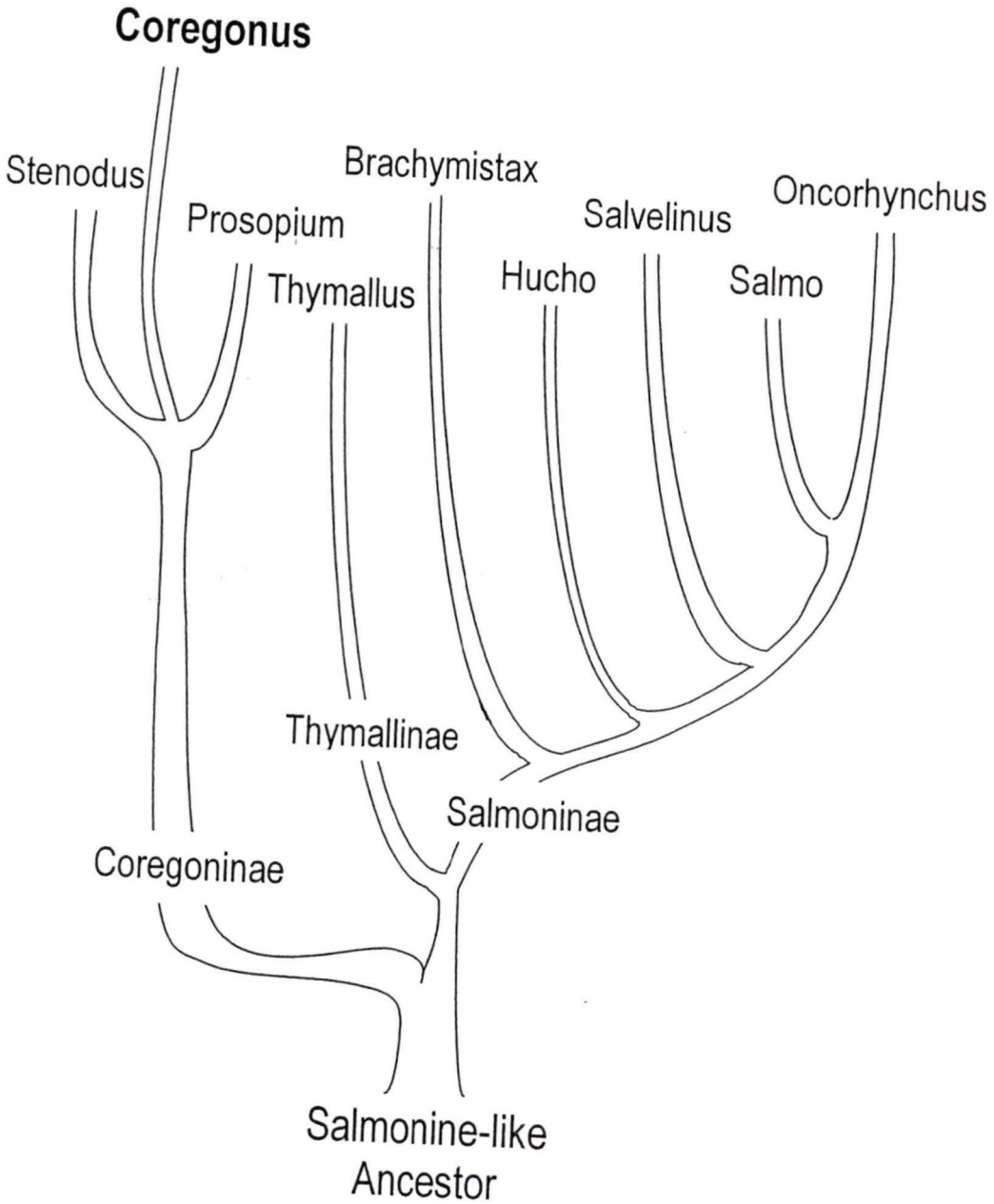
Our previous protein work determined the GnRH forms found in lake whitefish based on high-pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) techniques (Adams *et al.*, 2002). It was surprising to find that HPLC and RIA detected three forms of GnRH (sGnRH, cGnRH-II, and a new form of GnRH) as all other salmonids studied only show two (sGnRH and cGnRH-II). This lead to the isolation and sequencing of the novel peptide we labeled whitefish (wf)GnRH. This was the first salmonid reported to have three GnRH forms and we concluded that because lake whitefish is a basal salmonid and shows three forms, it is likely the tetraploid salmonid ancestor had at least three GnRH forms.

Salmonid tetraploidy event

Lake whitefish are part of the family Salmonidae whose ancestor underwent genome duplication causing a tetraploidy event. All salmonid subfamilies (Coregoninae, Thymallinae and Salmoninae) were affected by this event. This genomic duplication allowed the salmonids to be phenotypically plastic as duplicate genes would be free to assume new functions. The evidence supporting the tetraploid salmonid ancestor comes

Figure 1.7. Cladogram of Salmoniformes.

A cladogram that shows the order Salmoniformes (family Salmonidae), including the salmonid ancestor. The genus for lake whitefish, *Coregonus* (bold), appears early in the evolution of salmonids (drawn by Gresham Smith).


Coregonus
Stenodus
Prosopium
Brachymistax
Thymallus
Salvelinus
Oncorhynchus
Hucho
Salmo
Thymallinae
Salmoninae
Coregoninae
Salmonine-like
Ancestor

from Allendorf and Thorgaard (1984). For example, the salmonid genome size is approximately twice that of closely related fish with respect to both the amount of DNA per cell and chromosome arm number. Lake whitefish have a chromosome number of 2n=80 and chromosome arm number of 100 (Phillips and Rab, 2001). Phillips and Rab (2001) think that the tetraploid salmonid ancestor had a chromosome number of 2n=96 and chromosome arm number of 96. They concluded that lake whitefish chromosomes evolved by Robertsonian type centric fusions decreasing chromosome number while retaining chromosome arm number close to the hypothesized tetraploid ancestor. It appears that salmonids are undergoing karyotype orthoselection where Robertsonian fusions are causing lower chromosome numbers. These chromosome rearrangements are either occurring by random drift and population bottlenecks without selective advantage or by selective advantage where rearrangements are adapting the species to its environment (Phillips and Rab, 2001). With respect to the GnRH gene, if the diploid ancestor had two forms of GnRH then the tetraploid fish would express four (as each GnRH form is encoded on a separate gene). So far, the closest to this number is found in lake whitefish, the only salmonid to express three GnRH forms. The fourth form may be expressed but identical to its duplicate, or not yet detected, or mutated so that the gene is neither expressed nor functional. However, if the diploid ancestor had three GnRH forms as in the herring, then the tetraploid ancestor should have had six forms of GnRH, which leaves some of these genes undetected.

Hypotheses and Objectives

I formulated two hypotheses for this research based on literature available for GnRH. The first is that cDNA sequences obtained from lake whitefish will have similar structure to other known GnRH cDNAs. The second is that wfGnRH is the form responsible for gonadotropin release in lake whitefish.

There are three objectives in this study: (1) to obtain the cDNA sequences of each form of GnRH in lake whitefish, (2) to determine the releasing potential of wfGnRH on pituitary cells, and (3) to determine the location of each GnRH form in the whitefish brain using *in situ* hybridization.

In the first objective, I continued the characterization of GnRH in lake whitefish by sequencing the cDNA of each GnRH form found in this fish. The nucleotide sequence of each cDNA reveals how GnRH is encoded within the precursor and shows whether the gene is transcribed as mRNA in the brain. The cDNA sequences are needed to show that the gene is transcribed, and to make specific probes for localization of each GnRH form by *in situ* hybridization. The *in situ* method is better than immunocytochemistry in distinguishing the three GnRH forms. Furthermore, the cDNA sequences can be used for phylogenetic analysis (such as trees).

In the second objective I determined the biological activity of wfGnRH by measuring its releasing effect on pituitary cells. This is preliminary work to determine whether wfGnRH causes gonadotropin release *in vitro*. It examines whether wfGnRH is bioactive within salmonids and potentially could be used in fish aquaculture.

In the third objective, I determined the brain location of neurons that are expressing each of the different GnRH forms. With more than one GnRH form being

expressed in the brain, it is of interest to determine the location of these forms to see if there is any overlap. Also, the location within the brain may provide some insight into the function of each GnRH form.

All of the above data provide valuable information for GnRH characterization, especially in fresh water teleosts, and may shed some light onto the evolution of this peptide, and the tetraploidy event in salmonids.

Chapter 2

cDNA structure of three GnRH forms from a basal salmonid, lake whitefish (*Coregonus clupeaformis*)

Introduction

GnRH has been well characterized among salmonids with salmon (s)GnRH and chicken (c)GnRH-II as the two forms found to date. These forms are also common to other species. For example, cGnRH-II is found in cartilaginous fish, bony fish, amphibians, reptiles, birds and mammals, including humans. Salmon GnRH is more specific appearing in most teleosts such as bony tongue fish, herring, goldfish, carp, salmonids, pacu, rockfish, sea bream, cichlid and winter flounder. The amino acid sequence of the cGnRH-II region and cut site is the same in every species that expresses cGnRH-II. The signal peptide and GAP regions are slightly different across species due to differences in nucleotide sequences. Salmon GnRH follows the same pattern. It is also interesting to note that the same GnRH forms show more nucleotide similarities in different species than different GnRH forms found within a species. That is, the similarity of the nucleotide sequence of rainbow trout sGnRH cDNA compared to sea bream sGnRH cDNA is greater than that of than rainbow trout sGnRH compare to rainbow trout cGnRH-II. This is useful knowledge when designing primers for GnRH forms not yet known. I used rainbow trout sGnRH and cGnRH-II cDNA sequences to design primers to isolate lake whitefish sGnRH and cGnRH-II, respectively. Two sets of primers, one for sGnRH and different ones for cGnRH-II, were needed because I expected each GnRH form to be encoded on a different gene. This assumption was based on the knowledge that multiple GnRH forms expressed in vertebrate species are encoded on different genes (Chow *et al.*, 1998).

To completely characterize GnRH, the amino acid sequence of the peptide, cDNA precursor nucleotide sequence, and the complete gene sequence should be identified. The protein (i.e. amino acid sequence) is the biologically active form of GnRH and is needed for physiological studies. The cDNA sequence, which is essentially the mRNA, can be used to make probes (either DNA or RNA) that would show tissue location and when (i.e. different seasons or during development) the GnRH transcript is expressed. The gene sequence would be present in every cell, but is needed to study GnRH regulation. In this study, cDNA sequences were obtained, as we previously identified the amino acid (protein) structures. It is important to determine the exact mRNA (cDNA) sequence for each form of GnRH in lake whitefish, as it allows primers and probes that exactly match the endogenous GnRH forms to be designed. These are needed to study tissue expression and localization of GnRH. For example, von Schalburg and Sherwood (1999) found two different mRNA sequences in rainbow trout that both encoded sGnRH on duplicated genes. In addition, alternative splicing of one of the primary transcripts of sGnRH resulted in different mRNA transcripts in the brain and gonad.

Also, mRNA sequences have provided us with some information on the evolution of GnRH. Messenger RNA sequence similarity can be compared between species to reveal conserved portions of the gene with survival importance. Less conserved areas are able to change (mutate) over time without harming the species. For example, exon 2 of the GnRH gene encodes the GnRH hormone, cut site and beginning of the GAP. It is this exon that shows the most sequence similarity among different GnRH forms and also among genes coding the same forms found in different species. This shows the importance of this area for species survival. In contrast, changes are often seen within

both the 5' and 3' UTR regions, the signal peptide and GAP regions indicating that among species, strict conservation of these areas are less important for function.

Our previous protein work revealed for the first time three GnRH forms in a salmonid (Adams *et al.*, 2002). We used high-pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) techniques to characterize the three GnRH forms in lake whitefish: sGnRH, cGnRH-II and a novel form we labeled whitefish (wf)GnRH. Lake whitefish is a basal salmonid as it branches early from the common ancestor (see Figure 1.7). The objective of this part of my project was to determine the cDNA structure of each GnRH form found in lake whitefish as this is the first salmonid found to have three forms of GnRH instead of two. This information not only further characterizes GnRH in lake whitefish but is also necessary for making probes for future work such as Northern blot and *in situ* hybridization analysis. Furthermore, it allows for phylogenetic analysis (such as trees) to study the relationship of the novel wfGnRH to the other two salmonid forms and to other GnRHs.

Materials and Methods

Tissue Collection and RNA Isolation

For sGnRH and cGnRH-II isolation, twelve lake whitefish (*Coregonus clupeaformis*) brains (six male, six female) from the Freshwater Research Institute in Winnipeg were used. Brain tissue was ground to a powder using a cold mortar and pestle in liquid nitrogen. Total RNA was isolated using TRIzol RNA isolation reagent (Life Technologies, Burlington, ON), based on the acid guanidinium thiocyanate-phenol-

chloroform extraction method (Chomczynski and Sacchi, 1987). Messenger RNA was extracted from total RNA using Ambion's Micro Poly(A)$^+$ Pure kit (Ambion Inc., Austin, TX) as per manufacturer's directions. The concentration of mRNA was quantified by a spot test on an ethidium bromide agar plate. Messenger RNA was stored at -80°C.

For wfGnRH isolation, several collections of whitefish brains were required. No wfGnRH cDNA was found in tissue used for the sGnRH and cGnRH-II isolation from the Winnipeg hatchery (these fish were not kept under a normal photoperiod and would never spawn), or from a collection done at Exeter Lake in 100 Mile House, BC in November 2001 (n=2, both females). Rather, wfGnRH cDNA was isolated from RNA obtained from two (one male, one female) of the four lake whitefish brains (one male, three female) that were gill netted in June 2002 at Exeter Lake. Brain tissue was powdered as above and mRNA was extracted directly from ground tissue, quantified, and stored as above.

General cDNA synthesis and amplification protocol

For sGnRH and cGnRH-II isolation, both 5' and 3' rapid amplification of cDNA ends (RACE) products were done using SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA) by following the manufacturer's instructions. For wfGnRH isolation, 5' and 3' RACE products were made using Ambion's FirstChoice RNA Ligase Mediated (RLM)-RACE (Ambion Inc.) as per the manufacturer's directions. The 3' RACE procedure used a gene-specific forward primer and a universal anchor primer to obtain the 3' end of all three GnRH cDNAs. Similarly, the 5' RACE procedure used a reverse gene-specific primer and a universal anchor primer to obtain the 5' end of all three GnRH cDNAs. Generally, PCR mixtures contained 8µl of 10mM dNTP, 5µl of

10x PCR buffer (200 mM Tris-HCl pH 8.4, 500mM KCl and no Mg), 2μl of 50 mM $MgCl_2$, 0.5μl TAQ DNA Polymerase (Gibco, Burlington, ON), 1μl of each primer, 1μl of template DNA, topped to 50μl with sterile water. One drop of mineral oil was added to each tube to prevent evaporation of the mixture during amplification.

sGnRH cDNA

For 3' RACE first round PCR, a degenerate gene-specific primer was designed against the cDNA sequence of other known salmonid sGnRH regions. One μl (20μM) of this primer, salmon forward 1 better (sf1B), was used with 5μl of Clontech's 10x universal primer mix (see Table 2.1 for primer sequence, and Figure 2.1 for primer binding sites) following the protocol above except that the water volume was adjusted due to increased volume of primer. This product was used as DNA template for nested PCR with a second, gene-specific primer also made against sGnRH including the cut site from other salmonid cDNA sequences. One μl of 20μM salmon forward 2 better (sf2B) primer and 1μl of 10μM Clontech's nested universal primer (Table 2.1, Figure 2.1) were used for nested PCR. First round consisted of one 3 minute period at 94°C, followed by 35 rounds of a three step cycle including, denaturation at 94°C, annealing at 45°C, and extension at 72°C, 30s each, and finished with one 7 minute extension at 72°C. The nested round was similar except with an annealing temperature of 50°C. Nested products were separated on an electrophoresis gel and the bands between 200-400bp in length were ligated into the pGEM T-Vector plasmid (Promega, Madison, WI). Next, plasmid DNA was incorporated into *E. coli* XL-1 blue competent cells (Stratagene, Cedar Creek, TX) by electrotransformation. Colonies were grown on antibiotic treated plates (100

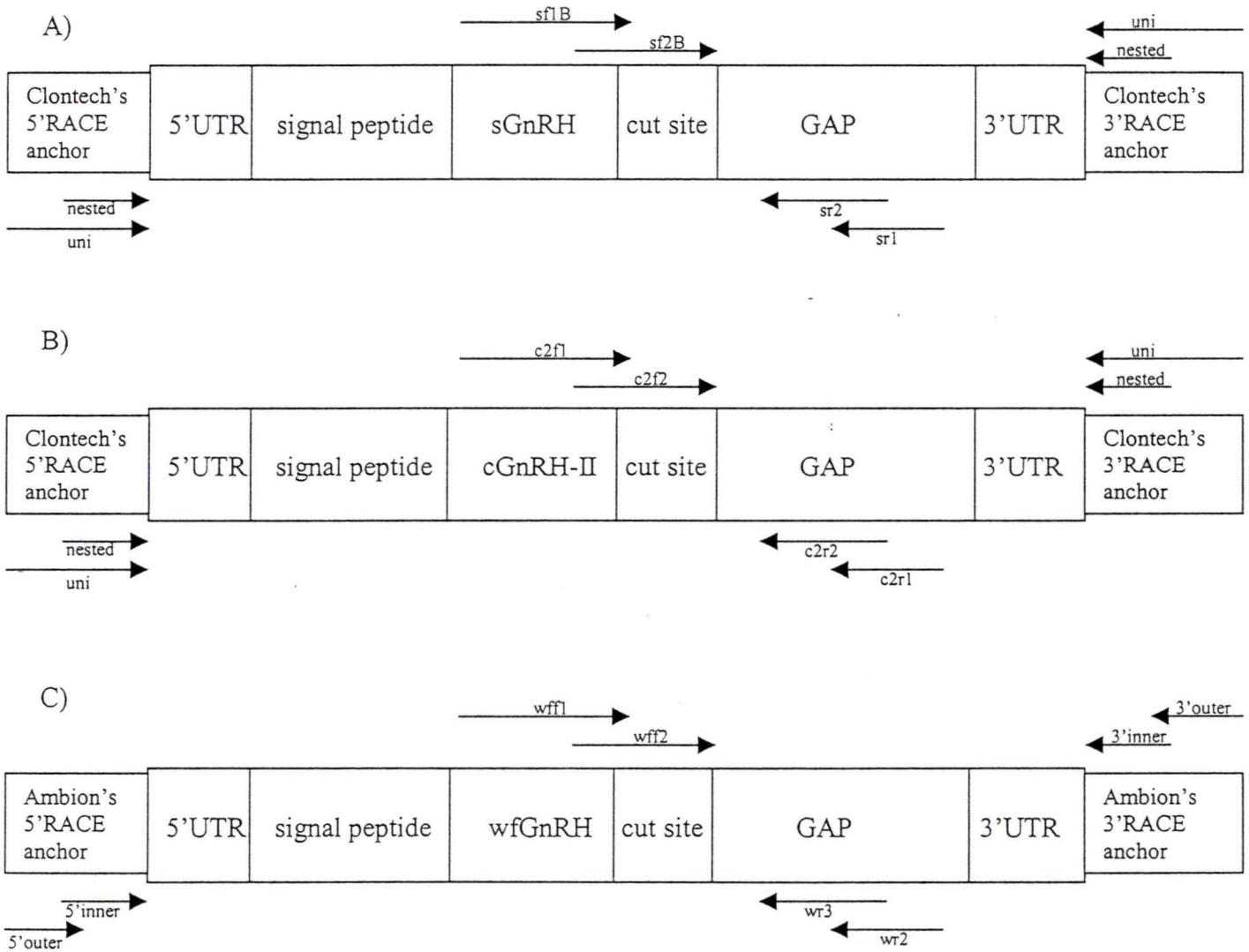
Table 2.1. 5' and 3' PCR primer sequences.

A list that shows the primers used in 5' and 3' RACE for amplifying and isolating sGnRH, wfGnRH, and cGnRH-II from lake whitefish. 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.

Primer Name	Primer Sequence
sf1B - salmon forward 1 better	5'-cagcactggtcgtatggvtgg-3'
sf2B - salmon forward 2 better	5'-ggctacctggaggraagagaa-3'
sr1 - salmon reverse 1	5'-cttcacctcctgtgtccatcatc-3'
sr2 - salmon reverse 2	5'-atcctgatggtggcctccagctc-3'
uni - Clontech's 10x universal primer mix	long (0.2uM) : 5'-ctaatacgactcactatagggcaagcagtggtaacaacgcagagt-3' short (1uM): 5'-ctaatacgactcactatagggc-3'
nested - Clontech's universal nested	5'-aagcagtggtaacaacgcagagt-3'
c2f1 - chicken II forward 1	5'-cagcactggtcncayggntggta-3'
c2f2 - chicken II forward 2	5'-tggtcncayggntggtayccngg-3'
c2r1 - chicken II reverse 1	5'-cttcgctgtggtctcaggtagct-3'
c2r2 - chicken II reverse 2	5'-ctgcattctcctgcctcacaga-3'
3' outer - Ambion's 3'RACE outer primer	5'-gcgagcacagccttaatacgact-3'
3' inner - Ambion's 3'RACE inner primer	5'-cgcggatccgaattcatacgactcactatagg-3'
5' outer - Ambion's 5'RACE outer primer	5'-gctgatggcgatgaatgaacactg-3'
5' inner - Ambion's 5'RACE inner primer	5'-cgcggatccgaacactgcgtttgctggctttgatg-3'
wff1 - whitefish forward 1	5'-cagcactggtcgtatggvatg-3'
wff2 - whitefish forward 2	5'-atgaaycctggaggvaagagr-3'
wr2 - whitefish reverse 2	5'-gcaagtaatgcctcagcct-3'
wr3 - whitefish reverse 3	5'-gcctgtacattttggcatgag-3'

Figure 2.1. cDNA gene-specific primers.

A diagram that shows where gene-specific primers bind to GnRH mRNA. Clontech's anchor sections were added to mRNA during rapid amplification of cDNA ends (RACE) preparation. A) sGnRH mRNA with 3' RACE primers are indicated by top arrows, and 5' RACE primers are indicated by bottom arrows. B) cGnRH-II mRNA with 3' RACE primers are indicated by top arrows, and 5' RACE primers are indicated by bottom arrows. C) wfGnRH mRNA with 3' RACE primers indicated by top arrows, and 5' RACE primers indicated by bottom arrows. Where s = salmon, c = chicken, wf = whitefish; salmon forward primers are sf1 and sf2, salmon reverse primers are sr1 and sr2; chicken-II forward primers are c2f1 and c2f2, chicken-II reverse primers are c2r1 and c2r2; Clontech's primers are universal mix (uni) and universal nested (nested); whitefish forward primers are wff1 and wff2, whitefish reverse primers are wr2 and wr3; and Ambion's primers are 3' RACE outer (3'outer) and inner (3'inner) primers and 5' RACE outer (5'outer) and inner (5'inner) RACE.


A)
sf1B
sf2B
uni
nested
Clontech's 5'RACE anchor
5'UTR
signal peptide
sGnRH
cut site
GAP
3'UTR
Clontech's 3'RACE anchor
nested
uni
sr2
sr1
B)
c2f1
c2f2
uni
nested
Clontech's 5'RACE anchor
5'UTR
signal peptide
cGnRH-II
cut site
GAP
3'UTR
Clontech's 3'RACE anchor
nested
uni
c2r2
c2r1
C)
wff1
wff2
3'outer
3'inner
Ambion's 5'RACE anchor
5'UTR
signal peptide
wfGnRH
cut site
GAP
3'UTR
Ambion's 3'RACE anchor
5'inner
5'outer
wr3
wr2

mg/ml Ampicillin, 0.1M I-PTG, 20 mg/ml X-Gal), then recombinant colonies were picked and grown overnight in LB broth (with 100 mg/ml Ampicillin). All DNA was isolated using Qiagen's QIAprep Spin Miniprep kit (Qiagen, Chatsworth, CA) as per manufacturer's instructions. A small amount of the DNA was digested using the restriction enzymes Pst-1 and Sph-1 at 37°C for two hours, and the digest was separated on a 1.5% agarose gel. Cut DNA was stained in ethidium bromide and inserts of desired size were sent for sequencing (Glickman lab at the Centre for Biomedical Research, University of Victoria). Sequencing was done in both forward and reverse directions.

The 5' RACE PCR protocol was very similar to above except different gene-specific primers and annealing temperatures were used. For first round amplification, salmon reverse 1 (sr1) primer, made against the GAP region of lake whitefish sGnRH sequenced above, was used with the universal primer mix. For nested PCR, salmon reverse 2 (sr2) primer, again made against the GAP region of whitefish sGnRH slightly upstream of reverse 1, was used with the universal nested primer (Table 2.1, Figure 2.1). A first round annealing temperature of 50°C and nested round annealing temperature of 56°C were used. Nested round PCR products were sequenced as above.

cGnRH-II cDNA

3' and 5' RACE PCR used the same protocol as above but with different gene-specific primers and annealing temperatures. In first round 3' PCR, primer chicken 2 forward 1 (c2f1), made against cGnRH-II regions of other salmonid cDNA sequences was used with an annealing temperature of 50°C. For the nested round, primer chicken 2 forward 2 (c2f2), made against the GnRH and cut site regions of salmonid cGnRH cDNA

sequences was used with an annealing temperature of 55°C (Table 2.1, Figure 2.1). Nested round PCR products were cloned and sequenced as described above. To amplify the 5' end of cGnRH-II, primer chicken 2 reverse 1 (c2r1), made against the GAP region of lake whitefish cGnRH-II sequenced above was used at an annealing temperature of 52°C for first round amplification. Chicken 2 reverse 2 (c2r2), made slightly upstream from reverse 1, was used with an annealing temperature of 58°C for nested PCR (Table 2.1, Figure 2.1). Nested PCR products were cloned and sequenced.

wfGnRH cDNA

3' and 5' RACE were also performed to isolate the wfGnRH sequence, but a slightly different protocol was used. For 3' RACE first round PCR, a degenerate gene-specific primer was designed against the first 7 amino acids of wfGnRH (Table 2.1, Figure 2.1). One μl (20μM) of this primer, whitefish forward 1 better (wff1B), was used with 2μl of Ambion's 10μM 3' RACE outer primer (Table 2.1, Figure 2.1) following the protocol above except the water volume was adjusted due to increased volume of primer. This product was used as DNA template for nested PCR with a second, gene-specific primer also made against the amino acid sequence of wfGnRH (amino acids 6-10) and the first two amino acids of the cut site from other salmonid cDNA sequences. One μl of 20μM whitefish forward 2 better (wff2B) primer and 2μl of Ambion's 10μM 3' RACE inner primer (Table 2.1, Figure 2.1) were used for nested PCR. First round consisted of one 3 minute period at 94°C, followed by 35 rounds of a three step cycle including, denaturation at 94°C, annealing at 50°C, and extension at 72°C, 30s each, and finished with one 7 minute extension at 72°C. The nested round was similar except with an annealing temperature of 55°C. Nested products were separated on an electrophoresis gel

and the band of desired size was ligated into pGEM T-Vector, amplified and sequenced as described above with the sGnRH cDNA methods.

The 5' RACE PCR protocol was very similar to above except that different gene-specific primers and annealing temperatures were used. For first round amplification, whitefish reverse 2 (wr2) primer, made against the GAP region of lake whitefish wfGnRH sequenced above, was used with the 5' RACE outer primer. And for nested PCR, whitefish reverse 3 (wr3) primer, again made against the GAP region of whitefish wfGnRH slightly upstream of reverse 2, was used with the 5' RACE inner primer (Table 2.1, Figure 2.1). A first round annealing temperature of 55°C and nested round annealing temperature of 57°C were used. Once again the PCR product was ligated into pGEM T-Vector, amplified, and sequenced in both directions as above.

Results

I have isolated three cDNA sequences that encode sGnRH, wfGnRH, and cGnRH-II separately from lake whitefish using 5' and 3' RACE and nested PCR with gene-specific oligonucleotide primers and universal anchor primers.

The cGnRH-II cDNA was discovered by separately sequencing both the 3' and 5' ends. The 3' end product was 428bp and spanned the last 8 amino acids of cGnRH-II, the cut site, GAP, and the 3'UTR. The 5' end product was 441bp and spanned the 5'UTR , signal peptide, cGnRH-II, cut site and into the GAP. The whole cGnRH-II cDNA was formed by overlapping the 3' and 5' sequences. It is 673bp long, containing a 239bp 5'UTR, a 258bp open reading frame (encoding the signal peptide, cGnRH-II, cut site, and GAP), a stop codon (TAA), and a 193bp 3'UTR (Figure 2.2). The open reading

Figure 2.2. Lake whitefish derived cGnRH-II cDNA

Nucleotide and amino acid sequence encoding the cGnRH-II 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. Chicken GnRH-II and the nucleotides corresponding to the polyadenylation signal (aataa) are in bold. The dash (-) indicates the stop codon.

```
acgcgggaagcactggtatcaacgcagagtacgcggggagagatatcatcagaggccagctctcta  66

caacactcatcgtcgcctcaggaaaagtgatttgtcactgagttgctggataccaacaggttaata  132

ataggcagaagtgaacagcgggcacacagacgtacctggcggacatcaacagtgataaccgattcc  198

ataccacagtgattatcagactaagggtctaagctccagtg ATG GTC AGT GTG GCT AGA   257
                                            M   V   S   V   A   R     6

CTG GTG TTT ATG CTG GGG CTG CTG CTG TGT CTG GGA GCC CAG CTG TCC TCC  308
 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  356
 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  407
 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  458
 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  511
 L   L   D   A   L   A   R   E   F   E   K   R   K   -                86

tggttgacttaaatccttccttgtctatctgtttattccatatgaccactattttaatcttatgtcc  578

tttgatccaattttgtggtttatccattttgataataatgtagcatcactcctttacccttgcaccc  645

tctttattgtccctttttccgctgtag                                          673
```

frame (ORF) starts at the first ATG codon, finishes at the stop codon, and contains a polyadenylation signal (aataa) that is 57bp upstream from the poly A tail.

The sGnRH was also identified by sequencing separately 3' and 5' ends. The 3' end product was 382bp and encoded the last 4 amino acids of sGnRH, the cut site, GAP and 3'UTR. The 5' end was 207bp and encoded the 5'UTR, signal peptide, sGnRH, cut site, and 3 amino acids of the GAP region. The whole sGnRH cDNA was formed by overlapping regions of the 5' and 3' sequences to give a 491bp cDNA that consists of a 44bp 5'UTR, a 249bp ORF, a stop codon (TAA), and a 195bp 3'UTR (Figure 2.3). Similar to above, the ORF starts at the first ATG codon, finishes at the stop codon and contains a polyadenylation signal that is only 9bp upstream from the poly A tail.

The full-length wfGnRH cDNA was obtained by overlapping the separately sequenced 3' and 5' ends. The 3' end product was 402bp and spanned the last 3 amino acids of wfGnRH, the cut site, the GAP region, and the 3'UTR. The 5' end product was 293bp and spanned the 5'UTR , signal peptide, wfGnRH, cut site, and into the GAP. The full-length wfGnRH cDNA is 511bp long, containing a 67bp 5'UTR, a 279bp ORF (encoding the signal peptide, wfGnRH, cut site, and GAP), a stop codon (TAA), and a 162bp 3'UTR (Figure 2.4). The ORF starts at the first ATG codon, finishes at the stop codon, and contains a polyadenylation signal (aataaa) that is 20bp upstream from the poly A tail.

At the DNA level, the ORF of lake whitefish cGnRH-II showed the greatest nucleotide identity (88%) with rainbow trout cGnRH-II, as indicated by a BLAST search (www.ncbi.nlm.nih.gov/blast/); the ORF of lake whitefish sGnRH is most like Atlantic

Figure 2.3. Lake whitefish derived sGnRH cDNA.

The nucleotide and 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 2.4. Whitefish GnRH cDNA.

The nucleotide and amino acid sequence that encodes the wfGnRH cDNA 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. 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
```

salmon sGnRH (93% nucleotide identity), and the ORF of the novel wfGnRH had less than 10% nucleotide sequence identity when compared to sbGnRH (*Haplochromis burtoni*). However, when the nucleotide sequence is translated into amino acid sequence, wfGnRH shows 60% amino acid identity (76% similarity) with *Haplochromis burtoni* sbGnRH; lake whitefish sGnRH has 79% amino acid identity (81% similarity) with Atlantic salmon sGnRH; and lake whitefish cGnRH-II has 79% amino acid identity (82% similarity) with rainbow trout cGnRH-II.

Discussion

I determined the full-length cDNA sequences for sGnRH, wfGnRH, and cGnRH-II from the brains and pituitaries in lake whitefish by RT-PCR using overlapping 5' and 3' RACE sequences. The GnRH cDNA sequences for both the sGnRH and cGnRH-II forms in lake whitefish are similar to those found in other teleosts, especially salmonids, which is not surprising considering that lake whitefish is a basal salmonid. One of the most interesting results was that wfGnRH tested with the BLAST database did not show significant nucleotide sequence identity with any other known GnRH sequences. However, the translated amino acid sequence confirmed its identity as a GnRH. It was also unexpected that wfGnRH was similar to sbGnRH (evolutionary implications are discussed later). Structurally, all three cDNAs have similar organization to all other GnRH cDNA sequences. Each sequence consists of 5'UTR, signal peptide, GnRH, GnRH-associated peptide (GAP), and 3'UTR, as seen in other vertebrate GnRH

sequences. The regions that are most conserved encode the GnRH peptide and the cut site.

Among the GnRH cDNA sequences from lake whitefish, cGnRH-II is the longest at 673bp, then wfGnRH at 511bp, and sGnRH at 491bp. The major length differences are found within the 5'UTR. For example, the 5'UTR in whitefish cGnRH-II is 239bp, whereas the same region is only 44bp in sGnRH, and 67bp in wfGnRH. This difference could be a result of mutations/deletions over evolution in the 5'UTR regions. The difference may also be explained if the isolation of the sGnRH 5'UTR region were incomplete, or if there were some intron sequence included in the cGnRH-II 5'UTR. The latter seems most likely when the salmon and chicken-II 5'UTR regions are compared to other species. The 5'UTR region for sGnRH is similar in length in Atlantic salmon (49bp) and in lake whitefish (44bp), whereas the 5'UTR of rainbow trout cGnRH-II is reported to be only 27bp (Penlington *et al.*, 1998) which is much shorter than whitefish cGnRH-II 5'UTR of 239bp. The short 5'UTR of rainbow trout cGnRH-II may be due to the method used for cDNA isolation, which was reverse transcription from mRNA only. The complete lake whitefish cGnRH-II gene needs to be sequenced to determine whether some of the cGnRH-II 5'UTR is intron or not. Differences in 5'UTR regions have been described before. For example, Torgersen and coworkers (2002) reported a sGnRH 5'UTR from zebrafish of only 9 nucleotides. The fact that there are variations in the 5'UTR region indicates that this region is more difficult to isolate. This may be due to the fact that there is not always selection for amplification of fragments corresponding to the actual 5' ends.

When the precursors are compared, the length of the signal peptide region is similar among all three lake whitefish GnRH cDNAs; the length of the GnRH is the same; and the length of the GAP region is between 47 and 57 amino acids. The lake whitefish cGnRH-II precursor has 88% nucleotide and 79% amino acid identity with rainbow trout cGnRH-II; the differences are only one amino acid within the signal peptide, no amino acids within GnRH, and 6 amino acids within the GAP region. The lake whitefish sGnRH precursor shows 93% nucleotide and 79% amino acid identity when compared to Atlantic salmon sGnRH with only 3 amino acid differences in the signal peptide, none in the GnRH region, and a 3 amino acid variation within the GAP region. These similarities suggest two things. One, the functions of the signal peptide and GAP are important. The signal peptide directs the hormone to the endoplasmic reticulum so it can be secreted from the cell. The GAP is thought to have some function in correct processing and packaging of GnRH (see Sherwood *et al.*, 1993). Both the above functions are essential for GnRH to be secreted properly. Two, the signal peptide and GAP regions, along with the GnRH peptide and cut site regions, may be used to determine how closely related two species are. Although sequence identity of the ORF is now being used to determine relatedness between species, the full genomic sequences in all organisms is known for only a few species. In such a case where the organism genome is not known, evolutionary clues can be obtained from comparing only one gene sequence between species. The GnRH gene is excellent for such a comparison as it is essential for species reproduction and ultimately species survival. We know that GnRH is present in every vertebrate species tested from the seven classes of vertebrates.

Sequencing and comparing GnRH cDNA sequences among species may provide clues to species relatedness.

The novel wfGnRH precursor reveals little nucleotide sequence identity to any known GnRH sequences within the database but shows 60% amino acid identity with *Haplochromis burtoni* sbGnRH. Within the GnRH coding region there is 80% amino acid sequence identity between wfGnRH and sbGnRH. This suggests that sbGnRH may have evolved from wfGnRH or vice versa. For this to occur the methionine and asparagine in positions 7 and 8 of wfGnRH would be substituted for leucine and serine in sbGnRH, respectively. The frequency of exchange from Leu↔Met based on a substitution frequency at an evolutionary distance of 2 accepted point mutations per 100 residues is 1.4% from Leu→Met and is 9.9% from Met→Leu (Schulz and Schirmer, 1979). The substitution would only require a single nucleotide change (TTG or CTG↔ATG). The second substitution of Ser↔Asn would also involve only one nucleotide (AGT↔AAT or AGC↔AAC) and has a forward exchange frequency of 5.1% and a backward exchange frequency of 6.7% (Schulz and Schirmer, 1979). This suggests a higher probability that sbGnRH evolved from wfGnRH and not vice versa. Of course there is always the possibility that both wfGnRH and sbGnRH evolved separately from a common ancestral GnRH form, such as salmon, mammalian, or herring.

Finding the different cDNA sequences in lake whitefish that encode different GnRH forms, supports the idea that multiple GnRH forms are encoded on different genes in vertebrates. Both lake whitefish sGnRH and cGnRH-II sequences are sufficiently similar to the corresponding GnRH sequences from rainbow trout (a closely related species) to conclude that I did isolate and sequence the GnRH cDNAs successfully. This

adds information to the evolution of GnRH by suggesting that the salmonid ancestor had both sGnRH and cGnRH-II, and that the cDNA sequences changed slightly as other salmonids evolved. The fact that wfGnRH could not be isolated from wild fish (taken in November from Exeter Lake) and hatchery fish (from Winnipeg) that could not spawn suggests the expression of wfGnRH may depend on season and/or sexual maturity. This does not seem to be the case for sGnRH and cGnRH-II expression as both were obtained from the above fish. The discovery of the novel wfGnRH cDNA suggests that the salmonid ancestor had at least three GnRH forms encoded on three separate genes with the potential of up to six forms, depending on the tetraploidy event. This is the first report of a salmonid expressing three GnRH forms and future work should be done to determine if other species closely related to lake whitefish express the wfGnRH form. It may be that other salmonids have encoded wfGnRH, but it is no longer expressed due to rearrangements within the chromosome or deletions/mutations within the gene or its promoter.

Chapter 3

Biological activity of wfGnRH and cGnRH-II from lake whitefish

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Introduction

The amino acid sequences for the GnRH forms in lake whitefish provide the information needed to make synthetic peptides to test for biological activity. This step will help to answer questions, such as, what is the function of each GnRH form in the brain? Do all three contribute to the release of gonadotropins from the pituitary or is just one form required? Various studies can be performed to obtain clues. The pituitary levels of each GnRH form, the binding affinity of each form to the pituitary GnRH receptor, and the ability of each form to stimulate the release of pituitary gonadotropins (GtH) can be measured. A combination of these types of experiments has revealed that both sGnRH and cGnRH-II potentially have equal releasing roles in goldfish. Chang and coworkers (1990) found both are equally effective at causing the release of GtH *in vitro* and Yu *et al.* (1988) reported similar pituitary levels for both in goldfish. However, in salmonids even though both sGnRH and cGnRH-II cause GtH pituitary release *in vitro*, cGnRH-II is not detected in the pituitary by immunocytochemistry (Amano *et al.*, 1991) suggesting only sGnRH has a physiological role. In the eel, mGnRH and cGnRH-II have the same GtH release activity *in vitro* (Montero, 1994) but pituitary levels of mGnRH are more abundant (Dufour *et al.*, 1993). Mammalian GnRH is concluded to have a major role in GtH control in the eel, whereas cGnRH-II may have a minor one. Catfish is completely different as cGnRH-II is more effective at releasing GtH in vivo than the cfGnRH form (Ngamvongchon *et al.*, 1992b). This is explained by the observations that cGnRH-II had a higher binding affinity for the GnRH receptor than cfGnRH (Schulz *et al.*, 1993). It is interesting to note that the same study revealed equal binding affinity of

cGnRH-II, sGnRH and mGnRH even though the last two forms are not found in catfish. Because cfGnRH is more abundant than cGnRH-II in the pituitary but cGnRH-II has a greater releasing potential it is suggested that both forms have a physiological role in catfish (Schulz *et al.*, 1993). From all the above studies, the general pattern that emerges is the form in the preoptic area is responsible for most of the GtH control with cGnRH-II playing a minor role. This pattern appears in species with only two GnRH forms. To answer the same questions for species with three forms, physiological studies are combined with brain location studies.

The objective of this project is to provide preliminary information on the physiological role of whitefish (wf)GnRH in lake whitefish. We tested the ability of wfGnRH and cGnRH-II to cause GtH release from pituitary cells *in vitro*. This shows whether wfGnRH is biologically active and provides a comparison with cGnRH-II. Remaining studies need to be done to test sGnRH releasing ability, pituitary levels and binding affinity of all three forms.

Materials and Methods

Usually biological activity is determined by injecting synthetic peptide into the fish and measuring the release of gonadotropins from the pituitary. However, we decided to measure the amount of GtH mRNA present in pituitary cells instead of measuring proteins because neither lake whitefish nor rainbow trout gonadotropins have been isolated as proteins, which are needed to prepare a radioimmunoassay. We used rainbow trout pituitary cells instead of lake whitefish because trout were more available. This was

a viable substitution as both species are closely related. We developed an assay for rainbow trout pituitary cell dispersion based on the procedures in Chang and Jobin (1994) with slight modifications. The University of Victoria Animal Care Committee approved all procedures. Trout were anesthetized in 50mg/L Eugenol and killed by decapitation. Pituitaries were removed, washed and treated using a combination of physical fragmentation and a trypsin/DNase treatment. Cells were washed three times in dispersion media (Medium 199 with Hanks' salts, 25mM HEPES, 2.2g/L sodium bicarbonate, 0.3% bovine serum albumin, 100 000 U/L penicillin, and 100 mg/L streptomycin, pH 7.2) for 5 minutes in a 18°C shaking water bath, cells were pelleted by a brief centrifugation at 2000xg and supernatant was removed. Cells were dispersed by three similar washes in trypsin type II (25mg in 10ml dispersion media), trypsin inhibitor (25mg in 10ml dispersion media) and DNAse II (0.1mg in 10ml dispersion media). Fragments were further dispersed in calcium-free media (Dubelco's PBS, 25mM HEPES, 2.2g/L sodium bicarbonate, 0.3% bovine serum albumin, 100 000 U/L penicillin, and 100 mg/L streptomycin, pH 7.2) by washing with 1mM EGTA in calcium-free media. This was followed by three washes of DNAse II in calcium free media (0.1mg in 10ml calcium-free media) with slow trituration, cells were allowed to settle before supernatant was removed. Cell yield and viability were determined at the time cells were harvested. They were then resuspended in culturing media, that contained Medium 199 with Earle's salts (Life Technologies, Burlington, ON), 25mM HEPES, 2.2 g/L $NaHCO_3$, 100 000 U/L penicillin and 100 mg/L streptomycin, pH 7.2. Cells were added to a culture plate (140 000 to 180 000 cells/well) and incubated at 18°C, 5% CO_2, and saturated humidity. After 2 hours, 1% horse serum (Life Technologies) was added to each well and cells were

incubated overnight under the same conditions as above. There were only enough cells to perform 4 treatments. Five wells of cells received no treatment (control), five wells were treated with synthetic wfGnRH 10^{-9}M, six wells with wfGnRH 10^{-7} M, and five wells (although one well was lost) with synthetic cGnRH-II 10^{-7} M for comparison. The synthetic peptides were prepared by Dr. J. Rivier at the Salk Institute (La Jolla, CA). All treatments lasted 12 hours, after which cells from each well were harvested separately for total RNA extraction as in the methods of Chapter 2. RNA concentration was quantitated by measuring absorbance at A_{260} on the spectrophotometer.

Total RNA (5ug) was run on a 1.5% agarose/formaldehyde gel at 30 volts for 4-5 hours. RNA from the gel was transferred to a GeneScreen plus nylon membrane (New England Nuclear, Boston, MA) using the capillary method with 0.1N NaOH. The membrane containing RNA was rinsed and then fixed by baking for 1 hour. Probes were made for the rainbow trout α-subunit that is shared by both gonadotropin (GtH) and thyroid stimulating hormone (TSH). Probes were purified cDNA fragments labeled with Random Primers DNA Labeling System (Life Technologies) with [α-^{32}P]-deoxycytidine 5'-triphosphate (dCTP) (3000 Ci/mmol) (New England Nuclear). For comparison, an 18S rRNA probe was made using an end-labeling kit (Amersham Pharmacia Biotech, Baie d'Urfé, QC) with [γ-^{32}P]-deoxyadenosine 5'triphosphate (dATP) (3000 Ci/mmol) (New England Nuclear). Next, the membrane was prehybridized at 55°C for 1.5-2 hours in 30ml of ULTRAhyb (Ambion, Austin, TX). Probes were added to the membrane in solution and hybridized overnight at 55°C. The following day the membrane was washed twice in 2% SSC and 0.2% SDS for 15 minutes each, followed by a 60 minute wash in 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) and signals were analyzed with ImageQuant software. Messenger RNA levels were compared to the 18S rRNA signal obtained for each lane and reported as a percentage with respect to the control. ANOVA and Student's t-test were performed to detect significant differences between groups ($p<0.05$).

Results

Figure 3.1 shows the bands from the Northern blot analysis. 18S rRNA bands were present in all three treatments and control, showing RNA isolation and transfer worked. Bands for 10^{-9}M wfGnRH and those for 10^{-7}M cGnRH-II appear similar to those in the control group, showing no significant increase of GtH/TSH-α subunit mRNA expression. However, bands for 10^{-7}M wfGnRH appear darker than control bands in the GtH/TSH-α subunit treatment revealing an increase in mRNA expression.

Results for the ANOVA and Student's t-test are reported as percent change compared to control. 10^{-7}M wfGnRH had the greatest effect on GtH/TSH-α subunit mRNA expression where it significantly increased expression by 90%. Although cGnRH-II (10^{-7}M) caused a 30% increase compared to the control, it was not statistically significant. Similarly, 10^{-9}M wfGnRH did not increase mRNA expression (Figure 3.2). When comparing the three treatments it appears that 10^{-7}M wfGnRH had the greatest effect followed by 10^{-7}M cGnRH-II, then 10^{-9}M wfGnRH.

Figure 3.1. GnRH northern blot analysis.

Messenger RNA bands for GtH/TSHα from northern blot analysis are shown after treatment of anterior pituitary cells with whitefish (wf)GnRH at 10^{-9} M, and 10^{-7} M, and chicken (c)GnRH-II at 10^{-7} M. Twenty separate wells were used with pituitary cells and the treatments shown. The mRNA from each well was isolated and individually loaded in one of the lanes. Lines and n number indicate number of individual experiments (bands) per treatment and control. 18S rRNA is shown as a positive control. The darkest bands within GtH/TSHα subunit mRNA appear from wfGnRH (10^{-7} M) treatment compared to control.

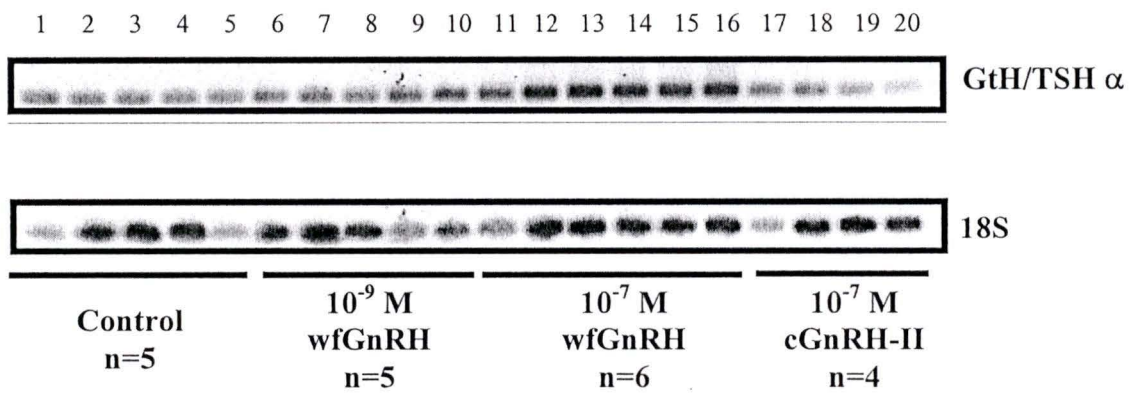
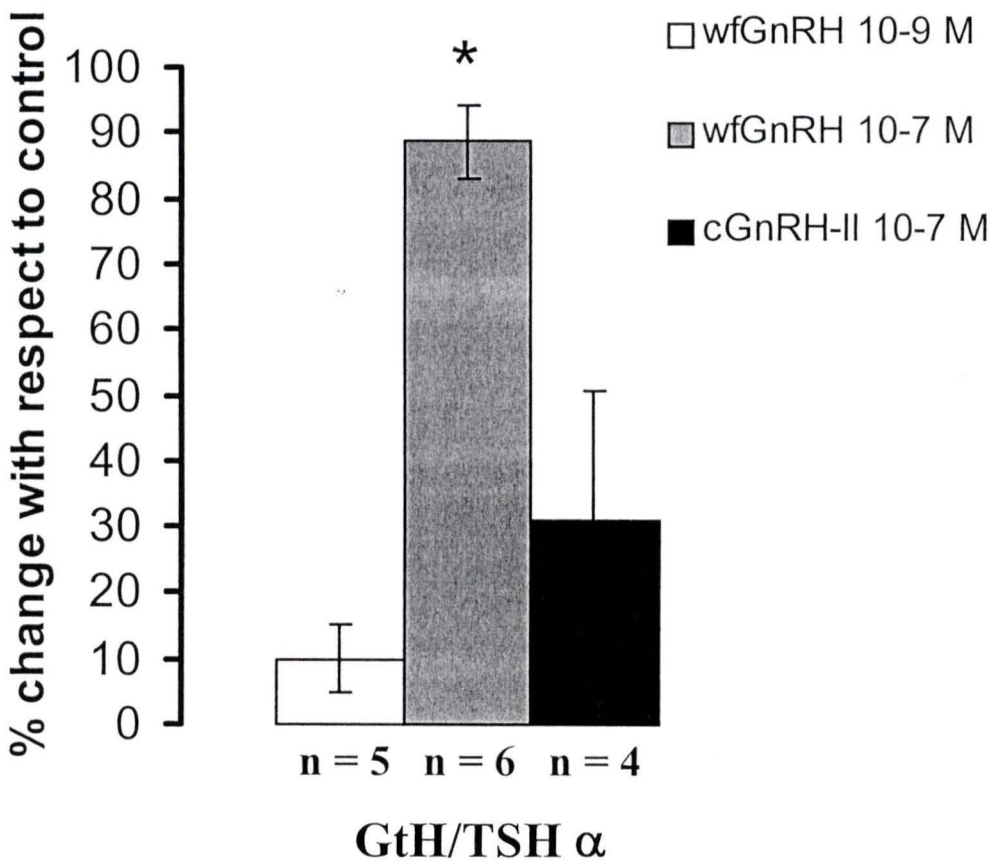

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
GtH/TSH α
18S
Control
n=5
10-9 M
wfGnRH
n=5
10-7 M
wfGnRH
n=6
10-7 M
cGnRH-II
n=4

Figure 3.2. Pituitary GtH mRNA expression.

Bar graph showing the expression of GtH/TSH α subunit mRNA from dispersed rainbow trout pituitary cells as percent change (±SEM) with respect to controls when treated with whitefish (wf)GnRH at 10^{-9} M and 10^{-7} M, and with chicken (c)GnRH-II at 10^{-7} M. The asterisk indicates a significant increase compared to control where $p>0.05$.


% 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
n = 5
n = 6
n = 4
GtH/TSH α

Discussion

We found that wfGnRH (10^{-7}M) was more effective at increasing GtH mRNA expression from rainbow trout (a closely related species to whitefish) pituitary cells *in vitro* than cGnRH-II (10^{-7}M). This assay examines the first step in pituitary activation, which is an increase in GtH mRNA. It will be important to confirm these results by measuring the level of GtH protein synthesis and release from lake whitefish pituitaries when this assay becomes available.

Other studies done on species with three GnRH forms expressed in the brain found slightly different results. One of the major problems in assessing the effect of GnRH in fish on the synthesis or release of GtHs is that purified GtH and an antibody are available for only a few fish. The most common assay system is the goldfish, which only biologically express sGnRH and cGnRH-II. This leads to problems detecting accurate releasing effects for any other GnRH form. For example, in pejerrey which express sGnRH, cGnRH-II and pejerrey (pj)GnRH, Montaner *et al.* (2001) found that sGnRH caused the greatest GtH release from goldfish pituitary cells *in vitro*, followed by pjGnRH, then cGnRH-II. However, an assay developed for pejerrey pituitary cells instead of goldfish may give results that would be slightly different and the species-specific pattern would emerge. It is unlikely that GnRH receptors in goldfish have a high binding affinity for pjGnRH because they do not express it. The pituitary levels of each form have been measured and only pjGnRH is present (Stefano *et al.*, 1997). This suggests that only pjGnRH plays a role in pituitary GtH release in pejerrey. Specific

brain locations have not been identified for each form yet. In herring the biological activity of the three GnRH forms, sGnRH, cGnRH-II and herring (hr)GnRH, have been reported (Carolsfeld *et al.*, 2000). Again these GnRH forms were tested with GtH release from goldfish pituitary cells *in vitro*. Carolsfeld and coworkers (2000) found cGnRH-II was most effective at releasing GtH followed by hrGnRH, then sGnRH. The same study also found that the non-native sea bream (sb)GnRH caused GtH release but was the least effective of the four GnRH forms. However, when herring pituitary content was measured, hrGnRH was the most abundant followed by sGnRH and cGnRH-II, suggesting hrGnRH plays the major role in herring and sGnRH and cGnRH-II play minor ones.

We suggest that because wfGnRH caused the greatest GtH mRNA synthesis in pituitary cells from rainbow trout, that it plays a major role in GtH release in lake whitefish. This preliminary study follows the general pattern observed in species with only two GnRH forms. The next step is to determine how effectively sGnRH activates GtH mRNA synthesis. Brain location studies would also provide clues as to specific GnRH function of each form found in lake whitefish. The combination of biological activity of wfGnRH in the pituitary and location of cell bodies in the preoptic area of the brain near to the pituitary for wfGnRH would help to establish wfGnRH as the main neuropeptide in the control of reproduction in lake whitefish. Also, extracting GnRH peptide from pituitaries would determine the form responsible for gonadotropin-release. Another interesting study would be to test the binding affinity for each GnRH form to any GnRH receptors found in the pituitary of lake whitefish. This would also provide information on GnRH function.

Chapter 4

Localization of brain GnRH in lake whitefish

Introduction

Localizing GnRH forms in the brain of lake whitefish should provide more insight into the function of each GnRH form found in the brains. However, it is critical that each form is detected by *in situ* hybridization for specificity of mRNA in cell bodies and by immunocytochemistry for peptide detection in cell bodies and neurites. In general, expression patterns for species expressing three GnRH forms are similar in fish, with sGnRH being expressed in the forebrain, cGnRH-II in the midbrain, and the third form in the preoptic area. Within this pattern the neurons expressing the third GnRH form usually have axons that extend into the pituitary gland. For example, in the sea bass sGnRH mRNA was localized in the olfactory bulb, sbGnRH in the preoptic area, and cGnRH-II in the midbrain by *in situ* hybridization (Zmora *et al.*, 2002). The same study revealed that immunocytochemistry detected sGnRH peptide in the forebrain, sbGnRH in the preoptic area, mediobasal hypothalamus and pars distalis of the pituitary, and cGnRH-II fibers distributed widely in the posterior brain (Zmora *et al.*, 2002). The authors then suggested that the third form is responsible for gonadotropin (GtH) release, whereas the other two forms have neurotransmitter roles or other roles related to reproduction.

For those species with only two GnRH forms, like masu salmon, sGnRH (which doubles as the third form) is found from the olfactory nerve and bulb to the preoptic area and into the hypothalamus. For salmonids, the sGnRH location has been confirmed by *in situ* hybridization, but cGnRH-II has not been studied using *in situ* hybridization techniques (see Table 1.2). Chicken GnRH-II follows the general pattern and is found in

the midbrain tegmentum (Amano *et al.*, 1991). Immunocytochemistry techniques located only axons containing sGnRH extending into the pituitary in masu salmon (Amano *et al.*, 1991). Similar patterns have been observed in both Atlantic salmon and rainbow trout where sGnRH is located in the olfactory bulb, telencephalon, and preoptic area and cGnRH-II is located in the midbrain tegmentum (Bailhache *et al.*, 1994; Penlington *et al.*, 1998). These studies suggest again that the preoptic form (sGnRH) is responsible for GtH release, whereas cGnRH-II may function as a neuromodulator. There is one species known that does not follow the pattern. Goldfish express both sGnRH and cGnRH-II throughout the brain and both forms have axons that extend into the pituitary (Kim *et al.*, 1995), although only immunocytochemistry was used for detection in this study. It should be noted that *in situ* hybridization techniques have not been done for goldfish GnRH localization (see Table 1.2).

The objective of this section is to determine the brain location of neurons that are expressing each of the different GnRH forms in lake whitefish. Although sGnRH has been localized by *in situ* hybridization in salmonids, cGnRH-II and wfGnRH have not. It is of interest to determine the location of these forms to see if there is any overlap. Also, the location within the brain may provide some insight into the function of each GnRH form. Even though *in situ* hybridization is highly dependent on gene expression and regulation, I chose to perform *in situ* hybridization to localize GnRH forms in the brain because each probe can be prepared to detect only one form. In immunohistochemistry the cross reactivity of antibodies to different GnRH forms, makes it difficult to determine which GnRH form a neuron contains.

Methods and Materials

Probes

Forward and reverse primers were made to amplify 250 bp within the 5'UTR region of the cGnRH-II cDNA for the probe, 270 bp within the GAP/3'UTR of wfGnRH for the probe, and 250 bp within the GAP/3'UTR region of sGnRH for the probe (see Table 4.1 for primer sequences and Figure 4.1 for primer location). Both the 5'UTR and the GAP/3'UTR regions have enough sequence variation that only the target GnRH form is detected. All PCR protocols for amplification were the same with a 3 minute denaturation at 94°C followed by 35 cycles of 30 seconds each at 94°C, 55°C and 72°C, and an extension round of 7minutes at 72°C. Amplified DNA was then separated by an electrophoresis gel and PCR products were cloned into pGEM-T vector (Promega, Madison, WI) with T7 and SP6 RNA polymerase promoter sites. Plasmid DNA was incorporated into cells; colonies were grown and picked; and DNA was isolated as per methods in chapter 2.

For both the cGnRH-II and sGnRH probes, DNA (20μg) was linearized separately with two different digests. Pst-1 was used to create the antisense strand for both cGnRH-II and sGnRH probes, whereas Sph-1 was used to create the sense strand for both probes. DNA (66μl) was added to 10μl of either Pst-1 or Sph-1 and 10μl of the enzyme's buffer, along with 14μl of double sterilized water. DNA was digested at 37°C for three hours, then heated to denature at 65°C for 15 minutes. An 8μl aliquot was removed and run on a 1.5% agarose electrophoresis gel to check that the DNA was linearized. DNA was then digested with proteinase K (20mg/ml) at 50°C for 40 minutes.

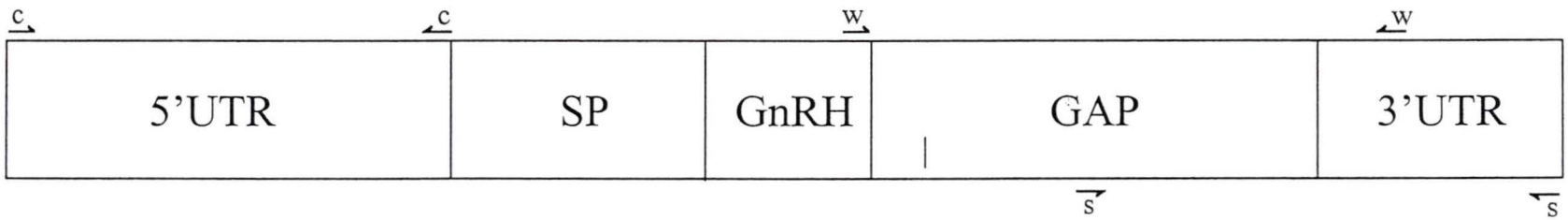
Table 4.1. GnRH probe primer sequences.

A list is provided of the different primer sequences used to obtain whitefish GnRH, salmon GnRH, and chicken GnRH-II probes for *in situ* hybridization of lake whitefish brains.

Probe	Forward Primer Sequence	Reverse Primer Sequence
chicken-II GnRH	5'-acgcgggaagcactggtatca-3'	5'-ctagcccactgaccatcact-3'
whitefish GnRH	5'-atgaaycctggaggvaagagr-3' where y=c+t, v=a+c+g, r=a+g	5'-gacccccaacaaagaccacc-3'
salmon GnRH	5'-gtctcagaaagactgagacca-3'	5'-aatcactctttattacaattt-3'

Figure 4.1. GnRH probe primer location.

A diagram showing the general GnRH cDNA structure with probe primer locations indicated by half arrows, where arrows pointing right represent forward primers, arrows pointing left are reverse primers, c = chicken GnRH, w = whitefish GnRH, and s = salmon GnRH.


c
c
w
w
5'UTR
SP
GnRH
GAP
3'UTR
s
s

DNA was then extracted by two phenol:chloroform:isoamylalcohol (25:24:1) extractions and precipitated in 100% ethanol (2.5 times volume) and 5M ammonium acetate (one tenth volume) at -80°C overnight. DNA was then resuspended in 20µl of double sterilized water; 1µl was removed, diluted in 99µl of water and the concentration was determined by the absorbance readings from a spectrophotometer. DIG labeled probes (Roche, Laval, Quebec) were made by transcribing 1µg of template DNA to make the cRNA probes by adding 2µl each of NTP labeling and transcription buffer, 1µl of either T7 polymerase (antisense probe), or SP6 (sense probe), and 1µl of RNAse inhibitor. Probes were then incubated at 37°C for three hours before 2µl of DNAse 1 (RNAse free) was added and probes underwent another incubation for 15 minutes at 37°C. The transcription reaction was stopped by adding 2µl of 0.2M EDTA, pH8.0 (in DEPC-treated water). The RNA probes were precipitated by adding 2.5µl 4M LiCl, 75µl 100% cold EtOH and incubating for 30 minutes at -80°C. The RNA was pelleted by spinning at 12,000g for 30 minutes. After which, most of the ethanol was removed (pellet was left covered) and 70% EtOH was added. The tubes were vortexed to resuspend the pellets, recentrifuged, and excess ethanol was removed. The RNA probes were resuspended in 50µl of DEPC-treated water with 1µl of RNase inhibitor and incubated at 65°C until the pellets dissolved.

A spot assay was done to estimate the concentration of the probes before the *in situ* hybridization took place. I spotted 1µl of a serial dilution (1ng/µl, 100pg/µl, 10pg/µl, 1pg/µl, 0.1pg/µl and 0.01pg/µl) of stock RNA that was DIG (Roche) labeled, and 1µl of a serial dilution of the RNA probes (undiluted, 1/50, 1/500, 1/5000, 1/50,000 and 1/500,000) on nylon membrane. Spots were baked on the membrane at 120°C for 40

minutes, and washed briefly in 10 ml wash buffer (TBS, 0.2% Tween-20). The membrane was incubated in 10ml of blocking buffer (TBS, 0.2% Tween-20, 10% serum) for 30 minutes at room temperature while gently shaking. Next, antibody (anti-digoxigenin-alkaline phosphatase) (Roche) was added to fresh blocking buffer (1:5000) and the membrane was incubated for 30 minutes at room temperature while shaking. The membrane was washed twice in washing buffer for 15 minutes each at room temperature and then incubated in detection buffer (100mM Tris-HCl pH 9.5, 100mM NaCl) for 2 minutes. Colour substrate solution was made fresh by mixing 45µl of nitro blue tetrazolium (NBT) (75mg/ml in 70% dimethylformamide) (Roche) and 35 µl of X-phosphate (BCIP) (50mg/ml in 100% dimethylformamide) (Roche) in 10mls of detection buffer. The membrane was incubated in colour substrate overnight at room temperature in the dark without shaking. The colour reaction was stopped the following day by washing the membrane in sterile water for 5 minutes. Spots were compared and 100ng of probe was used per 200µl hybridization buffer.

Tissue Preparation

For sGnRH and cGnRH-II, lake whitefish from the Freshwater Research Institute in Winnipeg (n=7, 3 male, 4 female) were anesthetized and perfused through the heart with a saline solution followed by 4% buffered paraformaldehyde. Brains and pituitaries were removed and shipped to Victoria in 30% sucrose solution. The brains were then cut once 2mm to the side of the midline so they could be frozen in O. C. T. Tissue Tek (Sakura, Torrance, CA), and then cryosectioned sagittally into 20µm sections. The sections were transferred onto clean, charged, Fisherbrand Superfrost/Plus slides (Fisher), and stored at -80°C.

For studies to localize wfGnRH, lake whitefish were gill netted in August, 2002 at Exeter Lake in 100 Mile House (n=4, 3 female, 1 male), because wfGnRH could not be isolated in fish from the Fresh Water Institute. Fish were anesthetized in 50mg/L MS-222 (Syndel, Vancouver, BC), and perfused intracardially with 10 ml of 0.85% NaCl solution followed by 10ml of buffered 4% paraformaldehyde. The heads were detached and cut sagitally to expose the brain, which was transferred into a 2ml tube and fixed in 4% paraformaldehyde. The fixative was replaced with clean solution 12 hours, and 24 hours after collection. Brains were then incubated in 30% sucrose solution until they sank (72 hours). Tissue was frozen, sectioned, and stored as above.

In situ hybridization

Slides were removed from the -80°C freezer and air dried completely (20 minutes). They were placed in a staining rack and post-fixed in 4% buffered paraformaldehyde at room temperature for 1 hour. Slides were then washed twice in 1X PBS, 5 minutes per wash. Sections were treated with 0.1μg/ml proteinase K in TE buffer (100mM Tris-HCl, 50mM EDTA) pH8.0 for 10 minutes at 37°C, then washed in DEPC water for 5 minutes at room temperature. Next, slides were washed in 0.1M TEA buffer, pH8.0, for 5 minutes and again in fresh TEA with acetic anhydride (2.5μl/ml buffer) for 10 minutes. Sections were then washed twice in 2XSCC for 5 minutes and dehydrated in graded ethanol (50%, 60%, 70%, 80% and 90%) made with DEPC water for 3 seconds each and air dried. Sections were prehybridized for 10 minutes by adding hybridization buffer (50% formamide, 5X SSC, 50μg/ml heparin, 500μg/ml tRNA, 0.1% Tween-20, 0.1M citric acid brought up to 50ml in sterile water) directly on sections and incubating at 37°C. Excess buffer was poured off slides and sections were hybridized overnight by

adding hybridization buffer and probe (100ng probe/200 µl buffer) directly to sections that were cover slipped (VWR, West Chester, PA) and incubated at 42°C in a container that was kept moist to prevent slides drying. Control slides with sense probe and no probe were included in the hybridization.

Slides were soaked in 2X SSC for 5 minutes to remove cover slips and washed in 2X SSC for 15 minutes at room temperature. Sections were treated with RNase A (40µg/ml in TBS) for 45 minutes at 37°C. Slides then underwent a series of washes: 2X SSC for 10 minutes at room temperature, 1X SSC for 10 minutes, 0.5X SSC for 10 minutes, 0.1X SSC for 45 minutes at 60°C, and twice in TBS for 10 minutes at room temperature. Sections were blocked by adding blocking buffer (TBS, 0.1% Tween-20, 10% serum) to slides and incubating for 30 minutes at room temperature. Antibody to the phosphatase was added to fresh blocking buffer (1:5000), placed over sections that were cover slipped and incubated at room temperature for 2 hours. Slides were washed twice in TBS 10 minutes each before incubating in coloration buffer (100mM Tris-HCl, pH9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20, brought up to 50ml in sterile water) for 10 minutes at room temperature. Fresh colour solution was made by adding nitro blue tetrazolium (NBT) and X-phosphate (BCIP) (Roche) in concentrations identical to the spot assay above and slides were incubated in colour solution overnight at room temperature in the dark.

The colour reaction was stopped by washing slides in TE buffer (10mM Tris-HCl, 1mM EDTA) for 20 minutes at room temperature. Graded ethanol was made with sterile water for dehydration of the sections. Slides were air dried before aqueous mounting

media (60% water, 8g gelatin, 40% glycerin, and 0.1g phenol as a preservative) was added. Sections were cover slipped, and left until mounting media was dry.

Results

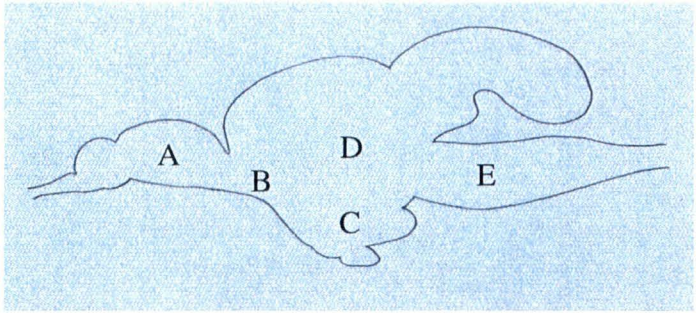
In situ hybridization with three different probes, each recognizing a different GnRH form in lake whitefish, revealed that sGnRH was mainly located in the olfactory bulb/forebrain area; cGnRH-II was in the midbrain; and wfGnRH was in the forebrain/preoptic area/hypothalamic regions.

Antisense riboprobes made against the GAP/3'UTR region of lake whitefish sGnRH were used to locate cells within the forebrain and the olfactory bulb (Figure 4.2). Also, there appeared to be some staining in the preoptic area (POA) and in the hypothalamus. Cells that were stained were often clustered and appeared large and roundish. There was no staining in the midbrain, optic tectum or hindbrain. There was faint staining with the sGnRH sense probe and no staining with the negative control (no probe).

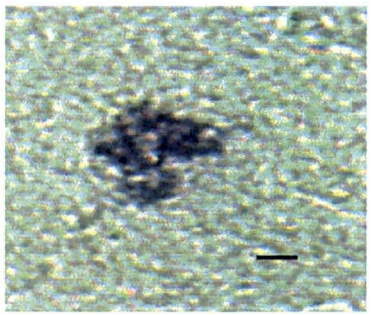
Antisense riboprobes made against the 5'UTR region of lake whitefish cGnRH-II were used to detect cells in the midbrain, optic tectum and hindbrain (Figure 4.2). There was no cGnRH-II staining in the forebrain or hypothalamus. Again clusters of cells were observed but cGnRH-II individual cells in the midbrain appeared smaller than those expressing sGnRH in the forebrain. There was no staining with either the cGnRH-II sense probe, or the negative control.

Figure 4.2. *In situ* hybridization showing GnRH location.

The brain location is shown for each GnRH form found in lake whitefish. A) Salmon GnRH labeled cells found in the ventral telencephalon. B) Salmon GnRH labeled cells located in the preoptic area. C) Whitefish GnRH stained cells in the hypothalamus. D) Chicken GnRH-II stained neurons in the midbrain. E) Chicken GnRH-II stained cells in the hindbrain. Scale bar is 10μm in each picture.



A)



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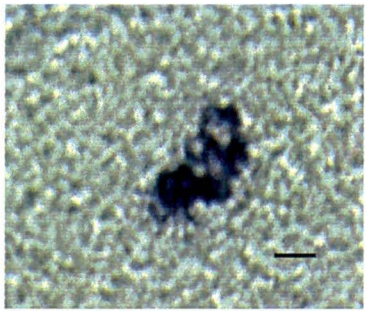

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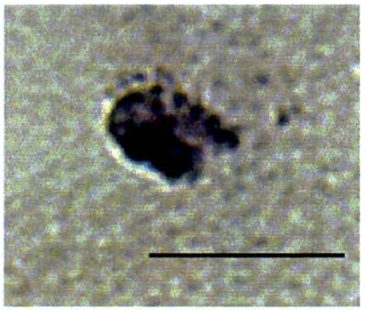

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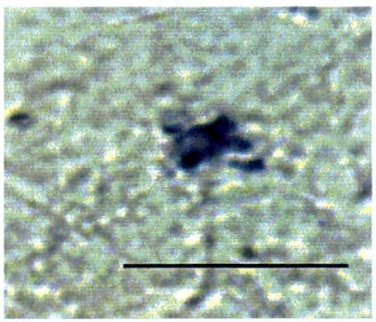
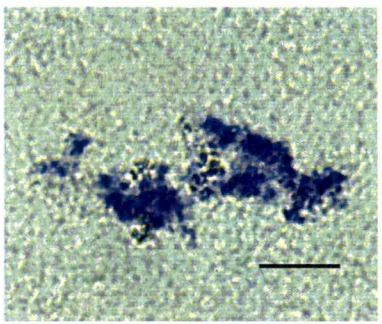


E)



Antisense riboprobes made against the GAP/3'UTR region of wfGnRH stained cells in the forebrain and POA (Figure 4.2). There were also a few cells stained in the olfactory bulb but none in the midbrain or hindbrain. Clusters of cells appeared quite small and staining was very dark. There was some background staining in all brain regions with the sense probe, but no staining with the negative control.

Discussion

The three different riboprobes were specific to each GnRH form found in lake whitefish as they were made from different regions of the different cDNAs and showed no sequence similarity to any other GnRH form.

The distribution of GnRH in lake whitefish brains agrees with other studies, as sGnRH was located in the forebrain, cGnRH-II was detected in the midbrain, and the third form (wfGnRH) was expressed in the POA (see Table 1.2 for comparison). We found that only cells in the midbrain expressed cGnRH-II. This is in agreement with the literature as the cGnRH-II form is restricted to the posterior brain in all vertebrates gnathostomes studied to date (Muske, 1993; Lescheid *et al.*, 1997; Parhar, 1997; Stefano *et al.*, 2000; Dubois *et al.*, 2001). The only exception is goldfish where the cGnRH-II is also expressed in some neurons in the forebrain (Kim *et al.*, 1995); however, *in situ* hybridization studies have not been done on goldfish. Immunocytochemistry performed on lake whitefish also detected cGnRH-II in midbrain neurons only (Laberge, personal communication). We found lake whitefish cGnRH-II peptide caused an increase in gonadotropin mRNA expression in dispersed pituitary cells of rainbow trout (Adams *et*

al. 2002). However, cGnRH-II probably does not have a gonadotropin-releasing role but rather a neuromodulatory role, as it was found only in the midbrain of lake whitefish.

Cells expressing sGnRH were mainly visualized in the olfactory bulb, and ventral telencephalon, although a few cells were found in the POA. Salmon GnRH located in the olfactory bulb would have a neuromodulatory role as these cells are most likely involved in responding to pheromones and other gonadal steroid hormones (see Laberge and Hara, 2001). It was interesting to find sGnRH in the ventral telencephalon and POA, which was confirmed by immunocytochemistry work that located sGnRH neurons in the same regions (Laberge, personal communication). The presence of sGnRH in the ventral telencephalon suggests it plays a role in gonadotropin-release in lake whitefish.

Neurons expressing wfGnRH were visualized in the ventral telencephalon, POA and hypothalamus and suggest that wfGnRH has a gonadotropin-releasing role in lake whitefish. This observation is supported by our observation that wfGnRH caused a significant increase in gonadotropin mRNA expression in dispersed rainbow trout pituitary cells (Adams *et al.*, 2002).

It is of interest to note that regions expressing sGnRH or wfGnRH overlapped. This overlapping has not been observed frequently, but was confirmed by immunocytochemistry, which also detected sGnRH overlap with wfGnRH in lake whitefish brains (Laberge, personal communication). Recent studies of the European sea bass (*Dicentrarchus labrax*) suggest that the distribution of cells expressing salmon or seabream GnRH overlap in the olfactory bulb, ventral telencephalon, and POA (Gonzalez-Martinez, 2001; 2002). The situation could be similar in lake whitefish where both salmon and whitefish GnRH-containing neurons could be present around the

olfactory bulb, in the ventral telencephalon and in the POA. It is possible that both sGnRH and wfGnRH have gonadotropin-releasing roles in lake whitefish. This would be advantageous to the more recently evolved salmonids that have lost or no longer express the third GnRH form (wfGnRH). In these salmonids (e.g. sockeye, Atlantic, trout) only 2 forms of GnRH are detected and sGnRH appears to be the releaser of gonadotropins from the pituitary.

Literature Cited

Abe H., and Oka Y. 2000. Modulation of pacemaker activity by salmon gonadotropin-releasing hormone (sGnRH) in terminal nerve (TN)-GnRH neurons. Journal of Neurophysiology **83**: 3196-3200.

Adams B.A., Vickers E.D., Warby C., Park M., Fischer W.H., Craig A.G., Rivier J.E., and Sherwood N.M. 2002. Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, *Coregonus clupeaformis*. Biology of Reproduction **67**: 232-239.

Allendorf F.W., and Thorgaard G.H. 1984. Tetraploidy and the evolution of salmonid fishes. In 'Evolutionary genetics of fishes'. Plenum Press, New York, pg 1-10.

Alok D., Hassin S., Kumar R.S., Trant J.M., Yu K., and Zohar Y. 2000. Characterization of a pituitary GnRH-receptor from a perciform fish, *Morone saxatilis*: functional expression in a fish cell line. Molecular and Cellular Endocrinology **168**: 65-75.

Amano M., Oka Y., Aida K., Okumoto N., Kawashima S., and Hasegawa Y. 1991. Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, *Oncorhynchus masou*. Journal of Comparative Neurology **314**: 587-597.

Amano M., Takahashi A., Yamanome T., Okubo K., Aida K., and Yamamori K. 2002. Molecular cloning of three cDNAs encoding different GnRHs in the brain of barfin flounder. General and Comparative Endocrinology **126**: 325-333.

Bailhache T., Arazam A., Klungland H., Alestrom P., Breton B., and Jego P. 1994. Localization of salmon gonadotropin-releasing hormone mRNA and peptide in the brain of Atlantic salmon and rainbow trout. Journal of Comparative Neurology **347**: 444-454.

Batten T.F.C., Cambre M.L., Moons L., and Vandesande F. 1990. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, *Poecilia latipinna*. Journal of Comparative Neurology **302**: 893-919.

Blalock J.E., and Costa O. 1989. Immune neuroendocrine interactions: implications for reproductive physiology. Annals of the New York Academy of Sciences **564**: 261-266.

Blomenrorh M., Bogerd J., Leurs R., Schulz R.W., Tensen C.P., Zandbergen M.A., and Goos H.J.Th. 1997. Differences in structure-function relations between nonmammalian and mammalian gonadotropin-releasing hormone receptors.

Bogerd J., Li K.W., Janssen-Dommerholt C., Goos H. 1992. Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). Biochemical and Biophysical Research Communications **238**: 517-522.

Braun C.B., Wicht H., and Northcutt R.G. 1995. Distribution of gonadotropin-releasing hormone immunoreactivity in the brain of the Pacific hagfish, *Eptatretus stouti* (Craniata: Myxinoidea). Journal of Comparative Neurology **353**: 464-476.

Burgus R., Butcher M., Amoss M., Ling N., Monahan M., Rivier J., Fellows R., Blackwell R., Vale W., and Guillemin R. 1972. Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor. Proceedings of the National Academy of Sciences USA **69**: 278-282.

Cameron C.E., Mackie G.O., Powell J.F.F., Lescheid D.W., and Sherwood N.M. 1999. Gonadotropin-releasing hormone in mulberry cells of Saccoglossus and Ptychodera (Hemichordata: Enteropneusta). General and Comparative Endocrinology **114**: 2-10.

Carolsfeld J., Powell J.F.F., Park M., Fischer W.H., Craig A.G., Chang J.P., Rivier J.E., and Sherwood N.M. 2000. Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from ancient teleost, herring. Endocrinology **141**: 505-512.

Chang J.P., Cook H., Freedman G.L., Wiggs A.J., Somoza G.M., de Leeuw R., and Peter R.E. 1990. Use of a pituitary cell dispersion method and primary cell culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carrassius auratus*. *In* 'Initial morphological, static, and cell column perifusion studies'. General Comparative Endocrinology **77**: 256-273.

Chang J.P., Jobin R.M., and Wong A.O.L. 1993. Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus*. Fish Physiology and Biochemistry **11**: 25-33.

Chang J.P., and Jobin R.M. 1994. Teleost pituitary cells: isolation, culture and use. *In* 'Biochemistry and molecular biology of fishes, vol. 3'. Elsevier, Amsterdam, pg 205-213.

Cheng K.W., Chow B.K.C., and Leung P.C.K. 2001. Functional mapping of a placenta-specific upstream promoter for human gonadotropin-releasing hormone receptor gene. Endocrinology **142**: 1506-1516.

Chomczynski P., and Sacchi N. 1987. Single-step method of RNA isolation by acid guanidnium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry **162**: 156-159.

Chow M.M., Kight K.E., Gothilf Y., Alok D., Stubblefield J., and Zohar Y. 1998. Multiple GnRHs present in a teleost species are encoded by separate genes: analysis of the sbGnRH and cGnRH-II genes from the striped bass, *Morone saxatilis*. Journal of Molecular Endocrinology **21**: 277-289.

Coe I.R., von Schalburg K.R., and Sherwood N.M. 1995. Characterization of the pacific salmon gonadotropin-releasing hormone gene, copy number and transcription start site. Molecular and Cellular Endocrinology **115**: 113-122.

Cornea A., Janovick J., Maya-Nunez G., and Conn P.M. 2001. Gonadotropin-releasing hormone receptor microaggregation. Journal of Biological Chemistry **276**: 2153-2158.

Craig A.G., Fischer W.H., Park M., Rivier J.E., Musselman B.D., Powell J.F.F., Reska-Skinner S.M., Prakash M.O., Mackie G.O., Sherwood N.M. 1997. Sequence of two gonadotropin-releasing hormones from tunicate suggest an important role of conformation in receptor activation. Federation of European Biological Societies Letters **413**: 215-225.

Demski L.S., Bauer D.H., and Gerald J.W. 1975. Sperm release evoked by electrical stimulation of the fish brain: a functional-anatomical study. Journal of Experimental Zoology **191**: 215-232.

Di Fiore M.M., Rastogi R.K., Ceciliani F., Messi E., Botte V., Pinelli C., D'Aniello B., and D'Aniello A. 2000. Mammalian and chicken I forms of gonadotropin-releasing hormone in the gonads of a protochordate, *Ciona intestinalis*. Proceedings of the National Academy of Sciences **97**: 2343-2348.

Duan W.R., Ito M., Park Y., Maizels E.T., Hunzicker-Dunn M., and Jameson L.J. 2002. GnRH regulates early growth response protein 1 transcription through multiple promoter elements. Molecular Endocrinology **16**: 221-233.

Dubois E.A., Zandbergen M.A., Peute J., Hassing I., van Dijk W., Schulz R.W., and Goos H.J.Th. 2000. Gonadotropin-releasing hormone fibers innervate the pituitary of the male African catfish (*Clarias gariepinus*). Neuroendocrinology **72**: 252-262.

Dubois E.A., Zandbergen M.A., Peute J., Bogerd J., and Goos H.J.Th. 2001. Development of three distinct GnRH neuron populations expressing two different GnRH forms in the brain of the African catfish (*Clarias gariepinus*). Journal of Comparative Neurology **437**: 308-320.

Dufour S., Monter M., Le Belle N., Basompierre M., King J., Millar R., Peter R., and Fontaine, Y.V. 1993. Differential distribution and response to experimental sexual maturation of two forms of brain gonadotropin-releasing hormone (GnRH) in the European eel, *Anguilla anguilla*. Fish Physiology and Biochemistry **11**: 99-106.

Fink G., and Jamieson M.G. 1976. Immunoreactive luteinizing hormone releasing factor in rat pituitary stalk blood: effects of electrical stimulation of the medial preoptic area. Journal of Endocrinology **68**: 71-87.

Goldberg J.A., Garofalo R., Price C.J., and Chang J.P. 1993. Presence and biological activity of a GnRH-like factor in the nervous system of *Helisoma trivolvis*. Journal of Comparative Neurology **336**: 571-582.

Gonzalez-Martinez D., Madigou T., Zmora N., Anglade I., Zanuy S., Zohar Y., Elizur A., Munoz-Cueto J.A., and Kah O. 2001. Differential expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). Journal of Comparative Neurology **429**: 144-155.

Gonzalez-Martinez D., Zmora N., Zanuy S., Sarasquete C., Elizur A., Kah O., and Munoz-Cueto J.A. 2002. Developmental expression of three different prepro-GnRH (gonadotropin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). Journal of Chemical Neuroanatomy **23**: 225-267.

Goos H.J.Th., and Murathanoglu O. 1977. Localization of gonadotropin-releasing hormone (GnRH) in the forebrain and neurohypophysis of the trout (*Salmo gairdneri*): an immunofluorescence study. Cell and Tissue Research **181**: 163-168.

Goos H.J., de Leeuw R., de Zoeten-Kamp C., Peute J., and Blahser S. 1985. Gonadotropin-releasing hormone-immunoreactive neuronal structures in the brain and pituitary of the African catfish, *Clarias gariepinus* (Burchell). Cell and Tissue Research **241**: 593-596.

Gorbman, A. 1980. Evolution of the brain-pituitary relationship: evidence from the Agnatha. Canadian Journal of Fisheries and Aquatic Sciences **37**: 1680-1686.

Gothilf Y., Munoz-Cueto J.A., Sagrillo C.A., Selmanoff M., Chem T.T., Kah O., Elizur A., and Zohar Y. 1996. Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): Complementary deoxyribonucleic acid characterization and brain localization. Biology of Reproduction **55**: 636-645.

Gray S.L., Adams B.A., Warby C.M., von Schalburg K.R., and Sherwood N.M. 2002. Transcription and translation of the salmon gonadotropin-releasing hormone (GnRH) genes in brain and gonads of sexually maturing rainbow trout (*Oncorhynchus mykiss*). Biology of Reproduction **67**: 1621-1627.

Grober M.S., Bass A.H., Burd G., Marchaterre M.A., Segil N., Scholz K., and Hodgson T. 1987. The nervus terminalis ganglion in *Anguilla rostrata*: an immunocytochemical and HRP histochemical analysis. Brain Research **436**: 148-152.

Grober M.S., and Bass A.H. 1991. Neuronal correlates of sex/role change in labrid fishes: LHRH-like immunoreactivity. Brain, Behavior and Evolution **38**: 302-312.

Halpern-Sebold L., and Schreibman M.P. 1983. Ontogeny of centers containing luteinizing hormone-releasing hormone in the brain of platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. Cell and Tissue Research **229**: 75-84.

Herbison A.E., Robinson H.F. and Skinner D.C. 1993. Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone releasing hormone. Neuroendocrinolgy **57**: 751-759.

Hillensjo T., and LeMaire W.J. 1980. Gonadotropin releasing hormone agonists stimulate meiotic maturation of follicle-enclosed rat oocytes in vitro. Nature **287**: 145-146.

Iwakoshi E., Takuwa-Kuroda K., Fujisawa Y., Hisada M., Ukena K., Tsutsui K., and Minakata H. 2002. Isolation and characterization of a GnRH-like peptide from *Octopus vulgaris*. Biochemical and Biophysical Research Communications **291**: 1187-1193.

Jan Y.N., Jan L.Y., and Kuffler K.W. 1979. A peptide as a possible transmitter in sympathetic ganglia of the frog. Proceedings of the National Academy of Sciences USA **76**: 1501-1505.

Jan Y.N., Bowers C.W., Branton D., Evans L., and Jan L.Y. 1983. Peptides in neuronal function: studies using frog autonomic ganglia. Cold Spring Harbor Symposium Quant Biology **48**: 363-374.

Jimenez-Linan M., Rubin B.S., and King J.C. 1997. Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. Endocrinology **138**: 4123-4130.

Joss J.M.P., King J.A., and Millar R.P. 1994. Identification of the molecular forms of and steroid hormone response to gonadotropin-releasing hormone in the Australian Lungfish, *Neoceratodus forsteri*. General and Comparative Endocrinology **96**: 392-400.

Kah O., Chambolle P., Dubourg P., and Dubois M.P. 1984. Immunocytochemical localization of luteinizing hormone-releasing hormone in the brain of the goldfish, *Carassius auratus*. General Comparative Endocrinology **53**: 107-115.

Kah O., Breton B., Dulka J.G., Nunez-Rodriguez J., Peter R.E., Corrigan A., Rivier J.E., and Vale W.W. 1986. A reinvestigation of the Gn-RH (gonadotropin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. Cell Tissue Research **244**: 327-337.

Kah O., Zanuy S., Mananos E., Anglade I., and Carrillo M. 1991. Distribution of salmon gonadotrophin releasing-hormone in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). Cell and Tissue Research **266**: 129-136.

Khan I.A., and Thomas P. 1993. Immunocytochemical localization of seratonin and gonadotropin-releasing hormone in the brain and pituitary gland of the Atlantic croaker *Micropogonias undulatus*. General Comparative Endocrinology **91**: 167-180.

Kim M., Oka Y., Amano M., Kobayashi M., Okuzawa K., Hasegawa Y., Kawashima S., Suzuki Y., and Aida K. 1995. Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. Journal of Comparative Neurology **356**: 72-82.

Klungland H., Lorens J.B., Andersen O., Kisen G.O., and Alestrom P. 1992. The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. Molecular and Cellular Endocrinology **84**: 167-174.

Klungland H., Andersen O., Kisen G., Alestrom P., and Tora L. 1993. Estrogen receptor binds to the salmon GnRH gene in a region with long palindromic sequences. Molecular and Cellular Endocrinology **95**: 147-154.

Laberge F., and Hara T.J. 2001. Neurobiology of fish olfaction: a review. Brain Research Reviews **36**: 46-59.

Lepretre E., Anglade I., Williot P., Vandesande F., Tramu G., and Kah O. 1993. Comparative distribution of mammalian GnRH (gonadotropin-releasing hormone) and chicken GnRH-II in the brain of the immature Siberian sturgeon (*Acipenser baeri*). Journal of Comparative Neurology **337**: 568-583.

Lescheid D.W., Terasawa E., Abler L.A., Urbanski H.F., Warby C.M., Millar R.P., and Sherwood N.M. 1997. A second form of gonadotropin-releasing hormone (GnRH) with characteristics of chicken GnRH-II is present in the primate brain. Endocrinology **138**: 5618-5629.

Lovejoy D.A., Sherwood N.M., Fischer W.H., Jackson B.C., Rivier J.E., and Lee T. 1991. Primary structure of gonadotropin-releasing hormone from the brain of a Holocephalan (Ratfish: *Hydrolagus colliei*). General and Comparative Endocrinology **82**: 152-161.

Lovejoy D.A., Ashmead B.J., Coe I.R., and Sherwood N.M. 1992a. Presence of gonadotropin-releasing hormone immunoreactivity in dogfish and skate brains. Journal of Experimental Zoology **263**: 272-283.

Lovejoy D.A., Fischer W.H., Ngamvongchon S., Craig A.G., Nahorniak C.S., Peter R.E., Rivier J.E., and Sherwood N.M. 1992b. Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into gonadotropin-releasing hormone evolution. Proceedings of the National Academy of Sciences USA **89**: 6373-6377.

Lin X., and Peter R.E. 1995. Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. General and Comparative Endocrinology **101**: 282-296.

Madigou T., Mananos-Sanchez E., Hulshof S., Anglade I., Zanuy S., and Kah O. 2000. Cloning, tissue distribution, and central expression of the gonadotropin-releasing hormone receptor in the rainbow trout (*Oncorhynchus mykiss*). Biology of Reproduction **63**: 1857-1866.

Magliulo-Cepriano L., Schreibman M.P., and Blum V. 1994. Distribution of variant forms of immunoreactive gonadotropin-releasing hormone and β-gonadotropins I and II in the platyfish, *Xiphophorus maculates*, from birth to sexual maturity. General and Comparative Endocrinology **94**: 135-150.

Marchant T.A., Chang J.P., Nahorniak C.S., and Peter R.E. 1989. Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology **124**: 2509-2518.

Matsuo H., Baba Y., Nair R.M.G., Arimura A., and Schally A.V. 1971. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochemical and Biophysical Research Communications **43**: 1334-1339.

Millan M., Aguilera G., Wynn P.C., Mendelsohn F.A.O., and Catt K.J. 1986. Autoradiographic localization of brain receptors for peptide hormones: angiotensin II, corticotropin-releasing factor, and gonadotropin-releasing hormone. Methods in Enzymology **124**: 590-606.

Millar R.P., and King J.A. 1988. Evolution of gonadotropin-releasing hormone: multiple usage of a peptide. News in Physiology **3**: 49-53.

Millar R., Lowe S., Conklin D., Pawson A., Maudsley S., Troskie B., Ott T., Millar M., Lincoln G., Sellar R., Faurholm B., Scobie G., Kuestner R., Terasawa E., and Katz A. 2001. A novel mammalian receptor for the evolutionarily conserved type II GnRH. Proceedings of the National Academy of Sciences USA **98**: 9636-9641.

Miller K.E., and Kriebel R.M. 1986. Peptidergic innervation of caudal neurosecretory neurons. General Comparative Endocrinology **64**: 396-400.

Miyamoto K., Hasegawa Y., Igarashi M., Chino N., Sakakibara S., Kangawa K., and Matsuo H. 1983. Evidence that chicken hypothalamic luteinizing hormone-releasing hormone is (Gln^8)-LH-RH. Life Science **32**: 1341-1347.

Miyamoto K., Hasegawa Y., Nomura M., Igarashi M., Kangawa K., and Matsuo H. 1984. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proceedings of the National Academy of Sciences USA **81**: 3874-3878.

Montaner A.D., Park M.K, Fischer W.H., Craig A.C., Chang J.P., Somoza G.M., Rivier J.E., and Sherwood N.M. 2001. Primary structure of a novel gonadotropin-releasing hormone in the brain of a teleost, pejerrey. Endocrinology **142**: 1453-1460.

Montero M., Vidal B., King J.A., Tramu G., Vandesande F., Dufour S., and Kah O. 1994. Immunocytochemical localization of mammalian GnRH (gonadotropin-releasing hormone) and chicken GnRH-II in the brain of the European silver eel (*Anguilla anguilla* L.). Journal of Chemical Neuroanatomy **7**: 227-241.

Moss R.L., and McCann S.M. 1973. Induction of mating behavior in rats by luteinizing hormone-releasing factor. Science **181**: 177-179.

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

Muske L.E. 1993. Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. Brain, Behavior and Evolution **42**: 215-230.

Muske L.E., and Moore F.L. 1987. Luteinizing hormone-releasing hormone-immunoreactive neurons in the amphibian brain are distributed along the course of the nervus terminalis. *In* 'The Terminal Nerve (Nervus Terminalis). Structure, Function and Evolution'. Annals of the New York Academy of Sciences **519**: 433-446.

Muske L.E., and Moore F.L. 1990. Ontogeny of immunoreactive gonadotropin-releasing hormone neuronal systems in amphibians. Brain Research **534**: 177-187.

Nabissi M., Pati D., Polzonetti-Magni A.M., and Habibi H.R. 1997. Presence and activity of compounds with GnRH-like activity in the ovary of seabream *Sparus aurata*. American Journal of Physiology **272**: 111-117.

Neill J.D., Duck L.W., Sellers J.C., and Musgrove L.C. 2001. A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates. Biochemical and Biophysical Research Communications **282**: 1012-1018.

Neill J.D. 2002. Mammalian gonadotropin-releasing hormone (GnRH) receptor subtypes. Archives of Physiology and Biochemistry **110**: 129-36.

Nelson J.S. 1994. Fishes of the World 3rd edition. John Wiley and Sons, Inc. New York, NY, pg 189-191.

Nelson S., R.D. Horvat, J. Malvey, D.A. Roess, B.G. Barisas, and C.M. Clay. 1999. Characterization of an intrinsically fluorescent gonadotropin-releasing hormone receptor and effects of ligand binding on receptor lateral diffusion. Endocrinology **140**: 950-957.

Nevitt G.A., Grober M.S., Marchaterre M.A., and Bass A.H. 1995. GnRH-like immunoreactivity in the peripheral olfactory system and forebrain of Atlantic salmon (*Salmo salar*): a reassessment at multiple life history stages. Brain, Behavior and Evolution **45**: 350-358.

Ngamvongchon S., Lovejoy D.A., Fischer W.H., Craig A.G., Nahorniak C.S., Peter R.E., Rivier J.E., and Sherwood N.M. 1992a. Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. Molecular and Cellular Neurosciences **3**: 17-22.

Ngamvongchon S., Rivier J.E., and Sherwood N.M. 1992b. Structure-function studies of five natural, including catfish and dogfish, gonadotropin-releasing hormones and eight analogs on reproduction in Thai catfish (*Clarias macrocephalus*). Regulatory Peptides **42**: 63-73.

Nozaki M., Tsukahara T., and Kobayashi H. 1984. An immunocytochemical study on the distribution of neuropeptides in the brain of certain species of fish. Biomedical Research **4**(Suppl.): 135-145.

Nunez Rodriguez J., Kah O., Breton B., and Le Menn F. 1985. Immunocytochemical localization of GnRH (gonadotropin releasing hormone) systems in the brain of a marine teleost fish, the sole. Experientia **41**: 1574-1576.

Okubo K., Suetake H., and Aida K. 1999. Expression of two gonadotropin-releasing hormone (GnRH) precursor genes in various tissues of the Japanese eel and evolution of GnRH. Zoological Science **16**: 471-478.

Okubo K., Amano M., Yoshiura Y., Suetake H., and Aida K. 2000. A novel form of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. Biochemical and Biophysical Research Communications **276**: 298-303.

Okubo K., and Aida K. 2001. Gonadotropin-releasing hormones (GnRHs) in a primitive teleost, the arowana: phylogenetic evidence that three paralogous lineages of GnRH occurred prior to the emergence of teleosts. General and Comparative Endocrinology **124**: 125-133.

Okuzawa K., Granneman J., Bogerd J., Goos H.J.Th., Zohar Y., and Kagawa H. 1997. Distinct expression of GnRH genes in the red seabream brain. Fish Physiology and Biochemistry **17**: 71-79.

O'Neill D.F., Powell J.F.F., Standen E.M., Youson J.H., Warby C.M., and Sherwood N.M. 1998. Gonadotropin-releasing hormone (GnRH) in ancient teleosts, the bonytongue fishes: putative origin of salmon GnRH. General and Comparative Endocrinology **112**: 415-425.

Page L.M., and Burr B.M. 1991. A Field Guide to Freshwater Fishes. Houghton Mifflin Company, Boston, pg 40.

Parhar I.S., Koibuchi N., Sakai M., Iwata M., and Yamaoka S. 1994. Gonadotropin-releasing hormone (GnRH): expression during salmon migration. Neuroscience Letters **172**: 15-18.

Parhar I.S., Soga T., and Skuma Y. 1996a. In situ hybridization for differentially expressed GnRH genes following estrogen and triiodothyronine treatment in the brains of juvenile tilapia (cichlid). Neuroscience Letters **218**: 135-138.

Parhar I.S., Pfaff D.W., and Schwanzel-Fukuda M. 1996b. Gonadotropin-releasing hormone gene expression in teleosts. Molecular Brain Research **41**: 216-227.

Parhar I.S. 1997. GnRH in tilapia: three genes, three origins and their roles. *In* 'GnRH Neurons: Gene to Behaviour'. Brain Shuppan, Tokyo, pg 99-116.

Pati D., and Habibi H.R. 1998. Presence of salmon gonadotropin-releasing hormone (GnRH) and compounds with GnRH-like activity in the ovary of goldfish. Endocrinology **139**: 2015-2024.

Pati D., and Habibi H.R. 2000. Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis. Molecular and Cellular Endocrinology **160**: 75-88.

Pazos A.J., and Mathieu M. 1999. Effects of five natural gonadotropin-releasing hormones on cell suspensions of marine bivalve gonad: stimulation of gonial DNA synthesis. General and Comparative Endocrinology **113**: 112-120.

Penlington M.C., Harden N., Garside J., Hoole D., and Arme C. 1998. Isolation and characterization of mRNA encoding the chicken-II type gonadotropin-releasing hormone (GnRH) precursor in the rainbow trout. Journal of Fish Biology **53**: 614-623.

Phillips R., and Rab P. 2001. Chromosome evolution in the salmonidae (Pisces): an update. Biological Reviews of the Cambridge Philosophical Society **76**: 1-25.

Powell J.F.F., Reska-Skinner S. M., Prakash M.O., Fischer W.H., Park M., Rivier J.E., Craig A.G., Mackie G.O., and Sherwood N.M. 1996. Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proceedings of the National Academy of Sciences USA **93**: 10461-10464.

Powell J.F.F., Zohar Y., Elizur A., Park M., Fischer W.H., Craig A.G., Rivier J.E., Lovejoy D.A., and Sherwood N.M. 1994. Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proceedings of the National Academy of Sciences USA **91**: 12081-12085.

Quanbeck C., Sherwood N.M., Millar R.P., and Terasawa E. 1997. Two populations of luteinizing hormone-releasing hormone neurons in the forebrain of the rhesus macaque during embryonic development. Journal of Comparative Neurology **380**: 293-309.

Rastogi R.K., and Iela L. 1994. Gonadotropin-releasing hormone: present concepts, future directions. Zoological Science **11**: 363-373.

Rissman E.F., Li X., King J.A., and Millar R.P. 1997. Behavioral regulation of gonadotropin-releasing hormone production. Brain Research Bulletin **44**: 459-464.

Robinson T.C., Tovet S.A., Chase C., Waldron T., and Sower S.A. 2000. Gonadotropin-releasing hormones in the brain and pituitary of the teleost, the white sucker. General and Comparative Endocrinology **117**: 381-394.

Rosenblum P.M., Goos H.J.Th., and Peter RE. 1994. Regional distribution and in vitro secretion of salmon and chicken-II gonadotropin-releasing hormones from the brain and pituitary of juvenile and adult goldfish, *Carassius auratus*. General Comparative Endocrinology **93**: 369-379.

Schafer H., Schulz R., and Blum V. 1989. Immunoreactivity to gonadotropin-releasing hormone and gonadotropic hormone in the brain and pituitary of the rainbow trout. Cell and Tissue Research **257**: 227-235.

Schally A.V., Arimura A., and Kastin A.J. 1973. Hypothalamic regulatory hormones. Science **179**: 341-350.

Schreibman M.P., Halpern L.R., Goos H.J.Th., and Margolis-Kazan H. 1979. Identification of luteinizing hormone-releasing hormone (LH-RH) in the brain and pituitary gland of a fish by immunocytochemistry. Journal of Experimental Biology **210**: 153-159.

Schreibman M.P., Halpbern-Sebold L., Ferin M., Margolis-Dazan H., and Goos H.J.Th. 1983. The effect of hypophysectomy and gondatropin administration on the distribution and quantity of LHRH in the brains of platyfish: a combined immunocytochemistry and radioimmunoassay study. Brain Research **267**: 293-300.

Schulz G.E., and Schirmer R.H. 1979. Principles of Protein Structure. Springer-Verlag, New York, pg. 171-172.

Schulz R.W., Bosma P.T., Zandbergen M.A., van der Sanden M.C.A., van Dijk W., Peute J., Bogerd J., and Goos H.J.Th. 1993. Two gonadotropin-releasing hormones in the African catfish, *Clarias gariepinus*: localization, pituitary receptor binding, and gonadotropin release activity. Endocrinology **133**: 1569-1577.

Scott W.B., and Crossman E.J. 1973. Freshwater Fishes of Canada. Bulletin 184, Fisheries Research Board of Canada, Ottawa, pg 269-275.

Sherwood, N.M. 1986. Evolution of a neuropeptide family: gonadotropin-releasing hormone. American Zoology **26**: 1041-1054.

Sherwood N., Eiden L., Brownstein M., Spiess J., Rivier J., and Vale W. 1983. Characterization of a teleost gonadotropin-releasing hormone. Proceedings of the National Academy of Sciences USA **80**: 2794-2798.

Sherwood N.M., Sowers S.A., Marshak D.R., Fraser B.A., and Brownstein M.J. 1986. Primary structure of gonadotropin-releasing hormone from lamprey brain. The Journal of Biological Chemistry **261**: 4812-4819.

Sherwood N.M., Doroshov S., and Lance V. 1991. Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: Reedfish (*Calamoichthys calabaricus*), Sturgeon (*Acipenser transmontanus*), and Alligator Gar (*Lepisosteus spatula*). General and Comparative Endocrinology **84**: 44-57.

Sherwood N.M., Lovejoy D.A., and Coe I.R. 1993. Origin of mammalian gonadotropin-releasing hormones. Endocrine Reviews **14**: 241-254.

Sherwood N.M., von Schalburg K., and Lescheid D.W. 1997. Origin and evolution of GnRH in vertebrates and invertebrates. *In* 'GnRH Neurons: Gene to Behaviour'. Brain Shuppan, Tokyo, pg 3-25.

Shivers B.D., Harlan R.E., Morrell J.I., and Pfaff D.W. 1983. Absence of estradiol concentration in nuclei of LHRH immunoreactive neurons. Nature **304**: 345-347.

Silverman A.J., Jhamandas J., and Renaud L.P. 1987. Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. Journal of Neuroscience **7**: 2312-2319.

Sower S.A., Chiang Y-C., Lovas S., and Conlon J.M. 1993. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. Endocrinology **132**: 1125-1131.

Stefano A.V., Canosa L.F., D'Eramo J.L., Fridman O., Affanni J.M., and Somoza G.M. 1997. GnRH molecular variants in the brain and pituitary gland of pejerrey, *Odontesthes bonariensis* (Atheriniformes). Immunological and chromatographic evidence for the presence of a novel molecular variant. Comparative Biochemistry and Physiology **118**: 335-345.

Stefano A.V., Aldana-Marcos H.J., Affanni J.M., and Somoza G.M. 2000. Gonadotropin-releasing hormone (GnRH) neuronal systems in the pejerrey, *Odontesthes bonariensis* (Atheriniformes). Fish Physiology and Biochemistry **23**: 215-223.

Stell W.K., Walker S.E., Chohan K.S., and Ball A.K. 1984. The goldfish nervus terminalis: a luteinizing hormone-releasing hormone and molluscan cardioexcitatory peptide immunoreactive olfactoretinal pathway. Proceedings of the National Academy of Sciences USA **81**: 940-944.

Stopa E.G., Koh E.T., Svendsen C.N., Rogers W.T., Schwaber J.S., and King J.C. 1991. Computer –assisted mapping of immunoreactive mammalian gonadotropin-releasing hormone in adult human basal forebrain and amygdala. Endocrinology **128**: 3199-3207.

Subheader N., and Krishna N.S.R. 1988. Immunocytochemical localization of LH-RH in the brain and pituitary of the catfish, *Clarias batrachus* (Linn.). General Comparative Endocrinology **72**: 431-442.

Sullivan K.E., Witkin J.W., Ferin M., and Silverman A.J. 1995. Gonadotropin-releasing hormone neurons in the rhesus macaque are not immunoreactive for the estrogen receptor. Brain Research **685**: 198-200.

Suzuki M., Hydod S., Kobayashi M., Aida K., and Urano A. 1992. Characterization and localization of mRNA encoding the salmon-type gonadotrophin-releasing hormone precursor of the masu salmon. Journal of Molecular Endocrinology **9**: 73-82.

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

Tsutsui H., Yamamoto N., Ito H., and Oka Y. 1998. Gonadotropin releasing hormone-immunoreactive neuronal system in the presumptive ancestral chordate *Ciona intestinalis* (Ascidian). General and Comparative Endocrinology **112**: 426-432.

Van der Kraak G., Dye H.M., and Donaldson E.M. 1984. Effects of LH-RH and Des-Gly10[D-Ala6]LH-RH-ethylamide on plasma sex steroid profiles in adult female coho salmon (*Oncorhynchus kisutch*). General Comparative Endocrinology **55**: 36-45.

von Schalburg K.R., and Sherwood N.M. 1999. Regulation and expression of gonadotropin-releasing hormone gene differs in brain and gonads in rainbow trout. Endocrinology **140**: 3012-3024.

von Schalburg K.R., Harrower W.L., and Sherwood N.M. 1999. Regulation and expression of gonadotropin-releasing hormone in salmon embryo and gonad. Molecular and Cellular Endocrinology **157**: 41-54.

Weber G.M., Powell J.F.F., Park M., Fischer W.H., Craig A.G., Rivier J.E., Nanakorn U., Parhar I.S., Ngamvongchon S., Grau E.G., and Sherwood N.M. 1997. Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. Journal of Endocrinology **155**: 121-132.

White R.B., and Fernald R.D. 1998. Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. General Comparative Endocrinology **112**: 17-25.

White S.A., Bond C.T., Francis R.C., Kasten T.L., Fernale R.D., and Adelman H.R. 1994. A second gene for gonadotropin-releasing hormone: cDNA and expression pattern in the brain. Proceedings of the National Academy of Sciences USA **91**: 1423-1427.

White S.A., Kasten T.L., Bond C.T., Adelman J.P., and Fernald R.D. 1995. Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. Proceedings for the National Academy of Sciences USA **92**: 8363-8367.

White R.B., Eisen J.A., Kasten T.L., and Fernald R.D. 1998. Second gene for gonadotropin-releasing hormone in humans. Proceedings of the National Academy of Sciences USA **95**: 305-309.

Wirsig-Wiechmann C.R. 2001. Function of gonadotropin-releasing hormone in olfaction. Keio Journal of Medicine **50**: 81-85.

Witkin J.W. 1990. Access of luteinizing hormone-releasing hormone neurons to the vasculature in the rat. Neuroscience **37**: 501-506.

Wray S. 2001. Development of luteinizing hormone releasing hormone neurons. Journal of Neuroendocrinology **13**: 3-11.

Wright D.E., and Demski L.S. 1991. Gonadotropin hormone-releasing hormone (GnRH) immunoreactivity in the mesencephalon of sharks and rays. Journal of Comparative Neurology **307**: 49-56.

Yamamoto N., Oka Y., Amano M., Aida K., Hasegawa Y., and Kawashima S. 1995. Multiple gonadotropin-releasing hormone (GnRH)-immunoreactive systems in the brain of the dwarf gourami, *Colisa lalia*: Immunohistochemistry and radioimmunoassay. Journal of Comparative Neurology **355**: 354-368.

Yoo M.S, Kang H.M., Choi H.S., Kim J.W., Troskie B.E., Millar R.P., and Kwon H.B. 2000. Molecular cloning, distribution and pharmacological characterization of novel gonadotropin-releasing hormone ([Trp^8] GnRH) in frog brain. Molecular and Cellular Endocrinology **164**: 197-204.

Yu K.L., Sherwood N.M., and Peter R.E. 1988. Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). Peptides **9**: 625-630.

Yuanyou L., and Haoran L. 2000. Differences in mGnRH and cGnRH-II contents in pituitaries and discrete brain areas of *Rana rugulosa W.* according to age and stage of maturity. Comparative Biochemistry and Physiology Part C **125**: 179-188.

Zandbergen M.A., Kah O., Bogerd J., Peute J., and Goos H.J.Th. 1995. Expression and distribution of two gonadotropin-releasing hormones in the catfish brain. Neuroendocrinology **62**: 571-578.

Zentel H.J., Jennes L., Reiboth R. Stumpf W.E. 1987. Ontogeny of gonadotropin releasing hormone and gonadotropin immunoreactivity in brain and pituitary of normal and estrogen-treated guppies, *Poecilia reticulata* Peters. Cell and Tissue Research **249**: 227-234.

Zmora N., Gonzalez-Martinez D., Munoz-Cueto J.A., Madigou T., Kah O., Mananos-Sanchez E., Doste S.Z., Zohar Y., and Elizur A. 2002. The GnRH system in the European sea bass, *Dicentrarcrhus labrax.* Journal of Endocrinology **172**:105-116.

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Adams BA, Lescheid DW, Vickers ED, Crim LW, and 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**.

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Characterization of gonadotropin-releasing hormone (GnRH) from lake whitefish (*Coregonus clupeaformis*): structure, function, and location.

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