EVOLUTION OF THREE NEUROPEPTIDES ISOLATED FROM THE BRAIN OF STURGEON

David William Lescheid
B.Sc., Simon Fraser University, 1989

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in the Department
of
Biology

We accept this dissertation as conforming
to the required standard

Dr. N. M. Sherwood (Dept. of Biology)

Dr. R. D. Burke (Dept. of Biology)

Dr. S. Misra (Dept. of Biochemistry and Microbiology)

Dr. T. W. Pearson (Dept. of Biochemistry and Microbiology)

Dr. E. M. Donaldson (External examiner)

ABSTRACT

In vertebrates the brain superimposes control on fundamental processes such as reproduction and growth. Neuropeptides secreted from the brain initiate a cascade of events that affect these processes. In this thesis three neuropeptides are examined to determine their structures and patterns in the context of vertebrate evolution.

Reproduction in vertebrates is controlled by the neuropeptide gonadotropin-releasing hormone, GnRH, a decapeptide belonging to a peptide family of twelve known members. One common theme in vertebrates is that there is usually more than one form of GnRH in the brain of a single species; often each form of GnRH has a separate location in the brain and therefore, an implied distinct function. In this thesis, the brain of Siberian sturgeon, *Acipenser gueldenstaedti*, initially was examined for GnRH using reversed-phase high performance liquid chromatography, HPLC, and radioimmunoassay, RIA, with specific antisera and was shown to contain mammalian (m)GnRH by chemical sequence analysis and by accurate determination of the molecular mass. In addition, another form of GnRH, termed chicken (c)GnRH-II, was found in the sturgeon brain. This is the first report to show that the primary structure of GnRH is identical in an evolutionarily-ancient fish and in mammals including humans. Further, the second form of GnRH, cGnRH-II, was identified for the first time in the brain of adult stumptail monkeys (*Macaca speciosa*) as well as in adult and fetal rhesus monkey (*Macaca mulatta*) brains. This study implies that at least two forms of GnRH are found in the brain of most vertebrate species including mammals.

In cartilaginous fish that evolved earlier than sturgeon, the same HPLC and RIA methods were used to demonstrate that regions of the brain and pituitary of skate, *Raja canebensis*, also contained cGnRH-II but dogfish (df)GnRH rather than mGnRH. By the same criteria, teleost fish like whitefish (*Prosopium williamsoni*), platyfish (*Xiphophorus maculatus*), green swordtail (*Xiphophorus hellerei)* and sablefish (*Anoplomia fimbria*)

were shown to have cGnRH-II and salmon (s)GnRH, as well as one or two more immunoreactive variants of GnRH with novel or seabream (sb)GnRH-like properties, within their brain. The identity of at least three types of immunoreactive GnRH molecules in the brain of these fish species suggests that three forms of GnRH in the brain is an early condition in teleost evolution.

Ancestral sturgeon emerged at a branch point between the bony fish lineage and the tetrapod lineage and therefore, it is useful to compare the neuropeptide structures found in their brain with those both in fish and more evolutionarily-advanced vertebrates. Several tetrapod species were examined to determine if the forms of GnRH found in the sturgeon brain had been retained in their evolution. In contrasts to studies in our laboratory and by others showing that most amphibians, reptiles and birds contain two forms of GnRH, the present research shows that the brain of the green anole lizard, *Anolis carolinensis*, contained only cGnRH-II within its brain. In addition, my HPLC and RIA studies showed that only mGnRH was present in the brain of guinea pig, hamster and rat suggesting that there are some species which function with only one form of GnRH in their brain. Also, there were no distinguishable forms of GnRH in a human placenta, demonstrating that the type(s) of GnRH might be tissue-specific.

Two neuropeptides associated with growth also were isolated from the sturgeon brain. A cDNA encoding growth hormone-releasing factor, GRF, and pituitary adenylate cyclase-activating polypeptide, PACAP, was isolated and sequenced using the polymerase chain reaction, PCR, and other molecular biology methods. In contrast to mammals where GRF and PACAP are encoded on separate genes, in sturgeon, GRF and PACAP are encoded in tandem on a single mRNA.

In this thesis, I establish the structure of GnRH, GRF, and PACAP in sturgeon, a species that evolved near a critical branching point between bony fish and tetrapods. These structures are used as a focal point for comparison to those of other vertebrates. This comparative evolutionary approach is an important step toward understanding the evolution of these important neuropeptides as well as enhancing our knowledge of general principles in the endocrine systems controlling reproduction and growth.

Examiners:

Dr. N.M. Sherwood (Dept. of Biology)

Dr. R. D. Burke (Dept. of Biology)

Dr. S. Misra (Dept. of Biochemistry and Microbiology)

Dr. T. W. Pearson (Dept. of Biochemistry and Microbiology)

Dr. E.M. Donaldson (External examiner)

TABLE OF CONTENTS

ABSTRACT.....ii

TABLE OF CONTENTS.....v

LIST OF FIGURES.....viii

LIST OF TABLES.....xii

LIST OF ABBREVIATIONS.....xiv

ACKNOWLEDGEMENTS.....xvi

CHAPTER 1: General Introduction.....1

GnRH peptide structure.....8
GnRH: function and location.....11
GnRH mRNA and gene structure.....12
GRF peptide structure.....16
GRF: location and function.....19
GRF mRNA and gene structure.....19
PACAP peptide structure.....21
PACAP: location and function.....21
GRF-like/ PACAP and PACAP mRNA and gene structure.....23
Glucagon superfamily.....24
Transport of GnRH, GRF and PACAP in the brain of vertebrates.....25
GnRH receptor.....26
GRF and PACAP receptor superfamily.....27
GRF receptor.....27
PACAP receptor.....28
Overlapping functions.....29
Prevertebrate history.....30
Purpose of thesis.....30
Literature cited.....33

CHAPTER 2: Determination of the primary structure for mGnRH from the brain of Siberian sturgeon, *Acipenser gueldenstadtii*.....47
Introduction.....48
Materials and Methods.....50
Results.....55
Discussion.....65
Literature cited.....68

CHAPTER 3: Identification of the forms of GnRH present in the brain of a cartilaginous fish, the skate, as well as in the brains of four teleosts: whitefish, platyfish, green swordtail and sablefish.....73
Introduction.....74
Materials and Methods.....78
Results.....88
Discussion.....112
Literature cited.....119

CHAPTER 4: Identification of cGnRH-II but not cGnRH-I or other forms of GnRH in the brain of a lizard, *Anolis carolinensis*.....126
Introduction.....127
Materials and Methods.....129
Results.....132
Discussion.....139
Literature cited.....145

CHAPTER 5: Identification of two forms of GnRH in the brain of adult and fetal monkeys and examination of a human placenta for GnRH immunoreactivity.....149
Introduction.....150
Materials and Methods.....152
Results.....158
Discussion.....183
Literature cited.....189

CHAPTER 6: Identification of mGnRH but not cGnRH-II or other forms of GnRH in the brains of guinea pig, hamster and rat.....195
Introduction.....196
Materials and Methods.....197
Results.....202
Discussion.....216
Literature cited.....220

CHAPTER 7: Isolation and characterization of the cDNA(s) encoding GRF/ PACAP from the brain of white sturgeon, *Acipenser transmontanus*.....223
Introduction.....224
Materials and Methods.....226
Results.....232
Discussion.....235
Literature cited.....250

CHAPTER 8: General conclusions.....254
Sturgeon mGnRH: past, present and future.....255
Anolis GnRH: loss or alteration of cGnRH-I, a mGnRH derivative.....258
cGnRH-II: pre-sturgeon to mammals.....259
Teleosts: three forms of GnRH in the brain of a single species.....261
Identification of a sturgeon GRF-like/ PACAP precursor.....264
Evolution: GnRH and GRF/ PACAP.....269
Literature cited.....277

LIST OF FIGURES

Chapter 1

Figure 1-1: Schematic diagram showing the phylogeny of vertebrates.....4, 5

Figure 1-2: The twelve GnRH peptide structures isolated to date.....10

Figure 1-3: GnRH, GRF and PACAP gene structures.....15

Figure 1-4: The GRF peptide structures isolated to date.....18

Chapter 2

Figure 2-1: Initial study of sturgeon GnRH.....57

Figure 2-2: Purification of sturgeon GnRH.....60, 61

Figure 2-3: Absorbance spectra of sturgeon GnRH eluted from a narrow-bore C-18 HPLC column.....64

Chapter 3

Figure 3-1: Percent cross-reactivity between (A) antisera GF-4, B-6 or Bla -5 and (B) antisera 7CR-10 or Adams-100 with 11 different synthetic GnRH peptides.....86, 87

Figure 3-2: Immunoreactive GnRH in different regions of skate brain and pituitary after HPLC elution.....92, 93

Figure 3-3: Immunoreactive GnRH in whitefish pituitary extracts after HPLC elution.....96

Figure 3-4: Immunoreactive GnRH in whitefish brain extracts after HPLC elution.....100,101

Figure 3-5: Immunoreactive GnRH in platyfish brain extracts.....103

Figure 3-6: Immunoreactive GnRH in green swordtail brain extracts after HPLC elution.....106

Figure 3-7: Immunoreactive GnRH from sablefish brain extracts after HPLC elution.....110, 111

Chapter 4

Figure 4-1: Immunoreactive GnRH after HPLC elution of *A. carolinesis* brain extracts.....136

Figure 4-2: Immunoreactive GnRH in HPLC fractions of *A. carolinensis* brain extracts.....138

Chapter 5

Figure 5-1: Immunoreactive GnRH in adult stumptail monkey brain extracts showing HPLC elution positions.....161

Figure 5-2: Immunoreactive GnRH in adult stumptail monkey brain extracts showing HPLC elution positions.....163

Figure 5-3: Mammalian GnRH in fetal (E72-E77) rhesus monkey after HPLC elution.....166

Figure 5-4: Chicken GnRH-II in fetal (E125) rhesus monkey after HPLC elution.....168

Figure 5-5: HPLC elution of mGnRH from adult stumptail monkey brain extracts from the diencephalon, cortex and brainstem regions.....171

Figure 5-6: HPLC elution of cGnRH-II in adult stumptail monkey brain extracts from the diencephalon, cortex and brainstem regions.....173

Figure 5-7: Immunoreactive GnRH in human placental extracts after HPLC elution.176

Figure 5-8: Examples of immunostained cells for chicken GnRH-II or mammalian GnRH.....179

Figure 5-9: Effect of intravenous administration of cGnRH-II (at Time 0) on plasma LH concentrations in adult female rhesus macaques.....182

Chapter 6

Figure 6-1: Immunoreactive GnRH following HPLC elution of guinea pig brain extracts.....204, 205

Figure 6-2: Immunoreactive GnRH in hamster brain extracts following elution from HPLC columns.....208, 209

Figure 6-3: Immunoreactive GnRH in maternal rat brain extracts after HPLC elution.....212

Figure 6-4: Immunoreactive GnRH following elution of fetal rat brain extracts from a C-18 HPLC column.....214, 215

Chapter 7

Figure 7-1: The regions of the sturgeon GRF-like/ PACAP precursor that were amplified by PCR.....228

Figure 7-2: Nucleotide sequence and the corresponding amino acid sequence of the white sturgeon GRF-like/ PACAP precursor.....234

Figure 7-3: Comparison of domains in the precursors of GRF-like/ or PRP/ PACAP.....237

Figure 7-4: A comparison of the known GRF and GRF-like peptides to human GRF.....240

Figure 7-5: White sturgeon GRF-like peptide compared to glucagon superfamily members.....247

Figure 7-6: White sturgeon PACAP peptide compared to glucagon superfamily members.....249

Chapter 8

Figure 8-1: Comparison of the precursor structures of mammalian PRP/ PACAP and non-mammalian GRF-like/ PACAP precursors with the precursor to human PRP/ PACAP.....267

Figure 8-2: Schematic diagram showing the phylogeny of GnRH.....272

Figure 8-3: Schematic diagram showing the phylogeny of GRF/ PACAP.....275

LIST OF TABLES

Table 2-1 : Sequence of HPLC steps in the purification of GnRH.....53

Table 3-1A: Skate HPLC and RIA summary.....79

Table 3-1B: Teleost species HPLC and RIA summary.....80

Table 3-2: HPLC programs used for the elution of fish extracts.....83

Table 3-3: Antisera characteristics.....89

Table 4-1: Antisera characteristics for Anolis study.....131

Table 4-2: Antisera cross-reactivity for Anolis study.....133

Table 5-1: Amount (ng) of immunoreactive GnRH in primate brain.....159

Table 6-1: Rodent HPLC and RIA summary.....198

LIST OF ABBREVIATIONS

Gonadotropin-releasing hormone (GnRH) family:

mGnRH: mammalian GnRH
(hyp9)mGnRH: hydroxyproline-9 mammalian GnRH
cGnRH-II: chicken GnRH-II
cGnRH-I: chicken GnRH-I
sGnRH: salmon GnRH
cfGnRH: catfish GnRH
hGnRH: herring GnRH
sbGnRH: sea bream GnRH
dfGnRH: dogfish GnRH
lGnRH-I: lamprey GnRH-I
lGnRH-II: lamprey GnRH-II
tGnRH-I: tunicate GnRH-I
tGnRH- II: tunicate GnRH-II

Other related terms:

LH, GTH-I: luteinizing hormone, gonadotropin-I
FSH, GTH-II: follicule stimulating hormone, gonadotropin-II
GAP: GnRH-associated peptide
irGnRH: immunoreactive GnRH
AA: amino acids

Techniques and columns used in the isolation of GnRH:

HPLC: high performance liquid chromatography
RIA: radioimmunoassay
SepPak C-18: 10 SepPak C-18 cartridges (Waters, Milford, MA) in which plastic connections were trimmed to remove dead space and remaining cartridges were held together in series by shrink wrap tubing
LC-18: Supelcosil long chain C-18 column (25cm x 4.6mm x 5μm particle size, Supleco, Bellefontaine, PA)
Vydac: Vydac diphenyl column (25cm x 4.6mm x 5μm particle size, Vydac, Nesperia, CA)

Solvents used in the isolation of GnRH:

mqH_20:	Milli-Q H_2O (Millipore, Bedford, MA)
CH_3CN:	acetonitrile
ACN:	acetontirile
HFBA:	heptafluorobutyric acid
TEAF:	triethylammonium formate* (brought to pH 6.5 by triethylamine)
TEAP:	triethylammonium phosphate* (brought to pH 6.5 by triethylamine)
TFA:	trifluoroacetate
PBS:	phosphate-buffered saline

Glucagon superfamily peptides:

GRF:	growth hormone-releasing factor
PACAP:	pituitary adenylate cyclase-activating polypeptide
GIP:	glucose-dependent insulin-releasing peptide
VIP:	vasoactive intestinal polypeptide
PHM:	peptide histidine methionine
PHI:	peptide histidine isoleucine
GIP:	glucose-dependant insulin-releasing peptide
GLP-I, GLP-II:	glucagon-like peptide (I and II)

Other related terms:

PRP:	PACAP-related peptide
GH:	growth hormone
IGF:	insulin-like growth factor
PRL:	prolactin
AA:	amino acids
nt:	nucleotides
DNA:	deoxyribonucleic acid
cDNA:	complementary DNA
mRNA:	messenger ribonucleic acid
dATP:	deoxyadenosine triphosphate
dNTP:	deoxynucleotide triphosphate
UTR:	untranslated region

Techniques and chemicals used in the isolation of GRF/ PACAP:

PCR:	polymerase chain reaction
RACE:	rapid amplification of cDNA (or conserved) ends
$MgCl_2$:	magnesium chloride
EDTA:	disodium ethylenediamine tetraacetic acid
DTT:	dithiothreitol
KCL:	potassium chloride
Tris HCl:	tris (hydroxymethyl) aminomethane buffered with 0.1N HCL
TdT:	terminal deoxynucleotidyl transferase

ACKNOWLEDGEMENTS

An educational journey of this magnitude could not have been accomplished without the support of friends and family. Undoubtedly there are some people who have helped me along the way whose names I will fail to mention; you know who you are and from the bottom of my heart I thank you. I especially would like to thank my family for providing a loving support group. Also, mom and dad, thanks for giving me the stubborn gene that helped me persevere despite what at times seemed to be overwhelming odds. The following "Sherwood lab rats" deserve special credit: 1) Drs. Imogen Coe and David Parker for teaching me about the wonders of molecular biology and Drs. David Lovejoy and Jim Powell for instructing me in the ways of fish and protein chemistry, 2) Sandra Krueckl and Kris von Schalburg, you have been true friends along every step of the way, God bless you in your future endeavours, and 3) the lab rookies, Erica, Kevin, Dan, Sarah, and Nola; the thesis road ahead might be long and arduous but the light is on, just keep moving towards it. Carol Warby, our technician, deserves special recognition for her patience in teaching me HPLC and RIA and her tremendous help in many of the projects. I am also indebted to Dr. Joachim Carolsfeld (Yogi) for his timely advice and words of wisdom. Annika Stein, Kathryn Clark, Michael Koch, Amanda Bridge, Emily Standen, David Young and Robin Munro, also are people who have worked in the Sherwood lab and to whom I am grateful for making it easier to go to work every day. Three special people that made it easier to leave the lab every day were Cathy, Val and Jeanie. Thank-you so much; your love, support and kindness will not be forgotten.

I also would like to acknowledge the critters that were used in these studies, especially the sturgeon, I sincerely hope that some of the knowledge that has been gained in this thesis will prove beneficial to their survival.

Finally, I want to thank my supervisor, Dr.Nancy Sherwood, for being a true mentor, providing support and encouragement over the years and never ceasing to amaze me with her undying drive and passion for this work.

Chapter 1

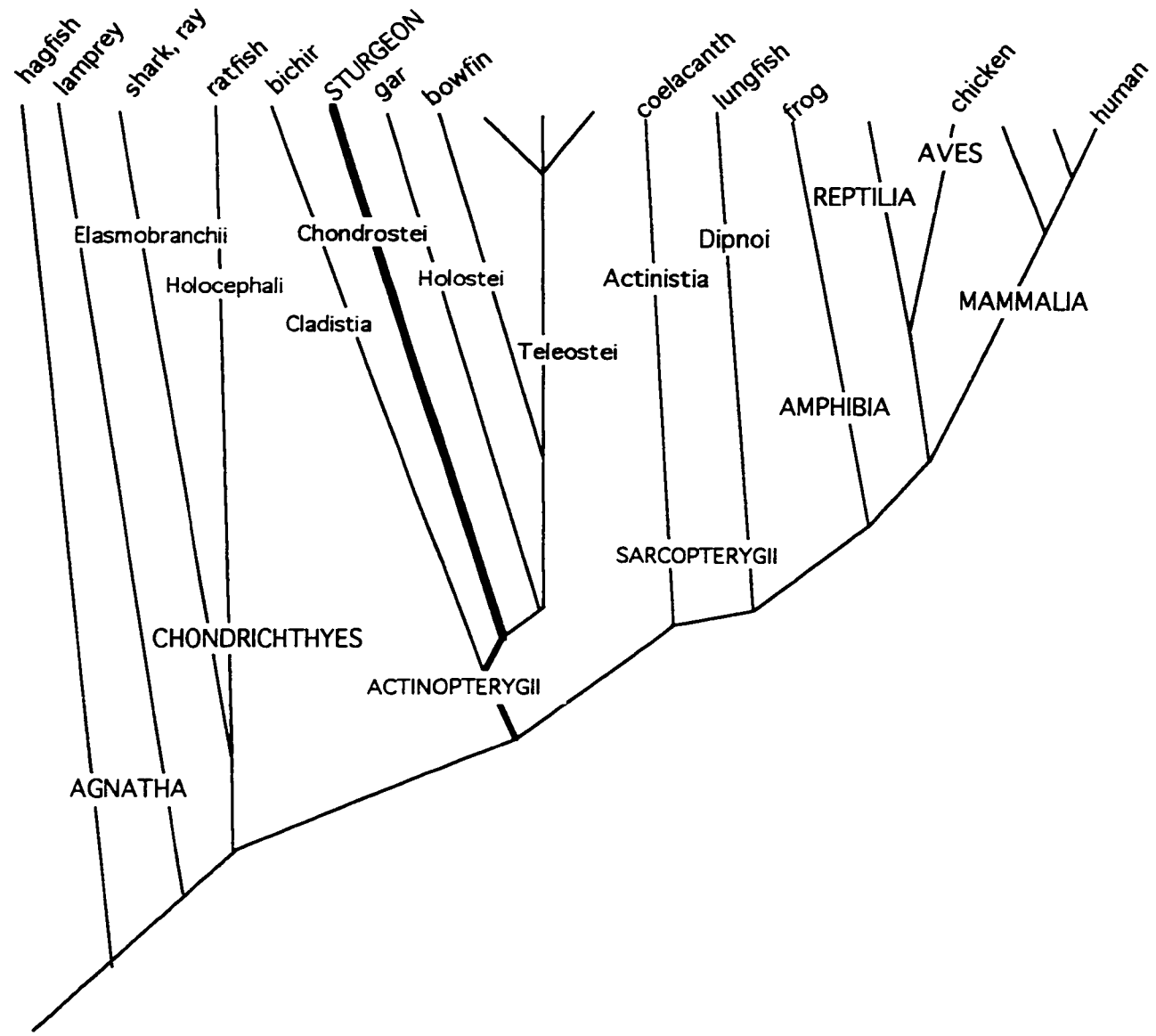
General Introduction

The process and products of evolution can be examined using both macroscopic and microscopic methods. A combination of both methods is useful in drawing conclusions about the phylogeny of species because the methods are so inextricably linked; most of the directly observed (macroscopic) characteristics of a species are produced co-operatively by the interaction between genetic (microscopic) and environmental factors. In evolution, stem groups include the more primitive ancestors of a distinct lineage. Ancestral sturgeon were part of the stem group for bony fish that emerged near the transition between the major lineages of bony fish and tetrapods. Living sturgeon have retained many of the primitive features of the stem group.

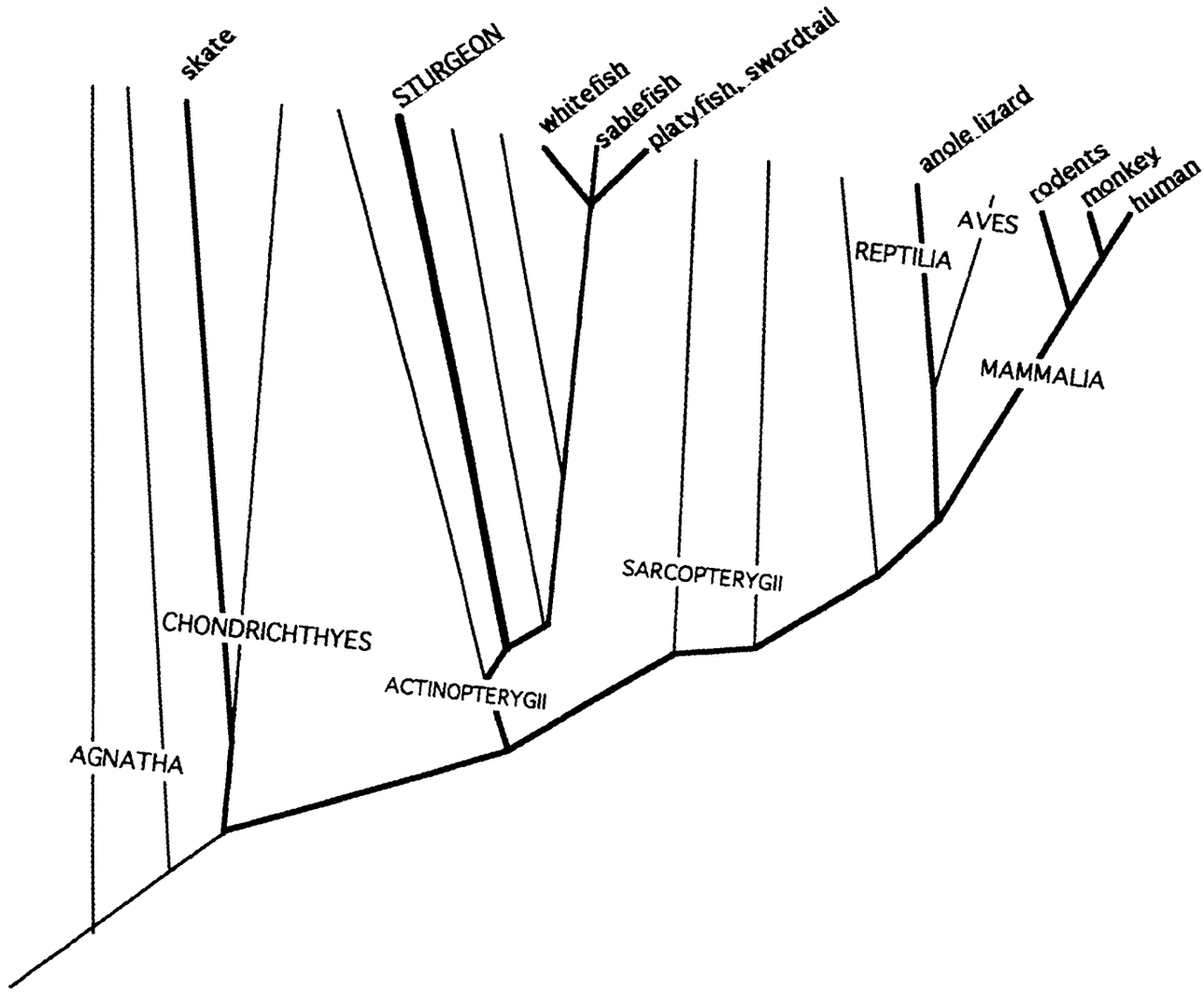
Sturgeon are of particular interest in phylogenetic studies because of their ancient origin and possible relationship to the tetrapod line (Fig. 1-1). Sturgeon-like fossils have been dated to the early Jurassic period and therefore, sturgeon ancestors emerged over 200 million years ago. These sturgeon ancestors, the Chondrostei, are survived by two orders and three families: order Palaenoiscoidei, the bichirs and order Acipenseroidei, the sturgeons and paddlefish (Young, 1981). Although extant species of sturgeon have evolved and changed considerably from their ancestors, they retain some features of primitive fish such as a heterocercal tail, bony external scutes and rhomboidal scales, an almost wholly cartilaginous skull and skeleton, and an unrestricted notochord. Some of their internal digestive, circulatory and buoyancy organs are also more primitive than those in more evolutionarily-advanced fish. Surprisingly, sturgeons have some internal anatomical characteristics that are more like tetrapods than like modern bony fish. For example, they have a rudimentary median eminence and hypothalamo-hypophyseal portal system, an acrosome sperm that undergoes an acrosomal reaction to enter the egg (even though the egg contains a micropyle), a Muellerian duct and an egg that undergoes holoblastic cleavage during development. In sturgeon, the presence of both primitive and advanced anatomical and physiological characteristics suggest that they represent an important evolutionary transition point.

Fig. 1-1. Schematic diagram showing the phylogeny of vertebrates. The major groups of vertebrates are identified by uppercase lettering, whereas lesser taxa are in lowercase with the first letter in uppercase; representative species from those orders are in lower case only. A) Bold lettering and bold lines identifies sturgeon. B) Bold underlined lettering identify sturgeon as well as other species studied in this thesis.

A.


hagfish
lamprey
shark, ray
ratfish
bichir
STURGEON
gar
bowfin
coelacanth
lungfish
frog
chicken
human
AVES
REPTILIA
MAMMALIA
Elasmobranchii
Holocephali
Chondrostei
Holostei
Cladistia
Teleostei
Actinistia
Dipnoi
AMPHIBIA
SARCOPTERYGII
CHONDRICHTHYES
ACTINOPTERYGII
AGNATHA

B.


skate
STURGEON
whitefish
sablefish
platyfish, swordtail
anole lizard
rodents
monkey
human
AVES
REPTILIA
MAMMALIA
SARCOPTERYGII
CHONDRICHTHYES
ACTINOPTERYGII
AGNATHA

Anatomical and physiological features are not the only tools that have proven useful in determining the phylogeny of species. Protein and peptide structure, and the sequence of the encoding gene and cDNA, also have become important in establishing the relatedness of species and formulating hypotheses about their origin. Although there have been few reported sequences from sturgeon, these have provided additional information suggesting that sturgeon arose close to a branching point in evolution between the emergence of bony fish and and the line that led to tetrapods. For example, sturgeon growth hormone, GH, has immunological and biological properties that are closer to those in more recently-evolved vertebrates like coelacanths, lungfish and tetrapods than to those of teleost GHs (Bewley and Papkoff, 1987; Farmer *et al.*, 1981; Hayashida, 1977; see Rubin and Dores, 1994). The peptide structures of sturgeon GHs have recently been characterized and as predicted they have higher sequence identity with tetrapod GHs than with teleost GHs (Yasuda *et al.*, 1992). The sturgeon liver also contains receptors that bind mammalian GHs with characteristics more closely resembling mammalian GH receptors than teleost GH receptors (Tarpey and Nicoll, 1985), suggesting parallel evolution between the peptide and its binding site. These authors suggest that sturgeon retain an ancestral form of GH that was conserved throughout the evolutionary lineage leading to mammals. However, during the course of teleost evolution, the stringent structure/ function relationship of the GH molecule was "relaxed", allowing rapid mutation to a more chemically and structurally unique form. In contrast, sturgeon prolactin, PRL, a peptide thought to share a common ancestry with GH, has a slightly higher sequence identity with teleost PRLs than with tetrapod PRLs. However, sturgeon PRLs have a putative tertiary structure that is more like the structure in lungfish and tetrapods than that in bony fish (Noso *et al.*, 1993). Another sturgeon protein that might have followed an evolutionary path more similar to that in teleosts than to that in tetrapods is thrombin B which has 82.5% amino acid sequence identity with rainbow trout thrombin B but only 64-72% identity with that in other vertebrates,

including hagfish, chicken and various tetrapods (Banfield and MacGillivray, 1992). There also is a sturgeon peptide, urotensin II (UII) that was shown to have a higher degree of relatedness with phylogenetically ancient fish than with teleosts (McMaster *et al.*, 1992; Waugh *et al.*, 1995). Finally, the sturgeon pituitary contains two neurohypophysial hormones; vasotocin, which has been conserved in hagfish, lampreys, cartilaginous fish, euteleosts and some mammals, and an oxytocin-like molecule, which is more like that present in some metatherian and placental mammals than in other vertebrates (Acher *et al.*, 1973, Rouille *et al.*, 1991). The aforementioned studies in sturgeon demonstrate that protein and peptide sequences can be useful tools in proposing the relatedness of species but they also show some of the limitations of using this method to establish phylogeny. Sturgeon contain peptides that are structurally related to evolutionarily-ancient vertebrates as well as to more recently-evolved vertebrates and hence phylogeny based solely on the peptide sequences they contain would be difficult. Therefore, it is clear that the phylogenetic information obtained from peptide sequences, and/or the cDNAs and genes that encode them, is most useful if used in conjunction with anatomical, physiological and fossil evidence.

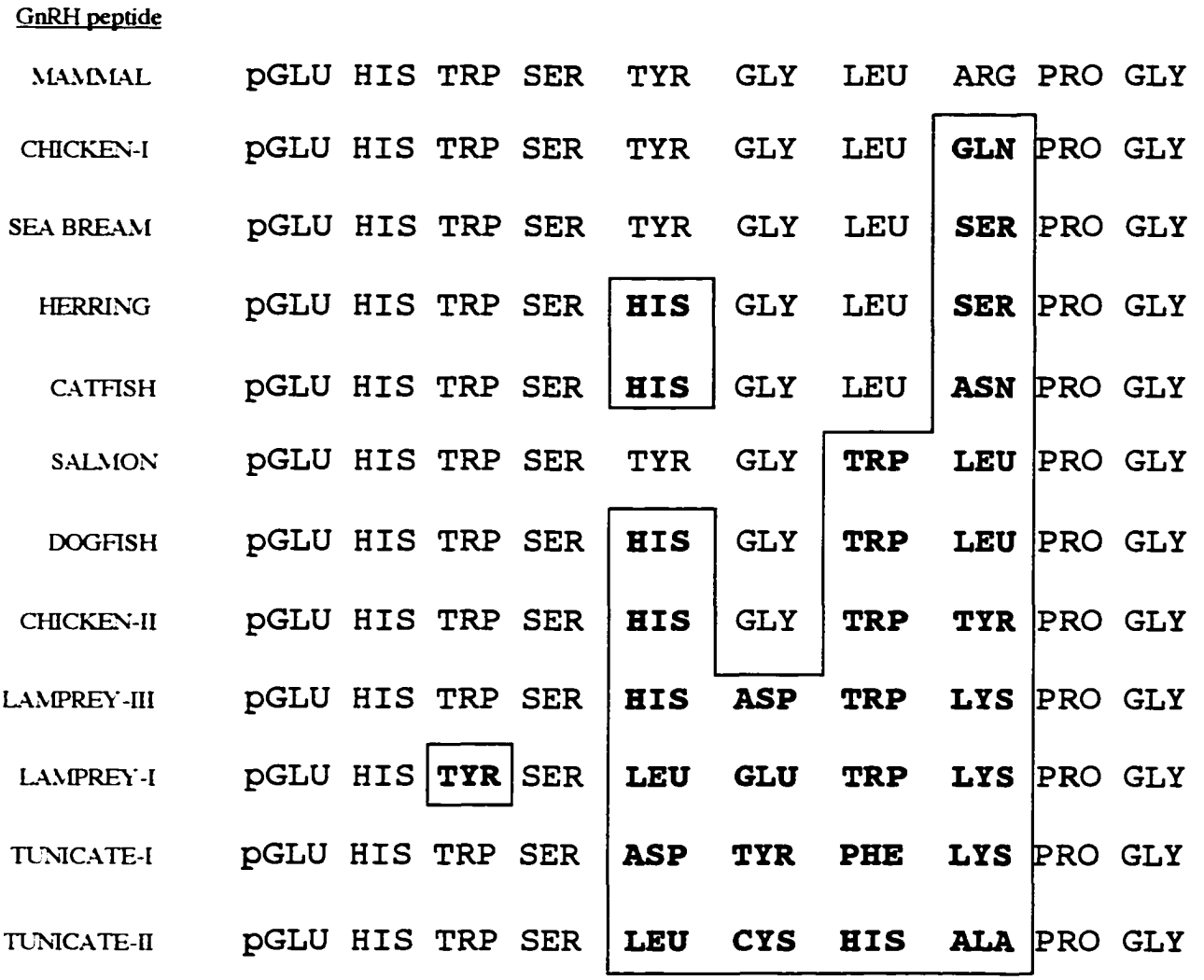
It might be expected that peptides central to major physiological processes would be highly conserved in evolution and therefore, be valuable as molecular tools to help determine phylogeny. Two such peptides are gonadotropin-releasing hormone, GnRH, and growth hormone-releasing factor, GRF; these two neuropeptides are synthesized in the brain and other tissues of vertebrates and are fundamental to the regulation of reproduction and growth, respectively. Another peptide with a high degree of sequence identity in vertebrates, but with many functions including the release of growth hormone, is pituitary adenylate cyclase-activating polypeptide, PACAP. GnRH and GRF are useful as phylogenetic probes because they are easily identified by structure and function. The high conservation of PACAP throughout vertebrate evolution also makes it favourable for helping to establish relatedness among species.

GnRH peptide structure

GnRH was first isolated from pig and sheep hypothalami (Burgus *et al.*, 1972; Matsuo *et al.*, 1971). Since that time, there have been twelve different GnRH peptides isolated; ten from vertebrates and two from invertebrates. Each GnRH is a decapeptide with a pyroglutamyl modified amino terminus, an amidated carboxy terminus, conserved amino acids 1, 2, 4, 9 and 10 (Fig. 1-2) and at least 50% sequence identity to other GnRH family members (Sherwood *et al.*, 1997). Most of the differences between GnRHs occurs in amino acids 5-8 but, additional structural diversity is also achieved by post-translational modification, as shown by hydroxylation of proline in position 9 in mammalian(m) GnRH (Gautron *et al.*, 1991) or by dimerization, as shown by tunicate(t)GnRH-II (Powell *et al.*, 1996).

One common theme among vertebrates is that more than one form of GnRH exists within the brain of a single species. The most ubiquitous form of GnRH is chicken (c)GnRH-II, which has been found in the brain of species from all classes of vertebrates except for Agnatha. Other frequently found forms of GnRH include mGnRH, which is in the brain of mammals, amphibians and possibly some evolutionarily-ancient bony fish. Salmon (s)GnRH is in the brain of all teleosts except for two species of catfish and some early-evolved teleosts like eels and butterflyfish (Powell, 1995; see Sherwood *et al.*, 1997; Standen, 1995). Within the brain, the two or three forms of GnRH are usually found in different locations and therefore, might have a separate function.

Fig. 1-2. The twelve GnRH peptide structures isolated to date. The amino acid differences from mammalian GnRH are outlined and in bold. The GnRH structures are named after the animal from which they were first isolated, but also are found in other species.


GnRH peptide
MAMMAL pGLU HIS TRP SER TYR GLY LEU ARG PRO GLY
CHICKEN-I pGLU HIS TRP SER TYR GLY LEU GLN PRO GLY
SEA BREAM pGLU HIS TRP SER TYR GLY LEU SER PRO GLY
HERRING pGLU HIS TRP SER HIS GLY LEU SER PRO GLY
CATFISH pGLU HIS TRP SER HIS GLY LEU ASN PRO GLY
SALMON pGLU HIS TRP SER TYR GLY TRP LEU PRO GLY
DOGFISH pGLU HIS TRP SER HIS GLY TRP LEU PRO GLY
CHICKEN-II pGLU HIS TRP SER HIS GLY TRP TYR PRO GLY
LAMPREY-III pGLU HIS TRP SER HIS ASP TRP LYS PRO GLY
LAMPREY-I pGLU HIS TYR SER LEU GLU TRP LYS PRO GLY
TUNICATE-I pGLU HIS TRP SER ASP TYR PHE LYS PRO GLY
TUNICATE-II pGLU HIS TRP SER LEU CYS HIS ALA PRO GLY

Function and location

GnRH can act as either a hypophysiotropic hormone or a neuromodulator in the brain depending on where it is located. Cells containing mGnRH, cGnRH-I, sGnRH, and sea bream (sb) GnRH are found in the forebrain-septo-preoptic system. The majority of the axons from these cells extend to hypothalamo-hypophyseal portal vessels in tetrapods or into the pituitary in teleosts. Therefore, these types of GnRH predominantly act as hypophysiotropic hormones stimulating the synthesis and release of the gonadotropins (GtHs), luteinizing hormone (LH), and follicle stimulating hormone (FSH). In addition, some axon branches terminate on neurons in the brain and therefore, these forms of GnRH might have a neuromodulatory as well as hypophysial function.

The role of cGnRH-II in reproduction is curious in that the synthetic form is a potent releaser of gonadotropins, but the anatomical position of axons containing cGnRH-II suggests that the peptide might not reach the portal vessels. cGnRH-II cells are most abundant in the mid-brain, with the majority of fibers extending to extra-hypothalamic parts of the brain and to the spinal cord (see Muske, 1993). The ability of chicken GnRH-II to release gonadotropins has been most clearly demonstrated in birds, where administration of either cGnRH-II or cGnRH-I can stimulate the release of LH and FSH (Sharp *et al.*, 1990). However, the role of gonadotropin releaser is assigned to cGnRH-I partially because of the anatomical location of neurons containing cGnRH-I; their axons terminate on the hypophysial portal system that perfuses the pituitary. The other reason is that active immunization against cGnRH-I, but not against cGnRH-II, results in a regression of the reproductive system and suppression of plasma LH (Sharp *et al.*, 1990). Hence, in most vertebrates the spatial isolation of cGnRH-II-containing cells from gonadotropin-synthesizing cells in the pituitary suggests that the predominant role of cGnRH-II is neuromodulation. This putative function has been demonstrated in the bullfrog where the sympathetic ganglia have strong binding sites for cGnRH-II and also where changes in potassium currents (Jones, 1987) or in late, slow post-synaptic

potentials (Hsueh and Schaeffer, 1985) occur upon exposure to cGnRH-II. Other indications of neuromodulation are that injection of GnRH into the midbrain enhances lordosis and female receptivity in rats (Pfaff *et al.*, 1993) and turtle doves (H.R. Besmer, unpublished data, Millar and King, 1994).

Other less well-known functions of GnRH include growth hormone and prolactin release from the pituitary of some species of fish as well as release of human chorionic gonadotropin, hCG, from the placenta and embryo development and growth in mammals (Sherwood *et al.*, 1997). GnRH mRNA transcripts also have been found in the placenta, mammary gland, ovary and testis of some species, suggesting that GnRH might be important in the development and maturation of these reproductive tissues (Sherwood *et al.*, 1997). The maintenance of proper gonadal development and function might be an ancestral function for GnRH because in adult tunicates, injection of either tGnRH-I or tGnRH-II into the visceral blood sinus increased the estradiol content in the gonads (Powell *et al.*, 1996).

GnRH mRNA and gene structure

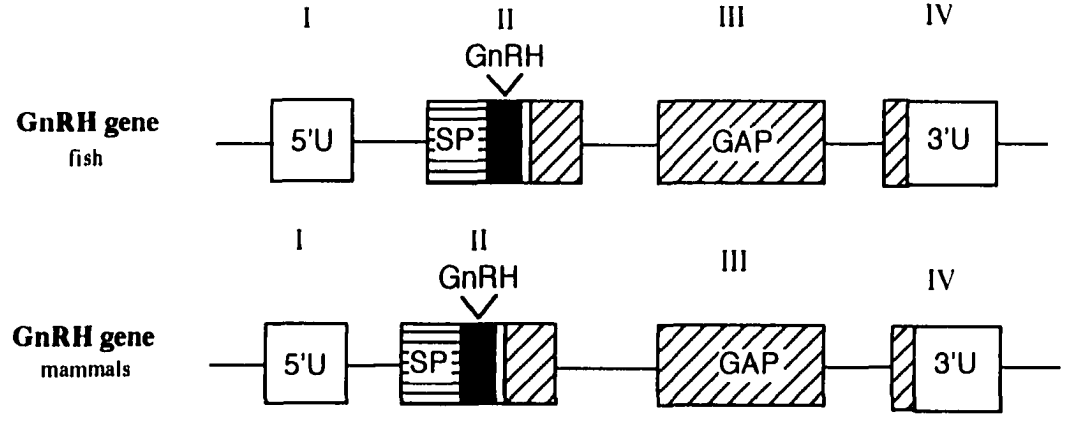
The cDNAs encoding six of the twelve known GnRH peptides have been isolated: 1) mGnRH, from the brain of human, rat, hamster and frog as well as from human placenta and rat ovary and lymphocyte, 2) cGnRH-II, from the brain of tree shrew, catfish, sea bream and cichlid and from the ovary of goldfish, 3) sGnRH, from the brain of seven salmonids, cichlid, sea bream and midshipman; also, two separate cDNAs encoding sGnRH have been reported in salmon, 4) catfish (cf) GnRH, from the brain of catfish; two separate cDNAs encoding cfGnRH also have been reported in catfish, 5) cGnRH-I, from the brain of chicken, and 6) sbGnRH, from the brain of gilthead sea bream and cichlid (Sherwood *et al.*, 1997). Each cDNA encodes a preprohormone precursor that includes a 23 amino acid (AA) signal peptide with a conserved hydrophobic core, a 10 AA GnRH with a deduced amino acid sequence identical to the

corresponding isolated peptide primary structure and a 46-56 AA GnRH-associated peptide (GAP). The GAP region is not only variable in length depending on species but also has little sequence identity among the various GnRH cDNAs except for 80% conservation among the seven salmonids. The function of GAP is not known but, it may be important for proper folding of the prohormone for processing (Sherwood *et al.*, 1997).

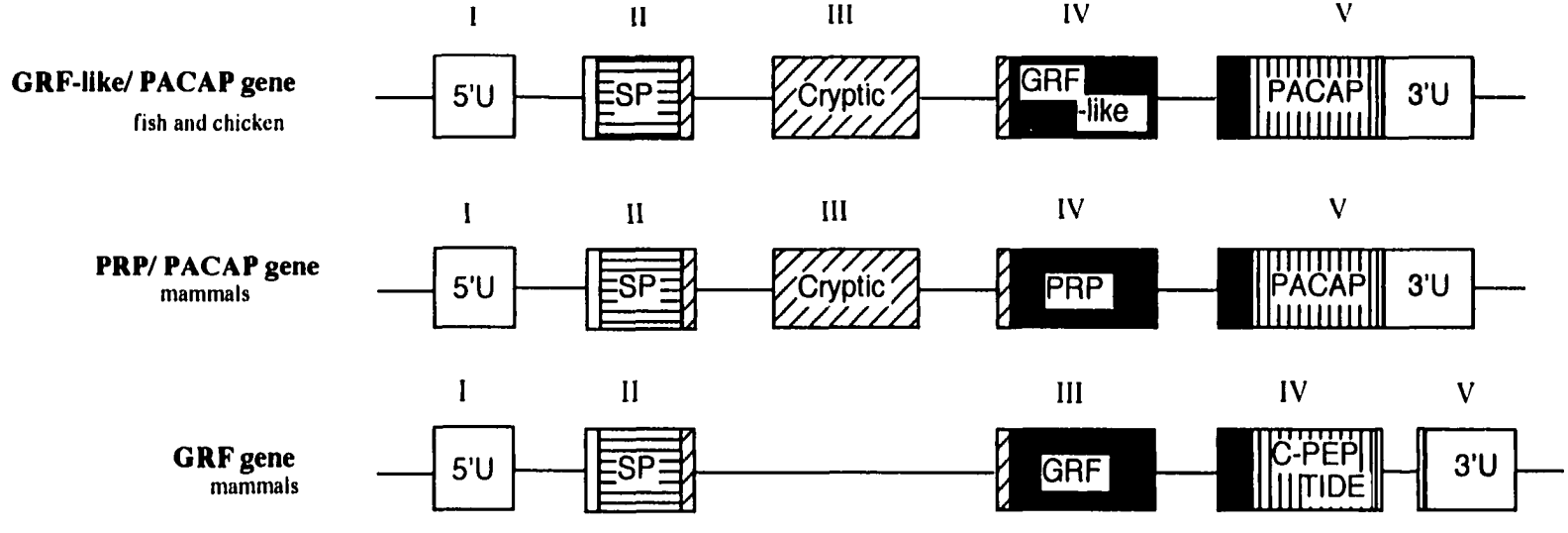
Currently, only the genes encoding mGnRH, cGnRH-I, cGnRH-II, sbGnRH and sGnRH are known. The gene from which mGnRH is transcribed was identified in the brain of human, rat, and mouse whereas, the gene encoding cGnRH-I was isolated from the brain of chicken (Sherwood *et al.*, 1997). The genes encoding cGnRH-II, sbGnRH and sGnRH were recently isolated from the striped bass (Chow *et al.*, 1997). The sGnRH gene was isolated from the brain of Atlantic salmon and Pacific salmon. Each gene has a similar structural pattern of four exons separated by three introns. In each gene, exon I encodes the 5' untranslated region (5' UTR); exon II encodes the signal peptide, GnRH, a Gly-Lys-Arg enzymatic cleavage site and the first eleven AA of the GAP; exon III encodes the majority of the GAP and; exon IV encodes the remainder of the GAP and the 3' untranslated region, 3' UTR (Fig. 1-3). The structural organization of the GnRH gene as well as domains encoded by each individual exon are conserved among species, but there is considerable sequence variability in the 5' regulatory region and in the size of the introns (Sherwood *et al.*, 1997). Each form of GnRH is encoded by a separate gene as there are no reports of two or more GnRH sequences encoded in tandem in a single precursor. Therefore, it is clear that other GnRH genes and cDNAs remain to be isolated.

Figure 1-3: GnRH, GRF, and PACAP gene structures. A). Comparison of the GnRH genes of fish and mammals. Exons I, II, II, and IV are labelled. Introns are shown as single lines. Exons, introns and peptide domains are not drawn to scale. (adapted from Sherwood *et al.*, 1994). B). Comparison of the GRF-like/ PACAP genes of fish and chicken with the PACAP and GRF genes of mammals. Exons I, II, III, IV and V are labelled. Introns are shown as single lines. Exons, introns and peptide domains are not drawn to scale (adapted from Parker *et al.*, 1997). Abbreviations are: 5'U, 5' untanslated region; SP, signal peptide; GAP, GnRH-associated peptide; 3'U, 3' untranslated region, cryptic, cryptic region; GRF, growth hormone-releasing factor; GnRH, gonadotropin-releasing hormone; PRP, PACAP-related peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; C-peptide, cryptic peptide

A.


I
II
III
IV
GnRH
GnRH gene
fish
5'U
SP
GAP
3'U
I
II
III
IV
GnRH
GnRH gene
mammals
5'U
SP
GAP
3'U

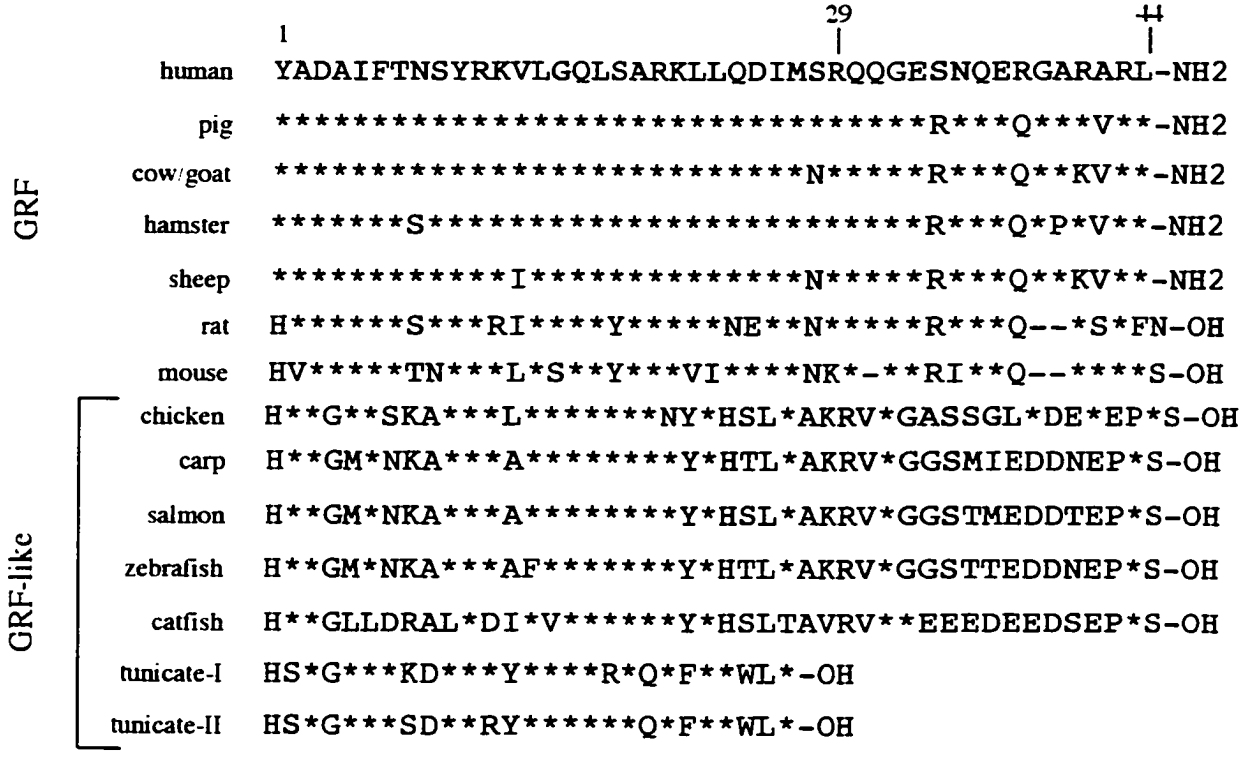
B.


I
II
III
IV
V
GRF-like/ PACAP gene
fish and chicken
5'U
SP
Cryptic
GRF
-like
PACAP
3'U
I
II
III
IV
V
PRP/ PACAP gene
mammals
5'U
SP
Cryptic
PRP
PACAP
3'U
I
II
III
IV
V
GRF gene
mammals
5'U
SP
GRF
C-PEP
TIDE
3'U

GRF peptide structure

Growth hormone-releasing factor, GRF, was one of the last hypothalamic neuropeptides to be identified. The peptide was initially isolated in 1982 from human pancreatic tumors (Guilleman *et al.*, 1982; Rivier *et al.*, 1982). It was present in three different forms, a forty-four amino acid amidated form, GRF_{1-44} NH2, and two non-amidated forms of forty amino acids and thirty seven amino acids each, GRF_{1-40} OH and GRF_{1-37} OH; only GRF_{1-44} NH2 was isolated from mammalian brain tissue (Rivier *et al.*, 1984). Since then six other mammalian GRF peptides and one non-mammalian GRF peptide have been isolated and sequenced (see Sherwood *et al.*, 1994). The cDNA encoding a GRF-like peptide has also been isolated from the brain of chicken (McRory *et al.*, 1997), salmon (Parker *et al.*, 1993), catfish (McRory *et al.*, 1995), zebrafish (Delgado *et al.*, 1996) and tunicate (McRory and Sherwood, 1997). Therefore, the mature GRF can be predicted from the nucleotide sequence (Fig. 1-4). The GRF peptides range in size from 46 (or 43) AA, in chicken, 45 AA in salmon, catfish and zebrafish, 44 AA in human, pig, cow, sheep, goat, and hamster, 43 AA in rat, 42 AA in mouse and 27 AA in tunicate (Fig. 1-4). In addition to a variation in size, the carboxy and amino termini of GRF are different among species. An aromatic amino acid in the first position is common to all GRFs but tyrosine is the initial amino acid in most mammals and histidine is the first amino acid in rat, mouse and the non-mammalian species. The carboxy termini of most mammalian GRFs is amidated but in the free acid form in rat, mouse and other GRFs. Phenylalanine in position 6 has been retained in all GRFs except for catfish GRF which has leucine in this position (Fig. 1-4; Campbell and Scanes, 1992; Ono *et al.*, 1994). It is this variability in size and termini among GRF peptides, as well as the relatively low sequence identity among them, that might explain why a molecule with structural similarity to mammalian GRFs was not reported in other vertebrates until 1992.

Fig. 1-4. The GRF peptide structures isolated to date. The peptides are listed in order of their sequence identity with human GRF except for tunicate GRFs, which are listed at the end because the peptides are shorter. The starred amino acids represent identity with human GRF. The bracket identifies the peptides that are termed 'GRF-like' because there is not sufficient functional evidence to date to categorize them as GRF.


1
29
44
GRF
GRF-like
human YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH2
pig **********R***Q***V**-NH2
cow/goat **********N*****R***Q**KV**-NH2
hamster *******S**********R***Q*P*V**-NH2
sheep **********I**********N*****R***Q**KV**-NH2
rat H******S***RI****Y*****NE**N*****R***Q--*S*FN-OH
mouse HV*****TN***L*S**Y***VI****NK*-**RI**Q--****S-OH
chicken H**G**SKA***L*******NY*HSL*AKRV*GASSGL*DE*EP*S-OH
carp H**GM*NKA***A********Y*HTL*AKRV*GGSMIEDDNEP*S-OH
salmon H**GM*NKA***A********Y*HSL*AKRV*GGSTMEDDTEP*S-OH
zebrafish H**GM*NKA***AF*******Y*HTL*AKRV*GGSTTEDDNEP*S-OH
catfish H**GLLDRAL*DI*V******Y*HSLTAVRV**EEEDEEDSEP*S-OH
tunicate-I HS*G***KD***Y****R*Q*F**WL*-OH
tunicate-II HS*G***SD**RY******Q*F**WL*-OH

Location and function

GRF is expressed primarily in the arcuate nucleus of the medial basal hypothalamus but GRF peptides and/or mRNA transcripts also have been found in the placenta, ovary, and testis of human, rat and mouse as well as in human pancreatic tumors (Sherwood *et al.*, 1994). The primary role of GRF is to stimulate the synthesis and release of GH from the pituitary. GH then circulates to the liver to augment the synthesis and release of the insulin-like growth factor -I, IGF-I, which functions as the final biological effector of the GRF message by inducing division and growth of cells and tissues (Deseva *et al.*, 1992; Frohman and Jansson, 1986; Rappaport, 1985). The effect of GRF on growth is held in check not only by feedback mechanisms involving GRF, GH and IGF but also by another neuropeptide, somatostatin, which inhibits the synthesis and release of GH from the pituitary. GRF has been reported to regulate somatotroph proliferation in the pituitary, to promote growth and differentiation of the fetus (either directly or indirectly by inducing the production of other placental hormones) and to augment steroidogensis in the ovary and testis (Mayo *et al.*, 1996). Immunoreactive GRF-containing axons are present in areas of the brain not directly associated with the hypophysial-portal system suggesting that GRF may have neurotransmitter or neuromodulatory functions affecting the release or activity of neurons. The observed effect of intracerebroventricular injection of GRF on the feeding behaviour of mice indicates that GRF has direct central actions on neural systems other than those directly associated with GH release (Vaccarino *et al.*, 1989). It is evident that GRF has multiple functional roles.

GRF mRNA and gene structure

For mammalian GRF, only two species-specific genes and two cDNAs are reported. The GRF-like precursors reported for fish and chicken are more closely related in organization to the PACAP precursors and will be discussed later (Fig. 1-4). The

human GRF gene is ten kilobases and contains five exons, one of which encodes the biologically active core of the GRF. Within the hypothalamus and in pancreatic tumors, the GRF gene is transcribed to a 750 nucleotide mRNA and translated into a 107 or 108 amino acid preprohormone containing the signal peptide, the mature GRF peptide and two cryptic peptides of unknown function (Frohman *et al.*, 1989; Mayo *et al.*, 1986). The rat GRF gene is of similar size and exon/ intron structure as the human gene but is spliced differently, resulting in a 104 amino acid precursor with little sequence identity to the human precursor at the carboxy-terminal end of the GRF domain, the cryptic region and the 3'UTR (Mayo *et al.*, 1985).

The cDNA encoding mouse GRF contains a 103 AA GRF precursor that has similar structure to the human and rat GRF precursors but has higher sequence identity to the rat GRF precursor, especially in the 5' and 3 ' UTRs and in the carboxy terminus of the GRF domain (Suhr *et al.*, 1989). The hamster GRF cDNA has a similar structural organization as the human, rat, and mouse GRF precursors but is closer in size and amino acid identity to the human GRF precursor than to the rodent precursors (Ono *et al.*, 1994).

In addition to its high expression in the brain, GRF mRNA transcripts can be found in the rat and mouse testes (Barry and Pescovitz, 1988; Barry and Pescovitz, 1990), rat ovaries (Bagnato *et al.*, 1992) and human, rat and mouse placenta (Gonzalez-Crespo and Boronat, 1991). These GRF transcripts are not identical in size or structure to those in the hypothalamus and vary considerably in quantity during development, suggesting that the regulation of the single copy GRF gene within these organs is tissue-specific. Placental GRF in particular seems to be regulated by an alternative promoter (Gonzalez-Crispo and Boronat, 1991; Mizobuchi *et al.*, 1991). This tissue specific expression suggests that GRF can also have localized functions which are physiologically significant at specific times of development.

PACAP peptide structure

PACAP was first isolated from sheep hypothalami as a 38 amino acid peptide with the ability to increase the accumulation of cyclic adenosine monophosphate, cAMP, in cultured rat pituitary cells (Miyata *et al.*, 1989). Since then the 38 AA peptide has been isolated from the brain of frog (Chartrel *et al.*, 1991) and a 27 AA PACAP peptide, potentially a cleavage product of $PACAP_{1-38}$, has been isolated from sheep hypothalami (Miyata *et al.*, 1990). The cDNA structure encoding PACAP has been characterized in human, sheep, rat, chicken, salmon, catfish, zebrafish and tunicate. Therefore, the structure of the mature PACAP peptides can be predicted from the nucleotide sequences. All of the predicted PACAP peptides in vertebrates are 38 AA and include a putative 27 AA peptide. The predicted tunicate PACAPs are only 27 AAs (McRory and Sherwood, 1997). All of the PACAPs have similar amino and carboxy termini and over 90% sequence identity among them.

The PACAP peptide has been sequenced from the testis of human, rat, and mouse (Hurley *et al.*, 1995; Sherwood *et al.*, 1994).

PACAP location and function

Immunoreactive PACAP cells and axons have been demonstrated in the paraventricular nucleus, supraoptic nucleus, preoptic area and median eminence of human, monkey, sheep, and rat brain as well as in the gut and testis of rat (see Arimura *et al.*, 1992: Sherwood *et al.*, 1994; Vigh *et al.*, 1991).

The evidence to date suggesting that PACAP might function as a hypophysiotropic hormone is based primarily on the location of irPACAP fibers in the median eminence abutting hypophysial portal vessels (Miyata *et al.*, 1990), the presence of PACAP specific receptors in pituitary cell membranes (Gotschall *et al.*, 1990) and the

ability of PACAP to act as a highly potent stimulator of cAMP synthesis in cultured rat pituitary cells (Miyata *et al.*, 1990). Also, if PACAP is administered in a pulsatile manner it can act as a releasing hormone in a rat cell perfusion assay, stimulating the release of GH, PRL, adrenocorticotropic hormone (ACTH) and LH. PACAP also will stimulate the release of some neuropeptides from dispersed pituitary cell cultures and pituitary clonal cell lines but not from static pituitary cell cultures (Sherwood *et al.*, 1994). PACAP's ability to stimulate LH and FSH release from rat anterior pituitary cells is enhanced if used in conjunction with mGnRH but there is no synergistic effect on the release of GH, ACTH , or thyroid stimulating hormone (TSH) if PACAP is applied together with their respective releasing factors (Culler and Paschall, 1991). PACAP might also affect the growth process in rats because it has been shown to stimulate GH release and increase the number of pituitary GH-secreting cells in a hemolytic plaque assay. This effect was specific to PACAP because the stimulatory effect was through pituitary receptors that were not GRF receptors (Goth *et al.*, 1992). In addition, PACAP has been shown to be a potent stimulator of GH release from salmon pituitary cell cultures (Parker *et al.*, 1997).

Other reported functions of PACAP in different species include stimulation of insulin secretion, vasodilation, interleukin-6 release, amylase secretion, modulation of GI tract motility and ion secretion, reduction of food intake, elevation of heart rate as well as the ability to stimulate cell proliferation in cultured cells and in transplanted tumors (Arimura, 1992; Rawlings and Hezareh, 1996; Sherwood *et al.*, 1994). Recently, PACAP also has been shown to inhibit the proliferation of embryonic rat cortical neuroblasts and enhance neuronal differentiation, suggesting that it might serve as a signal triggering the transition from proliferation to differentiation (Lu and DiCicco-Bloom, 1997). In fish and chicken, GRF-like/ PACAP mRNAs are expressed not only in the brain, but in the testis, ovary, and GI tract and in tunicate, a GRF-like/ PACAP mRNA is detected in both the neural ganglion and dorsal strand (McRory *et al.*, 1995,

1997; Parker *et al.*, 1997; McRory and Sherwood, 1997). Therefore, the ability of PACAP to function outside the brain seems to be conserved in evolution in non-mammalian vertebrates and invertebrates.

GRF-like/ PACAP and PACAP mRNA and gene structure

mRNAs encoding a GRF-like peptide and PACAP in tandem have been isolated from chicken (McRory *et al.*, 1997), salmon (Parker *et al.*, 1993), catfish (McRory *et al.*, 1997), zebrafish (Delgado *et al.*, 1996), and tunicate (McRory and Sherwood, 1997). In vertebrates , these mRNA precursors encode a preprohormone containing: a 22-26 AA signal peptide; a cryptic peptide of approximately 50 AAs; a 43-46 AA GRF-like peptide, including a 29 AA peptide and a 38AA PACAP, which includes a putative 27 AA form. Two different GRF-like/ PACAP cDNAs have been isolated from tunicate. Unlike the vertebrate precursors, the GRF-like and PACAP regions in tunicate both encode only 27AAs and one of the cDNAs does not encode a cryptic peptide (McRory and Sherwood, 1997). In chicken and salmon, there is an additional truncated GRF-like/ PACAP mRNA reported that results from alternative splicing; the transcript is similar in structure and sequence to the longer one described above except for the omission of the nucleotides encoding the first 32 AA of the GRF-like peptide. (McRory *et al.*, 1997; Parker *et al.*, 1997). The structural organization of each GRF-like/ PACAP precursor in different species is similar but there are considerable amino acid differences especially in the 5' UTR, 3' UTR and cryptic regions.

Human, sheep, and rat PACAP cDNAs are similar to the GRF-like/ PACAP precursor in tunicate, fish and chicken because the mammalian precursors also encode a peptide immediately upstream of the PACAP sequence; this 29 (or more likely 48) amino acid peptide is called PACAP- related peptide, PRP (Kimura *et al.*, 1990; Ogi *et al.*, 1990). Immunoreactive PRP cells and fibers and the processed peptide have been found in the median eminence and anterior pituitary (Hannibal *et al.*, 1994; Mikkelsen *et al.*,

1995) suggesting that it could potentially be a hypophysiotropic factor. However, there are currently no reports of a function for the PRP, either in the release of pituitary hormones or in the stimulation of cAMP *in vitro* (Mikkelsen *et al.*, 1995) despite its sequence similarity to GRF and other glucagon superfamily members. The substitution of the first amino acid in PRP to one (Asp) different from all other superfamily members (His or Tyr) might explain the difference in function (Sherwood *et al.*, 1994).

The mammalian gene encoding human PRP/ PACAP has a similar structural organization to the genes encoding salmon and chicken GRF-like/ PACAP but encodes PRP rather than GRF. Nonetheless, each gene contains five exons: exon I, encodes most of the 5' UTR; exon II, has the signal peptide; exon II, encodes the cryptic peptide; exon IV, encodes the PRP in mammals or the GRF-like peptide in fish and chicken and exon V, encodes the remaining portion of GRF in addition to PACAP and the 3' UTR (Hosoya *et al.*, 1992; McRory *et al.*, 1997; Okhubo *et al.*, 1992; Parker *et al.*, 1997). Two partial gene structures encoding GRF-like/ PACAP in tunicate have been isolated; one of these genes contains only three exons encoding the signal peptide, a 27AA GRF-like peptide and a 27AA PACAP whereas, the other gene has four exons encoding the same three domains as well as a cryptic domain (McRory and Sherwood, 1997). One of the common features of each of these genes is that the biologically active domains are encoded by separate exons; this is also characteristic of GRF and the genes encoding other members of the glucagon superfamily.

Glucagon superfamily

GRF and PACAP belong to the glucagon superfamily of peptides because they retain critical amino acids and a similar gene structure as well as a few overlapping functions (Bell, 1986). Other members include glucagon, glucagon-like peptide-I (GLP - I), GLP-2, secretin, vasoactive intestinal polypeptide (VIP), peptide histidine methionine (PHM), peptide histidine isoleucine (PHI), glucose-dependent insulin-releasing peptide

(GIP), PRP and helodermin, helospectin and exendin-3 (Campbell and Scanes, 1992). Further comparisons of different members of this glucagon superfamily will be useful in determining structure/function relationships as well as potential evolution of these neuropeptides.

Transport of GnRH, GRF and PACAP in the brain of vertebrates

GnRH, GRF and PACAP are transported from the site of synthesis in the brain to the site of action in the pituitary in different ways depending on the vertebrate species. In mammals, birds, reptiles and amphibians, these neuropeptides are released in a pulsatile manner from axon terminals of hypothalamic neurons into the hypophysial-portal system and then carried to their effector cells in the pituitary. In most teleosts, the link between brain and pituitary is more direct with the axons that contain GnRH, GRF, and PACAP terminating directly in the pituitary. Sturgeon have an intermediary transport system, using mostly axons to carry these neuropeptides to the pituitary but also using a rudimentary hypophysial portal system for some of the transport (Polenov and Pavlovic, 1978; Polenov *et al.*, 1976). There is also a recent report of a similar system in the tarpon, *Megalops cyprinoides* (Baskaran and Sathyanesan, 1992) suggesting that this method of transporting neuropeptides might be conserved in early-evolved teleost species. In cartilaginous fish, there is no evidence of a hypophysial portal system or of immunoreactive(ir) GnRH fibers impinging on the pituitary. However, GnRH and a GnRH-binding protein are found in the plasma of these fish and therefore, GnRH (and possibly the other neuropeptides) are carried to the pituitary by systemic circulation. In jawless fish, the method by which GnRH and other neuropeptides reach the pituitary is unknown; the current hypothesis is that diffusion occurs or blood vessels in the neurohypophysis or cerebral spinal fluid in the third ventricle of the brain carry GnRH to the anterior pituitary (Sherwood *et al.*, 1997). In tunicates, because there is no homologue to the vertebrate pituitary and because irGnRH is found in fibers that directly

innervate the sinus near the gonads and other tissues, GnRH and other neuropeptides might be carried close to their target organs by the axons that contain them (Powell *et al.*, 1996).

Once they reach the pituitary, GnRH, GRF and PACAP each bind separate types of seven transmembrane domain receptors that are guanine (G) protein-coupled to carry out their respective actions. However, although the receptors for each neuropeptide might have some similarities in structure, they usually bind only one type of hormone with high affinity.

GnRH receptor

The GnRH receptor, GnRHR, is located primarily on gonadotrope cells in the anterior pituitary, but there have been reports of cDNAs encoding the GnRHR in human breast and ovarian tumors and rat gonads; these extrapituitary receptors are identical in structure to the pituitary GnRHR of the corresponding species. The cDNAs encoding GnRH receptors have been isolated from the pituitary of human, sheep, cow, pig, mouse, rat, and catfish (Sealfon *et al.*, 1997). The predicted GnRHR proteins in mammals are 327-328 amino acids and have over 85% sequence identity among them, with the highest conservation in the seven transmembrane region. Also, unlike all other G protein coupled receptors of the same type, the mammalian GnRHR does not have an intracellular carboxy- terminal domain. In contrast, the predicted catfish GnRHR is 370 amino acids and does contain an intracellular carboxy- terminal domain (Sealfon *et al.*, 1997).

GRF and PACAP receptor superfamily

There is evidence to suggest that during evolution a family of receptors emerged in parallel with the glucagon super family of related peptides. For example, VIP and glucagon receptors have similar structure, similar exon/ intron organization of the genes, conserved critical amino acids as well as a common starting point in the post receptor cascade involving G proteins and adenylate cyclase (Laburthe *et al.*, 1996).

GRF receptor

To date, only the human, rat, and mouse cDNAs encoding the GRF pituitary receptors have been cloned. The GRF receptors predicted from the cDNA clones are approximately 400 AA with 80% sequence identity among them, the highest identity being in the seven transmembrane spanning regions. The rat GRFR mRNA is found in the anterior pituitary, placenta and kidney but not in the gonads. In contrast, the mRNA encoding GRF is found in the gonads as well as in the anterior pituitary and placenta, but not in the kidney (see Mayo *et al.*, 1996). This difference in distribution between the expression of the GRF receptor and peptide suggest that like PACAP, there also might be type(s) of GRF receptors that are tissue specific.

It is interesting to note that GRF will not bind the GRF receptor if there is a point mutation in amino acid 60 of the extracellular domain; this mutation results in the deficient GH production and the decreased number of pituitary somatotrophs characteristic of the dwarf phenotype of the little (*lit*) mouse (Mayo *et al.*, 1996). Amino acid 60 is the only completely conserved charged residue in the glucagon super family of receptors suggesting that it is essential for maintaining proper ligand/ receptor interaction.

In the pituitary GRF binds its receptor and activates the G proteins, which alter membrane ion channels, either by direct binding or by cAMP-induced phosphorylation of ion channel proteins, and ultimately cause the membrane potential changes that trigger GH release.

PACAP receptor

The cDNAs encoding the PACAP receptor have been isolated from human, cow, rat, and mouse. Unlike the receptors for GnRH and GRF, at least three different types of PACAP receptors are present in each species. Each of these receptors is structurally similar, with the highest sequence identity in the predicted seven transmembrane hydrophobic domains. However, they differ in their affinities for PACAP and VIP as well as in their tissue distribution. The PACAP type I receptor binds PACAP with much higher affinity than VIP and is found primarily in the brain, pituitary, adrenal medulla and testis whereas the type II PACAP receptor binds PACAP and VIP with equally low affinities and is found in the lung, liver and GI tract. The most recently isolated PACAP receptor, PACAP type III, binds both PACAP and VIP with high affinities and is located predominately in the pancreatic islet cells, but in low concentration in the lung, brain, stomach, colon and heart. The diversity of signal transduction by PACAP in various tissues can be further enhanced by alternative splicing of the receptor(s) during RNA processing, resulting in receptor sub-types with different affinities for PACAP1-38, PACAP1-27 and VIP as well as different abilities to couple to G proteins (Hezareh *et al.*, 1996; Laburthe *et al.*, 1996; Pisenga *et al.*, 1996; Rawlings and Hezareh, 1996). It is interesting to note that in goldfish, sGnRH and cGnRH-II bind a similar receptor on the pituitary gonadotropes but they activate different post-receptor pathways (Chang and Jobin, 1991). This implies that like the receptors for PACAP, there are receptor sub-

types for GnRH that have the ability to couple preferentially to different signalling pathways.

Identifying the receptors for GnRH, GRF and PACAP in different species and tissues is an important first step in understanding the specific function of these neuropeptides, which might have overlapping locations and common functions.

Overlapping functions

There is evidence to suggest that GnRH, GRF and PACAP neuropeptides have overlapping functions in certain tissues. For example, in several fish species GnRH can stimulate the release of GH *in vitro* (Lovejoy *et al.*, 1992; Marchant *et al.*, 1989; Ngamvongchon *et al.*, 1992). PACAP also has been shown to stimulate GH release under some experimental conditions but not others (Sherwood *et al.*, 1994). PACAP's potential effect on somatic growth has been further demonstrated in salmon where sPACAP was more effective than salmon GRF in stimulating the release of GH from cultured coho salmon pituitary cells (Parker *et al.*, 1997). PACAP also could potentially affect reproduction because PACAP can stimulate LH release in some experimental conditions (Hart *et al.*, 1992; Miyata *et al.*, 1989; Osuga *et al.*, 1992;) but not in others (Arimura *et al.*, 1992; Miyata *et al.*, 1989). In addition, PACAP has been shown to act synergistically with GnRH to stimulate gonadotropin release from rat anterior pituitary cell cultures (Culler and Paschall, 1991; Winters *et al.*, 1996;) but not *in vivo* in human (Hammond *et al.*, 1993). Furthermore, GnRH can inhibit PACAP stimulated cAMP accumulations in gonadotrope tumor cells (αT3-1) (McArdle *et al.*, 1994). However, GnRH and PACAP act through distinct receptors and therefore, the functional overlap between PACAP and GnRH in gonadotrope cells occurs at the intracellular level, possibly through competition for shared signalling molecules (McArdle, 1996). Distinct GRF and PACAP receptors also are present on somatotrope cells suggesting that the

cross-talk between signalling pathways is responsible for the overlapping functions of the neuropeptides in these cells.

Prevertebrate history

There is evidence that the emergence of GnRH, GRF and PACAP in evolution predates the emergence of the vertebrates. For example, two different GnRH peptides (Powell *et al.*, 1996) and two separate cDNAs encoding a GRF-like/ PACAP precursor (McRory *et al.*, 1997) have been isolated from the neural ganglion of the tunicate, *Chelyosoma productum*. One of the tunicate PACAP cDNAs was used as a probe and hybridized to the DNA of rat, starling, chicken, alligator, salmon, catfish, tunicate, reedfish and sea urchin (McRory and Sherwood, 1997); positive hybridization of the tunicate PACAP probe to sea urchin DNA suggests that PACAP-like molecules evolved even prior to the tunicates. Immunoreactive GnRH-like molecules have been shown to exist in the central nervous system (CNS) of the gastropod mollusc, *Helisoma trivolvis* (Goldberg *et al.*, 1993) and in acorn worm, *Saccoglossus* (Cameron *et al.*, 1997). Yeast α-mating factor has been shown to release LH from rat pituitary cells at a dose of 1000X compared to mGnRH, but no GnRH has been found in yeast (Loumaye *et al.*, 1982). There also is a report of an immunoreactive PACAP-like molecule in the CNS of *Drosophila* (Zhong *et al.*, 1995).

Purpose of thesis

Sturgeon, *Acipenser sp.*, are an excellent model for increasing our knowledge of the molecular aspects of reproduction and growth because of their evolutionary position, close to the branching point between bony fish and tetrapod ancestors. Sturgeon also is an interesting species to study the neuropeptides controlling reproduction and growth because these processes are unusual in sturgeon compared to other fish. For example, sturgeon take many years to become sexually mature; 8-14 years for females and 8 years

for males (Rochard *et al.*, 1990). This lengthy time to reproductive maturity is not due to a lack of reproductive hormones (GnRH is present in the brain) but the release of gonadotropins is dependent on the fish reaching a certain size, suggesting that growth and reproduction are tightly linked. The growth process in sturgeon is also interesting; not only do the fish have the ability to reach over six meters in length but also they have highly variable growth rates as juveniles. Isolating and characterizing the neuropeptides central to the control of reproduction and growth in sturgeon is an important first step toward understanding these processes in this little understood species and toward drawing general conclusions about the evolution of the control of these endocrine systems in vertebrates.

In this thesis, the primary structure of GnRH was determined first from the brain of an early-evolved vertebrate, the sturgeon, using high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) with anti-GnRH antisera. The results of this study were startling in that one of the two forms of GnRH was identical to human GnRH, but the second form in sturgeon seemed to be missing in humans. This led me to consider the origin and expression of GnRH in more evolutionarily-ancient as well as more recently-evolved vertebrates than sturgeon. The purpose of this part of the thesis was to use sturgeon GnRH peptides as a focal point for comparison to GnRH peptides in the evolutionary line leading to sturgeon and to the subsequent lines leading to teleosts or to mammals. To this end, the brain of a cartilaginous fish (skate) and teleost fishes (whitefish, platyfish, green swordtail and sablefish) as well as the brain of tetrapods (green anole lizard, guinea pig, hamster, rat and monkey) were examined for the type of GnRH present (see Fig. 1-1). Also, the brain of skate was divided into seven parts and the brain of adult monkey into three parts; each region was analyzed separately to establish whether distinct parts of the brain contained different types and quantities of GnRH. In addition, the pituitary of whitefish, the fetal tissue of rat and monkey and the placenta of human were analyzed for immunoreactive (ir) GnRH to determine whether

there was a difference between the type of GnRH present in the brain and that present in other tissues.

In the second part of this thesis, the sturgeon was again used but this time to isolate the complementary DNA (cDNA) structure encoding GRF and PACAP from the brain using the polymerase chain reaction (PCR) and additional molecular biology methods. The isolation of the GRF/ PACAP precursor in sturgeon, which has retained many primitive traits from the stem ancestors can be beneficial in deciding if there is a difference in the conservation of distinctive parts of the precursor; this information might help establish the functional importance of each respective domain.

Determining the structure of GnRH, GRF and PACAP in sturgeon and establishing the presence of irGnRH molecules in animals representing different vertebrate classes is useful in determining the evolutionary relationships of these important peptides among species. In addition, a phylogenetic study of GnRH, GRF and PACAP allows the deduction of general principles about neuropeptide structure, function and the regulation of reproduction and growth.

Literature cited

Acher, R., Chauvet, J., and Chauvet, M-T. (1973). Phylogeny of neurohypophysial hormones. The active peptides of a primitive fish, the sturgeon (*Acipenser* sp.). *Eur. J. Biochem.* **40**, 585-589.

Arimura, A. (1992). Pituitary adenylate cyclase-activating polypeptide (PACAP): discovery and current status of research. *Regul. Pept.* **37**, 287-304.

Arimura, A., Somogyvari-Vigh, A., Miyata, A., Mizuno, K., Coy, D.H. and Kitada, C. (1991). Tissue distribution of PACAP as determined by RIA: Highly abundant in the rat brain and testes. *Endocrinology* **129**, 2787-2789.

Bagnato, A., Moretti, C., Ohnishi, J., Frajese, G. and Catt, K. (1992). Expression of the growth hormone-releasing hormone gene and its peptide product in the rat ovary. *Endocrinology* **130**, 1097-1102.

Banfield, D.K., and MacGillivray, R.T.A. (1992). Partial characterization of vertebrate prothrombin cDNAs: amplification and sequence analysis of the B chains of thrombin from nine different species. *Proc. Natl. Acad. Sci. (U.S.A.)* **89**, 2779-2783.

Baskaran, G., and Sathyanesan, A.G. (1992). Tetrapod-like hypothalamo-hypophysial portal system in the teleost *Megalops cyprinoides* (Broussonet). *Gen. Comp. Endocrinol.* **86**, 211-219.

Bell, G.I. 1986. The glucagon superfamily: precursor structure and gene organization. *Peptides* **7**, 27-36.

Berry, S.A. and Pescovitz, O.H. (1988). Identification of a GHRH-like substance and its messenger RNA in rat testis. *Endocrinology* **123**, 661-663.

Berry, S.A. and Pescovitz, O.H. (1990). Ontogeny and pituitary regulation of testicular growth hormone-releasing hormone-like messenger ribonucleic acid. *Endocrinology* **127**, 1404-1411.

Bewley, T.A. and Papkoff, H. (1987). Conformation of sturgeon somatotropin. *Int. J. Peptide Protein Res.* **29**, 568-573.

Böhlen, P., Brazeau, P., Ling, N., Gaillard, R. and Guillemin, R. (1983a). Human hypothalamic growth hormone releasing factor (GRF): Evidence for two forms identical to tumor derived GRF-44-NH2 and GRF-40. *Biochem. Biophys. Res. Commun.* **114**, 930-936.

Böhlen, P., Esch, F., Brazeau, P., Ling, N. and Guillemin, R. (1983b). Isolation and characterization of the porcine hypothalamic growth hormone releasing factor. *Biochem. Biophys. Res. Commun.* **116**, 726-734.

Böhlen, P., Wehrenberg, W.B., Esch, F., Ling, N., Brazeau, P.and Guillemin, R. (1984). Rat hypothalamic growth hormone-releasing factor: isolation, sequence analysis and total synthesis. *Biochem. Biophys. Res. Commun.* **125**, 1005-1012.

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 ovine hypothalamic luteinizing hormone-releasing factor (LRF). *Proc. Natl. Acad. Sci. (U.S.A.)* **69**, 278-282.2

Cameron, C.B., Mackie, G.O., Powell, J.F.F., Lescheid, D.W., and Sherwood, N.M. (1997). Gonadotropin-releasing hormone in mulberry cells of Saccoglossus and Ptychodera (Hemichordata: Enteropneusta). (manuscript in preparation)

Campbell, R.M., and Scanes, C.G. (1992). Evolution of the growth hormone-releasing factor (GRF) family of peptides. *Growth Regul.* **2**, 175-191.

Chang, J.P. and Jobin., R.M. (1994). Regulation of gonadotropin release in vertebrates: a comparison of GnRH mechanisms of action. In: Davey, K. G., Peter, R. E., Tobe, S. S. (eds) Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa: 41-51.

Chartrel, N., Tonon, M-C., Vaudry, H. and Conlon, J.M. (1991). Primary structure of frog pituitary adenylate cyclase-activating polypeptide (PACAP) and effects of ovine PACAP on frog pituitary. *Endocrinology* **129**, 3367-3371.

Chow, M. M., Kight, K., Deoraj, A., Gothilf, Y. and Zohar, Y. (1997). Multiple GnRHs present in a teleost species are encoded by separate genes. In: Advances in the molecular endocrinology of fish: 2nd IUBS Toronto symposium, Toronto , Canada, May 16-19, 1997. p. 14 (abstract).

Culler, M.D. and Paschall, C.S. (1991). Pituitary adenylate cyclase-activating polypeptide (PACAP) potentiates the gonadotropin-releasing activity of luteinizing hormone- releasing hormone. *Endocrinology* **129**, 2260-2262.

Delgado, E., Wei, Y, Martin, S.C., Hienrich, and Mojsov, S. (1996). Cloning and characterization of PACAP peptides in zebrafish. Endocrine society meetings, San Francisco, CA, June 12-13. Abstract P2-367.

Devesa, J., Lima, L. and Tresguerres, J.A.F. (1992). Neuroendocrine control of growth hormone secretion in humans. *Trends Endocrinol. Metab*. **3**, 175-183.

Esch, F., Böhlen, P., Ling, N., Brazeau, P., Wehrenberg, W., Thorner, M., Cronin, M. and Guillemin, R. (1982). Characterization of a 40 residue peptide from a human pancreatic tumor with growth hormone releasing activity. *Biochem. Biophys. Res. Commun.* **109**, 152-158.

Farmer, S.W., Hayashida, T., Paploff H., and Polenov, A.L. (1981). Characteristics of growth hormone isolated from sturgeon (*Acipenser güldenstädti*) pituitaries. *Endocrinology* **108**, 377-381.

Frohman, L.A. and Jansson, J-O. (1986). Growth hormone-releasing hormone. *Endocrine Reviews* **7**, 223-253.

Frohman, M.A., Downs, T.R., Chomczynski, P. and Frohman, L.A. (1989). Cloning and characterization of mouse growth hormone-releasing hormone (GRH) complementary DNA: increased GRH messenger RNA levels in the growth hormone-deficient lit/lit mouse. *Mol. Endocrinol.* **3**, 1529-1536.

Gautron, J-P., Pattou, E., Bauer, K. and Kordon, C. (1991). (Hydroxyproline9) luteinizing hormone-releasing hormone: a novel peptide in mammalian and frog hypothalamus. *Neurochem. Int.* **18**, 221-235.

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*. *J. Comp. Neurol.* **336**, 571-582.

Gonzàlez-Crespo, S. and Boronat, A. (1991). Expression of the rat growth hormone-releasing hormone gene in placenta is directed by an alternative promoter. *Proc. Nat. Acad. Sci.(U.S.A.)* **88**, 8749-8753.

Goth, M.I., Lyons, C.E., Canny, B.J. and Thorner, M.O. (1992). Pituitary adenylate cyclase activating polypeptide, growth hormone (GH)-releasing peptide and GH-releasing hormone stimulate GH release through distinct pituitary receptors. *Endocrinology* **130**, 939-944.

Gottschall, P.E., Tatsuno, I., Miyata, A. and Arimura. A. (1990). Characterization and distribution of binding sites for the hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide. *Endocrinology* **127**, 272- 277.

Gubler, U., Monahan, J.J., Lomedico, P.T., Bhatt, R.S., Collier, K.J., Hoffman. B.J., Böhlen, P., Esch, F., Ling, N., Zeytin, F., Brazeau, P., Poonian, M.S. and Gage, L.P. (1983). Cloning and sequence analysis of cDNA for the precursor of human growth hormone-releasing factor, somatocrinin. *Proc. Nat. Acad. Sci.(U.S.A.)* **80**, 4311-4314.

Guillemin, R. (1986). "The Sherrington Lectures. XVIII. Hypothalamic Control of Pituitary Functions: The Growth Hormone Releasing Factor." Liverpool University Press, Liverpool, pp. 1-73.

Guillemin, R., Brazeau, P., Böhlen, P., Esch, F., Ling, N. and Wehrenberg, W. (1982). Growth hormone releasing factor from a human pancreatic tumor that caused acromegaly. *Science* **218**, 585-587.

Hammond, P.J., Talbot, K., Chapman, R., Ghatei, M.A., and Bloom, S.R. (1993). Vasoactive intestinal peptide, but not pituitary adenylate cyclase-activating polypeptide, modulates the responsiveness of the gonadotroph to LHRH in man. *J. Endocrinol.* **137**, 529-532.

Hannibal, J., Mikkelsen, J.D., Clausen, H., Holst, J.J., Wuff, B.S., Fahrenkrug, J. (1995). Gene expression of pituitary adenylate cyclase activating polypeptide (PACAP) in the rat hypothalamus. *Regul. Peptides* **55**, 133-148.

Hart, G.R., Gowing, H., and Burrin, J.M. (1992). Effects of a novel hypothalamic peptide, pituitary adenylate cyclase-activating polypeptide, on pituitary hormone release in rats. *J. Endocrinol.* **134**, 33-41.

Hayashida, T. (1977). Immunochemical and biological studies with growth hormone in a pituitary extract of the coleacanth, *Latimeria chalumnae* Smith. *Gen. Comp. Endocrinol.* **32**, 221-229.

Hezareh, M., Journot., L., Bépoldin, L., Schlegel, W., and Rawlings, S.R. (1996). PACAP/VIP receptor subtypes, signal transducers, and effectors in pituitary cells. In " VIP, PACAP, and related peptides. Second international symposium (A. Arimura and S.I. Said, Eds). *Annals New York Acad. Sci.* **805**, 315-327

Hosoya, M., Kimura, C., Ogi, K., Ohkubo, S., Miyamoto, Y., Kugoh, H. Shimizu, M., Onda, H., Oshimura, M., Arimura, A.and Fujino, M. (1992). Structure of the human pituitary adenylate cyclase activating polypeptide (PACAP) gene. *Biochim. Biophys. Acta* **1129**, 199- 206.

Hseuh, A. J. W., and Schaeffer, J. M. (1985). Gonadotropin-releasing hormone as a paracrine hormone and neurotransmitter in extrapituitary sites. *J. Steroid Biochem.* **23**, 757-764

Hurley, J.D., Gardiner, J.V., Jones, P.M., and Bloom, S.R. (1995). Cloning and molecular characterization of complementary deoxyribonucleic acid corresponding to a novel form of pituitary adenylate cyclase-activating polypeptide messenger ribonucleic acid in the rat testis. *Endocrinology* **136**, 550- 557.

Jones, S.W. (1987). Chicken II luteinizing hormone-releasing hormone inhibits the M-current of bull frog sympathetic neurons. *Neurosci Lett.* **80**, 180-184.

Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R.R., Stibbs, H.H., Arimura, A., and Fujino, M. (1990). A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs. *Biochem. Biophys. Res. Commun.* **166**, 81-89.

King, J. A., and Millar, R. P. (1995). Evolutionary aspects of gonadotropin-releasing hormone and its receptor. *Cell. Mol. Endocrinol.* **15**, 5-23.

Laburthe, M., Couvinaeu, A., Gaudin, P., Maoret, J-J., Rouyer-Fessard, C., and Nicole, P. (1996). Receptors for VIP, PACAP, Secretin, GRF, Glucagon, GLP-1, and other members of their new family of G-protein-linked receptors: structure-function relationships with special reference to the human VIP-1 receptor. In" VIP, PACAP, and related peptides, second international symposium, (A. Arimura and S.J. Said, Eds.)". Annals New York Acad. Sci. 805, 94-111.

Loumaye, E., Thorner, J., and Catt, K.J. (1982). Yeast mating pheromone activates mammalian gonadotrophs: Evolutionary conservation of a reproductive hormone? *Science* **218**, 1323-1325.

Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E., and Sherwood, N.M. (1992). Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Natl. Acad. Sci.(U.S.A.)* **89**, 6373-6377

Lu, N., and DiCicco-Bloom, E. (1997). Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells. *Proc. Natl. Acad. Sci. (U.S.A.)* **94**, 3357-3362.

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**, 2509-2518.75

Margioris, A.N., Brockmann, G., Böhler, H.C.L., Grino, Jr. M., Vamvakopoulos, N. and Chrousos, G.P. (1990). Expression and localization of growth hormone-releasing hormone messenger ribonucleic acid in rat placenta: *In vitro* secretion and regulation of its peptide product. *Endocrinology* **126**, 151-158.

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. *Biochem. Biophys. Res. Commun.* **43**, 1334-1339.1

Mayo, K.E., Cerelli, G.M., Lebo, R.V., Bruce, B.D., Rosenfeld, M.G. and Evans, R.M. (1985a). Gene encoding human growth hormone-releasing factor: structure, sequence, and chromosomal assignment. *Proc. Nat. Acad. Sci. (U.S.A.)* **82**, 63-67.

Mayo, K.E., Cerelli, G.M., Rosenfeld, M.G. and Evans, R.M. (1985b). Characterization of cDNA and genomic clones encoding the precursor to rat hypothalamic growth hormone-releasing factor. *Nature* **314**, 464-467.

Mayo, K.E., Miller, T. E., DeAlmeida, V., Zheng, J., and Godfrey, P. A. (1996). The growth hormone-releasing hormone receptor: signal transduction, gene expression, and physiological function in growth regulation. In" VIP, PACAP, and related peptides, second international symposium, (A. Arimura and S.J. Said, Eds.)". *Annals New York Acad. Sci.* **805**, 184-203.

Mayo, K.E., Vale, W., Rivier, J., Rosenfeld, M.G. and Evans, R.M. (1983). Expression-cloning and sequence of a cDNA encoding human growth hormone-releasing factor. *Nature* **306**, 856-88.

McArdle, C. A. (1996). Functional interaction between gonadotropin-releasing hormone and PACAP in gonadotropes and αT3-1 cells. In" VIP, PACAP, and related peptides, second international symposium, (A. Arimura and S.J. Said, Eds.)". *Annals New York Acad. Sci.* **805**, 112-121.

McArdle, C. A., Poch, A., Chomerus, E., and Kratzmeier, M (1994). Pituitary adenylate cyclase-activating polypeptide effects in pituitary cells: modulation by gonadotropin-releasing hormone in αT3-1 cells. *Endocrinology* **134**, 2599-2605.

McArdle, C.A., and Counis, R. (1996). GnRH and PACAP action in gonadotropes: cross-talk between phosphoinositidase C and adenyly cyclase mediated signaling pathways. *Trends Endocrinol. Metab.* **7**, 168-175.

McMaster, D., Belenky, M.A., Polenov, A.L., and Lederis, K. (1992). Isolation and amino acid sequence of urotensin II from the sturgeon *Acipenser ruthenus*. *Gen. Comp. Endocrinol.* **87**, 275-285.

McRory, J. E., and Sherwood, N. M. (1997). Two protochordate genes encode pituitary adenylate cyclase-activating polypeptide and related family members. *Endocrinology* **138**, 2380-2390.

McRory, J.E., Parker, D.B., Ngamvongchon, S., and Sherwood, N.M. (1995). Sequence and expression of cDNA for pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH)-like peptide in catfish. *Mol. Cell. Endocrinol.* **108**, 169-177.

McRory, J.E., Parker, R.L. and Sherwood, N.M. (1997). Expression and alternate processing of a chicken gene encoding both growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide. *DNA Cell. Biol.* **16**, 95-102

Mikkelsen, J.D., Hannibal, J., Fahrenkrug, J., Larsen, P.J., Olcese, J., and McArdle, C. (1995). Pituitary adenylate cyclase activating peptide-38 (PACAP-38), PACAP-27, and PACAP-related peptide (PRP) in the rat median eminence and pituitary. *J. Neuroendocrinol.* **7**, 47-55.

Millar, R. P., and King, J. A. (1994). Plasticity and conservation in gonadotropin-releasing hormone structure. In: "Perspectives in Comparative Endocrinology (Davey, K. G., Peter, R. E., Tobe, S. S., Eds)". National Research Council of Canada, Ottawa: 129-136

Miyata, A., Arimura, A., Dahl, R.R., Minamino, N., Uehara, A., Jiang, L., Culler, M.D. and Coy, D.H. (1989). Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem. Biophys. Res. Commun.* **164**, 567-574.

Miyata, A., Jiang, L., Dahl, R.D., Kitada, C., Kubo, K., Fujino, M., Minamino, N. and Arimura, A. (1990). Isolation of a neuropeptide corresponding to the N- terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP- 38). *Biochem. Biophys. Res. Commun.* **170**, 643-648.

Mizobuchi, M., Frohman, M.A., Downs, T.R. and Frohman, L.A. (1991). Tissue-specific transcription initiation and effects of growth hormone (GH) deficiency on the regulation of mouse and rat GH-releasing hormone gene in hypothalamus and placenta. *Mol. Endocrinol.* **5**, 476-484.

Muske, L.E. (1993). Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. *Brain Behav. Evol.* **42**, 215-230.39

Ngamvongchon, S., Sherwood, N.M., Warby, C.M., and Rivier, J.E. (1992). Gonadotropin-releasing hormone from the Thai catfish: chromatographic and physiological studies. *Gen. Comp. Endocrinol.* **87**, 266-274.

Noso, T., Nicoll, C.S., Polenov, A.L., and Kawauchi, H. (1993). The primary structure of sturgeon prolactin: phylogenetic implication. *Gen. Comp. Endocrinol.* **91**, 90-95.

Ogi, K., Kimura, C., Onda, H., Arimura, A. and Fujino, M. (1990). Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP). *Biochem. Biophys. Res. Commun.* **173**, 1271-1277.

Ohkubo, S., Kimura, C., Ogi, K., Okazaki, K., Hosoya, M., Onda, H., Miyata, A., Arimura, A. and Fujino, M. (1992). Primary structure and characterization of the precursor to human pituitary adenylate cyclase activating polypeptide. *DNA Cell. Biol.* **11**, 21-30.

Ono, M., Miki, N., Demura, H., Tadokoro, K., Nagafuchi, S., and Yamada, M. (1994). Molecular cloning of cDNA encoding the precursor for hamster hypothalamic growth hormone releasing factor. *DNA Sequence-J. Seq. Map.* **5**, 93-102.

Osuga, Y., Mitsuhashi, N., and Mizuno, M. (1992). In vivo effect of pituitary adenylate cyclase activating polypeptide 38 (PACAP 38) on the secretion of luteinizing hormone (LH) in male rats. *Endocrinol. Jpn.* **39**, 153-156.

Parker, D.B., Power, M.E., Swanson, P., Rivier, J., and Sherwood, N.M. (1997). Exon skipping in the gene encoding pituitary cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* **138**, 414-423.

Pfaff, D. W., Schwanzel-Fukuda, M., Parhar, I. S., Lauber, A. H., McCarthy, M. M., Kow, L-M. (1994). GnRH neurons and other cellular and molecular mechanisms for simple mammalian reproductive behaviors. *Rec. Prog. Horm. Res.* **49**, 1-25

Pisenga, J.R., Moody, T.W., and Wank, S.A. (1996). Differential signalling and immediate early gene activation by four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor (hPACAP-R). In " VIP, PACAP, and related peptides. Second international symposium (A. Arimura and S.I. Said, Eds). *Annals New York Acad. Sci.*, New York pp. 54-64.

Polenov, A.L., and Pavlovic, M. (1978). The hypothalamo-hypophysial system in acipenseridae. *Cell and Tissue Research* **186**, 559-570.

Polenov, A.L., Belenky, M.A., Garlov, P.E., and Konstantinova, M.S. (1976). The hypothalamo-*hypophysial* system in Acipenseridae, VI. The proximal neurosecretory contact region. *Cell Tiss. Res.* **170**, 129-144.

Powell, J. F. F., Reska-Skinner, S., Prakash, M. O., Fischer, W. H., Park., M. Rivier, Craig, G. A., Mackie, G. O. and Sherwood, N. M. (1996). Gonadotropin-releasing hormone in a tunicate and evidence regarding its ancestral mode of action. *Proc. Natl. Acad. Sci. (U.S.A.)* **93**, 10461-10464.

Powell, J.F.F. (1995) The origin and evolution of gonadotropin- releasing hormone in boney fishes. Ph. D thesis, University of Victoria, B. C.

Powell, J.F.F., Standen, E.M., Carolsfeld, J., Borella, M.I., Gazola, R., Fischer, W.H., Park, M., Craig, A.G., Warby, C.M., Rivier, J.E., Val-Sella, M.V., and Sherwood, N.M. (1997). Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. *Reg. Pept.* **68**, 189-195.

Rappaport, R. (1988). Endocrine Control of Growth. In: "Linear Growth: Retardation in Less Developed Countries, (John C. Waterlow, Ed.)." Vevey /Raven Press, Ltd., New York, New York. pp 109 -126.

Rawlings, S.R., and Hezareh, M. (1996). Pituitary adenylate cyclase-activating polypeptide (PACAP) and PACAP/ vasoactive intestinal polypeptide receptors: actions on the anterior pituitary gland. *Endo. Reviews* **17**, 4-29

Rivier, J., Spiess, J., Thorner, M. and Vale, W. (1982). Characterization of a growth hormone-releasing factor from a human pancreatic islet tumor. *Nature* **300**, 276-278.

Rivier, J., Spiess, J., and Vale, W. (1984). Isolation of two hypothalamic growth releasing factors. In: "Peptide Chemistry 1983" (E. Munekata, Ed.). Protein Research Foundation, Osaka, pp. 11-17.

Rouille, Y., Chauvet, J., Chauvet, M.T., Acher, R., and Polenov, A.L. (1991). Neurohypophysial hormones as molecular evolutionary tracers: investigations on the sturgeons *Acipenser stellatus* and *Acipenser guldenstadti.* *Comp. Biochem. Physiol.* **100**, 721-726.

Rochard, E., Castelnaud, G. and Lepage, M. (1990). Sturgeons (Pisces: Acipenseridae): threats and prospects. *J. Fish Biol.* **37**, 123-132.

Rubin, D.A., and Dores, R.M. (1994). Cloning of a growth hormone from a primitive bony fish and its phylogenetic relationships. *Gen. Comp. Endocrinol.* **95**, 71-83.

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

Sherwood, N. M., von Schalburg, K. R., and Lescheid, D. W. (1997). Origin and evolution of gonadotropin-releasing hormone in vertebrates and invertebrates. In: Parhar, I.S., Sakuma, Y. (eds) GnRH: Gene to Behaviour. Brain Shippon Publishers, Tokyo, Japan (in press)

Sherwood, N.M., Parker, D.P., McRory, J.E. and Lescheid, D.W. (1994). Molecular evolution of growth hormone-releasing hormone and gonadotropin-releasing hormone. In Fish Physiology. Vol. XIII, (ed. by N.M. Sherwood and C.L. Hew), Academic Press, Inc., San Diego, California. pp. 3-66.

Standen, E.M. (1995). Gonadotropin-releasing hormone: three primary structures in pacu fish and characterization in early teleosts. Honour's Thesis, University of Victoria, B.C.

Suhr, S.T., Rahal, O.J., Mayo, K.E. (1989). Mouse growth hormone-releasing hormone: precursor structure and expression in brain and placenta. *Mol. Endocrinol.* **3**, 1693-1700

Tarpey, J.F. and Nicoll, C.S. (1985). Characterization of hepatic growth hormone binding sites in two fish species, *Gillichthys mirabilis* (Teleostei) and *Acipenser transmontanus* (Chondrostei). *Gen. Comp. Endocrinol.* **60**, 39-50.

Vaccarino, F.J. (1990). Growth hormone-releasing factor and feeding. Behavioral evidence for direct central actions. In: "A decade of neuropeptides: Past, present, and future (G.F. Koob, C.A. Sandman and F.L. Strand, Eds.)". *Annals New York Acad. Sci.* **579**, 227-232.

Vaughan, J.M., Rivier, J., Spiess, J., Peng, C., Chang, J.P., Peter, R.E. and Vale, W. (1991). Isolation and characterization of a teleost hypothalamic growth hormone releasing factor from carp, *Cyprinus carpio*. *Neuroendocrinology* **56**, 539-549.

Vigh, S., Arimura, A., Köves, K., Somogyvàri-Vigh, A., Sitton, and Fermin, C.D. (1991). Immunohistochemical localization of the neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), in human and primate hypothalamus. *Peptides* **12**, 313-318.

Waugh, D., Youson, J., Mims, S.D., Sower, S., and Conlon, J.M. (1995). Urotensin II from the river lamprey (*Lampetra fluviatilis*), the sea lamprey (*Petromyzon marinus*), and the paddlefish (*Polydon spathula*). *Gen. Comp. Endocrinol.* **99**, 323-332.

Winters, S.J., Tsufii, T., and Attardi, B. (1996). Effects of GnRH and PACAP on gonadotropin secretion and subunit messenger RNAs. In" VIP, PACAP, and related peptides, second international symposium, (A. Arimura and S.J. Said, Eds.)". *Annals New York Acad. Sci.* **805**, 343-354.

Yasuda, A., Yamaguchi, K., Nosos, T., Papkoff, H., Polenove, A.L., Nicoll, C.S., and Kawauchi, H. (1992). The complete amino acid sequence of growth hormone from sturgeon (*Acipenser guldenstadti*). *Biochim. Biophys. Acta* **1120**, 297-234.

Young, J. Z. (1981). The life of vertebrates. Oxford University Press, New York.

Zhong, Y., and Peña, L.A. (1995). A novel synaptic transmission mediated by a PACAP-like neuropeptide in Drosophila. *Neuron* **14**, 527-536.

Chapter 2

Determination of the primary structure of mGnRH from the brain of Siberian sturgeon, *Acipenser gueldenstadtii*.

A modified version of this chapter has been published as:

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

Introduction:

Mammalian GnRH (mGnRH) was the first gonadotropin-releasing hormone to be identified (Burgus *et al.*, 1972; Matsuo *et al.*, 1971); there are currently 11 other GnRH peptides known. The location, form and function of mGnRH has been studied best in mammals, but there is little direct evidence concerning the existence and function of mGnRH in other vertebrates. The only mGnRH peptide to be isolated from a non-mammalian vertebrate was from the amphibians, *Rana catesbeiana* (Rivier *et al.*, 1981) and *Rana ridibunda* (Conlon *et al.*, 1993). There is considerable indirect evidence from HPLC, RIA and immunohistochemical investigations indicating that a GnRH-like molecule, immunologically similiar to mGnRH, is present in a variety of species. For example, some marsupials (King *et al.*, 1989; King *et al.*, 1990a), frogs, *Rana pipiens*, *Hyla regilla*, a newt, *Taricha granulosa*, a salamander, *Ambystoma gracile* (Sherwood *et al.*, 1986a), and a toad, *Xenopus laevis* (King and Millar,1981) contain mammalian-like immunoreactive molecules.

A previous report by Sherwood et al. (1991) had demonstated that one of the forms of GnRH within the brain of white sturgeon, *Acipenser transmontanus*, was immunologically and chromatographically indistinguishable from mGnRH. Mammalian GnRH-like molecules also have been reported in the brain of other evolutionarily-ancient bony fish in addition to sturgeon: reedfish, *Calamoichthys calabaricus*, the alligator gar, *Lepisosteus spatula*, the butterflyfish, *Pantodon bucholzi* (Powell, 1995; Standen, 1995) and two species of eel, *Anguilla anguilla* (King *et al.*, 1990b) and *Muraena militaris* (Powell, 1995; Standen, 1995). The holostean fish, *Amia calva*, may contain mammalian-like immunoreactive GnRH fibers within its brain and gut (Crim *et al.*, 1985) and the lungfish, *Neoceratodus forsteri* may contain immunoreactive mGnRH within its brain (King and Millar, 1992). Even species as evolutionarily-ancient as the gastropod mollusc, *Helisoma trivolvis*, have been suggested to contain a mammalian-like GnRH form (Goldberg *et al.*, 1993). However, it is important to establish a phylogenetic history of this

molecule based on primary structure and not solely on immunological data. The small size of GnRH means that antisera (particularly polyclonal antisera) raised against a specific GnRH form, may also cross-react to some degree with the other known GnRH family members. In addition, differences in experimental design of HPLC elution profiles and radioimmunoassays can result in misleading conclusions about the presence of mGnRH in other species.

The importance of sturgeon as a species that occupies a phylogenetic position close to the bifurcation leading to bony fish or mammals led me to choose it for a study to determine the GnRH structure present in the brain and therefore, provide valuable information of how GnRH could have evolved in vertebrates. The origin of mGnRH in phylogeny was interesting at the time I began this study not only because it was widely distributed among the vertebrates but also because it appeared to be the sole form of GnRH in most placental mammals, unlike the situation in most other vertebrates where multiple forms of GnRH existed.

This chapter presents the isolation and primary structure of mGnRH from the brains of the Siberian sturgeon, *Acipenser gueldenstaedti* . Although the previous report of a mGnRH-like molecule in sturgeon was done using white sturgeon, *Acipenser transmontanus*, Siberian sturgeon were chosen for this study because it is relatively easier to obtain sufficient brains of this species for the determination of peptide hormone primary structure. This work was done in collaboration with Jim Powell at the University of Victoria and with M. Park, W. Fischer and A.G. Craig at the Clayton Foundation for Peptide Biology, Salk Institute, La Jolla, California. Also, I.A. Bukovskaya and O. Barranikova at the University of St. Petersburg collected and sent the sturgeon brains from Russia.

Materials and Methods

Collection of brains

Brains were collected from adult pre-spawning male and female Russian sturgeon (*Ascipenser gueldenstaedti* Brandt) during the period of anadromous migration in the Volga river, April, 1992. The size of fish ranged from 110 to 130 cm in males and from 120 to 150 cm in females. The age of the fish is unknown. Brains were frozen and shipped on dry ice from St. Petersburg, Russia to Victoria, B.C., Canada. The tissue was stored at -80°C on arrival. Pituitaries were not collected with the brains.

Extraction

Frozen brains (485g) were processed using similar extraction techniques as described previously (Sherwood *et al.*, 1986). Briefly, frozen brains were crushed with a cold mortar and pestle, powdered with liquid nitrogen in a Waring blender and then added to a cold 1N HCL and acetone mixture (3:100, v/v). For every gram of brain tissue extracted, 5 ml of the acid:acetone mixture was used. The extraction mixture was stirred on ice for 3 h and then filtered through a Buchner funnel and filter (Whatman No.1). Insoluble material was extracted again for a further 5 minutes with continuous stirring in a 0.01N HCL and acetone mixture (1:4, v/v), and then refiltered. The volume of the acid: acetone mixture used in this additional extraction was 40% of the volume used in the initial extraction. The filtrates were combined and then treated with 5 consecutive applications of petroleum ether (4:1, v/v) to remove acetone, lipids and other hydrophobic substances. The final aqueous phase (800 ml) was evaporated in a vacuum centrifuge to 200 ml prior to application onto the SepPak cartridges.

SepPak and High performance liquid chromatography (HPLC)

For the preliminary study, sturgeon brains (11g) were treated as described above for the extraction of peptides. After solvents had been evaporated, the aqueous extract was filtered through a low protein absorbing filter (45 μm, μCoStar LB ™, Keenebunk, ME)

and then adjusted to pH 2.5 using 5N NaOH.

Initially, a blank sample of 600 μl of Milli-Q water (Millipore, Bedford, MA) was injected through a 1 ml injection loop onto a Supelco C-18 HPLC column (25.0 cm x 0.46 cm x 5 μm particle size, Supelco Canada, Oakville, Ont.), attached to a guard column (0.46 cm x 2 cm) of the same material and a Beckman HPLC System Gold, solvent Module 125 and UV detector Module 166. The following HPLC program was used to elute any potentially immunoreactive proteins: 10 min at 5% B (80% acetonitrile, CH_3CN; 20% 0.1M heptafluorobutyric acid, HFBA) and 95% A (0.1M HFBA), followed by an increase of 1.4% per min of solution B for 50 min. Sixty fractions of 1 ml each were collected in borosilicate glass tubes; 500 μl was removed from each fraction, vacuum dried, reconstituted in phosphate buffered saline (PBS) with 1% gelatin and assayed for immunoreactive GnRH with an RIA using antisera GF-4 and Bla-4. The cross-reactivities of these antisera are as reported previously by Kelsall et al. (1990) and described in chapter 3 of this thesis. GnRH immunoreactivity was not detected in this blank run (results not shown) and therefore, four aliquots of 800 μl of sturgeon brain extracts were applied at two minute intervals to the HPLC column and eluted using the same HFBA program that was used in the blank run. Sixty fractions of 1 ml each were collected in polyallomer tubes and treated as above, except only 100 μl fractions were assayed for immunoreactive GnRH in the RIAs.

For the large scale brain extraction, an additional purification step was done prior to injection onto the C-18 HPLC column using 10 SepPak C-18 cartridges held together in series by shrink wrap tubing. The SepPak column was primed with methanol and washed with Milli-Q water according to the manufacturer's instructions. The brain extracts were brought to pH 2.5 with 5N NaOH and then pumped onto the SepPak column using a peristaltic pump at a flow rate of 1.5 ml/min. The SepPak column was then attached to the same HPLC system as described above except that the detector was bypassed.

Proteinaceous material was eluted using the following HPLC program: initial conditions of 5% B (0.05% trifluoroacetic acid, TFA, in 80% acetonitrile, ACN) and 95% A (0.05% TFA in Milli-Q water) followed by a gradient increase to 80% B over 55 min and a final 5 min at 80% B (20% A). Sixty fractions of 1 ml each were collected in polyallomer tubes and treated as above, except only 50 μl fractions were assayed for immunoreactive GnRH in the RIAs. Immunoreactive fractions following elution of the sturgeon brain extracts from the SepPak column were combined and vacuum concentrated to remove any residual acetonitrile prior to their application on the C-18 HPLC column.

Purification of GnRH

Procedural steps for the purification of GnRH are as described in Ngamvongchon *et al.* (1992). Briefly, the purification of GnRH-like peptides was done using three successive HPLC stages utilizing a C_{18} Supelco column with varying solvents and ion-pairing agents (Table 2-1). These HPLC runs were followed by an HPLC run using a different type of column,Vydac phenyl, which should further separate peptides. Aliquots of 10 μl were used to determine the amount of immunoreactive GnRH (irGnRH). Fractions with irGnRH were present in only one or two fractions at each successive step. These fractions were then applied to the HPLC in the next step of the purification. Only one peak from the initial SepPak was detected and purified. Aliquots (10 μl) of fractions from the final step of the purification (Vydac phenyl column) were additionally assayed with antiserum B-6, which is specific for mGnRH.

Table 2-1: Sequence of HPLC steps in the purification of GnRH

[1] HPLC	[2]Solvent A	[2]Solvent B	pH	Column type	
1	0.05% TFA	0.05% TFA in 80% ACN	2.5	SepPak	C18
2	0.1M HFBA	0.1M HFBA in 80% ACN	2.0	Supelco	C18
3	0.012M TEAF	ACN	2.5	Supelco	C18
4	0.013M TEAP	ACN	6.5	Supelco	C18
5	0.05% TFA	0.05% TFA in 80% ACN	2.5	Vydac	Phenyl

[1] Consecutive HPLC steps are shown above. Immunoreactive fractions detected by antisera GF-4 and Bla-5 in each stage of purification were combined and reduced in volume before being used in the next step of HPLC and RIA.

[2] Chemical abbreviations are: trifluoroacetic acid, TFA; acetonitrile, ACN; heptafluorobutyric acid, HFBA; triethylammonium phosphate, TEAP; triethylammonium formate, TEAF.

Standards

Seven synthetic standards were combined and applied to the column at a concentration of 200 ng/ ml each immediately after HPLC and RIA analyses of the brain extracts were completed. The elution positions of the standards on the chromatograph were confirmed by absorbance peaks (A= 280 nm) and by GnRH-specific RIA using antisera GF-4, Bla-4 and B-6.

Radioimmunoassay (RIA)

Aliquots of 10 μl from fractions collected at each successive stage in the purification were assayed for irGnRH by methods previously reported (Sherwood *et al.* , 1983) and described below. Briefly, 300 μl of 10 mM phosphate buffered saline, PBS (pH 7.0) was added to the sample in a 5 ml polyallomer tube. A standard curve of nine different concentrations, ranging from 1 ng to 250 ng, was made by serially diluting a stock solution of synthetic mGnRH standard to a final volume of 300 μl using the PBS buffer. Reference tubes were prepared in triplicate and included: 1) zero binding tubes that contained 400 μl of PBS and 100 μl of radioactive tracer but no diluted antiserum, 2) maximal binding tubes that contained 300 μl of PBS, 100 μl of radioactive tracer, and 100 μl of diluted antiserum and 3) total radioactivity tubes that also contained 400 μl of PBS and 100 μl of radioactive tracer but no diluted antiserum and were used later to determine percent maximal binding(B/B_0). The radioactive tracer added to the sample, standard and reference tubes was ^{125}I-mGnRH; a 100 μl aliquot of approximately 6000 cpm was used. The tubes were incubated overnight at 4^0C and then all tubes, except the total radioactivity reference tubes, received 1 ml of 2.5% charcoal (w /v) , 0.25% dextran (w/ v) in 10 mM PBS. The tubes were vortexed vigorously and then incubated for a further 10 min at 4^0C before centrifugation for 15 min at 3000g at 4^0C (Centra-7R, Intl. Equip. Company, Needham Hts., Mass). The supernatants, representing the unbound fraction, were decanted into an additional set of labelled borosilicate glass tubes and placed in a gamma counter (LKB

Minigamma counter, Model 1275, Wallac Oy, Finland) for determination of radioactivity. The amount of irGnRH in the samples was determined by comparison of radioactivity to the standards.

The RIAs with antisera GF-4, Bla-4 and B-6 used mGnRH as the labeled hormone and standards. Antiserum GF-4 (raised against salmon GnRH) was used in a dilution of 1: 50,000 resulting in 22-32% binding of ^{125}I-mGnRH. Antiserum Bla-4 (made against lamprey GnRH-I) was used in a dilution of 1:10,000 resulting in 9-17% binding of ^{125}I-mGnRH. Seven of the known GnRH forms are recognized by these two antibodies, GF-4 and Bla-4. Limits of detection (B/B_o= 80%) averaged 10.4 pg for GF-4 and 47.6 pg for Bla-4. Antiserum B-6, although a polyclonal antibody, is specific for mGnRH and does not cross-react with any other known form. B-6 had a binding of 52% of ^{125}I-mGnRH and a detection limit (B/B_o= 80%) of 9.4 pg.

Characterization of the primary structure

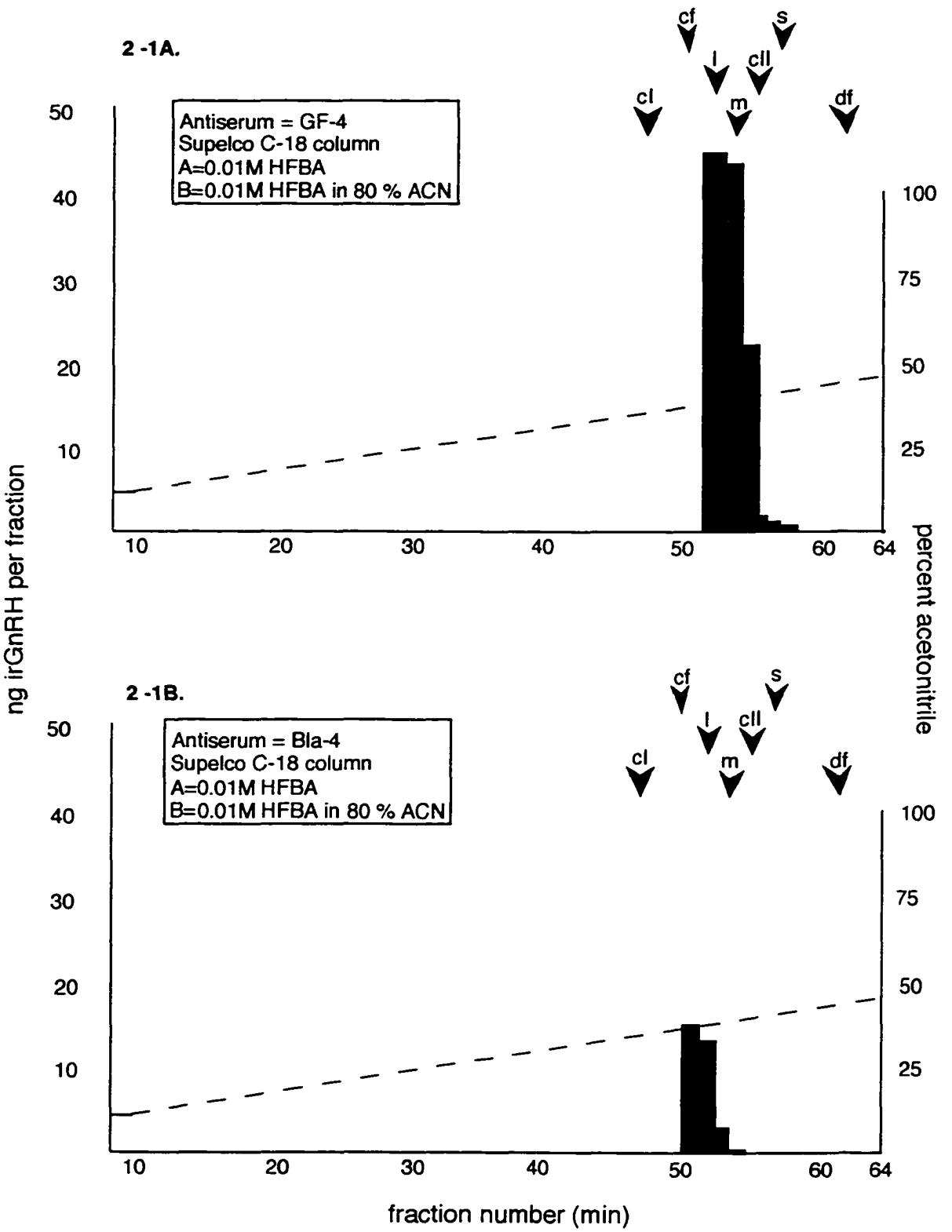
Sequence analysis was attempted on 10% (by volume) of the HPLC-purified sample before digestion. The lack of sequence data suggested that the peptide possessed a blocked N terminus. Fifty percent of the sample was dried and digested with calf liver pyroglutamate aminopeptidase (Boehringer Mannheim Biochemicals). The details of this procedure have been reported elsewhere (Lovejoy *et al.*, 1991b). The digested peptide was sequenced using an Applied Biosystems Protein Sequencer (Model 470A). A sample of the purified peptide was analyzed on the mass spectrometer as described (Ngamvongchon *et al.*, 1992).

Results

HPLC

Our small-scale preliminary investigation detected two forms of irGnRH using HFBA and 80% acetonitrile on a C_{18} analytical column (Fig. 2-1A, 2-1B).

Fig. 2-1. Initial study of sturgeon GnRH. (A) Elution of crude brain extract, assayed using GF-4 antiserum (1:50,000 dilution). (B) Elution of crude brain extract, assayed using Bla-4 antiserum (1:10,000 dilution). One immunoreactive GnRH peak was detected using both antisera. A second ir-peak that is not detected by Bla 4 can be deduced by noting a decreased quantity of GnRH in fraction 54. Refer to Table 2-1 for a description of the HPLC program used. The dashed line indicates percent acetonitrile. The mobile phase is indicated by A and B; HFBA, heptafluorobutyric acid and ACN, acetonitrile. The elution position of seven of the known forms of GnRH on the same HPLC program is shown above the figures. cI= chicken I; cf= catfish; l= lamprey; m= mammalian; cII= chicken II; s= salmon; df= dogfish GnRH.

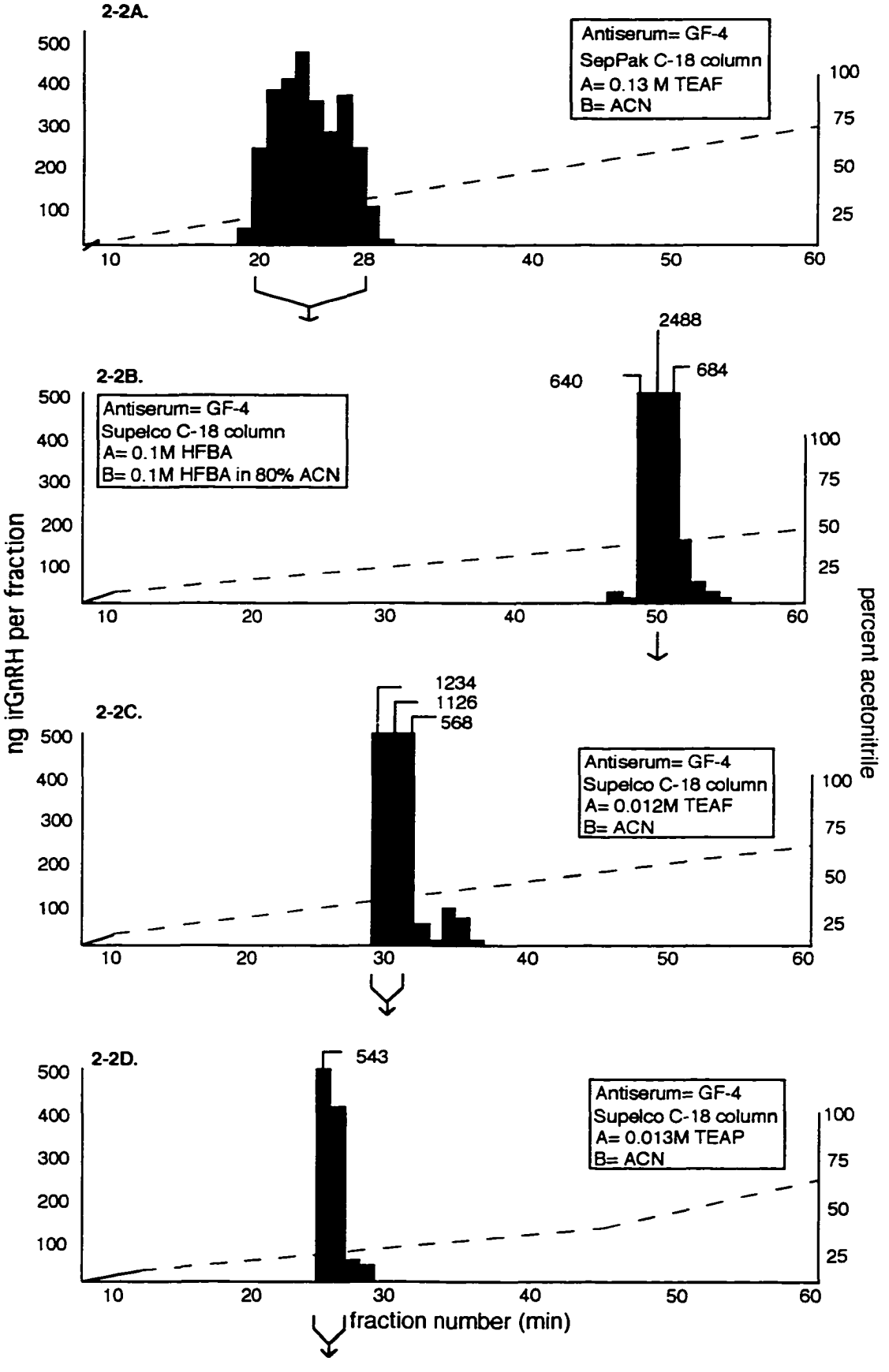

2 -1A.
Antiserum = GF-4
Supelco C-18 column
A=0.01M HFBA
B=0.01M HFBA in 80 % ACN
cf
s
I
cII
cI
m
df
50
40
30
20
10
100
75
50
25
10
20
30
40
50
60
64
2 -1B.
Antiserum = Bla-4
Supelco C-18 column
A=0.01M HFBA
B=0.01M HFBA in 80 % ACN
ng irGnRH per fraction
percent acetonitrile
fraction number (min)

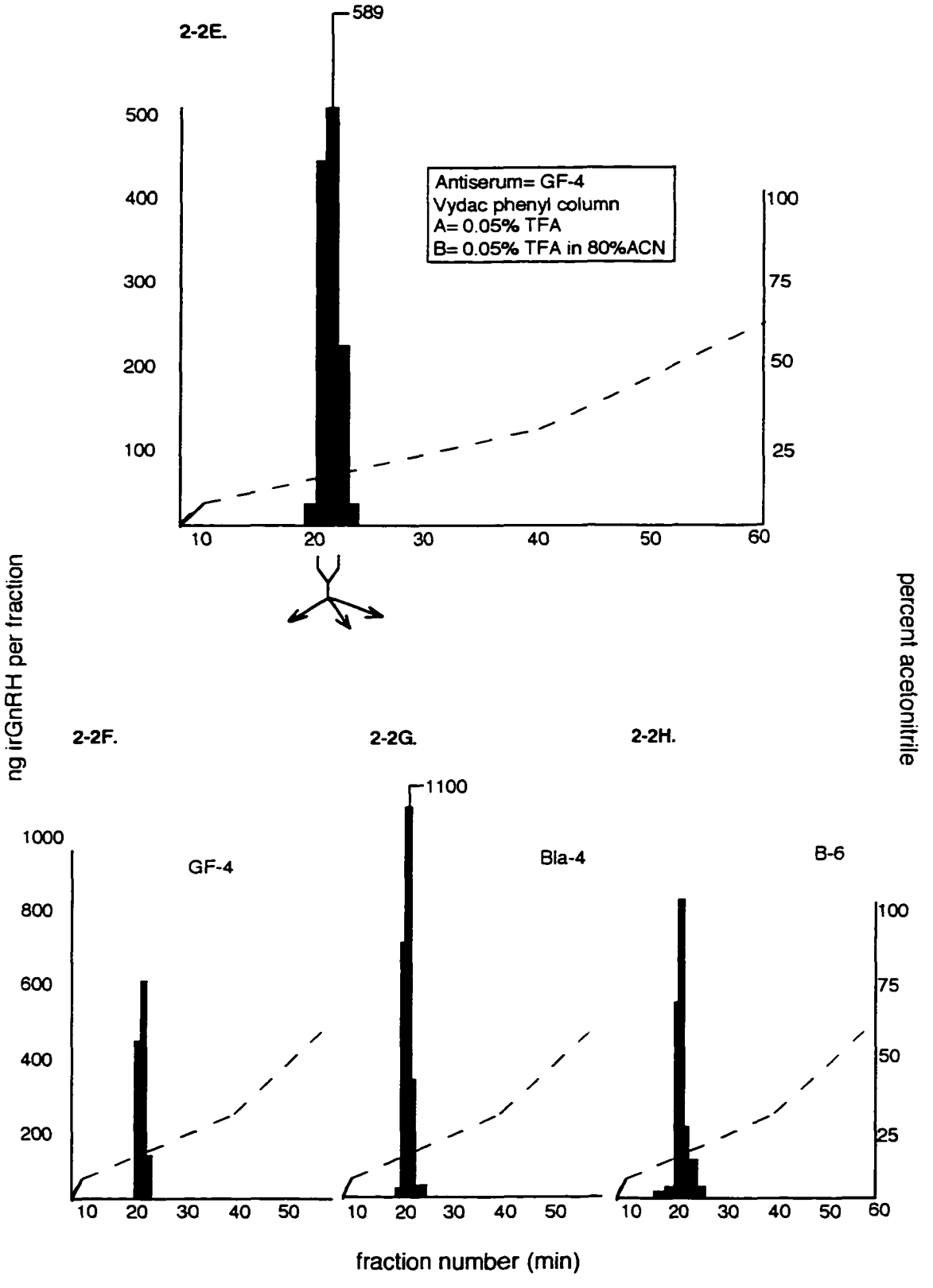
The material was tentatively assigned as mGnRH and cGnRH-II based on elution positions and antisera cross-reactivity.

Assay of fractions from the large-scale SepPak HPLC detected irGnRH in fractions 19-29 with antiserum GF-4 and fractions 14-41 with antiserum Bla-4. Fractions 20-28 (37-44% ACN) were selected as having the highest amount of irGnRH (Fig. 2-2A). The acetonitrile was evaporated in a vacuum centrifuge and the fractions combined before application to the HPLC. Elution of these fractions from a Supelco C_{18} analytical column using 0.1M heptafluorobutyric acid (HFBA) yielded irGnRH activity in fractions 43-62 (Fig. 2-2B). One major peak (2,488ng) was observed in fraction 50 as detected by both antisera. The total irGnRH detected represents 5.4ng GnRH /g sturgeon brain. Fraction 50 of this HPLC run was reduced in volume and applied to the same column with triethylammonium formate (TEAF) (Rivier, 1978). The irGnRH eluted as one peak in fractions 29 and 30 (30% ACN) with an irGnRH content of 2,360 ng/ 2ml (Fig. 2-2C). The immunoreactive peak at fractions 34 and 35 as detected by antiserum GF-4 and not by antiserum Bla-4 (data not shown), indicates the presence of a chicken GnRH-II-like molecule.

The acetonitrile in fractions 29 and 30 was removed before they were combined and applied to the same HPLC column in the fourth step of the purification. Fractions 26 and 27 (26% ACN) were found to contain 900 ng (543 + 375 ng) of irGnRH activity by antisera GF-4 (Fig. 2-2D) and 1, 306 ng by antiserum Bla-4 (not shown). The acetonitrile was removed from these fractions and they were applied to a Vydac phenyl HPLC column. Fractions 21 and 22 from the phenyl column (Fig. 2-2E) were used for structural and mass analysis. In addition, an aliquot from all fractions that eluted from the phenyl column were assayed with three antisera (Fig. 2-2F to 2-2H). Fractions 21 and 22 were found to contain a total of 1,200 (GF-4), 1,850 (Bla-4) and 1,400ng (B-6) irGnRH (Figs. 2-2F to 2-2H).

Fig. 2-2 . Purification of sturgeon GnRH. (A) Fractions 20-28 were used for the next step of purification. (B) Fraction 50 contained the largest quantity of ir-GnRH and was further purified. (C) Fractions 29, 30 and (D) Fractions 26, 27 were further purified. (E) Fraction 22 from the Vydac phenyl column was used for the structural characterization. (F, G, H) Fractions shown in Fig. 2-2E. were assayed using two additional antisera to confirm the presence of mGnRH. Refer to Table 2-1 for a description of the HPLC program used.


2-2A.
Antiserum= GF-4
SepPak C-18 column
A= 0.13 M TEAF
B= ACN
2-2B.
Antiserum= GF-4
Supelco C-18 column
A= 0.1M HFBA
B= 0.1M HFBA in 80% ACN
2488
640
684
2-2C.
Antiserum= GF-4
Supelco C-18 column
A= 0.012M TEAF
B= ACN
1234
1126
568
2-2D.
Antiserum= GF-4
Supelco C-18 column
A= 0.013M TEAP
B= ACN
543
ng irGnRH per fraction
percent acetonitrile
fraction number (min)


2-2E.
589
500
400
300
200
100
Antiserum= GF-4
Vydac phenyl column
A= 0.05% TFA
B= 0.05% TFA in 80%ACN
100
75
50
25
10
20
30
40
50
60
ng irGnRH per fraction
percent acetonitrile
2-2F.
2-2G.
2-2H.
1100
1000
800
600
400
200
GF-4
Bla-4
B-6
fraction number (min)

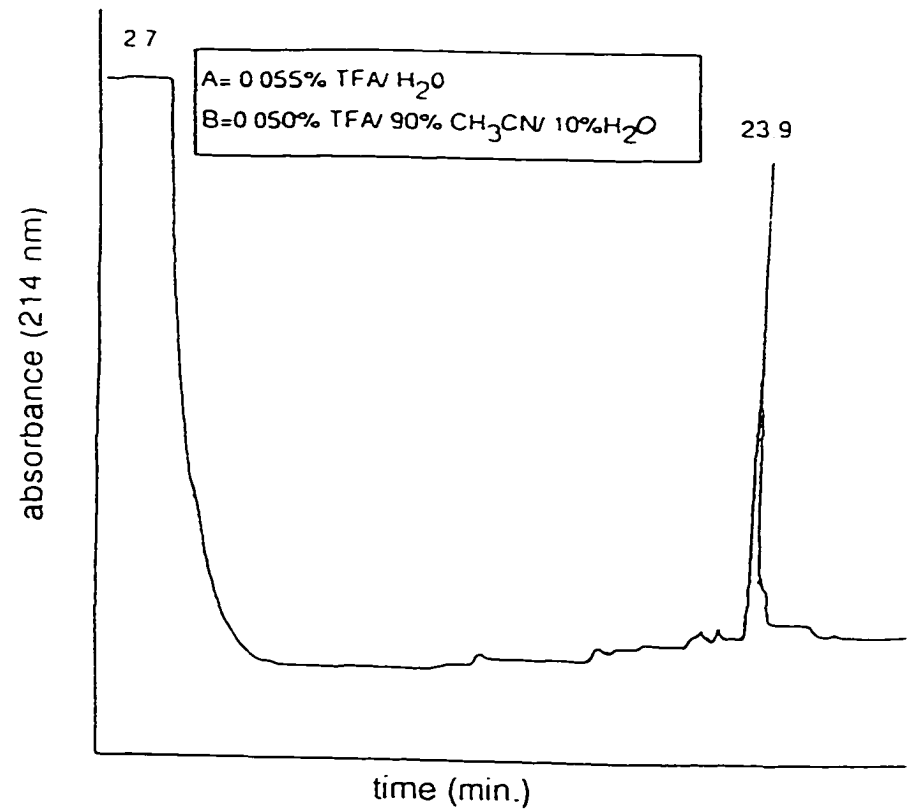
Sequence and Mass Spectral Analysis

A major peak eluted at 23.9 min from a narrow-bore HPLC system (Fig. 2-3A) after application of an aliquot (10%) of the irGnRH material from the previous purification step (phenyl column HPLC). Another aliquot (50%) of the immunoreactive material from the phenyl column HPLC separation was concentrated to dryness and subjected to pyroglutamyl aminopeptidase digestion as described (Fischer and Park, 1992). The digested sample eluted at 22.4 min, whereas the remaining undigested material eluted at 24.9 min (Fig. 2-3B). Edman degradation of the digested material that eluted at 22.4 minutes (Fig. 2-3B) was applied to an automated gas phase sequenator and yielded the following PTH amino acid derivatives in consecutive cycles: His (31.7 pmol), Trp (159.2 pmol), Ser (26.7 pmol), Tyr (116.7 pmol), Gly (80.1 pmol), Leu (101.0 pmol), Arg (30 pmol), Pro (28.7 pmol), and Gly (23.3 pmol). No other significant signal was observed in any of the cycles. The following sequence was assigned: His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.

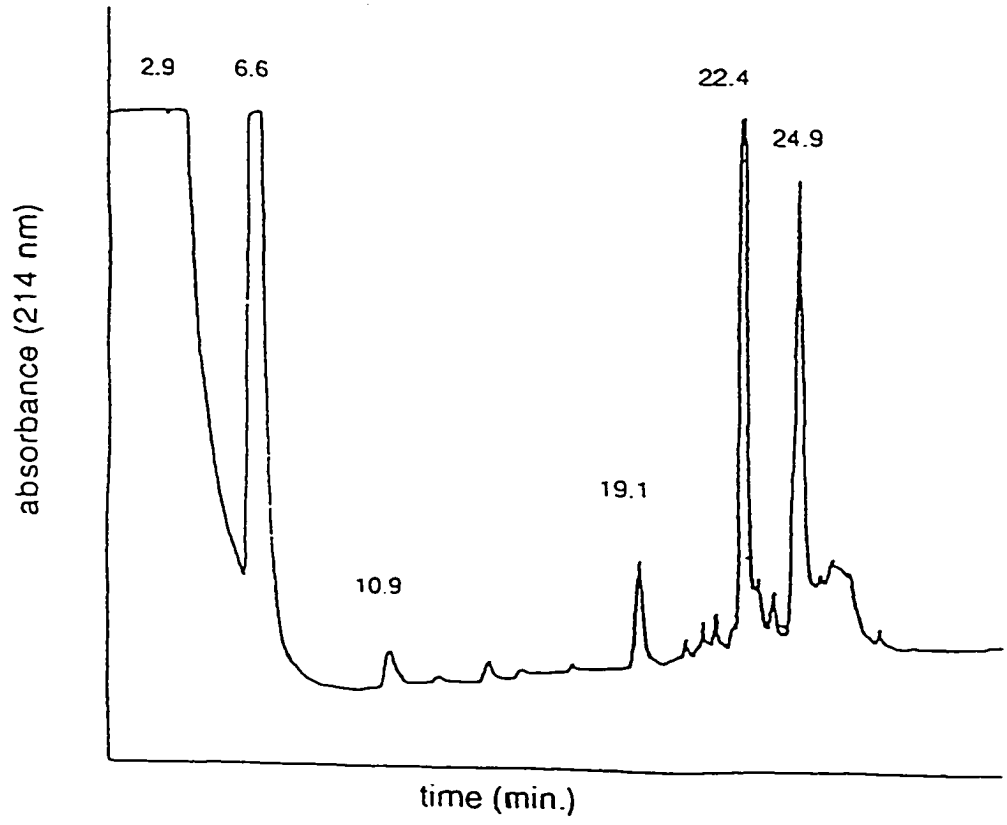
Another aliquot was further purified by reversed-phase HPLC and analyzed by liquid secondary ion mass spectrometry on a double focusing magnetic sector instrument (Jeol HX 110). The determined mass (m/z 1182.5) is consistent with the C-terminally amidated form of mammalian GnRH (MH^+=1182.58 Da). The complete sequence assignment is thus pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.

Fig. 2-3. Absorbance of sturgeon GnRH eluted from a narrow-bore C-18 HPLC column. (A) HPLC profile of undigested material. The intact sturgeon GnRH eluted at 23.9 min; (B) HPLC profile of sturgeon GnRH after digestion with pyroglutamate aminopeptidase. The digested peptide eluted at 22.4 min, whereas the undigested peptide eluted at 24.9 min. The fraction with the digested peptide was used for sequence analysis.

A


2 7
A= 0 055% TFA/ H_2O
B=0 050% TFA/ 90% CH_3CN/ 10%H_2O
23 9
absorbance (214 nm)
time (min.)

B


2.9
6.6
22.4
24.9
19.1
10.9
absorbance (214 nm)
time (min.)

Discussion:

This is the first report to determine the primary structure of mGnRH in a fish. The peptide was isolated from the brains of Siberian sturgeon, an evolutionarily-ancient bony fish. The identity of the purified peptide as mGnRH was established by chemical sequence analysis (after removal of the N-terminal pyroglutamate with a specific enzyme) and by accurate determination of the molecular mass.

Although immunoreactive mammalian-type GnRH molecules can be detected in various non-mammalian vertebrates, the definitive proof that this form was present at least 200 million years ago when the order Acipenseriformes evolved (see Birstein and Vasiliev, 1987) depends on the determination of primary structure. Relatively large quantities of mGnRH per brain, 5.4 ng/brain, were isolated from these fish. Usually, fish contain less GnRH per brain (Lovejoy and Sherwood, 1989). This relatively large quantity likely indicates that these fish were harvested at a stage of the life cycle when the endogenous levels of this peptide were elevated. Alternatively, the differences in brain sizes between species and variation in cross-reactivity among antisera may also contribute to the quantity of GnRH detected.

Mammalian GnRH is known to affect the reproductive physiology of sturgeons. Intramuscular injection of synthetic mGnRH or [D Ala^6, Pro^9 N Et] mGnRH to stellate (*A. stellatus*) and Siberian (*A. gueldenstaedtii*) sturgeons caused activation and exhaustion of pituitary gonadotropic cells and a corresponding rise in spermiation, ovulation and blood plasma levels of gonadotropins (Barannikova *et al.*, 1982; 1989; Barannikova and Bukovskaya, 1990, 1991). Injections of synthetic mGnRH agonists, in particular [D-Ala^6] mGnRH, were also successful in inducing ovulation in white sturgeon (Doroshov and Lutes, 1984, Gontcharov *et al.*, 1989), indicating that not only the structure but also the function of mGnRH has been conserved in these primitive fish.

My results suggest that the sturgeon brain also contains a small amount of cGnRH-II. We previously detected cGnRH-II in white sturgeon, *A. transmontanus*, with

chromatographic and immunological methods (Sherwood *et al.*, 1991). However, I was unable to isolate sufficient quantities from the brains of the fish in the present study to purify and characterize the peptide. cGnRH-II may not be present in sufficient levels at the stage of collection used here or may be masked by the presence of relatively high amounts of mGnRH. Nonetheless, my study as well the HPLC/ RIA study by Sherwood et al. (1991) and the immunocytochemical study by Lepretre et al. (1993) demonstrate that cGnRH-II exists with mGnRH in the brain of sturgeon. The ancient origin of cGnRH-II has been established further by chromatographic and immunological data from the brain of evolutionarily-ancient bony fish like reedfish and gar (Sherwood *et al.*, 1991) but most clearly by primary structure from the brain of fish that evolved prior to the sturgeon such as the dogfish shark, *Squalus acanthias* (Lovejoy *et al.*, 1992b) and the ratfish, *Hydrolagus colliei* (Lovejoy *et al.*, 1991b).

Lepretre and co-workers (1993) used immunocytochemistry to examine the differential distribution of mGnRH and cGnRH-II in the brain of the same species of sturgeon studied here, *A. baeri* . Mammalian-type immunoreactive cell bodies and fibers were concentrated in the forebrain region, whereas chicken II-type GnRH neurons were found mostly in the midbrain tegmentum. This distribution parallels that which has been reported for teleosts (Yu *et al.*, 1988; King *et al.*, 1990b; Okuzawa *et al.*, 1990) and birds (Mikami et al, 1988; Katz *et al.*, 1990; Sharp *et al.*, 1990). Cell bodies immunoreactive to mGnRH were also found in the brain of sterlet, *A. ruthenus* Linnaeus (Kulik *et al.*, 1992). The form of GnRH that predominates in the preoptic tissues and which has a primary role as a stimulant of pituitary gonadotropin synthesis and release, may vary in structure in different species. However, the GnRH form that is concentrated in the mesencephalon (cGnRH-II) has a potential neuromodulatory role (see King and Millar, 1992). In addition, a few species have cGnRH-II axons that provide a minor innervation of the portal vessels or pituitary and hence may have a more traditional gonadotropin-releasing role. This cGnRH-II form is highly conserved in that it is distributed throughout jawed vertebrates,

except for most placental mammals. However, the brain location of mGnRH within Siberian sturgeon suggests that it is this form and not cGnRH-II that is primarily responsible for pituitary gonadotropin release.

Determination of the primary structure of the mammalian form of GnRH in sturgeon clearly establishes mGnRH as one of the ancestral forms of GnRH. Nucleotide substitutions in the mGnRH gene could have led to one or more different forms of GnRH during evolution. For example, salmon GnRH (sGnRH) may be an evolutionary derivative of mGnRH because mGnRH is present in sturgeon but not in teleosts, except in early-evolving teleosts like the eel (King *et al.*, 1990b; Powell, 1995; Standen, 1995) and butterflyfish (Powell, 1995; Standen, 1995). Likewise, a change from mGnRH to cGnRH-I is thought to have occurred in an ancestral reptile (see Sherwood *et al.*, 1993). It also is clear from this thesis that the sturgeon brain contains the same form of GnRH that is found in the brain of humans. This evidence in addition to those reports described in chapter one of other peptides as well as morphological and physiological characters of sturgeon with mammalian-like properties, help to strengthen the hypothesis that these fish are derived from ancestors that evolved close to the stem line of tetrapod evolution.

Literature cited

Adelman, J.P., Mason, A.J., Hayflick, J.S. and Seeburg, P.H. (1986). Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proc. Natl. Acad. Sci. (U.S.A.)* **83**, 179-183.

Azad, N., Emanuele, N.V., Halloran, M.M., Tentler, J., Kelley, M.R. (1991). Presence of luteinizing hormone-releasing hormone (LHRH) mRNA in rat spleen lymphocytes. *Endocrinology* **128**, 1679-1681.

Barannikova, I.A., Boev, A.A., Bukovskaya, O.S., Efimova, N.A., and Presnov, D.I. (1989). Analysis of sturgeon (*Acipenser gueldenstaedti* Brandt) maturation induced by LH-RH and the possibilities of its application in sturgeon culture. *Vopr. Ichtiol.* **29**, 439-447 (in Russian).

Barannikova, I.A., and Bukovskaya, O.S. (1991). Hormonal control of sturgeon (Acipenseridae) reproduction. From the Proceedings of the 4th International Symposium on Reproductive Physiology of Fish, Norwich, U.K., 7-12 July 1991, p. 22-24.

Barannikova, I.A., and Bukovskaya, O.S. (1990). Influence of luliberin on pituitary gonadotropocytes state and dynamics of serum gonadotropin concentrations in stellate sturgeon (*Acipenser stellatus* Ballus). *Vopr. Ichtiol.* **30**, 126-136 (in Russian).

Barannikova, I.A., Bukovskaya, O.S. and Efimova, N.A. (1982). Hormonal control of the reproductive function of sturgeons (Chondrostei). From the Proceedings of the international symposium on reproductive physiology of fish, Wageningen, The Netherlands, 2-6 August, 1982, p. 49

Birstein, N. and Vasiliev, V.P. (1987). Tetraploid-octoploid relationships and karyological evolution in the order Acipenseriformes (Pisces). Karyotypes, nucleoli, and nucleolus-organizer regions in four acipenserid species. *Genetica* **72**, 3-12.

Bond, C.T., Hayflick, J.S., Seeburg, P.H., and Adelman, J.P. (1989). The rat gonadotropin-releasing hormone: SH locus: Structure and hypothalamic expression. *Mol. Endocrinol.* **3**, 1257-1262.

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 ovine hypothalamic luteinizing hormone-releasing factor (LRF). *Proc. Natl. Acad. Sci. (U.S.A.)* **69**, 278-282.

Conlon, J.M., Collin, F., Chiang, Y-C., Sower, S.A. and Vaudry, H. (1993). Two molecular forms of gonadotropin-releasing hormone from the brain of the frog, *Rana ridibunda*:, Purification, characterization, and distribution. *Endocrinology* **132**, 2117-2122.

Crim, J.W., Rajjo, I., and Vigna, S.R. (1985). Brain-gut peptides in holostean fish. 10th Internatl. Sympos. Comp. Endocrinol., Copper Mountain, CO, 21-26 July (Abstract).

Doroshov, S.I., and Lutes, P.B. (1984). Preliminary data on the induction of ovulation in white sturgeon (*Acipenser transmontanus* Richardson). *Aquaculture* **38**, 221-227.

Fischer, W.H. and Park, M. (1992). Sequence analysis of pyroglutamyl peptides: Microscale removal of pyroglutamate residues. *J. Prot. Chem.* **11**, 366.

Gautron, J.P., Pattou, E., Bauer, K. and Kordon, C. (1991). (Hydroxyproline^{9}) luteinizing hormone-releasing hormone: a novel peptide in mammalian and frog hypothalamus, Neurochem. Int. **18**, 221-235.

Goldberg, J.I., 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* . *J. Comp. Neurol.* **336**, 571-582.

Gontcharov, B.F., Igoumnova, L.V., Poloupan, I.S. and Savelieva, E.A. (1989). Induced oocyte maturation, ovulation and spermiation in sturgeons (Acipenseridae) using syntheic analogue of gonadotropin-releasing hormone, from Acipenser. 1st International symposium on the sturgeon, summaries of oral communications, posters, films, Bordeaux, France, 3-6 October.

Hayflick, J.S., Adelman, J.P., and Seeburg, P.H. (1989). The complete nucleotide sequence of the human gonadotropin-releasing hormone gene. *Nucleic Acids Res.* **17**, 6403-6404.

Katz, I.A., Millar, R.P., and King, J.A. (1990). Differential regional distribution and release of two forms of gonadotropin-releasing hormone in the chicken brain. *Peptides* **11**, 443-450.

Kelsall, R., Coe, I.R. and Sherwood, N.M. (1990). Phylogeny and ontogeny of gonadotropin-releasing hormone: Comparison of guinea pig, rat, and a protochordate. *Gen. Comp. Endocrinol.* **78**, 479-494.

Kepa, J.K., Wang, C., Neeley, C.I., Raynolds, M.V., Gordon, D.F., Wood, W.M., and Wierman, M.E. (1992). Structure of the rat gonadotropin releasing hormone (rGnRH) gene promoter and functional analysis in hypothalamic cells. *Nucleic Acids Res.* **20**, 1393-1399.

King, J.A., Hinds, L.A., Mehl, A.E., Saunders, N.R. and Millar, R.P. (1990a). Chicken GnRH II occurs together with mammalian GnRH in a South American species of marsupial (*Monodelphis domesticus*). *Peptides* **11**,521-525.

King, J.A. and Millar, R.P. (1990b). Geneaology of the GnRH family. *Prog. Clin. Biol.* **342**, 54-59

King, J.A. and Millar, R.P. (1992). Evolution of gonadotropin-releasing hormones, *Trends Endocrinol. Metabol.* **3**, 339-346.

King, J.A. and Millar, R.P. (1981). TRH, GH-RIH, and LH-RH in metamorphosing *Xenopus Laevis. Gen. Comp. Endocrinol.* **44**, 20-27.

King, J.A., Dufour, S., Fontaine, Y-A., Millar, R.P. (1990b). Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* **11**, 507-514.

King, J.A., Mehl, E.I., Tyndale-Biscoe, C.H., Hinds, L. and Millar, R.P. (1989). A second form of gonadotropin-releasing hormone (GnRH), with chicken GnRH II-like properties, occurs together with mammalian GnRH in marsupial brains. *Endocrinology* **125**, 2244-2252.

Kulik, V.V., Sharov, A.B., Slerko, B. and Polenov, A.L. (1992). Immunocytological determination of luliberinergic structures in the brain of sterlet, *Acipenser ruthenus*. From the Proceedings of the 8th Conference on Ecological Physiology and Biochemistry of Fish, Petrozavdsk, Russia, 30 Sept.- 3 Oct., 1992, p. 169-170.

Leprêtre, E., Anglade, I., Williot, P., Vandesande, F., Tramu, G., and Kah, O. (1993). Comparative distribution of mammalian GnRH (Gonadotrophin-releasing hormone) and chicken GnRH-II in the brain of the immature siberian sturgeon (*Acipenser baeri*). *J. Comp. Neurol.* **337**, 568-583.

Lovejoy, D.A., and Sherwood, N.M. (1989). Gonadotropin-releasing hormone in ratfish (*Hydrolagus colliei*): Distribution between the sexes and possible relationship with chicken II and salmon II forms. *Comp. Biochem. Physiol.* **92B**, 111-118.

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. *J. Exp. Zool.* **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 GnRH evolution. *Proc. Natl. Acad. Sci. (U.S.A.)* **89**, 6373-6377.

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*). *Gen. Comp.Endocrinol.* **82**, 152-161.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young III, W.S., Phillips, H.S., Nikolics, K., Seeburg, P.H. (1986). A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse, *Science* **234**, 1366-1371.

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. *Biochem. Biophys. Res. Commun.* **43**, 1334-1339.

Mikami, S., Yamada, S., Hasegawa, Y.,and Miyamoto, K. (1988). Localization of avian LHRH-immunoreactive neurons in the hypothalamus of the domestic fowl, *Gallus domesticus* , and the Japanese quail, *Coturnix coturnix* *Cell Tissue Res.* **251**, 51-58.

Millar, R.P., and King, J.A. (1987). Structural and functional evolution of gonadotropin-releasing hormone. *Int. Rev. Cytol.* **106**, 149-182.

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

Oikawa, M., Dargan, C., Ny, T., and Hsueh, A.J.W. (1990). Expression of gonadotropin-releasing hormone and prothymosin-messenger ribonucleic acid in the ovary, *Endocrinology* **127**, 2350-2356.

Okuzawa, K., Amano, M., Kobayashi, M., Aida, K., Hanyu, I., Hasegawa, Y., and Miyamoto, K. (1990). Differences in salmon GnRH and chicken GnRH-II contents in discrete brain areas of male and female rainbow trout according to age and stage of maturity. *Gen. Comp. Endocrinol.* **80**, 116-126.

Powell, J.F.F. (1995) The origin and evolution of gonadotropin- releasing hormone in boney fishes. Ph. D thesis, University of Victoria, B. C.

Powell, J.F.F., Zohar, Y., 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. *Proc. Natl. Acad. Sci. (U.S.A.)* **91**, 12081-12085.

Radovick, S., Wondisford, F.E., Nakayama, Y., Yamada, M., Cutler, Jr. G.B., and Weintraub, B.D. (1990). Isolation and characterization of the human gonadotropin-releasing hormone gene in the hypothalamus and placenta. *Mol. Endocrinol.* **4**, 476-480.

Rivier, J. (1978). Use of trialkylammonium phosphate (TEAP) buffers in reverse phase HPLC for high resolution and high recovery of peptides and proteins *J. Liq. Chromatog.* **1**, 343-367.

Rivier, J., Rivier, C., Branton, D., Millar, R., Spiess, J., and W., Vale. (1981). HPLC purification of ovine CRF, rat extra hypothalamic brain somatostatin and frog brain GnRH. *In* "Peptides: Synthesis- Structure- Function, Proc. 7th American Peptide Symposium" (D.H. Rich, and E. Gross .Eds.), Rockford ,Ill. pp. 771-776.

Seeburg, P.H. and Adelman, J.P. (1984). Characterization of cDNA for precursor of human luteinizing hormone-releasing. *Nature* **311**, 348-364.

Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M., and Huskisson, N.S. (1990). Physiological roles of chicken LHRH-I and -II in the control of gonadotrophin release in the domestic chicken. *J. Endocrinol.* **124**, 291-299.

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*). *Gen. Comp. Endocrinol.* **84**, 44-57.

Sherwood, N., Eiden, L., Brownstein, M., Spiess, J., Rivier, J., and Vale, W. (1983). Characterization of a teleost gonadotropin-releasing hormone, *Proc. Natl. Acad. Sci.(U.S.A.)* **80**, 2794-2798.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., and Brownstein, M.J. (1986b). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J.Biol. Chem.* **261**, 4812-4819.

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

Standen, E.M. (1995). Gonadotropin-releasing hormone: three primary structures in pacu fish and characterization in early teleosts. Honour's Thesis, University of Victoria, B.C.

Tan, L., and Rousseau, P. (1982). The chemical identity of the immunoreactive LHRH-like peptide biosynthesized in the human placenta. *Biochem. Biophys. Res. Commun.* **109**, 1061-1071.

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.

Chapter 3

Identification of the forms of GnRH present in the brain of a cartilaginous fish, the skate, as well as in the brains of four teleosts: whitefish, platyfish, green swordtail and sablefish.

Parts of this chapter has been submitted for publication in a modified form as:

Lescheid, D.W., Schreibman, M.P., Warby, C.M. and Sherwood, N.M. A novel form of gonadotropin-releasing hormone (GnRH) is present in the brain of platyfish and green swordtail. *Gen. Comp. Endocrinol.* (submitted)

Introduction

Heterogeneity of GnRH structures within the brain of a single species is common in vertebrates. Teleost fish make excellent model species to examine this diversity of GnRH structures within the brain because they are such a large and diverse group of vertebrates, utilizing a wide array of different reproductive strategies. Although it is possible that a single form of GnRH is the central regulator of reproduction in all fish, it is more likely that multiple forms of GnRH are present, each with a unique function. Indeed, seven of the twelve known forms of GnRH were first isolated from fish and four forms of GnRH (sGnRH, sbGnRH, hGnRH and cfGnRH) are only found in teleosts. Two of the most evolutionarily-conserved forms of GnRH, cGnRH-II and mGnRH, also are reported to be present in the brain of some teleosts.

The presence of more than one form of GnRH within a single brain, with non-overlapping cell locations, also suggests that there is more than one function for this peptide. In most teleosts, there is a direct neuronal link between the brain and pituitary, rather than the intermediate vascular medium between the brain and pituitary that is present in other vertebrates. Consequently, in teleosts the identification of GnRH in the pituitary or in regions of the brain that have fibers extending to the pituitary, provides strong anatomical evidence that a particular form of GnRH functions as the gonadotropin releaser.

Once I had established the presence of both mGnRH and cGnRH-II in the brain of sturgeon, my next objective was to determine if these two forms of GnRH had been conserved in evolution. Therefore, I used HPLC and RIA with specific anti-GnRH antisera to search for mGnRH, cGnRH-II or other forms of GnRH in the brain of a fish derived from a group that had diverged before sturgeon, the skate, as well as in the brain of fish derived from groups that had diverged after sturgeon, such as whitefish, platyfish, green swordtail and sablefish. I also examined six different regions of the brain and pituitary of skate as well as the pituitary of whitefish with the same HPLC and RIA

methods to determine if the area of the brain examined or the type of tissue examined made a difference in the form and amount of GnRH detected. My second objective was to determine whether two forms of GnRH or three forms of GnRH in the brain of a single species of teleosts represents the ancestral or derived condition. Also, by using whitefish as one of the species examined in this study, I could compare my results to those reported previously for Arctic char and salmon (which belong to two different, but closely related subfamilies to whitefish) and therefore, determine any differences in the type of GnRH present in the brain of closely related species of fish.

There have been only a few previous reports of GnRH-like substances within the brain of skates and rays, species that belong to the order Rajiformes, a more recently evolved order of elasmobranchs than the order Squaliformes, which includes the sharks. These reports include HPLC and RIA studies of the brain of the torpedo ray (King *et al.*, 1992) and skate (Bolduc *et al.*, 1988; Calvin *et al.*, 1993) as well as immunocytochemical studies of the brain of black skate, *Bathyraja kincaidi* (Lovejoy *et al.*, 1992) and rays (Wright and Demski, 1992). Determination of the identity of molecular forms of GnRH in the brain of an evolutionarily ancient vertebrate like skate would help ascertain which forms of GnRH in the brain of sturgeon are ancestral or derived.

My second question was whether two or three forms of GnRH in the brain of a single species is the ancestral condition in teleosts. The most evolutionarily-ancient teleosts that have been examined for GnRH in their brain are the knifefish, *Xenomystus nigri*, and the butterflyfish, *Pantodon bucholzi* ; both are members of the earliest-diverged order of teleosts, the Osteoglossiformes. Only two forms of GnRH were shown by HPLC and RIA to be present in the brain of these fish; mGnRH and cGnRH-II in butterflyfish and sGnRH and cGnRH-II in knifefish (Powell, 1995; Standen, 1995). The brain of two species of eel, *Muraena militaris* and *Anguilla anguilla* was shown also to contain only mGnRH-like and cGnRH-II-like molecules (King *et al.*, 1990; Powell, 1995; Standen, 1995) suggesting that two forms of GnRH were also present in the order

Anguilliformes, another early-evolved order of teleosts. Although the above evidence suggests that two forms of GnRH in the brain of a single species is the more ancestral condition in teleosts, there is more conclusive evidence that some early-evolved teleosts species contained three forms of GnRH in their brain.

Recently, three forms of GnRH have been isolated and sequenced from the brain of herring, *Clupea harengus* (Carolsfeld *et al.*, 1997) and pacu, *Piaractus mesopotomicus* (Powell *et al.*, 1997); these species belong to orders of teleost fish that emerged relatively close to the Anguilliformes. The later two reports proved unequivocally that three different GnRH structures are present in the brain of some early-evolved teleost species. Three forms of GnRH in the brain of a single species also has been confirmed by primary structure in tilapia, *Oreochromis niloticus* (Weber *et al.*, 1997) and by a combination of primary structure and/or cDNA sequence in cichlid *Haplochromis burtoni* (Powell *et al.*, 1995; White *et al.*, 1995) and gilthead sea bream, *Sparus aurata* (Powell *et al.*, 1994; Gothilf *et al.*, 1995); these three species belong to one of the most recently evolved orders of teleosts, the Percifomes. Therefore, the presence of three forms of GnRH in the brain of a single species has been confirmed in fish representing both early-evolved and recently-evolved orders of teleosts.

In this thesis, I examined the brain of whitefish, platyfish, green swordtail and sablefish, partly because they are from orders of teleosts that are widely separated by evolutionary time. Therefore, these species might be useful in answering the question of whether three forms or two forms of GnRH in the brain of a single species is the ancestral condition in teleosts. The additional reasons why these species were chosen for this study are included below.

The Rocky Mountain whitefish, *Prosopium williamsoni,* is a freshwater salmonid native to northern British Columbia. Whitefish are members of the Coregoninae, one of the earliest subfamilies to evolve within the Salmonidae family of fish. The other two subfamilies, Thymallinae (which includes graylings) and Salmoninae (which includes

chars, trouts and salmon) are thought to have evolved later and include somewhat more specialized fish. The brain of a species of Thymallinae and the brain and pituitary of different species of Salmoninae have been previously examined for GnRH-like immunoreactivity. However, there have been no previous reports of GnRH in species of Coregoninae. Fish species from each of the subfamilies of salmonids live in northwestern North America, making them accessible to study closely related species in a single family .

Platyfish, *Xiphophorus maculatus* and green swordtails, *Xiphophorus hellerei*, are members of the Cyprinodontiformes order of teleost fish, which also includes guppies, mollies, mummichogs and medaka. Cyprinodont fish are among the most widely used fish in laboratory studies of genetics, behavior, toxicology and reproduction; this is largely due to the relative ease with which they can be maintained in breeding aquaria and their rapid development (Parenti, 1994). Some of the important biological advances that were made using these fish as models include the first demonstration of: 1) a direct neuroanatomical connection between GnRH systems in the brain and pituitary gland in teleost fish (Schreibman *et al.*, 1979), 2) immunoreactive (ir) GnRH-containing cells in the nucleus olfactoretinalis of fish, now named the terminal nerve ganglion (Münz *et al.*, 1981), 3) a nucleus of irGnRH-containing cells in the midbrain with axonal projections to optic tectum, cerebellum, and medulla oblongata (Münz *et al.*, 1981), and 4) a system of irGnRH-containing nerves and fibers between the brain mesencephalon and caudal spinal cord of fish (Miller and Kriebel, 1986). These latter observations were among the first to suggest that GnRH is not only a hypophysiotropic factor but is also involved in integrating visual, olfactory, and motor input with the central control of reproduction. Studies on platyfish species have been useful also in demonstrating that genetic switches may exist that are responsible for determining the age of sexual maturation (Kallman and Borkoski, 1978; Kallman and Schreibman, 1973) and more recently, suggesting that certain cancers may be heritable. It is surprising, considering

the important role of these fish as models (especially in terms of the central control of reproduction), that the molecular forms of GnRH have been examined in only three species, mollies (Coe *et al.*, 1990), mummichog (Knox and Sower, 1991), and medaka (Powell *et al.*, 1997). The diversity of reproductive strategies in fish within the order Cyprinodontiformes makes this an interesting taxon in which to search for diverse forms of GnRH within the brain.

The sablefish or black cod, *Anoploma fimbria*, is a member of the Scorpaeniformes order of teleosts; this taxon diverged from the teleost lineage close to the Perciformes order. Only one previous report has examined the brain of species from this order of fish for GnRH; Powell *et al.*, 1997 demonstrated by HPLC and RIA that the brain of grass rockfish contained three distinct irGnRH peaks identified as sbGnRH, cGnRH-II, and sGnRH based on chromatographic and immunological characteristics. Also, a previous report by Solar *et al.*, 1990 demonstrated that ovulation of sablefish could be induced following oral administration of a sGnRH analogue. It has been demonstrated previously that native forms of GnRH might be most effective in releasing gonadotropins and therefore, an additional directive of this study was to isolate the forms of GnRH present in the brain and pituitary of sablefish so that they might be used in future commercial applications.

The sablefish study was done in collaboration with Jim Powell at the University of Victoria who also collected the whitefish brains and pituitaries. Also, platyfish brains were collected by Dr. M. Schreibman at Brooklyn College, New York and skate brains were collected and dissected by Dr. L. Demski at the University of Florida.

Materials and methods

Details of the species and amount of tissue used, the extraction method, the HPLC programs for purification, the antiserum for RIA and the deduced forms of irGnRH are summarized in Table 3-1A and 3-1B.

Table 3-1A: **Skate HPLC and RIA summary**

Species	[1]Tissue (grams)	[2]GF-4 irGnRH elution peaks	[2]7CR-10 irGnRH elution peaks	Inferred forms of irGnRH
Skate				
(Raja canebensis)	olfactory bulb/ terminal nerve (1.94g)	33-34 min	24-26 min 33-34min	cGnRH-II dfGnRH
	telencephalon (1.82g)	30-31 min 33-35 min	25-27 min 33-35 min	cGnRH-II unknown dfGnRH
	midbrain (0.85g)	25-26 min 30-31 min 32-33 min	25-26 min 32-34 min	cGnRH-II* unknown dfGnRH*
	inferior lobe (0.27g)	24-26 min 29-31 min 33-34 min	26 min 34-35 min	cGnRH-II unknown* dfGnRH
	pituitary (0.03g)	25 min 32-33 min	32-33 min	cGnRH-II dfGnRH
	cerebellum (0.50g)	25-26 min 30 min 33-34 min	25-26 min 33-34 min	cGnRH-II unknown dfGnRH
	medulla (1.35g)	25-26 min 29-30 min 32-33 min	25-26 min 32-33 min	cGnRH-II unknown dfGnRH

[1] Each tissue was extracted with an acid-acetone mixture. The extracts were then loaded onto HPLC and eluted with a 17-24% TEAF program combined with acetonitrile.
[2] The RIAs of the HPLC fractions used either antiserum GF-4 (1: 25,000 final concentration) or antiserum 7CR-10 (1: 37,500 final concentration).
* The starred forms of GnRH indicate where the highest concentration of immunoreactive GnRH was detected.

Table 3-1B. **Teleost species HPLC and RIA summary**

Species	[1]Tissue (grams)	HPLC purification steps	[2]Antisera in RIA	Inferred forms of irGnRH
Rocky mountain whitefish *(Prosopium williamson)*	brains (38.05g)	SepPak TFA TEAF 17-24% TEAP TFA Vydac TFA	GF-4	sGnRH cGnRH-II novel
	pituitaries (0.45g)	TEAF 17-24% TEAP TFA Vydac TFA	GF-4 BLA-5	sGnRH novel
Platyfish *(Xiphophorus maculatus)*	brains + pituitaries (0.35g)	TEAF 17-24%	GF-4 BLA-5	cGnRH-II sGnRH novel
Green swordtail *(Xiphophorus hellerei)*	brains + pituitaries (12.00g)	SepPak TFA TEAF 17-24% TEAP TFA Vydac TFA	GF-4 BLA-5	cGnRH-II sGnRH novel
Sablefish *(Anoplopoma fimbria)*	brains + pituitaries (215.0g)	SepPak TFA TEAF 17-24% TEAP TFA Vydac TFA	GF-4	cGnRH-II sGnRH sbGnRH

[1] Each tissue was extracted with an acid: acetone mixture

[2] Antisera characteristics are summarized in Table 3-3.

Tissue collection

Skate, whitefish, sablefish and platyfish

Juvenile skates were collected in Florida. Later, their pituitaries and brains were removed separately and divided into six different parts at Dr. Leo Demski's laboratory, University of South Florida, Florida City. Male and female, non-spawning Rocky Mountain whitefish (n= 75) were captured near Fort St. John, B.C. and their brains and pituitaries removed. Male and female sablefish brains including pituitaries were collected by H. Kreiberg and D. Murie of the Pacific Biological Station, Nanaimo, B.C. Male and female platyfish brains including pituitaries (n= 35) were collected at Dr. Martin Schreibman's laboratory, Brooklyn College, New York. The brain parts, pituitaries and whole brains from the above species were quickly frozen, shipped on dry ice to the University of Victoria, Victoria, B.C., Canada and stored at -80C.

Green Swordtail

Male and female green swordtail fish (n= 1000) were grown at the Tropical Gardens Fish Farm, Florida and shipped to the University of Victoria. The original fish came from a pure wild stock collected in Princess Margaret Creek in Costa Rica. The fish were kept in ponds in Florida with no other *Xiphophorus* spp., ensuring that there was no interbreeding and that the swordtail stock remained pure. After anesthesia, the fish were decapitated and the brains including pituitaries were removed, quickly frozen on dry ice and stored at -80C.

Brain and/or pituitary extraction

The total frozen mass of the tissues extracted in this study are shown in Table 3-1A and 3-1B. Each tissue extract was processed separately using similar extraction techniques as reported previously (Ngamvongchon *et al.*, 1992) and described in Chapter 2.

Partial purification of GnRH-like substances

The extracts of skate brain and pituitary, whitefish pituitary and platyfish brain and pituitary were filtered through a low protein absorbing filter (45 μm, μCoStar LB ™, Keenebunk, ME) and then adjusted to pH 2.5 using 5N NaOH prior to their application onto the C-18 HPLC column.

For the brain extracts of whitefish, green swordtail and sablefish, an additional purification step was done prior to injection onto the C-18 HPLC column. These brain extracts were brought to pH 2.5 with 5N NaOH, pumped onto 10 SepPak C-18 cartridges, and then eluted, collected and assayed by RIA as described previously in this thesis for the purification of sturgeon GnRH.

A blank sample of 600μl of Milli-Q water was applied to the HPLC column between consecutive applications of the different tissue extracts. The blank was eluted and aliquots were assayed by RIA to ensure that there was no contaminating immunoreactive material from the previous HPLC runs. Aliquots of the tissue extracts were injected sequentially in a volume not more than 600 μl at 2 minute intervals for multiple injections onto the C-18 HPLC column. The HPLC programs used for the purification of each tissue extract are listed in Table 3-1 and described in detail in Table 3-2. It should be noted that the details of the HPLC programs used for purification of skate and teleost tissue extracts are reported in detail in this chapter because they are different than those used for the purification of sturgeon GnRH in Chapter 2.

Standards

After the HPLC and RIA analyses of skate and teleost brain extracts were complete, 150 ngs each of 11 synthetic GnRH forms: mammalian (m)GnRH, hydroxy-proline^{9} mammalian (hydroxy-Pro^{9})GnRH, seabream (sb)GnRH, chicken(c)GnRH-I, salmon (s)GnRH, chicken (c)GnRH-II, dogfish (df)GnRH, catfish (cf)GnRH, lamprey (l)GnRH-I, tunicate (t)GnRH-I and tGnRH-II were loaded onto the same HPLC column.

Table 3-2: **HPLC programs used for elution of fish extracts**

HPLC program	[4]Mobile Phase	Elution profile
[1]SepPak TFA	A= 0.05% TFA/ H_2O B= 0.05% TFA/ 80% CH_3CN	T= 0 min 5%B T= 55 min 80%B T= 60 min 80%B
[2]TEAF 17-24%	A= 0.25M TEAF/ H_2O B= CH_3CN	T= 0 min 17%B T= 10 min 17%B T= 17 min 24%B T= 60 min 24%B
[2]TEAP	A= 0.12M TEAP/ H_2O B= CH_3CN	T= 0 min 5%B T= 5 min 5%B T= 15 min 20%B T= 55 min 40%B T= 60 min 70%B
[2]TFA	A= 0.05% TFA/ H_2O B= 0.05% TFA/ 80% CH_3CN	T= 0 min 5%B T= 5 min 5%B T= 55 min 80%B T= 60 min 80%B
[3]Vydac TFA	A= 0.05% TFA/ H_2O	T= 0 min 5%B T= 5 min 5%B T= 15 min 20%B T= 55 min 60%B T= 60 min 80%B

[1] Column = 10 SepPak C-18 cartridges (Waters, Milford, MA) in which plastic connections were trimmed to remove dead space and remaining cartridges were held together in series by shrink wrap tubing.

[2] Column = Supelcosil LC-18 (25 cm x 4.6 mm x 5 μm particle size, Supelco, Bellefonte, PA)

[3] Column = Vydac diphenyl (25 cm x 4.6 mm x 5 μm particle size, Vydac, Hesperia, CA)

[4]TFA = trifluoroacetic acid

H_2O = Milli-Q water (Millipore, Bedford, MA)

TEAF = triethylammonium formate (brought to pH 6.5 by triethylamine)

TEAP = triethylammonium phosphate (brought to pH 6.5 by triethylamine)

CH_3CN = acetonitrile

The standards were eluted using TEAF 17-24%, an HPLC program identical to the one described in Table 3-2 and assayed using antiserum GF-4. The elution positions of the standards on the chromatograph were confirmed by absorbance peaks (A= 280 nm) and by GnRH-specific RIA. It should be noted that salmon GnRH elutes in two positions even though the primary structure does not change. It also should be noted that the elution positions of the standards in this chapter are different than those shown in Chapter 2 because a different HPLC program was compared to the one used in the purification of sturgeon GnRH.

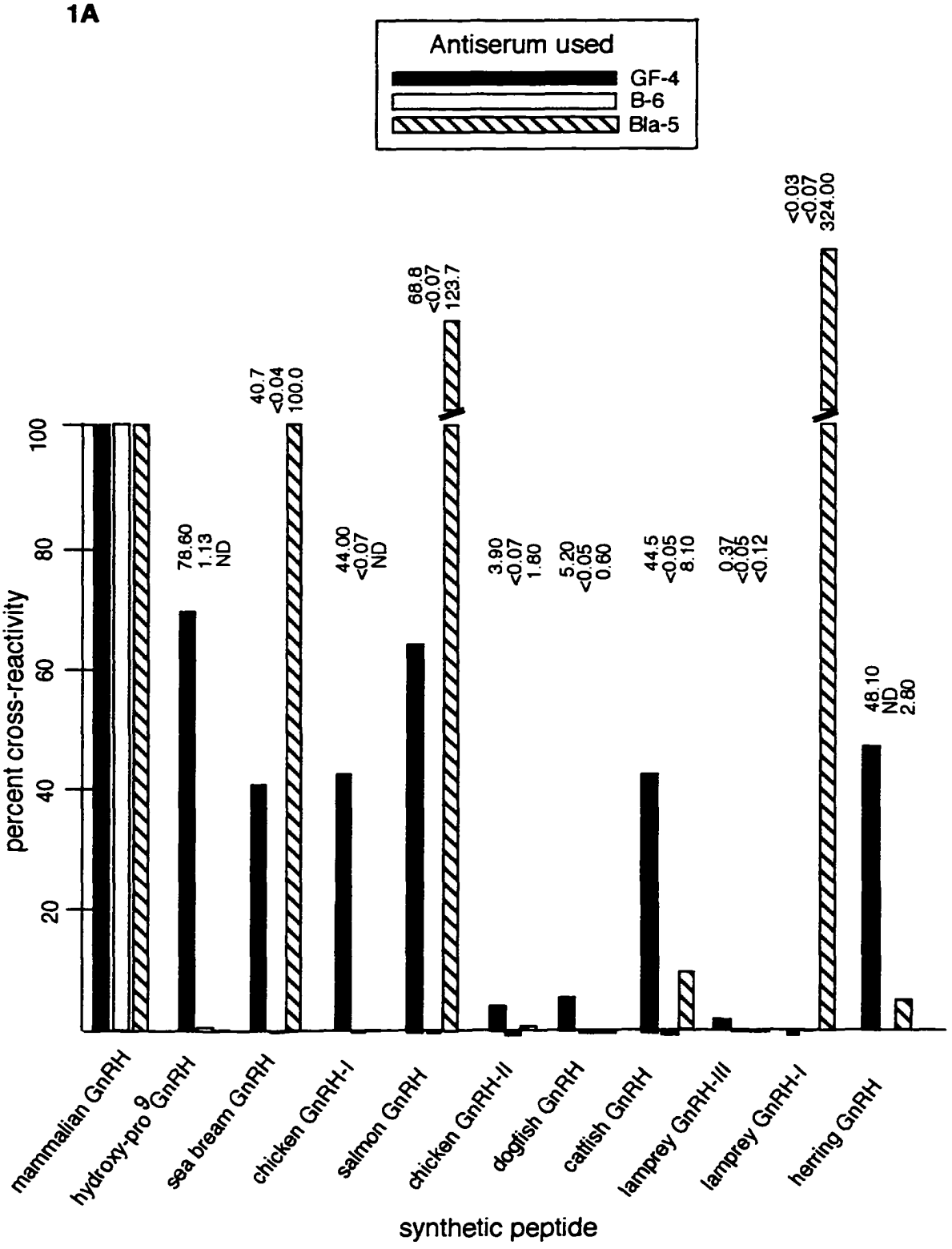
Cross-reactivity

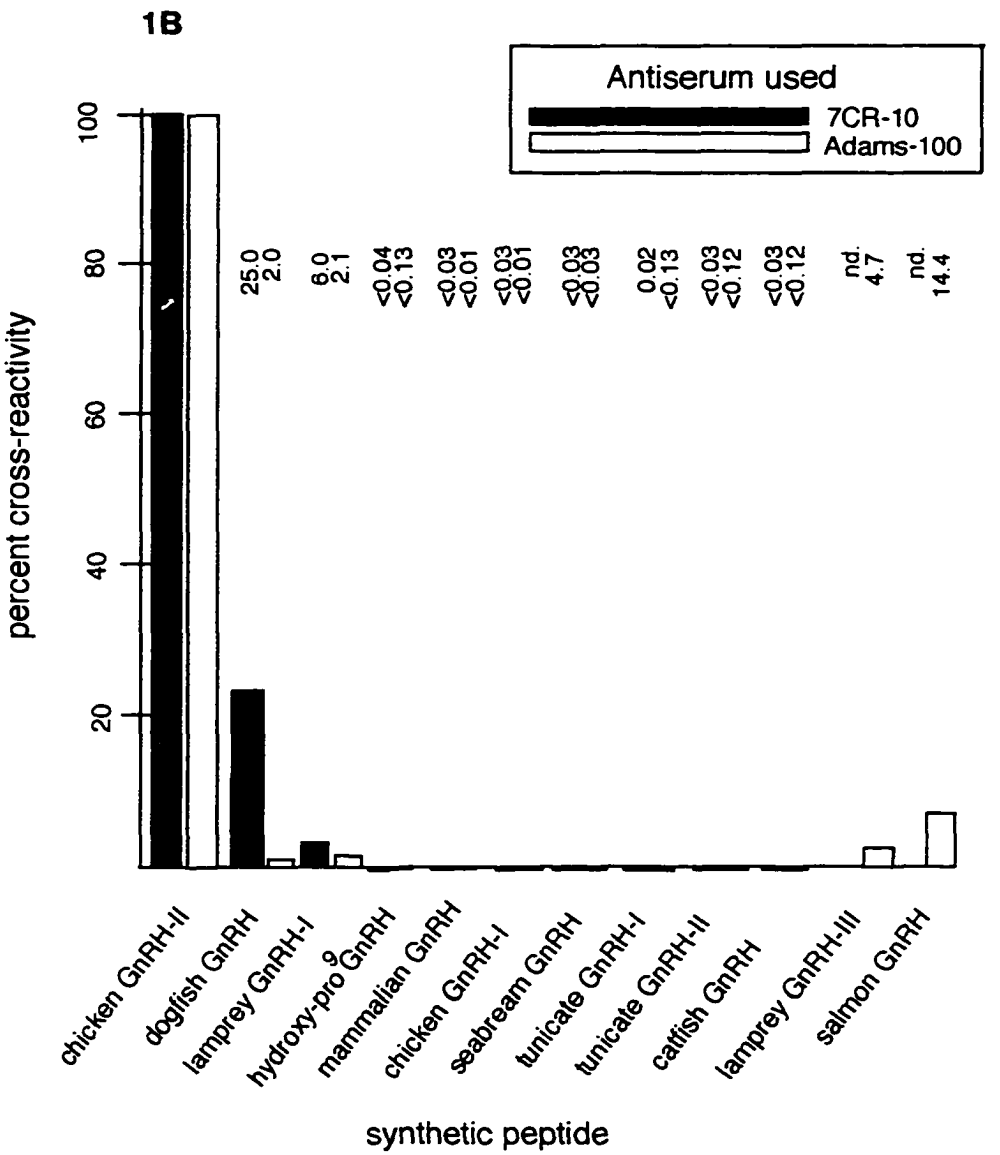
The cross-reactivity of antisera GF-4, B-6, Bla-5, 7CR-10 and Adams-100 with 11 synthetic GnRH peptides (mGnRH, hydroxy-Pro^{9}-mGnRH, sbGnRH, cGnRH-I, sGnRH, cGnRH-II, dfGnRH, cfGnRH, lGnRH-III, lGnRH-I and hGnRH) is shown in Fig. 3-1. Antisera GF-4, B-6, Bla-5 and 7CR-10 were raised in rabbits in our laboratory against salmon GnRH (GF-4), mammalian GnRH (B-6), lamprey GnRH-I (Bla-5) and dogfish GnRH (7CR-10) (Table 3-3). Antiserum Adams-100 (a gift from Dr. T. Adams, University of California, Davis) was raised in rabbits against cGnRH-II (Table 5-1). Adams-100 antiserum was not used until later in this thesis study but was included here for completeness.

Briefly, RIAs with GF-4, B-6 and Bla-5 (which cross-react with mGnRH) were homologous, using ^{125}I-labelled mGnRH tracer and mGnRH standard. Although antisera GF-4 and Bla-5 were raised in rabbits against sGnRH and lamprey (l) GnRH-I respectively, they both cross-react with a broad spectrum of GnRH forms as shown in Fig. 3-1A. The 7CR-10 RIA was heterologous using a ^{125}I-labelled l-GnRH-I trace and a cGnRH-II standard. The lGnRH was used as a tracer because is was considerably more stable and easier to iodinate than cGnRH-II. However, the Adams-100 RIA was homologous, using ^{125}I-labelled cGnRH-II tracer and cGnRH-II standard.

Fig. 3-1. Percent cross-reactivity between (A) antisera GF-4, B-6 or Bla -5 and (B) antisera 7CR-10 or Adams-100 with 11 different synthetic GnRH peptides. Mammalian GnRH was used as the reference peptide for antisera GF-4, B-6 and Bla-5, whereas cGnRH-II was used as the reference peptide for antisera 7CR-10 and Adams-100 in the calculation of percent cross-reactivity. Relative cross reactivity (%) is expressed as the number of picomoles of reference peptide at 50% B/B_0 divided by the number of picomoles of the other peptide at 50% B/B_0. ^{125}I-mGnRH was the tracer for the assays using antisera GF-4, B-6 and Bla-5. ^{125}I-lGnRH-I was the tracer for assays with 7CR-10, whereas ^{125}I-cGnRH-II was the tracer for assays with Adams-100. Final concentrations of GF-4, B-6, Bla-5, 7CR-10 and Adams-100 were 1: 25,000, 1: 5,000, 1: 5,000, 1: 37,500 and 1:25000, respectively.

1A


Antiserum used
GF-4
B-6
Bla-5
percent cross-reactivity
20
40
60
80
100
78.60
1.13
ND
40.7
<0.04
100.0
44.00
<0.07
ND
68.8
<0.07
123.7
3.90
<0.07
1.80
5.20
<0.05
0.60
44.5
<0.05
8.10
0.37
<0.05
<0.12
<0.03
<0.07
324.00
48.10
ND
2.80
mammalian GnRH
hydroxy-pro 9GnRH
sea bream GnRH
chicken GnRH-I
salmon GnRH
chicken GnRH-II
dogfish GnRH
catfish GnRH
lamprey GnRH-III
lamprey GnRH-I
herring GnRH
synthetic peptide


1B
Antiserum used
7CR-10
Adams-100
percent cross-reactivity
100
80
60
40
20
25.0
2.0
6.0
2.1
<0.04
<0.13
<0.03
<0.01
<0.03
<0.01
<0.03
<0.03
0.02
<0.13
<0.03
<0.12
<0.03
<0.12
nd.
4.7
nd.
14.4
chicken GnRH-II
dogfish GnRH
lamprey GnRH-I
hydroxy-pro9GnRH
mammalian GnRH
chicken GnRH-I
seabream GnRH
tunicate GnRH-I
tunicate GnRH-II
catfish GnRH
lamprey GnRH-III
salmon GnRH
synthetic peptide

Five concentrations of each synthetic peptide (100, 500, 1000, 5000 and 10000 pg) were tested in triplicate for their ability to displace the tracer from the respective antiserum. Relative cross-reactivity is expressed as picomoles of the reference peptide at 50% B/Bo divided by picomoles of the test peptide at 50% B/Bo multiplied by 100.

RIA

The HPLC fractions were assayed for GnRH immunoreactivity as reported previously (Sherwood *et al.*, 1986) and described in Chapter 2. Essentially, 25-100 μl aliquots of each semi-pure fraction from the HPLC run were assayed for GnRH immunoreactivity. Antisera characteristics are described in Table 3-3. In HPLC fractions where tracer binding exceeded detection limits, aliquots were diluted serially and the value closest to 50% B/Bo, the most sensitive portion of the curve was used to estimate the quantity of irGnRH present.

Results

Cross-reactivity of antisera

Characteristics of the five antisera used in this chapter are shown in Table 3-3. The cross reactivity with 11 different GnRH peptides is shown for antisera GF-4, B-6 and Bla-5 in Fig. 3-1A and for antisera 7CR-10 and Adams-100 in Fig. 3-1B. It is clear that antiserum B-6 is specific for mGnRH (Fig 3-1A); antisera Adams-100 and 7CR-10 are nearly specific for cGnRH-II (Fig. 3-1B); and antiserum GF-4 cross-reacts with many forms of GnRH such as mGnRH, hydroxy-Pro^9-mGnRH , sbGnRH, cGnRH-I, sGnRH, cGnRH-II, dfGnRH, cfGnRH and lGnRH-III. Antiserum Bla-5 also cross-reacts in varying degrees with different GnRHs but is most useful in detecting lGnRH-I because none of the other antisera cross-react significantly with this form of GnRH. These five antisera detect, in varying degrees, a wide range (n =9) of GnRH molecules that have previously been reported for fish.

Table 3-3: Antisera characteristics

[1] Antisera	Raised against	Final concentration	Percent binding	Limits of Detection (B/Bo=80 %)
GF-4	sGnRH	1:25,000	21.9	3.20 pg
Bla-5	lGnRH-I	1: 5,000	19.7	2.30 pg
B-6	mGnRH	1: 5,000	55.2	7.81 pg
7CR-10	dfGnRH	1:37,500	12.5	21.37 pg
Adams-100	cGnRH-II	1:25,000	7.2	1.10 pg

[1] In the RIAs for antisera GF-4, Bla-5 and B-6, ^{125}I-labelled mGnRH tracer with a mGnRH standard was used. In the RIA for antiserum 7CR-10, ^{125}I-labelled lGnRH-I tracer and cGnRH-II standard were used, whereas in the RIA with Adams-100, ^{125}I-labelled cGnRH-II tracer and a cGnRH-II standard were used.

Standards

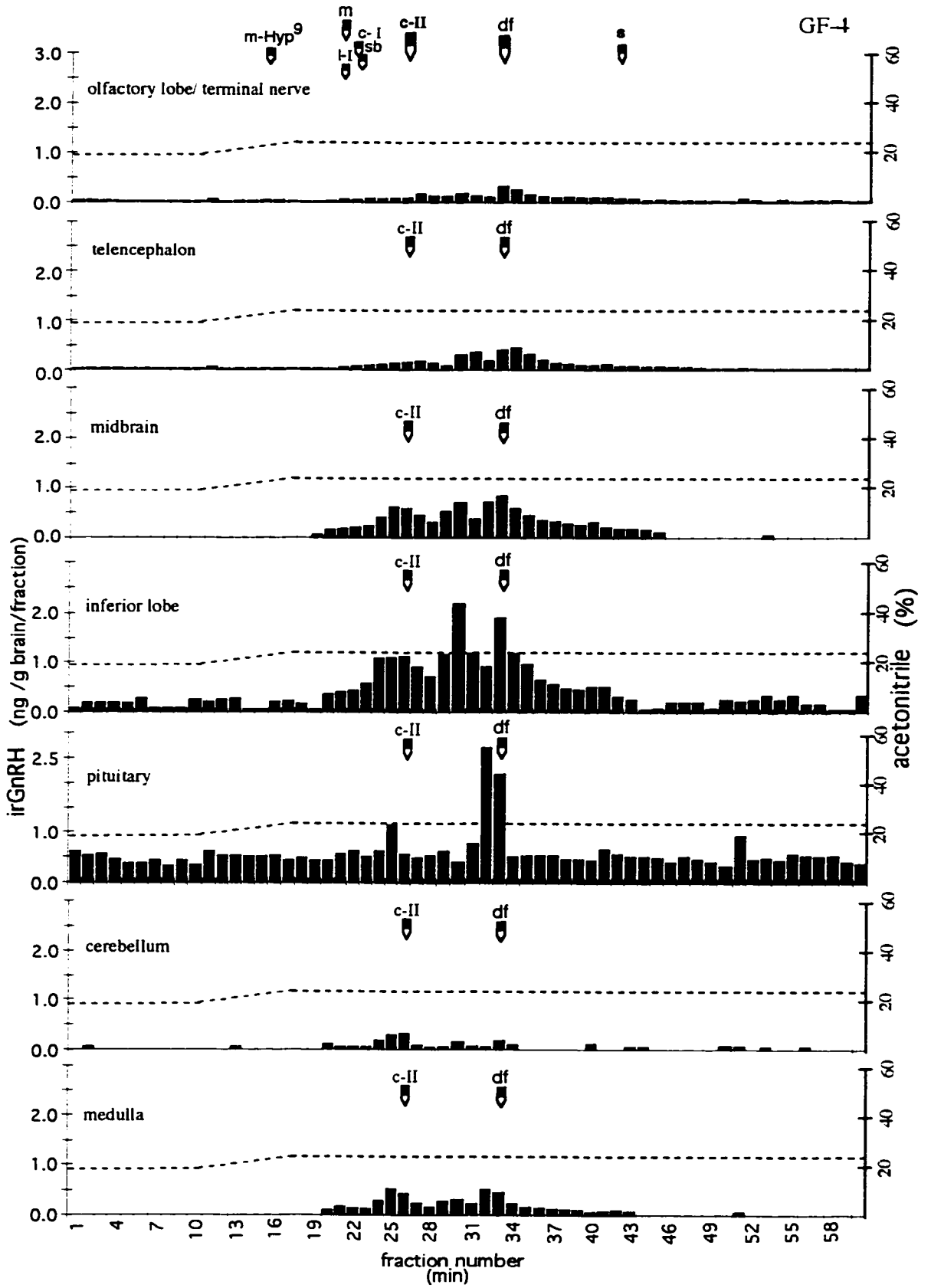
The elution position from HPLC of the synthetic peptide standards was detected first by an absorbance peak (280 nm) and then by RIA. For the standard peptides used for fish studies, an absorbance peak occurred at 21 min for mGnRH, 26 min for cGnRH-II, 33 min for dfGnRH and at 42-48 min for sGnRH. These absorbance peaks paralleled the immunoreactive peaks of synthetic peptides in fractions 19-20, 26, 33-34, and 41-42 as detected by antiserum GF-4 (Fig. 3-2 and 3-3; shown by the darkened arrows above the HPLC elution profile) and by antiserum Bla-5 (Fig. 3-5, 3-6B, 3-7B). This isocratic HPLC program has been used often in our laboratory with many different fish brain extracts and has proven useful in definitively separating both native and synthetic forms of teleost GnRHs: hGnRH (17min), cfGnRH (18min), mGnRH (21 min), lGnRH-I (21.1min), sbGnRH (23min), cGnRH-II (26min) dfGnRH (34min), and sGnRH (42 and 47-49min) (Fig. 3-3). These elution times are very consistent, within 1min, as long as the same type of column and HPLC system are used. Only one synthetic peptide, sGnRH, has a variable time of elution using this method, but it always elutes at least eight min later than dfGnRH, the closest eluting synthetic GnRH.

Skate brain regions

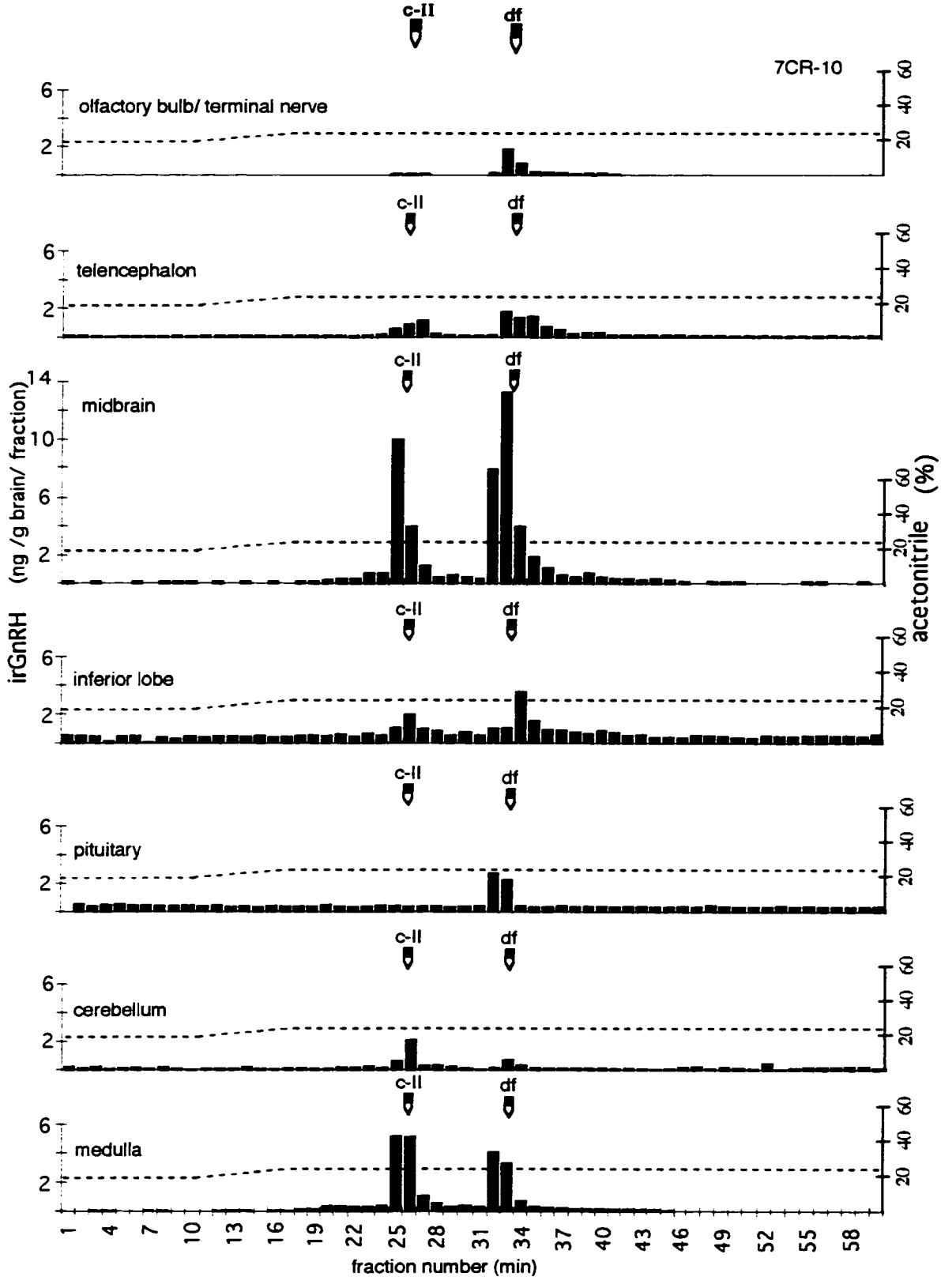
Antiserum GF-4 detected GnRH immunoreactivity in the 7 brain regions of the skate (Fig. 3-2A). The highest overall concentration of GnRH immunoreactivity was present in the inferior lobe, whereas the highest peak of irGnRH, 5.1 ng, was present in the pituitary (Fig. 3-2A). There were 3 irGnRH peaks detected by this antiserum; these peaks eluted at similar positons with cgnRH-II and dfGnRH or in the novel position of 29-30 min. In contrast, antiserum 7CR-10 detected two separate peaks of irGnRH in 5 skate regions and one peak in the pituitaries, olfactory bulbs/ terminal nerve. One peak was in HPLC fractions 25-26 and the other was in HPLC fractions 32-33 (Fig. 3-2).

Fig. 3-2. Immunoreactive GnRH in different regions of skate brain and pituitary after HPLC elution. The GnRH in each fraction (min) was detected by (A) antiserum GF-4 and (B) antiserum 7CR-10 following elution of the tissue extracts from the C-18 column using the TEAF 17-24% HPLC program. Small arrows above the HPLC elution profile represent the elution position of synthetic peptides under similar HPLC conditions. Dashed line indicates the percent acetonitrile used in elution.

A.


m-Hyp9
m
c-I
sb
I-I
c-II
df
s
GF-4
olfactory lobe/ terminal nerve
telencephalon
midbrain
inferior lobe
pituitary
cerebellum
medulla
irGnRH (ng /g brain/fraction)
acetonitrile (%)
fraction number (min)

B.

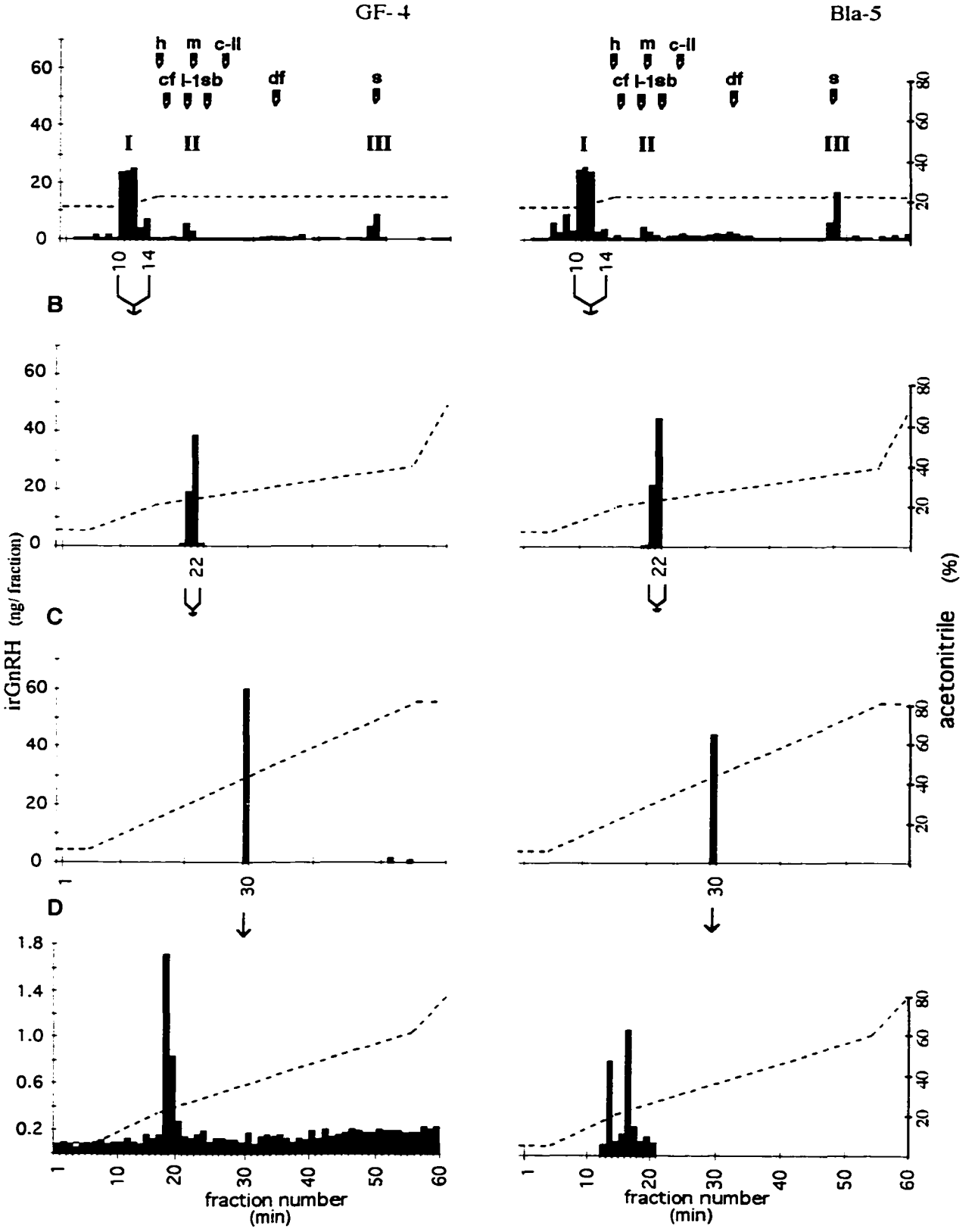

c-II
df
7CR-10
olfactory bulb/ terminal nerve
telencephalon
midbrain
inferior lobe
pituitary
cerebellum
medulla
irGnRH (ng /g brain/ fraction)
acetonitrile (%)
fraction number (min)

There was a difference in the concentration of GnRH depending on the brain region analyzed (Fig. 3-2B), with the highest concentration of irGnRH in both peaks in the midbrain (31.3ng) and the least in early peak in the olfactory bulb/ terminal nerve (3.4ng) and in the late peak in the cerebellum (2.7ng). Also, there was variability in the amount of each peak detected by antiserum 7CR-10. The pituitary and olfactory bulb/ terminal nerve regions were distinct from other tissue parts in that there was little or no irGnRH in the early peak (fractions 25-26) but detectable amounts in the late peak of 5.7 ng for the pituitary and 3.1 ng for the olfactory bulb/ terminal nerve (Fig. 3-2B). There was a pattern to the relative abundance of each irGnRH peak detected by antiserum 7CR-10, with more immunoreactivity in the late peak than in the early peak in the olfactory bulb/ terminal nerve, telencephalon and midbrain, but the opposite ratio in the cerebellum and medulla (Fig. 3-2B). Using antiserum B-7, I did not detect irGnRH in or near the HPLC elution position expected for mGnRH, except for a small peak, 0.08 ng/ g brain/ fraction in medulla (data not shown).

Whitefish pituitary

Using both antisera GF-4 and Bla-5, I detected three immunoreactive peaks in whitefish pituitary extracts: peak I, fractions 10-14; peak II, fractions 20-21 and peak III, fractions 48-49. The quantity of irGnRH detected in these three peaks was 65.7 ng, 8.2 ng, 13.2 ng by antiserum GF-4 and 78.0, 6.6 and 21.5 ng by antiserum Bla-5 (Fig. 3-3A). Peak I was purified further and irGnRH was detected with GF-4 in fractions 22, 30 and 19-20 after elution using the HPLC programs TEAP, TFA and VydacTFA, respectively (Fig. 3-3B to 3-3D); irGnRH was detected with antiserum Bla-5 in fractions 22, 30, and 14/ 17, respectively.

Fig. 3-3. Immunoreactive GnRH in whitefish pituitary extracts after HPLC elution. The amount of irGnRH is shown on the left and the fraction number below. The results with antiserum GF-4 are shown in the left figures and with antiserum Bla-5 in the right figures. Small arrows above the HPLC elution profile represent where synthetic peptides elute under similar HPLC conditions. The dashed line represents the percent acetonitrile used in elution. The pattern of irGnRH is shown after elution with (A) TEAF 17-24% HPLC program, (B) TEAP HPLC program, (C) TFA HPLC program and (D) Vydac TFA HPLC program from a Vydac diphenyl column.


A
GF-4
Bla-5
h
m
c-II
cf
I-1sb
df
s
I
II
III
10
14
B
22
C
1
30
D
irGnRH (ng/fraction)
acetonitrile (%)
fraction number
(min)

The chromatographic and immunological properties of peak III suggest that it represents a sGnRH-like peptide. This suggestion is based on the evidence that both antisera cross-react with sGnRH and also that antiserum Bla-5 detects more irGnRH in peak III than antiserum GF-4 (Fig. 3-3A), as can be expected by their relative cross-reactivity with sGnRH, 123.7% and 68.8% for Bla-5 and GF-4, respectively. Also, over a period of ten years we have found that sGnRH, the most hydrophobic of the 12 known forms of GnRH, is detected in fractions 42-55 after elution from the same type of column and HPLC system. There are no other known GnRH forms that elute this late under these HPLC conditions.

The identity of peak II is more difficult to establish because lGnRH-I, sbGnRH and mGnRH elute close together under similar HPLC conditions. Nevertheless, GF-4 does not cross-react with lGnRH-I and, therefore, detection of irGnRH in fractions 20-21 by antiserum GF-4 (Fig. 3-3A) suggests that lGnRH-I is not present. Furthermore, there have been no previous reports of lGnRH-I in teleost fish, except in one HPLC and RIA study in the pituitary of platyfish (Magliulo-Cepriano *et al.*, 1994) where proper controls were not done. If peak II were sbGnRH, relatively more irGnRH would be detected by antiserum Bla-5 than by antiserum GF-4 because of the relative differences in their cross-reactivity with synthetic sbGnRH; 100% and 40.7%, respectively. However, antiserum GF-4 detects more irGnRH, 8.2 ng, than antiserum Bla-5, 6.6 ng, in the HPLC elution position expected for sbGnRH (Fig. 3-3A) suggesting that sbGnRH is not present. Peak II also is not mGnRH because no immunoreactivity was detected using B-7, our mGnRH specific antiserum (data not shown). Therefore, it is possible that peak II is a novel form of GnRH.

The identity of peak I also is difficult to determine without first characterizing the primary structure. The immunoreactive peaks detected after elution using HPLC programs TEAF 17-24% and TEAP are in similar positions as peak I in the purification of GnRH from the pituitary of Pacific herring (Carolsfeld *et al.*, 1997) but they are

chromatographically distinct in the next two purification steps. This suggests that although peak I in the whitefish pituitary has hGnRH-like properties, it represents a different form of GnRH. Also, it is noted that the final Vydac TFA program resulted in decreased yield to approximately 4% as detected by antiserum GF-4 (Fig. 3-3D).

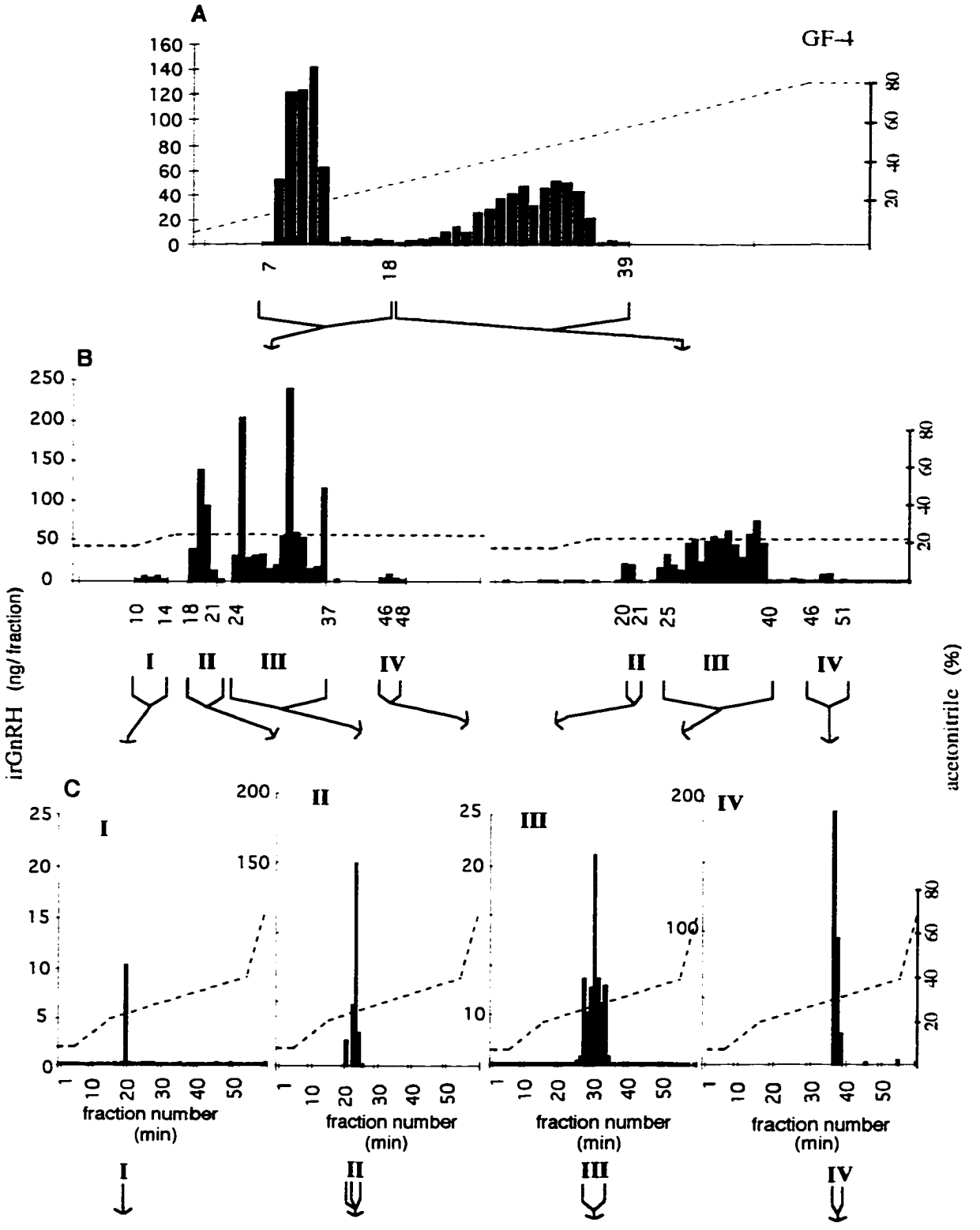
Whitefish Brains

Using antiserum GF-4, I detected 523.6 ng of GnRH-like immunoreactivity in fractions 8-12 and 471.7ng of irGnRH in fractions 25-35 after elution of whitefish brain extracts from a SepPak column (Fig. 3-4A). Each of these irGnRH peaks was combined and purified further by HPLC using a C-18 column and elution with the TEAF 17-24% program. Four peaks of irGnRH then were detected using antiserum GF-4 after fractions 7-18 were eluted and a number of peaks after fractions 19-39 were eluted (Fig. 3-4B). The irGnRH peaks were combined as shown and treated separately in further purification steps (Fig. 3-4B to 3-4E). In the final Vydac TFA purification step (Fig. 3-4E), four distinct peaks of irGnRH were present: whitefish (wf) I at 24 min, wf II at 27 min, wf III at 34 min and wf IV at 36 min (Fig. 3-4E). The largest amount of irGnRH was in peak IV in the final elution: wf I (12.6 ng), wf II (121.9 ng), wf III (17.6ng) and wf IV (240.6 ng). The HPLC elution characteristics of the immunoreactive peaks in each step of purification suggest that wf I is sbGnRH-like, wf II is cGnRH-II-like, and wf III is sGnRH-like. However, wf IV elutes at a distinct positon compared to other GnRHs purified in our laboratory and may be a novel form.

Platyfish

For platyfish brain extracts, there were three irGnRH peaks detected by antiserum Bla-5 after elution from the HPLC column: Peak I, fractions 25-27; Peak II, fractions 30-32 and Peak III, fractions 45-48 (Fig. 3-4).

Fig. 3-4. Immunoreactive GnRH in whitefish brain extracts after HPLC elution. Antsierum GF-4 was used for detection of irGnRH. The dashed line represents the percent acetonitrile used in elution The pattern of irGnRH after elution from (A) SepPak column, (B) C-18 column using the TEAF 17-24% HPLC program, (C) the TEAP HPLC program, (D) the TFA HPLC program and (E) from the Vydac diphenyl column using the Vydac TFA HPLC program.


A
GF-4
160
140
120
100
80
60
40
20
0
80
60
40
20
7
18
39
B
250
200
150
100
50
0
irGnRH (ng/ fraction)
acetonitrile (%)
10 14 18 21 24 37 46 48
20 21 25 40 46 51
I II III IV
II III IV
C
I
II
III
IV
200
150
100
25
20
15
10
5
0
1 10 20 30 40 50
fraction number (min)

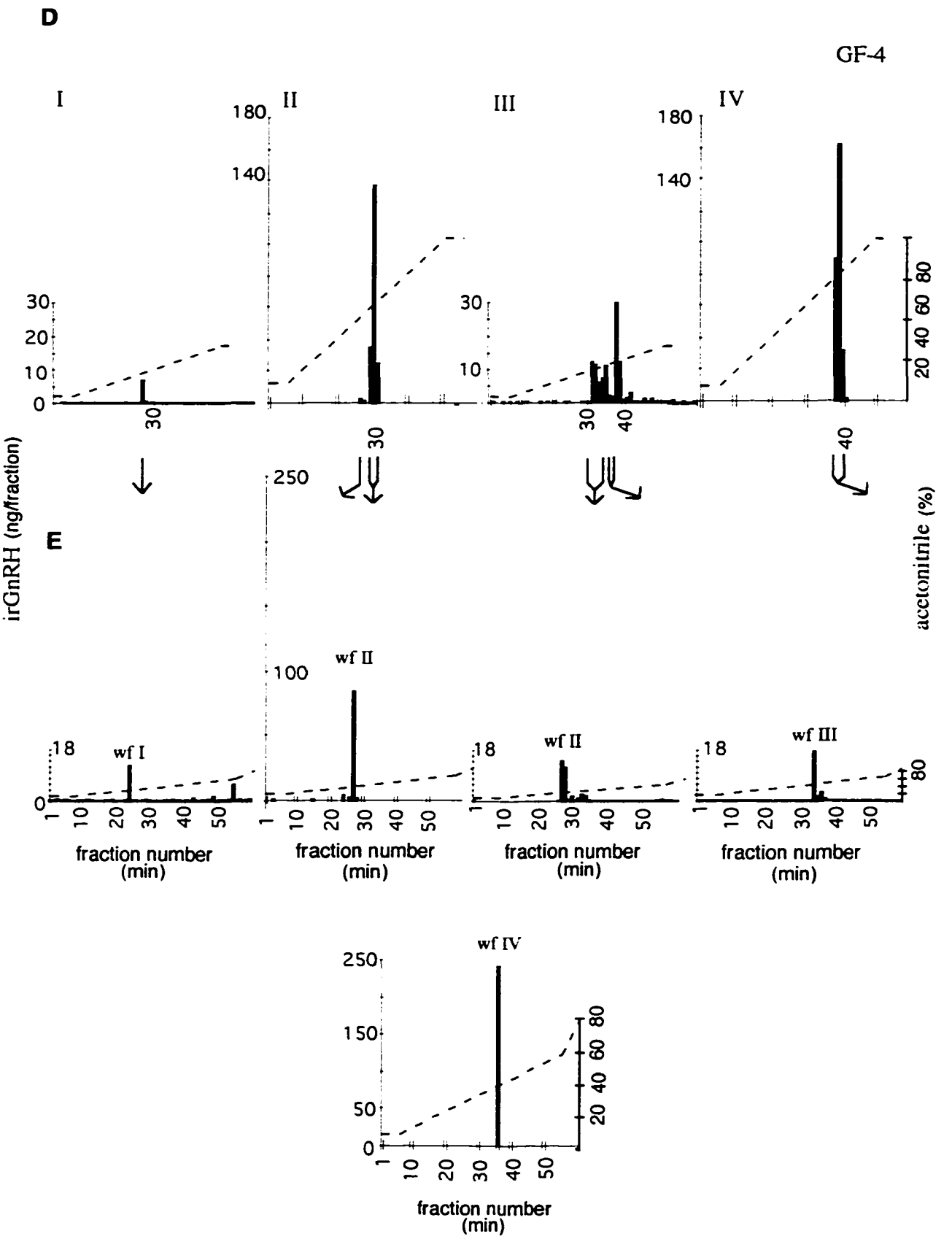
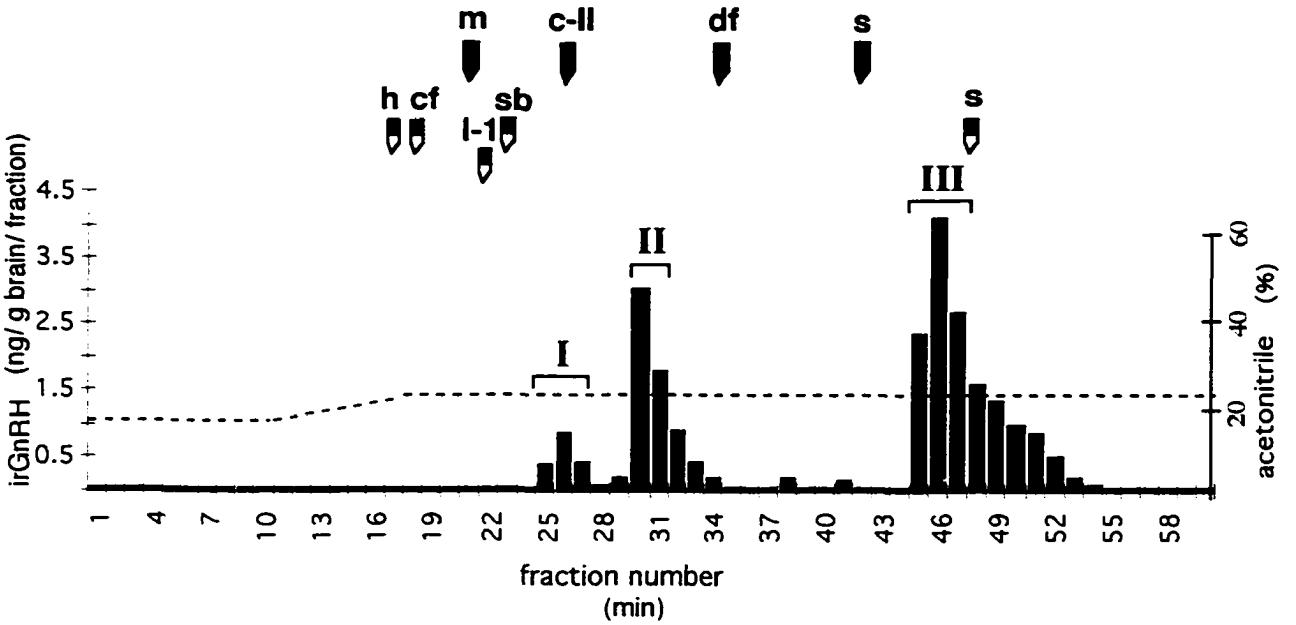

D
GF-4
I
II
III
IV
irGnRH (ng/fraction)
acetonitrile (%)
E
wf I
wf II
wf III
wf IV
fraction number (min)

Fig. 3-5. Immunoreactive GnRH in platyfish brain extracts after HPLC elution. Small darkened arrows above the HPLC elution profile represent the elution position for synthetic GnRH standards. Small, two-toned arrows above the HPLC elution profile represent the elution position of other synthetic GnRH standards tested previously under the same HPLC conditions. Note that salmon GnRH can elute in two positions even though the primary structure does not change. Dashed line represents the percent acetonitrile used for elution.

Bla-5


m
c-II
df
s
h cf
sb
I-1
s
III
II
I
irGnRH (ng/ g brain/ fraction)
4.5
3.5
2.5
1.5
0.5
60
40
20
acetonitrile (%)
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
fraction number
(min)

Green Swordtail

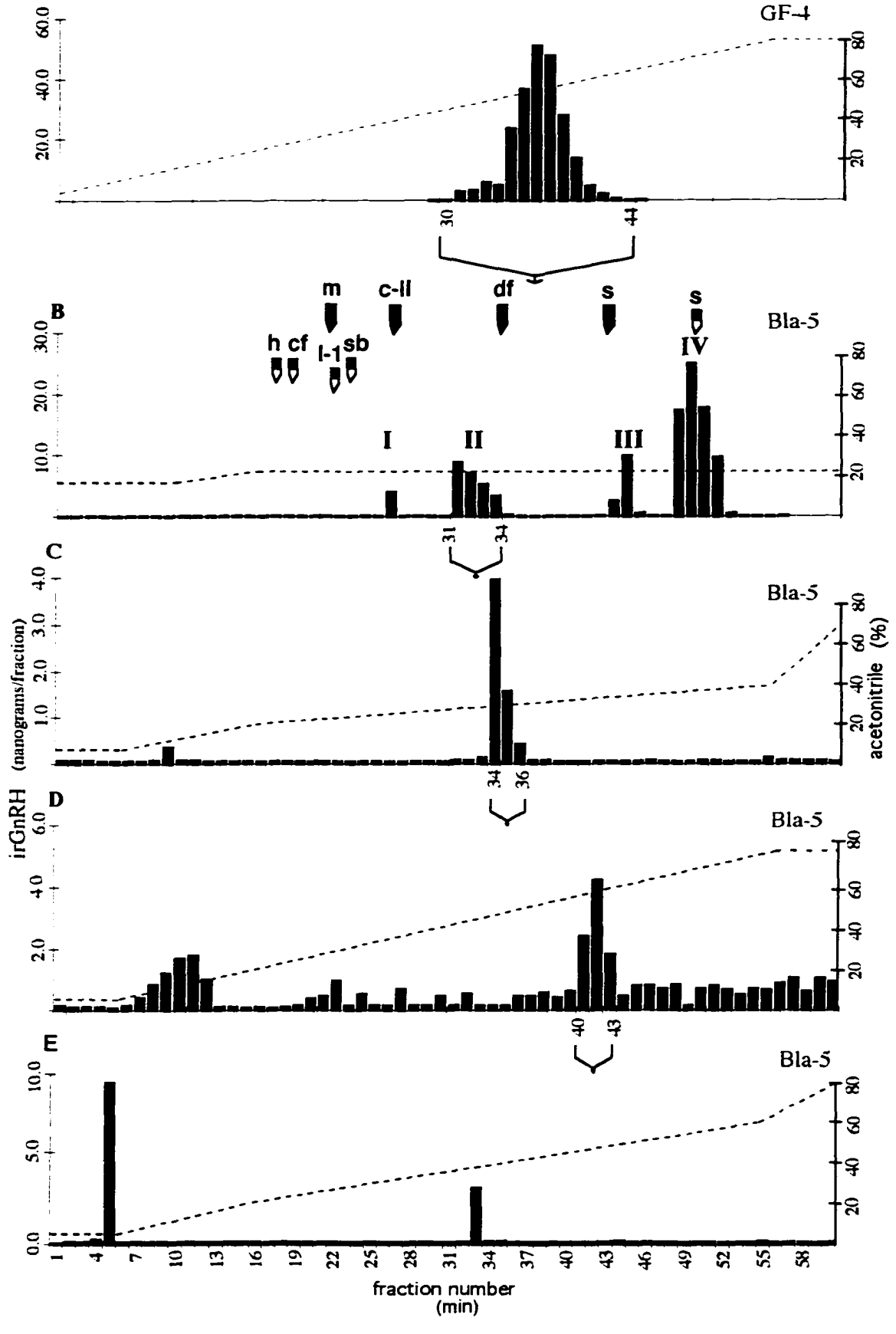
For green swordtail brain extracts, there was 228.5 ng of irGnRH-like material detected by antiserum GF-4 in fractions 30-44 following elution from the SepPak column (Fig. 3-6A). Four distinct peaks of immunoreactivity were detected by antiserum Bla-5 after elution using the TEAF 17-24% HPLC program: Peak I, fraction 26; Peak II, fractions 31-34; Peak III, fractions 43 and 44 and Peak IV, fractions 48-51 (Fig. 3-6B). The immunoreactive fractions from Peak II were further purified because of their novel position; TEAP, TFA , and Vydac TFA HPLC programs were used (Fig. 3-6C to 3-6E). In the final step of HPLC purification, irGnRH peaks were detected by antiserum Bla-5 in HPLC fraction 5 (9.7ng) and in fraction 33 (3.6ng), (Fig. 3-6E).

Platyfish and Green Swordtail

The elution position of irPeak I in both platyfish and green swordtail brain extracts is the same as the elution position of synthetic cGnRH-II under identical HPLC conditions, (Fig. 3-5 and Fig. 3-6B), suggesting that cGnRH-II is present. Detection of cGnRH-II-like immunoreactivity in the present study in small amounts, 0.35 ng total in the platyfish brains and 4.15 ng total in green swordtail brains, is valid in that the level of detection was 0.003 ng for antiserum GF-4 and 0.002ng for antiserum Bla-5. The low levels reflect that the antisera had low cross-reactivity with cGnRH-II, 3.90% with GF-4 and 1.80% with Bla-5, respectively (Fig. 3-1A).

GnRH immunoreactivity in HPLC fractions 45-48 and 48-51 in platyfish brain and swordfish brain extracts, respectively (Fig. 3-5 and Fig. 3-6B), suggests the presence of sGnRH. This evidence is based on the cross-reactivity of antisera Bla-5 and GF-4 with sGnRH, 123.7% and 68.8%, respectively. Also, sGnRH is the only known GnRH form to have this late of an elution time, greater than 42 min, under these HPLC conditions.

Fig. 3-6. Immunoreactive GnRH in green swordtail brain extracts after HPLC elution. The antisera were GF-4 and Bla-5. Small darkened arrows above the HPLC elution profile show where synthetic GnRH standards used in this study eluted. Small, two-toned arrows show where other GnRH standards eluted under the same HPLC conditions in our earlier studies. The dashed line represents the percent acetonitrile used for elution. abbreviations: m = mammalian GnRH, c-II = cGnRH-II, df= dogfish GnRH, s= sGnRH, h= herring GnRH, cf= catfish GnRH, l-I= lamprey GnRH-I, sb= seabream GnRH. The pattern of irGnRH detected by (A) antiserum GF-4 after elution from the SepPak column, (B) antiserum Bla-5 after elution from the C-18 column using using the TEAF 17-24% HPLC program, (C) antiserum Bla-5 after elution using the TEAP HPLC program, (D) antiserum Bla-5 after elution using the TFA HPLC program and (E) antiserum Bla-5 after elution from the Vydac diphenyl column using the Vydac TFA HPLC program.


GF-4
B
Bla-5
m
c-II
df
s
s
h cf
l-1
sb
IV
I
II
III
C
Bla-5
(nanograms/fraction)
acetonitrile (%)
irGnRH
D
Bla-5
E
Bla-5
fraction number
(min)

Immunoreactive GnRH in Peak III, fractions 43 and 44, of green swordtail brain extracts (Fig. 3-6B) most likely represents a shift in sGnRH even though the primary structure does not change, as has been demonstrated previously in our laboratory using the chemical sequence and mass spectrometry of extracts from various salmon (Powell, unpublished observations). The relatively wide peaks of sGnRH-like immunoreactivity are commonly observed in material that elutes late from the HPLC column.

The immunoreactive GnRH-like material detected by antisera Bla-5 and GF-4, in Peak II, HPLC fractions 30-32, in platyfish brain extracts and 31-34 in green swordtail brain extracts (Fig. 3-5 and Fig. 3-6B), is likely to be a novel form of GnRH and will be referred to as swordtail GnRH in the remainder of this chapter. However, it is noted that unknown irGnRH also elutes in this position in skate and whitefish. This immunoreactivity is unlike the other forms of GnRH isolated from teleost brain because of the elution position on the TEAF 17-24% HPLC program. This isocratic HPLC program has been used often in our laboratory with many different fish brain extracts and has proven useful in separating many native and synthetic forms of teleost GnRHs at times between 17 and 49 min (see page 90) (Fig. 3-3). These elution times are very consistent, within 1 min, as long as the same type of column and HPLC system is used. Only one synthetic peptide, sGnRH, has had a variable time of elution using this method, but it always elutes at least eight min later than dfGnRH, the closest eluting synthetic GnRH.

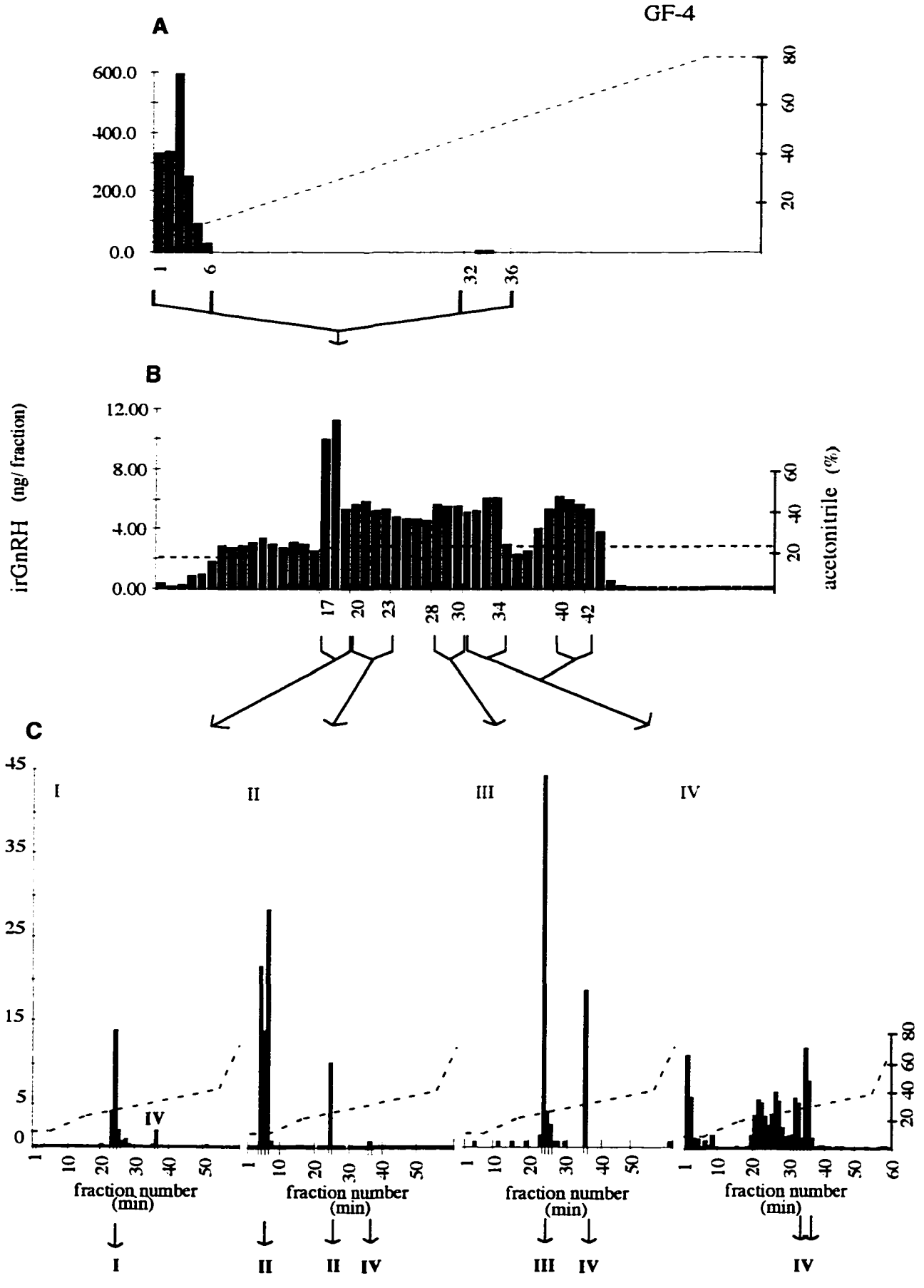
The amount of GnRH in the brain of all species in this chapter is not an absolute amount because of the differing cross-reactivities of the antisera. From platyfish brains, the amounts of irGnRH per gram of brain detected by antiserum GF-4 were 14.00 ng (sGnRH), 6.00 ng (swordtail GnRH) and 1.71 ng (cGnRH-II) per gram brain, (Fig. 3-5). In swordtail brain extracts the amounts of irGnRH detected per gram of brain by antiserum Bla-5 were 5.83 ng (sGnRH), 2.08 ng (swordtail GnRH), and 0.35 ng (cGnRH-II) (Fig. 3-6B). Therefore, there was a higher concentration of each of the respective

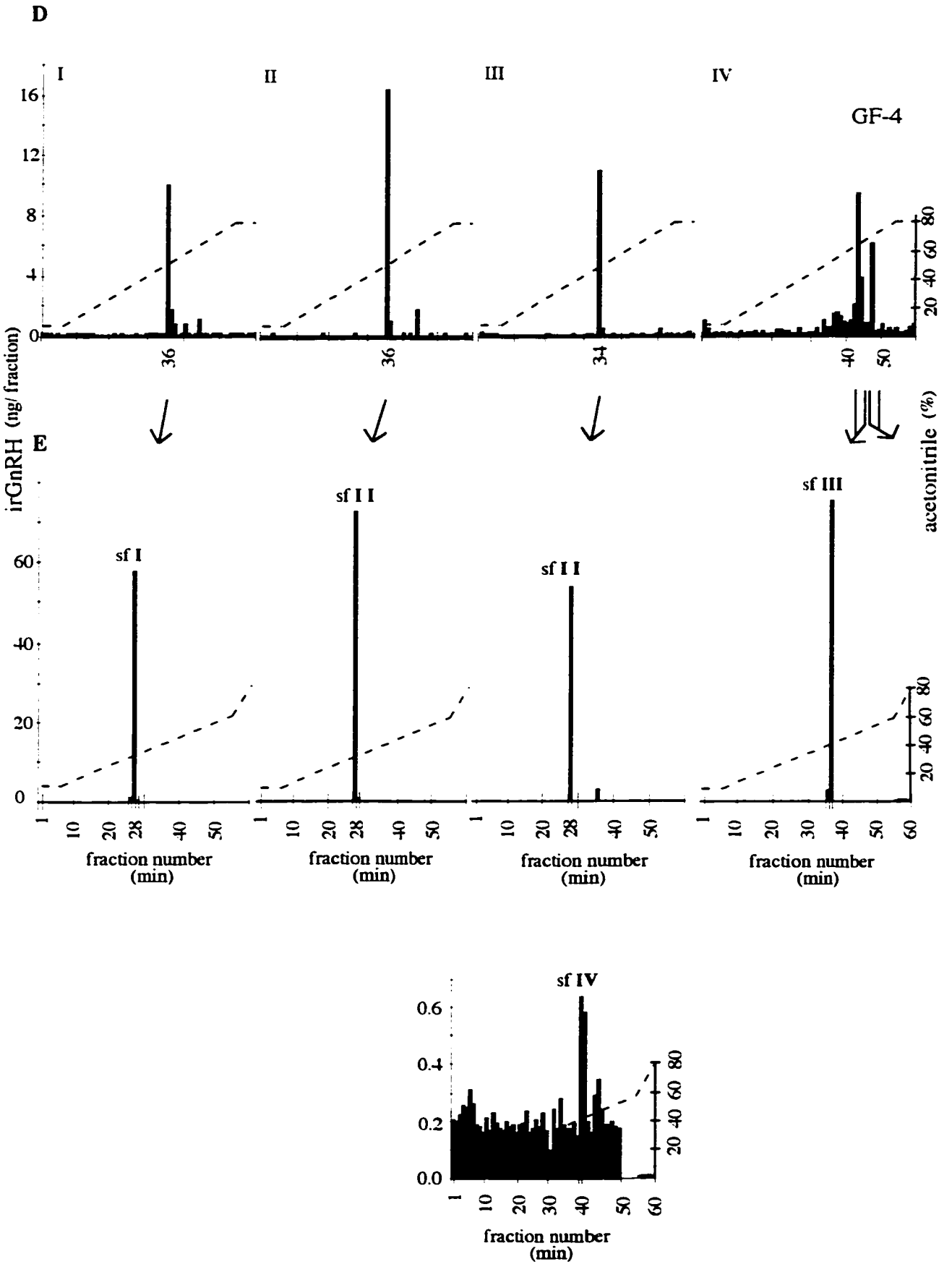
forms of GnRH in the brain of platyfish than of green swordtail. Clearly there is more cGnRH-II present than is detected by antisera Bla-5 or GF-4. Also, the accurate quantitation of the novel form of GnRH can not be determined until the structure is identified.

Sablefish Brains

Antiserum GF-4 detected 1622.4 ng and 10.2 ng of irGnRH in fractions 1-6 and fractions 32-36 respectively, following elution of sablefish brain extracts from SepPak (Fig. 3-7A). These irGnRH peaks were combined, vacuum concentrated, injected onto the C-18 HPLC column and eluted using the TEAF 17-24% HPLC program. The immunoreactive GnRH fractions used for additional steps of purification were chosen according to the predicted forms of GnRH present and were treated separately in HPLC. These irGnRH fractions were further purified using the TEAP, TFA, and Vydac TFA HPLC programs (Fig. 3-7B, C, D, E). In the final purification step (Fig. 3-7E), four distinct peaks of irGnRH were present in sablefish: sf I, 28 min (57.9ng); sf II, 28 min (125.7ng), sf III, 37min (35.2 ng) and sf IV, 39- 40 min (1.2ng). The chromatographic and immunological characteristics of these irGnRH peaks during purification suggest that sf I and sf II are sbGnRH, sf III is cGnRH-II, and sf IV is sGnRH.

Fig. 3-7. Immunoreactive GnRH from sablefish brain extracts after HPLC elution. Dashed line represents the percent acetonitrile used in elution. Elution from an HPLC column using a specific program is shown in (A) SepPak column with the SepPak TFA HPLC program, (B) C-18 column with the TEAF 17-24% HPLC program, (C) C-18 column with the TEAP HPLC program, (D) C-18 column with the TFA HPLC program and (E) Vydac diphenyl column with the Vydac TFA HPLC program.


GF-4
A
600.0
400.0
200.0
0.0
1
6
32
36
80
60
40
20
B
irGnRH (ng/fraction)
12.00
8.00
4.00
0.00
17
20
23
28
30
34
40
42
acetonitrile (%)
C
I
II
III
IV
45
35
25
15
5
0
fraction number (min)
I
II
II IV
III IV
IV


D
I
II
III
IV
GF-4
irGnRH (ng/ fraction)
acetonitrile (%)
E
sf I
sf I I
sf I I
sf III
sf IV
fraction number (min)

Discussion

In this study I have demonstrated that multiple forms of GnRH are present in the brain of skate, whitefish, platyfish, swordtail and sablefish using HPLC and RIA with GnRH antisera. Comparison of the elution positions of synthetic GnRH standards under identical HPLC conditions, the cross-reactivity with different GnRH antisera and previous reports from the literature has led me to conclude that cGnRH-II and dfGnRH are present in the brain of skate, but the skate pituitary only contains dfGnRH. By the same criteria, cGnRH-II and sGnRH are conserved in the brain of all of the teleosts studied here. The identity of the third (or fourth) form of GnRH in the brain of these species was not clear. In platyfish and swordtail brains the additional form appeared to be novel, whereas in sablefish brain the third form had sbGnRH-like chromatographic and immunological properties. In whitefish, detection of at least three forms of GnRH in the brain is unlike other salmonid HPLC and RIA studies.

dfGnRH and cGnRH-II are expressed early in development of skate brain

My HPLC and RIA study on skate was done using a small number of brains dissected into 7 regions from sexually immature skates. I have demonstrated that GnRH-like immunoreactivity is widespread throughout the brain of skate, as has been shown previously in adult black skate using immunocytochemistry with the same antiserum, GF-4 (Lovejoy *et al.*, 1992). I also have shown using both antisera GF-4 and 7CR-10 that irGnRH is present in the telencephalon and olfactory bulbs/ terminal nerve area of skate brain. This evidence of irGnRH in the terminal nerve and forebrain is similar to that reported for other cartilaginous fish (D'Antonio *et al.*, 1995 ; Sherwood and Lovejoy, 1993).

There have been only three previous HPLC and RIA studies on skates and rays. In the two skate studies, cGnRH-II-like, mGnRH-like, and lGnRH-I-like peptides were reported (Bolduc *et al.*, 1988; Calvin *et al.*, 1993) whereas in the ray study, cGnRH-II

and two novel forms of GnRH (one with sGnRH-like properties) were identified (King *et al.*, 1992). In the study by Calvin *et al.*, 1993, a sGnRH-like molecule and a novel form of GnRH also were reported. Although my results also show that multiple forms of GnRH are present in the brain of skate, the most clearly distinguishable forms were cGnRH-II and dfGnRH; I did not find any evidence of mGnRH or sGnRH using either antiserum GF-4 or B-7. The explanation may be that the dogfish form of GnRH was not isolated and sequenced until after the initial two reports in 1988 and 1993. I also did not find any evidence of lGnRH-I, but the antisera used have very limited cross-reactivity with it. It is unlikely that lGnRH-I exists in this species because our antisera did not detect it in large samples of other cartilaginous fish (ratfish and dogfish). I did not detect mGnRH in the brain of skate but because immature skates were used in this study it is possible that this form of GnRH is only expressed later in the development of this species. Alternatively, the mGnRH-like peaks previously reported in the brain of dogfish and skate might represent cross-reactivity with GnRH-like molecules that are not mGnRH but are closely related immunologically. The cGnRH-II and dfGnRH structures have already been isolated from the brain of dogfish and sequenced (Lovejoy *et al.*, 1992). Therefore, the detection of cGnRH-II and dfGnRH in the brain of skate suggests that these two forms of GnRH are present in species from groups of cartilaginous fish. The presence of dfGnRH, but not cGnRH-II, in the pituitary of skate suggests that dfGnRH is the gonadotropin releaser in these fish. However, if GnRH is carried in the blood to the pituitary of cartilaginous fish, as has been previously suggested (D'Antonio *et al.*, 1995; Lovejoy and Sherwood, 1993), then the presence of GnRH in the pituitary suggests a second route for GnRH to reach the pituitary.

Three forms of GnRH may be the ancestral condition in salmonids

An important question in GnRH research is the number and function of GnRH forms in one brain. Originally only one form was thought to be present but later, two forms were detected in fish and chicken, and now it is evident that three forms are present

in a number of teleost species. In this thesis research I also have shown by indirect chromatographic and immunological methods that there are at least three different immunoreactive variants of GnRH in the brain and pituitary of the whitefish, *Prosopium williamsoni*, a species belonging to the Coregoninae subfamily of salmonids. In a previous report by Young (1991) three irGnRH peaks were detected in the brain of Arctic grayling, *Thymallus arcticus*, a member of the Thymallinae subfamily. In contrast, other HPLC/ RIA studies detected only two GnRH-like peaks in the brain of species from the Salmoninae subfamily like Arctic char, *Salvelinus alpinus*, chinook salmon, *Oncorhynchus tshawytscha* , rainbow trout, *Oncorhyncus mykiss* and chum salmon, *Oncorhynchus keta* (Sherwood *et al.*, 1984; Young, 1991). Whitefish and Arctic char are difficult to obtain in large numbers, but further studies to isolate and characterize the primary structure of the forms of GnRH in whitefish and Arctic char are needed to determine whether three forms of GnRH in the brain is ancestral to the Salmonidae family and retained in the early-evolving subfamilies but lost in the later-evolving subfamily.

sGnRH and cGnRH-II are highly conserved in teleosts

Two of the most likely forms of GnRH to be present in the brain of the fish that I examined in this study are sGnRH and cGnRH-II. In salmonids, the sGnRH peptide has been confirmed by primary structure in chum salmon, *O. keta* (Sherwood *et al.*, 1983; Powell, 1995), by cDNA in masou salmon, *O. masou* (Suzuki *et al.*, 1992) and by cDNA and gene in Pacific salmon, *O. nerka* (Coe *et al.*, 1992), rainbow trout, *O. mykiss* (Ashihara *et al.*, 1993; Bailhache *et al.*, 1994; Klungland *et al.*, 1992) and Atlantic salmon, *Salmo salar* (Klungland *et al.*, 1992). The structures of sGnRH peptides also have been isolated and sequenced from the brain of herring, pacu, and tilapia (Carolsfeld *et al.*, 1997; Powell *et al.*, 1997; Weber *et al.*, 1997); cDNA structures encoding sGnRH have been isolated from the brain of cichlid, seabass and sea bream (Bond *et al.*, 1991; Gothilf *et al.*, 1993; Gothilf *et al.*, 1993; White *et al.*, 1995) as well as from the ovary of

goldfish (Lin and Peter, 1996). cGnRH-II is probably also present in the brain of salmonids because all of the antisera used in previous RIAs detected an irGnRH peak in the HPLC elution position expected for cGnRH-II (see Sherwood *et al.*, 1997). Furthermore, cGnRH-II is the most conserved form of GnRH in vertebrates and has been confirmed by primary structure in the brain of seabream, tilapia, herring, pacu and catfish (Bogerd *et al.*, 1994; Carolsfeld *et al.*, 1997; Powell *et al.*, 1995; Powell *et al.*, 1997; Ngamvongchon *et al.*, 1994; Weber *et al.*, 1997) and by cDNA in the brain of catfish (Bogerd *et al.*, 1992; Bogerd *et al.*, 1994;) and the ovary of goldfish (Lin and Peter, 1996). Also, recently the gene encoding cGnRH-II as well as the genes encoding sGnRH and sbGnRH were isolated from the brain of striped bass (Chow *et al.*, 1997).

Evidence that novel third forms of GnRH are present in teleost brains

I also have demonstrated in this study that three forms of GnRH in the brain of a single species are present in the brain of other teleosts like platyfish, green swordtail and sablefish. To date, two different third forms of GnRH are identified in fish: one is termed seabream GnRH and sequenced from seabream, cichlid, pacu, striped bass and tilapia; the other is termed herring GnRH and sequenced only from herring. In addition, teleosts that contain at least three variants of GnRH that have not been sequenced include: milkfish, *Chanos chanos* (Sherwood *et al.*, 1984); sabalo, *Prochilodus lineatus* (Somoza *et al.*, 1994); whitefish, *Prosopium williamsoni* (this thesis); grayling, *Thymallus articus*, arctic char, *Salvelinus alpinus* and chinook salmon, *Oncorhynchus tschawytsha* (Young, 1991); plainfin midshipman, *Porichthys nonatus* (Young, 1991); green molly, *Poecilia latipinna* (Coe *et al.*, 1990); platyfish, *Xiphophorus maculatus* and green swordtail, *Xiphophorus hellerei* (this thesis) and grass rockfish, *Sebastes rastrelliger* (Powell *et al.*, 1996). Although, none of the three irGnRH variants from the brain of these fish has been confirmed by primary structure, my report combined with the evidence from these other reports, provides further evidence that three forms of GnRH in the brain of a single species might be the ancestral condition in teleosts.

A. A novel GnRH in cyprinodonts

The identity of the third form of GnRH present in the brain of the teleosts studied in this thesis is more speculative. In platyfish and green swordtail, I detected GnRH-specific immunoreactivity in an HPLC elution position (fractions 30-31) unlike that of other known GnRHs, suggesting that a novel form of GnRH is present in the brain of these species. The only form of GnRH that elutes close to where swordtail GnRH elutes is synthetic dogfish (df)GnRH, which elutes at 34 min under the same HPLC conditions. It is unlikely that dfGnRH is present in green swordtail brain because this form has been isolated previously only from the brain of dogfish, a cartilaginous fish (Lovejoy *et al.*, 1991). Furthermore, I attempted to isolate the peptide of swordtail GnRH using additional HPLC programs. In each of these further purification steps, swordtail GnRH eluted in unique positions compared to other GnRH peptides detected in our laboratory using different teleost brain extracts and similar antisera and HPLC/ RIA protocols. It is interesting to note that the brain of a closely related species of fish, the molly (*P. latipinna*), was shown previously in our laboratory to contain irGnRH in HPLC fractions 29 and 30 using an identical HPLC program and antiserum GF-4 (Coe *et al.*, 1990). This study, as well as the evidence presented by the current study, suggests that a novel form of GnRH may be common to several members of this order of fish. It should be noted that the brain of another cyprinodont fish, the mummichog, *Fundulus heteroclitus*, has been shown by HPLC and RIA to have only sGnRH-like and mGnRH-like peptides in its brain (Knox and Sower, 1991) but it is not clear whether the antisera used in their study could detect cGnRH-II. Furthermore, the brain of another closely related species of fish, the Japanese medaka, was shown to contain only two forms of GnRH (cGnRH-11 and sGnRH) by the same methodology (Powell *et al.*, 1996); irGnRH in the novel GnRH elution position shown in this thesis study and in the Coe *et al.* study was not detected in medaka.

B. sbGnRH in sablefish

The third form of GnRH most likely present in the brain of sablefish is sea bream (sb)GnRH. The sbGnRH peptide is one of the latest forms of GnRH to be isolated. To date, sbGnRH has been characterized by primary structure in the brain of perciform fish like gilthead sea bream (Powell *et al.*, 1994) and tilapia (Weber *et al.*, 1997) and in the brain and pituitary of cichlid (Powell *et al.*, 1995) as well as in the brain of pacu (Powell *et al.*, 1997), a fish from an order that evolved before Perciformes. The cDNA structure encoding sbGnRH also has been isolated from the brain of gilthead seabream (Gothilf *et al.*, 1995, 1996), sea bass (Chow *et al.*, 1995) and African cichlid (White *et al.*, 1995) and the gene encoding sbGnRH from the striped bass (Chow *et al.*, 1997). Chromatographic and immunological evidence for a sbGnRH-like molecule is demonstrated also in the brain of perciform fish like pumpkinseed (Powell *et al.*, 1995), grass rockfish (Powell *et al.*, 1997), snook (Sherwood *et al.*, 1993a), mullet (Sherwood *et al.*, 1993b), red-spotted grouper, blue-fin tuna, black sea bream, and red seabream (Okuzawa *et al.*, 1993). Other more evolutionarily advanced fish than Perciformes like winter flounder (Andersson *et al.*, 1992; Idler *et al.*, 1987) and Japanese flounder (Okuzawa *et al.*, 1993) also have a third form of GnRH that is more hydrophilic than cGnRH-II and sGnRH and therefore, may be sbGnRH. The presence of sbGnRH in the brain of fish more evolutionarily ancient than the Perciformes, like pacu, suggests that it might represent one of the ancestral forms of GnRH from which other GnRHs have been derived. Alternatively, because sbGnRH is absent from the brain of herring, an order of fish that evolved earlier than that containing pacu, it is possible that sbGnRH is a derived form of GnRH. The two most likely forms of GnRH that sbGnRH could have evolved from are sGnRH and cGnRH-II, two of the most conserved GnRHs in teleosts. There are fewer amino acid and nucleotide differences between sbGnRH and sGnRH than between sbGnRH and cGnRH-II; therefore, it has been suggested that sbGnRH is more likely to be a derivative of sGnRH than of cGnRH-II (Powell *et al.*, 1994). The evidence presented in this thesis

as well as that reported previously by Standen, 1995 and Powell *et al.*, 1997 demonstrate that sbGnRH is conserved in the order Scorpaeniformes (sablefish, rockfish), a recently evolved order of teleost fish. The presence of sbGnRH in the order Characiformes (pacu) (Powell *et al.*, 1997b) also suggests that sbGnRH is not restricted to perciform fish but is present in fish that are more phylogenetically ancient.

Teleosts are important for tracing GnRH ancestry

The evidence presented in this study supports the suggestion that the ancestral condition in early teleosts was to have three forms of GnRH in the brain of a single species. Teleosts which have been shown to contain only two forms of GnRH may represent a derived condition where one form of GnRH is either lost in evolution or is altered into a novel form that can not be isolated and characterized by current methods. Alternatively, the third form of GnRH in some teleosts may have only a short period of expression during development and therefore, might be undetectable at the time of the tissue collection in studies that reported only two forms of GnRH in the brain. Another possibility is that tetraploid fish have lost the third form of GnRH as a result of chromosome loss that occurs after genome duplication.

Future structural studies are needed to confirm that cGnRH-II and dfGnRH are present in the brain of skate and that sGnRH and cGnRH-II are present in the brain of whitefish, platyfish, green swordtail and sablefish. Also, the third form of GnRH in the brain of these species should be isolated and characterized to determine whether it is a novel form of GnRH, as shown in platyfish and green swordtail and suggested in whitefish, or a previously described form of GnRH, as shown in sablefish. Once this third, most variable form, of GnRH has been isolated from the brain and/or pituitary of these fish, it can be determined which form of GnRH is the most important gonadotropin releaser.

Literature cited

Andersson, E, Bogerd, J., Borg, B., Sharp, P.J., Sherwood, N.M. and Goos, H.J.Th. (1995). Characterization and localization of gonadotropin-releasing hormone in the brain and pituitary of the three-spined stickleback, *Gasterosteus aculeatus*. *Cell. Tiss. Res.* **279**, 485-493.

Ashihara, M., Suzuki., M., Kubokawa, K., Yoshiura, M., Kobayashi, M., Urano, A. and Aida, K. (1993).Two differing precursor genes for the salmon-type gonadotropin-releasing hormone exist in salmonids. *J. Mol. Endocrinol.* **15**, 1-9.

Bailhache, T., Arazam, A., Klungland, H., Alëstrom, 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. *J. Comp. Neurol.* **347**, 444-454.

Barnett, F.H., Sohn, J., Reichlen, S., and Jackson, I.M.D. (1982). Three luteinizing hormone-releasing hormone like substances in a teleost fish brain: none identical with the mammalian LH-RH decapeptide. *Biochem. Biophys. Res. Commun.* **105**, 209-216.

Bogerd, J., Li, K.W., Janssen-Dommerholt, C. and Goos, H. (1992). Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). *Biochem. Biophys. Res. Commun.* **187**, 127-134.

Bogerd, J., Zandbergen, T., Andersson, E. and Goos, H. (1993). Isolation, characterization and expression of cDNAs encoding the catfish-type and chicken-II-type gonadotropin-releasing-hormone precursors in the African catfish. *Eur. J. Biochem.* **222**, 541-549.

Bolduc, T.G., Slater, C.H., and Sower, S.A. (1988). Multiple forms of gonadotropin-releasing hormone (GnRH) in the skate, ocean pout and atlantic salmon. *Am. Zool.* (abstract) **28**, 146A.

Bond, C.T., Francis, R.C., Fernald, R.D., and Adelman, J.P. (1991). Characterization of complementary DNA encoding the precursor for gonadotropin-releasing hormone and its associated peptide from a teleost fish. *Mol. Endocrinol.* **5**, 931-937.

Calvin, J.A., Slater, C.H., Bolduc, T.G., Laudano, A.P., and Sower, S.A. (1993). Multiple molecular forms of gonadotropin-releasing hormone in the brain of an elasmobranch: evidence for ir-lamprey GnRH. *Peptides* **14**, 725-729.

Carolsfeld, J., Powell, J.F.F., Park, M., Fischer, W.H., Craig, A.C., Rivier, J.E. and Sherwood, N.M. (1997). Primary structure of a novel form of gonadotropin releasing hormone from a primitive teleost, the Pacific herring *Clupea harengus pallasi*. *J. Mol. Evol*. (submitted)

Chow, M. M., Kight, K., Deoraj, A., Gothilf, Y. and Zohar, Y. (1997). Multiple GnRHs present in a teleost species are encoded by separate genes. *In*: Advances in the molecular endocrinology of fish: 2nd IUBS Toronto symposium, Toronto , Canada, May 16-19, 1997. p. 14 (abstract).

Coe, I.R., Grier, H., and Sherwood, N.M. (1992). Gonadotropin-releasing hormone in the molly *Poecilia latipinna*: molecular form, quantity, and location. *J. Exper. Zool.* **261**, 414-423.

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. *Mol. Cell. Endocrinol.* **115**, 113-122.

D'Antonio, M., Vallarino, M., Lovejoy, D.A., Vandesande, F., King, J.A., Pierantoni, R.,and Peter, R.E. (1995). Nature and distribution of gonadotropin-releasing hormone (GnRH) in the brain, and GnRH and GnRH binding activity in the serum of the spotted dogfish *Scyliorhinus canicula*. *Gen. Comp. Endocrinol.* **98**, 35-49.

Gothilf, Y, Muñoz-Cueto, J.A., Sagrillo, C.A., Selmanoff, M., Chen, T.T., Kah, O., Elizur, A., Zohar, Y. (1996). Three forms of gonadotropin-releasing hormone in a perciforme fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. *Biol. Reprod.* **55**, 636-645

Idler, D.R. and Everard, B.A. (1987). Mammalian, salmon, and chicken-like LHRHs from hypothalami of winter flounder (*Pseudopleuronectes americanus*) as evidenced by chromatographic mobility and immunoreactivity. In: "Proceeding of the third international symposium on the reproductive physiology of fish" (D.R. Idler, L.W. Crim and J.M. Walsh, *Eds.*). Marine Science Research Laboratory, Memorial University of Newfoundland, Newfoundland, Canada. p30. *J. Endocrinol.* **149**,

Kallman, K.D., and Borkoski, V. (1978). A sex-linked gene controlling the onset of sexual maturity in female and male platyfish (*Xiphophorus maculatus*), fecundity in females and adult size in males. *Genetics* **39**, 79-119.

Kallman, K.D., and Schreibman, M.P. (1973). A sex-linked gene controlling gonadotrope differentiation and its significance in determining the age of sexual maturation and size of the platyfish, *Xiphophorus maculatus*. *Gen. Comp. Endocrinol*. **21**, 287-304.

King, J.A., Dufour, S., Fontaine, Y.A., Millar, R.P. (1990). Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* **11**, 507-514.

King, J.A., Steneveld, A.A., Millar, R.P., Fasano, S., Romano, G., Spagnuolo, A., Zanetti, L., and Pierantoni, R. (1992). Gonadotropin-releasing hormone in elasmobranch (electric ray, *Torpedo maramorata*) brain and plasma: chromatographic and immunological evidence for chicken II and a novel molecular form. *Peptides* **13**, 27-35.

Klungland, H., Andersen, Ø. and Aleström, P. (1992a). The salmon gonadotropin-releasing hormone encoding gene in salmonids. *Mol. Mar. Biol. Biotech.* **1**, 420-425.

Klungland, H., Lorens, J.B., Andersen, Ø., Kisen, G.O., and Aleström, P. (1992b). The Atlantic salmon prepro-gonadotropin releasing hormone gene and mRNA. *Mol. Cell. Endocrinol.* **84**, 167-174.

Knox, C.J. and Sower, S.A. (1991). Multiple froms of gonadotropin-releasing hormone (GnRH) in four species of teleosts. *Am. Zool.* **31**, 20A (abstract).

Lin, X-W. and Peter, R.E. (1996). Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. *Gen. Comp. Endocrinol.* **101**, 282-296.

Lovejoy, D.A., and Sherwood, N.M. (1989). Gonaotropin-releasing hormone in ratfish (*Hydrolagus colliei*): distribution between sexes and possible relationship with chicken II and salmon II forms. *Comp. Biochem. Physiol. B* **92**, 111-118.

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

Lovejoy, D.A., King, J.A., Sherwood, N.M., and Peter, R.E. (1993). Identification of gonadotropin-releasing hormone and associated binding substances in the blood serum of a holocephalan (*Hydrolagus colliei*). *Peptides* **14**, 1237-1243.

Lovejoy, D.A., Sherwood, N.M., Fischer, W.H., Jackson, B.C., Riveir, J.E., and Lee, T. (1991). Primary structure of gonadotropin-releasing hormone from the brain of a holocephalan (ratfish: *Hydrolagus colliei*). *Gen. Comp. Endocrinol.* **82**, 152-161.

Lovejoy, D.A., Stell., W.K., and Sherwood, N.M. (1992). Partial characterization of four forms of immunoreactive gonadrotopin-releasing hormone in the brain and terminal nerve of the spiny dogfish (Elasmobranchii; *Squalus acanthius*). *Regul. Peptides* **37**, 39-48.

Magliulo-Cepranio, L., Schreibman, M.P., and Blüm, V. (1994). Distribution of variant forms of immunoreactive gonadotropin-releasing hormone and ß-gonadotropins I and II in the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. *Gen. Comp. Endocrinol.* **94**, 135-150.

Millar, R.P. and King, J.A. (1994). Plasticity and conservation in gonadotropin-releasing hormone structure. *In*: "Perspectives in Comparative Endocrinology" (K.G. Davey, R.E. Peter, and S.S.Tobe, Eds.), pp. 129-136. National Research Council of Canada, Ottawa.

Miller, K.E., and Kriebel, R.M. (1986). Peptidergic innervation of caudal neurosecretory neurons. *Gen. Comp. Endocrinol.* **64**, 396-400.

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

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

Okuzawa, K., Amano, M., Aida, K., Hasegawa, Y., Tanaka, H., and Kagawa, H. (1993). Chromatographic and immunological identification of gonadotropin-releasing hormone in five marine teleosts. *Fish Physiol. Biochem.***12**, 337-345.

Parenti, L.R. (1994). Killifish and ricefish. In "Encyclopedia of fishes" (J.R. Paxton and W.N. Eschmeyer, Eds.), pp.148-152. Academic Press Inc., San Diego, CA. pituitary gland of a fish by immunocytochemistry. *J. Exper. Zool.* **210**, 153-160.

Powell, R.C., Millar, R.P., and King, J.A. (1986). Diverse molecular forms of gonadotropin-releasing hormone in an elasmobranch and a teleost fish. *Gen. Comp. Endocrinol.* **63**, 77-85.

Powell, J.F.F. (1995) The origin and evolution of gonadotropin- releasing hormone in boney fishes. Ph. D thesis, University of Victoria, B. C.

Powell, J.F.F., Fischer, W.H., Park, M., Craig, A.G., Rivier, J.E., White, S.A., Francis, R.C., Fernald, R.D., Licht, P., Warby, C., and Sherwood, N.M. (1995). Primary structure of solitary form of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain and pituitary of cichlid and pumpkinseed fish. *Regul. Peptides* **57**, 43-53.

Powell, J.F.F., Krueckl, S.L., Collins, P.M., and Sherwood, N.M. (1996). Molecular forms of GnRH in three model fishes: rockfish, medaka and zebrafish. *J. Endocrinol.* (in press)

Powell, J.F.F., Reska-Skinner, S.M., Prakash, M.O., Fischer, W.H., Park, M., Rivier, J.E., Craig, G.A., Mackie, G.O., Sherwood, N.M. (1996). Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc. Natl. Acad. Sci. (U.S.A.)* **93**, 10461-10462

Powell, J.F.F., Standen, E.M., Carolsfeld, J., Borella, M.I., Gazola, R., Fischer, W.H., Park, M., Craig, A.G., Warby, C.M., Rivier, J.E., Val-Sella, M., and Sherwood, N.M. (1997). Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. *Regul. Peptides* **68**, 189-195.

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 gonadotopin-releasing hormone characterized from brains of one species. *Proc. Natl. Acad. Sci. (U.S.A.)* **91**, 12081-12085.

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. *J. Exper. Zool.* **210**, 153-160.

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 (*lLpisosteus spatula*). *Gen. Comp. Endocrinol.* **84**, 2-15.

Sherwood, N.M. and Lovejoy, D.A. (1993). Gonadotropin-releasing hormone in cartilaginous fishes: structure, location, and transport. *Enviro. Biol. Fishes* **38**, 197-208.

Sherwood, N.M., and Lovejoy, D.A. (1989). The origin of the mammalian form of GnRH in primitive fish. *Fish Physiol. Biochem.* **7**, 85-93.

Sherwood, N.M., Eiden, L., Brownstein, M., Spiess, J., Rivier, J. and Vale, W. (1983). Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci.* (USA) **80**, 2794-2798.

Sherwood, N.M., Grier, H.J., Warby, C., Peute, J., and Taylor, R.G. (1993). Gonadotropin-releasing hormones, including a novel form, in snook *Centropomus undecimalis*, in comparison with forms in the black sea bass *Centropristus striata*. *Regul. Peptides* **46**, 523-534.

Sherwood, N.M., Harvey, B., Brownstein, and Biden, L.E. (1984). Gonadotropin-releasing hormone (Gn-RH) in striped mullet (*Mugil cephalus*), milkfish (*Chanos chanos)*, and rainbow trout (*Salmo gairdneri*): comparison with salmon Gn-RH. *Gen. Comp.Endocrinol.* **55**, 174-181.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., Brownstein, M.J. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem.* **261**, 4812-4819.

Sherwood, N.M., von Schalburg, K.R., Lescheid, D.W. (1997). Origin and evolution of gonadotropin-releasing hormone in vertebrates and invertebrates. In "GnRH: Gene to Behaviour" (1. S. Parhar and Y. Sakuma, Eds.) Brain Shippon Publishers, Tokyo, Japan. (in press).

Solar, I.I., McLEan, E., Baker, I.J., Sherwood, N.M., and Donaldson, E.M. (1990). Short communication: induced ovulation of sablefish (*Anoplopoma fimbria*) following oral administration of des Gly^{10}-(D-Ala^{6})LH-RH ethylamide. *Fish Physiol. Biochem.* **8**, 497-499.

Somoza, G.M., Stéfano, A., D'Eramo, J.L., Canosa, L.F., and Fridman, O. (1994). Immunoreactive GnRH suggesting a third form of GnRH in addition to cIIGnRH and sGnRH in the brain and pituitary gland of *Prochilodus lineatus* (Characiformes). *Gen Comp. Endocrinol.* **94**, 44-52.

Standen, E.M. (1995). Gonadotropin-releasing hormone: three primary structures in pacu fish and characterization in early teleosts. Honour's Thesis, University of Victoria, B.C.

Suzuki, M., Hyodo, 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. *J. Mol. Endocrinol.* **9**, 73-82.

Weber, G.M., Powell, J.F.F., Park, M., Fischer, W.H., Rivier, J.E., Nanakorn, U., Ngamvongchon, S., Parhar, I.S., Grau, G., Sherwood, N.M. (1997). Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (*Oreochromis massambicus*) and primary structures for three native GnRH molecules. *J. Endocrinol.* (submitted)

White, S.A., Kasten, T.L., Bond, C.T., Adelman, J.P., Fernald, R.D. (1995). Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. *Proc. Natl. Acad. Sci.* (U.S.A.) **92**, 8363-8367.

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

Young, D.S. (1991). Partial characterization of gonadotropin-releasing hormone (GnRH) in three salmonid species and the plainfin midshipman; re-examination of GnRH in the pacific hagfish. Honour's thesis, University of Victoria, B.C.

Chapter 4

Identification of cGnRH-II but not cGnRH-I or other forms of GnRH in the brain of a lizard, *Anolis carolinensis*.

A modified version of this chapter has been submitted for publication as:

Lescheid, D.W., Rosen, G.J., Bridge, A.E., Jones, R.E, Warby, C.M and Sherwood, N.M. Immunoreactive gonadotropin-releasing hormone (GnRH) is detected in the form of chicken (c) cGnRH-II but not cGnRH-I within the brain of the green anole, *Anolis carolinensis* . *Gen. Comp. Endocrinol.* (submitted)

Introduction

My next objective was to determine which form(s) of GnRH found in sturgeon were retained in the evolution of the line leading to mammals. My hypothesis was that the species chosen for study would have at least two forms of GnRH in their brain; cGnRH-II, which is found in most vertebrates, and either mGnRH or a derivative of it. The first species chosen for my study using HPLC and RIA with GnRH antisera was the green anole lizard, *A. carolinensis*. This species was chosen partly because a previous immunocytochemical report had shown only one form of GnRH in the brain of *A. carolinensis* and partly because the evolution of GnRH in the brain of reptiles, especially squamate reptiles, is poorly understood.

There have been several reports that reptiles like other vertebrates have multiple forms of GnRH within the brain. In one report, two forms of GnRH were isolated and sequenced; this provided definitive proof that both cGnRH-I and cGnRH-II were present in at least one reptile, the American alligator, *Alligator mississippiensis* (Lovejoy *et al.*, 1991). In other studies using HPLC and RIA, cGnRH-I and cGnRH-II were shown to exist in the brain of slider turtle, *Pseudemys scripta* (Sherwood and Whittier, 1988), freshwater turtle, *Trachemys scripta* (Tsai and Licht, 1993a) and crocodile, *Crocodylus niloticus* (see Millar and King, 1994). Chicken GnRH-II and sGnRH have been reported for girdle-tailed lizard, *Cordylis nigra*, and ruin lizard, *Podarcis sicula* (Powell *et al.*, 1985, 1986). Several unidentified GnRH-like forms were noted also in *P. sicula* (Powell *et al.*, 1985, 1986) as well as in striped skink, *Mabuya capensis* (King and Millar, 1979; King and Millar, 1980;) and tortoise, *Chersine angulata* (King and Millar, 1979). In addition, cGnRH-I-like peptide and other small immunoreactive GnRH-like peaks were detected in the brain of the Canadian red-sided garter snake, *Thamnophilis sirtalis* (Sherwood and Whittier, 1988).

Immunocytochemical studies also support the idea that GnRH exists within the brain of snakes (*T. sirtalis*; Smith *et al.*, 1997 and *Elaphe climacophora*, *E. quadrivirgata* ,

E.conspicillata, Rhabdophis tigrinus ; Nozaki *et al.*, 1984); lizards such as chameleon (*Chameleon chameleo* ; Bennis *et al.*, 1989), skinks (*Chalcides ocellatus;* Powell *et al.*, 1986, *Eumeces okadae* ; Nozaki *et al.*, 1984); and turtle, (*Geoclemys reevesii* ; Nozaki *et al.*, 1984). All of the above studies, except the alligator study, have used indirect means (immunology, chromatography or a combination of methods such as HPLC/ RIA) to identify GnRH and therefore, other forms may be present and not detected by the antisera that were used. Nonetheless, it is evident that there are species-specific differences in the type of GnRH present in the brain of a reptile, and that cGnRH-I and cGnRH-II are the most clearly established forms.

It is puzzling to note that sGnRH has been reported in the brain of 2 lizard species, *C. nigra* and *P. sicula* (Powell *et al.*, 1985, 1986) using HPLC/ RIA. The presence of sGnRH has been only clearly established by structural studies for teleosts and not for any other vertebrate taxa. Therefore, if sGnRH is present in the brain of reptiles it would have evolved twice, which is unlikely. Other evidence for the presence of sGnRH in lizards is not very strong; that is, three immunocytochemical studies used antiserum raised against sGnRH to identify GnRH-like molecules in the brain of lizards. However, in each of these studies, the cross-reactivity reported was non-specific, with a similar staining pattern seen by another antiserum raised against mGnRH (Bennis *et al.*, 1989). Also, in one report preabsorption of tissues with another GnRH antigen (cGnRH-II) eliminated staining suggesting that the antiserum was not specific to sGnRH. (D'Aniello *et al.*, 1994). The same antiserum that was used in the D'Aniello study was used to report the presence of sGnRH in another lizard (Masucci *et al.*, 1992) but preabsorption controls were not done. In light of the above evidence it is more likely that the sGnRH-like immunoreactivity reported in the above studies represents non-specific crossreactivity with cGnRH-I, cGnRH-II or an as yet unidentified GnRH.

A previous immunocytochemical study in *A. carolinensis* found GnRH-staining cells only in the midbrain and hindbrain (Rosen *et al.*, 1997) an area of the brain where

cGnRH-II has been previously reported for most vertebrates. This immunocytochemical study was striking in that GnRH was not detected in the forebrain or olfactory region of *A. carolinensis* . All vertebrates studied to date have GnRH in the forebrain and all species, except for a few exceptions, have been shown to have at least two forms of GnRH in their brain (Sherwood *et al.*, 1997). Therefore, the lack of GnRH in the forebrain of *A. carolinensis* and the suggestion that only one form of GnRH existed within the brain was unexpected and necessitated further study. I used a sensitive HPLC/ RIA method with specific antisera to determine whether there was only a single form of GnRH within the brain of *A. carolinensis* and whether this form was cGnRH-II. If cGnRH-II is the sole form of GnRH in the brain of one or more lizards, then *A. caroliensis* would be a useful model for studying the function of this nearly ubiquitous, but poorly understood neuropeptide. The *A. carolinensis* brains used in this study were collected by Greta Rosen at the University of Colorado and the study was done in collaboration with Amanda Bridge at the University of Victoria.

Materials and Methods

Tissue collection

The brains (including pituitaries, N= 21) of *A. carolinensis* were collected in Lousiana and quickly frozen. Brains were shipped on dry ice to the University of Victoria, Canada and stored at -80 C. The brains of males undergoing gonadal recrudescence and females about to undergo their first ovulation were used because we predicted that GnRH synthesis in the forebrain would be maximal in the period leading up to the release of gonadotropins.

Extraction

The brains (total frozen mass of 0.83 g), were extracted as reported previously (Ngamvongchon *et al.*,1992) and as outlined in Chapter 2. The brain extract was vacuum dried to 2 ml, filtered through a low protein binding filter (45 μm, μCoStar LB ™,

Keenebunk, ME) and brought to pH 2.5 with 5N NaOH immediately prior to loading onto the HPLC column.

HPLC

A similar HPLC column and HPLC system was used as described in Chapter 2 of this thesis. Initially, a blank sample of 600 μl of Milli-Q water (Millipore, Bedford, MA) was injected through a 1 ml injection loop onto the column and eluted using the TEAF HPLC program described in Table 3-2. Sixty fractions of 1 ml each were collected and 500 μl was removed and assayed for immunoreactive GnRH using RIA as described in Chapter 2. GnRH immunoreactivity was not detected in this blank run and therefore, samples of *A. carolinensis* brain extract were injected sequentially in 600 μl volumes at 2 minute intervals for three injections. The same HPLC program (TEAF 17-24%) was used for elution. Sixty fractions of 1 ml each were collected and treated as described previously, except only 80-100 μl fractions were assayed for immunoreactivity in the RIAs.

Standards

Synthetic cGnRH-I and cGnRH-II, 150ngs each, were loaded onto the same HPLC column and eluted using an identical 17 -24% TEAF HPLC elution program as the one described above. Sixty fractions of 1ml each were collected and aliquots assayed using either Adams-100 or Adams-81 antiserum.

RIA

The HPLC fractions were assayed for GnRH immunoreactivity as reported previously (Sherwood *et al.*, 1986) and as described in Chapter 2. Essentially, 80-100 μl aliquots of each semi-pure fraction from the HPLC run were assayed for GnRH immunoreactivity using various antisera and ^{125}I-labelled synthetic GnRH tracers in a competitive RIA. In fractions where tracer binding exceeded detection limits (B/Bo= 80%; Table 4-1), 50 μl aliquots were diluted serially and the value closest to 50% B/Bo was used to estimate the quantity of irGnRH present. RIA and antisera characteristics are described in Table 4-1.

Table 4-1: Antisera characteristics for Anolis study

Antiserum	Raised against	Final concentration	Percent binding	Limits of Detection (B/Bo=80 %)
[1] GF-4	sGnRH	1:25,000	18.80	2.15 pg
[1] Bla-5	lGnRH-I	1: 5,000	16.90	1.92 pg
[2]7CR-10	dfGnRH	1:37,500	12.51	21.37 pg
[3]Adams-100	cGnRH-II	1:25,000	6.92	76.20 pg
[3] Adams-81	cGnRH-I	1:25,000	73.70	22.92 pg

[1] For RIAs using GF-4 and Bla-5, ^{125}I-labelled mGnRH was used as a tracer with a synthetic mGnRH standard.

[2] The RIA for 7CR-10 used a ^{125}I-labelled lGnRH-I tracer and a cGnRH-II standard.

[3] Both Adams-100 and Adams-81 RIAs were homologous with an ^{125}I-labelled cGnRH-II tracer and a cGnRH-II standard for Adams-100 and an ^{125}I-labelled cGnRH-I tracer and cGnRH-I standard used for Adams -81.

Cross-reactivity

Antisera GF-4, 7CR-10 and Bla-5 were raised in rabbits in our laboratory against salmon GnRH (GF-4), lamprey (l)GnRH-I (Bla-5) and dogfish GnRH (7CR-10). Antisera Adams-100 and Adams-81 (gifts from Dr. T. Adams, University of California, Davis) were raised in rabbits against cGnRH-II and cGnRH-I, respectively. Antisera GF-4 and Bla-5 cross-react in varying degrees with all of the 12 known forms of GnRH (Chapter 3) and therefore, are useful in determining which forms of GnRH are present. Antisera 7CR-10 and Adams-100 are specific for cGnRH-II, although they both have limited cross-reactivity with one other GnRH form. Adams-81 is specific for cGnRH-I.

The RIAs for antisera Adams-100, 7CR-10, GF-4 and Bla-5 have been previously described in Chapter 3. A homologous RIA was used for Adams-81, with ^{125}I-labelled cGnRH-I tracer and cGnRH-I standard. Five concentrations of each synthetic peptide (100, 500, 1000, 5000 and 10,000 pg) were tested in triplicate for their ability to displace the tracer from the respective antisera. Relative cross-reactivity is expressed as picomoles of the reference peptide at 50% B/Bo divided by picomoles of the test peptide at 50% B/Bo multiplied by 100.

Results

The cross-reactivity of antisera GF-4, Bla-5, 7CR-10, Adams-100 and Adams-81 with 4 synthetic GnRH peptides, mGnRH, cGnRH-I, cGnRH-II and sGnRH is shown in Table 4-2. It is clear that 7CR-10 is the most specific antiserum for cGnRH-II, whereas Adams-81 is the most specific for cGnRH-I (Table 4-2).

For HPLC, the elution position of the synthetic standards was detected first by an absorbance peak (280 nm) and then by RIA. For the synthetic standards, an absorbance peak occurred at 26 min for cGnRH-II and at 22 min for cGnRH-I. These absorbance peaks paralleled the immunoreactive peaks of synthetic standards detected in fractions 26 and 27 by Adams-100 and in fraction 22 by Adams-81. The elution positions of all 8 standards is shown in Fig. 4-1A and in Chapter 3.

Table 4 -2: Antisera cross-reactivity for Anolis study

Standard	[1]Antiserum crossreactivity (percent)				
	GF-4	Bla-5	7CR-10	Adams-100	Adams-81
mGnRH	100.00	100.00	< 0.03	< 0.04	<0.15
cGnRH-I	44.00	77.00	< 0.03	< 0.04	100.00
cGnRH-II	3.90	1.80	100.00	100.00	<0.15
sGnRH	68.80	123.70	< 0.03	14.40	<0.09

[1] Relative cross-reactivity (%) is picomoles of the reference peptide at 50 % binding (0.5 B/Bo) divided by picomoles of the other peptide at 50% binding (0.5B/Bo) multiplied by 100. mGnRH was used as the reference peptide for antisera GF-4 and Bla-5 in the calculation of percent cross-reactivity. cGnRH-II was used for antisera Adams-100 and 7CR-10 and cGnRH-1 was used for Adams-81.

For the brain extracts, both antisera GF-4 and Bla-5 detected immunoreactivity in fractions 26 and 27, corresponding to the elution position of synthetic cGnRH-II (Fig.4-1A, B). The presence of immunoreactive peaks in the same HPLC fractions using cGnRH-II-specific antisera, Adams-100 and 7CR-10, confirmed that cGnRH-II-like molecules exist (Fig. 4-2). Quantitatively, GF-4 detected 2.1 ng of ir-cGnRH-II in these peaks whereas Bla-5 detected 0.5 ng (Fig. 4-1). Antiserum Adams-100 detected about 5.0 ng and antiserum 7CR-10 detected 11.5 ng (Fig. 4-2).

Although both GF-4 and Bla-5 can cross-react with cGnRH-I (44.0% and 77.0%, respectively, Table 4-2 and Fig. 3-1), there was no detectable immunoreactivity in position 22 where synthetic cGnRH-I elutes under identical HPLC conditions (Fig. 4-1). Furthermore, a cGnRH-I-specific antiserum, Adams-81, did not detect any immunoreactivity (Fig. 4-1), suggesting that cGnRH-I was not present. Also, using these 5 GnRH-specific antisera (GF-4, Bla-5, 7CR-10, Adams-100, and Adams-81) in RIA, I did not find any evidence of sGnRH or other novel GnRH forms.

Fig. 4-1. Immunoreactive GnRH after HPLC elution of *A. carolinesis* brain extracts. GnRH was detected by antisera (A) GF-4, (B) Bla-5 and (C) Adams-81, respectively in HPLC fractions (each collected for 1 min). Small, darkened arrows above the HPLC elution profiles represent the elution positions of synthetic standards used in this study whereas, the small, two-toned arrows indicate the elution positions of other standards used in earlier studies in our laboratory with the same HPLC conditions. The dotted line represents the percentage of acetonitrile in the mobile phase during elution.

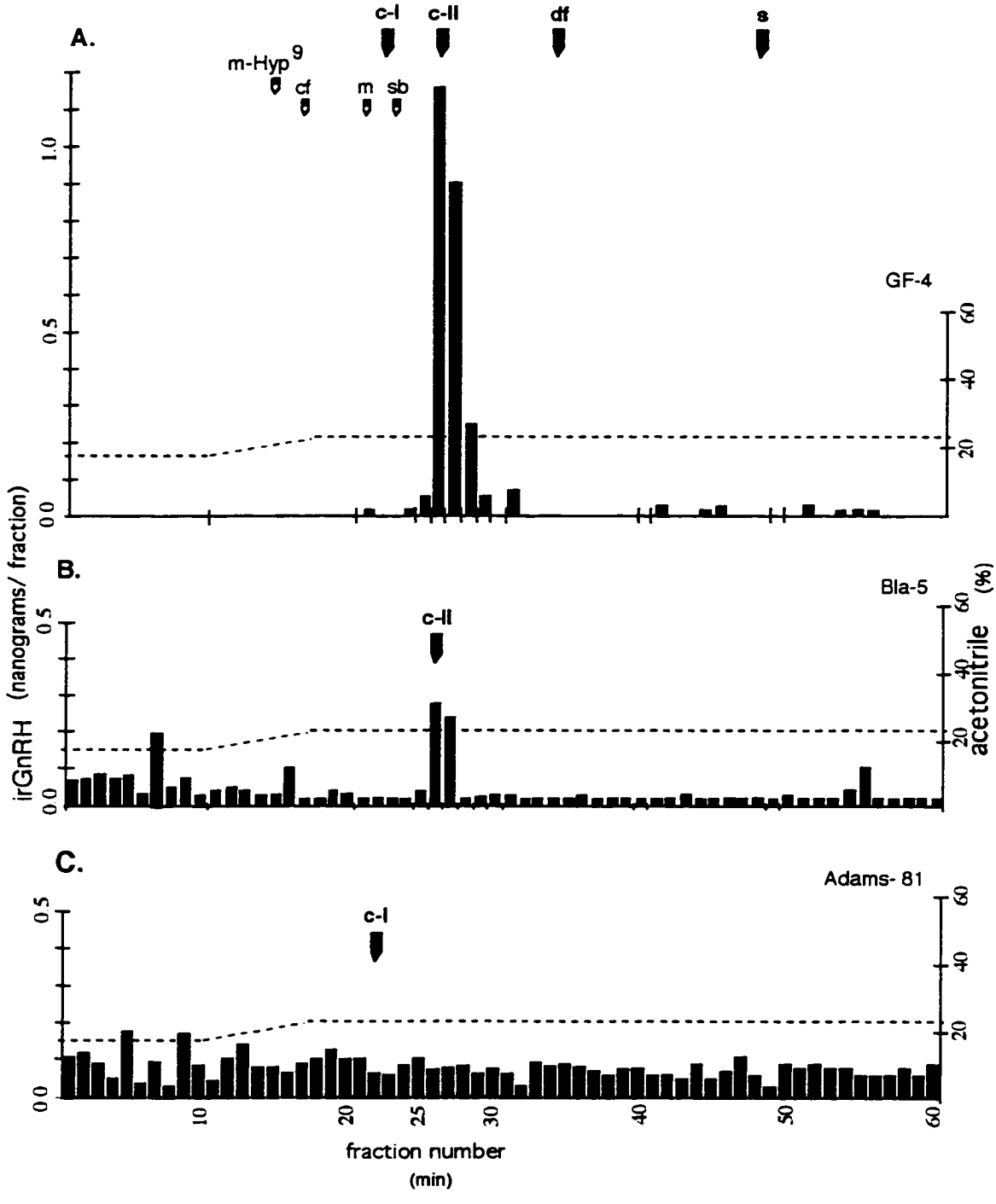
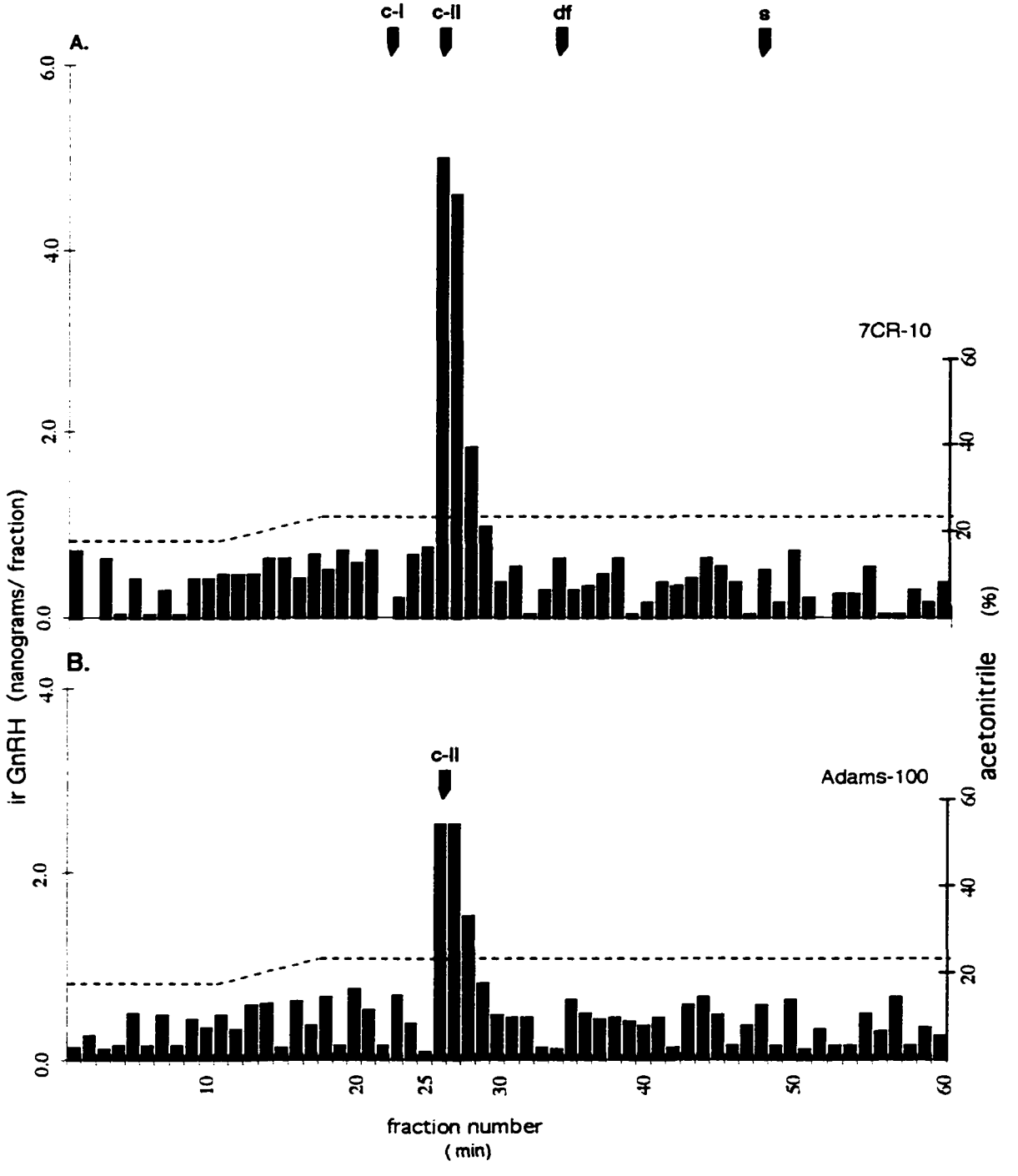

A.
m-Hyp 9
cf
m
sb
c-I
c-II
df
s
GF-4
1.0
0.5
0.0
60
40
20
B.
Bla-5
c-II
irGnRH (nanograms/ fraction)
0.5
0.0
60
40
20
acetonitrile (%)
C.
Adams- 81
c-I
0.5
0.0
60
40
20
10
20
25
30
40
50
60
fraction number
(min)

Fig. 4-2. Immunoreactive GnRH in HPLC fractions of *A. carolinensis* brain extracts. GnRH was detected by antisera (A) 7CR-10 and (B) Adams-100. Small darkened arrows above the HPLC elution profile represent the elution position of synthetic standards using similar HPLC conditions. The dotted line represents the percentage of acetonitrile in the mobile phase during elution.


A.
c-I
c-II
df
s
6.0
4.0
2.0
0.0
7CR-10
60
40
20
(%)
B.
4.0
2.0
0.0
c-II
Adams-100
acetonitrile
ir GnRH (nanograms/ fraction)
10
20
25
30
40
50
60
fraction number
(min)

Discussion

I have demonstrated that a cGnRH-II-like peptide, but not a cGnRH-I-like peptide is present in the brain of *A. carolinensis* using HPLC and RIA with five different GnRH antisera. The combination of my data with that in the literature supports the hypothesis that some lizards do not express the cGnRH-I peptide within the brain making them quite distinct from the other major groups of reptiles including snakes, turtles and alligators.

The strongest evidence to date that cGnRH-I is not present in the brain of certain lizards is based on studies in which HPLC elution position of the peptides is coupled with cross-reactivity results using well-characterized antisera. In addition to the present study of *A. carolinensis*, there are two other HPLC/ RIA studies supporting the conclusion that cGnRH-I is not present in the skink (*C. ocellatus*), the ruin lizard (*P. sicula*) and the girdle-tailed lizard (*C. nigra*) (Powell *et al.*, 1985; 1986). Hence, the absence of cGnRH-I is documented for four species of lizards that represent four different families.

Evidence to date suggests that some lizards such as *A. carolinensis* lack a detectable form of GnRH in the forebrain, but other lizards have an unknown form of GnRH in the forebrain. However, there are no reports of GnRH in the olfactory bulbs or terminal nerve; this distinguishes the lizards from most other vertebrates. GnRH cells in most vertebrates can be separated into two categories: GnRH cells in the septo-preoptic area that are olfactory-derived, and GnRH cells in the midbrain that are probably ependymally derived (Muske, 1993). In lizards, a variety of antisera have been used, but evidence to date shows that GnRH is predominately in the midbrain, although studies on some species also report GnRH-containing cells in the hypothalamus or in the preoptic-septal region (Bennis et al., 1989; D'Aniello et al., 1994; Demski and Wright, 1992; Doerr-Schott and Dubois, 1978; Münz *et al.*, 1981; Masucci *et al.*, 1992; Nozaki and Kobayashi, 1979; Nozaki *et al.*, 1984; Rosen *et al.*, 1997). None of the studies report GnRH cell bodies in the terminal nerve or olfactory bulbs, a situation that might be unique to lizards because an extensive GnRH system in this region of the brain has been reported for most tetrapods. The

importance of this observation is that the form of GnRH in the anterior brain is associated with neurons that originate in the olfactory placode and migrate before birth or hatching into the anterior brain coming to rest in the terminal nerve ganglion, anterior telencephalon and in the preoptic-septal-hypothalamic region in all vertebrates tested to date from fish to humans (see Muske, 1993). In adult snakes (Smith *et al.*, 1997) and turtles (Tsai and Licht, 1993a) and in developing and adult birds (Norgren and Lehman, 1991), the anterior form of GnRH is known to be cGnRH-I. The only developmental study of GnRH in reptiles is that of the ruin lizard (*P. sicula*) in which embryos were collected from day 21 of incubation until adulthood. GnRH was never detected in the olfactory bulbs or terminal nerve cells at any stage of development. Rather, cells staining with a cGnRH-II antiserum appeared in the mesencephalon on day 45 of incubation and in the infundibulum on day 52 of incubation (D'Aniello *et al.*, 1994). These immunocytochemical studies are useful in localizing GnRH-containing cell bodies or fibers, but rarely identify the form of GnRH that is present in the cells, because the antisera cross-react in varying degrees with the other members of the GnRH family. In the present study, our battery of antisera detect the 12 known forms of GnRH (see Chapter 3, Fig. 3-1) and hence, it is clear that cGnRH-I, sGnRH and related family members are not present.

If cGnRH-I does not exist in the lizard forebrain, then there are three possibile ways that gonadotropin release can be maintained. The first is that cGnRH-I is replaced by an unknown form of GnRH. In this case, nucleotide base substitution in the cGnRH-I gene is assumed to occur, resulting in a novel form of GnRH that is undetectable by current antisera and hence can not be identified. The second possibility is that cGnRH-II fibers terminate on the hypophysial portal vessels for the control of gonadotropin release from the pituitary gland, a role that has been previously assigned to cGnRH-I in reptiles and birds. The third possibility is that another neuropeptide acts as a gonadotropin-releaser, although there is currently no proof that reproduction continues if all forms of GnRH are knocked out.

Support for the idea that cGnRH-II may terminate on hypophysial portal vessels and control gonadotropin release in lizards is indirect. To date, irGnRH has been found in nerve cells or fibers of the infundibulum or median eminence in six species of lizard: *A. sagrei* (Demski and Wright, 1992), *P. siculus* (Doerr-Schott and Dubois, 1978; D'Aniello *et al.*, 1994; Masucci *et al.*, 1992), *E. okadae* (Nozaki *et al.*, 1984), *G. gecko* (Nozaki *et al.*, 1984; Nozaki and Kobayashi, 1979), *E. laticepes* and *S. undulatus* (Rosen *et al.*, 1997). The only exceptions in which irGnRH fibers were not present in the infundibulum, were *A. carolinensis* (Münz *et al.*, 1981; Rosen *et al.*, 1997) and *C. chameleo* (Bennis *et al.*, 1989), suggesting that there might be species specificity in the location of GnRH in the brain. Four lizard species (*P. sicula*, *C. nigra*, *C. ocellatus* and *A. carolinensis*) have been shown to lack cGnRH-I by HPLC studies and hence, I suggest that the immunoreactive median eminence fibers in some lizards contain cGnRH-II, the only other form known to be present. Also, two studies on the ruin lizard (*P. sicula*) report the usual cluster of GnRH neurons in the midbrain plus a cluster in the infundibulum (D'Aniello *et al.*, 1994, Masucci *et al.*, 1992). These results are the best indication that cGnRH-II may innervate the portal system.

In contrast to *A. carolinensis*, additional irGnRH cells are detected anterior to the median eminence, in the preoptic-septal region in brown anole, *A. sagrei* (Demski and Wright, 1992), the eastern fence lizard, *Sceloporous undulatus* (Rosen *et al.*, 1997) and two skinks, *Eumeces laticeps* (Rosen *et al.*, 1997) and *E.. okada* (Nozaki *et al.*, 1984). HPLC studies have not been done on these latter four species and hence we can not be sure of the GnRH identity in these cells that are just anterior to the hypothalamus, but not in the most anterior portion of the brain. Also, GnRH-positive cells, thought to contain cGnRH-II, appeared transiently in the telencephalon in juvenile lizards, but disappeared in the adults (D'Aniello *et al.*, 1994).

It is possible only to speculate on the reason for the absence of a second form of GnRH in *A. carolinesis*. Some possibilities are that 1) the neuroblasts containing GnRH

are blocked from migrating into the brain, or 2) a change occurs in the nucleotides in the regulatory or peptide-encoding region of the cGnRH-I gene, or 3) cGnRH-I is expressed only during a particular season or developmental stage.

For the first option, a similar situation has been described in humans where mGnRH cells did not migrate from the olfactory placode into the brain (Schwanzel-Fukuda *et al.*, 1989) due to deletions or point mutations in a specific gene, *kalig-1* (Ballabio *et al.*, 1989; Franco *et al.*, 1991; Hardelin and Petit, 1995). This resulted in anosmia, hypogonadism and sterility, which are characteristic of Kallman's Syndrome. If cGnRH-I did not migrate in *A carolinensis*, one might expect reproduction to cease unless some cGnRH-II or other novel GnRH-like fibers innervated the median eminence to stimulate gonadotropin release. In *A. carolinensis*, reproduction obviously continues but studies are needed to determine if there are cGnRH-I cells in the olfactory epithelium that have not migrated.

The second option is that cGnRH-I is not expressed in the brain due to a change in the regulatory region of the gene or in the region encoding cGnRH-I. This is the case in the hypogonadal mouse (Mason *et al.*, 1986) where a deletion in the gene results in a prohormone precursor that can not be processed; sexual development fails. In my study I used a variety of polyclonal antisera in RIA and therefore, there should be some cross-reactivity unless GnRH was not cleaved from the precursor or there was a mutation in a critical nucleotide in the region of 30 nucleotides encoding cGnRH-I. Also, a change in the regulatory or promoter-enhancer region of the gene might prevent transcription of the gene and hence lack of expression.

The third option is that cGnRH-I is expressed only at specific stages. This seems unlikely as cGnRH-I was not detected in the HPLC studies done on four different species that lack cGnRH-I at different stages. It also appears that once GnRH is expressed in the brain during development, expression continues. In the only developmental study on lizards, *P. sicula*, it was concluded that "the number of GnRH neurons and fibers, and the

intensity of immunostaining, increased during the period prior to hatching to the juvenile stage. Juveniles were similar to adults" (D'Aniello *et al.*, 1994). The same laboratory detected similar GnRH cells in adults at two different reproductive seasons (April and November) (Masucci *et al.*, 1992). The one exception to the pattern described above is that a cluster of GnRH cells appeared in the central telencephalon in juvenile lizards and disappeared in adults (D'Aniello *et al.*, 1994). Hence, in theory there could be a window during development when cGnRH-I is expressed, but this has not been proven.

There is not yet sufficient evidence to correlate the absence of cGnRH-I in the forebrain of *A. carolinesis* with its reduced vomeronasal and olfactory systems (Halpern, 1992; Young, 1981). In this lizard, vision and proprioception might be more important than olfaction for reproductive success as shown by arboreal mating preceded by vivid, colorful displays and elaborate head bobbing in the trees. Lack of evolutionary pressure to maintain a forebrain GnRH system might have resulted in the loss of cGnRH-I in this species, but cGnRH-I is reported also absent in several species, *C. ocellatus*, *P. sicula*, *C. nigra* , that are terrestrial and have well-developed vomeronasal and olfactory systems. Specifically, a combination of HPLC and immunocytochemical studies are needed for species that have either well-developed or poorly developed vomeronasal systems to determine if forebrain GnRH correlates with a vomeronasal system. It is interesting to note that unlike most lizards, the snake, *T. sirtalis*, has GnRH immunoreactivity predominately in the forebrain, and cGnRH-I as the only identified form of GnRH in its brain. Smith *et al.* (1997) used immunocytochemistry with a specific antiserum to show that cGnRH-I cells occurred in the anterior brain including the terminal nerve ganglion, telencephalon, preoptic area and hypothalamus. A cGnRH-II-specific antiserum used in the same study did not detect any immunoreactive cells or fibers. Likewise, Sherwood and Whittier (1988) did not detect cGnRH-II in the brain of the same species by HPLC/ RIA but did detect cGnRH-I and two small unknown GnRH peaks.

In conclusion, my results and those of Rosen et al. (1997) confirm that, in the

lizard *A. carolinesis*, only midbrain cGnRH-II is present in the brain. That is, cGnRH-I (or any other GnRH) is absent from the terminal nerve, septo-preoptic region, hypothalamus, and median eminence. Therefore, this species of lizard is similar to sturgeon in that it contains cGnRH-II in its brain, but unlike sturgeon because mGnRH or any other form of GnRH is not present in its brain.

It is interesting to note that *A. carolinensis* has been shown to contain only cGnRH-II in its brain by HPLC/ RIA and no detectable GnRH-like fibers in the hypothalamus or median eminence by immunocytochemistry. The major question that arises is how this lizard species continues to reproduce with only cGnRH-II in its brain, in a location that apparently is separated from any potential contact with gonadotropin-synthesizing cells in the pituitary. Further immunocytochemical studies at different times of the year are needed to determine if axon terminals with cGnRH-II are present in the median eminence.

Literature cited

Ballabio, A., Bardoni, B., Carrozzo, R., Andria, G., Bick, D., Campbell, L., Hamel, B., Ferguson-Smith, M.A., Gimelli, G., Fraccaro, M., Maraschio, P., Zuffardi, O., Guioli, S., and Camerino, G. (1989). Contiguous gene syndromes due to deletions in the distal short arm of the human X chromosome. *Proc. Natl. Acad. Sci. (USA)* **86**, 10001-10005.

Bennis, M., Dubourg, P., Gamrani, H., Calas, A., and Kah, O. (1989). Existence of a GnRH immunoreactive nucleus in the dorsal midbrain tegmentum of the chameleon. *Gen. Comp. Endocrinol.* **75**, 195-203.

Ciarcia, G., Paolucci, M., and Botte, V. (1989). Effects of gonadotrophin-releasing hormone variants on reproductive organs and plasma testosterone in the male lizard, *Podarcis s. sicula*. *J. Neuroendocinology* **1**, 205-208.

D'Aniello, B., Pinelli, C., King, J.A., and Rastogi, R.K. (1994). Neuroanatomical organization of GnRH neuronal systems in the lizard (*Podarcis s. sicula*) brain during development. *Brain Res.* **657**, 221-226.

Demski, L.S., and Wright, D.E. (1992). GnRH immunoreactivity in the brain of the brown Anole, *Anolis sagrei*. *Soc. Neurosci. Abstr.*, 18. 143.13

Doerr-Schott, J., and Dubois, M.P. (1978). Immunohistochemical localization of different peptidergic substances in the brain of amphibians and reptiles. *In* "Comparative endocrinology" (P.J. Gaillard and H.H. Boer, Eds.), pp.367-370. Elsevier, Amsterdam.

Franco, B., Guioli, S., Pragliola, A., Incerti, B., Bardoni, B., Tonlorenzi, R., Carrozzo, R., Màestrini, Pieretti, M., Taillon-Miller, P., Brown, C. J., Willard, H.F., Lawrence, C., Graziella Persico, M., Camerino, G., and Ballabio, A. (1991). A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature* **353**, 529-536.

Gautron, J-P., Pattou, E., Bauer, K., Kordon, C. (1991). (Hydroxyproline^9) luteinizing hormone-releasing hormone: a novel peptide in mammalian and frog hypothalamus. *Neurochem. Int* .**18**, 221-235

Halpern, M. (1992). Nasal chemical senses in reptiles. *In* "Biology of the reptiles Vol. 18 Physiology E., Hormones, brain and behaviour" (C. Gans and D. Crews, Eds.), pp. 424-523. The University of Chicago Press Ltd., Chicago.

Hardelin, J.P., and Petit, C. (1995). A molecular approach to the pathophysiology of the X chromosome-linked Kallmann's syndrome. *Baillieres Clin. Endocrinol. Metab.* **9**, 489-507.

King, J. A. and Millar, R.P. (1979). Heterogeneity of vertebrate luteinizing hormone-releasing hormone. *Science* **206**, 67-69.

King, J.A. and Millar, R.P. (1980). Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny. *Endocrinology* **106**, 707-717.

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

Licht, P., and Porter, D. (1985b). LH secretion in response to gonadotropin releasing hormone (GnRH) by superfused pituitaries from two species of turtle. *Gen. Comp. Endocrinol.* **59**, 442-448.

Licht, P., and Porter, D.A. (1985a). *In vivo* and *in vitro* responses to gonadotropin releasing hormone in the turtle, *Chrysemys picta*, in relation to sex and reproductive age. *Gen. Comp. Endocrinol.* **60**, 75-85.

Licht, P., Khorrami-Yaghoobi, P., and Porter, D.A. (1985). Effects of gonadectomy and steroid treatment on plasma gonadotropins and the response of superfused pituitaries to gonadotropin-releasing hormone in the turtle, *Sternotherus odoratus*. *Gen. Comp. Endocrinol.* **60**, 441-449.

Licht, P., Porter, D., and Millar, R.P. (1987). Specificity of amphibian and reptilian pituitaries for various forms of gonadotropin-releasing hormones *in vitro*. *Gen. Comp. Endocrinol.* **66**, 248-255.

Lovejoy, D.A., Fischer, W.H., Parker, D.B., McRory, J.E., Park, M., Lance, V., Swanson, P., Rivier, J.E., and Sherwood, N.M. (1991). Primary structure of two forms of gonadotropin-releasing hormone from the brains of the American alligator (*Alligator mississippiensis*). *Regul Peptides* **33**, 105-116.

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

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young III, W.S., Phillips, H.S., Nikolics, K., and Seeburg, P.H. (1986). A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the *hpg* mouse. *Science* **234**, 1366-1371.

Masucci, M., D'Aniello, B., Iela, L.,Ciarcia, G., and Rastogi, R.K. Immunohistochemical demonstration of the presence and localization of diverse molecular forms of gonadotropin-releasing hormone in the lizard (*Podarcis s. sicula*) brain. *Gen. Comp.Endocrinol.* **86**, 81-89.

Millar, R.P. and King, J.A. (1994). Plasticity and conservation in gonadotropin-releasing hormone structure. *In*: "Perspectives in Comparative Endocrinology" (K.G. Davey,

R.E. Peter, and S.S.Tobe, Eds.), pp. 129-136. National Research Council of Canada, Ottawa.

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

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

Norgren, R.B. Jr., and Lehman, M.N. (1991). Neurons that migrate from the olfactory epithelium in the chick express luteinizing hormone-releasing hormone. *Endocrinology* **128**, 1676-1681.

Nozaki, M., and Kobayashi, H. (1979). Distribution of LHRH-like substance in the vertebrate brain as revealed by immunohistochemistry. *Arch. Histol. Jpn.* **42**, 201-219.

Nozaki, M., Tsukahara, T., and Kobayashi, H. (1984). Neuronal systems producing LHRH in vertebrates. *In* "Endocrine correlates of reproduction" (K. Ochiai, Ed.), pp. 3-27. Verlag Press, New York.

Phillips, J.A., Alexander,N., Karesh, W.B., Millar, R.P., Lasley, B.L. (1985). Stimulating male sexual behaviour with repetitive pulses of GnRH in female green iguanas, *Iguana iguana.* *J. Exper. Zool.* **234**, 481-484.

Phillips, J.A., and Lasley, B.L. (1987). Modification of reproductive rhythm in lizards via GnRH therapy. *In* "The terminal nerve (nervus terminalis): Structure, function and evolution" (L.S. Demski and M. Schwanzel-Fukuda, Eds.). *Annals New York Acad. Sci.* **519**, 128-136.

Phillips, J.A., Frye, F., Bercovitz, A., Calle, P., Millar, R., Rivier, J., Lasley, B.L. (1987). Exogenous GnRH overrides the endogenous annual reproductive rhythm in green iguanas, *Iguana iguana. J.Exper. Zool* . **241**, 227-236.

Powell, J.F.F., Reska-Skinner, S., Prakash, M.O., Fischer, W.H., Park, M., Rivier, J.E., Craig, G.A., Mackie, G.O. and Sherwood, N.M. (1996). Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Pro. Natl. Acad. Sci. (USA)* **93**, 10461-10462.

Powell, R.C., Ciarcia, G., Lance, V., Millar, R.P., and King, J.A. (1986). Identification of diverse forms of GnRH in reptile brain. *Peptides* **7**, 1101-1108.

Powell, R.C., King, J.A., and Millar, R.P. (1985). [Trp^{7}, Leu^{8}] LH-RH in reptilian brain. *Peptides* **6**, 223-227.

Rosen, G., Sherwood, N.M., and King, J.A. (1997). Immunoreactive gonadotropin-releasing hormone (GnRHir) is associated wtih vestibular structures in the Green Anole (*Anolis carolinensis*). *Brain Behav. Evol.* (in press)

Schwanzel-Fukuda, M., Bick, D., and Pfaff, D.W. (1989). Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallman) syndrome. *Mol. Brain Res.* **6**, 311-326.

Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M., Huskisson, N. S. (1990). Physiological roles of chicken LHRH-I and -II in the control of gonadotropin release in the domestic chicken. *J. Endocrinol.* **124**, 291-299.

Sherwood, N.M., and Whittier, J.M. (1988). Gonadotropin-releasing hormone from the brains of reptiles: turtles (*Pseudemys scripta*) and snakes (*Thamnophilis sirtalis parietalis*). *Gen. Comp. Endocrinol.* **69**, 319-327.

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., Brownstein, M.J. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem.* **261**, 4812-4819.

Sherwood, N.M., von Schalburg, K.R., and Lescheid, D.W. (1997, in press). Origin and evolution of gonadotropin-releasing hormone in vertebrates and invertebrates. *In* "GnRH: Gene to Behaviour" (I. S. Parhar and Y. Sakuma, Eds.). Brain Shippon Publishers, Tokyo, Japan.

Smith, M.T., Moore, F.L., and Mason, R.T. (1997). Neuroanatomical distribution of chicken-I gonadotropin-releasing hormone (cGnRH-I) in the brain of the male red-sided garter snake. *Brain Behav. Evol.* **49**, 137-148.

Tsai, P-I., and Licht, P. (1993a). Differential distribution of cGnRH-I and cGnRH-II in the turtle brain. *Peptides* **14**, 221-226.

Tsai, P-S., and Licht, P. (1993b). *In vivo* responsiveness of LH secretion in the female turtle, *Trachemys scripta*, in relation to the reproductive stage. *Gen. Comp. Endocrinol.* **90**, 328-337.

Young, J.Z. (1981). The life of vertebrates, 3rd edition. Oxford University Press, New York, NY. pp. 292-322.

Chapter 5

Identification of two forms of GnRH in the brain of adult and fetal monkeys and examination of a human placenta for GnRH immunoreactivity.

Parts of this chapter have been modified and accepted for publication as:

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 with characteristics of chicken GnRH-II is present in the primate brain. *Endocrinology* (accepted).

Introduction

In Chapter 2 of this thesis, I demonstrated that the sturgeon brain contains both mGnRH and cGnRH-II. Primates also have mGnRH in their brains but it is not clear whether a second form of GnRH exists within the brain of these recently-evolved placental mammals. The most likely second form of GnRH present in the brain of primates is cGnRH-II because it is highly conserved in vertebrates and also because there have been previous reports of immunoreactive cGnRH-II-like molecules in the brain of early-evolved placental mammals like the musk shrew and tree shrew; a cDNA encoding cGnRH-II also had been isolated from the brain of tree shrew. During my thesis research, I developed two cGnRH-II specific RIAs (Chapter 4) which allowed me to use HPLC and these cGnRH-II specific RIAs to determine if I could detect cGnRH-II in the brain of adult stumptail monkeys (*Macaca speciosa*) as well as in adult and fetal rhesus monkey (*Macaca mulatta*) brains. Also, other RIAs and immunocytochemistry with different anti-GnRH antisera were used to characterize cGnRH-II-like as well as mGnRH-like immunoreactivity in the brains of these primates. In addition, the *in vivo* effect of the synthetic cGnRH-II-like peptide on the release of luteinizing hormone at different times in the reproductive cycle is reported.

Over the past 26 years since the discovery of mGnRH, there have been nine other GnRH peptides isolated from the brain of different vertebrates and two from the neural ganglia of an invertebrate, the tunicate (see thesis Chapter 1 and Sherwood *et al.*, 1997). The brain of a vertebrate usually has two or three forms of GnRH; these different GnRHs usually are present in separate locations in the brain and therefore, might have different functions. Until recently, it was thought that multiple forms and functions of GnRH within the brain of a single species did not exist in mammals (King *et al.*, 1988). However, in 1989 it was shown that primitive metatherian mammals, like marsupials (tammar wallaby, *Macropus eugenii* ; short-nosed bandicoot, *Isoodon macrourus* and eastern quoll, *Daspyurus viverrinus*) and a monotreme (echidna, *Tacyglossus aculeatus*),

have both immunoreactive mGnRH and cGnRH-II forms within their brain (King *et al.*, 1989; King *et al.*, 1990). In a later paper, the same group (King *et al.*, 1994) presented chromatographical and immunological data suggesting that mGnRH-like and cGnRH-II-like immunoreactive molecules were present within the brains of the adult and joey brushtail possums, *Trichosurus vulpecula* , as well as in eutherian mammals like the musk shrew, *Suncus murinus* and golden mole, *Chrysochloris asiatica*. Surprisingly, they could not find the cGnRH-II form in the bat, *Miniopterus shreibersii*, which is a eutherian mammal that has retained primitive characteristics. Dellovade et al. (1993) used HPLC and RIA and Rissman et al. (1995) used immunocytochemistry to confirm that musk shrew brains contain both mGnRH and cGnRH-II-like molecules. Definitive proof that placental mammals had more than one form of GnRH in their brain was not available until 1996 when two separate cDNAs, one encoding mGnRH and the other encoding cGnRH-II, were isolated from the brain of the tree shrew and shown to be in the forebrain and midbrain neurons, respectively (Kasten *et al.*, 1996). However, the tree shrew is one of the most early-evolved placental mammals, separated in evolution from the primates by at least 40 million years. Recently, evidence for a form of GnRH other than mGnRH (but not cGnRH-II) in the primate forebrain also has been presented (Quanbeck *et al.*, 1997). Despite these reports showing two forms of GnRH within the brain of some placental mammals, there was no clear evidence that more recently-evolved mammals like primates had an additional form of GnRH such as cGnRH-II within their brains.

Because of the previous reports of mGnRH and cGnRH-II-like immunoreactivity in the brain of early-evolved placental mammals it can be predicted that a similar combination of GnRHs exists within the brain of humans, which are also placental mammals. However, it is difficult to obtain human brain tissue for HPLC/ RIA studies. Nonetheless, human placental tissue has been shown to contain mGnRH with similar immunological properties (Gibbons *et al.*, 1975; Khodr and Siler-Khodr, 1980) and the same primary structure (Tan and Rousseau, 1982) as hypothalamic mGnRH. Therefore, I chose the

human placenta as a more readily available alternative than human brain. The placental GnRH molecule previously has been shown using *in situ* hybridization studies to be synthesized by cells in the placenta (Duello *et al.*, 1993; Kelly *et al.*, 1991); the peptide also is made in the brain of the mother but not carried across the placenta. The cDNA encoding mGnRH has been isolated from the placenta of human (Kelly *et al.*, 1991; Seeburg and Adelman, 1984) and rat (Adelman *et al.*, 1986) and has been shown to encode a GnRH identical to that of other mammals despite differences in the 5' UTR region (Radovick *et al.*, 1990) and the transcriptional start site (Dong *et al.*, 1993). It also was recently demonstrated that a cDNA encoding cGnRH-II, as well as a cDNA encoding sGnRH, were both present in the goldfish ovary (Lin and Peter, 1996). This was the first report of cGnRH-II outside the vertebrate brain, and provides further evidence that other vertebrate reproductive tissues, like human placenta, might also contain cGnRH-II-like molecules. Therefore, to establish that my assays could detect mGnRH in human placenta tissue and to investigate whether a cGnRH-II-like molecule was present outside the brain of recently-evolved placental mammals, I used HPLC and RIA with mGnRH-specific and cGnRH-II-specific antisera to search for GnRH(s) in a human placenta.

The primate study was done in collaboration with Dr. E. Terasawa and L.A. Abler at the University of Wisconsin and Dr. Urbanski at the University of Oregon. The primate brains used in this study were collected at the University of Wisconsin and sent to the University of Victoria.

Materials and Methods

Animals and tissue preparation

Stumptail (*Macaca speciosa*) and rhesus monkeys (*Macaca mulatta*), were born and raised at the University of Wisconsin Regional Primate Research Center (for immunocytochemical or chromatographic studies) or at the University of Oregon Regional Primate Center (for physiological studies). The animal protocols of these studies were

reviewed and approved by the institutional Animal Care Committee at the University of Wisconsin or at the University of Oregon. All experiments in this study were conducted under the guidelines established by the NIH and USDA.

Adult whole brains

For chromatographic studies, whole brains (one male at 19, two females at 16 and 14 years of age) were removed by necropsy from three adult stumptail monkeys. The brains (total mass= 297g) were quickly frozen and stored at -80 C. Later, the brains were treated and the proteins extracted as described in Chapter 2. The brain extract was reduced in volume in a vacuum centrifuge to approximately 3 ml and filtered through a 45 μm filter.

Fetal brains

Fetal brains were removed from five rhesus monkey fetuses on 72-125 embryonic days (two females at E75, E125, three males at E72, E76, and E77). Fetuses were delivered by cesarian section. The brains were removed, quickly frozen and stored at -80 C. Four of the brains (total mass=18.7g), were pooled (E72, E75, E76 and E77) and treated as above for the adult whole brains. At a later date, the brain, 35.0g, from the E125 fetus was prepared and treated in the same way as above.

Dissected adult brain

A dissected brain was prepared from one adult stumptail monkey (male, 19 years of age). The brain was collected, quickly frozen and later partially thawed to allow sectioning into three pieces using a razor knife. First, the brainstem region, 14.0 g, was removed with a single cut. Second, the diencephalic region, 7.7 g, (including the thalamus, hypothalamus and pituitary stalk) was cut from the telencephalon. Third, the remaining brain tissues, 68.0 g, including the telencephalon with frontal cortex, parietal lobes, temporal lobes, occipital lobes and cerebellum were combined. Each region was treated as above, with the same extraction, HPLC and RIA procedures as for the whole brain studies.

Placenta

One human placenta was collected from the U.B.C. hospital, Vancouver, B.C., after patient consent was obtained. The tissue was quickly frozen, and brought on dry ice to the University of Victoria and treated as described above for adult monkey brains. The placenta was collected after a live birth, following a full-term pregnancy.

Adult monkey brains: immunocytochemistry

For immunocytochemistry, two adult female rhesus monkeys kept under the housing conditions described previously (Saitoh *et al.*, 1995) and four rhesus monkey fetuses at E34, E50, E70, and E85 from time-mated pregnancies (Terasawa *et al.*, 1993) were used in this study. Adult monkeys were anesthetized with ketamine and pentobarbital sodium (50mg/kg), perfused with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.6) and then the brain was immersed in the same solution for several hours. The brainstem was removed and placed in 30% sucrose in PBS for 2-3 days (Saitoh *et al.*, 1995). Meninges were carefully removed under a stereomicroscope, and frozen serial sections were cut at 50 μm in the frontal plane.

Fetal monkey brains: immunocytochemistry

Fetuses were delivered by cesarian section under halothane anesthesia. The whole body (fetuses at E34) or brain (fetuses at E50 and E70) were immersed in 4% paraformaldehyde in PBS (pH 7.6) for 1-5 hours, time varying with tissue size. The oldest fetus (E85) was perfused with 4% paraformaldehyde and the brain was then immersed in the same solution for 4h. None of these monkeys was alive after delivery. After fixation, tissue was placed in 30% sucrose in PBS (pH 7.6) until fully saturated as evidenced by sinking. Tissue was then frozen and sectioned by cryostat at 15 μm either in the sagittal plane (E34) or in the frontal plane (E50, E70 and E85), and thaw-mounted onto gelatin-coated slides. Slides were stored at -20^{0}C until immunostaining.

Adult monkeys: physiology

Six adult female rhesus macaques (6-9 years of age) were used for the *in vivo* study.

High performance liquid chromatography (HPLC)

Monkey brain extracts

An HPLC column and HPLC system similar to the one described in Chapter 2 was used in this study. For a blank run, two consecutive 600 μl volumes of Milli-Q water were loaded at 2 min intervals onto the C-18 column and assayed for irGnRH in RIA as described in Chapter 2. A blank run was repeated between each application of brain extract to ensure that the column was free of any contaminating residual GnRH from previous HPLC analyses. GnRH standards had never been previously injected onto this column, also ensuring that results were not affected by any residual synthetic GnRH peptides.

The injection volumes were 3 ml or less and were applied as multiple injections of up to 700 μl each. These repeated injections were at 2 min intervals onto a 1 ml loop; the material in the loop was loaded onto the column using the TEAF HPLC program described in Chapter 3, Table 3-3. A 100 μl aliquot was removed from each of the sixty collected consecutive fractions and assayed for GnRH immunoreactivity as decsribed in Chapter 2.

Placental extracts

The human placental extracts were treated exactly as decribed above except they were semi-purified by SepPak (as described in Chapter 1) prior to their application onto the HPLC column. The HPLC column was washed extensively between successive applications of tissue: also, blank samples of Milli-Q water were applied betweeen each sample, eluted and assayed as described previously, to ensure that there was no contaminating GnRH-like material from the previous HPLC.

Standards

Synthetic standards were applied to the column after brain extracts were completed as described in Chapter 3. The elution positions of the standards on the chromatograph

were confirmed by absorbance peaks (A= 280 nm) and by GnRH-specific RIA.

Radioimmunoassay

The details of the RIA are reported elsewhere (Sherwood *et al.*, 1986) and described in chapter 2. Essentially, 100 μl aliquots of each semi-pure fraction from the different HPLC runs were assayed for GnRH immunoreactivity using various antisera and ^{125}I-labelled synthetic GnRH tracers in a competitive RIA. In fractions where tracer binding exceeded detection limits (B/Bo= 80%; Table 3-3), 50 μl aliquots were diluted serially and the value closest to 50% B/Bo was used to estimate the quantity of irGnRH present.

Immunocytochemistry

Adult brain sections were immunostained with a free-float method (Saitoh *et al.*, 1995) whereas fetal brain sections were stained on the slide, as described previously (Quanbeck *et al.*, 1997). Endogenous peroxidase was deactivated in sections by washing with PBS (pH 7.6) for 1 hour (4X15min) and treating with a 0.03% hydrogen peroxide in methanol solution. To further remove non-specific background staining, sections were washed with PBS for 1 hour (4X15min) and then blocked with a 0.5% normal goat serum in PBS for two hours (2X 60min). The sections were then exposed to antisera specific to chicken GnRH-II, antiserum 675 at 1: 2,500-5,000 dilution (J.A. King and R. Millar) or Adams-100 at 1: 5,000-10,000 dilution. A small number of sections were also exposed to antiserum GF-6 at 1: 6,000 dilution (our laboratory) or antiserum LR-1 at 1: 10,000 dilution (a gift from R. Benoit, University of Montreal). The sections were then incubated at 0-40°C for 40h. Sections were washed with PBS for one hour (4X15min) and exposed to the second antibody (biotinylated goat anti-rabbit IgG, Vector Labs, Burlingame, CA) for 1.5 hours at room temperature. This was followed by a PBS wash (2X15min) and exposure to avidin-biotin peroxidase complex solution (ABC, Vector Labs, Burlingame, CA) for 1.5 hours. After a final wash with a 0.05M Tris-buffered saline solution (TBS, pH 7.6, 2X15min), the final reaction product was visualized with a 3,3'-diaminobenzidine

(DAB) solution (0.5% DAB with 0.06% hydrogen peroxide in 0.1 M TBS at pH 7.6). Adult brain sections were mounted on gelatin-coated slides. All sections were coverslipped with glycerol jelly.

For specificity testing, each antiserum was preabsorbed with mammalian or chicken GnRH-II at concentrations ranging from 1-200 μg/ml for 24 hours prior to application. In addition, to eliminate the possibility of non-specific reactions with the second antibody, the primary antibody was omitted in a few cases.

Physiology

Effects of cGnRH-II on LH release were examined in six adult female rhesus monkeys. The synthetic peptide (Peninsula Laboratories, Belmont, CA) was infused intravenously, either during the mid-follicular phase of the menstrual cycle (Day 6 on average) or during the luteal phase (i.e. after the mid-cycle pre-ovulatory surge). Blood samples (1ml) were collected from conscious, unrestrained animals using a remote blood sampling system as described (Urbanski *et al.*, 1997). The assay procedure has been reported (Ellinwood and Resko, 1980). The same animals had been used to determine the effect of mGnRH (50 ng/kg BW, i.v.) on plasma LH concentrations during the mid-follicular and luteal phase of the menstrual cycle; these findings have been reported (Urbanski *et al.*, 1997). Plasma LH levels were measured using a mouse Leydig cell bioassay and the results were expressed in terms of the cynomolgus LH RP-I standard. The assay detection limit was 3 ng/ml; intra-assay coeffecient of variation was 12%; and inter-assay coeffecient of variation was 20%. Effects of synthetic mGnRH, 50 ng/kg, on LH release were similarly examined in the same monkeys for comparative purposes. At a later date, the *in vivo* chicken GnRH-II experiments were repeated exactly as above but using a 2,000 ng/kg dose. Mean plasma LH concentrations were analysed by ANOVA, followed by the Student-Newman-Keuls test.

Results

Cross-reactivity of antisera

The cross-reactivity and characteristics of the antisera used in this chapter, GF-4, B-6, 7CR-10 and Adams-100, are shown in Chapter 3, Table 3-3, Fig. 3-1A, 1B). The cross reactivity with 11 different GnRH peptides is shown for GF-4 and B-6 in Fig 3 -1A and for 7CR-10 and Adams-100 in Fig. 3-1B. It is clear that B-6 is specific for mGnRH (Fig. 3-1A); Adams-100 and 7CR-10 are nearly specific for cGnRH-II (Fig. 3-1B); and GF-4 cross-reacts with many forms of GnRH such as mGnRH, hydroxy-Pro^9-mGnRH , sbGnRH, cGnRH-I, sGnRH, cGnRH-II, dfGnRH, cfGnRH and lGnRH-III.

Adult whole brain

With antisera that detect mGnRH, GF-4 detected 22.2 ng and B-6 detected 21.7 ng of immunoreactive GnRH in fraction 22 (Fig. 5-1, Table 3-3). With cGnRH-II specific antisera, 7CR-10 detected 7.9 ng and Adams-100 detected 2.6 ng of irGnRH (Table 5-1). Both antisera detected this immunoreactivity in HPLC elution positions 26 and 27 (Fig. 5-2). Synthetic cGnRH-II eluted in fraction 26 under the same conditions. The specific antisera, B-6 (for mGnRH) and Adams-100 (for cGnRH-II), detected 73.1 and 8.8 pg irGnRH per gram of whole brain tissue, respectively. Thus, there was 8 times more mGnRH than cGnRH-II at the time of brain collection as measured by these antisera. The immunoreactivity detected by 7CR-10 in early eluting fractions was significantly reduced as shown after buffering of HPLC fractions to pH 6 with 5 M NaOH before assaying (Fig. 5-2).

Our mammalian-specific antiserum, B-6, also showed GnRH immunoreactivity in fractions 16-21 (Fig. 5-1). This immunoreactivity does not appear to be hydroxy-Pro^9-mGnRH because B-7 (the next bleed from the same rabbit and an antibody with identical characteristics as B-6), has only 1% cross-reactivity with synthetic hydroxy-Pro^9-mGnRH, compared to mGnRH.

Table 5 -1 : Amount (ng) of immunoreactive GnRH in primate brain

	mGnRH		cGnRH-II	
Whole Brain	[1] **GF-4**	**B-6 or B-7**	**7CR-10**	**Adams-100**
Adult (♀14,16yr ♂19yr)	22.2 ng	[2] 21.7 ng (73.1 pg/g)	7.9 ng	2.6 ng (8.8 pg/g)
Fetal (♀E75 ♂E72, E76, E77)	4.3 ng	3.0 ng (160.0 pg/g)	[3] ND	ND
Fetal (♀ E125)	9.2 ng	23.0 ng (657.1 pg/g)	0.4 ng	0.5 ng (12.9 pg/g)
Dissected Brain (Adult ♂ 19yr)				
Diencephalon	19.1 ng	4.7 ng (610.4 pg/g)	0.9 ng	2.2 ng (285.7 pg/g)
Forebrain-cortex -cerebellum	0.8 ng	0.9 ng (13.2 pg/g)	0.5 ng	0.3 ng (4.4 pg/g)
Brainstem	1.4 ng	2.2 ng (157.1 pg/g)	0.7 ng	0.7 ng (50.0 pg/g)

1 Antisera are GF-4, B-6, 7CR-10 and Adams-100.
2 Values in parentheses (pg/g) represent the irGnRH concentration detected by the most specific antiserum (B-6 or B-7 and Adams-100) in those particular brain extracts.
3 ND= not done.

Fig. 5 -1. irGnRH in adult stumptail monkey brain extracts showing HPLC elution positions. Antisera are (A) GF-4 and (B) B-6. Small arrows above HPLC elution profiles represent the different forms of GnRH detected by that specific antiserum as well as where those synthetic forms would elute under the same HPLC conditions. m-Hyp^{9} = hydroxyproline-^{9}-mammalian GnRH, **m**= mammalian GnRH, c-I= chicken GnRH-I, sb= sea bream GnRH, c-II= chicken GnRH-II, and s= salmon GnRH.

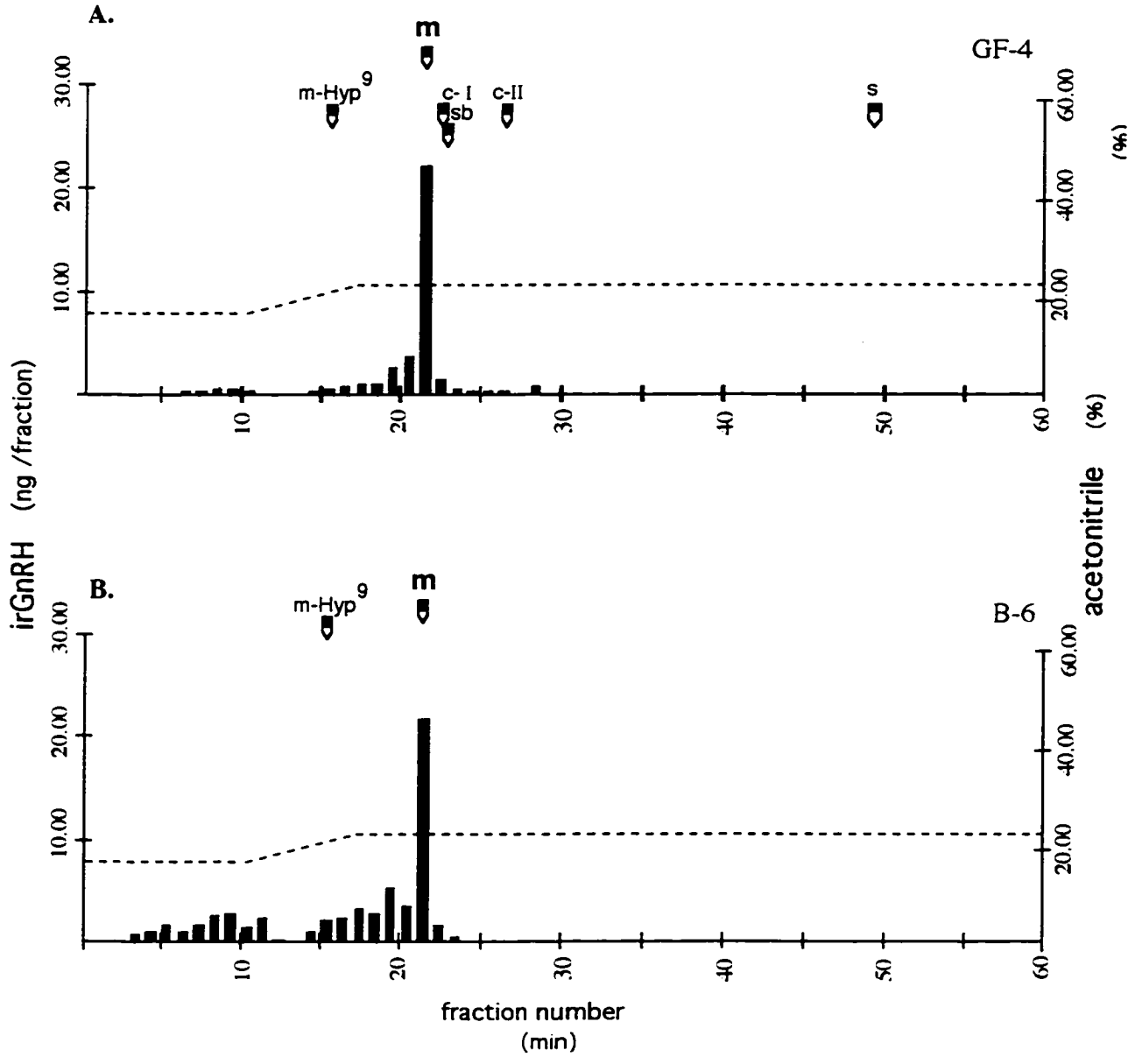
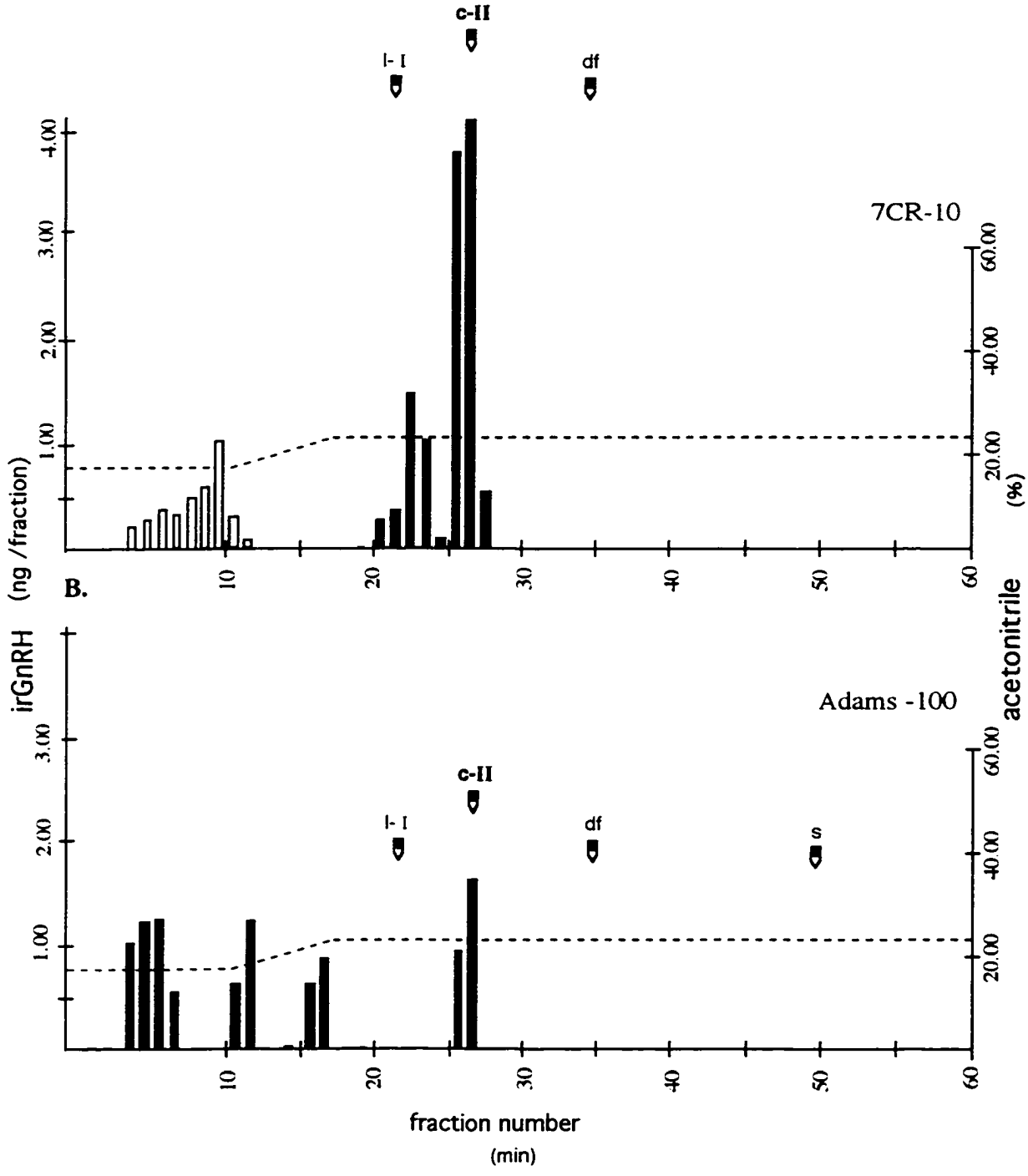

A.
m
GF-4
m-Hyp9
c-I
c-II
sb
s
30.00
20.00
10.00
60.00
40.00
20.00
irGnRH (ng /fraction)
acetonitrile (%)
10
20
30
40
50
60
B.
m-Hyp9
m
B-6
fraction number
(min)

Fig. 5 -2. irGnRH in adult stumptail monkey brain extracts showing HPLC elution positions. Antisera were (A) 7CR-10 and (B) Adams-100. In the assay with 7CR-10, open bars represent immunoreactivity in RIA tubes in which the final pH was changed to 6 with 5M NaOH. Changing the pH did not affect the assay with Adams-100 antiserum. Small arrows above HPLC elution profiles represent the different forms of GnRH detected by that specific antiserum as well as where those synthetic forms elute under the same HPLC conditions. l-I= lamprey GnRH-I, c-II= chicken GnRH-II, df= dogfish GnRH, s= salmon GnRH

A.


c-II
l- I
df
7CR-10
4.00
3.00
2.00
1.00
(ng /fraction)
60.00
40.00
20.00
(%)
10
20
30
40
50
60
B.
irGnRH
Adams -100
acetonitrile
c-II
l- I
df
s
3.00
2.00
1.00
60.00
40.00
20.00
fraction number
(min)

Fetal whole brain

IrGnRH, 4.3 ng, was detected in fractions 20 and 21 using antiserum GF-4 (Fig. 5-3). The antiserum (B-7) that is more specific for mGnRH than GF-4 detected 3 ng of mGnRH, 160 pg/g whole brain tissue (pooled samples of E72, E75, E76, E77), in the same fractions. There was 160 pg irGnRH per gram of fetal brain tissue compared to 73.1 pg/g adult brain tissue as detected by B-7 or B-6. Analysis of another fetal brain (E125) also showed immunoreactivity in elution positions 20 and 21 as detected by GF-4 and B-7 confirming the presence of mGnRH (data not shown). There was much more irGnRH detected in this fetal brain extract, 657.1 pg/g, than in the previous pooled fetal brain extracts, 160.0 pg/g, or adult brain extracts, 73.1 pg/g. Also the cGnRH-II specific antisera, 7CR-10 and Adams-100, detected immunoreactivity in positions 25 and 26 (Fig. 5-4). Adams-100 detected 0.45 ng cGnRH-II-like immunoreactivity,whereas 7CR-10 detected 0.37 ng. Therefore, there was 12.9 pg cGnRH-II per gram of fetal (E125) brain tissue as detected by Adams-100 antiserum. If compared to adult rhesus brains (8.8 pg cGnRH-II per gram tissue) there was a 1.5 fold increase in concentration of cGnRH-II-like immunoreactivity in fetal rhesus monkeys.

Fig. 5-3. mGnRH in fetal (E72-E77) rhesus monkey after HPLC elution. The antisera were (A) GF-4 and (B) B-7. Small arrows above the HPLC elution profles represent the elution positions of synthetic peptides under the same HPLC conditions. The dashed line indicates the pecentage acetontrile used for elution.

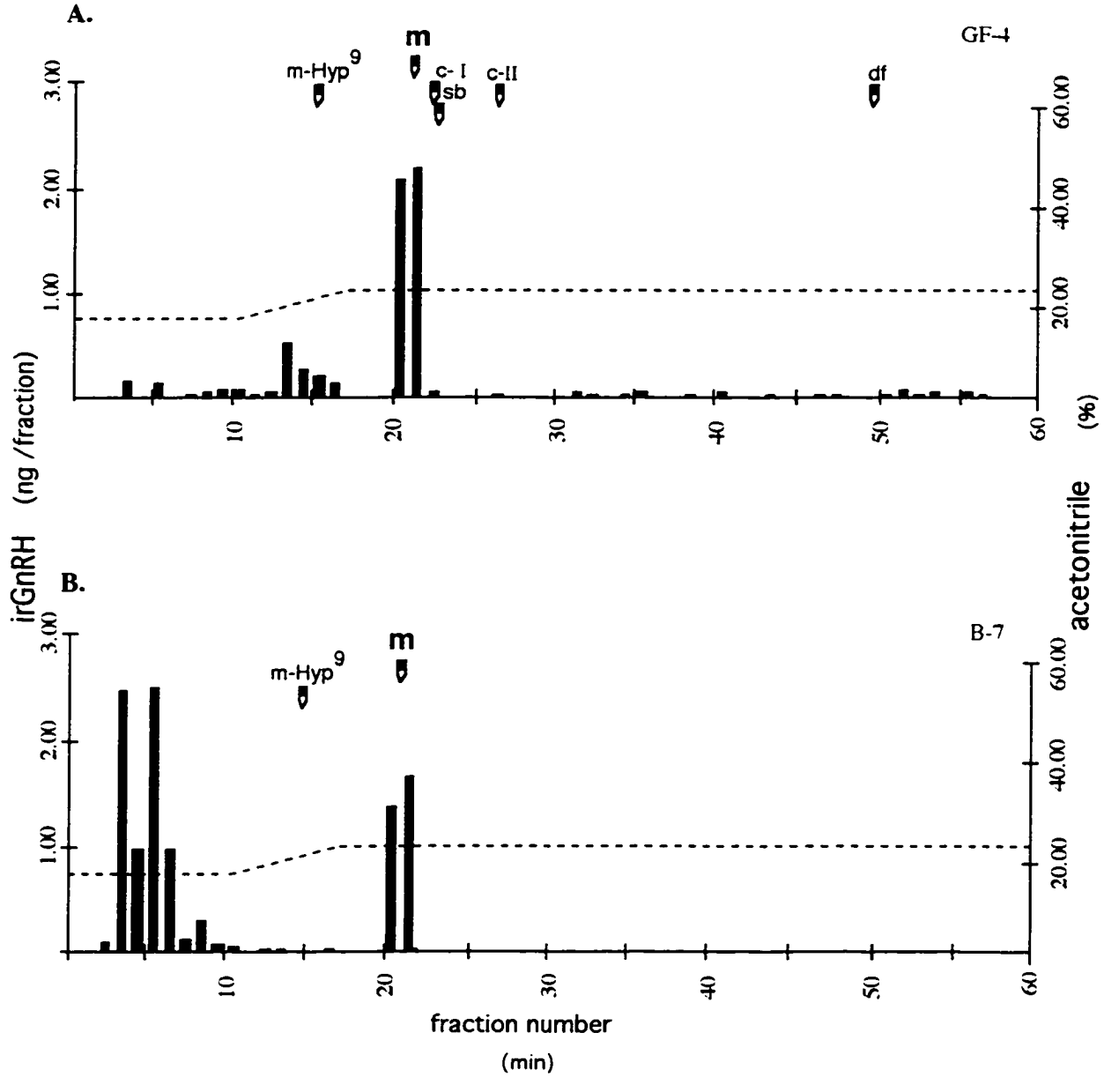
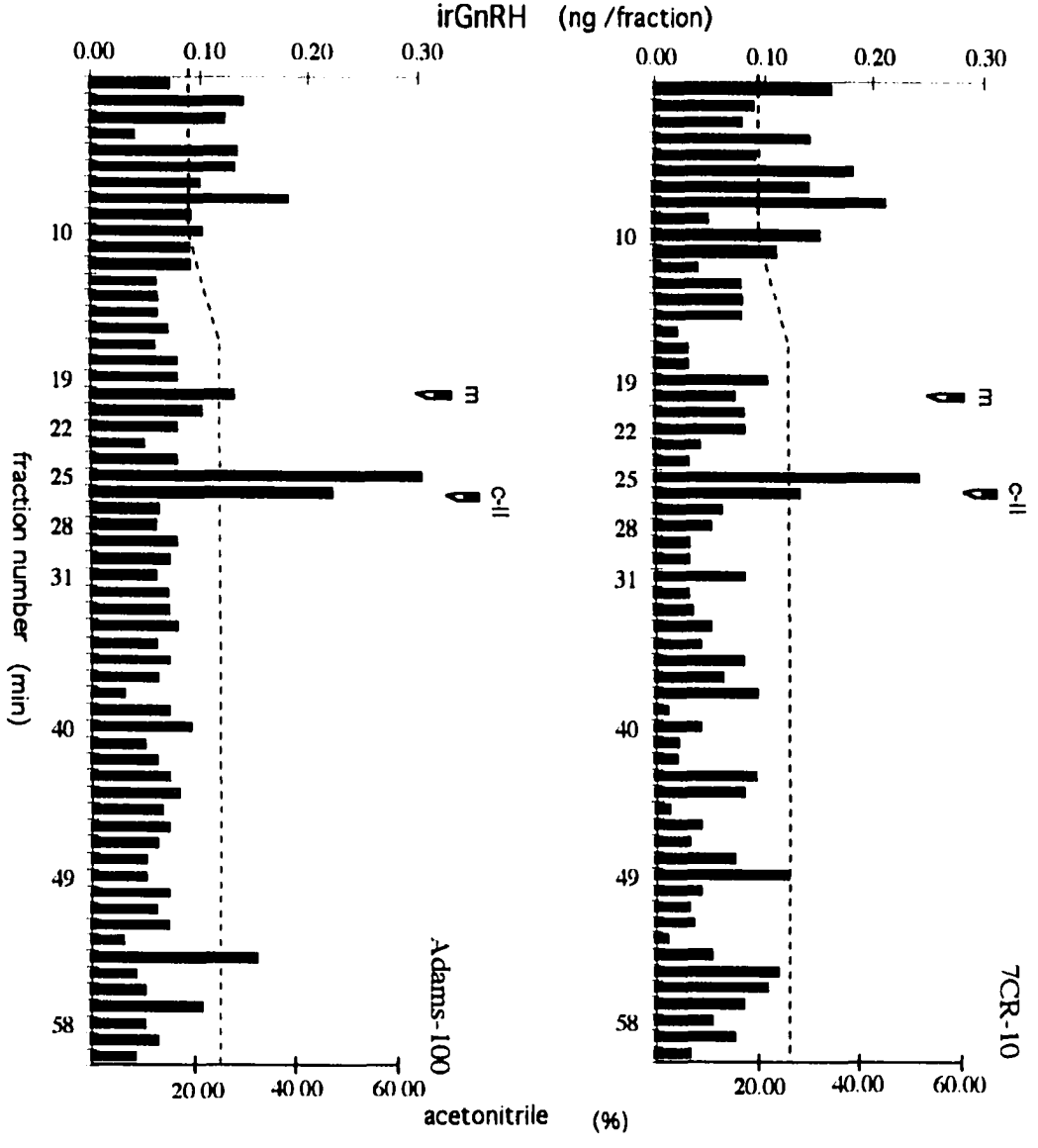

A.
m
GF-4
m-Hyp9
c- I
sb
c-II
df
3.00
2.00
1.00
60.00
40.00
20.00
10
20
30
40
50
60
(%)
irGnRH (ng /fraction)
acetonitrile
B.
B-7
m
m-Hyp9
fraction number
(min)

Fig. 5-4. cGnRH-II in fetal (E125) rhesus monkey brain extracts after HPLC elution. Antisera were (A) 7CR-10 and (B) Adams-100. Small arrows above the HPLC elution profles represent the elution positions of synthetic peptides under the same HPLC conditions. The dashed line indicates the pecentage acetontrile used for elution.


irGnRH (ng /fraction)
0.00
0.10
0.20
0.30
10
19
22
25
28
31
40
49
58
fraction number (min)
m
c-II
Adams-100
20.00
40.00
60.00
acetonitrile (%)
7CR-10

Dissected adult brain

All three dissected parts of the brain (diencephalon, forebrain-cortex-cerebellum, and brainstem) contained GnRH-like immunoreactivity primarily in fractions 20-21 (Fig. 5-5) and 26 (7CR-10) or 26 and 27 (Adams-100) (Fig. 5-6) corresponding to the elution positions of synthetic mGnRH and cGnRH-II, respectively. RIA analysis with antisera GF-4 detected 19.1 ng irGnRH in the diencephalon, 0.8 ng immunoreactivity in the cortex region, and 1.4 ng irGnRH in the brainstem (Fig. 5-5A). The more specific mammalian antiserum, B-6, detected 4.7, 0.9, and 2.2 ng immunoreactive GnRH per fraction in the same three brain regions, respectively (Fig. 5-5B). Fraction 26 contained 0.9, 0.5 and 0.7 ng irGnRH as detected by the 7CR-10 antiserum (Fig. 5-6A) and 2.2, 0.3 and 0.7 ng/fraction immunoreactivity as detected by antiserum Adams-100 (Fig. 5-6B). Quantitatively, B-6 detected more irGnRH /gram brain tissue, 610.4 pg/g, in the diencephalon, 13.2 pg/g in the cortex, and 157.1 pg/g in the brainstem. These results suggest a relative abundance of mGnRH in the diencephalon. Antiserum Adams-100 detected 285.7 pg/g, 4.4 pg/g, and 50.0 pg/g immunoreactivity in the diencephalon, cortex, and brainstem, respectively. Hence, immunoreactive mGnRH and ir-cGnRH-II are more concentrated in the diencephalon than in the brainstem or the cortical regions.

Fig. 5-5. HPLC elution of mGnRH from adult stumptail monkey brain extracts from the diencephalon, cortex and brainstem regions. Antisera were (A) GF-4 and (B) B-6. The pH of each fraction was adjusted to 6 with 5M NaOH before assaying for irGnRH. Small arrows above the HPLC elution profles represent the elution positions of synthetic peptides under the same HPLC conditions. The dashed line indicates the percentage acetonitrile used for elution.

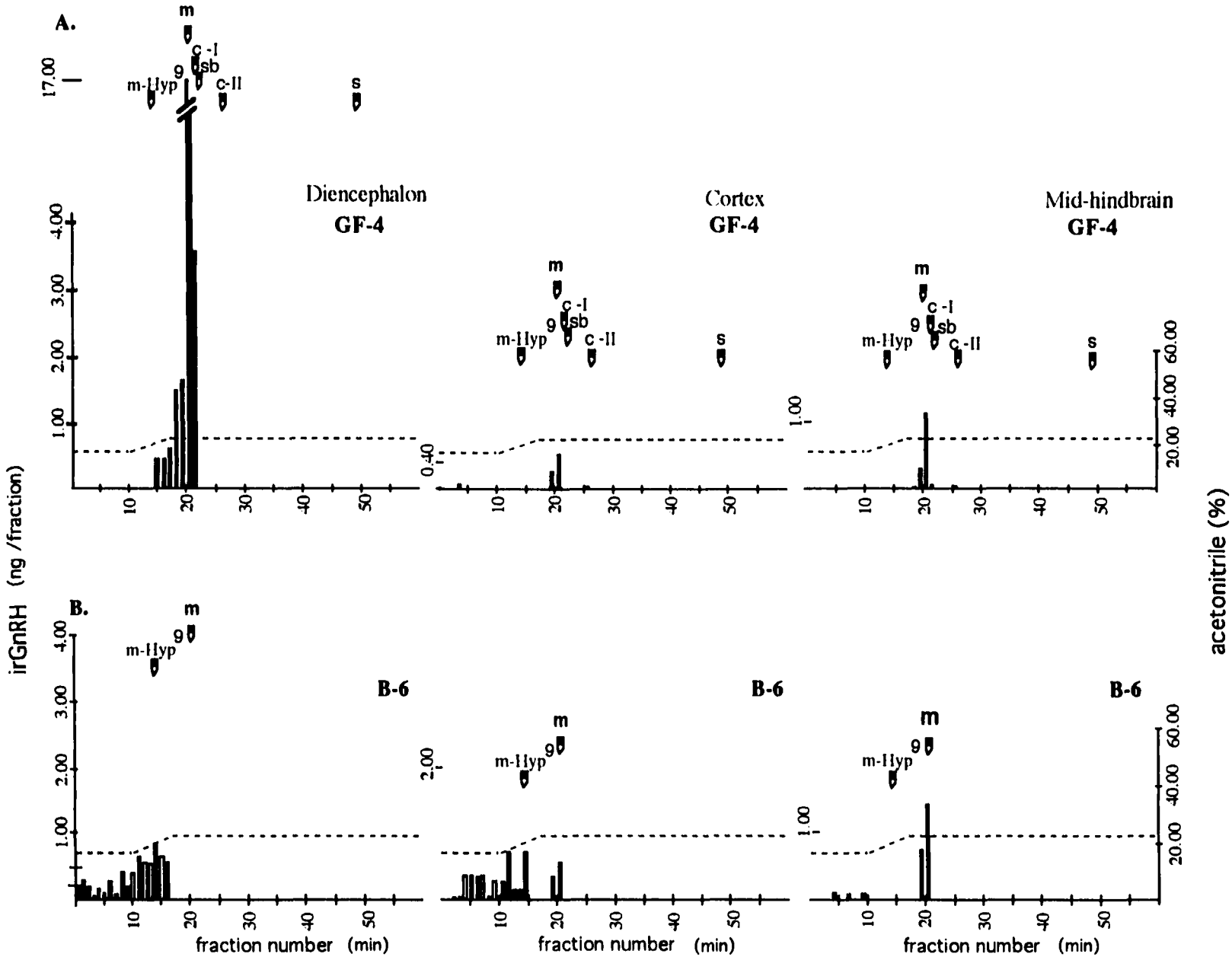
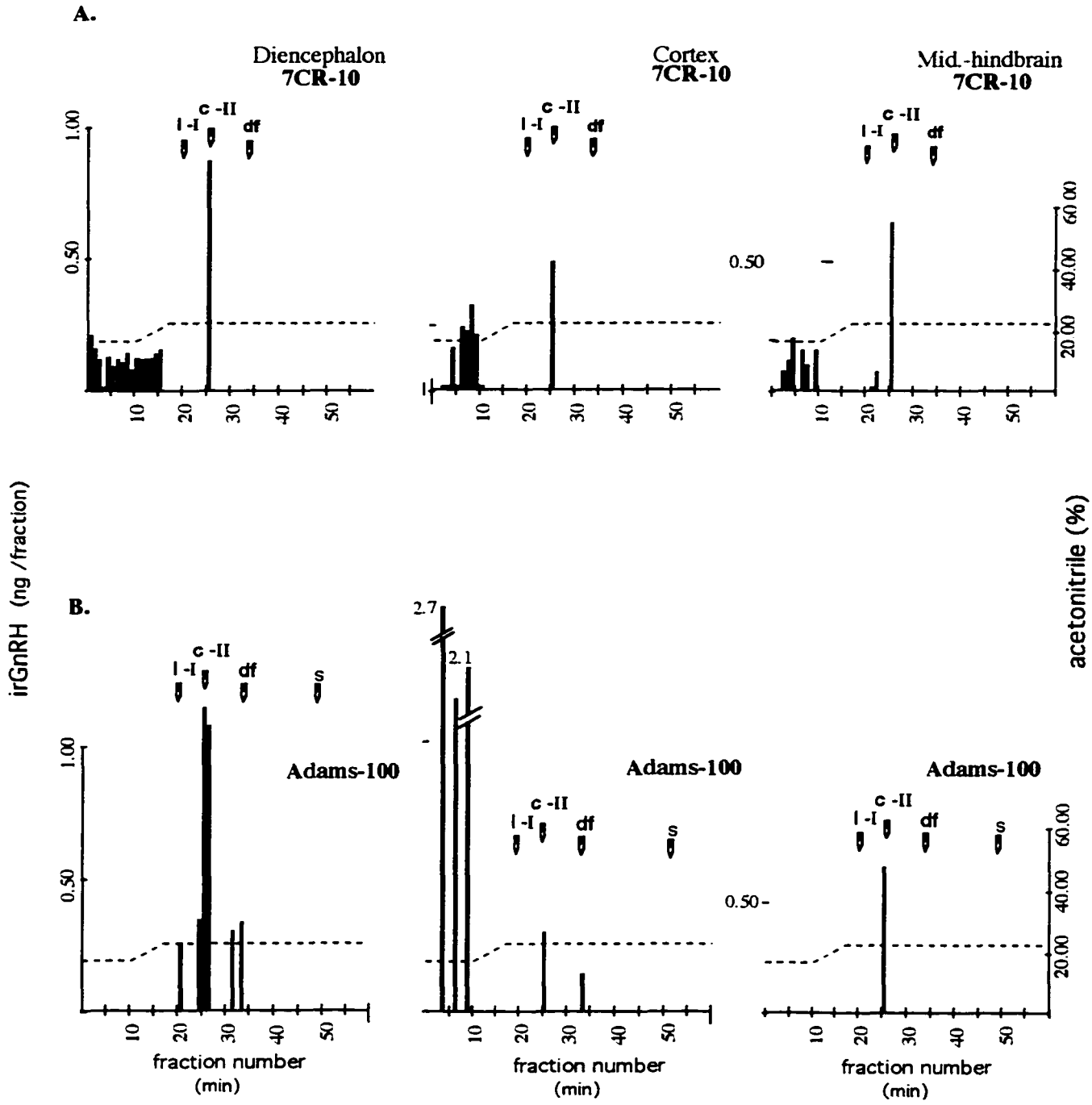

A.
B.
m
c-I
sb
9
m-Hyp
c-II
s
Diencephalon
GF-4
Cortex
GF-4
Mid-hindbrain
GF-4
B-6
irGnRH (ng /fraction)
acetonitrile (%)
fraction number (min)

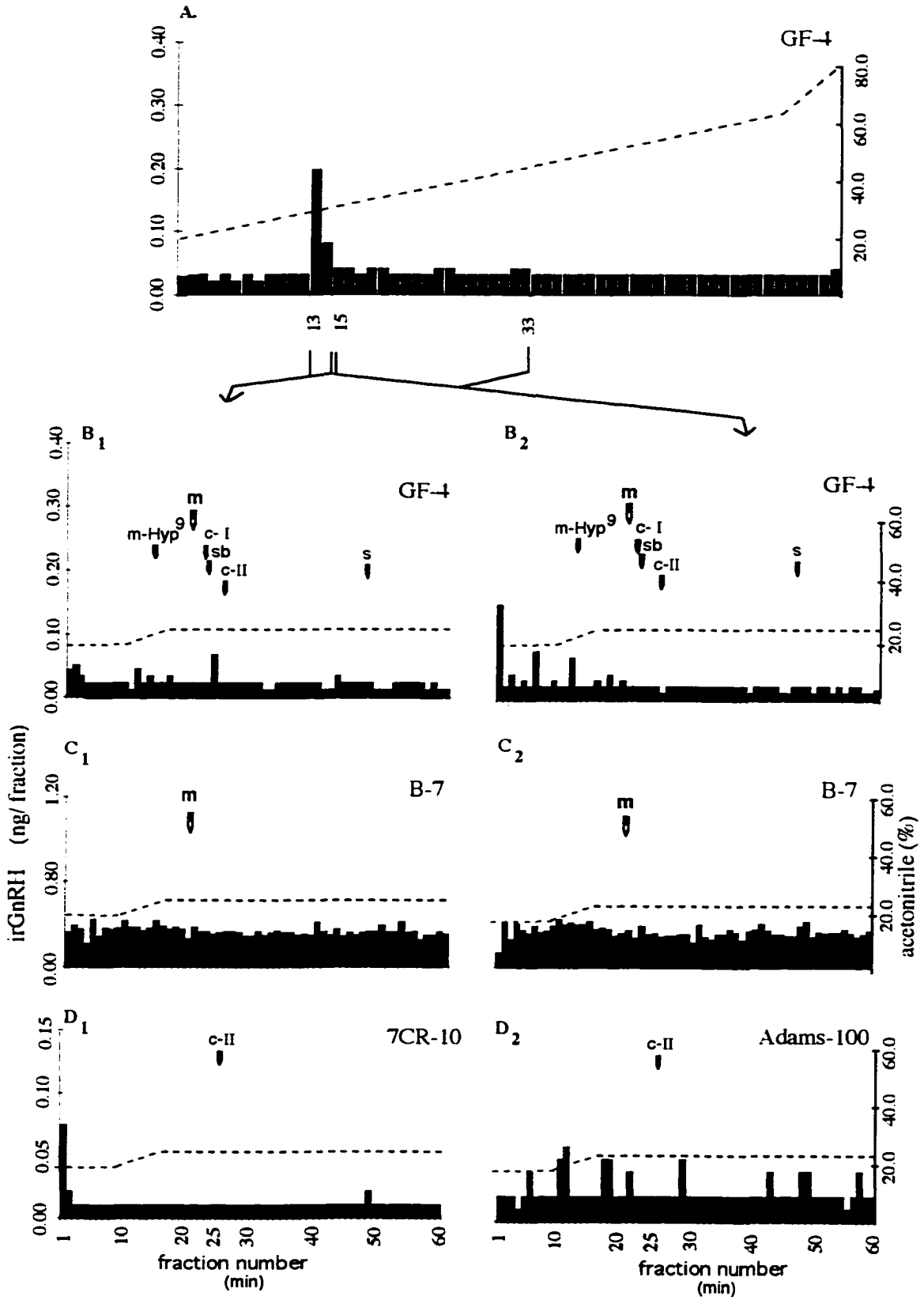
Fig. 5-6. HPLC elution of cGnRH-II in adult stumptail monkey brain extracts from the diencephalon, cortex and brainstem regions. Antisera were (A) 7CR-10 and (B) Adams-100. In all fractions, the pH was adjusted to 6 with 5M NaOH before assaying for irGnRH. Small arrows above the HPLC elution profles represent the elution positions of synthetic peptides under the same HPLC conditions. The dashed line indicates the pecentage acetonitrile used for elution.


A.
Diencephalon
7CR-10
Cortex
7CR-10
Mid.-hindbrain
7CR-10
c -II
I -I
df
1.00
0.50
60.00
40.00
20.00
10
20
30
40
50
B.
s
Adams-100
2.7
2.1
irGnRH (ng /fraction)
acetonitrile (%)
fraction number
(min)

Placenta

Only a small amount, 0.27 ng, of irGnRH was detected from the SepPak column in fractions 13 and 14 using antiserum GF-4 following elution of the human placental extracts (Fig. 5-7A). These two fractions were combined, vacuum-concentrated, injected onto the C-18 HPLC column, and eluted using the TEAF 17-24% HPLC program. Antiserum GF-4 only detected 0.06 ngs of irGnRH in fraction 24, and scattered amounts throughout the remaining sixty HPLC fractions (Fig. 5-7B). Further RIAs using antisera B-7 (Fig. 5-$7C_1$) and 7CR-10 (Fig. 5-$7D_1$) did not detect either mGnRH-like or cGnRH-II-like immunoreactivity. RIAs using antisera GF-4, B-7 and Adams-100 also did not detect irGnRH in the elution positions where the expected forms, mGnRH and cGnRH-II, would elute after SepPak fractions 15-33 were combined and eluted from the C-18 column using TEAF 17-24% (Fig. 5-$7B_2$, C_2, D_2, respectively). The quantities of irGnRH detected by GF-4 after elution from the C-18 HPLC column were too small (Fig. 5-$7B_1$, B_2) to suggest that additional forms of GnRH were present in the placental extracts.

Fig. 5-7. Immunoreactive GnRH in human placental extracts after HPLC elution. Small arrows above the HPLC elution profiles represent the elution position of synthetic peptides under similar HPLC conditions. Abbreviations are: m-Hyp9, hydroxyproline-^{9}-mammalian GnRH; m, mammalian GnRH; c-I, chicken GnRH-I; sb, sea bream GnRH; c-II, chicken GnRH-II and s, salmon GnRH. Dashed line represents the percent acetonitrile used in elution. irGnRH was detected by (A) antiserum GF-4 after elution from a SepPak column, (B_1) antiserum GF-4 after elution of SepPak fractions 13-15 from a C-18 column, (B_2) antiserum GF-4 after elution of SepPak fractions 16-33 from a C-18 column, (C_1) antiserum B-7 after elution of SepPak fractions 13-15 from a C-18 column, (C_2) antiserum B-7 after elution of SepPak fractions 16-33 from a C-18 column, (D_1) antiserum 7CR-10 after elution of SepPak fractions 13-15 from a C-18 column, (D_2) antiserum Adams-100 after elution of SepPak fractions 16-33 from a C-18 column.

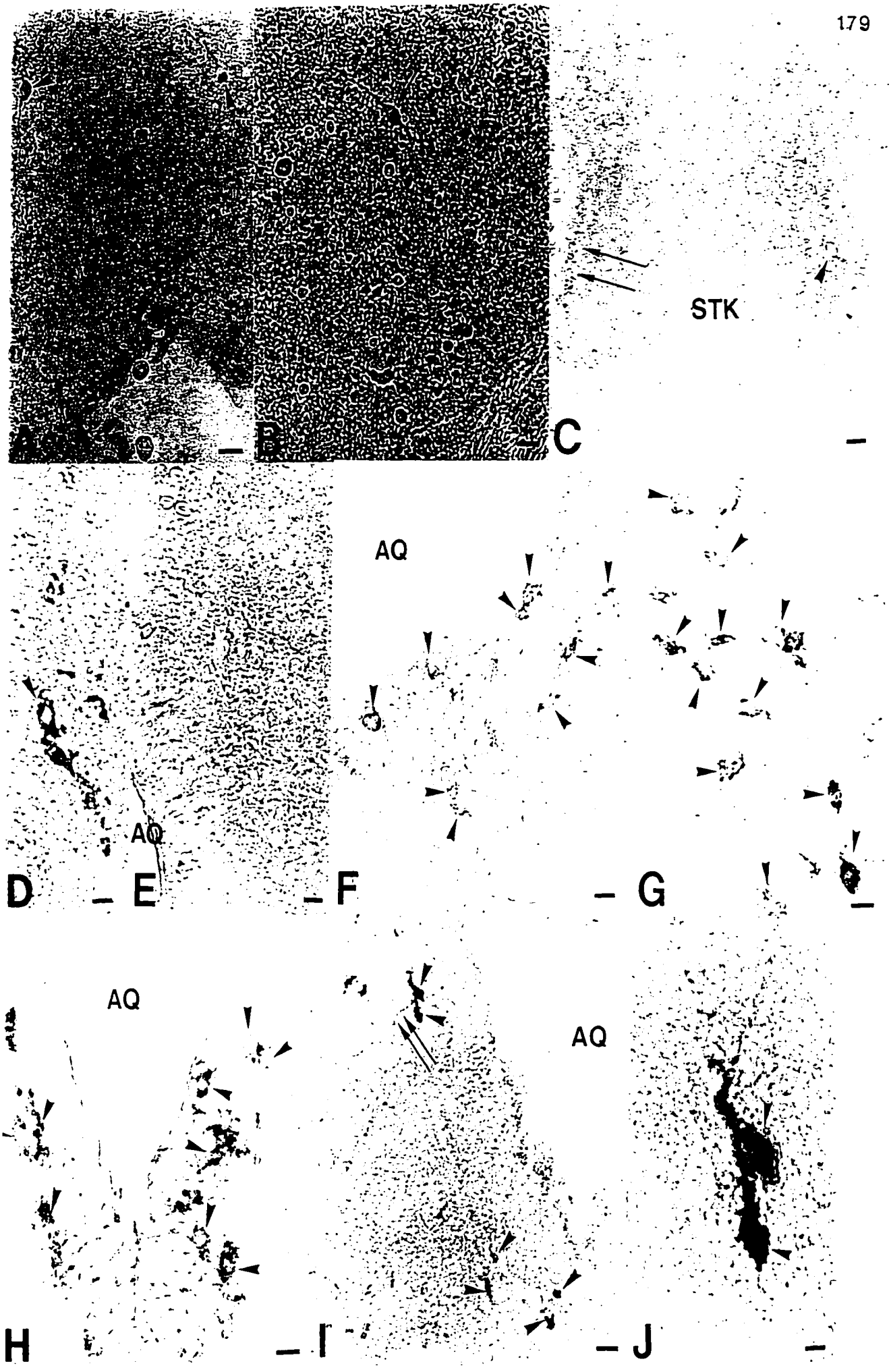

A
GF-4
0.00
0.10
0.20
0.30
0.40
20.0
40.0
60.0
80.0
13
15
33
B1
B2
GF-4
m
m-Hyp9
c-I
sb
c-II
s
C1
C2
B-7
irGnRH (ng/fraction)
acetonitrile (%)
0.00
0.80
1.20
D1
D2
7CR-10
Adams-100
0.00
0.05
0.10
0.15
1
10
20
25
30
40
50
60
fraction number
(min)

Immunocytochemistry

In the fetuses at stages E50, E70 and E85, chicken GnRH-II positive cells were seen in the basal region of the posterior hypothalamus, median eminence, pituitary stalk, and periaqueductal regions in the midbrain (Fig.5-8A, C, D, F, G, H) and chicken GnRH-II positive fibers were observed in the basal hypothalamus and pituitary stalk (Fig. 5-8A, C). In the adult brainstem, chicken GnRH-II positive cells were distributed in the periaqueductal regions (Fig. 5-8I, J). Chicken GnRH-II positive cells were found in the periventricular region of the posterior hypothalamus in the E34 fetus (not shown). Generally, chicken GnRH-II positive cells (~17 μm x 10 μm) were round in shape and smaller in size (Fig. 5-8A, C, F, G and H) than mammalian GnRH positive cells (22 μm x 10 μm), which had a fusiform shape (Fig. 5-8B). However, in the adult midbrain there were some large chicken GnRH-II cells (Fig. 5-8I and J). Although thick neurites were commonly observed for mammalian GnRH positive cells (Fig. 5-8B), generally chicken GnRH-II positive cells possessed fine short neurites (Fig. 5-8A, C and I).

Absorption tests indicated that tissues exposed to antiserum 675, which had been pretreated with chicken GnRH-II peptide (2μg/ml for 24h), did not have any immunostained cells (Fig. 5-8E). However, if the tissues were exposed to antiserum Adams-100, which had been pretreated with the chicken GnRH-II peptide (5-200 μg/ml for 24h), chicken GnRH-II positive cells were still present (not shown). Preabsorption with mGnRH did not alter immunoreactivity.

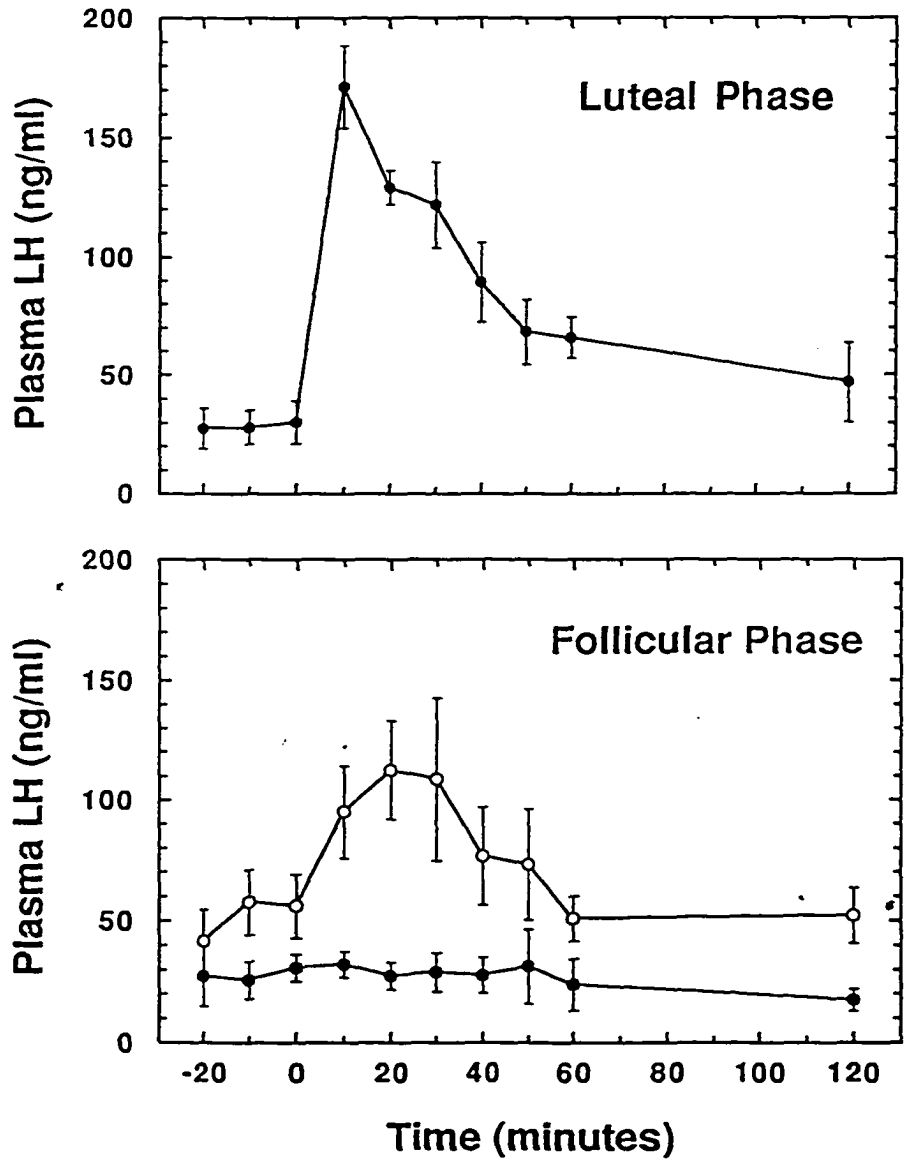
Fig. 5-8 Examples of immunostained cells for chicken GnRH-II, indicated by arrowheads (**A**, **C**, **D**, **F**, **G**, **H**, **I**, and **J**) or mammalian GnRH, indicated by arrows (**B**). In the medial basal hypothalamus (**A**) and the pituitary stalk (**C**) of the monkey fetus at E85, chicken GnRH-II immunopositive cells stained with antiserum 675 are seen. In the medial basal hypothalamus (**B**) of the adjacent section in the same monkey fetus, mammalian GnRH positive cells stained with antiserum GF-6 are also seen. The shape of mammalian GnRH positive cells is different from that of chicken GnRH-II positive cells. Chicken GnRH-II positive cells stained with antiserum 675 (**D**) or with antiserum Adams-100 (**F**, **G**, and **H**) are seen in the midbrain periaqueductal region of fetuses at E85 (**D**) at E50 (**F** and **G**). and at E70 (**H**). In the adjacent section of the fetus at E85 (**E**), there are no immunopositive cells with antiserum 675 when preabsorbed with chicken GnRH-II peptide. Chicken GnRH-II positive cells are seen in the adult midbrain of 2 monkeys (**I** and **J**). Two immunopositive cells shown in **I** were magnified in **J**. Chicken GnRH-II immunopositive fibers indicated by long double arrows are also seen in the pituitary stalk (**C**) and periaqueductal region (**I**). Abbreviations: AQ=aqueduct, STK=pituitary stalk. Scale bars: A, B, C, D, E, F, G and J = 20μm; I= 40μm; J= 10μm.


STK
AQ
AQ
AQ
AQ
A
B
C
D
E
F
G
H
I
J

Physiology

The intravenous administration of cGnRH-II (50 ng/kg BW) to rhesus macaques during the luteal phase of the menstrual cycle resulted in a marked increase ($P<0.01$) in plasma LH concentrations, which was sustained ($P<0.05$) for at least 40 min (Fig. 5-9). In contrast, the same dose of cGnRH-II failed to induce a significant increase ($P>0.05$) during the mid-follicular phase of the cycle. This finding is similar to that previously reported for differential effects of mGnRH on LH during the macaque's menstrual cycle (Urbanski *et al.*, 1997). A higher dose of cGnRH-II (2μg/kg BW) during the mid-follicular phase produced a higher plasma LH concentration ($P<0.05$) than that produced by 50 ng/kg BW, although the LH levels were still significantly lower than those induced by the 50ng/kg BW of cGnRH-II during the luteal phase ($P<0.01$).

Fig. 5-9. Effect of intravenous administration of cGnRH-II (at Time 0) on plasma LH concentrations in adult female rhesus macaques. Serial blood samples were collected from each animal either during the mid-follicular (lower panel) or luteal phase (upper panel) of the menstrual cycle. A significant LH increase was detected 10 min after administration of cGnRH-II (50ng/kg BW, solid symbols) during the luteal phase ($P<0.01$) but not during the mid-follicular phase ($P>0.05$); in the latter case, however, a higher dose of cGnRH-II (2μg/kg BW, open symbols) resulted in a significantly greater increase ($P<0.05$). Each point represents the mean LH concentration from six animals and the SEM are shown as vertical lines.


Luteal Phase
Follicular Phase
Plasma LH (ng/ml)
Plasma LH (ng/ml)
200
150
100
50
0
200
150
100
50
0
-20
0
20
40
60
80
100
120
Time (minutes)

Discussion

This is the first report that a second form of GnRH, with characteristics like cGnRH-II, is present within primate brains. HPLC elution position, as well as cross-reactivity with specific cGnRH-II antiserum in RIA and immunocytochemistry, suggest that the primate brain contains both mGnRH and a cGnRH-II-like molecule. In contrast, by the same criteria a full-term human placenta contained neither mGnRH nor cGnRH-II-like molecules.

cGnRH-II is conserved from jawed fish to primates

The chicken-II form of GnRH has been conserved in a variety of vertebrates, from the first jawed fish to primitive placental mammals. The primary structure for cGnRH-II has been obtained from chicken, alligator, frog, sea bream, tilapia, pacu, catfish, herring, dogfish, and ratfish (Powell *et al.*, 1996; Sherwood *et al.*, 1997). In addition, the sequence of the cDNA that encodes cGnRH-II is reported for several fish (Bogerd *et al.*, 1994; Gothilf *et al.*, 1996; White *et al.*, 1994) and the tree shrew (Rissman *et al.*, 1995). These data confirm the presence of cGnRH-II in representative members of each vertebrate class, except for jawless fish (Sherwood *et al.*, 1986; Sower *et al.*, 1996), and firmly establish it as the most conserved of the GnRH forms.

The combination of cGnRH-II and mGnRH in the brain of one species is not unique to primates, but has been detected for a variety of vertebrates. For example, mGnRH-like and cGnRH-II-like immunoreactivity in the brain has been shown in primitive bony fish like sturgeon (Lepretre *et al.*, 1993; this thesis, Chapter 2), reedfish and alligator gar (Sherwood *et al.*, 1991); teleosts like eels (King *et al.*, 1990); tetrapod ancestors like lungfish (Joss *et al.*, 1994) , amphibians like *Xenopus* (Conlon *et al.*, 1993) and *Ranid* frogs (Conlon, *et al.*, 1993; Licht *et al.*, 1994) and primitive placental mammals like musk shrew (Dellovade *et al.*, 1993).

cGnRH-II neurons in primates are conserved in location

I have shown using a combination of HPLC and RIA with specific antiserum that cGnRH-II-like and mGnRH-like immunoreactivity exist throughout the brain of stumptail and rhesus monkeys. Thus, primates are like other vertebrates in that cGnRH-II is distributed throughout the brain due to the wide distribution of axons. Immunocytochemistry also has shown that there are some cGnRH-II-like immunopositive cells and fibers in the basal hypothalamus, in close proximity to the hypophysial portal system. However, the relative scarcity of cGnRH-II-like immunopositive cells and fibers in the basal hypothalamus compared to mGnRH suggests that mGnRH functions as the primary gonadotropin releaser.

It is surprising that cGnRH-II has not been detected before in vertebrates such as sheep, rats and humans (King *et al.*, 1988). Differences in methodology, like specificity and sensitivity of cGnRH-II antisera or use of primate whole brain rather than just diencephalon, may have contributed to detection of this form. Alternatively, cGnRH-II may have a relatively small window of maximal expression and therefore, the timing of tissue collection may be crucial in its detection. Another technical problem is that our two cGnRH-II-specific antisera stained cells in the midbrain but one (Adams-100) was not blocked by the cGnRH-II peptide, whereas the other (675) was blocked. However, the specificity of cGnRH-II staining is established by antiserum 675 for the hypothalamus, pituitary stalk and midbrain as shown by Fig 8A, C, D, E, I and J.

The present study suggests that the GnRH systems in primates are not dissimilar from other vertebrates: i.e., there is 1) an anterior system of neurons that express one GnRH form (eg. mGnRH, sGnRH, cfGnRH, sbGnRH or cGnRH-I) with fibers projecting to the pituitary or median eminence for LH and FSH release, and 2) a posterior system of neurons that express cGnRH-II, with fibers projecting throughout the brain, including the hind-brain, posterior pituitary and spinal cord. In the musk shrew, cGnRH-II neurons are localized in a discrete cluster within the midbrain. Most of the fibers appear

to end in the medial habenula region, but some are widely scattered in the forebrain as well as in the median eminence, arcuate nucleus and infundibular stem (Rissman *et al.*, 1995). In contrast, mGnRH is found primarily in the forebrain (Dellovade *et al.*, 1993).

cGnRH-II is expressed early in development in primates

In the present study, cGnRH-II was detected as early as E125 (there was an insufficient quantity of the E72 -E77 brains for testing with Adams-100 or 7CR-10 antisera) by specific antisera with HPLC, and as early as E34 by immunocytochemistry. This timing of the origin of cGnRH-II-like cells is as early as that of the two populations of anterior mGnRH cells that migrate from the olfactory placode into the telencephalon and diencephalon in rhesus monkeys (Quanbeck *et al.*, 1997). The earliest migrating cells containing GnRH were detected in the telencephalon of the rhesus monkey as early as E30; the form of GnRH in the cells is detected only by GF-6. A recent prelimionayr study indicates that these cells contain fragments of GnRH (Teresawa *et al.*, 1997) but the molecular form is yet to be determined. These neurons ultimately settled in the lateral septum and amygdala, but not in the hypothalamus. The second type of cells containing mGnRH were detected by several antisera including GF-6; these cells originated at E32 - E36 and migrated into the forebrain at E38 -E42, about 1-2 weeks after the first type of cells. These late-migrating mGnRH neurons settle in the medial septum, preoptic area and anterior hypothalamus. Therefore, cGnRH-II positive cells do not originate in the olfactory placode. At present, the origin of cGnRH-II cells is unknown. One possibility is that the cGnRH-II neurons originate in the ventricular wall of the posterior hypothalamus and the mid-hindbrain area, and migrate only a short distance.

The function of cGnRH-II in all vertebrates remains an enigma

The fact that cGnRH-II has been conserved in species separated by such wide evolutionary time suggests that it may have important functional significance. In this study a low dose of synthetic cGnRH-II stimulated LH release in adult rhesus monkeys when administered during the mid-luteal phase. There was no significant increase in plasma LH

if the injection took place during the mid-follicular phase, unless a high dose (40x) of cGnRH-II was used. In fact, administration of mGnRH at the same dose to the same monkeys showed a similar differential pattern of LH release between the luteal phase and mid-follicular phase of the menstrual cycle (Urbanski *et al.*, 1997).

These data accord with high binding affinity of cGnRH-II for the GnRH receptor in humans (Davidson *et al.*, 1988) as well as in non-primate mammals. Other studies also show that cGnRH-II is effective in releasing LH, FSH or gonadotropins (Millar and King, 1995; Miyamoto *et al.*, 1990; Sharp *et al.*, 1990; Sherwood *et al.*, 1997; Zohar *et al.*, 1995) but it is not clear that the fibers containing cGnRH-II are present or reach the portal vessels or pituitary in all vertebrates. Hence, cGnRH-II may play a different physiological function. Indeed, cGnRH-II administered to chickens was effective in releasing LH and FSH, but blockage of the peptide with antisera *in vivo* did not alter the reproductive cycle (Sharp *et al.*, 1990). The location of cGnRH-II in the mid-hindbrain is one of the few clues about its possible functions. Despite these studies showing that more than one form of GnRH can stimulate gonadotropin release from the pituitary, mGnRH is usually more effective (Gautron *et al.*, 1992; Millar and King, 1995; Miyamoto *et al.*, 1990; Sherwood *et al.*, 1997). A unique chicken GnRH-II receptor has not been isolated and sequenced in any vertebrate to date and hence, the possibility remains that a single receptor type may exist that binds both mGnRH and cGnRH-II. King and Millar (1988) have presented evidence that a single receptor type binds both cGnRH-I and cGnRH-II in chicken pituitaries (King *et al.*, 1988).

The presence of a few cGnRH-II neurons in the basal hypothalamus of the rhesus monkey suggests that the cGnRH-II form may play a minor hypophysiotropic role, aiding in the stimulus of LH synthesis and/ or release during certain developmental periods or reproductive states. Alternatively, the presence of cGnRH-II in this brain area may not affect LH or FSH release, but function in the control of reproductive behavior (Millar and King, 1995). Other vertebrate studies suggest cGnRH-II is a neuromodulator that affects

reproductive behavior. This hypothesis is strengthened by evidence that cGnRH-II binds to high affinity sites in bullfrog sympathetic ganglia membranes resulting in altered potassium currents (Jones, 1987) or in late, slow post-synaptic excitatory potentials (Hseuh *et al.*, 1985; Jan *et al.*, 1979). Behavioral studies show that injection of GnRH into the midbrain, resulted in enhanced lordosis and female receptivity in rats (Ogawa, *et al.*, 1992; Sakuma *et al.*, 1980; Silver *et al.*, 1993; Pfaff *et al.*, 1994) and turtle doves. Intraperitoneal injection of cGnRH-II into a reptile, *Iguana iguana*, altered plasma steroid levels and female sexual receptivity (Phillips *et al.*, 1987). Furthermore, immunoreactive GnRH cells in the midbrain of the sting ray, *Urolophus halleri*, project to motor neurons associated with the clasper appendage that is involved in mating behavior (Demski, 1984).

The question of the presence of multiple forms of GnRH within the vertebrate brain is important for our understanding of the control of reproductive physiology and behavior. The discovery of cGnRH-II-like substance in the brain of adult and fetal monkeys suggests that an anterior and posterior GnRH system exists within the brains of evolutionarily advanced vertebrates, possibly even in humans. Each of the multiple forms of GnRH might be involved in the control of reproductive physiology and behavior or some of the GnRH forms might have a function unrelated to reproduction.

GnRH is not detectable in human placenta at term

After my discovery of a cGnRH-II-like molecule in primate brain, I predicted a similar combination of mGnRH and cGnRH-II-like immunoreactivity in the human placenta. However, I did not detect any irGnRH using antiserum GF-4 or any of our GnRH-specific antisera, B-7, 7CR-10 and Adams 100, in the placenta in this study. The absence of detectable mGnRH in the placenta in this study compared to other studies may not be surprising when the function of GnRH in the placenta is considered. In the placenta, GnRH functions as a paracrine hormone binding mGnRH-specific receptor sites (Bramley *et al.*, 1993; Currie *et al.*, 1981) to stimulate the synthesis and release of human chorionic gonadotropin, hCG, from cells in the same tissue (Belisle *et al.*, 1984; Kelly *et*

al., 1991; Petraglia *et al.*, 1989; Siler-Khodr and Khodr, 1981). hCG also functions in a paracrine fashion in the placenta by maintaining the endometrial wall during pregnancy. Therefore, hCG levels are highest in the first trimester and decline as pregnancy progresses. If GnRH exists in the placenta primarily to regulate hCG, it can be expected that the increase and decrease of GnRH levels would occur in parallel with the levels of hCG. Consequently, it might be predicted that GnRH levels in the placenta would be low at the time of birth, the time that the tissue for this study was collected. The importance of the timing of tissue collection to the detection of GnRH in the placenta is shown in a previous report by Seppala *et al.*, 1980 who demonstrated that GnRH-specific immunostaining was present in first and second trimester placentas but not in placentas collected after full-term pregnancies. The high sensitivity of some of our antisera made me hopeful that different forms of GnRH might still be detected at term, but this is not the case.

Literature cited

Adelman, J.P., Mason, A.J., Hayflick, J.S., and Seeburg, P.H. (1986). Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin-release inhibiting factor in human and rat. *Proc. Natl. Acad. Sci. (U.S.A.)* **83**, 179-183.

Belisle, S., Guevin, J-F., Bellabarba, D., Lehoux, J-G. (1984). Luteinizing hormone-releasing hormone binds to enriched human placental membranes and stimulates *in vitro* synthesis of bioactive chorionic gonadotropin. *J. Clin. Endocrinol. Metab.* **59**, 119-126.

Bogerd, J., Zandenberg, T., Andersson, E., and Goos, H. (1994). Isolation, characterization and expression of the cDNAs encoding the catfish-type and chicken-II-type gonadotropin-releasing hormone precursors in the African catfish. *Eur. J. Biochem.* **222**, 541-549

Bramley, T.A., McPhie, C.A., and Menzies, G.S. (1992). Human placental gonadotophin-releasing hormone (GnRH) binding sites: I. characterization, properties and ligand specificity. *Placenta* **13**, 555-581.

Conlon, J.M., Collin, F., Chiang, Y-C., Sower, S.A., and Vaudry, H. (1993). . Two molecular forms of gonadotropin-releasing hormone from the brain of the frog, *Rana ridibunda*: purification, characterization and distribution. *Endocrinology* **132**, 2117-2123.

Currie, A.J., Fraser, H.M., and Sharpe, R.M. (1981). Human placental receptors for luteinizing hormone releasing hormone. *Biochem. Biophys. Res. Commun.* **99**, 332-338.

Davidson, J.S., McArdle, C.A., Davies, P., Elario, R., Flanagan, C.A., and Millar, R.P. (1996). Asn ^{109} of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycamide. *J. Biol. Chem.* **271**,15510-15514

Dellovade, T.L., King, J.A., Millar, R.P., and Rissman, E.F. (1993). Presence and differential distribution of distinct forms of immunoreactive gonadotropin-releasing hormone in the musk shrew brain. *Neuroendocrinology* **58**, 166-177

Demski, L.S. (1984). The evolution of neurological substrates of reproductive behavior: Sex-steroid and LHRH-specific pathways including the terminal nerve. *Am. Zool.* **24**, 809-830

Dong, K-W, Yu, K-L, and Roberts, J.L. (1993). Identification of a major upstream transcription start site for the human progonadotropin-releasing hormone gene used in reproductive tissues and cell lines. *Mol. Endocrinol.* **7**, 1654-1666.

Duello, T.M, Tsai, S-J.,and Van Ess, P.J. (1993). *In situ* demonstration and characterization of progonadotropin-releasing hormone messenger ribonucleic acid in first trimester human placentas. *Endocrinology* **133**, 2617-2623.

Ellinwood, W.E., and Resko, J.A. (1980) Sex differences in biologically active and immunoreactive gonadotropins in the fetal circulation of rhesus monkeys. *Endocrinol* **107**, 902-907

Gautron J.P., Leblanc P., Bluet-Pajot M.T., Pattou E., L'Héritier A., Mounier F., Ponce G., Audinot V., Rasolonjanahary R., and Kordon, C. (1992). A second endogeneous molecular form of mammalian hypothalamic luteinizing hormone-releasing hormone (LHRH), (hydroxyproline^{9}) LHRH, releases luteinizing hormone and follicle-stimulating hormone *in vitro* and *in vivo*. *Mol. Cell. Endocrinol.* **85**, 99-107

Gautron, J-P., Pattou, E., Bauer, K., and Kordon, C. (1991). (Hydroxyproline^{9}) luteinizing hormone-releasing hormone: a novel peptide in mammalian and frog hypothalamus. *Neurochem. Int.* **18**, 221-235.

Gibbons, J.M., Mitnick, M., Chieffo, V. (1975). In vitro biosynthesis of TSH and LH-releasing factors by the human placenta. *Am. J. Obstet. Gynecol.* **121**, 127-131.

Gothilf, Y., Muñoz-Cueto, J.A., Sagrillo, C.A., Selmanoff, M., Chen, T.T., Kah, O., Elizur, A., and Zohar, Y. (1996). Three forms of gonadotropin-releasing hormone in a perciforme fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. *Biol. Reprod.* **55**, 636-645.

Hseuh, A.J.W., Schaeffer, J.M. (1985). Gonadotropin-releasing hormone as a paracrine hormone and neurotransmitter in extrapituitary sites. J. *Steroid Biochem.* **23**, 757-764.

Jan, Y.N., Jan, L.Y., and Kuffler, S.W. (1979). A peptide as a possible transmitter in sympathetic ganglia of the frog. *Proc. Natl. Acad. Sci. (U.S.A.)* **76**, 1501-1505

Jones, S.W. (1987). Chicken II luteinizing hormone-releasing hormone inhibits the M-current of bull frog sympathetic neurons. *Neurosci. Lett.* **80**, 180-184.

Joss, J.M.P., King, J.A., and Millar, R.P. (1994). Identification of the molecular forms of and steroid hormone responses to gonadotropin-releasing hormone in the Australian lungfish, *Neoceratodus forsteri*. *Gen. Comp. Endocrinol.* **96**, 392-400

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

Kelly, A.C., Rodgers, A., Dong, K.W., Barrezueta, N.X., Blum, M. and Roberts, J.L. (1991). Gonadotropin-releasing hormone and chorionic gonadotropin gene expression in human placental development. *DNA Cell. Biol.* **10**, 411-421.

Khodr, G.S, and Siler-Khodr, T.M. (1980). Placental luteinizing hormone releasing factor and its synthesis. *Science* **207**, 315-317.

King, J.A., and Millar, R.P. (1985). Identification of His^5, Trp^7, Tyr^8-GnRH (chicken GnRH-II) in amphibian brain. *Peptides* **7**, 827-834

King, J.A., Davidson J.S., and Millar R.P. (1988). Interaction of endogenous chicken gonadotrophin-releasing hormone-I and -II on chicken pituitary cells. *J. Endocrinol.* **117**, 43-49

King, J.A., Dufour, S., Fontaine, Y-A., and Millar, R.P. (1990). Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* **11**, 507-514

King, J.A., Hassan, M.F., Mehl, A.E.I., and Millar, R.P. (1988) . Gonadotropin-releasing hormone molecular forms in mammalian hypothalamus. *Endocrinology* **122**, 2742-2752.

King, J.A., Hinds, L.A., Mehl, A.E.I., Saunders, N.R., and Millar, R.P. (1990) Chicken GnRH II occurs together with mammalian GnRH in a South American species of marsupial (*Monodelphis domestica*). *Peptides* **11**, 521-525

King, J.A., Mehl, A.E.I., Tyndale-Biscoe, C.H., Hinds, L.A., and Millar, R.P. (1989). A second form of gonadotropin-releasing hormone (GnRH), with chicken GnRH II-like properties, occurs together with mammalian GnRH in marsupial brains. *Endocrinology* **125**, 2244-2252

King, J.A., Steneveld, A.A., Curlewis, J.D., Rissman, E.F., and Millar, R.P. (1994). Identification of chicken GnRH II in brains of metatherian and early-evolved eutherian species of mammals. *Regul. Peptides* **54**, 467-477

Leprêtre, E., Anglade, I., Williot, P., Vandesande, F., Tramu, G., and Kah, O. (1993). Comparative distribution of mammalian GnRH (Gonadotrophin-releasing hormone) and chicken GnRH-II in the brain of the immature siberian sturgeon (*Acipenser baeri*). *J. Comp. Neurol.* **337**, 568-583

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

Licht, P., Pei-San, T., and Sotowska-Brochocka, J. (1994) .The nature and distribution of gonadotropin-releasing hormones in brains and plasma of ranid frogs. *Gen. Comp. Endocrinol.* **94**, 186-198.

Lin, X-W.,and Peter, R.E. (1996). Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. *Gen. Comp. Endocrinol.* **101**, 282-296.

Lovejoy, D.A., Fischer, W.H., Ngamvongchon, S., Craig, A.G., Nahorniak, C.S., Peter, R.E., Rivier, J.E., and Sherwood, N.M. (1992). Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Natl. Acad. Sci. (U.S.A.)* **89**, 6373-6377

Millar, R.P., and King, J.A. (1994). Plasticity and conservation in gonadotropin-releasing hormone structure. *In*: "Perspectives in Comparative Endocrinology" (K.G. Davey, R.E. Peter, S.S. Tobe, Eds.). National Research Council of Canada, Ottawa: 129-136.

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. *Proc. Natl. Acad. Sci. (U.S.A.)* **81**, 3874-3878

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

Ogawa, S., Kow, L.M., and Pfaff, D.W. (1992). Effects of lordosis-relevant neuropeptides on midbrain periaqueductal gray neuronal activity in vitro. *Peptides* **13**, 965-975

Petraglia, F., Vaughan, J, and Vale, W. (1989). Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. *Proc. Natl. Acad. Sci. (U.S.A.)* **86**, 5114-5117.

Pfaff D.W., Schwanzel-Fukuda, M., Parhar, I.S., Lauber, A.H., McCarthy, M.M., and Kow L-M. (1994). GnRH neurons and other cellular and molecular mechanisms for simple mammalian reproductive behaviors. *Rec. Prog. Horm. Res.* **49**, 1-25.

Phillips, J.A., Frye F., Bercovitz A., Calle P., Millar R., Rivier J., and Lasley B.L. (1987). Exogenous GnRH overrides the endogenous annual reproductive rhythm in green iguanas, *Iguana iguana. J. Exper. Zool.* **241**, 227-236

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. *Pro. Natl. Acad. Sci. (U.S.A.)* **91**, 12081-12085.

Quanbeck, C.D., 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. *J. Comp. Neurol.* **380**, 293-309.

Radovick, S., Wondisford, F.E., Nakayama, Y., Yamada, M., Cutler, Jr. G.B., and Weintraub, B.D. (1990). Isolation and characterization of the human gonadotropin-releasing hormone gene in the hypothalamus and placenta. *Mol. Endocrinol.* **4**, 476-480.

Rissman, E.F., Alones, V.E., Craig-Veit, C.B., Millam, J.R. (1995). Distribution of chicken-II gonadotropin-releasing hormone in mammalian brain. *J. Comp. Neurol.* **357**, 524-531

Saitoh, Y., Luchansky, L.L., Claude, P., and Terasawa, E. (1995). Transplantation of the olfactory placode restores reproductive cycle in female rhesus monkeys (*Macaca mulatta*) bearing lesions in the medial basal hypothalamus. *Endocrinology* **136**, 2760-2769

Sakuma. Y., and Pfaff, D.W. (1980). LH-RH in the mesencephalic central grey can potentiate lordosis reflex of female rats. *Nature* **283**, 566-567.

Seeburg, P.H., and Adelman, J.P. (1984). Characterization of cDNA for precursor of human luteinizing hormone releasing hormone. *Nature* **311**, 666-668.

Seppälä, M., Wahlstrom, T., Lehtovirta, P., Lee, J.N., and Leppalouto, J. (1980). Immunohistochemical demonstration of luetinizing hormone-releasing factor like material in human syncytiotrophoblast and trophoblastic tumours. *Clin. Endocrinol.* **12**, 441-451.

Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M., Huskisson, N.S. (1990) .Physiological roles of chicken LHRH-I and -II in the control of gonadotropin release in the domestic chicken. *J. Endocrinol.* **124**, 291-299.

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 (*Lepissosteus spatula*). *Gen. Comp. Endocrinol.* **84**, 44-57.

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

Sherwood, N.M., Sower, S.A., Marshak, D.R., Fraser, B.A., Brownstein, M.J. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem.* **261**, 4812-4819.

Sherwood, N.M., von Schalburg, K.R., and Lescheid, D.W. (1997). Origin and evolution of gonadotropin-releasing hormone in vertebrates and invertebrates. In: "GnRH: Gene to Behaviour" (I.S. Parha and Y. Sakuma Eds.). Brain Shippon Publishers, Tokyo, Japan (in press).

Siler-Khodr, T.M., and Khodr, G.S. (1981). Dose response analysis of GnRH stimulation of hCG release from human term placenta. *Biol. Reprod.* **25**, 353-358.

Silver R., Ramos C.L., and Silverman A.J. (1993). Sexual behavior triggers appearance of non-neuronal cells containing gonadotropin-releasing hormone-like immunoreactivity. *J. Neuroendocrinol.* **4**, 207-210.

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.

Tan, L. and Rousseau, P. (1982). The chemical identity of the immunoreactive LHRH-like peptide biosynthesized in the human placenta. *Biochem. Biophys. Res. Commun.* **109**, 1061-1071.

Terasawa, E., Quanbeck, C.D., Schulz, C.A., Burich, A.J., Luchansky, L.L., and Claude, P. (1993). A primary cell culture system of luteinizing hormone releasing hormone neurons derived from embryonic olfactory placode in the rhesus monkey. *Endocrinology* **133**, 2379-2390.

Teresawa, E., Sherwood, N.M., Millar, R.P., Jennes, L., and Glucksman, M.J. (1997). The presence of a luteinizing hormone releasing hormone (LHRH) fragment in the fetal monkey forebrain. The 79th Annual Meeting of the Endocrine Sovvciety, Minneapolis, MN, June 11-14 (No. P2-383).

Urbanski, H.F., Garyfallou, V.T., Kohama, S.G., and Hess D.L. (1997). Alpha-adrenergic receptor antagonism and N-methyl-D-aspartate (NMDA) induced luteinizing hormone release in female rhesus macaques. *Brain Research* **744**, 96-104.

White, S.A., Bond, C.T., Francis, R.C., Kasten, T.L., Fernald, R.D., and Adelman, J.P. (1994). A second gene for GnRH: cDNA and pattern of expression in the brain. *Proc. Natl. Acad. Sci. (U.S.A.)* **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. *Proc. Natl. Acad. Sci. (U.S.A.)* **92**, 8363-83676

Zohar, Y., Elizur, A., Sherwood, N.M., Powell, J.F.F., Rivier, J.E., and Zmora, N. (1995). Gonadotropin-releasing activities of the three native forms of gonadotropin-releasing hormones present in the brain of gilthead seabream, *Sparus aurata*. *Gen. Comp. Endocrinol.* **97**, 289-299.

Chapter 6

Identification of mGnRH but not cGnRH-II or other forms of GnRH in the brains of guinea pig, hamster and rat.

Introduction

I have shown in Chapter 5 of this thesis that the brain of adult stumptail monkeys as well as adult and fetal rhesus monkey contains a cGnRH-II-like molecule in addition to mGnRH. However, although a combination of HPLC elution position and cross-reactivity with specific antisera provides strong evidence as to which forms of GnRH are present in the brain, more definitive proof would be to characterize GnRH by primary structure or by molecular biology methods. It is difficult to obtain enough primate tissue to determine the protein structure of GnRH and therefore, I decided to examine the brain of mammals that are more readily available than primates, such as guinea pigs (*Cavia porcellus*), hamsters (*Mesocritus auratus*) and adult and fetal rats (*Rattus rattus*). I hypothesized that mGnRH and/or cGnRH-II immunoreactivity would be present in the brain of these species with sufficient quantity to determine the protein sequence of both GnRHs detected and therefore, I would be able to clearly identify cGnRH-II in recently-evolved placental mammals. I also examined rat tissue at different times of development to determine if the form(s) of GnRH present is ontogenetically expressed and if cGnRH-II is present in fetal rodent brains as was shown in fetal primate brains in the previous chapter.

Previous HPLC and RIA studies have shown that mGnRH-like immunoreactivity exists within the brain of rodents like rats (Gautron *et al.*, 1991; Kelsall *et al.*, 1990; King *et al.*, 1990) and guinea pigs (Kelsall *et al.*, 1990). In addition, the cDNA encoding the mGnRH peptide has been isolated from the brain of rat (Adelman *et al.*, 1986) and hamster (Jansen *et al.*, 1996) and the mGnRH gene has been isolated from the brain of rat (Adelman *et al.*, 1986; Bond *et al.*, 1989; Kepa *et al.*, 1992) and mouse (Mason *et al.*, 1986). The cDNA encoding mGnRH also has been isolated from the placenta (Adelman *et al.*, 1986) and ovary (Oikawa *et al.*, 1990) of rat suggesting that mGnRH also might function outside the brain of this species. An additional form of GnRH that might be present in rodents is hydroxy-pro^9-mGnRH, a post-translational derivative of mGnRH

with hydroxylated proline in position 9, because this form has also been found in the brain of rats in addition to the brain of frogs (Gautron *et al.*, 1991; Sherwood *et al.*, 1997). The HPLC/ RIA evidence as well as reports that synthetic mGnRH stimulates the release of LH and FSH from the pituitary in rat, mouse, and human (Sherwood *et al.*, 1997) suggests that the gene is expressed and the mature peptide is present and functional in the brain of some rodent species.

There have been no previous reports of a cGnRH-II peptide cDNA or gene in the brain of rodents. However, lack of a specific antiserum or probe might account for the negative results. In our laboratory, I had three different cGnRH-II-specific antisera, 7CR-10, Adams-100, Millam and one mGnRH-specific antiserum, B-6 (or B-7) to use. In addition, I also had antiserum GF-4, which is capable of cross-reacting with eight of the twelve known forms of GnRH and therefore, is useful in searching for additional types of GnRH. In this chapter of my thesis, I used HPLC and sensitive RIAs with the battery of antisera available to me to search for mGnRH, cGnRH-II and other forms of GnRH in the brain of guinea pigs, hamsters and rats.

The rat study was done in collaboration with Elizabeth Logan at the Universtiy of Victoria, whereas the hamster brains used in this study were collected by Dr. David Lovejoy at the University of Manchester.

Materials and Methods

The details of the tissue, extraction technique, HPLC purification steps and antisera are summarized in Table 6-1. The details of the HPLC programs used are summarized in Chapter 3, Table 3-2.

Table 6-1: **Rodent HPLC and RIA summary**

Species	Tissue (grams)	Extraction technique	HPLC purification steps	Antisera used in RIA	Forms of irGnRH present
Guinea pig *(Cavia porcellus)*	adult brains (male/female) (85.0g)	acid: acetone	TEAF 17-24%	GF-4 B-7 Adams-100	mGnRH
Hamster *(Mesocriteus auratus)*	adult brains (male/female) (25.0g)	acid: acetone	TEAF 17-24%	GF-4 B-7 7CR-10 Millam	mGnRH
	adult brains (male/female) (252.0g)	TFA: ACN	SepPak TFA TEAF 17-24%	GF-4 B-7 Adams-100	mGnRH
Rat *(Rattus rattus)*	adult brains (female) (6.8g)	acid: acetone	TEAF 17-24%	GF-4 B-6 7CR-10 Gertie	mGnRH
	E19.5 heads (male/female) (13.8g)	acid: acetone	TEAF 17-24%	GF-4 B-6 7CR-10	?mGnRH
	E18.5 heads (male/female) (11.1g)	acid: acetone	TEAF 17-24%	GF-4 B-6 7CR-10	?mGnRH
	E14.5 fetuses (3.0g)	acid: acetone	TEAF 17-24%	GF-4 B-6 7CR-10	?
	E12.5 fetuses (7.0g)	acid: acteone	TEAF 17-24%	GF-4 B-6 7CR-10	?

Tissue Collection

Guinea Pig and Hamsters

Twenty-five adult male and female guinea pigs,*Cavia porcellus*, (Hartley 1986 strain) were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA) and Pel Freeze (Rogers, AR). The brains were from animals that ranged from 200g weanlings to 300-400g mature adults to 1000g retired breeders. In the hamster, *Mesocriteus auratus* , studies, 25 and 100 adults of both sexes were used in the initial and second study, respectively. All hamsters were collected in Dr. David Lovejoy's laboratory at the University of Manchester, England. In all three studies, animals were anesthetized using carbon dioxide; the brains were removed and quickly frozen in liquid nitrogen. Frozen brains were then shipped on dry ice to University of Victoria and stored at -80°C.

Rats

Six adult female rats, *Rattus rattus*, (Wistar strain) were time mated at Charles River (Montreal, QC) and shipped to University of Victoria where they were housed in our Animal Care Facility. At specific time intervals, embryonic days (E)12.5, E 14.5, E 16.5, E 18.5, and E 19.5, individual female rats were anesthetized using halothane gas and fetuses were removed by cesarian section. The whole fetus, including the placenta, was collected at E12.5; the whole fetus, excluding the placenta, was collected at E14.5; whereas, the head only was collected for E16.5, E18.5 and E19.5. The maternal rat brain also was collected after removal of the fetuses . All tissues were quick frozen in liquid nitrogen and stored at -80°C.

Tissue Extraction

Rat fetuses, guinea pig brains, rat brains, and a portion of the hamster brains were treated as reported previously (Ngamvongchon *et al.*, 1992) and described in Chapter 2. For extraction, tissue was pooled as follows: 1) rat fetuses from different days of development (5 pools), 2) adult brains pooled from each species (rat, guinea pig, hamster) and 3) additional hamster brains (1 pool).

In the second hamster study, an alternative method (Lovejoy *et al.*, 1992) was used to determine if the extraction procedure might affect the type and quantity of GnRH eluted in HPLC and detected by RIA. Briefly, the brains were crushed and powdered as described previously but then were added to a 0.05% TFA/80% ACN (1:5 v/v) mixture, which was stirred continuously for three hours. Then the extracts were centrifuged (JA-14 rotor, Beckman JA-21 preparative centrifuge, 20 000g, 5°C) for 15 minutes. The supernatant was removed and stored on dry ice, while the solids were re-extracted in 0.05% TFA /80% ACN (40% of the original volume) for a further 20 minutes with continuous stirring. Following centrifugation for another 15 minute, the supernatants were combined and extracted five consecutive times with petroleum ether (5:1, v/v) to remove lipids and large proteins.

Each tissue extract was vacuum concentrated (Savant Speed-Vac concentrator) to 10% of its original volumes following extraction to remove petroleum ether and then brought to pH 3 using 5N NaOH.

SepPak and HPLC

The details of the solvents used in the HPLC procedures are outlined in Table 6-1, whereas the details of the HPLC programs used are described in Chapter 3, Table 3-2.

For the tissue extracts from the guinea pig, rat and the initial hamster study, residual debris from the extraction procedure was removed by filtration through a low protein binding filter (45 μm, μCoStar LB ™, Keenebunk, ME) prior to their injection onto a similar C-18 HPLC column as described in Chapter 2. Brain extracts from the

second hamster study were semipurified initially by SepPak as described in Chapter 2 of this thesis.

Before every tissue sample, a blank sample of 600 μl of Milli-Q water (Millipore, Bedford, MA) was injected onto the C-18 HPLC column, then eluted and assayed by RIA as described previously in chapter two. If GnRH immunoreactivity was not detected after this blank sample was assayed, aliquots of not more than 800μl of tissue extract were injected at 2 minute intervals onto the same column, eluted using the same TEAF 17-24% HPLC program and collected in sixty consecutive fractions of 1 ml each. For each tissue extract, 50-100 μl aliquots were removed from each HPLC fraction and assayed for GnRH-like immunoreactivity with two or more antisera: GF-4, B-7, Adams-100, 7CR-10, or Millam as described in Chapter 2. The HPLC column was washed extensively between successive applications of different tissue and blank samples of Milli-Q water were applied as noted above to ensure that there was no contaminating GnRH-like material from the previous HPLC.

RIA

RIA and antisera characteristics are described in the Chapter 3 of this thesis.

Cross-reactivity

Characteristics of the RIAs using antisera GF-4, B-6 (or B-7), 7CR-10, and Adams-100 have been described in Chapter 3 (Fig. 3-1). Antiserum Gertie was raised in rabbits against lamprey (l)GnRH-III and was a gift of Dr. Stacia Sower (University of New Hampshire), whereas antiserum Millam was raised in rabbits against cGnRH-II and was a gift from Dr. Jim Millam (University of California, Davis). The Millam antiserum was added to this study because antisera 7CR-10 and Adams-100 are specific for cGnRH-II, but they both have limited cross-reactivity with one or two other GnRH forms. Also, antiserum Gertie is specific for lGnRH-III whereas, antiserum B-6 (or B-7) is specific for mammalian GnRH. Finally, antiserum GF-4 cross-reacts in varying

degrees with 10 of the 12 known forms of GnRH (see Chapter 3) and therefore, is useful in determining which forms of GnRH are present.

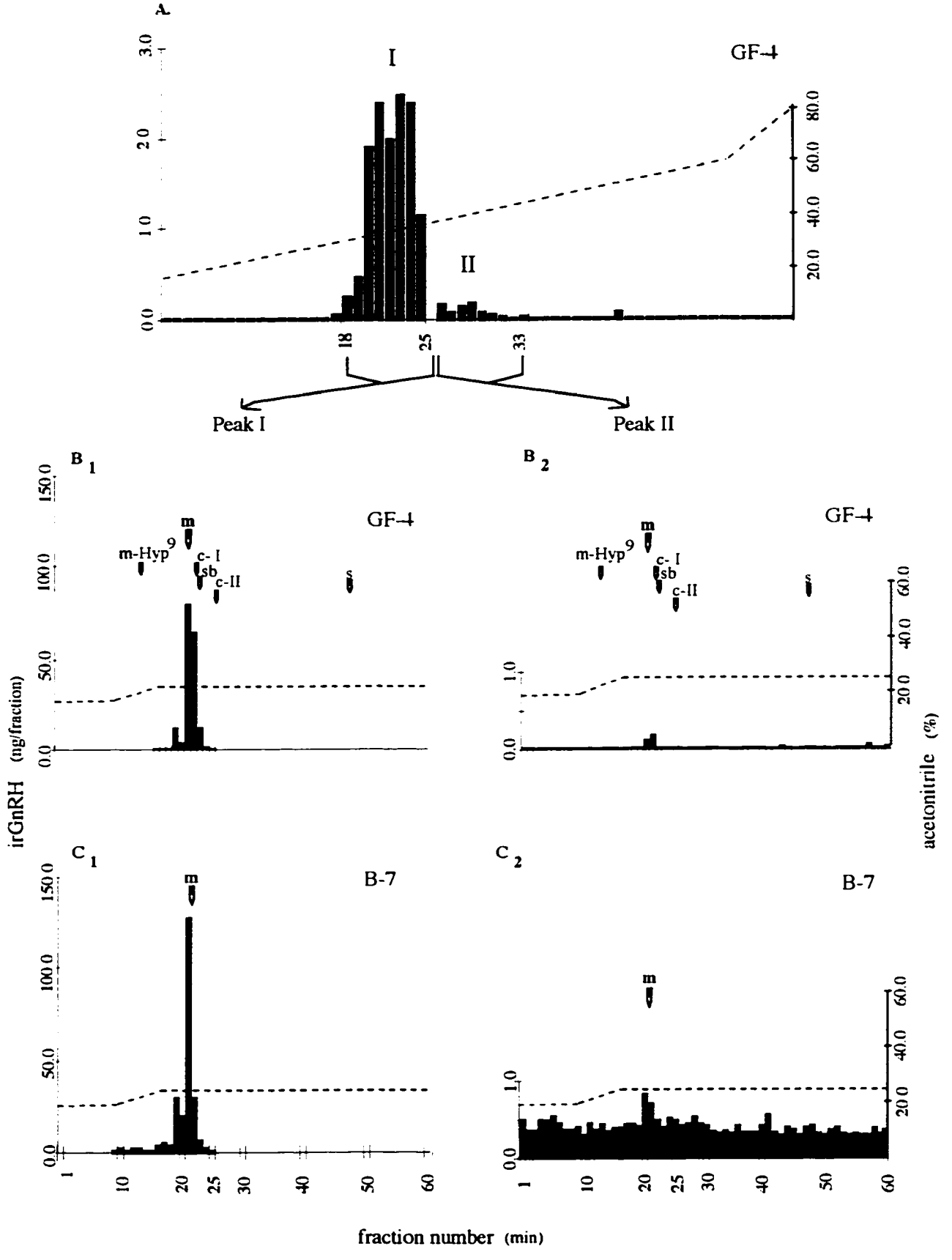
Homologous RIAs were used for antisera Millam and Gertie with ^{125}I-labelled cGnRH-II tracer and cGnRH-II standard used with Millam and ^{125}I-labelled lGnRH-III tracer and a lGnRH-III standard was used for antiserum Gertie.

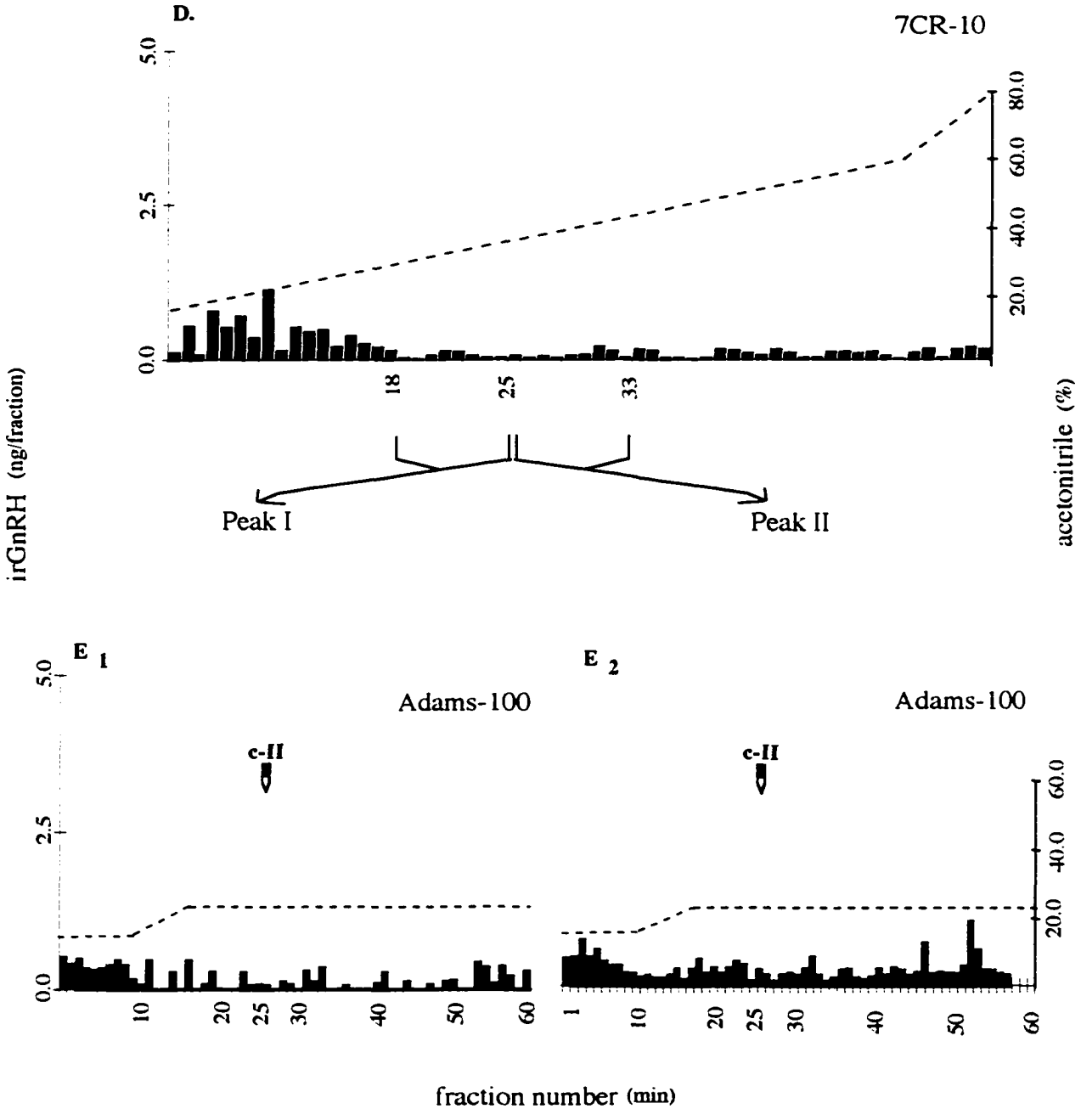
Results

Guinea Pig

For guinea pig brain extracts, the highest concentration of irGnRH was detected by antiserum GF-4 in HPLC fractions 18-25. These fractions were labelled as Peak I following elution from SepPak (Fig. 6-1A). There were also minor amounts of irGnRH in fractions 26-30, Peak II (Fig. 6-1A). Peak I and Peak II were treated separately in HPLC but both peaks were further purified by injection onto a C-18 HPLC column and by elution using the TEAF 17-24% HPLC program described in Table 3-2. Antiserum GF-4 detected 134.8 ng of irGnRH in fractions 22 and 23 after elution of Peak I, but only 1.04 ng of irGnRH in fraction 21 for Peak II (Fig. 6-1B $_1$, B$_2$). Using antiserum B-7, I detected 127.6 ng in fraction 22 after the elution of Peak I and 1.56 ng of irGnRH for Peak II (Fig. 6-1C$_1$, C$_2$). Although antiserum 7CR-10 detected some immunoreactivity in fractions 4-15 following elution of guinea pig brain extracts from SepPak (Fig. 6-1D), the amount of immunoreactivity was so small that the same SepPak fractions that were immunopositive to antiserum GF-4 were collected and used for further purification. RIA with the cGnRH-II specific antiserum, Adams-100, did not detect irGnRH peaks in any eluted HPLC fractions after either Peak I (Fig. 6-1E$_1$) or Peak II (Fig. 6-1E$_2$) were applied to the C-18.

Fig. 6-1. Immunoreactive GnRH following HPLC elution of guinea pig brain extracts. The antiserum were (A) GF-4 for fractions obtained from a SepPak column., (B_1) GF-4 for fractions 18-25, and (B_2) 26-33 from the HPLC C-18 column, (C_1) B-7 for fractions fractions 18-25 and (C_2) 26-33 from the HPLC C-18 column, (D) 7CR-10 for brain extracts from SepPak, (E_1) Adams-100 for fractions 18-25 and (E_2) 26-33 from the C-18 column. Small arrows above the HPLC elution profiles represent where synthetic peptides elute under similar HPLC conditions. Abbreviations are: m-Hyp9, hydroxyproline-^{9}-GnRH) mammalian GnRH; m, mammalian GnRH; c-I, chicken GnRH-I; sb, sea bream GnRH; c-II, chicken GnRH-II and s, salmon GnRH. Dashed line represents the percent acetonitrile used in elution.


A
I
II
GF-4
Peak I
Peak II
B 1
B 2
GF-4
m-Hyp9
c- I
sb
c-II
irGnRH (ng/fraction)
acetonitrile (%)
C 1
C 2
B-7
fraction number (min)

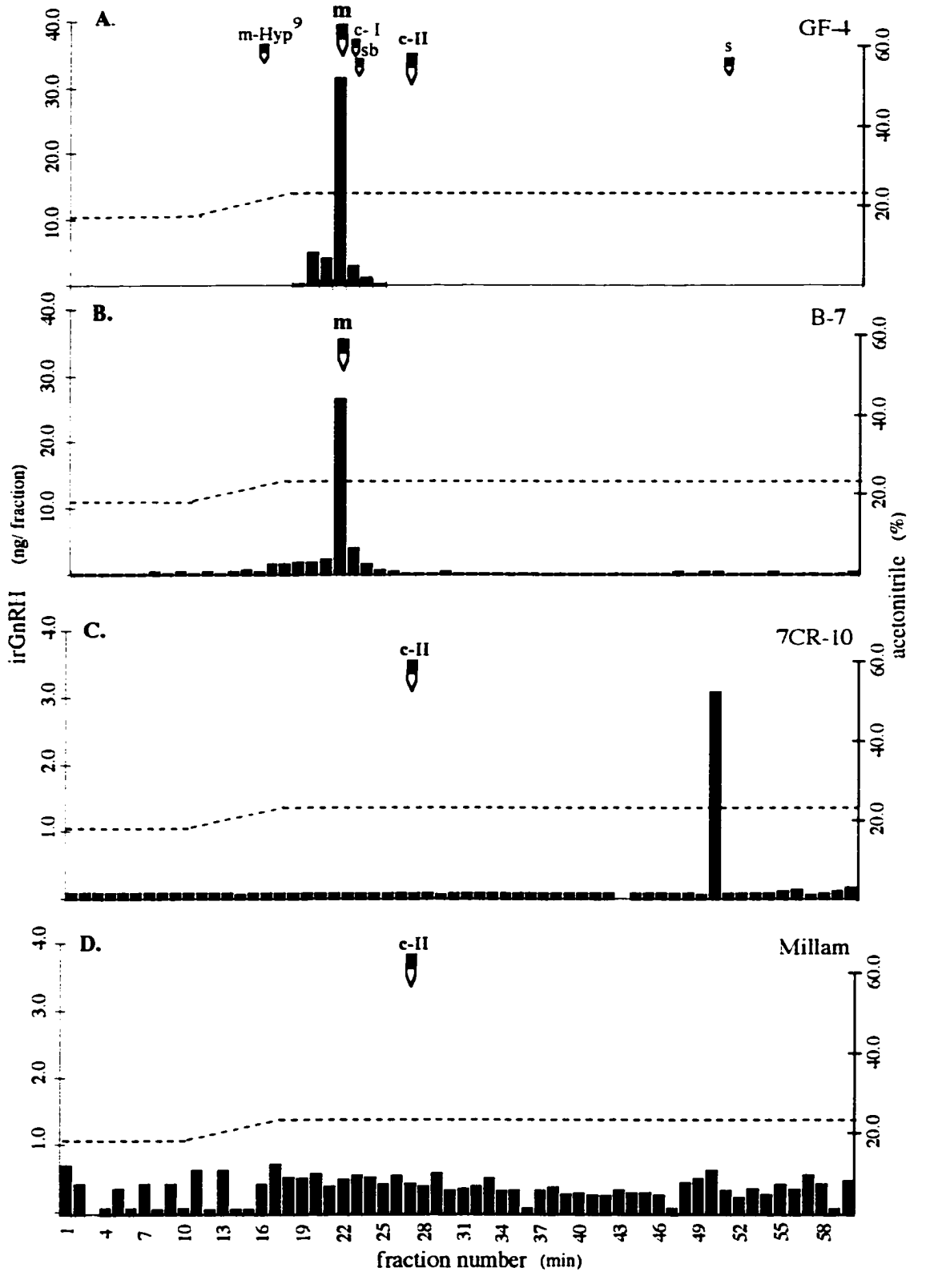

D.
7CR-10
5.0
2.5
0.0
80.0
60.0
40.0
20.0
18
25
33
Peak I
Peak II
irGnRH (ng/fraction)
acetonitrile (%)
E 1
Adams-100
c-II
E 2
Adams-100
c-II
10
20
25
30
40
50
60
1
fraction number (min)

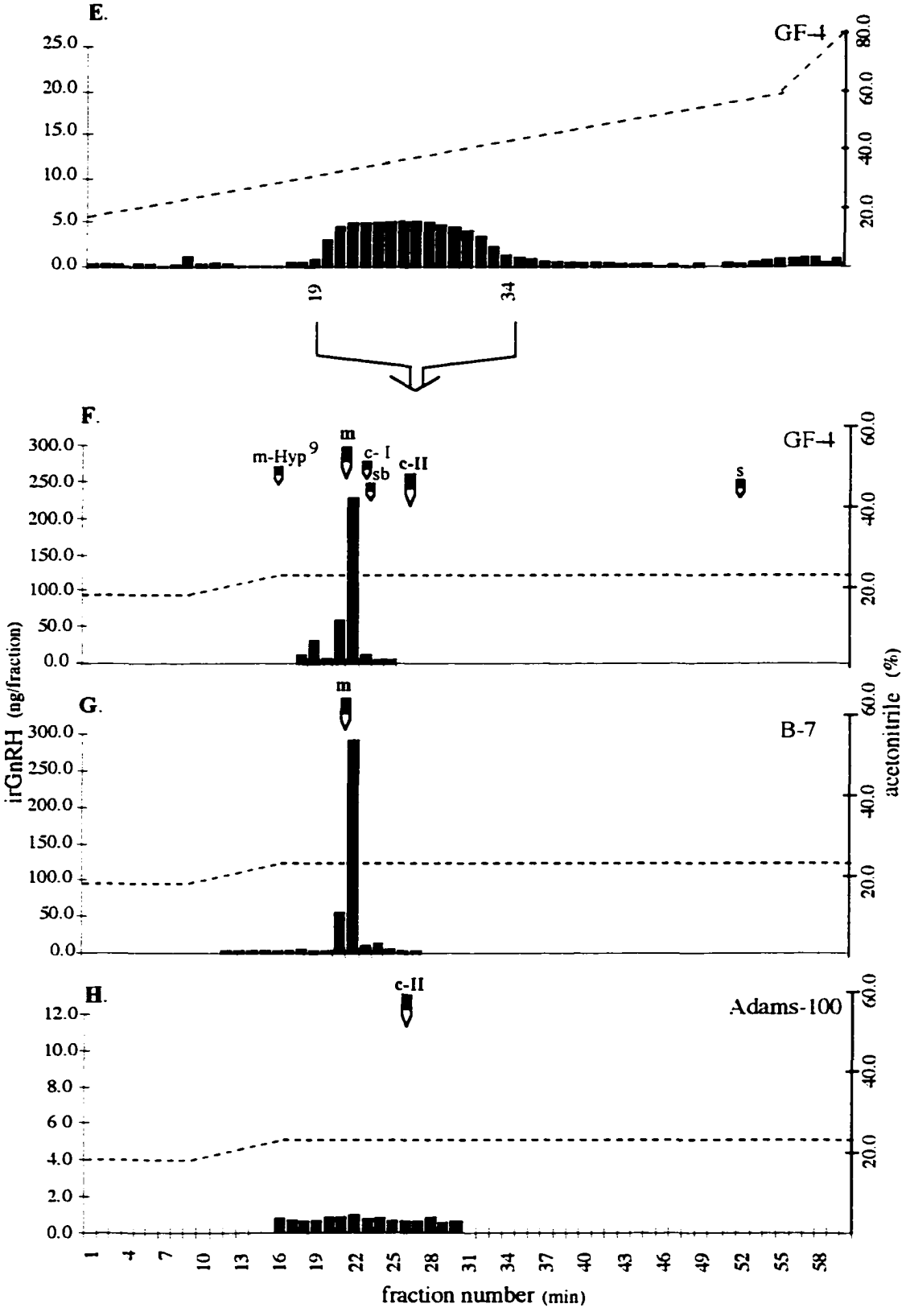
Hamster

In the initial hamster study, fraction 21 contained the highest quantity of irGnRH, 31.4 ng, as detected by antiserum GF-4 and 27.0 ng by antiserum B-7 (Fig. 6-2A, B). However, RIA with the cGnRH-II specific antisera, 7CR-10 and Millam, did not detect GnRH-like immunoreactivity in the fractions expected for cGnRH-II (Fig. 6-2C, D). The immunoreactive peak detected by antiserum 7CR-10 in fraction 50 (Fig. 6-2C) is probably an anomaly since this antiserum does not normally detect immunoreactivity in this fraction and also, because only synthetic sGnRH (unlikely to be present in hamsters) elutes this late using these HPLC conditions.

In the second hamster study, using the alternate TFA: ACN extraction method, irGnRH was detected in fractions 20-33 by antiserum GF-4 following elution from SepPak (Fig. 6-2E). Antiserum GF-4 detected 230.4 ng and antiserum B-7 detected 275.2 ng of irGnRH in HPLC fraction 22 after the immunoreactive SepPak fractions were combined and eluted from the C-18 HPLC column using the TEAF 17-24% HPLC program (Fig. 6-2F, G). In contrast, antiserum Adams-100 did not detect cGnRH-II-like immunoreactivity in the HPLC fractions expected for cGnRH-II (Fig. 6-2H). These are the same fractions shown in Fig. 6-2F and G. If the values are recalculated as irGnRH per gram of brain tissue, then antiserum B-7 detected 1.13 ng irGnRH per gram of hamster brain when the acid-acetone extraction method was used (Fig. 6-2B) and 1.09 ng irGnRH when the TFA-acetonitrile extraction method was used (Fig. 6-2G). The detection of similar amounts of mGnRH by antiserum B-7 regardless of the extraction method does not necessarily mean that the cGnRH-II molecule is similarily unaffected by extraction methods. However, cGnRH-II was not detected in the brain of hamster brain extract regardless of the extraction method or antisera used (Fig. 6-2C, D, H).

Fig. 6-2. Immunoreactive GnRH in hamster brain extracts after elution from HPLC columns. Antisera and HPLC columns were (A) that cross-reacts with many different forms of GnRH for fractions from a C-18 column, (B) B-7 that is specific for mGnRH was used for fractions that elute from a C-18 column, (C) 7CR-10 that is specific for cGnRH-II for fractions that elute from a C-18 column, (D) Millam that is specific for cGnRH-II for fractions that elute from a C-18 column, (E) GF-4 for fractions that elute from a SepPak column after a TFA: acetontitrile extraction, (F) GF-4 for fractions that elute from a C-18 column, (G) B-7 that is specific for mGnRH for fractions that elute from a C-18 column and (H) Adams-100 that is specific for cGnRH-II for fractions that elute from a C-18 column. Small arrows above the HPLC elution profile represent where synthetic peptides elute under similar HPLC conditions. Abbreviations are: m-Hyp9, hydroxyproline-^{9}-mammalian GnRH; m, mammalian GnRH; c-I, chicken GnRH-I; sb, sea bream GnRH; c-II, chicken GnRH-II and s, salmon GnRH. Dashed line represents the percent acetonitrile used in elution.


A.
m
m-Hyp9
c- I
sb
c-II
s
GF-4
B.
m
B-7
C.
c-II
7CR-10
D.
c-II
Millam
irGnRH (ng/ fraction)
acetonitrile (%)
fraction number (min)


E.
GF-4
25.0
20.0
15.0
10.0
5.0
0.0
80.0
60.0
40.0
20.0
19
34
F.
GF-4
m-Hyp9
m
c-I
sb
c-II
s
300.0
250.0
200.0
150.0
100.0
50.0
0.0
60.0
40.0
20.0
G.
B-7
m
irGnRH (ng/fraction)
acetonitrile (%)
H.
Adams-100
c-II
12.0
10.0
8.0
6.0
4.0
2.0
0.0
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
fraction number (min)

Rat (Adult)

In the adult female rat, GnRH-like immunoreactivity was detected in fraction 20 by both antisera GF-4 and B-6 (Fig. 6-3A, B). With antiserum GF-4, I detected 5 ng of irGnRH in fraction 20 (Fig. 6-3A) whereas I detected 9.2 ng of irGnRH in the same fraction using antiserum B-6 (Fig. 6-3B). Although there was some immunoreactivity detected by antisera GF-4 and B-6 in fractions 13-15 (Fig. 6-3A, B) the quantity was too small to suggest that it may be an additional GnRH-like form. There were no irGnRH-like peaks detected by either antisera 7CR-10 or Gertie (Fig. 6-3C, D).

Rat (Fetal)

Using antiserum GF-4, I detected 0.3 ng of GnRH-like immunoreactivity in fractions 19- 21 of E19.5 fetal rat heads (Fig. 6-4A4); 1.0 ng of irGnRH in fractions 21 and 22 of E18.5 fetal heads (Fig. 6-4A3); but no irGnRH in these HPLC fractions after elution of tissue extracts from E14.5 or E12.5 rat fetuses (Fig. 6-4A2, A1). Antiserum 7CR-10 did not detect cGnRH-II in HPLC fractions 25-26 in fetal extracts from E12.5, E14.5, E18.5 or E19.5 (Fig. 6-4B1, B2, B3, B4). All fractions also were assayed with antiserum B-6 but the background levels were too high for the RIAs to be useful.

Fig. 6-3. Immunoreactive GnRH of maternal rat brain extracts after HPLC elution. Antisera were (A) GF-4, (B) B-6, (C) 7CR-10 and (D) Gertie. Small arrows above the HPLC elution profile represent the elution position of synthetic peptides under similar HPLC conditions. Abbreviations are: m-Hyp9, hydroxyproline-^{9}-mammalian GnRH; m, mammalian GnRH; c-I, chicken GnRH-I; sb, sea bream GnRH; c-II, chicken GnRH-II and s, salmon GnRH. Dashed line represents the percent acetonitrile used in elution.

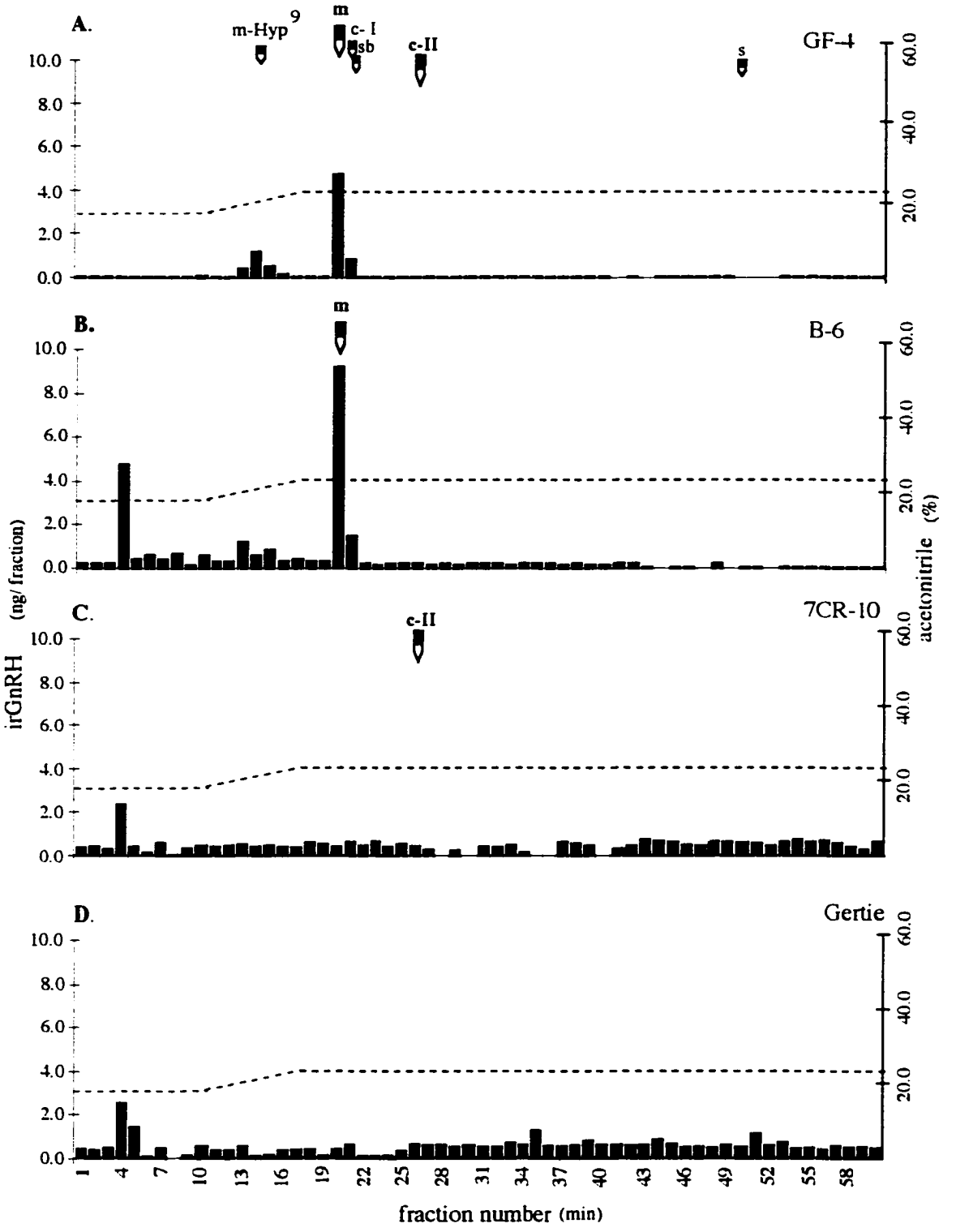
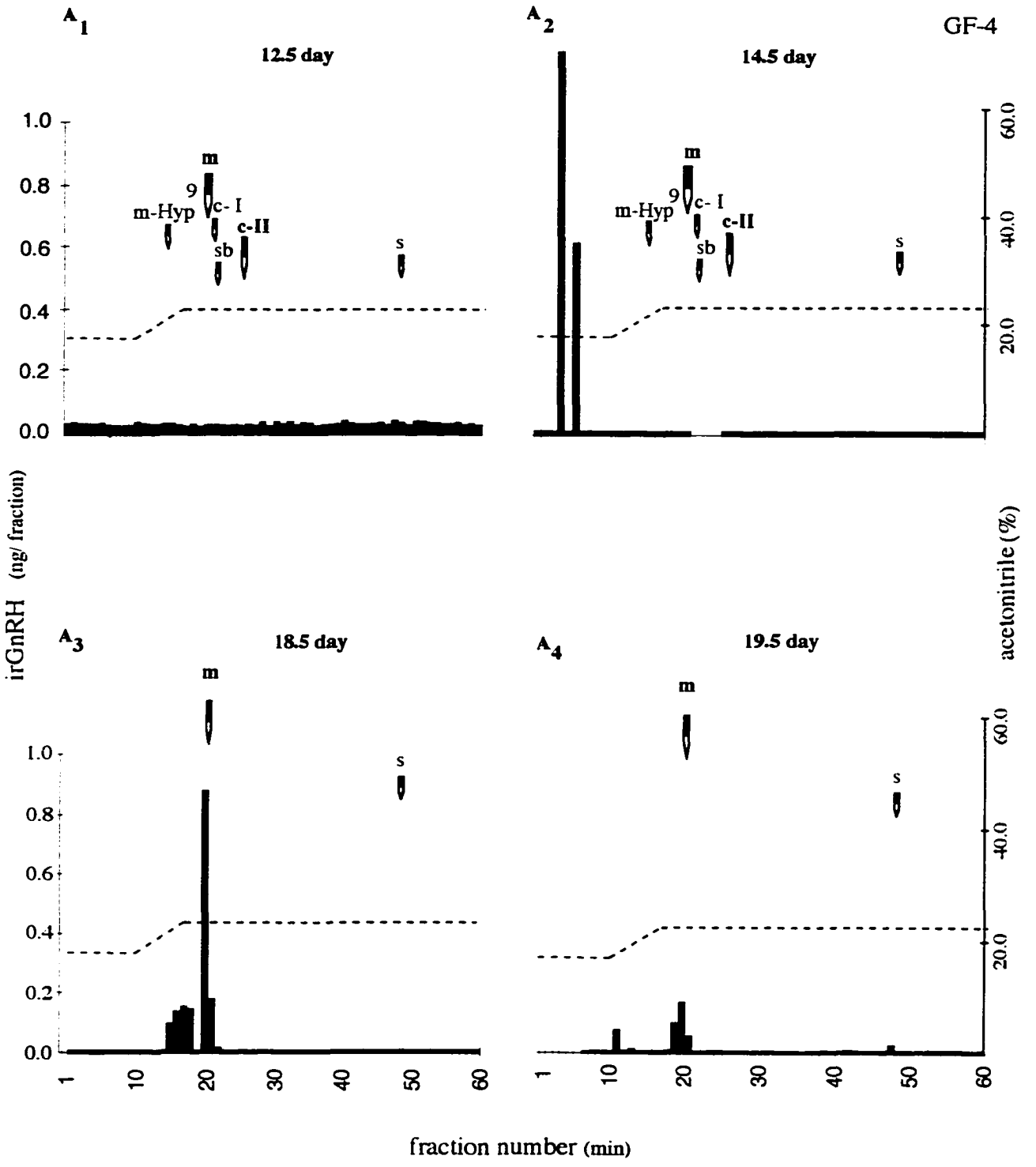
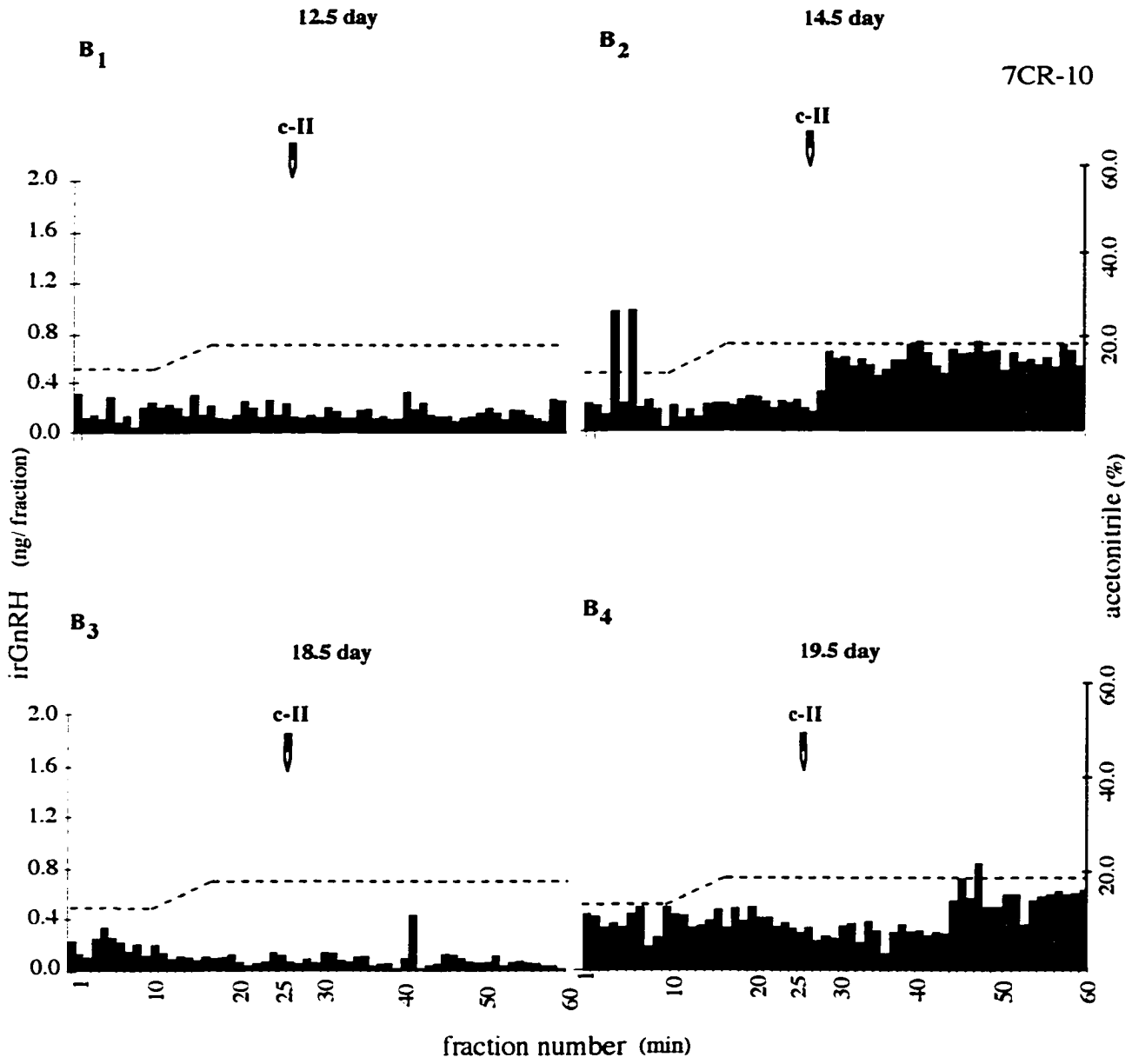

A.
m-Hyp 9
m
c- I
sb
c-II
s
GF-4
B.
m
B-6
C.
c-II
7CR-10
D.
Gertie
irGnRH (ng/ fraction)
acetonitrile (%)
fraction number (min)

Fig. 6-4. Immunoreactive GnRH following elution of fetal rat brain extracts from a C-18 HPLC column. The tissue and antisera were (A_1) 12.5 day fetal brain extract, antiserum GF-4, (A_2) 14.5 day fetal brain extract, antiserum GF-4, (A_3) 18.5 day fetal brain extract, antiserum GF-4, (A_4) 19.5 day fetal brain extract, antiserum GF-4, (B_1) 12.5 day fetal brain extract, antiserum 7CR-10, (B_2) 14.5 day fetal brain extract, antiserum 7CR-10, (B_3) 18.5 day fetal brain extract, antiserum 7CR-10 and (B_4) 19.5 day fetal brain extract, antiserum 7CR-10. Small arrows above the HPLC elution profile represent the elution position of synthetic peptides under similar HPLC conditions. Abbreviations are: m-Hyp9, hydroxyproline-^{9}-mammalian GnRH; m, mammalian GnRH; c-I, chicken GnRH-I; sb, sea bream GnRH; c-II, chicken GnRH-II and s, salmon GnRH. The dashed line indicates the percent acetonitrile used in elution.



Antiserum GF-4 was used for all of these RIAs.



Antiserum 7CR-10 was used for all of these RIAs

Discussion

By the criteria of HPLC elution position and cross-reactivity with specific GnRH antisera, I detected mGnRH but not cGnRH-II in the brain of guinea pig, hamster and adult rat. In the fetal rat study, detection of an immunoreactive peak by antiserum GF-4 in the HPLC elution position expected for mGnRH, suggests that mGnRH is present as a mature peptide in the brain of E 18.5 and E19.5 fetuses but not in tissues from E12.5 or E14.5 fetuses. There was no evidence of cGnRH-II-like immunoreactivity in any of these developmental stages. Detection of only mGnRH in the brain of these rodents was unlike the previous study in primate where I detected both mGnRH and a cGnRH-II-like peptide in fetal and adult stages of development.

The guinea pig, *Cavia porcellus*, is a good species to investigate whether structural variants of GnRH exist within the brain of mammals because in guinea pigs the structures of other peptides like insulin (Chan *et al.*, 1984), glucagon (Seino *et al.*, 1986) and vasoactive intestinal polypeptide (Du *et al.*, 1985) differ from other mammals The data from this thesis concurs with that of a previous study that demonstrated that the dominant form of GnRH within the brain of guinea pigs was mGnRH using both antiserum R-42 (another GnRH antiserum, like GF-4, that is capable of cross-reacting with many different GnRH forms) and antiserum B-6 (Kelsall *et al.*, 1990). Although both antisera R-42 and GF-4 cross-react with cGnRH-II; 58.1% with R-42 (Kelsall *et al.*, 1990) and 3.9% for GF-4 (see Chapter 3, Fig. 3-1A) neither of these two antisera detected cGnRH-II-like immunoreactivity in the two studies on guinea pig. Furthermore, in the present study I could not detect cGnRH-II-like immunoreactivity even with two cGnRH-II-specific antisera, also suggesting that cGnRH-II is absent from the brain of guinea pig.

The golden hamster, *Mesocriteus auratus*, was chosen to search for cGnRH-II in the brain of a mammal because I had access to a relatively large number of brains and therefore, I potentially could obtain primary structure if the initial HPLC/ RIA results were promising. There have been only a few reports of GnRH in the brain of hamster.

Because the hamster is a seasonal breeder, with reproductive receptiveness influenced by environmental cues, the changes in GnRH and GAP content in the brain of hamster following an alteration in photoperiod have been studied (Ronchi *et al.*, 1992). The most recent report was by Jansen *et al.* (1996) who isolated from the brain of hamster a partial cDNA that had 93-98% nucleotide sequence identity with the GnRH region for human, rat and mouse GnRH and 100% amino acid identity to mGnRH. My present HPLC and RIA study, using two separate studies and two different extraction techniques, also provides evidence that mGnRH is present in the brain of hamster. In addition, I have shown that cGnRH-II is not detected in the brain of hamster using three cGnRH-II-specific antisera in my RIA/ HPLC study.

In 1988, Judy King's group demonstrated that although there were several different bioactive GnRH-like immunoreactive peaks in the hypothalamus of adult rats, sheep and humans, the predominant form of GnRH present was mGnRH. The minor immunoreactive peaks reported in their study were thought to be modified forms of mGnRH, possibly altered during extraction procedures or HPLC. I also have shown using a mGnRH-specific antiserum, B-6, that the major form of GnRH in the brain of adult rats is mGnRH. Moreover, because I could not detect cGnRH-II-like immunoreactivity using both antisera GF-4 and 7CR-10, I suggest that cGnRH-II is absent or below detectable levels from the brain of adult rat and only mGnRH is present.

There have been several previous studies of the ontogeny of GnRH-containing neurons in the rat brain. However, my study is the first to look for a cGnRH-II-like peptide at early stages of rat development. It was demonstrated previously, using a mGnRH specific antiserum in immunocytochemistry, that irGnRH was present in the fetal rat brain at day 15, but not at day 13 or day 14 of development (Schwanzel-Fukuda *et al.*, 1981). Radioimmunoassay of extracts of rat hypothalami also detected small amounts of irGnRH in tissue extracts from day 15 (Chiappa and Fink, 1977). My data, which showed mGnRH-like immunoreactivity in tissue extracts from embryonic days

18.5 and 19.5 of development but not in those from days 12.5 or 14.5, is in agreement with these earlier reports. I also could not detect cGnRH-II-like immunoreactivity at any stage of development using both antisera GF-4 and 7CR-10, suggesting that if there were an additional form of GnRH in the rat brain during development, it is not like cGnRH-II.

It is interesting to note that I detected a cGnRH-II-like molecule in the brain of adult and fetal monkey but not in the brain of guinea pig, hamster or adult and fetal rat even though they are all placental mammals and even though similar methodology and antisera were used in the studies. It is possible that cGnRH-II is expressed in the rodent brain at different times of development than in the brain of monkey and therefore, I could not detect cGnRH-II in the present rodent studies. However, because I used a relatively large number of guinea pig, rat and hamster brains, and because I collected rat brains at different times in their development, I should have been able to detect cGnRH-II if it were present in the brain of at least one of the species studied. Additional explanations for the lack of detection for cGnRH-II in rodents are that the cGnRH-II gene has been altered or lost from the rodent genome in evolution.

Although the evidence from this chapter of my thesis suggests that only one of the GnRH peptides found in sturgeon, mGnRH, is retained in the evolution of rodents, it is important to note that my HPLC and RIA studies are at best a "snap-shot" of the forms of GnRH present within the brain of that species at the time of collection. The expression of GnRH within the brain is temporal, with maximal levels of one form or another achieved in different sexes at different times of development or reproductive states. Therefore, the likelihood of detecting specific GnRH forms using these methods is largely dependent on when the brains are collected. Detection of cGnRH-II, which is at best expressed in minute quantities in the brain, might be influenced by this methodological problem. The most definitive proof of multiple forms of GnRH within the brain of different mammals is the characterization of cDNAs or genes encoding different GnRH peptides using more sensitive molecular biology techniques like PCR or genomic and cDNA library

screening. However, a combination of HPLC elution position and cross-reactivity with GnRH specific antisera is an important step in establishing which forms of GnRH are present as mature peptides in the brain and which tissue should be collected for cDNA library preparation.

Literature cited

Adelman, J.P., Mason, A.J., Hayflick, J.S., and Seeburg, P.H. (1986). Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin-release inhibiting factor in human and rat. *Proc. Natl. Acad. Sci. (U.S.A.)* **83**, 179-183.

Bond, C.T., Hayflick, J.S., Seeburg, P.H., and Adelman, J.P. (1989). The rat gonadotropin-releasing hormone: SH locus: Structure and hypothalamic expression. *Mol. Endocrinol.* **3**, 1257-1262.

Chiappa, S.A., and Fink, G. (1977). Releasing factor and hormonal changes in the hypothalamic-pituitary-gonadotrophin and -adrenocorticortrophin systems before and after birth and puberty in male, female and androgenized female rats. *J. Endocrinol.* **71**, 211-224.

Dellovade, T.L., King, J.A., Millar, R.P., and Rissman, E.F. (1993). Presence and differential distribution of distinct forms of immunoreactive gonadotropin-releasing hormone in the musk shrew brain. *Neuroendocrinology* **58**, 166-177.

Gautron, J-P., Pattou, E., Bauer, K. and Kordon, C. (1991). (Hydroxyproline^9) luteinizing hormone-releasing hormone: a novel peptide in mammalian and frog hypothalamus. *Neurochem. Int.* **18**, 221-235.

Jansen, H.T., Berriman, S.J., Stevens, P.J., Zeietler, P. and Lehman, M.N. (1995). Cloning and sequencing of cDNA for hamster GnRH. Society for Neuroscience Meetings, Nov. 11-16, San Diego, California (abstract 745.4).

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

Kelsall, R., Coe, I.R., and Sherwood, N.M. (1990). Phylogeny and ontogeny of gonadortopin-releasing hormone: comparison of guinea pig, rat, and a protochordate. *Gen. Comp. Endocrinol.* **78**, 479-494.

Kepa, J.K., Wang, C., Neeley, C.I., Raynolds, M.V., Gordon, D.F., Wood, W.M., and Wierman, M.E. (1992). Structure of the rat gonadotropin releasing hormone (rGnRH) gene promoter and functional analysis in hypothalamic cells. *Nucleic Acids Res.* **20**, 1393-1399.

King, J.A., Dufour, S., Fontaine, Y.A., Millar, R.P. (1990). Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* **11**, 507-514.

King, J.A., Hassan, M.F., Meh,. A.E.I., Millar, R.P. (1988) Gonadotropin-releasing hormone molecular forms in mammalian hypothalamus. *Endocrinology* **122**, 2742-2752.

King, J.A., Hinds, L.A., Mehl, A.E.I., Saunders, N.R. and Millar, R.P. (1990). Chicken GnRH II occurs together with mammalian GnRH in a South American species of marsupial (*Monodelphis domestica*). *Peptides* **11**, 521-525.

King, J.A., Mehl, A.E.I., Tyndale-Biscoe, C.H., Hinds, L.A. and Millar, R.P. (1989). A second form of gonadotropin-releasing hormone (GnRH), with chicken GnRH II-like properties, occurs together with mammalian GnRH in marsupial brains. *Endocrinology* **125**, 2244-2252.

King, J.A., Steneveld, A.A., Curlewis, J.D., Rissman, E.F. and Millar, R.P. (1994). Identification of chicken GnRH II in brains of metatherian and early-evolved eutherian species of mammals. *Regul. Peptides* **54**, 467-477.

Lovejoy, D.A., Stell., W.K., and Sherwood, N.M. (1992). Partial characterization of four forms of immunoreactive gonadrotopin-releasing hormone in the brain and terminal nerve of the spiny dogfish (Elasmobranchii; *Squalus acanthius*). *Regul. Peptides* **37**, 39-48.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young III, W.S., Phillips, H.S., Nikolics, K., Seeburg, P.H. (1986). A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science* **234**, 1366-1371.

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

Oikawa, M., Dargan, C., Ny, T., and Hsueh, A.J.W. (1990). Expression of gonadotropin-releasing hormone and prothymosin-messenger ribonucleic acid in the ovary. *Endocrinology* **127**, 2350-2356.

Ronchi, E., Aoki, C., Krey, L.C., and Pfaff, D.W. (1992). Immunocytochemical study of GnRH and GnRH-associated peptide in male syrian hamsters as a function of photoperiod and gonadal alterations. *Neuroendocrinology* **55**, 134-145.

Schwanzel-Fukuda, M., and Silverman, A.J. (1980). The nervus terminalis of the guinea pig: a new luteinizing hormone-releasing hormone (LHRH) neuronal system. *J. Comp. Neurol.* **191**, 213-225.

Seeburg, P.H., Mason, A.J., Stewart, T.A. and Nikolics, K. (1987). The mammalian GnRH gene and its pivotal role in reproduction. *Recent Prog. Hormone Res.* **43**, 69-90.

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.R., Lescheid, D.W. (1997). Origin and evolution of gonadotropin-releasing hormone in vertebrates and invertebrates. In GnRH: Gene to Behavior (I. S. Parhar and Y. Sakuma, Eds.) Brain Shippon Publishers, Tokyo, Japan. (in press).

Chapter 7

Isolation and characterization of the cDNA(s) encoding GRF/ PACAP from the brain of white sturgeon, *Acipenser transmontanus*.

Introduction

In the final study of this thesis, I shifted my focus from studying GnRH to studying growth hormone-releasing factor, GRF, one of the central neuropeptide regulators of somatic growth in vertebrates. GRF also is useful as a molecular tool to help establish the phylogeny of species because the DNA and protein structures of GRF have been established in several species and can be used for comparative purposes.

Although there have been many reports of GnRH in non-mammalian vertebrates, there is much less known about GRF in species other than mammals. In teleosts, the only peptide with GRF sequence identity isolated to date is from carp (Vaughan *et al.*, 1991) but there are reports of cDNAs encoding a GRF-like peptide in salmon (Parker *et al.*, 1993), catfish (McRory *et al.*, 1995) and zebrafish (Delgado *et al.*, 1996) suggesting that the hormone is likely to be present in other fish species. An interesting discovery in teleosts was that the mRNA precursor encoding the GRF-like peptide also encoded another peptide in tandem; this peptide was pituitary adenylate cyclase-activating polypeptide, PACAP. This discovery was surprising because PACAP is encoded by a separate gene in mammals. In mammals the PACAP mRNA precursor also encodes PACAP-related peptide, PRP; which despite being found in the median eminence and despite having some sequence identity to GRF, has no known function (Mikkelsen *et al.*, 1995; Sherwood *et al.*, 1994). The organization of the mRNA makes the GRF-like/ PACAP presursor more similar to the mammalian PRP/ PACAP precursor than to the mammalian GRF precursor. Furthermore, the GRF-like peptide in teleosts has sequence identity to both PRP in mammals and mammalian GRF. The question is whether the GRF-like/ PACAP precursor in teleosts is ancestral to both PACAP and GRF in mammals or whether there is a separate gene encoding GRF in non-mammalian vertebrates that has not been discovered. A GRF-like/PACAP molecule recently has been isolated from the neural ganglion of tunicate (McRory and Sherwood, 1997), suggesting that GRF and PACAP encoded together on a single mRNA precursor is indeed the ancestral condition, predating the emergence of

vertebrates. Also, a GRF-like / PACAP precursor has been isolated from the brain of chicken (McRory *et al.*, 1997) further suggesting that having GRF and PACAP encoded on separate genes is a relatively recent event in evolution, occurring after the emergence of reptilian and avian ancestors. However, it is still possible that the GRF-like/ PACAP precursor in teleosts is ancestral to only the mammalian PACAP precursor and that there is another precursor ancestral to GRF in the more ancient species. Additional proof would be to determine if the GRF-like peptides predicted from the mRNA precursors in fish and chicken effectively function as true growth hormone releasers in these species. To date, only one functional study in salmon has been done and shows that both GRF and PACAP release GH *in vitro* (Parker *et al.*, 1997)

Sturgeon is an evolutionarily ancient fish that has ancestors that emerged at a branch point between the mammalian lineage and the teleostean lineage. Therefore, because of the importance of sturgeon as a species representing a transition point in evolution, characterization of the precursor encoding GRF and/or PACAP from the brain would be useful in determining the phylogeny of these important neuropeptides. The sturgeon also makes an interesting species from which to isolate these putative growth regulators because they have an unusual growth process for fish, with highly variable growth among juveniles and a continuous growth in adults until they reach an enormous size for fish, up to six meters in length. It would be interesting to determine whether the uniqueness in their growth process is reflected in a difference in their GRF/ PACAP structures relative to other species. My approach was to use the polymerase chain reaction (PCR) with oligonucleotide primers based on conserved sequences from other species to isolate the cDNA(s) encoding GRF/ PACAP from the brain of white sturgeon, *Acipenser transmontanus*. The tissue and cDNA used for part of this study was collected and prepared by Dr. P. Danielson at the University of Denver.

Materials and Methods

This project was completed in two separate stages. In stage 1, two separate cDNAs were isolated; one encoded a region from the middle of the signal peptide to the beginning of PACAP and one encoded the remainder of PACAP and part of the 3' untranslated region, 3' UTR (Fig. 7-1A). In stage 2, the sequences from stage 1 were confirmed and the sequences encoding all of the 3'UTR, the remainder of the signal peptide and some of the 5' UTR were determined (Fig. 7-1B).

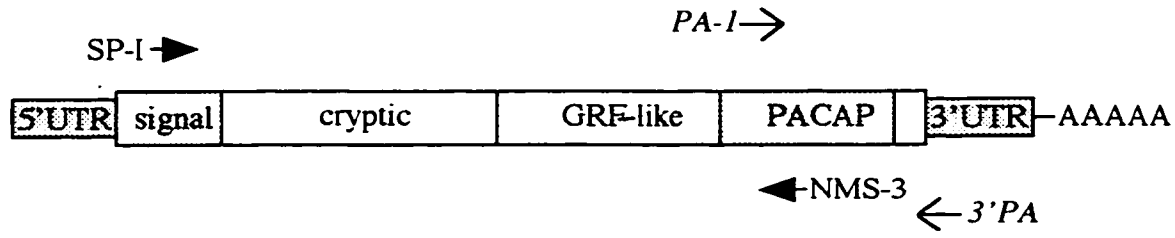
mRNA purification and cDNA synthesis

Stage 1

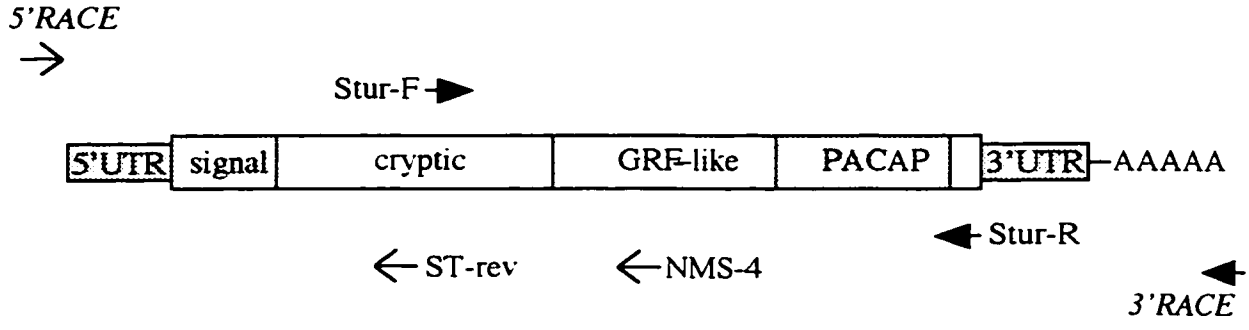
White sturgeon, *A. transmontanus*, were anesthetized in MS-222 and the brains removed and immediately frozen in liquid nitrogen. The brains were then shipped on dry ice to the University of Victoria, B.C. and stored at -80°C. RNA was isolated from the brains using the single step acidic-guanidium-isothiocyanate method of Chomczynski and Sacchi (1987) and then polyadenylated (poly A^+) mRNA was purified using oligo deoxythymidine (dT) cellulose pellets and the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). First strand cDNA was synthesized in a total volume of 20 μl using 400 ng of poly A^+ mRNA, 4 μl of 5x reverse transcriptase buffer (BRL H^- buffer), 4 μl of 10 mM deoxynucleotide triphosphates (dNTPs), 2μl of 100 mM dithiothreitol (DTT), 200 enzyme units (U) of SuperScript ribonuclease H^- reverse transcriptase (Life Technologies, Rockville, MD) and 0.5 μl of either 20 μM NMS-3 or 0.5 μl of 20 μM 3' PA as anchor primers. The reaction conditions were 25°C for 10 min, 42°C for 50 min, 90°C for 5 min and 4°C for 10 min; then 2U of RNAse H were added and the reaction was incubated for 20 min at 37°C.

Fig. 7. The regions of the sturgeon GRF-like/ PACAP precursor that were amplified by PCR. (A) In stage 1, oligonucleotide primers SP-1 and NMS-3 were used in combination in one PCR, whereas primers PA-1 and 3' PA were used in combination in another PCR. (B) In stage 2, oligonucleotide primers Stur-F and Stur-R were used in combination in the initial PCR, whereas primers Stur-F and 3' RACE, primers 5'RACE and NMS-4, and primers 5'RACE and St-rev were used in combination in other PCRs. The abbreviations are: 5' UTR, 5' untranslated region; signal, signal peptide; cryptic, cryptic peptide; GRF-like, growth hormone-releasing factor-like peptide; PACAP, pituitary adenylate cyclase activating polypeptide; 3' UTR, 3' untranslated region; AAAA, poly-adenylated tail.

A


PA-1
SP-I
5'UTR
signal
cryptic
GRF-like
PACAP
3'UTR
AAAAA
NMS-3
3'PA

B.


5'RACE
Stur-F
5'UTR
signal
cryptic
GRF-like
PACAP
3'UTR
AAAAA
Stur-R
ST-rev
NMS-4
3'RACE

Single stranded cDNA, 100 ng, was used as a template in PCR. The nucleotide primers Sp-1 (5'-GGAATCATAATGCACTACAGTGTC-3') and NMS-3 (5'-TCGGTAGCGGCTGTAGCTATCTG-3') were synthesized to match the signal peptide region and PACAP regions of the sockeye salmon GRF-like/ PACAP cDNA sequence, respectively (Parker *et al.*, 1993). The degenerate nucleotide primers PA-1 (5'-CA[TC]TCGGA[TC]GGGATCTTCACGGA[TC]AG-3') and 3' PA (5'-CATGTTTGGAIAGAAACACAIGAGCG-3') were based on conserved regions in the the salmon PACAP cDNA sequence (Fig. 7-1A) and were used at a later date in PCR.

DNA was amplified in a PCR cocktail containing 1x Promega buffer (50 mM KCl, 10 mM Tris-HCL, 0.1% Triton X-100), 50 pmols of the primers Sp-1 and NMS-3 (or PA-1 and 3' PA, at a later date), 8.0 μl of 1.25 mM dNTPs, 3.0 μl of 25 mM $MgCl_2$ and 2.5 U of Taq DNA polymerase (Promega, Madison, WI) in a 50 μl volume. PCR was performed in an automated thermocycler (Perkin-Elmer/ Cetus, Norwalk, CT) using a step program of one cycle at 94°C for 1.5 min, 55°C for 2.5 min, 72°C for 8 min; 29 cycles at 94°C for 1.5 min, 52°C for 2.5 min, 72°C for 3 min and a final cycle at 94°C for 1.5 min, 51°C for 2.5 min and 72°C for 5.5 min.

PCR products were separated by electrophoresis in 1.5% agarose gels, the bands excised, and DNA recovered by electroelution in dialysis tubing (Spectra Por 6, Spectrum Medical Inc.) with 1x buffer (40 mM Tris-acetate, pH 8.0; 2 mM ethylenediamine tetraacetic acid, EDTA). The eluted DNA was phenol: chloroform extracted, ethanol precipitated and then blunt-end ligated into pBluescript II KS+ plasmids (Stratagene, La Jolla, CA). Plasmids then were electroporated into *Escherichia coli* XL-1 Blue cells (Stratagene) using a Bio-Rad Gene Pulser following the manufacturer's instructions. Recombinant colonies were selected by blue and white selection and the bacteria were grown overnight at 37°C. Plasmid DNA was recovered using alkaline hydrolysis (Birnboin, 1983) and digested with Pvu II (Pharmacia, Bail d'Urfé, Québec) to identify recombinants. Recombinant plasmids with inserts of the predicted size were then

sequenced using the Sanger dideoxy chain termination method (Sanger *et al.*, 1977), [^{35}S] dATP (Dupont, NEN Life Science, Boston, MA) and the Sequenase Version 2.0 kit according to the manufacturer's instructions (U.S. Biochemical Corp., Cleveland, Ohio).

Stage 2

Tissue extraction and cDNA synthesis was performed in the laboratory of Dr. Dores, University of Denver, U.S.A. White sturgeon brains were excised, flash frozen and then crushed to a powder on dry ice. Poly A^+ was isolated by direct capture of mRNA onto oligo dT-25 coated paramagnetic beads (see Danielson and Fogleman, 1997). First strand cDNA was primed from the poly A tail using the primer 5'-GACTCGAGTCGTCGGATCCATCGAT$_{17}$-3', and the SuperScript II first strand cDNA synthesis kit according to the manufacturer's instructions (Gibco BRL, Grand Island, NY). The cDNA was then shipped on dry ice to the University of Victoria.

Amplification of cDNA, 60 ng, was done with the primers Stur-F (5'-GACTTGGCTTTTGACAGTG-3') and Stur-R (5'-TACAAATAGGTCAGTCGGC'3) which are identical to part of the cryptic region and part of the 3' end respectively, of the sturgeon GRF-like/ PACAP cDNA clones isolated in stage 1. PCR was performed using a similar reaction buffer as described previously, but with an initial cycle of 94°C for 2 min, 58°C for 1 min, 56°C for 1 min, 72°C for 3 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and a final cycle of 94°C for 1 min, 55°C for 1 min and 72°C for 7 min. PCR products were separated and excised as described previously, but DNA was recovered by centrifugation through ProSpin columns (Millipore Corp., Bedford, MA). The eluted DNA was phenol chloroform extracted, ethanol precipitated with 1 μl glycogen (10-20 mg/ml) and then ligated into pGEM Vector-T plasmid (Promega). Plasmids were electroporated as described previously, but XL-2-Blue MRF' cells (Stratagene) were used. Recombinant plasmids were selected, miniprepped and Pvu II digested as described

previously but inserts were sequenced using the Circumvent Thermocycle Sequencing kit (New England Biolabs, Boston, MA) according to the manufacturer's instructions.

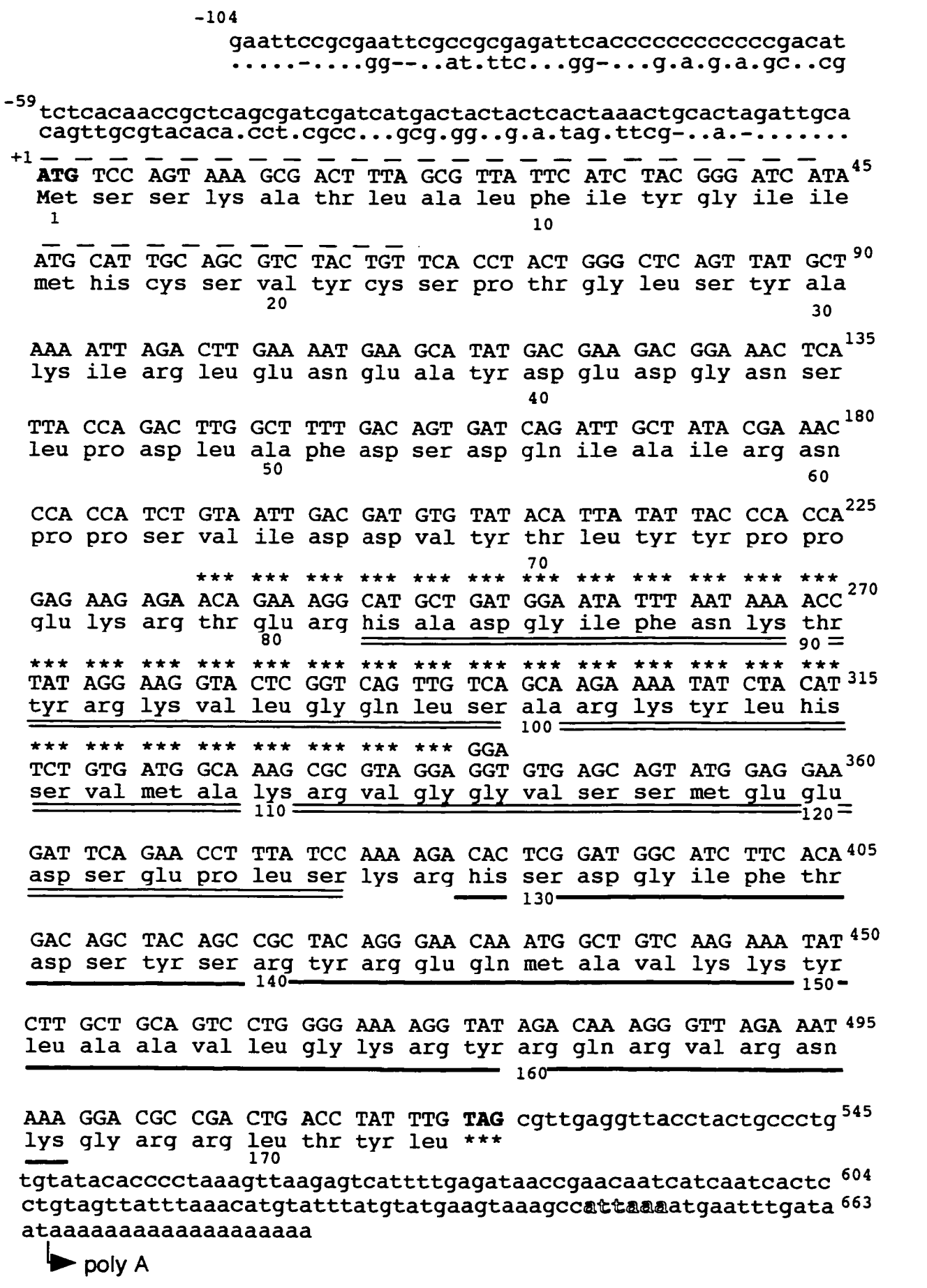
For 3' rapid amplification of cDNA ends (3' RACE, Frohman *et al.*, 1988) 60 ng of cDNA was amplified in PCR using the same reaction buffer as described previously but using the primer Stur-F and a 3' RACE primer (5'-GACTCGAGTCGGATCCATCGAT-3') designed to bind to the anchor primer used in the synthesis of first strand cDNA. PCR cycling conditions were one cycle of 94°C for 5 min, 58°C for 5 min and 72°C for 5 min, 31 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2.5 min and one cycle of 94°C for 1 min, 58°C for 1 min and 72°C for 10 min. PCR products were treated and sequenced exactly as outlined previously.

For 5' RACE (Frohman *et al.*, 1988) a poly-guanine, poly G, tail was first added to 500 ng cDNA using 8.0 μl 5x terminal deoxynucleotidyl transferase, TdT, buffer (500 mM sodium cacodylate pH 7.2, 1 mM 2-mercaptoethanol, 20 mM $MgCl_2$), 0.2 μl of 100 mM deoxyguanine triphosphate, dGTP, and 15 U of TdT (Gibco BRL) in a total volume of 40 μl . The reaction mixture was incubated at 87°C for 2 hours and then the poly G-tailed cDNA was ethanol-precipitated. cDNA was also sham-tailed for a negative control using the same reaction conditions as above but with TdT omitted. cDNA was diluted 1:10 and 1 μl used with similar PCR reaction buffers and cycling conditions as described for 3' RACE. The primers used in PCR were 5' RACE (5'-GAATTCGCGGCCGCTTCAGTC$_{10}$-3') and either NMS-4 (5'-CTACACGCTTTGCCATCAGAGA-3') or St-Rev (5'-CCATCTGTAATTGACGATGTG-3'); the latter two primers were designed according to the cDNA sequences of the salmon GRF-like domain or the sturgeon cryptic region, respectively. PCR products were treated and sequenced as described previously.

Results

I have isolated a white sturgeon GRF /PACAP cDNA that encodes a GRF-like peptide and PACAP molecule in tandem by using PCR with a variety of oligonucleotide primer combinations. In the initial PCR, two nondegenerate primers, Sp-1 and NMS-3, were used to amplify a 372 bp fragment (Fig. 7-1A; Fig. 7-2, nucleotides, nt, 40- 412) that had sequence similarity to the signal peptide, cryptic, GRF-like and PACAP regions of the salmon GRF-like/ PACAP cDNA. An additional PCR, using two degenerate primers, PA-1 and 3' PA, amplified a 233 bp fragment which encoded most of the PACAP region and some of the 3' UTR (Fig. 7-1A, Fig. 7-2, nt 382- 615). At a later date, two other primers, Stur-F and Stur-R, were used in PCR to amplify a 380 bp fragment (Fig. 7-1B, Fig. 7-2, nt 141- 521) which confirmed the sequence data obtained from the two initial positive PCR clones. A 3' RACE reaction using the primers Stur-F and 3' RACE was then used to amplify a 424 bp cDNA fragment which included sequences from the cryptic peptide to the poly A tail (Fig. 7-1B; Fig. 7-2, nt 141-665). Finally, in 5' RACE reactions, the primers 5' RACE and NMS-4 or ST-rev were used to confirm the signal and cryptic peptide sequences and amplify 104 bps of the 5' UTR (Fig. 7-1B; Fig. 7-2). In 5' RACE, two separate clones with nucleotide differences in the 5' UTR were isolated and sequenced (Fig. 7-2). The full length cDNA encoding sturgeon GRF-like/ PACAP was confirmed by the overlapping sequence data obtained from the various PCR fragments.

Fig.7-2. Nucleotide sequence and the corresponding amino acid sequence of the white sturgeon GRF-like/ PACAP precursor. Dashed line above amino acids 1-22 represents the predicted signal peptide; the double underlined region is the predicted GRF-like region and the single underlined region is the predicted PACAP peptide. Lower case letters represent the 5' untranslated region and 3' untranslated region. Amino acids in bold, **ATG** and **TAG**, are the start and stop codons, respectively. The sequence attaaa , nucleotides 645-650, is the putative polyadenylation signal. The starred amino acids, 79-113, are omitted from the truncated sturgeon GRF-like/ PACAP precursor due to alternative splicing.


-104
gaattccgcgaattcgccgcgagattcacccccccccccgacat
.....-....gg--..at.ttc...gg-...g.a.g.a.gc..cg
-59 tctcacaaccgctcagcgatcgatcatgactactactcactaaactgcactagattgca
cagttgcgtacaca.cct.cgcc...gcg.gg..g.a.tag.ttcg-..a.-.......
+1
ATG TCC AGT AAA GCG ACT TTA GCG TTA TTC ATC TAC GGG ATC ATA 45
Met ser ser lys ala thr leu ala leu phe ile tyr gly ile ile
1 10
ATG CAT TGC AGC GTC TAC TGT TCA CCT ACT GGG CTC AGT TAT GCT 90
met his cys ser val tyr cys ser pro thr gly leu ser tyr ala
20 30
AAA ATT AGA CTT GAA AAT GAA GCA TAT GAC GAA GAC GGA AAC TCA 135
lys ile arg leu glu asn glu ala tyr asp glu asp gly asn ser
40
TTA CCA GAC TTG GCT TTT GAC AGT GAT CAG ATT GCT ATA CGA AAC 180
leu pro asp leu ala phe asp ser asp gln ile ala ile arg asn
50 60
CCA CCA TCT GTA ATT GAC GAT GTG TAT ACA TTA TAT TAC CCA CCA 225
pro pro ser val ile asp asp val tyr thr leu tyr tyr pro pro
70
*** *** *** *** *** *** *** *** *** *** *** ***
GAG AAG AGA ACA GAA AGG CAT GCT GAT GGA ATA TTT AAT AAA ACC 270
glu lys arg thr glu arg his ala asp gly ile phe asn lys thr
80 90
*** *** *** *** *** *** *** *** *** *** *** *** *** ***
TAT AGG AAG GTA CTC GGT CAG TTG TCA GCA AGA AAA TAT CTA CAT 315
tyr arg lys val leu gly gln leu ser ala arg lys tyr leu his
100
*** *** *** *** *** *** *** *** GGA
TCT GTG ATG GCA AAG CGC GTA GGA GGT GTG AGC AGT ATG GAG GAA 360
ser val met ala lys arg val gly gly val ser ser met glu glu
110 120
GAT TCA GAA CCT TTA TCC AAA AGA CAC TCG GAT GGC ATC TTC ACA 405
asp ser glu pro leu ser lys arg his ser asp gly ile phe thr
130
GAC AGC TAC AGC CGC TAC AGG GAA CAA ATG GCT GTC AAG AAA TAT 450
asp ser tyr ser arg tyr arg glu gln met ala val lys lys tyr
140 150
CTT GCT GCA GTC CTG GGG AAA AGG TAT AGA CAA AGG GTT AGA AAT 495
leu ala ala val leu gly lys arg tyr arg gln arg val arg asn
160
AAA GGA CGC CGA CTG ACC TAT TTG TAG cgttgaggttacctactgccctg 545
lys gly arg arg leu thr tyr leu ***
170
tgtatacacccctaaagttaagagtcattttgagataaccgaacaatcatcaatcactc 604
ctgtagttatttaaacatgtatttatgtatgaagtaaagccattaaaatgaatttgata 663
ataaaaaaaaaaaaaaaaaa
poly A

Discussion

Once I had isolated the full-length cDNA encoding sturgeon GRF-like/ PACAP, I compared the predicted protein domains to those previously reported for GRF/ PACAP in fish and chicken as well as made comparisons to the peptides encoded by the mammalian GRF and PACAP precursors. The sturgeon precursor encodes a 173 amino acid peptide that contains four separate domains: a 22 amino acid signal peptide, a 59 amino acid cryptic peptide, a 45 amino acid GRF-like region and a 38 amino acid PACAP region (Fig. 7-2).

The sturgeon signal peptide, like the salmon signal peptide, is 22 amino acids, AA, which is smaller in size than the zebrafish and catfish signal peptides of 23 AA, and also than the human, ovine and rat signal peptides of 24 AA. The salmon signal peptide has 73% amino acid identity with the sturgeon signal peptide, whereas there is only 48% to 64% sequence identity if the signal peptides of zebrafish, catfish, chicken and human are compared to sturgeon (Fig. 7-3A). However, despite having low sequence identity in this region, the signal peptide of sturgeon maintains the core of hydrophobic amino acids that is common to the fish, bird and mammalian signal peptides.

The cryptic peptide region has no clearly defined function but has been postulated to maintain the precursor structure in a form that is necessary for proper enzymatic processing. The cryptic regions of human, chicken, and catfish are similar in size, but there is only 35% -39% sequence identity with the sturgeon region (Fig. 7-3A) suggesting that there are few functional constraints except for folding on this part of the precursor, allowing rapid mutation and change in evolution. It is interesting to note that, in contrast to the other cryptic peptides, there is 81% sequence identity between sturgeon and salmon cryptic regions and 59% identity between sturgeon and zebrafish cryptic regions. (Fig. 7-3A).

The sturgeon GRF-like peptide is flanked by a single arginine processing site (Fig. 7-2, AA 81) and a dibasic Lys-Arg enzymatic processing site (Fig. 7-2, AA 127- 128) predicting a 45 amino acid sturgeon GRF with a free acid hydroxylated carboxy-terminus.

Fig.7-3. Comparison of domains in the precursors of GRF-like/ PACAP or PRP/ PACAP. (A) The percent sequence identity between the domains of the white sturgeon GRF-like/ PACAP precursor to those of the human PRP/ PACAP precursor or the chicken, zebrafish, catfish, salmon and tunicate GRF-like/ PACAP precursors. (B) The percent sequence identity between the domains of human PRP/ PACAP precursor to those of chicken, sturgeon, zebrafish, catfish, salmon or tunicate GRF-like/ PACAPs. Abbreviations are: signal, signal peptide; cryptic, cryptic peptide; GRF-like, growth hormone-releasing factor-like peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; 3' UTR, 3' untranslated region.

A.

Sturgeon
GRF-like/PACAP

	signal	cryptic	GRF-like		PACAP		3'UTR
			29	45	27	38	142
human	64	35	62	59	97	92	74
chicken	48	37	83	76 or 81	90	90	30
zebrafish	52	59	79	76	97	92	25
catfish	57	39	59	62	97	92	22
salmon	73	81	86	82	97	95	79
tunicate-I	62	-----	63		96	-----	-----
tunicate-II	15	-----	59		85	-----	-----

B.

Human
PRP/PACAP

	signal	cryptic	PRP		PACAP	
			29	48	27	38
chicken	48	35	55	58	96	97
sturgeon	**64**	**39**	**59**	**54**	**96**	**92**
zebrafish	39	25	48	50	93	84
catfish	52	26	45	46	100	90
salmon	68	35	59	52	100	92
tunicate-I	67	-----	41	-----	96	-----
tunicate-II	20	-----	45	-----	85	-----

The length of sturgeon GRF, 45 AA, is similar to that of salmon, catfish, zebrafish and carp GRFs but unlike chicken GRF of 43 or 46 AA, human, pig, cow, goat, sheep and hamster GRFs of 44 AA, rat GRF of 43 AA, and mouse GRF of 42 AA. The presence of histidine in position 1 and a free acid carboxy -terminus in sturgeon GRF is like the fish GRFs, chicken GRF and rat and mouse GRF but unlike other mammalian GRFs that have tyrosine in position 1 and an amidated carboxy terminus (Fig. 7-4). Sturgeon GRF_{1-45} not only has an aromatic amino acid in the first position, shown to be critical in receptor binding, but also contains phenylalanine in position 6 which is found in all other GRFs except for catfish (Fig. 7-4) and is essential for full biological activity (Coy *et al.*, 1986; Ling *et al.*, 1984a). If the first 44 amino acids of GRF-like peptides are aligned among species, the sturgeon GRF-like peptide has 48% sequence identity to human GRF_{1-44} compared to 32% - 45% sequence identity between human GRF_{1-44} and the GRF-like peptides of chicken, carp, salmon, zebrafish and catfish (Fig. 7-4).

There is another Lys-Arg enzymatic processing site present within the sturgeon GRF peptide (Fig. 7-2, AA 110 -111) and therefore, a 29 amino acid peptide might also be produced. A 29 amino acid peptide potentially can be cleaved from all other known GRF peptides and might be more useful than the full length peptide in determining the relatedness of GRF between species because it is the biologically active core of the molecule (Ling *et al.*, 1984b) and also is encoded on one exon; the most variable region of GRF, AA 33-44 in human (Mayo *et al.*, 1985), AA 33-45 in salmon (Parker *et al.*, 1997) or AA 33-46 in chicken (Parker *et al.*, 1997), is encoded by a separate exon. A comparison of the GRF-like peptides of fish shows that sturgeon GRF_{1-29} has 86% amino acid identity with salmon GRF_{1-29}, 79% with zebrafish GRF_{1-29} and 59% with catfish GRF_{1-29}, respectively (Fig. 7-3A).

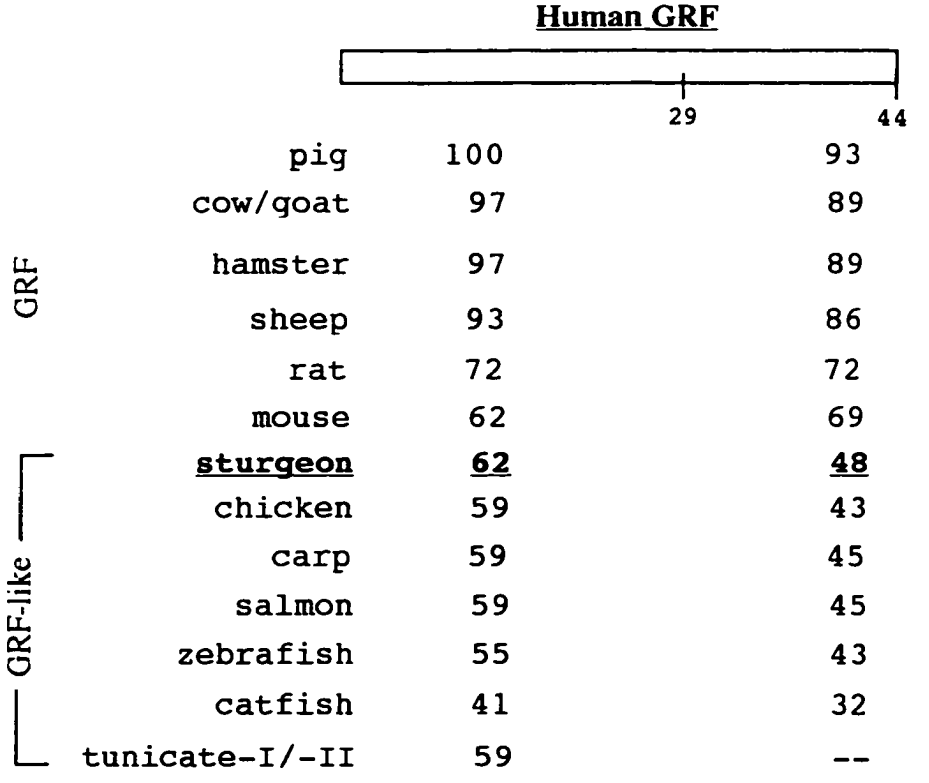
Fig.7-4. A comparison of the known GRF and GRF-like peptides to human GRF. The complete amino acid sequences are shown in the top portion of the figure whereas, the percent amino acid sequence identity is shown in the lower portion.

A.

GRF peptides compared to human GRF

		1 29 44
GRF	human	YADAIFTNSYRKVLGQLSARKLLQDIMS-RQQGESNQERGARARL-NH2
	pig	**********-*****R***Q***V**-NH2
	cow/goat	**********N-*****R***Q**KV**-NH2
	hamster	*******S**********-*****R***Q*P*V**-NH2
	sheep	**********I**********N-*****R***Q**KV**-NH2
	rat	H******S***RI****Y*****NE**N-*****R***Q--*S*FN-OH
	mouse	HV*****TN***L*S**Y***VI****N-K*-**RI**Q--****S-OH
GRF-like	**sturgeon**	**H**G**NKT**********Y*HSV*AK*VG*V*SM*EDSEPLS-OH**
	chicken	H**G**SKA***L*******NY*HSL*AK*VG*A*SGLGDEAEPLS-OH
	carp	H**GM*NKA***A********Y*HTL*AK*VG*G*MI*DDNEPLS-OH
	salmon	H**GM*NKA***A********Y*HSL*AK*VG*G*TM*DDTEPLS-OH
	zebrafish	H**GM*NKA***AF*******Y*HTL*AK*VG*G*TT*DDNEPLS-OH
	catfish	H**GLLDRAL*DI*V******Y*HSLTAV*VGE*EED*EDSEPLS-OH
	tunicate-I	HS*G***KD***Y****R*Q*F**WL*KR-OH

B.



Surprisingly, the first 29 amino acids of sturgeon GRF have higher sequence identity with human GRF_{1-29}, 62%, and chicken GRF $_{1-29}$, 83%, than with catfish GRF_{1-29}, 59%, (Fig. 7-3A) even though sturgeon are closer in evolutionary origin to catfish than to humans or chickens.

The GRF/ PACAP precursors of sturgeon, salmon, catfish, zebrafish and chicken are more similar in organization to mammalian PRP/ PACAP than they are to mammalian GRF. Also, the full length GRFs are closer in identity to PRP than to GRF (Fig. 7-3B and Fig. 7-4B). Contrarily, PRP lacks the Tyr or His as the essential first AA in the molecule and was not functional when tested for activation of cAMP (Mikkelson *et al.*, 1995). Therefore, comparing the biologically active core of the first 29 amino acids of their GRF-like peptides with human PRP_{1-29} might also provide clues to the history of the mammalian GRF and/ or PACAP peptides. The sequence similarities of the GRF $_{1-29}$ peptides of sturgeon, salmon and chicken are a little closer to human PRP $_{1-29}$ (55-59%), than those of zebrafish, catfish and tunicate (41%-48%) (Fig. 7-3B). The sequence identity of the sturgeon GRF_{1-29} is 62% with human GRF_{1-29} and only 59% with human PRP_{1-29}, but this difference is not striking enough to make conclusions about the evolution of GRF or PACAP. Future studies comparing the functions of sturgeon GRF_{1-29} with the functions of human GRF_{1-29} and human PRP_{1-29} would be more useful in establishing the relatedness of these molecules. Meanwhile, the relative closeness in sequence identity between the first 29 amino acids of the sturgeon GRF-like, human GRF and human PRP peptides provides additional support for the hypothesis that the the three peptides share a common ancestry.

The sturgeon GRF-like/ PACAP precursor also encodes a 38 amino acid PACAP peptide that is amidated, if Lys^{128} / Arg^{129} and Gly^{167} / Arg^{168} / Arg^{169} (Fig. 7-2) are used as enzymatic cleavage (Lys-Arg) and amidation (Gly) sites. Also, a 27 amino acid PACAP peptide that is amidated could be processed from PACAP $_{1-38}$ if the cleavage site

at Gly^{156} / Lys^{157} / Arg^{158} is used (Fig. 7-2). The structure of sturgeon PACAP, with putative 38 or 27 amino acid peptides that have amidated carboxy-termini is identical to the PACAPs isolated from other vertebrate species. In addition, there is 90%-95% sequence identity of sturgeon PACAP to human, chicken, zebrafish, catfish or salmon PACAPs (Fig. 7-3A). $PACAP_{1-27}$ is also highly conserved, 90%-97%, in non-mammalian vertebrates when compared to sturgeon PACAP $_{1-27}$ (Fig. 7-3B). Even tunicate $PACAP_{1-27}$ has over 85% sequence identity with vertebrate $PACAP_{1-27}$ (Fig. 7-3A) suggesting that they share a common ancestry and that this peptide has been conserved for over 600 million years (McRory and Sherwood, 1997). It is interesting to note that even though the PACAP domain is the most highly conserved domain in the precursor (Fig. 7-3A) the major function of PACAP is not yet clear in any vertebrate.

Two GRF/ PACAP precursors were isolated from sturgeon brain in this study. One cDNA was 105 bp shorter than the other and the nucleotides encoding the last 3AA of the cryptic peptide and the first 32 AA of GRF were missing (Fig. 7-2). The putative protein products from this truncated cDNA would include the signal, cryptic, and PACAP peptides but not the active core of the GRF peptide. This phenomenon has been described previously in five species of salmon and the domestic chicken and has been attributed to exon skipping during processing of the RNA transcript. If exon IV of the GRF/ PACAP gene in chicken and salmon were excised during mRNA processing the last three amino acids of the cryptic peptide and the first 32 amino acids of the GRF-like molecule would not be present (McRory *et al.*, 1997; Parker *et al.*, 1997). This alternative processing would result in the production of only PACAP peptides and therefore, might provide a means for increasing the relative amount of PACAP produced compared to the amount of GRF peptides produced. An increased amount of PACAP relative to GRF might have functional implications during development or in different tissues (McRory *et al.*, 1997; Parker *et al.*, 1997). Tissue specificity in alternative processing of precursors also has been described for the PHI/ VIP gene of turkeys and chickens, resulting in a shorter

mRNA transcript in the optic nerve and small intestine but not in the brain (Seugkivon *et al.*, 1995; Talbot *et al.*, 1995). The presence of a truncated GRF/ PACAP cDNA precursor in sturgeon suggests that exon skipping also occurs during mRNA processing and that this method of increasing the amount of one peptide relative to another might be an evolutionarily ancient mechanism.

Some interesting comparisons can be made if the nucleotide regions flanking the protein encoding domains of the GRF-like/ PACAP or PRP/ PACAP precursors of the different species are aligned. The 3' UTR of sturgeon GRF-like/ PACAP is 142 bp (Fig. 7-2), which is similar in size to the 3' UTR of salmon GRF-like/ PACAP, but is considerably shorter than the 3' UTR of zebrafish, catfish and chicken GRF-like/ PACAPs (355 bp, 1581 bp, 556 bp, respectively) and the 3' UTR of human PRP/ PACAP (1400 bp). One possibility for the relatively short 3'UTR in sturgeon GRF-like PACAP is that several polyadenylation signals exist in the gene, but the first one had been used for the cDNA isolated in this study. The short 3' UTR of sturgeon GRF-like/ PACAP appears to be complete because of the presence of a poly A tail and a putative polyadenylation site, *ATTAAA*, 16 bp upstream (Fig. 7-2). A similar polyadenylation signal is found in the cDNAs encoding salmon and zebrafish GRF-like/ PACAP, but not in the catfish and chicken GRF-like/ PACAP cDNAs or in human and ovine PRP/ PACAP precursors which use *AGTAAA* as polyadenylation signals (Delgado *et al.*, 1996; McRory *et al.*, 1997; see Parker *et al.*, 1997). If the first 142 bp of the 3' UTR of fish and chicken GRF-like/ PACAP and human PRP/ PACAP are aligned, the sturgeon 3' UTR has 79% nucleotide sequence identity with the 3' UTRs of salmon and 74% with human. In contrast, the first 142nts of the 3' UTRs of chicken, zebrafish and catfish have only 22%-30% sequence identity with the 3' UTR of sturgeon GRF/ PACAP (Fig. 7-3A).

The sturgeon signal, cryptic, GRF-like, PACAP and 3' UTRs have higher sequence identity to the same domains in the salmon precursor than those of zebrafish and catfish. The most striking difference among the fish precursors is in the 3' UTR which has

79% sequence identity between salmon and sturgeon but only 25% between zebrafish and sturgeon and 22% between catfish and sturgeon (Fig. 7-3A). There is also 74% sequence similarity between the first 142bp of the 3' UTR of human PRP/ PACAP and the 3' UTR of sturgeon GRF-like/ PACAP (Fig. 7-3B) which is relatively high compared to the same region in other GRF-like precursors except for salmon. The 3' UTR has been implicated in the control of mRNA transcription and stability (Kozak, 1991) and therefore, relatively high sequence identity in this region among sturgeon, salmon and human precursors might suggest a conservation of function.

Two separate 5' UTRs were characterized for sturgeon GRF/ PACAP. These 5' UTRs were 104 bp and 98 bp and had only 43.3% sequence identity to each other (Fig. 7-2). The length of these 5' UTRs is short relative to the 5' UTRs of zebrafish, catfish, chicken and salmon GRF-like/ PACAPs (211 bp, 327 bp, 250 bp and 473 bp, respectively). It is possible that the 5' UTR of the sturgeon GRF-like/ PACAP precursor is incomplete as truncated 5' UTRs also have been reported for salmon GRF-like/ PACAP (Parker *et al.*, 1993) and human PRP/ PACAP cDNAs (Okhubo *et al.*, 1992; Kimura *et al.*, 1990); these truncated 5' UTRs may reflect limitations of 5' RACE methodology. The presence of more than one potential 5' UTR for a single preprohormone precursor as shown in this study has been described previously in salmon GRF-like /PACAP (Parker *et al.*, 1993), human GRF (Gubler *et al.*, 1983), rat insulin-like growth factor I (Lowe *et al.*, 1987) and suggests differences in the regulation of the molecule.

Three structural features of the sturgeon GRF-like/ PACAP precursor show that it belongs in the glucagon superfamily of peptides. First, sturgeon GRF retains two critical amino acids common to other glucagon superfamily members: a Histidine in position 1 (Fig. 7-5), which is required for receptor binding (Ling *et al.*, 1984; Coy *et al.*, 1987) and a phenylalanine in position 6 (Fig. 7-5), which is important for biological activity (Coy *et al.*, 1987). Second, the structure of the sturgeon GRF-like/ PACAP precursor, with two separate peptides encoded in tandem in a single mRNA, is similar to other glucagon

superfamily members like PHM-27/ VIP (Bodner *et al.*, 1985; Lamperti *et al.*, 1991), PRP/ PACAP (Hosoya *et al.*, 1992; Kimura *et al.*, 1990; Ogi *et al.*, 1990; Ohkubo *et al.*, 1992), and glucagon/ GLP-1/ GLP-2 (Heinrich *et al.*, 1984). Third, there is over 50% sequence identity between sturgeon GRF or PACAP and some of the glucagon superfamily members. For example, sturgeon GRF_{1-27} has 63% sequence identity with human PHM-27 (Fig. 7-5) whereas sturgeon $PACAP_{1-27}$ has 57% and 64% sequence identity with dogfish VIP and human VIP (Fig. 7-6), respectively. The high sequence identity between these peptides relative to other superfamily members suggests that the sturgeon GRF-like/ PACAP precursor is most closely related to the PHM/ VIP precursor and that these two molecules diverged more recently than the other members of this superfamily.

The sturgeons are of particular interest in phylogenetic studies because of their ancient origin and possible relationship to the tetrapod line. Sturgeon-like fossils have been dated to the early Jurassic period, suggesting that ancestors to modern day sturgeons evolved at least 200 million years ago. In mammals, PACAP and GRF are encoded on separate genes, whereas in chicken, salmon, catfish, zebrafish and tunicate, the GRF-like and PACAP peptides appear to be encoded in tandem in the same precursor. In this chapter of my thesis, I have shown that GRF and PACAP are also encoded by a single mRNA in an evolutionarily ancient species like sturgeon, strengthening the idea that a precursor encoding both GRF and PACAP represents the ancestral condition.

Fig.7-5. White sturgeon GRF-like peptide compared to glucagon superfamily members. The complete amino acid sequences are shown in the top portion of the figure, whereas the percent amino acid sequence identity is shown in the lower portion. The calculated percent identity is based on the number of AA in the shorter molecule being compared.

Sturgeon GRF-like peptide compared to glucagon superfamily members

	1	29
GRF-like sturgeon	HADGIFNKTYRKVLGQLSARKYLNSVMAKRVGGVSSMEE--D-SEPLS
PHM-27 human	****V*TSDFS*L******K***E*L*
PRP human	DV*H**L*EA*****D****G*H*Q*LV*RG***-*LGGGAG*DA****
PHI-27 rat	****V*TSD*SRL***I**K***E*LI
GRF human	Y**A**TNS***********L*QDI*SRQQ*E-*NQ*RGARARL
VIP human	*S*AV*TDN*TRLRK*MAMK*****ILN
VIP dogfish	*S*AV*TDN*SRIRK*MAVK**IN*LL*
PACAP human	*S****TDS*S*YRK*MAVK***AA*LGKRYKQPVKNK
GLP-1 paddlefish	****TYTSDASSF*-*EQ*ARDFI*WLK*GQ
glucagon human	*SQ*T*TSD*S*Y*DSRR*QDFVQWL*NT
glucagon salmon	*SE*T*SND*S*Y*ETRR*QDFVQWL*NS
secretin human	*S**T*TSKLSR-*RE-G**LQRLYQGLV
GLP-2 human	****S*SDEMNTI*DN*AT*DFI*WLIQTKITDKK
GLP-1 human	**E*T*-TSDVSSYLEGQ*A*EFIAWLVKGRG
GIP human	Y*E*T*ISD*SIAMDKIRQQDFV*WLL*QKGKKSDWKHNITQ

Sturgeon GRF-like peptide
sequence identity with glucagon superfamily members

	(%)
PHM-27 human	63
PRP-48 human	58
PHI-27 rat	56
GRF human	48
VIP human	36
VIP dogfish	32
PACAP human	32
GLP-1 paddlefish	30
glucagon human	28
glucagon salmon	28
secretin human	26
GLP-2 human	26
GLP-1 human	29
GIP human	14

Fig.7-6. White sturgeon PACAP peptide compared to glucagon superfamily members. The complete amino acid sequences are shown in the top portion of the figure, whereas the percent amino acid sequence identity is shown in the lower portion. The calculated percent identity is based on number of AA in the shorter molecule being compared.

Sturgeon PACAP compared to glucagon family members

```
                  1                                  27
PACAP sturgeon HSDGIFTDSYSRYREQMAVKKYLAAVLGKRYRQRVRNK
    VIP dogfish ***AV***N***I*K*******INSL*A
      VIP human ***AV*T*N*T*I*K***M****NSL*A
secretin human ****T**SKL**L**G-*RLQR*LQG*V
  PHM-27 human *A**V**SDF**LLG*LSA****ESLI
    PHI-27 rat *A**V*SSD***LLQ*ISA****ESLI
glucagon human **QGT**SD*SK*LDSRRAQDFVQWLMNT
```

Sturgeon PACAP sequence identity with glucagon family members

(%)

VIP dogfish	64
VIP human	57
secretin human	48
PHM-27 human	44
PHI-27 rat	44
glucagon human	21

Literature cited

Birnboim, M. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* **100**, 243-255.

Bodner, M., Fridkin, M. and Gozes, I. (1985). Coding sequences for vasoactive intestinal peptide and PHM-27 peptide are located on two adjacent exons in the human genome. *Proc. Natl. Acad. Sci. (U.S.A.)* **82**, 3548-3551.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **62**, 156-159.

Coy, D.H., Murphy, W.A. and Lance, V.A. (1987). Observations on the chemistry and biology of growth hormone releasing factor. In: "Growth Hormone, Growth Factors, and Acromegaly (K. Lüdecke and G. Tolis, Eds.)" Raven Press, New York, pp. 13-20.

Danielson, P.B., and Fogleman, J.C. (1997). Isolation and sequence analysis of cytopchrome P450 12B1: the first mitochondrial insect P450 with homology to alpha, 25 dihydroxy-d3 24-hydroxylase. *Insect Biochem. Mol. Biol.* (in press)

Delgado, E., Wei, Y, Martin, S.C., Hienrich, and Mojsov, S. (1996). Cloning and characterization of PACAP peptides in zebrafish. Endocrine Society Meetings, San Francisco, CA, June 12-13. Abstract P2-367.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. (U.S.A.)* **85**, 8998-9002.

Gubler, U., Monahan, J.J., Lomedico, P.T., Bhatt, R.S., Collier, K.J., Hoffman. B.J., Böhlen, P., Esch, F., Ling, N., Zeytin, F., Brazeau, P., Poonian, M.S. and Gage, L.P. (1983). Cloning and sequence analysis of cDNA for the precursor of human growth hormone-releasing factor, somatocrinin. *Proc. Natl. Acad. Sci. (U.S.A.)* **80**, 4311-4314.

Heinrich, G., Gros, P. and Habener, J.F. (1984). Glucagon gene sequence. *J. Biol. Chem.* **259**, 14082-14087.

Hosoya, M., Kimura, C., Ogi, K., Ohkubo, S., Miyamoto, Y., Kugoh, H. Shimizu, M., Onda, H., Oshimura, M., Arimura, A. and Fujino, M. (1992). Structure of the human pituitary adenylate cyclase activating polypeptide (PACAP) gene. *Biochim. Biophys. Acta* **1129**, 199-206.

Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R.R., Stibbs, H.H., Arimura, A., and Fujino, M. (1990). A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs. *Biochem. Biophys. Res. Commun.* **166**, 81-89.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867-19870.

Lamperti, E.D., Rosen, K.M. and Villa-Komaroff, L. (1991). Characterization of the gene and messages for vasoactive intestinal polypeptide (VIP) in rat and mouse. *Mol. Brain Res.* **9**, 217-231.

Ling, N., Baird, A., Wehrenberg, W.B., Ueno, W., Munegun, T., and Brazeau, P. (1984a). Synthesis and *in vitro* bioactivity of C-terminal deleted analogs of human growth hormone-releasing factor. *Biochem. Biophys. Res. Commun.* **123**, 854-861.

Ling, N., Baird, A., Wehrenberg, W.B., Ueno, N., Munegumi, T., Chiang, T.C., Regno, M., and Brazeau, P. (1984b). Synthesis and *in vitro* bioactivity of human growth hormone-releasing fsctor analogs substituted at position-1. *Biochem. Biophys. Res. Commun.* **122**, 304-310.

Lowe, W.L., Roberts, C.T., Lasky, S.R. and Roith, D. (1987). Differential expression of alternative 5' untranslated regions in mRNAs encoding rat insulin-like growth factor I. *Proc. Natl. Acad. Sci. (U.S.A.)* **84**, 8946-8950.

Lu, N., and DiCicco-Bloom, E. (1997). Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells. *Proc. Natl. Acad. Sci. (U.S.A.)* **94**, 3357-3362.

Mayo, K.E., Cerelli, G.M., Lebo, R.V., Bruce, B.D., Rosenfeld, M.G., and Evans, R.M. (1985). Gene encoding growth hormone-releasing factor precursor: structure, sequence and chromosomal assignment. *Nature* **306**, 86-88.

McRory, J. E., and Sherwood, N. M. (1997). Two protochordate genes encode pituitary adenylate cyclase-activating polypeptide and related family members. *Endocrinology* **138**, 2380-2390.

McRory, J.E., Parker, D.B., Ngamvongchon, S., and Sherwood, N.M. (1995). Sequence and expression of cDNA for pituitary adenylate cyclase-activating polypeptide (PACAP) and growth hormone-releasing hormone (GHRH)-like peptide in catfish. *Mol. Cell. Endocrinol.* **108**, 169-177.

McRory, J.E., Parker, R.L. and Sherwood, N.M. (1997). Expression and alternate processing of a chicken gene encoding both growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide. *DNA Cell. Biol.* **16**, *95-102*

Mikkelsen, J.D., Hannibal, J., Fahrenkrug, J., Larsen, P.J., Olcese, J., and McArdle, C. (1995). Pituitary adenylate cyclase activating peptide-38 (PACAP-38), PACAP-27, and PACAP-related peptide (PRP) in the rat median eminence and pituitary. *J. Neuroendocrinol.* **7**, 47-55.

Ogi, K., Kimura, C., Onda, H., Arimura, A. and Fujino, M. (1990). Molecular cloning and characterization of cDNA for the precursor of rat pituitary adenylate cyclase activating polypeptide (PACAP). *Biochem. Biophys. Res. Commun.* **173**, 1271-1275.

Ohkubo, S., Kimura, C., Ogi, K., Okazaki, K., Hosoya, M., Onda, H., Miyata, A., Arimura, A. and Fujino, M. (1992). Primary structure and characterization of the precursor to human pituitary adenylate cyclase activating polypeptide. *DNA Cell Biol.* **11**, 21-30.

Parker, D.B., Coe, I.R., Dixon, G.H., and Sherwood, N.M. (1993). Two salmon neuropeptides encoded by one brain cDNA are structurally related to members of the glucagon superfamily. *Eur. J. Biochem.* **215**, 439-448.

Parker, D.B., Power, M.E., Swanson, P., Rivier, J., and Sherwood, N.M. (1997). Exon skipping in the gene encoding pituitary cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* **138**, 414-423.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. (U.S.A.)* **74**, 5463-5467.

Seungkwon, Y., Silsby, J.L., Farris, J., Foster, D.N., and El Halawani, M.E. (1995). Tissue specific alternative splicing of turkey preprovasoactive intestinal peptide messenger ribonucleic acid, its regulation and correlation with prolactin secretion. *Endocrinology* **136**, 2602-2610.

Sherwood, N.M., Parker, D.P., McRory, J.E. and Lescheid, D.W. (1994). Molecular evolution of growth hormone-releasing hormone and gonadotropin-releasing hormone. *In* "Fish Physiology. Vol. XIII, (N.M. Sherwood and C.L. Hew, Eds.)", Academic Press, Inc., San Diego, California. pp. 3-66.

Talbot, R.T., Dunn, I.C., Wilson, P.W., Sang, H.M., and Sharp, P. (1995). Evidence for alternative splicing of the chicken vasoactive intestinal peptide gene transcript. *J. Mol. Endocrinol.* **15**, 81-91.

Vaughan, J.M., Rivier, J., Spiess, J., Peng, C., Chang, J.P., Peter, R.E. and Vale, W. (1992). Isolation and characterization of hypothalamic growth hormone-releasing factor from Common carp, *Cyprinus carpio*. *Neuroendocrinology* **56**, 539-549.

Chapter 8

General conclusions:

In this thesis, I have determined the primary structure of the GnRH peptide(s) from sturgeon and identified GnRHs from a variety of species of vertebrates using HPLC and RIA with specific antisera. This research includes the first identification of a second form of GnRH with charcteristics of cGnRH-II in primates. I also have isolated the cDNA encoding a GRF-like/ PACAP precursor from sturgeon brain using PCR with various oligonucleotide primers. The isolation of both GnRH and GRF from the brain of sturgeon provides an opportunity to compare the structures of the peptide(s) controlling reproduction or growth with similar peptides in other species. The evolutionary position of sturgeon makes them a good species with which to compare these important neuropeptides in more evolutionarily-ancient or -recent species and speculate about their evolution. The evidence presented in this thesis, combined with earlier studies on GnRH, GRF and PACAP in vertebrates and invertebrates, has not only given us a more comprehensive knowledge of the evolution of these important peptides but also increased our understanding of the endocrine systems controlling reproduction and growth in various species.

Sturgeon mGnRH: past, present and future

Prior to this thesis, HPLC and RIA studies had shown that mGnRH-like molecules did exist in descendants of bony fish that evolved before teleosts; reedfish, sturgeon and alligator gar (Sherwood *et al.*, 1991) and bowfin (Crim *et al.*, 1985). In this thesis, purification and sequencing of the mGnRH peptide from the brain of the Siberian sturgeon, *Acipenser gueldenstadtii*, demonstrated that this form of GnRH has been conserved in evolution for at least 250 million years and that the structure of GnRH is identical to that in the human brain. The presence of mGnRH in sturgeon but not in most bony fish suggests that nucleotide substitutions in the mGnRH gene have occurred in the evolution of teleosts. This may have resulted in the sGnRH form found in the brain of most teleosts (Sherwood *et al.*, 1993). The point in bony fish evolution where mGnRH disappears and sGnRH appears is not clear because there are also reports of immunoreactive mGnRH-like molecules in the brain of early-evolved teleosts, like butterfly fish (Powell, 1995; Standen,

1995) and eels (King *et al.*, 1990). Although sturgeon represent the most evolutionarily-ancient vertebrate where the presence of mGnRH has been confirmed by primary structure, there are several reports of small amounts of mGnRH-like molecules in the brain of three species of dogfish (D'Antonio *et al.*, 1995; Lovejoy *et al.*, 1991; 1992; 1993; Powell *et al.*, 1986; Lovejoy, 1996) but this form of GnRH has not been isolated and sequenced from cartilaginous fish. This indirect evidence suggests that mGnRH may have arisen in cartilaginous fish prior to the emergence of the sturgeon. However, there is a family (order Polypteriformes) of bony fish that are thought to have evolved even before sturgeon: the order has only a few living species but mGnRH-like molecules have been shown to be present in the two species studied: reedfish, *Calamoichtys calabaricus*, (Sherwood *et al.*, 1991) and bichir, *Polypterus palmas* (Wright and Demski, 1996). It is clear from my thesis research that mGnRH is present in the brain of a species of Acipenseriformes, an order of fish that diverged soon after the polypterus fish and includes the sturgeons and paddlefish. Futhermore, in this thesis, various sections of the brain of skate, another cartilaginous fish, were examined by HPLC and RIA with a mGnRH specific antiserum, B-7. Mammalian GnRH-like immunoreactivity was not detected in this study, supporting the likelihood that bony fish like sturgeon and polypterus species are the earliest-evolved vertebrates to contain mGnRH in their brain. Nevertheless, it must be noted that only small amounts of juvenile skate brains were used in the study and therefore, it is plausible that mGnRH was not detected or alternatively, mGnRH might only be expressed later in the development of cartilaginous fish.

Confirmation of the mGnRH structure in sturgeon also might explain why mGnRH is effective in teleosts and other non-mammalian vertebrates that do not appear to have mGnRH in their brain. An endocrine peptide and its receptor must both be present for function and therefore, identifying mGnRH in sturgeon suggests that the mGnRH receptor also is evolutionarily ancient. If the non-mammalian GnRH receptors continue to retain the binding site they would still recognize mGnRH in addition to the more recently evolved

forms of GnRH. This promiscuity of non-mammalian receptors also might explain why application of non-endogeneous forms of GnRH stimulate gonadotropin release in some species. For example, sGnRH is a more effective stimulant of LH and FSH release than cGnRH-I in birds despite the lack of sGnRH in the avian brain. Similarily, dfGnRH is more effective at inducing ovulation in the Thai catfish than cfGnRH, one of the endogenous forms of GnRH (Sherwood *et al.*, 1993).

The recent cloning of GnRH receptors from mammals has made it possible to identify both the peptide and receptor in specific tissues, providing additional proof of the prospective function of GnRH within that tissue. Furthermore, receptor knock-out studies can now be done, which would aid in our understanding of the distinct functions of each of the multiple forms of GnRH within the brain or other tisssues. The sturgeon would make an interesting species to characterize the GnRH receptor(s) present because it is an evolutionarily- ancient species that contains both mGnRH and cGnRH-II peptides. There has been only one type of GnRH receptor isolated from mammals to date; this receptor has been shown to be specific for mGnRH in the sense that it does not bind sGnRH, cGnRH-I or lGnRH-I. However, there also might be an additional GnRH receptor that is specific for cGnRH-II because cGnRH-II and dfGnRH will release LH and FSH in rats (Sherwood *et al.*, 1997). In chapter five of this thesis I have shown using HPLC, RIA, immunoctyochemical and *in vivo* studies that a cGnRH-II-like molecule with the potential to release LH is present in the brain of monkeys. This suggests that a cGnRH-II specific receptor also is present in primates. In teleost fish, mGnRH, cGnRH-II and sGnRH will cause the release of gonadotropins. The question is whether the structure and selectivity of the mGnRH and cGnRH-II receptor(s) are retained in an evolutionarily-ancient species like sturgeon.

The gene and mRNA encoding mGnRH have been isolated from mammals. In this thesis, I have determined that the mature mGnRH peptide is present in the brain of

sturgeon. Further studies to isolate the mGnRH cDNA and gene from sturgeon would give clues to the evolution of the structure, regulation and processing of this important gene.

Anolis GnRH: loss or alteration of cGnRH-I, a mGnRH derivative

The presence of mGnRH in sturgeon, amphibians and mammals suggests that this form of GnRH has been retained in the evolution of vertebrates leading to tetrapods (Fig. 8-2). In reptiles and birds studied to date, cGnRH-I but not mGnRH is present within the brain. This evidence coupled with the fact that there is only a single amino acid difference between mGnRH and cGnRH-I suggests that cGnRH-I arose after the emergence of reptilian ancestors, following nucleotide substitutions in the mGnRH gene (Sherwood *et al.*, 1997). Birds and non-squamate reptiles like turtles and alligators have retained cGnRH-I as well as cGnRH-II (Fig. 8-2). However, the evolutionary story in squamate reptiles is not clear with some species containing only cGnRH-I, some species containing only cGnRH-II and some species having an unidentified form of GnRH (but not mGnRH) in addition to either cGnRH-I or cGnRH-II.

In this thesis, it was demonstrated that only cGnRH-II but not cGnRH-I was present in the brain of one species of lizard, *Anolis carolinensis*. Future studies in *Anolis* should now concentrate on the mechanisms controlling gonadotropin release, the function of the midbrain cGnRH-II neurons, as well as the ontogeny of the GnRH cells in this lizard. Several other reports also have provided evidence for a solitary form of GnRH in the brain of several lizard species. However, while it is evident that cGnRH-I is not present in the brain of all lizard species examined to date, there have been some reports of more than just cGnRH-II-like immunoreactivity in the brain of certain species of lizard. Therefore, it is possible that there are species-specific differences in the type and number of GnRH forms present in the brain of lizard. It is interesting to note that only cGnRH-I was reported in the brain of *T. sirtalis*, the only snake species where the identity of GnRH has been established by HPLC/ RIA and by immunocytochemistry. Furthermore,the predominant location of irGnRH cells and fibers in *T. sirtalis* was in the terminal nerve and

olfactory bulb region (Smith *et al.*, 1997), an area of the brain with no detectable GnRH-like immunoreactivity in lizards examined to date. Additional HPLC and RIA or *in situ* hybridization studies on more snake and lizard species are needed to determine whether this opposing pattern of GnRH form(s) and distribution is common among members of the Serpentes and Suarian orders of reptiles.

The evidence presented in this thesis suggests that in the evolution of some reptiles the cGnRH-I gene either has been lost from the genome or has been altered to a form of GnRH that is not detectable by current antisera. Interestingly, the study by Smith *et al.*, (1997) suggests that the cGnRH-I gene is retained but the cGnRH-II gene is lost or modified in the evolution of garter snake. Therefore, the study in this thesis coupled with the study in snake and previous studies of reptiles, suggests that cGnRH-I and cGnRH-II are retained in non-squamate reptiles like turtles and alligators while some species of squamate reptiles have lost one of the two forms of GnRH, resulting in a solitary form of GnRH within their brain. An important question is how these species continue to reproduce despite having only one form of GnRH in their brain. Future studies, examining the brain of different squamate reptiles at various stages of development for GnRH using more sensitive molecular biology techniques like *in situ* hybridization or PCR would be useful in determining whether other forms of GnRH are present but expressed only at specific times.

cGnRH-II: pre-sturgeon to mammals

During purification of mGnRH from sturgeon brain, it was clear that a cGnRH-II-like molecule was also present. This confirms an earlier report in sturgeon (Sherwood *et al.*, 1991) and helps establish cGnRH-II as the most conserved GnRH in vertebrate evolution, present in representative species of all vertebrate classes except for agnathans (see Sherwood *et al.*, 1997). In this thesis study, two new cGnRH-II specific RIAs were established, one using antiserum 7CR-10 and one using antiserum Adams-100. These two antisera were used to search the brain of skate, a vertebrate more evolutionarily-ancient than

sturgeon, as well as the brains of vertebrate species more evolutionarily-recent than sturgeon for the presence of cGnRH-II. Using these specific antisera, a cGnRH-II-like molecule was established in the brain of skate, providing further proof that cGnRH-II has an origin at least as old as cartilaginous fish. The skate brain also contained a GnRH-like molecule with dfGnRH immunological properties, suggesting that dfGnRH might be present in cartilaginous fish other than sharks. This thesis also shows that cGnRH-II is present in the brain of more recently-evolved bony fish than sturgeon, like whitefish, platyfish, green swordtail and sablefish emphasizing that this form of GnRH has been retained in the evolution of most teleosts.

In my examination of the brain and other tissues of mammals, a cGnRH-II-like molecule was absent from the brain of guinea pig, hamster and rat. I also did not detect cGnRH-II in fetal rat tissue or in human placenta. However, I was able to demonstrate that cGnRH-II was present in the brain of fetal and adult rhesus and stumptail monkeys. These thesis data represent the first time that the brain and tissues of these recently-evolved placental mammals have been examined for cGnRH-II using a combination of HPLC and RIA with cGnRH-II-specific antisera. The presence of cGnRH-II in monkey brain, but not human placenta might be due to the time of collection at term. However, the presence of cGnRH-II in monkey but not in rodents suggests that although this molecule has been retained in the evolutionary line leading to primates, it has been significantly altered or lost in species from the rodent lineages or is expressed at times not studied.

The discovery of a cGnRH-II-like molecule in the fetal and adult brain of monkey represents the most recently-evolved vertebrate to date that has been shown to have cGnRH-II in its brain. My evidence that both mGnRH and cGnRH-II are present in the brain of monkey clearly establishes that the presence of multiple forms of GnRH in the brain of a single species is not restricted to vertebrates that diverged prior to early-evolved placental mammals, as was previously thought, but has been retained in the evolution of primates. I also have shown that the presence of two forms of GnRH in a recently-evolved

placental mammal is not limited to the adult brain but is also found in the fetal brain.

Meanwhile, my results in Chapter 5 showing that cGnRH-II peptide blocked antiserum 675 but not Adams-100, both of which are specific for cGnRH-II, suggests that it is important to determine the primary structure or cDNA of the cGnRH-II-like molecule in primates to confirm its identity.

Teleosts: three forms of GnRH in the brain of a single species

The largest group of vertebrates are the teleosts, with over 27,000 species described (Young, 1981). With this vast number of species, each employing different reproductive strategies, perhaps it is not surprising that there have been four novel GnRH structures (cf, s, sb, hGnRH) in addition to cGnRH-II isolated from teleosts as well as reports of immunoreactive mGnRH-like and novel GnRH-like molecules. Representative species from this vertebrate taxon also have been useful in determining that at least two forms of GnRH are present in the brain of most vertebrates; usually each form has a distinct location and sometimes a unique function in the brain and pituitary of these species. In this thesis, I used HPLC and RIA with the battery of antisera available to me in our laboratory to search for GnRH in the brain of whitefish, platyfish, green swordtail, and sablefish. These fish are members of three separate teleost orders: Salmoniformes (whitefish), Cyprinodontiformes (platyfish and green swordtail), and Scorpaeniformes (sablefish). These fish were chosen partly because they represent teleost species whose ancestors are separated by a relatively large period of evolutionary time and therefore, the GnRH structures isolated from them would be helpful in determining how this peptide family evolved in teleosts.

There were three forms of GnRH, with immunological and chromatographic properties of cGnRH-II, sGnRH and a novel form, in the brain of whitefish, *Prosopium williamsoni*; these three forms also were present in the pituitary. Whitefish belong to the Coregoninae subfamily of Salmoniformes fish. Representative species from the two other subfamilies of Salmoniformes are Salmoninae (sockeye salmon, Atlantic salmon, rainbow

trout, masou salmon, chinook salmon) and Thymallinae (Arctic grayling); they have already been examined for the presence of GnRH within their brain. Therefore, if I combine the data gained from previous studies on Salmoniformes and this thesis study on whitefish, this represents the first time that all of the subfamilies within a single order of vertebrates has been examined for GnRH. It appears that at least three forms of GnRH present in the brain of a single species is common to the early-evolved species of the Salmoniformes family, although the characterization of primary structures or cDNA sequences would provide more definitive proof. Other molecular biological studies have shown only cGnRH-II and sGnRH in the brain of species in Salmoninae. Therefore, at some time during evolution of salmonids, possibly after the tetraploid event, one form of GnRH might have been lost or altered to a form that is undetectable by current methods.

I detected cGnRH-II, sGnRH and a novel form of GnRH within the brain of two other teleosts, platyfish and green swordtail. Two other species within the same order, Cyprinodontiformes, have been examined for the presence of GnRH using HPLC and RIA with GnRH antisera. In the brain of green molly, the same three forms of GnRH that were present in the brains of platyfish and green swordtail were present (Coe *et al.*, 1990); however, in Japanese medaka, only cGnRH-II and sGnRH were detected (Powell *et al.*, 1996). There are three possibilities for the presence of three forms of GnRH in the brain of platyfish, green swordtail and mollies and only two forms of GnRH in the brain of medaka, a closely related fish. First, medaka, although closely related to cyprinodont fish, are sometimes placed in a separate order, Beloniformes. It is possible that the novel form of GnRH arose after the medaka lineage split from the Cyprinodontiformes lineage and therefore, might be order specific. Second, it has been shown previously in the platyfish, molly and guppy that there is a distinct developmental timing in the appearance of each of the GnRH-producing centers in the brain of these fish. Therefore, it is possible that there is a narrow window of expression of the novel form of GnRH and that the medaka brains were collected at a time when the novel (termed swordtail) GnRH was present in

undetectable amounts. However, the platyfish, green swordtail and molly studies used brains that were collected at four different times of year and the novel form of GnRH still was detected by the methods used. Also, similar quantities of adult brains were used in all of the studies so the absence of swordtail GnRH in medaka was probably not due to inadequate amounts of tissue or the use of juvenile fish. The third possibility is that platyfish, green swordtail and mollies are live-bearers whereas medaka are egg layers (Parenti, 1994). Therefore, the difference in the number of GnRH forms present in their brain might reflect the differences in their reproductive strategies.

Three forms of GnRH with cGnRH-II-like, sGnRH-like and sbGnRH-like chromatographic and immunological properties were present in the brain of sablefish, as shown by this thesis study. The detection of these three forms of GnRH in sablefish as well as previous reports in a closely related fish, the grass rockfish (Powell *et al.*, 1996) and in an early-evolved telost, the pacu fish (Powell *et al.*, 1997) confirm that sbGnRH arose prior to the emergence of Perciform fish.

Sablefish belong to one order, whereas platyfish and green swordtails belong to a separate order but all three species are members of the Acanthopterygii superorder of euteleost fish. It is interesting to note that although all three fish species have cGnRH-II and sGnRH in their brain, they have a different third form. In fish species where three forms of GnRH have been clearly established by primary structure or *in situ* hybridization, cGnRH-II and sGnRH are present in different locations in the brain, whereas the third form is found predominately in the preoptic area with axons in the pituitary and therefore, is thought to be the primary gonadotropin releaser. In this thesis study, pituitaries were not analyzed separately from the brain tissue of these species in HPLC and RIA and *in situ* hybridization was not used so it is difficult to know which form of GnRH might be the gonadotropin releaser. However, if future studies show that sbGnRH or swordtail GnRH are concentrated in nerve terminals in the pituitary of sablefish and green swordtail, it might be hypothesized that these forms of GnRH are the true gonadotropin releasers.

All of the four teleost species studies in this thesis have at least three different forms of GnRH detected within their brain. This evidence, coupled with previous reports of three forms of GnRH present in the brain of other teleosts more early-evolved and more recently-evolved than the fish studied here suggest that three forms of GnRH in the brain of a single species is an ancestral condition in bony fish. Teleosts that have been shown to have only two forms of GnRH in their brain might represent a derived condition where one form of GnRH is lost or altered to an extent that it is not detectable by current methods. The evidence from this thesis as well as other reports also suggests that although cGnRH-II and sGnRH are likely to be present in the brain of most teleosts, there are additional novel forms of GnRH present in teleosts but with a more restricted species-specific or order-specific distribution.

Identification of an immunoreactive GnRH molecule with novel chromatographic properties in the brain of platyfish, green swordtail and whitefish suggest that the GnRH family is not limited to twelve members. Also, there are reports of novel GnRH-like molecules in other teleosts as well as in lamprey, dogfish, lizard and primate. These predicted novel forms of GnRH could result from one of at least three separate events: 1) a posttranslational modification of one of the existing GnRH peptides or 2) nucleotide base pair substitution in one of the known GnRH genes or 3) an additonal exon or gene duplication sometime in the evolution of GnRH. Future studies to determine the primary structure and cDNA or gene sequences of these novel forms of GnRH would help ascertain which of these three possibilities is most likely.

Identification of a sturgeon GRF-like/ PACAP precursor

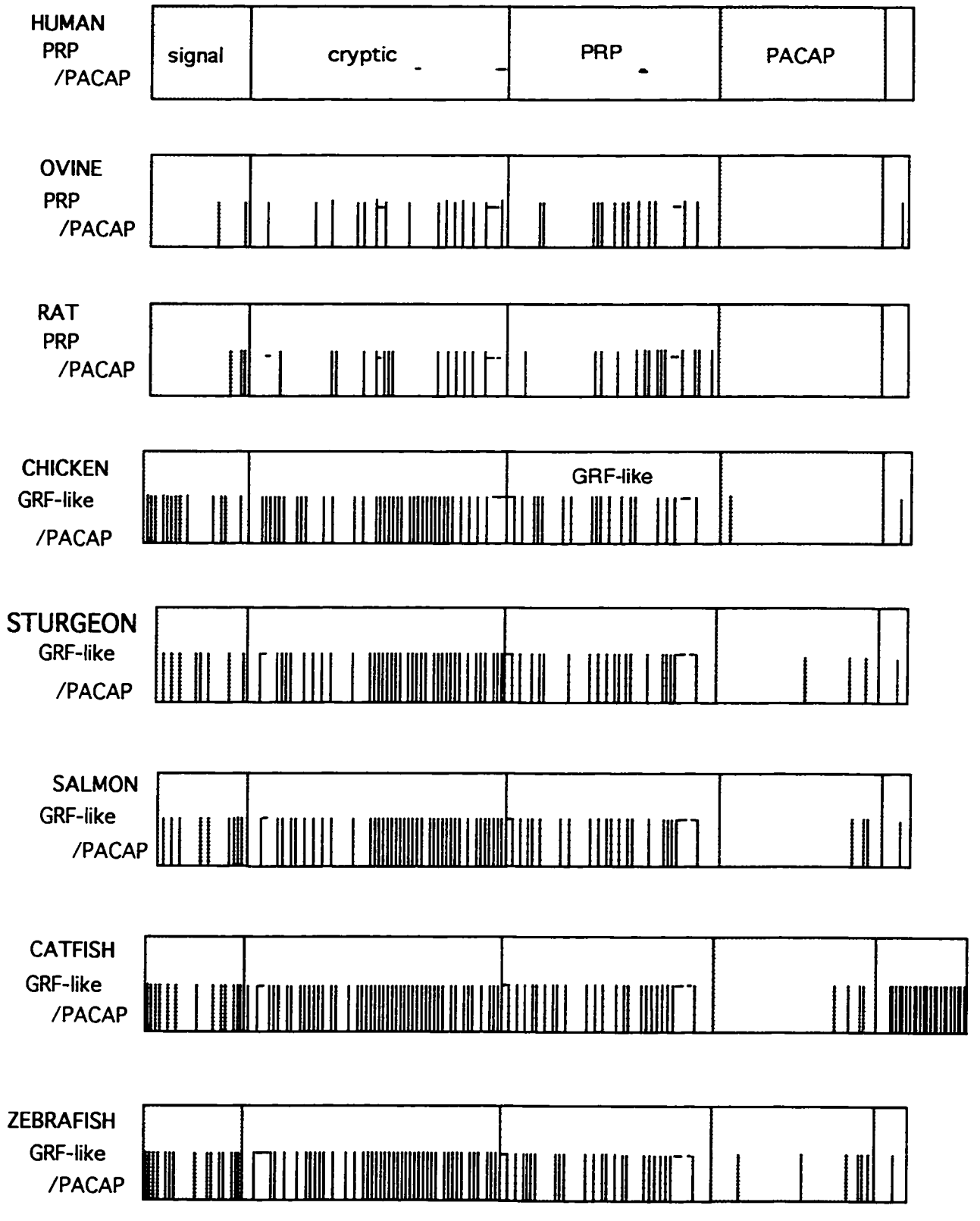
In this thesis, two different cDNAs encoding GRF-like/ PACAP were isolated from the brain of sturgeon; one that encoded the full-length precursor and one that encoded a shortened precursor missing the first 32 amino acids of GRF, which are encoded by exon 4 of the GRF-like /PACAP gene. This exon-skipping phenomenon has been previously described in the GRF-like /PACAP precursors of sockeye salmon (Parker *et al.*, 1997) and

chicken (McRory *et al.*, 1997). The presence of exon-skipping in an early-evolved species like sturgeon suggests that this mechanism has been retained in vertebrate evolution and might provide an alternate means of increasing the expression of a functionally important molecule. For example, in sturgeon, salmon, and chicken, the PACAP peptide can be produced not only when the full-length precursor is transcribed but also if the shortened precursor is transcribed. However, the GRF-like peptide will only be expressed if the full-length precursor is transcribed. The importance of exon skipping is not yet known, but because the presence of full-length or shortened GRF-like /PACAP precursors varies depending on the tissue examined in salmon (Parker *et al.*, 1992) and chicken (McRory *et al.*, 1997), it might be related to tissue-specific processing, providing a different balance of GRF and/or PACAP peptides depending on the functional demands of the tissue. Future studies using various sturgeon tissues and sensitive PCR methods with specific oligonucleotide primers could be done to determine if this tissue specific processing of the GRF-like/ PACAP precursor has been conserved in sturgeon.

Comparison of the sturgeon GRF-like /PACAP precursor with other GRF-like /PACAP or PRP /PACAP precursors demonstrates that specific regions of the molecules have a greater evolutionary plasticity than others (Fig. 8-1), possibly because of varying functional constraints placed upon them. A difference in the rate of evolution of various domains of preprohormone precursors has already been described for other neuropeptide families like corticotropin-releasing factor and somatostatin (see Sherwood and Parker, 1990). Variability in the degree of conservation of various regions of neurohormone precursors, as shown here by the sturgeon GRF-like /PACAP precursor, may be common to neuropeptide families.

Fig. 8-1: Comparison of the precursor structures of mammalian PRP/ PACAP and non-mammalian GRF-like/ PACAP precursors with the precursor to human PRP/ PACAP. Vertical lines indicate amino acid differences from the human PRP/ PACAP precursor. Abbreviations are: signal, signal peptide; cryptic, cryptic peptide; PRP, PACAP-related peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; GRF-like, growth hormone-releasing factor-like.

Structural Comparison of precursor molecules for GRF /PACAP



Comparison of GnRH and vasotocin precursor structure among vertebrates show a similar pattern as sturgeon GRF-like /PACAP with the highest conservation in the domain with the greatest perceived functional importance, followed by the signal peptide and lastly by the cryptic peptide (Coe, 1995). If this were the case, it can be suggested that PACAP, which has the highest conservation of all the precursor domains (Fig. 8-1), has an important functional role. However, PACAP has diverse functions in vertebrates; most recently PACAP was shown to induce a shift from mitosis to differentiation in cultured rat neuroblasts from the cerebral cortex. This suggests that PACAP might have a critical role early in development (Lu and DiCicco-Bloom, 1997). The signal peptide sequence is important for ensuring that the preprohormone can dock with and enter the endoplasmic reticulum to be processed into its mature bioactive forms and therefore, its main functional constraint is to retain the hydrophobic core necessary for insertion into the endoplasmic reticulum membrane. There are some amino acid changes between the putative signal peptide of sturgeon and the signal peptides of other GRF-like /PACAP or PRP /PACAP precursors (Fig. 8-1). However, the changes are conservative and the required hydrophobicity is maintained. Another region of the sturgeon GRF-like /PACAP precursor with relatively high sequence identify to other known precursors is the 3' UTR. This region has been shown to be important in proper expression and stability of the mRNA transcript (Kozak, 1991); important functions that might have led to enhanced conservation.

The greatest amino acid differences between the GRF-like/ PACAP and PRP/ PACAP precursors is in the cryptic peptide region (Fig. 8-1), a domain with no well-established function except a recent report that the cryptic peptide encoded by the human GRF gene can stimulate Sertoli cell activity in the testes (Breyer *et al.*, 1996). It is more likely that that the cryptic region of sturgeon GRF-like/ PACAP serves a conformational rather than a physiological function as has already been suggested for the C-peptide in insulin (Eipper *et al.*, 1986) and the cryptic peptide of GnRH, GAP. Although GAP and

GAP-related peptides have been shown to co-exist with GnRH in hypothalamic neurons and to inhibit prolactin release or promote gonadotropin release in some circumstances (Nikolics *et al.*, 1985; Thomas *et al.*, 1988; Yu *et al.*, 1988) the inconsistency of these results as well as the low sequence identity of GAP among vertebrates suggest that it is unlikely to function as a neuropeptide (see Sherwood *et al.*, 1994). In addition, if the region encoding the cryptic region of GnRH (exons 3 and 4) is deleted from the GnRH gene, an endocrine disorder will develop in mice. These "hypogonadal mice" do not appear to release GnRH although their GnRH gene still contains the exon that encodes the functional protein. This results in abnormally low levels of gonadotropins and sexually immature animals (Seeburg *et al.*, 1987) providing strong evidence that the cryptic region is important for effective precursor processing.

It is interesting to note that relative sequence identity among the GRF domain of the sturgeon precursor and the other vertebrate GRF or PRP precursors is not high compared to PACAP (Fig. 8-1). GRF's importance as a key neuropeptide regulator of growth in mammals is well established. The question is therefore, why a domain with such important physiological functions is not more tightly conserved. However, although the overall amino acid sequence of GRF has had numerous substitutions amongst vertebrate precursors, the amino acids critical for receptor binding and optimal functionality are highly conserved even in tunicates.

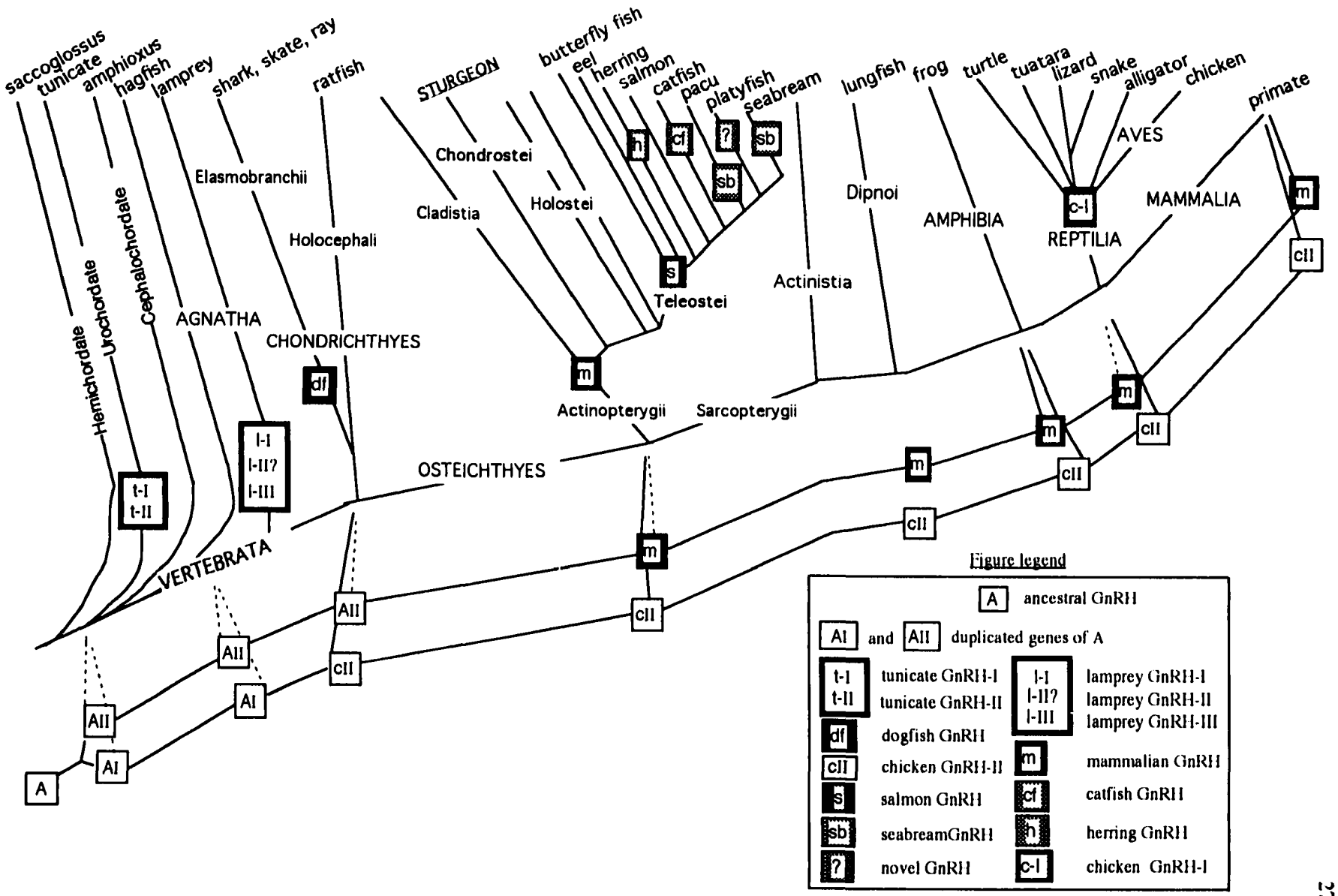
Evolution: GnRH and GRF/ PACAP

Gene duplication is an important mechanism in the evolution of peptides with new functions. In this situation, one of the gene duplicates continues to encode a protein that performs the original function, whereas the additional copy undergoes more rapid evolutionary change because of lessened functional constraints, eventually resulting in peptides with a novel function (Ohno, *et al.*, 1968; Ohta, 1994). This gene duplication mechanism has been described for a number of different neuropeptides including glucagon and insulin, in addition to GnRH and GRF. Another means of increasing the amount of

genetic material available for the evolution of peptides with new functions is to duplicate the entire genome. This mechanism has been described in salmonids where a genome duplication event occurred after the emergence of salmonid ancestors, resulting in tetraploid extant species. If this evolutionary mechanism is employed, there are additional alleles available for mutations that could result in peptides with new functions. Sturgeons are highly polyploid with conditions of 4n to 8n described for certain species (Birstein and Vaseliev, 1987). If only one copy of a gene were conserved to perform a critical function, the high redundancy of their genome would mean that there were many other gene copies available to mutate into genes encoding proteins with novel functions or alternatively, into pseudogenes with no particular function.

A current evolutionary scheme for the GnRH family is that the gene encoding the ancestral GnRH duplicated sometime prior to the emergence of tunicates, over 500 million years ago (Fig. 8-2). Subsequent nucleotide substitutions or further duplication of these ancestral genes gave rise to two of the most conserved forms of GnRH: 1) cGnRH-II, which arose during or before the emergence of cartilaginous fish and 2) mGnRH, which arose during or before the emergence of bony fish (Fig. 8-2). The other forms of GnRH originated after the ancestors for a vertebrate class had already diverged from the main evolutionary line. "Hence, lGnRH-I and lGnRH-III are only in jawless fish; dfGnRH is only found in cartilaginous fish; sGnRH, cfGnRH, sbGnRH and hGnRH are only in teleost bony fish and cGnRH-I is only found in the reptile-bird branch" (Fig. 8-2; see Sherwood *et al.*, 1997). These additional forms of GnRH are the result of nucleotide substitutions in one of the conserved forms of GnRH or are products of a further gene duplication. Because cGnRH-II is conserved in all vertebrates except for jawless fish and one species of snake, the mGnRH gene (which is not expressed in teleosts and reptiles) was most likely the template for the nucleotide substitutions that gave rise to the new forms of GnRH. Therefore, sGnRH, or derivatives of sGnRH, replaced mGnRH in most of the more evolutionarily-recent species of teleosts (Fig. 8-2).

Fig. 8-2. Schematic diagram showing the phylogeny of GnRH. Solid lines in lower part of diagram indicate the form(s) of GnRH that were conserved in the evolution of a particular vertebrate class, whereas dashed lines indicate that one or more of the ancestral forms of GnRH was altered to a new form after the vertebrate class diverged from the main evolutionary line. In bony fish, mGnRH is present in species that evolved early and then the gene was altered or not expressed in species that evolved after the origin of teleosts.

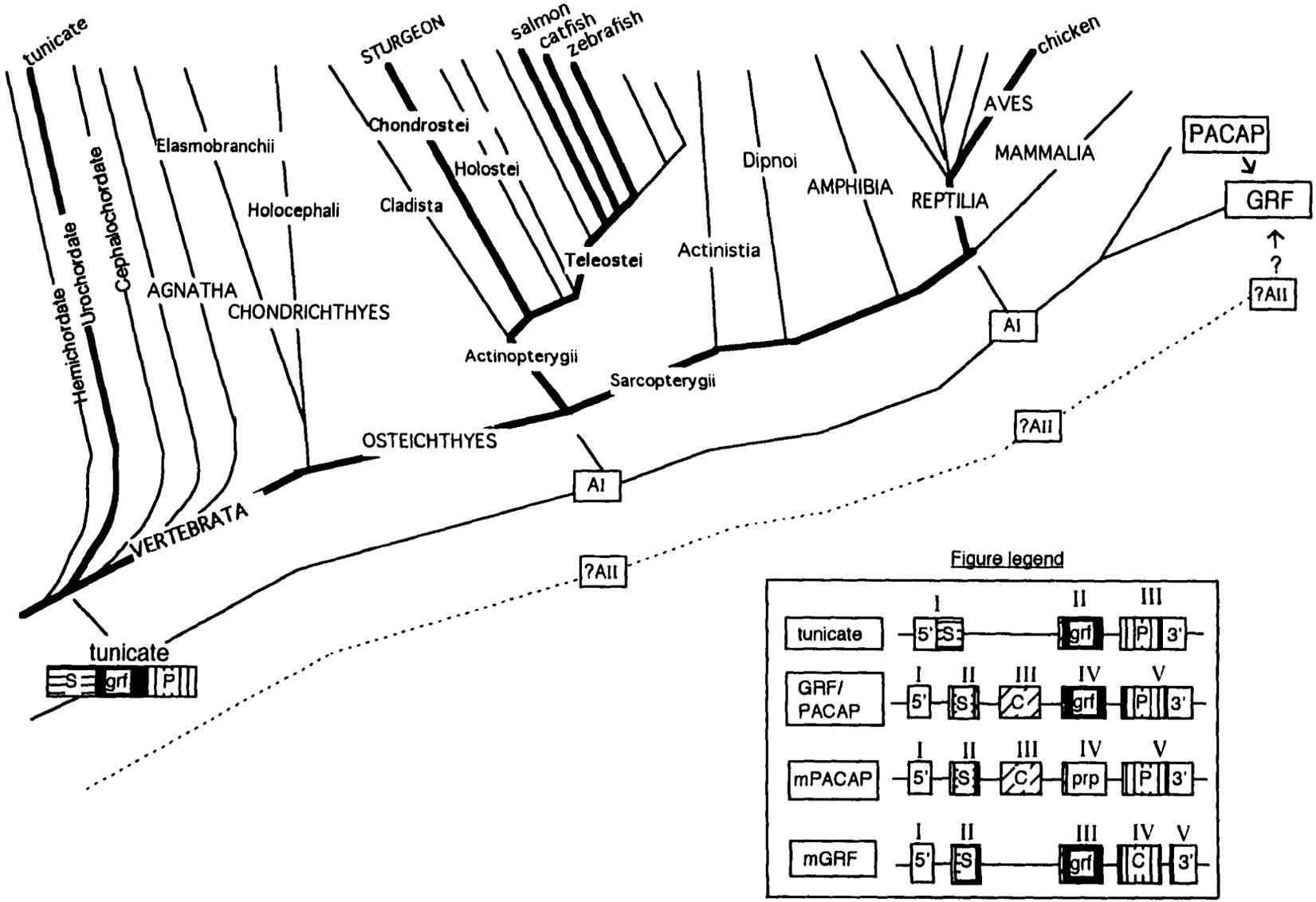

saccoglossus
tunicate
amphioxus
hagfish
lamprey
shark, skate, ray
ratfish
STURGEON
butterfly fish
eel
herring
salmon
catfish
pacu
platyfish
seabream
lungfish
frog
turtle
tuatara
lizard
snake
alligator
chicken
primate
AVES
Chondrostei
Elasmobranchii
Cladistia
Holostei
Holocephali
Dipnoi
AMPHIBIA
REPTILIA
MAMMALIA
Teleostei
Actinistia
Hemichordate
Urochordate
Cephalochordate
AGNATHA
CHONDRICHTHYES
Actinopterygii
Sarcopterygii
OSTEICHTHYES
VERTEBRATA
t-I
t-II
I-I
I-II?
I-III
df
h
cf
?
sb
s
m
c-I
cII
AII
AI
A
Figure legend
A ancestral GnRH
AI and AII duplicated genes of A
t-I tunicate GnRH-I
t-II tunicate GnRH-II
I-I lamprey GnRH-I
I-II? lamprey GnRH-II
I-III lamprey GnRH-III
df dogfish GnRH
cII chicken GnRH-II
m mammalian GnRH
s salmon GnRH
cf catfish GnRH
sb seabreamGnRH
h herring GnRH
? novel GnRH
c-I chicken GnRH-I

Also, mGnRH was probably replaced by cGnRH-I early in the phylogeny of reptiles because there is only a single amino acid difference between them and also because mGnRH is present in ancient fish, amphibians and mammals but not in reptiles and birds (Fig. 8-2).

The evolution of GRF and PACAP is not as well established as the evolution of GnRH. The GFR-like/ PACAP precursors are structurally more similar to the PRP/ PACAP precursors than to the GRF precursors and the PACAP domains are conserved among species. Therefore, PACAP, is most likely a more ancestral molecule than GRF. The recent isolation of two cDNAs encoding GRF-like/ PACAP precursors in tunicates suggest that a gene duplication event took place prior to the emergence of veretebrates (Fig. 8-3). The general precursor structure, encoding both a GRF-like and PACAP in tandem, was retained in the evolution of bony fish as well as in the evolution of reptiles and birds but some nucleotide substitutions also occurred during this time (Fig. 8-3). Alternative splicing, resulting in the shorter precursor with the first 32 amino acids of the GRF-like peptide skipped, arose prior to the emergence of bony fish and is retained in the evolution of birds (Fig. 8-3). An additional gene duplication and possibly an exon loss might have occurred prior to or during the emergence of mammals resulting in two separate genes encoding GRF and PACAP (Fig. 8-3).

It is possible that a separate gene encoding a GRF-like peptide was present prior to the fish- tetrapod split but has not been found (Fig. 8-3, AII). Further proof of the ancestry of the GRF-like/ PACAP precursor in sturgeon, chicken and fish would be to synthesize the peptides predicted from the nucleotide sequence and use them in functional studies to determine their effects in growth or release of growth hormone. Initial studies have been done *in vitro* in chicken. In sturgeon, a functional study is currently in progress where synthetic forms of GRF_{1-29}, GRF_{1-45}, PACAP $_{1-27}$, PACAP $_{1-38}$, and GnRH are being tested for their ability to release GH *in vivo* as well as stimulate growth and food conversion in juvenile sturgeon.

Fig. 8-3: Schematic diagram showing the phylogeny of GRF and PACAP. Bold, thickened lines indicate where cDNAs encode GRF-like/ PACAP peptides in tandem in the precursors studied to date. Abbreviations are: P, pituitary adenylate cyclase-activating polypeptide; S, signal peptide; grf, GRF-like peptide; C, cryptic peptide; AI, ancestral GRF-like/ PACAP precursor; ?AII, ancestral GRF gene; mPACAP, mammalian pituitary adenylate cyclase-activating polypeptide; mGRF, mammalian growth hormone-releasing factor; prp, PACAP-related peptide; 5', 5' untranslated region; 3', 3' untranslated region.


tunicate
Hemichordate
Urochordate
Cephalochordate
AGNATHA
Elasmobranchii
Holocephali
CHONDRICHTHYES
STURGEON
Chondrostei
Holostei
Cladista
Teleostei
salmon
catfish
zebrafish
Actinopterygii
Actinistia
Dipnoi
Sarcopterygii
OSTEICHTHYES
AMPHIBIA
REPTILIA
AVES
chicken
MAMMALIA
VERTEBRATA
AI
?AII
PACAP
GRF
?
?AII
AI
?AII
tunicate
S
grf
P
Figure legend
tunicate
GRF/ PACAP
mPACAP
mGRF
I
II
III
IV
V
5'
S
C
grf
prp
P
3'

It is interaction between environmental influences and the hereditary information contained within the genes of organisms that determines how an animal looks, functions, reproduces and grows. Some of the questions pertaining to the different reproductive strategies and growth rates between species can be at least partially answered by comparing both the presence and the activity of the various genes and factors involved in regulating reproduction and growth. In addition, this comparative approach, using the molecules integral to reproduction and growth from a variety of species, is helpful in interpreting evolutionary changes to endocrine systems as well as in giving some indication of the relatedness among members of peptide families and superfamilies.

Literature cited

Birstein, V.J., and Vasiliev, V.P. (1987). Tetraploid-octaploid relationships and karyological evolution in the order Acipenseriforms (Pisces): karyotypes, nucleoli, and nucleolus-organizer regions in four acipenserid species. *Genetica* **71**, 3-12.

Breyer, P.R., Rothrock, J.K., Neaudry, N., and Pescovitz, O.H. (1996). A novel peptide from the growth hormone releasing gene stimulates Sertoli cell activity. *Endocrinology* **137**, 2159-2162.

Coe, I.R. (1992). Molecular evolution of neuropeptides isolated from the brain of the Pacific salmon (*Oncorhynchus spp.*). Ph.D. Thesis, University of Victoria, Victoria, B.C. pp. 231.

Coe, I.R., Grier, H., and Sherwood, N.M. (1992). Gonadotropin-releasing hormone in the molly *Poecilia latipinna*: molecular form, quantity, and location. *J. Exper. Zool.* **261**, 414-423.

Crim, J.W., Rajjo, I., and Vigna, S.R. (1985). Brain-gut peptides in holostean fish. *10th Int. Symp. Comp. Endocrinol.* **37**, 294-305

D'Antonio, M., Vallarino, M., Lovejoy, D.A., Vandesande, F., King, J.A., Pierantoni, R.,and Peter, R.E. (1995). Nature and distribution of gonadotropin-releasing hormone (GnRH) in the brain, and GnRH and GnRH binding activity in the serum of the spotted dogfish *Scyliorhinus canicula*. *Gen. Comp. Endocrinol.* **98**, 35-49.

Eipper, B.A., Mains, R.E. and Herbert, E. (1986). Peptides in the nervous system. *Trends Neurosci.* **9**, 463-468.

King, J.A., Dufour, S., Fontaine, Y.A., Millar, R.P. (1990). Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* **11**, 507-514.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867-19870.

Lovejoy, D.A. (1996). Peptide hormone evolution: functional heterogeneity within GnRH and CRF families. *Biochem. Cell Biol.* **74**, 1-7.

Lovejoy, D.A., King, J.A., Sherwood, N.M., and Peter, R.E. (1993). Identification of gonadotropin-releasing hormone and associated binding substances in the blood serum of a holocephalan (*Hydrolagus colliei*). *Peptides* **14**, 1237-1243.

Lovejoy, D.A., Sherwood, N.M., Fischer, W.H., Jackson, B.C., Riveir, J.E., and Lee, T. (1991). Primary structure of gonadotropin-releasing hormone from the brain of a holocephalan (ratfish: *Hydrolagus colliei*). *Gen. Comp. Endocrinol.* **82**, 152-161.

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

Lu, N., and DiCicco-Bloom, E. (1997). Pituitary adenylate cyclase-activating polypeptide is an autocrine inhibitor of mitosis in cultured cortical precursor cells. *Proc. Natl. Acad. Sci. (U.S.A.)* **94**, 3357-3362.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young, W.S., Phillips, H.S., Nikolics, K., and Seeburg, P.H.(1986). A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science* **234**, 1366-1374.

McRory, J.E., Parker, R.L., and Sherwood, N.M. (1997). Expression and alternate processing of a chicken gene encoding both growth hormone-releasing hormone and pituitary adenylate cyclase-activating polypeptide. *DNA Cell. Biol.* **16**, *95-102*

Nokolics, K., Mason, A.J., Szonyi, E., Ramachandran, J., and Seeburg, P.H. (1985). A prolactin inhibiting factor within the precursor for human gonadotropin-releasing hormone. *Nature* **316**, 511-517.

Ohno, S., Wolf, U., and Atkin, N.B. (1968). Evolution from fish to mammals by gene duplication. *Hereditas* **59**, 169-187.

Ohta, T. (1994). Further examples of gene duplication revealed through DNA sequnce comparisons. *Genetics* **138**, 1331-1337.

Parenti, L.R. (1994). Killifish and ricefish. In "Encyclopedia of fishes" (J.R. Paxton and W.N. Eschmeyer, Eds.), pp.148-152. Academic Press Inc., San Diego, CA.

Parker, D.B., Power, M.E., Swanson, P., Rivier, J., and Sherwood, N.M. (1997). Exon skipping in the gene encoding pituitary cyclase-activating polypeptide in salmon alters the expression of two hormones that stimulate growth hormone release. *Endocrinology* **138**, 414-423.

Powell, J.F.F. (1995) The origin and evolution of gonadotropin- releasing hormone in boney fishes. Ph. D. thesis, University of Victoria, B. C.

Powell, J.F.F., Krueckl, S.L., Collins, P.M., and Sherwood, N.M. (1996). Molecular forms of GnRH in three model fishes: rockfish, medaka and zebrafish. *J. Endocrinol.* (in press)

Powell, J.F.F., Standen, E.M., Carolsfeld, J., Borella, M.I., Gazola, R., Fischer, W.H., Park, M., Craig, A.G., Warby, C.M., Rivier, J.E., Val-Sella, M., and Sherwood, N.M. (1997). Primary structure of three forms of gonadotropin-releasing hormone (GnRH) from the pacu brain. *Regul. Peptides* **68**, 189-195.

Powell, R.C., Millar, R.P., and King, J.A. (1986). Diverse molecular forms of gonadotropin-releasing hormone in an elasmobranch and a teleost fish. *Gen. Comp. Endocrinol.* **63**, 77-85.

Seeburg, P.H., Mason, A.J., Stewart, T.A., and Nokilics, K. (1987). The mammalian gene and its pivotal role in reproduction. *Recent. Prog. Hormone Res.* **43**, 69-90.

Sharp, P.J., Talbot, R.T., Main, G.M., Dunn, I.C., Fraser, H.M., and Huskisson, N. S. (1990). Physiological roles of chicken LHRH-I and -II in the control of gonadotropin release in the domestic chicken. *J. Endocrinol.* **124**, 291-299.

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 (*lLpisosteus spatula*). *Gen. Comp. Endocrinol.* **84**, 2-15.

Sherwood, N. M., von Schalburg, K. R., and Lescheid, D. W. (1997). Origin and evolution of gonadotropin-releasing hormone in vertebrates and invertebrates. In: "GnRH: Gene to Behaviour." (Parhar, I.S. and Sakuma, Y., Eds) Brain Shippon Publishers, Tokyo, Japan (in press)

Sherwood, N.M., and Lovejoy, D.A. (1993). Gonadotropin-releasing hormone in cartilaginous fishes: structure, location, and transport. *Enviro. Biol. Fishes* **38**, 197-208.

Sherwood, N.M. and Parker, D.B. (1990). Neuropeptide families: An evolutionary perspective. *J. Exper. Zool. Suppl.* **4**, 63-71.

Smith, M.T., Moore, F.L., and Mason, R.T. (1997). Neuroanatomical distribution of chicken-I gonadotropin-releasing hormone (cGnRH-I) in the brain of the male red-sided garter snake. *Brain Behav. Evol.* **49**, 137-148.

Standen, E.M. (1995). Gonadotropin-releasing hormone: three primary structures in pacu fish and characterization in early teleosts. Honour's Thesis, University of Victoria, B.C.

Thomas. G.B., Cummins, J.T., Doughton, B.W., Griffin, N., Millar, R.P., Milton, R.C.deL., and Clarke, I.J. (1988). Gonadotropin-releasing hormone associated peptide (GAP) and putative processed peptides do not release luteinizing hormone or follicle stimulating hormone or inhibit prolactin secretion in the sheep. *Neuroendocrinology* **48**, 342-350.

Wright, D.E., and Demski, L.S. (1996). Organization of GnRH and FMRF-amide systems in two primitive bony fishes (order Polypteriformes). *Brain Behav. Evol.* **47**, 267-278.

Young, J. Z. (1981). The life of vertebrates. Oxford University Press, New York.

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 *Carassius aurata*. *Peptides* **9**, 625-630.