National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

STRUCTURE-ACTIVITY STUDIES ON GONADOTROPIN-RELEASING HORMONE ANALOGS IN GOLDFISH

by

© C. K. MURTHY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1994

ISBN 0-315-95237-7

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: **C. K. MURTHY**

TITLE OF THESIS: **STRUCTURE-ACTIVITY STUDIES ON GONADOTROPIN-RELEASING HORMONE ANALOGS IN GOLDFISH**

DEGREE: **DOCTOR OF PHILOSOPHY**

YEAR THIS DEGREE GRANTED: **1994**

C. K. MURTHY
Sunkardy,
Thekkur Post
SRINGERI- 577 139
Karnataka state
INDIA

Date: May 16, 1994

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and research for acceptance, a thesis entitled: **STRUCTURE-ACTIVITY STUDIES ON GONADOTROPIN-RELEASING HORMONE ANALOGS IN GOLDFISH** submitted by **C. K. MURTHY** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY.**

(Dr. R. E. Peter, Supervisor)

(Dr. G. R. Foxcroft, Committee Member)

(Dr. J. P. Chang, Committee Member)

(Dr. N. E. Stacey, Committee Member)

(Dr. Y. Zohar, External Examiner)

(Dr. C. Strobeck, Chairman and Examiner)

Date: 16 May 1994

This thesis is dedicated to

my loving mother

and

fond memories of my father

ABSTRACT

In goldfish, salmon gonadotropin (GTH)-releasing hormone (sGnRH) and chicken GnRH-II (cGnRH-II) stimulate the secretion of GTH-II and growth hormone (GH). The objective of the present study was to study the structure-activity relations of GnRH analogs in the goldfish. The structure-function studies indicate that the structural requirements to develop potent GnRH antagonists in goldfish are different from those of mammals. Many 'putative' GnRH antagonists, especially those having D-arginine^{6}, stimulate GTH-II and GH release in goldfish, contrary to their actions in mammals. [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-mGnRH (Analog E) inhibited GTH-II and GH stimulatory actions of both sGnRH and chicken GnRH-II both *in vivo* and *in vitro*, without showing independent stimulatory actions The GTH-II release inhibition by analog E varied with the reproductive status of fish. being highest in sexually regressed fish. Analog E appears to inhibit GTH-II and GH release by competitively binding to GnRH receptors at the pituitary cells level. Analog E suppressed basal plasma GTH-II and GH levels, indicating the involvement of endogenous GnRH peptides in regulation of GTH-II and GH release. Exposure of male goldfish to a female sex pheromone results in increases in plasma GTH-II levels and endogenous GnRH mediates at least part of this response. Further, studies using many analogs and various other approaches, also indicate that the two native peptides act through a single population of receptors on gonadotrophs and through a functionally different population on somatotrophs.

In conclusion, [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-mGnRH (Analog E) inhibits native GnRH peptide-stimulated GtH-II and GH release both *in vivo* and *in vitro*. This GnRH antagonist will serve as an effective tool in future studies to understand the role of GnRH peptides in reproduction and growth.

Acknowledgement

I am greatly indebted to Dr. Dick Peter, my research supervisor, for providing me with an opportunity to work in his world renowned lab and to make use of his personal collection of books, journals and papers. I very much appreciate his excellent guidance and constant encouragement during my Ph.D. program. Dick is not only a great scientist, but also a great person with wonderful sense of humor and openness. If I can emulate a small part of his achievements as a scientist and as a person, I will be very happy in my life.

I greatly appreciate Dr. Norm Stacey, Dr. John Chang and Dr. G. Foxcroft, my supervisory committee members, for their suggestions and comments on my thesis. Special thanks to John and Norm for their help outside the lab and making our stay in Edmonton memorable. I am thankful to Dr. Yonathan Zohar, my external examiner, for his enjoyable discussions and valuable comments on my work.

My research would not have been possible without constant supply of GnRH analogs from Dr. J. E. Rivier, at The Salk Institute, La Jolla, USA. I am thankful to Dr. Rivier and also to Dr. J. J. Nestor Jr., The Institute of Bio-Organic Chemistry, Syntex Research, Palo Alto, USA for their generous gifts of GnRH analogs. I thank Dr. H. R. Habibi, University of Calgary for teaching me receptor binding techniques. Special thanks to Dr. N. G. N. Prasad, Department of Statistics for all the advice on statistical analysis.

I appreciate the help of Carol Nahorniak in the lab for iodinations, developing a wonderful goldfish GH assay, those 'gossip times' which made RIAs work really well and evening rides to Michener Park in her cozy Pontiac Grand Prix. During my stay in Dick's lab I was lucky to have friendship with many labmates across the world and they have contributed directly or indirectly to my research program. Hence I am thankful to my friends Vance Trudeau, Anderson Wong, Wei Ge, Joe Dulka, Duff Sloley, Kuni Suzuki, Makito Kobayashi, Chun Peng, and Wenbin Zheng. I appreciate the assistance of Bruce Wakeford in maintenance of fish. Special thanks to Richard Turner and Dr. Prasada Rao for working with me for two summers. Thanks also to Cathy and Shelley for their technical help.

A great deal of my success is due to the constant support of my wife, Jayashree. I appreciate Jayashree, Archana and Adithya for bearing with me and for their love and

affection. I also acknowledge the support of my brothers, mother, father and mother-in-law, and many friends back in India.

I thank all the tax (including GST!) payers of Canada for the financial support in the form of Canadian Commonwealth Scholarship. I acknowledge the opportunity provided by the Department of Fisheries, Government of Karnataka, India to undertake this program.

Special thanks to Dick and Leona for those enjoyable evenings at their house, especially Christmas parties, which we will sourly miss in India.

CONTENTS

List of Tables

List of Figures

List of abbreviations

Abbreviation	Description
General abbreviations:	
DA	dopamine
E2	estradiol
GABA	γ-aminobutyric acid
GH	growth hormone
GnRH	gonadotropin-releasing hormone
cGnRH-I	chicken gonadotropin-releasing hormone-I
cGnRH-II	chicken gonadotropin-releasing hormone-II
lGnRH	lamprey gonadotropin-releasing hormone
mGnRH	mammalian gonadotropin-releasing hormone
sGnRH	salmon gonadotropin-releasing hormone
GSI	gonadosomatic index
GTH	gonadotropin
GTH-I	gonadotropin-I
GTH-II	gonadotropin-II
SRIF	somatostatin
TRH	thyrotropin-releasing hormone
Abbreviations used in GnRH structure:	
Ac	Acetyl
h-Arg(Et_2)	Dialkyl-D-homoarginine
Lys(atz)	N^{ε}-5'-(3-amino-1*H*-1,2,4-triazole) lysine
Lys(Nic)	N^{ε}-Nicotinoyl lysine
Lys (iPr) or lLys	N^{ε}-Isopropyl lysine
Nal(2)	3-(2-naphthyl) alanine
Pal(3)	3-(3) pyridyl alanine
4Cl-D-Phe	para chloro-D-phenylalanine
4FD-Phe	para fluoro-D-phenylalanine
Δ^3-Pro	$\Delta^{3,4}$-dehydro proline

Chapter One

General Introduction

1. Gonadotropin-releasing hormone:

In the field of reproductive endocrinology, two historic events during the last fifty years merit special attention. In 1947, Green and Harris proposed the 'portal vessel chemotransmitter' hypothesis which states that the functions of the pituitary gland are regulated by regulatory substances produced in the brain and released into the pituitary portal blood (Green and Harris, 1947). Nearly twenty five years later, after highly competitive and prolonged research by scientists of the groups of Dr. Andrew Schally and Dr. Roger Guillemin, a decapeptide of identical structure was purified from porcine brain (Matsuo et al., 1971; Schally et al., 1971a) and ovine brain (Burgus et al., 1972). This peptide, called gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone (LHRH), was shown to stimulate gonadotropin secretion (Schally et al., 1971b). Since then many alternative forms of GnRH have been purified from brain of chicken (chicken GnRH-I and -II; King and Millar, 1982, Miyamoto et al., 1982, 1984), salmon (salmon GnRH; Sherwood et al., 1983), catfish (catfish GnRH; Ngamvongchon et al., 1992a; Bogerd et al., 1992), dogfish (dogfish GnRH; Lovejoy et al., 1992) and lamprey (lamprey GnRH-I, III; Sherwood et al., 1986; Sower et al., 1993). The structure of these peptides are shown in Table 1.1.

Lamprey GnRH-II form, for which only the amino acid composition but not sequence is known, has Ile, Phe and His instead Glu, Lys, and Tyr in lGnRH-I (Sower et al., 1993). All the known forms of GnRH have a similar decapeptide structure, with pyroglutamic acid ($pGlu^1$) at the amino terminus and Glycine amide(Gly^{10}-NH_2) at the carboxy terminus. The amino acids at position 1, 2, 4, 9 and 10 are identical in all the known forms. The variations in amino acid sequence are at positions 3, 5, 6, 7, and especially at position 8. Among the GnRH peptides mGnRH and cGnRH-II have wide

distribution in vertebrate species (for reviews see King and Millar, 1987, 1992; Sherwood et al., 1993).

In the literature it is well documented that GnRH regulates the synthesis and release of gonadotropins from the gonadotroph cells in the pituitary gland of vertebrates (for reviews see Jinnah and Conn, 1988; Lincoln, 1989; Peter et al., 1990a; Marshall et al., 1991; Barbieri, 1992; King and Millar, 1992). GnRH also stimulates growth hormone release in many teleost fish like goldfish, *Carassius auratus* (Marchant et al., 1989a), common carp, *Cyprinus carpio* (Lin et al., 1993), rainbow trout (Le Gac et al., 1993) and hybrid tilapia (Melamed et al., 1993). GnRH analogs have vast potential in treatment of many endocrine disorders and in pro- and anti-fertility therapy in humans (Vickery, 1986; Santen and Bourguignon, 1987; Jinnah and Conn, 1988; Schally et al., 1989; Barbieri, 1992) and in livestock management (Schanbacher, 1987). In aquaculture GnRH analogs are mainly used to induce maturation and ovulation of culturable fishes (Crim et al., 1987; Peter et al., 1988; Zohar, 1989).

1.1. Multiple forms of GnRH:

Probably with the exception of advanced mammal species (King et al., 1988b), the existence of multiple forms of GnRH is common in a species including marsupials (King et al., 1990) and the musk shrew, a primitive placental mammal (Dellovade et al., 1993). However, the physiological significance of the presence of multiple forms of GnRH is not clear. In general, cGnRH-II form is present in species of cartilaginous fish, bony fish, amphibians, reptiles, birds and lower mammals (for review see, Sherwood and Lovejoy, 1989; King and Millar, 1992; Sherwood et al., 1993). Although cGnRH-II has strong gonadotropin releasing activity in most of the vertebrates tested (except mammals), in general, it is predominantly located in extra-hypothalamic regions of the brain (King and Millar, 1992) and the other form of GnRH (sGnRH or mGnRH or cGnRH-I) is found in the hypothalamic region. On this basis, King and Millar (1992) suggested that, in general, cGnRH-II may not be involved in the regulation of release of gonadotropic hormones. In support of this, in chicken only cGnRH-I is present in, and is released from, the median eminence (Katz et al., 1990). Similarly, both sGnRH and cGnRH-II are found in the brain of rainbow trout, *Oncorhynchus mykiss* (Okuzawa et al., 1990) and masu salmon, *Oncorhynchus masou* (Amano et al., 1992), but only sGnRH is present in the pituitary

gland, implying the involvement of only sGnRH in regulation of gonadotropin-II (GTH-II) release in these two fish species. However, non-involvement of cGnRH-II in reproduction may not be true for all species. In goldfish, chromatographic and immunological studies have indicated the presence of sGnRH and cGnRH-II in the brain and pituitary (Yu et al., 1988). In the study by Yu et al. (1988), and also in the immunocytochemical study by Kim et al. (1993), both sGnRH and cGnRH-II were detected in different brain areas and in the pituitary, suggesting involvement of both peptides in the regulation of pituitary hormone secretion (reviewed by Peter et al., 1990a). In African catfish (*Clarias gariepinus*), cfGnRH and cGnRH-II are located in the same granules of axon terminals near the pituitary gonadotrophs (Zandbergen et al., 1992; Schulz et al., 1993), and cGnRH-II is more potent than cfGnRH in stimulating GTH-II release in African catfish (Schulz et al., 1993) and in inducing ovulation in Thai catfish, *Clarias macrocephalus* (Ngamvongchon et al., 1992b). In the frog, *Rana rodobinda*, the cGnRH-II form but not the mGnRH form is present in the hypothalamic region and suggested to be involved in regulation of GTH release (Conlon et al., 1993).

1.2. Anatomical distribution:

The distribution of GnRH has been extensively studied, especially in mammalian species (Silverman, 1988), which will not be covered here. In goldfish, sGnRH-immunoreactive (ir) cell bodies were found in the area between the olfactory nerve and the olfactory bulb (the terminal nerve), the ventral telencephalon, the preoptic area and the hypothalamus. cGnRH-II-ir cell bodies were observed in the same areas as those of sGnRH and also in the midbrain tegmentum. Both sGnRH and cGnRH-II-ir fibers were located widely in the brain areas and in the pituitary gland (Kah, 1986a; Kim et al., 1993). The measurable sGnRH and cGnRH-II content in different brain areas of goldfish shows a pattern of distribution of two native GnRH peptides similar to that described by immunocytochemical studies (Yu et al., 1988).

1.3. Biosynthesis of GnRH:

GnRH is synthesized as part of a larger precursor, GnRH preprohormone, having 92 amino acids in humans and rats (for review see Seeburg et al., 1987). Preprohormone in

rats consists of a signal sequence of 23 amino acids followed by the decapeptide, a Gly-Lys-Arg sequence for enzymatic processing, and a 56 amino acid C-terminal sequence (for review see Nikolics and Seeburg, 1987; Seeburg et al., 1987). The cDNA coding sequence has been identified for the precursor of mGnRH in rat (see Seeburg et al., 1987) and for sGnRH in African cichlids, *Haplochromis burtoni* (Bond et al., 1991), Atlantic salmon, *Salmo salar* (Klungland et al., 1992), masu salmon, *Oncorhynchus masou* (Suzuki et al., 1992) Pacific salmon, *Oncorhynchus nerka* (Coe et al., 1992) and for cGnRH-I in chicken (Dunn et al., 1992). The genes of mGnRH, sGnRH, cGnRH-I contain 4 exons [except in Pacific salmon, which has only three exons (Coe et al., 1992)], of which the second exon codes for a signal peptide of 21-23 amino acids, the GnRH decapeptide, a cleavage site, and part of a GnRH associated peptide (GAP). The nucleotide sequence of GAP of masu salmon shows considerable variation from the sequence of GAP in other species (Suzuki et al., 1992).

1.4. GnRH receptors:

GnRH binds to receptor protein on the target cell to elicit its physiological action. Among the vertebrates, mammalian GnRH receptor is specific; only mGnRH exhibits high gonadotropin-releasing activity (for review see King and Millar, 1987, 1992). In birds, reptiles and amphibians all the vertebrate GnRH peptides tested (except lGnRH) are biologically active (King and Millar, 1987, 1992) and in goldfish seven forms of GnRHs (lGnRH-III not tested) are active in stimulating GTH-II release (Peter et al., 1987a; Habibi et al., 1992; Lovejoy et al., 1992; Ngamvongchon et al., 1992a).

1.4.1. Receptor binding studies:

In mammals, many excellent reviews have covered the receptor binding characteristics of GnRH, molecular and structural characteristics of GnRH receptors and the mechanism of GnRH action (Clayton and Catt, 1981; Conn, 1986; Clayton, 1988; Hazum and Conn, 1988; Lincoln, 1989). In general, only a single class of high affinity binding site has been described in rat pituitary (Clayton and Catt, 1981). Binding of GnRH peptides or presence of mRNA for GnRH receptors has also been observed in various extra-hypothalamic tissues of mammals, such as brain, spinal cord, gonads, breast, lymphocytes, placenta, and breast and pancreatic tumor cells (for review see Bramley, 1987; Jones and Hsueh,

1987; Chieffi et al., 1991; King and Millar, 1992; Sherwood et al., 1993).

In recent years the GnRH receptor binding characteristics of many fish species has been studied (for review see Habibi and Peter, 1991; Habibi and Pati, 1993). Characterization of GnRH receptors in the goldfish pituitary demonstrated two classes of GnRH binding sites, high affinity, low capacity sites, and low affinity, high capacity sites (Habibi et al., 1987, 1989b). A protein band of 51,000 Mr associated with the high affinity binding sites in the pituitary of goldfish was evidenced by photoaffinity labelling studies (Habibi et al., 1990) and appears to mediate the biological function of GnRH in goldfish (Habibi et al., 1989b, 1992). In many other fish species such as winter flounder, *Pseudopleuronectes americanus* (Crim et al., 1988; Weil et al., 1992), three-spined stickleback, *Gasterosteus aculeatus* (Andersson et al., 1989), and gilthead seabream, *Sparus auratua* (Pagelson and Zohar, 1992) only one class of high affinity GnRH binding sites were demonstrated. In contrast, in sea lamprey, *Petromyzon marinus*, two classes of high affinity GnRH binding sites have been reported (Knox et al., 1994).

Limited studies in fish indicate regulation of GnRH receptor number by various factors. In goldfish, GnRH receptor numbers in the pituitary increased during sexual maturity, along with a parallel increase in the GTH-II response to GnRH (Habibi et al., 1989a). Similarly, in winter flounder the GnRH binding level was higher in prespawning, compared to post spawning females (Crim et al., 1988). Dopamine down regulates the GnRH receptor number (De Leeuw et al., 1989) consistent with its inhibitory actions on GTH-II release in goldfish (Peter et al., 1991). In catfish, castration results in an increase of GnRH binding sites, while androstenedione reversed this effect (Habibi et al., 1989a). However, implantation of testosterone to gonad intact goldfish had no effect on GnRH binding sites or affinity (Trudeau et al., 1993a). Multiple injections of GnRH over a 24 hr period enhances GnRH receptor numbers, but continuous treatment, at least under *in vitro* conditions, causes desensitization and reduction in the pituitary receptor content (Habibi, 1991a, b; Habibi and Peter, 1991).

In goldfish, GnRH acts through high affinity binding sites to stimulate GTH-II and GH release (Peter et al., 1990a, 1991; Habibi et al., 1992). So far, receptor binding studies using crude goldfish pituitary membrane preparations have not indicated the presence of more than one class of high affinity binding sites (Habibi and Peter, 1991). However, in

photoaffinity labeling studies, a protein band of approximate molecular weight of 51,000 having high affinity to GnRH, appeared as two closely associated bands on gel electrophoresis (Habibi et al., 1990). These two bands were suggested to represent GnRH receptors on gonadotrophs and somatotrophs. In electron microscope studies, sGnRH and cGnRH-II both displaced avidin gold-labelled biotinylated [D-Lys^6, Pro^9- NHEt]-sGnRH from the surfaces of immunohistochemically identified gonadotrophs and somatotrophs (Cook et al., 1991) indicating the presence of GnRH receptors on these two cell types.

Although it is common to find multiple forms of GnRH in a given species, it is not known whether they act through the same or different populations of GnRH receptors to stimulate gonadotropin release. In chicken, both cGnRH-I and -II are biologically active (but only cGnRH-I is a physiological regulator of GTH release), and they act through the same population of pituitary GnRH receptors (King et al., 1988a). In goldfish, both sGnRH and cGnRH-II displaced bound ^{125}I-[D-Arg^6, Pro^9-NHEt]-sGnRH from the crude goldfish pituitary membrane preparations (Habibi et al., 1987, 1989b; Habibi and Peter, 1991) and avidin gold-labelled biotinylated [D-Lys^6, Pro^9-NHEt]-sGnRH from the surfaces of immunohistochemically identified gonadotrophs and somatotrophs (Cook et al., 1991). These results suggest that the two native peptides are capable of interacting with receptors on gonadotrophs and somatotrophs. In contrast to these results, desensitization studies in goldfish show differences in the action of sGnRH and cGnRH-II (Habibi, 1991a, b; Khakoo et al., 1994). Further, in stimulating expression of mRNA of GTH-IIα and GTH-IIβ subunits, cGnRH-II was more potent than sGnRH in sexually mature goldfish, but less potent in sexually regressed goldfish, and thus sGnRH and cGnRH-II were suggested to regulate synthesis and release of GTH-II through different 'receptor-effector' mechanisms (Khakoo et al., 1994).

In recent years specific binding sites for GnRH have been demonstrated in the ovary of goldfish, common carp, and African catfish, as well as in the testis, brain, liver, and kidney of goldfish (Pati and Habibi, 1993; for review Habibi and Pati, 1993). In goldfish, the presence of GnRH-immunoreactive materials in the ovary and direct actions of GnRH on the ovary have been demonstrated (Habibi et al., 1988, 1989c; Habibi and Pati, 1993) indicating a possible paracrine action.

1.4.2. Structure of GnRH receptor:

Recently cDNA coding for GnRH receptors has been sequenced from a mouse αT3 cell line (Tsutsumi et al., 1992; Reinhart et al., 1992), sheep (Brooks et al., 1993), human (Kakar et al., 1992; Chi et al., 1993), and rat (Eidne et al., 1992). The receptors share considerable homology between species and have 327-328 amino acids with seven putative transmembrane domains, characteristic of G-protein-coupled receptors. The mRNA of human GnRH receptor was observed to be present in human pituitary, ovary, testis, breast and prostate, and in the MCF-7 breast cancer cell line, but not in liver and spleen (Kakar et al., 1992). Transfection of cDNA into the COS-7 cell line resulted in expression of human GnRH receptor with high affinity for GnRH analogs (Kakar et al., 1992). Further studies in human have indicated the presence of two genes and three mRNAs encoding for GnRH receptor proteins (Kakar et al., 1993). In sheep pituitary also three mRNAs encoding GnRH receptors have been identified and all three forms of mRNA are regulated similarly by steroids and GnRH, indicating the functional importance of each (Miller et al., 1993). However, the physiological relevance of these findings is not known at present. The cDNA sequence of non-mammalian GnRH receptors has not been reported.

1.5. Structure-activity studies of GnRH:

1.5.1. GnRH agonists:

The isolation and characterization of GnRH has led to an exciting phase of structure-function-activity studies both in mammalian and non-mammalian species (Millar and King, 1984; Peter et al., 1985; Karten and Rivier, 1986; Peter, 1986). The ideas behind development of potent GnRH agonists were to increase the receptor binding affinity, and to increase the resistance to enzymatic degradation in order to extend the plasma half-life of the analogs (Karten and Rivier, 1986, Gordon and Hodgen, 1992). This goal was achieved mainly by substituting Gly^6 with a D-amino acid (preferably hydrophobic) and/or replacement of the C-terminal glycine-amide residue by an ethylamide group (Karten and Rivier, 1986). The structure-function relationship of GnRH analogs in teleosts has been extensively studied using goldfish as a model (for review Peter, 1986; Peter et al., 1987a, 1990a, 1991). Although there are variations in the apparent potency of a given analog between fish species, in general, similar to mammalian studies, analogs with [D-X amino $acid^6$, Pro^9-NHEt] are the most potent in stimulating GTH-II release. However, a

hydrophobic amino acid at position 6 may not be as important for GTH-II releasing activity in goldfish, compared to mammals (Peter, 1986). In goldfish, [D-Arg^6, Pro^9-NHEt]-sGnRH is the most potent analog tested (Peter et al., 1991). The importance of receptor binding affinity (Habibi et al., 1989b) and resistance to enzymatic degradation (Zohar et al., 1990a, b; Goren et al., 1990) in determining the bioactivity of GnRH analogs have been indicated. The presence of serum GnRH binding protein in goldfish (Huang et al., 1991a, b) may extend the biological half-life and duration of action of GnRH in the blood.

1.5.2. GnRH antagonists:

Antagonistic analogs of GnRH competitively bind to GnRH receptors with high affinity, without activating the receptor, thereby inhibiting GTH secretion stimulated by native GnRH. The impetus for development of potent GnRH antagonists is in fertility control, in treatment of various endocrine disorders and steroid associated cancerous growth (Gordon and Hodgen, 1992).

During GnRH structure-function activity studies in mammals, a histidine residue at position 2 was found to be the key for intrinsic activity in GnRH analogs. Omission of histidine at position 2 or substitution by other smaller amino acids resulted in analogs having weak GnRH antagonistic properties (Vale et al., 1972). Further, Rees et al. (1974) found that insertion of a D-aromatic amino acid residue at position 2 enhanced the antagonistic potency of analogs. Based on the idea that D-amino acid substitution at position 6 increases resistance to enzymatic cleavage and increases receptor binding affinity of agonistic analogs, antagonists having D-amino acids at positions 2 and 6 were developed (Yardly et al., 1975). Thus, [D-Phe^2, D-Ala^6]-GnRH was the first antagonist to inhibit ovulation in rat (Yardley et al., 1975). Since many position 2 modified analogs still had weak intrinsic activity, the attention was then focused on position 3 (review see Nestor, 1984). Thus [D-Phe^2, D-Trp^3, D-Phe^6]-GnRH was effective in inhibiting ovulation by a single injection of 1 mg/rat (Nestor et al., 1984; Karten and Rivier, 1986). Based on energy minimization calculations, Rivier and Vale (1978) introduced D-pGlu at position 1 resulting in [D-pGlu^1, D-Ph^2, D-Trp^{3,6}]-GnRH. Subsequent studies showed that substitution of D-pGlu^1 with Ac-Pro^1 or Ac-Δ^3-Pro^1 would enhance the antagonistic potency of analogs (Humphries et al., 1978). In order to increase the hydrophobicity, analogs with 4-chloro- (4Cl) or 4-fluoro-(4F) Phe^2 were developed and in this series [Ac-

Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-GnRH was potent with high receptor binding affinity (Rivier et al., 1983). This analog was extensively tested for *in vivo* activity in mammals including humans (Pavlou et al., 1987).

One approach for the further development of antagonists was to augment the hydrophobicity of the peptide. The increased hydrophobicity was expected to have two non-specific effects (i) to decrease the rate of clearance of the peptide from the circulation, and (ii) to alter the apparent binding constant to membrane bound receptors by increasing the affinity for hydrophobic plasma membrane compartments (Nestor et al., 1984). The substitutions were mainly done at positions 1, 2, 3 and 6. Originally the most hydrophobic natural amino acid, tryptophan was substituted at positions 3 and 6 and later tryptophan was replaced by 3-(2-naphthyl)-D-Alanine (D(2)-Nal) for enhanced activity of GnRH antagonists (Nestor et al., 1984). The most potent analogs found in this series of studies were [N-Ac-Δ^3-Pro^1, D-pF-Phe^2, D-Nal(2)^{3,6}]-GnRH and [N-Ac-Pro^1, D-pF-Phe^2, D-Nal(2)^{3,6}]-GnRH.

The solubility problem of highly hydrophobic analogs led to the reexamination of substitution of polar amino acids like lysine and arginine in position 6. Such substitutions not only reduced the hydrophobicity, but also enhanced the potency of the analogs (Coy et al., 1982). [N-Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Trp^3, D-Arg^6, D-Ala^{10}] GnRH is one of the potent antagonists developed in this series (Rivier et al.,1984). Based on the observation of the presence of hydrophobic tryptophan in position 7 in chicken-II and salmon GnRHs, tryptophan was introduced to position 7 resulting in [N-Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal(3)^3, D-Arg^6, Trp^7, D-Ala^{10}]-GnRH, another highly potent antagonist ("Nal-Arg" analog; Rivier et al., 1984).

Observing the effects on potency of D-Arginine at position 6, a series of unnatural amino acids were designed and synthesized. These amino acids, having dialkyl homoarginines [hArg(Et$_2$)], combine the positive charge of arginine (electrostatic) with stabilization of membrane binding owing to interaction of alkyl groups with hydrophobic phospholipids in the membrane (Nestor et al., 1984; Nestor, 1987). The example is [N-Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}] GnRH, commonly known as Detirelix or RS-68439. This analog, having longer duration of action with a half-life of > 48 hr in man, has been under clinical pharmacological studies as an antifertility agent

(Nestor, 1987).

In many studies, in animals as well as humans, "Nal-Arg" type of analogs and Detirelix were found to induce histamine release from mast cells resulting in transient edema of the face and hence were withdrawn from clinical trials (Rivier et al., 1991). So the next phase of antagonist design and synthesis was directed at producing analogs with reduced histamine releasing activity, keeping the potency intact. In the next series [Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal^{3,6}, Arg^5, D-Ala^{10}] GnRH, and [Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal^3, Arg^5, D-Glu6(AA), D-Ala^{10}]-GnRH ("Nal-Glu" analog) with high potency and low histamine releasing activity were developed (Folkers et al., 1987; Rivier et al., 1991).

To further reduce the histamine release activity of GnRH antagonists, Ljungqvist et al. (1988) developed "Nal-Lys" analog or 'Antide', [Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal^3, Lys(Nic)^5, D-Lys(Nic)^6, Lys(iPr)^8, D-Ala^{10}]-GnRH. Antide is a potent antagonist in rat both *in vivo* (Wallen et al., 1991) and *in vitro* (Danforth et al., 1991a, b), and has a prolonged duration of action in monkey, probably owing to its ability to bind a 66, 000 da protein in serum (Danforth et al., 1990). But Antide is not easily soluble in aqueous buffers at pH higher than 4.0 to 5.0. Hence Rivier's group developed 'Azaline', [Ac-D-Nal^1, 4FD-Phe^2, D-Pal^3, Lys(atz)^5, D-Lys(atz)^6, ILys^8, D-Ala^{10}]-mGnRH and related analogs (Rivier et al., 1991; Theobald et al., 1991). Nestor's group has developed Ganirelix, [Ac-D-Nal^1, D-pCl-Phe^2, D-Pal^3, D-hArg(Et$_2$)^6, D-hArg(Et$_2$)^8, D-Ala^{10}]-mGnRH (Nestor et al., 1992). All these analogs are currently under different phases of clinical trials.

Information on antagonistic studies in non-mammalian species is scarce. In fishes, Crim et al. (1981) have shown that the GnRH antagonist [D-Phe^{2,6}, Phe^3]-mGnRH inhibits mGnRH stimulated gonadotropin release. Recently, Habibi (1991a) has observed that [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH can partially suppress both sGnRH and cGnRH-II actions on GTH-II release *in vitro* in goldfish. However, none of these studies provides information on the structure-activity relationships of GnRH antagonists in teleost fish, and also the antagonists used in these studies did not completely block the actions of native GnRH peptides.

1.6. Regulation of gonadotropin hormone and growth hormone release in fish:

Since the present study involves characterization of GnRH antagonists for their ability

to inhibit GTH-II and GH release stimulated by sGnRH and cGnRH-II, the regulation of GTH-II and GH will be briefly reviewed mainly using goldfish as a model species.

1.6.1. Gonadotropin hormones

1.6.1.1. Structure of gonadotropins:

Early studies on purification of gonadotropins demonstrated the presence of two types of GTH, a carbohydrate-poor, vitellogenic GTH and carbohydrate-rich, maturational GTH (for review, Idler and Ng, 1983). However, recent studies by Dr. Kawauchi and co-workers have revealed the presence of two glycosylated GTHs in salmonoid fish, GTH-I and -II (Suzuki et al., 1988; Kawauchi et al., 1989; Swanson, 1991). Duality of GTHs has also been reported in non-salmonoid fishes including common carp (Van Der Kraak et al., 1992), bonito, a marine fish (Kawauchi et al., 1991), red sea bream (Tanaka et al., 1993, and Atlantic croaker (Copeland and Thomas, 1993). A series of excellent studies in salmonoid fishes have shown that GTH-I and II are produced by separate pituitary cell types (Nazaki et al., 1990) and are differentially secreted during sexual maturation (Naito et al., 1991). Further studies have indicated that GTH-I acting through GTH-receptor type-I (GTH-RI) stimulates steroid production and vitellogenin uptake by maturing oocytes, while during final sexual maturation GTH-II acting through GTH-RII stimulates steroid production and final maturation (for references, see Miwa et al., 1994). In terms of stimulation of steoidogenesis, GTH-I and -II were equipotent during earlier stages of gametogenesis, while GTH-II was more potent in matured fish (Swanson, 1991). In goldfish, carp GTH-I and -II were equipotent in terms of stimulation of testicular and ovarian steroidogenesis, induction of oocyte final maturation (Van Der Kraak et al., 1992), ovarian growth, plasma vitellogenin level and vitellogenin uptake by follicles (Nunez Rodriguez et al., 1992).

1.6.1.2. Regulation of gonadotropin release in teleosts:

The hormonal regulation of gonadotropin release in many teleost species has received considerable attention over the last three decades (Peter, 1983; Peter et al., 1986, 1990a, b, 1991; Goos, 1991). GnRH peptides stimulate GTH-II release in almost all the species tested. In goldfish, two native forms, sGnRH and cGnRH-II stimulate GTH-II synthesis

(Khakoo et al., 1994) and release (Chang et al., 1990; Peter et al., 1991). cGnRH-II is more potent than sGnRH in stimulating GTH-II release *in vitro* (Chang et al., 1990; Habibi, 1991a). The GTH-II response to GnRH peptides appears to be maximal in sexually mature fish (Habibi et al., 1989a).

Dopamine acts as an endogenous inhibitor of gonadotropin release in many teleost species including goldfish (for review see Peter et al.,1986, 1991). *In vivo* studies with dopamine receptor antagonists indicate that dopamine inhibits both spontaneous and GnRH peptide stimulated GTH-II release (Chang and Peter, 1983; Omeljaniuk et al., 1987). The studies with African catfish, *Clarias gariepinus* have indicated a similar dual control of GTH-II, stimulation by GnRH peptides and inhibition by dopamine (Goos, 1991). Dopamine inhibits GTH-II secretion by acting directly at the pituitary level both in goldfish (Chang et al., 1984) and in African catfish (De Leeuw et al., 1986). In these two fish species, immunocytochemical studies have indicated the direct dopaminergic innervation of gonadotroph cells (Kah et al., 1986b; Peute et al., 1987). The GnRH receptor studies have indicated that at least part of the dopaminergic inhibition is mediated through down-regulation of the pituitary GnRH receptors (De Leeuw et al., 1989). In goldfish, dopamine also inhibits the release of GnRH peptides from pituitary fragments and preoptic-anterior hypothalamic slices (Yu et al., 1992). The injection of the dopamine D2 type receptor antagonists pimozide or domperidone potentiates the effects of sGnRH analog on GTH-II levels in blood, and combined treatment of D2 antagonist and GnRH agonist analogs is highly effective in inducing ovulation in fish (Peter et al., 1991).

Gonadal steroids have both negative (Kobayashi and Stacey, 1990) as well as positive effects (Trudeau et al., 1991). Recent studies in goldfish have indicated that at least part of the inhibitory effect of steroids is due to inhibition of the stimulatory effects of γ-amino butyric acid (GABA) (Kah et al., 1992) and an increase in dopamine turnover (Trudeau et al., 1993b; for review see Peter et al., 1991). In the European eel (*Anguilla anguilla*) at the silver stage, testosterone or estradiol strongly stimulated the expression of mRNA GTH-II β subunit (Quérat et al., 1991) and pituitary GTH content (Dufour et al., 1983). Similarly there was an increase in pituitary GTH content in juvenile male and female rainbow trout following testosterone implantation (Crim and Evans, 1979).

Neuropeptide Y stimulates GTH-II by acting directly on pituitary cells and by stimulating release of GnRH peptides (Peng et al., 1993). The gonadal peptides inhibin

and activin act directly on pituitary cells to stimulate GTH-II release (Ge et al., 1992). Cholecystokinin (CCK) stimulates GTH-II release in a dose-dependent fashion and CCK/gastrin like immunoreactivity has been demonstrated in goldfish pituitary (Himick et al., 1993).

1.6.2 Growth hormone:

1.6.2.1. Structure of growth hormone:

The primary structure of GH and the gene sequence encoding GH has been reviewed recently by Rand-Weaver et al. (1993). The primary sequence of GH has been identified from more than 15 fish species and in general, they have 183-188 amino acids with Mr of 20, 000 to 22, 000 da (Rand-Weaver et al., 1993). Owing to the importance of GH in possible enhancement of growth in aquaculture, the cDNA encoding GH peptides have been sequenced in many fish species (Ho et al., 1989; Fine et al., 1993).

1.6.2.2. Regulation of growth hormone release:

Similar to other vertebrates, growth hormone release in teleosts is controlled by multihormonal factors (Harvey, 1993). The inhibitory effects of somatostatin (SRIF) on GH release has been demonstrated in many fish species (Marchant et al., 1987, 1989b; for review see Harvey, 1993). A functional relationship between circulating GH levels and endogenous brain and pituitary SRIF immunoreactivity is indicated in goldfish, in which seasonal increases in GH secretion occur when SRIF concentrations are lowest, and vice versa (Marchant et al., 1989b).

A physiological role for growth hormone-releasing hormone (GRF or GHRH) in regulation of GH release of teleosts has been suggested based on the presence GRF-immunoreactivity in fish hypothalamus, presence of GRF-like peptides and stimulation of GH release by mammalian GRF or carp GRF (for references, see Harvey, 1993).

As mentioned earlier, GnRH also stimulates growth hormone release in many teleosts including goldfish (Marchant et al., 1989), common carp (Lin et al., 1993), rainbow trout (Le Gac et al., 1993) and tilapia (Melamed et al., 1993). In goldfish, both sGnRH and cGnRH-II stimulate GH release and sGnRH is slightly more potent than cGnRH-II stimulating GH release (Chang et al., 1990; Peter et al., 1990a, 1991).

In goldfish, GH release is also under the stimulatory control of thyrotropin-releasing hormone (Trudeau et al., 1992), neuropeptide Y (Peng et al., 1993) cholecystokinin (Himick et al., 1993), and bombesin (Himick and Peter, 1994). Dopamine, a neurotransmitter, stimulates GH release in a dose-related fashion by acting through dopamine D1 type receptors (Wong et al., 1992, 1993a) and the GH stimulatory actions of dopamine is highest in sexually regressed goldfish (Wong et al., 1993b).

1.7. Objectives of the present study:

Although the structure-activity relations of GnRH antagonists has been extensively studied in mammals, there are no systemic studies of this nature in non-mammalian species. Finding a potent GnRH antagonist will be useful (i) as a probe in studies on regulation of GTH-II and GH secretion by hypothalamic and gonadal factors in fish; (ii) in studies on GnRH receptors and associated intracellular second messenger systems; (iii) possibly in controlling the reproduction of fish in aquaculture, and, (iv) such structure activity studies may provide useful information to develop more potent GnRH agonists and antagonists. Based on the results from initial screening studies, the ability of selected GnRH antagonists to inhibit the GTH-II and GH release stimulated by native sGnRH and cGnRH-II peptides, both *in vivo* and *in vitro,* was tested. The actions of GnRH antagonists were demonstrated directly at the pituitary cell level and in competitive binding to GnRH receptors in crude goldfish pituitary membrane preparations. Based on the differential actions of several analogs, functional differences in GnRH receptors on gonadotrophs and somatotrophs are suggested.

Table 1. The amino acid sequence of eight known forms of GnRH in vertebrate species.

	1	2	3	4	5	6	7	8	9	10	
Mammal	pGlu	-His	-Trp	-Ser	-Tyr	-Gly	-Leu	-Arg	-Pro	-Gly-NH_2	(mGnRH)
Chicken I	pGlu	-His	-Trp	-Ser	-Tyr	-Gly	-Leu	-Gln	-Pro	-Gly-NH_2	(cGnRH I)
Catfish	pGlu	-His	-Trp	-Ser	-His	-Gly	-Leu	-Asn	-Pro	-Gly-NH_2	(cfGnRH)
Chicken II	pGlu	-His	-Trp	-Ser	-His	-Gly	-Trp	-Tyr	-Pro	-Gly-NH_2	(cGnRH II)
Dogfish	pGlu	-His	-Trp	-Ser	-His	-Gly	-Trp	-Leu	-Pro	-Gly-NH_2	(dfGnRH)
Salmon	pGlu	-His	-Trp	-Ser	-Tyr	-Gly	-Trp	-Leu	-Pro	-Gly-NH_2	(sGnRH)
Lamprey III	pGlu	-His	-Trp	-Ser	-His	-Asp	-Trp	-Lys	-Pro	-Gly-NH_2	(lGnRH-III)
Lamprey I	pGlu	-His	-Tyr	-Ser	-Leu	-Glu	-Trp	-Lys	-Pro	-Gly-NH_2	(lGnRH-I)

References

Andersson, E., Borg, B., and De Leeuw, R. (1989). Characterization of gonadotropin-releasing hormone binding sites in the pituitary of the three-spined stickleback, *Gasterosteus aculeatus*. Gen. Comp. Endocrinol. 76, 41-45.

Amano, M., Aida, K., Okumoto, N., and Hasegawa, Y. (1992). Changes in salmon GnRH and chicken GnRH-II contents in the brain and pituitary, and GtH contents in the pituitary in female masu salmon, *Oncorhynchus masou*, from hatching through ovulation. Zool. Sci. 9, 375-386.

Barbieri, R. L. (1992). Clinical applications of GnRH and its analogues. Trends Endocrinol. Metab. 3, 30-34.

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.

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.

Bramley, T. A. (1987). LHRH binding sites in human tissues. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 123-139. MTP Press Ltd., Boston.

Brooks, J., Taylor, P. L., Saunders, P. T. K., Eidne, K. A., Struthers, W. J., and McNeilly, A. S. (1993). Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. Mol. Cell. Endocrinol. 94, R23-R27.

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. USA. 69, 278-282.

Chang, J. P., and Peter, R. E. (1983). Effects of dopamine on gonadotropin release in female goldfish, *Carassius auratus*. Neuroendocrinology, 36, 351-357.

Chang, J. P., Cook, H., Freedman, G. L., Wiggs, A. J., Somoza, G. M., De Leeuw, R., and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture

system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Chang, J. P., MacKenzie, D. S., Gould, D. R., and Peter, R. E. (1984). Effects of dopamine and norepinephrine on *in vitro* spontaneous and GnRH induced gonadotropin release by dispersed cells or fragments of the goldfish pituitary. Life Sci. 2027-2033.

Chi, L Zhou, W., Prikhozhon, A., Flanagan, C., Davidson, J. S., Golembo, M., Illing, N., Millar, R. P., and Sealfon, S. C. (1993). Cloning and characterization of the human GnRH receptor. Mol. Cell. Endocrinol. 91, R1-R6.

Chieffi, G., Pierantoni, R., and Fasano, S. (1991). Immunoreactive GnRH in hypothalamic and extrahypothalamic areas. Int. Rev. Cytol. 127, 1-55.

Clayton, R. N. (1988). Mechanisms of GnRH action in gonadotrophs. Human Reprod. 3, 479-483.

Clayton, R. N., and Catt, K. J. (1981). Gonadotropin releasing hormone receptor characterization, physiological regulation and relationship to reproduction. Endocrine Rev. 2, 186-209.

Coe, I. R., von Schalburg, K., Adelman, J. P., and Sherwood, N. M. (1992). Evolution of the gonadotropin-releasing hormone family: Peptides and genes. In "Program & Abstracts", 74th annual meeting, The Endocrine Society. p 97 (Abstract # 183).

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.

Conn, M. P. (1986). The molecular basis of gonadotropin-releasing hormone action. Endocrine Rev. 7, 3-10.

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J. P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul. Peptides, 36, 369-378.

Copeland, P. A., and Thomas, P. (1993). Isolation of gonadotropin subunits and evidence

for two distinct gonadotropins in Atlantic Croaker (*Micropogonias undulatus*). Gen. Comp. Endocrinol. 91, 115-125.

Coy, D. H., Horvath, A., Nekola, M. V., Coy, E. J., Erchegyi, J., and Schally, A. B. (1982). Peptide antagonists of LH-RH: Large increases in antiovulatory activities produced by basic-D-amino acids in the six position. Endocrinology, 110, 1445-1447.

Crim, L. W., and Evans, D. M. (1979). Stimulation of pituitary gonadotropin by testosterone in juvenile rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol. 37, 192-196.

Crim, L. W., Evans, D. M., Coy, D. A., and Schally, A. V. (1981). Control of gonadotropic hormone release in trout: Influence of synthetic LH-RH and LH-RH analogues *in vivo* and *in vitro*. Life Sci. 28, 129-136.

Crim, L. W., Peter, R. E., and Van Der Kraak, G. (1987). The use of LHRH analogs in Aquaculture. In "LHRH and its analogs: Contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr., Eds.), pp 489-498. MTP Press Ltd., Boston.

Crim, L. W., St. Arnaud, R., Lavoie, M., and Labrie, F. (1988). A study of LHRH receptors in the pituitary gland of the winter flounder (*Pseudopleuronectes americanus* Walbaum). Gen. Comp. Endocrinol. 69, 372-377.

Danforth, D. R., Gordon, K., Leal, J. A., Williams, R. F., and Hodgen, G. D. (1990). Extended presence of Antide (NAL-LYS GnRH antagonist) in circulation: Prolonged duration of gonadotropin inhibition may derive from Antide binding to serum proteins. J. Clin. Endocrinol. Metab. 70, 554-556.

Danforth, D. R., Williams, R. F., Gordon, K., and Hodgen, G. D. (1991a). Inhibition of pituitary gonadotropin secretion by the gonadotropin-releasing hormone antagonist Antide. I. *In vitro* studies on mechanism of action. Endocrinology, 128, 2036-2040.

Danforth, D. R., Williams, R. F., Gordon, K., Leal, J. A., and Hodgen, G. D. (1991b). Inhibition of pituitary gonadotropin secretion by the gonadotropin-releasing hormone antagonist Antide. II. Development of an *In vitro* bioassay for characterization of pharmaco-kinetics and pharmacodynamics of Antide in circulation. Endocrinology, 128, 2041-2044.

Dellovade, T. L., King, J. A., Millar, R. P., and Rissman, E. F. (1993). Presence of differential distribution of distinct forms of immunoreactive gonadotropin-releasing

hormone in the musk shrew brain. Neuroendocrinology, 58, 166-177.

De Leeuw, R., Goos, H. J. Th., and Van Oordt, P. G. W. J. (1986). The dopaminergic inhibition of the GnRH induced gonadotropin release. An *in vitro* study with fragments and cell suspensions from pituitaries of the African catfish, *Clarias gariepinus* (Burchell). Gen. Comp. Endocrinol. 63, 171-177.

De Leeuw, R., Habibi, H. R., Nahorniak, C. S., and Peter, R. E. (1989). Dopaminergic regulation of gonadotropin releasing hormone receptor activity in the goldfish (*Carassius auratus*). J. Endocrinol. 121, 239-247.

Dunn, I. C., Sharp, P. J., and Sang, H. M. (1992). Evolution of GnRH peptides. In "Proceedings of the British Neuroendocrine Group Meeting", Edinburgh, Scotland. (Quoted in King and Millar, 1992).

Dufour, S., Delerue-Le Belle, N., and Fontaine, Y. A. (1983). Effects of steroid hormones on pituitary immunoreactive gonadotropin in European fresh-water eel, *Anguilla anguilla L.* Gen. Comp. Endocrinol. 52, 190-197.

Eidne, K. A., Sellar, R. E., Couper, G., Anderson, L., and Taylor, P. L. (1992). Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Mol. Cell. Endocrinol. 90, R5-R9.

Fine, M., Sakal, E., Vashdi, D., Daniel, V., Levanon, A., Lipshitz, O., and Gertler, A. (1993). Recombinant carp (*Cyprinus carpio*) growth hormone: expression, purification, and determination of biological activity *in vitro* and *in vivo*. Gen. Comp. Endocrinol. 89, 51-61.

Folkers, K., Bowers, C., Tang, P. -F. L., Feng, D., Okamoto, T., Zhang, Y., and Ljungqvist, A. (1987). Specificity of design to achieve antagonists of LHRH of increasing effectiveness in therapeutic activity. In "LHRH and its analogs: Contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr. Eds.), pp 25-36. MTP Press Ltd., Boston.

Ge, W., Chang, J. P., Peter, R. E., Vaughan, J., Rivier, J., and Vale, W. (1992). Effects of porcine follicular fluid (pFF), inhibin A, and activin A on goldfish gonadotropin release *in vitro*. Endocrinology, 131, 1922-1929.

Goos, H. J. Th. (1991). Fish gonadotropins and GnRH's: Fundamental aspects and practical applications. Bull. Inst. Zool. Academia Sinica, Monograph, 16, 119-137.

Gordon, K., and Hodgen, G. D. (1992). Evolving role of gonadotropin-releasing

hormone antagonists. Trends Endocrinol. Metab. 3, 259-263.

Goren, A., Zohar, Y., Fridkin, M., Elhanati, E., and Koch, Y. (1990). Degradation of gonadotropin-releasing hormones in the gilthead seabream, *Sparus aurata.* I. Cleavage of native salmon GnRH and mammalian LHRH in the pituitary. Gen. Comp. Endocrinol. 79, 291-305.

Green, J. D., and Harris, G. W. (1947). Neurovascular link between neruohypophysis and adenohypophysis. J. Endocrinol. 5, 136-146.

Hazum, E., and Conn, P. M. (1988). Molecular mechanism of gonadotropin releasing hormone (GnRH) action. I. GnRH receptor. Endocrine Rev. 9, 379-386.

Habibi, H. R. (1991a). Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: Effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod. 44, 275-283.

Habibi, H. R. (1991b). Desensitization to native molecular forms of gonadotropin-releasing hormone in the goldfish pituitary: Dependence on pulse frequency and concentration. Gen. Comp. Endocrinol. 84, 199-214.

Habibi, H. R., and Pati, D. (1993). Endocrine and paracrine control of ovarian function: Role of compounds with GnRH-like activity in goldfish. In "Cellular Communication in Reproduction". (F. Facchinette, I. W. Henderson, R. Pierantoni, and A. M. Polzonetti-Magni, Eds.), pp 59-70. Journal of Endocrinology Ltd., Bristol, UK.

Habibi, H. R. and Peter, R. E. (1991). Gonadotropin-releasing hormone (GnRH) receptors in teleosts. In "Proceedings of the 4th International symposium, Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 109-113. FishSymp 91, Sheffield, UK.

Habibi, H. R., De Leeuw, R., Nahorniak, C. S., Goos, H. J. Th., and Peter, R. E. (1989a). Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: Seasonal and gonadal effects. Fish Physiol. Biochem. 7, 109-118.

Habibi, H. R., Peter, R. E., and Hazum, E. L. I. (1990). Photoaffinity labelling of pituitary gonadotropin-releasing hormone receptors in goldfish (*Carassius auratus*). Biol. Reprod. 43, 1006-1011.

Habibi, H. R., Peter, R. E., Nahorniak, C. S., Milton, R. C. L., and Millar, R.P. (1992).

Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides, 37, 271-284.

Habibi, H. R., Marchant, T. A., Nahorniak, C. S., Van Der Loo, H., Peter, R. E., Rivier, J. E., and Vale, W. W. (1989b). Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon GnRH in the pituitary of goldfish (*Carassius auratus*). Biol. Reprod. 40, 1152-1161.

Habibi, H. R., Peter, R. E., Sokolowska, M., Rivier, J. E., and Vale, W. W. (1987). Characterization of GnRH binding to pituitary receptors in goldfish (*Carassius auratus*). Biol. Reprod. 36, 844-853.

Habibi, H. R., Van Der Kraak, G., Bulanski, E., and Peter, R. E. (1988). Effects of teleost GnRH on reinitiation of oocyte meiosis in goldfish, *in vitro*. Am. J. Physiol. 255, R-268-R273.

Habibi, H. R., Van Der Kraak, G., Fraser, R., and Peter, R. E. (1989c). Effects of a teleost GnRH analog on steroidogenesis by the follicle-enclosed goldfish oocytes, *in vitro*. Gen. Comp. Endocrinol. 76, 95-105.

Harvey, S. (1993). Growth hormone secretion in poikilotherms and homeotherms. In "The endocrinology of growth, development, and metabolism in vertebrates". (M. P. Schreibman, C. G. Scanes, and P. K. T. Pang, Eds.), pp 151-182. Academic Press, New York.

Ho, W. K. K., Tsang, W. H., and Dias, N. P. (1989). Cloning of grass carp growth hormone cDNA. Biochem. Biophys. Res. Commun. 161, 1239-1243.

Himick, B. A., and Peter, R. E. (1994). Bombesin acts to suppress feeding behavior and alter serum growth hormone in goldfish. Physiol. Behavior, 55, 65-72.

Himick, B. A., Golosinski, A. A., Jonsson, A. -C., and Peter, R. E. (1993). CCK/ Gastrin-like immunoreactivity in the goldfish pituitary: Regulation of pituitary hormone secretion by CCK-like peptides *in vitro*. Gen. Comp. Endocrinol. 92, 88-103.

Huang, Y. P., Guo, D. C., and Peter, R. E. (1991a). Isolation and characterization of a gonadotropin-releasing hormone binding protein in goldfish serum. Gen. Comp. Endocrinol. 84, 58-66.

Huang, Y. P.. Peng, C., and Peter, R. E. (1991b). Metabolism of gonadotropin-releasing hormone in goldfish: Serum clearance and tissue uptake studies. Gen. Comp.

Endocrinol. 84, 67-75.

Humphries, J., Wasiak, T., Wan, Y. P., Folkers, K., and Bowers, C. Y. (1978) An antiovulatory decapeptide of higher potency which has an L-amino acid (Ac-Pro) in position 1. Biochem. Biophys. Res. Commun. 85, 709-713.

Idler, D. R., and Ng, T. B. (1983). Teleost gonadotropins: Isolation, biochemistry, and function. In "Fish Physiology: Reproduction". Vol IX. (W. S. Hoar, D. J. Randall, and E. M. Donaldson. Eds.), pp 187-221. Academic Press, New York.

Jinnah, H. A., and Conn, P. M. (1988). GnRH action at the pituitary: Basic research and clinical applications. In "Peptide hormone: Effects and Mechanisms of action". (A. Negro-Vilar, and P. M. Conn, Eds.), pp 119-142. CRC press, Boca Rotan, Florida.

Jones, P. B. C., and Hsueh, A. J. W. (1987). Direct antigonadal actions of LHRH. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 1. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 163-180. MTP press Ltd., Boston.

Kah, O., Breton, B., Dulka, J. G., Nunez Rodriguez, J., Peter, R. E., Corrigan, A., Rivier, J. E., and Vale, W. W. (1986a). A reinvestigation of the GnRH systems in the goldfish brain using antibodies to salmon GnRH. Cell Tissue Res. 244, 327-337.

Kah, O., Dubourg, P., Onteniente, B., Geffard, and M., Calas, A. (1986b). The dopaminergic innervation of the goldfish pituitary: An immunocytochemical study at the electron-microscope level using antibodies against dopamine. Cell Tissue Res. 244, 577-582.

Kah, O., Trudeau, V. L., Sloley, B. D., Chang, J. P., Dubourg, P., Yu, K. L., and Peter, R. E. (1992). Influence of GABA on gonadotrophin release in the goldfish. Neuendocrinology, 55, 396-404.

Kakar, S. S., Musgrove, L. C., Devor, D. C., Sellers, J. C., and Neill, J. D. (1992). Cloning, sequencing , and expression of human gonadotropin releasing hormone (GnRH) receptors. Biochem. Biophys. Res. Comm. 189, 289-295.

Kakar, S. S., Musgrove, L. C., Sellers, J. C., and Neill, J. D. (1993). Human gonadotropin releasing hormone (GnRH) receptor: Further molecular characterization. In "Program & Abstracts", 75th annual meeting, The Endocrine Society. pp 339 (Abstract # 1153).

Karten, M. J., and Rivier, J. E. (1986). Gonadotropin releasing hormone analog design.

Structure-function studies towards the development of agonists and antagonists: Rationale and perspectives. Endocrine Rev. 7, 44-66.

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.

Kawauchi, H., Itoh, H., and Koide, Y. (1991). Additional evidence for duality of fish gonadotropins. In "Proceedings, 4th International symposium on the The Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 2-7. FishSymp 91, Sheffield, UK.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M., Nakai, Y., and Itoh, S. (1989). The duality of teleost gonadotropins. Fish Physiol. Biochem. 7, 29-38.

Khakoo, Z., Bhatia, A., Gedamu, L., and Habibi, H. R. (1994). Functional specificity for salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II coupled to the gonadotropin release and subunit messenger ribonucleic acid level in the goldfish pituitary. Endocrinology, 134, 838-847.

Kim, M., Oka, Y., Amano, M., Kobayashi, M., Aida, K., and Kawashima, S. (1993). Immunocytochemical localization of sGnRH and cGnRH-II in the goldfish brain and effects of olfactory tract section on the distribution of GnRH. In "Program, XII International Congress of Comparative Endocrinology". pp A-82 (abstract).

King, J. A., and Millar, R. P. (1982). Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization. J. Biol. Chem. 257, 10729-10732.

King, J. A., and Millar, R. P. (1987). Phylogenetic diversity of LHRH. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 53-73. MTP Press Ltd., Boston.

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

King, J. A., Davidson, J. S., and Millar, R. P. (1988a). Interaction of endogenous chicken gonadotrophin-releasing hormone-I and -II on chicken pituitary cells. J. Endocrinol. 177, 43-49.

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., Hassan, M. F., Mehl, A. E., and Millar, R. P. (1988b). Gonadotropin releasing hormone molecular forms in mammalian hypothalamus. Endocrinology, 122, 2472-2752.

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

Knox, C. J., Boyd, S. K., and Sower, S. A. (1994). Characterization and localization of gonadotropin-releasing hormone receptors in the adult female sea lamprey, *Petromyzon marinus*. Endocrinology, 134, 492-498.

Kobayashi, M., and Stacey, N. E. (1990). Effects of ovariectomy and steroid hormone implantation on serum gonadotropin levels in female goldfish. Zool. Sci. 7, 715-721.

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P. Y., Loir, M., Mourot, B., and Weil, C. (1993). Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem. 11, 219-232.

Lin, X. W., Lin, H. R., and Peter, R. E. (1993). Growth hormone and gonadotropin secretion in the common carp (*Cyprinus carpio* L): *In vitro* interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen. Comp. Endocrinol. 89, 62-71.

Lincoln, D. W. (1989). Luteinizing hormone-releasing hormone. In "Endocrinology". Vol I. (L. J. DeGroot, Ed.), pp 142-151. W. B. Saunders Co., Philadelphia.

Ljungqvist, A., Feng, D. M., Hook, W., Shen, Z, X., Bowers, C., and Folkers, K. (1988). Antide and related antagonists of luteinizing hormone release with long action and oral activity. Proc. Natl. Acad. Sci. USA. 85, 8236-8240.

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.

Marshall, J. C. Dalkin, A. C., Haisenlender, D. J., Paul, S. J., Ortolano, G. A., and Kelch, R. P. (1991). Gonadotropin-releasing hormone pulses: regulators of

gonadotropin synthesis and ovulatory cycles. Recent Prog. Horm. Res. 47, 155-189.

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. Proposed aminoacid sequence. Biochem. Biophys. Res. Commun. 43, 1334-1339.

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

Marchant, T. A., Dulka, J. G., and Peter, R. E. (1989b). Relationship between serum growth hormone levels and the brain and pituitary content of immunoreactive somatostatin in the goldfish, *Carassius auratus L.* Gen. Comp. Endocrinol. 73, 458-468.

Marchant, T. A., Fraser, R. A., Andrews, P. C., and Peter, R. E. (1987). The influence of mammalian and teleost somatostatins on the secretion of growth hormones from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul. Pepties, 17, 41-52.

Melamed, P., Eliahou, N., Sivan, B., and Yaron, Z. (1993). Hypothalamic factors regulating GH secretion in tilapia. In "Program, XII International Congress of Comparative Endocrinology", pp A-104 (abstract).

Millar, R. P., and King. '. A. (1984). Structure-activity relations of LHRH in birds. J. Exp. Zool. 232, 42. 430.

Miller, W. L., Wu, J. C., and Sealfon, S.C. (1993). Estradiol, progesterone, inhibin and GnRH regulate GnRH receptor mRNA(s) in ovine pituitary cultures. In "Program & Abstracts", 75th annual meeting, The Endocrine Society. pp 61 (Abstract # 41).

Miyamoto, K., Hasegawa, Y., Minegishi, T., Nomura, M., Tkahashi, Y., Igarashi, M., Kangawa, K., and Matsuo, H. (1982). Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. Biochem. Biophys. Res. Commun. 107, 820-827.

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. USA. 81, 3874-3878.

Miwa, S., Yan, L., and Swanson, P. (1994). Localization of two gonadotropin receptors

in the salmon gonad by *in vitro* ligand autoradiography. Biol. Reprod. 50, 629-642.

Naito, N., Hyodo, S., Okumoto, N., Urano, A., and Nakai,Y. (1991). Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. Cell Tissue Res. 266, 457-467.

Nazaki, M., Naito, N., Swanson, P., Miyata, K., Nakai, Y., Oots, Y, Suzuki, K., and Kawauchi, H. (1990). Salmonoid pituitary gonadotrophs I. Distinct cellular distributions of two gonadotropins, GTH-I and GTH II. Gen. Comp. Endocrinol. 77, 348-357.

Nestor, J. J. Jr. (1984). Development of agonists of LHRH analogs. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 3-10. MTP Press Ltd., Boston.

Nestor, J. J. Jr. (1987). Development of LHRH antagonists. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 3-16. MTP Press Ltd., Boston.

Nestor, J. J. Jr., Ho, T. L., Tahilramani, R., Mc Rae, G. I., and Vickery, B. H. (1984). Long acting LHRH agonists and antagonists. In "LHRH and its analogs". (F. Labrie, A. Belanger, and A. Dupont, Eds.). pp 24-35. Elsevier Science Press, Amsterdam.

Nestor, J. J. Jr., Tahilramani, R., Ho, T. L., Goodpasture, J. C., Vickery, B. H., and Ferrandon, P. (1992). Potent gonadotropin-releasing hormone antagonists with low histamine-releasing activity. J. Med. Chem. 35, 3942-3948.

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

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

Nikolics, K., and Seeburg, P. H. (1987). Biosynthesis of LHRH. In "LHRH and its analogs: Contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J.

Nestor Jr., Eds.), pp 37-51. MTP Press Ltd., Boston.

Nunez Rodriguez, J., Suzuki, K. S., Peter, R. E., and Kawauchi, H. (1992). Effects of common carp GTH-I and GTH-II on vitellogenesis and vitellogenin uptake in goldfish (*Carassius auratus*). In "Abstacts", 2nd International Symposium on Fish Endocrinology, Saint-Malo, France. pp L51 (abstract).

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.

Omeljaniuk, R. J., Shih, S. H., and Peter, R. E. (1987). In vivo evaluation of dopamine receptor-mediated inhibition of gonadotrophin secretion from the pituitary gland of the goldfish, *Carassius auratus*. J. Endocrinol. 114, 449-458.

Pagelson, G., and Zohar, Y. (1992). Characterization of gonadotropin-releasing hormone binding to pituitary receptors in the gilthead seabream (*Sparus aurata*). Biol. Reprod. 47, 1004-1008.

Pati, D., and Habibi, H. R. (1993). Characterization of gonadotropin-releasing hormone (GnRH) receptors in goldfish ovary: variation during follicular development. Am. J. Physiol. 264, R227-R234.

Pavlou, S. N., Wakefield, G. B. and Kovacs, W. J. (1987). LHRH antagonists in normal men. In "LHRH and its analogs:Contraceptive and Therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 245-256. MTP Press Ltd., Boston.

Peng, C., Chang, J. P., Yu, K. L., Wong, A. O.-L., Van Goor, F., Peter, R. E. and Rivier, J. E. (1993). Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary: Involvement of both presynaptic and pituitary cell actions. Endocrinology, 132: 1820-1829.

Peter, R. E. (1983). The brain and neurohormones in teleost reproduction. In "Fish Physiology: Reproduction". Vol IX. (W. S. Hoar, D. J. Randall, and E. M. Donaldson. Eds.), pp 97-135. Academic Press, New York.

Peter, R. E. (1986). Structure-activity studies on gonadotropin-releasing hormone in teleosts, amphibians, reptiles and mammals. In "Comparative Endocrinology: Developments and Directions" (C. L. Ralph, Ed.), pp 75-93. Alan R. Liss, Inc., New York.

Peter, R. E., Chang, J. P., Nahorniak, C. S., Omeljaniuk, R. J., Sokolowska, M., Shih, S. H., and Billard, R. (1986). Interaction of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog. Horm. Res. 42, 513-548.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990a). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In:"Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, and M. H. Stetson, Eds.), pp. 393-398. Wiley-Liss Inc., NY.

Peter, R. E., Habibi, H. R., Marchant, T. A., and Nahorniak, C. S. (1987a). Vertebrate GnRH: Phylogeny and structure-function relationships. Ann. NY. Acad. Sci. 519:299-309.

Peter, R. E., Lin, H. R., and Van Der Kraak, G. (1988). Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH analogues and dopamine antagonists. Aquaculture, 74, 1-10.

Peter, R.E., Nahorniak, C. S., Shih, S., King, J. A., and Millar, R. P. (1987b). Activity of position 8-substituted analogs of mammalian gonadotropin releasing hormone (mGnRH) and chicken and lamprey gonadotropin releasing hormones in goldfish. Gen. Comp. Endocrinol. 65, 385-393.

Peter, R. E., Nahorniak, C. S., Sokolowska, M., Chang, J. P., Rivier, J. E., Vale, W. W., King, J. A., and Millar, R. P. (1985). Structure-activity relationships of mammalian, chicken and salmon gonadotropin releasing hormones *in vivo* in goldfish. Gen. Comp. Endocrinol. 58, 231-242.

Peter, R. E., Trudeau V. L., and Sloley, B. D. (1991). Brain regulation of reproduction in teleosts. Bull. Inst. Zool. Academia Sinica, Monograph, 16, 89-118.

Peter, R. E., Yu, K. L., Marchant, T. A., and Rosenblum, P. M. (1990b). Direct neural regulation of the teleost adenohypophysis. J. Exp. Zool. Suppl. 4, 84-89.

Peute, J., Schild, R. G., Schild, V. A., Buijs, R., Van Asselt, L. A. C., and Van Oordt, P. G. W. J. (1987). Immunocytochemical evidence for peptidergic gonadotropin releasing hormone and dopaminergic innervations of the gonadotropic cells in the pituitary of the African catfish, *Clarias gariepinus*. Gen. Comp. Endocrinol. 67, 303-310.

Quérat, B., Hardy, A., and Fontaine, Y. A. (1991). Regulation of the type-II

gonadotrophin α and β subunit mRNAs by oestradiol and testosterone in the European eel. J. Mol. Endocrinol. 7, 81-86.

Rand-Weaver, M., Kawauchi, H., and Ono, M. (1993). Evolution of the structure of the growth hormone and prolactin family. In "The endocrinology of growth, development, and metabolism in vertebrates". (M. P. Schreibman, C. G. Scans, and P. K. T. Pang, Eds.), pp 13-42. Academic Press, New York.

Reinhart, J., Mertz, L. M., and Catt, K. J. (1992). Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. J. Biol. Chem. 267, 21281-21284.

Rees, R. W., Foell, T. J., Chia, S. -Y., and Grant, N. (1974). Synthesis and biological activities of analogs of LHRH modified in position 2. J. Med. Chem. 17, 1016-1019.

Rivier, C., Rivier, J., Perrin, M., and Vale, W. (1983). Comparison of the effect of several gonadotropin releasing hormone antagonists on luteinizing hormone secretion, receptor binding and ovulation. Biol. Reprod. 29, 374-378.

Rivier, J. E., and Vale, W. W. (1978). [D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}]-LRF. A potent luteinizing hormone releasing factor antagonist *in vitro* and inhibitor of ovulation in the rat. Life Sci. 23, 869-876.

Rivier, J., Rivier, C., Perrin, M., Porter, J., Vale, W. W. (1984). LHRH analogs as anti-ovulatory agents. In "LHRH and its analogs:Contraceptive and therapeutic applications". Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 11-22. MTP Press Ltd., Boston.

Rivier, J., Theobald, P., Porter, J., Perrin, M., Gunnet, J., Hahn,D. W., and Rivier, C. (1991). Gonadotropin releasing hormone antagonists: Novel structures incorporating N$^{\omega}$-cyano modified guanidine moieties. Biochem. Biophys. Res. Comm. 176, 406-412.

Santen, R. J., and Bourguignon, J. -P. (1987). Gonadotropin releasing hormone: Physiological and therapeutic aspects, agonists and antagonists. Hormone res. 28, 88-103.

Schally, A. V., Arimura, A., Baba, Y., Nair, R. M. G., Matsuo, H., Redding, T. W., Debeljuk, L., and White, W. F. (1971a). Isolation and properties of the FSH and LH releasing hormone. Biochem. Biophys. Res. Commun. 43, 393-399.

Schally, A. V., Arimura, A., Kastin, A. J., Matsuo, H., Baba, Y., Redding, T. W., Nair,

R. M. G., and Debeljuk, L. (1971b). Gonadotropin releasing hormone: One peptide regulates secretion of luteinizing and follicle stimulating hormones. Science 173, 1036-1039.

Schally, A. V., Bajusz, S., Redding, T. W., Zalatnai, A., and Comaru-Schally, A. M. (1989). Analogs of LHRH: The present and the future. In "GnRH analogs in cancer and human reproduction". Vol I. (B. H. Vickery, and B. Lunenfeld, Eds.), pp 5-31. Kluwer Academic Publishers, Boston.

Schanbacher, B. D. (1987). LHRH and analogs in relation to livestock. In "LHRH and its analogs: Contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr., Eds.), pp 385-396. MTP Press Ltd., Boston.

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

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. Horm. Res. 43, 69-98.

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. USA. 80, 2794-2798.

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

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., and Brownstein, M. J. (1986). Primary structure of gonadotropin-releasing hormone from lamprey brain. J. Biol. Chem. 261, 2812-4819.

Silverman, A. J. (1988). The gonadotropin-releasing hormone (GnRH) neuronal systems: immunocytochemistry. In "The Physiology of Reproduction". (E. Knobil, and J. Neill, Eds.), pp 1283-1304. Raven Press, New York.

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.

Suzuki, K., Kawauchi, H., and Nagahama, Y. (1988). Isolation of two distinct salmon gonadotropins from chum salmon pituitary glands. Gen. Comp. Endocrinol. 71, 292-301.

Suzuki, M., Hyodo, S., Kobayashi, M., Aida, K., and Urano, A. (1992). Characterization and localization of mRNA encoding the salmon-type gonadotropin-releasing hormone precursor of the masu salmon. J. Mol. Endocrinol. 9, 73-82.

Swanson, P. (1991). Salmon gonadotropins: reconciling old and new ideas. In "Proceedings, 4th International symposium on the Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 2-7. FishSymp 91, Sheffield, UK.

Tanaka, H., Kagawa, H., Okuzawa, K., and Hirose, K. (1993). Purification of gonadotropins (pmGTH I and II) from red seabream (*Pagrus major*) and development of a homologous radioimmunoassay for pmGTH II. Fish Physiol. Biochem. 10, 409-418.

Theobald, P., Porter, J., Rivier, C., Corrigan, A., Hook, W., Siraganian, R., Perrin, M., Vale, W., and Rivier, J. (1991). Novel gonadotropin-releasing hormone antagonists: Peptides incorporating modified N^{ω}-guanoguanidino moieties. J. Med. Chem. 34, 2395-2402.

Trudeau, V. L., Murthy, C. K., Habibi, H. R., Sloley, B. D., and Peter, R. E. (1993a). Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. Biol. Reprod. 48, 300-307.

Trudeau, V. L., Peter, R. E., and Sloley, B. D. (1991). Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44, 951-960.

Trudeau, V. L., Sloley, B. D., Wong, A. O. L., and Peter, R. E. (1993b). Interaction of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. Gen. Comp. Endocrinol. 89, 39-50.

Trudeau, V. L., Somoza, G. M., Nahorniak, C. S., and Peter, R. E. (1992). Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology, 56,

483-490.

Tsutsumi, M., Zhou, W., Millar, R. P., Mellon, P. L., Roberts, J. L., Flanagan, A., Dong, K., Gillo, B., and Sealfon, S. C. (1992). Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol. Endocrinol. 6, 1163-1169.

Vale, W., Grant, G., Rivier, J., Monahan, M., Amoss, M., Blackwell, R., Burgus, R., and Guillemin, R. (1972). Synthetic polypeptide antagonists of the hypothalamic luteinizing hormone-releasing factor. Science, 176:933-934.

Vickery, B. H. (1986). Comparison of the potential for therapeutic utilities with gonadotropin releasing hormone agonists and antagonists. Endocrine Rev. 7, 115-124.

Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

Wallen, K., Eisler, J. A., Tannenbaum, P. L., Nagell, K. M., and Mann, D. R. (1991). Antide (NAL-LYS GnRH antagonist) suppression of pituitary-testicular function and sexual behavior in group-living Rhesus Monkeys. Physiol. Behavior 50, 429-435.

Weil, C., Crim, L. W., Wilson, C. E., and Cauty, C. (1992). evidence of GnRH receptors in cultured pituitary cells of the winter flounder (*Pseudopleuronectes americanus W.*). Gen. Comp. Endocrinol. 85, 156-164.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1992). Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology, 130, 1201-1210.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1993a). *In vitro* and *in vivo* evidence that dopamine exerts growth hormone-releasing activity in goldfish. Am. J. Physiol. 264, E925-932.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1993b). Interactions of somatostatin, gonadotropin-releasing hormone, and the gonads on dopamine-stimulated growth hormone release in the goldfish. Gen. Comp. Endocrinol. 92, 366-378.

Yardley, J. P., Foell, T. J., Beattie, C. W., and Grant, N. (1975) Antagonism of luteinizing hormone release and of ovulation by an analog of the luteinizing hormone-releasing hormone. J. Med. Chem. 18, 1244-1247.

Yu, K. L., and Peter, R. E. (1992). Adrenergic and dopaminergic regulation of gonadotropin-releasing hormone release from goldfish preoptic-anterior hypothalamus

and pituitary *in vitro*. Gen. Comp. Endocrinol. 85, 138-146.

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.

Zandbergen, M. A., Peute, J., Verkley, A. J., and Goos H. J. Th. (1992). Application of cryosubstitution in neurohormone-and neurotransmitter-immnocychemistry. Histochemistry, 97, 133-139.

Zohar, Y. (1989). Fish reproduction: Its physiology and artificial manipulation. In "Fish culture in warm water systems: Problems and trends". (M. Shilo, and S. Sarig, Eds.), pp 65-119. CRC Press Inc., Florida.

Zohar, Y., Goren, A., Fridkin, M., Elhanati, E., and Koch, Y. (1990a). Degradation of gonadotropin-releasing hormones in the gilthead seabream, *Sparus aurata*. II. Cleavage of native salmon GnRH and mammalian LHRH and their analogs in the pituitary, kidney, and liver. Gen. Comp. Endocrinol. 79, 306-319.

Zohar, Y., Goren, A., Tosky, M., Pagelson, G., Leibovitz, D., and Koch, Y. (1990b). The bioactivity of gonadotropin releasing hormones and its regulation in the gilthead seabream, *Sparus aurata*: *in vivo* and *in vitro* studies. Fish Physiol. Biochem. 7, 59-67.

Chapter Two

In vitro characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish, *Carassius auratus*[1]

Introduction

As in vertebrates, gonadotropin-releasing hormone (GnRH) is involved in the regulation of gonadotropin (GTH-II) secretion in teleost fish (for review Peter et al., 1986, 1990a, b, 1991). In goldfish brain and pituitary two forms of GnRH, namely salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II) have been identified (Yu et al., 1988). In addition to stimulating GTH-II secretion, both forms of GnRH stimulate growth hormone (GH) release in goldfish (Marchant et al., 1989; Chang et al., 1990; Habibi et al., 1992). During spawning of goldfish there is a significant decrease in total GnRH levels in the pituitary and brain starting from about 12-16 h prior to ovulation, corresponding with the onset of the preovulatory surges in serum GTH-II and GH concentrations; a few hours after ovulation, brain and pituitary GnRH levels, and serum GTH-II and GH levels return to near normal preovulatory values (Yu et al., 1991). The negative correlation between GnRH content in the pituitary and brain, and serum GTH-II and GH concentrations suggests a causal relationship (Yu et al., 1991). Treatment with superactive analogs of GnRH is being used in aquaculture to induce maturation and ovulation of cultured fishes (Crim et al., 1987; Peter et al., 1988, 1990a, 1991). Repeated injection of superactive GnRH analogs also enhances body growth (Marchant et al., 1989). Thus, GnRH appears to be important in regulation of both reproduction and growth in fish.

In extensive studies on mammals, GnRH antagonistic analogs have been developed by modifying the GnRH sequence especially at positions 1, 2, 3, and 6 (for review Karten and Rivier, 1986). There is, however, very little information available on the structure-activity

[1]A version of this chapter has been published: CK Murthy, CS Nahorniak, JE Rivier, RE Peter (1993). Endocrinology, 133, 1633-1644.

relations of GnRH antagonists in fish or any other non-mammalian vertebrate. Crim et al. (1981) have shown that [D-Phe^{2,6}, Phe^{3}]- GnRH can block the increase in plasma GTH-II levels induced by injection of mammalian GnRH (mGnRH) in male trout *in vivo*. Recently, Habibi (1991) has observed that [D-p-Glu^{1}, D-Phe^{2}, D-Trp^{3,6}]-mGnRH can partially suppress both sGnRH and cGnRH-II actions on GTH-II release *in vitro* in goldfish. However, none of these studies provides information on the structure-activity relationships of GnRH antagonists in teleost fish, and also the antagonists used in these studies did not completely block the actions of sGnRH and cGnRH-II. Finding a potent GnRH antagonist will be useful (i) as a probe in studies on regulation of GTH and GH secretion by hypothalamic and gonadal factors in fish; (ii) in studies on GnRH receptors and associated intracellular second messenger systems; and, (iii) possibly also in controlling the reproduction of fish in aquaculture. The objective of the present study was to identify and characterize GnRH antagonists for their ability to block both sGnRH- and cGnRH-II-induced GTH-II and GH release in goldfish using an *in vitro* pituitary fragments perifusion system.

Materials and Methods

Experimental animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries, Stoutland, MO. The fish were maintained in flow-through aquaria (1800 liters) at 17 ± 1° C under a simulated natural photoperiod of Edmonton for at least 2-3 weeks prior to experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish of both sexes, with body weight ranging from 20-35 g were used in the present study. The sexual maturity of fish was assessed by measuring the gonadosomatic index (GSI= weight of gonad/total body weight X 100). During initial screening of GnRH analogs, fish in a sexually regressed state or early gonadal recrudescence were used.

Reagents and test substances sGnRH, cGnRH-II, and lamprey GnRH (lGnRH) (purchased from Peninsula Laboratories Inc., Belmont, CA) were dissolved in 0.1 M acetic acid and stored at -25° C as aliquots of 50 µM. The aliquots were diluted with perifusion medium to required concentrations immediately prior to use in experiments. GnRH analog [D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}]-mGnRH was purchased from Sigma Chemical Co., St. Louis, MO. All other analogs (Table 2.1) were the gift of Dr. J. E. Rivier, The Clayton

Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA. GnRH analogs were dissolved in a primary solvent containing propylene glycol and physiological saline at a ratio of 60:40, and subsequently diluted to required concentrations with perifusion medium. The final propylene glycol concentration was less than 0.5%.

Perifusion of the pituitary fragments *In vitro* experiments were conducted using a validated pituitary fragments perifusion system (Marchant et al., 1989). Briefly the pituitary glands, collected from goldfish, were chopped into fragments (0.2 mm^3) using a McIlwain tissue chopper. The fragments were washed with medium 199 and placed between two layers of Cytodex beads (purchased from Sigma Chemical Co., St. Louis, MO) in the perifusion columns (3 pituitary equivalents per column) with a continuous flow of incubation medium (medium 199) at 18° C. The fragments were perifused overnight (at least 15 h) at a flow rate of 5 ml/h. Thereafter, the incubation was continued using Hank's balanced salt solution supplemented with 25 mM HEPES and 0.1% BSA at a flow rate of 15 ml/h for 2 h before starting the experiment. Five minute fractions of perifusate were collected, frozen and stored at -25 C for hormonal assay. During initial screening, GnRH analogs were tested for their possible stimulatory actions on GTH-II and GH secretion under 2 min pulse and 30 or 35 min continuous treatment conditions.

Radioimmunoassay GTH-II levels in perifusate were measured by using a validated radioimmunoassay specific for goldfish GTH-II (Peter et al., 1984; Van Der Kraak et al., 1992). The GH levels were measured by using a radioimmunoassay similar to that described by Marchant et al. (1989), except that standard and labeled ligand were goldfish GH (gGH), purified following the technique described by Rand-Weaver and Kawauchi (1992). The antibody raised against gGH has <0.01% cross reactivity with carp and goldfish prolactin, and carp somatolactin. Carp GTH-I and GTH-II caused no displacement of iodinated gGH from the antibody. The ED20, ED50 and ED80 values for the assays were 83.8 ± 2.2, 36.4 ± 1.3 and 16.7 ± 1.0 ng/ml, respectively (n=10). The intra-assay and inter-assay variability were 3.04% (n=9) and 12.68% (n=8), respectively.

Data analysis The results of initial screening of GnRH analogs are presented as ng/ml for

a single column out of 2-4 columns. To provide a mean response, the GTH-II or GH values were expressed as percentage of pretreatment mean (of 3 fractions prior to first pulse) for each column and pooled from 4-6 columns. The quantification of hormone response was done as described by Peng et al. (1990). Briefly, the average hormone level of 3 fractions immediately preceding each pulse (prepulse mean) was treated as a basal hormone level and the hormone values following a GnRH pulse were expressed as a percentage of the prepulse mean. Post pulse fractions with hormone contents greater than one standard error (SEM) above the prepulse mean were considered to be a part of the response. The response (expressed as % prepulse) above the pretreatment mean from the fractions considered as part of the response were added to get the net response in a particular column.

To establish a dose-response relationship, the ability of each dose of antagonist to block 20 nM sGnRH (or cGnRH-II) stimulated hormone release (GTH-II and GH) was calculated in each column as follows:

R = (T/S) 100, where R = the hormonal response for 20 nM sGnRH (or cGnRH-II) in the presence of a given concentration of GnRH antagonist expressed as a percentage of the standard response; S = Net response of hormone release under 20 nM sGnRH (or cGnRH-II) pulse for 2 min in the absence of any antagonist (taken as standard response for that particular column); and T = Net response of hormone release under 20 nM sGnRH (or cGnRH-II) pulse in presence of X nM antagonist.

The 'R' values obtained for a given dose of antagonist from 4-6 perifusion columns under the same treatment regime were averaged and expressed as mean ± SEM, and were plotted against the dose of analog. Using a computerized ALLFIT program (De Lean et al., 1978) ED50 ± approximate error value for each antagonist [effective dose for 50% reduction of the hormone release induced by 20 nM sGnRH (or cGnRH-II)] was calculated as a measure of potency of that analog. The difference in responses between treatment and control was assessed by ANOVA followed by Student's t-test ($p < 0.05$). The dose-response curves were compared either following $\sin^{-1}(\sqrt{X/100})$ transformation by ANOVA and Fisher's LSD test ($p < 0.05$) or by constructing 95% confidence intervals.

Results

Initial screening and characterization studies During the initial screening, analogs based

on mGnRH, sGnRH and lGnRH were studied to determine their ability to stimulate GTH-II and GH release, and to block sGnRH-stimulated GTH-II release. Each analog was tested at various concentrations (20 nM to 10 μM) for its actions on GTH-II and GH release at least 2-3 times, with different protocols using pituitary fragments collected from fishes in various stages of sexual maturity. Since sGnRH is known to stimulate GTH-II release from goldfish pituitary fragments, a 2 min pulse of 20 nM sGnRH was given, or in some cases 20 nM and 100 nM pulses were given, at the start in every column to demonstrate that the fragments were responding normally. The GTH-II release profile of a single representative column under 2 μM dose of each analog has been presented in left panels of Fig. 2.1. Owing to limited amounts of GH tracer, normally only the perifusion fractions during and around the 30 or 35 min continuous analog treatment were analysed for GH content and are shown on right side panels of Fig. 2.1. Most of the analogs tested except for a few indicated below, had some GTH-II and GH releasing activity. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C, Fig. 2.1c), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E, Fig. 2.1e) and [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH (Analog M, Fig. 2.1m) did not increase the release of GTH-II either under 2 min pulse treatment (20 nM and 100 nM) or under 30 or 35 min continuous treatment at 2 μM concentration. In the presence of analogs C, E, and M, GTH-II release stimulated by 20 nM sGnRH was strongly reduced when compared to that of sGnRH alone. Analogs E (Fig. 2.1e) and M (Fig. 2.1m) had no effects on GH release. However, analog C had strong GH stimulatory activity (Fig. 2.1c). [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH (Analog K, Fig. 2.1k) and [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH (Analog J, Fig. 2.1j) under continuous treatment at 2 μM concentrations strongly stimulated both GTH-II and GH release. lGnRH (Fig. 2.1n) and analogs based on lGnRH structure, specifically analogs G, H, and I, and analog D weakly stimulated GTH-II and GH release from perifused pituitary fragments under 30 or 35 min continuous treatment at 2 μM concentration (Fig. 2.1g, h, i, and d, respectively). [Ac-D-4Cl-Phe^{1,2}, D-Trp^3, D-Lys^6]-mGnRH (analog A) weakly stimulated GTH-II release and more strongly stimulated GH release (Fig. 2.1a); whereas, analogs B and F more strongly stimulated GTH-II release and analog B, but not F, weakly stimulated GH release. Analog N was not tested in this series of experiments. A

qualitative summary of the actions of various analogs on GTH-II and GH release is presented in Table 2.2.

Analogs based on mGnRH or sGnRH structure having D-arginine at position 6 (analogs B, D, H, J and K) all have stimulatory actions on GTH-II and GH release (Fig. 2.1; Table 2.2); analog F had stimulatory actions on GTH-II release but no effects on GH release. Analog L with arginine at position 5 also increased GTH-II release, but significantly suppressed basal GH release exhibiting differential actions on GTH-II and GH release. Although, many of these analogs stimulated GTH-II and GH release, further stimulation by sGnRH was abolished in their presence. Normally, GTH-II stimulatory activity of an analog was observed under 30 or 35 min continuous treatment, but not under 2 min pulse.

Further characterization of the actions of analogs C and E as GnRH antagonists was carried out using fragments collected from fish in the final stages of gonadal recrudescence or sexually mature fish (Figs. 2.2, 2.3). In control columns, 20 nM sGnRH (Fig. 2.2a, d) and cGnRH-II (Fig. 2.3a, d) caused repeatable increases of similar magnitude in GTH-II. In the presence of analog E (Fig. 2.2b, e), GTH-II release stimulated by 20 nM sGnRH was suppressed by about 86 ± 3%. At 90 min after termination of analog E treatment, 20 nM sGnRH induced a magnitude of GTH-II release similar to that of the initial pulse (Fig. 2.2e). Analog C at 2 μM concentration inhibited sGnRH actions on GTH-II release with a lesser efficiency (63 ± 3% inhibition, Fig. 2.2c, f); at 90 min after termination of exposure to analog C, the response to 20 nM sGnRH was significantly lower compared to the initial pulse, indicating that the duration of action of analog C was prolonged relative to that of analog E. Analogs C and E inhibited cGnRH-II-induced GTH-II release by about 67 ± 4% and 85 ± 2% respectively (Fig. 2.3a to f). Analog E suppressed 20 nM sGnRH (86 ± 3%) and cGnRH-II (85 ± 2%) actions on GTH-II release equally (Fig. 2.2e, 2.3e). Similar equipotency was exhibited by analog C (Figs. 2.2f, 2.3f). Analog E, at 2 μM concentration, had a significantly greater inhibition of sGnRH actions on GTH-II release than analog C (86 ± 3% vs 63 ± 3%; Figs. 2.2e, f). Similarly, in suppressing cGnRH-II induced GTH-II release, analog E (85 ± 2%) was significantly more effective than analog C (67 ± 4%; Fig. 2 3e, f).

In a similar experiment, a 2 μM dose of analogs M and N, [Ac-D(2)Nal^{1}, 4Cl-D-Phe^{2}, D-(3)Pal^{3,6}]-mGnRH also suppressed 50 nM sGnRH-induced GTH-II release by

81 ± 4% and 98 ± 2% respectively from the pituitary fragments collected from sexually regressed fish (Fig. 2.4). This inhibitory action by analog N was significantly greater than that of analog M. The inhibitory effects of analog N, but not M were observed even at 90 min after termination of analog treatment.

Dose-response studies sGnRH (20 nM for 2 min) given at 90 min intervals elicited GTH-II responses of similar magnitude in pituitary fragments obtained from post-spawning fish (Fig. 2.5a, b). In the presence of increasing concentrations (2 to 2000 nM) of analog E the GTH-II responses to 20 nM sGnRH gradually decreased (Fig.2.6a, c). In the reciprocal experiments with decreasing concentrations of analog E, sGnRH stimulated GTH-II release gradually increased (Fig. 2.6b, d). The GTH-II responses were quantified and the pooled data showed a dose-related inhibition of sGnRH stimulated GTH-II release by analog E (Fig. 2.7a), with an ED50 (effective dose to inhibit 20 nM sGnRH stimulated GTH-II release by 50%) of 242 ± 48 nM. At a 2 μM concentration analog E inhibited the GTH-II response to 20 nM sGnRH by 94 ± 2%. In sexually regressed fish, analog E also suppressed 20 nM sGnRH induced GTH-II release in a dose-dependent fashion with an ED50 of 128 ± 82 nM (Fig. 2.7b). A similar approach was used to investigate the antagonist activity of analog E on the GTH-II releasing activity of cGnRH-II in sexually regressed fish. The dose-related inhibition of cGnRH-II action by analog E had an ED50 of 169 ± 17 nM (Fig. 2.8). At a dose of 2 μM, analog E completely blocked the stimulatory action of cGnRH-II on GTH-II release. Although ED50 comparisons do not show any significant differences, ANOVA on transformed dose response data shows that analog E has a significantly greater inhibition of sGnRH induced GTH-II release in sexually regressed fish (Fig. 2.7b) than in post-spawning fish (Fig. 2.7a). Similarly, analog E (2 μM) totally blocked 20 nM cGnRH-II action in regressed fish (Fig. 2.8) as against an inhibition of 85 ± 2% in sexually mature fish (Fig. 2.3).

Analog M also suppressed sGnRH induced GTH-II release from pituitary fragments collected from sexually regressed fish in a dose-dependent fashion, with an approximate ED50 of 326 ± 96 nM (Fig. 2.9a). Under similar experimental conditions, analog M blocked 20 nM cGnRH-II stimulated GTH-II release in a dose-related manner with an ED50 of 249 ± 74 nM (Fig. 2.9b). At a 2 μM concentration analog M inhibited 20 nM

sGnRH and cGnRH-II induced GTH-II release by 88 ± 3% and 88 ± 2%, respectively. Comparison of ED50 values along with confidence intervals, or of responses at different doses by ANOVA following $\sin^{-1}(\sqrt{X/100})$ transformation, did not reveal any significant differences in the ability of analog M to suppress sGnRH and cGnRH-II induced GTH-II release.

Antagonism of GH response to GnRH Repeated 2 min pulses of 20 nM sGnRH (Fig. 2.10a) or cGnRH-II (Fig. 2.10b) given either in the presence or in the absence of solvent, induced similar increases in GH release from the pituitary fragments collected from fishes in the final stages of gonadal recrudescence. In the presence of analog E (2 μM), the GH release stimulated by both 20 nM sGnRH (Fig. 2.10c) and 20 nM cGnRH-II (Fig. 2.10d) was reduced compared to initial pulses. In a similar study using sexually regressed fish, analogs E (Fig. 2.11a, d), M (Fig. 2.11b, e) and N (Fig. 2.11c, f) at 2 μM concentration significantly suppressed 50 nM sGnRH induced GH secretion without showing any GH stimulatory activity. The inhibitory effects of analogs E and N, but not of M, were still evident even at 90 min after termination of analog treatment.

sGnRH (20 nM for 2 min) given at 90 min intervals elicited GH responses of similar magnitude in pituitary fragments obtained from post-spawning fish (Figs. 2.12a and 2.13a). The GH responses to sGnRH (20 nM) in post spawning fish were gradually increased in the presence of decreasing concentrations of analog E (Fig. 2.12b, c) and gradually decreased in the presence of increasing concentrations of analog E (data not shown). This dose-related suppression of GH release by analog E had an ED50 of 128 ± 74 nM (Fig. 2.13b). Analog E also blocked 20 nM sGnRH induced GH release from pituitary fragments obtained from sexually regressed fish, in a dose-related manner (Fig. 2.13c); at a 20 nM dose, analog E suppressed 20 nM sGnRH action on GH release by more than 50%.

Repeated 2 min pulses of 20 nM cGnRH-II elicited GH release of similar magnitude from the pituitary fragments collected from sexually regressed goldfish (Fig. 2.14a). Analog E suppressed 20 nM cGnRH-II induced GH release in a dose-related fashion with an ED50 of 157 ± 67 nM (Fig. 2.14b).

Comparison of dose-response data by ANOVA indicated that analog E was more effective in suppressing sGnRH induced GH release (Fig. 2.13c) than GTH-II release

(Fig. 2.7b) in sexually regressed fish. Similarly analog E had a greater inhibition of cGnRH-II induced GH release (Fig. 2.14b) than GTH-II release (Fig. 2.8). However, analog E at the 2 μM concentration showed carry over effects on sGnRH induced GH release at 90 min after termination of analog E treatment. This may influence the dose response studies making it difficult to compare the ability of analog E to inhibit GTH-II and GH release especially at the high doses. However, in single dose studies, analog E generally suppressed the GH release response to sGnRH to a greater extent than that of GTH-II (Figs. 2.2b and 2.10c). Similarly, analog M was more effective in inhibiting the GH release response to sGnRH compared to GTH-II release response to sGnRH (Figs. 2.4c vs 2.11e). The dose dependent inhibition of analog M on sGnRH or cGnRH-II stimulated GH release was not tested.

Discussion

Amongst all the GnRH analogs tested in the present study, only four exhibited pure antagonism to sGnRH and cGnRH-II stimulated GTH-II release from the perifused goldfish pituitary fragments without some accompanying agonistic action on GTH-II release. All of the purely antagonistic analogs, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C), [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH (analog M), and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) have modifications at positions 1, 2, 3 and 6 of mGnRH or sGnRH or cGnRH-II. Similar modifications produce potent GnRH antagonists in mammalian studies (for review Karten and Rivier, 1986). Vale et al. (1972) reported that des-His^2-GnRH had no agonistic activity, but reduced the GnRH stimulated LH release from the dispersed rat pituitary cells. Substitution of D-phenylalanine at position 2 resulted in a weak GnRH antagonist (Rees et al., 1974). Further, introduction of D-Ala^6 along with D-Phe^2 resulted in an antagonist which inhibited ovulation in rats at a dose of 6 mg (Yardley et al., 1975). Further enhancement of potency of GnRH antagonists by modification of the amino acid at position 3, preferably by substitution of a D-amino acid, was attributed to a decrease in residual intrinsic activity along with a greater resistance to enzymatic degradation (Rivier and Vale, 1978). Introduction of D-pGlu (Rivier and Vale, 1978) or Ac-Pro at position 1, caused an additional increase in potency of GnRH antagonists;

injection of 7.5 μg of [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) at noon on the proestrus day completely inhibited ovulation in rats (Rivier et al., 1984).

In mammals, the substitution of D-Trp^6 with D-Arg^6 in [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH along with the very hydrophobic residue Ac-D(2)-Nal^1 greatly increased antagonist potency (Rivier et al., 1984). In contrast, in the present study analogs of both mGnRH (analog F) and sGnRH (analog B) having [Ac-D(2)-Nal^1, 4FD-Phe^2, D-Trp^3, D-Arg^6] were stimulatory to GTH-II release; analog B was also stimulatory to GH release. [Ac-4Cl-Phe^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, D-Ala^{10}]-mGnRH at 1.5 -3.0 μg in corn oil per rat caused 100% inhibition of ovulation (Coy et al., 1982). Further studies by Horvath et al. (1982) showed that antagonist [Ac-(2)-D-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, D-Ala^{10}]-mGnRH having Ac-(2)-D-Nal instead of Ac-4Cl-Phe at position 1, was approximately 3 times more potent than the parent analog. This antagonist also exhibited a longer duration of inhibition, suppressing LH levels for more than 30 h in ovariectomized rats when injected at a dose of 100 μg (Nekola and Coy, 1984). A similar strong GnRH inhibitory activity has also been reported for [Ac-(2)-D-Nal^1, D-pCl-Phe^2, D-Trp^3, D-h-Arg(Et$_2$)^6, D-Ala^{10}]-mGnRH and for [Ac-(2)-D-Nal^1, D-4Cl-Phe^2, D-Trp^3, D-h-Arg(Et$_2$)^6, Trp^7, D-Ala^{10}]-mGnRH (Nestor et al., 1988). In goldfish, [D-Arg^6, Pro^9 NHEt]-sGnRH has a high receptor affinity (Habibi et al., 1989c), and is the most potent GTH-releasing analog studied to date both *in vivo* (Peter et al., 1985) and *in vitro* (Habibi et al., 1989c). In the present study the D-Arg^6 substituted analogs B, D, H, J, and K were all stimulatory to both GH and GTH-II release; analog F was the only exception, with only GTH-II releasing activity. Other stimulatory analogs, without D-Arg^6, showed only a weak GH and GTH-II stimulation (analogs A, D, G, I, and L); analog L was inhibitory on GH release. Only analogs E, and M with hydrophobic D-Trp^{3,6} or D-(3)Pal^{3,6} (analog N) acted as pure GnRH antagonists on GH and GTH-II release; uniquely, analog C was antagonistic on GTH-II release, and stimulatory on GH release. These results demonstrate that position 6 generally plays an important role in determining the nature of intrinsic activity of GnRH peptides in the goldfish.

Structure-activity studies of GnRH agonistic analogs using the goldfish pituitary indicate that there is less specificity in ligand requirements to activate receptors compared to that of mammals (Habibi et al., 1989c). In goldfish, all of the known GnRH peptides tested to date, including mGnRH, sGnRH, cGnRH-I, cGnRH-II, lGnRH (Habibi et al.,

1992), catfish GnRH (Ngamvongchon et al., 1992), and dogfish GnRH (Lovejoy et al., 1992) stimulate GTH-II release. However, in the rat, beside mGnRH, only cGnRH-II shows any significant GTH stimulatory activity (32% and 41% potency of mGnRH in stimulating LH and FSH release, respectively; Miyamoto et al., 1984). It may be that in the goldfish, since the ligand structure requirements for receptor activation are not highly rigid, the requirements for receptor antagonists may be more restrictive. In support of this, many of the analogs tested in the present study known to be potent GnRH antagonists in mammals were stimulatory to GTH-II release in the goldfish.

The inhibitory action of antagonists is presumably due to competitive binding to GnRH receptors in the pituitary. Analog E displaced bound ^{125}I-sGnRH-A from a crude goldfish pituitary membrane preparation (see Chapter 6); analog M also showed similar displacement properties, with a receptor binding affinity greater than that of the two native peptides (Habibi, 1991). In the present study, although many analogs induced increased GTH-II release, in the presence of these stimulatory analogs, a pulse of sGnRH or cGnRH-II often failed to further stimulate GTH-II release indicating that the analogs also act through the same population of receptors. In mammals it has been extensively documented that GnRH antagonists bind competitively to GnRH receptors to exert inhibitory action on agonists (for review Clayton, 1989; Karten and Rivier, 1986; Vickery, 1987).

Comparison of GTH-II response data by ANOVA following transformation showed that analog E caused a significantly greater suppression than analog M of both sGnRH and cGnRH-II induced GTH-II release in sexually regressed fish. The GTH-II responses to 20 nM sGnRH in presence of analog E (Fig. 2.7b) and analog M (Fig. 2.9a) were significantly different, specifically at 20, 200, 500, and 1000 nM doses of analogs. Similarly, compared to analog M (Fig. 2.9b), analog E (Fig. 2.8) showed a greater inhibition of cGnRH-II actions on GTH-II release, specifically at 200, 500, 1000, and 2000 nM doses. In mammalian studies substitution of D-p-Glu^1 with Ac-Pro^1 (Humphries et al., 1978) and of D-Phe^2 with D-chloro (or fluoro)-Phe^2 (Coy et al., 1979) was found to enhance the potency of GnRH antagonists. These modifications also appear to be favorable in increasing the GnRH antagonist potency in goldfish.

Analog C, based on sGnRH structure, having similar modifications as that of analog E, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH, was a significantly less potent antagonist

of sGnRH stimulated GTH-II release compared to analog E. Similarly, the antagonistic ability of analog C on cGnRH-II action was significantly lower compared to analog E. However, at 90 min after termination of analog C treatment, the sGnRH and cGnRH-II induced GTH-II response was significantly less than the initial pulse, whereas responsiveness was entirely regained following exposure to analog E under a similar protocol. Since sGnRH is more hydrophobic than mGnRH, it may be that analog C has a higher dissociation constant than analog E.

There are seasonal changes in the GTH-II responses to GnRH peptides in goldfish with the greatest responses occurring in sexually mature fish (Habibi et al., 1989b). The ability of GnRH antagonists to inhibit this response also appears to vary with reproductive developmental stage of goldfish. Analog E had a greater inhibition of sGnRH action in sexually regressed fish compared to post spawning fish. Similarly, analog E (2 μM) totally blocked 20 nM cGnRH-II action in regressed fish as compared to an inhibition of 85 ± 2% in sexually mature fish. A similar tendency was also exhibited by analog M. Habibi et al. (1989b) have shown that the pituitary GnRH receptor number is highest in sexually mature fish and lowest in sexually regressed fish. Accordingly, the results indicate that less GnRH antagonist is required to occupy the receptors in sexually regressed fish, providing the basis for the seasonal changes in apparent activity of the antagonists.

The two native forms of GnRH peptides present in goldfish, sGnRH and cGnRH-II (Yu et al., 1988), are assumed to stimulate GTH-II release via the same population of receptors on gonadotrophs (Habibi et al., 1988, 1989a, 1992). Analog E at a 2 μM dose antagonized the actions of both sGnRH and cGnRH-II on GTH-II release equally. Similar results were observed in the case of analog C. The sGnRH based analog C at 50, 300, and 1000 nM concentration blocked 50 nM sGnRH and cGnRH-II induced GTH-II release with a similar efficacy (see Chapter 3). In the dose response studies, the ability of analog M, at doses ranging from 2 nM to 2 μM, to inhibit sGnRH and cGnRH-II stimulation on GTH-II release was not significantly different. Habibi (1991) also noted similar results using [D-p-Glu^{1}, D-Phe^{2}, D-Trp^{3,6}]-mGnRH (analog M in the present study). The results support the idea that the two native forms of GnRH peptides act through the same population of receptors on gonadotrophs. This is further supported by the fact that both sGnRH and cGnRH-II displace bound ^{125}I-sGnRH-A from the pituitary membrane

preparations (Habibi et al., 1988, 1989a, 1992; Peter et al., 1990a) and avidin gold labelled biotinylated sGnRH analog from the gonadotrophs (Cook et al., 1991). Interestingly, there are distinct differences in the intracellular mechanisms mediating the action of sGnRH and cGnRH-II on GTH-II release (Chang and Jobin, 1991; Chang et al., 1993). cGnRH-II action on GTH-II release is mainly mediated by internalization of extracellular calcium, without involving mobilization of intracellular calcium and arachidonic acid metabolism (Chang et al., 1993). However, sGnRH stimulated GTH-II release is mediated by all these second messenger components.

Analog E effectively blocked sGnRH and cGnRH-II induced GH release. This is the first report showing a GnRH antagonist blocking actions of GnRH peptides on somatotrophs. Analogs M and N also significantly suppressed 50 nM sGnRH induced GH release. Analogs E, M, and N at a given concentration exhibited a greater suppression of GH than GTH-II release stimulated by sGnRH and cGnRH-II. Analog E had greater suppression of GH than GTH-II release induced by sGnRH in sexually regressed and post-spawning fish, and by cGnRH-II in sexually regressed fish. Analogs M and N, at least at 2 μM concentrations, caused greater inhibition of GH than GTH-II release induced by 50 nM sGnRH. These results indicate that a lower dose of analogs is required to inhibit GnRH induced GH compared to GTH-II release, and implies that there are differences in the properties of GnRH receptors on GTH and GH cells. In support of this hypothesis, a 2 μM dose of analog E at 90 min after termination of analog E treatment exhibited carry over effects on GH release but not on GTH-II release. The possibility of persistent antagonism by this high dose of analog E on GH release makes it difficult to compare these particular parts of the dose response inhibition curves of GTH-II and GH by analog E.

[Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C) had stimulatory actions on GH release, but not on GTH-II release. In contrast to this, [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L) inhibited GH release, but stimulated GTH-II release. Similarly, [Ac-D(2)-Nal^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH (analog F) also showed differential GTH-II and GH release action. Additional studies are required to determine if other antagonists have differential actions on GH versus GTH-II release. Nevertheless the results further indicate differences in the structure-function relationships between GnRH receptors on somatotrophs and gonadotrophs.

In conclusion the results presented here indicate that GnRH antagonists can inhibit

GnRH and cGnRH-II stimulated GTH-II and GH release *in vitro*. The inhibition is influenced by the sexual development stage of the fish. Both sGnRH and cGnRH-II actions on gonadotrophs are equally suppressed by antagonists, indicating that the native peptides act through the same population of receptors on the gonadotrophs. The properties of GnRH receptors on GTH-II and GH cells may however, be different, as some antagonists exhibit a greater ability to suppress GH than GTH-II release. Further, a few analogs show differential actions on GTH-II and GH release. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH is the most potent antagonist studied to date. The substitution of D-Arg^6 normally results in stimulatory analogs.

Fig. 2.1 Effects of analogs A to M and of lGnRH (see Table 2.1 for analog structures) pulse treatment (2 min at 20 nM or 100 nM) or continuous exposure (30 or 35 min at 2 μM) on unstimulated and 20 nM sGnRH stimulated GTH-II release from the perifused pituitary fragments (left panels). Effects of analogs A to M and of lGnRH at 2 μM concentration under continuous exposure on GH release from the perifused pituitary fragments (right panels). Results of a representative column out of 2-4 similar columns for each peptide are presented.

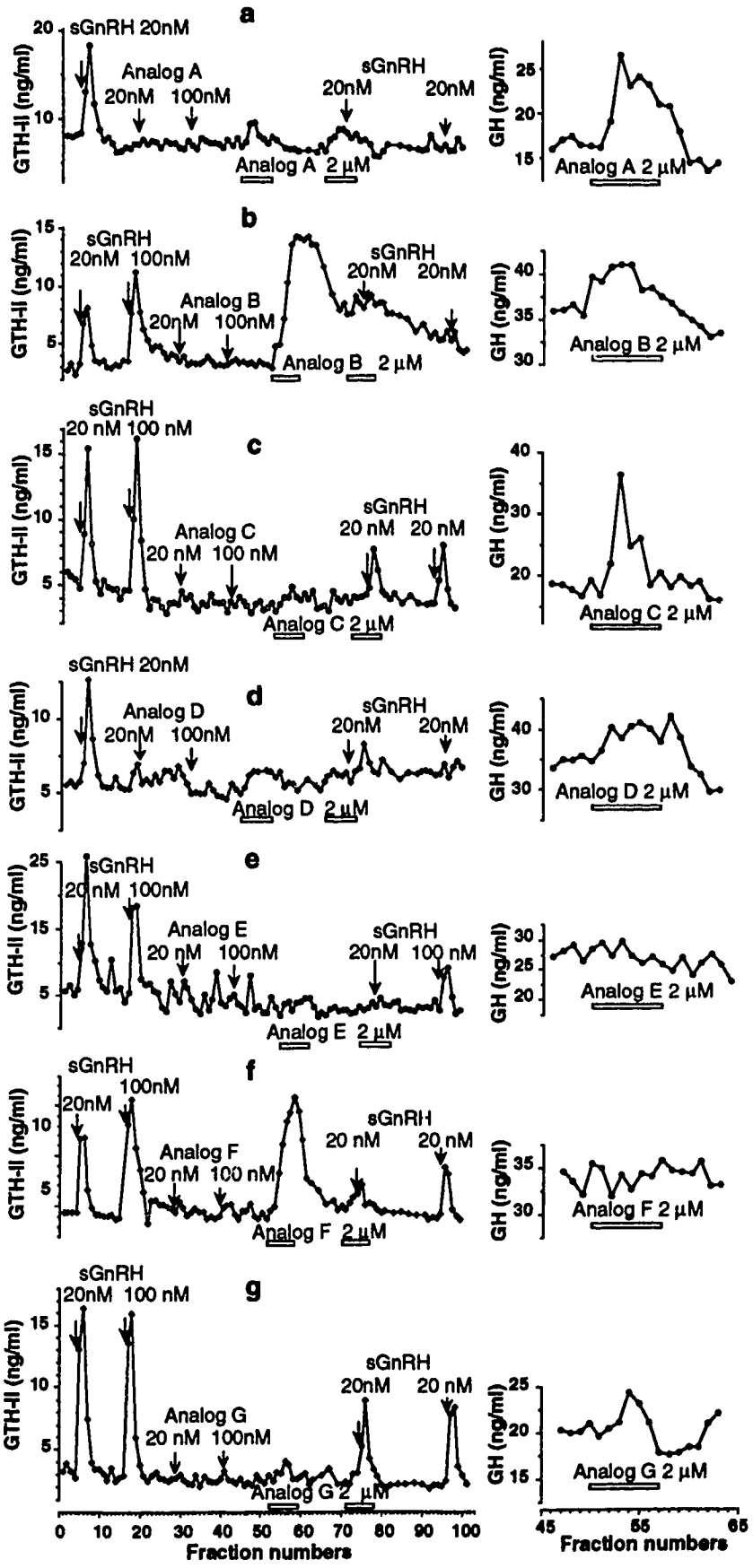
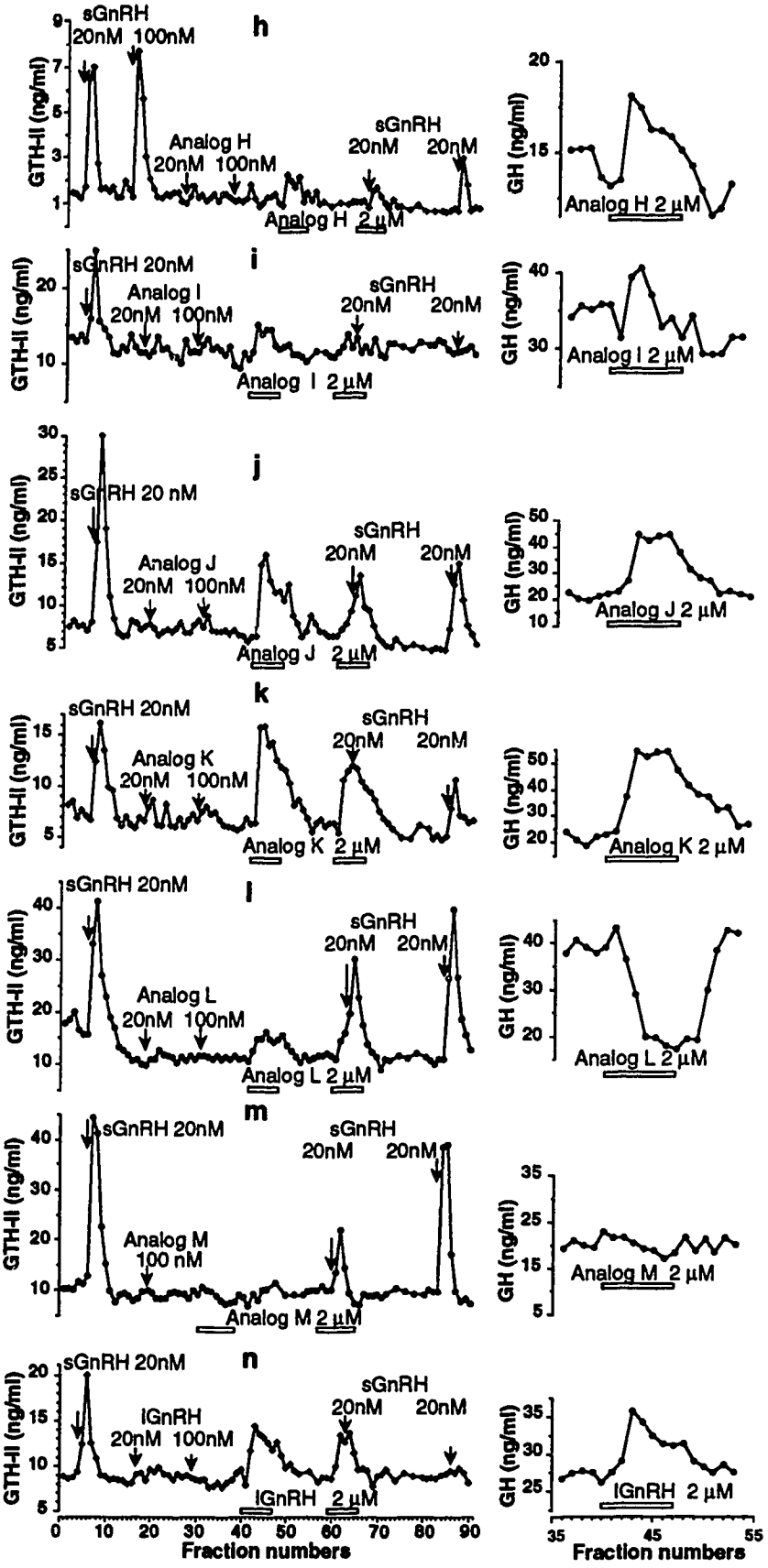

a
sGnRH 20nM
Analog A
20nM 100nM
sGnRH
20nM 20nM
Analog A 2 μM
GTH-II (ng/ml)
GH (ng/ml)
b
sGnRH
20nM 100nM
Analog B
20nM 100nM
sGnRH
20nM 20nM
Analog B 2 μM
c
sGnRH
20 nM 100 nM
Analog C
20 nM 100 nM
sGnRH
20 nM 20 nM
Analog C 2 μM
d
sGnRH 20nM
Analog D
20nM 100nM
sGnRH
20nM 20nM
Analog D 2 μM
e
sGnRH
20 nM 100nM
Analog E
20 nM 100nM
sGnRH
20nM 100 nM
Analog E 2 μM
f
sGnRH
20nM
100nM
Analog F
20 nM 100 nM
sGnRH
20 nM 20 nM
Analog F 2 μM
g
sGnRH
20nM 100 nM
Analog G
20 nM 100nM
sGnRH
20nM 20 nM
Analog G 2 μM
Fraction numbers
Fraction numbers

Fig. 2.1 Continued



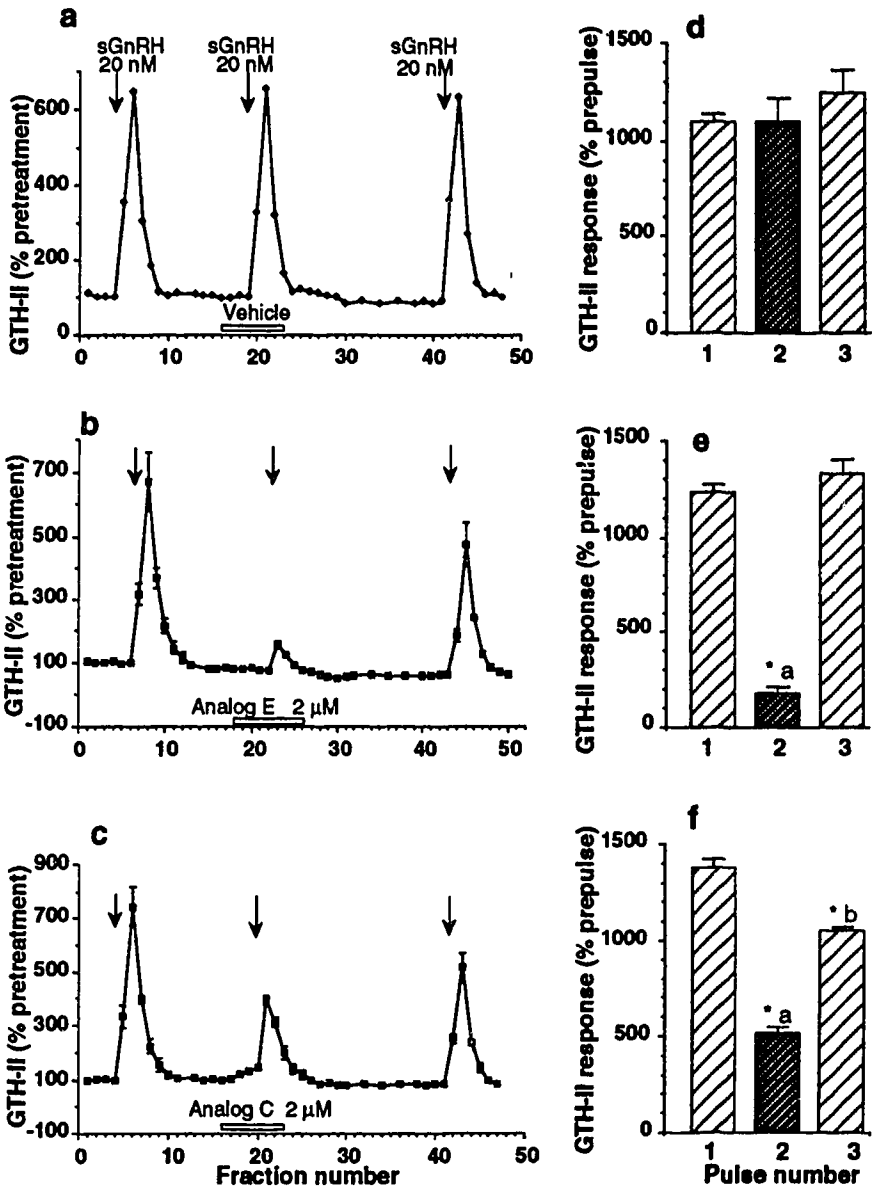


Fig. 2.2 The effects of repeated 2 min pulses (arrows) of 20 nM sGnRH on GTH-II release with the second pulse given in presence (bars) of vehicle (a), 2 μM of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; b), or 2 μM of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-sGnRH (analog C; c). The data were transformed as % pretreatment and expressed as mean ± SEM (n=4). The GTH-II responses were quantified as % prepulse and presented as mean ± SEM for 20 nM sGnRH in presence of vehicle (d), 2 μM of analog E (e), and 2 μM of analog C (f).

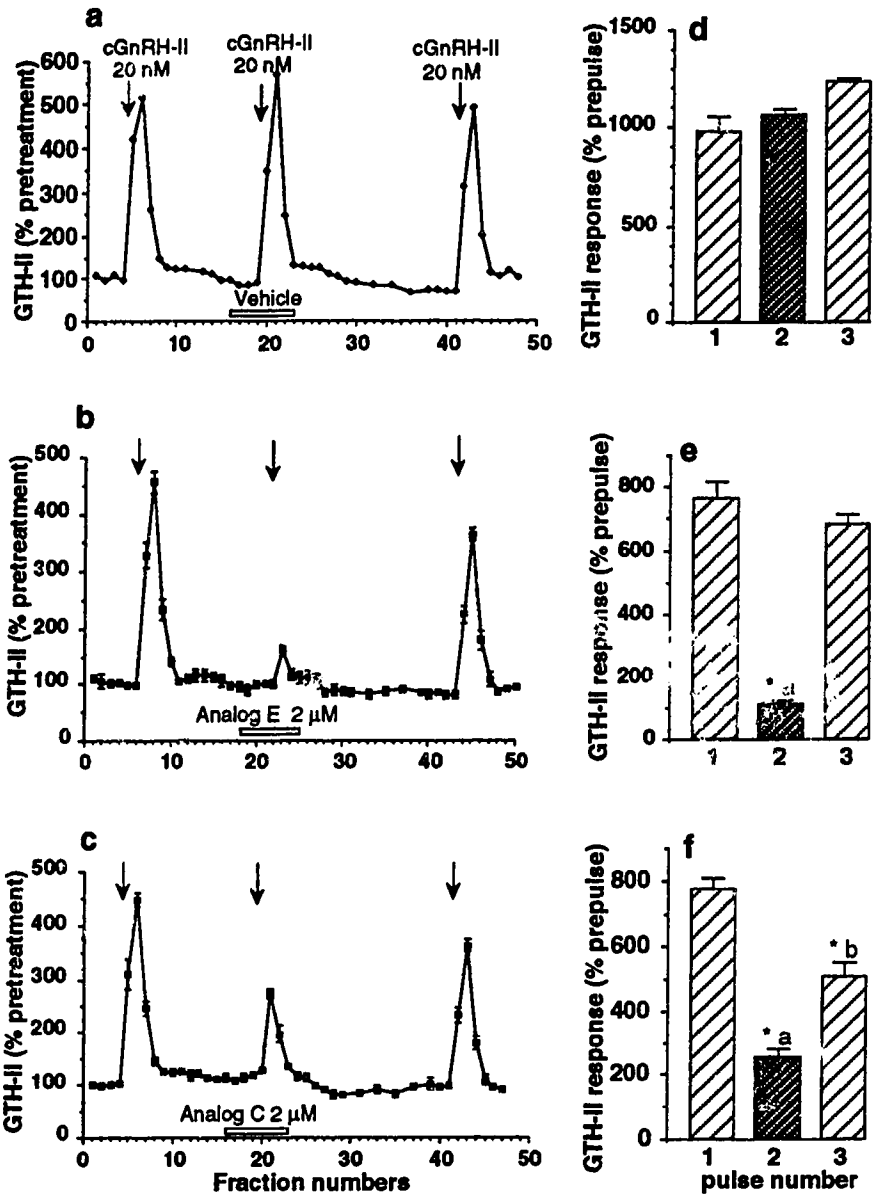


Fig. 2.3 The effects of repeated 2 min pulses (arrows) of 20 nM cGnRH-II on GTH-II release from the pituitary fragments collected from sexually recrudescent goldfish (GSI = 8.9 ± 0.7%), with the second pulse given in presence (bars) of vehicle (a), 2 μM of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; b), or 2 μM of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-sGnRH (analog C; c). The data were transformed as % pretreatment and expressed as mean ± SEM (n=4). The GTH-II responses were quantified as % prepulse and presented as mean ± SEM for 20 nM cGnRH-II in presence of vehicle (d), 2 μM of analog E (e), and 2 μM of analog C (f).

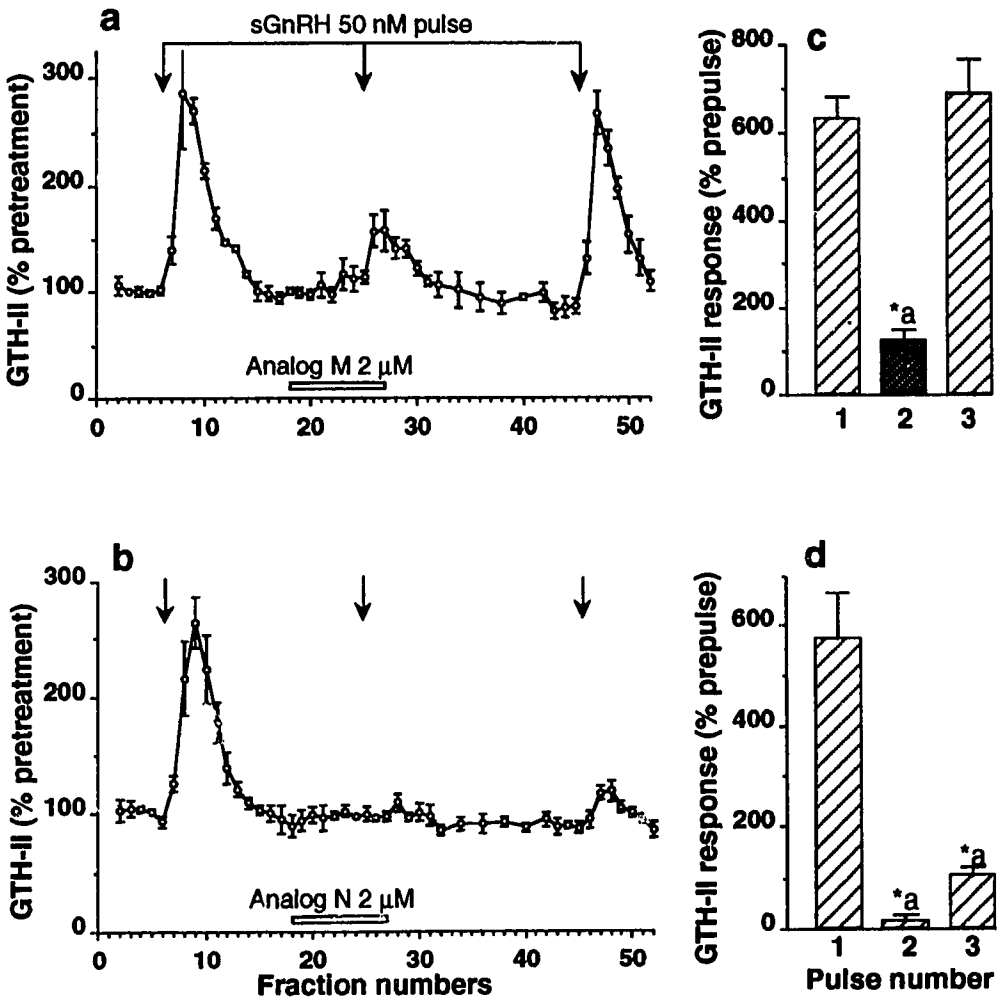


Fig. 2.4 The effects of repeated 2 min pulses (arrows) of 50 nM sGnRH on GTH-II release from the pituitary fragments collected from sexually regressed goldfish (GSI = <2%), with the second pulse given in presence (bars) of 2 µM of [D-p-Glu1, D-Phe2, D-Trp3,6]-mGnRH (analog M; a), or 2 µM of [Ac-D(2)-Nal1, 4Cl-D-Phe2, D-(3)Pal3,6]-cGnRH-II (analog N; b). The data were transformed as % pretreatment and expressed as mean ± SEM (n=4). The GTH-II responses were quantified as % prepulse and presented as mean ± SEM for 50 nM sGnRH in presence of 2 µM of analog M (c), and 2 µM of analog N (d).

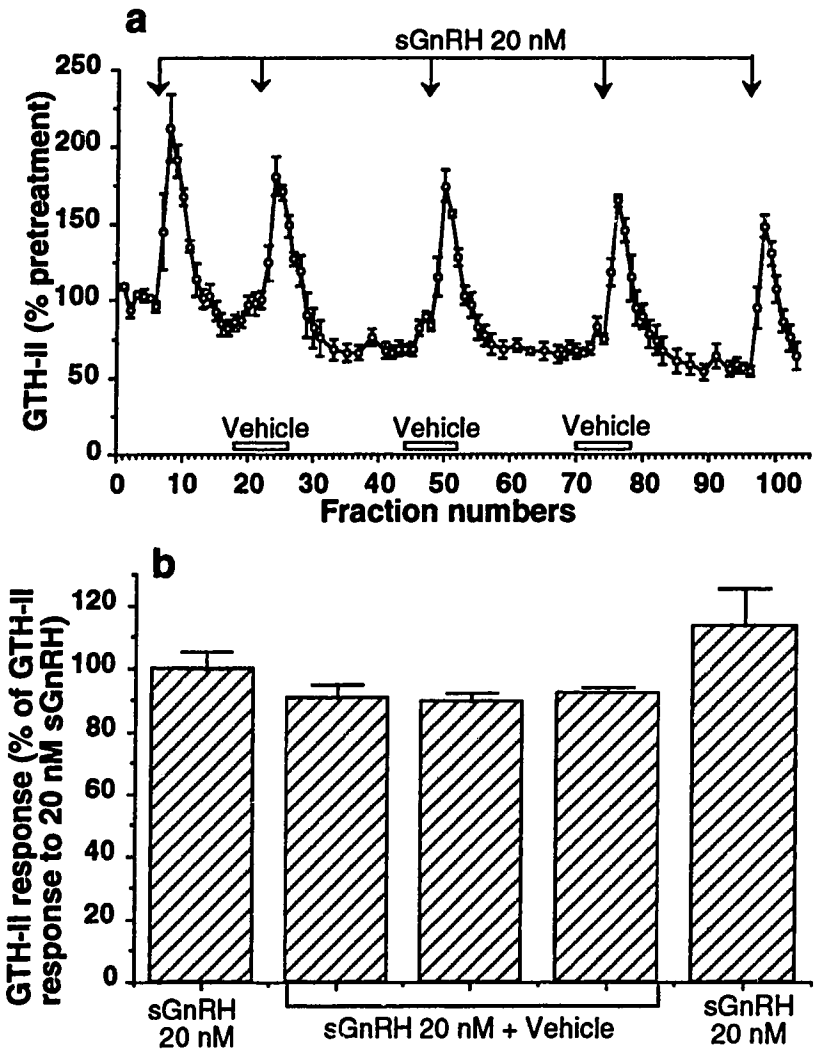


Fig. 2.5 The effects of repeated 2 min pulses (arrows) of 20 nM sGnRH on GTH-II release from the pituitary fragments collected from post-spawning goldfish (GSI = 8.3 ± 1.3%), in presence (bars) of vehicle; data were transformed as % pretreatment (a) and quantified as % prepulse (b).

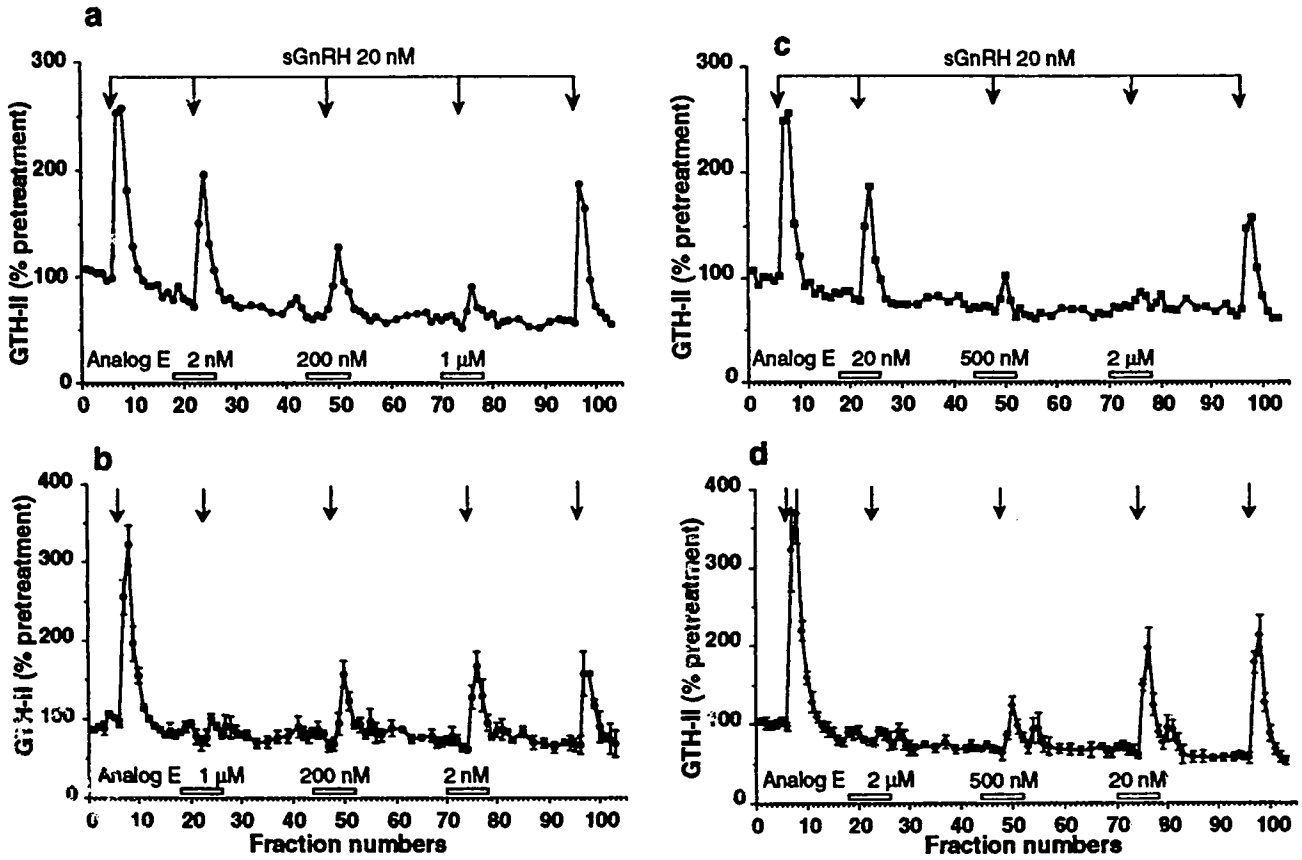


Fig. 2.6 The inhibition of 20 nM sGnRH (2 min; arrows) induced GTH-II release from the pituitary fragments collected from post-spawning goldfish (GSI = 8.3 ± 1.3%), by various doses of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E) given in either increasing concentrations (a,c) or decreasing concentrations (b, ♂). The GTH-II values in ng/ml were transformed as % pretreatment, and presented mean (a, c, n=2) or as mean ± SEM (b, d, n=4).

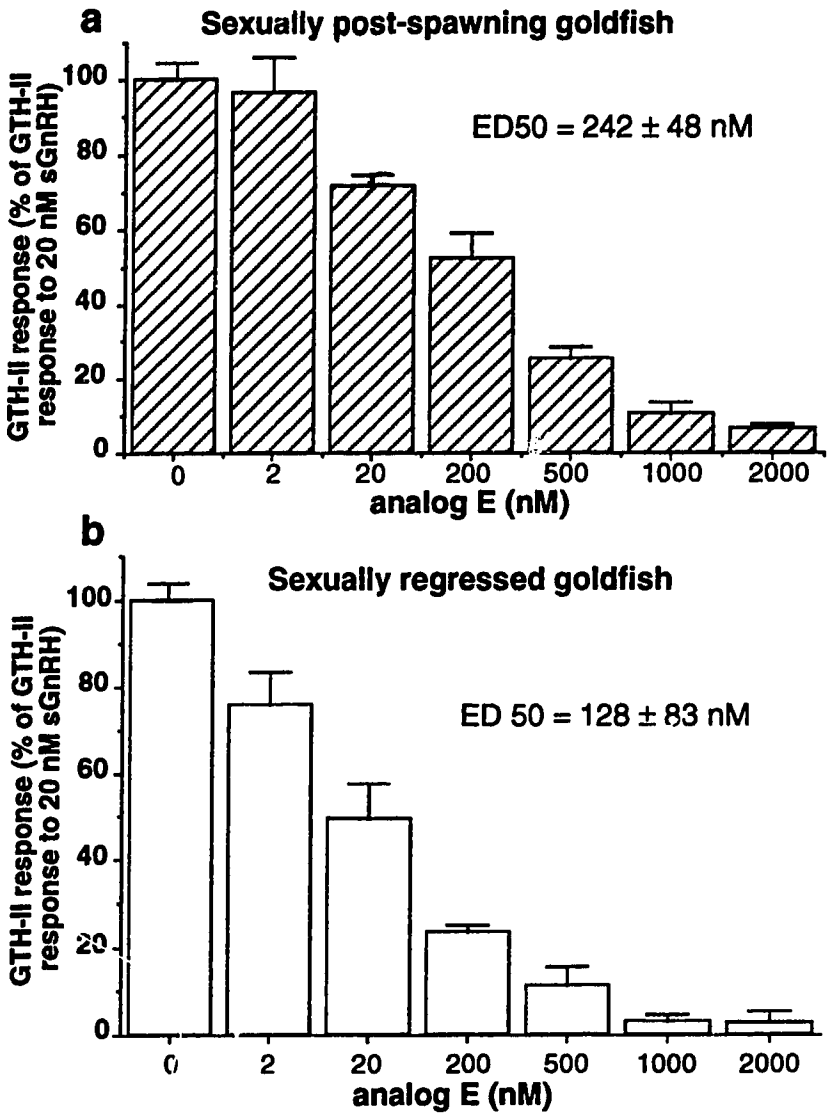


Fig. 2.7a). The GTH-II responses presented in Fig. 2. 6 were quantified as % prepulse, and presented as % of standard GTH-II response to 20 nM sGnRH alone (n = 6). b). The GTH-II responses of pituitary fragments obtained from sexually regressed goldfish (GSI = <1.0%), to 20 nM sGnRH (2 min) in presence of various doses of [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) given in decreasing concentrations were quantified as % prepulse, pooled, and presented as % of standard GTH-II response to 20 nM sGnRH alone (n = 6).

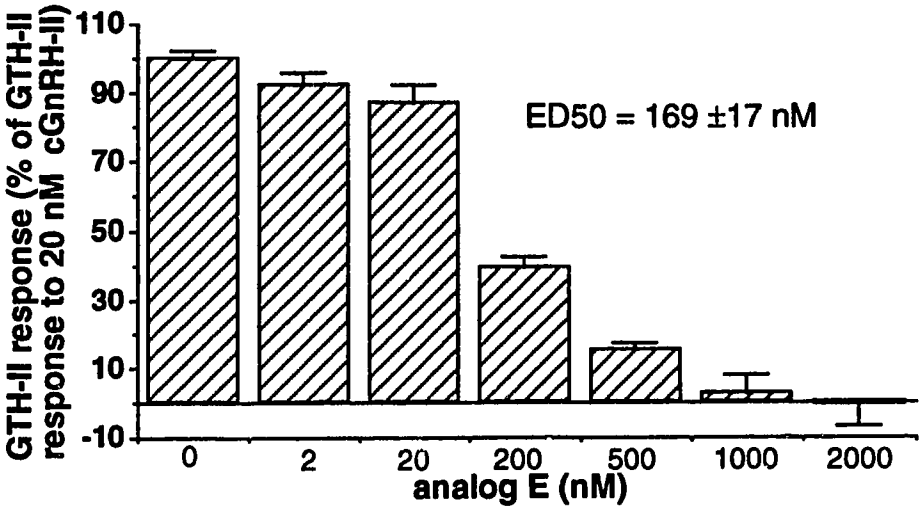


Fig. 2.8 The GTH-II responses of pituitary fragments obtained from sexually regressed goldfish (GSI = <1.0%), to 20 nM cGnRH-II (2 min) in presence of various doses of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E) given in increasing and decreasing concentrations were quantified as % prepulse, pooled, and presented as % of standard GTH-II response to 20 nM cGnRH-II alone (n = 6).

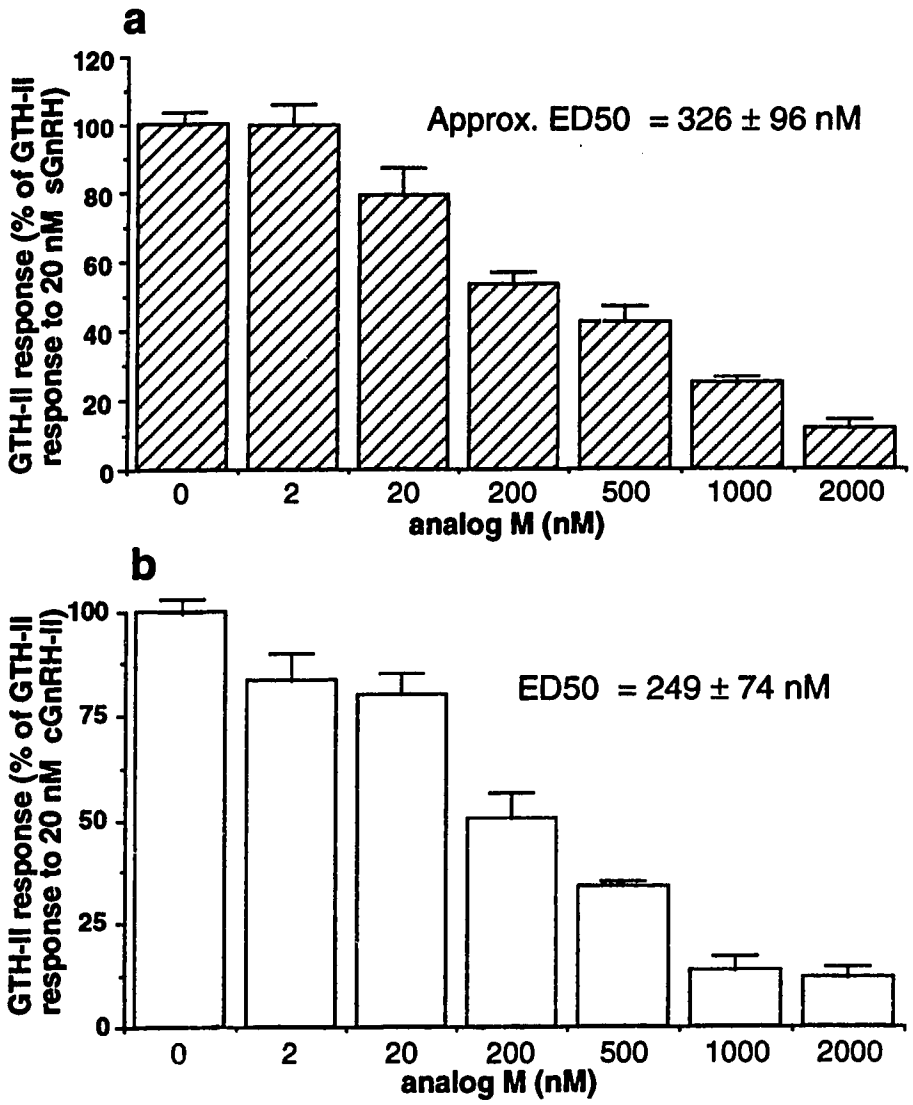


Fig. 2.9 The GTH-II responses of pituitary fragments obtained from sexually regressed goldfish (GSI = <1.0%), to 20 nM sGnRH (a) or to cGnRH-II (b) in the presence of different concentrations of [D-p-Glu1, D-Phe2, D-Trp3,6]-mGnRH (analog M) were quantified as % prepulse, pooled and presented as % of standard GTH-II response to 20 nM sGnRH or cGnRH-II alone (n = 4).

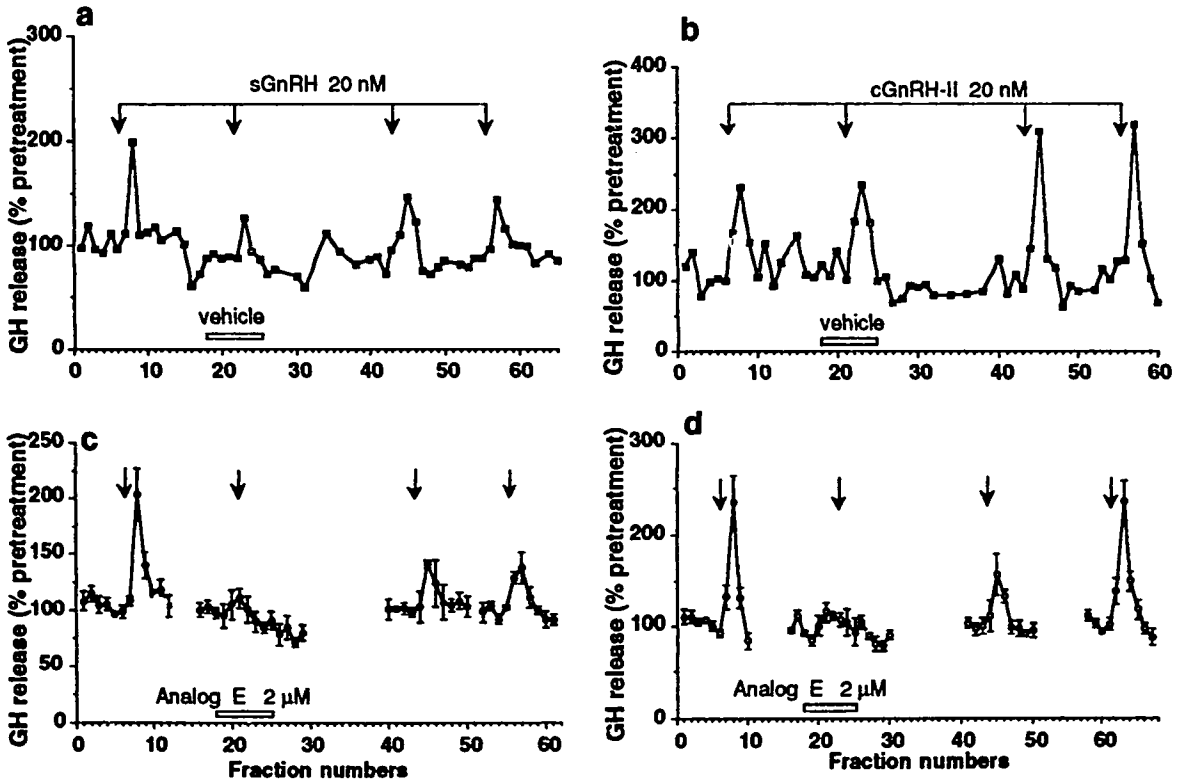


Fig. 2.10 The effects of repeated 2 min pulses (arrows) of 20 nM sGnRH (a, c) or cGnRH-II (b, d) on GH release with the second pulse given in the presence (bars) of vehicle or a 2 µM concentration of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E). The GH values in ng/ml were transformed as % pretreatment, pooled and presented as mean (a, b, n=2) or as mean ± SEM (c, d, n=4).

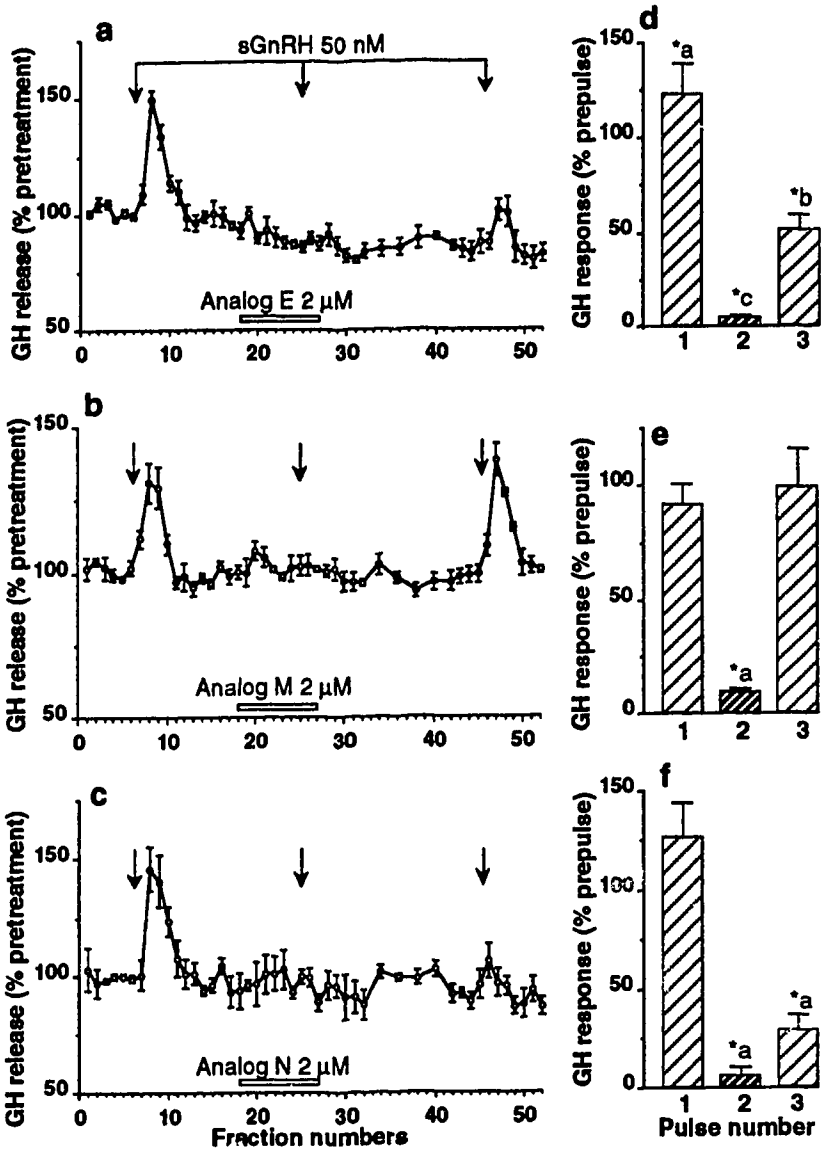


Fig. 2.11 The effects of repeated 2 min pulses (arrows) of 50 nM sGnRH on GH release with the second pulse given in presence (bars) of 2 µM of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; a), or 2 µM of [D-p-Glu1, D-Phe2, D-Trp3,6]-mGnRH (analog M; b), or 2 µM of [Ac-D(2)-Nal1, 4Cl-D-Phe2, D-(3)-Pal3,6]-cGnRH-II (analog N; c). The data were transformed as % pretreatment and expressed as mean ± SEM (n=4). The GH responses were quantified as % prepulse and presented as mean ± SEM for 50 nM sGnRH in presence of 2 µM of analog E (d) 2 µM of analog M (e), and 2 µM of analog N (f).

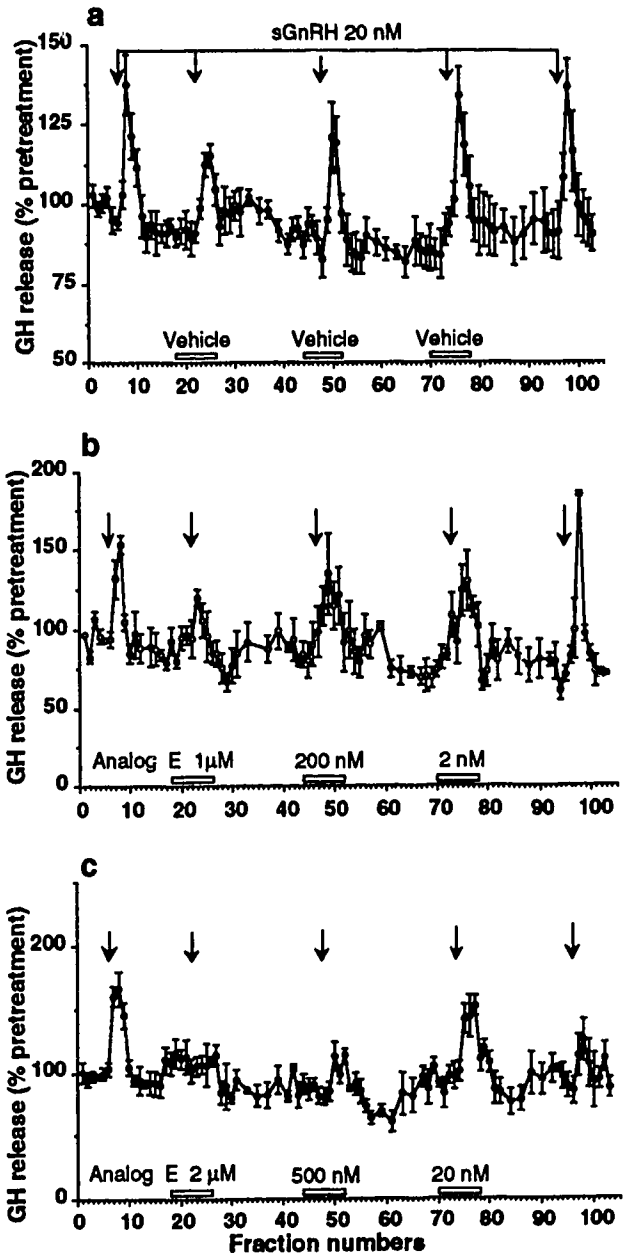


Fig. 2.12 The effects of repeated 2 min pulses (arrows) of 20 nM sGnRH on GH release from the pituitary fragments collected from post-spawning goldfish (GSI = 8.3 ± 1.3%), in the presence (bars) of vehicle (a) or various doses of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; b, c). The GH values in ng/ml were transformed as % pretreatment, and presented as mean ± SEM (n = 4).

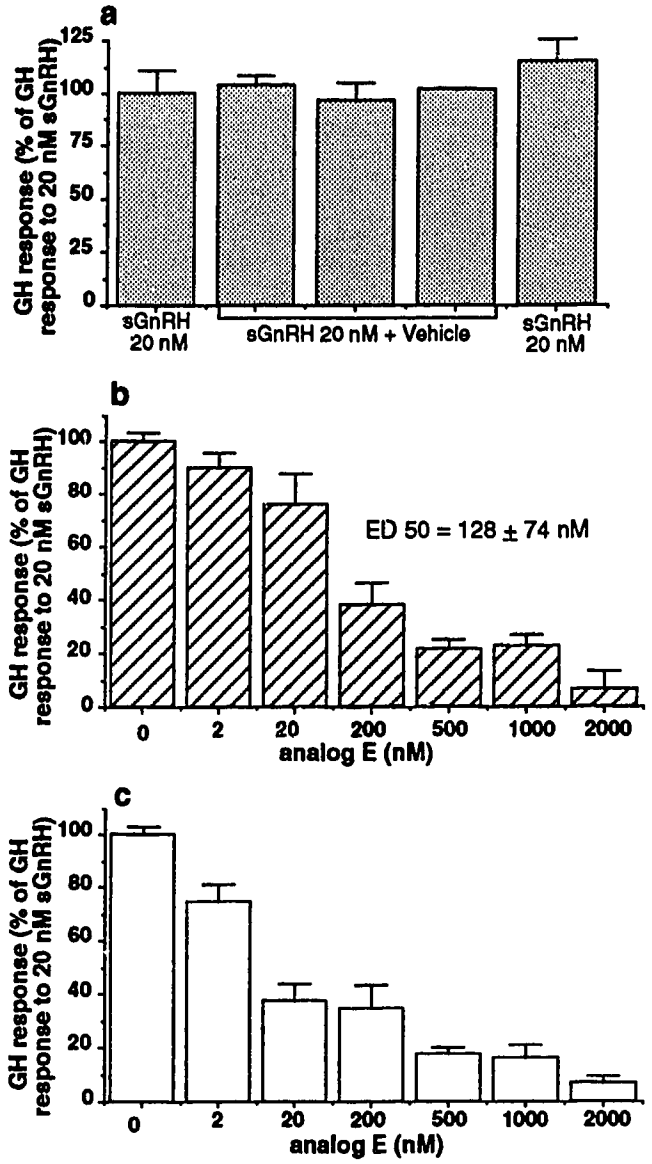


Fig. 2.13 The GH responses (presented in Fig. 2.12) to 20 nM sGnRH (2 min) in presence of vehicle or different concentrations of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E) were quantified as % prepulse, and presented as % of standard GH response to 20 nM sGnRH alone (a and b respectively). In a similar experiment, the effects of various doses of analog E on 20 nM sGnRH induced GH release from pituitary fragments obtained from sexually regressed fish were measured. The GH responses were quantified as % prepulse and expressed as % of GH response to 20 nM sGnRH alone (c).

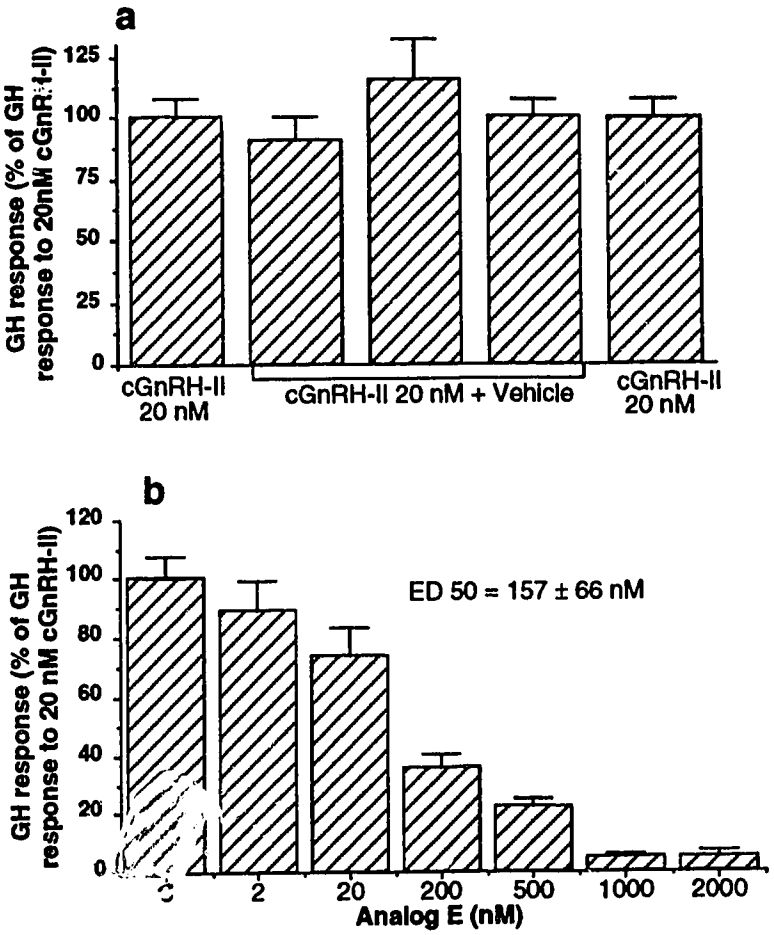


Fig. 2.14 The effects of repeated 2 min pulses of 20 nM cGnRH-II on GH release from the pituitary fragments collected from sexually regressed goldfish (GSI = <1%), in the presence of vehicle (n = 4) or various doses of [Ac-Δ^3-Pro^1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; n = 6) were measured. The GH responses were quantified as % prepulse, and presented as % of standard GH response to 20 nM cGnRH-II alone (a and b respectively).

Table 2.1. A list of GnRH peptides and analogs used in the study:

---

mGnRH: [pGlu^1, His^2, Trp^3, Ser^4, Tyr^5, Gly^6, Leu^7, Arg^8, Pro^9, Gly^{10}-NH_2]

sGnRH: [pGlu^1, His^2, Trp^3, Ser^4, Tyr^5, Gly^6, **Trp**^{7}, **Leu**^{8}, Pro^9, Gly^{10}-NH_2]

cGnRH-II: [pGlu^1, His^2, Trp^3, Ser^4, **His**^{5}, Gly^6, **Trp**^{7}, **Tyr**^{8}, Pro^9, Gly^{10}-NH_2]

lGnRH: [pGlu^1, His^2, **Tyr**^{3}, Ser^4, **Leu**^{5}, **Glu**^{6}, **Trp**^{7}, **Lys**^{8}, Pro^9, Gly^{10}-NH2]

Analog A: [Ac-D-4Cl-Phe^{1,2}, D-Trp^3, D-Lys^6]-mGnRH

Analog B: [Ac-D(2)-Nal^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-sGnRH

Analog C: [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH

Analog D: [Ac-D(2)-Nal^1, 4FD-Phe^2, D-Trp^3, D-Arg^6, NMe Leu^8]-sGnRH

Analog E: [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH

Analog F: [Ac-D(2)-Nal^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH

Analog G: [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Tyr^3, Leu^5, D-Gln^6, Trp^7, Lys^8]-mGnRH

Analog H: [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Tyr^3, Leu^5, D-Arg^6, Trp^7, Lys^8]-mGnRH

Analog I: [Ac-D(2)-Nal^{1,6}, 4Cl-D-Phe^2, D-Tyr^3, Leu^5, Trp^7, Lys^8]-mGnRH

Analog J: [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH

Analog K: [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH

Analog L: [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH

Analog M: [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH

Analog N: [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II

[Ac = Acetyl; h-Arg (Et_2) = Dialkyl-D-homoarginine; Nal(2) = 3-(2-naphthyl) alanine; Pal(3) = 3-(3) pyridyl alanine; 4Cl-D-Phe = para chloro phenylalanine; 4FD-Phe = para fluoro phenylalanine; Δ^3-Pro = $\Delta^{3,4}$-dehydro proline].

Table 2.2 Effects of various GnRH analogs when perifused alone for 30 or 35 min on GTH-II and GH release from goldfish pituitary fragments.

Analog	GTH-II release	GH release
A	+	++
B	+++	+
C	0	+++
D	+	+
E	0	0
F	++	0
G	+	+
H	+	+
I	+	+
J	+++	+++
K	+++	+++
L	+	---
M	0	0
N	0	0
lGnRH	++	++

+ = Stimulation, - = Inhibition, and 0 = No stimulation or inhibition of release; +++ (or ---) = strong; ++ (or --) = moderate; and + (or -) = weak effects

References

Chang, J. P., and Jobin, R. M. (1991). Two native GnRH peptides stimulate gonadotropin release in goldfish by different second messenger components. In "Proceedings, 4th International Symposium on the Reproductive Physiology of Fish", (A. P. Scott, J, P. Sumpter, D. E. Kime, M. Rolfe, Eds.), pp 221-223. FishSymp 91, Sheffield, UK.

Chang, J. P., Cook, H., Freedman, G.L., Wiggs, A. J., Somoza, G. M., De Leeuw, R., and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static. and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

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

Clayton, R. N. (1989). Gonadotropin-releasing hormone: its actions and receptors. J. Endocrinol. 120, 11-19.

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J.P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul. Peptides, 36, 369-378.

Coy, D. H., Horvath, A., Nekola, M. V., Coy, E. J., Erchegyi, J., and Schally, A. V. (1982). Peptide antagonists of LH-RH: large increases in antiovulatory activities produced by basic D-amino acids in the six position. Endocrinology, 110, 1445-1447.

Coy, D. H., Mezo, I., Pedroza, E., Nekola, M. V., Vilchez, J., Piyachaturawat, P., Schally, A. V., Seprodi, J., and Teplan, I. (1979). LHRH antagonists with potent antiovulatory activity. In "Peptides:Structure and Biological Function". (E. Gross, and J. Meinhofer, Eds.), pp 775-780. Pierce Chemical Co., Rockford, IL.

Crim, L. W., Evans, D. M., Coy, D. H., and Schally, A. V. (1981) Control of gonadotropic hormone release in trout: Influence of synthetic LH-RH and LH-RH analogues *in vivo* and *in vitro*. Life Sci. 28, 129-136.

Crim, L. W., Peter, R. E., and Van Der Kraak, G. (1987). The use of LHRH analogs in

aquaculture. In "LHRH and its analogs: contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr., Eds.), pp 489-498. MTP Press Ltd., Boston.

De Lean, A., Munson, P. J., and Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: Application of bioassay radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235, E97-E102

Habibi, H. R. (1991). Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: Effects of native GnRH peptides and a synthetic GnRH antagonist. Biol. Reprod. 44, 275-283

Habibi, H. R., De Leeuw, R., Nahorniak C. S., Goos, H. J. Th., and Peter, R. E. (1989b). Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. Fish Physiol. Biochem. 7, 109-118.

Habibi, H. R., Marchant, T. A., Nahorniak C. S., Van Der Loo, H., Peter, R. E., Rivier, J. E., and Vale, W. W. (1989c). Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius auratus*). Biol. Reprod. 40, 1152-1161.

Habibi, H. R., Nahorniak, C. S., Van Der Loo, H., Peter, R. E., King, J. A , and Miller, R. P. (1989a). Gonadotropin-releasing hormone (GnRH) receptor binding and biological activity for chicken-II GnRH analogues in the goldfish pituitary. 11th International Symposium on Comparative Endocrinology, Malaga, Spain, p 148 (Abstract).

Habibi, H. R., Peter, R. E., Nahorniak C. S., Milton, R. C. L., and Millar, R. P. (1992). Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides, 37, 271-284.

Habibi, H. R., Van Der Loo, H., Marchant, T. A., Nahorniak, C. S., Peter, R. E., King, J. A , Miller, R. P., Rivier, J. E., and Vale, W. W. (1988). Studies on the molecular requirement for gonadotropin-releasing hormone (GnRH) receptor recognition in the goldfish pituitary. 8th International Congress of Endocrinology, Kyoto, Japan, p 127 (abstract).

Horvath, A., Coy, D. H., Nekola, M. V., Coy, E. J., Schally, A.V., and Teplan, I.

(1982) Synthesis and biological activity of LH-RH antagonists modified in position 1. Peptides, 3, 969-973.

Humphries, J., Wasiak, T., Wan, Y. P., Folkers, K., and Bowers, C. Y. (1978). An antiovulatory decapeptide of higher potency which has an L-amino acid (Ac-Pro) in position 1. Biochem. Biophys. Res. Commun. 85, 709-713.

Karten, M. J., and Rivier, J. E. (1986). Gonadotropin-releasing hormone analog design. Structure-function studies towards the development of agonists and antagonists-rationale and prospectives. Endocrine Rev. 7, 44-66.

Lovejoy, D. A., Fisher, 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.

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 hormone in the goldfish. Endocrinology, 124, 2509-2518.

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. USA. 81, 3874-3878.

Nekola, M. V., and Coy, D. H. (1984). LHRH antagonists in females. In "LHRH and its analogs-contraceptive and therapeutic applications" Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 125-136. MTP Press Ltd., Boston.

Nestor, J. J. Jr., Tahilramani, R., Ho, T. L., McRae, G. I., and Vickery, B. H. (1988). Potent, long-acting luteinizing hormone-releasing hormone antagonists containing new synthetic amino acids: N, N'-Dialkyl-D-homoarginines. J. Med. Chem. 31, 65-72.

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

Peng, C., Huang, Y. P., and Peter, R. E. (1990). Neuropeptide Y stimulates growth

hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology, 52, 28-34.

Peter, R. E., Chang, J. P., Nahorniak, C. S., Omeljaniuk, R. J., Sokolowska, M., Shih, S. H., and Billard, R. (1986). Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog. Horm. Res. 42, 513-548.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990a). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In:"Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, and M. H. Stetson, Eds.), pp. 393-398. Wiley-Liss Inc. NY.

Peter, R. E., Lin, H. R., and Van Der Kraak, G. (1988). Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH analogues and dopamine antagonists. Aquaculture, 74, 1-10.

Peter, R. E., Nahorniak, C. S., Chang, J. P., and Crim, L. W. (1984). Gonadotropin release from the pars distalis of goldfish, *Carassius auratus* transplanted beside the brain or into the brain ventricles; Additional evidence for gonadotropin release-inhibitory factor. Gen. Comp. Endocrinol. 55, 337-346.

Peter, R. E., Nahorniak, C. S., Sokolowska, M., Chang, J. P., Rivier, J. E., Vale, W. W., King, J. A., and Millar, R. P. (1985). Structure-activity relationships of mammalian, chicken and salmon gonadotropin releasing hormones *in vivo* in goldfish. Gen. Comp. Endocrinol. 58, 231-242.

Peter, R. E., Trudeau, V. L., and Sloley, B. D. (1991). Brain regulation of reproduction in teleosts. Bull. Inst. Zool. Academia Sinica, Monograph, 16, 89-118.

Peter, R.E., Yu, K. L., Marchant, T. A., and Rosenblum, P. M. (1990b). Direct neural regulation of the teleost adenohypophysis. J. Expt. Zool. Supp. 4, 84-89.

Rand-Weaver, M., and Kawauchi, H. (1992). A rapid procedure for the isolation of bioactive growth hormone. Gen. Comp. Endocrinol. 85, 341-345.

Rees, R. W., Foell, T. J., Chia, S. -Y., and Grant, N. (1974). Synthesis and biological activities of analogs of LHRH modified in position 2. J. Med. Chem. 17, 1016-1019.

Rivier, J. E., and Vale, W. W. (1978). [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-LRF. A potent luteinizing hormone releasing factor antagonist *in vitro* and inhibitor of ovulation in the rat. Life Sci. 23, 869-876.

Rivier, J., Rivier, C., Perrin, M., Porter, J., and Vale, W. W. (1984). LHRH analogs as

anti-ovulatory agents. In "LHRH and its analogs: contraceptive and therapeutic applications". Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 11-22. MTP Press Ltd., Boston.

Vale, W., Grant, G., Rivier, J., Monahan, M., Amoss, M., Blackwell, R., Burgus, R., and Guillemin, R. (1972). Synthetic polypeptide antagonists of the hypothalamic luteinizing hormone-releasing factor. Science, 176, 933-934.

Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

Vickery, B. H. (1987). Reproductive physiology and general pharmacology of LHRH antagonists. In "LHRH and its analogs: contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr., Eds.), pp 201-215. MTP Press Ltd., Boston.

Yardley, J. P., Foell, T. J., Beattie, C. W., and Grant, N. (1975) Antagonism of luteinizing hormone release and of ovulation by an analog of the luteinizing hormone-releasing hormone. J. Med. Chem. 18, 1244-1247.

Yu, K. L., Peng, C., and Peter, R. E. (1991) Changes in brain level of gonadotropin-releasing hormone and serum gonadotropin and growth hormone in goldfish during spawning. Can. J. Zool. 69, 182-188

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 Three

Functional evidence regarding receptor subtypes mediating the actions of native gonadotropin-releasing hormones (GnRH) in goldfish, *Carassius auratus*[1]

Introduction

Gonadotropin-releasing hormone (GnRH) is involved in the regulation of gonadotropin (GTH) secretion in vertebrates. In most of the vertebrates, with the possible exception of higher mammals (King et al., 1988), existence of multiple forms of GnRH in a given species is common (for review Sherwood and Lovejoy, 1989; King and Millar, 1992). In goldfish, chromatographic and immunological studies have indicated the presence of [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II, cGnRH-II) (Yu et al., 1988). In the study by Yu et al. (1988), and also in the immunocytochemical study by Kim et al. (1993), both sGnRH and cGnRH-II were detected in different brain areas and in the pituitary, suggesting involvement of both peptides in the regulation of pituitary hormone secretion (review Peter et al., 1990). Indeed, both sGnRH and cGnRH-II have been shown to stimulate GTH-II and growth hormone (GH) release in goldfish (Marchant et al., 1989; Chang et al., 1990; Habibi et al., 1992). In contrast, although cGnRH-I and cGnRH-II stimulate LH and FSH release in chicken, only cGnRH-I is found in the median eminence of the hypothalamus (Katz et al., 1990). Similarly only cGnRH-I is released from the chicken median eminence, suggesting that cGnRH-I is the regulator of gonadotropin release in chicken (Katz et al., 1990). Although both sGnRH and cGnRH-II are found in the brain of rainbow trout (Okuzawa et al., 1990) and masu salmon (Amano et al., 1992), only sGnRH is present in the pituitary gland, implying the involvement of only sGnRH in regulation of GTH release in these two

[1]A version of this chapter has been published: CK Murthy, RE Peter (1994). Gen. Comp. Endocrinol., 94, 78-91.

fish species.

In receptor studies on the goldfish pituitary, two classes of binding sites have been described, high affinity/low capacity and low affinity/high capacity binding sites (Habibi et al., 1987, 1990). Both sGnRH and cGnRH-II displaced bound ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH (sGnRH-A) from the crude goldfish pituitary membrane preparations (Habibi et al., 1987, 1988; Habibi and Peter, 1991) suggesting that both native forms of GnRH act through the same population of receptors. In further support of this hypothesis, in electron microscope studies Cook et al. (1991) found that sGnRH and cGnRH-II both displaced avidin gold-labelled biotinylated [D-Lys^{6}, Pro^{9}-NHEt]-sGnRH from the surfaces of immunohistochemically identified gonadotrophs and somatotrophs. Chang and co-workers showed that the two native GnRHs in goldfish activate somewhat different second messenger components in stimulating GTH-II release (Chang et al., 1991a, 1991b). They proposed a novel hypothesis that two closely related peptides can compete for the same receptors on gonadotrophs and stimulate hormone release via activation of different post-receptor messenger systems (Chang and Jobin, 1991). In contrast to these results, continuous exposure of pituitary fragments to sGnRH or cGnRH-II (10^{-7} M) caused desensitization, and under such conditions cGnRH-II treated fragments had significantly lower receptor content than those treated with sGnRH (Habibi, 1991a). In a further study, Habibi (1991b) observed that cGnRH-II exerted a greater degree of desensitization than sGnRH when administered either continuously or as 2-min pulses every 20 min, whereas sGnRH exerted a greater degree of desensitization when given as 2-min pulses every 60 min. These results are not fully compatible with the view that sGnRH and cGnRH-II interact with the same receptor population on gonadotrophs (Habibi and Peter, 1991). In a photoaffinity labelling study in goldfish, a protein band of Mr 51,000 exhibiting a high affinity for GnRH was found to be present as two closely associated bands on the gel (Habibi et al., 1990), and these two bands of protein were suggested to represent GnRH receptors on gonadotrophs and somatotrophs.

In a previous study with goldfish pituitary fragments, [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-sGnRH (analog C) stimulated GH, but not GTH-II release (Murthy et al., 1993, see Chapter 2), suggesting differences in the properties of GnRH receptors on somatotrophs and gonadotrophs. In a similar study, [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3}, D-Arg^{6}]-mGnRH (analog F) stimulated GTH-II, but not GH release, and [Ac-D(2)-Nal^{1},

4Cl-D-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L) weakly stimulated GTH-II release, but strongly suppressed GH release. However, the possible duality of GnRH receptors in goldfish pituitary has not been studied in detail. Although the desensitization of GTH-II response to native GnRH peptides in goldfish is well documented (Habibi, 1991a, b), possible desensitization of the GH response to GnRH has not been studied.

The objectives of the present study were 1) to determine whether there are differences in the GH and GTH-II responses to sGnRH and cGnRH-II (i) in the presence of sGnRH and cGnRH-II based antagonists, (ii) under desensitizing conditions with prolonged sGnRH or cGnRH-II treatment and (iii) in the presence of sGnRH and cGnRH-II. 2) To confirm the differential actions of selected 'putative' GnRH antagonists on GTH-II and GH release.

Materials and Methods

Experimental animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries, Stoutland, MO. The fish were maintained in flow-through aquaria (1800 liters) at 17 ± 1°C under a simulated natural photoperiod of Edmonton for at least 2-3 weeks prior to experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish of both sexes, with body weight ranging from 20-35 g were used in the present study.

Reagents and test substances Salmon GnRH and chicken cGnRH-II (Peninsula Laboratories Inc., Belmont, CA) were dissolved in 0.1 M acetic acid and stored at -25°C as aliquots of 50 μM. The aliquots were diluted with perifusion medium to required concentrations immediately prior to use in experiments. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C of Chapter 2), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH (analog F of Chapter 2), and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N of Chapter 2) were gifts of Dr. Jean E. Rivier, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA. All analogs were dissolved in a primary solvent containing propylene glycol and physiological saline at a ratio of 60:40, and subsequently diluted to required concentrations with perifusion medium; the final propylene glycol concentration was less than 0.5%.

Perifusion of the pituitary fragments and cells *In vitro* experiments were conducted using a validated pituitary fragments perifusion system (Marchant et al., 1989). Briefly the pituitary glands, collected from goldfish, were chopped into fragments (0.2 $mm^{3)}$ using a McIlwain tissue chopper. The fragments were washed with medium 199 and placed between two layers of Cytodex beads (Sigma Chemical Co., St. Louis, MO) in the perifusion columns (3 pituitary equivalents per column) with a continuous flow of incubation medium (Medium 199) at 17°C. The fragments were perifused overnight (at least 15 h) at a flow rate of 5 ml/h. The following morning, the incubation was continued using Hank's balanced salt solution supplemented with 25 mM HEPES and 0.1% BSA at a flow rate of 15 ml/h for 2 hr before starting the experiment. Fractions of perifusate were collected for 5 or 10 min intervals, frozen and stored at -25°C for hormonal assay.

The enzymatically dispersed pituitary cells (for procedure see Chang et al., 1990) were incubated with preswollen cytodex beads (Cytodex I, Sigma) at 28°C under 5% CO_2 and saturated humidity. After overnight incubation, cytodex beads with pituitary cells attached were loaded into perifusion columns and the experiment was conducted as described above.

Radioimmunoassay GTH-II levels in perifusate were measured by using a validated carp GTH-II specific radioimmunoassay (Peter et al., 1984; Van Der Kraak et al., 1992). The GH levels were measured by using a radioimmunoassay described earlier (Murthy et al., 1993) using goldfish GH as standard and labeled ligand.

Data analysis For presentation the GTH-II values were expressed as the percentage of pretreatment mean (3 fractions prior to first pulse) for each column and pooled from 4-6 columns. The quantification of hormone response was as described by Peng et al. (1990). Briefly, the average hormone level of 3 fractions immediately preceding each pulse (prepulse mean) was defined as the basal hormone level. The hormone values following a GnRH pulse were expressed as a percentage of the prepulse mean. Post-pulse fractions with hormone contents greater than one standard error of the mean (SEM) above the prepulse mean were considered to be a part of the response. The response (expressed as % prepulse) above the pretreatment mean from the fractions, considered as part of the response, were added to get the net response in a particular column.

Significant differences between treatments and controls were analysed by ANOVA followed by Fisher's LSD test or Student's t-test ($p < 0.05$).

Results

GTH-II response: The efficacy of [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C) and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) to inhibit the action of the two native peptides on gonadotrophs was tested. Analog C at a dose of 50 nM (ratio of 1:1 of agonist : antagonist), 300 nM (1:6 ratio) and 1000 nM (1: 20 ratio) suppressed both 50 nM sGnRH and 50 nM cGnRH-II stimulated GTH-II release in a dose dependent manner (Fig. 3.1a, b). The results from two experiments (n=5-8 columns) were pooled and compared. The inhibition of sGnRH and cGnRH-II action was not significantly different at a given dose of analog C, when the GTH-II response was expressed either as % of standard response (GTH-II response to 50 nM sGnRH or cGnRH-II in the absence of analog C; not shown) or as % of GTH-II response to 50 nM sGnRH (Fig. 3.1c). [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) at a dose of 50 nM, 300 nM, and 1000 nM, suppressed both 50 nM sGnRH and 50 nM cGnRH-II stimulated GTH-II release (Fig. 3.2a, b). The inhibition by analog N was not as clearly dose-dependent as for analog C (compare Fig. 3.1c to Fig. 3.2c). However, at a given dose analog N equally suppressed GTH-II release induced by both sGnRH and cGnRH-II (Fig. 3.2c).

In the next series of experiments, the ability of sGnRH to stimulate GTH-II release when given as a pulse during the continuous treatment of cGnRH-II, and vice versa, was tested. As evident from Fig. 3.3a, 100 nM sGnRH and cGnRH-II stimulated GTH-II release from pituitary fragments either in the presence or absence of vehicle. However, under continuous treatment of 100 nM sGnRH (Fig. 3.3b), a 2 min pulse of 100 nM sGnRH or cGnRH-II did not cause any further increase in GTH-II release. At 60 min after termination of continuous sGnRH exposure, the GTH-II responses to both sGnRH and cGnRH-II were significantly lower compared to the initial pulse. Similarly, during continuous treatment of cGnRH-II (Fig. 3.3c), a 2 min pulse of cGnRH-II or sGnRH did not further stimulate GTH-II release; at 60 min after cGnRH-II exposures, the GTH-II responses to the two native peptides were again significantly reduced.

The effects of desensitization caused by prolonged exposure of pituitary fragments to sGnRH or cGnRH-II on the response to subsequent pulses of either of the two peptides was further examined. In control columns the magnitude of sGnRH and cGnRH-II induced GTH-II release from pituitary fragments before and after 90 min vehicle exposure was not significantly different (Fig. 3.4a, d). At 60 min after continuous exposure to 100 nM sGnRH for 90 min, there was a lower GTH-II response to both sGnRH (Fig. 3.4b, e) and cGnRH-II (Fig. 3.4c, f). There were no significant differences in the GTH-II responses to the two native peptides after desensitization with sGnRH (response to sGnRH was 44 ± 3% of initial sGnRH pulse and the response to cGnRH-II was 47 ± 5% of initial cGnRH-II pulse; Fig. 3.4e, f). Under similar conditions, pituitary fragments desensitized with continuous exposure to 100 nM of cGnRH-II also had lower responses to a subsequent pulse of the two native peptides (Fig. 3.5a, b); the desensitization process reduced the GTH-II responses to sGnRH and cGnRH-II equally (the GTH-II responses to sGnRH and cGnRH-II were 57 ± 3% and 49 ± 9%, respectively, of their initial pulses; Fig. 3.5c, d).

GH response: The GH responses to repeated pulses of 50 nM sGnRH in the absence and presence of various doses of analog C and N were measured. The initial and final pulses (2 min) of 50 nM sGnRH stimulated GH release (Fig. 3.6a, b). Interestingly, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C) itself stimulated GH release in a dose dependent manner with the highest GH stimulation at 1 μM. In continued presence of analog C, GH response to a pulse of 50 nM sGnRH was suppressed in a dose-dependent manner. At 1 μM dose, analog C completely inhibited additional GH release response to sGnRH (Fig. 3.6a, b). [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) at a dose of 50 nM, 300 nM, and 1000 nM, suppressed both 50 nM sGnRH and 50 nM cGnRH-II stimulated GH release (Fig. 3.7a, b). At a given dose analog N equally suppressed GH release induced by both sGnRH and cGnRH-II, without showing any preferential inhibition (Fig. 3.7c).

Similar to GTH-II responses presented in Fig. 3.3a, 100 nM sGnRH and cGnRH-II stimulated GH release either in the presence or absence of vehicle (Fig. 3.8a). Under continuous treatment of 100 nM sGnRH a 2 min pulse of 100 nM cGnRH-II caused a small, but insignificant (either by comparison of post-pulse fractions to prepulse fractions,

or by comparing the net GH response in terms of total area under curve) increase in GH release (Fig. 3.8b). At 60 min after termination of continuous sGnRH exposure, the GH responses to both sGnRH and cGnRH-II were significantly lower compared to the initial pulses. Similarly, during continuous treatment of cGnRH-II (Fig. 3.8c), 2 min pulse of sGnRH caused a small, but insignificant increase in GH release; at 60 min after cGnRH-II exposure, the GH responses to sGnRH and cGnRH-II were again significantly reduced.

The magnitude of sGnRH and cGnRH-II induced GH release responses before and after 90 min vehicle exposure were not significantly different (Fig. 3.9a, d). At 60 min after continuous exposure to 100 nM sGnRH, the GH responses to both sGnRH (Fig. 3.9b, e) and cGnRH-II (Fig. 3.9c, f) were equally reduced (response to sGnRH was 46 ± 3% of initial pulse, Fig. 3.9e, and the response to cGnRH-II was 47 ± 1% of initial pulse, Fig. 3.9f). Under similar conditions, continued exposure of pituitary fragments to 100 nM of cGnRH-II also reduced the GH responses to subsequent pulses of 100 nM sGnRH (42 ± 4% of initial pulse) and 100 nM cGnRH-II (41 ± 2 % of initial pulse; Fig. 3.10) equally.

Differential release responses of GTH-II and GH: The differential release responses of GTH-II and GH to [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C) were further tested. Exposure of pituitary fragments to 2 μM concentration of analog C for 30 min caused no significant change in GTH-II release, but stimulated GH release (Fig. 3.11). Further, in presence of analog C (2 μM) 50 nM sGnRH induced GTH-II and GH release was significantly suppressed compared to responses to initial pulse of 50 nM sGnRH. At 90 min after termination of analog C treatment, GTH-II and GH responses to 50 nM sGnRH were significantly lower than those to the initial sGnRH pulse (Fig. 3.11c, d). To find out the possible site of action of analog C, enzymatically dispersed pituitary cells were used. sGnRH (50 nM) stimulated both GTH-II and GH release from the dispersed pituitary cells (Fig. 3.12a, b). Analog C (2 μM) had no significant effect on GTH-II release, but significantly stimulated GH release, indicating the direct action of analog C at the pituitary cell level.

Additional differences in the GTH-II and GH responses were noticed during prolonged (35 min) treatment of pituitary fragments with [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH (analog F). Analog F at 100 nM and 1000 nM concentrations

significantly stimulated GTH-II release in a dose dependent fashion (Fig. 3.13a, c), with a slight, but significant inhibition of GH release (Fig. 3.13b, c). However, the inhibition of GH release by analog F was not dose-dependent and was significant only at higher doses.

Discussion

In the present study both sGnRH and cGnRH-II increased the release of GTH-II and GH in agreement with earlier observations on the GTH-II and GH releasing ac[illegible] these peptides (Chang et al., 1990; Peter et al.,1990; Habibi 1991a; Habibi e[illegible]. To test whether these two native peptides act through the same population of G[illegible] receptors on gonadotrophs, two GnRH antagonists based on the structure of native GnRH peptides, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C) and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}]-cGnRH-II (analog N) were used to determine whether the actions of sGnRH and cGnRH-II could be differentially inhibited. Analog C, at low, intermediate and high dosages relative to sGnRH and cGnRH-II, inhibited the GTH-II releasing actions of both native peptides with equal efficacy. Analog N also suppressed the GTH-II stimulatory actions of sGnRH and cGnRH-II equally; however, analog N appears to have persistent antagonism resulting in [illegible]ng of a clear dose-related inhibition of the GTH-II responses to sGnRH and cGnRH[illegible] earlier studies, analog C (at 2 μM dose), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-n[illegible]nalog E; at 2 μM dose) and [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH (analog M; a[illegible]oses ranging from [illegible] to 2 μM) suppressed sGnRH and cGnRH-II action on GTH-II release equally (Murthy et al., 1993; see Chapter 2). These results support the idea that the two native GnRH peptides act through the same population of receptors on gonadotrophs.

This hypothesis was further tested using another approach. It was further reasoned that if there is more than one population of GnRH receptors being preferential or selective to each peptide, then in the presence o[illegible] submaximal dose of one peptide, the other should be able to further stimulate GTH-II release. However, in this study when a pulse of cGnRH-II was given during continuous treatment with sGnRH, there was no additional increase in GTH-II release in response to cGnRH-II; similarly sGnRH cau[illegible]no additional GTH-II release in the presence of cGnRH-II. Habibi (1991a) has shown [illegible]ntinuous exposure of pituitary fragments to 100 nM of sGnRH or cGnRH-II for 60 min causes desensitization of receptors to subsequent GnRH treatment, and that at least part of the

desensitization is due to a decline in binding capacity. The influence of desensitization following continued exposure of pituitary fragments to 100 nM sGnRH (or cGnRH-II) for 90 min or 150 min on the response to a subsequent pulse of these two peptides was tested and there was no preferential reduction in the response to any one peptide. It should be noted that the decrease in responsiveness following continued exposure to sGnRH or cGnRH-II is not due to depletion of GTH-II in gonadotrophs; Habibi (1991a) found that under similar experimental conditions the calcium ionophore (A23187) could still induce GTH-II release. Together these results support the hypothesis that sGnRH and cGnRH-II act through a single population of receptors on gonadotrophs. In support of this hypothesis, in static incubation studies using dispersed goldfish pituitary cells the GTH-II response to maximally effective doses of sGnRH and cGnRH-II are not additive (Chang et al., 1993)

The results on GH release in all above discussed experiments were similar to that of GTH-II release, and indicate that sGnRH and cGnRH-II act via the same population of receptors on somatotrophs. In support of this hypothesis, analog N equally suppressed actions of both sGnRH and cGnRH-II on GH release, without showing any preferential inhibition. Interestingly, analog C stimulated GH release while acting as a 'true' antagonist on GTH-II release (see discussions below). A pulse of cGnRH-II given during continuous treatment with sGnRH, caused a small, but not significant additional GH release; similarly sGnRH caused no significant additional GH release in the presence of cGnRH-II. As in the case of GTH-II, continued exposure of pituitary fragments for 90 min and 150 min to 100 nM sGnRH or 100 nM cGnRH-II results in reduction of GH responses to a subsequent pulse of 100 nM sGnRH and cGnRH-II. However, following continuous treatment with sGnRH or cGnRH-II there was no preferential reduction in the response to any one peptide.

Although the results of the present study do not provide evidence for different populations of GnRH receptors on either gonadotrophs or somatotrophs, there is evidence, however, of differences between the GnRH receptors on somatotrophs and GnRH receptors on gonadotrophs. Analog C stimulated GH release, without causing a significant increase in GTH-II release. The differential actions of analog C on GTH-II and GH release were observed both in pituitary fragments and dispersed pituitary cells, indicating a direct

action of analog C at the pituitary cell level. This functional difference in receptors is further substantiated by the results with analog F, which on long term treatment induced a dose dependent GTH-II release, with a slight, but significant inhibition of GH release. In an earlier study analog F also stimulated GTH-II release, without apparent GH stimulation (Murthy et al., 1993; see Chapter 2). [Ac-D(2)-Nal^{1}, 4Cl-D-Phe^{2}, D-(3)Pal^{3,6}, Arg^{5}, D-Ala^{10}]-mGnRH (analog L) (2 μM) showed GTH-II stimulatory versus GH inhibitory activities (Murthy et al., 1993; see Chapter 2). The differential actions of analog L on GTH-II and GH release were dose dependent and could also be observed in the dispersed pituitary cells (see Chapter 4). These results indicate the direct, but differential actions of these GnRH analogs on GTH-II and GH release, suggesting functional differences in the properties of GnRH receptors on gonadotrophs and somatotrophs.

In the *in vitro* pituitary fragment perifusion studies a lower dose of analog E was required to block sGnRH induced GH release compared to GTH-II release; the dosages of analog E to inhibit 20 nM sGnRH induced GTH-II and GH release from pituitary fragments obtained from post-spawning fish by 50 % were 241.9 ± 48.4 nM and 128 ± 74 nM, respectively (Murthy et al., 1993; see Chapter 2). In a recent structure-activity study of GnRH analogs using the goldfish pituitary fragment perifusion system, it was found that analogs substituted with histidine, leucine, methionine or tyrosine at position 8 exhibited significantly lower GTH-II releasing activity compared to that of sGnRH or cGnRH-II (Habibi et al., 1992). In the same study, the efficiency of these position 8 substituted analogs in terms of stimulation of GH release was not significantly different from the two native peptides, suggesting a differential requirement of ligand structure for optimal receptor activation. Among the native peptides, sGnRH has a slightly greater GH but weaker GTH-II release potential than cGnRH-II (Chang et al., 1990; Peter et al., 1990; Habibi et al., 1992). Photoaffinity labelling studies indicated that the high affinity binding protein responsible for biological action, has a Mr of 51, 000 dalton, and appeared as two closely associated bands on the gels (Habibi et al.,1990). These two bands were suggested to represent the high affinity binding sites on gonadotrophs and somatotrophs (Peter et al., 1990). Together these results strengthen the idea that the GnRH receptors on gonadotrophs and somatotrophs are distinct.

Extensive work by Habibi (1991a, b) has shown that prolonged exposure of pituitary fragments to sGnRH or cGnRH-II results in desensitization of the GTH-II response to a

subsequent pulse of these two peptides. At least a part of the reduced response was due to a decrease in binding capacity. This is the first report showing desensitization of the GH response to GnRH treatment. Exposure of pituitary fragments to 100 nM of sGnRH or cGnRH-II for 90 min or 150 min resulted in significant reduction in the GH response to a subsequent pulse of both sGnRH and cGnRH-II. However, the actual mechanism of desensitization of the GH response to GnRH peptides is not clear from these experiments.

In conclusion, the two native peptides sGnRH and cGnRH-II appear to act through the same population of GnRH receptors on gonadotrophs to stimulate GTH-II release. Likewise, sGnRH and cGnRH-II appear to act through the same population of GnRH receptors on somatotrophs to stimulate GH release. However, the GnRH receptors on the somatotrophs appear to be functionally distinct from that on gonadotrophs.

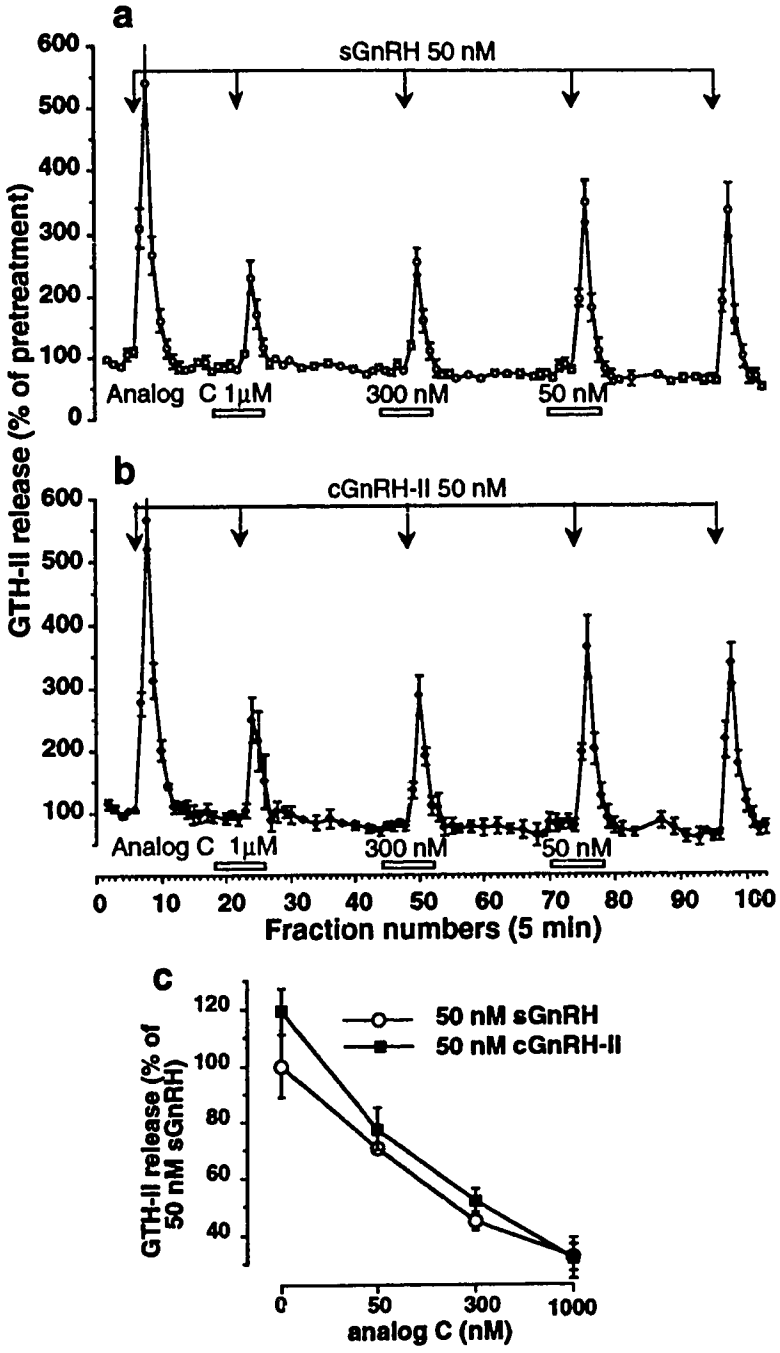


Fig. 3.1 The GTH-II responses to 50 nM sGnRH (a) and to 50 nM cGnRH-II (b) in the absence and presence of different concentrations of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-sGnRH (analog C). The data were transformed as % pretreatment, pooled and expressed as mean ± SEM (n = 4). GTH-II responses were quantified as % prepulse, pooled from two experiments (n = 5-7) and expressed as % of response to 50 nM sGnRH (c).

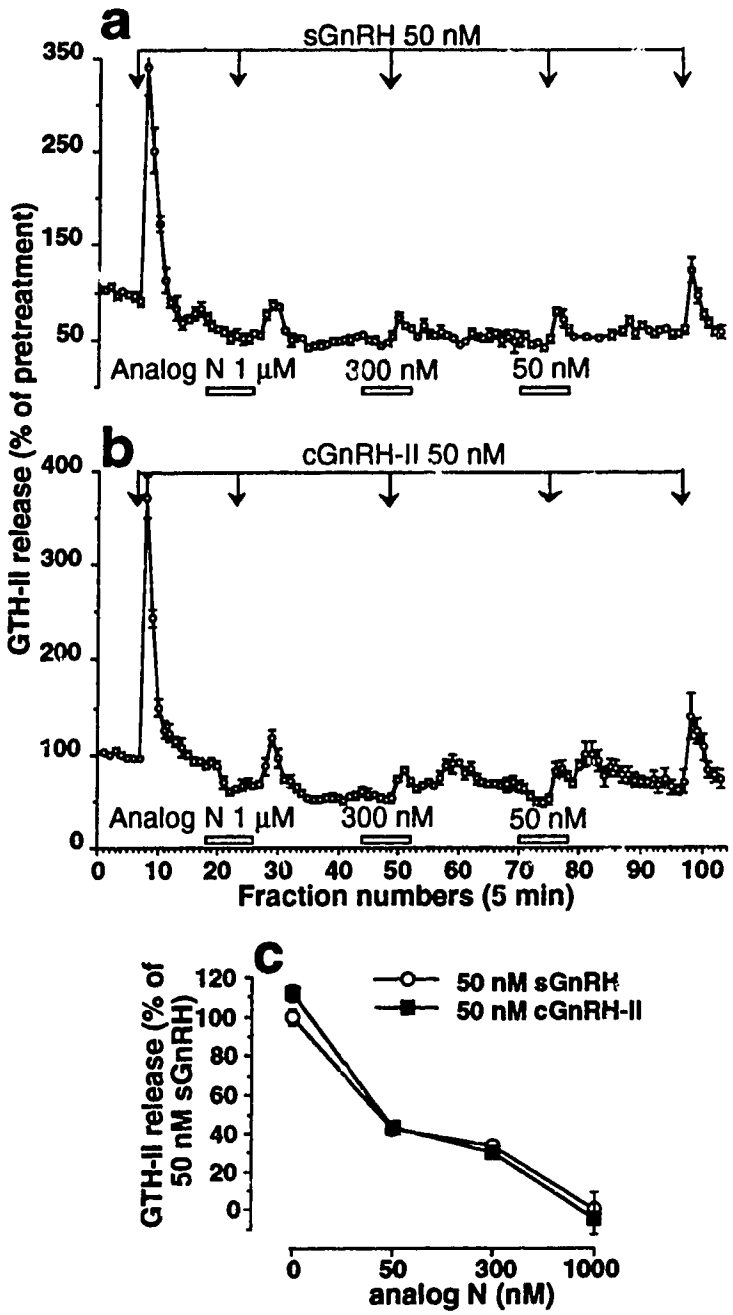


Fig. 3.2 The GTH-II responses to 50 nM sGnRH (a) and to 50 nM cGnRH-II (b) in the absence and presence of different concentrations of [Ac-D(2)Nal1, 4Cl-D-Phe2, D(3)-Pal3,6]-cGnRH-II (analog N). The data were transformed as % pretreatment, pooled and expressed as mean ± SEM (n = 4). GTH-II responses were quantified as % prepulse and expressed as % of response to 50 nM sGnRH (c).

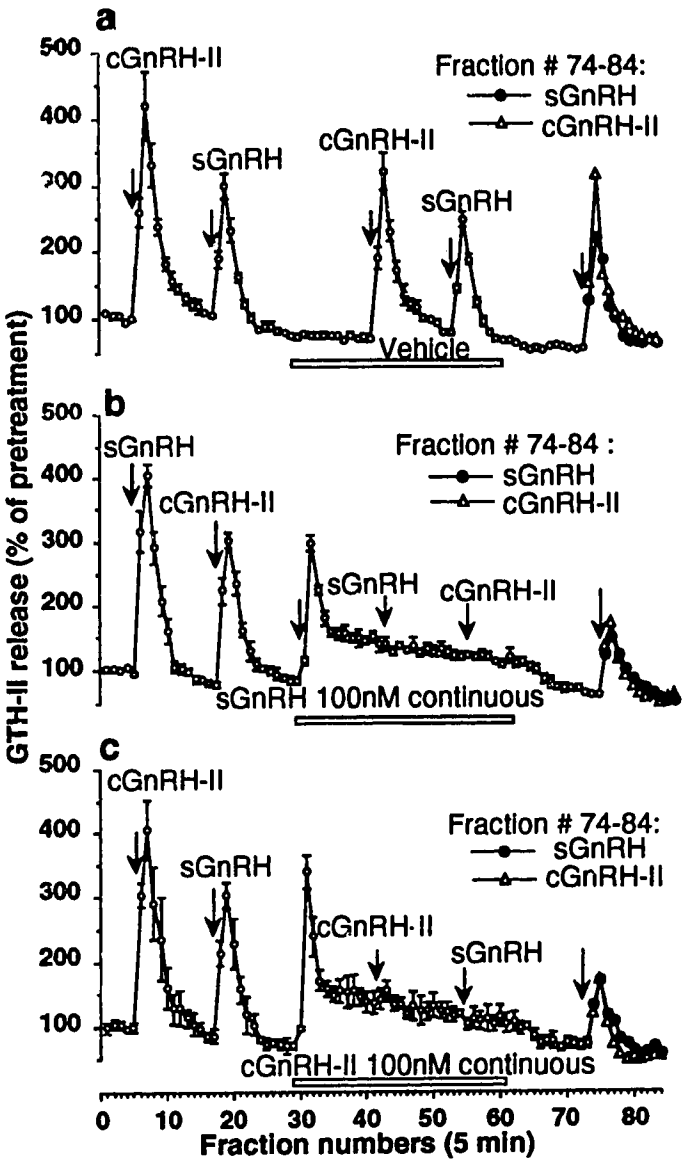


Fig. 3.3 The GTH-II responses of pituitary fragments to 100 nM pulse of sGnRH and cGnRH-II, before, during and after continuous treatment of vehicle (a), 100 nM sGnRH (b) and 100 nM cGnRH-II (c). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4).

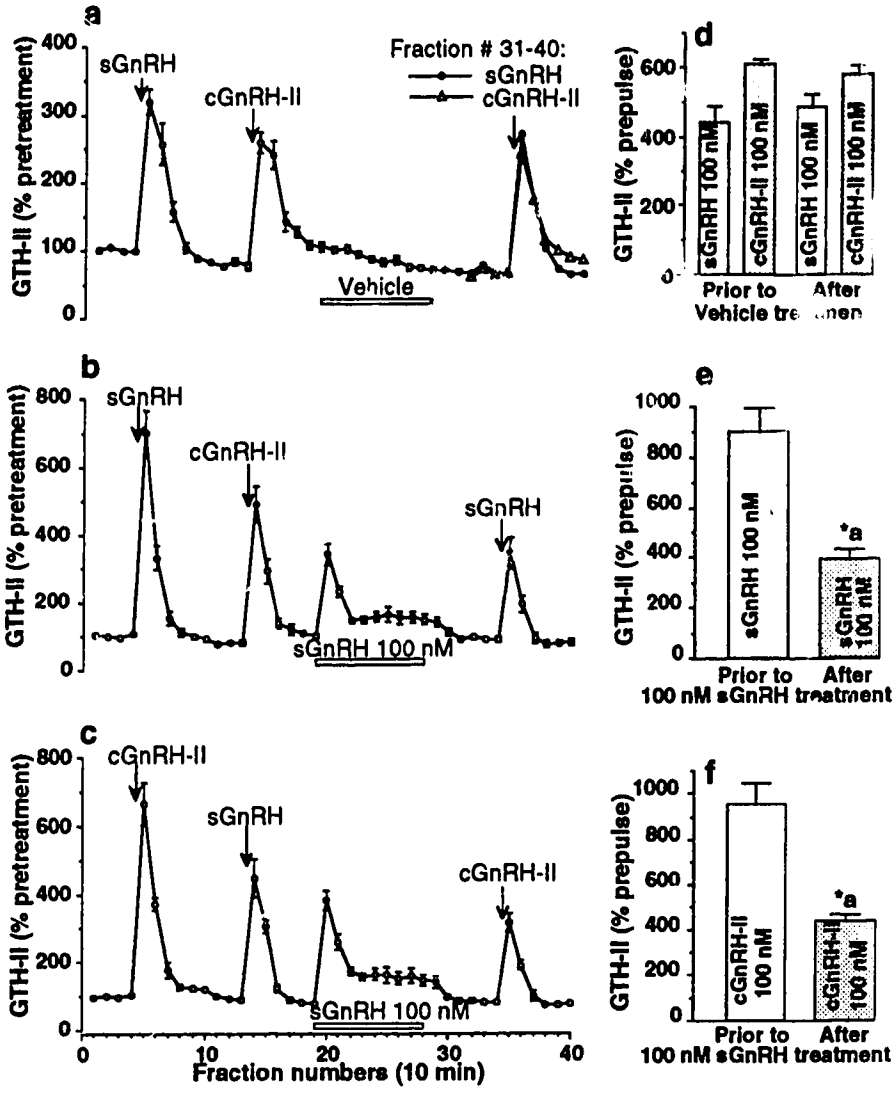


Fig. 3.4 The GTH-II responses of the pituitary fragments to 100 nM sGnRH and cGnRH-II, prior to and after vehicle (a), or 100 nM sGnRH treatment for 90 min (b, c). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). These responses were quantified as % prepulse and presented in Fig. d, e and f, respectively.

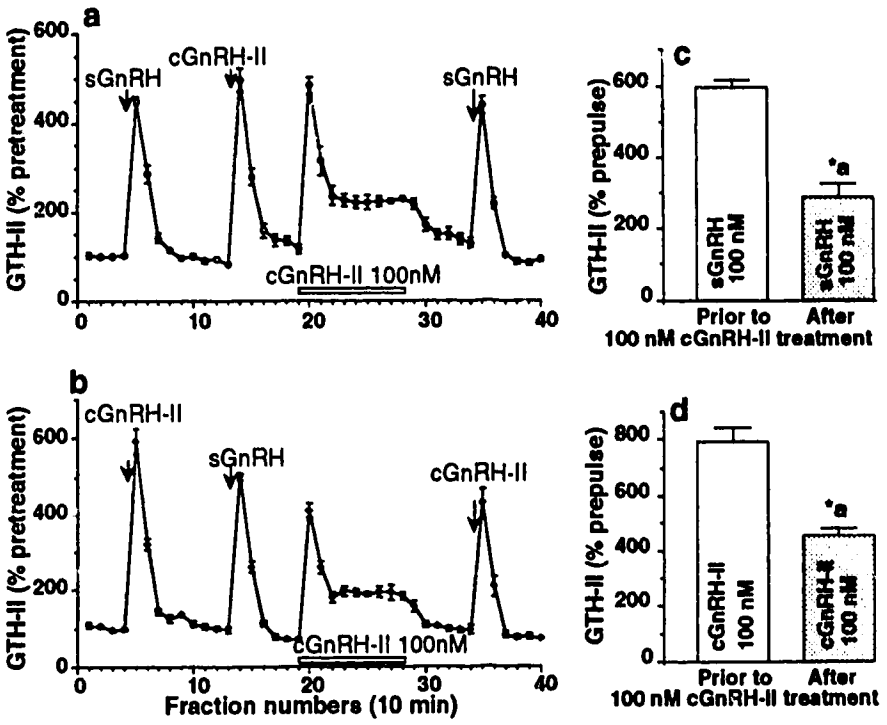


Fig. 3.5 The GTH-II responses of the pituitary fragments to 100 nM sGnRH and cGnRH-II, prior to and after 100 nM cGnRH-II treatment for 90 min (a, b). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). These responses were quantified as % prepulse and presented in (c) and (d), respectively.

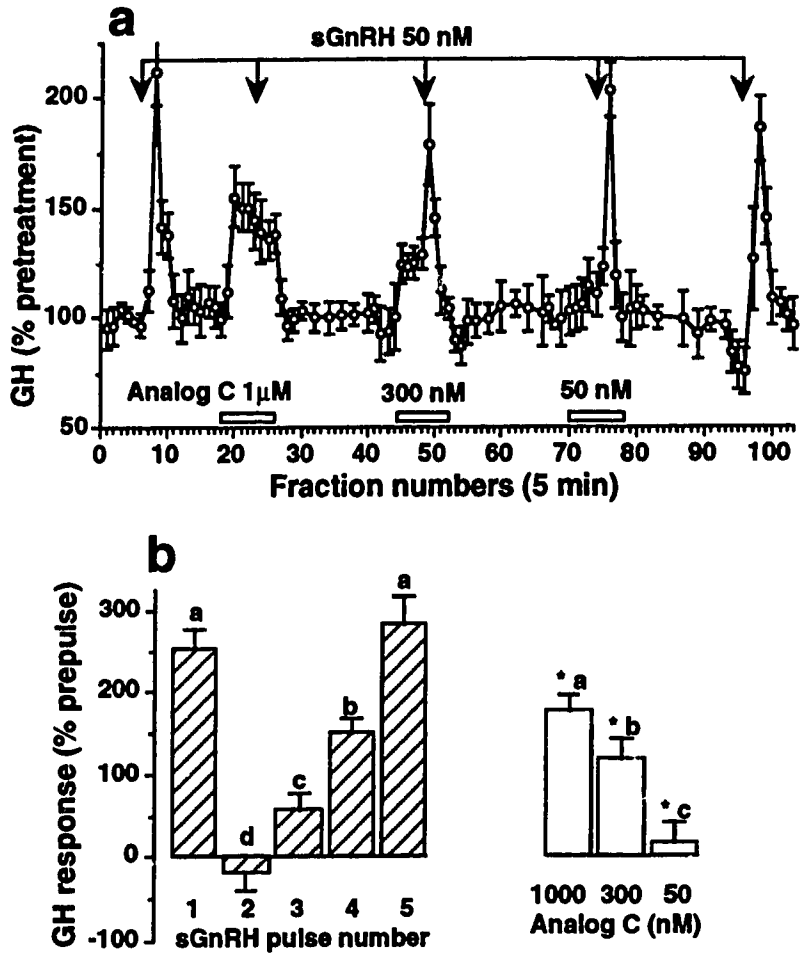


Fig. 3.6 The GH responses of the pituitary fragments to 50 nM sGnRH in the absence and presence of different concentrations of [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C; a). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). GH responses (during 20 min post-pulse) to repeated pulses of 50 nM sGnRH were quantified as % prepulse and presented in the left hand panel of Fig. b as mean ± SEM. Similarly, GH release responses during 20 min treatment of different doses of analog C (before sGnRH pulse) were quantified as % prepulse and presented in the right hand panel of Fig. b.

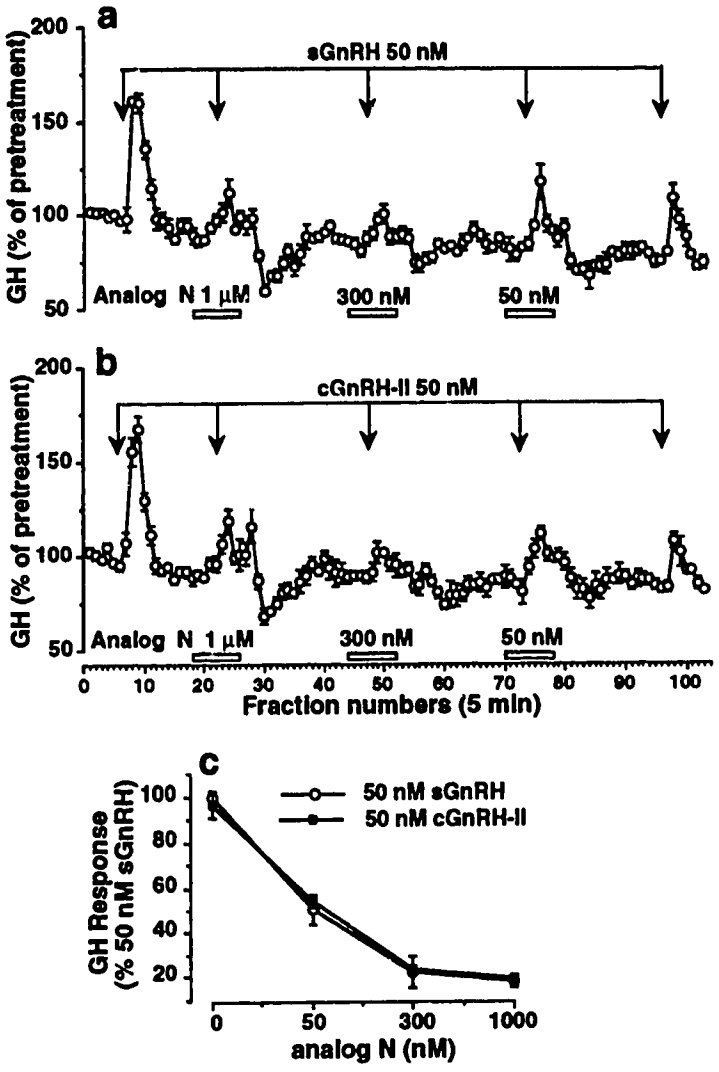


Fig. 3.7 The GH responses of the pituitary fragments to 50 nM sGnRH (a) and to 50 nM cGnRH-II (b) in the absence and presence of different concentrations of [Ac-D(2)Nal1, 4Cl-D-Phe2, D(3)-Pal3,6]-cGnRH-II (analog N). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). GH responses were quantified as % prepulse and expressed as % of response to 50 nM sGnRH (c).

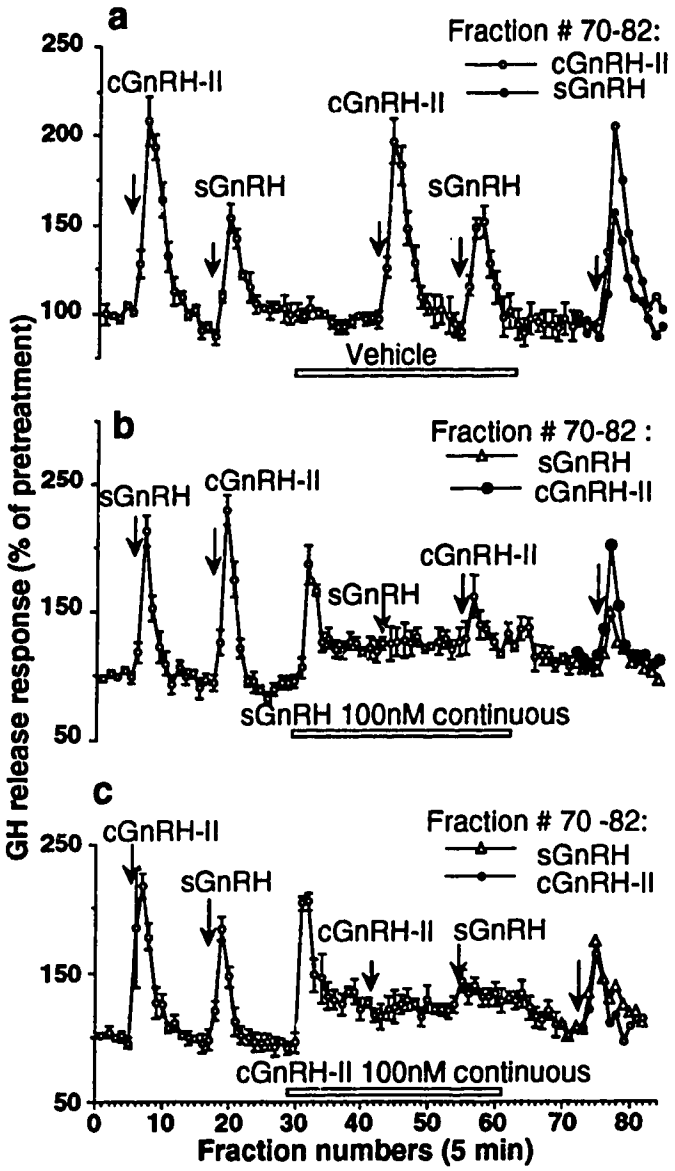


Fig. 3.8 The GH responses of the pituitary fragments to 100 nM pulse of sGnRH and cGnRH-II, before, during and after continuous treatment of vehicle (a), 100 nM sGnRH (b) and 100 nM cGnRH-II (c). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4).

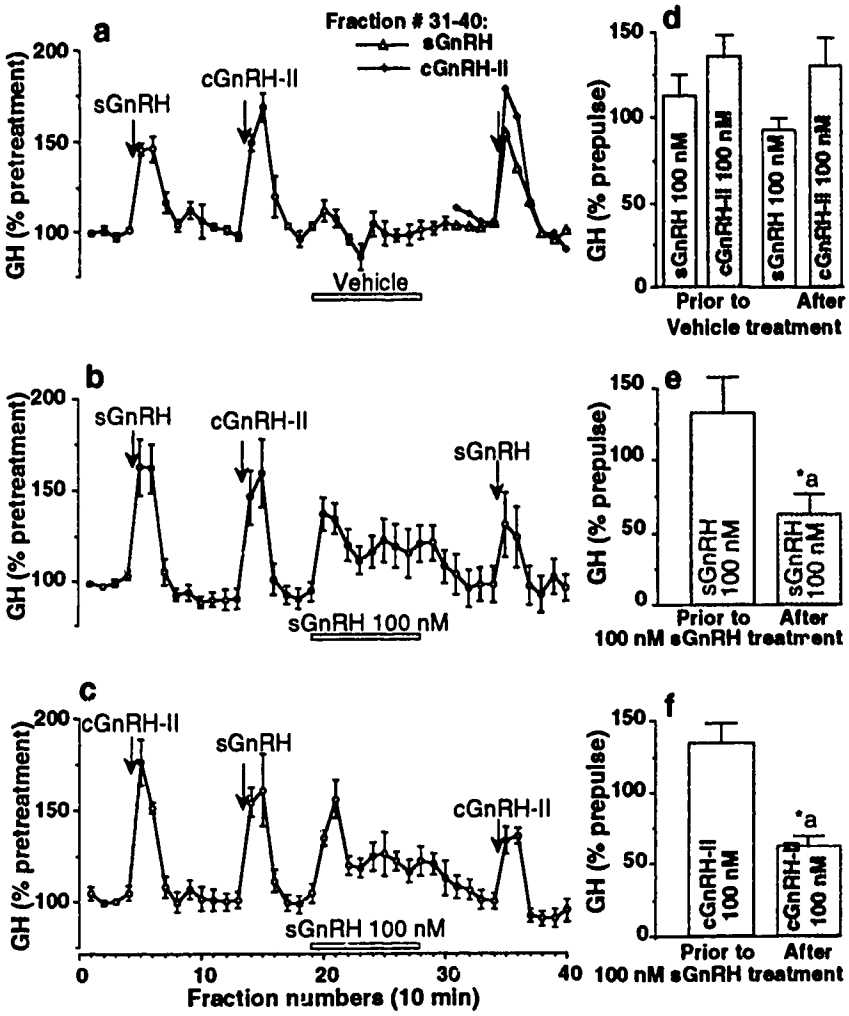


Fig. 3.9 The GH responses of the pituitary fragments to 100 nM sGnRH and cGnRH-II, prior to and after vehicle (a), or 100 nM sGnRH treatment for 90 min (b, c). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). These responses were quantified as % prepulse and presented in d, e and f, respectively.

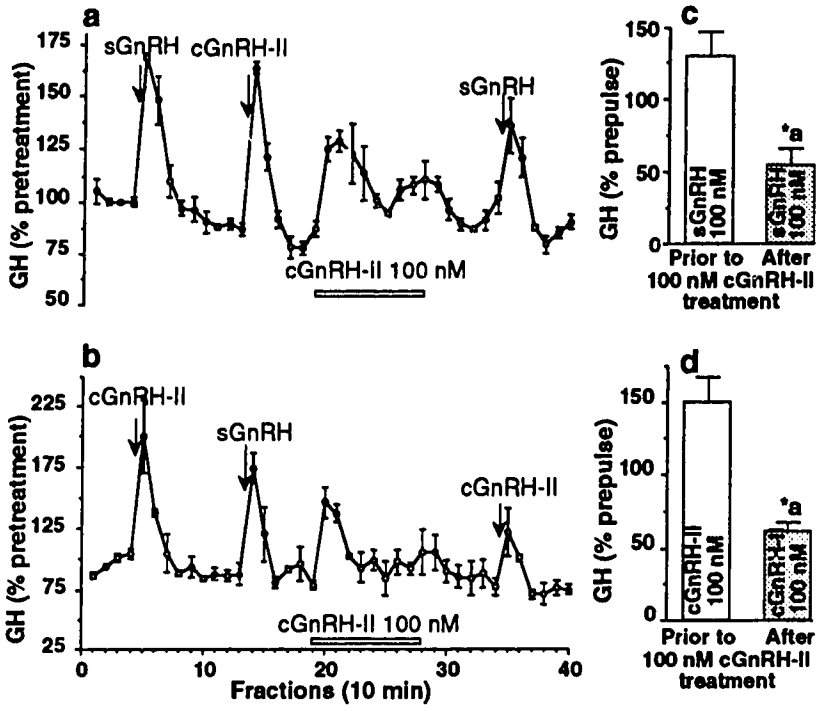


Fig. 3.10 The GH responses of the pituitary fragments to 100 nM sGnRH and cGnRH-II, prior to and after 100 nM cGnRH-II treatment for 90 min (a, b). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). These responses were quantified as % prepulse and presented in Fig. c and d, respectively.

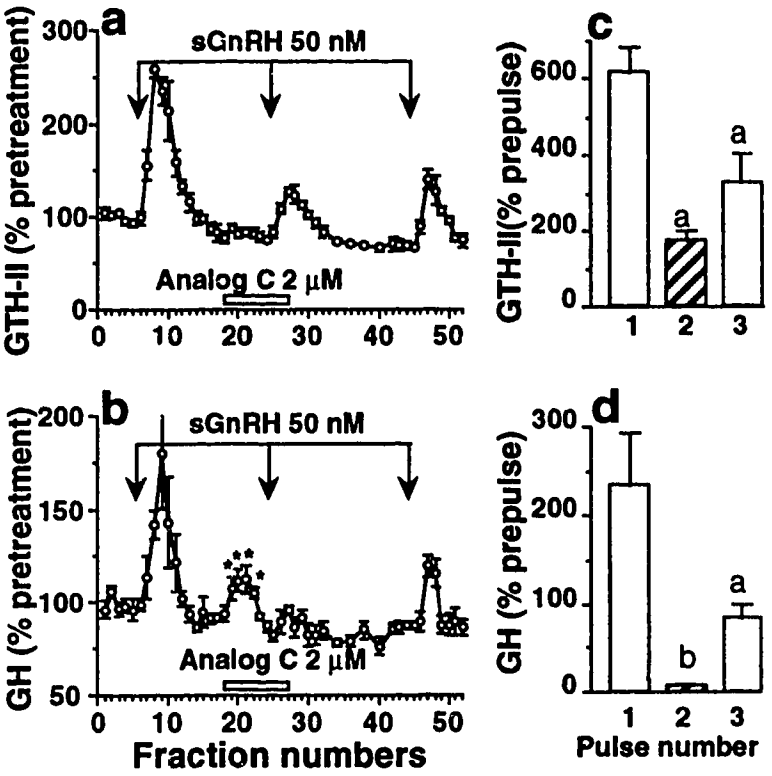


Fig. 3.11 The GTH-II (a, c), and GH (b, d) release responses of the pituitary fragments to repeated pulses of 50 nM sGnRH given in the absence or presence of analog C (2 μM). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). The GTH-II and GH responses were quantified as a percentage prepulse and presented as mean ± SEM (c, and d).

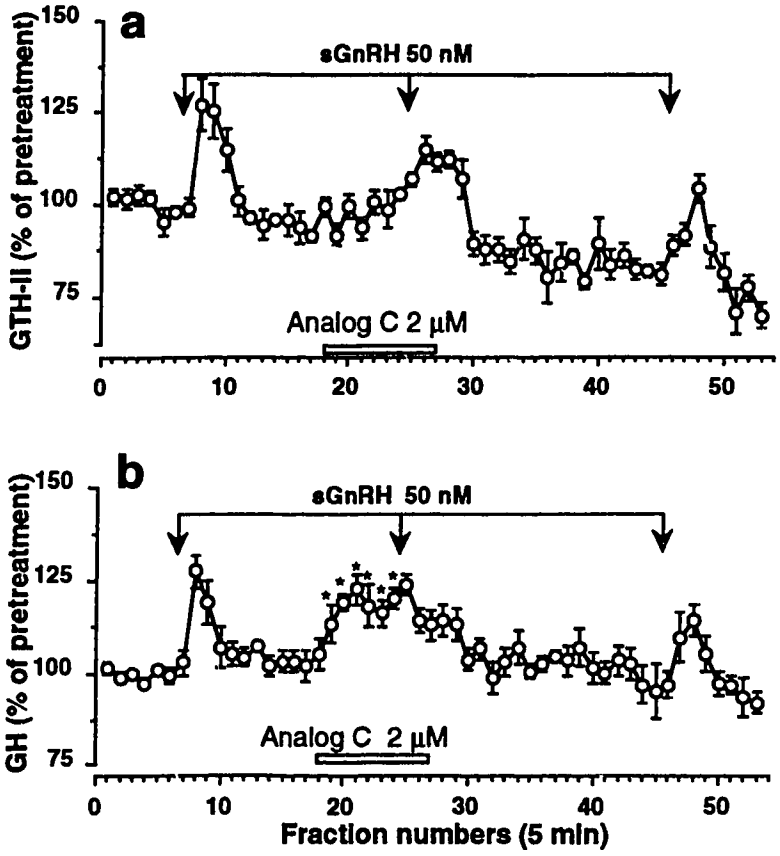


Fig. 3.12 The GTH-II (a), and GH (b) release responses of the dispersed pituitary cells to repeated pulses of 50 nM sGnRH given in the absence or presence of analog C (2 µM). The data were transformed as % pretreatment, and expressed as mean ± SEM (n = 4). A significant increase in GH release in response to analog C treatment, compared to pretreatment level (ANOVA followed by Fisher's LSD test) is indicated by * mark.

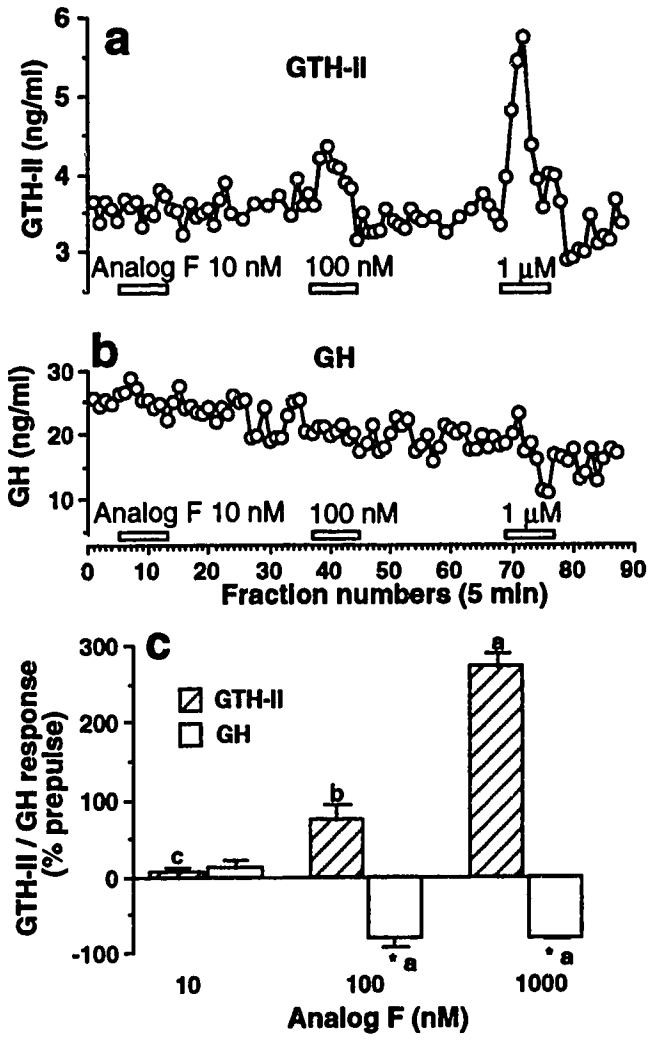


Fig. 3.13 The actions of different dosages of [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3, D-Arg6]-mGnRH (analog F), under continuous treatment (35 min) on GTH-II (a) and GH (b) release from the pituitary fragments. The results are from a representative column out of 3 or 4 similar columns and hormonal levels are expressed as ng/ml. The GTH-II and GH release responses to different doses of analog F were quantified as % prepulse, pooled and presented in (c). A significant difference in GTH-II release response to different doses of analog F is indicated by different letters. Similarly, a significant decrease GH release response to analog F is indicated by * mark.

References

Amano, M., Aida, K., Okumoto, N., and Hasegawa, Y. (1992). Changes in salmon GnRH and chicken GnRH-II contents in the brain and pituitary, and GtH contents in the pituitary in female masu salmon, *Oncorhynchus masou*, from hatching through ovulation. Zoological Sci. 9, 375-386.

Chang, J. P., and Jobin, R. M. (1991). Two native GnRH peptides stimulate gonadotropin release in goldfish by different second messenger components. In: "Proceedings of the 4th International Symposium on the Reproductive Physiology of Fish" (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp. 221-223. FishSymp 91, Sheffield, UK.

Chang, J. P., Cook, H., Freedman, G. L., Wiggs, A. J., Somoza, G. M., De Leeuw, R., and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Chang, J. P., Jobin, R. M., and De Leeuw, R. (1991a). Possible involvement of protein kinase C in gonadotropin and growth hormone release from dispersed goldfish pituitary cells. Gen. Comp. Endocrinol. 81, 447-463.

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

Chang, J. P.,Wildman, B., and.Van Goor, F. (1991b). Lack of involvement of arachidonic acid metabolism in chicken gonadotropin-releasing hormone-II (cGnRH-II) stimulation of gonadotropin secretion in dispersed pituitary cells of goldfish, *Carassius auratus*. Identification of a major difference in salmon GnRH and chicken GnRH-II mechanism of action. Mol. Cell. Endocrinol. 79, 75-83.

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J. P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul. Peptides, 36, 369-378.

Habibi, H. R. (1991a). Homologous desensitization of gonadotropin-releasing hormone

(GnRH) receptors in the goldfish pituitary : Effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod. 44, 275-283.

Habibi, H. R. (1991b). Desensitization to native molecular forms of gonadotropin-releasing hormone in the goldfish pituitary: dependence on pulse frequency and concentration. Gen. Comp. Endocrinol. 84, 199-214.

Habibi, H. R. and Peter, R. E. (1991). Gonadotropin-releasing hormone (GnRH) receptors in teleosts. In: "Proceedings of the 4th International Symposium on The Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 109-113. FishSymp 91, Sheffield, UK.

Habibi, H. R., Peter, R. E., and Hazum, E. L. I. (1990). Photoaffinity labelling of pituitary gonadotropin-releasing hormone receptors in goldfish (*Carassius auratus*). Biol. Reprod. 43, 1006-1011.

Habibi, H. R., Peter, R. E., Nahorniak, C. S., Milton, R. C. L., and Millar, R.P. (1992). Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides. 37, 271-284.

Habibi, H. R., Peter, R. E., Sokolowska, M., Rivier, J. E., and Vale, W. W. (1987). Characterization of gonadotropin-releasing hormone (GnRH) binding to pituitary receptors in goldfish (*Carassius auratus).* Biol. Reprod. 36, 844-853.

Habibi, H. R., Van Der Loo, H., Marchant, T. A., Nahorniak, C. S., Peter, R. E., King, J. A., Miller, R. P., Rivier, J. E., and Vale, W. W. (1988). Studies on the molecular requirement for gonadotropin-releasing hormone (GnRH) receptor recognition in the goldfish pituitary. In: "Proceedings, 8th International Congress of Endocrinology", Kyoto, Japan". p127. Japan Convention Services Inc., Kyoto. (Abstract 03 -29-028).

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.

Kim, M., Oka, Y., Amano, M., Kobayashi, M., Aida, K., and Kawashima, S. (1993). Immuno-cytochemical location of sGnRH and cGnRH-II in the goldfish brain and effects of olfactory tract section on the distribution of GnRH. In "Program, XII International Congress of Comparative Endocrinology". Toronto, Canada, p A-82 (abstract).

King, J. A., and Millar, R. P. (1987). Phylogenetic diversity of LHRH. In: "LHRH and its analogs-contraceptive and therapeutic applications", Part 2. (B. H. Vickery, and J. J. Nestor Jr, Eds.), pp 53-73. MTP Press Ltd., Boston.

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

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.

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

Murthy, C. K., Nahorniak, C. S., Rivier, J. E., and Peter, R. E. (1993). *In vitro* characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish, *Carassius auratus*. Endocrinology 133, 1633-1644.

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.

Peng, C., Huang, Y. P., and Peter, R. E. (1990). Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology 52, 28-34.

Peter, R. E., Nahorniak, C. S., Chang, J. P., and Crim, L. W. (1984). Gonadotropin release from the pars distalis of goldfish, *Carassius auratus* transplanted beside the brain or into the brain ventricles; Additional evidence for gonadotropin release-inhibitory factor. Gen. Comp. Endocrinol. 55, 337-346.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In:"Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, M. H. Stetson, Eds.), pp. 393-398. Wiley-Liss Inc. NY.

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

Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

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 Four

Differential actions of a mammalian gonadotropin-releasing hormone (GnRH) antagonist on gonadotropin-II and growth hormone release in goldfish, *Carassius auratus*[1]

Introduction

Gonadotropin-releasing hormone (GnRH) peptides stimulate gonadotropin-II (GTH-II) and growth hormone (GH) secretion in goldfish (Marchant et al., 1989; for review Peter et al., 1990), common carp (Lin et al., 1993), and rainbow trout (Le Gac et al., 1993). Probably with the exception of higher mammals (King et al., 1988b) presence of multiple forms of GnRH in a given species of vertebrate is common (for review Sherwood and Lovejoy, 1989; King and Millar, 1992; Sherwood et al., 1993). In goldfish two forms of GnRH, salmon GnRH (sGnRH) and chicken GnRH (cGnRH-II) have been demonstrated in the brain and pituitary (Yu et al., 1988; Kim et al., 1993). Both sGnRH and cGnRH-II stimulate GTH-II and GH release *in vivo* and *in vitro* in goldfish (Marchant et al., 1989; Habibi et al., 1992; for review Peter et al., 1990). The GTH-II and GH stimulatory actions of the two native peptides were also observed with dispersed goldfish pituitary cells in static culture and in perifusion, indicating the direct action at the level of pituitary cells (Chang et al., 1990). Both sGnRH and cGnRH-II displaced ^{125}-I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH (sGnRH-A) bound to crude goldfish pituitary membrane preparations (Habibi et al., 1987; for review Habibi and Peter, 1991). Further, both sGnRH and cGnRH-II also displaced avidin gold-labelled biotinylated [D-Lys^{6}, Pro^{9}-NHEt]-sGnRH from the surface of gonadotrophs and somatotrophs (Cook et al., 1991), confirming a direct action of GnRH on these cells.

Structure-function activity studies of GnRH analogs in mammals indicate that

---

[1]A version of this chapter has been accepted for publication: CK Murthy, RJ Turner, AOL Wong, PD Prasada Rao, JE Rivier, RE Peter (1994). Neuroendocrinology (in press).

modifications of the amino acid sequence especially at positions 1, 2, 3, and 6 results in potent antagonists (for review Karten and Rivier, 1986). The potency of GnRH antagonists was further enhanced by substitution of D-Arg^6 along with a hydrophobic aromatic N-terminus (Nekola et al., 1982, Rivier et al., 1984). To reduce the *in vivo* release of histamine by D-Arg^6 substituted analogs (for review Karten and Rivier, 1986; Karten et al., 1987), the "Arg^5, Arg^8" series of antagonists with D-3-Pal^6, were developed (Folkers et al., 1986; Nestor, 1987; for review Folkers et al., 1987). One of the analogs in this series, [N-Ac-D(2)Nal^1, D-pCl-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-GnRH was highly potent in inhibiting ovulation in rats (Folkers et al., 1986), with only moderate histamine release activity.

In our previous studies on the characterization of GnRH antagonists in goldfish (Murthy et al., 1993; see Chapter 2), many mammalian GnRH antagonists, especially those with D-Arg^6, stimulated both GTH-II and GH release from perifused goldfish pituitary fragments. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E of Chapter 2) inhibited both sGnRH and cGnRH-II induced GTH-II and GH release in a dose dependent fashion (Murthy et al., 1991, 1993; see Chapter 2). Interestingly, some mammalian GnRH antagonists differentially regulated GTH-II and GH release. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH (analog F of Chapter 2) stimulated GTH-II, but not GH release (see Chapters 2, 3). [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L of Chapter 2), a potent GnRH antagonist in mammals (Folkers et al., 1987), weakly stimulated GTH-II release, but strongly suppressed GH release from the perifused pituitary fragments. In order to further characterize the differential actions of analog L on GTH-II and GH release in goldfish, we have studied its dose-dependent actions on GTH-II and GH release, and specificity of inhibition of native GnRH actions on GTH-II and GH release. We also tested whether analog L acts directly at the pituitary cell level.

Materials and Methods

Experimental animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries, Stoutland, MO. The fish were maintained in flow-through aquaria (1800 liters) at 17 ± 1°C under a simulated natural photoperiod of Edmonton for at least 2-3 weeks prior to experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish of both sexes, with body weight ranging from 20-35 g were used in the present study.

The sexual maturity of fish was assessed by measuring the gonadosomatic index (GSI= weight of gonad/total body weight X 100).

Reagents and test substances sGnRH, cGnRH-II (Peninsula Laboratories Inc., Belmont, CA) were dissolved in 0.1 M acetic acid and stored at -25°C as aliquots of 50 μM. The aliquots were diluted with perifusion medium to required concentrations immediately prior to use in experiments. [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L of Chapter 2) and [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E of Chapter 2) were gift of Dr. J. E. Rivier, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA, and were dissolved in 0.3-0.5 ml of primary solvent containing propylene glycol and physiological saline at a ratio of 60:40, and subsequently diluted to required concentrations with perifusion medium. The final propylene glycol concentration was less than 0.5%. SKF38393 (dopamine D1 type receptor agonist; Research Biochemicals Inc., Wayland, MA) was dissolved in 200 μl of dimethyl sulfoxide (DMSO) and diluted to required concentrations with perifusion medium. The final DMSO concentrations were less than 0.1%. Thyrotropin-releasing hormone (TRH; Peninsula Laboratories Inc., Belmont, CA) was dissolved in perifusion medium.

Perifusion of pituitary fragments and cells *In vitro* experiments were conducted using a validated pituitary fragments perifusion system described earlier (Marchant et al., 1989) with minor changes. Briefly, the pituitary glands, collected from goldfish, were chopped into fragments (0.2 mm^3) using a McIlwain tissue chopper. The fragments (three pituitary equivalents per chamber) were washed with medium 199 and placed in 0.5 ml micro-chambers (Endotronics Inc., Minneapolis, MN) between two 13 mm filters; 8 micron pore size for inlet (Millipore Inc., Bedford MA) and 5 micron pore size for outlet (Micron Separations Inc., Westboro, MA) with a continuous flow of medium (M199) at 18°C. These filters were supported by circular stainless steel screens with a pore size of 450 micron. The reservoirs, holding perifusion medium or drugs were attached to a 3-way valve. From the 3-way valve the drug or medium was delivered to the micro-chambers by a tubing passing through an ISMTEC multichannel cartridge pump (Cole-Parmer Instruments Corporation, Chicago, Illinois). The fragments were perifused overnight (at

least 15 h) at a flow rate of 5 ml/h. Thereafter, the incubation was continued using Hank's balanced salt solution supplemented with 25 mM HEPES and 0.1% BSA at a flow rate of 15 ml/h for 2 h before starting the experiment. Five minute fractions of perifusate were collected, frozen and stored at -25°C for hormonal assay.

The enzymatically dispersed pituitary cells (for procedure see Chang et al., 1990) were incubated with preswollen cytodex beads (Cytodex I, Sigma Chemical Co., St. Louis, MO) at 28°C under 5% CO_2 and saturated humidity. After overnight incubation, cytodex beads with pituitary cells attached were loaded into 0.5 ml micro-chambers and the experiment was conducted as described above.

GnRH receptor study The GnRH receptor studies were conducted using goldfish crude pituitary membrane preparations. The iodination of [D-Arg^6, Pro^9-NHEt]-sGnRH (sGnRH-A), crude pituitary membrane preparation and displacement studies were conducted as described by Habibi et al. (1987). The displacement by 10^{-6}M sGnRH-A was taken as non-specific binding in all the experiments. The displacement curves were analysed by a non-linear weighted least-square curve fitting method (Munson and Rodbard, 1980; McPherson, 1985), using the computer program LIGAND (Biosoft, Cambridge, UK). The data analysis was done in two steps; the initial affinity constant and binding capacity values were obtained by Equilibrium Binding Data Analysis (EBDA) and based on these initial estimates, the final estimates were obtained by LIGAND. The displacement data of analog L from individual experiments were analysed individually and then data from four individual experiments were analysed simultaneously to obtain the final estimates of equilibrium association constant (Ka).

Radioimmunoassay GTH-II levels in perifusate were measured by using a validated radioimmunoassay specific for goldfish GTH-II (Peter et al., 1984; Van Der Kraak et al., 1992). The GH levels were measured by using a goldfish GH radioimmunoassay described by Murthy et al. (1993).

Data analysis To provide a mean response, the GTH-II or GH values were expressed as percentage of pretreatment mean (of 6 fractions prior to first pulse) for each column and pooled from 4 columns. The quantification of the hormone response was done as

described by Peng et al. (1990). Briefly, the average hormone level of the 3 fractions immediately preceding each pulse (prepulse mean) was treated as basal, and the hormone values following a GnRH pulse were expressed as a percentage of this prepulse mean. Post pulse fractions with hormone contents greater than one standard error (SEM) above the prepulse mean were considered to be a part of the response. The response (expressed as % prepulse) above the pretreatment mean from the fractions considered as part of the response were added to get the net response in a particular column. Using a computerized ALLFIT program (De Lean et al., 1978) ED50 ± approximate error values for analog L to stimulate GTH-II release and to inhibit GH release were calculated as a measure of potency. The difference in responses between treatment and control was assessed by ANOVA followed by Fisher's LSD test or Student's t-test ($p < 0.05$).

Results

Dose response studies To compare the GTH-II and GH releasing potencies of analog L and sGnRH, the dose-related effects of 2 min pulse treatments of analog L on GTH-II and GH release from perifused pituitary fragments was tested. Analog L given as 2 min pulses at 73 min intervals stimulated GTH-II release (Fig. 4.1a, c) and inhibited GH release (Fig. 4.1b, d). The effects of analog L on hormone release were very distinct at higher concentrations of 1 μM and 10 μM. Analog L stimulated GTH-II release with an approximate ED50 of 1018 ± 756 nM and maximum net response of 388 ± 82% of prepulse. The minimum effective concentration of analog L to cause a significant increase in GTH-II release was 1000 nM. In the same experiment sGnRH stimulated GTH-II release with an ED50 of 17.2 ± 10.6 nM and caused a maximum response of 1993 ± 299% of prepulse. sGnRH at a minimum concentration of 10 nM significantly increased GTH-II release (see Chapter 5). Thus, analog L was 60-100 times less potent than sGnRH in stimulating GTH-II release. Interestingly, analog L on its own suppressed the unstimulated GH release from the perifused pituitary fragments in a dose related manner, with inhibition being significant at 1 μM and 10 μM concentrations. Analog L at 10 μM concentration suppressed the unstimulated GH release by 111 ± 10% prepulse (Fig. 4.1d). The inhibition of unstimulated GH release by analog L was transient and the GH levels returned to prepulse values quickly (Fig. 4.1b).

In our preliminary studies the actions of analog L were distinctly noted under prolonged (30-35 min) treatment conditions. Hence we tested the dose-related effects of various concentrations of analog L ranging from 1 nM to 3 μM for 30 min at 90 min intervals. Under these treatment conditions analog L stimulated GTH-II release in a dose related manner with an ED50 of 976 ± 436 nM; the minimum effective concentration being 10 nM (Fig. 4.2). Analog L inhibited GH release with an ED50 of 922 ± 318 nM and the minimum effective concentration was 300 nM (Fig. 4.3). Similar to the previous experiment, the inhibition of unstimulated GH release by analog L was transient with the basal GH values returning to prepulse levels (Fig. 4.3a, b). At 90 min after termination of analog L treatment, a pulse of 50 nM sGnRH stimulated GH release from the pituitary fragments.

Inhibition of sGnRH and cGnRH-II actions by analog L Since analog L strongly inhibited unstimulated GH release, we wanted to test the effects of analog L on GH and GTH-II release induced by 50 nM sGnRH (Fig. 4.4) and 50 nM cGnRH-II (Fig. 4.5). Similar to earlier experiments, exposure of pituitary fragments to analog L at 2 μM for 30 min resulted in increased GTH-II release (Fig. 4.4a, c) and reduced GH release (Fig. 4.4b, d). In the presence of analog L (2 μM), the GTH-II and GH release responses to a 2 min pulse of 50 nM sGnRH were significantly suppressed compared to the responses to the initial pulse of sGnRH (GTH-II, 69 ± 5% inhibition; GH, 79 ± 3% inhibition; Fig. 4.4c, d). At 90 min after termination of analog L treatment, the GTH-II and GH release responses to a 2 min pulse of 50 nM sGnRH were similar to that of the initial sGnRH pulse.

In a similar experiment, a 2 μM concentration (45 min) of analog L inhibited 50 nM cGnRH-II induced GTH-II (59 ± 3% inhibition; Fig. 4.5a, c) and GH (78 ± 6% inhibition; Fig. 4.5b, d) release. Again, at 90 min after the termination of analog L treatment, the GTH-II and GH responses to a pulse of cGnRH-II were not significantly different from the responses to the initial pulse of cGnRH-II.

Specificity of inhibition of GnRH actions by analog L In the previous experiments analog L significantly suppressed both sGnRH and cGnRH-II stimulated GTH-II and GH release. In these experiments the specificity of analog L to suppress the GH releasing effects of the

GH secretogogues SKF38393, a dopamine D1 type receptor agonist, and TRH was tested. In the presence or absence of vehicle repeated 2 min pulses of 0.5 μM SKF38393 elicited GH release responses of similar magnitude from perifused pituitary fragments (Fig. 4.6a, c). Similarly, the GH responses (quantified as % prepulse) to 0.5 μM SKF38393 in the presence of 2 μM analog L were not significantly different from the GH responses to SKF38393 in the presence or absence of vehicle (Fig. 4.6b, d). In another experiment, the GH release responses to 2 min pulse of 1 μM concentration of TRH in the presence of either vehicle or analog L (2 μM) were not significantly different (Fig. 4.7), indicating the inhibitory actions of analog L only on GnRH actions.

Site of action of analog L These experiments explored the possible sites of action mediating the differential effects of analog L on GTH-II and GH release, and in inhibiting the hormonal release responses elicited by native forms of GnRH. In the first experiment, exposure of pituitary fragments to 2 μM of analog L for 30 min in the continuous presence of vehicle caused a significant increase in GTH-II release (Fig. 4.8a), and a significant decrease in GH release (Fig. 4.8d). However, in the continuous presence of 2 μM [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; a 'true' GnRH antagonist, [Murthy et al., 1993; see Chapter 2]), the GTH-II release stimulation by analog L was completely inhibited (Fig. 4.8b, c). Under similar conditions, analog E partially, but significantly inhibited the GH release suppression by analog L (Fig. 4.8e, f).

In the next experiment, the effects of analog L treatment on GTH-II and GH release from enzymatically dispersed goldfish pituitary cells were tested. As observed with pituitary fragments, the exposure of dispersed pituitary cells to 2 μM analog L for 45 min resulted in significantly enhanced GTH-II (Fig. 4.9a) and suppressed GH release (Fig. 4.9b; quantification of data not shown). Further, in the presence of analog L, GTH-II and GH release stimulated by a 2 min pulse of 50 nM sGnRH was significantly inhibited, indicating the actions of analog L directly at the pituitary cell level (quantification of data not shown).

In the GnRH receptor binding studies, incubation of crude pituitary membrane preparations with ^{125}-I-sGnRH-A and with increasing concentrations (10^{-12} to 10^{-6} M, in triplicate) of 'cold' analog L resulted in a dose-dependent displacement of bound ^{125}-I-

sGnRH-A (Fig. 4.10). The profiles of ^{125}I-sGnRH-A displacement curves by analog L, sGnRH-A, and sGnRH were similar (see Chapter 6), indicating the binding of analog L to GnRH receptors in the pituitary membrane preparations. The analysis of the displacement curves for analog L for two binding sites was statistically a better fit than for a single binding site, similar to previous findings regarding GnRH receptors in the goldfish pituitary (Habibi et al., 1987; Habibi and Peter, 1991). The simultaneous analysis of four displacement curves by LIGAND indicated equilibrium association constants (Ka) of 4.17 $\pm$ 1.01 X 10^{11}, M^{-1} (95 % confidence limits being 2.45 - 7.10 X 10^{11}, M^{-1}) and 0.63 $\pm$ 0.05 X 10^{7}, M^{-1} (95 % confidence limits being 0.53 - 0.75 X 10^{7}, M^{-1}) for high and low affinity binding sites, respectively.

Discussion

[Ac-D(2)Nal^{1}, 4Cl-D-Phe^{2}, D(3)-Pal^{3,6}, Arg^{5}, D-Ala^{10}]-mGnRH (analog L) stimulated GTH-II release and inhibited GH release in a dose-dependent manner. These results support our earlier report (Murthy et al., 1993; see Chapter 2) on differential actions of analog L on GTH-II and GH release. However, the actions of analog L in goldfish are contrary to results in mammalian studies. Folkers et al. (1986) have shown that analog L acts as a potent GnRH antagonist in rat; analog L inhibits ovulation by 60, 80 and 100% at 0.125, 0.25 and 0.5 µg/rat doses, respectively. Analog L was the most potent antagonist known in 1986, with significantly lower histamine release (Folkers et al., 1987). Similar to the present results, many mammalian GnRH antagonists stimulated LH release from chicken gonadotrophs (Millar and King, 1984; King et al., 1988a). These results indicate that the ligand structure requirements for GnRH antagonists are different for rat and goldfish, and also between other species.

The observed GTH-II stimulation by analog L cannot be due to contamination of analog L with GnRH agonists such as mGnRH, because the time course of GTH-II stimulation by agonists and analog L are different. Normally, agonists stimulate GTH-II release immediately after exposure to goldfish pituitary fragments and dispersed cells. Analog L, given as a 2 min pulse at a high concentration or continuously at a lower concentration, stimulated GTH-II release 10-15 min after initial exposure to pituitary fragments or cells. Secondly, GnRH agonists stimulate both GTH-II and GH release in goldfish. In contrast, analog L stimulated GTH-II, but inhibited GH release.

It is interesting to note that analog L stimulated GTH-II release, but suppressed GH release. In addition, analog L (2 μM) was found to have a greater ability to suppress sGnRH and cGnRH-II induced GH release compared to inhibition of GTH-II release induced by sGnRH and cGnRH-II. A similar tendency has also been noticed for some other GnRH antagonists in goldfish (Murthy et al., 1993; see Chapter 2). Further, analog E (2 μM) completely blocked the GTH-II stimulatory actions of analog L (Fig. 4.8a, b, c), but only partially suppressed GH release inhibitory actions of analog L (Fig. 4.8d, e, f). These results suggest that the structure-activity relations of GnRH receptors on gonadotrophs and somatotrophs in goldfish are different. In support of this, in an earlier study in goldfish, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C) acted as a 'true' antagonist on GTH-II release, but stimulated GH release on its own (Murthy et al., 1993; see Chapters 2, 3). In contrast, [Ac-D(2)-Nal^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH (analog F) strongly enhanced GTH-II release, but weakly inhibited GH release (see Chapters 2, 3). Results from other GnRH structure-activity studies in goldfish indicate that the requirements for superactive GnRH agonists for GTH-II and GH are also different (Peter et al., 1990; Habibi et al., 1992).

Analog L, under both 2 min pulse treatment and 30 min prolonged exposure, stimulated GTH-II release in a dose dependent manner. However, in terms of ED50 and the maximum GTH-II release response, the GTH-II stimulatory ability of analog L was 60-100 times lower than that of sGnRH. Although analog L stimulated GTH-II release, in continuous presence of analog L both sGnRH and cGnRH-II actions on GTH-II and GH release were significantly suppressed. These results suggest that analog L acts as a 'partial antagonist' (an antagonist with weak agonistic activity) on GTH-II release in goldfish. In the preliminary screening studies, many of the mammalian GnRH antagonists, especially those with D-Arg^6, which exhibited weak to strong stimulatory actions on GTH-II release (Murthy et al., 1993; see Chapter 2) also showed inhibitory actions on GnRH induced GTH-II release. Similar to these results, many mammalian antagonists are reported to act as 'partial antagonists' in chicken pituitary cells (Millar and King, 1984; King et al., 1988a).

We tested the hypothesis that analog L does not interact with receptors of other ligands acting on somatotrophs in the goldfish. In goldfish, GH release is under the stimulatory

control of multiple hypothalamic factors (Marchant et al., 1989; Chang et al., 1990; Peng et al., 1990; Habibi et al.,1992; Trudeau et al., 1992; Wong et al., 1992, 1993a, b, c; Peng et al., 1993). One of these factors, dopamine has been shown to act via D1 type of receptors to stimulate GH release (Wong et al., 1992; 1993a, b, c). Similarly, TRH stimulates GH secretion from the perifused goldfish pituitary fragments in a dose dependent manner (Trudeau et al., 1992). When tested, both SKF38393 (dopamine D1 type agonist) and TRH stimulated GH secretion from the perifused pituitary fragments. Analog L at 2 μM had no significant effects on GH release induced either by SKF38393 or by TRH. These results support the concept that analog L specifically acts via GnRH receptors on somatotrophs

The next series of studies were directed to test the hypothesis that analog L acts through GnRH receptors on the GTH and GH cells. Analog E acts as a 'true' antagonist to inhibit sGnRH and cGnRH-II actions on GTH-II and GH release in goldfish pituitary fragments (Murthy et al., 1991, 1993; see Chapter 2) as well as dispersed pituitary cells (see Chapter 6), without any independent effects on basal secretion of GTH-II and GH. In the present study, in the continuous presence of analog E the ability of analog L to stimulate GTH-II and to inhibit GH release were significantly suppressed. Secondly, analog L acted directly on the enzymatically dispersed pituitary cells to stimulate GTH-II and inhibit GH release, and, in the continuous presence of analog L, sGnRH stimulated GTH-II and GH release from the pituitary cells was significantly suppressed. Thirdly, in the receptor binding studies analog L displaced ^{125}-I-sGnRH-A bound to crude pituitary membrane preparations in a dose dependent manner. ^{125}-I-sGnRH-A displacement profile of analog L was similar to that of sGnRH and sGnRH-A. The equilibrium association constant (Ka_1) of analog L to high affinity site [Ka_1= 4.17 ± 1.01 (95% confidence limits: 2.45 - 7.10) X 10^{11}, M^{-1}] was significantly higher than that of sGnRH [Ka_1 = 3.41 ± 0.5 (2.43 - 4.78) X 10^{10}, M^{-1}) and sGnRH-A [Ka_1 = 4.97 ± 0.27 (4.41 -5.60) X 10^{10}, M^{-1}]. However, the equilibrium association constant (Ka_2) of analog L to low affinity site [0.63 ± 0.05 (0.53 - 0.75) X 10^7, M^{-1}) was lower than that of both sGnRH [3.14 ± 0.71 (1.87 - 5.27) X 10^7, M^{-1}] and sGnRH-A [8.92 ± 0.47 (7.94 - 10.03) X 10^7, M^{-1}]. Taken together the results indicate that the actions of analog L are due to binding to GnRH receptors in the pituitary cells and not by a non-specific interaction with any other protein on the cell surface. Recently Schutz and Freissmuth (1992) suggested the possible

existence of 'neutral antagonists" (without any intrinsic activity) and 'negative antagonists' (with negative intrinsic activity or suppression of basal activity). It may be interesting to know whether the inhibition of unstimulated GH release from dispersed pituitary cells by analog L is due to negative intrinsic activity.

Like in other vertebrates, somatostatin (SRIF) can inhibit both unstimulated and stimulated GH release in goldfish (Marchant et al., 1987; Peng et al., 1993; Wong et al., 1993b). However, the ability of analog L to suppress the unstimulated GH release is not by stimulation of SRIF release, as SRIF inhibits GH stimulatory activity of both dopamine (Wong et al., 1993b) and TRH (V.Trudeau, C. Peng and R. Peter, unpublished results). Also, analog L had no significant effects on the GH stimulatory actions of both dopamine and TRH in the goldfish. Further, the actions of analog L are not through activation of SRIF receptors as SRIF has no action on GTH-II release (Marchant et al., 1989), while analog L stimulates GTH-II release. The stimulatory actions of GnRH on GH release may not be restricted to teleost fish. mGnRH has been found to stimulate GH release from the rat pituitary that has been anatomically and/or functionally disconnected from the CNS (Panerai et al., 1976) and by perifused rat pituitary cells (Badger et al., 1987). In humans mGnRH stimulates GH release under many pathological conditions such as acromegaly, mental depression, anorexia nervosa, and schizophrenia (for review Müller, 1987).

In mammals, many GnRH antagonists, especially those with D-Arg^6, stimulate histamine release *in vivo* (for review Karten and Rivier, 1986; Karten et al., 1987; Nestor, 1987). Histamine appears to have inhibitory actions on stimulated GH release *in vivo* in rats and humans (for review Müller, 1987). However, it is unlikely that the inhibition of GH release from goldfish pituitary fragments and dispersed cells by analog L is due to histamine release. In rats histamine and histamine receptor antagonists have no direct actions on GH release *in vitro* (for review Müller, 1987). Further, [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH, which has a much greater histamine release activity than analog L in rats (Nestor, 1987), strongly stimulated GH as well as GTH-II release from perifused goldfish pituitary fragments (Murthy et al., 1993; see Chapters 2, 5) suggesting that histamine release by GnRH antagonists and the suppression of GH release in goldfish by analog L are independent.

In conclusion, [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L) differentially acts on GTH-II and GH release, suggesting differences in the

properties of the GnRH receptors on GTH and GH cells. Contrary to its actions as a potent GnRH antagonist in mammals, analog L has independent GTH-II stimulatory actions in goldfish. Analog L also independently suppresses GH release in goldfish. Analog L exerts its actions directly at the pituitary cell level, by binding to GnRH receptors. Analog L also specifically inhibits sGnRH and cGnRH-II actions on GTH-II and GH release, without any significant effects on GH release induced by DA and TRH.

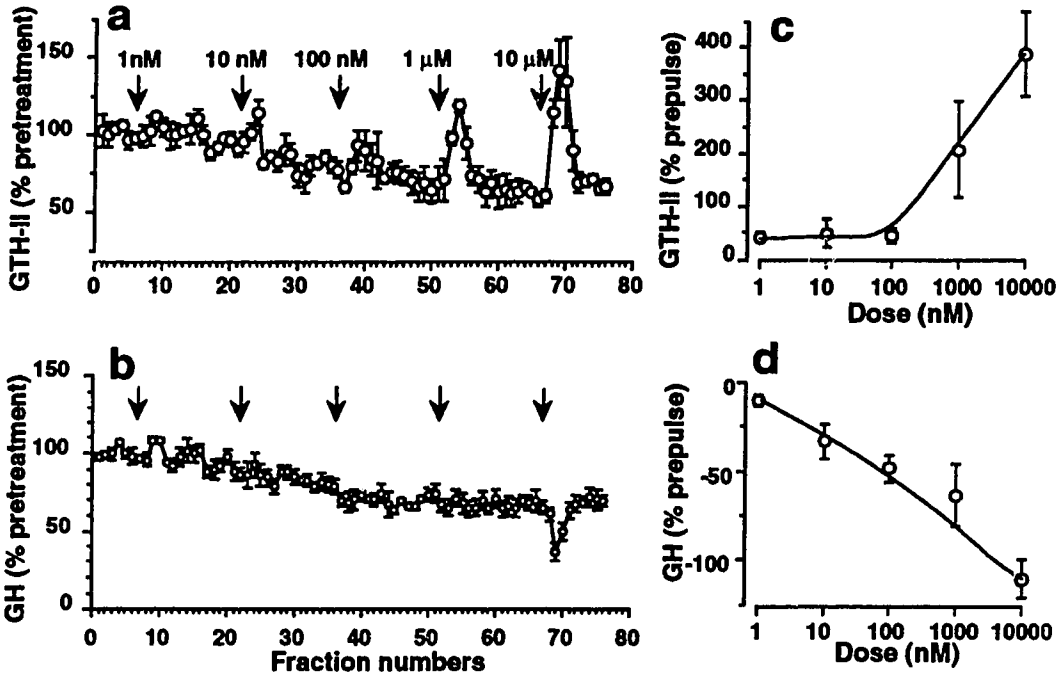


Fig. 4.1 The effects of repeated 2 min pulses (arrows) of different concentrations (from 1 nM to 10 μM) of [Ac-D(2)Nal1, 4Cl-D-Phe2, D(3)-Pal3,6, Arg5, D-Ala10]-mGnRH (analog L) on GTH-II (a) and GH (b) release from perifused pituitary fragments collected from sexually mature goldfish (GSI = 8.3 ± 1%). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n = 4). The GTH-II and GH release responses to different concentrations of alanlog L were quantified as % prepulse and presented as mean ± SEM (c, d).

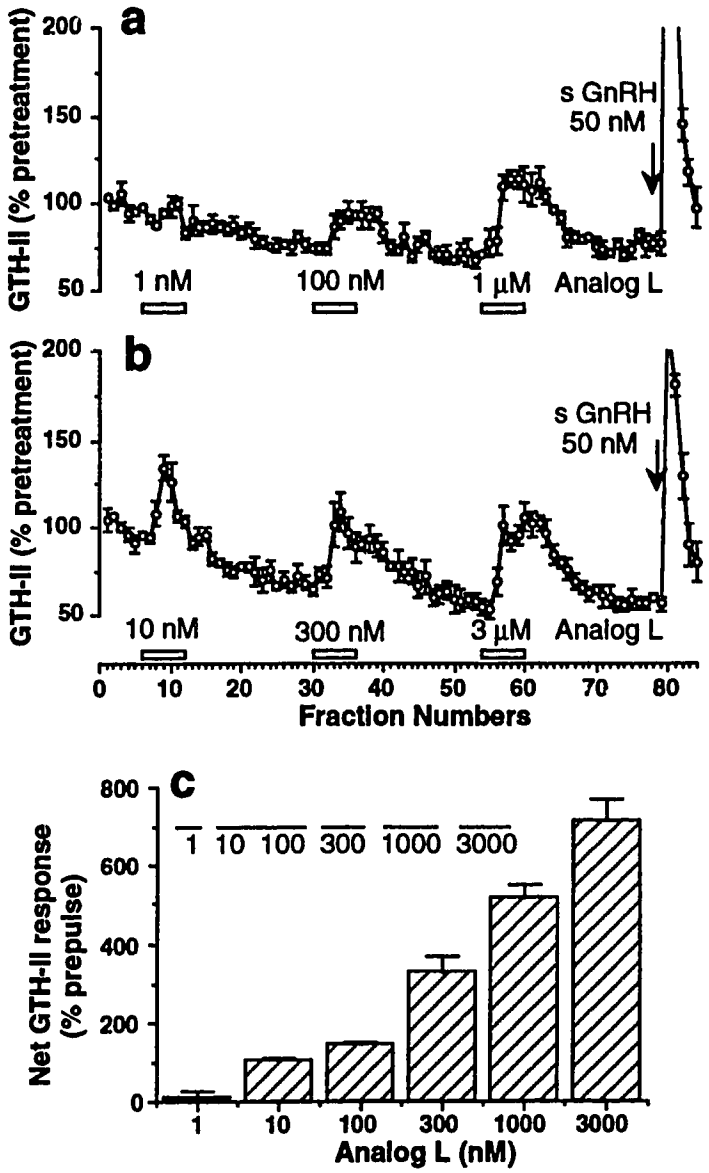


Fig. 4.2 The effects of prolonged treatment (30 min; open boxes) of different concentrations (from 1 nM to 3 µM) of [Ac-D(2)Nal[1], 4Cl-D-Phe[2], D(3)-Pal[3,6], Arg[5], D-Ala[10]]-mGnRH (analog L) on GTH-II release (a, b) from perifused pituitary fragments collected from sexually recrudescent goldfish (GSI = 3.8 ± 0.5%). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II release responses to different concentrations of analog L were quantified as % prepulse and presented as mean ± SEM (c). Significant differences between responses at a given concentration are indicated by separate lines on top of each concentration (ANOVA and Fisher's LSD test).

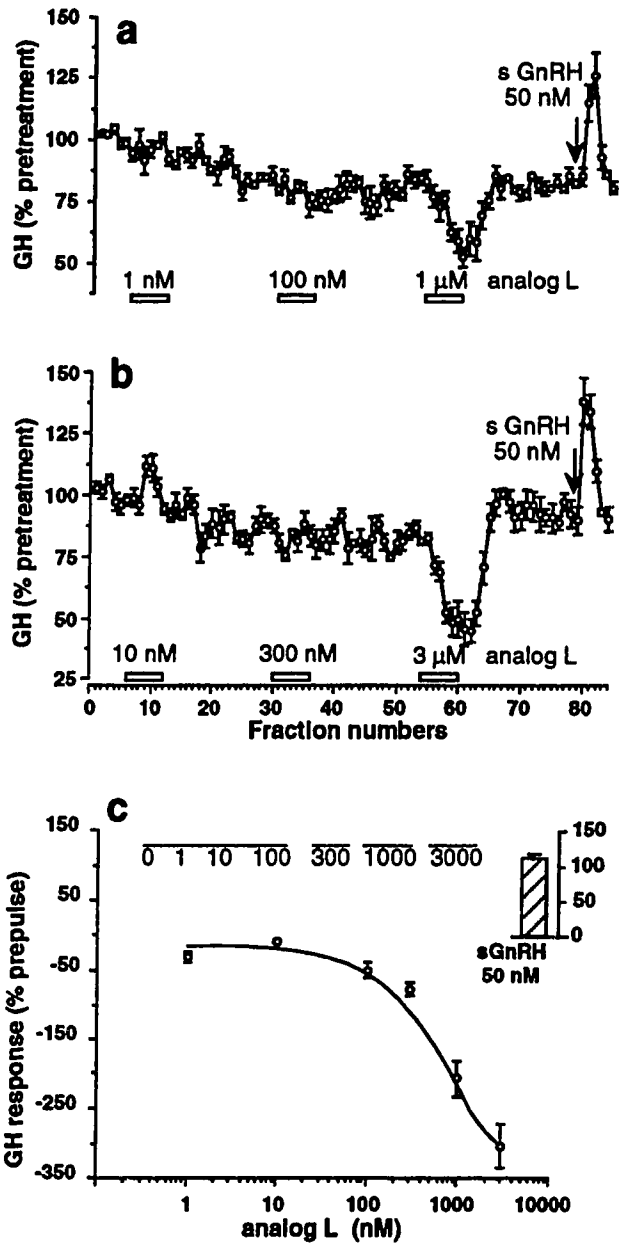


Fig. 4.3 The effects of prolonged treatment (30 min; open boxes) of different concentrations (from 1 nM to 3 µM) of [Ac-D(2)Nal1, 4Cl-D-Phe2, D(3)-Pal3,6, Arg5, D-Ala10]-mGnRH (analog L) on GH release (a, b) from perifused pituitary fragments collected from sexually recrudescent goldfish (GSI = 3.8 ± 0.5%). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GH release responses to different concentrations of analog L were quantified as % prepulse and presented as mean ± SEM (c). Significant differences between responses at a given concentration are indicated by separate lines on top of each concentration (ANOVA and Fisher's LSD test).

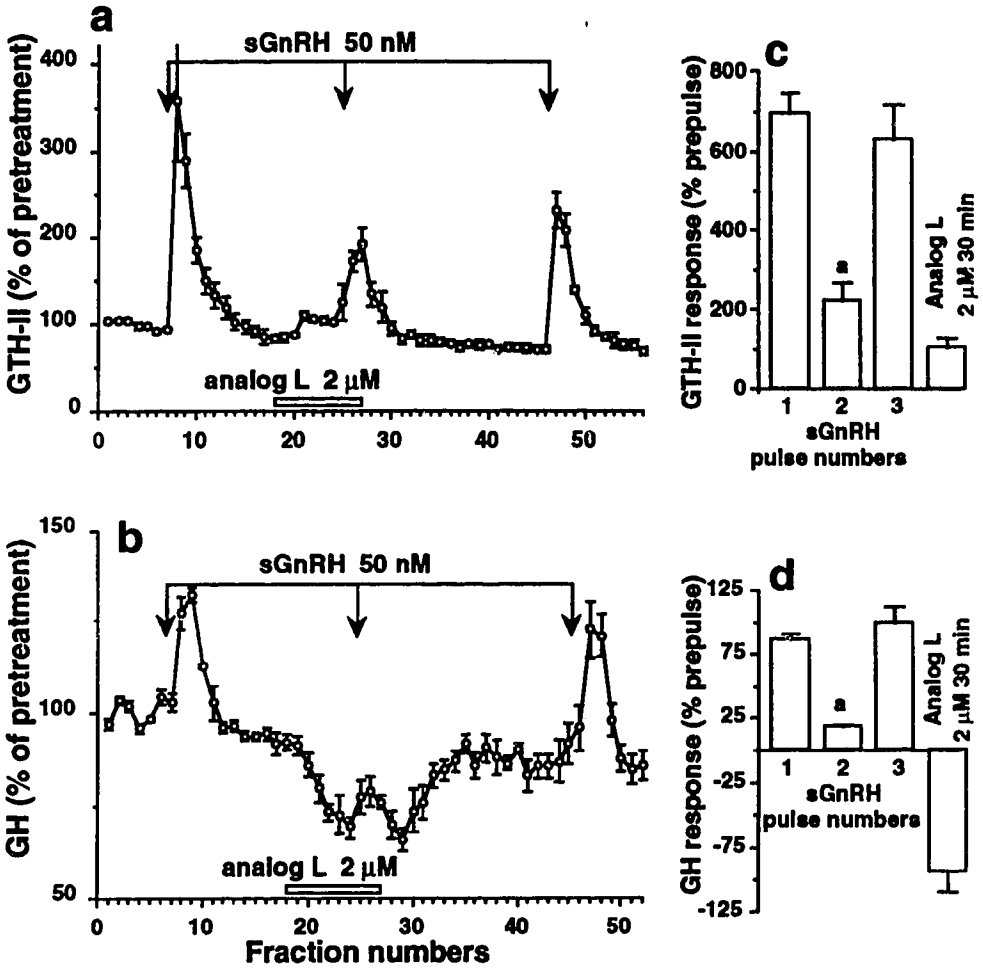


Fig. 4.4 The effects of repeated 2 min pulses (arrows) of 50 nM sGnRH on GTH-II release (a) and GH release (b) from perifused pituitary fragments collected from sexually regressed goldfish (GSI = <2%), with the second pulse given in the presence (45 min; open box) of 2 µM [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses to three repeated pulses of sGnRH were quantified as % prepulse and presented as mean ± SEM (shown in the first three columns in Fig. c, d). The hormonal responses to 2 µM concentration of analog L (first 30 min of exposure) were quantified as % prepulse and presented as mean ± SEM (shown in the fourth column in Fig. c, d).

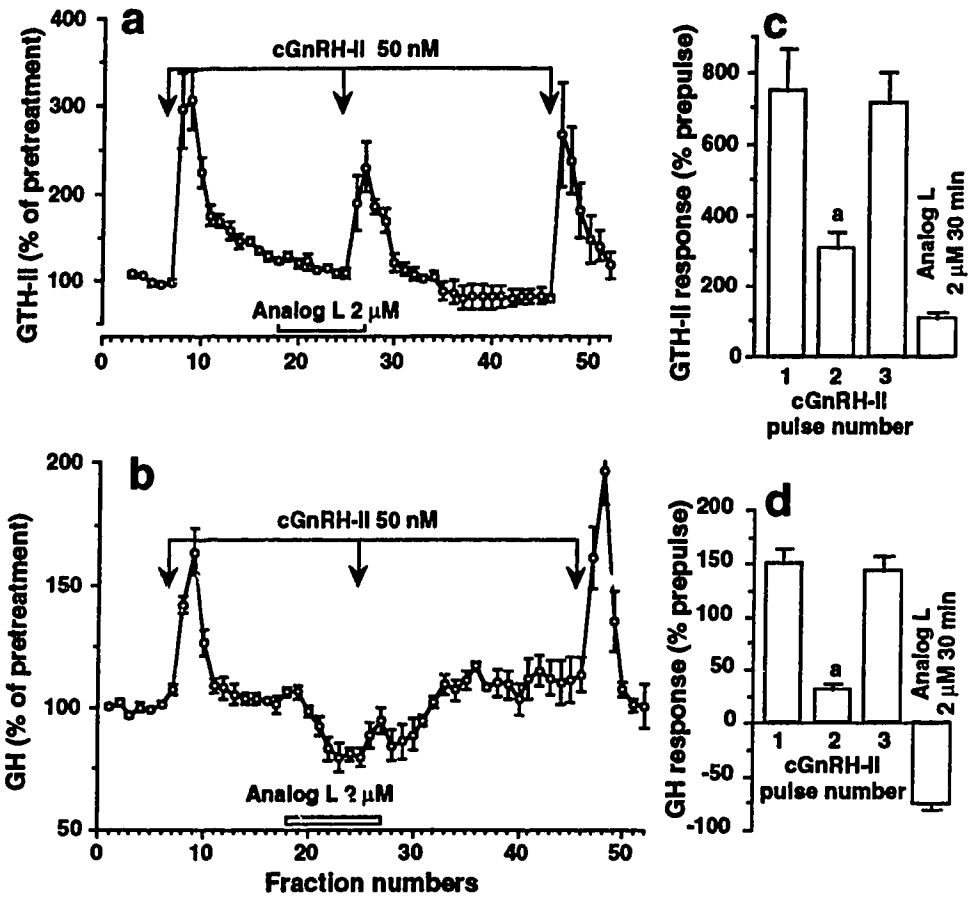


Fig. 4.5 The effects of repeated 2 min pulses (arrows) of 50 nM cGnRH-II on GTH-II release (a) and GH release (b) from perifused pituitary fragments collected from sexually regressed goldfish (GSI = <2%), with the second pulse given in the presence (45 min; open box) of 2 μM [Ac-D(2)Nal1, 4Cl-D-Phe2, D(3)-Pal3,6, Arg5, D-Ala10]-mGnRH (analog L). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses to three repeated pulses of cGnRH-II were quantified as % prepulse and presented as mean ± SEM (shown in the first three columns in Fig. c, d). The hormonal responses to 2 μM concentration of analog L (first 30 min of exposure) were quantified as % prepulse and presented as mean ± SEM (shown in the fourth column in Fig. c, d)

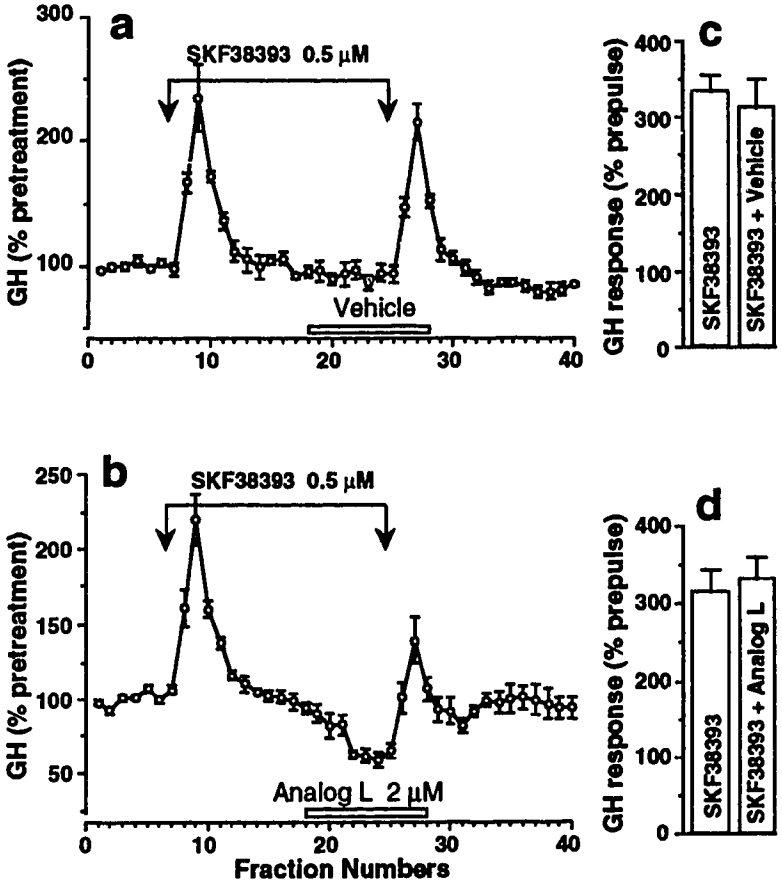


Fig. 4.6 The effects of repeated 2 min pulses (arrows) of 50 µM SKF38393 (dopamine D1 agonist) on GH release from perifused pituitary fragments collected from sexually recrudescent goldfish (GSI = 3.8 ± 0.5%), with the second pulse given in the presence (50 min; open box) of vehicle (a) or 2 µM [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L; b). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GH release responses to two repeated pulses of SKF38393 were quantified as % prepulse and presented as mean ± SEM (c, d). In quantification of GH response to SKF38393 in presence of analog L (b, d) the increase in GH following termination of analog L treatment (fraction 32 onwards) was not considered as part of the response.

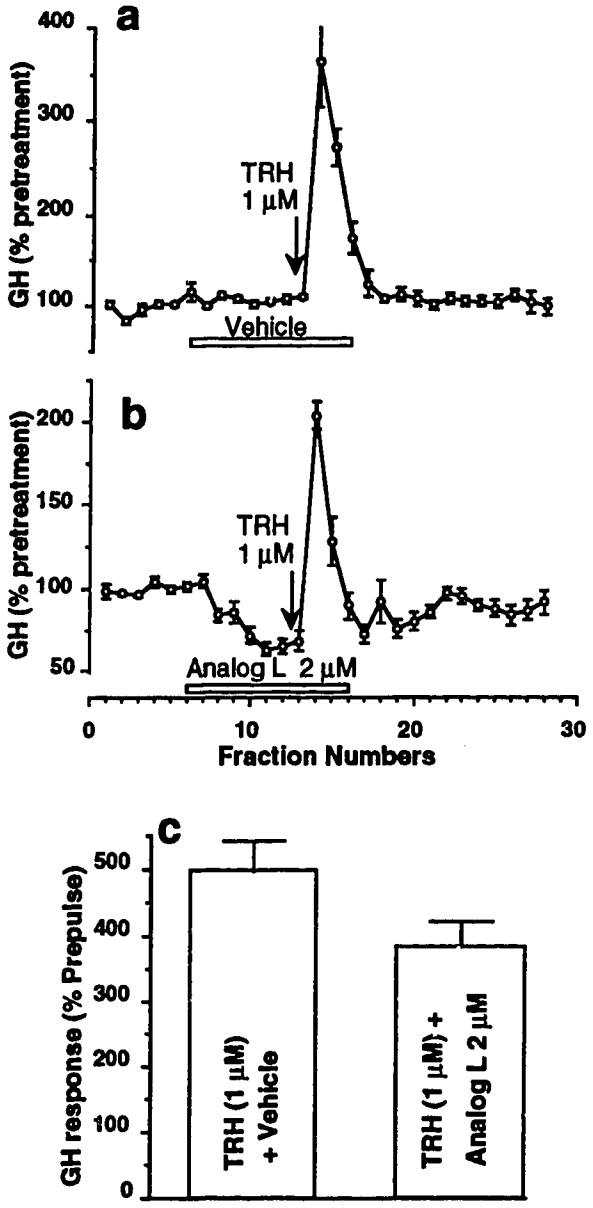


Fig. 4.7 The effects of 2 min pulses (arrows) of 1 μM thyrotropin-releasing hormone (TRH) on GH release from perifused pituitary fragments collected from sexually recrudescent goldfish (GSI = 3.8 ± 0.5%), given in the presence (50 min; open box) of vehicle (a) or 2 μM [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L; b). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GH release responses to pulses of TRH were quantified as % prepulse and presented as mean ± SEM (c). In quantification of GH response to TRH in the presence of analog L (b, c) an increase in GH release following termination of analog L treatment (fraction 18 onwards) was not considered as part of the response.

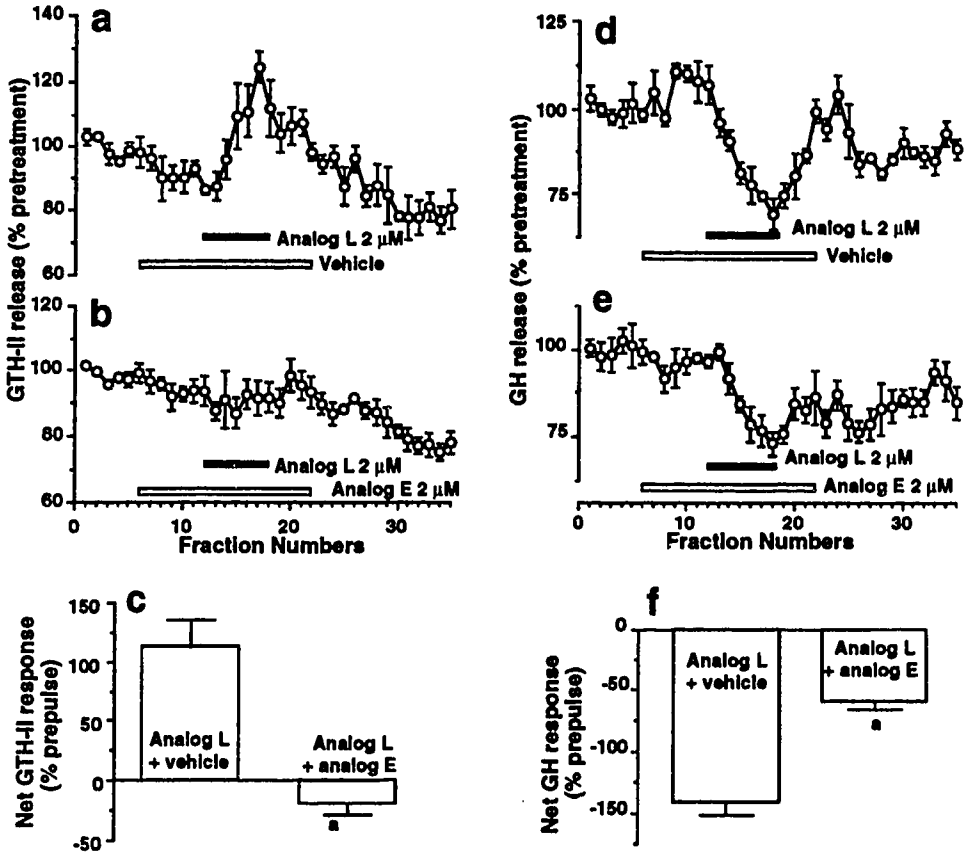


Fig. 4.8 The effects of prolonged treatment (30 min; closed box) of 2 μM [Ac-D(2)Nal1, 4Cl-D-Phe2, D(3)-Pal3,6, Arg5, D-Ala10]-mGnRH (analog L) given in the presence (80 min; open box) of vehicle (a, d) or 2 μM [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; b, e) on GTH-II (a, b) and GH release (d, e) from perifused goldfish pituitary fragments. The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses were quantified as % prepulse and presented as mean ± SEM (c, f).

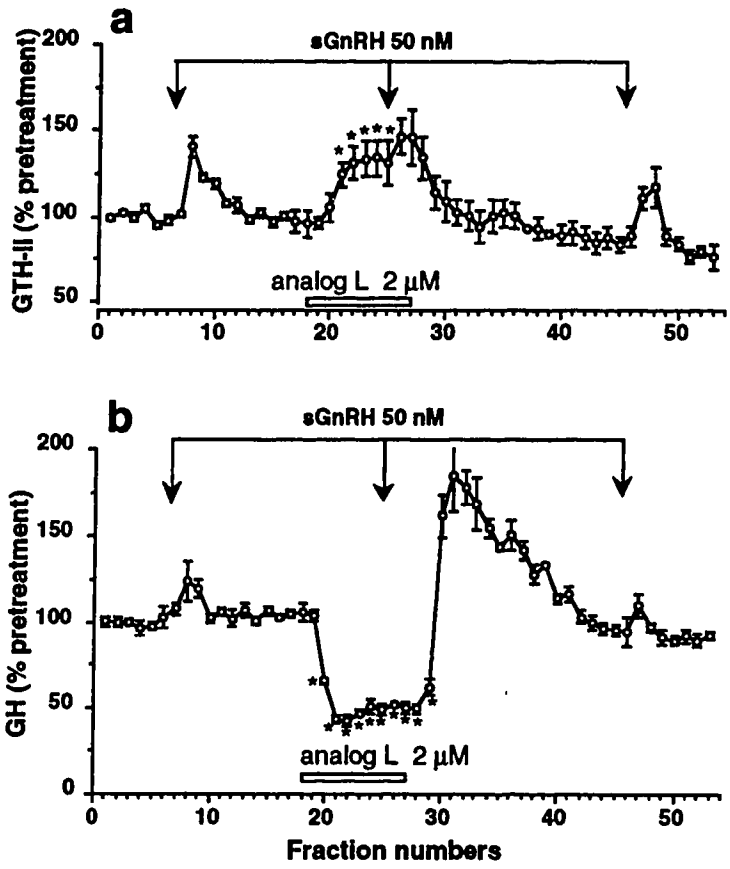


Fig. 4.9 The effects of repeated 2 min pulses (arrows) of 50 nM sGnRH on GTH-II release (a) and GH release (b) from enzymatically dispersed goldfish pituitary cells, with the second pulse given in the presence (45 min; open box) of 2 µM [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D(3)-Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). A significant increase in GTH-II release and a significant decrease in GH release in response to analog L treatment, compared to pretreatment level (ANOVA followed by Fisher's LSD test) is indicated by * mark.

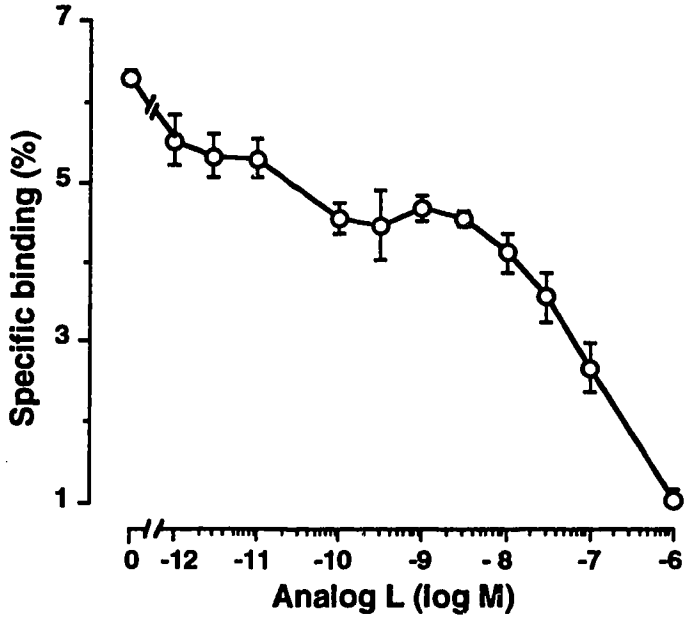


Fig. 4.10 The binding of ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH (sGnRH-A), in % of specific binding (% total binding - % binding in presence of 10^{-6} M sGnRH-A) to crude goldfish pituitary membrane preparations in the absence and presence of increasing concentrations of analog L. Each value represents the mean ± SEM of 9-12 treatments.

References

Badger, T. M., Crowley, W. F., Millard, W. J., Mak, P., and Babu, G. N. (1987). Growth hormone secretion following gonadotropin-releasing hormone or bombesin administration in perifused rat pituitary cells. In "Program and Abstracts", 69th annual meeting of the Endocrine Society, p48 (abstract # 109).

Chang, J. P., Cook, H., Freedman, G. L., Wiggs, A. J., Somoza, G. M., De Leeuw, R., and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J. P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul. Peptides, 36, 369-378.

De Lean, A., Munson, P. J., and Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: Application of bioassay radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235, E97-E102

Folkers, K., Bowers, C., Shao-bo, X., Tang, P. -F. L., Kubota, M. (1986). Increased potency of antagonists of the luteinizing hormone releasing hormone which have D-3-Pal in position 6. Biochem. Biophys. Res. Commun. 137, 709-715.

Folkers, K., Bowers, C., Tang, P. -F. L., Feng, D., Okamoto, T., Zhang, Y., and Ljungqvist, A. (1987). Specificity of design to achieve antagonists of LHRH of increasing effectiveness in therapeutic activity. In "LHRH and its analogs: Contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr. Eds.), pp 25-36. MTP Press Ltd., Boston.

Habibi, H. R. and Peter, R. E. (1991). Gonadotropin-releasing hormone (GnRH) receptors in teleosts. In: "Proceedings of the 4th International symposium on the Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 109-113. FishSymp 91, Sheffield, UK.

Habibi, H. R., Peter, R. E., Nahorniak, C. S., Milton, R. C. L., and Millar, R.P. (1992). Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino

acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides,. 37, 271-284.

Habibi, H. R., Peter, R. E., Sokolowska, M., Rivier, J. E., and Vale, W. W. (1987). Characterization of GnRH binding to pituitary receptors in goldfish (*Carassius auratus*). Biol. Reprod. 36, 844-853.

Karten, M. J., and Rivier, J. E. (1986). Gonadotropin releasing hormone analog design. Structure-function studies towards the development of agonists and antagonists: Rationale and perspectives. Endocrine Rev. 7, 44-66.

Karten, M. J., Hook, W. A., Siraganian, R. P., Coy, D. H., Folkers, K., Rivier, J. E., and Roeske, R. W. (1987). *In vitro* histamine release with LHRH analogs. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 179-190. MTP Press Ltd., Boston.

Kim, M., Oka, Y., Amano, M., Kobayashi, M., Aida, K., and Kawashima, S. (1993). Immunocytochemical localization of sGnRH and cGnRH-II in the goldfish brain and effects of olfactory tract section on the distribution of GnRH. In "Program, XII International Congress of Comparative Endocrinology". pp A-82 (abstract).

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

King, J. A., Davidson, J. S., and Millar, R. P. (1988a). Interaction of endogenous chicken gonadotrophin-releasing hormone-I and -II on chicken pituitary cells. J. Endocrinol. 177, 43-49.

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

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P. Y., Loir, M., Mourot, B., and Weil, C. (1993). Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem. 11, 219-232.

Lin, X. W., Lin, H. R., and Peter, R. E. (1993). Growth hormone and gonadotropin secretion in the common carp (*Cyprinus carpio* L): *In vitro* interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen. Comp. Endocrinol. 89, 62-71.

Marchant, T. A., Chang, J. P., Nahorniak, C. S., and Peter, R. E. (1989). Evidence that

GnRH also functions as a growth hormone releasing factor in the goldfish. Endocrinology, 124, 2509-2518.

Marchant, T. A., Fraser, R. A., Andrews, P. C., and Peter, R. E. (1987). The influence of mammalian and teleost somatostatins on the secretion of growth hormone from goldfish (*Carassius auratus L*) pituitary fragments *in vitro*. Regul. Peptides, 17, 41-52.

McPherson, G. A. (1985). Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. J. Pharmacol. Methods 14, 213-218.

Millar, R. P., and King, J. A. (1984). Structure-activity relations of LHRH in birds. J. Exp. Zool. 232, 425-430.

Müller, E. E. (1987). Neural control of somatotropic function. Physiol. Rev. 67, 962-1053.

Munson, P. J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand binding systems. Analytical Biochem. 107, 220-239.

Murthy, C. K., Nahorniak, C. S., Rivier, J. E., and Peter, R. E. (1993). *In vitro* characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish, *Carassius auratus*. Endocrinology, 133, 1633-1644.

Murthy, C. K., Peter, R. E., Rivier, J. E., and Vale, W. W. (1991). Characterization of gonadotropin-releasing hormone (GnRH) receptor antagonists in goldfish (*Carassius auratus*). In: "Proceedings of the 4th International Symposium on The Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 63. FishSymp 91, Sheffield, UK.

Nekola, M. V., Horvath, A., Ge, L. J., Coy, D. H., Schally, A. V.(1982). Suppression of ovulation in the rat by an orally active antagonist of luteinizing hormone-releasing hormone. Science, 218, 160-161.

Nestor, J. J. Jr. (1987). Development of LHRH antagonists. In "LHRH and its analogs-contraceptive and therapeutic applications" Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 3-16. MTP Press Ltd., Boston.

Panerai, A. E., Cocchi, D., Gil-ad, I., Locatelli, V., Rossi, G. L., and Müller, E. E. (1976). Stimulation of growth hormone release by luteinizing hormone-releasing hormone in the hypophysectomized rat bearing an ectopic pituitary. Clin. Endicrinol. 5,

717-722.

Peng, C., Huang, Y. P., and Peter, R. E. (1990). Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology, 52, 28-34.

Peng, C., Humphries, S., Peter, R. E., Rivier, J. E., Blomqvist, A. G., and Larhammar, D. (1993). Actions of goldfish neuropeptide Y on the secretion of growth hormone and gonadotropin-II in female goldfish. Gen. Comp. Endocrinol. 90, 306-317.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In:"Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, and M. H. Stetson, Eds.), pp. 393-398. Wiley-Liss Inc. NY.

Peter, R. E., Nahorniak, C. S., Chang, J. P., and Crim, L. W. (1984). Gonadotropin release from the pars distalis of goldfish, *Carassius auratus* transplanted beside the brain or into the brain ventricles; Additional evidence for gonadotropin release-inhibitory factor. Gen. Comp. Endocrinol. 55, 337-346.

Rivier, J., Rivier, C., Perrin, M., Porter, J., Vale, W. W. (1984). LHRH analogs as anti-ovulatory agents. In "LHRH and its analogs-contraceptive and therapeutic applications" Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 11-22. MTP Press Ltd., Boston.

Schutz, W., and Freissmuth, M. (1992). Reverse intrinsic activity of antagonists on G protein-coupled receptors. Trends Pharmacol. Sci. 131, 376-379.

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

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

Trudeau, V. L., Somoza, G. M., Nahorniak, C. S., and Peter, R. E. (1992). Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology, 56, 483-490.

Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1992). Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the dopamine D1 receptors. Endocrinology, 130, 1201-1210.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1993a). *In vitro* and *in vivo* evidence that dopamine exerts growth hormone-releasing activity in goldfish. Am. J. Physiol. 264, E925-932.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1993b). Interactions of somatostatin, gonadotropin-releasing hormone, and the gonads on dopamine-stimulated growth hormone release in the goldfish. Gen. Comp. Endocrinol. 92, 366-378.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1993c).Characterization of D1 receptors mediating dopamine-stimulated growth hormone release from pituitary cells of the goldfish, *Carassius auratus*. Endocrinology, 133, 577-584.

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 Five

A new gonadotropin-releasing hormone (GnRH) superagonist in goldfish: influence of dialkyl-D-homoarginine at position 6 on gonadotropin-II and growth hormone release[1]

Introduction

In goldfish and other teleost fishes gonadotropin-II (GTH-II) secretion is under dual control of stimulation by gonadotropin-releasing hormone (GnRH) and inhibition by dopamine (DA) (for review Peter et al., 1986, 1991). In goldfish, the two primary native forms of gonadotropin-releasing hormones (GnRH), salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II) (Yu et al., 1988) stimulate GTH-II and also growth hormone (GH) release both *in vitro* and *in vivo* (Marchant et al., 1989; Chang et al., 1990; Habibi et al., 1992; for review Peter et al., 1990, 1991). Structure-activity studies of GnRH analogs in goldfish have indicated that substitution of D-arginine^6 along with proline^9-NHEt enhances GTH-II releasing potency (Peter, 1986; Peter et al., 1991). Thus, [D-Arg^6, Pro^9-NHEt]-sGnRH (sGnRH-A) is the most potent GnRH analog known for stimulation of GTH-II release in goldfish.

In our previous study, initial screening of GnRH antagonists for their actions on GTH-II and GH release indicated that many mammalian GnRH antagonists, especially those having D-Arg^6, often have moderate to strong GTH-II and GH releasing activity (Murthy et al., 1993; see Chapter 2). Among the analogs tested [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH (analog J) and [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH (analog K) strongly stimulated GTH-II release (Murthy et al., 1993; see Chapter 2). In contrast to these results, both of these analogs act as potent GnRH antagonists in rat (Rivier et al., 1984; Nestor et al., 1988).

---

[1]A version of this chapter has been submitted for publication: CK Murthy, RJ Turner, JJ Nestor Jr., RE Rivier, RE Peter (1994), Regul. Peptides (submitted).

[Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH, known as Detirelix (analog K of Chapter 2), has potent, and prolonged inhibition of LH release in rat (Nestor et al., 1988, 1992), dog (Vickery et al., 1987), monkey (Bremner et al., 1987; Khurshid et al., 1991), and man (Pavlou et al., 1987).

In the present study, the GTH-II and GH stimulatory actions of analogs J and K were further tested under pulses and long term exposure using perifused goldfish pituitary fragments. Further, to study the site of action, the ability of a 'true' GnRH antagonist to inhibit analog K stimulated GTH-II and GH release was investigated. Since analog K, having [D-hArg(Et$_2$)^6], strongly stimulated GTH-II release, we hypothesized that [D-hArg(Et$_2$)^6]-substituted GnRH analogs may act as superagonists in goldfish. The relative potency of several [D-hArg(Et$_2$)^6] or [D-hArg(CH$_2$CF$_3$)$_2^6$] substituted GnRH analogs in stimulating GTH-II and GH release from the goldfish pituitary was determined.

Materials and Methods

Experimental animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries, Stoutland, MO. The fish were maintained in flow-through aquaria (1800 liters) at 17 ± 1°C under a simulated natural photoperiod of Edmonton for at least 2-3 weeks prior to experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish of both sexes, with body weight ranging from 20-35 g were used in the present study. The sexual maturity of fish was assessed by measuring the gonadosomatic index (GSI= weight of gonad/ total body weight X 100).

Reagents and test substances sGnRH, and cGnRH-II were purchased from Peninsula Laboratories Inc., Belmont, CA, and [D-Arg^6, Pro^9-NHEt]-sGnRH was a gift from Syndel laboratories Ltd., Vancouver, BC, Canada. [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH, [D-hArg(Et$_2$)^6]-cGnRH-I, [D-hArg(Et$_2$)^6]-mGnRH, [D-hArg(CH$_2$CF$_3$)$_2^6$]-mGnRH and [D-hArg(CH$_2$CF$_3$)$_2^6$, Pro^9-NHEt]-mGnRH were gift of Dr. J. J. Nestor Jr., The Institute of Bio-Organic Chemistry, Syntex Research, Palo Alto, CA., USA. All these peptides were dissolved in 0.1 M acetic acid and stored at -25°C as aliquots of 50 µM. The aliquots were diluted with perifusion medium to required concentrations immediately prior to use in experiments. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E), [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH (analog J) and [Ac-

D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH (analog K) were gift of Dr. J. E. Rivier, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA., USA. These analogs were dissolved in a primary solvent containing propylene glycol and physiological saline at a ratio of 60:40, and subsequently diluted to required concentrations with perifusion medium; the final propylene glycol concentrations were less than 0.5%.

Perifusion of the pituitary fragments *In vitro* experiments were conducted using a validated pituitary fragments perifusion system (Marchant et al., 1989) with minor changes described earlier (see Chapter 4). Briefly, goldfish pituitary glands were chopped into fragments (0.2 mm^3) using a McIlwain tissue chopper. The fragments (three pituitary equivalents per chamber) were washed with Medium 199 (M 199) and placed in 0.5 ml micro-chambers (Endotronics Inc., Minneapolis, MN) between two 13 mm filters; 8 microns pore size for inlet (Millipore Inc., Bedford, MA) and 5 microns pore size for outlet (Micron Separations Inc., Westboro, MA) with a continuous flow of medium (M199) at 18°C. These filters were supported by circular stainless steel screens with a pore size of 450 microns. The reservoirs, holding perifusion medium or drugs were attached to a 3-way valve. From the 3-way valve the drug or medium was delivered to the perifusion chambers by a tubing passing through an ISMTEC multichannel cartridge pump (Cole-Parmer Instruments Corporation, Chicago, IL). The fragments were perifused overnight (at least 15 h) at a flow rate of 5 ml/h. Thereafter, the incubation was continued using Hank's balanced salt solution supplemented with 25 mM HEPES and 0.1% BSA at a flow rate of 15 ml/h for 2 h before starting the experiment. Five minute fractions of perifusate were collected, frozen and stored at -25°C for hormone measurement.

Radioimmunoassay GTH-II levels in perifusate were measured by validated RIA specific for GTH-II (Peter et al., 1984; Van Der Kraak et al., 1992). The GH levels were measured by using a goldfish GH RIA described by Murthy et al. (1993).

Data analysis To provide a mean hormone release response, the GTH-II or GH values were expressed as percentage of pretreatment mean (of 6 fractions prior to first pulse) for

each column and pooled from 4 columns. The quantification of the hormone response was done as described earlier (Murthy et al. 1993; see Chapter 2). Briefly, the average hormone level of the 3 fractions immediately preceeding each pulse (prepulse mean) was treated as basal, and the hormone values following a GnRH pulse were expressed as a percentage of this prepulse mean. Post pulse fractions with hormone contents greater than one standard error (SEM) above the prepulse mean were considered to be a part of the response. The response (expressed as % prepulse) above the prepulse mean from the fractions considered as part of the response were added to get the net response in a particular column. Statistical differences between treatment and control groups were assessed by ANOVA followed by Fisher's LSD test ($p < 0.05$). For the dose-response studies, non-linear least square curve fitting program ALLFIT (De Lean et al., 1978) was used to obtain ED50 ± approximate error values as a measure of potency. When the hormone release response failed to reach a plateau, the response at the highest concentration of peptide tested was taken as maximum response. The significant differences between the potency of analogs were compared by constructing 95% confidence limits following log transformation.

Results

Dose-related actions of analogs J and K on GTH-II and GH release: The perifused goldfish pituitary fragments collected from sexually mature goldfish (GSI = 8.3 ± 1%) were exposed to 2 min pulses of increasing doses of analog J or K or native sGnRH at 75 min intervals. Analogs J and K, and sGnRH stimulated GTH-II and GH release in a dose-dependent manner (Figs. 5.1 and 5.2). Compared to sGnRH, analogs J and K were significantly lower in potency to stimulate GTH-II and GH release in terms of ED50, minimum dose required to cause significant hormone release and maximum response (Fig. 5.2a, b; Table 5.1); the minimum concentrations required to cause significant GTH-II and GH release were 1000 nM (analog J), 100 nM (analog K) and 1 nM (sGnRH). In terms of the maximum GTH-II and GH release responses, analogs J and K caused significantly lower magnitude of hormone release than sGnRH (Fig. 5.2a, b; Table 5.1), suggesting that analogs J and K are "partial antagonists" (antagonists with agonistic activity) on GTH-II and GH release in goldfish.

The effects of prolonged treatment (40 min exposures, 2 hr interval) of increasing

concentrations of analog K and sGnRH on GTH-II and GH release were tested. Both analog K and sGnRH stimulated GTH-II and GH release in a dose-dependent manner (Fig. 5.3). Although analog K was less effective in stimulating hormone release, at a 1000 nM concentration it caused GTH-II release response which was 63 ± 3 % of the GTH-II response to 1000 nM sGnRH (Fig. 5.3c). At 1000 nM concentration both analog K and sGnRH caused GH release of similar magnitude (Fig. 5.3f).

Probable site of action of analogs J and K: First, we tested the interactions of analogs J and K on native cGnRH-II stimulated GTH-II release. In control columns, three repeated pulses of 20 nM cGnRH-II caused GTH-II release of similar magnitude (results not shown). Both analogs stimulated GTH-II release from the goldfish pituitary fragments (Figs. 5.4a, b). Further, in continuous presence of 2 μM analog J or K, the GTH-II release responses induced by 20 nM of cGnRH-II were significantly reduced compared to the initial cGnRH-II pulses (Fig. 5.4). Similarly, both analogs suppressed cGnRH-II induced GH release (results not presented) indicating interaction of analogs J and K with GnRH receptors on gonadotrophs and somatotrophs in the goldfish pituitary. Even at 90 min after termination of treatment of analogs J and K, GTH-II responses to cGnRH-II pulse were significantly lower than that to the initial pulse, suggesting prolonged action of analogs J and K.

Further, we tested the ability of a 'true' GnRH antagonist in goldfish, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E ; see Chapter 2), to suppress GTH-II and GH release stimulated by analog K. Treatment of pituitary fragments with analog K (2 μM for 10 min) in continuous presence of vehicle caused an increase in GTH-II and GH release (Fig. 5.5a, d). However, in continuous presence of analog E, the release of GTH-II and GH stimulated by analog K were significantly inhibited (Fig. 5.5).

Actions of dialkyl-D-homoarginine^6 substituted analogs on GTH-II and GH release: Since D-homo arginine^6 substituted mammalian GnRH antagonists act as "partial antagonists" with strong GTH-II and GH releasing activity in goldfish, we postulated that incorporation of D-homoarginine^6 may favor developing potent GnRH agonists in the goldfish. The effects of 2 min pulses given at 90 min intervals of D-homoarginine^6

substituted sGnRH, mGnRH and cGnRH-I analogs on GTH-II and GH release from perifused goldfish pituitary fragments were tested. All the analogs tested stimulated GTH-II and GH release in dose-dependent manner (Figs. 5.6 to 5.9). In the first series of experiments [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH strongly stimulated GTH-II release in terms of both minimum concentrations required to elicit a significant response (1 nM as against 10 nM for sGnRH) and ED50 values (Fig. 5.6 and Table 5.2). The magnitude of GTH-II release at high concentrations (100 and 1000 nM) of [D-hArg(Et_2)^6]-cGnRH-I was similar to that of sGnRH, [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH; however, at lower concentrations of 1 and 10 nM [D-hArg(Et_2)^6]-cGnRH-I stimulated GTH-II release of significantly lower magnitude compared to [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH (Fig. 5.6). [D-hArg(Et_2)^6]-cGnRH-I was less potent than sGnRH, in terms of ED50, in stimulating GTH-II release (Table 5.2). In the next series of experiments only [D-hArg(Et_2)^6]-mGnRH was more potent than sGnRH in stimulating GTH-II release (Fig. 5.7; Table 5.2). The other two analogs of mGnRH, [D-hArg(CH_2CF_3)$_2^6$]-mGnRH and [D-hArg(CH_2CF_3)$_2^6$, Pro^9-NHEt]-mGnRH caused significantly lower GTH-II release at 1000 nM compared to sGnRH, and were equipotent (in terms of ED50) to sGnRH in stimulating GTH-II release (Fig. 5.7; Table 5.2). Overall, [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH was the most potent to stimulate GTH-II release in goldfish, being 2-3 times and 6-8 times more potent than [D-Arg^6, Pro^9-NHEt]-sGnRH and sGnRH, respectively (Table 5.2).

The growth hormone release responses to these peptides show significant differences from that of GTH-II release responses. Both [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH strongly stimulated GH release (Fig. 5.8), being more potent than sGnRH (Fig. 5.8; Table 5.2). cGnRH-I-A was significantly lower in potency in terms of minimum concentration required to cause significant GH release (100 nM as against 10 nM for sGnRH) and ED50 values (Fig. 5.8; Table 5.2). The three mGnRH analogs caused significant GH release only at higher concentrations of 100 and 1000 nM (Fig. 5.9), caused lower GH release at 1000 nM than sGnRH (except for [D-hArg(CH_2CF_3)$_2^6$, Pro^9-NHEt]-mGnRH, which had a maximum GH stimulation comparable to that of sGnRH), and were lower in potency compared to sGnRH (Table 5.2).

Discussion

Both [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH (analog J) and [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH (analog K) stimulated GTH-II and GH release in a dose-related fashion from the perifused goldfish pituitary fragments. However, the potencies of these two analogs to stimulate GTH-II and GH release, in terms of minimum effective dose, maximum response and ED50 values, were significantly lower than that of native sGnRH. In the continuous presence of analogs J and K, GTH-II and GH release stimulated by a pulse of cGnRH-II (present study) and sGnRH (Murthy et al., 1993; see Chapter 2) were significantly suppressed, implying the interaction of analogs J and K with GnRH receptors. This hypothesis is further supported by the observation that 2 μM analog E, a 'true' GnRH antagonist in goldfish (see Chapter 2), significantly suppressed the GTH-II and GH stimulatory actions of 2 μM analog K. In a preliminary study, analog K displaced ^{125}I-[D-Arg^6, Pro^9-NHEt]-sGnRH bound to crude goldfish pituitary membrane preparations (Murthy CK, Peter RE, unpublished results). Taken together, these results indicate that analogs J and K, by interacting with GnRH receptors on the goldfish gonadotrophs and somatotrophs, are "partial antagonists", antagonizing the actions of native GnRH peptides and having some independent agonist activity.

Interestingly, both analogs J and K act as potent GnRH antagonists in mammals (Rivier et al., 1984; Nestor et al., 1988, 1992; see Introduction). Similar to our findings, many mGnRH antagonists stimulate LH release from chicken gonadotrophs (Millar and King, 1984; King et al., 1988). The differences in the actions of mGnRH antagonists between species suggests functional differences in GnRH receptors between species, and supports our hypothesis that the ligand structure requirements for GnRH antagonists are different for rat and goldfish, and also between other species (see Chapter 4). One important observation is that most of the mGnRH antagonists having agonistic activity in goldfish have D-arginine or D-homoarginine at position 6 (see Chapter 2; present study). In mammals, modifications at position 6 plays a critical role in enhancing the potency of both agonists and antagonists by increasing resistance to enzymatic cleavage, providing structural stability, and increasing the receptor binding affinity (Loumaye et al., 1982; for review Karten and Rivier. 1986). Recent studies in fishes have indicated similar

importance of position 6 substitution in enhancing the receptor binding affinity (Habibi et al., 1989) and resistance to enzymatic degradation (Zohar et al., 1990; for review Peter et al., 1991). The agonistic activity of D-Arg^6 substituted mGnRH antagonists in goldfish demonstrates that modifications at this position also play an important role in determining the nature of intrinsic activity of GnRH peptides in goldfish. In lampreys substitution of naturally occurring Glu^6 (in lamprey GnRH-I) with Gly^6 resulted in total loss of biological activity (unpublished results, quoted in Sower et al., 1993). In rats substitutions at position 6 are also suggested to alter the intrinsic activity of reduced size GnRH peptides (Haviv et al., 1989a, b).

Since analog K, having [D-hArg(Et$_2$)^6], exhibited relatively strong GTH-II releasing activity, we postulated that incorporation of [D-hArg(Et$_2$)^6]-may enhance the agonistic activity of other analogs. The results demonstrated that [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH is a potent stimulator of GTH-II release from the perifused goldfish pituitary fragments. In stimulating GTH-II release, [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH is at least 2-3 times more potent than [D-Arg^6, Pro^9-NHEt]-sGnRH, which was previously the most potent GnRH analog known in goldfish (for review Peter et al., 1990, 1991), and at least 8-10 times more potent than native sGnRH. The rank order of potency to stimulate GTH-II release was [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH > [D-Arg^6, Pro^9-NHEt]-sGnRH > [D-hArg(Et$_2$)^6]-mGnRH > sGnRH = [D-hArg(CH$_2$CF$_3$)$_2^6$]-mGnRH = [D-hArg(CH$_2$CF$_3$)$_2^6$, Pro^9-NHEt]-mGnRH > [D-hArg(Et$_2$)^6]-cGnRH-I. In rainbow trout and landlocked salmon also [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH were among the most potent GnRH peptides in stimulating an increase in plasma GTH levels (Crim et al., 1988). In the rat estrus suppression bio-assay [D-hArg(Et$_2$)^6, Pro^9-NHEt]-mGnRH was at least 150 times more potent than mGnRH (Nestor et al., 1984). Since [D-hArg(Et$_2$)^6] substituted analogs, besides being highly potent, are also known to have long lasting effects in rat (Nestor et al., 1984), it would be of interest to test the duration of action and potency of [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH *in vivo* in goldfish.

There are significant differences in the potency of analogs to stimulate GH release compared to GTH-II release (Table 5.2). While equipotent, both [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH were more potent than sGnRH in stimulating GH release. All other analogs, based on mGnRH and cGnRH-I were

significantly less potent in stimulating GH release, both in terms of ED50 and the maximum GH release response, compared to sGnRH. The differences in potency of these analogs in stimulating GTH-II and GH release support our earlier hypothesis that the GnRH receptors on GTH and GH cells in goldfish are functionally different (Habibi et al., 1992; see Chapter 3). It is interesting to note that among the native peptides tested sGnRH is the most potent in stimulating GH release (Chang et al., 1990; Peter et al., 1990; Habibi et al., 1992). In the present study only sGnRH, but not mGnRH, based analogs, were potent GH stimulators. This suggests that the Trp^7, Leu^8 residues in sGnRH are important in stimulating GH release and may also be essential for superactive analogs in stimulating GH release in goldfish.

In conclusion, in contrast to their actions in mammals, [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH (analog J) and [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et_2)^6, D-Ala^{10}]-mGnRH (analog K) are "partial antagonists" in goldfish, interacting with GnRH receptors in the goldfish pituitary to stimulate GTH-II and GH release independently, as well as antagonizing the actions of native GnRH peptides. These results suggest that ligand structure requirements for GnRH antagonists are different for rat and goldfish, and also between other species. The amino acid residue at position 6 appears to play a crucial role in determining the nature of intrinsic activity of an analog, besides increasing its receptor binding affinity and stability. [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH is the most potent analog in stimulating GTH-II release in goldfish; [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH and [D-Arg^6, Pro^9-NHEt]-sGnRH are equipotent in stimulating GH release.

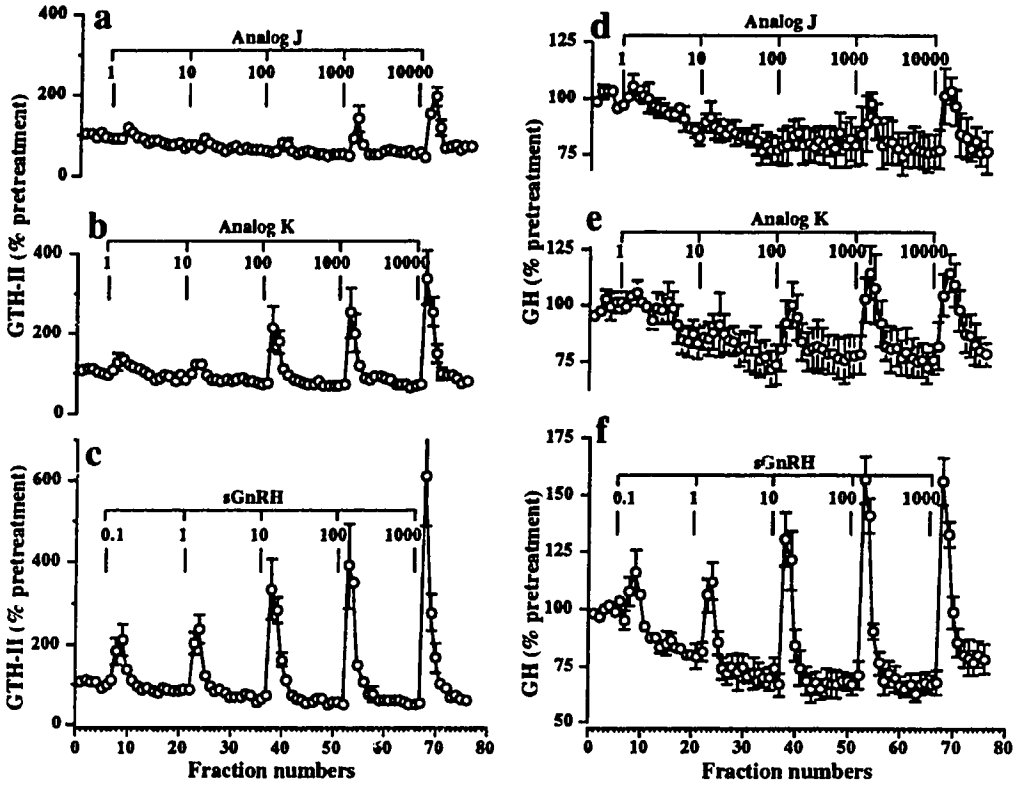


Fig. 5.1 The effects of 2 min pulses (lines) at 75 min intervals of different concentrations (from 0.1 nM to 10 μM) of [Ac-D(2)-Nal1, 4Cl-D-Phe2, D-Trp3, D-Arg6, Trp7, D-Ala10]-mGnRH (analog J), [Ac-D(2)-Nal1, 4Cl-D-Phe2, D-Trp3, D-hArg(Et_2)6, D-Ala10]-mGnRH (analog K), and sGnRH on GTH-II (a, b, c) and GH (d, e, f) release from the perifused pituitary fragments collected from sexually mature goldfish (GSI = 8.3 ± 1%). The hormone release data in ng/ml were transformed as % of pretreatment mean, pooled and expressed as mean ± SEM (n = 4).

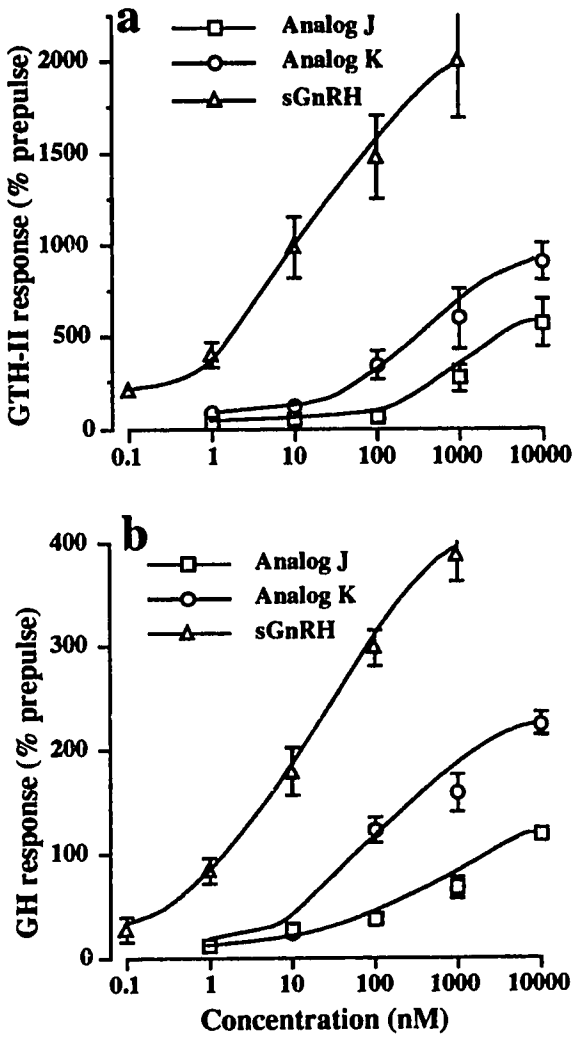


Fig. 5.2 The GTH-II and GH release responses shown in Fig. 5.1 were quantified as % prepulse and presented as mean ± SEM (a, b).

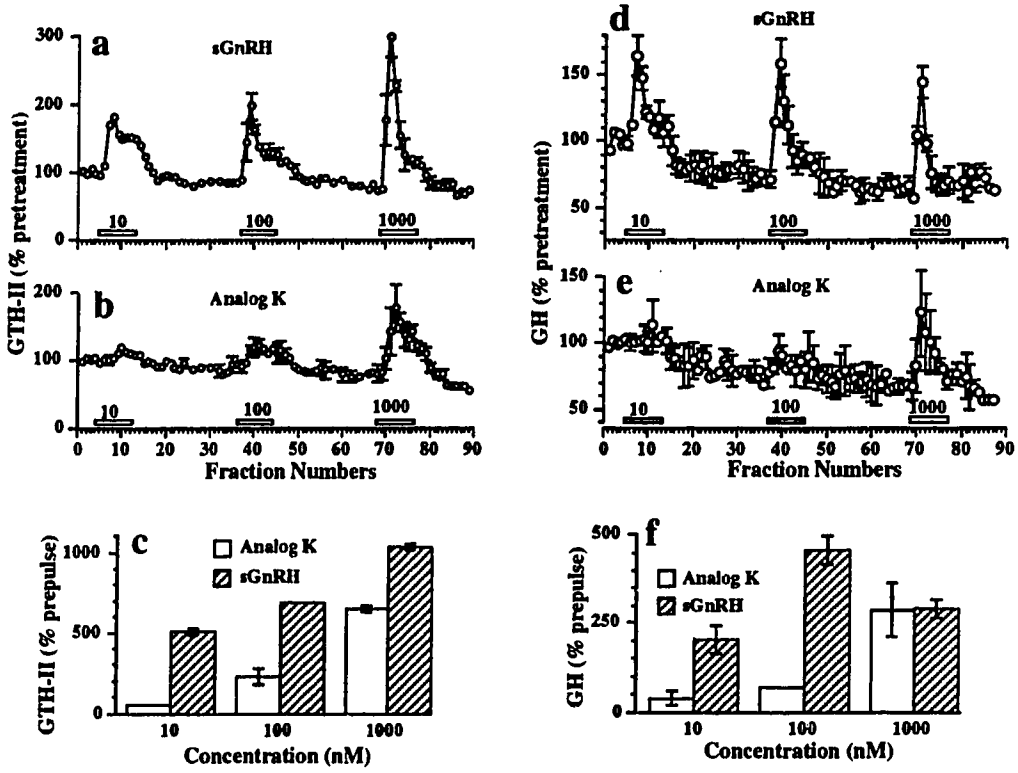


Fig. 5.3 The effects of prolonged exposure (40 min; open boxes) at 120 min intervals of increasing concentrations (from 10 to 1000 nM) of [Ac-D(2)-Nal1, 4Cl-D-Phe2, D-Trp3, D-hArg(Et2)6, D-Ala10]-mGnRH (analog K), and sGnRH on GTH-II (a, b) and GH (d, e) release from the perifused pituitary fragments obtained from sexually regressed goldfish (GSI < 2.0%). The hormone release data in ng/ml were transformed as % of pretreatment mean, pooled and expressed as mean ± SEM (n = 4). The GTH-II and GH release responses to treatments were quantified as % prepulse and presented as mean ± SEM (c, f).

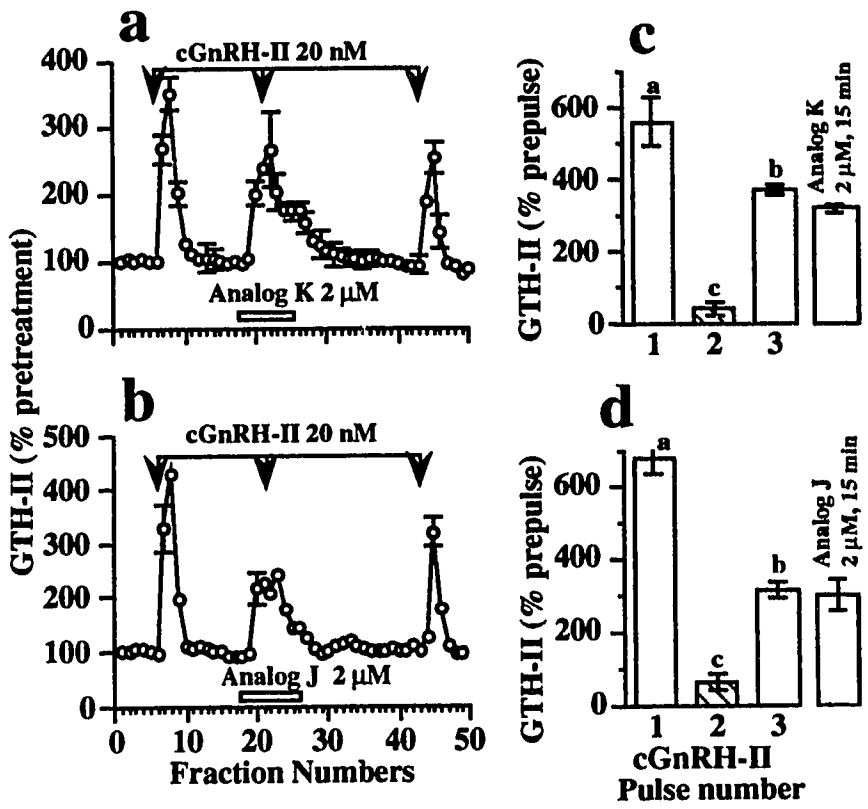


Fig. 5.4 The effects of three repeated 2 min pulses (arrows) of 20 nM cGnRH-II on GTH-II release from perifused pituitary fragments (from sexually recrudescent goldfish; GSI = 6.7 ± 0.5), with the second pulse given in the presence (45 min; open box) of 2 μM [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et$_2$)^6, D-Ala^{10}]-mGnRH (analog K; a) or [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-Arg^6, Trp^7, D-Ala^{10}]-mGnRH (analog J; b). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II release responses to three repeated pulses of cGnRH-II were quantified as % prepulse and presented as mean ± SEM (shown in the first three columns in Fig. c, d). The GTH-II responses to 2 μM concentration of analogs J and K (first 15 min of exposure) were quantified as % prepulse and presented as mean ± SEM (shown in the fourth column in Fig. c, d).

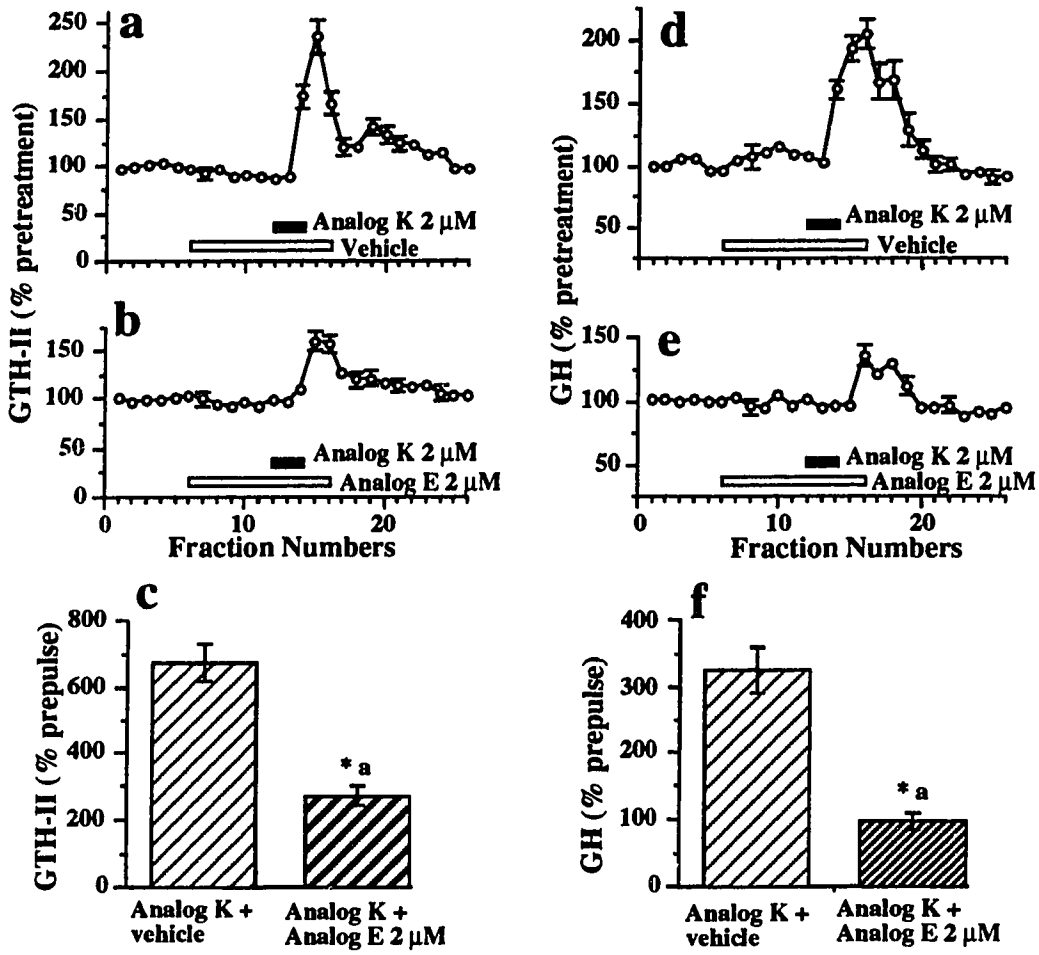


Fig. 5.5 The effects of 10 min treatment (closed box) of 2 μM [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-Trp^3, D-hArg(Et2)^6, D-Ala^{10}]-mGnRH (analog K) given in the presence (50 min; open box) of vehicle (a, d) or 2 μM [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; b, e) on GTH-II (a, b) and GH release (d, e) from perifused pituitary fragments (from sexually recrudescent goldfish; GSI = 6.7 ± 0.5). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses were quantified as % prepulse and presented as mean ± SEM (c, f).

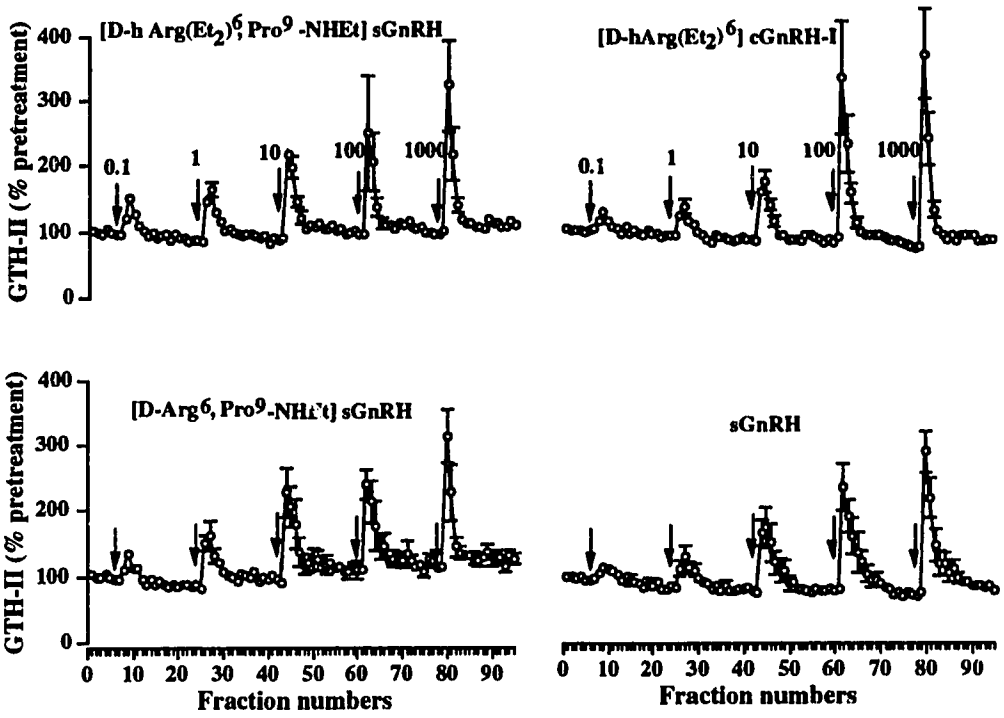


Fig. 5.6 The effects of 2 min pulses (arrows) at 90 min intervals of increasing concentrations (from 0.1 to 1000 nM) of [D-hArg(Et2)6, Pro9-NHEt]-sGnRH, [D-hArg(Et2)6]-cGnRH-I, [D-Arg6, Pro9-NHEt]-sGnRH and sGnRH on GTH-II release from the perifused pituitary fragments from sexually regressed goldfish (GSI < 2%). The hormone release data in ng/ml were transformed as % of pretreatment mean, pooled and expressed as mean ± SEM (n = 4).

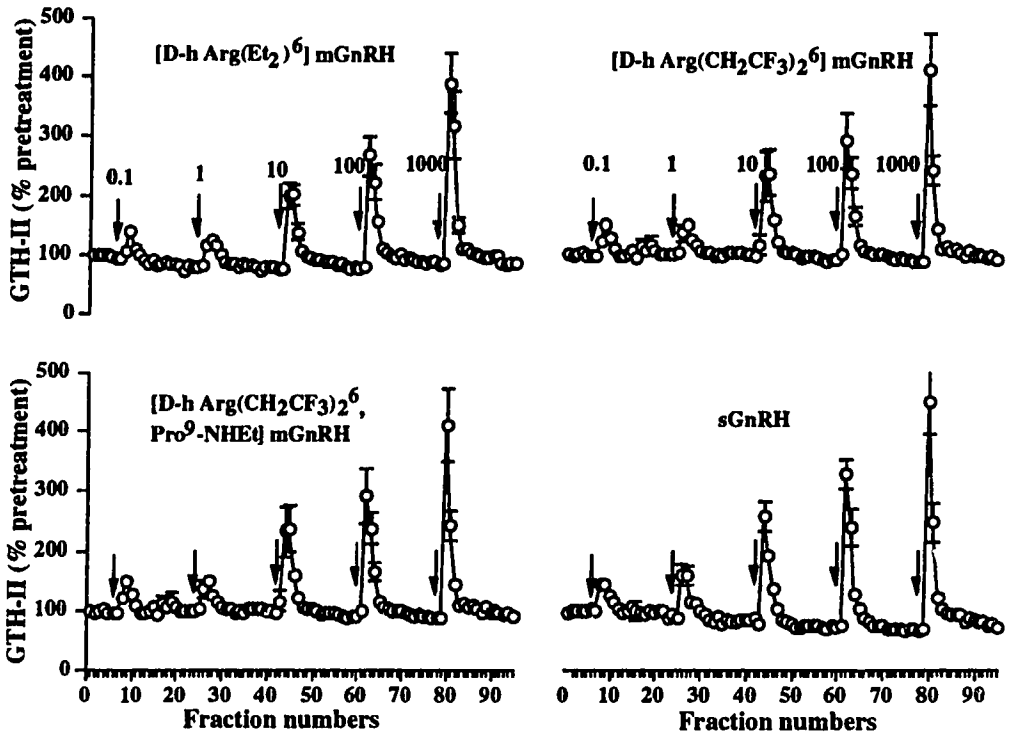


Fig. 5.7 The effects of 2 min pulses (arrows) at 90 min intervals of increasing concentrations (from 0.1 to 1000 nM) of [D-hArg(Et2)6]-mGnRH, [D-hArg(CH2CF3)26]-mGnRH, [D-hArg(CH2CF3)26, Pro9-NHEt]-mGnRH and sGnRH on GTH-II release from perifused pituitary fragments from sexually regressed goldfish (GSI < 2%). The hormone release data in ng/ml were transformed as % of pretreatment mean, pooled and expressed as mean ± SEM (n = 4).

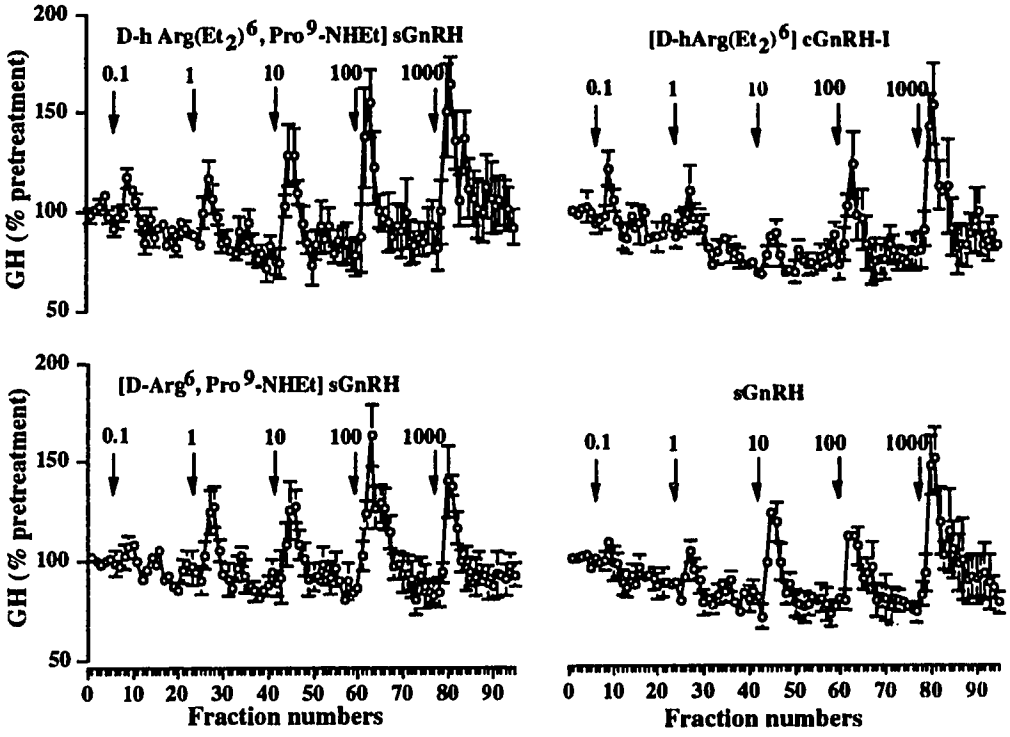


Fig. 5.8 The effects of 2 min pulses (arrows) at 90 min intervals of increasing concentrations (from 0.1 to 1000 nM) of [D-hArg(Et2)6, Pro9-NHEt]-sGnRH, [D-hArg(Et2)6]-cGnRH-I, [D-Arg6, Pro9-NHEt]-sGnRH and sGnRH on GH release from the perifused pituitary fragments from sexually regressed goldfish (GSI < 2%). The hormone release data in ng/ml were transformed as % of pretreatment mean, pooled and expressed as mean ± SEM (n = 4).

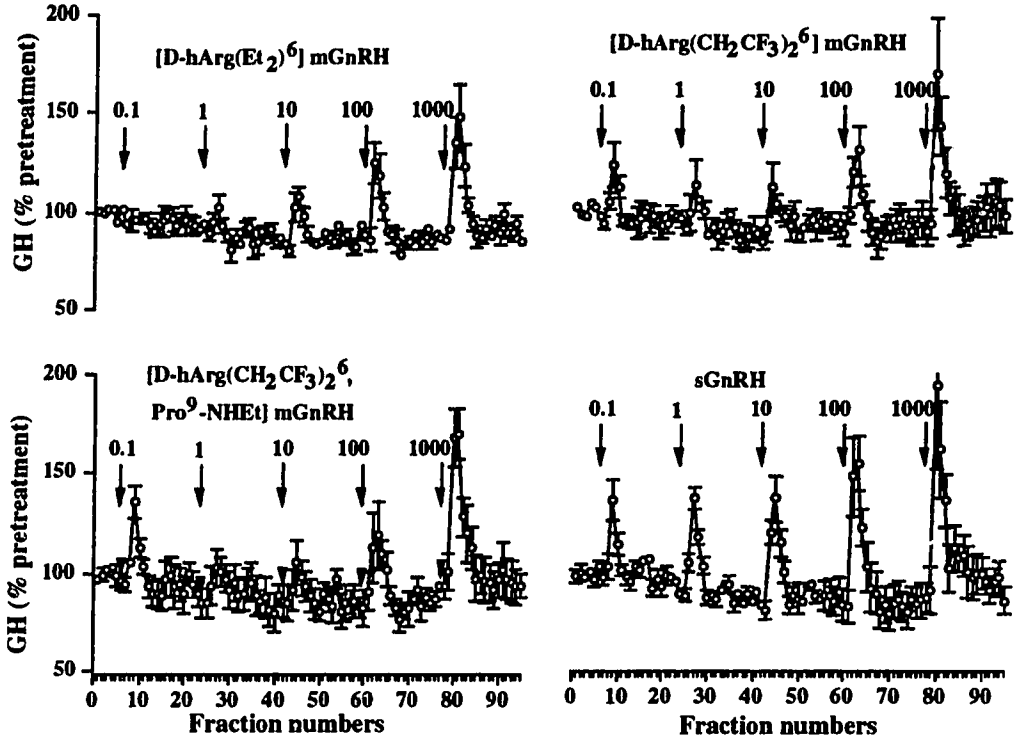


Fig. 5.9 The effects of 2 min pulses (arrows) at 90 min intervals of increasing concentrations (from 0.1 to 1000 nM) of [D-hArg(Et2)6]-mGnRH, [D-hArg(CH2CF3)26]-mGnRH, [D-hArg(CH2CF3)26, Pro9-NHEt]-mGnRH and sGnRH on GH release from the perifused pituitary fragments from sexually regressed goldfish (GSI < 2%). The hormone release data in ng/ml were transformed as % of pretreatment mean, pooled and expressed as mean ± SEM (n = 4).

Table 5.1 The ED50 estimates (in nM) of sGnRH and analogs J and K to stimulate gonadotropin-II (GTH-II) and growth hormone (GH) release from the perifused goldfish pituitary fragments. The ED50 values (with approximate SEM) were obtained by analyzing dose-response curves by ALLFIT, computerized non-linear least square curve fitting program. The minimum effective dose represents the lowest concentration observed to produce a significant hormone release, as determined by ANOVA followed by Fisher's LSD test. The maximum response represents the largest stimulation of hormone release observed in the dose-response curve.

Peptide	Minimal effective dose (nM)		Maximum response (% of prepulse) ± SEM		ED50 (nM) ± SEM	
	GTH-II	GH	GTH-II	GH	GTH-II	GH
Analog J	1000	1000	574 ± 129^{c}	117 ± 3^{C}	1602 ± 585^{a}	1301 ± 226^{A}
Analog K	100	100	903 ± 104^{b}	224 ± 11^{B}	319 ± 79^{b}	93 ± 38^{B}
sGnRH	1	1	1993 ± 299^{a}	387 ± 26^{A}	17.2 ± 6^{c}	28 ± 2^{C}

Table 5.2 The ED50 estimates (in nM) for sGnRH and the position 6 substituted analogs to stimulate gonadotropin-II (GTH-II) and growth hormone (GH) release from the perifused goldfish pituitary fragments. The ED50 values (with approximate SEM) were obtained by analyzing dose-response curves by ALLFIT, computerized non-linear least square curve fitting program. The maximum response represents the largest stimulation of hormone release observed in the dose-response curve. Different letter superscripts indicate significant differences in the ED50 values or in maximum responses ($p < 0.05$) within the experiment.

Peptides	ED50 (nM) ± SEM		Maximum response (% of prepulse) ± SEM	
	GTH-II	GH	GTH	GH
Experiment 1				
[D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH	3.3 ± 0.6^a	11.8 ± 3.1^A	957 ± 59	597 ± 102^A
[D-hArg(Et_2)^6]-cGnRH-I	39.5 ± 5.4^d	174.1 ± 29.9^C	943 ± 76	340 ± 48^B
[D-Arg^6, Pro^9-NHEt]-sGnRH	8.0 ± 0.2^b	17.7 ± 7.5^A	797 ± 96	432 ± 52^{AB}
sGnRH	23.6 ± 4.5^c	39.4 ± 7.9^B	887 ± 127	478 ± 86^{AB}
Experiment 2				
[D-hArg(Et_2)^6]-mGnRH	15.7 ± 2.8^a	141.4 ± 17.5^B	1143 ± 69	257 ±14^B
[D-hArg(CH_2CF_3)$_2^6$]-mGnRH	27.6 ± 1.7^b	146.7 ± 17.3^B	826 ± 31^a	248 ± 27^B
[D-hArg(CH_2CF_3)$_2^6$, Pro^9-NHEt]-mGnRH	29.9 ± 5.4^b	127.4 ± 13.6^B	865 ± 48^a	405 ± 32^A
sGnRH	28.5 ± 5.8^b	47.2 ± 6.7^A	1251 ± 63	486 ± 37^A

References

Bremner, W. J., Adams, L. A. and Steiner, R. A. (1987). Suppression of plasma gonadotropins, testosterone and sperm production in adult male monkeys by a potent inhibitory analog of LHRH. In "LHRH and its Analogs: Contraceptive and Therapeutic Applications". Part 2. (B. H. Vickery, and J. J. Nestor, Jr. Eds.), pp 217-225. MTP Press, Boston.

Chang, J. P., Cook, H., Freedman, G. L., Wiggs, A. J., Somoza, G. M., De Leeuw, R., and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Crim, L. W., Nestor, Jr. J. J. and Wilson, C. E. (1988). Studies of the biological activity of LHRH analogs in the rainbow trout, landlocked salmon, and the winter flounder. Gen. Comp. Endocrinol. 71, 372-382.

De Lean, A., Munson, P. J., and Rodbard, D. (1978). Simultaneous analysis of families of sigmoidal curves: Application of bioassay radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235, E97-E102

Habibi, H. R., Marchant, T. A., Nahorniak, C. S., Van Der Loo, H., Peter, R. E., Rivier, J. E., and Vale, W. W. (1989). Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon GnRH in the pituitary of goldfish (*Carassius auratus*). Biol. Reprod. 40, 1152-1161.

Habibi, H. R., Peter, R. E., Nahorniak, C. S., Milton, R. C. L., and Millar, R.P. (1992). Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides. 37, 271-284.

Haviv, F., Palabrica, C. A., Bush, E. N., Diaz, G., Johnson, E. S. and Greer, J.(1989a). Active reduced-size analogues. In "GnRH analogues in Cancer and Human Reproduction". Vol I. (B. H. Vickery, and B. Lunenfeld, Eds.), pp 45-52. Kluwer Academic Publishers, Boston.

Haviv, F., Palabrica, C. A., Bush, E. N., Diaz, G., Johnson, E. S., Love, S. and Greer, J. (1989b). Active reduced-size hexapeptide analogues of luteinizing hormone-releasing

hormone, J. Med. Chem. 32, 2340-2344.

Karten, M. J., and Rivier, J. E. (1986). Gonadotropin-releasing hormone analog design. Structure-function studies towards the development of agonists and antagonists-rationale and perspectives. Endocrine Rev. 7, 44-66.

Khurshid, S., Weinbauer, G. F. and Nieschlag, E. (1991). Effects of administration of testosterone and gonadotrophin-releasing hormone (GnRH) antagonist on basal and GnRH-stimulated gonadotrophin secretion in orchidectomized monkeys. J. Endocrinol. 129, 363-370.

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. 177, 43-49.

Loumaye, E., Naor, Z., and Catt, K. (1982). Binding affinity and biological activity of gonadotropin-releasing hormone agonists in isolated pituitary cells. Endocrinology, 111, 730-736.

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

Millar, R. P., and King, J. A. (1984). Structure-activity relations of LHRH in birds. J. Exp. Zool. 232, 425-430.

Murthy, C. K., Nahorniak, C. S., Rivier, J. E., and Peter, R. E. (1993). *In vitro* characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish, *Carassius auratus*. Endocrinology, 133, 1633-1644.

Nestor, Jr. J. J., Ho, T. L., Tahilramani, R., McRae, G. I. and Vickery, B. H. (1984). Long acting LHRH agonists and antagonists: Basic and clinical aspects. In "LHRH and its Analogues: Basic and Clinical Aspects". (F. Labrie, A. Belanger, A. Dupont, Eds.), pp 24-35. Elsevier Science Publishers, Amsterdam.

Nestor, J. J. Jr., Tahilramani, R., Ho, T. L., McRae, G. I., and Vickery, B. H. (1988). Potent, long-acting luteinizing hormone-releasing hormone antagonists containing new synthetic amino acids: N, N'-Dialkyl-D-homoarginines. J. Med. Chem. 31, 65-72.

Nestor, J. J. Jr., Tahilramani, R., Ho, T. L., Goodpasture, J. C., Vickery, B. H., and Ferrandon, P. (1992). Potent gonadotropin-releasing hormone antagonists with low histamine-releasing activity. J. Med. Chem. 35, 3942-3948.

Pavlou, S. N., Wakefield, G. B. and Kovacs, W. J. (1987). LHRH antagonists in normal men. In "LHRH and its analogs: Contraceptive and Therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 245-256. MTP Press Ltd., Boston.

Peter, R. E. (1986). Structure-activity studies on gonadotropin-releasing hormone in teleosts, amphibians, reptiles and mammals. In "Comparative Endocrinology: Developments and Directions". (C. L. Ralph, Ed.), pp 75-93. Alan R. Liss, Inc., New York.

Peter, P.. E., Chang, J. P., Nahorniak, C. S., Omeljaniuk, R. J., Sokolowska, M., Shih, S. H., and Billard, R. (1986). Interaction of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog. Horm. Res. 42, 513-548.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In"Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, and M. H. Stetson, Eds.), pp 393-398. Wiley-Liss Inc. NY.

Peter, R. E., Nahorniak, C. S., Chang, J. P., and Crim, L. W. (1984). Gonadotropin release from the pars distalis of goldfish, *Carassius auratus* transplanted beside the brain or into the brain ventricles; Additional evidence for gonadotropin release-inhibitory factor. Gen. Comp. Endocrinol. 55, 337-346.

Peter, R. E., Trudeau V. L., and Sloley, B. D. (1991). Brain regulation of reproduction in teleosts. Bull. Inst. Zool. Academia Sinica, Monograph, 16, 89-118.

Rivier, J., Varga, J., Porter, J., Perrin, M., Rivier, C., Vale, W., Struthers, S. and Hagler, A. (1984). Design of cyclic GnRH antagonists. In "LHRH and its Analogues: Basic and Clinical Aspects". (F. Labrie, A. Belanger, A. Dupont, Eds.), pp 19-23. Elsevier Science Publishers, Amsterdam.

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.

Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

Vickery, B. H., McRae, G. I. and Goodpasture, J. C. (1987). Clinical uses of LHRH

analogs in dogs. In "LHRH and its Analogs: Contraceptive and Therapeutic Applications". Part 2. (B. H. Vickery, and J. J. Nestor, Jr. Eds.), pp 517-543. MTP Press, Boston.

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.

Zohar, Y., Goren, A., Fridkin, M., Elhanati, E., and Koch, Y. (1990). Degradation of gonadotropin-releasing hormones in the gilthead seabream, *Sparus aurata.* II. Cleavage of native salmon GnRH and mammalian LHRH and their analogs in the pituitary, kidney, and liver. Gen. Comp. Endocrinol. 79, 306-319.

Chapter Six

Receptor binding of gonadotropin-releasing hormone (GnRH) antagonists that inhibit gonadotropin-II and growth hormone release in goldfish, *Carassius auratus*[1]

Introduction

In goldfish the two native forms of gonadotropin-releasing hormones (GnRH), salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II), stimulate gonadotropin-II (GTH-II) and growth hormone (GH) release both *in vitro* and *in vivo* (Marchant et al., 1989; Chang et al., 1990; Habibi et al., 1992; for review Peter et al., 1990a, 1991). The goldfish pituitary has two classes of GnRH binding sites, high affinity/low capacity sites and low affinity/high capacity binding sites (Habibi et al., 1987, 1990; for review Habibi and Peter, 1991). Both sGnRH and cGnRH-II displace bound ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH (sGnRH-A) from crude goldfish pituitary membrane preparations (Habibi et al., 1987, 1992; Habibi and Peter, 1991) suggesting that the labelled ligand binds to the same populations of receptors as sGnRH and cGnRH-II on gonadotrophs and somatotrophs, respectively. In electron microscope studies, Cook et al. (1991) found that sGnRH and cGnRH-II both displaced avidin gold-labelled biotinylated [D-Lys^{6}, Pro^{9}-NHEt]-sGnRH from the surfaces of immunohistochemically identified gonadotrophs and somatotrophs, also indicating that the latter analog binds to the same populations of receptors as sGnRH and cGnRH-II. In a photoaffinity labelling study in goldfish, a protein of molecular weight 51,000 dalton exhibiting a high affinity for GnRH was found to be present as two closely associated bands on the gel (Habibi et al., 1990), and these two bands of protein may represent different GnRH receptors on gonadotrophs and/ or somatotrophs

[1]A version of this chapter has been accepted for publication: CK Murthy, AOL Wong, HR Habibi, JE Rivier, RE Peter (1994) Biol. Reprod. (accepted).

In our previous study using a goldfish pituitary fragments perifusion system, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E of Chapter 2) inhibited sGnRH and cGnRH-II stimulated GTH-II as well as GH release in a dose dependent manner (Murthy et al., 1993; see Chapter 2). Two other GnRH analogs based on native GnRH peptide sequences, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C of Chapter 2), and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N of Chapter 2) inhibited GTH-II and GH release stimulated by sGnRH and cGnRH-II; however, analog C was found to have GH releasing actions on its own, indicating that it is not a 'true' antagonist on GH release (Murthy et al., 1993; see Chapters 2, 3). The differential action on GTH-II and GH release by analog C, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^3, D-Arg^6]-mGnRH (analog F; see Chapters 2, 3) and [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH (analog L; see Chapter 4) indicate the presence of functional differences in the properties of GnRH receptors on gonadotrophs and somatotrophs (see Chapter 3, 4). On this basis, one objective of the present study was to determine whether more than one population of GnRH receptors in goldfish pituitary membranes could be detected, based on the binding characteristics of analog C.

In mammalian studies, it has been extensively documented that GnRH antagonists inhibit mGnRH actions by competitively binding to GnRH receptors at the pituitary level (for review Karten and Rivier, 1986; Clayton, 1989; Gordon and Hodgen, 1992). Teleost fish including goldfish, in contrast to other vertebrates, lack a functional hypothalamo-hypophysial portal blood system and the adenohypophysis is directly innervated by GnRH neurons originating from preoptic area and the anteroventral hypothalamus (for reviews see Kah, 1986; Peter et al., 1990b, 1991). In rat, GnRH neurons terminating in medio-basal hypothalamus (releasing GnRH into portal blood system) contain GnRH receptors mediating autofeed back regulation (Sarkar, 1987; Zanishi et al., 1987). Presence of nerve terminals in the goldfish pituitary fragments may complicate the inference from our earlier dose-response studies on the site of action of GnRH antagonists, and hence we tested the direct action of GnRH antagonists at the pituitary cell level.

In goldfish, although sGnRH and cGnRH-II stimulate GTH-II and GH release (Peter et al., 1991), in the previous GnRH receptor binding studies only sGnRH-A was used as labelled ligand (Habibi et al., 1987, 1990). Here we have made an effort to determine whether there is any selective displacement of bound labelled sGnRH-A or [D-Arg6]-

cGnRH-II (cGnRH-A) by antagonists, especially those based on native GnRH molecules. The other objective of the present study was to test whether there is any correlation between the receptor binding affinity of an antagonist and its bioactivity in terms of the potency and apparent duration of action.

Materials and Methods

Experimental animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries, Stoutland, MO. The fish were maintained in flow-through aquaria (1800 liters) at 17 ± 1°C under a simulated natural photoperiod of Edmonton for at least 2-3 weeks prior to experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish of both sexes, with body weight ranging from 20-35 g were used in the present study. The sexual maturity of fish was assessed by measuring the gonadosomatic index (GSI= weight of gonad/ total body weight X 100%).

Reagents and test substances sGnRH, cGnRH-II (Peninsula Laboratories Inc., Belmont, CA), sGnRH-A (Syndel laboratories Ltd., Vancouver, BC, Canada) and (D-Arg^6]-cGnRH-II (cGnRH-II-A, gift from Dr. R. P. Millar, University of Cape Town, Cape Town, South Africa) were dissolved in 0.1 M acetic acid and stored at -25°C as aliquots of 50 μM (for perifusion) or 1 mM (for receptor binding studies). The aliquots were diluted with perifusion medium or with binding assay buffer to required concentrations immediately prior to use in experiments. (Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C), and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) were gift of Dr. J. E. Rivier, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA. All analogs were dissolved in a primary solvent containing propylene glycol and physiological saline at a ratio of 60:40, and subsequently diluted to required concentrations with perifusion medium or binding assay buffer; the final propylene glycol concentration was always less than 0.5%.

Perifusion of the pituitary fragments and cells *In vitro* experiments were conducted using a validated pituitary fragments perifusion system for the goldfish (Marchant et al., 1989)

with minor changes. Briefly, goldfish pituitary glands were chopped into fragments (0.2 mm^3) using a McIlwain tissue chopper. The fragments (three pituitary equivalents per chamber) were washed with Medium 199 (M 199) and placed in 0.5 ml micro-chambers (Endotronics Inc., Minneapolis, MN) between two 13 mm filters; 8 micron pore size for inlet (Millipore Inc., Bedford MA) and 5 micron pore size for outlet (Micron Separations Inc., Westboro, MA) with a continuous flow of medium (M199) at 18°C. These filters were supported by circular stainless steel screens with a pore size of 450 micron. The reservoirs, holding perifusion medium or drugs were attached to a 3-way valve. From the 3-way valve the drug or medium was delivered to the perifusion chambers by a tubing passing through an ISMTEC multichannel cartridge pump (Cole-Parmer Instruments Corporation, Chicago, Illinois). The fragments were perifused overnight (at least 15 h) at a flow rate of 5 ml/h. Thereafter, the incubation was continued using Hank's balanced salt solution supplemented with 25 mM HEPES and 0.1% BSA at a flow rate of 15 ml/h for 2 h before starting the experiment. Five minute fractions of perifusate were collected, frozen and stored at -25°C for hormone measurement.

Dispersed goldfish pituitary cells were prepared by controlled trypsin/ DNase treatment (for procedure see Chang et al., 1990) and were incubated with preswollen cytodex beads (Cytodex I, Sigma Chemical Co., St. Louis, MO) at 28°C under 5% CO_2 and saturated humidity. After overnight incubation, cytodex beads with pituitary cells attached were loaded into 0.5 ml perifusion chambers and the experiments were conducted as described above.

GnRH receptor study The GnRH receptor studies were conducted using goldfish crude pituitary membrane preparations. The iodination of sGnRH-A and [D-Arg^6]-cGnRH-II, and the crude pituitary membrane preparation and displacement studies were conducted as described by Habibi et al. (1987). The amount of ^{125}I-sGnRH-A bound to crude pituitary membrane preparations in the presence or absence of competitors was expressed as percentage specific binding by taking the binding in presence of unlabeled 10^{-6} M sGnRH-A as non-specific binding (% total binding - % non-specific binding). The displacement curves were analysed by a non-linear weighted least-square curve fitting method (Munson and Rodbard, 1980; McPherson, 1985a, b), using the computer program LIGAND (Biosoft, Cambridge, UK). The data analysis was done in two steps; the initial affinity

constant and binding capacity values were obtained by Equilibrium Binding Data Analysis (EBDA) and based on these initial estimates, the final estimates were obtained by LIGAND. The displacement data of native sGnRH, sGnRH-A, analogs C, E, and N from individual experiments were analysed individually and then data from two to four individual experiments were analysed simultaneously to obtain the final estimates of equilibrium association constant (Ka). The 95% confidence intervals were obtained following log transformation (McPherson, 1985b).

Radioimmunoassay GTH-II levels in perifusate were measured by validated RIA specific for GTH-II (Peter et al., 1984; Van Der Kraak, 1992). The GH levels were measured by using a goldfish GH RIA described by Murthy et al. (1993).

Data analysis To provide a mean hormone release response, the GTH-II or GH values were expressed as percentage of pretreatment mean (of 6 fractions prior to first pulse) for each column and pooled from 4 columns. The quantification of the hormone response was done as described by Peng et al. (1990). Briefly, the average hormone level of the 3 fractions immediately preceding each pulse (prepulse mean) was treated as basal, and the hormone values following a GnRH pulse were expressed as a percentage of this prepulse mean. Post pulse fractions with hormone contents greater than one standard error (SEM) above the prepulse mean were considered to be a part of the response. The response (expressed as % prepulse) above the prepulse mean from the fractions considered as part of the response were added to get the net response in a particular column. Statistical differences between treatment and control groups were assessed by ANOVA followed by Fisher's LSD test ($p < 0.05$).

Results

Actions of analog E on sGnRH-stimulated GTH-II and GH release from the perifused goldfish pituitary fragments: To test the antagonistic actions of [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) on goldfish pituitary fragments as well as cells, *in vitro* experiments were conducted using pituitary fragments and dispersed pituitary cells obtained from the same batch of goldfish. The exposure of perifused pituitary fragments obtained

from sexually regressed goldfish (GSI = < 2.0%) to 50 nM sGnRH for 2 min resulted in a significant increase in release of GTH-II (Fig. 6.1a, c) and GH (Fig. 6.1b, d). In the continuous presence of 2 μM analog E, sGnRH-stimulated GTH-II (98 ± 2% inhibition) and GH (97 ± 4 % inhibition) release were significantly suppressed (Fig. 6.1c, d). At 90 min after termination of analog E treatment, 50 nM sGnRH induced a magnitude of GTH-II release similar to that of the initial sGnRH pulse, indicating the transient nature of inhibition by analog E (Fig. 6.1c). However, the GH response to sGnRH at 90 min after the termination of analog E treatment was significantly lower in magnitude compared to the GH response to the initial sGnRH pulse (Fig. 6.1d).

In a similar experiment, analog C (2 μM) and analog N (2 μM) inhibited 50 nM sGnRH stimulated GTH-II release by 72 ± 2% and 98 ± 2% respectively (see Chapter 2). The inhibition of sGnRH stimulated GTH-II release by analog C was significantly lower than that by analogs E and N (Murthy et al., 1993; see Chapter 2). Analog N (2 μM) also suppressed 50 nM sGnRH stimulated GH release by 94 ± 3% (see Chapter 2).

In these experiments using pituitary fragments from sexually regressed goldfish (GSI = < 2.0%), analogs C and N were found to have persistent antagonism on GTH-II and GH release at least up to 90 min after termination of analog treatment, indicating the longer duration of action of analogs C and N (see Chapter 2). Analog E had no persistent antagonism on GTH-II release at 60 min (Murthy CK and Peter RE, unpublished results) and 90 min (see Chapter 2; present results) after termination of analog E treatment. However, all three analogs had persistent antagonism on GH release to sGnRH at least up to 90 min after termination of analog treatment (see Chapter 2; present results).

Actions of analog E on sGnRH- and cGnRH-II-stimulated GTH-II and GH release from the perifused dispersed goldfish pituitary cells: To test whether analog E can act directly on pituitary cells, the dispersed pituitary cells obtained from sexually regressed goldfish (GSI = < 2.0%) were exposed to 2 min pulses of 50 nM sGnRH in the presence and absence of 2 μM analog E. A two min pulse of 50 nM sGnRH significantly increased the release of both GTH-II and GH from the perifused dispersed pituitary cells (Fig. 6.2a, b). Similar to results obtained with perifused pituitary fragments, analog E at 2 μM concentration for 30 min (prior to sGnRH pulse) had no apparent effects on GTH-II and GH release from the perifused dispersed goldfish pituitary cells. In the presence of analog

E, sGnRH-stimulated GTH-II and GH release were significantly suppressed, indicating the direct actions of analog E on the pituitary cells (Fig. 6.2a-d). At 90 min after termination of analog E treatment, the GTH-II, but not the GH, response to sGnRH was normal, suggesting that the inhibitory actions of analog E on GTH-II release was transient (Fig. 6.2a, c). However, analog E had persistent antagonism on GH release at least up to 90 min after termination of analog E treatment (Fig. 6.2d).

In another experiment using a similar protocol as for Fig. 6.2, analog E significantly suppressed GTH-II and GH release from the perifused dispersed goldfish pituitary cells induced by 2 min pulse of 50 nM cGnRH-II (Fig. 6.3a-d), confirming the direct actions of analog E on goldfish pituitary cells. Again, the persistence of antagonism by analog E at 90 min after the termination of analog E treatment was present on GH release, but not on GTH-II release (Fig. 6.3b, d).

GnRH receptor binding studies: In the next series of experiments, the GnRH receptor binding characteristics of analog E, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C), and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) were tested using crude goldfish pituitary membrane preparations. In the presence of increasing concentrations of analog E (10^{-12} to 10^{-6} M), the % specific binding of ^{125}I-sGnRH-A to crude pituitary membrane preparations (Fig. 6.4) gradually decreased. The analysis of the displacement curves for native sGnRH, sGnRH-A, and analogs E, C, and N was best fitted with a two-site binding model, similar to previous findings regarding GnRH receptors in the goldfish pituitary (Habibi et al., 1987, 1990, 1992).

The displacement profile of analog E was similar to that of native sGnRH and sGnRH-A (Fig. 6.4) indicating that analog E competitively binds to the same classes of GnRH receptors on the crude pituitary membrane preparations. The receptor binding affinity (equilibrium association constant, Ka_1) of analog E for high affinity binding sites (10.79 ± 1.14; 95 % confidence intervals (CI), 8.55 - 13.60 X 10^{10} M^{-1}) was significantly higher than that of native sGnRH (3.41 ± 0.5; CI, 2.43 - 4.78 X 10^{10} M^{-1}) and sGnRH-A (4.97 ± 0.27; CI, 4.41 - 5.60 X 10^{10} M^{-1}; Table 6.1). However, the equilibrium association constant (Ka_2) of analog E for low affinity binding sites (0.59 ± 0.29 X 10^7 M^{-1}) was significantly lower than that of native sGnRH (3.14 ± 0.71 X 10^7 M^{-1}) and

sGnRH-A ($8.92 \pm 0.47 \times 10^7$ M^{-1}; Table 6.1).

The displacement profile of analog C (Fig. 6.5a) was similar to that of native sGnRH peptide, again indicating the competitive binding of analog C to GnRH receptors in the crude goldfish pituitary membrane preparations. The receptor binding affinity (Ka_1) of analog C for high affinity sites ($24.60 \pm 6.94 \times 10^{10}$ M^{-1}) was 5 - 10 times (significantly) higher than that of sGnRH and sGnRH-A (Table 6.1). The binding affinity (Ka_2) of analog C to low affinity binding sites was similar to that of sGnRH-A and slightly, but significantly, higher than that of sGnRH (Table 6.1). Analog N also displaced bound ^{125}I-sGnRH-A in a dose-dependent manner (Fig. 6.5b), and the binding affinity (Ka_1) of analog N for high affinity sites was at least 10 times higher than that of native sGnRH and sGnRH-A (Table 6.1). The Ka_1 of analog N, based on native cGnRH-II structure, was significantly higher than that of analogs C and E (Table 6.1).

Further, to test the possibility of selective binding, the ability of analogs to displace bound ^{125}I-[D-Arg^6]-cGnRH-II (cGnRH-II-A) from the crude goldfish pituitary membrane preparations was tested. Analogs C, E and N, sGnRH-A and cGnRH-II-A at 10^{-6} M concentrations displaced bound ^{125}I-cGnRH-II-A (Fig. 6.6). These results indicate that these analogs competitively bind to the same population of GnRH receptors on the crude goldfish pituitary membrane preparations.

Discussion

In the present study two native GnRH peptides, sGnRH and cGnRH-II, stimulated GTH-II and GH release from perifused pituitary fragments and dispersed cells of goldfish. These results confirm earlier studies (Marchant et al., 1989; Chang et al., 1990; Peter et al., 1990a, 1991; Habibi et al., 1992) suggesting the involvement of native GnRH peptides in the regulation of GTH-II and GH release. Similar to our earlier report, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) significantly suppressed 50 nM sGnRH stimulated GTH-II and GH release from perifused goldfish pituitary fragments. Although there are no indications of neuronal activity in the pituitary fragments after overnight preincubation in the perifusion system, presence of nerve terminals in the goldfish pituitary fragments complicates the inference on site of action of analog E (see Introduction). Analog E significantly suppressed both sGnRH and cGnRH-II stimulated GTH-II and GH release from dispersed goldfish pituitary cells, indicating the ability of analog E to act directly at the

pituitary cell level to inhibit native GnRH actions. In the receptor binding studies analog E competitively displaced bound ^{125}I-sGnRH-A from the crude goldfish pituitary membrane preparations (Fig. 6.4) and from dispersed goldfish pituitary cells (results not presented). The binding displacement profile of analog E is similar to that of native sGnRH and sGnRH-A. Analog E (2 μM) also displaced ^{125}I-cGnRH-II-A bound to crude pituitary membrane preparations. Similarly, analogs C and N (2 μM) displaced bound ^{125}I-sGnRH-A and ^{125}I-cGnRH-II-A. Taken together these results indicate that GnRH antagonists inhibit native GnRH peptide action by competitively binding to GnRH receptors on the goldfish pituitary cells. In agreement with these results, [D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}]-mGnRH which acts as a weak GnRH antagonist in goldfish (Murthy et al., 1993; see Chapter 2), competitively binds to goldfish pituitary GnRH receptors with an affinity 8-10 times higher than that of native sGnRH (Habibi, 1991).

In mammalian studies it is well established that GnRH antagonists competitively bind to GnRH receptors on the pituitary cells (for review Karten and Rivier, 1986; Clayton, 1989; Gordon and Hodgen, 1992). GnRH antagonists displace bound radioiodinated agonists and vice versa, suggesting that both agonists and antagonists act through the same population of GnRH receptors (Perrin et al., 1983). Antagonists also displace photolabeled GnRH analogs bound to crude rat pituitary membrane preparations (Hazum and Keinan, 1983). Similarly, photoaffinity labelled GnRH agonist or antagonist bound to rat pituitary membranes was displaced by unlabeled agonist or antagonist, indicating that agonists and antagonists bind to the same sites (Janovick et al., 1993).

The rank order of binding to the high affinity sites in the goldfish pituitary membrane preparations was analog N ≥ C > E. In the *in vitro* assay the antagonist activity on GTH-II release in goldfish was in the order of E = N > C (Murthy et al., 1991, 1993; see Chapter 2, 3), indicating a non-linearity of relationship between receptor binding affinity and antagonistic activity of these analogs. Although a positive correlation between binding affinities and the *in vitro* potencies of GnRH agonists and antagonists has been found in numerous studies in rats (Loumaye et al., 1982; for review Karten and Rivier, 1986), a number of reports showing non-linearity have also appeared. For example, in rat there was no linear correlation between receptor binding affinity of three GnRH antagonists having D-Trp^{6}, D-Nal(2)^{6} or D-Arg^{6}, and their *in vitro* potency (Rivier et al., 1983). A similar

lack of correlation between receptor binding affinity and *in vitro* biological activity has been reported by Bajusz et al. (1988). In goldfish, there was a significant correlation between binding to the high affinity sites and *in vitro* biological GTH-II releasing activity of position 6 and 10 substituted agonists of native sGnRH (R =0.94); however, there was only a weak correlation (R = 0.41) for similar analogs based on mGnRH peptide, suggesting a lower or lack of correlation for analogs based on the non-native mGnRH structure (Habibi et al., 1989). Further, the binding affinities of mGnRH analogs were significantly lower than that for sGnRH analogs, although many mGnRH analogs were as potent as sGnRH analogs in releasing GTH-II from perifused goldfish pituitary fragments (Habibi et al., 1989). Further, there was no correlation between GTH-II releasing activity and receptor binding affinity of position 5, 7, and 8 substituted mGnRH analogs in goldfish (Habibi et al., 1992). Similarly, in African catfish, *Clarias gariepinus*, although mGnRH analogs were more potent than sGnRH in stimulating GTH release, the receptor binding affinity of the mGnRH analogs was lower than for sGnRH (De Leeuw et al., 1988). In the present study, although analog E (based on mGnRH) was more potent than analog C (based on sGnRH) in inhibiting sGnRH stimulated GTH-II release, its binding affinity was significantly lower than that of analog C. In goldfish pituitary fragments perifusion, the rank order of *in vitro* GTH-II releasing potency is cGnRH-II ≥ sGnRH = mGnRH (Habibi, 1991; Habibi et al., 1992), while the rank order of binding affinity is cGnRH-II > sGnRH > mGnRH (Habibi et al., 1992), again indicating weaker ability of mGnRH in receptor binding, but not in terms of *in vitro* GTH-II releasing activity. Taken together these results indicate a lack of positive relation between receptor binding affinity and *in vitro* GTH-II release inhibiting (or stimulating) potency for analogs based on non-native GnRH peptides in goldfish. Further, the rank order of binding affinity of analogs C (sGnRH-a), E (mGnRH-a) and N (cGnRH-II-a) appears to be related to the rank order of binding affinity of parent molecules (cGnRH-II > sGnRH > mGnRH).

Both in pituitary fragments and in dispersed pituitary cells analog E at 2 μM concentration showed persistent antagonism even 90 min after termination of analog E treatment on GH release, but not on GTH-II release. A similar tendency was also noticed in our earlier studies (see Chapter 2), suggesting functional differences in the properties of GnRH receptors on gonadotrophs and somatotrophs in goldfish. Other evidence for functional differences is based on photoaffinity labeling studies (Habibi et al., 1990),

differential actions of some 'putative' GnRH antagonists (see Introduction) on GTH-II and GH release (Murthy et al., 1993; see Chapters 2, 3, 4) and a requirement for a lower concentration of analog E to block GH than GTH-II release.

In addition to the evidence for stimulation of GH release by GnRH peptides in goldfish, recent studies have confirmed a similar action of GnRH in common carp (Lin et al., 1993), tilapia (Melamed et al., 1993), and rainbow trout (Le Gac et al., 1993). In goldfish GnRH appears to act through high affinity binding sites to stimulate GH release (Habibi et al., 1990, 1992). Although functional differences in the properties of GnRH receptors on GTH and GH cells are suggested (see above), only one high affinity GnRH binding site has been demonstrated in the goldfish pituitary (Habibi et al., 1987, 1989; Habibi and Peter, 1991). Even in the present study, analog C, which stimulates GH release but not GTH-II release, did not distinguish more than one population of high affinity binding sites. It is possible that GnRH receptors on goldfish gonadotrophs and somatotrophs have different structure-function relationships, but have similar binding affinities to native GnRHs and their analogs. Molecular sequencing of GnRH receptors in goldfish will help to clarify this point.

In general, there seems to be a lack of correlation between GnRH receptor binding affinity and GH releasing (or inhibiting) potency of natural GnRH peptides and analogs. Habibi et al. (1992) did not find a correlation between *in vitro* GH releasing potency and receptor binding affinity (to either high affinity site or low affinity site) of mammalian GnRH, sGnRH, chicken GnRH-I, cGnRH-II, lamprey GnRH and position 5, 7 and 8 substituted mGnRH analogs. In the present study, analogs E and N have similar *in vitro* potency to inhibit GH release but analog N has at least 10 times higher receptor binding affinity to high affinity sites than analog E, suggesting no positive relation between receptor binding affinity and *in vitro* potency. Analog C has GH stimulatory actions and hence cannot be used in these correlations.

Although there is a lack of relation between receptor binding affinity and *in vitro* GTH-II releasing potency for analogs C, E and N, there appears to be a positive relationship between receptor binding affinity and duration of *in vitro* GTH-II release inhibitory action. The rank order of binding affinity and duration of action on GTH-II release was analog N ≥ C > E (see Chapters 2, 3; and present study). In addition, all three analogs exhibited persistent antagonism on GnRH stimulated GH release at least up to 90

min after termination of analog treatment. Because *in vivo* potency of agonists and antagonists is known to be influenced not only by receptor binding affinity but also by other factors such as absorption, distribution, resistance to degradation and elimination (Karten and Rivier, 1986), the ultimate usefulness of these antagonists in aquaculture remains to be investigated.

In the present study the receptor binding affinity (Ka_1) of sGnRH to high affinity binding sites was $3.41 \pm 0.5 \times 10^{10}\ M^{-1}$, which is higher than reported earlier ($0.67 \pm 0.2 \times 10^{10}\ M^{-1}$; Habibi et al., 1987). This difference may be due to seasonal variation in the binding affinity during the reproductive season. Further studies are needed to clarify this idea.

In conclusion, results presented in this study indicate that the [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) can inhibit native GnRH peptide actions on GTH-II and GH release by acting directly on the pituitary cells. Analogs C, E and N were able to displace bound ^{125}I-sGnRH-A and ^{125}I-cGnRH-II-A, indicating that these analogs exert inhibitory actions by competitively binding to GnRH receptors. There is no positive relation between receptor binding affinity of the three analogs tested and *in vitro* potency to inhibit GTH-II release; however, there appears to be a positive relationship with duration of antagonism on GTH-II and GH release.

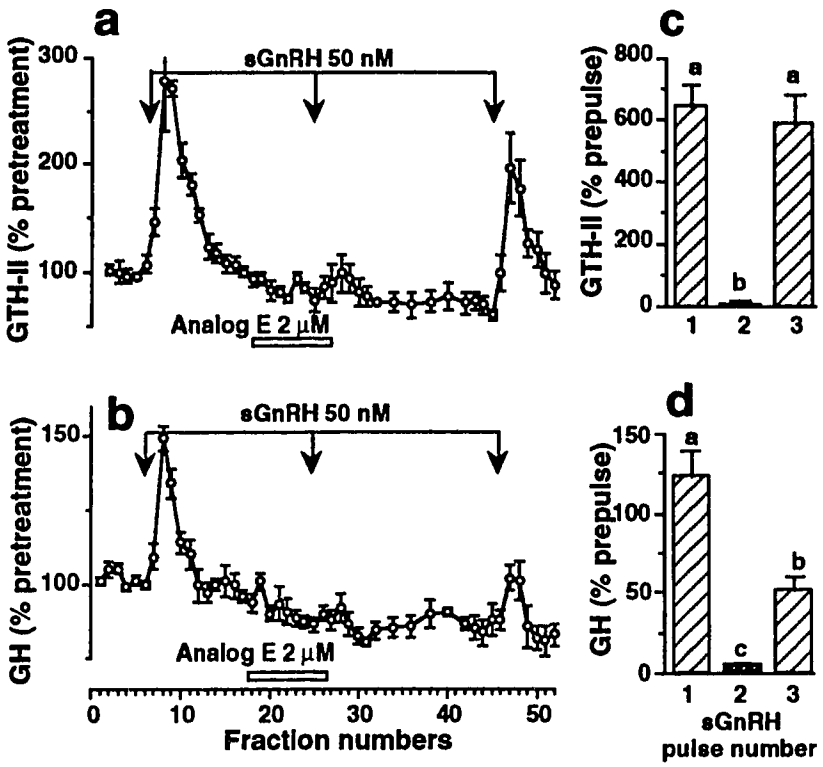


Fig. 6.1 The effects of repeated 2 min pulses (arrows) of 50 nM sGnRH on GTH-II release (a) and GH release (b) from perifused pituitary fragments obtained from sexually regressed goldfish (GSI = < 2.0%), with the second pulse given in the presence (45 min; open bar) of 2 μM [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses to three repeated pulses of sGnRH were quantified as % prepulse and presented as mean ± SEM (c, d). Significant differences in quantified GTH-II and GH release in response to three sGnRH pulses were indicated by different letters (ANOVA and Fisher's LSD test, $p < 0.05$).

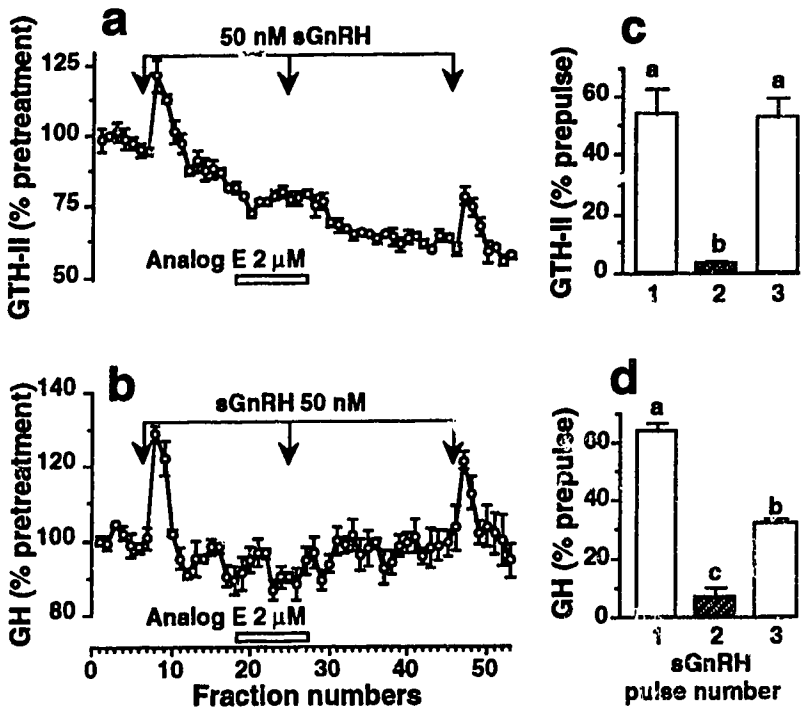


Fig. 6.2 The effects of repeated 2 min pulses (arrows) of 50 nM sGnRH on GTH-II release (a) and GH release (b) from perifused dispersed pituitary cells obtained from sexually regressed goldfish (GSI = < 2.0%), with the second pulse given in the presence (45 min; open box) of 2 μM [Ac-Δ^3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses to three repeated pulses of sGnRH were quantified as % prepulse and presented as mean ± SEM (c, d). Significant differences in quantified GTH-II and GH release in response to three sGnRH pulses were indicated by different letters (ANOVA and Fisher's LSD test, $p < 0.05$).

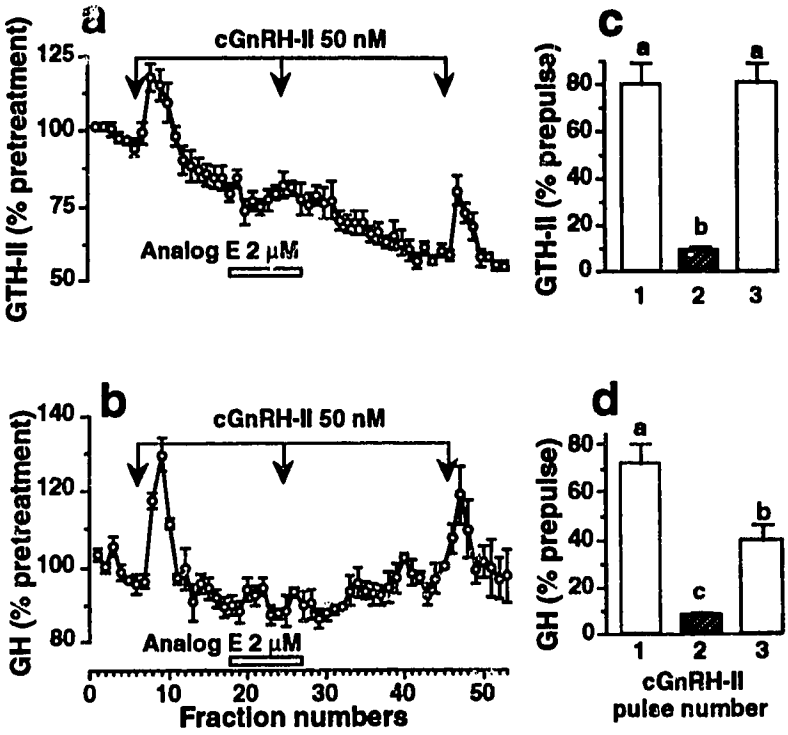


Fig. 6.3 The effects of repeated 2 min pulses (arrows) of 50 nM cGnRH-II on GTH-II release (a) and GH release (b) from perifused dispersed pituitary cells obtained from sexually regressed goldfish (GSI = < 2.0%), with the second pulse given in the presence (45 min; open box) of 2 μM [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E). The hormone release data in ng/ml were transformed as % pretreatment, pooled and expressed as mean ± SEM (n=4). The GTH-II and GH release responses to three repeated pulses of sGnRH were quantified as % prepulse and presented as mean ± SEM (c, d). Significant differences in quantified GTH-II and GH release in response to three cGnRH-II pulses were indicated by different letters (ANOVA and Fisher's LSD test, $p < 0.05$).

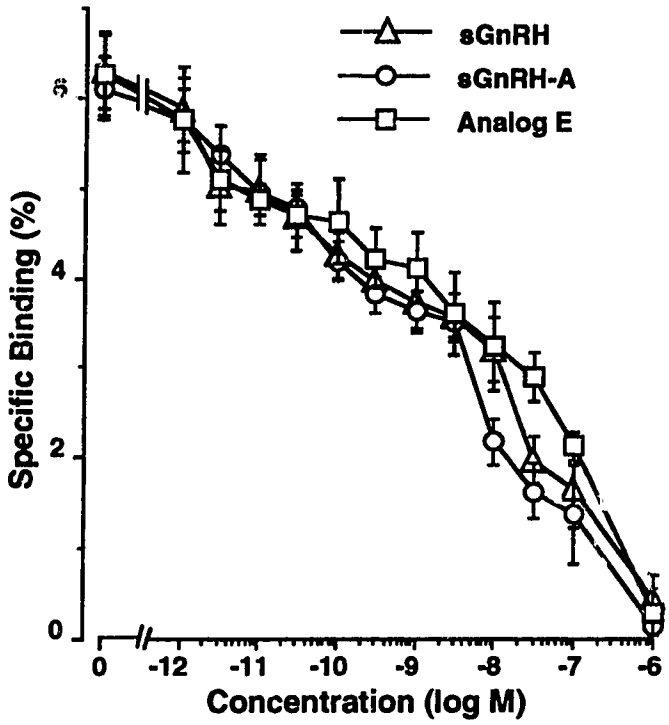


Fig. 6.4 Displacement of ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH bound to goldfish pituitary membranes by increasing concentrations of unlabeled sGnRH, [D-Arg^{6}, Pro^{9}-NHEt]-sGnRH (sGnRH-A) or [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-mGnRH (analog E). Values (mean ± SEM) represent % specific binding determined by subtraction of % non-specific binding in the presence of 10^{-6} M unlabeled sGnRH-A. Results were obtained by pooling from 3 or 4 similar experiments, each in triplicates, using goldfish that were in sexual recrudescent stage of development (GSI = 4.2 ± 0.5%).

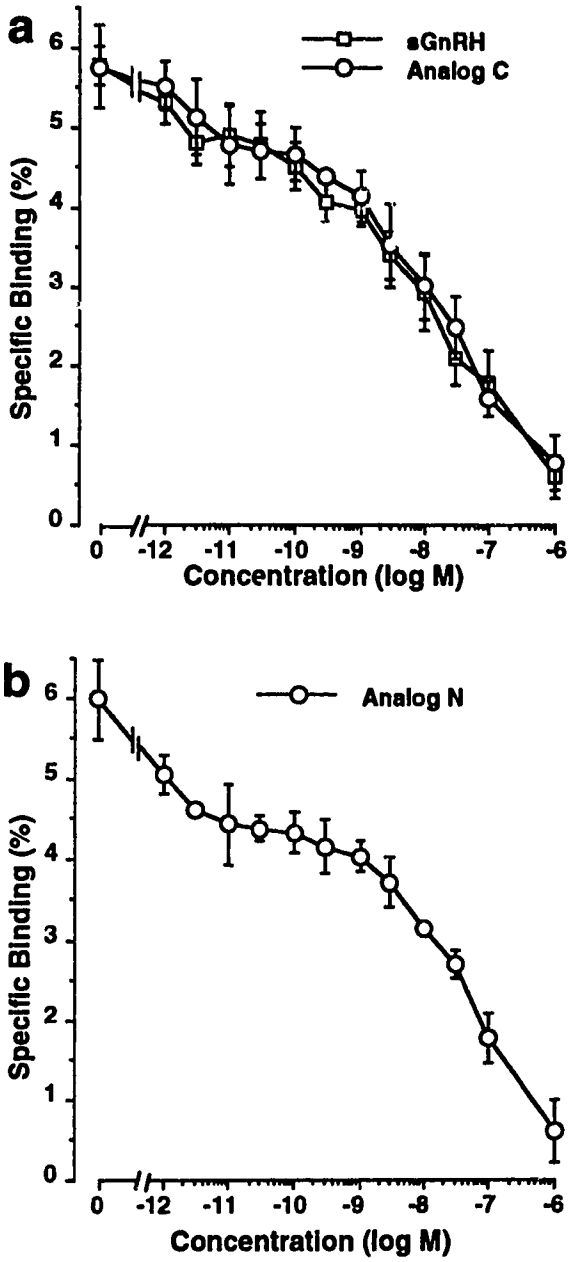


Fig. 6.5 Displacement of ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH bound to goldfish pituitary membranes by increasing concentrations of unlabeled sGnRH or [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-sGnRH (analog C; a) and [Ac-D(2)Nal^{1}, 4Cl-D-Phe^{2}, D-(3)Pal^{3,6}]-cGnRH-II (analog N; b). Values (mean ± SEM) represent % specific binding determined by subtraction of % non-specific binding in the presence of 10^{-6} M unlabeled sGnRH-A. Results were obtained by pooling from 2 or 3 similar experiments, each in triplicates, using goldfish that were in sexual recrudescent stage of development (GSI = 4.2 ± 0.5%).

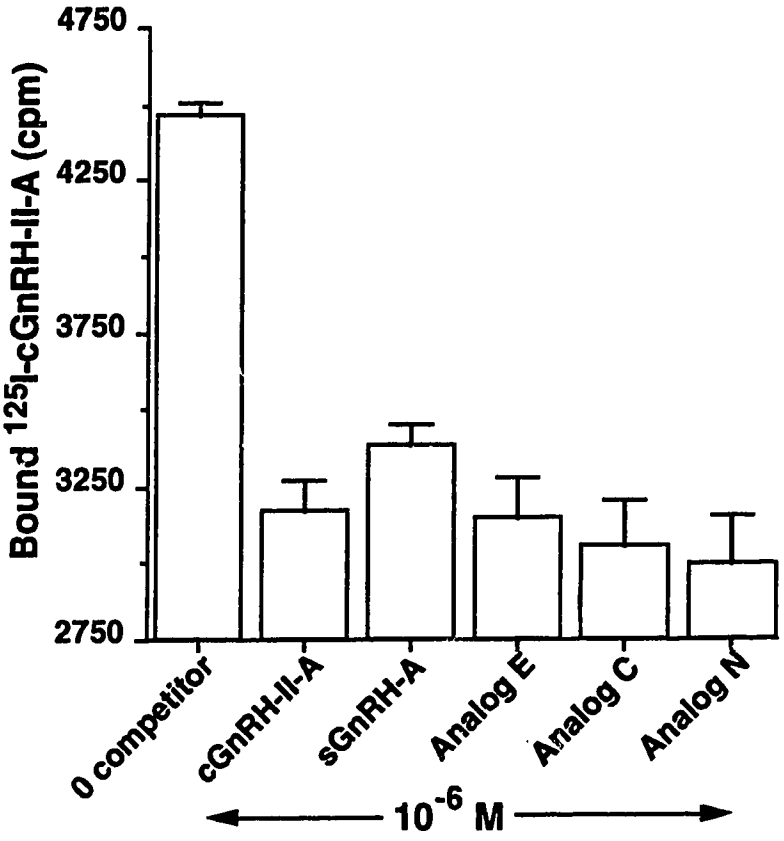


Fig. 6.6 Displacement of ^{125}I-[D-Arg^{6}]-cGnRH-II (cGnRH-II-A) bound to goldfish pituitary membrane by 10^{-6} M concentration of unlabeled cGnRH-II-A or [D-Arg^{6}, Pro^{9}-NHEt]-sGnRH or [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-mGnRH (analog E), or [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-sGnRH (analog C) or [Ac-D(2)Nal^{1}, 4Cl-D-Phe^{2}, D-(3)Pal^{3,6}]-cGnRH-II (analog N). Values (in cpm) represent mean ± SEM obtained from 2 similar experiments, in triplicate, using goldfish that were in sexual recrudescent stage of development (GSI = 4.2 ± 0.5%).

Table 6.1 Estimates of binding affinity (equilibrium association constant, Ka*) and 95 % confidence intervals of sGnRH and selected GnRH analogs to goldfish pituitary membrane preparations.

	Receptor binding affinity	
	High affinity sites	Low affinity sites
	Ka_1 10^{10} M^{-1} (95 % CI)	Ka_2 10^7, M^{-1} (95 % CI)
sGnRH	3.41 (2.43 - 4.78)	3.14 (1.87 - 5.27)
sGnRH-A	4.97 (4.41 - 5.60)	8.92 (7.97 - 10.03)
Analog C	24.60 (11.92 - 50.79)	7.85 (4.77 - 12.93)
Analog E	10.79 (8.55 - 13.60)	0.59 (0.2 - 1.74)
Analog N	46.22 (33.58 - 63.65)	1.21 (0.5 - 2.9)

*Ka values were estimated by using a non-linear least square curve-fitting computer program (LIGAND) from the displacement curves presented in Figs. 6.4 and 6.5, fitted for two classes of binding sites; data points obtained from 2-4 displacement curves, each in triplicate.

References

Bajusz, S., Csernus, V. J., Janaky, T., Bokser, L., Fekete, M., and Schally, A. V. (1988). New antagonists of LHRH. II. Inhibition and potentiation of LHRH by closely related analogues. Int. J. Peptide Protein Res. 32, 425-435.

Chang, J. P., Cook, H., Freedman, G.L., Wiggs, A. J., Somoza, G. M., De Leeuw, R., and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Clayton, R. N. (1989). Gonadotropin-releasing hormone: its actions and receptors. J. Endocrinol. 120, 11-19.

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J. P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul. Peptides, 36, 369-378.

De Leeuw, R., Van't Veer, C., Smit-Van Dijk, W., Goos, H. J. Th., and Van Oordt, P. G. W. J. (1988). Binding affinity and biological activity of gonadotropin-releasing hormone analogs in the African catfish, *Clarias gariepinus*. Aquaculture, 71, 119-131.

Kah, O. (1986). Central regulation of reproduction in teleosts. Fish Physiol. Biochem. 2, 25-34.

Karten, M. J., and Rivier, J. E. (1986). Gonadotropin-releasing hormone analog design. Structure-function studies towards the development of agonists and antagonists-rationale and perspectives. Endocrine Rev. 7, 44-66.

Gordon, K., and Hodgen, G. D. (1992). Evolving role of gonadotropin-releasing hormone antagonists. Trends Endocrinol. Metab. 3, 259-263.

Habibi, H. R. (1991). Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary : Effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod. 44, 275-283.

Habibi, H. R. and Peter, R. E. (1991). Gonadotropin-releasing hormone (GnRH) receptors in teleosts. In "Proceedings of the 4th International Symposium on The Reproductive Physiology of Fish" (A. P. Scott, J. P. Sumpter, D. E. Kime, and M.

Rolfe, Eds.), pp 109-113. FishSymp 91, Sheffield, UK.

Habibi, H. R., Marchant, T. A., Nahorniak, C. S., Van Der Loo, H., Peter, R. E., Rivier, J. E., and Vale, W. W. (1989). Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon GnRH in the pituitary of goldfish (*Carassius auratus*). Biol. Reprod. 40, 1152-1161.

Habibi, H. R., Peter, R. E., and Hazum, E. L. I. (1990). Photoaffinity labelling of pituitary gonadotropin-releasing hormone receptors in goldfish (*Carassius auratus*). Biol. Reprod. 43, 1006-1011.

Habibi, H. R., Peter, R. E., Nahorniak C. S., Milton, R. C. L., and Millar, R. P. (1992). Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides, 37, 271-284.

Habibi, H. R., Peter, R. E., Sokolowska, M., Rivier, J. E., and Vale, W. W. (1987). Characterization of gonadotropin-releasing hormone (GnRH) binding to pituitary receptors in goldfish (*Carassius auratus).* Biol. Reprod. 36, 844-853.

Hazum, E., and Keinan, D. (1983). Gonadotropin-releasing hormone receptors: Photoaffinity labeling with an antagonist. Biochem. Biophys. Res. Commun. 110, 116-121.

Janovick, J. A., Haviv, F., Fitzpatrick, T. D., and Conn, P. M. (1993). Differential orientation of a GnRH agonist and antagonist in the pituitary GnRH receptor. Endocrinology, 133, 942-945.

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P. Y., Loir, M., Mourot, B., and Weil, C. (1993). Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem 11, 219-232.

Lin, X. W., Lin, H. R., and Peter, R. E. (1993). Growth hormone and gonadotropin secretion in the common carp (*Cyprinus carpio* L): *In vitro* interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen. Comp. Endocrinol. 89, 62-71.

Loumaye, E., Naor, Z., and Catt, K. (1982). Binding affinity and biological activity of gonadotropin-releasing hormone agonists in isolated pituitary cells. Endocrinology, 111, 730-736.

Marchant, T. A., Chang, J. P., Nahorniak, C. S., and Peter, R. E. (1989). Evidence that

gonadotropin-releasing hormone also function as a growth hormone releasing hormone in the goldfish. Endocrinology, 124, 2509-2518.

McPherson, G. A. (1985a). Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. J. Pharmacol. Methods 14, 213-218.

McPherson, G. A. (1985b). Kinetic, EBDA, Ligand, Lowrey: A collection of radioligand binding analysis programs-Manual. Published by Biosoft, Cambridge, UK. pp 1-127.

Melamed, P., Eliahou, N., Sivan, B., and Yaron, Z. (1993). Hypothalamic factors regulating GH secretion in tilapia. In "Program, XII International Congress of Comparative Endocrinology", pp A-104 (abstract).

Munson, P. J., and Rodbard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand binding systems. Analytical Biochem. 107, 220-239.

Murthy, C. K., Nahorniak, C. S., Rivier, J. E., and Peter, R. E. (1993). *In vitro* characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish, *Carassius auratus*. Endocrinology, 133, 1633-1644.

Murthy, C. K., Peter, R. E., Rivier, J. E., and Vale, W. W. (1991). Characterization of gonadotropin-releasing hormone (GnRH) receptor antagonists in goldfish (*Carassius auratus*). In "Proceedings of the 4th International Symposium on The Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 63. FishSymp 91, Sheffield, UK.

Peng, C., Huang, Y. P., and Peter, R. E. (1990). Neuropeptide Y stimulates growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology, 52, 28-34.

Perrin, M. H., Haas, Y., Rivier, J. E., and Vale, W. W. (1983). Gonadotropin-releasing hormone binding to rat anterior pituitary membrane homogenates. Comparison of antagonists and agonists using radiolabeled antagonist and agonist. Mol. Pharmacol. 23, 44-51.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990a). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In "Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, and M. H. Stetson, Eds.). pp 393-398. Wiley-Liss Inc. NY.

Peter, R. E., Nahorniak, C. S., Chang, J. P., and Crim, L. W. (1984). Gonadotropin

release from the pars distalis of goldfish, *Carassius auratus* transplanted beside the brain or into the brain ventricles; Additional evidence for gonadotropin release-inhibitory factor. Gen. Comp. Endocrinol. 55, 337-346.

Peter, R. E., Trudeau, V. L., and Sloley, B. D. (1991). Brain regulation of reproduction in teleosts. Bull. Inst. Zool. Academia Sinica, Monograph, 16, 89-118.

Peter, R.E., Yu, K. L., Marchant, T. A., and Rosenblum, P. M. (1990b). Direct neural regulation of the teleost adenohypophysis. J. Expt. Zool. Supp. 4, 84-89.

Rivier, C., Rivier, J., Perrin, M., and Vale, W. (1983). Comparison of the effect of several gonadotropin releasing hormone antagonists on luteinizing hormone secretion, receptor binding and ovulation. Biol. Reprod. 29, 374-378.

Sarkar, D. K. (1987). In vivo secretion of LHRH in ovariectomized rats in regulated by a possible auto feedback mechanism. Neuroendocrinology, 45, 510-513.

Van Der Kraak, G., Suzuki, K., Peter, R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

Zanishi, M., Messi, E., Motta, M., and Martini, L. (1987). Ultrashort feedback control of luteinizing hormone-releasing hormone secretion *in vitro*. Endocrinology, 121, 2199-2204.

Chapter Seven

In vivo actions of a gonadotropin-releasing hormone (GnRH) antagonist on gonadotropin-II and growth hormone secretion in goldfish, *Carassius auratus*[1]

Introduction

In goldfish, the two native forms of GnRH, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) (Yu et al., 1988) stimulate gonadotropin-II (GTH-II) and growth hormone (GH) release both *in vivo* and *in vitro* (for review Peter et al., 1990a, 1991). The goldfish pituitary has two classes of GnRH binding sites, high affinity, low capacity sites and low affinity, high capacity binding sites (Habibi et al., 1987; Habibi and Peter 1991). Both sGnRH and cGnRH-II displace bound ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH from crude goldfish pituitary membrane preparations (Habibi and Peter, 1991). In addition, both sGnRH and cGnRH-II displace avidin gold-labelled biotinylated [D-Lys^{6}, Pro^{9}-NHEt]-sGnRH from the surfaces of immunohistochemically identified gonadotrophs and somatotrophs (Cook et al., 1991), indicating the presence of GnRH binding sites on both of these two cell types. The GTH-II responses to sGnRH and the GnRH receptor content increases with sexual development in goldfish (Habibi et al., 1989). At the onset of the preovulatory surges in plasma GTH-II and GH levels in goldfish, there is a corresponding decrease in brain and pituitary GnRH content (Yu et al., 1987). GnRH also stimulates GH release in common carp (Lin et al., 1993), rainbow trout (Le Gac et al., 1993), and tilapia (Melamed et al., 1993). Repeated injection of mGnRH-a results in enhancement of growth (Marchant et al., 1989). These results support the hypothesis that GnRH is involved in the regulation of both reproduction and growth of teleost fish.

[1]A version of this chapter has been submitted for publication: CK Murthy, W Zheng, VL Trudeau, CS Nahorniak, JE Rivier, RE Peter, Gen. Comp. Endocrinol.

The regulation of GTH-II release in goldfish is controlled by multiple hypothalamic factors (Peter et al., 1991). Besides GnRH, various other factors such as growth hormone-releasing hormone (GRF; Vaughan et al., 1992), neuropeptide Y (Peng et al., 1993), dopamine (Wong et al., 1993), thyrotropin-releasing hormone (Trudeau et al., 1992), and cholecystokinin (Himick et al., 1993) stimulate GH release. However, the relative importance of these various factors in regulation of GTH-II and GH release in fish is not clear. The two classical approaches to understand the role of endogenous factors in a given endocrine event are immunoneutralization and inhibition by antagonists. Unlike tetrapods, which have a well defined median eminence and a hypothalamic portal system, in teleosts the cell bodies of GnRH perikarya in the preoptic area project to the pituitary gland to directly innervate the GTH-II and GH cells (for review Peter et al., 1990b; Kah et al., 1993). This anatomical arrangement obviates passive immunoneutralization of hypothalamic factors to test their actions on the release of pituitary hormones.

In our previous *in vitro* study using perifused goldfish pituitary fragments, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) suppressed both GTH-II and GH release stimulated by sGnRH and cGnRH-II (Murthy et al., 1993; see Chapter 2). The inhibitory actions of analog E were observed in pituitary fragments collected from goldfish in different sexual development stages (Murthy et al., 1993; see Chapter 2). Analog E also inhibited GTH-II and GH release from dispersed goldfish pituitary cells stimulated by GnRH peptides, and displaced ^{125}I-[D-Arg^6, Pro^9-NHEt]-sGnRH bound to crude goldfish pituitary membrane preparations (see Chapter 6), indicating that analog E acts by binding to GnRH receptors.

In the present study, the ability of analog E to inhibit the increases in plasma GTH-II and GH levels induced by exogenous sGnRH and cGnRH-II in normal and dopamine depleted goldfish was demonstrated. Further, the importance of endogenous GnRH in regulation of basal plasma GTH-II and GH levels was examined. The involvement of GnRH in the increase in plasma GTH-II levels of sexually mature goldfish exposed to a female sexual pheromone was also demonstrated.

Materials and Methods

Experimental animals Goldfish of the common or comet varieties were purchased from Ozark Fisheries, Stoutland, MO. The fish were maintained in flow-through aquaria (1800

liters) at 17 ± 1°C under a simulated natural photoperiod of Edmonton for at least 2-3 weeks prior to experiments. The fish were fed to satiation daily with Ewos trout pellets. Fish weighing 20-35 g were used in the present study. Fish were tagged, transfered to smaller aquaria (100 liters) 5 days prior to experiment and were maintained at 20 ± 1°C. Fish were anesthetized by immersion in 0.05% solution of tricaine methane-sulfonate (Syndel Laboratories Ltd., Vancouver, BC, Canada) prior to all handling. During the experiments, blood samples were collected by puncture of the caudal vasculature using a 25-gauge needle attached to a 1-ml syringe rinsed with heparin (1000 Units/ml in distilled water; Sigma Chemical Co., St. Louis, MO). Plasma was collected following centrifugation, and frozen on dry ice and stored at -25°C until radioimmunoassay for sGnRH-II and GH levels. The sexual maturity of fish was assessed by measuring the gonadosomatic index (GSI= weight of gonad/ total body weight X 100). The reproductive developmental stage of fish was classified (using female fish as index) as sexually regressed (GSI = <2%), sexually recrudescent (GSI = 2 - 8%) and sexually mature (GSI = >8%). In the pheromone study, mature males with free flowing milt on slight pressure of the abdomen were used.

Reagents and test substances sGnRH, and cGnRH-II were purchased from Peninsula Laboratories Inc., Belmont, CA. [D-Arg^{6}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-mGnRH (analog E) was gift of Dr. J. E. Rivier, The Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA. All peptides were dissolved in a primary solvent containing propylene glycol and physiological saline at a ratio of 30:40, and subsequently diluted to required concentrations with saline. α-methyl-*p*-tyrosine methyl ester (α-MPT; Sigma Chemical Co., St. Louis, MO) was dissolved in saline prior to injection. Injections of all the peptides and chemicals were intraperitoneal in volumes of 5 µl/g body weight. In the pheromone study, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-P) (Sigma Chemical Co., St. Louis, MO) was dissolved in 100 µl of ethanol and added to the aquarium water to create a final water concentration of approximately 5 X 10^{-10} M (Dulka et al., 1992). Estradiol (E_2) was purchased from Sigma Chemical Co., St. Louis, MO.

Experimental protocols

The effects of analog E on increases in the plasma GTH-II and GH levels stimulated by sGnRH in normal goldfish: In the first experiment sexually mature females (GSI = 10 1%) were injected with sGnRH (0.1µg/g body weight), with or without analog E (1.0 µg/g), or with analog E (1.0 µg/g) alone. In the next experiment, sexually recrudescent goldfish (GSI = 2.4 ± 0.2%) were injected with vehicle (groups 1, 2) or analog E (1.0 µg/g; groups 3, 4) and one hour later (designated as time zero) injected with vehicle (groups 1, and 4) or sGnRH (1.0 µg/g; groups 2, and 3). In both experiments blood samples were taken at 0, 3, and 6 h post injection.

The effects of analog E on increases in plasma hormone levels stimulated by sGnRH in goldfish with depleted pituitary and brain dopamine levels: It is well documented that dopamine exerts a strong inhibitory effect on GTH-II release in teleost fish in general, and blockade of dopamine action by use of D2 type receptor antagonists or by suppression of dopamine synthesis results in potentiation of GnRH actions on GTH-II release (for review Peter et al., 1990a, 1991). Because it normally is difficult to induce a significant increase in plasma GTH-II levels in response to a low dose of GnRH (0.1 µg/g) in sexually regressed or early recrudescent goldfish due to the GTH-II release-inhibitory actions of dopamine, in this series of experiments the actions of analog E were tested in goldfish in which the inhibitory effects of dopamine were reduced. Following the protocol developed by Trudeau et al. (1993), fish were treated with α-MPT, a dopamine synthesis inhibitor (240 µg/g) and five days later the dopamine content in both the pituitary and brain were reduced by about 80 % (results not presented). Under these conditions fish were injected with vehicle, sGnRH or cGnRH-II (0.1 µg/g), sGnRH or cGnRH-II combined with analog E (1.0 µg/g), or analog E alone. Blood samples were collected at 0 and 3 h post injection.

Inhibitory effects of analog E on basal hormone levels: In two separate experiments, fish were injected with analog E (1.0 or 10.0 µg/g) and blood samples were taken at 1.5, 4, and 48 h post treatment.

The effects of analog E on the increased plasma GTH-II levels in sexually mature male

goldfish exposed to a female sexual pheromone: It is well documented that a preovulatory sexual pheromone, 17α, 20β-P, released from ovulating females, increases plasma GTH-II levels and milt volume in sexually matured male goldfish (for review Stacey et al., 1991). Following the protocol of Dulka et al. (1992) sexually mature male goldfish were placed in standing water aquaria (100 liters) and injected with either vehicle or analog E (1.0 μg/g), and one hour later (designated as time 0 h) fish were exposed for 6 h to ethanol (EtOH), or 10 μg of 17α, 20β-P dissolved in 100 μl of ethanol added to the water (approximately 5×10^{-10} M). Blood samples were collected at 6 h post pheromone exposure.

The effects of analog E on elevation in plasma GH levels stimulated by sGnRH in estrogen pretreated goldfish: Since estradiol (E_2) has been shown to enhance both basal and sGnRH stimulated GH release in goldfish (Trudeau et al., 1992), the effects of analog E on basal and sGnRH-stimulated plasma GH levels were tested. Solid silastic pellets containing E_2 or no steroid (blank) were prepared, washed in saline, and implanted intraperitoneally as described by Trudeau et al. (1991). Five days later, fish were injected with vehicle or analog E (2 μg/g), and one hour later (designated as time zero) injected with vehicle or sGnRH (0.5 μg/g). Blood samples were taken at 3 and 6 h post second injection.

<u>*Radioimmunoassay*</u> GTH-II levels in plasma sample were measured by validated RIA specific for GTH-II (Peter et al., 1984; Van Der Kraak et al., 1992). The GH levels were measured by using a goldfish GH RIA described by Murthy et al. (1993).

<u>*Data analysis*</u> The plasma GTH-II and GH levels in ng/ml were transformed as percentage of pretreatment (0 h) value and presented as mean ± SEM (Figs. 8.1, 8.2, 8.4, and 8.7). In the experiments where a 0 h blood sample was not taken, the plasma GTH-II and GH levels in ng/ml were simply averaged and presented as mean ± SEM (Figs 8.3, 8.5, 8.6, and 8.8). Statistical differences between treatment and control groups were assessed by either Student's t-test or ANOVA (on percentage pretreatment data or log transformed ng/ml data) followed by Fisher's LSD test. A p value of < 0.05 was considered as

statistically significant difference. However, higher level of significance, if found, was also indicated in the legends.

Results

GTH-II responses:

The effects of analog E on increases in the plasma GTH-II levels stimulated by sGnRH in normal goldfish: Compared to vehicle injected fish, sGnRH (0.1 μg/g) treated fish had significantly increased plasma GTH-II levels at 3 and 6 h post injection (Fig. 7.1). Co-injection of analog E (1.0 μg/g) with sGnRH significantly suppressed the sGnRH induced increase in plasma GTH-II levels at both 3 and 6 h post injection (Fig. 7.1a, b), indicating the ability of analog E to suppress the GTH-II stimulatory actions of exogenous sGnRH. Analog E treatment alone had no significant effects on plasma GTH-II levels at 3 and 6 h post injection.

In the second experiment, sGnRH (1.0 μg/g) again stimulated a significant increase in plasma GTH-II levels at both the 3 and 6 h sample times (Fig. 7.2a, b). However, in analog E (1.0 μg/g) pretreated fish, sGnRH (1.0 μg/g) failed to increase plasma GTH-II levels at both 3 and 6 h after the second injection (Fig. 7.2a, b). Similar to the first experiment, analog E alone had no significant effects on GTH-II levels.

The effects of analog E on the increases in plasma GTH-II levels stimulated by sGnRH in goldfish with depleted pituitary and brain dopamine levels: The basal GTH-II levels (pooled data from two groups each, n = 20-21) in α-MPT pretreated fish (8.2 ± 0.4 ng/ml) were slightly, but significantly higher than in the saline injected controls (6.2 ± 0.4 ng/ml). However, comparison of the basal GTH-II levels of individual groups (n = 10-11) did not show a significant difference between saline and α-MPT treatment (Fig. 7.3). In saline pretreated fish, injection of sGnRH (0.1 μg/g) caused a significant increase in plasma GTH-II levels 3 h post treatment (Fig. 7.3). In α-MPT pretreated fish, sGnRH (0.1 μg/g) stimulated significantly higher plasma GTH-II levels than in the saline pretreated sGnRH injected fish, indicating the potentiation of the GTH-II response to sGnRH in α-MPT pretreated fish (Fig. 7.3).

Treatment of α-MPT pretreated, sexually recrudescent goldfish with sGnRH (0.1 μg/g) resulted in a significant increase in plasma GTH-II levels at 3 h post-treatment

compared to vehicle treated fish (Fig. 7.4a). However, co-injection of sGnRH with analog E (1.0 μg/g) resulted in inhibition of the stimulatory effects of sGnRH on plasma GTH-II levels. Further, injection of analog E alone (1.0 μg/g) caused a significant suppression of basal GTH-II levels compared to control fish (Fig. 7.4a), indicating the involvement of endogenous GnRH in regulation of basal plasma GTH-II levels in dopamine depleted fish.

In a similar experiment, injection of cGnRH-II (0.1 μg/g) to α-MPT pretreated, sexually regressed goldfish (GSI = < 2%) induced a significant increase in plasma GTH-II levels at 3 h post injection (Fig. 7.4b). However, injection of cGnRH-II (0.1 μg/g) combined with analog E (1.0 μg/g) partially, but significantly reduced the elevation of plasma GTH-II release caused by cGnRH-II. Once again, analog E treatment alone reduced basal GTH-II levels at 3 h post-treatment, compared to control fish (Fig. 7.4b).

Inhibitory effects of analog E on basal plasma GTH-II levels: The involvement of endogenous GnRH in regulation of basal GTH-II levels in normal fish was further examined by using either a moderate dose of analog E (1.0 μg/g) in a large number of fish (n = 22-24) or a high dose (10 μg/g) in a small number of fish (n = 7-9). Injection of analog E (1.0 μg/g) to sexually recrudescent fish (GSI = 2.4 ± 0.2; n = 22-24) significantly reduced plasma GTH-II levels at 90 min post-injection compared to vehicle injected fish (Fig. 7.5a). In another experiment, injection of 10 μg/g of analog E caused a significant decrease (48% reduction compared to vehicle treated group) in plasma GTH-II levels in sexually recrudescent goldfish (GSI = 2.6 ± 0.6; n = 7-9) (Fig. 7.5b). The plasma GTH-II levels in analog E injected fish remained lower compared to vehicle injected fish at 4 h (7.5 ± 1.3 vs 12.8 ± 1.9 ng/ml), but not at 48 h post-treatment (10.1 ± 2.7 vs 11.8 ± 2.1 ng/ml).

The effects of analog E on the increased plasma GTH-II levels in sexually mature male goldfish exposed to a female sexual pheromone: In vehicle pretreated fish, exposure to 17α, 20β-P resulted in a significant increase in plasma GTH-II levels compared to ethanol exposed fish (Fig. 7.6). In analog E (1.0 μg/g) pretreated fish, exposure to 17α, 20β-P failed to cause a significant increase in plasma GTH-II levels compared to ethanol exposed sexually mature goldfish (Fig. 7.6). 17α, 20β-P had no significant effects on plasma GH levels in either vehicle or analog E pretreated male goldfish (results not shown).

GH responses:

The effects of analog E on increases in the plasma GH levels stimulated by sGnRH in normal goldfish: Injection of a high dose of sGnRH (1.0 μg/g) resulted in a significant increase in plasma GH levels at 3 h after second injection (Fig. 7.7a). In analog E (1.0 μg/g) pretreated fish, sGnRH (1.0 μg/g) failed to stimulate an increase in plasma GH levels (Fig. 7.7a). Analog E alone had no significant actions on GH levels. At 6 h after the second injection, vehicle plus sGnRH treated fish, but not analog E plus sGnRH treated fish, had significantly higher plasma GH levels compared to vehicle plus vehicle treated fish (results not shown), indicating a continued inhibition of sGnRH actions on plasma GH levels by analog E.

In sexually recrudescent goldfish (GSI = 4.1 ± 0.6%) pretreated with α-MPT, cGnRH-II (1.0 μg/g) caused a significant increase in plasma GH levels; however, co-injection of analog E (1.0 μg/g) with cGnRH-II inhibited the increase in the plasma GH levels (Fig. 7.7b).

The effects of analog E on elevation in plasma GH levels stimulated by sGnRH in estrogen pretreated goldfish: In goldfish implanted with blank pellets, injection of sGnRH stimulated an increase in plasma GH levels compared to vehicle injected fish (Fig. 7.7c). Implantation of E_2 pellets increased the basal plasma GH levels compared to fish implanted with blank pellets. In E_2 implanted fish, sGnRH injection stimulated significantly increased plasma GH levels compared to E_2 implanted vehicle injected fish, as well as blank implanted sGnRH injected fish (Fig. 7.7c). Notably, pretreatment of the E_2 implanted fish with analog E (1.0 μg/g) 1 h prior to sGnRH injection, resulted in inhibition of the GH stimulatory effects of sGnRH. Analog E alone had no significant effects on basal GH levels in E_2 primed fish (Fig. 7.7c).

Inhibitory effects of analog E on basal plasma GH levels: Similar to the results on GTH-II release, injection of a moderate dose of analog E (1.0 μg/g) in a large number of fish (n = 17-21) or a high dose (10.0 μg/g) in small number of fish (n = 7-9) significantly reduced plasma GH levels at 90 min post-injection, compared to vehicle injected fish (Fig. 7.8a, b). The plasma GH levels in analog E (10.0 μg/g) injected fish remained significantly lower

compared to vehicle injected fish at 4 h, but not at 48 h post-treatment (results not presented).

Discussion

The increases in plasma GTH-II levels stimulated by injection of sGnRH (0.1 and 1.0 µg/g body weight) in sexually mature female and sexually recrudescent goldfish were inhibited by co-injection or pretreatment (1 h prior to sGnRH injection) with [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E). Dosages of analog E to sGnRH at a ratio of 10:1 or 1:1 significantly suppressed such an increases in plasma GTH-II levels for at least 6 h after sGnRH injection. Further, analog E suppressed both sGnRH- and cGnRH-II-stimulated increases in plasma GTH-II levels in dopamine depleted sexually regressed or recrudescent goldfish. Analog E also inhibited the increase in plasma GH levels induced by sGnRH (in normal fish or E_2 primed fish) and cGnRH-II (in dopamine depleted fish). In any of these experiments analog E alone had no stimulatory actions on plasma GTH-II or GH levels. These results indicate that analog E can effectively suppress the increases in plasma GTH-II and GH levels caused by the injection of native GnRH peptides and the inhibitory ability of analog E can be observed throughout the reproductive developmental stages of goldfish. These results also support our *in vitro* observations that analog E acts as a 'true' GnRH antagonist in goldfish, inhibiting sGnRH- and cGnRH-II- stimulated GTH-II and GH release, without having independent stimulatory effects on either GTH-II or GH release (Murthy et al., 1993; see Chapter 2).

In rat, injection of 7.5 µg of analog E at noon on the day of proestrus inhibited ovulation by 100% (Rivier et al., 1984). Analog E exhibited acute and long term suppression of pulsatile gonadotropin secretion in adult male monkeys (Bercu et al., 1984). Similarly the gonadotropin inhibitory effects of analog E have also been demonstrated in normal men and postmenopausal women (for review Pavlou et al., 1987). Thus analog E acts as a GnRH antagonist in mammals as well as in fish.

Since analog E was able to suppress the actions of sGnRH and cGnRH-II on GTH-II and GH release, both *in vitro and in vivo*, the role of endogenous GnRH in regulation of basal plasma GTH-II and GH levels was examined by injecting analog E to inhibit endogenous GnRH actions. Injection of analog E at a moderate dosage (1.0 µg/g) to a large number of fish, or a high dosage (10.0 µg/g) to a small number of fish resulted in

significant decreases in basal plasma GTH-II and GH levels for at least up to 4 h after injection, indicating the regulatory importance of endogenous GnRH in both GTH-II and GH release. The decrease in plasma GTH-II and GH levels following analog E injection are not due to non-specific, toxic effects of analog E; at 48 h after injection of analog E (10.0 μg/g) plasma GTH-II and GH levels were similar to vehicle injected fish. Further, in dopamine depleted goldfish, a moderate dose of analog E (1.0 μg/g) caused a significant suppression of basal GTH-II levels (Fig. 7.4a, b), but not GH levels (Fig. 7.7b), indicating the facility of studying the role of endogenous GnRH in regulation of GTH-II release under a dopamine depleted state.

Similar to earlier studies (Marchant et al., 1989), both sGnRH and cGnRH-II injection caused an increase in plasma GH levels. Such an increase in plasma GH levels was inhibited by analog E. In goldfish, besides GnRH, various other hypothalamic peptides also stimulate GH release (see Introduction). An important question is why GnRH peptides are involved in the regulation of GH secretion in goldfish and other teleosts. GnRH has no effect on GH release in normal mammals, except under various pathological conditions (Müller, 1987), nor in birds and reptiles (for review Harvey, 1993). In goldfish sexual development involves growth of gonads from less than 1% of body weight to as much as 20% of body weight. Growth hormone potentiates GTH-II-stimulated steroid production by ovarian follicles of goldfish (Van Der Kraak et al., 1990). Recombinant salmon GH increases and modulates steroid production by rainbow trout and killifish gonads *in vitro* (Singh et al., 1988; Le Gac et al., 1992) and in spotted seatrout (*Cynoscion nebulosus*) (Singh and Thomas, 1993). High affinity, low capacity GH binding sites have been reported in trout testis (Le Gac et al., 1992). The plasma GH levels in adult Atlantic salmon, *Salmo salar*, increase with sexual development, and manipulation of photoperiod causing delay or advancement of sexual maturity results in parallel change in timing of the increases in plasma GH levels (Björnssson et al., 1994). Co-treatment of GH with E_2 was required for induction of vitellogenin synthesis in primary hepatocyte culture in eel, *Anguilla japonica* (Kwon and Mugiya, 1994). These results suggest the involvement of GH in reproductive activities, and that control of both GTH-II and GH by GnRH peptides is an integral function in regulation of reproduction in teleost fish.

In goldfish, the gonadal steroid 17α, 20β-P functions as a potent preovulatory female

sex pheromone in males to stimulate increases in serum GTH-II levels and subsequent increases in milt volume (Stacey et al., 1991). At least part of the neuroendocrine mechanisms involved in the increase in GTH-II release in response to exposure to 17α, 20β-P is a rapid reduction in the turnover of dopamine at the pituitary level (Dulka et al., 1992). This decrease in dopamine inhibitory action on gonadotrophs presumably serves to potentiate the actions of endogenous GnRH. In the present study exposure of vehicle injected mature male goldfish to 17α, 20β-P, resulted, as expected, in an increase of plasma GTH-II levels and milt volume (data not shown). Interestingly, preinjection of male fish with analog E resulted in a significant suppression in the increase in the plasma GTH-II levels caused by 17α, 20β-P, but not milt volume (data not shown). This indicates the involvement of endogenous GnRH in mediating the actions of 17α, 20β-P on GTH-II release. However, from our study it is not possible to determine whether the activation of the GnRH system by 17α, 20β-P is parallel to a reduction in dopamine turnover or subsequent to it. Because, dopamine also inhibits GnRH release from hypothalamic slices and pituitary fragments *in vitro* (Yu and Peter., 1991), it is possible that a reduction in dopamine turnover (Dulka et al., 1992) can cause an increase in GnRH release as well as potentiate the actions of GnRH peptides on GTH-II release, leading to an increase in plasma GTH-II levels. Alternatively, the pheromone may stimulate GnRH release, but simultaneously suppress dopamine release, resulting in increased GTH-II release. Anatomical evidence in catfish indicates the possibility of activation of the GnRH system in the preoptic area by pheromones acting through the medial olfactory tract (Resink et al., 1989). In the preoptic region of goldfish, perikarya and axons of GnRH are innervated by dopaminergic neurons and in turn GnRH neurons innervate dopamine perikarya and axons (Anglade et al., 1991; Kah et al., 1993), indicating a close interaction between GnRH and dopamine systems in regulation of GTH-II release

In the present study, there was no significant change in the plasma GH levels of males exposed to 17α, 20β-P. In goldfish, dopamine stimulates GH release both *in vivo* and *in vitro* by acting through DA D1 type receptors, and DA D1 type receptor antagonists reduce the plasma basal GH levels (Wong et al., 1993). A reduction in dopamine turnover in males following exposure to 17α, 20β-P would lead to a decrease in basal plasma GH levels. Since there is no change in plasma GH levels, another factor, probably GnRH,

must be causing an additional stimulation of GH release to compensate for the reduction in stimulation by dopamine. Results with GTH-II indicating the involvement of GnRH, supports the idea that 17α, 20β-P causes an increase in GnRH release, in addition to a decrease in dopamine turnover.

In conclusion, the present results suggest that [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) inhibits native GnRH actions on GTH-II and GH release *in vivo*, without showing any independent stimulatory actions. Inhibition of basal GTH-II and GH levels by analog E strongly suggests the importance of endogenous GnRH in regulation of both GTH-II and GH release in goldfish. In α-MPT pretreated (dopamine depleted) fish analog E inhibits the actions of both exogenous and endogenous GnRH peptides on GTH-II release. Increases in plasma GTH-II levels in male goldfish following exposure to 17α, 20β-P were also inhibited by analog E. The regulation of both GTH-II and GH release by GnRH may be essential owing to participation of both GTH-II and GH in various endocrine events during gonadal cycles, and ovulation, in fish.

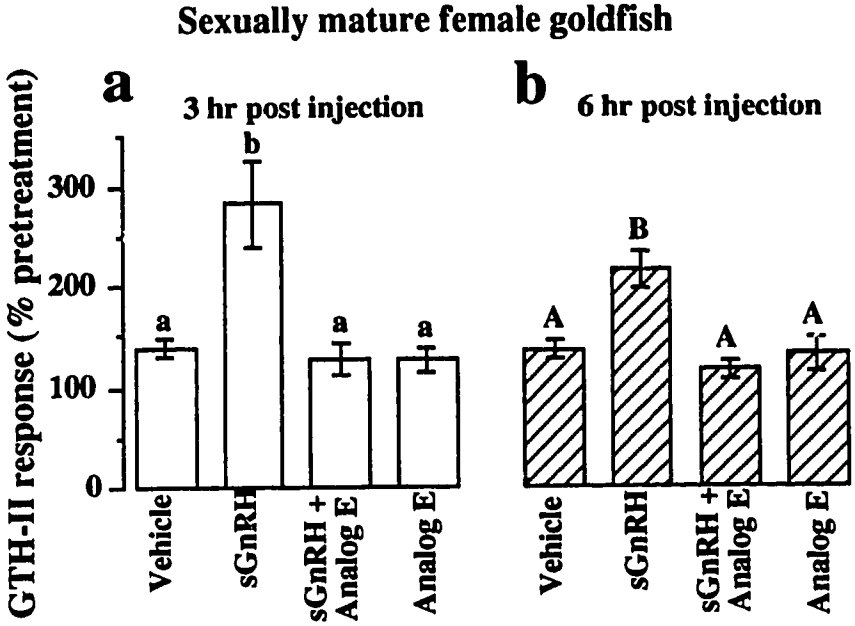


Fig. 7.1 The changes in the plasma GTH-II levels expressed as % of pretreatment mean ± SEM at 3 hr (a) and 6 hr (b) after injection of vehicle, sGnRH (0.1 μg/g body weight), sGnRH combined with [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; 1.0 μg/g) or analog E (1.0 μg/g) alone to sexually mature female goldfish (GSI = 10.5 ± 1%). The significant differences between treatment groups were analysed by ANOVA followed by Fisher's LSD test (n = 10) and indicated by different letter superscripts ($p < 0.001$).

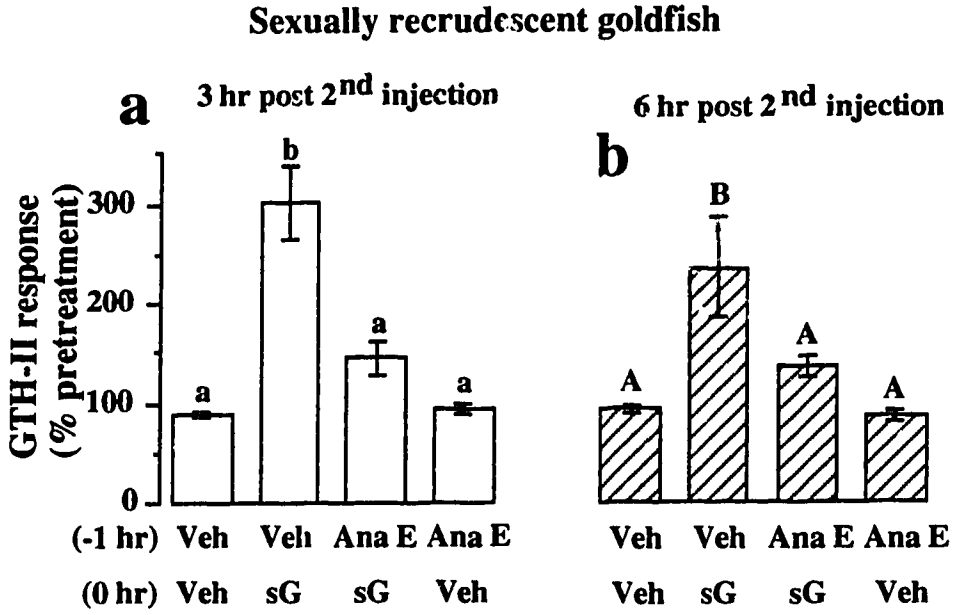


Fig 7.2 The changes in the plasma GTH-II levels expressed as % of pretreatment mean ± SEM at 3 hr (a) and 6 hr (b) after the second injection of vehicle + vehicle (Veh + Veh), vehicle + sGnRH (Veh + sG, 1.0 μg/g), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; 1.0 μg/g) plus sGnRH (ana E + sG), or analog E (1.0 μg/g) plus vehicle (ana E + Veh) to sexually recrudescent goldfish (GSI = 2.4 ± 0.2%). The significant differences between treatment groups were analysed by ANOVA followed by Fisher's LSD test (n = 11-12) and indicated by different letter superscripts ($p < 0.001$).

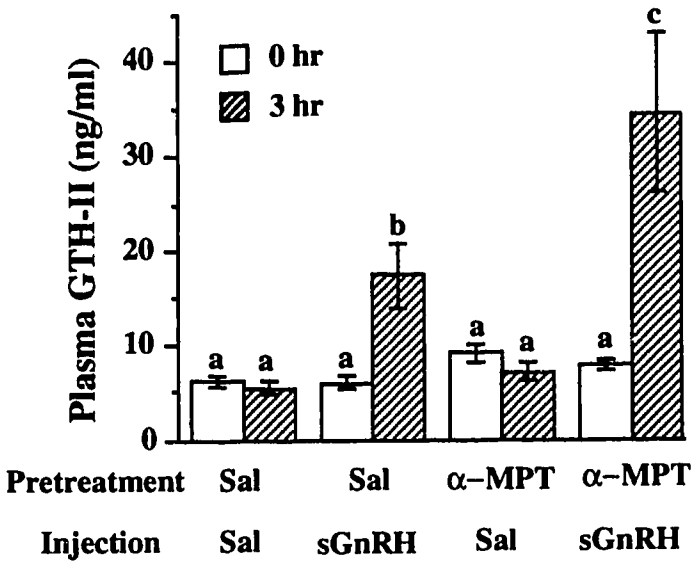


Fig. 7.3 The changes in the plasma GTH-II levels presented as mean (ng/ml) ± SEM at 0 hr and 3 hr after injection of saline (sal) or sGnRH (1.0 μg/g) to sexually recrudescent goldfish (GSI = 2.5 ± 0.2%), pretreated with α-methyl-p-tyrosine methyl ester (α-MPT; 240 μg/g) five days prior to experiment. The significant differences between treatment groups were analysed by ANOVA on log transformed data, followed by Fisher's LSD test (n = 10) and indicated by different letter superscripts ($p < 0.01$).

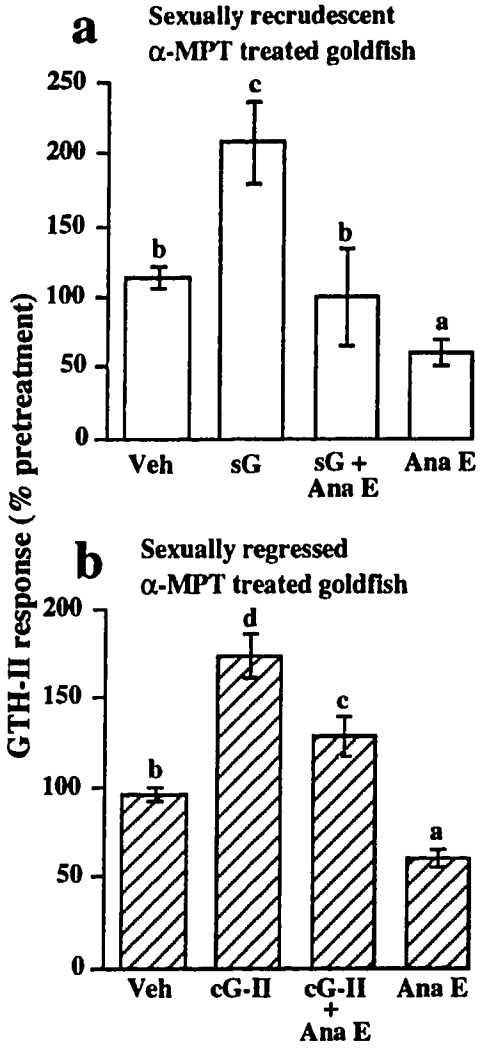


Fig. 7.4 The changes in the plasma GTH-II levels (expressed as % of pretreatment mean ± SEM) at 3 hr after injection of vehicle (Veh), sGnRH (sG; 0.1 μg/g body weight), sGnRH combined with [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; 1.0 μg/g), or analog E (ana E; 1.0 μg/g) alone in α-MPT (240 μg/g) pretreated sexually recrudescent goldfish (GSI = 5.4 ± 0.6%) (a). Similarly, the changes in the plasma GTH-II levels (expressed as % of pretreatment mean ± SEM) at 3 hr after injection of vehicle (Veh), cGnRH-II (cG-II; 0.1 μg/g body weight), cGnRH-II combined with analog E (1.0 μg/g), or analog E (ana E; 1.0 μg/g) alone in α-MPT (240 μg/g) pretreated sexually regressed goldfish (GSI = < 2 %) (b). The significant differences between treatment groups were analysed by ANOVA followed by Fisher's LSD test (n = 10-12) and indicated by different letter superscripts ($p < 0.01$).

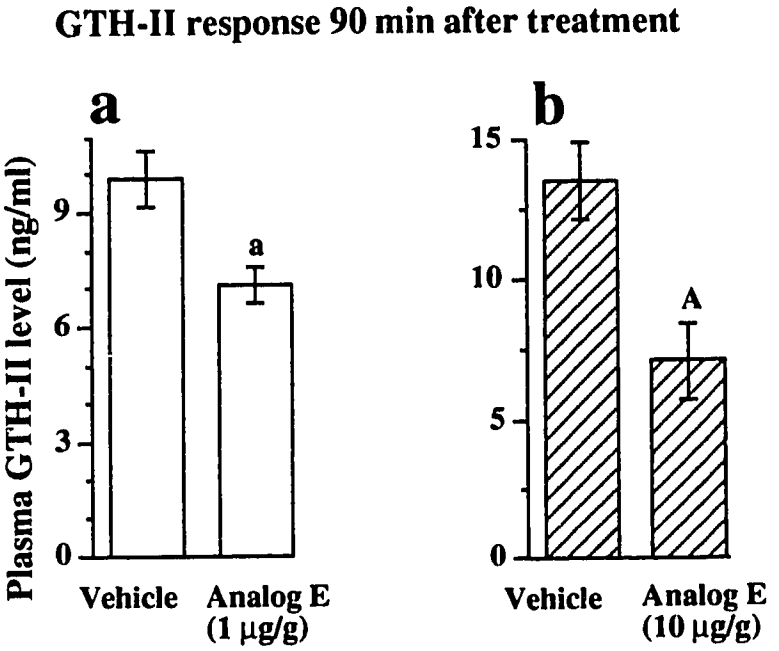


Fig. 7.5 The changes in the plasma GTH-II levels presented as mean (ng/ml) ± SEM at 1.5 hr after injection of (i) vehicle or [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; 1.0 µg/g) in sexually recrudescent goldfish (GSI = 2.4 ± 0.2; n = 22-24) (a) and (ii) vehicle or analog E (10.0 µg/g) in sexually recrudescent goldfish (GSI 2.6 ± 0.6; n = 7-9) (b). The significant difference between treatment and control groups in each experiment was analysed by Student's t-test and indicated by different letter superscripts ($p < 0.05$).

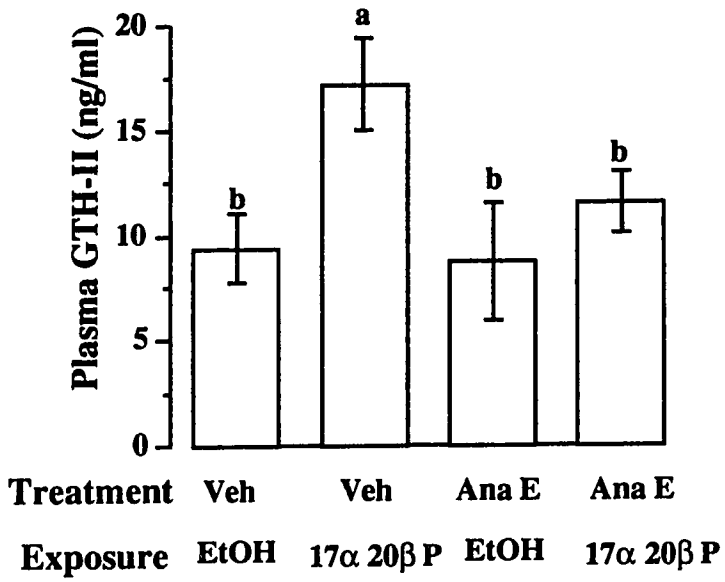


Fig. 7.6 The changes in the plasma GTH-II levels presented as mean (ng/ml) ± SEM at 6 hr after exposure to ethanol (control) or 17α, 20 β-dihydroxy-4-pregnen-3-one (17α, 20β-P) in sexually mature male goldfish pretreated (at -1 hr) with vehicle or [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E; 1.0 μg/g). The significant differences between treatment groups were analysed by ANOVA on log transformed data, followed by Student's t-test (n = 10) and indicated by different letter superscripts ($p < 0.01$).

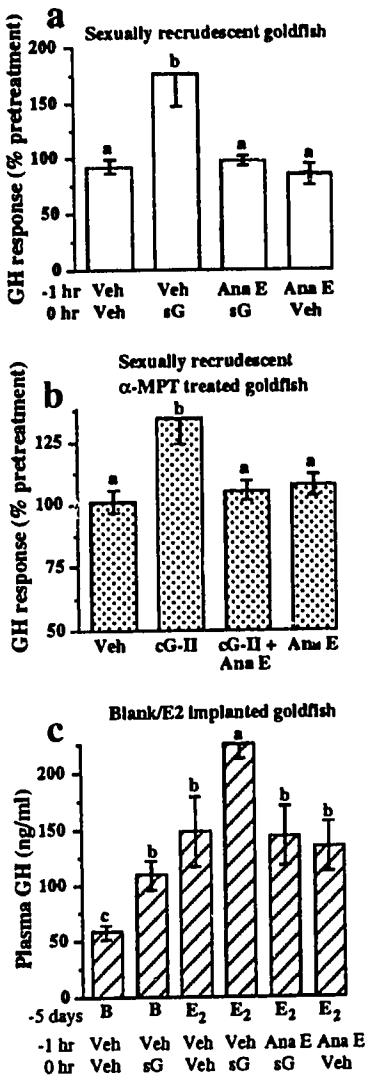


Fig. 7.7 The changes in the plasma GH levels expressed as % of pretreatment mean ± SEM at 3 hr after the second injection of vehicle + vehicle (Veh + Veh), vehicle + sGnRH (Veh + sG, 1.0 μg/g), [Ac-Δ3-Pro1, 4FD-Phe2, D-Trp3,6]-mGnRH (analog E; 1.0 μg/g) plus sGnRH (ana E + sG), or analog E (1.0 μg/g) plus vehicle (ana E + Veh) to sexually recrudescent goldfish (GSI = 2.4 ± 0.2%)(a). The changes in the plasma GH levels expressed as % of pretreatment mean ± SEM at 3 hr after injection of vehicle, cGnRH-II (cG-II; 0.1 μg/g), analog E (1.0 μg/g) combined with cGnRH-II (cG-II; 0.1 μg/g), or analog E (1.0 μg/g) alone in a-MPT (240 μg/g) pretreated sexually recrudescent goldfish (GSI =4.1 ± 0.6%)(b). The changes in the plasma GH levels presented as ng/ml (mean ± SEM) at 3 hr after injection of sGnRH-II (sG, 0.5 μg/g) pretreated or not with analog E (2.0 μg/g), or analog E (ana E, 2.0 μg/g) alone in blank (B) or estradiol (E2) implanted sexually recrudescent goldfish (GSI =2.5 ± 0.3 %)(c). The significant differences between treatment groups were analysed by ANOVA followed by Fisher's LSD test) and indicated by different letter superscripts ($p < 0.005$ for Figs. a, c and $p < 0.001$ for Fig. b).

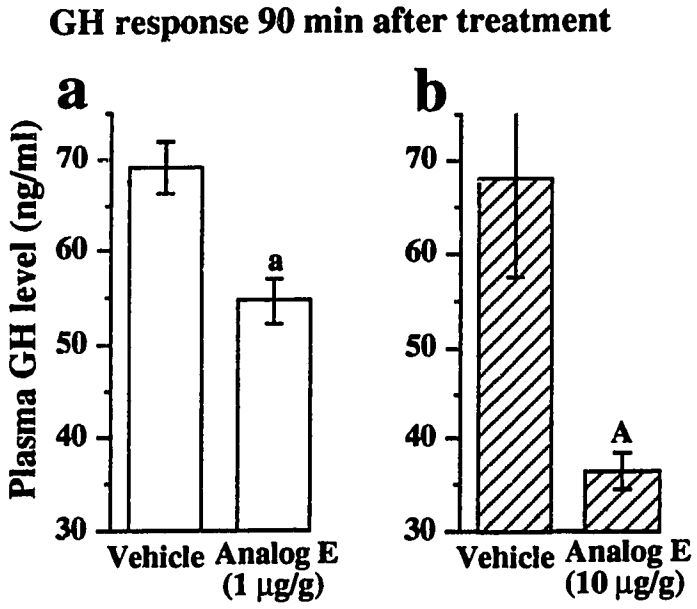


Fig. 7.8 The changes in the plasma GH levels presented as mean (ng/ml) ± SEM at 1.5 hr after injection of vehicle or [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E, 1.0 μg/g) in sexually recrudescent goldfish (GSI = 2.4 ± 0.2; n = 22-24) (a), and vehicle or analog E (10.0 μg/g) in sexually recrudescent goldfish (GSI 2.6 ± 0.6; n = 7-9) (b). The significant difference between treatment and control groups in each experiment was analysed by Student's t-test and indicated by different letter superscripts ($p < 0.05$ for Fig. a and $p < 0.01$ for Fig. b).

References

Anglade, I., Tramu, G., and Kah, O. (1991). Neuroanatomical substrate for dopamine-sGnRH (gonadotrophin-releasing hormone) interactions in the forebrain of the goldfish (*Carassius auratus*). In "Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish" (A. P. Scott, J. P. Sumpter, D. E. Kime, M. S. Rolfe, Eds.), pp 60. FishSymp 91, Sheffield, UK.

Bercu, B. B., Spiliotis, B. E., Lee, B. C., Brown, T. J., Vale, W., Rivier, J., Nixon, W. E., and Reid, R. (1984). Effects of a potent gonadotropin releasing hormone antagonist on pulsatile testosterone and gonadotropin secretion in the male nonhuman primate. Life Sci. 35, 381-387.

Björnsson, B. T., Taranger, G. L., Hansen, T., Stefansson, S. O., and Haux C. (1994). The interrelation between photoperiod, growth hormone, and sexual maturation of adult atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 93, 70-81.

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J. P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul. Peptides, 36, 369-378.

Dulka, J. G., Sloley, B. D., Stacey, N. E., and Peter, R. E. (1992). A reduction in pituitary dopamine turnover is associated with sex pheromone-induced gonadotropin secretion in male goldfish. Gen. Comp. Endocrinol. 86, 496-505.

Habibi, H. R, and Peter, R. E. (1991). Gonadotropin-releasing hormone (GnRH) receptors in teleosts. In "Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish" (A. P. Scott, J. P. Sumpter, D. E. Kime, M. S. Rolfe, Eds.), pp 109-113. FishSymp 91, Sheffield, UK.

Habibi, H. R., De Leeuw, R., Nahorniak, C. S., Goos, H. J. Th., and Peter, R. E. (1989). Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: Seasonal and gonadal effects. Fish Physiol. Biochem. 7, 109-118.

Habibi, H. R., Peter, R. E., Sokolowska, M., Rivier, J. E., and Vale, W. W. (1987). Characterization of gonadotropin-releasing hormone (GnRH) binding to pituitary receptors in goldfish (*Carassius auratus*). Biol. Reprod. 36, 844-853.

Harvey, S. (1993). Growth hormone secretion in poikilotherms and homeotherms. In "The

endocrinology of growth, development and metabolism in vertebrates" (M. P. Schreibman, C. G. Scanes, and P. K. T. Pang, Eds.), pp 151-176. Academic Press, New York.

Himick, B. A., Golosinski, A. A., Jonsson, A. -C., and Peter, R. E. (1993). CCK/ gastrin-like immunoreactivity in the goldfish pituitary: Regulation of pituitary hormone secretion by CCK-like peptides *in vitro*. Gen. Comp. Endocrinol. 92, 88-103.

Kah, O., Anglade, I., Lepretre, E., Dubourg, P., and de Monbrison, D. (1993). The reproductive brain in fish. Fish Physiol. Biochem. 11, 85-98.

Kwon, H-C. and Mugiya, Y. (1994). Involvement of growth hormone and prolactin in the induction of vitellogenin synthesis in primary hepatocyte culture in the eel, *Anguilla japonica*. Gen. Comp. Endocrinol. 93, 51-60.

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P.-Y., Loir, M., Mourot, B., and Weil, C. (1993). Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem. 11, 219-232.

Le Gac, F., Ollitrault, M., and Le Bail, P.-Y. (1992). Evidence for binding and action of growth hormone in trout testis. Biol. Reprod. 46, 949-957.

Lin, X. W., Lin, H. R., and Peter, R. E. (1993). Growth hormone and gonadotropin secretion in the common carp (*Cyprinus carpio* L): *In vitro* interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen. Comp. Endocrinol. 89, 62-71.

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 hormone in the goldfish. Endocrinology, 124, 2509-2518.

Melamed, P., Eliahou, N., Sivan, B., and Yaron, Z. (1993). Hypothalamic factors regulating GH secretion in tilapia. In: Program of XII International Congress of Comparative Endocrinology, Toronto, Canada, p A-104 (abstract).

Müller, E. E. (1987). Neural control of somatotropic function. Physiol. Rev. 67, 962-1053.

Murthy, C. K., Nahorniak, C. S., Rivier, J. E., and Peter, R. E. (1993). *In vitro* characterization of gonadotropin-releasing hormone (GnRH) antagonists in goldfish, *Carassius auratus*. Endocrinology, 133, 1633-1644.

Pavlou, S. N., Wakefield, G. B., and Kovacs, W. J. (1987). LHRH antagonists in

normal men. In "LHRH and its analogs: Contraceptive and Therepeutic applications". Part 2, (B. H. Vickery and J. J. Nestor, Jr. Eds.), pp 245-256. MTP Press, Boston.

Peng, C., Chang, J. P., Yu, K. L., Wong, A. O.-L., Van Goor, F., Peter, R. E., and Rivier, J. E. (1993). Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary: Involvement of both presynaptic and pituitary cell actions. Endocrinology, 132, 1820-1829.

Peter, R. E., Nahorniak, C. S., Chang, J. P., and Crim, L. W. (1984). Gonadotropin release from the pars distalis of goldfish, *Carassius auratus* transplanted beside the brain or into the brain ventricles; Additional evidence for gonadotropin release-inhibitory factor. Gen. Comp. Endocrinol. 55, 337-346.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P., and Marchant, T. A. (1990a). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In "Progress in Comparative Endocrinology" (A. Epple, C. G. Scanes, M. H. Stetson, Eds.), pp 393-398. Wiley-Liss Inc. New York.

Peter, R. E,. Trudeau, V. L., and Sloley, B. D. (1991). Brain regulation of reproduction in teleosts. Bull. Inst. Zool., Academia Sinica, Monograph, 16, 89-118.

Peter, R. E., Yu, K. L., Marchant, T. A., and Rosenblum, PM. (1990b). Direct neural regulation of the teleost adenohypophysis. J. Exp. Zool., Suppl. 4, 84-89.

Resink, J. W., Voorthuis, P. K.,Van den Hurk, R., Vullings, H. G. B., and Van Oordt, P. G. W. J. (1989). Pheromone detection and olfactory pathways in the brain of the female African catfish, *Clarias gariepinus*. Cell Tissue Res. 256, 337-345.

Rivier, C., Rivier, J., Perrin, M., and Vale, W. (1983). Comparison of the effect of several gonadotropin releasing hormone antagonists on luteinizing hormone secretion, receptor binding and ovulation. Biol. Reprod. 29, 374-378.

Singh, H., Griffith, R. W., Takahashi, A. P., Kawauchi, H., and Stegeman, J. J. (1988). Regulation of gonadal steroidogenesis in *Fundulus heteroclitus* by recombinant salmon growth hormone and purified salmon prolactin. Gen. Comp. Endocrinol. 72, 144-153.

Singh, H., and Thomas, P. (1993). Mechanisms of stimulatory action of growth hormone on ovarian steroidogenesis in spotted seatrout (*Cynoscion nebulosus*). Gen. Comp. Endocrinol. 89, 341-353.

Stacey, N. E., Sorensen, P. W., Dulka, J. G., Cardwell, J. R., and Irvine, A. S. (1991).

Fish sex pheronones: Current status and potential applications. Bull. Inst. Zool., Academia Sinica, Monograph, 16, 189-227.

Trudeau, V. L., Peter, R. E., and Sloley, B. D. (1991). Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol. Reprod. 44, 951-960.

Trudeau, V. L., Sloley, B. D., Wong, A. O. L., and Peter, R. E. (1993). Interaction of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. Gen. Comp. Endocrinol. 89, 39-50.

Trudeau, V. L., Somoza, G. M., Nahorniak, C. S., and Peter, R. E. (1992). Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology, 56, 483-490.

Van Der Kraak, G., Rosenblum, P. M., and Peter, R. E. (1990). Growth hormone-dependent potentiation of gonadotropin-stimulated steroid production by ovarian follicles of the goldfish. Gen. Comp. Endocrinol. 79, 233-239.

Van Der Kraak, G., Suzuki K., Peter R. E., Itoh, H., and Kawauchi, H. (1992). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

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.

Yu, K. L., Nahorniak, C. S., Peter, R. E., Corrigan, A., Rivier, J. E. and Vale, W. W. (1987). Brain distribution of radioimmunoassayable gonadotropin-releasing hormone in female goldfish: Seasonal variation and periovulatory changes. Gen. Comp. Endocrinol. 67, 234-246.

Yu, K. L., Rosenblum, P. M. and Peter, R. E. (1991). *In vitro* release of gonadotropin-releasing hormone from the preoptic-anterior hypothalamic region and pituitary female goldfish. Gen. Comp. Endocrinol. 81, 256-267.

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 Eight

General Discussions and Conclusions

The main aim of the present thesis was to characterize and study the structure-activity relations of GnRH antagonists in fish, using goldfish as a model. The other objectives were to use GnRH antagonists, thus characterized, to understand the role of GnRH in various activities associated with regulation of GTH-II and GH secretion. Further, it was also envisaged to use the information gained from structure-activity studies in designing and testing potent GnRH agonists and antagonists.

Characterization of GnRH antagonists:

The objectives of the initial 'screening' studies (Chapter 2) using the perifused goldfish pituitary fragments were (i) to test whether 'putative' GnRH antagonists have any effects on GTH-II and GH release and (ii) whether these analogs can inhibit GTH-II and GH release stimulated by sGnRH and cGnRH-II. Based on their actions the analogs can be grouped into three categories. The first group of analogs including analogs E, M, and N, had no actions on their own, on either GTH-II or GH release. However, in continuous presence of these analogs, sGnRH and cGnRH-II stimulation of GTH-II and GH release were significantly reduced, and hence these analogs are designated 'true' antagonists. The second group of analogs, including analogs A, B, D, G, H, I, J, and K had moderate to strong GTH-II as well as GH releasing activity on their own, and in continuous presence of these analogs, sGnRH and cGnRH-II stimulation of GTH-II and GH release were significantly reduced. These analogs are designated 'partial' antagonists (antagonists with agonistic activity). The third group of analogs including analogs C, F and L, on their own stimulated either GTH-II or GH release, but not both. These analogs also significantly suppressed sGnRH and cGnRH-II stimulation of GTH-II and GH release. This group of analogs do not fit any particular designation.

Among the 'true' antagonists [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) and [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH (analog M) were chosen for further dose response studies, because, unlike analog N, these two analogs had no persistent antagonism on GTH-II release. Both analogs E and M suppressed GTH-II release stimulated by 20 nM of sGnRH or cGnRH-II in a dose-dependent manner. Analog E was more potent than analog M in inhibiting the actions of the two native peptides on GTH-II release. The ability of a given dose of analog E to inhibit GTH-II release stimulated by sGnRH varied with the sexual developmental stage of goldfish, being highest in sexually regressed fish. The GnRH receptor content in goldfish is lowest in sexually regressed goldfish and highest in sexually mature goldfish (Habibi et al., 1989a). Hence, it was postulated that in sexually regressed fish, it takes less antagonist to saturate the receptors and thus the antagonist is apparently more effective at this sexual developmental stage.

GH release from perifused goldfish pituitary fragments, stimulated by either sGnRH or cGnRH-II was suppressed in a dose-dependent manner by analog E (Chapter 2). Analog E also inhibited the actions of the native GnRH peptides in dispersed pituitary cells (Chapter 6). Further, analog E displaced ^{125}I-[D-Arg^6, Pro^9-NHEt]-sGnRH and ^{125}I-[D-Arg^6]-cGnRH-II bound to crude goldfish pituitary membrane preparations, with a receptor binding affinity significantly higher than that of sGnRH (Chapter 6). Analog E was effective in inhibiting the increases in plasma GTH-II and GH stimulated by both sGnRH and cGnRH-II (Chapter 7). These results indicate that analog E is an antagonist capable of inhibiting the actions of the two native peptides on GTH-II and GH release both *in vivo* and *in vitro*, by competitively binding to GnRH receptors on the pituitary cells in goldfish.

Analogs C, M and N are also 'true' antagonists on GTH-II release, and analog N is a 'true' antagonist on GH release. The receptor binding affinities of analogs C, E and N were significantly higher than that of sGnRH (Chapter 6). There was no significant correlation between the receptor binding affinity and the *in vitro* GTH-II inhibitory potency of these three analogs. In general, there is a positive correlation between receptor binding affinity and *in vitro* potency of GnRH agonists and antagonists in mammals (Karten and Rivier, 1986). However, many studies also indicate a lack of correlation between receptor binding affinity and *in vitro* potency (Rivier et al., 1983; Bajusz et al., 1988). In goldfish, position 6 and 10 substituted sGnRH analogs, but not mGnRH

analogs, showed significant correlation between binding affinity and *in vitro* potency (Habibi et al., 1989b). Compared to sGnRH analogs, many mGnRH analogs had significantly lower binding affinity, but similar *in vitro* GTH-II releasing potency (Habibi et al., 1989b). In the present study, although analog E (based on mGnRH) was more potent than analog C (based on sGnRH) in inhibiting sGnRH stimulated GTH-II release, its binding affinity was significantly lower than that of analog C. These results indicate that although the GnRH receptors in goldfish are 'less specific' as all known forms of GnRH stimulate GTH-II release, they may 'prefer' native GnRH peptides and their analogs in terms of receptor binding. Further testing of analogs of cGnRH-II (the other native GnRH) for potency and binding affinity may provide additional support for this hypothesis.

<u>*Structure activity studies:*</u>

GnRH Antagonists: The early development of GnRH antagonists in mammals involved analogs having modifications at positions 2, 6, and 2, 3, 6 (for review see Karten and Rivier, 1986). There are no systematic studies in non-mammalian species to demonstrate the effectiveness of position 2, 6 modified antagonists. In male trout, [D-Phe^{2,6}, Phe^{3}]-mGnRH inhibited the increase in plasma GTH-II levels stimulated by mGnRH, indicating the effectiveness of an analog having modifications at positions 2, 3, 6 (Crim et al., 1981). In the present study analogs having modifications at positions 1, 2, 3, and 6, and, in a few cases, analogs with modifications at positions 1, 2, 3, 6 and 10 were tested. All of the purely antagonistic analogs, [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-sGnRH (analog C), [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH (analog M), and [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) have modifications at positions 1, 2, 3 and 6 of mGnRH, sGnRH or cGnRH-II. Similar modifications produce potent GnRH antagonists in mammals (Karten and Rivier, 1986). Habibi (1991a) earlier described that [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH (analog M) inhibited GTH-II release stimulated by sGnRH and cGnRH-II in goldfish and King et al. (1988) demonstrated that this analog inhibited LH release stimulated by cGnRH-I and -II in chicken.

In dose-response studies, compared to [D-p-Glu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH

(analog M), [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E), caused a significantly greater suppression of GTH-II release stimulated by sGnRH and cGnRH-II in sexually regressed fish. In mammalian studies substitution of D-p-Glu^1 with Ac-Pro^1 (Humphries et al., 1978) and of D-Phe^2 with D-chloro (or fluoro)-Phe^2 (Coy et al., 1979) was found to enhance the potency of GnRH antagonists. These modifications also appear to be favorable in increasing the GnRH antagonist potency in goldfish (see Chapter 2 for further discussion). Additional increase in potency in mammals was achieved by introduction of 3-(2-naphthyl) alanine [Nal(2)] at position 1 (Nestor, 1987) and Pal(3) at positions 3 and 6 (Folkers et al., 1987). [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}]-cGnRH-II (analog N) having similar modifications inhibited sGnRH and cGnRH-II actions on GTH-II and GH release (see Chapters 2, 3). The GTH-II release inhibitory potency of analog N was higher than that of analog C and similar to that of analog E (see Chapters 2, 3). Analog N also had significantly higher receptor binding affinity than analogs C and E.

Both analog C (based on sGnRH) and analog E (based on mGnRH) have similar modifications at positions 1, 2, 3, and 6, having [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]. Based on limited dose-response studies (see Chapters 2, 3) with analog C and full dose-response studies on analog E (see Chapter 2), analog E was significantly more potent than analog C in suppressing GTH-II release stimulated by both sGnRH and cGnRH-II (see Chapter 2 for further discussion). However, analog C has significantly higher receptor binding affinity than analog E (see Chapter 6). The reason for low potency of analog C may be 'excess hydrophobicity' as hypothesized for sGnRH agonistic analogs (Habibi et al., 1989b). Most of the potent agonists in mammals have hydrophobic D-Trp, D-Ser(tBu), D-His(Bzl) or D-Nal(2) at position 6 (Nestor, 1984). In goldfish, analogs of mGnRH and sGnRH having hydrophobic D-Trp^6 or D-His(Bzl)^6 were only 2-4 times more potent than sGnRH in stimulating GTH-II release (Habibi et al., 1989b). Further, [D-Ala^6, Pro^9-NHEt]-sGnRH had significantly higher receptor binding affinity, but a lower GTH-II release potency than [D-Ala^6, Pro^9-NHEt]-mGnRH (Habibi et al., 1989b). In the same study, compared to [D-Trp^6, Pro^9-NHEt]-mGnRH, [D-Trp^6, Pro^9-NHEt]-sGnRH had significantly higher receptor binding affinity, but a lower GTH-II release potency. Based on these results, it was hypothesized that substitution of a hydrophobic amino acid at position 6 of sGnRH, which already has tryptophan, a hydrophobic and aromatic amino acid at position 7 may result in 'excess hydrophobicity' compared to

mGnRH analogs having similar substitution. Interestingly, analog N having [D-(3)Pal^6] along with Trp^7 has higher receptor binding affinity compared to both analogs C and E. Further, the GTH-II release inhibitory efficacy of analog N is higher than that of analog C and similar to that of analog E. This may be due to presence of a basic and more hydrophilic moiety (compared to tryptophan) of D-(3)Pal at position 6 along with Trp^7. Since we have not tested mGnRH and sGnRH analogs having [Ac-D(2)Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}], it is not possible to compare the relative potency of these analogs. In mammalian studies also substitution of D-(3)Pal^6, Trp^7 resulted in potent antagonists (Nestor, 1984).

In mammals substitution of D-Arg^6 or D-hArg(Et$_2$)^6 resulted in significant enhancement of potency of GnRH antagonists (for review see Karten and Rivier, 1986; Nestor, 1987). In contrast to this, in the present study, mGnRH and sGnRH analogs with D-Arg^6 or D-hArg(Et$_2$)^6 (analogs B, F, J and K) stimulated GTH-II release. When tested further, two of these analogs, analogs J and K, which are 'true' antagonists in mammals (Rivier et al., 1984; Nestor, 1987; Nestor et al.,1992) stimulated both GTH-II and GH release in a dose-dependent manner, although with significantly lower potency than sGnRH in terms of minimum effective concentration, ED50 and maximum hormone release (see Chapter 5). The ability of these analogs to interact with GnRH receptors in eliciting an increase in GTH-II and GH release was demonstrated by two approaches. First, in continuous presence of analogs J and K, the GTH-II stimulatory actions of sGnRH and cGnRH-II were significantly suppressed. Second, the increase in GTH-II and GH release stimulated by analog K was significantly suppressed by the 'true' antagonist analog E. These results indicate that analogs J and K are 'partial antagonists' (antagonistic analogs with weak to moderate agonistic activity). In chicken also, two mammalian GnRH antagonists [D-Phe^{2,6}, D-Trp^3]-mGnRH and [Ac-D-Nal(2)^1, D-α-Me-4Cl-Phe^2, D-Trp^3, D-Arg^6, D-Ala^{10}]-mGnRH stimulated LH release (Millar and King, 1984; King et al., 1988). These results indicate that there are functional differences in the properties of GnRH receptors between goldfish and mammals, and the structure function requirements for antagonists are different. Further, analog A having D-Lys^6 also weakly stimulated GTH-II and GH release (see Chapter 2). Even analog L, [Ac-D(2)-Nal^1, 4Cl-D-Phe^2, D-(3)Pal^{3,6}, Arg^5, D-Ala^{10}]-mGnRH having arginine at position 5 weakly stimulated GTH-

II release and strongly inhibited GH release (see Chapter 4). In view of stimulatory actions in goldfish of these analogs, especially the "Nal-Arg" analogs, it will be interesting to test the actions of recently developed "Nal-Lys" analogs such as 'Antide', [Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal^3, Lys(Nic)^5, D-Lys(Nic)^6, Lys(iPr)^8, D-Ala^{10}]-mGnRH (Ljungqvist et al., 1988), and 'Azaline', [Ac-D-Nal^1, 4FD-Phe^2, D-Pal^3, Lys(atz)^5, D-Lys(atz)^6, ILys^8, D-Ala^{10}]-mGnRH and related analogs (Rivier et al., 1991; Theobald et al., 1991).

In goldfish, compared to sGnRH, lamprey GnRH-I (lGnRH-I) has significantly higher receptor binding affinity, but markedly lower GTH-II and GH releasing activity (Habibi et al., 1992). Based on these observations it was postulated that analogs based on lGnRH-I structure with modifications at positions 1, 2, 3 and 6 may be potent antagonists in goldfish. All three such analogs tested (analog G, H, and I) were 'partial antagonists' having weak GTH-II and GH stimulatory effects, while suppressing the actions of sGnRH. The receptor binding affinity of these analogs was not tested.

GnRH Agonists: Based on the observation that analog K, having [D-hArg(Et_2)^6], strongly stimulated GTH-II and GH release in goldfish, it was postulated that [D-hArg(Et_2)^6] substituted agonists may act as superagonists in goldfish. A series of GnRH analogs having [D-hArg(Et_2)^6] or [D-hArg(CH_2CF_3)$_2^6$] were tested for their ability to stimulate GTH-II and GH release from the perifused pituitary fragments. Among these peptides [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH is the most potent analog tested in stimulating GTH-II and GH release. [D-hArg(Et_2)^6, Pro^9-NHEt]-sGnRH is one of the most potent agonists in rainbow trout, landlocked salmon and winter flounder (Crim et al., 1988). [D-Arg^6, Pro^9-NHEt]-sGnRH is the most potent analog formerly known in goldfish in terms of stimulating GTH-II release and receptor binding affinity (for review see Peter et al., 1990, 1991). [D-Arg^6, Pro^9-NHEt]-sGnRH is also a superagonist in many teleost species (Crim et al., 1988; Goos, 1991). These results indicate that substitution of hydrophilic D-Arg^6 or D-hArg(Et_2)^6 helps in developing potent agonists to stimulate GTH-II release in teleost fish. Most of the potent agonists in mammals have hydrophobic D-Trp, D-Ser(tBu), D-His(Bzl) or D-Nal(2) at position 6 (Nestor, 1984). In goldfish, analogs of mGnRH and sGnRH having hydrophobic D-Trp^6 or D-His(Bzl)^6 were only 2-4 times more potent than sGnRH in stimulating GTH-II release (Habibi et al., 1989b). In the same study [D-Arg^6, Pro^9-NHEt]-sGnRH was 17 times more potent. In

stimulating GTH-II release, sGnRH analogs with a hydrophobic amino acid at position 6, were less potent compared to mGnRH analogs having the same substitution and this was suggested to be due to the presence of 'excess hydrophobicity' (see discussion above). Since cGnRH-II also has Trp^7, it will be interesting to test the role of hydrophobic or hydrophilic amino acids at position 6 of cGnRH-II analogs in determining the GTH-II release potency in goldfish. In the present study [D-hArg$(Et_2)^6$] substituted cGnRH-II analogs were not tested.

[D-hArg$(Et_2)^6$] substituted mGnRH analogs are very potent in estrus suppression assay in rat (for review Nestor et al., 1984). In contrast, [D-hArg$(Et_2)^6$] substituted mGnRH and cGnRH-II analogs were only 1-2 times more potent than the parent molecule in stimulating LH release in chicken pituitary cells (for review Millar and King, 1987). However, [D-hArg$(Et_2)^6$] substituted sGnRH analog was significantly more potent than corresponding mGnRH, cGnRH-I and cGnRH-II analogs in chicken (Millar and King, 1987). These results demonstrating marked differences in the potency of GnRH analogs between species, support the hypothesis that there are functional differences in the GnRH receptors between vertebrate species.

The potency of GnRH analogs to stimulate GH release compared to GTH-II release also showed marked differences, and is discussed in a later section.

Use of GnRH antagonists in physiological studies:

The main impetus for development of GnRH antagonists in mammals is their possible use as anti-fertility agents. However, antagonists are also useful in establishing specificity of GnRH actions in terms of receptor binding, activation of second messengers and biological activity. In the present study using analog E it has been shown that endogenous GnRH peptides are important in regulation of 'basal' plasma GTH-II and GH levels. Further, GnRH has been shown to mediate at least a part of the actions of a female sexual pheromone, 17α, 20 β-dihydroxy-4-pregnen-3-one (17α, 20β-P) on sexually mature males in stimulating an increase in plasma GTH-II levels (see Chapter 7 for discussion). By use of an antagonist, the involvement of GnRH in GTH-II release stimulated by neuropeptide Y (NPY) has been demonstrated in goldfish (Peng et al., 1993). Using a similar approach, the gonadal peptides inhibin-A and activin-A have been shown to

stimulate GTH-II release independent of GnRH in goldfish (Ge et al., 1992). Habibi (1991a) used a GnRH antagonist to demonstrate that prolonged exposure of goldfish pituitary fragments to native GnRH peptides, but not to a GnRH antagonist, caused down-regulation of receptor number and desensitization of the GTH-II response to subsequent challenge of GnRH peptides.

Functional differences in the properties of GnRH receptors on gonadotrophs and somatotrophs:

Although many species of vertebrates have more than one form of GnRH, it is not known whether these multiple forms act through the same or different populations of receptors. In chicken, both cGnRH-I and -II act on the same population of receptors (King et al., 1988). However, further studies indicated that only cGnRH-I is found in the hypothalamus and released into hypothalamo-hypophysial portal system, and thus only cGnRH-I is involved in the regulation of reproduction in chicken (Katz et al., 1990). In goldfish, receptor binding and electron microscopy studies indicate that both sGnRH and cGnRH-II are capable of displacing the receptor bound radioligand, ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH (Habibi et al., 1987; Habibi and Peter, 1991), or competing for receptor binding sites with avidin gold-labelled biotinylated [D-Lys^{6}, Pro^{9}-NHEt]-sGnRH (Cook et al., 1991). Furthermore, in static incubations of dispersed goldfish pituitary cells, the GTH-II responses to maximally effective doses of sGnRH and cGnRH-II were not additive (Chang et al., 1993). These results suggest that both native peptides bind to the same population of receptors.

In contrast to this hypothesis, compared to sGnRH, cGnRH-II is more potent in stimulating GTH-II release, has a higher binding affinity, and causes greater degree of desensitization and down regulation of receptor number (Habibi, 1991a). Further, in causing desensitization of the GTH-II response by perifused pituitary fragments, cGnRH-II is more effective than sGnRH when given either as continuous or extended pulses, or as a 2 min pulse every 20 min; on the other hand, sGnRH is more effective under 2 min pulses every 60 min (Habibi, 1991b). In addition, pulsatile alternate treatments with sGnRH and cGnRH-II (10 nM, every 30 min) caused lower desensitization compared to repeated pulses of sGnRH or cGnRH-II or repeated pulses of the two combined (Khakoo et al., 1994). In stimulating expression of mRNA of GTH-IIα and GTH-IIβ subunits,

cGnRH-II was more potent than sGnRH in sexually mature goldfish, but less potent in sexually regressed goldfish (Khakoo et al., 1994). Based on these results, sGnRH and cGnRH-II were suggested to regulate synthesis and release of GTH-II through different 'receptor-effector' mechanisms (Khakoo et al., 1994). Significant differences have also been found in the second messenger pathways mediating the increase in GTH-II release stimulated by sGnRH and cGnRH-II (for review see Chang and Jobin, 1991; Chang et al., 1993). Compared to sGnRH, cGnRH-II is more dependent on extracellular calcium and fails to mobilize calcium from intracellular sources. In addition, metabolism of arachidonic acid is involved in sGnRH, but not in cGnRH-II actions (Chang and Jobin, 1991; Chang et al., 1993).

However, these results do not necessarily indicate the presence of more than one population of GnRH receptors on gonadotrophs in goldfish. The results presented in chapter 3 indicate that antagonists (analogs C and N) based on sGnRH and cGnRH-II structures have no preferential inhibition on GTH-II stimulatory actions of either sGnRH or cGnRH-II. Similarly, t' ; mGnRH based analogs E and M also showed no preferential inhibition (see Chapter 2). In continuous presence of 100 nM sGnRH, a pulse of 100 nM cGnRH-II caused no significant additional GTH-II release and a sGnRH pulse given in continuous presence of cGnRH-II also caused no additional GTH-II release. Prolonged exposure of pituitary fragments to either sGnRH or cGnRH-II caused a significant decrease in the GTH-II response to subsequent pulse treatment with either sGnRH or cGnRH-II, without showing any preferential reduction in response to any one peptide. All these results support the hypothesis that both sGnRH and cGnRH-II act through the same population of receptors on gonadotrophs. It is well documented that GnRH receptors in goldfish are less 'specific' in terms of ligand requirement as demonstrated by the GTH-II releasing activity of all tested forms of GnRH (Habibi et al., 1992, Peter et al., 1991). In view of this, it will be interesting to know whether other forms of GnRH such as mGnRH, cGnRH-I, dogfish GnRH or catfish GnRH, follow sGnRH or cGnRH-II in terms of activation of 'receptor-effector' systems and second messenger components.

Our results with growth hormone release also indicate that both sGnRH and cGnRH-II act through the same population of receptors on somatotrophs. However, the results suggest that GnRH receptors on gonadotrophs and somatotrophs are functionally different.

Analog C stimulated GH release from the pituitary fragments and from dispersed pituitary cells, while acting as 'true' antagonist on GTH-II release (Chapters 2, 3). In contrast, analog F stimulated GTH-II in a dose dependent manner, but weakly suppressed GH release (Chapter 3). Further, analog L weakly stimulated GTH-II release in a dose dependent manner, while strongly inhibiting GH release (Chapter 4). The differential actions of analog L on GTH-II and GH release is through interaction with GnRH receptors as indicated by (i) the ability of analog L to significantly suppress the actions of sGnRH and cGnRH-II on GTH-II and GH release, (ii) the ability of analog L to displace ^{125}I-[D-Arg^{6}, Pro^{9}-NHEt]-sGnRH bound to crude pituitary membrane preparations, and (iii) a significant inhibition of the actions of analog L by a 'true' antagonist, analog E. Further, the inhibitory ability of analog L is specific to GnRH as it had no actions on the GH stimulatory actions of dopamine and thyrotropin-releasing hormone. An additional support for the functional differences in GnRH receptors on gonadotrophs and somatotrophs comes from the structure-activity studies of GnRH agonists in goldfish wherein significant differences in the potencies to stimulate GTH-II and GH release have been observed. Compared to sGnRH, [D-hArg(Et$_2$)^{6}]-mGnRH was significantly more potent in stimulating GTH-II release, but less potent in stimulating GH release (Chapter 5). Similarly, [D-hArg(CH$_2$CF$_3$)$_2^{6}$]-mGnRH and [D-hArg(CH$_2$CF$_3$)$_2^{6}$, Pro^{9}-NHEt]-mGnRH were equipotent to sGnRH on GTH-II release, but significantly less potent than sGnRH on GH release (Chapter 5). Substitution of position 8 in mGnRH with tyrosine, leucine, methionine or histidine resulted in loss of GTH-II, but not GH, releasing potency compared sGnRH (Habibi et al., 1992).

Interestingly, recent cloning of GnRH receptors has indicated the presence of two genes in human (Kakar et al., 1992; 1993), and three mRNAs in human (Kakar et al., 1992; 1993) and sheep (Miller et al., 1993) encoding for GnRH receptor proteins. Molecular cloning of GnRH receptors will provide further evidence regarding the presence or absence of multiple receptors in the pituitary cells of goldfish.

GnRH as regulator of GH release:

One of the interesting aspects of GnRH actions in goldfish is its stimulatory actions on GH release (Peter et al., 1990). sGnRH and cGnRH-II stimulate GH release *in vitro* from the goldfish pituitary fragments and dispersed pituitary cells (Marchant et al., 1989; Chang

et al., 1990; Habibi et al., 1992) and *in vivo* (Marchant et al., 1989). The direct action of GnRH on GH cells has been demonstrated by localization of avidin gold-labelled biotinylated [D-Lys^6, Pro^9-NHEt]-sGnRH on the cell surface of somatotrophs and that such binding is displaced by co-incubation with unlabeled sGnRH or cGnRH-II (Cook et al., 1991). In the present study also both sGnRH and cGnRH-II stimulated GH release *in vitro* (see Chapter 2, 3, and 4) and *in vivo* (see Chapter 7). Further, analog E suppressed the basal plasma GH levels, indicating the involvement of GnRH peptides in the regulation of GH secretion in goldfish. In support of this, during spawning in male goldfish, serum GH and GTH-II levels increased with a corresponding decrease in brain GnRH levels (Yu et al., 1991). Further, gonadal steroids modulate the GH response to sGnRH; gonadectomy reduces (Wong et al., 1993), while estradiol implantation to normal goldfish enhances (Trudeau et al., 1992) the GH responses to sGnRH or sGnRH analog. Further, the *in vitro* GH responses to sGnRH and cGnRH-II were maximum in sexually mature and lowest in sexually regressed goldfish (Murthy et al., 1993). GnRH binding capacity also increases during sexual maturity (Habibi et al., 1989b). Based on these observations endogenous GnRH was suggested to be an important regulator of GH release during sexual maturity (Wong et al., 1993) and spawning (Yu et al., 1991).

GnRH also stimulates GH release in common carp (Lin et al., 1993), rainbow trout (Le Gac et al., 1993), and tilapia (Melamed et al., 1993). Recent studies have demonstrated the involvement of GH in various activities during sexual development in fish. Growth hormone potentiates GTH-II-stimulated steroid production by ovarian follicles of goldfish (Van Der Kraak et al., 1990). Recombinant salmon GH increases and modulates steroid production by rainbow trout and killifish gonads *in vitro* (Singh et al., 1988; Le Gac et al., 1992) and in spotted seatrout (*Cynoscion nebulosus*) (Singh and Thomas, 1993). High affinity, low capacity GH binding sites have been reported in trout testis (Le Gac et al., 1992). The plasma GH levels in adult Atlantic salmon, *Salmo salar*, increase with sexual development, and manipulation of photoperiod causing delay or advancement of sexual maturity results in a parallel change in the timing of the increases in plasma GH levels (Björnssson et al., 1994). Co-treatment of GH with E_2 was required for induction of vitellogenin synthesis in primary hepatocyte culture in eel, *Anguilla japonica* (Kwon and Mugiya, 1994). Thus, the control of both GTH-II and GH by GnRH peptides

is an integral function in regulation of reproduction in teleost fish.

Conclusions:

In summary, the important conclusions based on the results presented in this thesis are as follows:

1. [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-mGnRH (analog E) is a 'true' GnRH antagonist in goldfish without any independent stimulation of GTH-II and GH release. Analog E is effective in suppressing GTH-II and GH release stimulated by sGnRH and cGnRH-II, both *in vitro* and *in vivo*. Analog E by competitively binding to GnRH receptors on pituitary cells, inhibits the actions of native GnRH peptides.

2. Endogenous GnRH regulates basal GTH-II and GH levels and such regulation of GTH-II and GH by endogenous GnRH is an integral part of sexual development in fish. Further, GnRH mediates at least part of the actions of female sex pheromone, 17α, 20 β-dihydroxy-4-pregnen-3-one on sexually mature males in stimulating an increase in plasma GTH-II levels.

3. Many mammalian GnRH antagonists, especially those with D-Arg^6, stimulate GTH-II and GH release in goldfish, indicating differences in the properties of the receptors between species and in ligand requirement to develop potent antagonists.

4. The two native GnRH peptides act through the same population of receptors on gonadotrophs; however, GnRH peptides act through functionally different receptor populations on somatotrophs and gonadotrophs.

5. [D-hArg(Et$_2$)^6, Pro^9-NHEt]-sGnRH is the most potent GnRH analog in stimulating GTH-II release in goldfish, and one of the most potent analogs in stimulating GH release.

References

Bajusz, S., Csernus, V. J., Janaky, T., Bokser, L., Fekete, M., and Schally, A. V. (1988). New antagonists of LHRH. II. Inhibition and potentiation of LHRH by closely related analogues. Int. J. Peptide Protein Res. 32, 425-435.

Björnsson, B. T., Taranger, G. L., Hansen, T., Stefansson, S. O., and Haux C. (1994). The interrelation between photoperiod, growth hormone, and sexual maturation of adult Atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 93, 70-81.

Chang, J. P., Cook, H., Freedman, G. L., Wiggs, A. J., Somoza, G. M., De Leeuw, R. and Peter, R. E. (1990). Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Chang, J. P., and Jobin, R. M. (1991). Two native GnRH peptides stimulate gonadotropin release in goldfish by different second messenger components. In "Proceedings of the 4th International Symposium on The Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 221-223. FishSymp 91, Sheffield, UK.

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

Cook, H., Berkenbosch, J. W., Fernhout, M. J., Yu, K. L., Peter, R. E., Chang, J. P., and Rivier, J. E. (1991). Demonstration of gonadotropin-releasing hormone receptors on gonadotrophs and somatotrophs of the goldfish: An electron microscope study. Regul. Peptides, 36, 369-378.

Crim, L. W., Evans, D. M., Coy, D. H., and Schally, A. V. (1981). Control of gonadotropic hormone release in trout: Influence of synthetic LH-RH and LH-RH analogues *in vivo* and *in vitro*. Life Sci. 28, 129-136.

Crim, L. W., Nestor, Jr. J. J. and Wilson, C. E. (1988). Studies of the biological activity of LHRH analogs in the rainbow trout, landlocked salmon, and the winter flounder. Gen. Comp. Endocrinol. 71, 372-382.

Folkers, K., Bowers, C., Tang, P. -F. L., Feng, D., Okamoto, T., Zhang, Y., and

Ljungqvist, A. (1987). Specificity of design to achieve antagonists of LHRH of increasing effectiveness in therapeutic activity. In "LHRH and its analogs: Contraceptive and therapeutic applications". Part 2. (B. H. Vickery, and J. J. Nestor Jr. Eds.), pp 25-36. MTP Press Ltd., Boston.

Ge, W., Chang, J. P., Peter, R. E., Vaughan, J., Rivier, J., and Vale, W. (1992). Effects of porcine follicular fluid, inhibin-A, and activin-A on goldfish gonadotropin-release *in vitro*. Endocrinology, 131, 1922-1929.

Goos, H. J. Th. (1991). Fish gonadotropins and GnRH's: Fundamental aspects and practical applications. Bull. Inst. Zool. Academia Sinica, Monograph, 16, 119-137.

Habibi, H. R. (1991a). Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: Effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod. 44, 275-283.

Habibi, H. R. (1991b). Desensitization to native molecular forms of gonadotropin-releasing hormone in the goldfish pituitary: Dependence on pulse frequency and concentration. Gen. Comp. Endocrinol. 84, 199-214.

Habibi, H. R. and Peter, R. E. (1991). Gonadotropin-releasing hormone (GnRH) receptors in teleosts. In "Proceedings of the 4th International Symposium on The Reproductive Physiology of Fish". (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. Rolfe, Eds.), pp 109-113. FishSymp 91, Sheffield, UK.

Habibi, H. R., De Leeuw, R., Nahorniak, C. S., Goos, H. J. Th., and Peter, R. E. (1989a). Pituitary gonadotropin-releasing hormone (GnRH) receptor activity in goldfish and catfish: seasonal and gonadal effects. Fish Physiol. Biochem. 7, 109-118.

Habibi, H. R., Marchant, T. A., Nahorniak, C. S., Van Der Loo, H., Peter, R. E., Rivier, J. E., and Vale, W. W. (1989b). Functional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the pituitary of goldfish (*Carassius auratus*). Biol. Reprod. 40, 1152-1161.

Habibi, H. R., Peter, R. E., Nahorniak, C. S., Milton, R. C. L. and Millar, R. P. (1992). Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7, and 8 in the goldfish pituitary. Regul. Peptides, 37, 271-284.

Habibi, H. R., Peter, R. E., Sokolowska, M., Rivier, J. E., and Vale, W. W. (1987).

Characterization of GnRH binding to pituitary receptors in goldfish (*Carassius auratus*). Biol. Reprod. 36, 844-853.

Kakar, S. S., Musgrove, L. C., Devor, D. C., Sellers, J. C., and Neill, J. D. (1992). Cloning, sequencing , and expression of human gonadotropin releasing hormone (GnRH) receptors. Biochem. Biophys. Res. Comm. 189, 289-295.

Kakar, S. S., Musgrove, L. C., Sellers, J. C., and Neill, J. D. (1993). Human gonadotropin releasing hormone (GnRH) receptor: Further molecular characterization. In "Program & Abstracts", 75th annual meeting, The Endocrine Society. pp 339 (Abstract # 1153).

Karten, M. J., and Rivier, J. E. (1986). Gonadotropin releasing hormone analog design. Structure-function studies towards the development of agonists and antagonists: Rationale and perspectives. Endocrine Rev. 7, 44-66.

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.

Khakoo, Z. Bhatia, A., Gedamu, L., and Habibi, H. R. (1994). Functional specificity for salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II coupled to the gonadotropin release and subunit messenger ribonucleic acid level in the goldfish pituitary. Endocrinology, 134, 838-847.

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. Endocr. 177, 43-49.

Kwon, H-C. and Mugiya, Y. (1994). Involvement of growth hormone and prolactin in the induction of vitellogenin synthesis in primary hepatocyte culture in the eel, *Anguilla japonica.* Gen. Comp. Endocrinol. 93, 51-60.

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P.-Y., Loir, M., Mourot, B., Weil, C. (1993). Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem. 11, 219-232.

Le Gac, F., Ollitrault, M., and Le Bail, P.-Y. (1992). Evidence for binding and action of growth hormone in trout testis. Biol. Reprod. 46, 949-957.

Lin, X. W., Lin, H. R., and Peter, R. E. (1993). Growth hormone and gonadotropin

secretion in the common carp (*Cyprinus carpio* L): *In vitro* interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen. Comp. Endocrinol. 89, 62-71.

Ljungqvist, A., Feng, D. M., Hook, W., Shen, Z, X., Bowers, C., and Folkers, K. (1988). Antide and related antagonists of luteinizing hormone release with long action and oral activity. Proc. Natl. Acad. Sci. USA. 85, 8236-8240.

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

Melamed, P, Eliahou, N, Sivan, B, and Yaron, Z. (1993). Hypothalamic factors regulating GH secretion in tilapia. In "Program, XII International Congress of Comparative Endocrinology", Toronto, Canada, p A-104 (abstract).

Millar, R. P., and King, J. A. (1984). Structure-activity relations of LHRH in birds. Exp. Zool. 232, 425-430.

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

Miller, W. L., Wu, J. C., and Sealfon, S.C. (1993). Estradiol, progesteron, inhibin and GnRH regulate GnRH receptor mRNA(s) in ovine pituitary cultures. In "Program & Abstracts", 75th annual meeting, The Endocrine Society. pp 61 (Abstract # 41).

Murthy, C. K., Wong, A. O. L., Turner, R. J., and Peter, R. E. (1993). Seasonal variations in growth hormone response to two native gonadotropin-releasing hormones (GnRH) in goldfish. In "Program, XII International Congress of Comparative Endocrinology". pp A-113 (abstract).

Nestor, J. J. Jr. (1984). Development of agonists of LHRH analogs. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 3-10. MTP Press Ltd., Boston.

Nestor, J. J. Jr. (1987). Development of LHRH antagonists. In "LHRH and its analogs-contraceptive and therapeutic applications". Part 2. (B. H. Vickery and J. J. Nestor Jr., Eds.), pp 3-16. MTP Press Ltd., Boston.

Nestor, Jr. J. J., Ho, T. L., Tahilramani, R., McRae, G. I. and Vickery, B. H. (1984). Long acting LHRH agonists and antagonists: Basic and clinical aspects. In "LHRH and its Analogues: Basic and Clinical Aspects". (F. Labrie, A. Belanger, A. Dupont, Eds.),

pp 24-35. Elsevier Science Publishers, Amsterdam.

Nestor, J. J. Jr., Tahilramani, R., Ho, T. L., Goodpasture, J. C., Vickery, B. H., and Ferrandon, P. (1992). Potent gonadotropin-releasing hormone antagonists with low histamine-releasing activity. J. Med. Chem. 35, 3942-3948.

Peng, C., Chang, J. P., Yu, K. L., Wong, A. O. L., Van Goor, F., Peter, R. E., and Rivier, J. E. (1993). Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary: Involvement of both presynaptic and pituitary cell actions. Endocrinology, 132, 1820-1829.

Peter, R. E., Habibi, H. R., Chang, J. P., Nahorniak, C. S., Yu, K. L., Huang, Y. P. and Marchant, T. A. (1990). Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. In "Progress in Comparative Endocrinology". (A. Epple, C. G. Scanes, and M. H. Stetson Eds.), pp 393-398. Alan R. Liss. Inc., New York.

Peter, R. E., Trudeau V. L., and Sloley, B. D. (1991). Brain regulation of reproduction in teleosts. Bull. Ins. Zool. Academia Sinica, Monograph, 16, 89-118.

Rivier, C., Rivier, J., Perrin, M., and Vale, W. (1983). Comparison of the effect of several gonadotropin releasing hormone antagonists on luteinizing hormone secretion, receptor bindingand ovulation. Biol. Reprod. 29, 374-378.

Rivier, J., Rivier, C., Perrin, M., Porter, J., and Vale, W. W. (1984). LHRH analogs as anti-ovulatory agents. In "LHRH and its analogs-contraceptive and therapeutic applications" Part 1. (B. H. Vickery, J. J. Nestor Jr., and E. S. E. Hafez, Eds.), pp 11-22. MTP Press Ltd., Boston.

Rivier, J., Theobald, P., Porter, J., Perrin, M., Gunnet, J., Hahn,D. W., and Rivier, C. (1991). Gonadotropin releasing hormone antagonists: Novel structures incorporating N^{ω}-cyano modified guanidine moieties. Biochem. Biophys. Res. Comm. 176, 406-412.

Singh, H., Griffith, R. W., Takahashi, A. P., Kawauchi, H. and Stegeman, J. J. (1988). Regulation of gonadal steroidogenesis in *Fundulus heteroclitus* by recombinant salmon growth hormone and purified salmon prolactin. Gen. Comp. Endocrinol. 72, 144-153.

Singh, H., and Thomas, P. (1993). Mechanisms of stimulatory action of growth hormone on ovarian steroidogenesis in spotted seatrout (*Cynoscion nebulosus*). Gen. Comp. Endocrinol. 89, 341-353.

Theobald, P., Porter, J., Rivier, C., Corrigan, A., Hook, W., Siraganian, R., Perrin, M., Vale, W., and Rivier, J. (1991). Novel gonadotropin-releasing hormone antagonists: Peptides incorporating modified N^{ω}-guanoguanidino moieties. J. Med. Chem. 34, 2395-2402.

Trudeau, V. L., Somoza, G. M., Nahorniak, C. S., and Peter, R. E. (1992). Interactions of estradiol with gonadotropin-releasing hormone and thyrotropin-releasing hormone in the control of growth hormone secretion in the goldfish. Neuroendocrinology, 56, 483-490.

Van Der Kraak, G., Rosenblum, P. M. and Peter, R. E. (1990). Growth hormone-dependent potentiation of gonadotropin-stimulated steroid production by ovarian follicles of the goldfish. Gen. Comp. Endocrinol. 79, 233-239.

Wong, A. O. L., Chang, J. P., and Peter, R. E. (1993). Interactions of somatostatin, gonadotropin-releasing hormone, and the gonads on dopamine-stimulated growth hormone release in the goldfish. Gen. Comp. Endocrinol. 92, 366-378.

Yu, K. L., Peng, C., and Peter, R. E. (1991). Changes in brain levels of gonadotropin-releasing hormone and serum levels of gonadotropin and growth hormone in goldfish during spawning. Can. J. Zool. 69, 182-188.