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DISSERTATION

IMMUNOLOGICAL DETECTION OF EXTRA-PITUITARY GONADOTROPIN-RELEASING HORMONE RECEPTORS

SUBMITTED BY

Jennifer Malvey McCallum

Department of Physiology

In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2001

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JENNIFER MALVEY M^{c}CALLUM ENTITLED "IMMUNOLOGICAL DETECTION OF EXTRA-PITUITARY GONADOTROPIN-RELEASING HORMONE RECEPTORS" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Adviser

Department Head

ABSTRACT OF DISSERTATION

IMMUNOLOGICAL DETECTION OF EXTRA-PITUITARY GONADOTROPIN-RELEASING HORMONE RECEPTORS

A high-titer antisera directed against amino acids 23-36 in the N-terminal of the GnRH receptor was generated and Western Blot analysis resulted in immunological detection in samples known to harbor GnRH receptors. Analysis of pituitary tissue from several species revealed the presence of a single 28 kD immunopositive band. Analysis of extra-pituitary tissues from the ovine, bovine, murine and lapine also resulted in consistent immunological detection of a 28 kD band in the adrenal, brain, endometrium, kidney, liver, myometrium and ovary. Heart and lung were devoid of product.

The product was smaller than either the predicted size of the peptide backbone (37,800) or GnRH receptors identified by photo-affinity labeling (32,000-62,000). *In vitro* transcription and translation of the cDNA resulted in a radiolabeled band with a Mr of 28,000. Western analysis of this product resulted in an immunopositive band of the same size. Therefore, the smaller is most likely due to aberrant migration of the peptide backbone in the polyacrylamide gel.

Southern blot analysis of reverse transcribed RNA was conducted and brain contained 3.7%, endometrium 1.9%, and myometrium 1.2%, respectively, of the pituitary. Thus, levels of GnRH receptor in these extra-pituitary tissues are extremely low when compared to the levels found in the pituitary.

Receptor assays were performed on partially purified membranes of ovine, rat and murine adrenal, brain, endometrium, kidney, liver and pituitary and rat testis. Ligand binding was demonstrated in the pituitary of all species and in the rat testis.

Larger sized receptors, like those reported from PAL studies, were not detected. This may be due to the location of two potential glycosylation signals close to the epitope our antibody recognizes (a.a. 23-36). If glycosylation occurs at either or both of these amino acids, the carbohydrate moieties may cause steric hindrance and inhibit the antibody from binding. Our results do not preclude the possibility that other forms of the receptor exist in any of the tissues surveyed. They may be present but not detected by our antibody in sufficient quantity to produce a positive signal.

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I would also like to thank all of the people that I have worked with over the years in our department. I have received invaluable technical assistance, support and guidance from so many of you. My sincerest thanks goes to Bernadette Meberg, Kit Sutherland, Xiaoming Sha, Bonnie Martin, Rick Silva, Louisa West, Keith Rollyson, Chris Quirk, Mike Gallegos, Dawn Duval, Scott Nelson and Matt Allen. These are but a few who contributed to my educational experience.

Lastly, I would like to thank my family and friends for their encouragement and support over the years during my academic endeavors. Special thanks goes to my husband, Greg, who has paid the bills all of these years and learned to amuse himself while I work towards my academic goals.

DEDICATION

This dissertation is dedicated to my family. To my parents who taught me not to be afraid to try new things; to my husband, Greg, who has always stood by my side and supported me; and to my dog, Max, who frequently reminds me that there is always time for a few minutes of fun.

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Chapter One

REVIEW OF LITERATURE

A. Gonadotropin-Releasing Hormone

GnRH was structurally characterized over 30 years ago from porcine and ovine hypothalamic tissues (Schally et al., 1971; Amoss et al., 1971). Since its isolation, it has been biochemically characterized in detail and much is known about the function of each of its 10 amino acids (Figure 1-1). Almost all GnRH sequences are identical with the exception of variant sequences in the salmon (Sherwood et al., 1983), lamprey (Sherwood et al., 1986) and chicken (King and Millar, 1982; Miyamoto et al., 1984).

Amino acid substitution studies have led to an understanding of the requirements for optimal binding of GnRH to its receptor and subsequent activation of the gonadotrope (Figure 1-2; Karten and Rivier, 1986). Substitution of the first three residues of the GnRH molecule ($Glu^1 - His^2 - Trp^3$) with hydrophobic D-amino acids results in analogs with antagonistic properties and all three residues appear to be important for receptor activation (Conn et al., 1984). When Glu^1 is replaced with Leu, Gly, Pro or Thr, there is almost a complete loss of bioactivity (Sandow et al., 1978; Fujino et al., 1972; Okada et al., 1973; Coy et al., 1973). Substitution studies indicate His^2 may interact with the receptor via its aromatic character, hydrogen-bonding capacity or acid-base properties and substitution of this residue with bulky hydrophobic residues

Figure 1-1: The Amino Acid Sequence of GnRH.

3

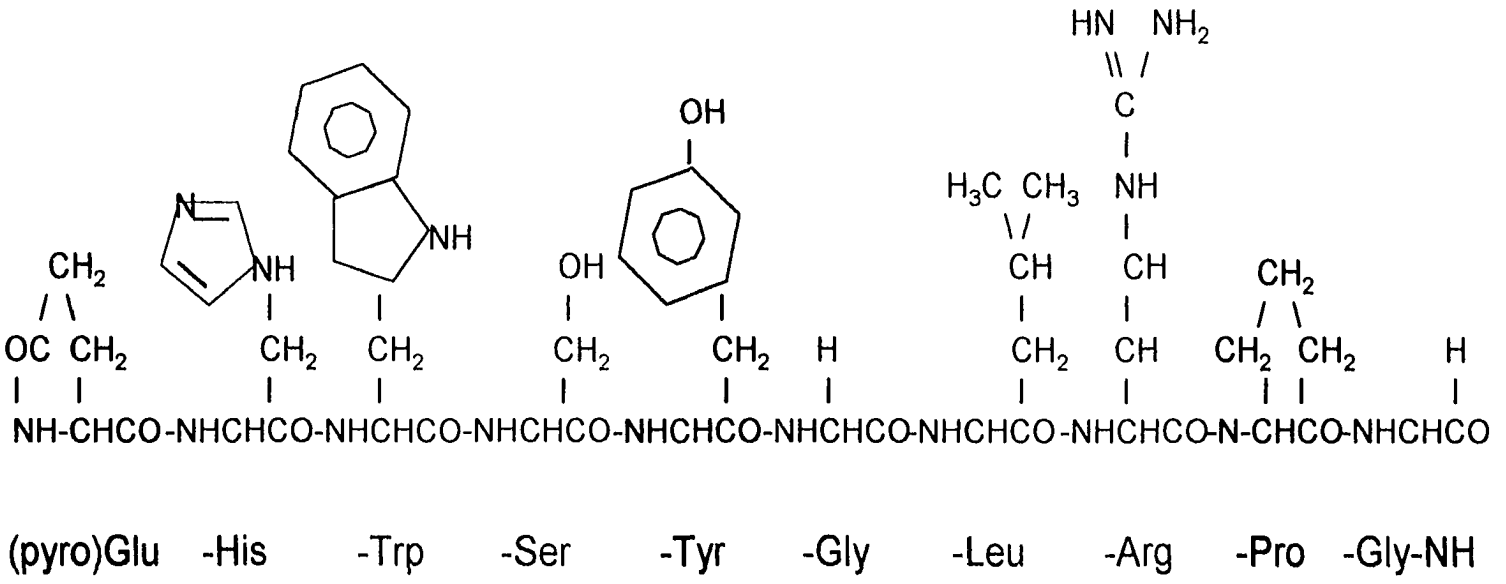
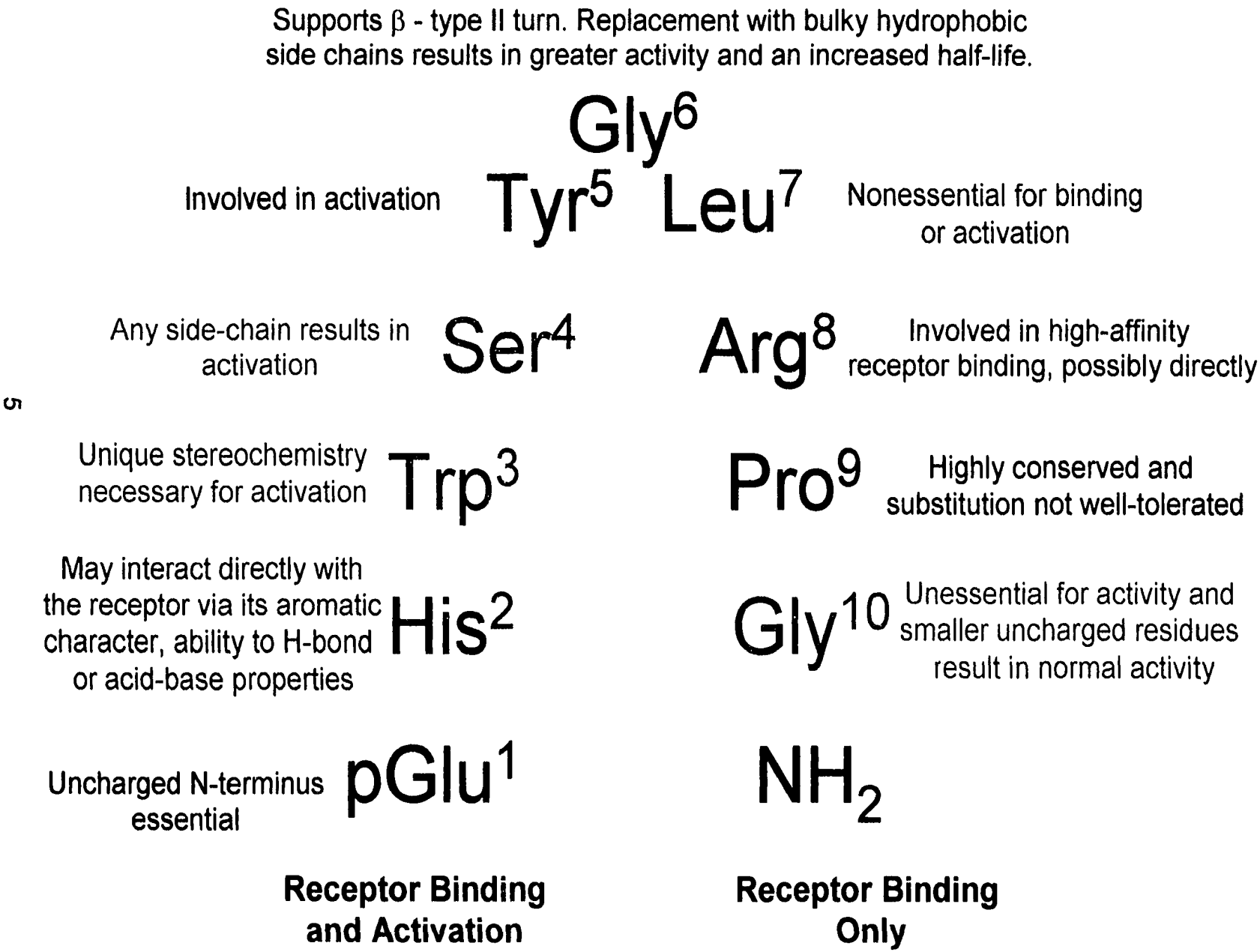


Figure 1-2: Proposed Function of Each Amino Acid of GnRH



makes potent antagonists (Rivier et al., 1992; Rivier et al., 1995; Janecka et al., 1984; Millar et al., 1987; Karten and Rivier; 1986). Mutagenic studies of the GnRH receptor itself indicates His^2 of the GnRH molecule may interact directly with Lys^{121} of the GnRH receptor (Zhou et al., 1995). The unique stereochemistry of Trp^3 also appears to be important for activation of the receptor as minor changes in this position result in high affinity binding but lowered activation of the receptor (Hirotsu et al., 1974). Substitutions at any of these three residues results in the production of antagonists. Combining the substitution with another at Gly^6 for a strong basic amino acid results in an extremely potent antagonist. Substitution of Ser^4 with a wide-variety of amino acids results in normal activity indicating this residue is not critical for receptor binding or activation (Conn et al., 1987).

The central area of the molecule containing, Tyr^5– Gly^6 – Leu^7– Arg^8, appears to have a wide-variety of functions (Figure 1-2). Substitution studies reveal the aromatic side chain of the non-conserved residue, Tyr^5, is required for activation of the receptor but the hydroxyl group is not necessary (Haviv et al., 1993; Millar et al., 1986; Fujino et al., 1972; Sandow et al., 1978). Other substitutions support the presumption that Gly^6 is involved in a β-II-type bend (Monahan et al., 1973). Replacement of this residue with D-amino acids with bulky hydrophobic side chains results in increased activity (Millar et al., 1987; Karten and Rivier; 1986). Leu^7 does not appear to be essential for binding to or activation of the receptor but both are enhanced by the presence of uncharged L-amino acids, like Leu itself (Millar et al., 1986; Folkers et al., 1986; Sandow et

al., 1978, Fujino et al., 1972). Manipulation or substitution of Arg^8 indicates the acidic residue is essential for high-affinity receptor binding except if there is a D-amino acid in position 6 (Janeck et al., 1991; Nikolics et al., 1989). Additionally, from cross-linking studies it appears to interact directly with the receptor itself (Nikolics et al., 1989).

The carboxyl terminus of GnRH contains Pro^9-Gly^{10}-NH_2. Pro^9 does not tolerate substitution well and results in low activity analogs (Figure 1-2; Sandow et al., 1978). This is not surprising as this is an extremely conserved residue among all GnRH sequences. The amino terminal Gly^{10}- amide appears unessential for activity and smaller uncharged residues are acceptable in this position (Fujino et al., 1972; Coy et al., 1973, 1975). Substitution with larger groups suggests they may sterically hinder binding to the receptor (Coy et al., 1973).

Native GnRH has a circulating half-life of less than 10 minutes in most mammals, due to degradation of the amino terminal portion of the molecule (Bennett and McMartin, 1979). Additionally, several enzymes with the ability to cleave GnRH have been isolated from the pituitary gland but it is unknown if they participate in inactivation (Handelsman and Swerdloff, 1986).

Substitutions at Gly^6 with hydrophobic D-amino acids results in an increased circulating half-life of the analog (Figure 1-2; Coy et al., 1976). Amino acid substitutions to increase the affinity of the analog for the receptor as well as increase its half-life in serum have resulted in many commercially available superagonists, such as D-Ser(tBu^6) GnRH Ethylamide (Buserelin, 17-19 times

more potent than GnRH, Hoechst), D-Leu^6 – GnRH Ethylamide (Leuprolide, >50 times more potent than GnRH, TAP) and 3 (2 naphthyl) Ala^6 – GnRH (Nafarelin, >200 times more potent than GnRH, Syntex). These superagonists have many important clinical and agricultural uses.

B. **Gonadotropin-Releasing Hormone Receptors**

Gonadotropes have a single class of high-affinity binding sites for GnRH on their plasma membranes (Clayton and Catt, 1981). Receptor number varies over the estrous cycle in direct response to gonadal steroids and hypothalamic GnRH (Clayton and Catt, 1981; Savoy-Moore et al., 1980; Bauer-Dantoin et al., 1993; Brooks et al., 1993). Additionally, increases in GnRH receptor number correspond to increases in receptor mRNA, suggesting control at the transcriptional level (Sakurai et al., 1997).

Estradiol increases the number of receptors and this increase is evidenced during the periovulatory period (Crowder and Nett, 1984; Brooks et al., 1993). This increase has been reported for many species, including sheep (Moss et al., 1981; Crowder and Nett, 1984), mice (Naik et al., 1985), and cattle (Schoenemann et al., 1985). Estradiol increases both the frequency and amplitude of GnRH pulses during the follicular phase. Conversely, high levels of progesterone during the luteal phase decrease the frequency of GnRH pulses which results in a decreased receptor number (Moss et al.,1981).

Other gonadal hormones also function to regulate the GnRH receptor. In rats, receptor synthesis is increased by activin A while inhibin decreases receptor

number but doesn't affect synthesis of new receptor (Braden and Conn, 1992; Wang et al., 1988; Braden et al., 1990).

Commonly, ligands will regulate their own receptor and the GnRH receptor is not different in this respect. Continuous delivery of GnRH to ovariectomized ewes results in a dramatic decrease in receptor number (Nett et al., 1981). Delivery in a manner which mimics the physiological pulsatile pattern results in an increase in receptor number (Turzillo et al., 1995). The GnRH receptor is unusual in that it is up- or down-regulated by GnRH depending on the duration and level of GnRH exposure. The increase in receptor number requires both protein and RNA synthesis (Loumaye and Catt, 1983; Conn et al., 1984a) while down-regulation does not (Conn et al., 1984a). The effect of a complete absence of GnRH is evidenced in a strain of mice that have a mutation in their gene for GnRH (Mason et al., 1986a+b). These mice have absolutely no circulating levels of LH or FSH and, hence, no gonadal function.

Thus, GnRH receptor numbers vary dramatically over the course of the estrous cycle. In the rat, the highest levels are observed between metestrus and early proestrus with levels declining rapidly during the preovulatory LH surge (Savoy-Moore et al., 1980). GnRH receptor mRNA has also been shown to fluctuate throughout the cycle and reach a maximum level on the afternoon of proestrus (Bauer-Danton et al., 1993). This fluctuation in receptor number can be duplicated in cultured gonadotrope cells. Cultures incubated with levels of progesterone comparable to those found midcycle, demonstrate approximately 500 plasma membrane receptors per cell while exposure to estradiol greatly

increases receptor number to 15,000 to 20,000 per cell (Laws et al., 1990b; Gregg et al., 1990, 1991).

C. Structural Analysis of the GnRH Receptor

I. Introduction

Many attempts have been made to obtain accurate structural information for the GnRH receptor. Size estimates have been relentlessly pursued by a wide variety of methods including photoaffinity-labeling, affinity-chromatography purification, radiation inactivation and immunological detection. Most of these methodologies are plagued by technical difficulties and have not resulted in acquisition of solid data pertaining to the size of the GnRH receptor.

II. Photoaffinity Labeling

Photoaffinity labeling (PAL) involves the covalent attachment of a photoreactive label to the GnRH receptor which is visualized after sodium didodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and subsequent exposure of the dried gel to x-ray film. Analysis of rat pituitary membranes revealed two bands with apparent molecular weights (Mr) of 60,000 and 48,000 (Figure 1-3; Hazum,1981). Examination of pituitary membranes from rats, rabbits and mice revealed a larger molecular weight component, with an apparent Mr of 52,000 to 62,000, and a smaller component, with an apparent molecular weight of 40,000 to 43,000 (Figure 1-3; Iwashita and Catt, 1985). Bovine and ovine pituitary GnRH receptors had an Mr of 42,000 and 39,000,

respectively (Ibid). Analysis of rat granulosa and Leydig cells revealed two bands in each tissue with Mr of 53,000 and 43,000. Human epithelial ovarian carcinoma has been examined with PAL and proteins with Mr of 63,000 and 46,000 were detected (Figure 1-3; Pahwa et al. 1989).

PAL was also utilized to determine if GnRH agonists and antagonists situate themselves similarly in the rat GnRH receptor (Figure 1-3; Janovick et al., 1993). The Mr of proteins in the lanes loaded with receptors bound with agonist were 60,000, 56,000, 52,000, 48,000, 46,000 and 44,000. The lanes loaded with receptors bound with antagonist revealed bands with Mr of 62,000, 58,000, 54,000, 52,000, 48,000 and 45,000. All bands could be electroeluted from the gels and re-electrophoresed to the same position. Portions of all eluted samples were treated with trypsin and electrophoresed again. The Mr of samples treated with PAL agonists all shifted greater than 10,000 and samples incubated with PAL antagonist all shifted much less than 10,000. The results indicate agonists and antagonists bind to the receptor in different orientations as different tryptic cleavage sites are available for proteolysis (Janovick et al., 1993).

PAL has not proven to be an effective method of accurately assessing the size of the GnRH receptor (Figure 1-3). Protein bands on gels are diffuse and difficult to identify resulting in inconsistent findings that are difficult to interpret Additionally, the majority of this work has been done using derivatives with the photoreactive group in the side chain of D-Lys^6-GnRH analogs (Hazum, 1981; Hazum and Keinan, 1983; Nikolics et al., 1989; Iwashita and Catt, 1985; Janovick et al., 1993). For PAL to accurately assess the size of a receptor itself,

Figure 1-3: Summary of the Results of Photo-Affinity Labeling Experiments

Photo-Affinity Labeling Experiments

Group	Tissue	Mr
Hazum, 1981	Rat Pituitary	60,000 + 48,000
Iwashita and Catt, 1985	Rat, Rabbit + Mouse Pituitary	52,000 - 62,000; 42,000 - 43,000
	Bovine + Ovine Pituitary	42,000 + 39,000
	Rat Granulosa Cells	53,000
	Rat Leydig Cells	43,000
Pahwa et. al., 1989	Human Epithelial Carcinoma	63,000 + 43,000
Janovick et. al., 1993	Rat Pituitary	
	-Treated with Agonist	60,000; 56,000; 52,000; 48,000; 46,000; 44,000
	-Treated with Antagonist	62,000; 58,000; 54,000; 52,000; 48,000; 45,000

it is important to choose ligands with the photo-reactive group in an optimal spatial arrangement that allows it to covalently bind the receptor (Bayley and Knowles; 1977). Substitution studies of GnRH at amino acid position 6 with large macromolecules such as Polyglutamic acid (Amoss et al., 1974), Polyethyleneglycol (Sandow et al., 1978) or Agarose (Conn and Hazum, 1981) suggest researchers have made a poor choice by inserting the photoreactive group in this position. All analogs with these substitutions maintain high-affinity binding to the GnRH receptor and support activation. This is logical as the GnRH molecule appears to bind its receptor in a horseshoe shape, with only the two ends contacting the receptor itself (Figure 1-2). This suggests amino acid position 6 is not close to the ligand binding domain of the receptor. Thus, when a photo-reactive group is attached in this position, it may not be binding the receptor itself and could be binding various membrane-associated proteins. This could account for the wide-variety of results from these studies.

III. **Radiation Inactivation**

Another method utilized to determine size of the GnRH receptor is radiation inactivation (Conn and Venter, 1985). This method is based on the theory that an inverse relationship exists between the dose-dependent inactivation of a macromolecule by radiation and the size of that molecule. This method permits analysis of the GnRH receptor while still in the membrane in its native state. Examination of the GnRH receptor in membrane preparations with

several doses of ionizing radiation resulted in a size estimate of 136,346, which could possibly be two receptors in a dimer formation.

Radiation inactivation is a useful method for determining molecular weight of a molecule and has been used successfully with several protein receptors (Venter et al., 1983). The size of the slow inward calcium channel and the muscarinic cholinergic receptor were determined by this method and validated by additional techniques of molecular weight assessment (Lo et al., 1982). Unfortunately, this method has not proven to be useful for determining molecular weight in all receptors and is a technique that is quite difficult to master. Currently, there is only one report of an attempt with the GnRH receptor.

IV. **Purification by Affinity Chromatography**

Many attempts have been made to purify the GnRH receptor using affinity chromatography (Figure 1-4). Hazum et al. (1986) solubilized the receptor from rat pituitary membranes in the zwitterionic detergent, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfate (CHAPS), and purified it with affinity chromatography. The purified receptor exhibited IC_{50} values identical to the membrane bound receptor when analyzed with GnRH, [D-Lys^6]-GnRH, [D-pGlu^1, D-Phe^2, D-Trp^3-D-Lys^6]-GnRH Buserelin and [biotinyl-D-Lys^6]-GnRH, which indicates the solubilized receptor has similar binding characteristics as the membrane bound receptor. The radiolabeled affinity purified receptor was analyzed by SDS-PAGE and autoradiography which resulted in two bands with Mr of 59,000 and 57,000. PAL of the same receptor produced bands of the

Figure 1-4: Summary of Results from Affinity Chromatography Experiments

Affinity Chromotography Experiments

Group	Tissue	Method	Mr
Hazum et. al., 1986	Rat Pituitary	Radiolabelling	57,000 + 59,000
		PAL	57,000 + 59,000
Iwashita et. al., 1988	Rat Pituitary	PAL - Native Conditions	700,000
		Sucrose Density Centrifugation	250,000
		PAL - Denaturing Conditions	52,000 + 62,000
Christiansen and Houen, 1994	Bovine Pituitary	Silver-Staining	60,000
Flanagan et. al., 1996	Ovine Pituitary	Silver-Staining	67,000

same size. Unfortunately, the purified receptor only retained 4-10% of initial activity in CHAPS extracts and yields were extremely low which prevented using this method to purify large amounts of the receptor.

Another group purified the rat pituitary receptor and retained 63% of the original membrane binding activity (Figure1- 4; Iwashita et al., 1988). Scatchard analysis revealed binding affinity of the purified receptor was the same as that found in pituitary membranes (Scatchard, 1949). Gel filtration of the receptor under native conditions after PAL revealed a product with an Mr of 700,000 when compared to protein standards. Sucrose density centrifugation was utilized to obtain a size estimate of the receptor and it had a Mr of 250,000 when compared to protein standards. When either the solubilized receptor or membrane bound receptor were PAL and denatured in SDS, the Mr was 60,000 and 52,000, which agreed with previous reports using PAL. This indicates the minimum Mr of the solubilized receptor under non-denaturing conditions is 250,000 suggesting it is associated with additional membrane components which appear to be necessary for proper ligand binding, as the denatured form of the receptor without additional membrane components is unable to bind ligand.

A GnRH binding protein has also been purified and characterized from the bovine pituitary (Figure 1-4; Houen et al., 1994). It was purified with affinity-chromatograpy after solubilization of bovine pituitary membranes using Triton X-100. The binding affinity of the binding protein was only slightly less than that reported for the membrane bound receptor. The binding protein was analyzed with SDS-PAGE which revealed a single protein with a Mr of 60,000 after silver-

staining. Tryptic cleavage of this gel piece followed by microsequencing revealed an unknown sequence with homology to other members of the G-Protein Coupled Receptor (GPCR) Superfamily, although comparison with the predicted peptide sequence of GnRH receptors did not result in homology.

A GnRH binding protein was also isolated from ovine pituitary membranes by solubilization with CHAPS and affinity purification (Figure 1-4; Flanagan et al., 1996). The CHAPS extract maintained 75% of the original membrane binding activity. The affinity purified binding protein was analyzed with SDS-PAGE which revealed a single band with a Mr of 67,000 after silver-staining. The band was microsequenced and found to have homology to the bovine GnRH binding protein isolated by Houen et al. which had no homology to any predicted peptide sequences of the GnRH receptor.

Large-scale purification of the GnRH receptor by affinity chromatography has been unsuccessful due to many inherent problems. Technical problems include the sheer amount of tissue necessary for purification and the extreme loss of receptor protein during the purification process. For example, purification of the ovine GnRH receptor required preparing 287 pituitaries producing 1 liter of homogenate from which greater than 90% of the activity was lost after running the homogenate over the affinity column.

V. **Immunological Methods**

A novel immunological approach was tested by Eidne et al. (1985) in which antibodies were raised to the GnRH molecule itself (Figure 1-5). Three classes were made directed against the N-terminal, C-terminal and middle portion of the GnRH molecule. Rat pituitary membranes and adrenal glands, and bovine adrenal glands, were analyzed with SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane before incubation with GnRH and the three antisera. Antisera directed against the middle portion of the molecule resulted in immunological detection of bands in the rat pituitary and adrenal gland and bovine adrenal gland with Mr of 60,000. Samples incubated with antisera directed against either end of the GnRH molecule did not result in immunological detection in any tissue. This is not surprising as GnRH appears to bind its receptor by its two ends which would render the epitopes unavailable for antibody to bind.

Hazum et al. (1987) generated a polyclonal antisera directed against his purified bovine pituitary receptor (Figure 1-5). It was able to immunoprecipitate many forms of the receptor including a PAL receptor, iodinated purified receptor and a receptor bound with a radiolabeled agonist. All forms of the receptor had a Mr of 60,000 when analyzed with SDS-PAGE. The results were identical in pituitary samples from both the bovine and rat. This antisera did not inhibit luteinizing hormone release in rat pituitary primary cultures indicating the antisera was not directed against the hormone binding domain of the receptor.

Figure 1-5: Summary of Experiments Utilizing an Immunological Approach

Summary of Immunological Experiments

Group	Method	Tissue	Technique	Mr/ICC Stain
Eidne et. al., 1985	Polyclonal Antibody	Rat Pituitary	Western Blot	60,000
		Rat Adrenal	Western Blot	60,000
Hazum et. al., 1987	Polyclonal Antibody	Bovine Pituitary	Western Blot	60,000
		Ovine Pituitary	Western Blot	60,000
Gorcs et. al., 1986	Complementary Peptide Approach	Rat Pituitary	ICC	Stained 10% of cells
Karande et. al., 1995	Monoclonal Antibody	Human Pituitary	Western Blot	60,000
			ICC	Stained 10% of cells

Gorcs et al. (1986) utilized the complementary peptide approach to generate an antisera to the GnRH receptor using rat hypothalamic GnRH and human placental GnRH sequences (Figure 1-5). This technique is based on the theory that complementary nucleic acid segments encode for the ligand and receptor binding site respectively (Arimura et al., 1985; Neri et al., 1991). It is thought that peptide ligands have well-defined amphiphilic conformations necessary for high-affinity binding to their receptor and during the receptor-ligand binding event, the two would need to be complementary to one another in their hydrophobic and hydrophilic domains (Snell, 1984). Thus, theoretically, two peptides generated by complementary nucleic acid strands should bind to each other because of the combined amphiphilic structure (Blalock and Smith; 1984; Bost and Blalock, 1986). This technique was successful in generating specific antisera to the ACTH receptor which was then used for affinity purification.

The technique was then attempted with GnRH and its receptor (Figure 1-5; Gorcs et al., 1986). Two peptides were synthesized with the sequences complementary to mRNA for rat hypothalamic GnRH and human placental GnRH. Antibodies were then generated against these peptides and analyzed for recognition of GnRH and its receptor. Antisera generated against human placental GnRH appeared to decrease plasma LH in ovariectomized rats and to stain approximately 10% of dispersed rat pituitary cells. It appeared to stain the receptor as it didn't bind GnRH or LH when tested by ELISA, or by a pre-absorption test with immunocytochemistry, but direct proof in the form of a dual-stain for LH was not obtained.

The synthetic peptide approach was used to generate a monoclonal antibody to the human GnRH receptor (Figure 1-5; Karande et al., 1995). A peptide corresponding to amino acids 1-29 in the amino-terminal extracellular domain of the receptor was generated and anti-peptide monoclonal antibodies were produced using the hybridoma technique (Kohler and Milstein, 1975; Westerwoudt et al., 1984). An antibody was produced that was able to recognize the GnRH receptor on human gonadotropes with immunocytochemistry and in pituitary membranes with Western Blotting which revealed the presence of a single band with Mr of 60,000.

Unfortunately, there have been many limitations with the antibodies produced thus far. In many cases the antibody supply has been limited which has hindered extensive research with any one antisera in particular and eliminated the possibility of corroboration. All reports have been single publications on limited samples.

D. **Isolation of cDNA Sequences for the GnRH Receptor**

I. **Introduction**

As this brief background suggests, there are many discrepancies in the reported size of the GnRH receptor. It wasn't until recently that additional tools were available with which to examine the receptor. The first cDNA for the murine GnRH receptor was reported in 1992 and was achieved by a homology screening strategy of the αT3 cell line (Tsutsumi et al., 1992). Degenerative oligonucleotide primers were designed to conserved transmembrane domain

regions of G-protein coupled receptors. Resultant polymerase-chain reaction (PCR) products were cloned and sequenced. Complementary oligonucleonucleotides of these sequenced products were injected into *Xenopus* oocytes with αT3 RNA and clones which produced GnRH-induced chloride channel activity were identified. These were utilized to identify a full length clone which was examined after transfection in Cos-1 cells. Binding characteristics and GnRH stimulated inositol phosphate production were similar to that reported in ovine primary cultures (Millar et al., 1993).

This report was quickly followed by the cloning of the rat (Eidne et al., 1992; Kaiser et al., 1992), human (Kakar et al., 1992; Chi et al., 1993), bovine (Kakar et al., 1993), and ovine (Brooks et al., 1993; Illing et al., 1993; Turzillo et al., 1994) cDNA sequences, making further structural analysis possible.

II. **Predicted Peptide Characteristics**

The predicted peptide sequence of the mouse and rat cDNA sequences are 327 amino acids and the cow, sheep and human are 328, due to an extra residue in transmembrane domain 2 (TMD2) (Figure 1-6). The predicted peptide sequence of all receptors demonstrates >85% homology with almost complete conservation throughout the TMDs. Hydrophobicity studies indicate the presence of TMDs which categorizes the receptor into the large G-protein coupled receptor (GPCR) superfamily. Actual structural information has only been obtained

Figure 1-6: Motifs Conserved Across Members of the G-Protein Coupled Receptor Superfamily Present in the GnRH Receptor

been obtained for two members of this superfamily, rhodopsin and bacteriorhodopsin, with cryo-electron microscopy of two-dimensional crystals (Henderson et al., 1990). Based on the presumed structural similarity between GPCRs and rhodopsin or bacteriorhodopsin, the transmembrane domains of all GPCR's are thought to be comprised of α-helices arranged in a circular bundle formation with a central hydrophilic pocket (Baldwin et al., 1993).

Many GPCRs have potential glycosylation sites which are utilized (Rands et al., 1990). The cow, sheep and human GnRH receptors contain two potential sites while the mouse and rat receptor have three (Figure 1-6). All sequences contain one in the amino terminus and one in the first extracellular loop (ECL1), while rodents have an additional potential glycosylation site in the amino terminus.

Another highly conserved feature of the GPCR superfamily is the presence of two cysteine residues in ECL1 and ECL2 which are presumed to be covalently linked via a disulfide bond (Probst et al., 1992; Baldwin, 1993). The GnRH receptor has the conserved cysteine residues and an additional two in the amino terminus and ECL2, respectively (Figure 1-6).

A conserved motif among members of the GPCR superfamily, found in the GnRH receptor, is a series of proline residues in TMD2, TMD4, TMD5, TMD6 and TMD7 which may function to induce kinks in the α-helices and be involved in ligand-induced signal transduction (Figure 1-6; Baldwin et al., 1993). This appears to occur in the 5HT-2 receptor which is structurally similar to the GnRH receptor (Zhang and Weinstein, 1993).

Figure 1-7: Unique Features of the GnRH Receptor Not Common to Other Members of the G-Protein Coupled Receptor Superfamily

Unique Features
of the GnRH Receptor

The GnRH receptor also has several unique features compared to other members of this superfamily (Figure 1-7). One is an extremely short intracellular carboxyl-terminus of 1-2 amino acid residues. This is in contrast to other members of the superfamily that have large carboxyl-termini which interact with G-proteins, causing an intracellular response to ligand binding (Tensen et al., 1997; Troskie et al., 1997; Wang et al., 1996; Lefkowitz et al., 1990).

A second unusual feature of the GnRH receptor is a short extra-cellular amino terminus of 35 amino acid residues (Figure 1-7). Other GPCRs, like the Luteinizing Hormone or Follicle Stimulating receptors, have long amino termini with 300 to 400 amino acid residues which are involved in ligand-binding (Segaloff and Ascoli, 1993).

A third feature is the absence of the conserved Asp-Arg-Tyr sequence at the juncture of TMD3 and the second intracellular loop. In its place is a Asp-Arg-Ser sequence which creates a site for potential phosphorylation. A fourth unique feature is the presence of a Asn^{87} in TMD2 and an Asp^{318} in TMD7. The exact opposite orientation (Asp^{87} in TMD2 and an Asn^{318} in TMD7) is highly conserved among the GPCR superfamily.

The unique motifs found in the GnRH receptor indicate it may function differently than other members of the GPCR superfamily (Figures 1-6 and 1-7). In most GPCRs, the amino terminus is involved in ligand binding which is most likely not the case with the GnRH receptor because of its diminutive length. It is more plausible that the extracellular loops and transmembrane domains are involved in forming the binding pocket Additionally, many GPCRs signal an

intracellular G-protein with their carboxyl terminus which is also not likely to happen in the GnRH receptor because of its composition of only 1 or 2 amino acid residues. These dramatic departures from the typical structure of a member of the GPCR superfamily suggest that the GnRH receptor functions very differently than the other GPCRs characterized to date.

III. **Site-Directed Mutagenesis**

Isolation of cDNA sequences for the GnRH receptor from several species allows researchers to investigate which regions of the receptor are involved in ligand binding, receptor internalization and signal transduction. These studies have involved replacing key amino acids by site-directed mutagenesis to determine the function of individual amino acids or particular regions of the receptor. Mutated cDNA's are then transfected into mammalian cell lines and the effect of the mutation on the receptor's characteristics can be analyzed. Receptor affinity can be assessed with ligand binding assays and receptor activation with inositol-phosphate production.

The potential reciprocal mutation of an Asn in TMD2 and Asp in TMD 7 has been extensively analyzed (Figure 1-8; Cook et al., 1993; Zhou et al., 1993; Awara et al., 1996). Cook et al. (1993) mutated the two residues individually (Asn^{87} to Asp and Asp^{318} to Asn) in the rat GnRH receptor as well as both mutations together to re-create the highly conserved orientation. The Asn^{318} mutation had no effect on ligand binding when transfected into Cos-1 cells but binding wasn't associated with GnRH-dependent inositol-phosphate production.

The Asp^{87} and $Asp^{87}Asn^{318}$ mutants demonstrated a complete lack of GnRH binding.

In contrast, Zhou et al. (1993) repeated a similar experiment in the mouse GnRH receptor and did not get the same results (Figure1- 8). The reciprocal mutation resulted in high-affinity binding, which was not obtained with the Asp^{87} mutant. The single Asn^{318} mutation had no effect on ligand binding. These results suggest Asn^{87} and Asp^{318} are in close proximity to one another, possibly creating an interaction which contributes to the structural and functional integrity of the receptor necessary for proper ligand binding and activation.

Stable transfected GH_3 cells were created with these mutations in the rat GnRH receptor (Figure 1-8; Awara et al., 1996). The Asn^{318} and $Asp^{87}Asn^{318}$ mutant receptors demonstrated normal ligand binding but Buserelin-stimulated IP production was attenuated. The Asp^{87} mutant was not examined. This study suggests Asn^{87} and Asp^{318} are not critical residues for high-affinity binding but are necessary for signal transduction.

The Asn residues in the three potential glycosylation sites in the mouse GnRH receptor (Asn^{4}, Asn^{18} and Asn^{102}) were mutated to Gln in order to determine if they were utilized and if so, their function (Figure 1-8; Davidson et al., 1995). Mutation of any of the three residues resulted in only slight decreases in binding affinity but a drastic reduction in the numbers of receptors binding ligand. This could have been due to a decrease in actual receptor number or a decrease in the number of receptors capable of binding ligand. All

Figure 1-8: Summary of Site-Directed Mutagenesis Studies

Summary of Site-Directed Mutagenesis Studies

Group	Mutation	Effect
Cook et al., 1993	Asn^{87} to Asp	No Detectable Ligand Binding
	Asp^{318} to Asn	Normal Ligand Binding
	Both together	No Detectable Ligand Binding
Zhou et al., 1993	Asn^{87} to Asp	Low Affinity Binding
	Asp^{318} to Asn	No Effect on Ligand Binding
	Both together	High Affinity Binding
Awara et al., 1996	Asn^{87} to Asp	Not Examined
	Asp^{318} to Asn	Normal Ligand Binding but Lowered IP Production
	Both together	Normal Ligand Binding but Lowered IP Production
Davidson et al., 1995	Asn^{8}, Asn^{18}, Asn^{102}	Glycosylation Important for Proper Trafficking to the Membrane
Flanagan et al., 1994	Acidic Residues	Gln^{301} Appears to Recognize Amino Acid Position 8 of GnRH
Arora et al., 1995	$Asp^{138}Arg^{139}Ser^{140}$	Unique Ser Residue Appears Unimportant for Signal Transduction
Eat et al., 1995	Lys^{121}	Involved in High-Affinity Agonist Binding
Arora et al., 1995	Asn-Pro-X_{1-2}-Tyr^{322}	Tyr Important for High Affinity Binding, Activation and Signal Transduction
Davidson et al., 1995	Asn^{102}	Discriminates for C-Terminal Glycinamides
Heding et al., 1998	Carboxy Terminus	Appears Important in some GPCRs for Rapid Desensitization and Endocytosis
Neill et al., 1997	Epitope Tagging	Amino Epitope Tag did not Affect Binding Characteristics
Cook and Eidne, 1997	Cys^{14}, Cys^{114}, Cys^{195}, Cys^{199}	Disulfide Bond Appears to Form Between Cys^{14} and Cys^{195}

receptors that did bind ligand were capable of GnRH-stimulated inositol phosphate production but levels were decreased in a manner predicted by the decrease in receptor number. This suggests that carbohydrate moieties are not involved in ligand binding but are instead critical for the proper trafficking and insertion of the receptor into the membrane.

Arg^8 of GnRH appears to be the key to the high-affinity binding of GnRH to its receptor. This may be due to an electrostatic interaction between Arg^8 and a negatively charged residue on the extracellular loops or TMD of the receptor. The hypothesis was tested by mutation of eight conserved acidic residues of the mouse GnRH receptor to isoteric Asn or Gln (Figure 1-8; Flanagan et al., 1994). All mutant constructs were tested for preference for agonists containing Arg^8 and activation of the receptor by inositol phosphate production. Mutation of Glu^{301} to Gln resulted in decreased affinity for GnRH and [Lys^8]-GnRH when compared to the wild-type receptor. Affinity for [Glu^8] GnRH was greater than 10-fold above the wild-type receptor. The Gln^{301} mutant receptor appeared to have lost the ability to discriminate between ligands with different amino acids in position 8, as discrimination was possible with ligands containing substitutions in amino acid positions 5,6 or 7. This indicates that Glu^{301} of the mouse GnRH receptor is involved in recognition of Arg^8 of GnRH, possibly through an electrostatic interaction.

The highly conserved Asp-Arg-Tyr sequence in the second intracellular loop (2IL) has been implicated in G-Protein coupling in other GPCRs (Dohlman et al., 1991; Moro et al., 1993, 1994). The GnRH receptor has a Asp-Arg-Ser

sequence in this position (Figure 1-7). The unique Ser^{140} was substituted with Tyr to assess the effect on ligand binding and signal transduction (Figure 1-8; Arora et al., 1996). Replacement with Tyr significantly increased binding affinity and internalization but had no effect on signal transduction when compared with wild-type. Thus, Ser^{140} appears to be unimportant for communication with intracellular G-proteins in the GnRH receptor.

A preliminary three-dimensional model of the GnRH receptor has been proposed which indicates Lys^{121} in TMD3 is located in the binding pocket of the receptor (Ballesteros and Weinstein, 1995). Lys^{121} is conserved across all mammalian GnRH receptors whose structures have been reported to date. This position is analogous to Asp^{113} of the β-adrenergic receptor which is required for high-affinity neurotransmitter binding. The purpose of Lys^{121} in the mouse GnRH receptor was examined by mutation (Figure 1-8). Replacement with Arg^{121} preserved high-affinity agonist binding but Gln^{121}, Leu^{121} and Asp^{121} completely abolished binding. Antagonist binding had comparable high affinities for the wild-type, Arg^{121}, and Gln^{121} mutant receptors indicating a charge-strengthened hydrogen bond donor is necessary in this position for high-affinity agonist binding but not antagonist binding to the mouse GnRH receptor.

The sequence of events which result in agonist-induced internalization of membrane bound receptors isn't clear but several conserved sequences appear to be involved. In the low-density lipoprotein and transferrin receptors, it appears to involve a Asn-Pro-X-Tyr motif (Collawn et al., 1990; Trowbridge et al., 1993). A similar motif, Asn-Pro-X_{2-3}-Tyr, is present in TMD7 of most GPCRs (Probst et

al., 1992). Mutation of the Tyr residue in the β-adrenergic receptor results in complete loss of receptor internalization and desensitization (Barak et al., 1994).

A slightly modified form of this sequence, Asn-Pro-X_{1-3}-Tyr, is found in the GnRH receptor. The role of the conserved aromatic Tyr^{322} was examined by mutation of the residue to Ala or Phe (Figure 1-8; Arora et al., 1995). Substitution with Ala resulted in high-affinity agonist binding but a complete loss of agonist-induced inositol phosphate production, and internalization rates were decreased to 50% of wild-type. Substitution with Phe, which maintained the aromatic nature in the position, resulted in no change in binding affinity, agonist induced inositol-phosphate production or internalization when compared to wild-type. This indicates the aromatic characteristics of Tyr are necessary for high-affinity binding of ligand to the receptor and proper agonist-induced receptor activation and signal transduction.

When Davidson et al. (1995) examined the role of potential glycosylation sites in the GnRH receptor, they found an increase in binding affinity when Asn^{102} was mutated to Gln. This suggests that Asn^{102} may be directly involved in ligand binding. Mutation of Asn^{102} to Ala resulted in decreased binding affinity for GnRH, as measured by stimulation of inositol phosphate (IP) production which was also seen with eight additional analogs containing a glycinamide C-terminus (Davidson et al., 1996). Analogs with ethylamide instead of a C-terminal glycinamide residue demonstrated that only small decreases in potency when compared to wild-type receptors. This indicates that Asn^{102} is key to discriminating specifically for ligands with C-terminal glycinamides but ligands

with C-terminal ethylamides are less dependent on Asn^{102}. This is consistent with early GnRH substitution studies which demonstrated removal of the C-terminal glycinamide resulted in a marked decrease in bioactivity, suggesting an interaction between the glycinamide and the receptor (Karten and Rivier, 1986). Replacement of the glycinamide C-terminus with an ethylamide caused an increase in biopotency (Karten and Rivier, 1986; Fujino et al., 1972).

The mammalian GnRH receptor is the only member of the GPCR superfamily lacking an intracellular carboxyl-terminus, although non-mammalian GnRH receptors still retain one (Figure 1-7; Eidne et al., 1992; Troskie et al., 1997). Other GPCRs show rapid desensitization upon prolonged stimulation with agonist which involves uncoupling of G-proteins, rapid down-regulation of effector systems, such as inositol phosphate, and receptor internalization (Nussenzveign et al., 1993; Davidson et al., 1994; Perlman and Gershengorn, 1991; Falck-Pedersen et al., 1994). In several GPCRs, desensitization has been tied to the phosphorylation of several amino acid residues in the cytoplasmic tail (Lefkowitz et al., 1990; Matus-Leibovitch, et al., 1995; Wang et al., 1996). In contrast, the mammalian GnRH receptor does not have a carboxyl-tail and does not show rapid desensitization of IP production while the catfish GnRH receptor which does have a carboxylic tail, undergoes rapid desensitization (Davidson et al., 1994; McArdle et al., 1995; Anderson et al., 1995).

A chimera of the rat GnRH receptor was created by fusion of the carboxyl-terminus of the thyrotropin-releasing hormone receptor (TRH-R) and the desensitization rates were compared to the catfish and rat GnRH receptor

(Figure 1-8; Heding et al., 1998). The TRH-R is known to undergo rapid desensitization of IP production, the same effector system utilized by the GnRH receptor (Davidson et al., 1994; Perlman and Gershengorn, 1991). Prolonged stimulation with agonist resulted in rapid desensitization and endocytosis of the rat GnRH-R/TRH-R chimera, the TRH-R, and the catfish GnRH receptor. Neither event occurred with the wild-type rat GnRH receptor, indicating the carboxyl-terminus is indeed important in some GPCRs for rapid desensitization and endocytosis.

Although there is abundant information pertaining to the structure of the GnRH receptor, many of the above-mentioned site-directed mutagenesis studies are prejudiced due to their dependence on binding assays. If binding does not occur several possibilities exist: 1) protein was not synthesized or folded correctly; 2) protein was not correctly trafficked to the membrane; 3) protein was incorrectly inserted into the membrane; 4) mutation disrupted optimal binding kinetics of the receptor; or 5) mutation disrupted structure of the receptor necessary for communication with second messengers. It is difficult to discern which of these possibilities exists without the ability to detect the unbound form of the receptor.

An attempt was made to overcome this difficulty by creating an epitope tagged GnRH receptor to be used as a model to further study the receptor (Neill et al., 1997). Influenza hemagglutinin (HA-1) epitope tags were attached to the amino- (N-) and carboxy- (C-) termini of the rat GnRH receptor cDNA sequence and transfected into Cos-1 cells for binding studies. Epitope tagging resulted in

functional receptors assessed by binding studies and inositol phosphate analysis. Due to its higher expression and conveniently located extracellular epitope, the N-terminal tagged receptor was utilized for the majority of studies. Western blots of transfected cells resulted in bands with Mr of 67,000, 57,000 and 32,000

The N-terminal epitope tagged receptor was also expressed in SF9 cells which produced a functional receptor, although binding characteristics were not similar to the native pituitary receptor. Expression was also extremely low which makes any type of analysis difficult. Western blots of SF9 cell membranes resulted in bands with Mr of 32,000, 56,000, 69,000 and 120-140,000 (Ibid).

The four cysteine residues in the extracellular portion of the receptor were examined for the presence of an integral disulfide bond, important for the proper three-dimensional structure of the receptor necessary for high-affinity ligand binding (Figure 1-8; Cook and Eidne, 1997). The β-adrenergic receptor also has four extracellular cysteine residues which form two disulfide bonds critical for high-affinity ligand binding (Dohlman et al., 1990; Noda et al., 1994). All four Cys residues; Cys^{14}, Cys^{114}, Cys^{195} and Cys^{199}, of the rat GnRH receptor were mutated to Ala and ligand binding and activation assessed (Cook and Eidne, 1997). An HA epitope tag was incorporated into the amino terminus of the receptor to assess cell-surface expression. Ala^{114} and Ala^{195} mutant receptors were unable to bind ligand while displaying levels of cell-surface expression similar to wild-type. Ala^{199} demonstrated comparable receptor binding and activation with the wild-type receptor while Ala^{14} also had functional ligand

binding and activation, although at a lower level. This suggests that the GnRH receptor does form a disulfide bond between Cys^{114} and Cys^{195} which is important for the proper structure of the receptor necessary for ligand binding.

E. **Extra-Pituitary Sources of GnRH Receptors**

GnRH receptors have been reported in a wide variety of extra-pituitary tissues, such as human placenta, ovary, testis, adrenal and in many carcinomas (Emons et al., 1993; Yano et al., 1994; Bramley et al., 1985, 1987, 1992; Currie et al., 1981; Petersson et al., 1989). Examples of carcinomas include breast, kidney, ovarian and prostate tumors as well as many immortalized reproductive tissue cancer cell lines (Fekete et al., 1989a-d; Eidne et al., 1985; Sion-Vardi et al., 1992; Emons et al., 1993; Segal-Abramson et al., 1992a+b; Yano et al., 1994; Eidne et al., 1987). The nucleotide sequence of mRNA for GnRH receptors isolated from the breast cancer cell line, MCF-7, and from an ovarian tumor showed complete homology with the human pituitary receptor (Kakar et al., 1994).

These extra-pituitary sites are of interest due to the large number of clinical patients currently receiving GnRH agonist therapy in the treatment of sex steroid-sensitive cancers such as breast cancer (Harris et al., 1989; Waxman et al., 1985), prostate cancer (Schroder,1991), and pancreatic cancer (Greenway et al., 1987). Traditionally the mechanism of action of GnRH therapy was thought to be desensitization of the pituitary to GnRH resulting in a decline in gonadotropin secretion and gonadal hormone production. Recent reports of a

direct action of GnRH through its receptor on breast, ovarian, endometrial and prostate tumor cell lines to inhibit their growth challenges the traditional theory (Miller et al., 1985; Eidne et al., 1987; Limonta et al., 1993a +b; Segal-Abramson et al., 1992; Emons et al, 1993, Yano et al., 1994).

F. **Statement of the Problem**

GnRH analogs have important applications in both the clinical and agricultural fields. Clinical use includes administration in the treatment of endometriosis, precocious puberty, prostate cancer, breast cancer, and infertility. Agricultural use includes treatment of cystic follicles in dairy cattle, induction of ovulation in estrous synchronization programs, induction of puberty, synchronization of follicle populations prior to initiating superovulatory regimes, and treatment of males undergoing testicular degeneration. Additionally, administration of GnRH agonists in a continual slow-release fashion is currently being examined as a method of fertility control in wild horses, deer, feral dogs, sea lions and other animals which are difficult to control by a hunting season (Irby et al., 1996; Cheville et al., 1998; U.S. Agricultural Statistics Service, 1993).

Reports of extra-pituitary receptors, which if substantiated, could have wide-ranging clinical and agricultural implications. Antibodies have been used with some degree of success to gain additional information about the location of extra-pituitary GnRH receptors (Eidne et al., 1985; Hazum et al., 1987; Gorcs et al., 1986; Karande et al., 1995). This method appears to result in greater clarity and consistency when combined with Western Blotting than PAL, which often

leads to multiple bands of varying sizes. The antibodies created thus far have all identified distinct single protein bands with Western Blotting techniques (Figure 1-5). The antibody approach may also be a useful method to determine the size of the GnRH receptor.

We have generated a large amount of antibody to the GnRH receptor using the synthetic peptide approach and used the antisera to determine the presence and size of the GnRH receptor in a wide-variety of pituitary tissues with Western Blotting. Use of the specific antibody towards the GnRH receptor can result in a greater understanding of the location of the GnRH receptor in extra-pituitary tissues. If GnRH receptors on carcinomas do function as moderators of cell growth, an easy screening method for human biopsy samples for receptors would result in appropriately prescribed GnRH therapy to patients with GnRH sensitive tumors.

Chapter Two

Immunological Detection Of Extra-Pituitary Gonadotropin-Releasing Hormone Receptors

Introduction

Gonadotropin-Releasing hormone (GnRH), along with its receptor, plays a key role in mammalian reproduction. It is synthesized by hypothalamic neurons and secreted into the hypothalamic portal vasculature in a pulsatile fashion. By binding to specific high-affinity receptors on gonadotropes in the anterior pituitary gland, GnRH stimulates the synthesis and secretion of luteinizing hormone and to a lesser extent, follicle stimulating hormone (Clayton, 1989).

GnRH was isolated nearly 40 years ago and has been biochemically characterized (Matsuo and Schally, 1971). In contrast, nothing was known about the structure of its receptor until 1992 when a murine pituitary cDNA was isolated (Tsutsumi et al., 1992, Reinhart et al., 1992). This report was quickly followed by cloning of the pituitary receptor from several species, including human, rat, ovine, and bovine (Brooks et al., 1993; Kakar et al., 1992; Kakar et al., 1993; Eidne et al., 1992; Kaiser et al., 1992). Hydrophobicity studies of the proposed amino acid sequence indicate that the receptor has seven transmembrane domains which categorizes it into the G-protein-coupled receptor superfamily. All cDNA

sequences predict a single polypeptide of 327-328 amino acids with an approximate molecular weight of 38 kDa.

There are two potential glycosylation sites in the ovine, bovine and human receptors and three in the murine and rat receptors. If glycosylation occurs, this could significantly increase the size of the receptor over that of the peptide backbone alone. Many attempts have been made to obtain accurate size estimates of the GnRH receptor. The most prevalent method has been photoaffinity labeling (PAL) which has resulted in size estimates of 32,000-64,000 molecular weight (Mr) (Pahwa et al., 1989; Iwashita and Catt, 1985; Hazum, 1981, Janovick et al., 1993; Hazum, 1983; Nikolics et al., 1989; Neill et al., 1997).

GnRH receptors have been reported in a wide-variety of extra-pituitary tissues such as the human placenta, ovary, testis, adrenal, and a wide variety of carcinomas (Emons et al., 1993; Yano et al., 1994; Bramley et al., 1985, 1987, 1992; Currie et al., 1981; Petersson et al., 1989). Examples of carcinomas include breast, kidney, ovary, and prostate tumors as well as many immortalized reproductive tissue cancer cell lines (Fekete et al., 1989a-d; Eidne et al., 1985, Sion-Vardi et al., 1992; Emons et al., 1993; Segal-Abramson et al., 1992; Yano et al., 1994; Eidne et al., 1987). The nucleotide sequence of GnRH receptors isolated from the breast cancer cell line, MCF-7, and from an ovarian tumor, showed complete homology with the human pituitary receptor (Kakar et al., 1994).

These extra-pituitary sites are of interest due to the large number of clinical patients currently receiving GnRH agonist therapy for the treatment of sex steroid-sensitive cancers such as breast cancer (Klijin et al., 1982; Harris et al., 1989; Waxman et al., 1985), prostate cancer (Schroder, 1991), uterine endometrial cancer (Gallagher et al 1991), ovarian cancer (Ibid.) and pancreatic cancer (Greenway et al., 1987). Traditionally the mechanism of action of GnRH therapy was thought to be desensitization of the pituitary to GnRH. This would result in a decline in gonadotropin secretion and gonadal hormone production. Recent reports of a direct action of GnRH to inhibit growth of breast, ovarian, endometrial and prostate tumor cell lines gives us reason to question the previous theory (Miller et al., 1985; Eidne et al., 1987; Limonta et al., 1992, 1993; Segal-Abramson et al., 1992; Emons et al., 1993, Yano et al., 1994). If the presence of GnRH receptors on carcinomas does moderate growth, an easy method for screening human cancer biopsies for the presence of GnRH receptors would result in appropriately prescribed GnRH therapy to patients with GnRH-sensitive tumors.

Identification of extra-pituitary GnRH receptors is valuable when considering GnRH therapy. Currently, little information is available concerning location of GnRH receptors or if these receptors function similarly to the pituitary receptor. This paper outlines a strategy employed to develop and validate an immunological probe to the GnRH receptor. An antiserum was generated and determined to be specific to the GnRH receptor. The antisera was then used to survey pituitary and extra-pituitary tissues for the presence of the GnRH receptor.

Extra-pituitary tissues were also analyzed with Southern blotting techniques to determine if GnRH receptor RNA was present. Receptor assays were employed to determine if the GnRH receptors in extra-pituitary tissues were capable of binding GnRH.

Materials and Methods

Peptide synthesis

A peptide comprised of amino acids 23-36 of the murine GnRH receptor (LIQGKLPTLTVSGL) was synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO). The peptide was determined to be >90% pure by reverse-phase high performance liquid chromatography and was linked to keyhole limpet hemocyanin (KLH) (Sigma Chemical Co., St. Louis, MO) using the crosslinker glutaraldehyde (Sigma Chemical Co., St. Louis, MO). Briefly, 21 mg GnRH receptor peptide was combined with 25 mg KLH in 2 ml of ammonium acetate buffer (0.1 M, pH 7.0). Two ml 0.02M glutaraldehyde were added dropwise. The solution was then stirred for 5 hours at room temperature before being dialyzed at 4° C against 0.1 M ammonium acetate buffer (pH 7.0) overnight. The dialyzed reaction mixture was then lyophilized.

Immunization

Adult rabbits were given intradermal injections of 1 mg GnRH receptor peptide-KLH dissolved in Freunds complete adjuvant (Pierce Rockville, IL). In three

weeks, booster immunizations were administered subcutaneously with 250 ug GnRH receptor peptide-KLH dissolved in Freunds incomplete adjuvant. Sera were collected via the jugular vein three weeks following booster immunization.

Tissue Collection and Cell Lines

All mice in this study were subjected to carbon monoxide prior to tissue collection. Ewes and rabbits were euthanized with an overdose of sodium pentobarbital prior to tissue collection. Bovine pituitaries were purchased from a slaughterhouse (Monsanto, CO). Additional bovine tissue was collected from a slaughterhouse (Petrie's Meat Packing Plant, Pierce, CO). All tissues were collected immediately after death, frozen on dry ice and stored at –70° C. The murine gonadotrope tumor cell line (αT3) was obtained from Dr. P. Mellon, University of California, San Diego, CA.. The human cervical cancer cell line (HeLa) and monkey kidney tumor cell line (Cos-7) were obtained from the American Type Culture Collection (ATCC). Cell lines were maintained according to the recommendations of ATCC. All procedures utilizing animals were approved by Colorado State University's Animal Care and Use Committee.

Sample Preparation

Tissues were homogenized with a free-standing Polytron homogenizer at 4° C in lysis buffer [0.1M SDS, 10 mM Tris, pH 7.5 (200-300mg tissue/ml)]. After homogenization, samples were boiled 5 minutes and then sheared through a 22 and then a 25 gauge needle. Membranes from bovine pituitary were prepared as

described previously (Crowder et al., 1984). Immortalized cell lines were homogenized with a hand-held Polytron homogenizer at 4° C in lysis buffer (1 x 10^6 cells/ 2 ml buffer).

Protein Determination

Total protein concentrations in all samples were determined using the Bio-Rad protein kit with bovine serum albumin as a standard (Hercules, CA).

SDS-Polyacrylamide gel electrophoresis and Western blotting

Gel electrophoresis was performed according to the method of Laemmli (1970) using 12.5% SDS-polyacrylamide gels. Multi-colored molecular weight markers were purchased from Novex (San Diego, CA.). The protein markers were myosin - 250 kDa, phosphorylase B - 148 kDa, glutamic dehydrogenase - 60 kDa, carbonic anhydrase - 42 kDa, myoglobin blue - 30kDa, myoglobin red - 22 kDa, lysozyme - 17 kDa, aprotinin - 6 kDa, and insulin 4 kDa. An equal volume of loading buffer (5% glycerol, 10% SDS, and bromophenol blue) and 5% β-mercaptoethanol was added to each sample before boiling for 3 min. Denatured proteins (10 μg/lane) were immediately electrophoresed and transferred to nitrocellulose membranes. After transfer, gels were stained with Coomassie Blue and membranes with Ponceau S to ensure optimal transfer of protein had occurred. Membranes were rinsed under tap-water to remove Ponceau S and incubated with 5% non-fat dry milk (NFDM) in TTBS [0.05% Tween-20 in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5)] for 60 min at room

temperature with shaking to block non-specific binding sites. Membranes were washed 3 x 5 min in TBS before incubation with the primary antibody (1:3000 antisera in 1 x TBS containing 5% NFDM) for 60 min at room temperature with shaking. The membranes were then incubated with alkaline-phosphatase goat anti-rabbit immunoglobin conjugate (Anti-Rabbit IgG Alkaline Phosphatase), (Novex, San Diego, CA) diluted 1:3000 in TBS containing 5% NFDM for 60 min at room temperature with shaking and then washed as above. Immunopositive bands were visualized after incubation with nitro blue tetrazolium (0.33 mg/ml, Promega, Madison, WI) and 5-bromo-4-chloro-3-indolyl-phosphate (0.165 mg/ml Promega, Madison, WI) in 100 mM Tris-Cl, 100 mM NaCl, 5 mM $MgCl_2$, (pH 9.5).

In-vitro transcription and translation

Murine GnRH receptor cDNA in pBK-CMV (Promega, Madison, WI) or CMV5 (C. Clay) was transcribed and translated *in vitro* using the rabbit reticulocyte lysate system from Promega (Madison, WI) according to the manufacturer's instructions. ^{35}S-methionine (Amersham, UK) was included in the reaction to label the receptors. Samples were analyzed by SDS-PAGE and dried gels were exposed to x-ray film. Western analysis of the products was performed as described above.

RNA extraction and RT-PCR:

Total cellular RNA was extracted and purified from ovine tissues as adapted from Chomczynski and Sacchi (1987). Briefly, approximately 200 mg of tissue was

homogenized in a guanidinium thiocyanate buffer using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Then acidic phenol plus chloroform and isoamyl alcohol was used to extract total RNA from the homogenates. After an incubation on ice for 15 minutes, the samples were centrifuged at 10,000 x g for 20 minutes at 4° C. The aqueous layer was mixed with an equivalent amount of -20° C isopropanol and then placed at -20° C for 2 hours to precipitate the RNA. Samples were centrifuged again and then washed with 70% ethanol. The resulting pellets were resuspended into DEPC treated water and quantified using a spectrophotometer. RNA was examined on a 6% formaldehyde-agarose gel containing ethidium bromide (EtBR) to evaluate the integrity of the RNA. RNA was transcribed into DNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) at 42° C for 90 minutes. The resulting DNA served as a template for polymerase chain reaction (PCR) using 2.5 U of *Taq* polymerase (Promega) according to manufacturer instructions. Forty cycles were performed at 95° C, 54° C and 72° C for 30s, 30s, and 1 min., respectively. The primers were designed based on the 5' flanking region 15 amino acids upstream of the intrinsic start site and the 3' flanking region 4 amino acids down stream of the stop codon (Forward primer: AGAAGTGCCAGAAACACAAG, reverse primer: GGATCTGACACCCTGTTTAAC). PCR products were electrophoresed onto a 0.8% agarose-tris acetate gel and stained with EtBR.

Southern Blot Hybridization:

The identity of the DNA generated by RT-PCR was confirmed by Southern blot analysis. PCR products in agarose gels were transferred to nitrocellulose membrane (NitroBind, MSI, Westboro, MA) by capillary action. After cross-linking the DNA to the membrane by exposure to UV light, the membrane was hybridized overnight at 42° C with a 701 bp ovine GnRH receptor cDNA probe (described in Turzillo et al., 1994). The hybridization buffer consisted of 50% formamide, 6X SSC (900 mM NaCl and 90 mM Na citrate), 5X Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 1% SDS and 0.2 mg/ml denatured salmon sperm DNA. The final wash was performed in 0.1X SSPE (15 mM NaCl, 1 mM Na phosphate and 0.1 mM EDTA, pH 7.4) and 1% SDS at 65° C for 1 hour. The membrane was then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) and analyzed using 1.55 NIH Image (National Institutes of Health, Bethesda, MD). Results were measured in arbitrary units and expressed as a percentage of the pituitary value.

Receptor Assays

Bovine pituitary membrane pools were prepared as described previously (Crowder et al., 1984). All other tissues were collected and frozen as described for Western analysis. Partially purified membranes were prepared by homogenization with a free-standing Polytron homogenizer at 4° C in receptor assay buffer (10 mM Tris Base, 1 mM $CaCl_2$, 0.1% BSA). Immediately after

homogenization, all samples were centrifuged 30,000 x g for 30 min. The supernatant was aspirated and the pellet resuspended in 1-2 ml receptor assay buffer. Partially purified membranes (50 μL, 10 μg total protein) were examined for the presence of GnRH receptors by incubation with 0.2 nM [^{125}I]D-Ala$_6$-GnRH-Pro$_9$-ethyl-amide ([^{125}I]D-Ala$_6$) with and without 35 ng unlabelled DAla$_6$ for 4 hours at 4° C . Bovine pituitary membranes served as a positive control. After incubation, all tubes were immediately centrifuged after addition of 3 ml of ice-cold receptor assay buffer. Amounts of specifically bound [^{125}I]D-Ala$_6$ were determined and data are presented in a qualitative manner.

Results

Antisera were initially validated by assaying samples that are well-characterized in our laboratory. Bovine pituitary membranes and αT3 cells, a murine pituitary tumor cell line, both have previously demonstrated the presence of functional GnRH receptors in radiolabelled ligand binding assays. As a negative control, Cos-7 cells, a monkey kidney tumor cell line, and HeLa cells, a human cervical cancer cell line, were included. Neither have functional GnRH receptors. Sodium-didodeceyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a nitrocellulose membrane and Western analysis resulted in immunopositive bands in the bovine pituitary membrane and αT3 cells with an apparent molecular weight (Mr) of 28,000 (Figure 2-1). The Cos-7 and HeLa cells were devoid of immunostaining. Evaluation of pituitary membranes

Figure 2-1: SDS-PAGE and Western Analysis of Bovine Pituitary, Cos-7 Cells, Hela Cells and αT3 Cells.

prepared from two different ewes analyzed alongside bovine pituitary membranes resulted in immunopositive bands with a Mr of 28,000 in all samples (Figure 2-2). Analysis of the same samples, with antibody previously exposed to excess peptide, resulted in the complete absence of immunopositive bands (Data not shown). Substitution of normal rabbit serum for the primary antibody also did not result in immunopositive bands (Data not shown).

Presence of extra-pituitary GnRH receptors was evaluated in the ewe. Endometrium, myometrium, brain, ovary, pituitary, heart, liver, kidney, adrenal and lung were examined (Figure 2-3). Immunopositive bands were present in the endometrium, myometrium, brain, ovary, pituitary, liver, kidney and adrenal tissue. All visualized bands had an apparent Mr of 28,000. Heart and lung lacked immunopositive bands. Examination of bovine liver, kidney, pituitary, heart, and lung tissue resulted in immunological detection in the liver, kidney and pituitary samples (Figure 2-4). Again, all bands had a Mr of 28,000 and heart and lung tissue were devoid of immunological staining.

Murine samples were analyzed in the same fashion and tissues surveyed included adrenal, lung, liver, heart, spleen, testis, brain, ovary and pituitary. Immunopositive bands appeared in the adrenal, liver, testis, brain, ovary and pituitary tissues with an apparent Mr of 28,000 (Figure 2-5). Bands were not present in the samples prepared from lung, heart or spleen. Analysis of rabbit tissue resulted in similar findings. Immunopositive bands were detected in the liver, kidney, brain, ovary, uterus and pituitary (Figure 2-6). All immunopositive

Figure 2-2: SDS-PAGE and Western Analysis of Ovine Heart, Lung and Pituitary alongside Bovine Pituitary.

SDS-PAGE and Western Analysis

Figure 2-3: SDS-PAGE and Western Analysis of Ovine Endometrium, Myometrium, Brain, Ovary, Pituitary, Heart, Liver, Kidney, Adrenal and Lung.

SDS-PAGE and Western Analysis

Ovine Tissue Homogenates

Figure 2-4: SDS-PAGE and Western Analysis of Bovine Liver, Kidney, Pituitary, Heart and Lung.

SDS-PAGE and Western Analysis

Bovine Tissue Homogenates

Figure 2-5: SDS-PAGE and Western Analysis of Mouse Adrenal, Lung, Liver, Heart, Spleen, Testis, Brain, Ovary and Pituitary.

Figure 2-6: SDS-PAGE and Western Analysis of Rabbit Liver, Kidney, Heart, Lung, Brain, Ovary, Uterus and Pituitary.

SDS-PAGE and Western Analysis

Rabbit Tissue Homogenates

bands had an apparent Mr of 28,000. No bands were present in the heart or lung tissue.

In vitro transcription and translation of the murine GnRH receptor cDNA inserted into two different vectors in the presence of ^{35}S-methionine resulted in a single radiolabeled band with a Mr of 28,000 (Figure 2-7). No additional fragments were visible. Western analysis of each reaction resulted in a single band with a Mr of 28,000 identical to that found in other immunopositive tissues (Figure 2-8). Reactions containing plasmids without the GnRH receptor cDNA did not produce any radiolabeled or immunodetectable products.

Total cellular RNA was extracted and purified from ovine adrenal, lung, liver, kidney, brain, endometrium, myometrium, ovary, pituitary, heart and skeletal muscle. Resulting RNA was transcribed into DNA and used as a template for PCR. These products were then analyzed for hybridization with a 701 bp ovine GnRH receptor cDNA probe and results were expressed as a percentage of the pituitary value, brain contained 3.7%, endometrium 1.9% and myometrium 1.2%, respectively, of the value found in the pituitary (Figure 2-9).

Receptor binding assays of partially purified membranes of ovine adrenal, brain, endometrium, kidney, liver and pituitary were performed, but specific binding of ([^{125}I]D-Ala$_6$) was detected only in the pituitary membranes. Receptor assays of partially purified membranes of rat brain, heart, kidney, liver, lung, pituitary and testis resulted in specific binding of ([^{125}I]D-Ala$_6$) only in the pituitary and testis.

Figure 2-7: In-vitro Transcription and Translation of pBKCMV, pBSKS, pBKCMV-GnRH Receptor, and pBSKS-GnRH Receptor in Rabbit Reticulocyte Lysate with ^{35}S-Methethionine.

In-Vitro Transcription/Translation in Rabbit Reticulocyte Lysate with ^{35}S-methionine

Figure 2-8: In-vitro Transcription and Translation of pBKCMV, Bovine Pituitary, pBKCMV-GnRH Receptor and pBSKS-GnRH Receptor

Figure 2-9: Southern Blot Analysis of Ovine Adrenal, Lung, Liver, Kidney, Brain, Endometrium, Myometrium, Ovary, Pituitary, Heart and Skeletal Muscle.

Southern Blot Analysis of Ovine Tissue

Discussion

Immunization of rabbits with a synthetic peptide comprised of amino acids 25-36 in the murine pituitary GnRH receptor resulted in a high titer antisera. Based on the results of initial experiments, the antisera appeared to be specific for the GnRH receptor. Use of the antisera in Western Blot analysis resulted in immunological detection in samples known to express receptors, such as bovine pituitary membranes and αT3 cells. All immunopositive bands had an apparent Mr of 28,000. Cos-7 and HeLa cell samples lacked immunological detection. Likewise, GnRH receptors are nondetectable in these cells by radiolabeled ligand binding assays.

Western analysis of pituitary tissue from several species revealed the presence of a single immunopositive band with a Mr of 28,000 . The size of the immunopositive band remained the same across all species examined.

Analysis of extra-pituitary tissues from the ovine, bovine, murine and rabbit resulted in consistent immunological detection in the adrenal, brain, endometrium, kidney, liver, myometrium, ovary, testis and uterus, although not all tissues from each specie were analyzed. All immunopositive bands had a Mr of 28,000. Heart and lung were consistently devoid of immunodetectable product.

The product observed by Western blot analysis is smaller than either the size of the peptide backbone predicted from the cDNA sequence (37,800) or that which has been detected by photo-affinity labeling (32,000-62,000) (Pawha et al., 1989; Iwashita and Catt, 1985; Hazum, 1981, Janovick et al., 1993; Hazum,

1983; Nikolics et al., 1984; Iwashita and Catt; 1986; Neil et al., 1997). One possible explanation for the smaller size is aberrant migration in the SDS polyacrylamide gel. Proteins can adopt a three-dimensional conformation conducive to fast migration. This can occur when proteins do not entirely unfold during treatment with SDS and β–mercaptoethanol or when they bind abnormally large amounts of SDS (Gardner et al., 2000). Either of these scenarios can cause them to migrate through the gel at an abnormal rate for their true size.

An alternative explanation for the size of the detected product is that cleavage occurs during sample preparation and Western analysis detects only the fragment of the GnRH receptor that contains the epitope for the antisera. To investigate the possibility of cleavage, *in vitro* transcription and translation was utilized with rabbit reticulocyte lysate and ^{35}S-methionine. The 12 methionine residues along the length of the receptor allowed visualization of the majority of the protein which, in turn, should result in a number of fragments should cleavage occur.

In vitro transcription and translation of the GnRH receptor cDNA in two different expression vectors resulted in a single radiolabeled band with a Mr of 28,000 (Figure 2-7). Western analysis of this product resulted in the same immunopositive band found in bovine pituitary membranes (Figure 2-8). Expression vectors which did not contain the GnRH receptor cDNA did not produce any products on either the x-ray film or in Western Analysis. Based on these results, it appears that degradation did not occur during sample

preparation. Most likely, the smaller size of the receptor is due to aberrant migration of the peptide backbone in the polyacrylamide gel.

Recently, Millar et al. (2000) investigated the effect of estradiol and inhibin on the size of the ovine GnRH receptor, as detected by photoaffinity labeling. They found that incubation of ovine pituitary cultures with estradiol or inhibin for 6-24 hours resulted in a 38 KDa form of the receptor. Incubation for an additional 24 hours with estradiol resulted in a 43KDa form of the receptor, which did not change size after incubation with phosphatase. But incubation with N-glycosidase F or tunicamycin resulted in a 29 KDa receptor which indicated the 38 and 43 KDa forms of the GnRH receptor were hyperglycosylated variations of the 29 KDa peptide backbone. This supports results obtained with the antiserum which indicate the GnRH peptide backbone demonstrates aberrant migration in a polyacrylamide gel.

The antisera appears to detect only the nonglycosylated form of the GnRH receptor. Larger sized receptors, like those reported from PAL studies, were never detected with the antiserum. This may be due to the location of two potential glycosylation signals (a.a. 4 + 18) which are close to the epitope of the antibody (a.a. 23-36). If glycosylation occurs at these amino acids, the carbohydrate moieties may inhibit the antibody from binding. The results do not preclude the possibility that other forms of the receptor exist in any of the tissues surveyed. They may be present but not detected by the antibody in sufficient quantity to produce a positive signal. The antiserum may merely be unable to detect these larger forms of the receptor.

Southern blot analysis of reverse transcribed RNA from extra-pituitary tissues was also conducted. Brain demonstrated 3.7%, endometrium 1.9%, and myometrium 1.2%, respectively, of the pituitary sample. These results indicate the levels of GnRH receptor in these extra-pituitary tissues is extremely low when compared to the levels found in the pituitary. Lack of detectable signal in ovary, liver, kidney and adrenal may merely indicate a level which is below the minimum level of detection in Southern Blot analysis.

Receptor analysis of numerous tissues from the ovine and rat indicate the GnRH receptor detected in many of the extra-pituitary tissues is either non-functional or below the limit of detection in the assay (80 pmole). Although many ovine tissues demonstrate the presence of the receptor with the antiserum, only the pituitary had functional GnRH receptors in ligand-binding assays. Many of the rat extra-pituitary tissues also demonstrated the presence of the GnRH receptor with the antiserum, although when the same tissues were analyzed with receptor assays, only the gonadal and pituitary receptors appeared to be functional. The GnRH receptor has previously been reported in the gonads of rats where it was found to have a similar affinity to the pituitary receptor (Clayton, 1980; Brown and Reeves, 1983).

The function of the GnRH receptor detected with the antisera remains unknown. Davidson et al. (1995) eliminated the potential glycosylation sites in the GnRH receptor cDNA and found that after transfection of this mutated receptor cDNA into Cos-1 cells, normal GnRH receptor binding characteristics and stimulation of second-messenger systems was demonstrated. Hence, it

appeared that carbohydrate moieties were not necessary for ligand binding. However, expression of these nonglycosylated forms of the receptor was significantly decreased, indicating carbohydrate moieties may be involved in receptor expression or stability. Keinan and Hazum (1985) investigated the effect of periodate on ligand binding and concluded the carbohydrate moieties were important for normal high-affinity binding.

In conclusion, a specific, high-titer antisera directed against amino acids 25-36 of the GnRH receptor was produced, which detects a single form of the receptor with an apparent Mr of 28,000. Additionally, much of the receptor detected with the antisera appears to be nonfunctional. Only the pituitary receptor of all species and the mouse and rat gonadal receptor were detectable in ligand-binding assays. Perhaps, the lack of glycosylation on the detected extra-pituitary receptors indicate a form of the receptor incapable of binding ligand. If this is true, the presence of these nonglycosylated GnRH receptors in extra pituitary tissues of patients currently receiving endogenous GnRH therapy will have no effect as moderators of cell growth.

Chapter Three

SUMMARY AND CONCLUSIONS

A. Summary

Gonadotropin-Releasing hormone (GnRH), along with its receptor, plays a key role in mammalian reproduction. It is synthesized by hypothalamic neurons and secreted into the hypothalamic portal vasculature in a pulsatile fashion. By binding to specific high-affinity receptors on gonadotropes in the anterior pituitary gland, it stimulates ~~the~~ synthesis and secretion of luteinizing hormone and to a lesser extent, follicle stimulating hormone.

GnRH receptors have also been reported in a wide-variety of extra-pituitary tissues such as human placenta, ovary, testis, adrenal and in many carcinomas. These extra-pituitary sites are of interest due to the large number of clinical patients currently receiving GnRH agonist therapy to treat sex steroid-sensitive cancers such as breast, prostate, and pancreatic cancer. .

A high-titer antisera directed against amino acids 23-36 in the N-terminal extracellular region of the GnRH receptor was generated by conjugating the synthetic peptide to keyhole limpet hemocyanin and using the conjugate to immunize rabbits. The antisera appeared specific for the GnRH receptor. Use of the antisera in Western Blot analysis resulted in immunological detection of a protein in samples known to harbor receptors, such as bovine pituitary membranes and αT3 cells. All immunopositive bands had an apparent Mr of

28,000. Cos-7 and HeLa cells did not have immunological detectable proteins using this antiserum. Likewise, GnRH receptors are nondetectable in these cells by radiolabeled ligand binding.

Western analysis of pituitary tissue from several species revealed the presence of a single band with a Mr of 28,000 independent of the species examined. Analysis of extra-pituitary tissues from the ovine, bovine, murine and lapine resulted in consistent immunological detection in the adrenal, brain, endometrium, kidney, liver, myometrium, ovary, testis and uterus although not all tissues from each specie were analyzed. All immunopositive bands had a Mr of 28,000. Heart and lung were consistently devoid of immundetectable product in all species.

The product observed by Western blot analysis was smaller than either the size of the peptide backbone predicted from the cDNA sequence (37,800) or GnRH receptors identified by photo-affinity labeling (32,000-62,000). One possible explanation for the smaller form of receptor detected in this study is that Western analysis was detecting only a fragment of the GnRH receptor that contained the epitope for the antisera. To investigate this possibility murine cDNA for GnRH receptor was transcribed and translated *in vitro* using rabbit reticulocyte lysate and ^{35}S-methionine was included in the reaction mixture. There are 12 methionine residues in the peptide backbone of the receptor. Therefore, if cleavage was occurring during the preparation of the protein for Western analysis it is likely that radioactive fragments of different sizes would be observed. *In vitro* transcription and translation of the GnRH receptor cDNA in

two different expression vectors resulted in a single radiolabeled band with a Mr of 28,000. Western analysis resulted in an immunopositive band of the same size as that found in bovine pituitary membranes. Expression vectors which did not contain the GnRH receptor cDNA did not result in products detected either by autoradiography or Western Analysis. Therefore, it seems unlikely that degradation occurred during sample preparation. The smaller size of the receptor is due to aberrant migration of the peptide backbone in the polyacrylamide gel.

Southern blot analysis of reverse transcribed RNA from extra-pituitary tissues was also conducted. The percentage of RNA specific to the probe in each extra-pituitary sample is compared to the amount of RNA specific to the probe found in the pituitary. Respectively, brain contained 3.7%, endometrium 1.9%, and myometrium 1.2% of the pituitary sample. These results indicate the levels of GnRH receptor in these extra-pituitary tissues are extremely low when compared to the levels found in the pituitary. Lack of detectable signal in ovary, liver, kidney and adrenal may merely indicate a level of mRNA which was below the minimum level of detection in the Southern Blot analysis.

Receptor assays were performed on partially purified membranes of ovine and murine adrenal, brain, endometrium, kidney, liver and pituitary. The same tissues were analyzed in the rat with the addition of testis. As expected, ligand binding was demonstrated in the pituitary of all species and in the rat testis but was not detectable in any other extra-pituitary tissues. This indicates the GnRH

receptor detected in many of the extra-pituitary tissues is either non-functional or below the limit of detection in our assay.

B. Conclusions

The actual function of the GnRH receptor we are detecting with our antisera remains unknown. Larger sized receptors, like those reported from PAL studies, were never detected. This may be due to the location of two potential glycosylation signals (a.a. 4 + 18) which are close to the epitope of our antibody (a.a. 23-36). If glycosylation occurs at either or both of these amino acids, the carbohydrate moieties may cause steric hindrance and inhibit the antibody from binding. Our results do not preclude the possibility that other forms of the receptor exist in any of the tissues surveyed. They may be present but not detected by our antibody in sufficient quantity to produce a positive signal. However, with the exception of the rat testis, they are either present in insufficient amounts to be detected by ligand binding, or are nonfunctional.

In concluding, a specific, high-titer antisera directed against amino acids 23-36 of the GnRH receptor was produced, which detects a single form of the receptor with an apparent Mr of 28,000. Based on the lack of receptor binding in most of the extra-pituitary tissues, the form of the receptor detected with our antisera appears nonfunctional. Perhaps, the lack of glycosylation of the extra-pituitary receptors indicate a form of the receptor incapable of binding ligand. If this is true, the presence of these nonglycosylated GnRH receptors in extra-

pituitary tissues of patients currently receiving endogenous GnRH therapy will have no effect as moderators of cell growth.

C. Recommendations

Several questions remain unanswered at the end of this dissertation.

The GnRH receptor levels detected with the antisera did not correlate with the mRNA levels detected with Southern blot analysis. Several untested possibilities exist for the different results obtained with the two methods. The first possibility is that Western blot analysis is not necessarily quantitative. The detection method utilized for this research was alkaline-phosphatase and the possibility exists that all experiments were conducted with an excess of antigen. In other words, across a wide range of antigen, the same amount of antibody was used for detecting that antigen, and, hence, revealed the same signal across many differing quantities of antigen. Antigen excess was not examined in the present study but could be analyzed by titrating the amount of sample to the point where a difference in alkaline-phosphatase signal was obtained over a range of antigen concentrations.

The second possibility is that there was a problem in the reverse-transcription reaction resulting in a lower amount of receptor mRNA detected in the Southern blot. There may have been a section of the mRNA with difficult secondary structure that did not allow the reaction to complete. This would cause the Southern blot to appear as if there was no detectable level in some of the samples, when in fact there was mRNA present but it was merely unable to

complete a reverse transcription reaction. Additionally, if levels of GnRH receptor mRNA were low in some of the tissues and there was a problem with the reverse-transcription procedure, it would be more likely to appear that there was no signal in those tissues.

A third possibility is that the mRNA for the GnRH receptor may not be stable in many of the extra-pituitary tissues. Hence, there is detectable protein via the Western blot analysis but mRNA is not detectable with Southern blot analysis. This possibility could be tested by running a RNA stability assay.

A definitive answer was not obtained at the end of this research to explain the function of the extra-pituitary GnRH receptors. One possibility is that these receptors have no physiological relevance. This is supported by the fact that many of these receptors in several tissues were are not detectable in ligand-binding assays. Additionally, the antisera used for this research appears to detect only a non-glycosylated form of the GnRH receptor in the extra-pituitary tissues and this nonglycosylated form may be unable to bind ligand. The carbohydrate moieties may be necessary for proper trafficking of the receptor to the cell membrane where ligand binding occurs, or integral for stability of the receptor. Additionally, carbohydrate moieties may be important for ligand binding itself.

A second possible function of these receptors is that they are playing a role in the degradation of GnRH or are mediating a peripheral reserve of GnRH. The evidence to support this possibility lies in the fact that GnRH has a half-life of 3-5 minutes in the peripheral plasma after leaving the portal vasculature and little is found excreted in the urine but when GnRH is placed directly in plasma, it has

bioactivity for over 90 minutes (Varkkunen et al., 1974). It appears a mechanism exists to clear GnRH from the system and these extra-pituitary receptors may be the mediators of that activity.

A third possibility is that these extra-pituitary GnRH receptors may function in a much different manner than the pituitary receptor. This is supported by reports of GnRH in extra-pituitary tissues, either by immunoprecipitation or RNA, which indicate this form is very different from the hypothalamic form. The extra-pituitary form of GnRH appears very thermal labile whereas the hypothalamic form is very thermal stabile. Additionally, sequencing of the extra-pituitary form indicates the presence of portions of the precursor molecule which is not present in the hypothalamic form. This extra-pituitary form of GnRH may be the form that interacts with extra-pituitary GnRH receptor. This may be why these receptors are unable to bind hypothalamic GnRH in ligand-binding assays.

Lastly, the antiserum may be detecting a similar sequence in a protein that was not the GnRH receptor.

As with all good research, there is never a definitive end and many more questions are generated by the results.

Chapter Four

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