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Gonadotropin-releasing hormone receptor: Cloning, characterization, and mutational investigation

Zhou, Wei, Ph.D.

City University of New York, 1995

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Gonadotropin-Releasing Hormone Receptor: Cloning, Characterization, and Mutational Investigation

by

Wei Zhou

A dissertation submitted to the Graduate Faculty in Biomedical Sciences

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy, The City University of New York

1995

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Approval Page

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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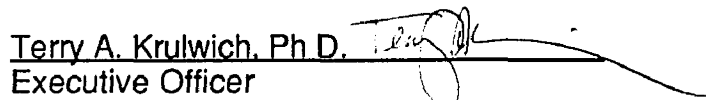

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Abstract

Gonadotropin-Releasing Hormone Receptor:
Cloning, Characterization, and Mutational Investigation
by
Wei Zhou

Adviser: Dr. Stuart C. Sealfon

Gonadotropin-releasing hormone (GnRH) plays a critical role in regulating the function of the mammalian reproduction system. GnRH exerts its effects by interacting with the gonadotropin-releasing hormone receptor (GnRHR). In this thesis work, molecular techniques were used to investigate the structure and function relationship of the GnRHR. The body of work consists of two major components: one encompassing the molecular cloning of the mouse GnRHR and the other, the characterization of a series of mutant receptors created by site-directed mutagenesis. The first part covers both the cDNA and the genomic cloning of the mouse GnRHR as well as the identification of variant transcripts of the receptor gene found in a mouse pituitary tumor cell line. The second part of the thesis involves the analysis of a series of residues important for receptor function. Several residues have been characterized that have critical roles in receptor binding and activation and the results support an interaction between transmembrane helices 2 and 7. These studies provide experimental evidence for defining the molecular model of the GnRHR and should lead to further understanding of the mechanism of the receptor function.

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Chapter 1

Background

Introduction

Gonadotropin-releasing hormone (GnRH) is a neuropeptide secreted by the GnRH neurons of the hypothalamus. As the key hormone of the hypothalamus-pituitary-gonad axis, GnRH secretion integrates signals from both neuronal and endocrine systems. The critical role of GnRH in regulating mammalian reproductive function underlines the importance of studying the interaction between GnRH and its receptor, the gonadotropin-releasing hormone receptor (GnRHR). Isolation of the mammalian GnRHR clone and characterization of its interaction with GnRH at the molecular level may help in rational drug design and the treatment of reproductive system diseases and provide insight for the study of other G protein-coupled receptors (GPCRs).

Prior to the molecular characterization of the GnRHR presented in the subsequent chapters, numerous studies have investigated the GnRH/GnRHR system focusing on the structure of GnRH and its analogs, the characteristics of GnRH binding to the receptor, and the signal transduction pathways in the gonadotrope following GnRH stimulation (for a review, see Hazum and Conn 1988; Stojilkovic, Reinhart et al. 1994). These studies provide important information about the system and constitute the fundamental background for this thesis project. They will be briefly reviewed at the beginning of the chapter.

Cloning of the mammalian GnRHRs has revealed that these receptors belong to the family of homologous seven-transmembrane receptors that couple to G proteins. These receptors carry a variety of important functions and have been investigated extensively using molecular biological techniques. The

accumulating information obtained on the structure and function relationship of GPCRs provides the guideline for the investigation of the GnRHR. Accordingly, the second part of the chapter is devoted to cover the recent advancement in the field with emphasis on studies related to this thesis project.

GnRH Structure and Function

The history of GnRH research is clinically related. Application of GnRH and its analogs in the treatment of disorders of the reproduction system provides a strong incentive to elucidate the structure of GnRH and to develop GnRH analogs.

Following the determination of the decapeptide sequence of GnRH (Matsuo, Baba et al. 1971; Burgus, Butcher et al. 1972), pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, both theoretical and experimental approaches have been used to study GnRH structure and function. The empirical energy calculation method used by Momany suggests that GnRH exists in a cyclic conformation (Momany 1976). This appears to agree with structural analysis of GnRH that identified a hydrogen bond between the pyrrolidone carbonyl (residue 1) and the glycinamide group (residue 10) (Nikolics, Coy et al. 1977). Shinitzky, on the other hand, proposed that the GnRH conformation is constrained by a ß-bend formed between Ser^4, Tyr^5, Gly^6, and Leu^7 (Shinitzky and Fridkin 1976; Shinitzky, Hazum et al. 1976). Designing GnRH analogs with a constrained conformation has proven a valid approach for the development of functional GnRH agonists and antagonists (Rivier, Varga et al. 1984; Struthers, Tanaka et al. 1990).

The results of the structure and function analysis of GnRH are consistent with a constrained GnRH conformation. *In vivo* the secreted GnRH has a half-life of only about 5-10 minutes in vertebrates (Redding, Kastin et al. 1973; Wagner, Adams et al. 1979). To render the resistance to proteases, which recognize the peptide backbone between Tyr^5, Gly^6 and Leu^7 (Griffiths and Kelly 1979), substitution of D-amino acids for Gly^6 has been made. The modification extends the half-life of GnRH to longer than 2 h in human (Coy and Nekola 1984). At the same time, It is found that a D-amino acid at position 6 also increases GnRH

potency in inducing ovulation (Fujino, Kobayashi et al. 1972; Monahan, Amoss et al. 1973), particularly when amino acids with large side-chains are used (Coy, Vilchez-Martinez et al. 1976; Vale, Rivier et al. 1976). The forced bend in GnRH by a D-amino acid and its correlation with the improved potency of the GnRH analog suggests that the constrained conformation is favored during GnRH binding to the receptor.

In addition to the potency improvement achieved with Gly^6 substitutions, other GnRH modification studies suggest that different residues of GnRH molecule confer distinct properties of GnRH. Successful improvement in drug potency for all GnRH analogs, following the replacement of the Gly^{10} with an ethylamine group (Fujino, Kobayashi et al. 1972; Rivier, Monahan et al. 1972), indicates that the carboxyl terminus of the GnRH molecule contributes to both agonist and antagonist function. On the other hand, modifications targeted at the N-terminus of GnRH usually alter the agonist function more than they do on antagonists, suggesting the N-terminal residues are involved in conferring agonist properties of GnRH (Karten and Rivier 1986). Understanding the structural basis for the distinction of agonists and antagonists is important for mapping the ligand binding pockets of the GnRHR and elucidating the mechanisms of ligand-receptor interaction at molecular level, as discussed in Chapter 6.

While early GnRH research was both driven and evaluated by drug development, the direct assessment of the interaction between GnRH and GnRHR remained unexplored until radiolabeled GnRH analog became available. The method of GnRH labeling usually takes advantage of the presence of a tyrosine residue in the GnRH. High specific activity radiolabeled ligands (> 1mCi/μg), usually obtained with the iodination of a GnRH analogue such as GnRH-A ([D-Ala^6]des-Gly^{10}-GnRH-ethylamide) or Buserelin ([D-Ser(t-

Bu)^6]des-Gly^{10}-GnRH ethylamide), are capable of detecting the GnRHR expressed on the pituitary cell membrane (Sandow and Konig 1979; Clayton and Catt 1980; Hazum 1982). Analysis of the differential modification by GTP analog on agonist and antagonist binding to the GnRHR indicates that the receptor is G protein coupled. While antagonist binding was not altered in the presence of Gpp(NH)p, a GTP analog, agonist binding to the pituitary membrane was decreased when Gpp(NH)p was incorporated in the binding assay. Moreover, reduced affinity rather than a decrease in the number of binding sites was responsible for this decrease in agonist binding (Perrin, Haas et al. 1989). The high and low affinity states of the GnRHR in binding to agonists and the specific modification of high affinity state by GTP or its analog are characteristic of receptors that couple to G proteins (Conklin and Bourne 1993).

GnRHR Signal Transduction Pathway

The signal transduction of the GnRHR upon agonist binding involves G protein, phospholipase C, and calcium. More than 10 subfamilies of G proteins have been identified (Birnbaumer 1990; Simon, Strathmann et al. 1991). The particular G proteins to which the GnRHR couples have been characterized in the αT3 cells, a tumor cell line derived from the transgenic mouse pituitary (Windle, Weiner et al. 1990). Immunodepletion (Hsieh and Martin 1992) and immunoblotting (Shah and Milligan 1994) studies with G protein-specific antibodies suggested that GnRHR couples to G_q and G_{11} subtypes of G protein. These two G protein subtypes are capable of activating the downstream phospholipase C_β (PLC_β) which generates diacylglycerol and inositol triphosphates (IP_3) by hydrolyzing phosphatidylinositol (Taylor and Exton 1991; Lee, Park et al. 1992). The increased IP_3 level switches on the rapid efflux of calcium from intracellular pool by turning on the calcium channel (Berridge and Irvine 1989). The peak of the calcium mobilization happens as fast as within 1 min of GnRH stimulation and is responsible for the rapid release of the luteinizing hormone (Clayton 1989). A secondary phase, occurring over the ensuring minutes, is probably augmented by calcium influx from extracellular pools (Naor, Katikineni et al. 1982).

The signal transduction pathway of the GnRHR has also been studied following the microinjection of the pituitary mRNA into the *Xenopus* oocytes. The calcium mobilization mediates the opening of the calcium-dependent chloride channel whose conductance could be measured electrophysiologically. This technique allows the detection of the GnRHR mRNA in a dose-dependent manner. In agreement with the GnRHR signal transduction pathway previously described, application of IP_3 alone induces an elevated chloride conductance (Sealfon, Gillo et al. 1990). Successful monitoring the oocyte expression system

contributed to the cloning of the first mammalian GnRHR (Tsutsumi, Zhou et al. 1992).

It is worth noting that unusual for a G protein coupled receptor, dimerization of the GnRHR appears to be sufficient for the receptor activation. It was found that divalent, but not monovalent antibodies against a GnRH antagonist, activated the antagonist-bound GnRHR, suggesting receptor dimerization as the mechanism of GnRHR activation (Conn, Rogers et al. 1982; Gregory and Taylor 1982). More surprisingly, a simple positively-charged polylysine alone induced a dose-dependent LH secretion from the pituitary cells (Conn, Rogers et al. 1984). The implications for these findings are largely unknown. However, studies attempted to elucidate the mechanisms of the findings should be facilitated by the availability of receptor clones.

Molecular Studies of GPCRs

Several hundred receptors that belong to the superfamily of G protein-coupled receptors have been cloned. These include receptors for neurotransmitters like acetylcholine, dopamine, norepinephrine, and 5-hydroxytryptamine (5HT) as well as peptides such as tachykinin, endothelin, vasopressin, and hypothalamic releasing hormones of thyrotropin and gonadotropin. The rapid identification of new receptors or receptor subtypes has been facilitated by the polymerase chain reaction (PCR) cloning based on homology among this family of receptors (for a review, see Probst, Snyder et al. 1992; Strader, Fong et al. 1994). Meanwhile, information regarding the structure and function of GPCRs has become available from the combination of molecular techniques and pharmacological characterization of engineered receptors. For historical reasons, such studies were originally targeted at neurotransmitter receptors, particularly adrenergic receptors, and the field is moving into other neurotransmitter and peptide receptors (reviewed by O'Dowd, Lefkowitz et al. 1989; Kobilka 1992; Strader, Fong et al. 1994). Below is a brief review of these studies:

Deletion and chimeric receptors revealed the general features of GPCRs

G protein-coupled receptors feature seven putative hydrophobic transmembrane domains across the cell membrane. In the absence of crystallographic information about any of the receptors, the general structure of GPCRs is modeled based on the structure of bacteriorhodopsin constructed from its two dimensional electron-density map (Henderson, Baldwin et al. 1990). The little homology between the bacteriorhodopsin and GPCRs in primary structure and the lack of G protein coupling by bacteriorhodopsin suggest that GPCRs may possess features of their own and the direct application of the structural information derived from bacteriorhodopsin may not

be merited (Pardo, Ballesteros et al. 1992).

The structure and function relationship of the GPCRs was initially analyzed by constructing deletion or chimeric receptors. Using the β-adrenergic receptor as a model, Strader's group compared the effects of deletions at the hydrophobic transmembrane helices and hydrophilic extracellular or intracellular loops (Dixon, Sigal et al. 1987a; Dixon, Sigal et al. 1987b). It was found that deletions made at the TMHs were associated with a loss of proper receptor processing or function while most of the hydrophilic loops could be deleted without much perturbation to ligand binding. Lefkowitz and colleagues constructed and analyzed a series of chimeric α_2/β_2 adrenergic receptors and concluded that the intracellular loops of the receptors, particularly the third intracellular loop, are critical for interacting with G proteins (Kobilka, Kobilka et al. 1988).

These initial studies were quite successful in developing a general understanding of GPCR structure and function relationships and served as the basis for later studies. However, interpretation of the experimental results obtained with deleted or chimeric receptors is difficult because of the large changes introduced. Additionally, problems arising from "molecular incompatibility" in these engineered receptors may obviate a clear interpretation of the results (Kobilka, Kobilka et al. 1988). Partly reflecting this concern, site-directed mutagenesis techniques are becoming widely used in most of the recent studies to investigate the function of individual residues.

Ligand binding pockets of the GPCRs

G protein coupled receptors include receptors for neurotransmitters and peptides. Although the early studies of GPCRs conducted on neurotransmitter receptors suggest that transmembrane helices are sufficient for ligand binding, accumulating evidence indicates that this is not the case with peptide receptors.

The present understanding of the binding sites of both receptors will be discussed.

Neurotransmitters bind to the transmembrane helices of their receptors

Most neurotransmitters have cationic amino head groups. This correlates with the finding that the receptors for these ligands have in their TMH 3 a conserved Asp that is not present in other receptors. The possible role of this Asp (Asp^{113}), among other acidic residues, in interacting with the ligand was first investigated in the β-adrenergic receptors. Of the mutant receptors studied, Asn^{113} totally abolished the specific binding to the radiolabeled antagonist [^{125}I]-iodocyanopindolol despite its normal antigenic expression. The result is consistent with the involvement of Asp^{113} in ligand binding and the charge interaction as the underlying mechanism for the function of this residue (Strader, Sigal et al. 1987). Supporting this hypothesis, substitutions at this locus with Ala, Asn, Glu, and Ser resulted in variable increase in EC_{50}, consistent with reduced affinity of these mutant receptors (Strader, Sigal et al. 1988; Strader, Candelore et al. 1989a; Strader, Gaffney et al. 1991). Similar to the findings with the ß-adrenoceptors, investigation of the corresponding locus in the GnRHR, occupied by a Lys (Lys^{121}), suggests that the Lys residue is likely to serve as a GnRH contact site (Chapter 6).

The role of the TMH 3 Asp in binding to the neurotransmitter ligand is supported by studies in other neurotransmitter receptors. Replacement of the Asp in α-adrenoceptors (Wang, Buck et al. 1991), m_1 muscarinic receptors (Fraser, Wong et al. 1989), and 5-HT_2 receptors (Wang, Gallaher et al. 1993) significantly decreased the affinity of the mutant receptors for the ligands. Moreover, chemical labeling studies of the receptor binding sites were found to be consistent with the mutagenesis results (Curtis, Wheatley et al. 1989; Tota and Strader 1990).

Unlike the TMH 3 Asp which is present in all neurotransmitter receptors, other transmembrane residues that have been identified as important for binding to their perspective receptor ligands are less conserved. Among these residues are two TMH 5 serines in the β-adrenoceptor. Removal of the hydroxyl side chain from either Ser^{204} or Ser^{207} by substitution of the Ser residues with alanines attenuated the activity of catecholamine agonists. The effects of these mutations on agonist activity were mimicked selectively by the removal of the each of the catechol hydroxyl moieties from the aromatic ring of the agonist. Coordinated modification at the ligand and the receptor suggested the spatial interaction between Ser^{204}, Ser^{207} of the receptor and *meta-, para*-hydroxyl group of the catecholamine, respectively (Strader, Candelore et al. 1989b). In a similar study, Wess et al. investigated the role of hydroxyl group-containing residues of m3 muscarinic receptors in agonists binding (Wess, Gdula et al. 1991; Wess, Maggio et al. 1992) and found substitution of Thr^{234} and Tyr^{506} of TMHs 5 and 6, respectively, caused a large decrease in affinity for agonists.

Many lines of evidence suggested that the TMH 7 of GPCRs may form part of the ligand binding pocket as it is involved in conferring the receptors agonist and antagonist binding specificity. Changes made in the upper face of TMH 7 normally conserve high affinity binding for ligands although the specificity, or the order of affinity for ligands, is frequently altered. A Phe residue in the α-adrenergic receptor (Phe^{412}), for example, which corresponds to an Asn in the 5-HT_{1A} and β-adrenergic receptors, and to a Val in the 5-HT_2 receptor, has been characterized in many studies. Substitution of the Asn for Val resulted in a mutant 5-HT_{1A} receptor which selectively decreased its affinity for β-antagonists of pindolol and other aryloxyalkylamines while conserving its affinity for other 5-HT agonists and antagonists (Guan, Peroutka et al. 1992). Similarly, when the Phe of α-adrenergic receptors was mutated to the Asn

found in β-adrenergic receptors, the mutant receptor gained affinity for β-antagonist alprenolol by 3000-fold and reduced affinity for the α-antagonist yohimbine by 350-fold (Suryanarayana, von-Zastrow et al. 1992). The locus is also responsible for determining the specificity for a series of 5-HT_{1B} receptor ligands. The profound differences between the human and rodent receptors in binding profile to many drugs disappeared after an exchange of the two residues between the two receptors (Oksenberg, Marsters et al. 1992).

Binding pocket of peptide receptors includes extracellular domains as well

Both extracellular and transmembrane helix residues of the peptide receptors have been reported to be required for binding. Glycohormone receptors, for example, have large N-terminal segments in addition to seven TMHs. The high affinity binding state of these receptors could be achieved by their N-termini alone (Tsai-Morris, Buczko et al. 1990; Nagayama, Russo et al. 1991). Additionally, the N-terminus of the peptide C5a receptor has been reported to be responsible for recognizing the C5a molecule as a whole (Siciliano, Rollins et al. 1994). Residues in the extracellular loops are also critical for peptide receptor binding. Various amino acids of the extracellular loops of the tachykinin receptors are believed to form the receptor binding pockets (Fong, Huang et al. 1992; Fong, Yu et al. 1992) (reviewed by Strader, Fong et al. 1994). Recently, an extracellular 3 Glu of the GnRHR has been reportedly implicated to interact with Arg^8 of GnRH by means of charge interaction (Flanagan, Becker et al. 1994).

Despite the evidence that the extracellular part of the peptide receptor forms part of the binding pocket, more residues in the transmembrane helices of the peptide receptors are yet to be identified for their involvement in ligand binding. One important question that remains to be addressed is whether binding to peptide receptors involves similar TMH regions to those found in

neurotransmitter receptors. So far as this question is concerned, the TMH 3 locus corresponding to the Asp in neurotransmitter receptors represents a special point to study. This locus has been investigated in the endothelin$_B$ receptor where a Lys residue is found. In support of the lack of a major role in endothelin binding, replacement of the Lys with an Asp caused only a 25-fold decrease in affinity for the endothelin1 (Zhu, Wu et al. 1992). In this thesis project a TMH 3 Lys residue (Lys^{121}) of the GnRHR, corresponding to the Asp in the neurotransmitter receptors, is studied and the results support this residue being involved in binding to GnRH and GnRH-A, a GnRH superagonist (Chapter 6).

Interhelical Interactions of TMHs

Understanding the mechanisms of helix:helix interaction and its alteration in response to agonist activation of the GPCR represents a very important aspect of studying the structure/function relationship and elucidating the functionality of the receptors. Unlike characterizing individual amino acids, identification of a helix:helix interaction requires a dynamic view of the receptor in its functional state. The elevated complexity may explain the lack of extensive experimental approach to address this question.

In addition to the patterns of helical interactions predicted by GPCR models (Findlay and Eliopoulos 1990; Hibert, Trumpp-Kallmeyer et al. 1991; MaloneyHuss and Lybrand 1992; Pardo, Ballesteros et al. 1992; Zhang and Weinstein 1993), certain means of helical arrangement are supported by experimental data. For example, in their characterization of chimeric α/β-adrenergic receptors, Lefkowitz's group suggested that TMH 7 should be close to TMHs 3 and 4 based on compatibility among chimeric receptors (Kobilka, Kobilka et al. 1988). Kobilka and his colleagues further investigated the helical arrangement using chimeric adrenergic receptors. When a TMH 7 Asn (Asn^{312})

of the β-adrenoceptor was replaced by the corresponding residue of a Phe in the α-receptor, the mutant receptor required the presence of both TMH 1 and TMH 2 of the α-adrenergic receptor for ligand binding, suggesting a proper contact between this TMH 7 locus and a residue(s) located in TMH 1 or 2 is necessary (Suryanarayana, von-Zastrow et al. 1992).

Wess et al have presented evidence that interhelical interaction could occur between separate receptor molecules. Co-expression of the mirror adrenergic/muscarinic chimeric receptors leads to the reconstitution of wild-type like function (Maggio, Vogel et al. 1993a; Maggio, Vogel et al. 1993b). Although more combinations of the chimeric receptors may reveal other possible helical arrangement in the receptors, these studies confirm that interaction between two receptor segments, TMHs 1 through 5 and TMHs 6 and 7, exist.

More precise information about interhelical interactions has been obtained from site-directed mutagenesis studies of rhodopsin. A salt bridge is proposed to form between two amino acids: a Schiff base donor of TMH 7 Lys and its counterion, a TMH 3 Glu (Glu^{113}). The salt bridege serves to constrain the inactive state of the rhodopsin (Robinson, Cohen et al. 1992). The TMH 3 and TMH 7 proximity predicted by the formation of the salt bridge was recently expanded to include TMH 2 as well. A TMH 2 mutation, with the substitution of an Ala for an Asp (Asp^{90}), recovers the loss of function after the TMH 3 Glu is replaced (Rao, Cohen et al. 1994). The results indicate that Asp^{90} may somehow compensate for the role of Glu^{113} in interacting with the TMH 7 Lys and consequently, TMHs 2, 3, and 7 should be close to each other.

Consistent with most of the helix:helix interaction studies discussed above, we have identified a possible TMH 2 and TMH 7 proximity in the GnRHR based on the analysis of the TMH 2 Asn^{87} and TMH 7 Asp^{318} mutant receptors (Chapters 4 and 5). Common to these approaches of studying the helix:helix

interaction is that the loss of receptor function caused by mutation of one TMH could be restored by certain mutations on another TMH. A broader and more versatile utilization of the approach may help delineate the interhelical interaction in more GPCRs and significantly advance our understanding of its role in receptor function.

Role of TMH 2 Asp and TMH 7 Asn of GPCRs

In this thesis, the roles of TMH 2 Asn (Asn^{87}) and TMH 7 Asp (Asp^{318}) and their potential interactions have been studied. Of the TMH 2 and TMH 7 loci, where an Asp and an Asn are found in most other GPCRs, respectively, the TMH 2 locus has been well characterized. The Asp residue is involved in a variety of receptor functions including allosteric modulation of agonist binding and proper G protein coupling.

Ligand binding to many GPCRs is modulated allosterically by monovalent cations. Receptors with distinct downstream effectors have been described sensitive to cation modulation in ligand binding. These include G_s-coupled ß-adrenergic receptors (Minuth and Jakobs 1986), G_i-coupled α-adrenergic (Limbird, Speck et al. 1982), dopamine D_2 (Neve 1991) and opioid (Ott, Costa et al. 1988) receptors, and receptors that stimulate PLC such as thyrotropin-releasing hormone (Hinkle and Kinsella 1984) and formyl peptide receptors (Gierschik, Sidiropoulos et al. 1989). The effect of cation modulation takes place in many forms and varies among the different systems studied. In general, the presence of sodium tends to decrease the affinity of the receptors for agonists whereas the affinity for antagonists are not changed or even increased (Limbird, Speck et al. 1982; Jagadeesh, Cragoe et al. 1990). Motulsky and Insel investigated the involvement of the intracellular and the extracellular sodium in the modulation of ligand binding to the α-adrenergic receptors expressed in the intact platelet cells and found that the intracellular

sodium is responsible for the allosteric regulation of the receptor (Motulsky and Insel 1983). Although the mechanism of sodium modulation awaits further exploration, this may not occur at the level of receptor coupling to G proteins primarily because GTP analogs and G protein-modifying agents have additive effects on decreasing agonist binding in the presence of sodium (Ott, Costa et al. 1988; Gierschik, Sidiropoulos et al. 1989; Urwyler 1989).

With the application of molecular techniques, the role of individual residues in sodium modulation of the receptors could be investigated. Limbird's group identified the highly consensus TMH 2 Asp as mediating the allosteric modulation of the α-adrenergic receptor (Horstman, Brandon et al. 1992). While the wild-type receptor displayed a five-fold decrease in its affinity for agonists in the presence of sodium, the mutant receptor with an Asn at the locus was insensitive to sodium modulation. In contrast to agonist binding, increased antagonist binding to [^{3}H]yohimbine was obtained with the wild-type receptor in the presence of sodium. However sodium did not affect the mutant receptor. Thus, although sodium exerted a differential role in modulating agonist and antagonist binding to the receptor, mutation at the TMH 2 Asp abolished both effects.

In addition to conferring sodium modulation to the receptor, the TMH 2 acidic residue is also critical for receptor activation. When the Asp of dopamine D_2 receptors was replaced by Ala and Glu, Neve et al. discovered that the mutation not only decreased the affinity of the receptors for antagonist epidepride and agonist dopamine by several fold but also uncoupled the receptor to adenylyl cyclase (Neve, Cox et al. 1991). Similar to this observation, mutation at the same locus in the luteinizing hormone/choriogonadotropin receptor decreased the affinity for luteinizing hormone and choriogonadotropin as well as attenuated the receptor's ability to stimulate choriogonadotropin-

dependent cAMP accumulation (Quintana, Wang et al. 1993).

Consistent with the impaired coupling of the TMH 2 mutant receptors, the mutation also affects the modulation of agonist binding by GTP or its analog in both the β-adrenergic (Chung, Wang et al. 1988) and α-adrenergic (Surprenant, Horstman et al. 1992) receptors. The varied effects of the TMH 2 Asp mutations in many GPCRs studied imply that this residue serves some fundamental function common to these GPCRs, a hypothesis supported by the evolutionary conservation of the Asp (Probst, Snyder et al. 1992).

Relatively less is known about the role of TMH 7 Asn and no connection has been suggested between TMH 2 Asp and TMH 7 Asn in other GPCRs. In a mutagenesis study of the 5-HT_{1C} receptor, the TMH 2 Asp and the TMH 7 Asn were substituted in separate constructs. None of the TMH 2 Asn and the TMH 7 Ala and Val mutant receptors had detectable antagonist binding. On the other hand, the TMH 7 Asn to Gln mutation did not alter the total binding for radiolabeled antagonist (Chanda, Minchin et al. 1993). No functional assay was reported on these TMH 7 mutant receptors and the results could not be compared to those described under Chapter 5 of the thesis. However, the study in this thesis work suggests that both of the conserved TMH 2 Asp and TMH 7 Asn should be critically important for GPCR function and more studies are necessary to characterize the role of the TMH 7 locus.

The thesis followed the above observations and investigated the structure and function implication of the interchange between the two residues in the GnRHR. The results indicate that like the conserved TMH 2 Asp in other GPCRs, Asp^{318} in the TMH 7 of the GnRHR is required for efficient receptor activation. The reversed functional role of Asn^{87} and Asp^{318} in the GnRHR supports an interaction between these two residues and a helix:helix interaction between TMHs 2 and 7 (Chapters 4 and 5).

Chapter 2

Cloning and Functional Expression of a Mouse Gonadotropin-Releasing Hormone Receptor

Abstract

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in the reproductive system and GnRH analogues have wide therapeutic applications ranging from the treatment of prostatic cancer to infertility. Determination of the predicted structure of the GnRH receptor (GnRHR) would illuminate the mechanisms of receptor activation and regulation and allow directed design of improved GnRH analogues. We report the cloning of a complementary DNA representing the mouse GnRHR and confirm its identity using *Xenopus* oocyte expression. Injection of sense RNA transcript leads to the expression of a functional, high-affinity GnRHR. Expression of the GnRHR using gonadotrope cell line RNA, however, is blocked by an antisense oligonucleotide. *In situ* hybridization in the rat anterior pituitary reveals a characteristic GnRHR distribution. The nucleotide sequence encodes a 327 amino acid protein which has the seven putative transmembrane domains characteristic of G protein-coupled receptors, but which lacks a typical intracellular C-terminus. The unusual structure and novel potential regulatory domain of the GnRHR may explain unique aspects of its signal transduction and regulation.

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Introduction

The GnRHR is a key mediator in the integration of the neural and endocrine systems. Normal reproduction depends on the pulsatile release of physiological concentrations of GnRH which binds to specific high affinity pituitary receptors and triggers the secretion of the gonadotropins LH and FSH. Whereas physiological concentrations of GnRH orchestrate normal reproduction, high levels of agonist lead to an opposite response, the suppression of gonadotropin secretion. The capacity of GnRH analogues both to activate and to inhibit the hypothalamic-pituitary-gonadal axis has led to their wide clinical utility in the treatment of a variety of disorders ranging from infertility to prostatic carcinoma.

The responsiveness and capacity of the gonadotrope GnRHR is influenced by agonist concentration and pattern of exposure (Clayton 1989). Both *in vivo* and *in vitro* studies have demonstrated that low concentration pulsatile GnRH is trophic to the receptor and that a high concentration of agonist induces receptor down-regulation and desensitization. The binding of GnRH to its receptor stimulates phospholipase C and generates inositol-1,4,5-trisphosphate and diacylglycerol (Huckle and Conn 1988). These second messengers, in turn, release calcium from intracellular stores and activate protein kinase C. Receptor up-regulation appears to involve both protein kinase C and calcium (Young, Naik et al. 1985; Huckle and Conn 1988; Huckle, McArdle et al. 1988). It is not certain which effectors underlie down-regulation.

While great progress has been made in understanding the mechanisms underlying GnRHR regulation and desensitization through receptor binding studies, direct measurement of GnRHR gene transcription and biosynthesis has not been possible. Cloning of the GnRHR cDNA would advance the study of GnRHR activation, regulation and uncoupling. Determining the primary

structure of the receptor would facilitate the directed design of improved analogues. Using a polymerase chain reaction (PCR) based strategy we have isolated a GnRHR cDNA from the gonadotrope cell line, αT3-1.

Materials and Methods

Drugs were obtained from the following sources: the GnRH antagonist [D-Phe^{2,6},Pro^{3}]-GnRH (Bachem, Torrance, CA), buserelin (D-Ser(But)^{6},Pro^{9}-N-ethylamide GnRH) was a gift of Hoerchst-Roussel Pharmaceuticals (Somerville, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). All animal care was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Oocyte Micro-Injection and Recording

Adult female *Xenopus laevis* (Nasco,Ft. Atkinson, WI) were kept at 18-20 C and a day/night cycle of 15h/9h. Oocytes were prepared for injection and the responses recorded as previously described (Sealfon, Gillo et al. 1990). Cells were placed in a 0.5 ml bath and voltage clamped at -70 mV using standard two electrode technique (Dascal 1987). Peptide ligands were diluted in the perfusion buffer and introduced into the bath. The clamp current was recorded using a chart recorder. Reversal potentials were determined by continuous ramping from -70 to +10 mV over 2 seconds with and without agonist through an IBM PC/AT system using the TL-1 interface and pCLAMP software from Axon Instruments (Burlingame, CA).

PCR Cloning and Hybrid Arrest Screening

RNA preparation and cDNA synthesis were performed as previously described (Sealfon, Gillo et al. 1990; Snyder, Roberts et al. 1991b). Subclones for hybrid arrest screening were isolated using PCR with a variety of degenerate oligonucleotides corresponding to conserved transmembrane domains of the GPCR superfamily. The oligonucleotides used to isolate the group of subclones including WZ7, modified from sequences of published oligomers (Zhou, Grandy et al. 1990), corresponded to transmembrane III (5'-GAGTCGACCTGTG(CT)G (CT)(GC)AT(CT)(AG)CNNT(GT)GAC(AC)G(CG)TAC-3') and transmembrane VI

(5'-CAGAATTCAG(AT)AGGGCANCCAGCAGAN(CG)(AG)(CT)GAA-3'). PCR was performed at low stringency. A portion of the reaction was reamplified at high stringency, digested with restriction enzymes, subcloned into pBluescript II KS+ (Stratagene) and sequenced. For hybrid-arrest assay, an antisense oligonucleotide corresponding to transmembrane II of the $5HT_{1C}$ receptor (5'-ATCAGCAATGGCTAG-3') (Julius, MacDermott et al. 1988) and an oligonucleotide corresponding to WZ7 (5'-AGCATGATGAGGAGG-3') were synthesized. A mixture of αT3-1 (1 mg/ml) and rat brain total RNA (1 mg/ml) was preincubated with antisense oligonucleotide (100 μg/ml) for 10 minutes at 37 C in a buffer containing 200 mM NaCl and 5 mM Tris, pH 7.4 in a 3 μl volume. *Xenopus* oocytes were injected with 50 nl of the mixture and incubated for 48 hours before recording.

Library Screening and Sequencing

10^6 plaques of a UniZap (Stratagene) αT3-1 cDNA library were screened with the insert of WZ7 which had been ^{32}P-labelled by random hexamer primers. 40 positive plaques were identified and 7 purified on secondary and tertiary screening. WZ25 was subcloned into pBluescript II SK+ by helper phage excision and both strands sequenced by the dideoxy-chain termination method with Sequenase T7 DNA polymerase (USB). Sequence was further confirmed by resequencing both strands using taq polymerase labelling and an Applied Biosystems automated sequencer. To exclude the possibility that the predicted cytoplasmic C-terminus was truncated due to a mutation in WZ25, the 3' sequence was confirmed in two additional independent clones. The nucleotide and amino acid sequence were analyzed using the Wisconsin GCG package on a VAX computer and MacVector (IBI) on a microprocessor.

Characterization of WZ25 RNA transcript

WZ25 in pBluescript II SK+ (Stratagene) was linearized and capped RNA transcript synthesized using T3 RNA polymerase (Stratagene). Oocytes were injected with 1.25ng of the resulting transcript and incubated for 48 hours before recording. Oocytes were pre-treated with either buffer or a GnRH antagonist (antagonist 6: [Ac-D-Nal(2)^1,D-a-Me-pCl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH; antagonist 27: [Ac-D-Nal(2)^1,D-a-Me-pCl-Phe^2,D-Trp^3,N-e-Ipr-Lys^5,D-Tyr^6,D-Ala^{10}]GnRH; (Van der Spuy, Pillay et al. 1987)) for 3 minutes prior to GnRH administration. To confirm receptor expression, oocytes were re-exposed to GnRH after a three minute washout of antagonist.

Radioligand Binding Assay

For membrane preparation, 500 oocytes were each injected with 2.5 ng synthetic WZ25 RNA. After 48 hours, oocyte membranes were prepared as described (Kobilka, MacGregor et al. 1987) and resuspended in binding buffer containing 10mM HEPES, 1mM EDTA, and 0.1% bovine serum albumin to give a final concentration of 20 oocytes/ml. The receptor binding assay using ^{125}I-[D-Ala^6, NaMe-Leu^7, Pro^9-NHEt]GnRH (GnRH-A) was based on that previously described for rat and sheep pituitary membranes (Millar, Flanagan et al. 1989). The binding in the presence of 10^{-6} M GnRH analogue was considered to represent non-specific binding. Average Bo (maximal binding) and non-specific binding values were 1429 and 662 cpm, respectively. The dissociation constant (K_d) for GnRH-A and GnRH was determined using Enzfitter (Elsevier-BIOSOFT).

Solution Hybridization, Northern Blot Analysis, and *in situ* Hybridization

A 399 nucleotide ^{32}P-labelled GnRHR and a 117 nucleotide 1B15 (cyclophilin internal standard) antisense cRNA probe were synthesized and hybridized to RNA in solution using described methods (Autelitano, Snyder et al. 1989). Northern blot analysis using poly(A)$^+$ αT3-1 RNA was performed as

described (Sambrook, Fritsch et al. 1989). *In situ* hybridization using ^{35}S-UTP labeled cRNA was performed on free-floating pituitary sections following published methods (Gall and Isackson 1989). Sections were mounted and exposed to Amersham Beta-max film for 3 days or dipped in radioactive emulsion and developed after 17 days.

Results and Discussion

RNA from the mouse gonadotrope cell line, αT3-1 (Windle, Weiner et al. 1990), which directs the expression of a functional GnRHR in *Xenopus* oocytes (Sealfon, Gillo et al. 1990), was used to synthesize cDNA for PCR with degenerate oligonucleotides corresponding to conserved motifs of the G protein-coupled receptors (GPCRs; Probst, 1992). PCR products were subcloned and sequenced, and antisense oligomers synthesized for a hybrid-arrest assay (Kawashi 1985). An oligonucleotide corresponding to clone WZ7, when co-injected with αT3-1 and rat brain RNA, completely abolished the expression of the GnRHR in oocytes but did not affect expression of the brain $5HT_{1C}$ receptor (Fig 1). A second antisense oligonucleotide, representing a different segment of WZ7, also completely and specifically eliminated GnRHR expression in all oocytes tested (n=16). Clone WZ7 was used as a probe to screen an αT3-1 bacteriophage cDNA library and seven positive plaques were purified.

To test whether the clone with the largest insert of 1.3 kb, WZ25, encodes a functional GnRHR, it was subcloned for RNA synthesis and oocyte expression. All synthetic RNA-injected oocytes (n>50), when exposed to GnRH, demonstrated a large depolarizing response characteristic of GnRHR expression (Fig. 2). The reversal potential (V_r) and calcium-dependence of the response to GnRH induced in oocytes by WZ25 RNA transcript were similar to those previously obtained using αT3-1 RNA (Sealfon, Gillo et al. 1990). The V_r of the current elicited by GnRH was -27±0.79 mV (n=7), consistent with that of the chloride ion in oocytes (Barish 1983). The GnRH-elicited response was completely abolished by preloading the oocyte with 5 mM EGTA one hour before recording (n=4), but was not significantly affected by the absence of Ca^{2+} in the perfusate (n=7). Thus the receptor expressed from clone WZ25

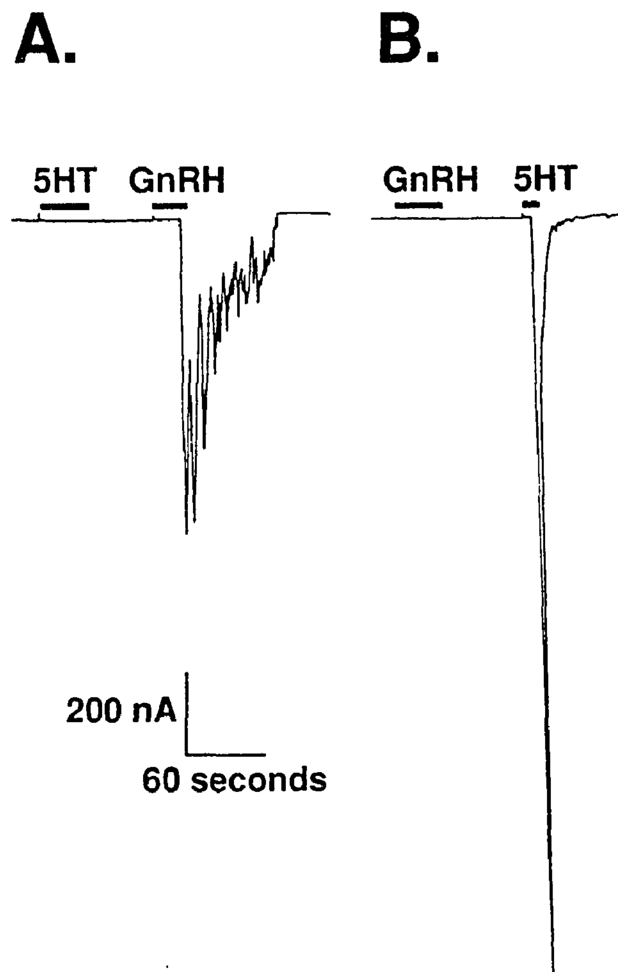

Figure 1 Hybrid-arrest of serotonin (5HT) receptor and GnRHR expression by antisense oligonucleotides.

100 nM 5HT or 200 nM GnRH were introduced into the bath at the horizontal lines. A, Response to 5HT and GnRH in oocytes previously injected with a mixture of rat brain RNA (for the 5HT response), αT3-1 RNA (for the GnRH response) and antisense $5HT_{1C}$ receptor oligonucleotide. 16 cells showed identical responses. B, Response to GnRH and 5HT in oocytes previously injected with a mixture of rat brain RNA, αT3-1 RNA and antisense WZ7 oligonucleotide. 24 cells had identical responses.

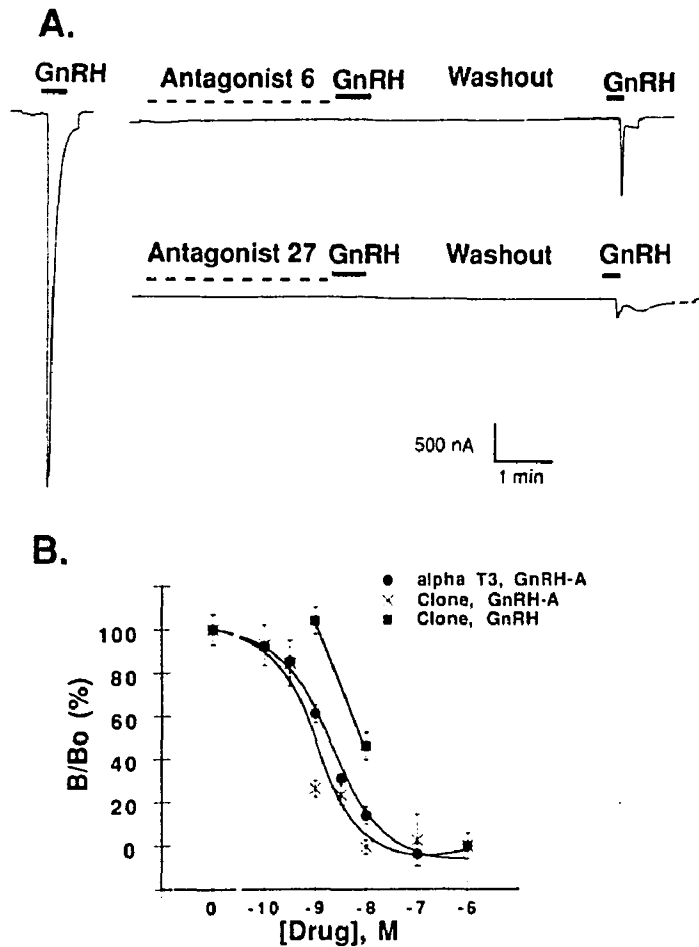

Figure 2 Characterization of clone WZ25 expressed in oocytes.

A, Electrophysiological responses to GnRH of oocytes injected with the WZ25 transcript in the absence (left) or presence (right) of GnRH antagonist. The three tracings shown are from different cells. Solid and dotted lines indicate GnRH and GnRH antagonist administration, respectively. Uninjected oocytes had no response to GnRH (n=12).

B, Displacement of ^{125}I-GnRH-A by GnRH-A and GnRH in membranes of oocytes injected with transcript from WZ25. A comparative displacement curve using αT3-1 cell membranes combined with membranes from uninjected oocytes is also shown (●). Error bars show SEM.

exhibited a response mediated through the activation of the oocyte's calcium-dependent chloride current by intracellular calcium, as is characteristic of receptors that cause phosphatidylinositol hydrolysis (Dascal 1987). The pharmacology of the response obtained was in agreement with expression of the mammalian GnRHR. The GnRH agonist [D-Ser(t-Bu)^{6},Pro^{9}-NHEt]GnRH (100 nM buserelin, n=6) elicited a depolarizing current in RNA-injected oocytes. In the presence of equimolar weak GnRH antagonist [D-Phe^{2,6}, Pro^{3}]GnRH, there was a 60% reduction in the response to GnRH, in comparison with the response to GnRH alone (1880±551 nA, n=5, and 4756±1082 nA, n=4, respectively). Two potent GnRH antagonists completely eliminated the GnRH-elicited current (Fig 2A).

To further characterize the receptor encoded by this cDNA clone, radioligand binding assays were performed on membranes purified from oocytes injected with the WZ25 RNA transcript. The GnRH agonist [D-Ala^{6}, NaMe-Leu^{7}, Pro^{9}-NHEt]GnRH (GnRH-A) bound with high affinity to membranes of oocytes injected with synthetic RNA (Fig 2B). Displacement of ^{125}I-GnRH-A by GnRH-A revealed similar Kds of 4.5 and 2.9 nM in WZ25 RNA-injected oocyte membranes and αT3-1 cell membranes respectively. Displacement by GnRH of GnRH-A bound to the cloned receptor was an order of magnitude less effective, as has been previously reported for αT3-1 membranes (Horn, Bilezikjian et al. 1991). Thus the hybrid-arrest and expression data confirm that clone WZ25 represents the mouse GnRHR.

The nucleotide and corresponding predicted amino acid sequence of clone WZ25 are shown in Figure 3. The longest open reading frame encodes a 327 amino acid protein (relative molecular mass, M_r=37,683). The larger size reported for the binding subunit of the solubilized rat GnRHR, M_r 50,000-60,000 (Hazum, Schvartz et al. 1986; Iwashita, Hirota et al. 1988), may be due to

CACGAGAGGGACTCCACTCTTGAAGCCTGTCCTTGGAGAAAT -1

ATGGCTAACAATGCATCTCTTGAGCAGGACCCAAATCACTGCTCGGCCATCAACAACAGC 60
M A N N A S L E Q D P N H C S A I N N S 20

ATCCCCTTGATACAGGGCAAGCTCCCGACTCTAACCGTATCTGGAAAGATCCGAGTGACC 120
I P L I Q G K L P T L T V S G K I R V T 40

GTGACTTTCTTCCTTTTCCTACTCTCTACTGCCTTCAATGCTTCCTTCTTGTTGAAGCTG 180
V T F F L F L L S T A F N A S F L L K L 60
I

CAGAAGTGGACTCAGAAGAGGAAGAAAGGAAAAAAGCTCTCAAGGATGAAGGTGCTTTTA 240
Q K W T Q K R K K G K K L S R M K V L L 80

AAGCATTTGACCTTAGCCAACCTGCTGGAGACTCTGATCGTCATGCCACTGGATGGGATG 300
K H L T L A N L L E T L I V M P L D G M 100
II

TGGAATATTACTGTTCAGTGGTATGCTGGGGAGTTCCTCTGCAAAGTTCTCAGCTATCTG 360
W N I T V Q W Y A G E F L C K V L S Y L 120

AAGCTCTTCTCTATGTATGCCCCAGCTTTCATGATGGTGGTGATTAGCCTGGACCGCTCC 420
K L F S M Y A P A F M M V V I S L D R S 140
III

CTGGCCATCACTCAGCCCCTTGCTGTACAAAGCAACAGCAAGCTTGAACAGTCTATGATC 480
L A I T Q P L A V Q S N S K L E Q S M I 160

AGCCTGGCCTGGATTCTCAGCATTGTCTTTGCAGGACCACAGTTATATATCTTCAGGATG 540
S L A W I L S I V F A G P Q L Y I F R M 180
IV

ATCTACCTAGCAGACGGCTCTGGGCCCACAGTCTTCTCGCAATGTGTGACCCACTGCAGC 600
I Y L A D G S G P T V F S Q C V T H C S 200

TTTCCACAGTGGTGGCATCAGGCCTTCTACAACTTTTTCACCTTCGGCTGCCTCTTCATC 660
F P Q W W H Q A F Y N F F T F G C L F I 220
V

ATCCCCCTCCTCATCATGCTAATCTGCAATGCCAAAATCATCTTTGCTCTCACGCGAGTC 720
I P L L I M L I C N A K I I F A L T R V 240

CTTCATCAAGACCCACGCAAACTACAGATGAATCAGTCCAAGAATAATATCCCAAGAGCT 780
L H Q D P R K L Q M N Q S K N N I P R A 260

CGGCTGAGAACGCTAAAGATGACAGTCGCATTCGCTACCTCCTTTGTCGTCTGCTGGACT 840
R L R T L K M T V A F A T S F V V C W T 280
VI

CCCTACTATGTCCTAGGCATTTGGTACTGGTTTGATCCAGAAATGTTGAACAGGGTGTCA 900
P Y Y V L G I W Y W F D P E M L N R V S 300

GAGCCAGTGAATCACTTTTTCTTTCTCTTTGCTTTCCTAAACCCGTGCTTCGACCCACTC 960
E P V N H F F F L F A F L N P C F D P L 320
VII

ATATATGGGTATTTCTCTTTGTAGTTGGGAGACTACACAAGAACTCAGATAGAAATAAGG 1020
I Y G Y F S L 327

TAACTAATTGCACCAATTGAGAATAAACTCAAAGCTTTTGACACACTTATATACAAGGCA 1080
GGGTTTAAGGTTAGATTATCAACCTTGTTTTTGTACAGAGTTTGTTGTTAGAGCTTCAGA 1140
AGACCTTCAAAAACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1185

Figure 3 Nucleotide and deduced amino acid sequences of clone WZ25. Numbering begins with the first methionine of the 981 base pair open reading frame. The deduced amino acid sequence is shown below the nucleotide sequence. Putative transmembrane regions I-VII are underlined. Symbols below the amino acid sequences indicate potential N-glycosylation sites (▲), and phosphorylation sites for protein kinase A (◆), Casein kinase 2 (●) and protein kinase C (✱) (Hubbard and Ivatt 1981; Kemp and Pearson 1990; Kennelly and Krebs 1991; Pearson and Kemp 1991). The nucleotide sequence has been submitted to Genbank under the accession number M93108.

receptor glycosylation. Three consensus N-linked glycosylation sites are present, two in the N-terminus and one in the putative first extracellular loop. The first ATG is believed to represent the translation initiation site because it closely approximates a Kozak consensus sequence (Kozak 1987) and a second cDNA clone with additional 5' sequence contains two nonsense codons in this reading frame at positions -54 and -57 (not shown). Thus translation initiating at any upstream start sites would terminate before reaching the correct open reading frame. There is no polyadenylation signal and the apparent poly(A) tail most likely represents oligo(dT) priming in the 3'-untranslated region during library construction. The functional GnRHR cDNA we have isolated is 1.3 kb whereas the mRNA containing this sequence is approximately 4.6 kb as determined by sucrose gradient (Sealfon, Gillo et al. 1990) and Northern blot analysis (Fig. 5B). PCR analysis of 40 positive plaques identified by primary library screening suggests that the GnRHR mRNA contains both additional 5'- and additional 3'-untranslated sequence.

Hydrophobicity analysis of the predicted protein demonstrates seven stretches of highly hydrophobic amino acids with 20-30% sequence similarity to

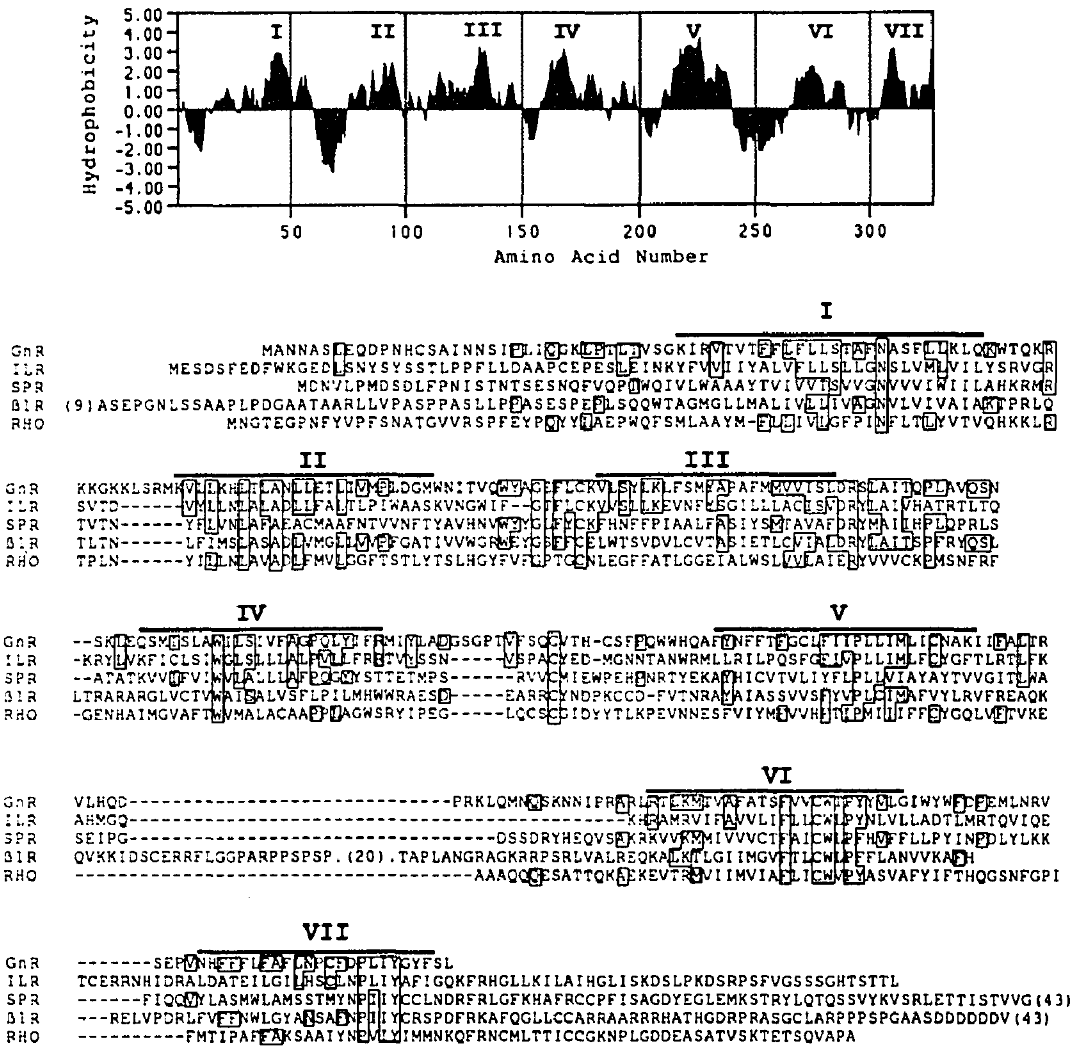

Figure 4 Hydrophobicity plot of the GnRHR and amino acid sequence alignments.

Alignment of: GnR, mouse gonadotropin-releasing hormone receptor; ILR, human interleukin-8 receptor (Murphy and Tiffany 1991); SPR, rat substance P receptor (Hershey and Krause 1990); b1R, human β1-adrenergic (Frielle, Collins et al. 1987); and RHO, human rhodopsin (Nathans, Hogness et al. 1984). I-VII denote putative transmembrane regions. Boxes indicate identical amino acid residues.

other GPRs with the highest degree of homology to the interleukin-8 receptor (Fig. 4). While several highly conserved residues are noted in the GnRHR, such as the cysteines present in each of the first two extracellular loops which stabilize many receptors, several features of the GnRHR are unusual. For example, the highly conserved transmembrane II aspartate/glutamate, which has been found to be essential for the function of many GPRs, is replaced by an asparagine. The GnRHR is nearly the smallest member of the GPR superfamily and, unlike any other GPCR, it lacks a polar cytoplasmic C-terminus. The putative first cytoplasmic loop is longer than any other GPR. Unique among GPCRs, the GnRHR may activate via dimerization (Conn, Rogers et al. 1982; Gregory and Taylor 1982). Its unusual structure may subserve this proposed mechanism of activation.

Another deviation from other GPCRs is the substitution of serine for the conserved tyrosine located adjacent to transmembrane III. This creates a potential phosphorylation site, unique to the GnRHR, in a domain critical for signal transduction of other GPCRs. Phosphorylation of the C-terminus, which is absent in the GnRHR, contributes to desensitization of several GPCRs (Probst, Snyder et al. 1992). It will be interesting to determine whether the novel phosphorylation site of the GnRHR mediates receptor desensitization. Other potential regulatory phosphorylation sites are also present (Fig. 3).

The presence of GnRHR mRNA in a variety of neuroendocrine cell lines was studied by solution hybridization/nuclease protection assay (Fig. 5A). GnRHR mRNA was detected in αT3-1 cells and in mouse pituitary, but not in GnRH neuron-derived (GT-1), corticotroph (AtT20) or somatolactotroph (GH3) cell lines at the limits of detection of the assay. The absence of detectable GnRH-R mRNA in the GT-1 and AtT-20 cell lines has been confirmed using higher concentrations of RNA in the solution-hybridization/nuclease protection

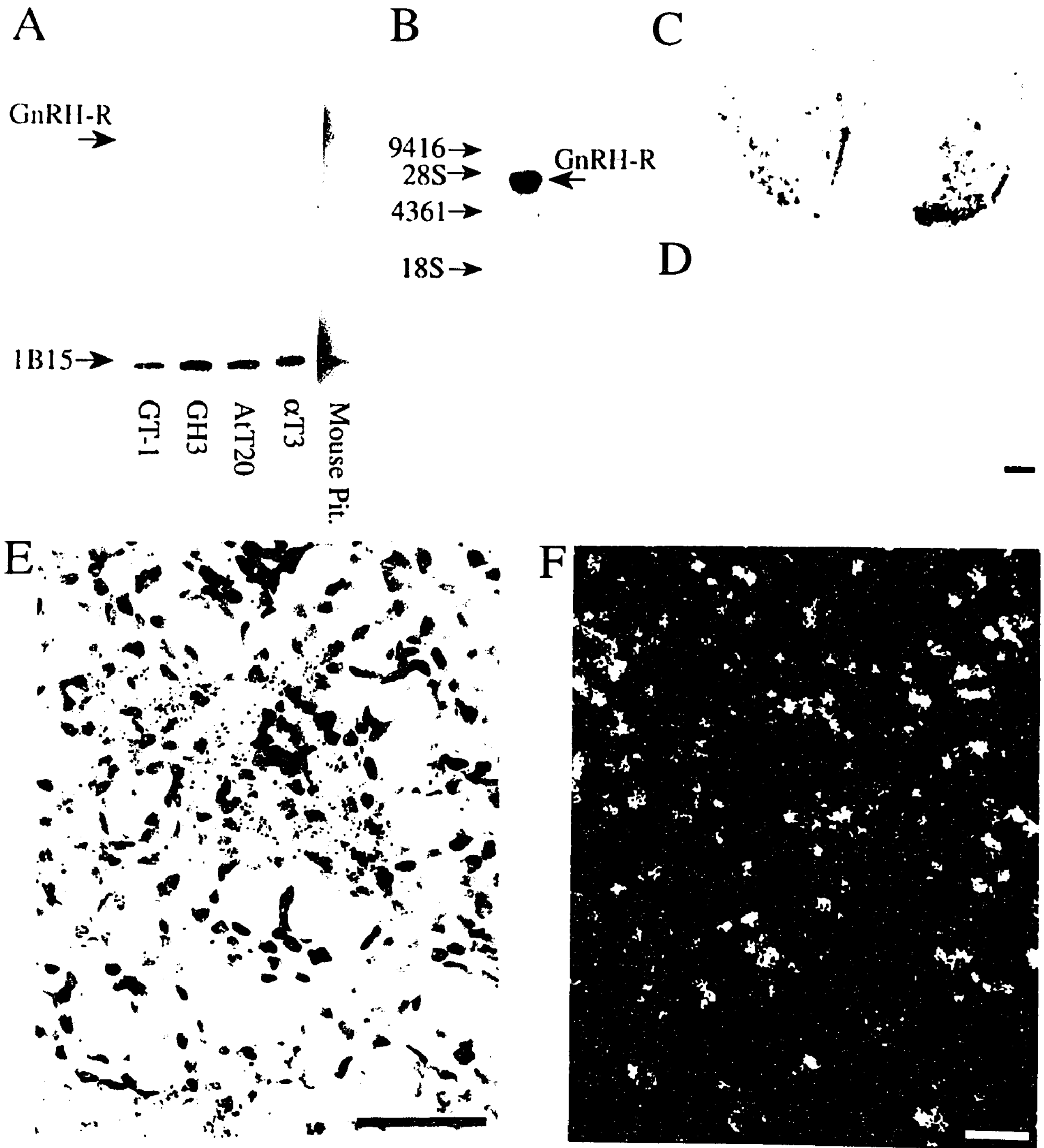
A
GnRH-R
1B15
GT-1
GH3
AtT20
αT3
Mouse Pit.
B
9416
28S
4361
18S
GnRH-R
C
D
E
F

Figure 5 Distribution of GnRHR mRNA.

Autoradiogram of A, solution hybridization assay using 2 μg of total mouse pituitary, GT-1, GH3, and AtT20 RNA and 625 ng of αT3-1 total RNA , B, Northern blot analysis with 3 μg of poly(A)+ αT3-1 RNA, and C-F, rat anterior pituitary *in situ* hybridization. C, antisense probe X-ray film autoradiography. D, sense probe control (calibration bar=450 μm). E,F, dark-field (calibration bar=50 μm), bright-field (calibration bar=100 μm) photomicrographs of emulsion-dipped anterior pituitary section. The molecular weight markers are Hind III digested lambda DNA.

assay (Dr. Andrea C. Gore, unpublished data). Figure 5C shows the distribution of the GnRHR mRNA in rat anterior pituitary. Labelling was heterogeneously distributed throughout the gland, a pattern previously observed by GnRHR autoradiography (Badr and Pelletier 1988). Bright-field and dark-field microscopy reveals clustering of the cells expressing the GnRHR mRNA (Fig 5 E, F).

The discovery of the sequence of GnRH heralded a major advance in reproductive endocrinology and revealed the complex nature of GnRHR signal transduction and regulation. Unlike most hormonal signals, GnRH is released in a pulsatile fashion, with the frequency and amplitude of the pulses conveying crucial information (Weiss, Jameson et al. 1990; Hasenleder, Dalkin et al. 1991). GnRHR binding capacity itself is either up- or down-regulated by agonists depending on duration of exposure and concentration (Loumaye 1982). The clinical utility of GnRH agonists, which help control a variety of human diseases, including prostatic hypertrophy, prostatic cancer, endometriosis and precocious puberty, depends on this induction of pituitary desensitization. The cloning of the GnRHR will lead to greater understanding of

the complex interplay of hypothalamic, pituitary and gonadal hormones which underlies both pharmacotherapy and reproduction.

Chapter 3

Structure of the Mouse Gonadotropin-Releasing Hormone Receptor Gene: Variant Transcripts Generated by Alternative Processing

Abstract

The mouse gonadotropin-releasing hormone receptor (GnRHR) is unique among G-protein coupled receptors in its lack of a putative intracellular C-terminal domain. A gonadotrope cell line cDNA library was screened in a search for alternative forms of the receptor transcript and 42 clones were obtained, representing a number of variant cDNAs. To determine the origin of these transcripts, the structure of the mouse gene was mapped from 11 distinct genomic clones. The gene contains three exons, spanning more than 22 kilobases. Exons 1, 2, and 3 encode, respectively, nucleotides +1 to +522, +523 to +739, and +740 to +981 of the open reading frame of the cDNA for the functional mouse GnRHR. Southern blot analysis with genomic DNA is consistent with the presence of a single gene. By comparison with the genomic sequence, the origins of the variant cDNAs isolated can be clarified. All the cDNAs contain the first exon and the majority (71%) encode the functional 327 amino acid receptor previously reported. One group of clones (14%), which contains exons 1 and 2, continues 700 bp past the exon 2 splice donor of the wild-type receptor. These clones terminate after a polyadenylation signal and have an open reading frame encoding a protein of only 261 amino acids. In a different group of transcripts (5%), exon 2 is absent, resulting in a shift in the reading frame and encoding a protein of 177 amino acids. These data support alternative processing of the mouse GnRHR gene.

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Introduction

The hypothalamic neuropeptide, gonadotropin-releasing hormone (GnRH), serves a key role in regulating mammalian reproductive function. GnRH activates high affinity receptors on the pituitary gonadotrope and elicits the release of the gonadotropins luteinizing hormone and follicle stimulating hormone.

We have previously reported the cloning of the mouse GnRH receptor (GnRHR) from a transgenic mouse-derived gonadotrope cell line, αT3-1 (Windle, Weiner et al. 1990; Tsutsumi, Zhou et al. 1992). The open reading frame of the cDNA for the functional receptor encodes a 327 amino acid protein which contains seven putative transmembrane helices (TMH), as is characteristic of G protein-coupled receptors (GPCR). Several features of the putative amino acid sequence for the GnRHR are unique, especially the lack of an intracellular C-terminal domain. The GnRHR, out of more than 200 cloned GPCRs, represents the only example of a receptor lacking a domain which plays a significant role in the regulation and coupling of many other receptors (O'Dowd, Lefkowitz et al. 1989; Probst, Snyder et al. 1992).

Alternative transcripts which alter the length of the mouse TRH receptor C-terminal domain have been reported (de-la-Pena, Delgado et al. 1992). In order to investigate the possibility that an alternative transcript for the GnRHR exists which encodes a C-terminal domain-containing receptor, a large number of cDNA clones were isolated and characterized and the mouse gene structure was investigated. Although cDNAs for the GnRHR have been isolated from 4 mammalian species (Kaiser, Zhao et al. 1992; Kakar, Musgrove et al. 1992; Tsutsumi, Zhou et al. 1992; Chi, Zhou et al. 1993; Illing, Jacobs et al. 1993), this is the first study of the gene structure in any species. We describe the organization of the mouse gene and the identification of several classes of

alternative transcripts encoding truncated proteins lacking several transmembrane domains.

Materials and Methods

Cloning of the Variant Mouse GnRHR cDNA Clones

A Uni-ZAP cDNA library (Stratagene, La Jolla, CA) constructed from the mouse pituitary tumor cell line αT3-1 (Windle, Weiner et al. 1990) was screened at high stringency with a 399 base pair cDNA clone encoding TMH 3 through TMH 6 as previously described (Tsutsumi, Zhou et al. 1992).

Genomic Library Screening

A mouse 129SV genomic library was purchased, which was constructed from female mice liver DNA partially digested with Sau3AI before cloning into the Xho I site of Lamda-FixII (Stratagene, La Jolla, CA). This library was screened twice using two separate ^{32}P-labeled cDNA probes by random hexamer priming (New England Biolabs, Beverly, MA). Bacteriophage DNA was transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) and hybridized in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate), 5 x Denhardt's, 0.1% SDS, and 100 µg/ml of salmon sperm DNA at 68C for 16h. Filters were washed twice at room temperature for 20 minutes in 1 x SSC and 0.1% SDS, once at 55C for 45 minutes, and once in 0.1 x SSC, 0.1% SDS at 68C for 1h and were exposed to Kodak XAR5 X-ray film for 5 days. Plaques were purified after 2 to 3 screenings. DNA sequencing was performed either manually (Sequenase, USB, Cleveland, OH) or by automated sequencer (Biorad, Richmand, CA).

Southern Blot Analysis

Mouse tail DNA and rat liver DNA were generous gifts of Drs. Kevin Kelley and James Roberts, respectively. 10 µg of mouse or rat genomic DNA were digested overnight with 20 units of restriction endonucleases, electrophoresed on a 0.8% agarose gel, and blotted to nitrocellulose filter (Schleicher & Schuell, Keene, NH). The membrane was hybridized with a

random primed, 501 bp EcoRI/HindIII fragment of the mouse GnRHR cDNA located within the first exon. Hybridization was carried out for 16 hours at 55C in the same buffer used for genomic screening. Filters were washed in a constant solution of 2 x SSC, 0.1% of SDS for 30 min each at 25C, 55C and 68C .

Polymerase Chain Reaction (PCR) Analysis of cDNA Clones

PCR was carried out with the positive cDNA clones in the αT3-1 cell library in order to examine their organization. 30 cycles were performed at 94C, 55C, and 72C for 1 minute each using 1 ml of phage suspension and primers corresponding to various regions of the functional GnRHR and the flanking vector sequences. These include (numbering refers to the functional cDNA previously reported (Tsutsumi, Zhou et al. 1992)): T3 (a vector sequence upstream to the insert) and 708 (+195--+209); 707 (+40--+56) and 710 (+947--+962); 707 and 720 (+745--+764); and 707 and T7 (a vector sequence downstream to the insert). The orientation of these primers and the different size of PCR bands are indicated in Fig.1. One-tenth of each PCR reaction was analyzed on a 1% agarose gel.

PCR Analysis of the Reverse-Transcribed mRNA (RT-PCR)

αT3-1 RNA was fractionated through a sucrose gradient and a high molecular weight fraction enriched for GnRHR mRNA was selected (Sealfon, Gillo et al. 1990). Enriched and total RNA was reverse-transcribed and used as a PCR template. In order to distinguish various receptor transcripts, oligonucleotides specific for different transcripts were used. These primers are (Fig.1): 707/710 for the functional receptor located within coding region); R15S2/R15AS2 (+1381--+1397 and +1947--+1931 in clone WZ15); and 713/R16AS1 (+717--+732 and +1293--+1278 in clone WZ16). PCR conditions were as described above.

Computer Analysis of DNA Sequences

All the DNA sequence analysis was performed on GCG (Genetic Computer Group) program from University of Wisconsin served by the Mount Sinai Vax. Genbank homology searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service with default parameters. All sequences obtained have been submitted to Genbank.

Result

<u>Identification of the Variant cDNA Clones:</u>

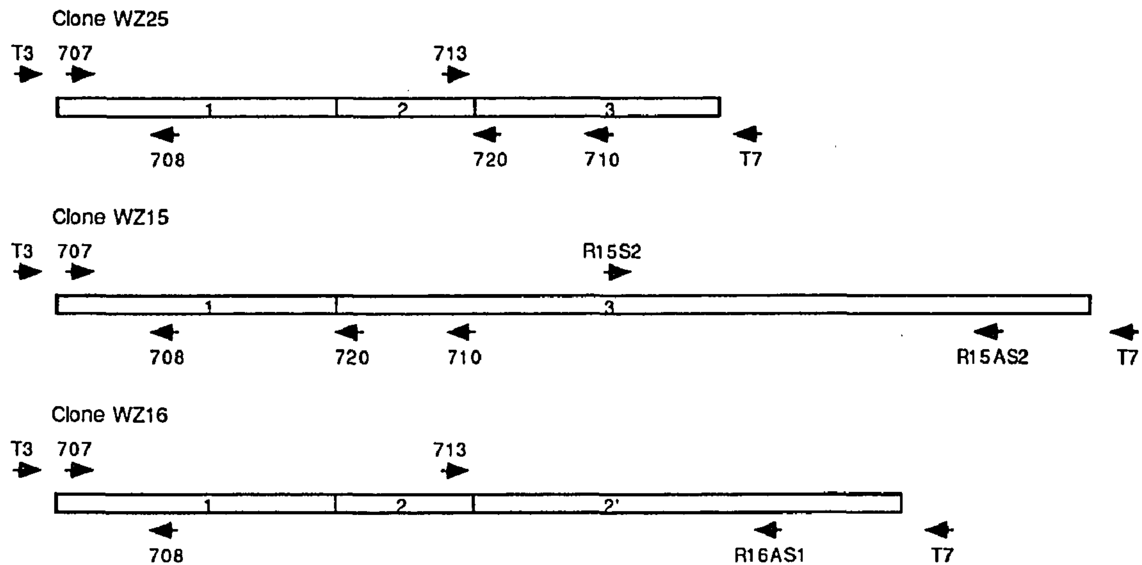
During cDNA cloning of the mouse GnRHR, 42 clones were identified from the primary library screening. These clones were initially mapped using multiple sets of PCR primers corresponding to different regions of the functional cDNA clone previously reported (WZ25; (Tsutsumi, Zhou et al. 1992)) as well as T3 and T7 primers flanking the phage multiple cloning sites (Fig.1).

The PCR reaction using the vector sequence T3 and T7 primers allowed all 42 clones to be screened for the presence additional 5'- and 3'-untranslated sequence by gel electrophoresis. Surprisingly, the 5'- end of all 42 clones fell within a range of 80 bp. The clone with the longest 5'- extent (WZ15) was sequenced and an additional (37 bp) of 5'- untranslated sequence was obtained.

Based on the PCR analysis, four types of clones could be distinguished. At least one representative clone from each group was completely sequenced. All the clones, while varying slightly in the length of the 5'-untranslated segments, were identical from the initiation codon to the sequence encoding TMH 4. The different cDNA clones varied significantly in the sequence encoding the carboxyl half of the receptor. Their sequence and structure will be described below.

<u>Characterization of the Genomic DNA Encoding the Mouse GnRHR:</u>

In order to elucidate the origin of these different cDNAs, the structure of the mouse gene was investigated. A mouse genomic library was first screened with the longest cDNA clone identified, WZ15. Out of 10^6 plaques screened, three plaques were purified through subsequent screens (MG221, MG331, and MG961, Fig.2). Southern blot analysis using ^{32}P-labeled 5'- and 3'- segments of the cDNA encoding the functional receptor, WZ25, indicated that at least one



Primer Pairs Used in PCR	Sizes of PCR Products (bp) WZ25	WZ15	WZ16
T3/708	3 10	3 50	3 40
707/720	7 20	500	-
707/710	920	700	-
707/T7	1 170	2 200	1 460

Fig. 1 PCR analysis of variant cDNA clones.

The variant cDNA clones are mapped by PCR using WZ25, WZ15, and WZ16 derived primers whose position and direction are indicated by arrows. T3 and T7 sequences are present in the vector, flanking the cloning sites. The exon boundaries are included in the schematic clones for references. This analysis reveals, at the lower panel of the figure, the size difference of the PCR bands. No bands are visualized when primers 707/720 and 707/710 are used to PCR clone WZ16.

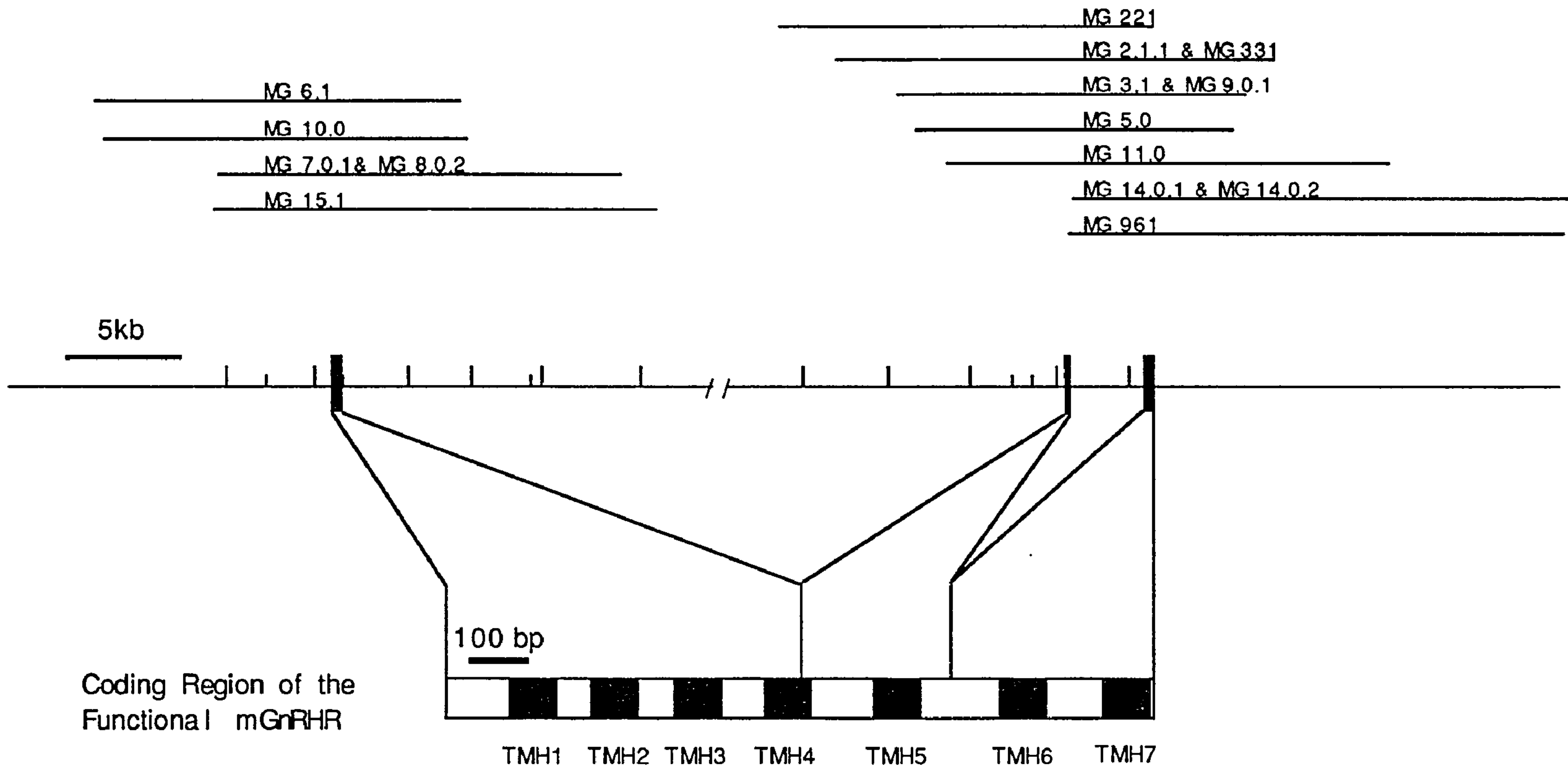

MG 221
MG 2.1.1 & MG 331
MG 3.1 & MG 9.0.1
MG 5.0
MG 11.0
MG 14.0.1 & MG 14.0.2
MG 961
MG 6.1
MG 10.0
MG 7.0.1& MG 8.0.2
MG 15.1
5kb
100 bp
Coding Region of the Functional mGnRHR
TMH1
TMH2
TMH3
TMH4
TMH5
TMH6
TMH7

Fig. 2 Genomic organization of the mouse GnRHR.

Genomic clones for the mouse GnRHR and the derived receptor gene structure are aligned to show their relative position. Exons responsible for the coding region of the receptor are represented by filled boxes and introns by thin lines. The genomic DNA is mapped by restriction enzyme XbaI. Hatch marks correspond to XbaI sites, short hatches indicate sites whose relative positions could not be definitely assigned. The filled boxes in the coding region of the functional receptor cDNA represent the putative transmembrane helices.

5' exon was missing in these three genomic clones. An additional 10^6 plaques of the same library were screened using ^{32}P-labeled WZ25 as a probe and 12 new genomic clones were isolated. Restriction mapping and Southern blot analysis revealed that the clones fell into two groups, one 5' and one 3' (Fig.2). In order to determine whether any of the 5'- and 3' genomic clones overlapped, all the 5'- clones were hybridized with ^{32}P-labeled XbaI-fragments of the 3' clone MG331. No hybridization was observed. DNA fragments which hybridized with the cDNA probes in the Southern blot were subcloned and sequenced.

Alignment of the cDNA and the genomic sequence established the organization of the mouse GnRHR gene. As shown in Fig.2, the gene spans at least 22 kb and consists of three exons and two introns. As there is no overlap between the two groups of genomic clones, the precise length of intron 1 cannot be determined, although it is larger than 15 kb. Intron 2 is ~ 5 kb in length.

The 5'-most sequence found in any genomic clones extends 560 nucleotides upstream from the initiation ATG. Although a TATA sequence is found at -180, primer extension of the αT3-1 RNA with several GnRHR primers within the coding sequence failed to yield a band or bands which would indicate the transcription start site of the mRNA (see Discussion). Furthermore,

computer analysis did not identify conserved binding motifs of common transcription factors in this region. Accordingly the possibility of an additional upstream exon(s) cannot be excluded by the present data, a difficulty encountered with other GPCR genes (Peralta, Winslow et al. 1987; Grandy, Marchionni et al. 1989).

Southern blot analysis was performed to determine whether there are multiple genes for the GnRHR. Mouse and rat genomic DNAs were hybridized with a ^{32}P-labeled HindIII/EcoRI exon 1 cDNA probe (nucleotides -46 to 460), a sequence which is found in all the receptor cDNAs identified (Figs.3 and 4). Two bands were visualized with PstI digested mouse and rat DNA, consistent with the exon 1 probe containing a PstI site. All other digestions yielded single bands (Fig.5). These data support the existence of a single GnRHR gene in the mouse and rat genome.

Alternative Transcripts of the Mouse GnRHR Gene

The structure of the mouse gene clarified the origin of the variant cDNAs isolated. The N-terminal sequence present in all the cDNAs was derived from exon 1 of the receptor gene. Most cDNAs isolated (71%) contain the coding region for all 7 TMH domains and represent the previously reported functional GnRHR. Three other groups of clones, however, differed in their sequence and putative protein structure (Figs.3 and 4).

Two clones, WZ15 and WZ19 contain only exons 1 and 3 and lack exon 2. Because of a change in the reading frame, these clones have a nonsense codon immediately following exon 1 and encode a truncated protein of 177 amino acids (Fig.4). Clone WZ19 terminates at the identical nucleotide of exon 3 as the previously published receptor cDNA (WZ25). The gene sequence is A-rich in this region (AAAAACAAAAACAAAAAACAAAAAA) and clones WZ19 and WZ25 were presumably generated by false priming of oligo-d(T) at this site

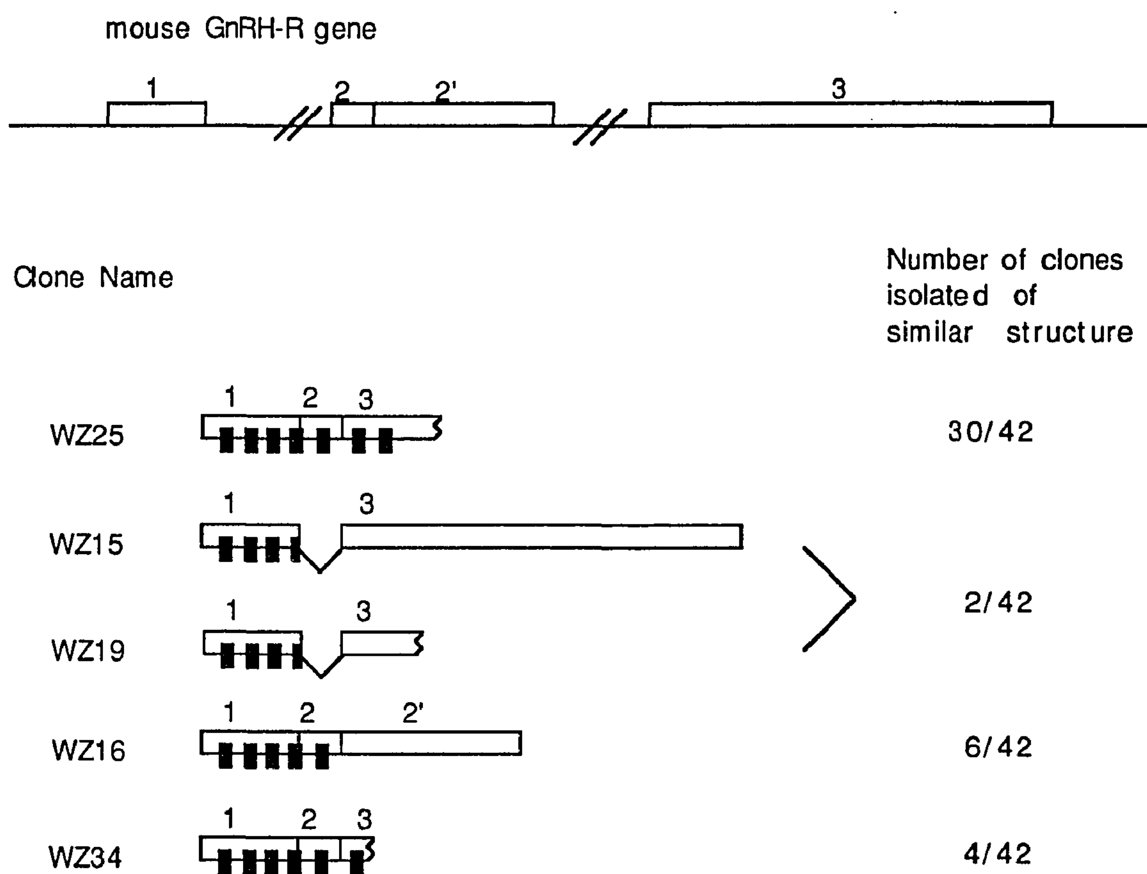


Fig. 3 Schematic of variant cDNA clones and their relationship to the gene structure.

The variant cDNA clones are shown with their exons and the frequency of their isolation from the cDNA library. For reference, the putative transmembrane helices encoded by all the represented transcripts are indicated by filled boxes.

```
      --------------EXON 1--------------------- ---------------EXON 2----------------
     481                                     522                                     560
WZ25 AGCCTGGCCTGGATTCTCAGCATTGTCTTTGCAGGACCACAGTTATATATCTTCAGGATGATCTACCTAGCAGACGGCTC...
WZ15 AGCCTGGCCTGGATTCTCAGCATTGTCTTTGCAGGACCACAG--------------------------------------------
WZ16 AGCCTGGCCTGGATTCTCAGCATTGTCTTTGCAGGACCACAGTTATATATCTTCAGGATGATCTACCTAGCAGACGGCTC...
WZ34 AGCCTGGCCTGGATTCTCAGCATTGTCTTTGCAGGACCACAGTTATATATCTTCAGGATGATCTACCTAGCAGACGGCTC...

     ---EXON 2---------- --------------EXON 3, OR exon 2'---------------------------
     721                 740                                                     800
WZ25 CTTCATCAAGACCCACGCAAACTACAGCTGAATCAGTCCAAGAATAATATCCCAAGAGCTCGGCTGAGAACGCTAAAGAT...
WZ15 -------------------AACTACAGCTGAATCAGTCCAAGAATAATATCCCAAGAGCTCGGCTGAGAACGCTAAAGAT...
WZ16 CTTCATCAAGACCCACGCAgtacgtattccttagatctaagaatcactgtagacaaacgaatttaacacttacacagagt
WZ34 CTTCATCAAGACCCACGCAAACTACAGCTGAATCAGTCCAAGAATAATATCCCAAGAGCTCGGCTGAGAACGCTAAAGAT...

      ----------------------------------EXON 3--------------------------------------
     881                                                                         960
WZ25 AAATGTTGAACAGGGTGTCAGAGCCAGTGAATCACTTTTTCTTTCTCTTTGCTTTCCTAAACCCGTGCTTCGACCCACTC...
WZ15 AAATGTTGAACAGGGTGTCAGAGCCAGTGAATCACTTTTTCTTTCTCTTTGCTTTCCTAAACCCGTGCTTCGACCCACTC...
WZ34 AAATGTTGAACAGGGTGTCAGAGCCAGTGAAAAAAAAAAAAAAAAAAAA

      ----------------------------------EXON 3--------------------------------------
     1121
WZ25 TTTGTTGTTAGAGCTTCAGAAGACCTTCAAAAACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA               (1185)
WZ15 TTTGTTGTTAGAGCTTCAGAAGACCTTCAAAAACAAAAACAAAAAACAAAAAAACCCGAAATGTGTCCTTACTCATAAGC (1200)

      ----------------------------------EXON 3--------------------------------------
     1201
WZ15 TTTCTAAACAATCCTCAGCCTTGTCGGATATTTTGCTGTATATGATTTAGGATTCTTTACTTCCTCCTTAGATCATAATA (1280)
     TATAAATACTTAAAATGACTACGTCCTAAGGTAAACAGTACCAAAAACAAAGGCTGGGAAAACACAACATATTCATAACA (1360)
     TCAGCACAGTTTGGGGAATTTTTTTTTTTCTCAAAGGGGGCTCAGCATCAATAACAGTCTGGCTCTATCCTAACACCTA (1440)
     CAGGTCATCAGCAAAAACAGATGCTATCCCAAGACCTAAAGCCCAGAGACAGAAGTATCACTGTCCATAGGTTAGCAGGA (1520)
     AAAACCGGTTGATGTTGTCCCAACACCTAAGGCACAGAGACGGTAGAATCAGTCACTGTCCACAGGTCAGGTTCTAGACA (1600)
     GGCTGATGCTATCACAGCCTAGAGCGCATACGAGCAGAATTACTGTCCAGAAGTCAATTCTTTAATCCGCAATGATTAAA (1680)
     GCACTTAGTTTTCTCTTTTACTTTAAAAGCTTTTTTGATTCTGATTCAGCAACAAATCCCACCAGCCTGGCAAGATTATT (1760)
     TACATGATAATACAAATCAGTCCCATTTACATGCTACAGATTGTGACACATCCCTACATAGTGGAGGAATTTAAAGGGAT (1840)
     AGATATTTTCCCCCATAGACTGTTAGACTATTCCTTAGAATATTTCATACTGTACAGTATATAGGGTTTATGTTAGCTGA (1920)
     CAGTCATGAGTAGTGTTTTAATATAATTAAGAAAATGCAGATAGGAGTTATCTTGTAAGAATAATATACAATCCACTTTA (2000)
     ACAACATGGTGGAACTTTTGTCTATCACCTTAACTACGGAATCATTTTCCACAAATAAATAAGGAAAGTTAATTTAAAAA (2080)
     AAAACATCAAGCAAATATACAACAGAAAGAGGAGGTTTGATGTATTAAGGGAACTCAAGGCCAGGAGAGCATAGAATTTC (2160)
     CCCTACACAGACCAAATCATAGCAGAGATATTAGAGAGGAGCGAAGTAAAACTCTACGTGTGAAAAAAACAACTACCGCT (2240)
     GAAAAAGAACGCAGTCCTGAAAAGTATGTCTGCAGAGTATCACGTGCTTTGTTCAACTCGTGAAAAACGGGAGGAGGTGA (2320)
     AAATTAGACTACAACACCCACTCACTCGGGAAAGTATGTGAACTCACATTCAATAATTACTAATAGACATTGATGTGAAC (2400)
     TACAAAAAAAAAAAAAAAAAA                                                           (2421)

      ----------------------------------EXON 2'-------------------------------------
     801
WZ16 ggtcgtgctttatagacggtggcaaacagctcacaaaggttaggtcatttgataactcacaatcacccccttgaaataat (880)
     atttttactatatactatagatgagatcctttactgacacctcatggtctcatttttcaccgtggaataaaggtcacata (960)
     agatctcaaaagtccccacacgtccttgaagatttatgcacagttaagagttgggggtacggggagacgttttcgacagt (1040)
     ggtgtagccacttcataaggtgccactgctcctacaagcaactgtaccgactggttttgtgtcaactcgacacaagctag (1120)
     agtcatcagagagaaagaagccttggtggaggaaattcttccatgagatccagctgtaaggcatttttttcaactagtgat (1200)
     caatgggggaagggccagcccattgtggtggcgccacccctggctggggccctgggttctgtaagaaagcagactaagca (1280)
     aactacaggaagcaggccagtaagcagcacccctccatggccagaagcatcgctcctgcctccaggttcctgtactactt (1360)
     gagttcctaacctggcttccctttggtaaagagcagtgatgtagaaatataagctgaataaatcctttcttcttcaactt (1440)
     aaaaaaaaaaaaaaaaaaaaaa                                                          (1463)
```

Fig. 4 The nucleotide sequence of the variant transcripts of the mouse GnRHR gene.

The variant transcripts, WZ15, WZ16, and WZ34, are aligned with the functional receptor clone, WZ25. Numbers start from the first ATG of the coding region. Only divergent sequences of the variant transcripts are shown. Sequence in lower case represents the exon 2' observed only in clone WZ16. The first stop codon of each transcripts is underlined. The polyadenylation signal sequence in WZ16 is in bold type. DNA sequence is determined using dideoxynucleotide-termination method.

during cDNA library construction. Clone WZ15, which lacks exon 2, contains additional 3'-untranslated sequence not found in other clones. The 1.2 kb of sequence distal to the A-rich region, also found in the mouse genome, most likely represents additional 3' untranslated sequence which was lost in the clone for the functional receptor because of internal oligo(dT) priming. Supporting the assignment of this sequence to exon 3 is the observation that it is 66% homologous to 3'-untranslated sequence of the human GnRHR cDNA (Chi, Zhou et al. 1993).

The third group of cDNAs characterized, represented by clone WZ16, contains the first two exons of the receptor gene. However, in this clone the genomic sequence is continuously transcribed following exon 2 where an additional 700 bp (exon 2') is found. Clones of this structure were present in 14% of cDNAs characterized (Fig.3). WZ16 contained an authentic polyadenylation signal sequence (AATAAA) 18 bp upstream from the poly(A) tail. As the genomic sequence at the location corresponding to the poly(A) tail of WZ16 is not A-rich (AATTTTGGTCATGGGTATTT), this cDNA must be generated by *in vivo* polyadenylation. The last group of cDNA clones characterized (10%),

represented by WZ34, are identical to the functional receptor cDNA (WZ25) until just before TMH 7, where they truncate. As the 3' end of these clones do not correspond with any intron/exon junctions or consensus splice site acceptor/donor sequences, this group of clones most likely represents an artifact of library construction.

To confirm that these alternative transcripts represent cytoplasmic mRNAs, a Northern blot analysis was carried out with the αT3-1 cytoplasmic mRNA. WZ15- and WZ16-specific probes were obtained by polymerase chain reaction of WZ15 and WZ16 cDNA templates using the primer pairs of R15S2/R15AS2 and 713/R16AS1, respectively (Fig.1) before random-primer labeling. In our hands, Northern blot analysis with the functional cDNA identifies one intense band at 4.3 kb and one smaller and one larger faint band not visualized in all blots (Tsutsumi, Zhou et al. 1992; Tsutsumi, Laws et al. 1993). The probes specific for the variant transcripts, however, did not detect any definite bands on Northern blot. Therefore a more sensitive RT-PCR analysis was performed. αT3-1 cell cDNA was subjected to PCR amplification using primers R15S2/R15AS2 (for clone WZ15), 713/R16AS1 (for clone WZ16), and 707/710 (for clone WZ25; see Fig.1). Probably reflecting their low abundance, the WZ15 and WZ16 primers generated faint bands of the expected size whereas the WZ25 primers generated an intense band. When size-fractionated αT3-1 cell mRNA, previously selected by oocyte recording to be enriched in mRNA for the functional receptor (Sealfon, Gillo et al. 1990), was reverse-transcribed and used as a PCR template, a band was detected only with the WZ25 primers (Fig.6). These data suggest that the WZ15- and WZ16-type transcripts exist in the cytoplasmic RNA and that they sediment in a different sucrose fraction from the RNA for clone WZ25.

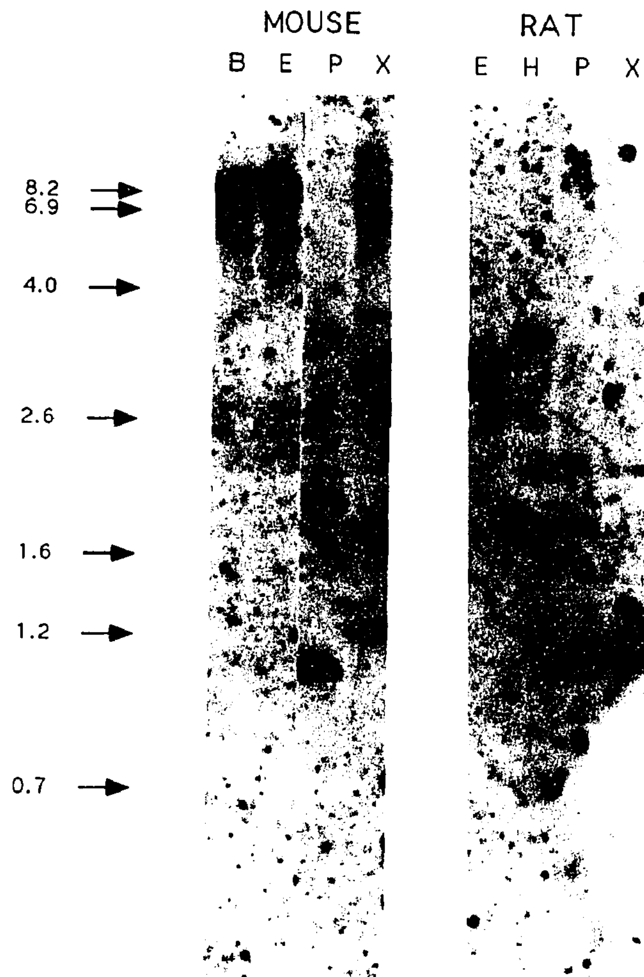


Fig. 5 Genomic Southern blot analysis of the GnRHR gene.

Mouse and rat genomic DNA digested with restriction endonucleases BamHI(B), EcoRI(E), HindIII(H), PstI(P), and XbaI(X) were hybridized with an exon 1 probe.

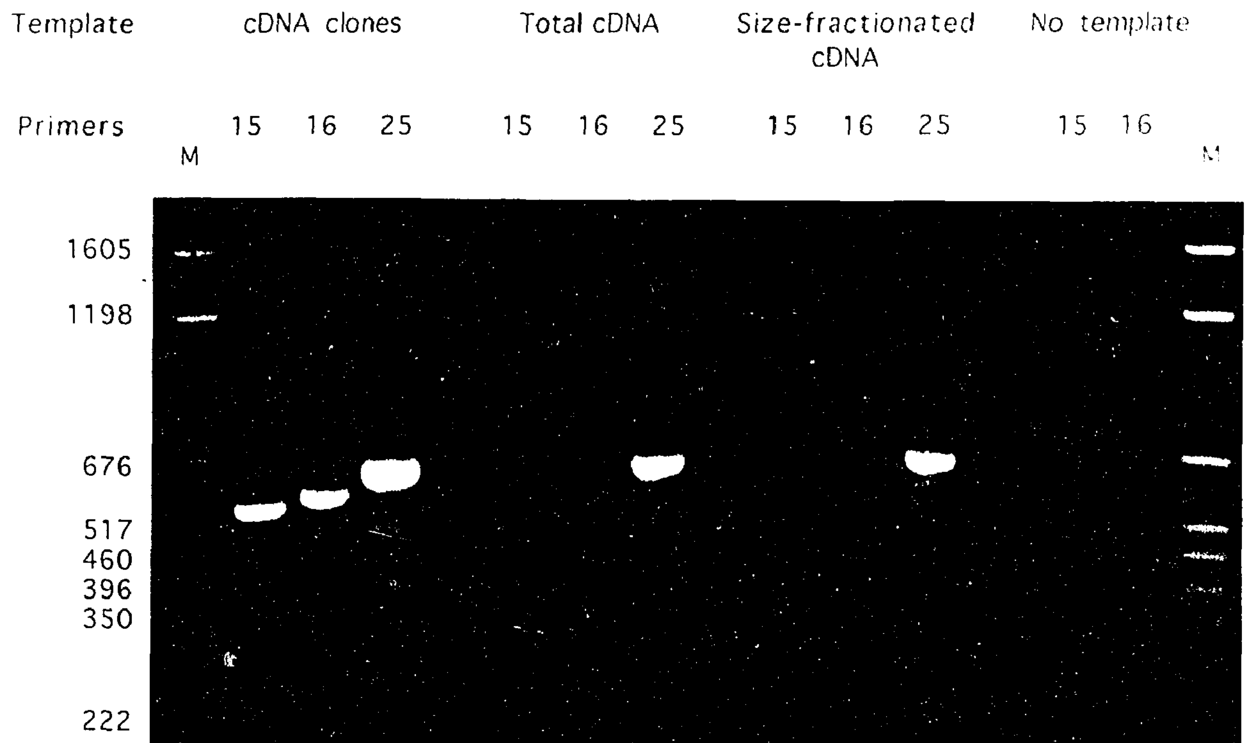


Fig. 6 Reverse-transcription PCR analysis of the variant transcripts of the mouse GnRHR.

Templates used for PCR include cDNA clones (positive control), cDNA reverse transcribed from total cytoplasmic RNA, cDNA reverse transcribed from size-fractionated cytoplasmic RNA enriched in functional GnRHR mRNA, and no cDNA templates (negative control). Primers specific for each of the WZ15, WZ16, and WZ25 clones are selected as described in the text. Sizes of the molecular weight markers (pGEM, Promega) are shown on the left.

Discussion

The coding region of the mouse GnRHR gene contains three exons and two introns. The present study and the cDNA cloning in other mammalian species (Kaiser, Zhao et al. 1992; Kakar, Musgrove et al. 1992; Chi, Zhou et al. 1993; Illing, Jacobs et al. 1993) have failed to identify any alternative forms encoding a functional receptor. Thus the sequence of the mouse exons confirm the unusual features of the mammalian receptor, including its lack of a C-terminal domain (Tsutsumi, Zhou et al. 1992).

The structure of the mouse GnRHR gene allows alternative mRNA processing to occur. In this report, several types of variant GnRHR transcripts are identified. One type of cDNA, represented by clone WZ16, contains exons 1 and 2 and continues 700 bp past the end of exon 2 (exon 2'). Part of this additional sequence (+1100 to +1230) is 78% homologous to the mouse Ins repetitive element (Sylla, Allard et al. 1984) which is associated with polyomavirus transformation. As the repetitive element is inserted within the 3' untranslated sequence of this transcript it is unlikely to contribute to its function. This is the first reported example of a GPCR gene containing an Ins insertion, although Ins insertions are frequently found within other mammalian genes (Beaubien, Rosinski-Chupin et al. 1991; Koop, Wilson et al. 1992). Clone WZ16 terminates immediately following a polyadenylation signal sequence (AATAAA) and represents the only GnRHR gene transcript identified which extends to its 3' polyadenylation site. The additional 1.2 kb of 3' untranslated sequence is believed to represent additional exon sequence because of homology with the untranslated 3'-sequence of the human GnRHR cDNA (Chi, Zhou et al. 1993).

Northern blot analysis previously reported identified several hybridization bands with αT3-1 RNA. The most intense signal is seen at ~4.3 kb. Faint bands at ~ 5 kb and ~2 kb are not visualized on all blots (Reinhart, Mertz et al. 1992;

Tsutsumi, Zhou et al. 1992; Tsutsumi, Laws et al. 1993). The alternative transcripts and exon sequence described in this report cannot definitively be correlated with the reported Northern blot analysis. The exons identified which can contribute to the mRNA encoding the functional receptor, exons 1, 2 and 3, represent 2.4 kb of sequence and could constitute the entire sequence of the ~ 2 kb transcript seen on Northern blot analysis. It is likely that additional 5'-untranslated sequence not found in the genomic clones identified represents the additional sequence contained in the larger Northern blot band. Perhaps tertiary structure in the 5'- untranslated region of this mRNA underlies the failure to obtain additional 5' cDNA sequences in all the 42 cDNA clones isolated and to identify the transcription start site by primer extension. Of note, the mouse GnRHR was subsequently cloned by two other laboratories and the 5'- extent of all clones isolated are remarkably similar (Reinhart, Mertz et al. 1992; Perrin, Bilezikjian et al. 1993).

Analysis of the gene structure of GPCRs may provide insight into the mechanisms underlying their evolutionary history (Patthy 1987; Rogers 1990). Many of these genes have no introns within the coding regions and are believed to have arisen by retroposition (Brosius 1991; Probst, Snyder et al. 1992). Among receptor genes with introns, most can be grouped by their intron positioning within the coding region. The pituitary glycohormone receptors such as follicle-stimulating hormone receptors and luteinizing hormone receptors have their coding region distributed among as many as 11 exons (Tsai-Morris, Buczko et al. 1991; Heckert, Daley et al. 1992). In this subfamily of receptors, the long extracellular termini are encoded by multiple exons whereas the receptor core, including all seven TMHs, is encoded by a single exon. This gene structure is believed to have arisen from the fusion of two genes (Tsai-Morris, Buczko et al. 1991). The opsin subfamily has intron insertions within the

coding region, with a conserved intron location within TMH 3 (Nathans, Hogness et al. 1984; Nathans, Thomas et al. 1986). Another group of receptor genes comprise the endothelin receptor genes (Hosoda, Nakao et al. 1992; Arai, Nakao et al. 1993), the tachykinin receptors (Shigemoto, Yokota et al. 1990; Hershey, Dykema et al. 1991) and the dopamine D_2, D_3, D_4 (Grandy, Marchionni et al. 1989; Giros, Martres et al. 1990; Van-Tol, Bunzow et al. 1991) receptors, which have introns separating many TMH domains and contain one intron at a conserved DRY sequence at the cytoplasmic side of TMH 3. Other receptors including the Drosophila muscarinic receptor gene (Shapiro, Wakimoto et al. 1989), the human neuropeptide Y receptor gene (Herzog, Baumgartner et al. 1993), the human 5-hydroxyltryptamine$_2$ receptor gene (Chen, Yang et al. 1992), and the mouse GnRHR gene now reported show little relationship to other receptors with respect to intron positioning. For example, of all other GPCR genes reported to date, no intron is found within the sequence encoding TMH 4 where the mouse GnRHR gene has an intron of >15 kb. As more receptor genes are characterized a clearer understanding of the various gene structures and their evolutionary relationships should emerge.

Alternative gene splicing is increasingly recognized as an important mechanism for generating different receptor gene products. For example, functionally and anatomically distinct glutamate receptor isoforms, termed "flip" and "flop," are formed by transcription of two different short exons (Sommer, Keinanen et al. 1990). $GABA_A$ ß subunit heterogeneity due to alternative splicing has also been reported (Bateson, Lasham et al. 1991). In the GPCR family, dopamine D_2 and D_3 receptor isoforms created by the insertion or deletion of a free standing exon have been identified (Giros, Sokoloff et al. 1989; Monsma, McVittie et al. 1989; Fishburn, Belleli et al. 1993). Alternative splicing also generates soluble hormone binding proteins from the luteinizing

hormone and thyroid-stimulating hormone receptor genes (Frazier, Robbins et al. 1990; Tsai-Morris, Buczko et al. 1990; Tsai-Morris, Buczko et al. 1991; Graves, Tomer et al. 1992). Recently thyrotropin-releasing hormone receptor variants in which the length of the C-terminal domains are altered by alternative RNA processing have been reported (de-la-Pena, Delgado et al. 1992). In addition, rat and human dopamine D_3 receptor gene transcripts which alter the reading frame and do not encode functional receptor proteins have been reported (Giros, Martres et al. 1991; Snyder, Roberts et al. 1991a).

None of the variant GnRHR gene transcripts identified would be expected to encode a functional receptor protein. However the existence of alternative splicing in the mouse raises the possibility that in other species alternative forms of the functional receptor may be identified. Furthermore, although not yet demonstrated to exist *in vivo*, it has recently been reported that functional GPCRs can assemble by intermolecular assembly of two subunits each containing different TMHs (Maggio, Vogel et al. 1993a; Maggio, Vogel et al. 1993b). Thus it is possible that the truncated proteins encoded by these alternative transcripts could function as subunits of heteromeric GPCRs.

Chapter 4

A Reciprocal Mutation Supports Helix 2 and Helix 7 Proximity in the Gonadotropin-Releasing Hormone Receptor

Abstract

The activation of the pituitary gondadotropin-releasing hormone receptor (GnRHR), a member of the seven-transmembrane G-protein coupled receptor (GPCR) family, triggers a cascade of events leading to gonadotropin release and stimulation of the reproductive system. An unusual feature of this receptor, observed in the mouse, rat, and human, is the presence of Asn^{87} in the second putative transmembrane helix at the location of a highly conserved Asp in the GPCR family, and of Asp^{318} in the putative seventh transmembrane helix where nearly all other GPCRs have Asn. The possibility that these residues interact was suggested by this reciprocal pattern and by a three-dimensional model of the GnRHR, and was investigated by site-directed mutagenesis. Replacing Asn^{87} in the second transmembrane domain by Asp eliminated detectable ligand binding. A second mutation generating the double mutant receptor $Asp^{87}Asn^{318}$, recreated the arrangement found in other GPCRs and re-established high affinity agonist and antagonist binding. The restoration of binding by a reciprocal mutation indicates that these two specific residues in helix 2 and 7 are adjacent in space and provides an empirical basis to refine the model of the receptor's transmembrane helix bundle.

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Introduction

The GPCRs comprise a large family of receptor proteins that mediate signaling by coupling to G-proteins (Kobilka 1992). All of the cloned GPCRs have seven hydrophobic domains considered to represent the transmembrane domains (TMH 1-7) which contain consensus amino acid motifs at corresponding positions (Probst, Snyder et al. 1992). Residues highly conserved among all GPCRs, which include receptors with widely divergent ligand structures and different coupling specificities to G-proteins, are likely to be essential structural determinants of receptor function. Two such residues are an Asp in TMH 2 and Asn in TMH 7, 98% and 95% conserved respectively (Probst, Snyder et al. 1992). However, these residues are not conserved in the mammalian GnRHR (Kaiser, Zhao et al. 1992; Tsutsumi, Zhou et al. 1992; Chi, Zhou et al. 1993) and, in fact, appear to be interchanged (Fig. 1).

The inversion of the conserved residues suggests that this receptor may represent a natural reciprocal mutation relative to other GPCRs and that an interaction between these two residues contributes to the protein's structural organization and functional integrity. To test this hypothesis, three mutant receptors, Asp^{87}, Asn^{318} and an $Asp^{87}Asn^{318}$ reciprocal mutant were produced and their properties compared to the wild-type GnRHR expressed in Cos-1 cells

Materials and Methods

Generation and expression of mutant constructs

The mouse GnRHR cDNA (Tsutsumi, Zhou et al. 1992) was subcloned into pSelect (Promega, Madison, WI) and mutations introduced by oligonucleotide-mediated mutagenesis. For expression in COS-1 cells, the inserts from sequence-identified mutants were excised and subcloned into pcDNAI/Amp (Invitrogen, San Diego, CA). The mutations were confirmed by sequencing both strands of the inserts in the final expression vector constructs. The wild-type and mutant receptors were transiently expressed in Cos-1 cells by transfection of constructs using DEAE-Dextran as described (Chi, Zhou et al. 1993).

Receptor binding

8-15 μg DNA was transfected in 10 cm dishes containing 3 x 10^6 Cos-1 cells (Millar, Flanagan et al. 1989; Chi, Zhou et al. 1993). Cell membranes were prepared by homogenization (Dounce) in binding buffer (10 mM HEPES, pH 7.4, 1 mM EDTA and 0.1% BSA) and centrifuged at 15 000 x g for 30 min at 4 C. The membrane pellet was resuspended in binding buffer and incubated with ^{125}I-GnRH agonist (D-Ala^{6}-N-Me-Leu^{7}-Pro^{9}-N-ethylamide GnRH; GnRH-A) and varying concentrations of unlabeled GnRH, Gln^{8}-GnRH, GnRH-A and GnRH antagonist 26 (Ac-D-4-Cl-Phe^{1,2}-D-Trp^{3}-D-Lys^{6}-D-Ala^{10}-NH_2 GnRH) for 60 min at 4 C. Binding (Bo) in mutant receptors is expressed as mean percentage of wild-type binding ± S.E.M. of 2 - 4 experiments, each done in triplicate.

Inositol phosphate

Inositol phosphate production was assayed as described (Davidson, Wakefield et al. 1990). DNA (2.5 μg) was used for 1.8 x 10^5 cells in 12 well plates. Transfected cells were labelled overnight with [^{3}H]-inositol and stimulated with varying concentrations of GnRH in the presence of LiCl. The

reaction was terminated by the addition of perchloric acid and phytic acid. After neutralizing with KOH the inositol phosphates were separated on a Dowex ion exchange column and counted. The mean maximum inositol phosphate production from dose-response curves of mutant receptors was expressed as a percentage of the inositol phosphate production (%IP) by the wild-type receptor in the same experiment. Maximal stimulation of the wild-type receptor induced phosphoinositol turnover of 7.0 ± 1.0 fold basal levels (3 experiments).

Molecular modeling

The model of the transmembrane helix bundle of the GnRHR was constructed according to the criteria and procedures described elsewhere (Ballesteros and Weinstein 1993; Zhang and Weinstein 1993) using structural inferences derived from the analysis of sequence conservation patterns (Donnelly, Johnson et al. 1989; Lesk and Boswell 1992), the physico-chemical properties of conserved and partially conserved residues (Eisenberg, Weiss et al. 1984; Baldwin 1993), and specific protein motifs such as Pro-kinks (Williams and Deber 1991; Ballesteros and Weinstein 1992; Sankararamakrishnan and Vishveshwara 1992). The predicted helix boundaries take into account the role of Arg and Lys residues at the membrane-cytoplasm interface, where these residues belong to the transmembrane helix acting as an anchor to the membrane through ionic pairs with phospholipid head-groups (Ballesteros and Weinstein 1992). Sequence alignments were generated using the Oxford Molecular Serratus software package, and the model was refined by energy minimization using the Quanta/CHARMm molecular modeling package (Molecular Simulation, Inc.)

Results and Discussion

With expression of the Asp^{87} mutant, binding was not detectable with either labeled agonist or antagonist. Mutation of the Asp in TMH 7 to Asn^{318} in conjunction with the TMH 2 Asp^{87} mutation ($Asp^{87}Asn^{318}$) restored high affinity binding of both agonist and antagonist to values that were similar to those obtained with the wild-type receptor (Table 1 and Fig 2).

The absence of detectable binding following expression of a receptor with a single mutation might be due to elimination of a direct ligand contact site. A direct role in ligand binding for Asn^{87}, however, is unlikely for the following reasons: *i.* The residue at this locus is highly conserved among GPCRs with structurally unrelated ligands. *ii.* All ligand contact sites that have been suggested from experiments are located nearer to the extracellular surface. Ligands covalently bound to the ß adrenergic receptor, for example, identify a TMH 2 attachment site located 14 residues above the corresponding GnRHR locus studied (Dohlman, Caron et al. 1988). *iii.* As reported here, agonist and antagonist affinities were nearly identical for the wild-type and $Asp^{87}Asn^{318}$ mutant. The agonists and antagonists evaluated must have multiple contact points on the receptor which should be asymmetrically distributed. Thus if Asn^{87} were a direct ligand contact site, the double mutant, introducing an Asn in a different location, would not be expected to reconstitute a binding site with properties indistinguishable from those of the wild-type receptor.

Thus the loss of binding of the Asp^{87} mutant must be due to a structural perturbation of the receptor which either distorts the binding site or, as determined by immunofluorescence microscopy for a $ß_2$ adrenergic receptor mutant, disrupts proper membrane insertion of the mutant receptor (Suryanarayana, Daunt et al. 1991). The present data do not allow discrimination between intracellular retention of the mutant receptor and

a

Receptor name	HELIX 2	HELIX 7
	80 87 90 100	310 318 325
GnRH receptor (Mus musculus)	KVLLKHLTLANLLETLIVMPLDGMWNIT	NHFFFLFAFLNPCFDPLIYGYFSL
Cholecystokinin receptor (Homo sapiens)	NAFLLSLAVSDLLLAVACMPFTLLPNLM	ISFIHLLSYASACVNPLVYCFMHR
GRP/bombesin receptor (Mus musculus)	NLFISSLALGDLLLLVTCAPVDASKYLA	SICAHLLAFTNSCVNPFALYLLSK
Neuromedin B receptor (Homo sapiens)	NIFISNLAAGDLLLLLTCVPVDASRYFF	TLVARVLSFGNSCVNPFALYLLSE
Neurotensin receptor (Homo sapiens)	HYHLGSLALSDLLTLLLAMPVELYNFIW	YMVTNALFYVSSTINPILYNLVSA
5HT1C receptor (Homo sapiens)	NYFLMSLAIADMLVGLLVMPLSLLAILY	LNVFVWIGYVCSGINPLVYTLFNK
beta-2 adrenergic receptor (Homo sapiens)	NYFITSLACADLVMGLAVVPFGAAHILM	YILLNWIGYVNSGFNPLIYCRSPD

b

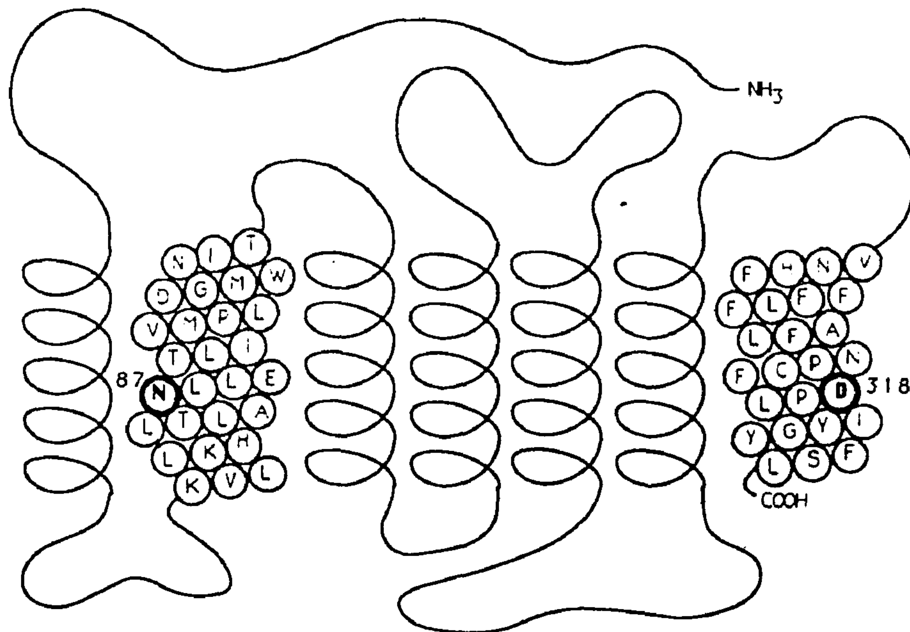


Fig. 1 Alignment of GnRHR sequences with other representative GPCR sequences, illustrating the exchange of the conserved TMH 2 aspartate and TMH 7 asparagine. a, TMH 2 and TMH 7 sequence alignments. Asn^{87} and Asp^{318} in the GnRHR are in bold type. GRP, gastrin-releasing peptide; 5HT, 5-hydroxytryptamine. b, Helical net schematic diagram of TMH 2 and TMH 7 of the GnRHR, showing the putative locations of the residues studied by mutagenesis.

membrane expression of a receptor which does not form a functional binding pocket. However, either explanation for the loss of binding supports the conclusion that the Asn-Asp single mutation in TMH 2 interferes with proper packing of the membrane helices and alters the essential overall structure of the receptor.

The restoration of binding to the Asp^{87} mutant with the introduction of the reciprocal Asn^{318} mutation indicates that these residues have a complementary role in maintaining the receptor's structure. A correlation between restoration of functional attributes by a reciprocal double mutation and spatial proximity of the targeted residues has been demonstrated with a zinc finger protein using 2-D nuclear magnetic resonance (Weiss and Keutmann 1990). Structural implications of double revertant mutants have been evaluated in terms of free energy considerations (Carter, Winter et al. 1984; Ward, Timms et al. 1990). Regaining binding for the double mutant indicates that the changes in the free energy of binding ($RT\ln K_d$) in each single mutant are not simply additive in the double mutant, but that the effect of one mutation is dependent on the residue at the other locus. Such non-additivity is expected if a single mutation causes a structural perturbation in the microenvironment of the other residue (Carter, Winter et al. 1984). It is therefore likely that Asn^{87} and Asp^{318} share a common microenvironment, a condition that could be fulfilled by direct hydrogen-bonding, as illustrated in Fig. 3A, but may involve more complex networks of interacting side chains in the two helices. Furthermore, while the present results indicate that Asn^{87} and Asp^{318} are adjacent in space and therefore interact, they do not prove that the residues are directly hydrogen-bonded.

Interhelical interactions have been studied in adrenergic receptors by substitution of a different TMH 7 locus that corresponds to Phe^{308} in the

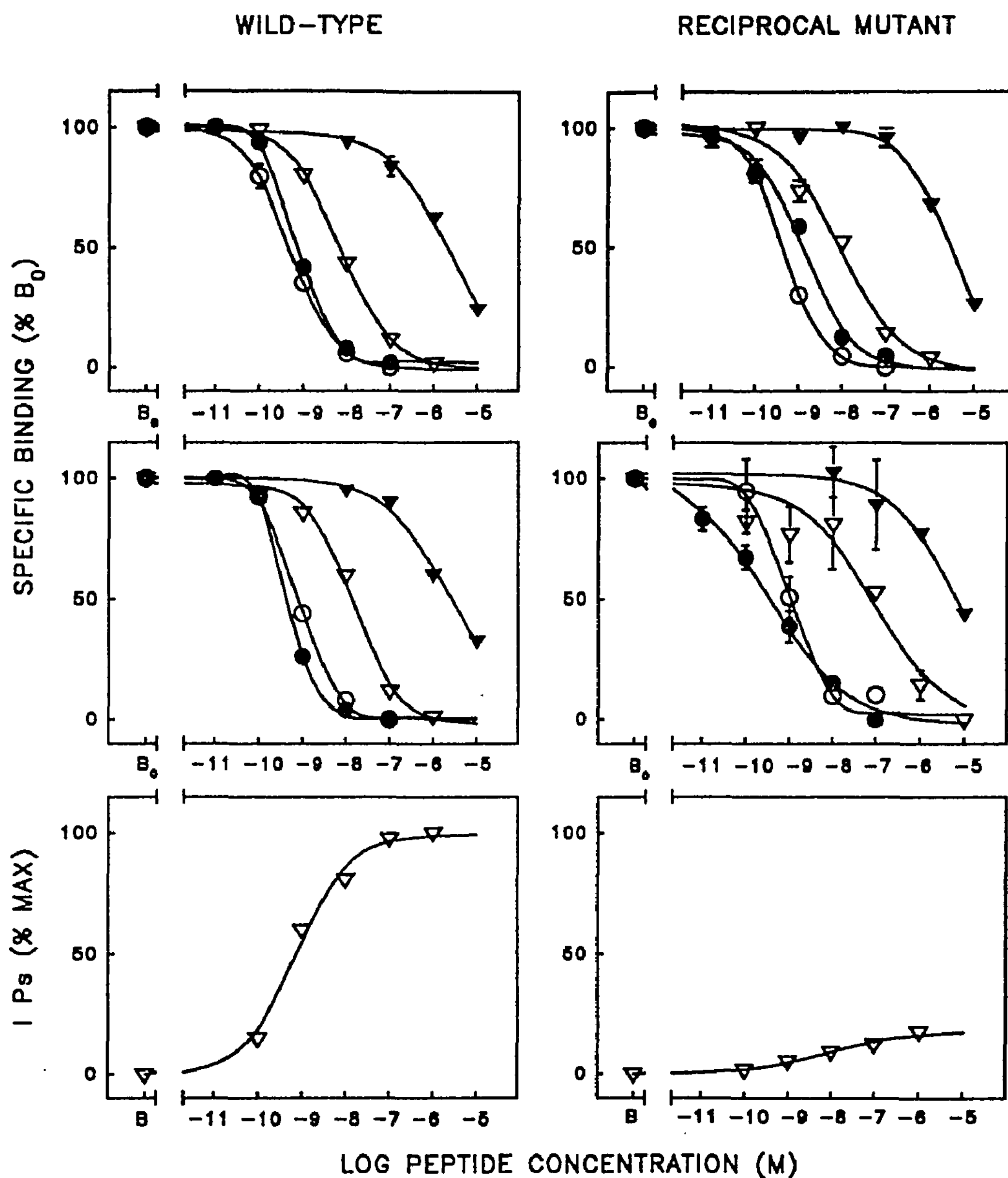


Fig. 2 Receptor binding and ligand stimulation of inositol phosphate producetion in COS-1 cells transfected with wild-type (left) and $Asp^{87}Asn^{318}$ reciprocal mutant (right) receptor constructs. Upper, competition binding of GnRH peptides with ^{125}I-GnRH-A. O, GnRH-A; ●, antagonist 26; Δ, GnRH; ▼, Gln^{8}-GnRH. Middle, competition binding of GnRH peptides with ^{125}I-GnRH antagonist26. Lower, stimulation by GnRH of total inositol phosphate (PI) production.

Table 1 Binding of wild-type and mutant receptors expressed in COS-1 cells. Wild-type and mutant receptors were generated and expressed as described in Materials and Methods. The ^{125}I-labled GnRH agonist used was GnRH-A; competition data were obtained with unlabled GnRH, Gln^{8}-GnRH, GnRH-A, and GnRH antagonist26

Construct	B_0 (%)	IC_{50} (nM)			
		GnRH	GnRH-A	Antagonist26	Gln^{8}-GnRH
Wild-type	100	15.9±5.6	1.3±0.7	2.7±1.5	2605±222
Asp^{87}	0.9±2.1	---	---	---	---
$Asp^{87}Asn^{318}$	39.7±8.2	21.7±16.7	1.3±0.4	2.3±1.4	4120±2030
Asn^{318}	69.7±7.4	10.1±1.4	0.6±0.2	0.9±0.4	1950±640

GnRHR. A mutation of Asn^{312} in the β_2 adrenergic receptor to Phe, which is found at this position in the α_2 adrenergic receptor, eliminates function and disrupts proper membrane localization (Suryanarayana, Daunt et al. 1991). The effect of this mutation , which presumably interferes with proper helix:helix packing, was corrected by exchanging both TMH 1 and TMH 2 with the homologous domains of the α_2 adrenergic receptor (Suryanarayana, von-Zastrow et al. 1992). These reports, together with the present data, are consistent with the proximity of TMH 2 and TMH 7 in the GPCRs.

The result that the side chains of Asn^{87} and Asp^{318} are in close proximity provides an empirical structural guide for assembling the model of the receptor. Fig 3B presents a model of the helix bundle in the transmembrane portion of the GnRHR constructed from an extensive set of considerations and criteria (Ballesteros and Weinstein 1993; Zhang and Weinstein 1993). The GnRHR template resembles the projection density map of rhodopsin (Baldwin 1993;

Schertler, Villa et al. 1993), and the proposed interactions between helix 2 and 7 is consistent with side chain interactions predicted from the model of the transmembrane helix bundle of the $5HT_2$ receptor reported recently (Zhang and Weinstein 1993).

The single mutation to Asn^{318} had no marked effect on binding (Table 1). The result that binding in the GnRHR is disrupted by a single Asp^{87} mutation but not by a single Asn^{318} mutation is consistent with the proposed structural model (Fig. 3). Asp is assumed to be ionized and in a hydrogen-bond interaction could only be an acceptor at the ε position of its side chain. Asn can act as both a hydrogen-bond acceptor (C=O) and as a donor (NH_2) at the ε position. Thus both the double mutant receptor and the Asn^{318} receptor would allow favorable interactions such as hydrogen-bonding to occur. In the Asp^{87} mutant, however, the side chains would electrostatically repulse and impair binding through disruption of receptor structure.

While the data indicate that the binding pocket of the wild-type and reciprocal mutant receptors are similar, the double mutant (Fig. 2) and the TMH 7 Asn^{318} mutant (data not shown) are poorly coupled to phophoinositol turnover compared to the wild-type receptor. The differences in coupling suggest that receptor activation requires other loci on the receptor to interact with one or both of these residues, an arrangement not replicated in the mutant receptors. The coordinated mutation of other highly conserved GPCR loci may reveal such interactions and help to elucidate the structural changes that accompany receptor activation (Neve, Cox et al. 1991).

The TMH 2 Asp conserved in virtually all other GPCRs (Asn^{87} in the GnRHR) has been extensively studied by mutagenesis of neurotransmitter receptors. Replacement of this residue in different receptors has been found to have a variety of functional effects, including reduced agonist affinity (Strader,

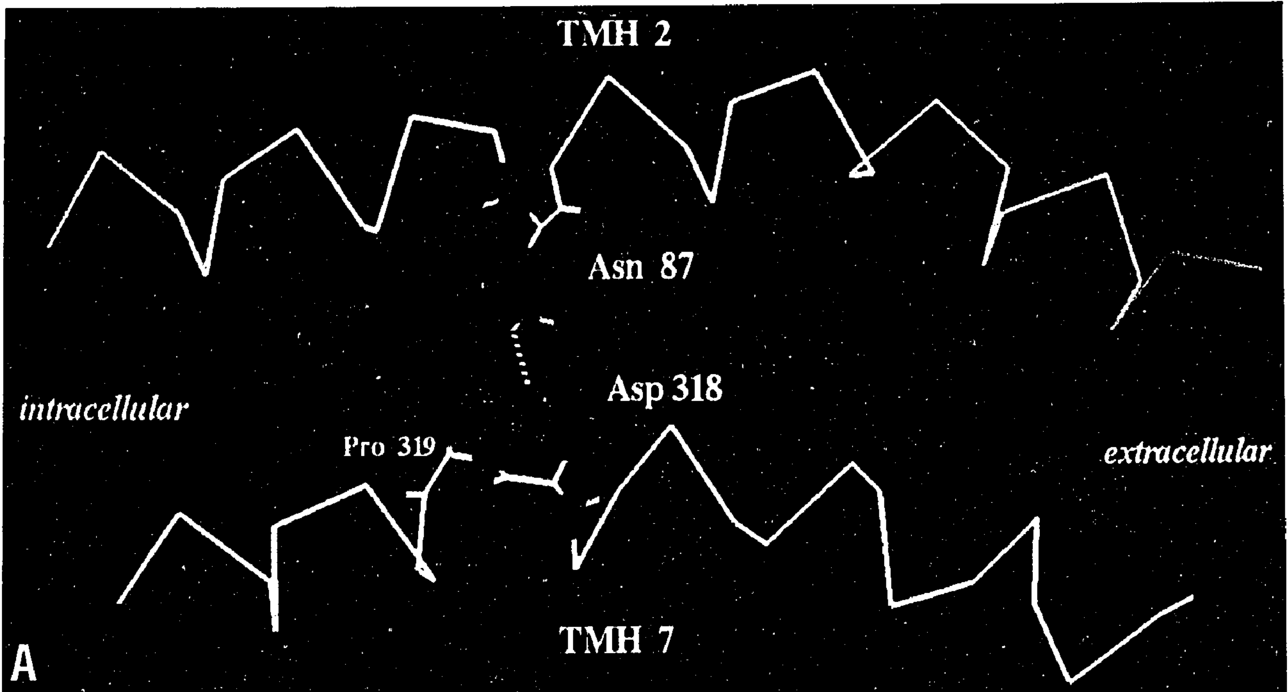

TMH 2
Asn 87
Asp 318
intracellular
Pro 319
extracellular
TMH 7
A
TMH 6
TMH 7
TMH 5
TMH 1
TMH 2
TMH 3
TMH 4
B

Fig. 3 Molecular modeling of the GnRHR.

A, view parllel to the membrane of a partial GnRHR model, showing spatial proximity of Asn^{87} in TMH 2 and Asp^{318} in TMH 7. Note that direct hydrogen bonding has been selected for illustrative puposes only. Other hydrogen-bonding pattern and interactions are also consistent with the present experimental data. B, Three-dimensional model of the transmembrane portion of the GnRHR, viewed from the extracellular side.

Sigal et al. 1987; Chung, Wang et al. 1988; Fraser 1989; Neve, Cox et al. 1991; Wang, Buck et al. 1991; Chanda, Minchin et al. 1993; Wang, Gallaher et al. 1993), loss of modulation of binding by pH (Neve, Cox et al. 1991), by sodium (Neve, Cox et al. 1991; Horstman, Brandon et al. 1992), or by GTP analogues (Chung, Wang et al. 1988; Surprenant, Horstman et al. 1992; Wang, Gallaher et al. 1993), as well as diminished or absent coupling (Chung, Wang et al. 1988; Fraser, Wong et al. 1989; Wang, Buck et al. 1991; Surprenant, Horstman et al. 1992). While the functional changes reported in different receptors are diverse, they are all consistent with an alteration in the native or allosterically modulated structure of these receptors associated with the loss of the acidic Asp side chain at this position. Our results, which indicate the proximity of this locus to a specific TMH 7 residue in the GnRHR, suggest the latter site as a novel locus to be probed in the attempt to clarify the structural basis of the complex effects reported for mutation of the conserved Asp in TMH 2 of GPCRs. To our knowledge only one study has reported the effects of substitution of the same TMH 7 Asn. Replacing Asn^{396} in the serotonin 5-HT_{1A} receptor with Ala, Phe or Val eliminated agonist binding, whereas binding was retained with Gln^{396} (Chanda, Minchin et al. 1993). These results are consistent with the present data and the preliminary receptor model in Fig. 3B.

In the absence of detailed structural data on any GPCR, there is a paucity of experimental information from which to infer intramolecular contacts. Our results provide data supporting the proximity of specific residues in different helices. Additional potential sites of interaction between side chains in different helices can be similarly identified from the sequence and the three-dimensional model, and tested to elicit experimental validation of an increasingly reliable molecular model of the GnRHR and other GPCRs.

Chapter 5

Functional Role of Interchanged Helix 2 and Helix 7 Residues of the Gonadotropin-Releasing Hormone Receptor

Abstract

An Asp and an Asn residue conserved, respectively, in the transmembrane helix two and helix seven domains of most G protein coupled receptors are interchanged in the gonadotropin-releasing hormone receptor. The restoration of receptor function obtained with reciprocal mutations at these two positions supports the proposal that these sites are adjacent in space (Zhou, W., C. Flanagan, J. A. Ballesteros, K. Konvicka, J. S. Davidson, H. Weinstein, R. P. Millar and S. C. Sealfon (1994) Mol. Pharmacol. 45,165-170). To clarify the functional and structural role of residues in these loci, the effects on receptor activity of a series of mutations at each position were examined. Substitution of Asn^{87} with either Gln, Asp or Ala led to a complete loss of high affinity receptor binding and of agonist-induced phosphoinositol hydrolysis. All the exchanges for Asp^{318}, including Glu, Asn and Ala, as well as the reciprocal mutation to $Asp^{87}Asn^{318}$, decreased E_{max} and increased EC_{50} while maintaining high affinity binding. These results are shown to be consistent with the hypothesis that efficient receptor activation requires a favorable interaction between Asn^{87} and Asp^{318}. However, the differing effects of substitutions at the two positions suggest that for functionally proper packing of the helices Asn^{87} interacts with at least one other receptor locus. These inferences agree with results from a three-dimensional model of the receptor.

Introduction

The neuropeptide gonadotropin-releasing hormone (GnRH) is released from the hypothalamus and serves a central role in regulating mammalian reproductive function. GnRH exerts its effects through binding to the pituitary gonadotropin-releasing hormone receptor (GnRHR) and stimulating the synthesis and secretion of luteinizing hormone and follicle stimulating hormone. Mammalian GnRHR cDNAs have been cloned from many species, including mouse (Reinhart, Mertz et al. 1992; Tsutsumi, Zhou et al. 1992), human (Kakar, Musgrove et al. 1992; Chi, Zhou et al. 1993) , rat (Eidne, Sellar et al. 1992; Kaiser, Zhao et al. 1992; Perrin, Bilezikjian et al. 1993), sheep (Illing, Jacobs et al. 1993), and cow (Kakar, Rahe et al. 1993) and belong to the family of G protein coupled receptors (GPCRs, reviewed in Probst, Snyder et al. 1992; Strader, Fong et al. 1994).

Nearly all cloned GPCRs have an Asp in the putative transmembrane helix (TMH) 2 and an Asn in TMH 7 at topologically corresponding locations in their sequences. The GnRHR is unusual in having these residues interchanged. The relationship between Asn^{87} in TMH 2 and Asp^{318} in TMH 7 of the GnRHR was studied previously by examining the properties of both Asp^{87} and Asn^{318} mutant receptors and a reciprocal mutation $Asp^{87}Asn^{318}$ of the receptor (Zhou, Flanagan et al. 1994). The Asp^{87} mutation caused a loss of radioligand binding. Introducing a second mutation at TMH 7 forms the $Asp^{87}Asn^{318}$ reciprocal mutant receptor which recreates the pattern found in other GPCRs. The reciprocal mutation led to restoration of high affinity binding. These findings are consistent with the suggestion that the two side-chains share the same microenvironment and are adjacent in space (Zhou, Flanagan et al. 1994). The proposed proximity of these loci in TMH 2 and 7 is consistent with a preliminary three-dimensional model of the receptor's

transmembrane helix bundle (Zhou, Flanagan et al. 1994).

In order to elucidate the nature of the interaction between these loci and to determine the side chain requirements and functional role of each site, the effects of a series of substitutions at each locus on receptor binding and response were characterized. The results reveal that the effects of effects of removing one of the interacting residues from the spatial proximity of the other, differ from those of modifying the hydrogen-bonding potentials of the side-chains at each loci. They suggest a network of hydrogen-bonding interactions involving these two side-chains that contributes to a mechanism of receptor activation in which TMHs 2 and 7 have essential roles.

Materials and Methods

GnRH and GnRH analogs

All chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO). GnRH (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and GnRH-A ([des-Gly^{10}, D-Ala^6, Pro-$ethylamide^9$]-GnRH), a potent GnRH agonist (Bachem, Torrance, CA) were used for ligand binding and phosphatidyl inositol turnover studies. Iodination of GnRH-A was catalyzed in the presence of Iodogen (Pearce Chemical Co., Rockford, IL) following published protocols (Laws, Beggs et al. 1990).

DNA Constructs and Transfection

Mutagenesis of the mouse GnRHR was performed in the pAlter vector (Promego, Madison, WI) and the receptor coding region was subsequently subcloned into the expression vector pcDNAI/Amp (Invitrogen, San Diego, CA). All the mutations were confirmed by automated DNA sequencing (Biorad, Hercules, CA). 10 µg of DNA construct and 100 µl of Lipofectamine (BRL, Gaithersburg, MD) were used to transfect each 100 mM plate of COS-1 cells (American Type Culture Collection, Rockville, MD) that had been seeded 24 hs before at $3x10^6$ cells per plate. Cells were maintained in Dulbecco-Modified Eagle's Medium containing 10% of fetal bovine serum.

Membrane Binding Assay

3 days following transfection, the cells were harvested and homogenized. After resuspension of the membrane preparation in the binding buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% BSA), 10-30 µg of membrane protein was used in each tube in a total volume of 0.4 ml. The reaction continued for at least one hour at 4 C. Non-specific binding was determined in the presence of 10^{-7} M of competing ligands. The specific binding, B, at a given concentration of competing ligand, D, was fitted by least

squares fit using Kaleidagraph (Synergy Software, Reading, PA) to $B = B_0/(1+D/IC_{50})$, where B_0 is the specific binding in the absence of competing ligand, and IC_{50} is the concentration of the competing ligand that gives half of the maximal specific binding.

Phosphatidylinositol Hydrolysis Assay

Following the transfection in 100 mM plates, the cells were harvested with trypsin and seeded in 2X12-well plates. 32 h later the medium was replaced by serum-free Dulbecco-Modified Eagle's Medium containing 0.5 μCi/ml of $[^3H]$*myo*-inositol (NEN, North Billerica, MA). 16 hs later, the cells were washed with serum-free medium and incubated with the desired concentrations of GnRH in the presence of 20 mM of LiCl for 45 minutes at 37 C. Cell extracts, in 10 mM of formic acid, were applied to the Dowex ion-exchange column before elution by buffer containing 1 M ammonium formate and 0.1 M formic acid. The procedure essentially followed published protocols (Berg, Clarke et al. 1994). After scintillation counting, the data were plotted using Kaleidagraph (Synergy Software, Reading, PA) and fitted to the equation $E = E_{max}/(1+EC_{50}/D)$, where the E_{max} is the maximum stimulation, D is the concentration of the agonist, and EC_{50} represents the agonist concentration that produces half-maximal stimulation.

Results

This study was designed to analyze the role of Asn^{87} and Asp^{318} in GnRHR function and to elucidate the side-chain properties required at each locus. The single substitutions studied included: Asp, Ala and Gln for Asn^{87} in TMH 2, and Asn, Ala and Glu for Asp^{318} in TMH 7. In addition, a reciprocal mutant receptor, $Asp^{87}Asn^{318}$, was constructed to form the structural pattern seen in other GPCRs.

Confirming our previous report (Zhou, Flanagan et al. 1994), substituting Asp for Asn^{87} eliminated detectable ligand binding and GnRH-stimulated PI hydrolysis. Remarkably, both the conservative substitution of Gln and the introduction of Ala at this position had the same effect. Both the detectable ligand binding and the high-affinity GnRH-stimulated PI hydrolysis were abolished by all three substitutions studied (Fig. 1). However, when the TMH 7 Asp^{318} was replaced by an Asn in the Asp^{87} mutant to obtain the $Asp^{87}Asn^{318}$ reciprocal mutant receptor, high affinity agonist ligand binding was restored (Fig 2, Table 1). The IC_{50} values for GnRH ($3.2 \pm 2.0 \times 10^{-8}$ M, 2 experiments) and GnRH-A ($1.3 \pm 0.8 \times 10^{-9}$ M, 2 experiments), a GnRH agonist, were comparable to those of the wild-type receptor ($2.0 \pm 1.5 \times 10^{-8}$ M, 4 experiments and $1.2 \pm 0.6 \times 10^{-9}$ M, 5 experiments, respectively). Ligand-stimulated PI hydrolysis has also restored, although the maximum response was reduced and the EC_{50} increased by 34-fold (Fig. 1, Table 1).

Unlike the TMH 2 mutants, the TMH 7 mutants had affinities comparable to the wild-type receptor. The IC_{50} values obtained for GnRH-A varied from 0.7 to 2.3×10^{-9} M for the mutants (Fig. 2; Table 1), compared to $1.2 \pm 0.6 \times 10^{-9}$ M for the wild-type receptor. The affinities for GnRH were also similar among this group of mutants (Table 1). However, the level of expression of the mutant receptors was lower for any of the mutants than for the wild-type receptor (B_0,

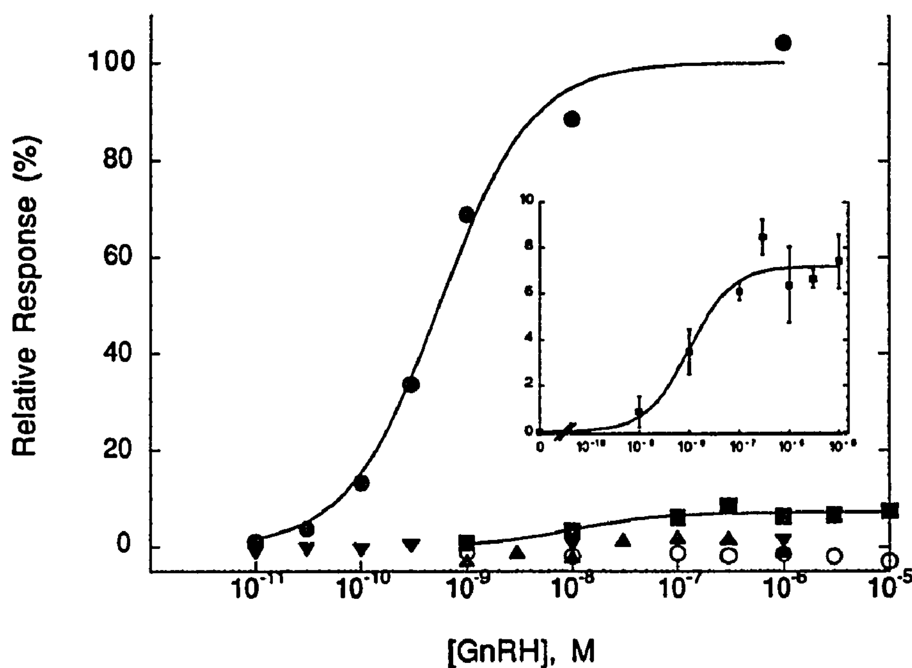


Fig. 1 GnRH-elicited PI hydrolysis in COS-1 cells transfected with the wild-type GnRHR (●), and TMH 2 mutant receptors of Ala^{87} (▼), Asp^{87} (O), Gln^{87} (▲), and $Asp^{87}Asn^{318}$ (■). The response was normalized to the maximum stimulation of the wild-type receptor. The concentration-response curve of $Asp^{87}Asn^{318}$ mutant receptor is also shown in the insert for clarity. Standard errors were within ±2% of the maximum wild-type response and are not shown in the main figure, which represents one of two to four replicate experiments, each done in triplicate.

Table 1, Characterization of the TMH 7 mutant GnRHRs

The affinity of GnRH-A for the wild-type and mutant receptors was determined by competition binding assay with transfected COS-1 cell membrane preparations. The maximum binding (B_0) and the maximal response of PI stimulation by the mutant receptors were normalized against those of the wild-type receptor obtained in the same experiment. N.D. denotes not determined due to insufficient specific binding. Values represent the mean ± standard error of 2-5 triplicate experiments.

Receptors	Binding			Coupling	
	B_0 (%)	IC_{50},*nM* GnRH	IC_{50},*nM* GnRH-A	EC_{50},*nM* GnRH	Maximum Response (%)
Wild-type	100	20±15	1.2±0.6	0.85±0.46	100
Ala^{318}	73.8±12.5	22±14	0.71±0.22	42±18	12±0.1
Glu^{318}	9.2±2.1	N.D.	2.3±1.4	20±10	36.6±6.5
Asn^{318}	62.4±5.0	27±18	0.97±0.23	45±29	38.9±11.7
$Asp^{87}Asn^{318}$	37.2±2.5	32±20	1.3±0.77	30±18	11.7±4.5

Table 1).

In order to investigate the contribution of the Asp^{318} locus to activation of the receptor, the concentration-response curve for coupling to PI hydrolysis was determined for each mutant. The wild-type receptor had an EC_{50} of 8.5±4.6 $\times10^{-10}$ M for GnRH, with the maximal response generally 5-10 fold above the basal level. In the TMH 7 mutant receptors and the reciprocal mutant $Asp^{87}Asn^{318}$ receptor, the EC_{50} was increased to a range of 2.0 to 4.5$\times10^{-8}$ M. The maximal response obtained varied from 12% of the wild-type maximum for the double mutant to 39% for the Asn^{318} construct (Fig. 3, Table 1). Notably, the EC_{50}

values obtained with all the mutants were not significantly different from each other and were comparable to the affinity of GnRH determined by radioligand binding. No correlation was observed between the level of receptor expression and the maximal stimulation obtained.

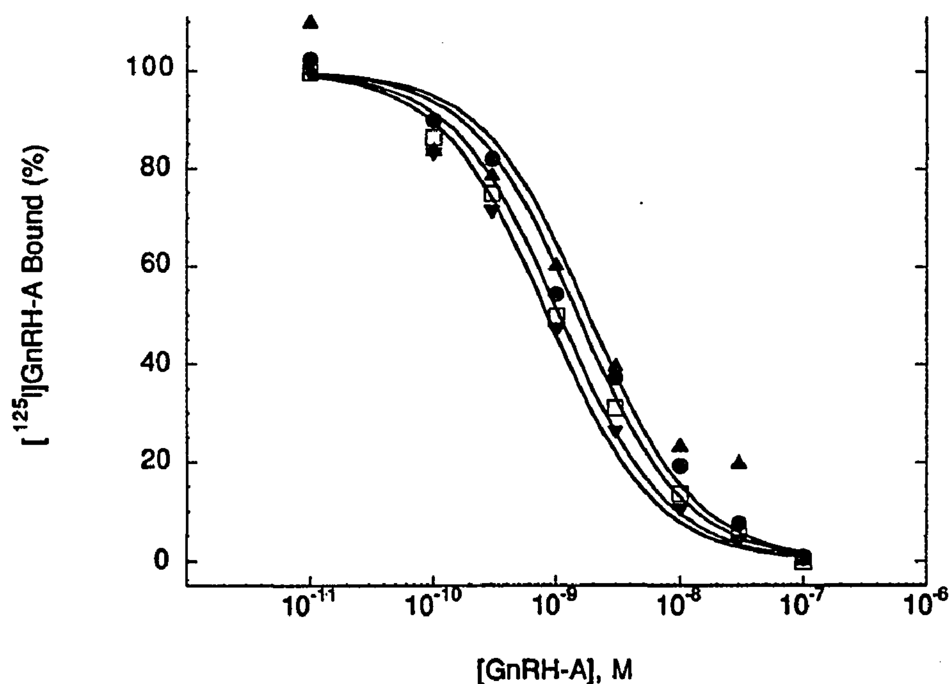


Fig. 2 Competition binding of the wild-type GnRHR (●) and the mutants, Ala^{318} (▼), Asn^{318} (□), and Asp^{87}Asn^{318} (▲). Each point represents the average of triplicate determinations. The experiment shown is representative of two to five data sets.

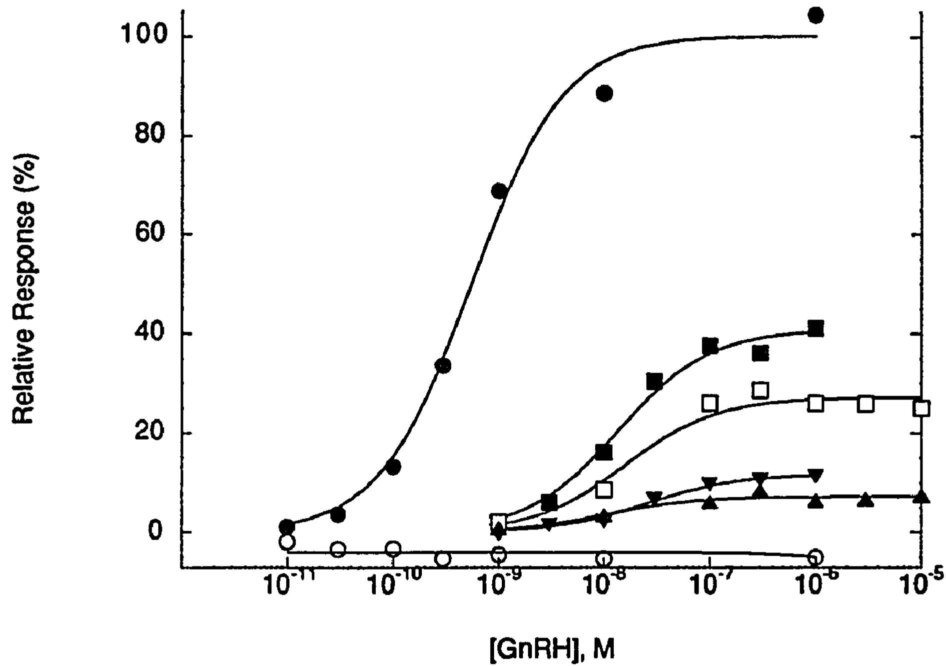


Fig. 3 GnRH-elicited PI hydrolysis in COS-1 cells transfected with the wild-type GnRHR (●), and TMH 7 mutant receptors of Ala^{318} (▼), Glu^{318} (■), Asn^{318} (□), and $Asp^{87}Asn^{318}$ (▲). Cells transfected with the expression vector pcDNAI/Amp alone (O) were also measured. The response is normalized to that of the wild-type receptor. The data is representative of two to four replicate experiments performed in triplicate.

Discussion

The TMH 2 and TMH 7 loci studied in this report contain an Asp and an Asn respectively in more than 95% of cloned GPCRs (Probst, Snyder et al. 1992). Mutation of the Asn in TMH 2 eliminates both detectable binding and stimulation of PI hydrolysis but introduction of a second mutation of Asn for Asp in TMH 7, which reverses these two residues and generates the pattern found in other GPCRs leads to a complete restoration of binding and a partial restoration of receptor coupling, confirming our previous report (Zhou, Flanagan et al. 1994). The demonstration of a reciprocal mutation which improves function disrupted by a single mutation is consistent with these loci being adjacent in space and provides a unique experimental system to characterize the inter-related functional roles of these two residues.

All the substitutions for Asp^{318} studied (Glu, Asn and Ala) generated receptors manifesting wild-type binding affinity and significant but reduced coupling to PI hydrolysis, consistent with the involvement of Asp^{318} in receptor activation. Interestingly, the substitutions for Asn^{318}, including the reciprocal mutant receptor, generated receptors with comparable EC_{50} values which were 24- to 53-fold higher than that of the wild-type receptor. All of the TMH 7 mutant receptors reduced the maximal response as well as the number of high-affinity binding sites, but no correlation was seen between the level of receptor expression and the maximal response obtained. Thus, the shift observed in EC_{50} is not likely to be directly attributable to the reduced level of receptor expression. None of the mutant receptors displayed significantly altered affinity for the labeled agonists. These observations suggest that the effect of the mutations in TMH 7 is a specific reduction of the efficiency of receptor activation that produces the rightward shift in EC_{50} and the reduction in maximal response. Therefore Asp^{318} must have a structural role during receptor

activation and its absence leads to the decreased signal transduction in the mutants.

With our previous demonstration of an Asn^{87} and Asp^{318} proximity and the restoration of high affinity binding by the reciprocal mutant receptor (Zhou, Flanagan et al. 1994), the structural role of Asp^{318} in GnRHR activation emerging from the present results suggests that a key function of Asp^{318} may be to maintain a proper interaction of TMH 7 with Asn^{87}. When this interaction is disrupted following the introduction of an Ala^{318}, the mutant receptor is reduced to a less efficient activation mechanism expressed in a 50-fold increase in EC_{50}. Other mutations introduced at the TMH 7 locus, including Glu, Asn, and the reciprocal mutation of $Asp^{87}Asn^{318}$, also fail to maintain the proper interaction due to differences in steric and hydrogen bonding properties, resulting in a similar degree of shift in EC_{50} and a reduction in E_{max}. Thus there is a lack of flexibility in the choice of amino acids at the TMH 7 locus required to maintain a favorable TMH 2 and TMH 7 interaction. It is noteworthy, however, that disruption of the interaction does not cause a complete loss of coupling.

The potential involvement of the TMH 2 Asn^{87} in interhelical interactions was also probed by this mutational study. Specifically, mutants were introduced to evaluate the side-chain properties such as its length, polarity, and steric exclusion. The differential effects of the substitution with these distinct amino acids serve to analyze the structural role of Asp^{318} and to deduce its mechanistic involvement. The surprisingly complete loss of measurable binding and coupling of the receptor with all the substitutions at this position indicate that all of the characteristics of Asn are required for the expression of a properly-folded, functional receptor. The lack of flexibility in side-chain requirements at this position is consistent with Asn serving a crucial role in helix:helix packing.

Analysis of the results obtained with the TMH 2 mutant receptors further

suggests that Asn^{87} in the GnRHR is involved in interactions with at least one other residue in addition to Asp^{318}. Whereas the Ala^{318} receptor retains wild-type binding affinities and the capacity to transduce signal, the Ala^{87} receptor is completely inactive. The very different effects of introducing an Ala at the two positions indicate that Asn^{87} is involved in a structurally critical interaction with a site in addition to Asp^{318}. Unlike the Asn^{87}-Asp^{318} interaction which is involved in optimized GnRHR activation, the Asn^{87} to non-Asp^{318} interaction seems to be critical for the functional expression of the GnRHR. A three-dimensional model of the receptor transmembrane helix bundle (Zhou, Flanagan et al. 1994) suggests that the conserved Asn in TMH 1 may be another residue interacting with Asn^{87}, a hypothesis that will require further experimental probing.

Both the stringent requirement for the residue at the TMH 2 locus and the capacity of the reciprocal mutation to restore function may be explained by the involvement of Asn^{87} in multiple interactions that serve distinct functions. Analysis of the results clearly shows that the reciprocal mutation did not confer any functional advantage over other TMH 7 mutant receptors by its ability to retain an optimized interaction between the TMH 2 and TMH 7 loci. Both the reciprocal mutant receptor and the Ala^{318} mutant receptor, which excludes a TMH 2 and TMH 7 interaction, displayed similar values in E_{max} and EC_{50} in the functional PI assay. On the other hand, the reciprocal mutation rescues the receptor from the complete lack of activity exhibited by the Asp^{87} mutation and is apparently favored in preserving receptor function compared to other TMH 2 mutant receptors. The results suggest that the correction by the reciprocal mutant receptor is not accomplished solely by restoring the TMH 2 and TMH 7 interaction. Rather, it is likely that the presence of a TMH 7 Asn makes possible an interaction with the Asp^{87} that also preserves the other hydrogen bond

required at this TMH 2 locus. Although an Asp^{87}-Asn^{318} interaction is not as favorable functionally as the wild-type Asn^{87}-Asp^{318}, it may be sufficient to recreate an Asn-like microenvironment at the TMH 2 locus by diminishing the effects of the negative charge of the Asp. In the Asp^{87} mutant receptor, the unfavorable interaction in the microenvironment containing Asp^{318} is likely to alter the positioning of the TMH 2 locus and disrupt its interaction with the additional site. Consistent with such precise spatial restrictions on the pattern of interaction with Asn^{87}, the Gln mutation, which has similar hydrogen bond potential to Asn, also causes a loss of receptor function. More dramatically, the Ala^{87} mutant receptor becomes totally inactive by eliminating any hydrogen bonding capacities in that locus.

As expected for a residue that serves multiple and critical functions in a receptor, the TMH 2 locus is highly conserved among GPCRs. Substitution of the conserved Asp has been shown to cause a variety of perturbations in other GPCRs studied, including a loss of GTP modulation of agonist binding (Chung, Wang et al. 1988; Surprenant, Horstman et al. 1992; Wang, Gallaher et al. 1993), poor receptor coupling (Chung, Wang et al. 1988; Fraser, Wong et al. 1989; Wang, Buck et al. 1991; Surprenant, Horstman et al. 1992), and a decrease in agonist affinity (Strader, Sigal et al. 1987; Chung, Wang et al. 1988; Fraser, Wong et al. 1989; Neve, Cox et al. 1991; Wang, Buck et al. 1991; Chanda, Minchin et al. 1993; Wang, Gallaher et al. 1993). Most of these results could be explained by a primary effect of the mutation on the mechanism of receptor activation which is impaired by the substitution of Asp. In the GnRHR, the replacement of the conserved TMH 2 Asp with an Asn is apparently compensated for by the appearance of a TMH 7 Asp in place of a conserved TMH 7 Asn. The involvement of other residues specific to the GnRHR in establishing the functionality of the GnRHR is very likely but the unique

structural interchange of the two residues in the GnRHR is consistent with a common activation mechanism in the GPCRs involving these two loci. Supporting the findings with the GnRHR, the mutational investigation of the serotonin receptor has indicated that the disruption of receptor function by the TMH 2 mutation can be restored by a reciprocal mutation involving the TMH 7 position (Chi, L., et al. manuscript in preparation).

Although the re-arrangement of the transmembrane helices is likely to be the mechanism underlying GPCR activation, there is only limited experimental data to support this hypothesis. Our study identifies two key residues, Asn^{87} and Asp^{318}, that are involved in GnRHR activation and implicated in a specific helix:helix interaction. The results supporting a role for the interaction between Asn^{87} and Asp^{318} in the mechanism of GnRHR activation also suggest that Asn^{87} forms a favorable interaction with at least one other locus that is absolutely required for the functional GnRHR expression. Further studies of such specific interactions should help evaluate the current understanding expressed in the three dimensional model of the receptor (Zhou, Flanagan et al. 1994) and reveal other crucial helix:helix interactions in the dynamic molecular process underlying receptor activation.

Chapter 6

A Locus of the Gonadotropin-Releasing Hormone Receptor Which Differentiates Agonist and Antagonist Binding Sites

Abstract

The decapeptide gonadotropin-releasing hormone (GnRH) binds to a heptahelical G protein coupled receptor and stimulates the hydrolysis of phosphatidyl inositol. Because a molecular model of the receptor predicted that Lys^{121} in the third transmembrane helix contributes to the binding pocket, the function of this side-chain was studied by site-directed mutagenesis. Substitution of Arg at this position preserved high agonist affinity and improved the efficiency of signal transduction. The maximal level of GnRH-stimulated inositol phosphate accumulation was significantly higher for the Arg^{121} mutant receptor, despite a lower level of expression compared to the wild-type receptor. Introduction of Gln at this position reduced binding below the limits of detection and led to a ~1000-fold increase in agonist EC_{50}. Antagonist affinity, in contrast, remained equivalent to that of the wild type receptor for both the Arg^{121} and Gln^{121} mutants. Leu and Asp at this position abolished both binding and signal transduction. The results indicate that Lys^{121} is involved in agonist, but not antagonist binding, and that ligand interaction at this site contributes to receptor activation.

Introduction

GnRH, a decapeptide secreted from neurons in the medial-basal hypothalamus, has a central role in regulating the mammalian reproductive system. GnRH induces its biological effect by interacting with high affinity pituitary receptors. cDNA cloning of the GnRH receptor (GnRHR) from five mammalian species (Eidne, Sellar et al. 1992; Kaiser, Zhao et al. 1992; Kakar, Musgrove et al. 1992; Reinhart, Mertz et al. 1992; Tsutsumi, Zhou et al. 1992; Chi, Zhou et al. 1993; Illing, Jacobs et al. 1993; Kakar, Rahe et al. 1993; Perrin, Bilezikjian et al. 1993) , has revealed that the receptor is a member of the large family of homologous seven transmembrane helix (TMH) G-protein-coupled receptors (GPCRs), which includes receptors for neurotransmitters and peptides (Kobilka 1992; Probst, Snyder et al. 1992; Strader, Fong et al. 1994).

Modulation of the pituitary-gonadal axis via the GnRHR has proven to be therapeutically important and extensive research has led to the development of several thousand peptide analogs (Karten and Rivier 1986). In contrast to some other peptides such as the tachykinins and cholecystokinin, for which small non-peptide analogs have been identified, all GnRHR ligands reported thus far are peptides. Delineation of the precise contact sites between GnRH and its receptor is critical for developing an understanding of the relationship of the GnRHR binding pocket to that of other neurotransmitter and peptide receptors, and for determining the molecular mechanisms underlying receptor activation. Ultimately this insight may lead to the design of novel GnRHR ligands.

We have previously reported that a mutation in TMH 7 of the GnRHR restored binding which had been eliminated by a mutation in TMH 2 (Zhou, Flanagan et al. 1994). The revertant character of mutations at these loci suggested that these two sites are in spatial proximity, a hypothesis that facilitated the refinement of a preliminary three-dimensional model of the

receptor helix bundle constructed according to a set of integrated methods (Ballesteros and Weinstein 1994). This receptor model predicts that Lys^{121}, located in the third TMH, is positioned in the ligand binding pocket of the receptor and would be accessible to GnRH. Lys^{121} is found in all five mammalian GnRH receptors at a locus which corresponds to the position of the conserved Asp of the cationic-amine receptors (Asp^{113} in the ß-adrenergic receptor), a residue required for high affinity neurotransmitter binding (Strader, Sigal et al. 1988; Fraser 1989; Strader, Fong et al. 1994). In order to investigate the role of Lys^{121} in ligand binding and activation of the GnRHR, a series of mutations were introduced at this position and the resulting receptors were expressed and characterized in COS-1 cells. The results identify the role of hydrogen-bonding at this position for the affinity of agonists, but not antagonists. Moreover the length of the side chain at this locus appears to determine the position of the agonist required for activation of the receptor.

Experimental Procedures

Reagents and peptides

All chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO). GnRH (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) and GnRH-A ([des-Gly^{10}, D-Ala^{6}, Pro-$ethylamide^{9}$]-GnRH), a GnRH agonist, were purchased from Bachem (Torrance, CA). Antagonist 27 ([Ac-D-$Nal(2)^{1}$,D-a-Me-pCl-Phe^{2},D-Trp^{3},N-e-Ipr-Lys^{5},D-Tyr^{6},D-Ala^{10}]-GnRH (Van der Spuy, Pillay et al. 1987) was a gift of R. Roeske.

Receptor constructs and site-directed mutagenesis

The coding region of the human GnRHR cDNA (Chi, Zhou et al. 1993) was digested with EcoRI/PstI and subsequently subcloned into the EcoRI/Xho I sites of the pAlter vector (Promego, Madison, WI). Mutations were introduced as previously reported (Zhou, Flanagan et al. 1994). Mutated receptor cDNA inserts, confirmed by automated DNA sequencing (Biorad, Hercules, CA) were isolated after EcoRI/BspI digestion and ligated to pcDNAI/Amp (Invitrogen, San Diego, CA) previously digested by the same restriction enzymes. One of the constructs studied, the Asp^{121} GnRHR, was generated from the mouse GnRHR using pAlter digested with EcoRI and XbaI (Zhou, Flanagan et al. 1994).

Tissue culture and DNA transfection

COS-1 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco-Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and seeded into 100 mM plates at 3 million cells per plate the day before the transfection. A mixture of 10 μg plasmid DNA and 100 μl lipofectamine (BRL, Gaithersburg, MD) was used to transfect each plate of cells in serum-free DMEM and an equal volume of media containing 20% FBS was added 5h after transfection.

Assay of inositol phosphate accumulation

Cells were harvested with trypsin 24 h following transfection and plated into 12-well dishes. 56 h following transfection, cells were labeled in DMEM containing 0.5 μCi/ml of [^{3}H]*myo*-inositol (NEN, North Billerica, MA). 12-16 h later, cells were washed and exposed to GnRH agonist (plus antagonist for Schild analysis) and 20 mM of LiCl for 45 min at 37C. Cell extracts in 10 mM formic acid at 4C were loaded on the Dowex ion-exchange column and eluted with 1 M ammonium formate and 0.1 M formic acid.

Radiolabeled ligand binding assay

Iodination of GnRH-A was catalyzed in the presence of Iodogen (Pearce Chemical Co., Rockford, IL) following published protocols (Laws, Beggs et al. 1990). 72 h after transfection, cells were scraped from the culture plates and centrifuged. Membrane pellets were stored at -80C for up to 2 months. 20-50 μg of crude membrane protein adjusted to 0.4 ml with assay buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% BSA) was incubated with 60,000 CPM of [^{125}I]GnRH-A (~2.8×10^{-11} M) at 4 C for 90 min and processed through a Brandel harvester (BRL, Gaithersburg, MD). Non-specific binding was determined in the presence of 10^{-7} M cold ligand.

Data and statistical analysis

The counts obtained from PI assays were plotted using Kaleidagraph software (Synergy Software, Reading, PA) on a Macintosh computer and fitted against the formula: $E = E_{max}/(1+EC_{50}/D)$, where E_{max} is the maximal response, EC_{50} is the concentration of the agonists that gives half of the maximal response, and D is the concentration of the agonist. To determine antagonist affinity, a Schild analysis (Arunlakshana and Schild 1959) was performed. The ratio of EC_{50} in the presence and absence of antagonist was plotted against the corresponding antagonist concentration and fitted to the

equation: $\log(EC_{50}'/EC_{50}-1) = \log[\text{antagonist}]-\log(Kd\ \text{antagonist})$. The slope of the best-fit line obtained was found not to be significantly different from the theoretical slope of 1 (two-tailed t-test). A paired t-test was used to evaluate the significance of differential PI stimulation by Arg^{121} and wild-type receptors. The IC_{50} was calculated by least squares fit to the equation $B = B_{max}/(1+D/IC_{50})$, where B_{max} is the total specific binding, IC_{50} is the concentration of the competing ligands that gives half of the total specific binding, and D is the concentration of the competing ligands added.

Results and Discussion

Affinity and Relative Efficacy of Agonists for the Wild-type and Mutant Receptors. In order to test the hypothesis that Lys^{121} interacts with GnRH, the effects on receptor function of substituting Arg, Gln, Leu and Asp at this position were investigated. These mutations were designed to probe the characteristics of this proposed interaction. Arg preserves a charged basic residue at this locus. Gln retains a hydrogen bond donor but not an ionic charge. Leu eliminates all polar and ionic side-chain interactions. Asp was introduced because it reverses the charge of Lys and is found in this position in neurotransmitter receptors. The progressive divergence from Lys of these substitutions allows the side-chain properties required at this locus to be evaluated.

Expression of the wild-type receptor in COS-1 cells generated high affinity binding of the radiolabeled agonist [^{125}I]GnRH-A (GnRH-A IC_{50}=2.26±0.85x10^{-9} M, 5 experiments). When Arg was substituted for Lys^{121}, affinity was comparable to that of the wild-type receptor (IC_{50}=2.24±1.30x10^{-9} M, 4 experiments, Table 1, Fig 1). However, with the substitution of Gln, Leu and Asp at this position, agonist binding was reduced below detectable limits. In order to provide further insight into the function of the mutant receptors, agonist-stimulated PI hydrolysis was also evaluated. The wild-type, Arg^{121} and Gln^{121} receptors were all able to mediate phosphatidyl inositol hydrolysis. No stimulation was detected in cells transfected with the Leu^{121} or Asp^{121} receptor mutants (Fig 2). The EC_{50} obtained with the Arg^{121} construct was comparable to that found with the expression of the wild-type receptor. The EC_{50} obtained with the Gln^{121} receptor, however, was three orders of magnitude higher (Fig 2 and Table 1). The EC_{50} values obtained for stimulation with GnRH-A of the wild-type, Arg^{121}, and Gln^{121} mutants showed the same trend, including the large

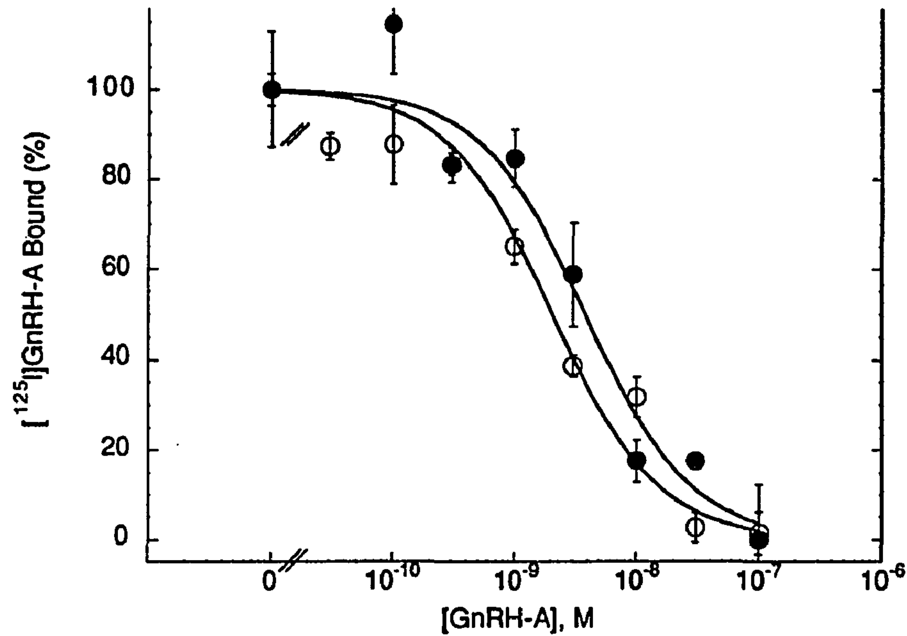


Fig. 1 Agonist binding to the wild-type (O) and Arg^{121} mutant (●) GnRHRs expressed in COS-1 cells.

The maximum binding of the Arg^{121} mutant receptor, at 30-40% of that of the wild-type receptor, was normalized to 100%. Data represent mean and standard error of triplicate determinations from one displacement binding experiment, as described under "Experimental Procedures". The data is representative of five replicate experiments.

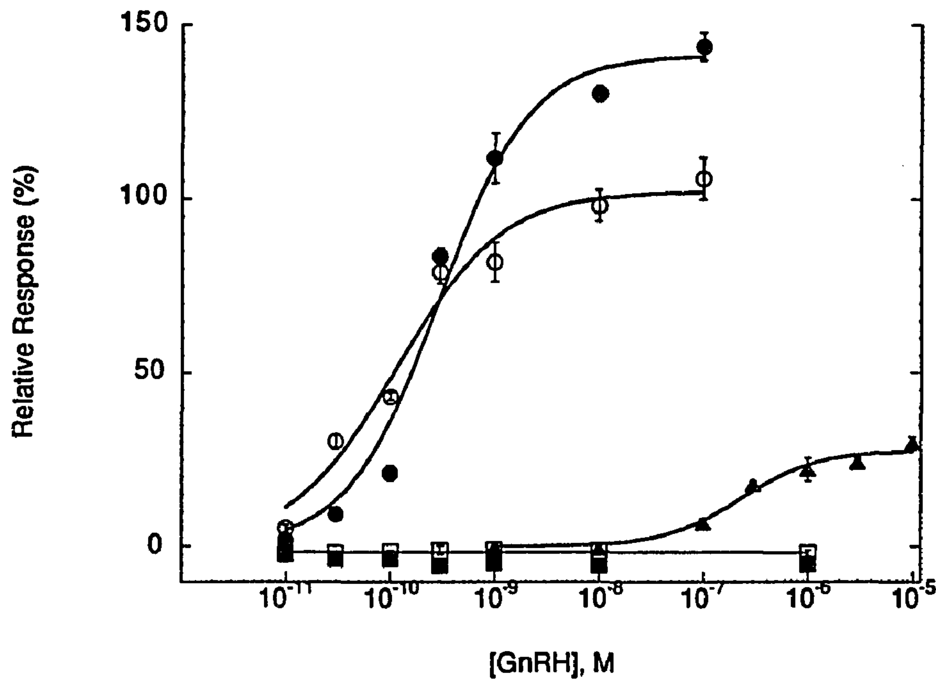


Fig. 2 GnRH-stimulated PI hydrolysis in COS-1 cells expressing mutant GnRHRs.

The cells were transfected with the expression vector pcDNAI/Amp (■), Leu^{121} (□), Gln^{121} (▲), Arg^{121} (●), or wild-type (O) GnRHRs and the resulting increase in intracellular inositol phosphates was measured. The response was normalized to that obtained in the wild-type receptor, which usually gave a five- to ten-fold stimulation over basal level. Data shown are the mean± standard error. Each curve is representative of four to five replicate experiments.

Table 1 Ligand binding and PI hydrolysis stimulation by wild-type and Lys[121] mutant GnRHRs.

IC_{50} values were obtained in GnRH-A binding to the receptors in competition with [^{125}I]GnRH-A as described under "Experimental Procedures". K_d values were calculated based on Schild analysis. N.D., not determined. No specific binding was obtained with Gln[121] mutant receptor (------). Data are presented as mean±standard error of two to five experiments, each performed in triplicate.

Receptors	Affinity (M) -log(IC_{50})	Affinity (M) -log(IC_{50})	Affinity (M) -log(K_d)	EC_{50} -log(EC_{50})	EC_{50} -log(EC_{50})
	GnRH	GnRH-A	Ant27	GnRH	GnRH-A
wild-type GnRHR	9.05±0.13	8.68±0.19	8.61±0.55	9.75±0.26	9.92±0.15
Arg[121] GnRHR	N.D.	8.81±0.53	8.43±0.17	9.63±0.14	9.54±0.23
Gln[121] GnRHR	------	------	9.14±0.55	6.81±0.47	6.35±0.13

relative increase for the Gln[121] mutant (Table 1).

To help evaluate the cause of the increased EC_{50} values obtained with the Gln[121] receptor, the relationship between receptor expression and EC_{50} was examined. In several receptor expression systems, the EC_{50} has been found to depend on the number of receptors expressed (Mei, Lai et al. 1989; Adham, Ellerbrock et al. 1993). Such findings are compatible with the presence of a large receptor reserve in these systems (Kenakin 1993). If the wild-type GnRHR was expressed in COS-1 cells with a high proportion of spare receptors, the increased EC_{50} observed with the Gln[121] receptor could in principle be due to a marked decline in the level of mutant receptor expression. To test this hypothesis, cells were transfected with decreasing amounts of wild-type DNA construct such that the maximal stimulation was reduced below that

obtained with the Gln^{121} receptor. No change was observed in the range of EC_{50} values, which varied only between 1.65 and $1.83x10^{-10}$ M (Fig. 3). Furthermore, the EC_{50} of 3 x 10^{-7} M obtained with the Gln^{121} receptor is much greater than the radioligand competition binding affinity of GnRH for the wild-type receptor ($IC_{50}=0.9x10^{-9}$ M, Table 1), a difference which can only be explained by reduced agonist affinity for the mutant receptor. Thus, little if any of the 1000-fold increase in EC_{50} observed with the Gln^{121} receptor is attributable to a loss of receptor reserve. Therefore, the increase in EC_{50} observed with the substitution of Gln must reflect decreased agonist affinity.

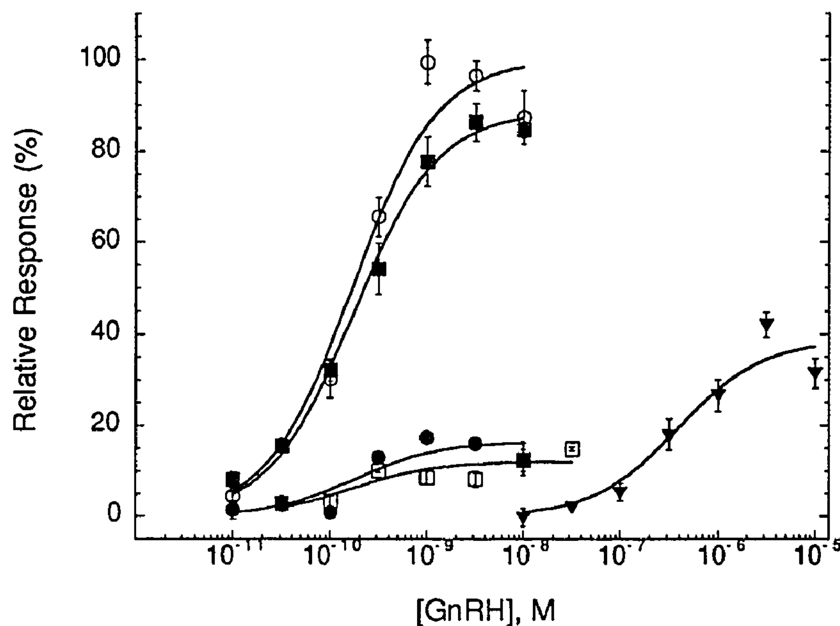
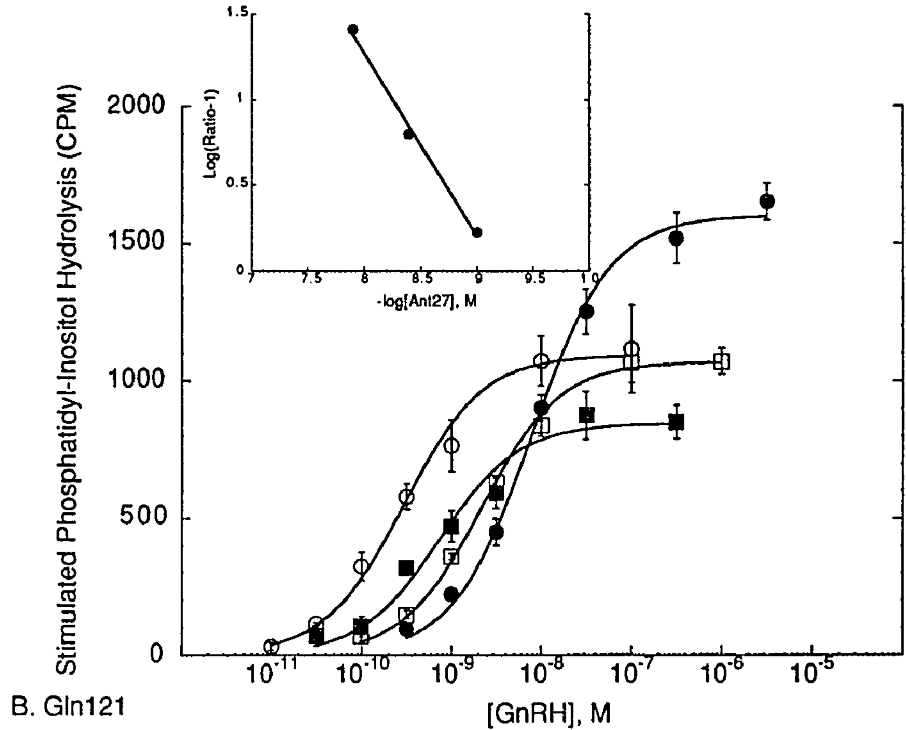


Fig. 3 Effect of varying receptor expression on PI hydrolysis dose-response relationships.
COS-1 cells were transfected with variant amount of the wild-type receptor DNA, from the standard 10 μg (○) to 3.3 μg (■), 1.3 μg (●), and 1 μg (□) per 100 mM plate, all at the same ratio of DNA/lipofectamine. In the control experiment, 10 μg of Gln^{121} mutant DNA construct (▼) was used for transfection as described under "Experimental Procedures". Data represent means and standard error of triplicate measurements.

Antagonist Binding Properties Suggest Differences From Agonist Recognition. The effects of the Lys^{121} substitutions on antagonist binding were also investigated. The lack of [^{125}I]GnRH-A binding to the Gln^{121} receptor prevented a direct measurement of its affinity for antagonist in competition binding. We have previously studied the expressed mouse GnRHR using radiolabeled antagonist (Zhou, Flanagan et al. 1994). This approach was not possible for the human receptor because of high non-specific binding obtained with labeled antagonist and a relatively lower level of human receptor expression. Therefore a Schild analysis was performed which allows the assessment of antagonist affinity from the functional response (Arunlakshana and Schild 1959). Whereas the agonist EC_{50} obtained with the Gln^{121} receptor was ~1000 fold higher than those obtained with the wild-type and Arg^{121} receptors, the antagonist affinities for the three receptors were indistinguishable (Fig. 4 and Table 1). In contrast to high affinity agonist binding which appears to require a positive charge at this position, antagonist binding is not sensitive to this locus of the receptor. Such a distinction of GnRHR binding sites for agonists and antagonists is consistent with recent photoaffinity-labeling studies of the GnRH receptor which suggested that agonists and antagonists are oriented

A. Wild-type



B. Gln121

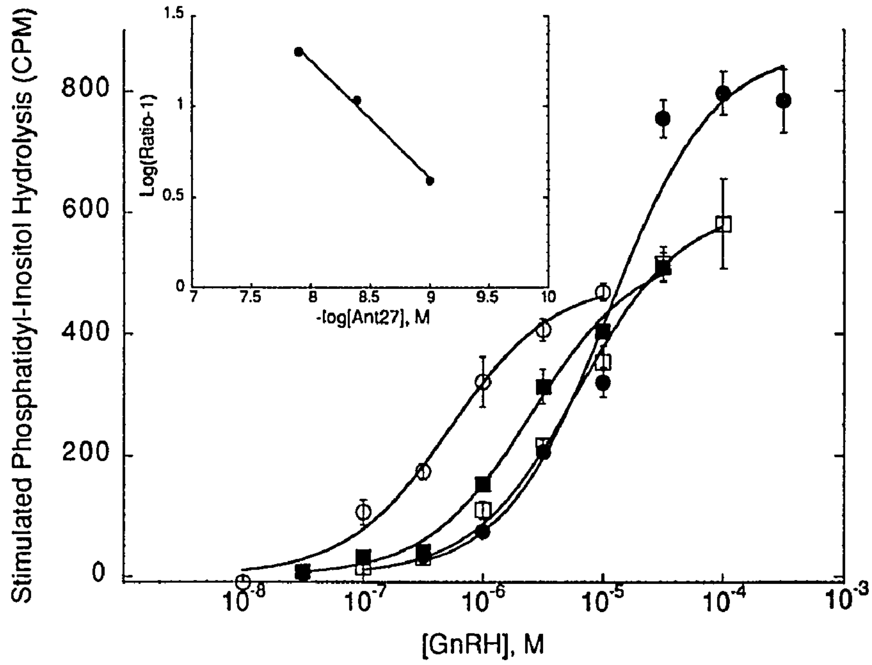


Fig. 4 Blockade by antagonist of GnRH stimulation of PI hydrolysis by the wild-type GnRHR (*panel A*) and Gln^{121} mutant receptor (*panel B*).
GnRH-stimulation of PI hydrolysis was performed in the absence of Ant27 (○), or in the presence of 10^{-9} M (■), $4X10^{-9}$ M (□), or $1.25X10^{-8}$ M (●) of Ant27. *Inset*, Schild plot of the data shown. The ratio represents EC'_{50}/EC_{50}, as described under "Experimental Procedures". Ant27 alone did not cause any PI stimulation at a concentration up to 10^{-6} M (data not shown). Data are representative one of three replicate experiments, each performed in triplicate.

differently in the receptor (Janovick, Haviv et al. 1993).

Mammalian GnRH contains an Arg in position 8, whereas chicken I GnRH, which has low affinity for the mammalian receptor, has a Gln in this position (Millar, Flanagan et al. 1989). We have previously found that an acidic residue in the third extracellular loop of the GnRH receptor is required for this selectivity for mammalian Arg^{8}-GnRH, and have proposed a direct interaction between Arg^{8} of GnRH and this extracellular locus (Flanagan, Becker et al. 1994). The effect of having an acidic residue at this position, however, is smaller for constrained GnRH agonist analogs like GnRH-A. Thus, substitution of Gln for Glu^{301} in the mouse GnRH receptor induces a 50-fold decrease in the affinity for GnRH but only a 4-5 fold change in the affinity for GnRH-A. These results are consistent with the presence of different, although presumably overlapping, binding sites for different classes of agonists. The binding site of GnRH includes the acidic residue in the third extracellular loop, whereas the binding site of GnRH-A has minimal involvement of this locus. Agonist interaction sites which play a fundamental role in positioning the pharmacophore for receptor activation, or in transmitting the activation itself, are likely to be conserved among all agonist classes. Because both GnRH and

GnRH-A activity require a positive charge at Lys^{121}, interaction with this site is likely to play a crucial role in receptor activation. Taken together, these considerations identify at least three types of determinants for ligand-receptor interaction: residues required for antagonist binding, residues required for binding of certain agonists, and residues involved in binding of all agonists. Clarification of the role of various receptor loci in ligand interaction should facilitate the understanding of the mechanism of agonist activation of the receptor.

The Role of the 121 Locus in GnRHR in Determining the Nature and Consequences of Interactions with Ligands. With mutation of the ligand docking site, the effects of each mutation must correlate with the altered energy of interaction arising from the side-chain properties of the amino acids introduced. Analysis of this correlation in the present study provides insight into the nature of the interaction between GnRH and the residue at position 121. A basic amino acid, either Lys or Arg, is required for high affinity agonist binding, yet a direct ionic interaction can be excluded by the lack of a suitable counterion on the ligand. The results, however, are consistent with a hydrogen bond interaction. Both Lys and Arg are strong hydrogen bond donors (Jeffrey and Saenger 1991). The presence of the weaker hydrogen-bond donor Gln at this position would reduce the strength of this interaction and lead to the decreased agonist affinity observed. The Leu and Asp mutant receptors lack hydrogen-bond donors at this position, and both demonstrate no detectable agonist binding or coupling. The results are therefore consistent with a charge-strengthened hydrogen bond as the underlying mechanism for Lys^{121} to interact with GnRH. Because Lys^{121} contributes to the high-affinity of agonists but not antagonists, a limited number of candidate hydrogen-bond acceptors in GnRH can be proposed . Structure-activity studies of GnRH have indicated the

importance of His[2] and Trp[3] for agonist activity (Karten and Rivier 1986) and these two residues, therefore, represent the best candidates for interacting with Lys[121]. In forming the hydrogen bond, Lys[121] could interact with the electron-dense aromatic rings of His[2] or Trp[3], the type of interaction postulated to be responsible for the binding of the non-peptide antagonist CP96345 to the substance P receptor (Fong, Cascieri et al. 1993). Alternatively, Lys[121] may hydrogen bond to the polar imino group of His[2]. Further molecular modeling and experimental studies should allow the precise site of interaction on GnRH to be determined.

The two contact sites between GnRH and the receptor identified to date, Lys[121] and Glu[301], have similarities in their tolerance for variability at each position. In both cases little alteration in affinity is induced by substitution with a similarly charged residue. The Glu in extracellular loop 3 of the mouse GnRHR, which is involved in high affinity GnRH binding, can be replaced by an Asp with little effect on receptor affinity (Flanagan, Becker et al. 1994). In the present study, we find that the Arg[121] substitution, presenting the hydrogen-bond donor group at either ε or η positions that can mimic the ξ position of the group in Lys, gives comparable affinity. In either locus of the GnRHR, substitution by Gln significantly decreases GnRH affinity. Thus alterations in the length of the side-chain of charged residues in TMH 3 and in the third extracellular loop are both well tolerated, suggesting flexibility in the spatial constraints for the binding interaction.

In contrast to affinity, the receptor activation mechanism appears to be sensitive to the length of the side chain at position 121. Thus, the maximal level of GnRH-stimulated inositol phosphate accumulation obtained with expression of the Arg[121] receptor was significantly higher than that generated by the wild-type receptor in all experiments (paired two-tailed t-test $p<.02$, $n=5$

experiments). Expression of the Gln^{121} receptor led to a lower maximal stimulation. Because the Gln^{121} receptor was undetectable in radioligand binding, the possibility that the reduction in Emax was due to a lower level of expression of this receptor cannot be definitively excluded. In the case of the Arg^{121} construct, however, the higher level of stimulation was accompanied by a more than 50% reduction in the level of receptor expression, indicating that the activated state of the Arg^{121} receptor is more efficient at G-protein coupling than the activated wild-type receptor.

A qualitatively similar effect of side chain length on efficacy was observed In the ß-adrenergic receptor. Asp^{113}, which is located at a position homologous to that of Lys^{121} in the GnRHR, serves as the counterion for binding of the catecholamine head group of the ligand. Substitution of Glu at this position led to the development of partial agonist activity from antagonists for the wild-type ß-adrenergic receptor (Strader, Candelore et al. 1989a). In the GnRHR, replacing Lys^{121} with Arg leads to an augmentation of the efficiency of signal transduction by GnRH. In both receptors, altering the length of this charged side chain leads to an alteration of measured drug efficacy. This similarity between a neurotransmitter and a peptide receptor suggests that the interaction with this locus can contribute to activation, possibly by serving to position the component of the ligand which triggers the receptor.

We have here identified a transmembrane domain contact site in the GnRHR that is specifically involved in docking GnRH agonists. We have previously provided evidence for the proximity of specific side chains in helix 2 and helix 7 (Zhou, Flanagan et al. 1994) and have proposed another site of GnRH docking in the third extracellular domain (Flanagan, Becker et al. 1994). These studies provide insight into the structure of the receptor and of the GnRH binding site. The results obtained constitute a useful basis for extending and

refining an experimentally testable model of the structure of the receptor-hormone complex that will make possible the elucidation of the molecular dynamics of receptor activation.

Chapter 7

Perspective and Conclusions

The work presented in previous chapters contains two major components, molecular characterization of the GnRHR and mutational investigation of the cloned receptor. In this section, the findings described previously will be summarized and placed within the context of what has been learned through the study of other G protein coupled receptors.

Cloning and Structural Features of the Mammalian GnRHRs

Characterization of the GnRHR dates back more than two decades, beginning with elucidation of the peptide sequence of GnRH (Matsuo, Baba et al. 1971; Burgus, Butcher et al. 1972). Major areas that were developed to study GnRH and its interaction with the GnRHR include *in vivo* and *in vitro* administration of GnRH analogs to modify gonadotropin release and pituitary membrane binding assays with radiolabeled GnRH analogs. These studies provided considerable insights into the biochemistry and pharmacology of the GnRHR and facilitated the GnRHR cloning. Fundamental insights about the signal transduction of the receptor such as its coupling to G proteins, phospholipase C, and calcium mobilization has been obtained (Stojilkovic, Reinhart et al. 1994).

Cloning of the first mammalian GnRHR was achieved by relying on the postulated homology between the GnRHR and other G protein-coupled receptors. Enriched mRNAs from αT3-1 cells, a transgenic gonadotrope tumor cell line (Windle, Weiner et al. 1990), allowed a sensitive detection of the GnRHR signal electrophysiologically when expressed in *Xenopus* oocytes (Sealfon, Gillo et al. 1990). Sets of PCR oligos compromising conserved

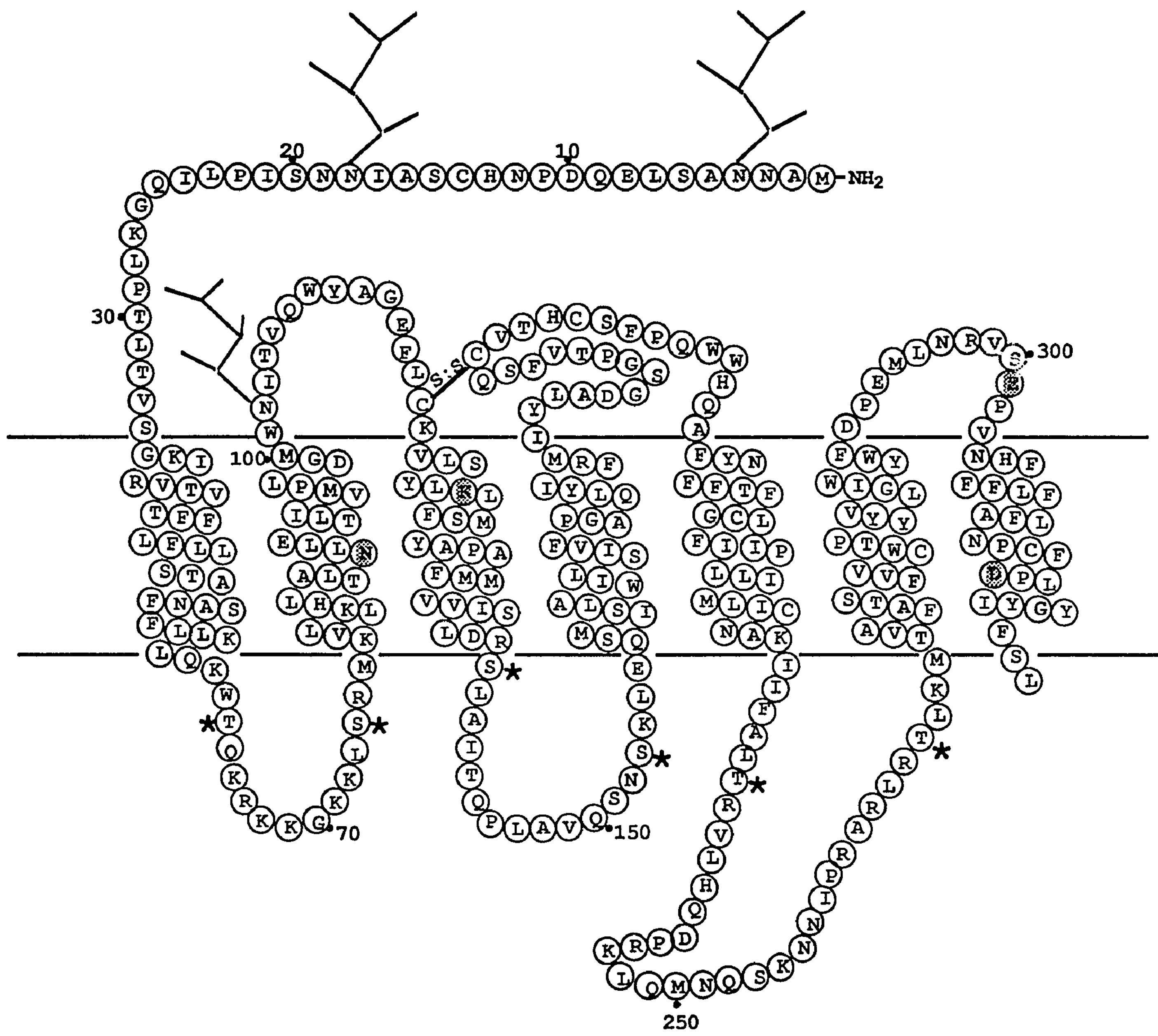

NH2
10
20
30
70
100
150
250
300
S:S

Fig. 1 Primary structure of the mouse GnRHR showing the proposed topology of the seven transmembrane helices.

The extracellular domain is at the top of the figure. The locations of Asn^{87}, Lys^{121}, Glu^{301}, and Asp^{318} studied or discussed in the this chapter are indicated by shadowed circles. The postulated membrane boundaries are represented by solid lines. Potential extracellular glycosylation sites and intracellular protein kinase phosphorylation sites are indicated by branches and stars, respectively. A disulfide bond bridge is shown to link the conserved Cys residues found in most GPCRs.

nucleotide sequences of the GPCRs amplified a cDNA fragment from the cytoplasmic mRNA of the αT3-1 cells which was later labeled to screen the αT3-1 cell cDNA library. Several positive clones with open reading frames encoding heptahelical receptor peptides were obtained. Microinjection of the mRNA derived from the cDNA clones into the *Xenopus* oocytes triggered a GnRH-dependent chloride channel conductance and resulted in a membrane preparation that contained high-affinity GnRH binding sites (Tsutsumi, Zhou et al. 1992).

The DNA sequence of the cloned mouse GnRHR was later independently confirmed by other researchers (Reinhart, Mertz et al. 1992) and in other species, including human (Kakar, Musgrove et al. 1992; Chi, Zhou et al. 1993), rat (Eidne, Sellar et al. 1992; Kaiser, Zhao et al. 1992; Perrin, Bilezikjian et al. 1993), sheep (Illing, Jacobs et al. 1993), cow (Kakar, Rahe et al. 1993), and pig (Weesner, G.D. and Matteri, R.L., 1994, from unpublished Genebank release). The deduced open reading frame of the GnRHR cDNA encodes a peptide of either 327 amino acids in mouse and rat, or 328 in non-rodent species (Fig. 1). Like other GPCRs, the primary structure of the cloned GnRHRs

```
                                                +----TMH 1----50
hGnRHR  MANSASPEQN QNHCSAINNS IPLMQGNLPT LTLSGK|IRVT VTFFLFLLSA
oGnRHR  ---GD--DQN E-------S- ---TP-S--- ------|---- ---------T
bGnRHR  ----D----- E-------S- ---TP-S--- ------|---- ---------T
pGnRHR  ---------- --------S- -L-T------ ----PN|---- ---------T
rGnRHR  ---N--L--D ---------- ---T--K--- ------|---- ---------T
mGnRHR  ---N--L--D P--------- ---I-GK--- --V---|---- ---------T
                                                +---------------

        ----------+        I1        +----------TMH 2-------100+
hGnRHR  TFNASFLLKL|QKWTQKKEKG KKLSRMK|LLL KHLTLANLLE TLIVMPLDGM|
oGnRHR  I--T------|-N---R---R ----K--|V-- ---------- ----------|
bGnRHR  I--T------|-N---R---R -------|--- ---------- ----------|
pGnRHR  A---------|-----R------------|V-- ---------- ----------|
rGnRHR  A--A---V--|-R----RK-- -------|V-- ---------- ----------|
mGnRHR  A---------|------RK-- -------|V-- ---------- ----------|
        ----------+                  +-------------------------+

                E1        +------------TMH 3-------+    I2     150
hGnRHR  WNITVQWYAG ELLCK|VLSYL KLFSMYAPAF MMVVISL|DRS LAITRPLALK
oGnRHR  ---------- -----|----- ---------- -------|--- ----------
bGnRHR  ---------- -----|----- ---------- -------|--- ----------
pGnRHR  ---------- -F---|----- ---------- -------|--- --------V-
rGnRHR  ---------- -----|----- ---------- -------|--- --V-Q---VQ
mGnRHR  ---------- -----|----- ---------- -------|--- --I-Q---VQ
                          +------------------------+

                +---------TMH 4------------+       E2        200
hGnRHR  SNSKVGQS|MV GLAWILSSVF AGPQLYIFRM I|HLADSSGQTK VFSQCVTHC
oGnRHR  ----L--F|-I ----L---I- --------G- -|----D----E G--------
bGnRHR  ----L--F|-I ----L---I- --------G- -|----D----E G--------
pGnRHR  ---RL-RF|-I ----L---I- ---------- -|---------E G--------
rGnRHR  -K--LER-|-I S--W---I-- ---------- -|Y---G--PA  ---------
mGnRHR  ----LE--|MT S------I-- ---------- -|Y---G--P-  ---------
                +--------------------------+

                     +----------TMH 5-----------+          250
hGnRHR  SFSQWWHQAF YN|FFTFSCLF IIPLFIMLIC NAKIIF|TLTRVLHQDPHELQ
oGnRHR  --P------- --|-------- ----L----- ------|-----------K--
bGnRHR  --P------- --|-------- ----L--V-- ------|-----------K--
pGnRHR  --P------- -D|-------- ----L----- ----M-|------Q----N--
rGnRHR  --P----E-- --|-------- ----L----- ------|A---------RK--
mGnRHR  --P------- --|----G--- ----L----- ------|A---------RK--
                     +--------------------------+

            I3            +---------TMH 6-----------+    E3   300
hGnRHR  LNQSKNNIPR ARLKT|LKMTV AFATSFTVCW TPYYVLGI|WYWFDPEMLNRL
oGnRHR  ---------Q ---R-|----- ---------- --------|------D-V--V
bGnRHR  ---------- ---R-|----- ---------- --------|------D-V--V
pGnRHR  ---------- ---R-|----- ----A-I--- ---L----|--------V--V
rGnRHR  ---------- ---R-|----- ------VI-- --------|-----------V
mGnRHR  M--------- ---R-|----- ------V--- --------|-----------V
                          +-------------------------+

              +--------TMH 7---------328
hGnRHR  SDPVNH|FFFL FAFLNPCFDP LIYGYF|SL
oGnRHR  ------|---- ---------- ------|--
bGnRHR  ------|---- ---------- ------|--
pGnRHR  ------|---- ---------- ------|--
rGnRHR  -E----|---- ---------- ------|--
mGnRHR  -E----|---- ---------- ------|--
              +----------------------+
```

Fig. 2 Structure comparison of the cloned mammalian GnRHRs.
The primary structure of the sheep (o), cow (b), pig (p), rat (r), and mouse (m) GnRHRs are compared to that of the human GnRHR (h) and different amino acids are shown. The putative transmembrane helices, separated by intracellular (I1-I3) and extracellular (E1-E3) loops, are boxed. Number refers to the human GnRHR. See text for references.

contains seven putative transmembrane helices (TMH) based on hydrophobicity analysis (Fig. 2).

Almost all GPCRs are glycoproteins (Kobilka 1992; Probst, Snyder et al. 1992). Consistent with this general observation, two consensus glycosylation sites, located at the receptor N-terminus and the first extracellular loop, respectively, are found in all cloned mammalian GnRHRs (Fig. 1) and a third one is found in rodent receptors at the beginning of the coding region. Like that of neurotransmitter receptors (Rands, Candelore et al. 1990; von Koppen and Nathanson 1990) and other peptide receptors (Liu, Davis et al. 1993), glycosylation of the GnRHR is not required for high affinity binding although mutation of both of the N-terminal glycosylation sites caused reduced expression to the cell surface of the mutant mouse GnRHRs (Davidson et al. 1994).

Structural comparisons reveal some unusual features of the GnRHR. With a short N-terminus and the lack of the cytoplasmic carboxyl tail, the GnRH receptor is the shortest receptor molecule in the GPCR family. The absence of the carboxyl terminus, which is present in all other cloned GPCRs and is believed to be involved in their coupling to G proteins and desensitization in response to agonist treatment (O'Dowd, Hnatowich et al. 1988; O'Dowd, Hnatowich et al. 1989; Sanchez-Yague, Rodriguez et al. 1992; Nussenzveig,

Heinflink et al. 1993), may contribute to the unique regulatory properties of the GnRHR (Davidson, Wakefield et al. 1994). Unique structure and function relationships in the GnRHR were also suggested by the replacement of conserved amino acids. For example, the TMH 2 Asp and TMH 7 Asn, represented in more than 95% of cloned GPCRs, are interchanged in the GnRHR. In TMH 3, the conserved Asp-Arg-Tyr motif found in most GPCRs is replaced by Asp-Arg-Ser in the GnRHR. The unusual structural features of the GnRHR present a unique system to investigate the structure and function relationship of the GPCR family.

Gene Structure of the GnRHR and the Alternative Transcripts

In order to confirm the cDNA sequence of the mammalian GnRHRs and to characterize the receptor promoter region and its unique regulatory properties, the gene structure of the GnRHR was analyzed. Unlike the genes encoding many GPCRs (reviewed by (Probst, Snyder et al. 1992)), the mouse GnRHR gene contains at least three exons and two introns (Zhou and Sealfon 1994). The gene spans at least 22 kb in the mouse genome as the first two exons are separated by a large intron longer than 15 kb (Fig. 2 of Chapter 3). A similar structural composition of the rat receptor gene is suggested by the Southern blot mapping of the rat genomic DNA (Zhou and Sealfon 1994). Recently, the gene structure of the human GnRHR has been reported. Similar to that of the mouse gene, the coding region of the human GnRHR is encoded by three exons with a large intron separating the first two exons (Fan, Jeung et al. 1994). The conserved genomic structure of mammalian GnRHRs so far cloned indicates a close evolutionary kinship of these receptors, which is consistent with the conserved primary structure of GnRH in mammalian species.

During cDNA cloning of the mouse GnRHR, several variant forms of the receptor were identified in the αT3-1 cell cDNA library (Zhou and Sealfon 1994), which represented alternative transcripts of the gene (Fig. 3 of Chapter 3). One of the alternative transcripts, WZ16, lacks exon 3 but contains an additional 800 bp of genomic sequence (exon 2') following exon 2. This alternative transcript resembles the structure of the dopamine D_3 receptor transcripts identified (Fishburn, Belleli et al. 1993). Like that of many alternative transcripts described in other GPCRs, the significance of the finding in the GnRHR remains to be elucidated. None of the alternative GnRHR transcripts contains a complete coding region for all seven transmembrane helices or is functional following transfection into the COS-1 cells (Zhou and Sealfon,

unpublished results). However, the possibility remains that partial receptors encoded by the alternative transcripts may play a role in regulating the function of other GPCRs through inter-molecular interactions. The *In vitro* study of intermolecular interactions between chimeric muscarinic and adrenergic receptors has indicated that reconstitution of functional seven transmembrane domain receptors from separate receptor subunits containing different helices is possible (Maggio, Vogel et al. 1993a; Maggio, Vogel et al. 1993b).

Ligand Binding Pockets of GnRHR

The potential contribution of the TMH 3 Lys^{121} to ligand binding was characterized in the human GnRHR (Chapter 6). Lys^{121} is located at a position homologous to that of the conserved Asp in neurotransmitter receptors. The strategy for this study was to analyze the requirement for the side-chain properties of Lys^{121} in preserving high-affinity ligand binding. In particular, the involvement of charge, hydrogen-bond formation, and hydrophobic exclusion of the Lys residue was examined by the substitution for Arg, Gln, Leu, and Asp. As described in Chapter 6, among the mutant receptors studied, only the Arg^{121} GnRHR preserves measurable agonist binding whereas Arg^{121} and Gln^{121} GnRHRs are the only two mutant receptors capable of eliciting PI hydrolysis. Although the Arg^{121} mutant receptor displays IC_{50} and EC_{50} values similar to those of the wild-type receptor, the Gln^{121} mutant receptor increases the EC_{50} by 1000 fold. The results indicate a loss of agonist affinity as a result of the substitution made at the TMH 3 locus and are consistent with a role of the Lys^{121} in binding to the GnRH agonists.

The lack of measurable binding by the Gln^{121} mutation prevents a direct measurement of the agonist affinity by membrane binding assay. However, conclusions about the affinity of GnRH agonists for this mutant can be derived from functional assays. The EC_{50} observed with Gln^{121} is approximately 100-fold larger than the IC_{50} value of the wild-type receptor. Even though a reduced expression of the Gln^{121} mutant receptor may be responsible for a reduction in E_{max} and an increase in EC_{50} according to the classical receptor theory (Clark 1926), the EC_{50} should not be larger than the affinity of the receptor for the agonist. Therefore, the large increase in EC_{50} cannot be fully accounted for by reduced receptor expression and indicates that the Gln mutation has reduced the affinity for GnRH agonists.

A parallelism between a gradual loss of agonist affinity and a weakening hydrogen-bond donor at the TMH 3 locus suggests the presence of a direct hydrogen bond formation between Lys^{121} and GnRH. However, alternative explanations are also compatible with the current data. For example, it is possible that Lys^{121} may interact with another residue that directly binds agonists. The loss of affinity with Lys mutation could also result from the loss of the structural contribution of Lys to the formation of the binding pocket. With additional studies such as chemical labeling and cooperative substitution of the GnRH agonists and the Lys^{121} residue, the mechanism of a direct versus an indirect role of Lys^{121} in ligand binding may be distinguished.

Structure and function analysis of GnRH molecules has indicated that Arg^{8}, the hallmark of the mammalian GnRH, is required for conferring high-affinity binding to the mammalian GnRHR (Millar, Flanagan et al. 1989). An acidic residue (Glu^{301}) located at the third extracellular loop of the mouse GnRHR has been studied and found to be conferring the specificity of GnRH analogs with substitutions at Arg^{8}. Mutation of this Glu to Gln reduces the affinity for GnRH dramatically whereas conversion of most other extracellular acidic residues into their respective Asn or Gln is without significant effect in GnRH binding (Flanagan, Becker et al. 1994). Unlike the wild-type GnRHR, the Gln^{301} mutant receptor loses the specificity for GnRH analogs with substitutions at the eighth position. The data support an ionic interaction between Arg^{8} of GnRH and Glu^{301} of the GnRHR (Flanagan, Becker et al. 1994).

Although the Gln^{301} substitution decreases the affinity for GnRH by 50-fold, the mutation has little effects on the affinity for GnRH superagonists in the membrane binding assay (Flanagan, Becker et al. 1994). As a distinct group of synthetic GnRH analogs, superagonists have in common a large D-amino acid at position 6 that usually improves their affinity and potency (Karten and Rivier

1986). It has been postulated that a D-amino acid stabilizes the β-bend formed among Ser^4-Tyr^5-Gly^6-Leu^7 of the natural GnRH molecule and favors the binding of GnRH to its receptor (Shinitzky and Fridkin 1976; Shinitzky, Hazum et al. 1976). Such a constrained conformation may minimize the requirement for Glu^{301} if the primary function of the residue is to induce a conformation change in GnRH to that already found in GnRH superagonists.

Thus two residues have been identified for their involvement in agonist binding, an acidic Glu^{301} on the extracellular loop 3 and a basic TMH 3 Lys^{121}. Both are charged residues and substitution with non-charged amino acids at either locus disrupts their normal function. The experimental results indicate that these two residues play distinct role in ligand binding. While substitution at the TMH 3 locus does not discriminate GnRH and GnRH-A, Gln^{301} mutation affects the affinity for the two agonists to quite different extent. Therefore, the Lys^{121} may be involved in the general mechanism of agonist binding to the GnRHR whereas the role of Glu^{301} is more restricted to binding to GnRH. Since Arg^{121} and Gln^{121} mutant receptors have similar high-affinity for GnRH antagonist, the TMH 3 locus is not involved in antagonist binding, at least for the antagonist tested. The distinct GnRHR binding sites for GnRH, GnRH-A, and GnRH antagonists revealed by the mutational analysis are supported by the chemical linking studies (Janovick, Haviv et al. 1993). Additional characterization to analyze the differential impairment to receptor function by mutations at Lys^{121} and Glu^{301} may reveal the distinct roles they play and the way they coordinate during receptor activation. This knowledge will certainly benefit rational GnRH drug design and help clarify the complicated mechanisms of peptide binding to GPCRs.

In the neurotransmitter receptors, the conserved TMH 3 Asp has been proposed to form an ionic interaction with the biogenic amine head groups of

neurotransmitters (Strader, Sigal et al. 1988). The similar role of Lys[121] of the GnRHR and the conserved Asp in neurotransmitter receptors in mediating high affinity agonist binding suggests that the third transmembrane domain of GPCRs may be conserved in evolution as the ligand binding pocket. Supporting this hypothesis, a TMH 3 Lys of the endothelin$_B$ receptor, corresponding to Lys[121] of the GnRHR, appears to mediate the specificity of binding to endothelins (Zhu, Wu et al. 1992). At a locus one residue away in TMH 3, a Tyr residue of the thyrotropin-releasing hormone receptor reportedly binds to Pro[3] of the hormone via a hydrogen bond (Perlman, Thaw et al. 1994). Despite the possible common role of the TMH 3 in ligand binding, the data accumulated to date clearly indicate a different binding pocket between neurotransmitter receptors and peptide receptors. Unlike the neurotransmitter receptors characterized by their well-defined binding pockets in their transmembrane domains (Strader, Sigal et al. 1987; Kobilka, Kobilka et al. 1988), the binding sites identified for the peptide receptors seem to be more diverse. Both extracellular and transmembrane residues have been identified for binding to peptide ligands. The glycohormone receptor subfamily, for example, mediates high affinity binding through the receptor's large N-terminus and shearing the transmembrane region is without effect (Frazier, Robbins et al. 1990; Tsai-Morris, Buczko et al. 1990). In the neurokinin receptor subfamily, the extra-cellular loops of the receptors are likely involved in binding to their peptide ligands (Fong, Cascieri et al. 1993; Fong, Huang et al. 1993). Moreover, even among the peptide receptors in which TMH 3 has been implicated in binding to their ligands, the nature of the interaction is more likely to be a hydrogen-bond interaction instead of electrostatic interaction as described in the neurotransmitter receptors. Further studies covering detailed analysis of peptide receptor binding may reveal other residues that form the binding pocket and

allow a better understanding of the distinction between these two receptor classes.

Helical Rearrangement and GnRHR Activation

One of the unique features of the GnRHR is that the highly conserved TMH 2 Asp and TMH 7 Asn found in most GPCRs are interchanged. Of the two residues, the TMH 2 Asp has been extensively studied in many other GPCRs. Mutation of the Asp causes a wide variety of effects including loss of GTP modulation of agonist binding (Chung, Wang et al. 1988; Surprenant, Horstman et al. 1992; Wang, Gallaher et al. 1993), poor G protein coupling (Chung, Wang et al. 1988; Fraser, Wong et al. 1989; Wang, Buck et al. 1991; Surprenant, Horstman et al. 1992), decrease in agonist affinity (Strader, Sigal et al. 1987; Chung, Wang et al. 1988; Fraser 1989; Neve, Cox et al. 1991; Wang, Buck et al. 1991; Chanda, Minchin et al. 1993; Wang, Gallaher et al. 1993), and resistance to allosteric modulation by cation (Neve, Cox et al. 1991; Horstman, Brandon et al. 1992; Kong, Raynor et al. 1993; Quintana, Wang et al. 1993). The simultaneous replacement and the phenotypic exchange of these two conserved residues in the GnRHR are unlikely to be coincidental and suggest an unexplored correlation.

The role of these two residues in the GnRHR was first investigated by examining the function of Asp^{87} and Asn^{318} mutant receptors as well as the reciprocal mutant $Asp^{87}Asn^{318}$ receptor. Whereas the Asp^{87} substitution eliminates detectable binding and PI coupling, a second mutation at the TMH 7 locus represented by the reciprocal mutant receptor restores high affinity ligand binding (Table 1 of Chapter 4). The results support the hypothesis that these two amino acids in the GnRHR can influence each other structurally and must share the same microenvironment. Based on these results, the proximity of the TMH 2 and TMH 7 has been proposed (Zhou, Flanagan et al. 1994).

Establishment of the structural proximity between Asn^{87} and Asp^{318} is of tremendous importance in elucidating the structure of the family of G protein

coupled receptors. Therefore a critical examination of the data obtained is necessary before the conclusion is made. The major problem associated with the study is a total lack of binding by the Asp^{87} mutant receptor which makes it impossible to ascertain whether the mutation disrupted receptor expression or reduced the affinity for ligands. The question is fundamental to the correlation of the role of Asn^{87} to receptor expression or structural integrity, or both. It is also possible that only one of several conformation states of the reciprocal mutant receptor mimics that of the wild-type receptor and is responsible for the measured binding and coupling. Unfortunately, despite the vigorous study of the TMH 2 Asp in other GPCRs, little information is available to help address the above questions which should be equally important for other GPCRs.

Further mutational investigation of the two residues has been carried out (Chapter 5) following the initial characterization. A series of substitutions are made at the two loci, including Ala, Asp, and Gln for Asn^{87} in TMH 2, and Ala, Asn, and Glu for Asp^{318}. All the TMH 7 mutant receptors, including the reciprocal mutant receptor, cause a similar degree of increase in EC_{50} without altering the high affinity binding. The EC_{50} values obtained with the mutant receptors, approximately 40 fold larger than that of the wild-type receptor, are independent of the substitution made despite the distinct side-chain properties of the amino acids. Mutations at the TMH 2 locus cause more dramatic effects as all the mutant receptors totally abolish measurable receptor binding and activation.

Among the longer list of TMH 2 mutant receptors, the $Asp^{87}Asn^{318}$ reciprocal mutant receptor remains to be the only one with measurable binding and coupling. Supporting the findings in Chapter 4, the data indicate a lack of flexibility at the TMH 2 locus and further support a functional relationship between these two residues which may underline the restorative nature of the

reciprocal mutant receptor.

With more extensive characterization of the mutant receptors, it becomes possible to speculate on the roles of these two residues and the interaction between them. Analysis of the mutation at the Asp^{318} locus suggests that this residue forms part of the receptor activation mechanism. Supporting this hypothesis, all the mutant receptors increased the EC_{50} in PI coupling without affecting high-affinity ligand binding. Assuming a receptor occupancy stoichiometry similar to that of the wild-type receptor, the mutant receptors have to be defective compared to the wild-type receptor in their transition to the active state at the same degree of receptor occupancy. On the other hand, an alternative hypothesis that the mutant receptors may have shifted the EC_{50} by decreasing receptor expression is not consistent with the membrane binding assay results which indicate no correlation between the B_0 and E_{max} or EC_{50}. However, a complete elimination of the alternative hypothesis awaits more experiments. For example, it is imperative to show that the mutant receptors do not turnover faster than does the wild-type receptor in the functional assay, a dynamic process which effectively reduces the amount of receptors available. Pertinent to the same issue, the relatively long time of agonist exposure in the normal PI assay, chosen for optimized wild-type response, may not be optimum for the mutant receptors if they internalize faster. Accordingly, it is important to characterize the basic biochemistry and pharmacology of the mutant receptors before comparing them to the wild-type receptor. In fact, the concerns raised here about the Asp^{318} mutant receptors should be shared by the mutational studies on other receptors as well.

The data obtained suggest that postulated Asn^{87}-Asp^{318} interaction contributes to GnRHR activation. Substituting the Asp^{318} with an Ala, presumably an ideal mutation that disrupts the specific interaction without

indirectly interfering with a TMH 2-TMH 7 proximity, causes a 40 fold shift in EC_{50}. Other TMH 7 mutations shift the EC_{50} to a similar extent and likely reveal the contribution of the interaction between these two helices. However, the study on the serotonin receptors seems to disagree with the limited role the TMH 2/7 interaction plays. In the human $5HT_2$ receptor, where an Asp and an Asn are found at the TMH 2 and TMH 7 position, respectively, introducing an Ala at either locus completely abolishes the receptor function (Chi et al, unpublished observations). It is not clear whether the TMH 2/7 interaction plays different role in these two receptors, or, in the GnRHR, the mutations tested at Asp^{318} happen to be compensated for by the presence of some residues unique to the receptor. Other substitutions at the TMH 7 locus in the GnRHR, with smaller or larger amino acids, may help a general understanding of the role the interhelical interaction.

Similar to the Asp^{87} mutant receptor initially described, all the new mutations introduced at the TMH 2 locus cause a complete loss of binding or coupling. The negative results make it difficult to dissect the functional role of the Asn^{87}. However, it seems likely that this residue is involved in interaction with a site other than Asp^{318} because of the more dramatic effects of mutations at this locus. The Ala^{87} mutant receptor, for instance, is more detrimental to the GnRHR function than the Ala^{318} mutant receptor. Presumably, the mutation elicits an additive effect in addition to the disruption of Asn^{87}-Asp^{318} interaction. For this hypothesis to be testified, further studies with substitutions in other helices may be required.

The results obtained to date are supportive of a TMH 2 and TMH 7 interaction which may play an important role and form part of the network in the GnRHR activation. Future studies on other GPCRs should reveal the universality of the findings in the GnRHR. In addition, with the identification of other

interhelical interactions, our understanding about the structure/function relationship of the of GPCRs and the signal transduction mechanisms of this family of receptors would be further enriched.

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