GONADOTROPIN RELEASING HORMONE RECEPTOR LIGAND INTERACTIONS

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CONTENTS

To the memory of my late husband, Philip Shapiro.

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ABBREVIATIONS

BSA	bovine serum albumin
$[Ca^{2+}]_i$	cytosolic free calcium concentration
cAMP	cyclic adenine monophosphate
cDNA	complimentary deoxyribonucleic acid
cGMP	cyclic guanine monophosphate
CG	chorionic gonadotropin
CHAPS	3-[(cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
EC_{50}	peptide concentration required to half-maximally stimulate release of LH or production of inositol phosphates
EDTA	ethylenediamine tetraacetic acid (disodium salt)
FSH	follicle stimulating hormone
G-protein	guanine nucleotide binding protein
GAP	GnRH associated peptide
GDP	guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
GnRH	gonadotropin releasing hormone
GnRHR	gonadotropin releasing hormone receptor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IC_{50}	peptide concentration required to half-maximally inhibit binding of labelled GnRH peptides

IP_3	inositol 1,4,5-trisphosphate
IP	inositol phosphates
K_d	dissociation constant
LH	luteinizing hormone
MEM	minimum essential medium
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phosphatidyl inositol-specific phospholipase C
PIP_2	phosphatidyl 4,5-bisphosphate
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TM	transmembrane helix
tris	2-amino-2-(hydroxymethyl)-1,3-propandiol

ABSTRACT

GONADOTROPIN RELEASING HORMONE RECEPTOR LIGAND INTERACTIONS

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The decapeptide, gonadotropin releasing hormone (GnRH), is the central regulator of reproductive function. It binds to receptors on the gonadotrope cells of the pituitary and stimulates release of luteinizing hormone (LH) and follicle stimulating hormone (FSH).

Eleven different structural forms of GnRH have now been identified in various animal species. Chimaeric analogues of some of the variant forms of GnRH were synthesized in order to study the functional significance of the most common amino acid substitutions, which occur in positions 5, 7 and 8. Peptide binding affinities for sheep and rat GnRH receptors and potencies in stimulating LH and FSH release from cultured sheep pituitary cells and LH release from cultured chicken pituitary cells were measured. Histidine in position 5 decreased LH releasing potency in chicken cells, but slightly increased receptor binding affinity in rat and sheep membranes. Tryptophan in position 7 had minimal effect on GnRH activity in mammals, but increased LH release in chicken cells. Although differences in the structural requirements of mammalian and chicken GnRH receptors were anticipated, it was also found that rat GnRH receptors exhibited higher affinity for analogues with Tryptophan in position 7, than did sheep GnRH receptors. Substitutions in position 8 revealed the most marked differences in the structural requirements of mammalian and chicken GnRH receptors. Arginine was required for high GnRH activity in mammalian systems, but analogues with neutral substitutions in position 8 were more potent in chicken pituitary cells. The tolerance of position 8 substitutions, combined with the relatively small effects, in chicken cells, of incorporating a D-amino acid in position 6, indicate that the chicken GnRH receptor is less stringent than mammalian receptors in its recognition of peptide conformation.

To examine how changes in ligand structure cause changes in receptor binding affinity and receptor activation, it was necessary to know the structures of the GnRH receptors. A protocol was developed for the purification of GnRH binding proteins from detergent-solubilized pituitary membranes, by affinity chromatography. This procedure yielded a protein which migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, but was different from the recently cloned GnRH receptor.

To test the proposal that the arginine residue in mammalian GnRH interacts with an acidic receptor residue, eight conserved acidic residues of the cloned mouse GnRH receptor were mutated to asparagine or glutamine. Mutant receptors were transiently expressed in COS-1 cells and tested for decreased preference for Arg^8-containing ligands by ligand binding and inositol phosphate production. One mutant receptor, in which the glutamate residue in position 301 was mutated, exhibited decreased affinity for mammalian GnRH. The mutant receptor also exhibited decreased affinity for [Lys^8]-GnRH, but unchanged affinity for [Gln^8]-GnRH compared with the wildtype receptor, and increased affinity for the acidic analogue, [Glu^8]-GnRH. This loss of affinity was specific for the residue in position 8, because the mutant receptor retained high affinity for analogues with favourable substitutions in positions 5, 6 and 7. Thus, the Glu^{301} residue of the GnRH receptor plays a role in receptor recognition of Arg^8 in the ligand, consistent with an electrostatic interaction between these two residues.

The Glu^{301} and Arg^8 residues were not required for the high affinity interactions of conformationally constrained peptides. This indicates that an interaction which involves these two residues may induce changes in the conformation of GnRH after it has bound to the receptor.

CHAPTER 1

STRUCTURE AND FUNCTION OF GONADOTROPIN RELEASING HORMONE AND THE GONADOTROPIN RELEASING HORMONE RECEPTOR

SUMMARY

Gonadotropin releasing hormone (GnRH) regulates reproductive function by controlling the release of the two gonadotropic hormones, luteinizing hormone and follicle stimulating hormone. GnRH binds to receptors on the plasma membrane of pituitary gonadotrope cells and activates G-proteins which include the G_q class. The G-proteins, in turn, stimulate the effector enzyme, phospholipase C, to hydrolyze a membrane phospholipid, phosphatidyl inositol bisphospate, to generate the second messengers, inositol trisphosphate and diacylglycerol, which regulate cytosolic free calcium levels and protein kinase C.

Eleven different GnRH structures have been identified in various animal species and they have varying activities in mammals and non-mammalian vertebrates. The GnRH peptide has no fixed conformation in solution. However, molecular dynamics simulations and molecular modelling, combined with the introduction of conformational constraints into synthetic GnRH analogues, have shown that GnRH probably interacts with its receptor in a conformation which incorporates a β bend centered around the glycine residue in position 6. Analysis of the contribution of individual amino acids to the activity of GnRH shows that, although no single residue is absolutely essential, the amino-terminal residues appear to have an important role in receptor activation, while the carboxy-terminal residues are necessary for high affinity binding to the receptor.

Cloning of the GnRH receptor showed its structure to be typical of G-protein coupled receptors, comprising a single polypeptide chain with seven hydrophobic segments. It also has some atypical features including the lack of a hydrophilic carboxy-terminal domain, an unusually long first intracellular loop and the apparent interchange of two highly conserved residues, an aspartate which usually occurs in the second transmembrane helix and an asparagine usually found in the seventh. This natural reciprocal mutation has been exploited in experiments to define the three-dimensional conformation of the GnRH receptor and other G-protein coupled receptors. A lysine residue in the third transmembrane helix has been shown to affect binding of GnRH agonists, but not antagonists. Like other peptide receptors, the ligand binding site extends to the extracellular domain of the GnRH receptor, where an acidic residue in the third extracellular loop is important for ligand binding. Site-directed mutagenesis and studies with proteolytic enzymes have shown that the ligand binding domain for GnRH antagonists differs from that for GnRH agonists.

INTRODUCTION

This chapter will only briefly describe the physiological role of GnRH in reproduction, its biosynthesis and the transduction of the GnRH signal to the interior of the cell. The structure of GnRH will be reviewed in more depth with a view to understanding which apsects of GnRH structure are important for its activity in binding and activating the pituitary GnRH receptor. The structure and function of the GnRH receptor will be discussed in comparison with the G-protein coupled receptors, to which it is related.

PHYSIOLOGY AND CELL BIOLOGY OF GnRH

The role of GnRH in reproduction

Gonadotropin releasing hormone (GnRH) plays a central role in regulating animal reproduction. It is synthesized in the hypothalamic neurosecretory cells and released into portal vessels which connect the hypothalamic region of the brain with the pituitary gland. Thus it integrates the neural and endocrine systems (Millar and King, 1988; Hazum and Conn, 1988). In the pituitary it provokes the gonadotrope cells to release both of the gonadotropic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Schally *et al.*, 1971a, 1971b, 1971c; Matsuo *et al.*, 1971; Burgus *et al.*, 1972) which in turn control the function of the gonads (ovaries and testes). LH stimulates ovulation and formation of the corpus luteum in females and androgen secretion in males, whereas FSH stimulates the growth and maturation of ovarian follicles in females and spermatogenesis in males.

In addition to stimulating release of gonadotropins, GnRH has other important actions on the pituitary. It regulates expression of GnRH receptors (Clayton, 1989; Young *et al.*, 1985) and modulates levels of GnRH receptor mRNA (Yasin *et al.*, 1995). GnRH also regulates biosynthesis of LH, increasing levels of mRNA for the β subunit of LH (Clayton, 1989 for review) and regulates glycosylation of LH, thus modifying its bioactivity (Azhar *et al.*, 1978; Ramey *et al.*, 1987). GnRH treatment also increases the size, secretory apparatus and number of gonadotropes (Clayton, 1989). GnRH actions are modulated by the gonadal steroids, estradiol, progesterone and testosterone (Clayton, 1989; Dalkin *et al.*, 1989), and by the gonadal polypeptides, activin and inhibin, named for their effect on FSH secretion (Seeburg *et al.*, 1987).

GnRH biosynthesis

In rat hypothalamus and in human hypothalamus and placenta, GnRH is synthesized as a 92-amino acid precursor (fig. 1.1) (Adelman *et al.*, 1986; Seeburg and Adelman, 1984). The first 23 amino acids comprise the signal sequence which features a typical hydrophobic

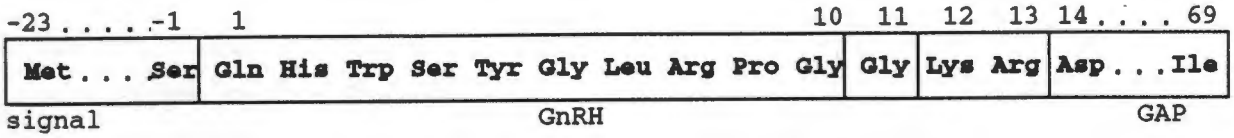


Figure 1.1 Schematic diagram of the human pre-pro-GnRH, showing the signal sequence (-23 to -1), the GnRH sequence, the Gly residue involved in carboxy-terminal amidation, the Lys-Arg processing site, and the 57-amino acid GnRH-associated peptide (GAP).

middle section. The signal sequence is 60% homologous between rat and human hypothalami, and identical in human hypothalamus and placenta. It is followed by the decapeptide GnRH sequence and the Gly-Lys-Arg sequence necessary for enzymatic processing. It is likely that the precursor is processed by trypsin-like cleavage at the pair of basic amino acids, followed by removal of the exposed basic amino acids by carboxypeptidase B-like activity and amidation utilizing Gly^{11} as the amide donor (Millar and King, 1987; Seeburg *et al.*, 1987). The amino-terminal pyro-glutamate is formed by spontaneous cyclization of Gln (Millar and King, 1987; Seeburg *et al.*, 1987). The remaining 57-amino acid GnRH-associated peptide has been reported to inhibit release of prolactin and to stimulate gonadotropin release (Nikolics *et al.*, 1985; Phillips *et al.*, 1985) via a mechanism independent of the GnRH receptor (Milton *et al.*, 1986; Millar *et al.*, 1986a). The effects of the GnRH-associated peptide are controversial and appear to require gap junctions and cell communication (Seeburg *et al.*, 1987).

Pulsatile release of GnRH

GnRH is released from the hypothalamus in pulses (Carmel *et al.*, 1976 and many others). The frequency and amplitude of these pulses vary, under neural control, with the oestrous and menstrual cycles (Fox and Smith, 1985; Dalkin *et al.*, 1989). Fast pulses of GnRH preferentially stimulate LH release. They also stimulate the synthesis of mRNA for the β subunit of LH and for the α subunit which is common to both gonadotropic hormones. In contrast, slower pulses of GnRH favour the release of FSH and the synthesis of FSH β subunit mRNA. As the frequency of pulses of GnRH secretion changes in normal physiology, frequency modulation may be the mechanism by which a single hypothalamic releasing hormone regulates differential secretion of both LH and FSH (Dalkin *et al.*, 1989).

Intracellular transduction of the GnRH signal

GnRH binds to a specific receptor on the plasma membrane of pituitary gonadotrope cells (Naor, 1990). Activation of the receptor is probably associated with a change in

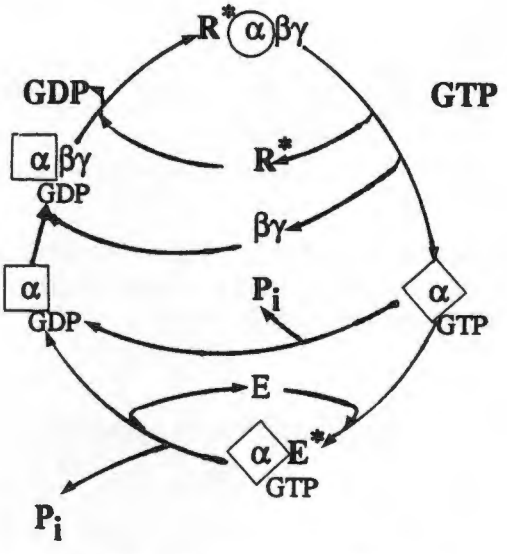


Figure 1.2 The G-protein activation cycle showing the 3 different conformations of G_α and interactions with receptor and effector during the GTPase cycle. See text for explanation. R^*, activated receptor; E, effector (based on Conklin and Bourne, 1993).

conformation (Samama *et al.*, 1993), which transmits the binding signal to the intracellular surface of the cell membrane and activates one or more guanine nucleotide binding proteins (G-proteins) (Andrews *et al.*, 1986; Perrin *et al.*, 1989; Limor *et al.*, 1989).

The G-proteins that transmit information from cell membrane receptors to their intracellular effectors belong to a large homologous family of heterotrimeric proteins, consisting of α, β and γ subunits. The different G-proteins are most readily distinguished by their α subunits (table 1.1), although there are also less well characterized structural and functional differences in their β and γ subunits (Taylor, 1990). The α subunits bind and hydrolyze GTP and contain the structural determinants of interaction with receptors and effector enzymes (Conklin and Bourne, 1993). The G-protein activation cycle is illustrated in fig 1.2. G-proteins bind to activated receptors in their trimeric form with GDP bound to the α subunit. Activated receptors catalyze activation of G-proteins by increasing the rate of GDP dissociation and allowing subsequent replacement of GDP with GTP (Taylor, 1990; Conklin and Bourne, 1993). Binding of GTP causes the α subunit to take on a new conformation and it rapidly dissociates from both the receptor and the β–γ complex (Conklin and Bourne, 1993). Both of the resulting free α-GTP and β–γ subunits regulate intracellular effectors (Sternweiss and Smrcka, 1992; Exton, 1994).

Although it has been suggested that multiple G-proteins are involved in GnRH signal transduction (Hawes and Conn, 1993; Hawes *et al.*, 1993), only two, G_q and G_{11}, have been identified and the GnRH receptor does not appear to discriminate between them (Hsieh and Martin, 1992; Shah and Milligan, 1993; Shah *et al.*, 1995). The GTP-bound α subunits of the G_q-proteins activate the β isoforms of the effector enzyme, phospholipase C (PLC). Activated α_q and α_{11} subunits stimulate the β1 isoenzyme of PLC (Sternweiss and Smrcka, 1992; Exton, 1994). PLC hydrolyzes the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate (PIP_2) to generate the second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Andrews *et al.*, 1986; Limor *et al.*, 1989; Naor, 1990). The involvement of PLC in GnRH signal transduction was supported by early work which showed

that GnRH increased phosphoinositide turnover (Kiesel and Catt, 1984; Andrews and Conn, 1986; Naor *et al.*, 1985), while later work demonstrated rapid hydrolysis of PIP_2 in response to GnRH (Naor *et al.*, 1986; Morgan *et al.*, 1987; Naor, 1990).

The LH secretory response to GnRH is biphasic: there is an initial rapid spike of LH release which is followed by a lower sustained plateau phase. The peak and plateau in LH release are preceded by similar changes in cytosolic free calcium concentration ($[Ca^{2+}]_i$). The peak and plateau in $[Ca^{2+}]_i$ reflect a temporal sequence of calcium mobilization from intracellular stores followed by an influx of extracellular calcium (Catt and Stojilkovic, 1989). IP_3, a second messenger generated by PLC, binds to intracellular receptors and mobilizes intracellular Ca^{2+} stores to produce the initial spike of $[Ca^{2+}]_i$ (Guilemette *et al.*, 1987; Naor *et al.*, 1988; Catt and Stojilkovic, 1989; Naor, 1990). The second, plateau, phase of $[Ca^{2+}]_i$ elevation results from influx of extracellular Ca^{2+} through at least two groups of "receptor-operated" calcium channels. About 50% of calcium influx occurs through L-type, dihydropyridine sensitive, voltage sensitive calcium channels, while the remainder enters through dihydropyridine insensitive channels (Tasaka *et al.*, 1988; Naor *et al.*, 1988; Davidson *et al.*, 1988; Catt and Stojilkovic, 1989; Naor, 1990; Davidson *et al.*, 1991). The increased $[Ca^{2+}]_i$ leads to activation of the calcium binding protein calmodulin (Conn *et al.*,

Table 1.1 The family of G-Protein α subunits in mammals
(based on Conklin and Bourne, 1993)

Subfamily	Gα	Effectors	Intracellular Message
s	α_s	adenyl cyclase (+)	cAMP
		Ca^{2+} channels (open)	membrane potential (-)
	α_{olf}	adenyl cyclase (+)	cAMP (+)
i/o/t	$\alpha_{i,i2,i3}$	K^+ channels (open)	membrane potential (+)
	α_o	Ca^{2+} channels (close)	membrane potential (-)
	α_z	adenyl cyclase (-)	cAMP (-)
	α_{t1}	cGMP-phosphodiesterase	cGMP (-)
	α_{t2}	cGMP-phosphodiesterase	cGMP (-)
	α_{gust}	unknown	unknown
q	$\alpha_{q,11,14,15,16}$	phospholipase C β	IP_3 (+), DAG (+)
12/13	$\alpha_{12,13}$	unknown	unknown

1981a, 1981b; Jennes *et al.*, 1985; Conn *et al.*, 1985a).

The other product of PLC activity, DAG, activates protein kinase C (PKC). However, there is controversy as to the importance of PKC activity in GnRH-stimulated LH release (Catt and Stojilkovic, 1989; Naor, 1990; Davidson *et al.*, 1991). In favour of a role for PKC is the demonstration that phorbol esters and DAGs (PKC activators) stimulated LH release (Smith and Conn, 1984; Conn *et al.*, 1985b; Harris *et al.*, 1986), as did insertion of the enzyme into permeabilized PKC-depleted cells (Naor, 1990). GnRH also stimulates redistribution of PKC from the cytosol to the plasma membrane, the main mechanism of its activation (Hirota *et al.*, 1985, Naor *et al.*, 1985). Similar phosphoprotein substrates were identified in cultured pituitary cells which were stimulated with GnRH and with a phorbol ester. Phosphorylation of the GnRH substrates was inhibited in PKC-depleted cells and in the presence of a PKC inhibitor (Strulovici *et al.*, 1987). However, in experiments using cells depleted of PKC by prolonged incubation with tetradecanoyl phorbol ester (TPA), a PKC activator and analogue of DAG, McArdle *et al.* (1987) reported that PKC depletion did not affect GnRH-stimulated LH release although others (Stojilkovic *et al.*, 1988; Dan-Cohen and Naor, 1990) reported decreased response to GnRH. In addition, it was shown that a phorbol ester stimulated release of more LH than did GnRH and that the effects of simultaneous administration of phorbol ester and GnRH was additive (Beggs and Miller, 1989; Kile and Nett, 1994) or synergistic (Johnson *et al.*, 1987). Also, inhibitors of PKC did not inhibit GnRH-stimulated LH release (Johnson *et al.*, 1987; Beggs and Miller, 1989). Thus, it is likely that both elevation of $[Ca^{2+}]_i$ and activation of PKC, are necessary to elicit the full GnRH response (Naor and Eli, 1985), and that PKC may amplify or modulate the calcium signal (Davidson *et al.*, 1991). The major function of PKC may be to regulate the longer-term effects of GnRH, in particular gonadotropin gene expression (Andrews *et al.*, 1988) and up-regulation of the GnRH receptor (Conn, 1989).

GnRH STRUCTURE AND THE RELATIONSHIP BETWEEN STRUCTURE AND ACTIVITY

Structures of naturally occurring GnRHs

The structure of GnRH was first determined from porcine hypothalamus (Matsuo *et al.*, 1971). Shortly thereafter, an identical structure was found in ovine (sheep) hypothalamus (Amoss *et al.*, 1971; Burgus *et al.*, 1972). The same GnRH structure was demonstrated in humans and rats (Adelman *et al.*, 1986). Post-translationally modified [hydroxyproline^9]-GnRH was also found in extracts of mammalian hypothalamus (Gautron *et al.*, 1991). Other forms of GnRH have been isolated from non-mammalian species and sequenced (table 1.2). Two forms were found in the chicken: [Gln^8]-GnRH (chicken GnRH I) (King and Millar,

residue #	1	2	3	4	5	6	7	8	9	10
species										
mammalian	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	$GlyNH_2$
hydroxy-Pro									HOPro	
chicken I								Gln		
sea bream								Ser		
salmon							Trp	Leu		
catfish					His			Asn		
chicken II					His		Trp	Tyr		
dogfish					His		Trp	Leu		
lamprey III					His	Asp	Trp	Lys		
lamprey I			Tyr		Leu	Glu	Trp	Lys		
tunicate I					Asp	Tyr	Phe	Lys		
tunicate II					Leu	Cys	His	Ala		

Table 1.2 Comparison of variant GnRH structures with mammalian GnRH

1982) and [His^5,Trp^7,Tyr^8]-GnRH (chicken GnRH II) (Miyamoto *et al.*, 1984). [Trp^7,Leu^8]-GnRH (salmon GnRH) was sequenced from salmon brain (Sherwood *et al.*, 1983) and [Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8]-GnRH (lamprey GnRH I) from lamprey (Sherwood *et al.*, 1986). More recently, three novel forms of GnRH, [His^5,Trp^7,Leu^8]-GnRH (dfGnRH) (Lovejoy *et al.*, 1992), [His^5,Asn^8]-GnRH (cfGnRH) (Bogerd *et al.*, 1992) and [Ser^8]-GnRH (sbGnRH) (Powell *et al.*, 1994), have been identified in dogfish, catfish and seabream respectively and another lamprey form ([His^5,Asp^6,Trp^7,Lys^8]-GnRH) has been found (Sower *et al.*, 1993). Two forms of GnRH, [Asp^5,Tyr^6,Phe^7,Lys^8]-GnRH and [Leu^5,Cys^6,His^7,Ala^8]-GnRH, have recently been identified in an invertebrate, the tunicate (Sherwood, 1995). The brains of non-mammalian vertebrates and some mammals (King *et al.*, 1989; King *et al.*, 1990; Dellovade *et al.*, 1993; King *et al.*, 1994) contain more than one molecular form of GnRH. It is believed that the different GnRHs have been co-opted for multiple functions in addition to gonadotropin release. Schemes suggesting the evolution of the different GnRH peptides have been presented (for reviews see King and Millar, 1987; Sherwood, 1987; King and Millar, 1990, 1992).

Certain structural features of the GnRH molecule have been conserved throughout the 500 years of evolution separating lampreys and humans. Peptide length is conserved, since all of the characterized GnRH structures are decapeptides. The $pGlu^1$-His^2 residues at the amino terminus, Ser^4, and the Pro^9-$Gly^{10}NH_2$ at the carboxy-terminus are conserved. The conservation of these features suggests that they could have significant functional roles (King and Millar, 1990).

Activities of naturally occurring GnRHs

In cultured mammalian (rat and sheep) pituitary cells, mammalian GnRH is highly potent and stimulates gonadotropin release at low doses. The LH releasing potency of chicken

GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) is about one third of that of mammalian GnRH, whilst the potencies of chicken GnRH I ([Gln^8]-GnRH) and salmon GnRH ([Trp^7,Leu^8]-GnRH) are lower at 1 to 3 % and 2 to 5 % that of mammalian GnRH and lamprey GnRH I is inactive. This order of potency is also apparent in the abilities of the naturally occurring GnRHs to compete for binding to mammalian GnRH receptors (Millar and King, 1983a, 1983b; Sherwood *et al.*, 1983; Miyamoto *et al.*, 1984; Hasegawa *et al.*, 1984). The reasons for these differences will be explored in chapter 2.

The relationships between peptide structure and receptor binding or gonadotropin releasing activity are less well characterized in non-mammalian systems. In birds, mammalian GnRH and chicken GnRH I ([Gln^8]-GnRH) have similar gonadotropin releasing activity *in vitro* (Millar and King, 1983a, 1983b; Hattori *et al.*, 1985, Hasegawa *et al.*, 1984, Johnson *et al.*, 1984) and *in vivo* (Johnson *et al.*, 1984). Chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) is the most active of the naturally occurring GnRH peptides in stimulating gonadotropin release from chicken pituitary cells (Millar *et al.*, 1986b; Chou *et al.*, 1985) while salmon GnRH ([Trp^7,Leu^8]-GnRH) is two to five times more active than chicken GnRH I (Millar *et al.*, 1986b). These results indicate that Trp in position seven may increase GnRH activity in the chicken. Lamprey GnRH I is inactive in cultured chicken pituitary cells, indicating that amino acids in positions 3 and 6 may be important for GnRH activity in birds (Sower *et al.*, 1987). Extrapolating from LH releasing experiments, the chicken GnRH receptor appears to have a broader specificity than that of mammals and a relatively low affinity (Hasegawa *et al.*, 1984). This apparent low affinity may explain why few workers have reported receptor binding experiments in birds. Receptor binding assays in mammalian systems became widely used only after the development of radiolabeled superactive agonists (Clayton *et al.* 1979). The activities of the naturally occurring GnRHs in mammalian and chicken systems will be explored more thoroughly in chapter 2.

High doses of GnRH are required for *in vivo* experiments in fish compared with those required for experiments in mammals (King and Millar, 1987). This is probably due to inhibitory activity of endogenous dopamine which can be counter-acted by pimozide, a dopamine antagonist (Peter *et al.*, 1985; Peter, 1986). The GnRHs which occur in higher vertebrates were equipotent *in vivo* in stimulating gonadotropin release in goldfish (Peter *et al.*, 1985; King and Millar, 1987; Sherwood, 1987; Horvath *et al.*, 1986). GnRH stimulates secretion of growth hormone in addition to gonadotropin in goldfish. Distinct GnRH receptors control the secretion of these two trophic hormones (Murthy and Peter, 1994). Mammalian GnRH, salmon GnRH and chicken GnRH II exhibited high potency in stimulating release of both hormones *in vitro* while chicken GnRH I was an order of magnitude less potent (Habibi *et al.*, 1992). The two receptors were distinguished by their responses to synthetic analogues of GnRH (Murthy and Peter, 1994; Habibi *et al.*, 1992). Specific, high affinity GnRH

receptors have been demonstrated in goldfish (Habibi *et al.*, 1987, 1989a, 1990) and in catfish, which had higher affinity for salmon GnRH than for analogues of mammalian GnRH which act as superagonists in mammals (De Leeuw *et al.*, 1988; Habibi *et al.*, 1989b).

Differences in activity of various GnRH peptides have not been demonstrated in either amphibians or reptiles (Peter, 1986; King and Millar, 1987 for reviews). It is clear that the structural requirements for GnRH activity in non-mammalian vertebrates are different from, and may be less specific than in mammals. Although the non-mammalian GnRH receptors share the characteristic of not discriminating between the different naturally occurring GnRHs, this does not necessarily mean the GnRH receptors in teleosts, amphibians and birds are more similar to each other than they are to mammalian GnRH receptors (Peter, 1986).

The biologically active conformation of GnRH

The solid phase method of peptide synthesis was developed by Merrifield in the 1960s (Merrifield, 1963), shortly before the structure of GnRH was first determined (Matsuo *et al.*, 1971). This new technology allowed rapid development of novel GnRH analogues for clinical and pharmaceutical applications (Karten and Rivier, 1986). Synthetic analogues of GnRH have also been useful as tools for investigating how GnRH interacts with its receptor.

The entire length of the GnRH peptide is required for full GnRH activity. A series of peptides successively shortened form the carboxy-terminus were essentially inactive except for des-Gly^{10}-GnRH which exhibited 10% potency (Rivier *et al.*, 1973). All other fragments and deletion analogues of GnRH were inactive (Sandow *et al.*, 1978; Karten and Rivier, 1986). Since it is unlikely that all of the sidechains of this relatively large ligand are in contact with the receptor, the requirement of the full peptide length suggests that the amino- and carboxy termini interact with the receptor and that the peptide backbone plays a role in positioning the functional groups which interact with the receptor.

The conformation of the GnRH molecule has been investigated by proton and ^{13}C nuclear magnetic resonance and by circular dichroism (Chary *et al.*, 1986, Deslauriers *et al.*, 1975, Cann *et al.*, 1979). The NMR studies showed that GnRH is highly flexible in solution and they do not indicate that it has any strong conformational preferences (Chary *et al.*, 1986; Deslauriers *et al.*, 1975). The circular dichroism study showed that GnRH exists in solution as a mixture of structural conformers which have different sensitivities to temperature and solvent composition (Cann *et al.*, 1979). In spite of its lack of defined secondary structure in solution, GnRH is likely to have a preferred conformation at the receptor (Deslauriers *et al.*, 1975).

The most abundant conformers of GnRH in solution are likely to be those which are

most stable and have the lowest free energy. One of these more abundant species could represent the bioactive conformation which interacts with the GnRH receptor (Morrison *et al.*, 1987). The computer-based techniques of molecular dynamics simulations and molecular modelling have been used to determine the likely conformations of GnRH. Momany (1976a) identified three low energy conformations of GnRH which are likely to occur in solution. All three of these structures "seem to be hinged at Gly^6". Substitution of the achiral Gly residue with a bulky L-amino acid would "open the structure up" and change the conformation around Gly^6. But the "hinge" would be stabilized by substitution of Gly^6 with a D-amino acid. The "hinge" consisted of a modified type II β-bend starting with Tyr in the *i*th position, Gly in the $i + 1$, Leu in the $i + 2$, and Arg in the $i + 3$. Comparison of the three calculated structures with experimental data obtained with synthetic analogues of GnRH, identified one conformer as the structure likely to interact with the GnRH receptor (Momany, 1976b). This structure indicated that the GnRH receptor might make contact with a face of the GnRH molecule consisting of the Arg^8 sidechain, Gly^{10}-NH_2, the cis peptide of $pGlu^1$ and the His^2 ring. Two other energy minimization studies have also identified conformers with turns around Gly^6 and Leu^7 (Struthers *et al.*, 1985; Morrison *et al.*, 1987). Shinitzky and Fridkin (1976) built a model of GnRH based on a proposed interaction between the sidechains of the residues in positions two, five and eight. Their model also contained a bend in the middle portion of the molecule. Knowledge-based computer modelling, in which the sequence of GnRH was compared with protein sequences of known structure, also generated a model of the active GnRH conformation which contained a β-turn (Gupta *et al.*, 1993). In this case, however, it was a type III turn formed by the Trp^3-Ser^4-Tyr^5-Gly^6 residues. This model predicted that the surface interacting with the receptor will consist of $pGlu^1$, $Gly^{10}NH_2$ and the sidechains of Arg^8 and Trp^3.

Experimental approaches to determining the biologically active conformation of GnRH have involved the introduction of conformational constraints into the GnRH molecule. Backbone conformational constraints limit the number of conformations available to the peptide and increase peptide potency by stabilizing biologically active conformers (Freidinger *et al.*, 1980) or decrease potency by stabilizing inactive conformers. Substitution of D-Ala for Gly^6 improved the activities of both agonist and antagonist analogues of GnRH, probably due to increased binding affinity (Monahan *et al.*, 1973). In contrast, L-Ala^6-substituted GnRH showed decreased potency. These data were considered to be consistent with GnRH having a type-II bend formed by Ser^4-Tyr^5-Gly^6-Leu^7 when it interacts with the receptor (Monahan *et al.*, 1973). However, substitution of Leu^7 with N-Me-Leu, which eliminated the possibility of a hydrogen bond between the N-H of Leu^7 and the C=O of Ser^4, did not affect GnRH potency (Ling and Vale, 1975). Thus, the enhanced potency of [D-Ala^6]-GnRH does not reflect stabilization of a β-bend incorporating a Ser^4-Leu^7 hydrogen bond, but it was consistent with a β-turn conformation of residues five to eight, as was described by energy minimization

studies (Momany, 1976a). When the proposed β-turn conformation of residues five to eight (Momany, 1976a) was stabilized by incorporation of a γ-lactam ring at the Gly-Leu peptide bond, the resulting analogue was nine times more active than GnRH *in vitro* (Freidinger *et al.*, 1980). The enhanced potencies obtained with three different conformational constraints (D-amino acids, N-Me-Leu^7 and γ-lactam), all of which stabilize a type-II β-turn structure, provide strong evidence for the existence of this type of structure in GnRH when it binds to the receptor (Freidinger *et al.*, 1980).

Attempts were also made to constrain the active conformation of GnRH by peptide cyclization. A peptide in which the sidechains of residues in positions four and seven were covalently linked (*cyclo*-[Glu^4,D-Ala^6,Orn^7]-GnRH) was inactive (Donzel *et al.*, 1977). This did not, however, disprove the hypothesis of a β-turn at residues Ser-Tyr-Gly-Leu, because the linear homologue, [Gln^4,D-Ala^6,Orn^7(Ac)]-GnRH, was also inactive. Two peptides cyclized through residues one and ten (*cyclo*-[β-Ala^1,D-Ala^6,Gly^{10}]-GnRH and *cyclo*-[6-aminohexanoyl^1,D-Ala^6,Gly^{10}]-GnRH) exhibited 1% of the activity of GnRH *in vivo* and were more active than the same peptides before cyclization (Seprodi *et al.*, 1978). Similar peptides, *cyclo*-[β-Ala^1,2-D-Nal^6,Gly^{10}]-GnRH, *cyclo*-[γ amino butyric acid^1,2-D-Nal^6,Gly^{10}]-GnRH and *cyclo*-[6 amino caproic acid^1,2-D-Nal^6,Gly^{10}]-GnRH were partial agonists with high affinity for the GnRH receptor (Rivier *et al.*, 1985). This suggests that some degree of peptide flexibility may be necessary for full agonist activity, or that free carboxy- or amino-terminal sidechains are necessary for receptor activation while not being necessary for high affinity binding. While peptide cyclization has not been useful in determining a conformation in which GnRH activates its receptor, it has been useful in the design of GnRH antagonists. A bicyclic GnRH antagonist with bridges between residues four and ten and between residues five and eight exhibited high affinity (K_d, 0.22 nM) for the GnRH receptor (Rivier *et al.*, 1992). GnRH and GnRH agonists may assume the same backbone conformation as this antagonist for binding to the receptor. Alternatively, since the binding sites for peptide agonists and antagonists are likely to be distinct, although overlapping (Fong and Strader, 1994), agonists may assume a different conformation at the receptor and agonist flexibility may be necessary for receptor activation.

The contribution of individual amino acid residues to GnRH activity

Many different *in vivo* and *in vitro* methods have been used to analyze the activities of GnRH agonists and antagonists. Early studies utilized *in vivo* assays which were relatively insensitive and did not distinguish changes in affinity from changes in efficacy. *In vivo* test systems are affected by factors such as resistance of analogues to proteolytic enzymes, binding of analogues to plasma proteins and peptide interactions with cell membrane phospholipids. These factors influence the survival of test peptides in the circulation (Karten

and Rivier, 1986) and provide an indication of how analogues will behave when administered pharmacologically. However, they result in relatively indirect estimates of the interactions of peptides with the GnRH receptor. *In vitro* assays are less subject to the pharmacokinetic properties of peptides and allow independent measurement of receptor binding activity and receptor activation as monitored by LH release or inositol phosphate production. As this review aims to analyze the interaction of GnRH with the receptor, it will emphasize the results of *in vitro* assays where they are available.

The amino-terminal pGlu residue was found to be important for the biological activity of GnRH during the original purification of the peptide (Amoss *et al.*, 1971). Subsequently, most substitutions for $pGlu^1$ caused complete loss of activity. Analogues with the cyclic $(O{=}C)Ser^1$ (Fujino *et al.*, 1972a), acylated Gly^1 (Okada *et al.*, 1973) or D-$pGlu^1$ retained some activity and indicated that the -CO-NHCHCO group is necessary for significant levels of GnRH activity (Coy *et al.*, 1975; Sandow *et al.*, 1978).

The imidazole ring of His^2 has a number of chemical features which make it a potential catalyst of receptor activation. These include its aromatic character, acid-base properties and capacity for hydrogen bonding (Coy *et al.*, 1975). The acid-base and hydrogen bonding properties of His^2 can be altered quite drastically with retention of considerable activity, as was demonstrated by [1-N^{im}-His^2]-GnRH (6% potency) and [β-Pyrazoly-3-Ala^2]-GnRH (19%) (Coy *et al.*, 1975), but aromaticity is necessary in position two for GnRH activity. Substitutions with non-aromatic amino acids resulted in analogues with less than 0.1% of the potency of GnRH, while [Phe^2]-GnRH had 2-4% of the potency of GnRH and [Trp^2]-GnRH 40% (Coy *et al.*, 1975; Karten and Rivier, 1986). These analogues were tested using *in vivo* assays which could not distinguish low affinity for the receptor from antagonist behaviour. The earliest *in vitro* assays of GnRH analogue activity showed that [Gly^2]-GnRH was a partial agonist, having about 50% of the intrinsic activity of GnRH, but also inhibiting GnRH-stimulated LH release to about 50% (Vale *et al.*, 1972). Des-His^2-GnRH was found to be a competitive inhibitor of GnRH and was thus the first GnRH antagonist described (Vale *et al.*, 1972). Substitutions for His^2, usually D-4-Cl-Phe or D-4-F-Phe, are characteristic of all highly active GnRH antagonists (Karten and Rivier, 1986; Ljungqvist *et al.*, 1988; Haviv *et al.*, 1993 for examples). The importance of the position 2 residue in antagonist design indicates that the aromatic sidechain or the peptide bond stereochemistry of His^2 plays a role in activating the GnRH receptor (Vale *et al.*, 1972).

Replacement of Trp^3 with non-aromatic amino acids resulted in almost complete loss of activity, while [Phe^3]-GnRH and [Tyr^3]-GnRH showed low activity (Coy *et al.*, 1975; Sandow *et al.*, 1978). Substitutions with the unnatural aromatic amino acids, 2-naphthyl-Ala and pentamethyl-Phe (Me_5-Phe), resulted in analogues with about 50% potency (Sandow *et al.*, 1978; Coy *et al.*, 1974). Since Me -Phe is quite different from Trp, but resembles it in its

ability to form π-π complexes with certain aromatic compounds, the high potency of [Me$_5$-Phe^3]-GnRH suggested that Trp^3 interacts with an electron acceptor group in the receptor. Replacement of the position 5 hydrogen atom, in the aromatic nucleus of Trp^3, with an electron-withdrawing fluorine atom resulted in an analogue, [5-F-Trp^3]-GnRH, with only 6% potency. This supported the proposal that electron transfer from the Trp^3 ring is important for GnRH activity (Coy *et al.*, 1974, 1975). [D-Trp^3]-GnRH exhibited low LH-releasing potency (0.13%) *in vivo,* and it was suggested that the altered stereochemistry prevented efficient binding to the receptor (Hirotsu *et al.*, 1974; Coy *et al.*, 1975). However, D-Trp^3 was later a component of many high affinity GnRH antagonists (Karten and Rivier, 1986 for review). This indicates that D-Trp^3-analogues of GnRH may bind to the receptor with high affinity, but that the L-stereochemistry is necessary for receptor activation. Thus, Trp^3 probably also plays a role in activating the GnRH receptor, possibly via a π-π interaction with an aromatic residue in the receptor. Alternatively, it may interact with an electron acceptor such as a basic amino acid, or an OH or NH group in the receptor.

Analogues in Ser^4 was substituted with residues which had small sidechains, Ala, Thr and Gln, retained activity (4 to 19% of GnRH) (Coy *et al.*, 1975; Sandow *et al.*, 1978), but larger substituents, Ser(Bu^t) or Leu, resulted in complete loss of activity (Sandow *et al.*, 1978). This suggests that there are spatial constraints in the folding of GnRH, or in the binding pocket of the receptor, which cannot accommodate the larger sidechains. This reasonably good tolerance of substitutions in position four contrasts with the 100% conservation of Ser^4 in all of the naturally occurring GnRH structures described to date. The high degree of conservation would indicate that Ser^4 has an important role in GnRH activity.

The few substitutions for Tyr^5 which were tested caused only small losses of GnRH activity (Coy *et al.*, 1975; Sandow *et al.*, 1978). This tolerance at position five has been exploited in the development of GnRH receptor binding assays, for which Tyr^5 is iodinated, and in attempts to decrease the histaminic response of GnRH antagonists (Roeske *et al.*, 1987).

The importance of the achiral Gly^6 residue lies in its ability to support the biologically active conformation of GnRH as discussed above. Its flexibility allows a bend in the peptide which is not energetically favoured by the stereochemistry of L-amino acids, but which is favoured by synthetic D-isomers (Momany, 1976a). D-amino acids with large sidechains can be incorporated in position six (Sandow *et al.*, 1978; Karten and Rivier, 1986 for reviews). This indicates that there is space in the ligand binding pocket of the receptor to accommodate these substitutions or that their sidechains may be oriented away from the receptor.

Most GnRH analogues with substitutions for Leu^7 retained moderate activity (Coy *et al.*, 1975), although analogues with small or charged sidechains (Sandow *et al.*, 1978) or with

D-Leu (Hirotsu *et al.*, 1974) in position seven were less active while [Pro^7]-GnRH was inactive (Sandow *et al.*, 1978). Thus, a relatively large, uncharged residue with L-stereochemistry is necessary in position seven for full GnRH activity.

The position eight residue is the most variable in the naturally occurring GnRHs and the basic guanidyl sidechain of the Arg^8 in mammalian GnRH is the determinant of the specificity of mammalian GnRH receptors for mammalian GnRH. Substitution with Lys yielded a peptide with approximately 10% of the LH-releasing activity and receptor binding affinity of GnRH (Shinitzky *et al.*, 1976; Milton *et al.*, 1983), while substitution with neutral Gln or hydrophobic Leu decreased LH-releasing activity and GnRH receptor binding affinity by two orders of magnitude (Sandow *et al.*, 1978; Milton *et al.*, 1983). Thus, the basic sidechain in position eight affects the affinity of binding of GnRH to its receptor. However, since position-eight substitutions had similar effects on receptor binding and LH release, Arg^8 does not appear to have a role, once the ligand is bound, in activating the receptor. Two hypotheses were put forward to explain the basis of the higher affinities of Arg^8-containing GnRHs. An ionic interaction of Arg^8 with one or more negatively charged residues, either an amino acid sidechain (Hazum, 1987) or a polysaccharide sialic acid residue (Keinan and Hazum, 1985), in the receptor, were proposed. The alternative proposal was that the sidechain of Arg^8 affects the structure of the ligand, stabilizing the active conformation of GnRH by hydrogen bonding with the sidechains of His^2 and Tyr^5 (Shinitzky and Fridkin, 1976; Shinitzky *et al.*, 1976; Hazum *et al.*, 1977). Low pK values were measured for His^2 and Tyr^5 in GnRH and it was suggested that the more acidic nature of these amino acid sidechains was due to their proximity to the cationic sidechain of Arg^8 (Shinitzky and Fridkin, 1976). GnRH analogues with neutral substitutions, Gln (Milton *et al.*, 1983) and ω-nitro-Arg (Shinitzky *et al.*, 1976), in position eight, exhibited normal, higher pK values for His^2 and Tyr^5, and extended titration ranges. These results indicate decreased interaction of the His^2 and Tyr^5 sidechains with the neutral substituents in position eight. The decreased sidechain interaction was proposed to decrease stabilization of the bioactive conformation and thus cause the lower bioactivity of the neutral GnRH analogues. The model of GnRH structure derived from these studies (Shinitzky and Fridkin, 1976), while not described in detail, has a bend in the middle of the molecule which allows close apposition of the amino- and carboxy-termini. In this respect it is similar to models of GnRH which were based on energy minimization and database sequence comparison (Monahan *et al.*, 1973; Momany, 1976a, 1976b; Struthers *et al.*, 1985; Gupta *et al.*, 1993). This thesis will address the functional importance of Arg^8 in chapters 2 and 4.

The rigidity imposed by Pro in position nine was expected to have important conformational effects (Coy *et al.*, 1975) and the few substitutions that were tested decreased GnRH activity (Sandow *et al.*, 1978). In contrast, the amino-terminal Gly-NH_2 residue can be

altered considerably without substantial loss of activity (Coy *et al.*, 1975). Substitutions with several small alkylamides increased GnRH activity (Fujino *et al.*, 1972b), while substitutions with larger amides (Fujino *et al.*, 1972b), Ala or D-amino acids (Coy *et al.*, 1975, Sandow *et al.*, 1978) decreased activity. However, the uncharged nature of the carboxy-terminal amide is important for LH-releasing activity. The free acid peptide was 1000-fold less active than GnRH (Sievertsson *et al.*, 1971).

In conclusion, much of the work on analogues of GnRH has been directed at the development of pharmacologically active peptides which were tested *in vivo*, rather than at dissecting the functional relevance of various structural features. All residues of GnRH can be substituted to some degree without total loss of activity. Thus, no single residue is absolutely crucial to GnRH activity. The amino-terminal residues His^2 and Trp^3 and the -CO-NHCHCO group of $pGlu^1$ appear to be important in activating the GnRH receptor, while the carboxy-terminal residues Arg^8, Pro^9 and $Gly^{10}NH_2$ are necessary for high affinity binding to the receptor. Gly^6 allows GnRH to assume the biologically active conformation which is required for high affinity interaction with the receptor.

THE GnRH RECEPTOR

During the last two and a half decades much has been learnt about the structural features of GnRH which are important for its function. However, this information has been accumulated from experiments based largely on only one side of the receptor-ligand interaction, because the structure of the receptor was unknown. Information about the structure of the receptor is crucial for understanding how GnRH binds to the receptor, how the receptor is activated and how it interacts with G-proteins. Knowledge of the GnRH receptor ligand binding site will facilitate the development of improved analogues for clinical applications and in time the development of non-peptide GnRH analogues is expected to revolutionize male and female contraception.

GnRH has its most important function in the pituitary. In mammals, the pituitary GnRH receptor is located exclusively on the gonadotrope cells which secrete LH and FSH (Naor, 1990). GnRH receptors have also been reported to occur in several non-pituitary tissues including rat and human gonads (Hazum and Keinan, 1984; Iwashita and Catt, 1985; Hazum and Nimrod, 1982; Latouche *et al.*, 1989) and human placenta (Iwashita *et al.*, 1986) and in human neoplasms (Miller *et al.*, 1985; Eidne *et al.*, 1985; Clayton, 1989; Kakar *et al.*, 1994). Since the physiological significance of the extra-pituitary actions of GnRH are unclear (Naor, 1990) this review will concentrate on the pituitary GnRH receptor.

Several attempts were made to purify the GnRH receptor by conventional biochemical methods. Jansem De Almeida Catanho *et al.* (1983) solubilized bovine pituitary membranes

in Triton X-100 and used concanavalin A affinity chromatography to partially purify an approximately 60kD GnRH binding protein. Hazum *et al.* (1986) purified two GnRH binding proteins with M_rs of 59 and 57 kD. They solubilized rat pituitary membranes in a zwitterionic detergent and isolated the receptor with an avidin affinity column to which [biotinyl-D-Lys^6]-GnRH was coupled. The bovine GnRH receptor was also purified by this procedure and was used to produce antibodies to the GnRH receptor (Hazum *et al.*, 1987). However, neither group published amino acid sequences of their GnRH binding proteins. Christiansen and Houen (1994) purified a 60kD protein from bovine pituitaries using similar methodology (solubilization in Triton X-114 followed by affinity chromatography on a GnRH-agarose column), but their partial amino acid sequence shows no homology with the deduced amino acid sequence of the recently cloned bovine GnRH receptor (Kakar *et al.*, 1993). Purification of GnRH binding proteins by affinity chromatography is discussed in more detail in chapter 3.

Primary structure: the GnRH receptor is a rhodopsin-type G-protein coupled receptor

The structure of the GnRH receptor was first elucidated when it was cloned from cDNA of the αT3 mouse gonadotrope cell line. A polymerase chain reaction (PCR)-based strategy was combined with hybrid-arrest screening in *Xenopus* oocytes (Tsutsumi *et al.*, 1992). The sequence was confirmed by Reinhart *et al.* (1992) and Perrin *et al.* (1993) and was used to clone GnRH receptors from human (Kakar *et al.*, 1992; Chi *et al.*, 1993), rat (Kaiser *et al.*, 1992; Eidne *et al.*, 1992; Perrin *et al.*, 1993), sheep (Brooks *et al.*, 1993; Illing *et al.*, 1993), bovine (Kakar *et al.*, 1993) and porcine (Weesner and Matteri, 1994) pituitaries. The sequences of the cloned GnRH receptors exhibit high homology with greater than 80% amino acid identity between any two sequences.

The cloned GnRH receptors have many of the characteristic features of G-protein coupled receptors (GPCRs). They consist of a single amino acid chain with an extracellular amino-terminal domain which does not have a terminal signal sequence, seven hydrophobic putative membrane-spanning segments and a cytosolic carboxy terminus. There are three major families of seven transmembrane GPCRs in mammals: the rhodopsin family which comprises at least 300 members, the secretin receptor family and the metabotropic glutamate receptor family (Schwartz, 1994; Baldwin, 1994). The GnRH receptors contain the distinctive sequence patterns characteristic of the rhodopsin group (fig. 1.3) (Baldwin, 1993). The high degree of conservation of particular amino acid residues amongst the rhodopsin family of GPCRs suggests that this family of receptors may have a common structure. The conserved residues probably have important roles in maintaining receptor structure (Findlay *et al.*, 1993).

The rat and mouse GnRH receptors contain three N-glycosylation consensus sequences

(Asn-X-Ser/Thr). Mutagenesis studies have shown that two of these sites, the Asn^4 and Asn^{18} residues, are glycosylated when the mouse receptor is transiently expressed in COS-1 cells, while Asn^{102} is not (Davidson *et al.*, 1995). However, the GnRH receptor in native pituitary membranes migrates with a lower apparent M_r than does the wildtype receptor expressed in COSM6 cells (Perrin *et al.*, 1993). This suggests that only one residue is glycosylated *in vivo*. As the Asn^4 site is not conserved in other species of GnRH receptor, it is possible that only Asn^{18} is glycosylated in the pituitary. While the polysaccharide moieties do not affect the affinity of ligand binding to the receptor, they do seem to affect receptor stability as mutations of Asn^4 or Asn^{18} decreased receptor expression (Davidson *et al.*, 1995). These results are consistent with an early study in which treatment of cultured pituitary cells with tunicamycin, an inhibitor of *N*-glycosylation, decreased the number of GnRH receptors without affecting binding affinity (Schvarz and Hazum, 1985) and with a subsequent study which showed that treatment of solubilized GnRH receptors with wheat germ agglutinin or with neuraminidase, which cleaves terminal sialic acid residues, did not affect ligand binding affinity (Hazum, 1987).

Unusual features of the GnRH receptor sequence

The cloned GnRH receptors also have some unique features. They are nearly the smallest of the GPCRs, with only 327 amino acids, in the case of the rat and mouse receptors, and 328 amino acids in the other species, which contain an insertion in the second extracellular loop. They lack the polar cytoplasmic C-terminal domain which is involved in the short term homologous desensitization of many GPCRs by receptor kinases (Hausdorf *et al.*, 1990; Palczewski and Benovic, 1991) and indeed, GnRH receptor-mediated inositol phosphate production does not exhibit homologous desensitization after stimulation with GnRH for more than 90 minutes (Davidson *et al.*, 1994a). Thus, the well described desensitization of physiological responses to GnRH administration (Clayton, 1989) must depend on other mechanisms, such as receptor internalization and intracellular responses distal to the generation of inositol phosphates (Davidson *et al.*, 1994a). Honda *et al.* (1995) have recently shown that another G_q-coupled receptor (platelet-activating factor receptor) is desensitized via down-regulation of the IP_3 receptor which regulates cytosolic Ca^{2+} levels. This mechanism might also account for desensitization of responses to GnRH.

The putative first cytoplasmic loop of the GnRH receptor is unusually long (Tsutsumi *et al.*, 1992). This long loop may substitute for the cytoplasmic C-terminal domain in functions, such as receptor internalization, which require the cytoplasmic C-terminal segment in other GPCRs (Nussenzvieg *et al.*, 1993; Thomas *et al.*, 1995). Alternatively, it may support one of the unusual functional characteristics of the GnRH receptor such as ligand-induced up-regulation of the receptor (Clayton, 1982; Loumaye and Catt, 1983) or receptor activation via

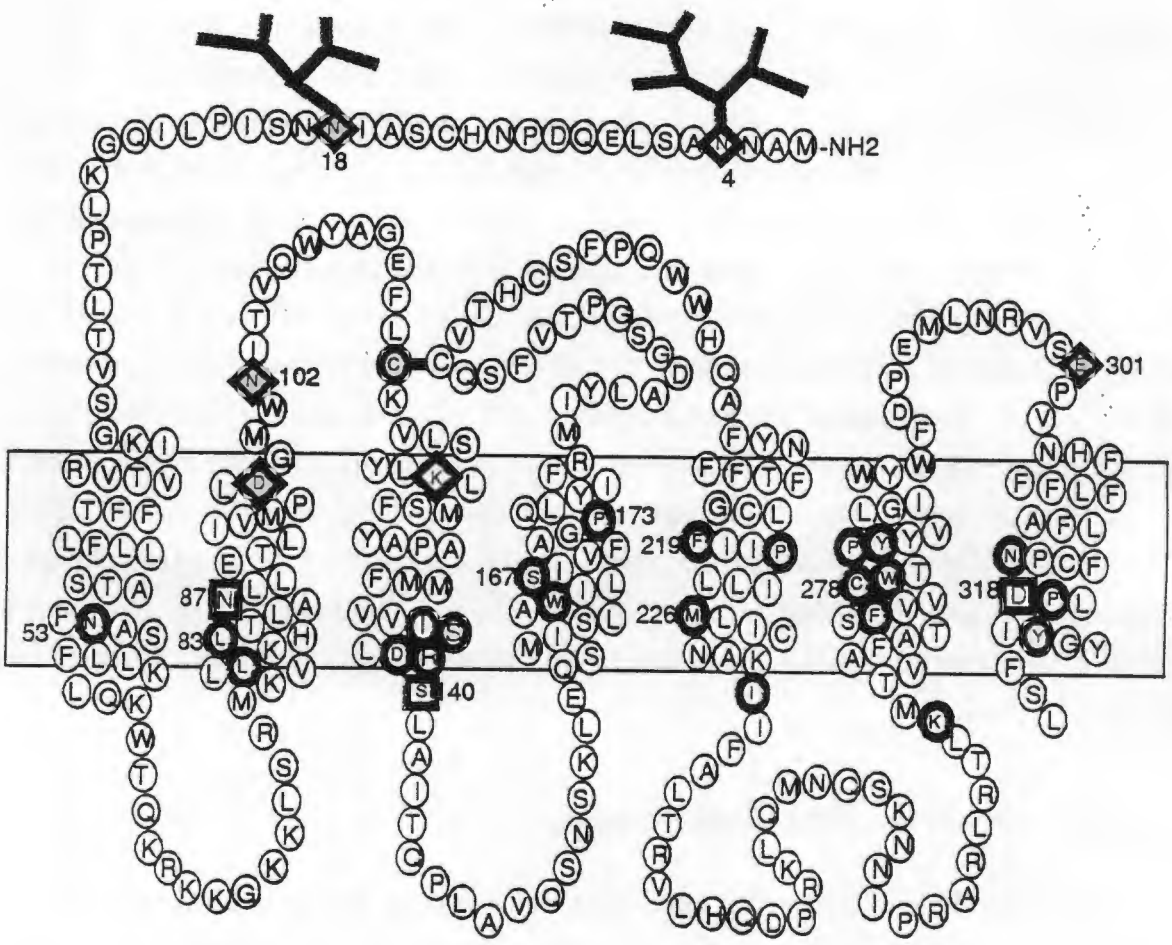


Figure 1.3 A two-dimensional diagram of the mouse GnRH receptor. Highly conserved key residues characteristic of the rhodopsin group of GPCRs are indicated by bold circles: TMI, N^{53}; TMII, L^{80}, L^{83}; TMIII, C^{114}, I^{135}-S-x-D-R^{139}; TMIV, W^{164}, S^{167}, P^{173}; TMV, F^{219}-x-x-P^{222}, M^{226}, I^{233}; TMVI, K^{266}, F^{275}-x-x-C-W-x-P-Y^{282}; TMVII, F^{310}, N^{314}, P^{319}-x-x-Y^{322}. Substitutions in positions of residues highly conserved in other GPCRs are indicated by squares: N^{87}, S^{140}, D^{318}. Other residues mentioned in the text are marked by diamond symbols: N^{4}, N^{18}, N^{102}, K^{121}, D^{98} and E^{301}.

dimerization (Tsutsumi *et al.*, 1992; Conn *et al.*, 1982; Gregory *et al.*, 1982; Hazum and Keinan, 1985).

Another unusual feature of the cytosolic surface of the GnRH receptor is Ser^{140} in the position of the highly conserved Tyr (Y) residue of the "DRY" motif (Probst *et al.*, 1992) at the cytosolic end of transmembrane helix III (fig. 1.3). The Asp/Glu and Arg residues of this motif have been implicated in the coupling of some GPCRs to their G-Proteins (Baldwin, 1994; Fahmy and Sakmar, 1993). It is possible that this coupling is modulated by phosphorylation of the Tyr residue and that substitution with Ser allows regulation by a serine-threonine kinase instead of by a tyrosine kinase. However, mutation of Ser^{140} to Ala had no obvious effect on GnRH receptor function (Davidson *et al.*, 1994b).

Secondary and tertiary structure, allosteric regulation and G-protein coupling

The three-dimensional structure of GPCRs is unknown. Their association with the membrane makes them difficult to isolate and manipulate without denaturation. The shell of detergent which is necessary to maintain receptor conformation impedes formation of the crystals needed for diffraction studies (Findlay and Eliopoulos, 1990). However, the structures of six other integral membrane proteins have been reported (Kuhlbrandt, 1995). These structures provide some information about the intramembrane domains of membrane proteins (Findlay and Eliopoulos 1990). Intramembrane regions are largely α-helical. The helices span the membrane and are connected by 'loops' which are exposed to the polar environment and located at the surface of the membrane bilayer or extending into the aqueous phase (Findlay and Eliopoulos 1990). The α-helices are not absolutely regular. Distortions may be caused by Pro residues (Findlay and Eliopoulos 1990; Baldwin, 1993) and by stretches of hydrophilic residues (Findlay and Eliopoulos 1990).

Like the GPCRs, bacteriorhodopsin, one of the integral membrane proteins for which the structure has been reported, contains seven stretches of hydrophobic amino acids, which span the membrane. Bacteriorhodopsin is also similar to rhodopsin in that it responds to light through isomerization of covalently bound retinal (Baldwin, 1993; Soppa, 1994). Because of these similarities, the structure of bacteriorhodopsin has been used as a basis for several models of GPCRs (Findlay and Eliopoulos, 1990; Hibert *et al.*, 1991; Trump-Kallmeyer *et al.*, 1992; Zhang and Weinstein, 1993; reviewed by Schwartz, 1994). However, unlike rhodopsin, bacteriorhodopsin does not activate a G-protein and it has no sequence similarity with any of the GPCRs (Soppa, 1994; Schwartz, 1994). A projection map of rhodopsin, recently determined by electron crystallography of two-dimensional crystals, shows that the structure of rhodopsin clearly differs from that of bacteriorhodopsin. The resolution of this map (9Å) is too low to allow identification of specific helices. However, compared with that of bacteriorhodopsin, the projection map of rhodopsin is less elongated and slightly wider and the helices are tilted differently. The map shows four helices nearly perpendicular to the membrane and three helices which are more tilted (Schertler *et al.*, 1993). As the general arrangement of helices is similar in rhodopsin and bacteriorhodopsin, conclusions from models based on bacteriorhodopsin as to which residues face the centre of the molecule, should be valid. However, conclusions regarding specific interactions such as ligand binding and receptor activation would be unreliable as these depend on detailed knowledge of the relative positions of amino acids contained in different helices (Baldwin, 1994).

The conservation of particular amino acids within the hydrophobic regions of the rhodopsin GPCRs implies conserved structural and functional roles for these residues and implies that the proteins of the family share a common structure and mechanism of activation. Roles of the conserved residues may include maintaining the integrity of helix packing or

providing a framework for the binding pocket (Findlay *et al.*, 1993). They may also relay conformational changes associated with receptor activation. A probable arrangement of the helices of the GPCRs has been proposed, taking into consideration the sites of the most conserved residues in 105 unique receptor sequences; the sites where variability is restricted; sites which contain polar residues; sites where differences in sequence occur between closely related receptors and the rhodopsin projection map (Baldwin, 1993). Highly conserved residues, because of their proposed role in maintaining molecular structure, are expected to face other helices or the inside of the helical bundle (Donnelly *et al.*, 1989). Conserved proline residues sometimes cause kinks in the helices and thus may be on any face of the helix. Baldwin's analysis shows that helices I, II, IV, V and VI have clearly defined hydrophilic and lipophilic surfaces while helices III and VII show less segregation. Hydrophilic surfaces are likely to face towards the interior of the molecule while lipophilic surfaces are likely to be in contact with the membrane lipids. Helix III has very little lipophilic area. This helix is thus likely to be somewhat buried within the molecule and less exposed to the membrane lipid. The helices are probably arranged anticlockwise as viewed from the extracellular side of the membrane (Baldwin, 1993; Schwartz, 1994).

The conserved Cys residue at the extracellular end of the third transmembrane helix in the GnRH receptor probably forms a disulphide bridge with one of the Cys residues in the second extracellular loop. This second Cys residue is highly conserved in occurrence but not in position (Schwartz, 1994). The disulphide bridge probably stabilizes the active conformation of GPCRs (Baldwin, 1994). The importance of a disulphide bridge in the GnRH receptor was shown by an early experiment in which treatment with the reducing agent dithiothreitol decreased the affinity of GnRH agonist binding (Keinan and Hazum, 1985).

Several models of specific receptors have recently been based on the proposed structure of rhodopsin (Findlay *et al.*, 1993; Zhou *et al.*, 1994). However, only a few studies have investigated specific contact points between the helices (Schwartz, 1994). Mutation of the Asn^{312} residue in helix VII of the β_2 adrenergic receptor to the Phe found in the α_2 receptor caused complete loss of function. Replacement of the first two helices of the mutant β_2 receptor with the corresponding α_2 receptor sequence restored activity. Similarly, replacing the equivalent Phe^{412} residue of the α_2 adrenergic receptor with the Asn found in the β_2 receptor caused a loss of agonist binding which could be rescued by helix I of the β_2 receptor. This is compatible with a model of these receptors in which there is interaction of the Phe/Asn residue in helix VII with helix I (Suryanarayana *et al.*, 1992). Similar 'rescue' experiments have also indicated contact between helix I and helix VII of the muscarinic receptors (Pittel and Wess, 1993). A mutant Asp residue in helix II of rhodopsin from a patient with stationary night blindness was found to substitute for the counterion (usually Glu in helix III) of the Schiff base in helix VII (Rao *et al.*, 1994). Thus the sidechains of these

three residues (the usual Gly in helix II, Glu in helix III and Lys in helix VII) project into the same space within the rhodopsin molecule. This finding supports the anticlockwise arrangement of the transmembrane helices (Schwartz, 1994).

The GnRH receptor has also been used as a model for the identification of interhelical interactions. Two of the highly conserved residues, Asp in helix II and Asn in helix VII (fig. 1.3), are not conserved in the GnRH receptor. In fact they appear to be interchanged, with Asn^{87} in the conserved position in helix II and Asp^{318} in the helix VII position. This suggested that the GnRH receptor might be a natural reciprocal mutant compared with the other GPCRs and that the interchange might indicate that the two residues interact with each other. Mutation of Asn^{87} in helix II to Asp abolished receptor function. A second mutation, recreating the arrangement found in other GPCRs ($Asp^{87}Asn^{318}$), regenerated ligand binding. This restoration of binding by the reciprocal mutation shows that the sidechains of two residues in helices II and VII have complimentary roles in maintaining the structure of the receptor and occupy the same microenvironment within the receptor (Zhou *et al.*, 1994). Subsequent work has shown that all mutations of Asn^{87} (Asp, Ala and Gln) without a compensatory mutation at position 318 abolish receptor function. A hydrogen bond which may form between Asn^{87} and Asp^{318} is not important for proper receptor structure as substitution of Asp^{318} with Ala (which does not form hydrogen bonds) had a minimal effect on receptor structure as monitored by ligand binding (W. Zhou, H. Weinstein, C. Flanagan, R. P. Millar and S. C. Sealfon, in preparation). This also shows that Asn^{87} must participate in an interaction with a residue, other than Asp^{318}, which is important for receptor structure. Substitution of Asp^{318} with Glu decreases receptor expression by 90% (W. Zhou, H. Weinstein, C. Flanagan, R. P. Millar and S. C. Sealfon, in preparation). This probably shows that the intramolecular environment is confined and cannot easily accommodate an extra methylene group.

All mutations of Asp^{318} result in decreased inositol phosphate production in response to GnRH. When the receptor binding levels are taken into account, the rank order of uncoupling is Ala^{318} > $Asp^{87}Asn^{318}$ (double mutant) > Asn^{318} > Glu^{318} (W. Zhou, H. Weinstein, C. Flanagan, R. P. Millar and S. C. Sealfon, in preparation). This rank order indicates that both the presumed negative charge and hydrogen bonding capacity of Asp^{318} are important for coupling of agonist binding to intracellular signal transduction.

The helix II Asp has been widely studied. In many receptors, mutation of the conserved Asp disrupts receptor-G-protein interactions. This uncoupling is characterized by a decrease in agonist binding affinity, loss of the allosteric effect of GTP, which decreases agonist binding affinity, and decreased activation of G-proteins (Chung *et al.*, 1988, β adrenergic receptor; Ceresa and Limbird, 1994, α_2 adrenergic receptor; Quintana *et al.*, 1993, LH/CG receptor). The uncoupling of GnRH receptors mutated at Asp^{318}, in helix VII, suggests that

the functional role of the helix II Asp in other GPCRs might indeed be located at the helix VII Asp in the GnRH receptor. Alternatively, both residues may function in coupling the agonist binding signal to G-protein activation. One study has analyzed the importance of the conserved Asn residue in helix VII. It appears to have a role in maintaining the structure of the human $5HT_{1A}$ serotonin receptor. Coupling to intracellular signal transduction was not investigated (Chanda *et al.*, 1993).

The conserved helix II Asp also mediates the regulation of agonist binding by sodium ions in many GPCRs (Horstman *et al.*, 1990; Ceresa and Limbird, 1994; Kong *et al.*, 1993; Quintana *et al.*, 1993). However, an exploratory experiment in our laboratory suggested that this function of the helix II Asp has not been transferred to the helix VII Asp of the GnRH receptor. Cations modulate GnRH receptor agonist binding (Hazum, 1981; Loumaye *et al.*, 1984; Keinan and Hazum, 1985; Wormald *et al.*, 1985; Hazum, 1987) and antagonist binding (Hazum, 1981; Loumaye *et al.*, 1984). Monovalent cations Na^+, K^+ and Cs^+ inhibit GnRH agonist binding with similar IC_{50}s in the range 10 to 25 mM while divalent cations are more potent inhibitors with IC_{50}s ranging from 0.025 mM for Cu^{2+} to 1 mM for Ca^{2+} (Keinan and Hazum, 1985; Hazum, 1987). It has been suggested that cations bind to carboxylic groups within the ligand binding site of the GnRH receptor (Keinan and Hazum, 1985; Hazum, 1987). However, examination of the inhibition curves shows that some cations do not completely inhibit agonist binding, while others (Cu^{2+} and Mn^{2+}) exhibit very steep inhibition curves. These steep curves indicate a complex mechanism of inhibition rather than simple competitive inhibition. Thus, while it is likely that cations interact with one or more acidic amino acid residues as proposed, the residues are unlikely to be directly involved in ligand binding. The effect of cations on receptor affinity is probably an allosteric effect which causes a change in receptor conformation.

The intracellular loops of the GPCRs, particularly the third intracellular loop, mediate coupling to G-proteins (Dohlman *et al.*, 1991; Conklin and Bourne, 1993). Peptides corresponding to short segments of the intracellular loops are sufficient to bind and activate G-proteins. Therefore, in the absence of agonist, the inactive receptor must be able to constrain or shield such peptide segments from productive interaction with G-proteins (Samama *et al.*, 1993). Thus it has been proposed that receptors exist in an equilibrium between two conformations R and R^*. Agonist binding shifts this equilibrium towards R^*, which can be regarded as the active conformation. The R^* conformation exhibits high affinity for agonist ligands, and, according to this theory, is the only form which can bind the G-protein (Samama *et al.*, 1993). The existence of at least two conformations of the β-adrenergic receptor are supported by the increased agonist binding affinity of a constitutively active mutant receptor (Samama *et al.*, 1993) and by the increased basal activity when the wildtype receptor is overexpressed (Bond *et al.*, 1995). Addition of GTP (or a non-

hydrolyzable GTP analogue which activates G-proteins) destabilizes the ternary complex of hormone-receptor-G-protein and allows the receptor to return to the R conformation which has low affinity for agonists (De Lean *et al.*, 1980).

The ligand binding domain

Receptor activation is initiated by binding of an agonist ligand. Consequently, the conformational changes associated with receptor activation must have their origin in the ligand binding pocket (Findlay and Eliopoulos, 1990). Because of the spectroscopic properties of its tethered ligand, the ligand binding site of rhodopsin is the most widely studied. The bound retinal is located approximately 22 Å deep within the membrane bilayer. When the structural similarities between the cloned β-adrenergic receptor and rhodopsin were noted, it was suggested that the conserved transmembrane domains of these receptors might provide a common site for agonist binding (Strader *et al.*, 1994).

The significant chemical features of adrenalin and noradrenalin, the natural ligands of the β-adrenergic receptors, consist of a basic amine group, a stereoselective β-hydroxyl group and a catechol ring of which both hydroxyl groups are necessary for full activity. Specific residues in the β-adrenergic receptor have been identified as contact points for each of these functional groups. Asp^{113} in transmembrane helix III of the β-adrenergic receptor has been shown to form a salt bridge with the amine group, while two Ser residues in helix V form hydrogen bonds with the catechol hydroxyl groups. A Phe residue in helix VI has been implicated in binding to the catechol ring and it has been proposed that a Ser residue in helix IV interacts with the β-hydroxyl group (Strader *et al.*, 1994 for review). It has been proposed that the different amino acid residues which occur in equivalent positions in other related receptors participate in equivalent interactions with their ligands (Hibert *et al.*, 1991). Sequence alignment shows that all receptors that bind biogenic amines have an Asp residue in helix III in the position corresponding to the Asp^{113} of the β-adrenergic receptor. Mutagenesis studies have shown that the helix III Asp plays a critical role in ligand binding by α_2-adrenergic, muscarinic and histamine receptors (Strader *et al.*, 1994 for review).

The GnRH receptor has a basic residue, Lys^{121}, in helix III at the position analogous to the Asp^{113} of the β-adrenergic receptor. Substitution of Lys^{121} with an uncharged Gln decreased the binding affinity of agonist analogues but did not affect antagonist binding affinity (Zhou *et al.*, 1995). This indicates that Lys^{121} may interact with agonist ligands, but not with antagonists. GnRH antagonists differ from agonists chiefly in their amino-termini, where the pGlu-His-Trp sequence of GnRH is substituted with aromatic D-amino acids. Thus, an amino-terminal residue of GnRH may interact with Lys^{121} of the receptor. The interaction clearly does not involve a salt bridge as there are no negative charges in GnRH. The

electropositive Lys^{121} sidechain may form a hydrogen bond with the electron-dense aromatic rings of the His^2 or Trp^3 residues of GnRH, the imino group of His^2 (Zhou *et al.*, 1995), or a carbonyl group in the peptide backbone. In an early attempt to determine the nature of GnRH receptor residues involved in GnRH binding using chemical modification of pituitary membranes, the *N*-hydroxysuccinimide ester of acetic acid which modifies free amino groups did not affect GnRH binding (Keinan and Hazum, 1985). The combination of this information with the mutagenesis results suggests that the Lys^{121} residue is relatively inaccessible to the solvent or that its environment within the receptor decreases its reactivity.

GnRH is a considerably larger ligand than are the biogenic amines. As a consequence, the binding pocket of the GnRH receptor probably extends beyond the transmembrane domain. If there is a common origin of the conformational changes associated with activation of the rhodopsin family of receptors, it is likely to be a site accessed by all ligands. Such a site would be contained in the membrane-spanning helical bundle. Thus, it can be expected that the amino-terminal portion of the GnRH ligand, which is responsible for receptor activation, is the part that interacts with the transmembrane portion of the ligand binding pocket. It has recently been suggested that the activating end of another peptide ligand (substance P) interacts with a residue in the transmembrane domain of its receptor (Huang *et al.*, 1994; Strader *et al.*, 1994).

Peptide ligands are more complex than the biogenic amines. Their larger size means that they have many more functional groups, both in the amino acid sidechains and in the peptide backbone, which can potentially interact with specific receptor residues. The ligand binding pockets of peptide receptors need to be larger than those of the biogenic amines in order to accommodate the larger size of their ligands. Consequently, in addition to residues in the transmembrane domains (Yokota *et al.*, 1992; Mauzy *et al.*, 1992; Huang *et al.*, 1994; Strader *et al.*, 1994; Krystek *et al.*, 1994; Zhou *et al.*, 1995), both the extracellular amino-terminal segments and the extracellular loops of peptide receptors have been implicated in binding of diverse peptide ligands (Fong *et al.*, 1992a; Walker *et al.*, 1994; DeMartino *et al.*, 1994; Hjorth *et al.*, 1994). Indeed, the ligand binding sites for the large glycoprotein hormones, LH, FSH and TSH, are largely contained within the extracellular amino-terminal domains of their receptors (Segaloff and Ascoli, 1993; Dallas *et al.*, 1994). It has, however, been suggested that after binding to the amino-terminal domain of its receptor, LH is positioned in contact with the transmembrane domain in order to initiate receptor activation (Ji and Ji, 1991a, 1991b; Ji *et al.*, 1993). This implies that the origin of receptor activation lies in the transmembrane domain and that the mechanism of activation might be the same as in receptors which bind small ligands.

There is also evidence that shows that the extracellular segments of the GnRH receptor are involved in ligand binding. Deletion of part of the amino-terminal segment of the receptor

abolishes ligand binding and GnRH-stimulated inositol phosphate production (S. C. Sealfon, unpublished results). Chemical modification of the GnRH receptor in pituitary membranes indicated that at least one and possibly two carboxyl groups are involved in GnRH binding (Hazum and Keinan, 1985). The carboxyl groups were attributed to Glu or Asp residues or to polysaccharide sialic acid residues (Keinan and Hazum, 1985; Schvartz and Hazum, 1985; Hazum, 1987). Mutagenesis of the glycosylation consensus sequences in the mouse GnRH receptor has shown that polysaccharides do not affect ligand binding affinity (Davidson *et al.*, 1995). However, two acidic amino acid residues which affect the binding of GnRH have been identified in the first and third extracellular loops of the mouse GnRH receptor. These residues, Asp^{98} and Glu^{301}, will be discussed in chapter 4.

Treatment of the GnRH receptor in rat pituitary membranes with 2-methoxy-5-nitrobenzyl bromide, which modifies the indole ring of Trp residues, decreased subsequent binding of a GnRH agonist. This suggests that a Trp residue has an important role in ligand binding (Keinan and Hazum, 1985). Similarly, treatment with *p*-diazobenzenesulphonic acid or iodoacetamide indicated that a Tyr residue, but not a His, participates in ligand binding (Keinan and Hazum, 1985). The specific Trp and Tyr residues have yet to be identified in the cloned GnRH receptors.

GnRH and many other peptides are highly flexible and exist as an equilibrium mix of conformers in solution (Karten and Rivier, 1986). The multiplicity of peptide conformations adds to the complexity of ligand binding interactions in peptide receptors. The complexity was revealed by studies utilizing chimaeric receptors, consisting of segments from different receptor subtypes. Subtypes are closely related receptors which recognize a single ligand or a family of structurally similar peptides. For example, the neurokinin receptor subtypes, NK1, NK2 and NK3, bind the neurokinin peptides substance P, neurokinin A and neurokinin B, which have the common Carboxy-terminus, Phe-Xaa-Gly-Leu-Met-NH_2. However, each receptor subtype recognizes the peptides with a different rank order of affinity. Chimaeric receptors, consisting of segments from different receptor subtypes, have been used to locate general regions involved in ligand-binding domains, prior to identification of the specific residues concerned by point mutations (Schwartz *et al.*, 1994; Strader *et al.*, 1994 for reviews). It would be expected that conserved residues in the peptide ligands would interact with receptor residues which are conserved across all subtypes. Similarly, variant ligand residues would be expected to interact with variant receptor residues. However, experience with the neurokinin receptors has shown that this is not necessarily true and it has been suggested that this probably relates to peptide conformation (Fong *et al.*, 1992a; Strader *et al.*, 1994; Huang *et al.*, 1994). Exchange of four variant residues in the amino-terminal and first extracellular loop regions of the NK1 receptor for the amino acids which occur in equivalent positions in the NK3 receptor did not affect subtype selectivity and decreased receptor affinity

for all peptide ligands (Fong *et al.*, 1992a). Clearly the divergent receptor residues do not affect only interactions with divergent residues in the ligands. Exchanging residues in the second and third extracellular loops of the NK1 receptor with equivalent residues of the NK3 receptor increased affinity of the mutant NK1 receptor for neurokinin B, the preferred ligand of the NK3 receptor. However, the exchange did not decrease affinity of the mutant NK1 receptor for its own ligand, substance P (Fong *et al.*, 1992a). Similarly, chimaeras of the NK1 and NK2 receptors showed that the epitope which determines high affinity binding of substance P is located in the region extending from transmembrane helix II to the second extracellular loop of the NK1 receptor. However, chimaeras incorporating the equivalent region of the NK2 receptor did not exhibit high affinity for neurokinin A (Yokota *et al.*, 1992). Thus the residues which determine the ligand selectivity of receptor subtypes are located at different positions in different receptors. Similarly, residues located at different sites in the ETA and ETB endothelin receptors have been shown to determine endothelin subtype selectivity (Mauzy *et al.*, 1992; Krystek *et al.*, 1992). It is now clear that the positions of ligand contact sites vary between subtypes of peptide receptors. Within a single receptor, both conserved and divergent residues appear to interact with conserved residues which occur in all peptide ligands. Divergent residues located in different regions of the different receptors determine subtype selectivity (Strader *et al.*, 1994). Probably, each receptor subtype recognizes a different peptide conformation (Fong *et al.*, 1992a). Different peptide conformations would position some of the ligand functional groups (both conserved and divergent) differently within the ligand binding pocket and cause them to interact with residues in different positions in the receptor sequences.

Several different forms of GnRH have been identified in various vertebrate species (see structures of naturally occurring GnRHs). The differences in their amino acid sequences are similar to the differences found in the sequences of the neurokinin peptides. This suggests that, in the absence of well described subtypes of the GnRH receptor, the relationship of the mammalian GnRH receptors to any one of the non-mammalian receptors may be similar to that found between subtypes of receptors for peptides such as the neurokinins. If this is true, amino acid sequence homology between mammalian and non-mammalian GnRH receptors should be about 60 to 80% in the transmembrane helices (Strader *et al.*, 1994). In addition, the non-mammalian receptors probably show selectivity for peptide conformations different from that preferred by the mammalian receptors. As discussed earlier in this chapter, the mammalian GnRH receptor appears to recognize GnRH in a conformation containing a β-II bend. Constraining this conformation enhances binding to the mammalian GnRH receptor, but is much less effective in increasing interaction with the chicken pituitary GnRH receptor (Millar *et al.*, 1986b; chapter 2). This indicates that the chicken GnRH receptor does not bind GnRH in the same conformation as do the mammalian GnRH receptors. The chicken GnRH receptor will be discussed in more detail in chapter 2.

In addition to the ability of receptors to recognize particular conformations of peptide ligands, it is possible that receptors cause the ligands to change conformation subsequent to binding. It has been proposed that binding of the glycopeptide ligand, C5a, to the amino-terminal segment of its receptor is followed by a change in the conformation of the ligand which exposes the carboxy-terminal portion of the ligand. This allows subsequent interaction of the exposed carboxy-terminus with the transmembrane domain of the receptor (DeMartino *et al.*, 1994). We have also proposed that the conformation of GnRH is modified by interaction with its receptor (Flanagan *et al.*, 1994; discussed in chapter 4).

The antagonist binding domain

It is becoming clear from studies with many GPCRs that agonist and antagonist ligands do not necessarily occupy the same ligand binding domain even though antagonists appear to be competitive inhibitors of agonist binding (Fong *et al.*, 1992a, 1992b; Gether *et al.*, 1993; Huang *et al.*, 1994; Strader *et al.*, 1994). It has been proposed that the competitive behavior arises from a volume exclusion effect in which agonist and antagonist binding pockets overlap in space, although agonist and antagonist ligands interact with different receptor residues (Fong *et al.*, 1992a; Huang *et al.*, 1994; Strader *et al.*, 1994). It has also been observed that some (non-peptide) antagonists are not simple competitive inhibitors of agonist binding (Huang *et al.*, 1994; Rosenkulde *et al.*, 1994), and it has been proposed that they inhibit agonist access by an allosteric mechanism (Rosenkulde *et al.*, 1994).

The GnRH receptor also appears to have different binding domains for agonist and antagonist ligands. Mutation of the Lys^{121} residue affects binding of agonists but not of antagonists (Zhou *et al.*, 1995). Thus, Lys^{121} may be an agonist contact site, but it is clearly not an antagonist binding site. Even before the cloning of the GnRH receptor allowed site-directed mutagenesis, there was evidence that the GnRH receptor binding sites for agonists and antagonists differed. Pretreatment of pituitary membranes with proteolytic enzymes, trypsin and chymotrypsin, decreased subsequent binding of labelled antagonist more than it did binding of labelled agonist (Hazum, 1981). This suggests that the agonist binding site was less exposed to the enzymes. The agonist binding site may, thus, be more buried within the membrane domain of the receptor. A different proteolytic approach was more recently used to compare the binding sites of photoactive agonist and antagonist analogues of GnRH (Janovick *et al.*, 1993). Radioiodinated photoactive agonist and antagonist peptides were covalently attached to the GnRH receptor in pituitary membranes. Both of the resulting labelled receptor-ligand complexes migrated as broad bands with a M_r range of 46-60 K on SDS-PAGE. The broadness of the bands is due to heterologous glycosylation of the receptor. After treatment of the receptor-ligand complexes with trypsin, the agonist label was still attached to the glycosylated amino-terminal fragment of the receptor which migrated with a

33-48 K range of M_r. In contrast, the antagonist label migrated with the dye front of the gel, showing that it was bound to a much smaller fragment of receptor which was not glycosylated (Janovick *et al.*, 1993). At the least, this shows that the environment of the photoactive group attached to a D-Lys in position 6 of the ligands is different for agonists and antagonists. Thus, two quite different approaches, mutagenesis of Lys^{121} and proteolytic cleavage of the receptor, show that the binding sites for GnRH agonists and antagonists differ.

Although it has been shown that most vertebrate species have more than one form of GnRH (King and Millar, 1992) and multiple receptor subtypes have been demonstrated (Murthy and Peter, 1994; Murthy *et al.*, 1994), the high sequence homologies and similar pharmacological behavior of the receptors cloned to date indicate that they all belong to a single subtype. Consequently, it has not been possible to use GnRH receptor subtype chimaeras to investigate ligand binding domains. However, in some receptor systems, chimaeras of the same receptor subtype from different species have been used to investigate binding of non-peptide antagonists (Fong *et al.*, 1992b; Sachais *et al.*, 1993; Jensen *et al.*, 1993). Non-peptide ligands have not yet been described for the GnRH receptor, but species differences have been reported in the recognition of peptide GnRH antagonists by human and rat receptors (Wormald *et al.*, 1985). Chimaeras of mammalian GnRH receptors may thus provide a useful tool for investigating the GnRH antagonist binding domain.

In other peptide receptor systems, non-peptide antagonists have provided useful pharmacological tools. Their binding sites are different from the binding sites of both agonists and peptide antagonists. Consequently, they exhibit different sensitivities to receptor mutagenesis and allow analysis of the ligand binding properties of mutant receptors which have low affinities for peptide ligands (Fong *et al.*, 1992a; Gether *et al.*, 1993). Development of non-peptide ligands (whether agonist or antagonist) for the GnRH receptor will facilitate studies of the receptor. The cloning of the GnRH receptor will allow establishment of systems to screen lead compounds for development of non-peptide analogues of GnRH. In turn the growing understanding of the receptor ligand binding domain will optimize the development of new GnRH analogues.

Development of non-peptide analogues of GnRH will revolutionize the pharmacological and prophylactic applications of GnRH analogues. Resistance of the new analogues, because of their non-peptide nature, to digestive enzymes will allow them to be administered orally. The new ease of administration is anticipated to make non-peptide GnRH analogues the basis of a new generation of non-steroidal oral contraceptives for both men and women.

CHAPTER 2

CHARACTERIZATION OF STRUCTURAL REQUIREMENTS FOR GnRH RECEPTOR BINDING AND GONADOTROPIN RELEASE USING CHIMAERAS OF VARIANT GnRH FORMS

SUMMARY

Eleven variant forms of GnRH have now been identified in various animal species. The most frequently substituted residues are those in positions 5, 7 and 8. Chimaeric analogues of 4 vertebrate forms of GnRH were synthesized in order to study the functional significance of the most common substitutions. Peptides were tested for their ability to stimulate release of LH and FSH in cultured sheep pituitary cells and LH in cultured chicken pituitary cells. Binding affinities for the GnRH receptors in sheep and rat pituitary cell membranes were also measured. Arginine was required in position 8 for high GnRH activity in mammalian systems, but analogues with neutral substitutions in position 8 were more potent in chicken pituitary cells. The chicken GnRH receptor appears to be less stringent than the mammalian receptors in its recognition of peptide conformation. Histidine in position 5 decreased LH releasing potency in chicken cells, but slightly increased receptor binding affinity in rat and sheep membranes. Tryptophan in position 7 had minimal effect on GnRH activity in mammals, but increased LH release in chicken cells. Some substitutions affected receptor binding affinity and receptor activation differently in sheep pituitaries. Peptides with histidine in position 5 had relatively high receptor binding potency compared with their LH releasing potency, while peptides with Trp^7,Tyr^8 substitutions exhibited relatively high gonadotropin releasing potencies and lower receptor binding affinities. Rat GnRH receptors exhibited higher affinity for analogues with tryptophan in position 7 than did sheep GnRH receptors.

INTRODUCTION

Although early studies suggested that the structure of GnRH was conserved throughout the vertebrates (Jeffcoate *et al.*, 1974), eleven different forms of GnRH have now been identified in the various animal species (see chapter 1, Structures of naturally occurring GnRHs). During the 500 million years of evolution separating the most primitive vertebrates, lampreys, from mammals, the GnRH residues in positions 5, 7 and 8 have been most subject to substitution.

It is well known that mammalian GnRH receptors require the Arg^8 residue found in mammalian GnRH (Sandow *et al.*, 1978). However, studies with chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) showed that it was more active than chicken GnRH I ([Gln^8]-

GnRH) in rat and sheep pituitaries as well as in chicken pituitaries (Miyamoto *et al.*, 1984; Millar *et al.*, 1986b). This indicated that analogues of GnRH with His^5 and/or Trp^7 substitutions might have increased gonadotropin releasing activity and high affinity for the GnRH receptor. Such analogues could form the basis of a new series of GnRH agonists and antagonists containing only naturally occurring L-amino acids.

In addition, a previous study reported that chicken GnRH II was more potent in stimulating FSH release than in stimulating LH release in cultured chicken pituitary cells (Millar *et al.*, 1986b). This suggested that chicken GnRH II might be the contentious specific FSH releasing peptide (reviewed in Mizunuma *et al.*, 1983).

In this study, chimaeras of naturally occurring GnRH mutants were synthesized in order to investigate the functional significance of the position 5, 7 and 8 substitutions. Chimaeras were based on mammalian GnRH, chicken GnRH I ([Gln^8]-GnRH), salmon GnRH ([Trp^7,Leu^8]-GnRH) and chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) because these structures were known at the time. Receptor binding and gonadotropin releasing properties of the chimaeras were studied in two mammalian systems (sheep and rat) and one non-mammalian system (chicken).

MATERIALS AND METHODS

Peptides

GnRH analogues [Trp^7,Leu^8]-GnRH and [Leu^8]-GnRH were gifts from Dr. R. Roeske. [D-Trp^6,Gln^8]-GnRH was synthesized by R. P. Millar. Other peptides were synthesized by R. Milton. Mammalian GnRH, lamprey GnRH I and [D-Trp^6]-GnRH were synthesized manually and the rest were synthesized on a Beckman System 990 peptide synthesizer using conventional solid phase methodology on *p*-methylbenzhydrylamine-1% divinylbenzene-styrene copolymer resin. Cleavage of the peptide-amide from the resin and concomitant deprotection were performed in redistilled hydrogen fluoride containing 1% anisole. Peptides were purified by preparative C18 reversed phase HPLC to greater than 96% purity (integrated area under the main peak vs the total integrated area recorded at 210 nm). Peptides were used as trifluoroacetic acid salts except for mammalian GnRH and [D-Trp^6]-GnRH which were converted to acetate form by chromatography on DEAE cellulose (Whatman DE52). Amino acid compositions were determined by gas phase hydrolysis in 6M HCl (Waters Pico-Tag system) followed by *o*-phthalaldehyde derivatization. Compositions were consistent with the predicted peptide sequences. Concentrations of peptides used in biological tests were calculated using the peptide contents determined by amino acid analysis.

Culture of chicken pituitary cells and stimulation of LH release

Anterior pituitaries were dissected from chicken heads (Golden Grove Poultry Co., Cape Town) which had been washed with disinfectant (Hibitane, 0.5% chlorhexidene gluconate in 80% methanol) and kept on ice for less than 2 h. Pituitaries were collected into HEPES-buffered (20 mM, pH 7.4) Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) to prevent desiccation. They were then minced with a scalpel blade and digested for 1 h at 37° C with continuous slow stirring in collagenase solution [0.9% w/v collagenase (155 U/mg, Worthington Biochemical Corp, Freehold, NJ), and 18 mg/l deoxyribonuclease (Miles laboratories, Elkhart, IN), in HEPES-BSA buffer (25mM HEPES, pH 7.2, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 0.36mM $CaCl_2$, 10mM glucose and 10g/l BSA, fatty acid free, Pentex fraction V, Miles Laboratories)]. Cell clumps were broken up by passage through a pipette at 10 min intervals. The cell suspension was centrifuged twice at 500 g for 5 min and the pellet resuspended each time in buffer A (140mM NaCl, 4mM KCl, 1.4mM Na_2HPO_4, 8.3mM glucose, 20mM HEPES, pH 7.4, and 6mg/l phenol red) to which 0.5mM EDTA and 0.3% (w/v) BSA were added. The cell suspension was then filtered through nylon gauze prior to dilution in DMEM containing 10% foetal calf serum (Gibco), penicillin (60 mg/l), streptomycin (100 mg/l), neomycin (100 mg/l) and amphotericin B (20 mg/l) and dispensed into 6-well tissue culture plates (Falcon, Oxnard, CA) at a density of 1.3 pituitary equivalents per well. One pituitary equivalent amounts to about 5 x 10^5 cells. Cells were cultured at 37° C in 5% CO_2 for 24 h, after which the medium was replaced with amphotericin B-free medium and culture was continued for a further 24 h. Prior to stimulation, cells were washed twice with buffer A containing 1mM $CaCl_2$ and 0.1% (w/v) BSA and preincubated for two periods of 5 min in the same buffer. Triplicate stimulations were performed for 30 min at 37° C with peptides dissolved in the same buffer. Medium was collected, centrifuged to remove any loose cells, and stored at -20° C until radioimmunassay for chicken LH. Mammalian GnRH was included in all bioassays as a reference standard for comparing the activity of GnRH analogues.

Culture of sheep pituitary cells and stimulation of gonadotropin release

The gonadotropin releasing activities of synthetic GnRH analogues were assessed in cultured sheep pituitary cells using a previously described method (Millar and King, 1983a; Millar *et al.*, 1986b). Briefly, anterior pituitaries were dissected from sheep heads within 30 min of slaughter, minced, and digested in collagenase solution as described for chicken pituitaries. The cells were resuspended in Eagle's Minimum Essential Medium with Hank's salts (MEM) (Gibco, Paisley, Scotland) containing 10% foetal calf serum and antibiotics, and cultured (10^5-10^6 cells/well) at 37° C in 5% CO_2 for 4 or 5 days with a medium change after 3 days. Cells were washed twice in MEM containing 10% foetal calf serum and 4 times with

serum-free MEM. Peptide stimulations were performed for 2h in 1ml serum-free MEM at 37° C in 5% CO_2. 0.9 ml of medium was collected into tubes containing 0.1 ml of HEPES-BSA buffer, centrifuged and stored (-20° C) until radioimmunoassay for sheep LH and FSH.

Gonadotropin radioimmunoassays

Purified sheep LH (LER-1056-C2, a gift from L. E. Reichert) was iodinated using chloramine T, and separated from free radioidide on a cellulose CF 11 (Whatman Inc., Clifton, NJ) column. Sheep LH radioimmunassays (RIA) were performed as previously described (Millar and Aehnelt, 1977) using unlabeled sheep LH (NIH-LH-S18) to generate the standard curve and antiserum GDN 15 (a gift from G. D. Niswender, Colorado State University) which had been raised against sheep LH. The sheep FSH RIA was performed according to instructions using oFSH-RP1 as standard, ^{125}I-oFSH-I-1 (radioiodinated as for LH) and anti-oFSH-1 antiserum (1:160 000 final dilution) which were supplied by S. Raiti (National Hormone and Pituitary Program, NIDDK). Chicken LH was measured as previously described (Follet *et al.*, 1972) using antiserum 16/6 and ^{125}I-(Sharp PRC AEI-1). For all 3 assays antiserum-bound label was precipitated using second antibody coupled to cellulose (Sac-Cel RD70, Wellcome Reagents Limited, Beckenham, England). All of the samples from each stimulation experiment were included in the same assay. Interasssay coefficients of variation were 13.7 % for chicken LH, 4.6% for sheep LH and 8.3% for sheep FSH.

GnRH receptor binding assays

Competitive binding of GnRH peptides to rat pituitary cell membranes was investigated as previously described (Millar and King, 1983a; Millar *et al.*, 1986b). In addition a sheep pituitary GnRH receptor binding assay was developed for this study. Anterior pituitaries from adult male rats (Long-Evans) or castrated adult male sheep were homogenized in ice-cold 10mM tris-HCl buffer containing 10mM tris, pH 7.4, 1mM dithiothreitol, 1mM EDTA and 0.1% (w/v) BSA. The rat pituitary homogenate was centrifuged for 30 min at 15 000 x g and 4° C and the resulting pellet was resuspended in tris-HCl buffer. The sheep pituitary homogenate was centrifuged first at 100 x g (5 min, 4° C), to remove a component which exhibited high non-specific binding, and the resulting supernatant was recentrifuged at 3 500 x g for 10 min at 4° C. The pellet was resuspended in tris-HCl buffer. The resuspended membranes (0.16 rat or 0.05 sheep pituitary equivalents per tube) were incubated (90 min on ice) with ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH (60 000 cpm, iodinated by the chloramine T method, specific activity 1000 Ci/g) and increasing concentrations of test peptides in a final volume of 0.5 ml. The incubation was terminated by addition of 3 ml of phosphate-buffered saline containing 1% (w/v) BSA (PBS-BSA) and immediate filtration,

under vacuum, through glass-fibre filters (GF/C, Whatman), presoaked in PBS-BSA. Filters were washed 3 times with 3 ml PBS-BSA and the retained radioactivity was counted in a gamma counter. Non-specific binding (2% in rat, 26% in sheep) was determined in the presence of 10^{-6}M [D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH and subtracted from all samples.

Data analysis

EC_{50} values (peptide concentrations required for half-maximal stimulation of gonadotropin release) and IC_{50}s (peptide concentrations required for half-maximal inhibition of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH binding) were determined using four-parametre nonlinear curve-fitting (ALLFIT) (DeLean *et al.*, 1978), forcing curves to share minimum and maximum values. Potencies were calculated relative to the EC_{50}s and IC_{50}s obtained with mammalian GnRH. All values presented are the means of at least 2 experiments performed in triplicate.

RESULTS

Mammalian systems

The non-mammalian GnRHs exhibited relatively low potencies in stimulating release of LH and FSH from cultured sheep pituitary cells (table 2.1, fig. 2.1A). They also exhibited low binding affinity for both sheep and rat GnRH receptors (table 2.1, figs. 2.1D, 2.2A). The common feature of these peptides is the substitution of a neutral residue for the basic Arg^8 of mammalian GnRH. The low gonadotropin releasing potencies and binding affinities of the position 8-substituted analogues [Leu^8]-GnRH and [Tyr^8]-GnRH (table 2.1, figs. 2.1B, 2.1E, 2.2B, 2.3) and the relatively low potencies of all chimaeras containing neutral residues in position 8 (table 2.1, fig 2.3) confirmed the importance of Arg^8 for GnRH activity in mammals.

The IC_{50} for GnRH in the sheep receptor binding assay was more than an order of magnitude higher than the EC_{50}s for LH and FSH release. This is consistent with the existence of a considerable receptor reserve in pituitary gonadotropes. Because of the differences in the absolute values of EC_{50}s and IC_{50}s, potencies were calculated relative to mammalian GnRH to allow comparison of the different assays. The relative binding affinities of the non-mammalian GnRHs correlated broadly with their relative LH and FSH releasing activities. However, their gonadotropin releasing potencies were an order of magnitude higher than their receptor binding potencies (table 2.1, figs. 2.4, 2.1A, 2.1D). Higher gonadotropin releasing potency than receptor binding potency was a characteristic of all peptides with neutral residues in position 8 (figs. 2.4, 2.1, table 2.1).

Figure 2.1 Stimulation of LH release and competition for binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH by GnRH analogues in sheep gonadotropes. LH release, left panels, GnRH receptor binding, right panels. Symbols are identified in the key in fig. 2.2 (facing page).

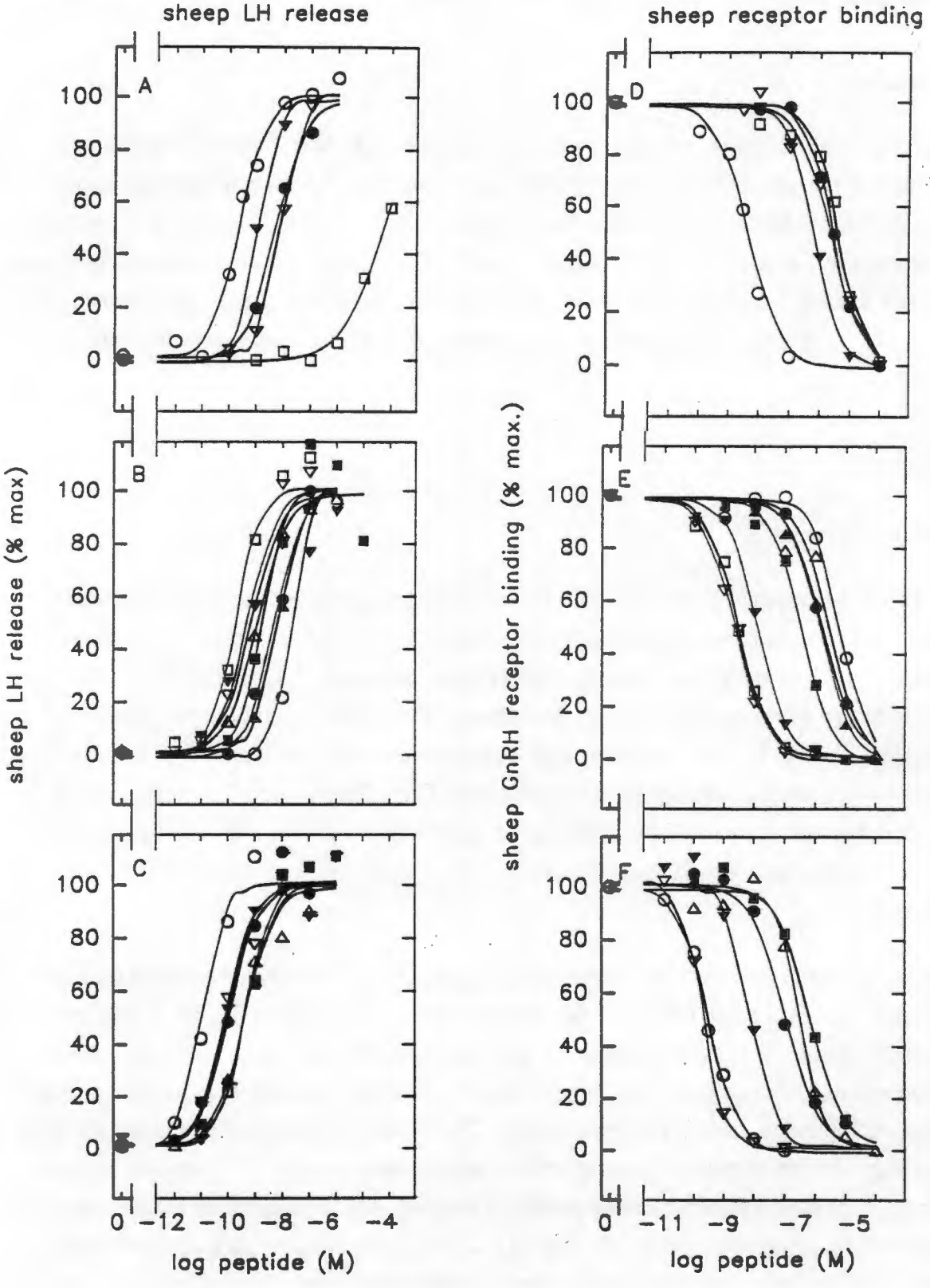


Figure 2.2 GnRH analogue competition for binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH in rat pituitary membranes.

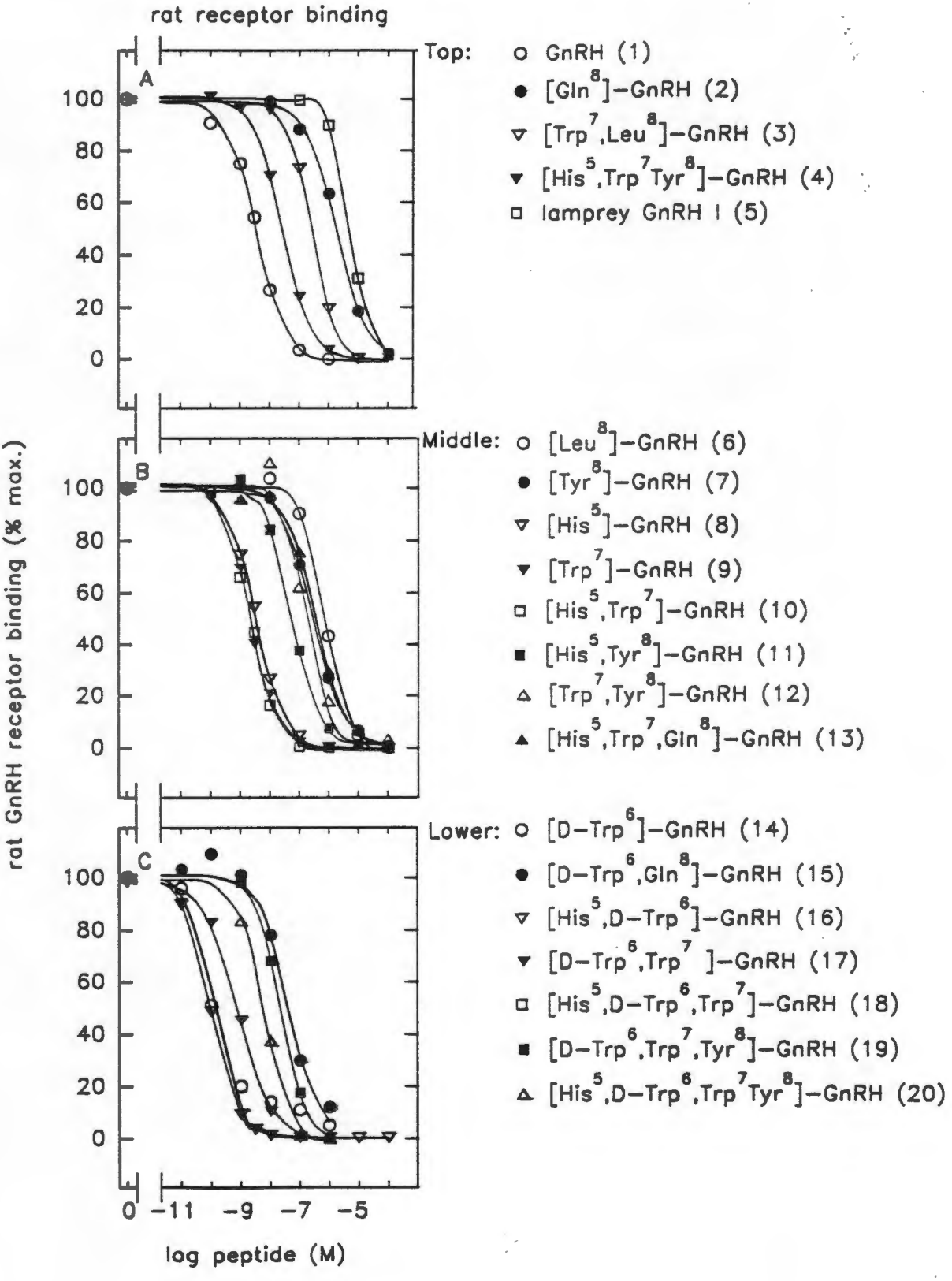


Table 2.1 Gonadotropin releasing activity and GnRH receptor binding of vertebrate GnRHs and chimaeric analogues in mammalian and chicken gonadotropes

PEPTIDE	HPLC retent time	GONADOTROPIN RELEASE						RECEPTOR BINDING			
		Chicken LH		Sheep LH		Sheep FSH		Sheep		Rat	
# substitutions	(min)	EC50(nM)	potency	EC50(nM)	potency	EC50(nM)	potency	IC50(nM)	potency	IC50(nM)	potency
1 GnRH (mammalian)	8.8	2.9 ± 0.40	1.0	0.21 ± 0.05	1.0	0.11 ± 0.26	1.0	4.1 ± 0.3	1.0	3.4 ± 0.2	1.0
2 Gln^8 (chicken I)	10.2	1.8 ± 0.30	1.6	6.3 ± 3.9	0.03	2.8 ± 1.6	0.04	2600 ± 400	0.002	1400 ± 10	0.002
3 Trp^7,Leu^8 (salmon)	21.4	0.40 ± 0.08	7.3	11 ± 3	0.02	4.3 ± 6.7	0.03	2700 ± 600	0.002	258 ± 203	0.013
4 His^5,Trp^7,Tyr^8 (chicken II)	12.2	0.35 ± 0.07	8.2	0.92 ± 0.26	0.23	1.6 ± 0.8	0.07	545 ± 91	0.008	22 ± 2	0.15
5 Tyr^3,Leu^5,Glu^6,Trp^7,Lys^8 (lamprey I)	9.8	23000 ± 10000	<0.001	39000 ± 15000	<0.001	13000 ± 8000	<0.001	3600 ± 500	<0.001	3300 ± 220	0.001
6 Leu^8	18.8	2.6 ± 0.7	1.1	20 ± 7	0.01	9.7 ± 2.4	0.01	4100 ± 600	0.001	590 ± 80	0.006
7 Tyr^8	14.3	0.71 ± 0.18	4.1	4.2 ± 2.3	0.05	1.1 ± 0.5	0.11	1500 ± 300	0.003	208 ± 31	0.016
8 His^5	2.0	30 ± 6	0.1	0.43 ± 0.10	0.49	0.39 ± 0.43	0.28	2.0 ± 0.3	2.1	2.0 ± 0.2	1.7
9 Trp^7	10.6	0.49 ± 0.10	5.9	0.19 ± 0.07	1.1	0.18 ± 0.12	0.62	11 ± 2	0.37	1.5 ± 0.2	2.3
10 His^5,Trp^7	6.0	9.6 ± 3.0	0.30	0.16 ± 0.06	1.3	0.25 ± 0.11	0.44	2.7 ± 0.4	1.5	1.5 ± 0.2	2.3
11 His^5,Tyr^8	7.9	3.0 ± 0.7	0.96	2.1 ± 0.6	0.10	3.7 ± 1.4	0.03	280 ± 54	0.015	31 ± 3.6	0.11
12 Trp^7,Tyr^8	17.5	0.19 ± 0.03	16	0.98 ± 0.24	0.22	0.49 ± 0.11	0.23	2000 ± 300	0.002	133 ± 17	0.026
13 His^5,Trp^7,Gln^8	6.5	1.4 ± 0.4	2.1	7.2 ± 2.4	0.03	9.7 ± 3.2	0.01	1300 ± 300	0.003	267 ± 39	0.013
14 D-Trp^6	18.8	0.55 ± 0.08	5.3	0.008 ± 0.002	27	0.005 ± 0.003	25	0.16 ± 0.03	26	0.07 ± 0.02	47
15 D-Trp^6,Gln^8	22.2			0.17 ± 0.04	1.3	0.20 ± 0.10	0.57	95 ± 20	0.043	36 ± 9	0.09
16 His^5,D-Trp^6	14.0	0.82 ± 0.28	3.5	0.050 ± 0.015	4.2	0.027 ± 0.011	4.2	0.14 ± 0.02	30	0.06 ± 0.01	57
17 D-Trp^6,Trp^7	21.5	0.63 ± 0.18	3.1	0.081 ± 0.015	2.6	0.12 ± 0.05	0.93	5.2 ± 0.9	0.80	0.41 ± 0.11	8.3
18 His^5,D-Trp^6,Trp^7	15.8	0.95 ± 0.33	3.1	0.14 ± 0.03	1.5	0.17 ± 0.08	0.65				
19 D-Trp^6,Trp^7,Tyr^8	26.6	0.32 ± 0.06	8.9	0.38 ± 0.09	0.55	0.35 ± 0.11	0.31	436 ± 96	0.009	14 ± 3	0.25
20 His^5,D-Trp^6,Trp^7,Tyr^8	21.3	0.42 ± 0.09	6.9	0.54 ± 1.43	0.39	0.71 ± 0.21	0.16	203 ± 39	0.020	3.5 ± 0.8	0.99

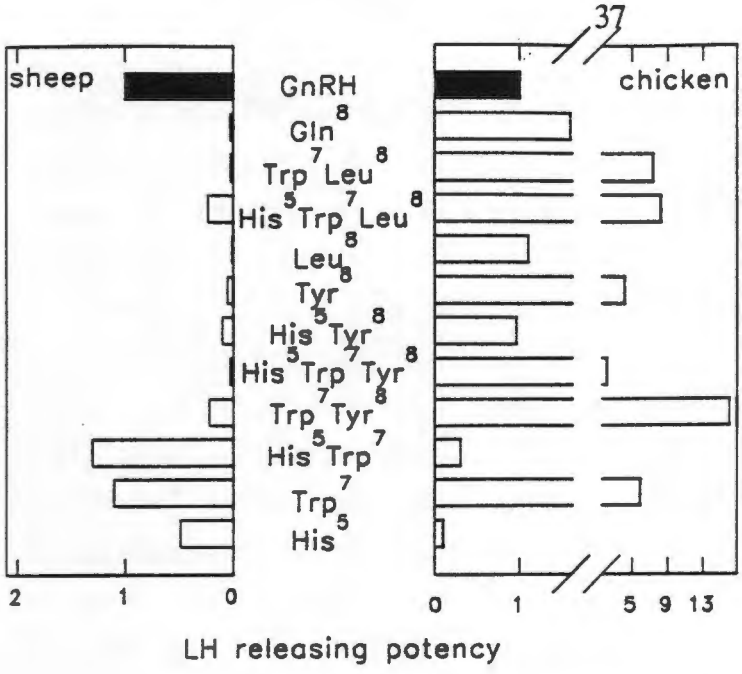


Figure 2.3 Comparison of LH releasing potencies of vertebrate GnRHs and chimaeras in sheep and chicken pituitary cells.

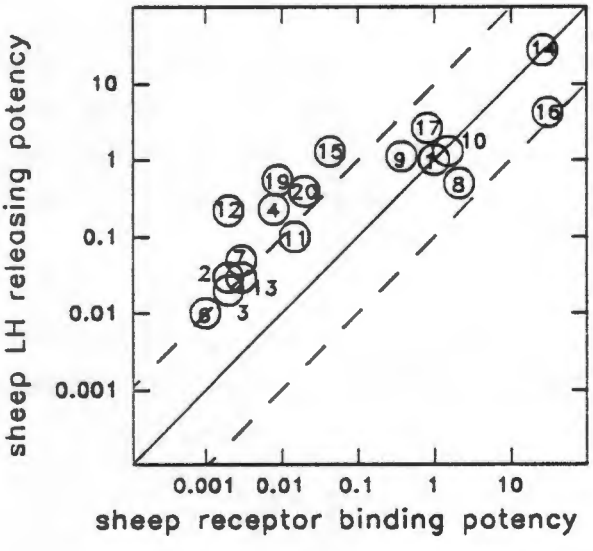


Figure 2.4 Relationship between the receptor binding potencies and LH releasing potencies of GnRH peptides in sheep. Numbers correspond to the peptides in table 2.1, (–) indicates a 1:1 relationship, (--) indicates an order of magnitude displacement from a 1:1 relationship.

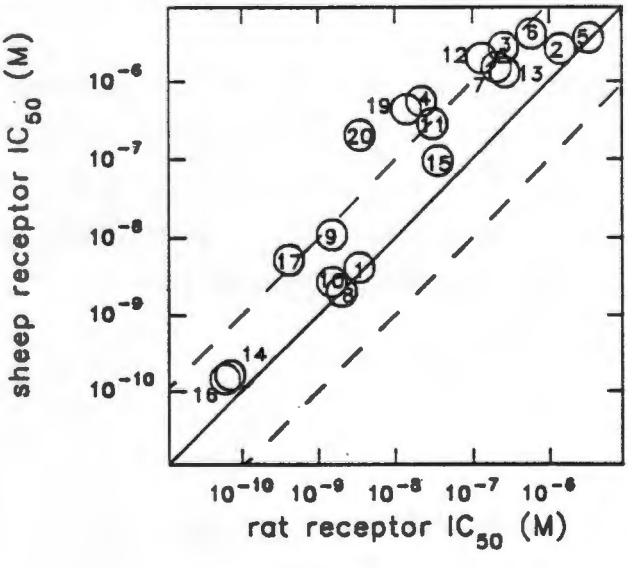


Figure 2.5 Relationship between IC_{50}s of GnRH analogues in rat and sheep pituitary membranes. Numbers correspond to the peptides in table 2.1, (–) indicates a 1:1 relationship, (--) indicates an order of magnitude displacement from a 1:1 relationship.

Chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) was the most active of the non-mammalian GnRHs in the sheep and rat test systems. Chimaeric peptides were synthesized to test the hypothesis that the His^5 or Trp^7 substitutions might account for the higher potency of chicken GnRH II compared with peptides containing substitutions only in position 8. Incorporation of His^5 into GnRH slightly increased binding affinity for sheep and rat GnRH receptors and slightly decreased sheep gonadotropin releasing activity (table 2.1, figs 2.1E, 2.2B, 2.1B). Trp^7 alone or in combination with His^5 (peptides 9 and 10 in table 2.1) had minimal effects on GnRH receptor binding affinity or gonadotropin release. In combination with Tyr^8, His^5 increased binding affinity (5-fold) without affecting gonadotropin release (peptide 11 compared with peptide 7 in table 2.1). In contrast, incorporation of Trp^7 enhanced the gonadotropin releasing activity of [Tyr^8]-GnRH without affecting binding affinity (peptides 12 and 7 in table 2.1). Thus, His^5 seems to account for the relatively high binding affinity of chicken GnRH II, while Trp^7 seems to account for its higher biological activity.

Comparison of the LH releasing potencies and receptor binding potencies of the GnRH analogues in the sheep pituitary (fig. 2.4) shows that peptides containing a His^5 substitution had higher binding potency than releasing potency (peptides 8 and 16). Conversely, peptides with the highest ratio of releasing potency to binding potency contained Trp^7,Tyr^8 substitutions (peptides 12 and 19).

D-Trp^6 incorporation markedly enhanced the gonadotropin releasing activities and receptor binding affinities of mammalian GnRH, chicken GnRH I and [His^5]-GnRH (peptides 14, 15 and 16 compared with peptides 1, 2, and 8) in the mammalian systems. However, it was much less effective in the presence of Trp in position 7 (table 2.2, figs. 2.1, 2.2).

The was no difference in potency for release of LH compared with FSH with any of the peptides tested (table 2.1).

Comparison of the results obtained in the receptor binding assays performed with sheep and rat pituitary membranes (fig. 2.5) shows that, while there is a general correlation of the affinities measured in both systems, some peptides exhibit distinctly different affinities for the two receptors. Peptides 3, 4, 12, 19 and 20 (table 2.1) exhibit more than 10-fold lower affinity for the sheep receptor than for the rat receptor. Common features of these peptides are Trp in position 7 and a hydrophobic residue (Tyr or Leu) in position 8.

Chicken LH release

Non-mammalian GnRHs were more potent than mammalian GnRH in stimulating release of LH from cultured chicken pituitary cells, except for lamprey GnRH I which was inactive (fig. 2.6A). As was anticipated from this result and previous studies (Miyamoto *et al.*, 1984; Millar

#	GnRH analogue	POTENCY RELATIVE TO PARENT COMPOUND LH sheep	FSH sheep	receptor binding sheep	receptor binding rat	LH chicken
14	D-Trp^6	26.7	25.0	25.9	47.1	5.3
15	D-Trp^6,Gln^8	37.1	14.2	27.0	38.9	
16	His^5,D-Trp^6	8.5	14.7	14.5	34.0	35.9
17	D-Trp^6,Trp^7	2.3	1.5	2.1	3.6	0.53
18	His^5,D-Trp^6,Trp^7	1.1	1.5			10.2
19	D-Trp^6,Trp^7,Tyr^8	2.5	1.4	4.5	9.6	0.57
20	His^5,D-Trp^6,Trp^7,Tyr^8	1.7	2.3	2.7	6.5	0.84

Table 2.2 Effect of D-Trp^6 incorporation on gonadotropin releasing and receptor binding potencies of GnRH analogues

et al., 1986b), GnRH analogues with substitutions in position 8 showed high activity in stimulating LH release from chicken pituitary cells (fig. 2.6B, table 2.1).

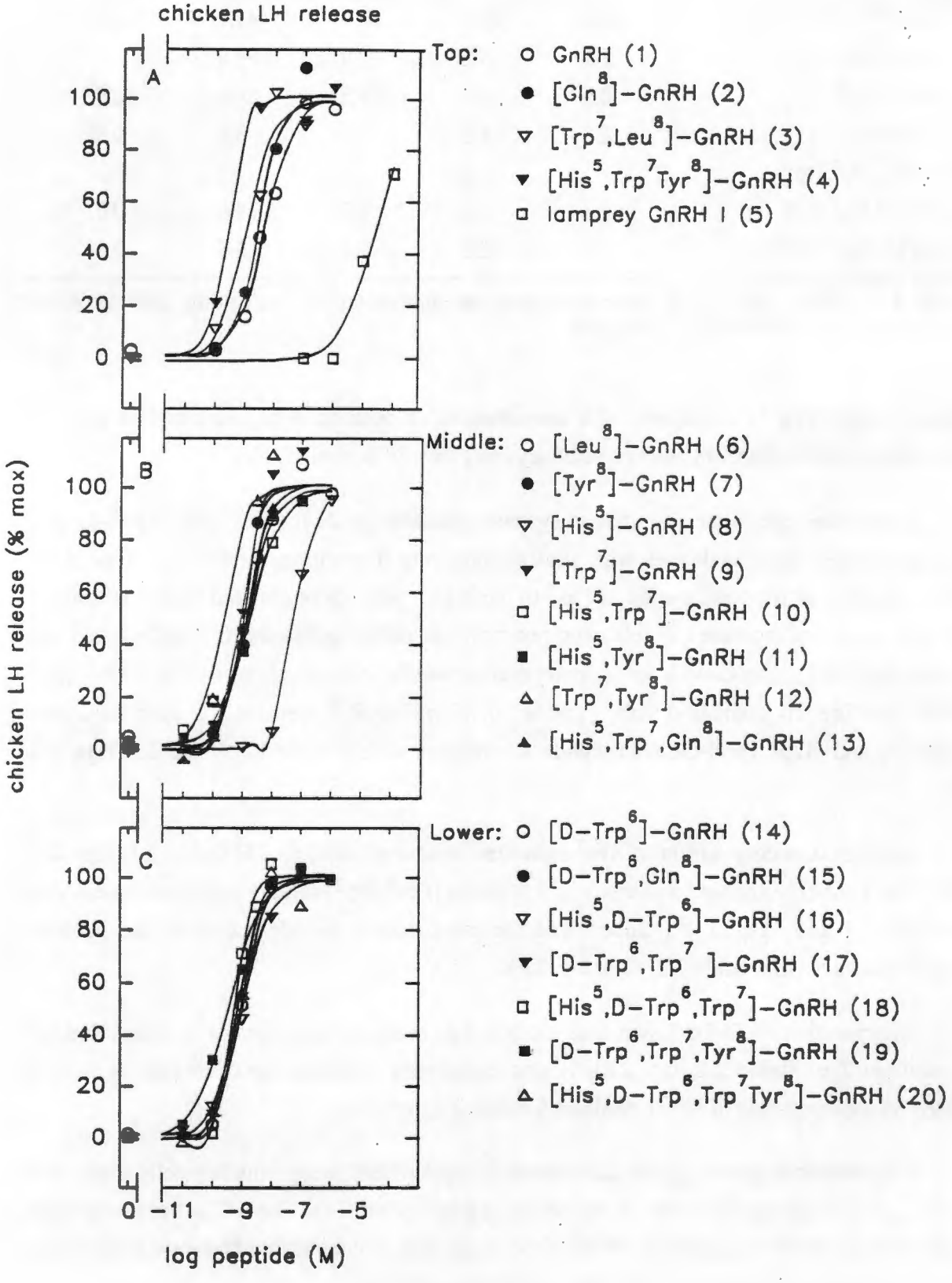
As was the case in the mammalian systems, chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) was more potent than analogues with substitutions only in position 8 (table 2.1, figs. 2.6A, 2.6B). Analysis of the contribution of His^5 to the higher potency of chicken GnRH II, showed that His^5 does not increase LH releasing potency, but rather decreases it. [His^5]-GnRH was 10-fold less active than GnRH, while incorporation of His^5 decreased the activities of [Trp^7]-GnRH (peptide 10 compared with peptide 9), [Tyr^8]-GnRH (peptide 11 compared with peptide 7) and [Trp^7,Tyr^8]-GnRH (peptide 4 compared with peptide 12) (table 2.1, figs. 2.3, 2.6).

In contrast, incorporation of Trp^7 enhanced release of chicken LH (table 2.1, figs. 2.3, 2.6). [Trp^7]-GnRH exhibited a potency of 5.9, while [His^5,Trp^7]-GnRH was more potent than [His^5]-GnRH and [Trp^7,Tyr^8]-GnRH was the most potent peptide tested in the cultured chicken pituitary cells (table 2.1, figs. 2.3, 2.6).

Incorporation of D-Trp^6 increased chicken LH releasing activities of peptides that did not contain Trp^7 (table 2.2, fig. 2.6). It also completely reversed the decreases in activity caused by incorporation of His in position 5 (table 2.2, fig. 2.6).

Comparison of the EC_{50}s for LH release in chicken and sheep pituitary cells shows that the EC_{50}s were generally lower in the sheep system (table 2.1). The EC_{50} for mammalian GnRH was an order of magnitude lower in the sheep than in the chicken and it was lower than all but one of the EC_{50}s measured in the chicken pituitary cells.

Figure 2.6 Stimulation of LH release by GnRH analogues in chicken gonadotropes.



Comparison of the EC_{50}s for LH release in chicken and sheep pituitary cells shows that the EC_{50}s were generally lower in the sheep system (table 2.1). The EC_{50} for mammalian GnRH was an order of magnitude lower in the sheep than in the chicken and it was lower than all but one of the EC_{50}s measured in the chicken pituitary cells.

Comparison of potencies for LH release in the two systems (fig. 2.3) shows that peptides containing a hydrophobic residue in position 8 have increased potency in the chicken, while the same peptides have decreased potency in sheep cells. The presence of His in position 5 markedly decreases potency in chicken cells, but has much less effect in sheep cells. Trp^7 causes increased potency in chicken cells, but does not affect potency in sheep cells.

DISCUSSION

Lamprey GnRH I exhibited low potency in stimulating gonadotropin release from both mammalian and chicken pituitary cells, consistent with a previous report (Sower *et al.*, 1987). Lamprey GnRH I is the most divergent of the non-mammalian GnRHs, containing more substitutions than occur in GnRHs from higher vertebrates. The differences in lamprey GnRH structure may reflect divergent evolution since the lampreys, which constitute part of the most ancient class of vertebrates, separated from the other vertebrates about 500 million years ago. The differences in GnRH structure are probably accompanied by differences in GnRH receptor structure.

The other vertebrate GnRHs exhibited high activity in stimulating LH release from chicken pituitary cells, but all except mammalian GnRH had low potencies in sheep and rat pituitaries, consistent with previous reports (Millar and King, 1983a; Hasegawa *et al.*, 1983; Millar *et al.*, 1986b). The common structural feature of these non-mammalian GnRHs is the substitution of Arg^8 with neutral amino acids and it has been suggested that this is responsible for the low affinities of these peptides for mammalian GnRH receptors (Milton *et al.*, 1983). The low receptor binding affinities and gonadotropin releasing potencies of the position 8-substituted analogues [Leu^8]-GnRH and [Tyr^8]-GnRH supports the importance of the basic Arg^8 residue in mammals. Other chimaeras with neutral residues in position 8, [His^5,Tyr^8]-GnRH and [Trp^7,Tyr^8]-GnRH, also exhibited relatively low activities in the mammalian test systems. In contrast, the position 8-substituted GnRH analogues all had high potency in stimulating release of chicken LH. These results support earlier suggesions that although mammalian GnRH receptors require Arg in position 8, the avian receptors are less specific, binding analogues with neutral or basic residues in position 8 (Hasegawa *et al.*, 1984; Millar and King, 1987, 1988). Fish GnRH receptors are also relatively non-specific with respect to amino acid residues position 8. Mammalian GnRH, salmon GnRH and chicken GnRH II all

have high gonadotropin releasing potency in goldfish (Habibi *et al.*, 1992). Thus, vertebrates in which the naturally occurring GnRH contains a neutral residue in position 8 appear to be tolerant of neutral or basic amino acid substitutions in position 8. In contrast, mammals, in evolving an Arg^8-containing GnRH, appear also to have evolved a receptor which requires a basic residue in position 8.

The positive charge of Arg^8 in mammalian GnRH may interact with a negatively charged residue in the mammalian receptor (Hasegawa *et al.*, 1984; Keinan and Hazum, 1985; Hazum, 1987). The identification of a negatively charged Glu residue which determines the specificity of the mouse GnRH receptor in recognizing mammalian GnRH will be discussed in chapter 4. Arg^8 has also been proposed to have a role in stabilizing the active conformation of GnRH. The sidechain of Arg^8 appears to interact with the sidechains of the His^2 and Tyr^5 residues in mammalian GnRH (Shinitzky and Fridkin, 1976). Neutral amino acid substituents in position 8 interact less with the sidechains of His^2 and Tyr^5 and therefore are probably less able to stabilize the active peptide conformation (Shinitzky *et al.*, 1976; Milton *et al.*, 1983). The decreased stabilization of a biologically active conformation of the peptide may account for the lower activity, in mammalian systems, of GnRH analogues with neutral residues in position 8. In contrast, since the chicken GnRH receptor does not require an Arg in position 8, it appears to be less stringent in its recognition of peptide conformation or it may require a different conformation from that required by mammalian receptors.

The importance of peptide conformation for interaction with mammalian GnRH receptors was confirmed by the effect of incorporating D-Trp in position 6 of GnRH. Substitution of Gly^6 with a D-amino acid stabilizes a β-turn conformation of the residues, Tyr^5-Gly^6-Leu^7-Arg^8, which forms an important component of the active conformation of GnRH (Momany, 1976a, b). In this study, incorporation of D-Trp in position 6 of mammalian GnRH increased receptor binding affinity and gonadotropin releasing potency more than 20-fold, in agreement with previous studies in the rat (Millar and King, 1983b). D-Trp^6 incorporation also increased the potencies of [Gln^8]-GnRH and [His^5]-GnRH by an order of magnitude (table 2.2). However, D-Trp^6 incorporation induced only small increases in the activities of peptides which contained Trp in position 7 (table 2.2). This may be a consequence of steric hindrance caused by the close proximity of 2 bulky sidechains (D-Trp^6,Trp^7) in these peptides. Alternatively, it may be a consequence of the hydrophobicity of the peptides (indicated by the high retention times of peptides 17, 18, 19 and 20 on reversed phase HPLC, table 2.1). It has been reported that increasing hydrophobicity of GnRH analogues is associated with increasing biological potency *in vivo* up to an optimum hydrophobicity, after which activity declines (Nestor *et al.*, 1984). In this light, it is interesting to note that incorporation of a charged D-amino acid, D-Arg, in position 6 increased the activity of the hydrophobic chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) 9-fold

(Millar *et al.*, 1986b), while in the current study incorporation of the hydrophobic D-Trp^6 increased its activity only 2-fold.

D-Trp^6 incorporation had much less effect in chicken pituitary cells. It resulted in small increases or decreases in LH releasing potency for most peptides (table 2.2). These findings are in agreement with earlier observations that substitutions of D-amino acids for Gly^6 give rise to only small increases in potency in the chicken (Hasegawa *et al.*, 1984; Millar *et al.*, 1986b). These results indicate that the chicken GnRH receptor is less stringent than the mammalian receptor in its requirement of a particular peptide conformation or that it requires a different peptide conformation from that stabilized by D-amino acids in position 6. Incorporation of D-Trp^6 did, however, reverse the decrease in activity caused by His^5, as it increased the potencies of the inactive His^5-containing peptides by an order of magnitude (table 2.2). This suggests that the low LH releasing potency in the chicken which results from incorporation of His^5 is caused by an effect on peptide conformation which can be reversed by a D-amino acid in position 6.

As previously reported, chicken GnRH II was more potent than chicken GnRH I in stimulating LH release from both chicken and mammalian pituitary cells (Millar *et al.*, 1986b). This observation suggested the possibility that substitution of His^5 and Trp^7 into GnRH, while retaining the important Arg^8, might produce GnRH analogues, consisting entirely of natural L-amino acids, with activities equal to or higher than mammalian GnRH (Folkers *et al.*, 1986; Millar *et al.*, 1986b). These substitutions had minimal effect on GnRH activity and result in peptides ([His^5]-GnRH, [Trp^7]-GnRH and [His^5,Trp^7]-GnRH) with receptor binding affinities and gonadotropin releasing potencies similar to mammalian GnRH in mammalian test systems. These results are in agreement with an *in vivo* study of the same analogues in rats (Folkers *et al.*, 1986). These are the first analogues of GnRH made up entirely of natural amino acids to exhibit activity as high as GnRH itself (Folkers *et al.*, 1986). These results do not explain why chicken GnRH II has higher GnRH activity than [Tyr^8]-GnRH. However, incorporation of His^5 into [Tyr^8]-GnRH increased receptor binding affinity, while Trp^7 increased the gonadotropin releasing activity of [Tyr^8]-GnRH. This suggests that some kind of interaction amongst the sidechains of the 3 residues accounts for the higher activity of chicken GnRH II.

In contrast to the mammalian systems, substitutions in positions 5 and 7 had marked effects in the chicken. Incorporation of His^5 caused a decline in LH releasing activity (up to 20-fold in the case of [His^5,Trp^7]-GnRH compared with [Trp^7]-GnRH) in chicken cells. We have not been able to develop a reliable chicken GnRH receptor binding assay, apparently due to the lack of an analogue with sufficiently high affinity for use as a tracer. In the absence of a receptor binding assay, it is not possible to tell whether the His^5-induced decrease in LH releasing potency is accompanied by a similar decrease in receptor binding affinity or by

increased binding affinity as was the case for the sheep pituitary. If receptor binding affinity is not decreased, incorporation of His^5 might provide a starting point for design of antagonists for the chicken GnRH receptor, since GnRH antagonists designed for mammalian receptors have very low affinity for the chicken receptor (Jacobs *et al.*, 1995). The increased chicken LH releasing potency of analogues with Trp in position 7, indicates that the high activity of chicken GnRH II in chicken cells is due to the incorporation of this residue.

Using full dose-response curves, no peptide was found to exhibit enhanced FSH releasing potency compared with LH release. Thus, this work in cultured sheep pituitary cells does not support a previous observation *in vivo* in rats that [His^5]-GnRH caused preferential FSH release (Folkers *et al.*, 1986).

The EC_{50} for stimulation of sheep LH release by GnRH was 20-fold lower than the IC_{50} for GnRH in the sheep receptor binding assay. This difference indicates the presence of spare receptors and suggests that occupancy of about one twentieth of the receptors by GnRH would be sufficient to stimulate a full LH response (Taylor and Insel, 1990). The EC_{50} for mammalian GnRH in the chicken bioassay was similar to the IC_{50} in the sheep receptor binding assay. This may indicate either that the chicken pituitary cells have a minimum of spare receptors, or that the chicken GnRH receptor has low affinity for mammalian GnRH (Hasegawa *et al.*, 1984).

The calculation of potencies relative to GnRH allows comparison of results obtained in the stimulation and binding assays in spite of the differences in peptide concentrations required for half-maximal responses. Comparison of the potencies obtained in the sheep LH release and receptor binding assays (fig. 2.4) shows that analogues containing His^5 in combination with Arg^8 ([His^5]-GnRH and [His^5,D-Trp^6]-GnRH) have high receptor binding potency relative to their LH releasing potency. In the absence of spare receptors, these peptides might act as partial agonists, stimulating less than maximum LH release at concentrations where all receptors are occupied. They may thus provide useful tools for the study of recombinant GnRH receptors. Some peptides exhibit the opposite characteristic, namely higher LH releasing potency than binding potency. These peptides contain neutral substitutions in position 8. It would appear from these results, that the neutral GnRH analogues, once bound, are better able to activate the GnRH receptor. This suggests that mammals, in evolving a charged ligand, may have evolved a high affinity ligand binding interaction at the expense of receptor activation. Such a system might conceivably allow a similar range of gonadotropin release in response to stimulation by lower levels of GnRH. Alternatively, it is possible that the neutral peptides stimulate gonadotropin release through a second receptor which is not detected in the receptor binding assay.

Differences in the structural requirements of the chicken and mammalian receptors for

GnRH were anticipated. However, this study has also demonstrated a difference in the requirements of rat and sheep receptors. Comparison of IC_{50}s obtained in the sheep and rat receptor binding assays (fig. 2.5), shows that the sheep receptor has lower affinity (higher IC_{50}s) for analogues which contain Trp in position 7 in combination with a hydrophobic residue in position 8. Species differences in ligand structural requirements between mammals have previously been demonstrated for an antagonist where human and rat pituitaries were compared (Wormald *et al.*, 1985).

In conclusion, this study has used chimaeric analogues of four vertebrate forms of GnRH to study the functional significance of amino acid substitutions in positions 5, 7 and 8. Arg^8 is required for GnRH activity in mammals, while analogues with neutral amino acids in position 8 are more active in chicken pituitary cells. This result, combined with the differential consequences of incorporating a conformational constraint in the form of D-Trp^6, supports earlier conclusions that the chicken and mammalian GnRH receptors differ in their recognition of ligand conformation. Incorporation of His in position 5 decreased LH releasing potency in chicken cells, but slightly increased affinity for mammalian GnRH receptors. Trp in position 7 increases GnRH activity in the chicken, but has minimal effect in mammals. No peptide could be identified as a preferential FSH releasing hormone, but substitution of some residues had differential consequences for receptor binding affinity and receptor activation. In addition to the expected difference between mammals and chickens, a species difference in peptide structural requirements between sheep and rats has been shown.

CHAPTER 3

DEVELOPMENT OF METHODS FOR PURIFICATION OF MEMBRANE-ASSOCIATED GONADOTROPIN RELEASING HORMONE BINDING PROTEINS

SUMMARY

This study describes the development of a protocol for use in purification of the GnRH receptor from sheep pituitary membranes. Several detergents were tested for their ability to solubilize GnRH binding activity. The zwitterionic detergent, CHAPS, gave the best recovery of binding activity in the soluble fraction. Solubilized membranes were applied to affinity columns prepared with a range of GnRH analogues. Only columns prepared with two high affinity analogues, [D-Lys^6,Pro^9-NHEt]-GnRH and antagonist 26, retained more than 50% of the applied GnRH binding activity. NaCl concentrations up to 0.5M did not promote dissociation of GnRH receptor prebound to labelled GnRH agonist. In contrast, low pH did promote dissociation of preformed receptor-ligand complexes. Thus, after application of solubilized membranes, columns were washed with a gradient of sodium chloride up to 0.4M and GnRH binding activity was eluted from the columns using an acidic buffer. This protocol eluted GnRH binding activity from the agonist affinity column, but not from the column prepared with antagonist 26. The [D-Lys^6,Pro^9-NHEt]-GnRH affinity column was therefore used for the subsequent purification. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the acid-eluted fraction revealed a major protein band with a molecular weight of 67 kD. Amino acid sequence analysis of the protein showed that it is different from the cloned GnRH receptor, but homologous with a GnRH binding protein recently purified from bovine pituitary. This protein may have a function which is modulated by binding of GnRH, GnRH fragments or GnRH-related peptides.

INTRODUCTION

Since the determination of the structure of GnRH, much has been learnt about the structural features of the GnRH molecule which are important for its function. However, the accumulation of this information has depended largely on experiments designed around only one side of the receptor-ligand interaction, because the structure of the receptor was not known. Information about the structure of the receptor, thus, became crucial for further understanding of how GnRH interacts with its receptor.

Several previous attempts have been made to purify the GnRH receptor by conventional biochemical methods. Solubilization of bovine pituitary membranes in Triton X 100, followed by concanavalin A affinity chromatography resulted in partial purification of a 60kD GnRH

binding protein (Jansem De Almeida Catanho *et al.*, 1983). Two GnRH binding proteins were purified from rat pituitaries. Membranes were solubilized in a zwitterionic detergent and the receptor was isolated on [biotinyl-D-Lys^6]-GnRH coupled to an avidin affinity column (Hazum *et al.*, 1986). GnRH receptor antibodies were prepared using a bovine receptor preparation purified by the same protocol (Hazum *et al.*, 1987). However, neither group reported amino acid sequences of their GnRH binding proteins. We used these previously described methods as a basis for the development of a protocol for the purification of the GnRH receptor from sheep pituitaries, anticipating that even a short segment of the amino acid sequence could be used in strategies to clone the GnRH receptor.

Although only a single GnRH receptor has been identified in mammalian species, there is a body of evidence indicating the existence of at least two forms of GnRH in most vertebrates (King and Millar, 1987, 1990, 1992) and the existence of GnRH receptor subtypes (Murthy and Peter, 1994; Murthy *et al.*, 1994). In particular, chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) is present in most vertebrates. It has been structurally conserved over 500 million years of evolution and appears to serve a neuromodulatory role. This peptide has more recently been identified in certain mammalian species (King *et al.*, 1989; King *et al.*, 1990; Dellovade *et al.*, 1993; King *et al.*, 1994). Since the receptor for chicken GnRH II has not been revealed by molecular cloning, it may have poor structural homology with the cloned mammalian receptors or even be structurally unrelated, as is the case for the nicotinic and muscarinic receptors which both bind acetyl choline, but belong to different receptor families. This novel receptor could potentially be identified by purification and partial amino acid sequencing followed by molecular cloning. In addition to GnRH receptors, other proteins may be regulated by binding of GnRH or GnRH breakdown products. Indeed, it has recently been demonstrated that the activity of a hypothalamic N-Methyl-D-Aspartate receptor is regulated by a GnRH fragment (Bourguinon *et al.*, 1994).

This study describes the development of a GnRH agonist affinity chromatography protocol for purification of proteins which bind GnRH, and the purification of a 67 kD protein from sheep pituitary membranes. The purified protein is different from the cloned GnRH receptors and may have a function in processes modulated by GnRH or by GnRH breakdown products, similar to the recently described regulation of hypothalamic N-Methyl-D-aspartate receptors by a GnRH fragment (Bourguignon *et al.*, 1994).

MATERIALS AND METHODS

Peptides

The GnRH agonist, [D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH, and the D-Lys^6-containing GnRH analogues, [His^5,D-Lys^6,Trp^7,Tyr^8]-GnRH, [D-Lys^6,Trp^7,Tyr^8]-GnRH, [D-

Lys^6,Trp^7,Gln^8]-GnRH and [D-Lys^6,Trp^7,Leu^8]-GnRH were prepared by R. Milton, using conventional solid phase peptide synthesis. [D-Lys^6,Pro^9-NHEt]-GnRH was a gift from J. Rivier and antagonist 26, [Ac-D-4-Cl-Phe^{1,2},D-Trp^3,D-Lys^6,D-Ala^{10}]-GnRH, was a gift from D. Coy.

Plasma membrane preparation and solubilization

Sheep pituitary membrane preparation was modified somewhat from that described in chapter 2. Sheep pituitaries were collected on to ice within 30 min of death at the Cape Town Municipal Abattoir. Anterior pituitaries were dissected free of bone and membranes within 2 hs and frozen at -70^o C for up to 30 days or used immediately for membrane preparation. Batches of up to 40 fresh or frozen pituitaries were minced with a scalpel blade and homogenized in ice cold HEPES buffer (10mM HEPES, 1mM EDTA, pH 7.4, 3ml/pituitary) using a Polytron (3 short bursts at moderate speed). We found higher binding in HEPES buffer compared with the tris-HCl buffer used in chapter 2. In addition, dithiothreitol was omitted because it has been reported to decrease receptor binding affinity (Keinan and Hazum, 1985). The homogenate was centrifuged for 10 min at 500 x g and 4^o C to remove debris. The resulting supernatant was layered on top of 50% sucrose (10 ml) and centrifuged for 30 to 40 min at 180,000 x g and 9^o C (45,000 rpm in a Beckman 50.2 Ti rotor). This step was necessary to remove the component which caused high non-specific binding in the ligand binding assay (chapter 2). The relatively high temperature was important because at lower temperatures (4^o C) the particulate material did not separate into discreet interface and pellet components, but was distributed throughout the sucrose. The opaque fraction at the interface between the buffer and sucrose was collected, diluted (approximately 4-fold) with HEPES buffer and recentrifuged for 30 to 40 min at 180,000 x g and 4^o C. The resulting pellets were resuspended in the same buffer (3 pituitary equivalents/ml) using a 0.8mm diameter needle and syringe, and stored at -70^o C or immediately solubilized.

Pituitary membranes were diluted to a final concentration of 1 pituitary equivalent/ml with HEPES buffer containing 4mM CHAPS (3-[(cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate, Sigma, St Louis, USA) and solubilized by shaking on ice for 1 h. In preliminary experiments CHAPS (1 - 100 mM), octyl glucopyranoside (Sigma, 12.5 -200 mM), Tween 20 (0.03 -1 %) and Triton X-100 (0.06 - 0.48 mM) were tested. The resulting mixture was diluted with an equal volume of HEPES buffer and centrifuged for 1 h at 180,000 x g and 4^o C to remove particulate material. The supernatant was used immediately for affinity chromatography or for binding experiments.

Affinity chromatography

For pilot experiments, GnRH analogues containing D-Lys in position 6 were coupled to Affi-Gel 10 beads (Biorad, Richmond, USA). Resin suspension (1 ml) was washed with ethanol then with HCl diluted to pH 2.5 - 3.0 and incubated at 4° C with peptides (5 - 30 nmol) in 1 ml 10mM HEPES (pH 7.4) for 2 - 4 h with shaking. Unreacted sites on the resin were blocked by incubation with 1M ethanolamine (1 h, room temperature) and the affinity resin was washed with, and stored in HEPES buffer. To test their capacity to bind GnRH receptor, affinity resins were mixed with solubilized pituitary membranes, diluted to 1mM CHAPS, and incubated for 1 h on ice with shaking. Suspensions were centrifuged (4° C, 10 min, 10,000 x g) and the supernatant tested for GnRH agonist binding.

For preparative affinity chromatography, the more rigid Affi-Prep 10 resin (Biorad, Richmond, USA) was used to allow the solubilized membrane preparation to be pumped on to the column at higher speed without compression of the resin. [D-Lys^6,Pro^9-NHEt]-GnRH (1.5 - 2.9 µmol in 2 - 10 ml of 50mM or 10mM HEPES, pH 7.4) was coupled to Affi-Prep 10 (4 - 8 ml) as described above for Affi-Gel 10 and stored in HEPES buffer.

Solubilized pituitary membranes (up to 40 pituitary equivalents) were diluted to a final CHAPS concentration of 1 mM and applied to the affinity column. Unbound material was washed off with approximately 3 ml of HEPES buffer containing 0.1 mM CHAPS and then with a 40 ml gradient of NaCl (0 - 0.4M) in 0.1mM CHAPS-HEPES buffer. Bound proteins were eluted with an acidic HEPES buffer (0.1mM CHAPS, 1mM EDTA, 10mM HEPES, pH 5.8). 4 ml fractions were collected and neutralized with NaOH, using 10 µl phenol red (0.4 %) as an indicator and tested for binding of GnRH agonist. Fractions which bound GnRH agonist were pooled and lyophilized.

GnRH agonist binding assay

Conventional GnRH receptor ligand binding assays depend on using filters to retain labelled ligand bound to particulate membrane receptors, while the soluble unbound ligand passes into the filtrate (Millar *et al.*, 1995). This protocol had to be modified to measure binding of the GnRH agonist, [D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH, to solubilized pituitary membrane preparations and affinity column eluates. 400 µl of solution was incubated on ice for 60 - 90 min with 100,000 cpm of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH (specific activity 500 µCi/µg) in a final volume of 1 ml of HEPES buffer. The reaction was terminated by addition of 1 ml of ice cold dextran-coated charcoal (0.1 % w/v Dextran T70, 1 % w/v activated charcoal in phosphate-buffered saline). The unbound ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH is adsorbed by the dextran-charcoal, while the ligand which is bound to proteins remains in solution. Tubes were mixed and centrifuged for 15 min at 4° C and 2,000

x g. Supernatants were counted in a gamma counter (Packard Crystal II). Non-specific binding was estimated in the presence of 5 x 10^{-7}M unlabelled [D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH. This lower than the usual (10^{-6} M) concentration of unlabelled ligand was used because higher concentrations displaced labelled ligand from the charcoal causing artifactually raised estimates of non-specific binding. Assays were performed in duplicate or triplicate. Results are presented as mean specific binding ± standard error.

[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH was iodinated by a modification of the chloramine-T method and the iodinated peptide was purified by C18 reversed phase HPLC (Millar *et al.*, 1995).

Dissociation of GnRH binding proteins

To test the ability of NaCl to promote dissociation of binding proteins prebound to labelled agonist, total and non-specific binding tubes were set up as above and preincubated for 1 h to allow binding to occur. After addition of 200 µl aliquots of NaCl to give the required concentrations, tubes were incubated for various times and treated with charcoal as above. A similar protocol was used to test the effect of changes of pH. Membranes were preincubated in tris-acetate (10mM, pH 7.5) before addition of previously determined volumes of tris-acetate buffers at pH 3.5 and pH 10.0 and incubated in a final volume of 2 ml before addition of dextran-coated charcoal. To test whether treatment with extremes of pH would affect the ability of the solubilized pituitary membrane preparation to subsequently bind GnRH agonist, membranes were solubilized in tris-acetate buffer (pH 7.5) and were not diluted prior to the centrifugation step. 1ml aliquots were adjusted to the pH indicated using tris or acetic acid and incubated for 4 h in a total volume of 4 ml. Samples were then neutralized using phenol red as an indicator and diluted to 5 ml. 1.2ml aliquots were used to measure agonist binding activity in a 1.5 ml final assay volume.

SDS polyacrylamide gel electrophoresis and western blotting

SDS polyacrylamide gels were run under reducing conditions according to the method of Laemmli (1970). Analytical gels were prepared with 7% acrylamide or a gradient of 7 - 11% and proteins were detected by silver staining (modified from Oakley *et al.*, 1980, J. Hapgood, personal communication). Briefly, gels were Coomassie-stained overnight and destained in 30% methanol/10% acetic acid, then incubated sequentially in 50% methanol/12% trichloroacetic acid/2% $CuCl_2$ (30 min); 10% ethanol/5% acetic acid; 0.01% $KMnO_4$; 10% ethanol/5% acetic acid; 10% ethanol; water; 0.1% $AgNO_3$ (15 min each) and washed briefly with water and 10% K_2CO_3. Colour was developed using 0.01% formaldehyde in 2% K_2CO_3. Gels used to estimate the protein concentration of the final

product and for western blotting contained 1 M tris in both the stacking and running gels. The higher buffer concentration helped to minimize the anomalies which arise from high electrolyte concentrations in samples. Proteins for molecular weight calibration and sheep serum were dissolved or diluted in "reconstitution buffer" (3.0M HEPES, pH 7.5, 0.3M CHAPS, 0.01% phenol red) to mimic the reconstituted lyophilized sample before being mixed with an equal volume of application buffer.

Since the affinity purified binding protein exhibited mobility similar to that of BSA in SDS-PAGE, western blotting experiments were performed to confirm that the purified protein was not sheep serum albumin. An aliquot of the purified protein and various dilutions of sheep serum were electrophoresed as described above and electroblotted (1 h, 4^{o} C, 0.5 mA) on to a Nytran N nylon membrane (Schleicher and Schuell, Dassel, Germany). The membrane was blocked for 3 hs in tris-buffered saline (TBS, 0.1 M NaCl, 50 mM tris, pH 8.0) containing 20% (w/v) fat free milk powder (Borden Foods, Braamfontein, South Africa), washed 3 times in TBS containing 5% milk powder and incubated overnight in 15 ml of the same buffer containing 250 µl of rabbit anti-sheep whole serum (Sigma, St Louis, USA). The membrane was washed 4 times and incubated for 30 min with horse radish peroxidase-conjugated goat anti-rabbit Ig G (100 µl in 15 ml, Bio-Rad, Richmond, USA). After washing with 5% milk TBS and with TBS, proteins were visualized with 4-chloro-1-naphthol (60 mg in 20% methanol/TBS containing 100 µl 30% H_2O_2). Albumin was the major protein in the sheep serum and, at the concentrations of sheep serum used, it was the only protein detected by the antiserum.

RESULTS AND DISCUSSION

Several detergents, CHAPS, octyl glucopyranoside, Tween 20 and Triton X 100, were tested at various concentrations for their ability to solubilize GnRH agonist binding activity. Only CHAPS allowed recovery of binding activity in the soluble fraction. Maximum solubilization of specific GnRH agonist binding was found after treatment with 4mM CHAPS (fig. 3.1), similar to the concentration shown to be optimal for solubilization of bovine and rat pituitary GnRH receptors (Winiger *et al.*, 1983; Perrin *et al.*, 1983; Hazum *et al.*, 1986), and this concentration was used for further studies. The CHAPS concentration was not adjusted to a defined concentration across all tubes in the binding assay, as this experiment was also intended to indicate the effect of the residual CHAPS on subsequent binding of the solubilized preparation to the GnRH analogues attached to the affinity columns.

Both the amino- and carboxy-termini of the GnRH molecule are blocked, and the peptide contains no residues with free carboxyl, amino or sulfhydryl groups. Thus, a reactive group must be introduced into the peptide to allow it to be coupled to affinity resins. GnRH is

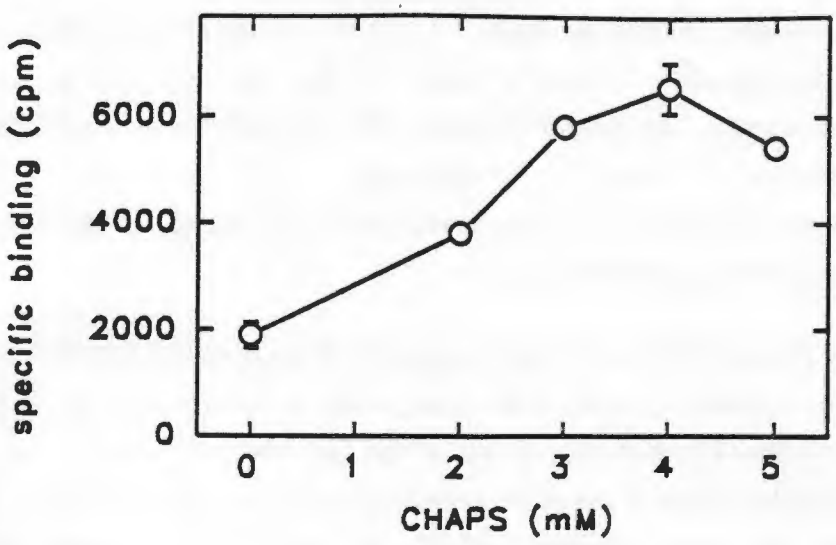


Figure 3.1 Solubilization of GnRH agonist binding activity. Sheep pituitary membranes were incubated, with shaking, in the indicated concentrations of CHAPS, diluted and centrifuged to remove particulate material. Binding assays were performed on the resulting supernatant.

believed to interact with its receptor in a folded conformation such that both termini are necessary for high affinity binding (Karten and Rivier, 1986). For this reason, modification of residues in the terminal regions of the molecule is undesirable. However, substitution of the achiral glycine residue in position 6 with D-amino acids stabilizes the preferred peptide conformation, increasing GnRH activity (Monahan *et al.*, 1973; Momany, 1976a) and increasing the affinity of GnRH binding to its receptor (Millar *et al.*, 1989; chapter 2). Six GnRH analogues, in which D-Lys, which contains a free amino group, was substituted for Gly^6, were tested for their ability to bind to the GnRH receptor. Two analogues, [D-Lys^6,Pro^9-NHEt]-GnRH and antagonist 26, exhibited high affinity for the receptor while the other peptides exhibited lower affinities (fig. 3.2a), characteristic of analogues with substitutions in position 8 (Millar *et al.*, 1989; chapter 2 of this thesis). Harsh conditions are frequently required to disrupt high affinity interactions in order to elute proteins of interest. These harsh conditions can denature the proteins. Thus, using an affinity column prepared with a lower affinity ligand might help reduce damage to the GnRH binding proteins. To test this proposal, affinity supports were prepared with each of the peptides. All supports bound GnRH receptor (fig. 3.2b). However, only the resins prepared with the two high affinity analogues bound more than 50% of the GnRH receptor applied (fig. 3.2b). Affinity columns were prepared with both of these resins.

If peptide ligands are used to elute the GnRH receptor from the affinity column, eluted fractions contain large amounts of peptide which interfere in the competitive binding assay used to quantify the receptor. Non-peptide eluting agents, which would allow subsequent

measurement of the receptor by ligand binding assays, were thus sought. Previous reports had shown that salts inhibit GnRH agonist binding (Hazum, 1987). We therefore tested whether incubation with NaCl would promote dissociation of the GnRH receptor prebound to a labelled GnRH analogue. Preformed complexes of GnRH receptor and ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH were stable for at least 30 min in the presence of up to 0.5M NaCl (fig. 3.3a). In contrast, increasing the hydrogen ion concentration did promote dissociation of prebound receptor-agonist complexes. Incubation for 30 min at pH 6 or lower

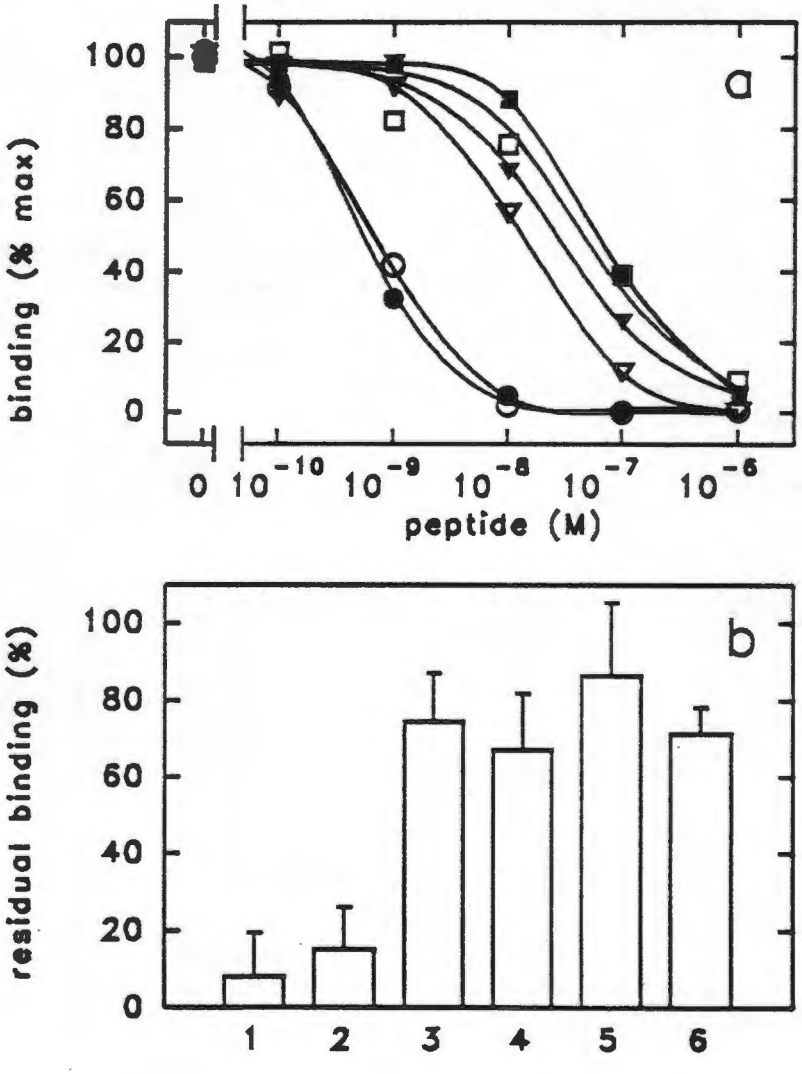
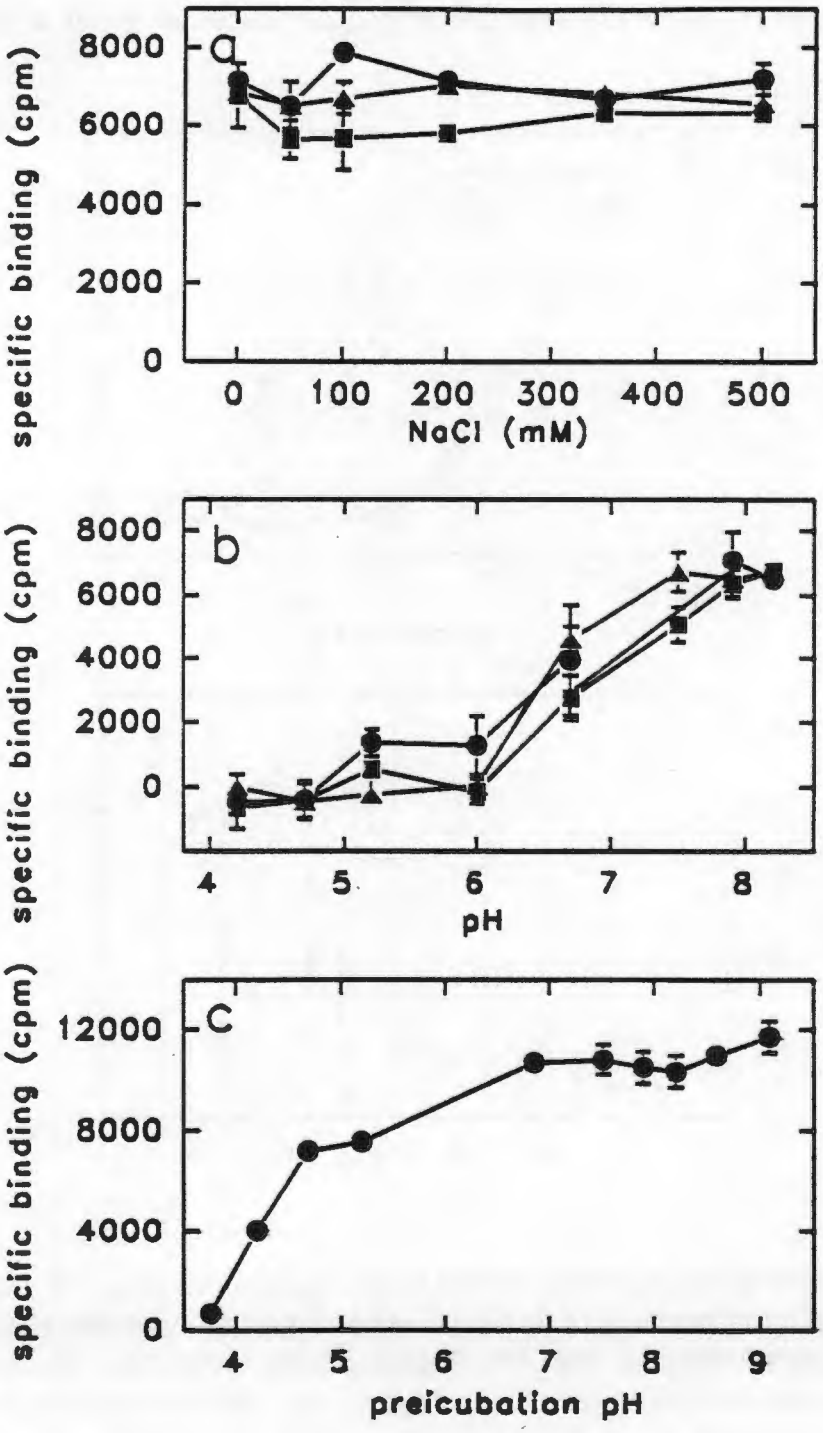


Figure 3.2 Competition binding assay of GnRH analogues containing D-Lys in position 6 and residual GnRH binding activity in solubilized pituitary membranes after incubation with affinity supports prepared with the same peptides. Competition binding assays (a) were performed as described in the text. Solubilized pituitary membranes were incubated with the indicated affinity supports (b). GnRH binding activity is expressed as the per cent of GnRH binding activity in a preparation incubated with affinity support to which no peptide was coupled. Peptides are: antagonist 26 (○, 1); [D-Lys^6,Pro^9-NHEt]-GnRH (●, 2); [His^5,D-Lys^6,Trp^7,Tyr^8]-GnRH (▽, 3); [D-Lys^6,Trp^7,Tyr^8]-GnRH (▼, 4); [D-Lys^6,Trp^7,Gln^8]-GnRH (□, 5) and [D-Lys^6,Trp^7,Leu^8]-GnRH (■, 6).

Figure 3.3 Effect of NaCl and pH on preformed GnRH receptor-agonist complexes and recovery of GnRH binding activity after incubation at low pH. Solubilized pituitary membranes were incubated with ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH, before addition of NaCl (a) to give the indicated concentrations and incubation for a further 5 min (●), 10 min (▲) or 30 min (■). After incubation to allow ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH to bind, pH was adjusted (b) as indicated and incubation continued for a further 20 min (●), 30 min (▲) or 1 h (■). Solubilized pituitary membranes were incubated at the pHs indicated (c) prior to neutralization and measurement of GnRH binding activity.



caused complete dissociation of labelled GnRH agonist (fig. 3.3b). To test whether high affinity binding could be recovered after treatment with acid, solubilized pituitary membranes were incubated at low pH and neutralized before GnRH agonist binding activity was measured. Binding activity was recovered in samples pre-incubated at pH values greater than

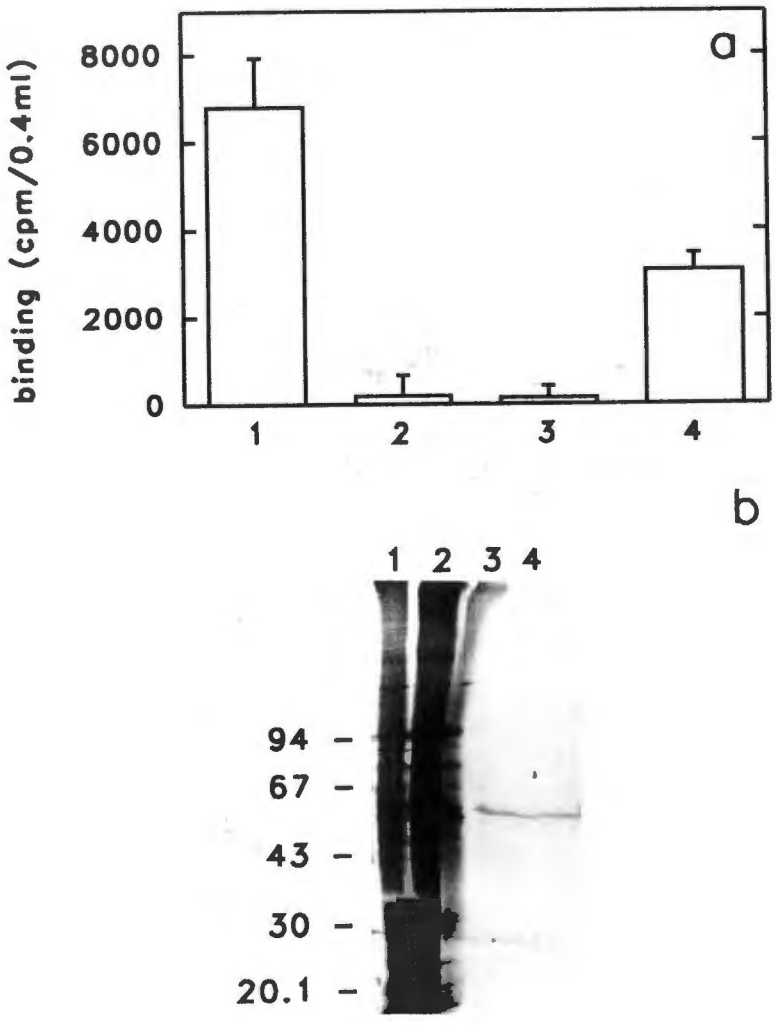


Figure 3.4 GnRH binding activity and SDS-PAGE analysis of affinity chromatography fractions. GnRH agonist binding assays (a) were performed on aliquots of the starting material (1), the same material after passage through the column (2), the pooled material collected during the NaCl gradient (3) and the acid-eluted fraction (4). SDS-PAGE utilized aliquots of the starting material (lane 1), the same material after passage through the column (lane 2), and the NaCl gradient (lane 3) and the entire acid-eluted fraction (lane 4).

4.5 (fig. 3.3c). The conclusions from these experiments were that since NaCl did not disrupt receptor ligand complexes, it might provide a useful column washing step to remove non-specifically bound proteins, while the GnRH binding activity could be eluted from the column with a low pH (between pH 4.5 and 6.0) buffer. Binding activity could then be measured in the acid-eluted fractions after neutralization.

Greater than 90% of the GnRH binding activity in solubilized pituitary membrane preparations applied to the affinity columns was retained by the columns (fig. 3.4a). GnRH agonist binding was not detected in fractions collected during the gradient of NaCl (fig. 3.4a). However, as anticipated, GnRH binding activity was eluted from the [D-Lys^6,Pro^9-NHEt]-GnRH column by the acidic binding buffer (fig. 3.4a). This protocol did not elute binding activity from the affinity column prepared with antagonist 26 (not shown). Antagonist 26 has been shown to form a "functionally irreversible complex" with the GnRH receptor (Loumaye *et al.*, 1984) and it is possible that low pH does not promote dissociation of this antagonist from the receptor in the same way as it does agonists. SDS-PAGE analysis of fractions

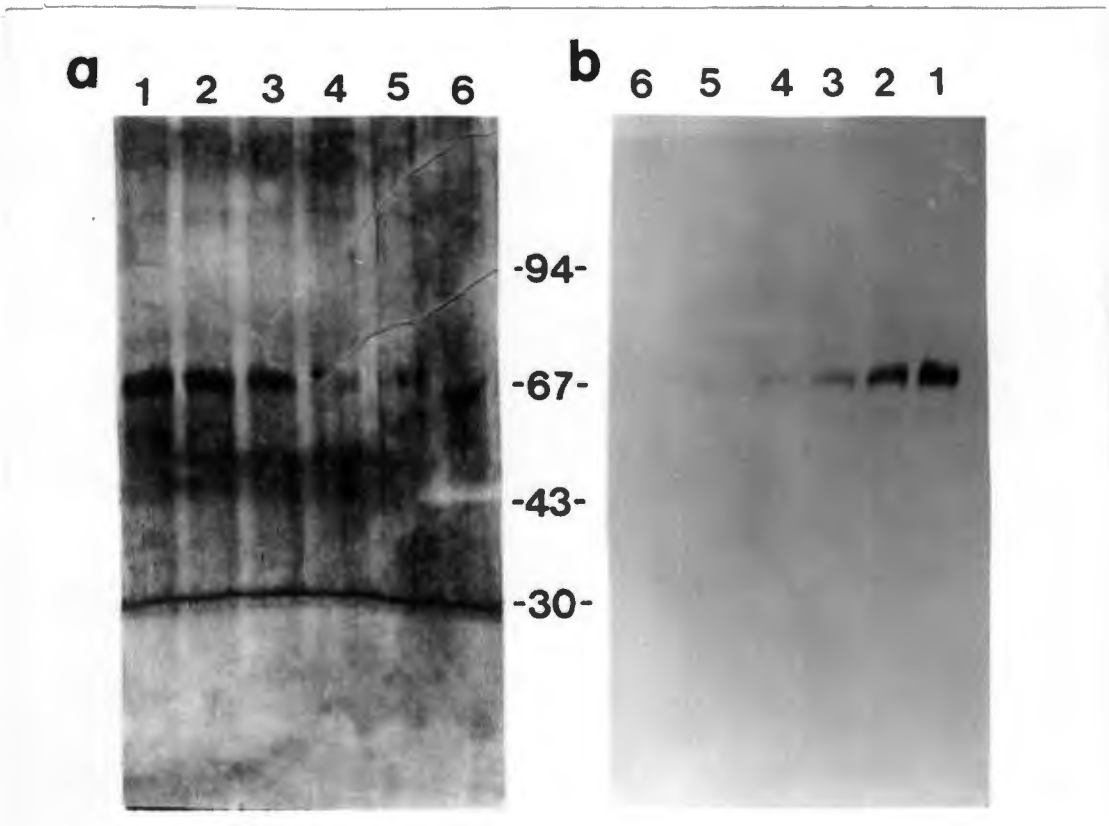


Figure 3.5 SDS-PAGE and western blot showing that the purified protein is not sheep serum albumin. Sheep serum at various dilutions and an aliquot of the pooled fractions eluted from the affinity columns were electrophoresed as described under experimental (a) and western blotted (b). Lanes marked 1 - 5 contain sheep serum diluted 1:4 000; 1:8 000; 1:16 000; 1:32 000 and 1: 64 000 while lane 6 contains the acid-eluted protein.

collected from the [D-Lys^6,Pro^9-NHEt]-GnRH affinity column showed that the NaCl gradient up to 0.4 M did indeed elute a large number of proteins which were probably only loosely bound to the column by non-specific interactions (fig. 3.4b). This therefore constituted an important, albeit unusual clean-up step in the purification.

The acid-eluted fractions, which were pooled and lyophilized to concentrate them, exhibited a major protein band and a minor band (fig. 3.4b). However, their apparent relative molecular weights of 59 kD and 56 kD are probably inaccurate because the high electrolyte concentration of the lyophilized sample caused this lane to run differently from the other lanes on the gel (fig. 3.4b). The lane was about 4 times wider than the other lanes and the dye front moved more slowly. For subsequent gels, molecular weight markers were dissolved in a buffer similar to the reconstituted lyophilized sample. We also found that increasing the buffer concentration in the gels, as described under "experimental", resulted in an even dye front and helped prevent lane broadening. However, resolution of the bands was compromised (fig. 3.5 for example). The [D-Lys^6,Pro^9-NHEt]-GnRH column was used for all subsequent purifications. Binding activity recovered in the acid-eluted fractions of subsequent affinity chromatography runs ranged from 3.5 - 16.2 % of that in the solubilized material applied to the columns.

Lyophilized acid-eluted fractions from seven affinity column runs (278 pituitaries) were pooled. The high concentrations of residual buffer components in the reconstituted mixture caused interference in conventional protein assay methods. Thus, protein concentration was estimated by comparison of an aliquot of the mixture with a BSA standard curve using modified SDS polyacrylamide gel electrophoresis. The mixture contained approximately 10 μg (equivalent to 150 pmol) of a protein which migrated as a single band with M_r 67 kD. This molecular weight is slightly higher than the 57 to 64 kD determined by photoaffinity labelling or western blotting of GnRH receptors in pituitaries of other mammalian species (Iwashita and Catt, 1985; Eidne *et al.*, 1985; Hazum *et al.*, 1986; Perrin *et al.*, 1993). It is also higher than the molecular weight predicted from the deduced amino acid sequence of the recently cloned sheep GnRH receptor (Illing *et al.*, 1993; Brooks *et al.*, 1993). Although the actual molecular weight of the cloned receptor is likely, due to post-translational glycosylation, to be higher than the predicted minimum, the cloned sheep GnRH receptor has fewer glycosylation consensus sequences than the rat and mouse receptors. It is, thus, unlikely to migrate with a higher apparent molecular weight than the rat and mouse GnRH receptors. In addition, the protein purified here does not exhibit the band broadening, characteristic of photoaffinity-labelled GnRH receptors, caused by heterogeneous glycosylation (Janovick *et al.*, 1993; Davidson *et al.*, 1995). However, it is similar to the 66kD protein which co-purified with the rat GnRH receptor (Hazum *et al.*, 1986) and which was thought, by the authors, to be residual BSA used to coat affinity columns. BSA was not used in the current study, but small

quantities of sheep serum albumin present in the pituitary tissue could have adhered to the column and eluted with the GnRH receptor. Western blotting showed that the protein is not ovine serum albumin (fig. 3.5).

Lyophilized fractions containing the affinity purified 67kD protein were resuspended in purified (Milli-Q) water (5ml) and dialyzed extensively against 0.02% SDS (in water) to remove detergent, buffer components and phenol red. The dialyzed sample was concentrated by vacuum centrifugation (Speed-Vac, Savant) and the 67kD protein resolved by SDS-Page under reducing conditions. The gel-purified protein (with an estimated recovery of less than 50 pmol) was subjected to *in situ* tryptic cleavage and peptides were recovered by HPLC extraction (Tetaz *et al.*, 1993). Microsequence analysis of several peaks revealed mainly sequences which were homologous to human epidermal type II keratins (Johnson *et al.*, 1985; Tyner *et al.*, 1985). However, subtraction of a keratin-derived sequence from one double sequence revealed the sequence LXYQL (X=unknown), which showed homology to the tryptic peptide sequence (LVYQL) derived from a GnRH-binding protein recently purified from bovine pituitary (Christiansen and Houen, 1994).

The GnRH binding protein purified from bovine pituitaries (Christiansen and Houen, 1994) exhibited properties similar to those of the protein described here. Its apparent M_r was estimated to be 60 kD by SDS-PAGE analysis of samples lyophilized in phosphate-buffered saline. The protein did not exhibit the band broadening characteristic of glycosylated GnRH receptors. In addition, partial amino acid sequencing showed that it was not the bovine GnRH receptor. However, it is probably the bovine homolog of the protein which we have purified from sheep pituitary as both proteins contain the tryptic peptide sequence LXYQL. This purification of the same protein from different, although closely related, species using different affinity chromatography protocols, suggests that the protein purified here may bind GnRH or part of the GnRH molecule. It may have a physiological role which is regulated by GnRH or, as has recently been described for an N-Methyl-D-aspartate receptor, by a fragment of GnRH (Bourguignon *et al.*, 1994)

In conclusion, we have purified a 67kD protein which bound to a GnRH agonist affinity column. It was not eluted from the affinity column by high salt concentrations, but was dissociated from the immobilized GnRH agonist by an acidic buffer. The eluted preparation retained GnRH binding activity. The protein does not exhibit the broad band on SDS-PAGE which is characteristic of the heterogeneously glycosylated GnRH receptor and amino acid sequence analysis showed that it is not the gene product of the recently cloned sheep GnRH receptor (Brooks *et al.*, 1993; Illing *et al.*, 1993). The sequence is, however, homologous with that of a bovine pituitary protein recently purified by a different GnRH affinity chromatography protocol and partially sequenced (Christiansen and Houen, 1994). Since both of these proteins bound to immobilized GnRH agonists, they may have a biological function

which is modulated by binding of GnRH or a GnRH fragment.

While we were in the process of developing a technique for separating the protein described in this study from the residual buffer components without losing it, the mouse GnRH receptor was cloned by our collaborators. I participated in studies to characterize the ligand binding properties of the first cloned GnRH receptor (Tsutsumi *et al.*, 1992) as well as those of the human (Chi *et al.*, 1993) and sheep GnRH receptors (Illing *et al.*, 1993), which were subsequently cloned form cDNA libraries (Also see chapter 6, Other studies conducted during the course of this thesis). The cloned mouse GnRH receptor was mutated in order to study the GnRH receptor ligand binding domain as described in chapter 4.

CHAPTER 4

GLUTAMATE^{301} OF THE MOUSE GnRH RECEPTOR CONFERS SPECIFICITY FOR ARGININE^{8} OF MAMMALIAN GnRH

SUMMARY

The Arg residue in position 8 of mammalian GnRH is necessary for high affinity binding to mammalian GnRH receptors. This requirement has been postulated to derive from an electrostatic interaction of Arg^{8} with a negatively-charged receptor residue. In order to identify such a residue, eight conserved acidic residues of the mouse GnRH receptor, Glu^{8}, Asp^{90}, Asp^{98}, Glu^{111}, Asp^{185}, Asp^{292}, Glu^{294} and Glu^{301}, were mutated to isosteric Asn or Gln. Mutant receptors were tested for decreased preference for Arg^{8}-containing ligands by ligand binding and inositol phosphate production.

One of the mutants, in which the Glu^{301} residue was mutated to Gln, exhibited a 56-fold decrease in apparent affinity for mammalian GnRH. The mutant receptor also exhibited decreased affinity for [Lys^{8}]-GnRH, but its affinity for [Gln^{8}]-GnRH was unchanged compared with the wildtype receptor. The apparent affinity of the mutant receptor for the acidic analogue, [Glu^{8}]-GnRH, was increased more than 10-fold. The mutant receptor did not, therefore, distinguish mammalian GnRH from analogues with amino acid substitutions in position 8 as effectively as did the wildtype receptor. This loss of discrimination was specific for the residue in position 8, because the mutant receptor did distinguish mammalian GnRH from analogues with favourable substitutions in positions 5, 6 and 7. These findings show that the Glu^{301} residue of the GnRH receptor plays a role in receptor recognition of Arg^{8} in the ligand and are consistent with an electrostatic interaction between these two residues.

The mutant receptor exhibited high affinity for conformationally constrained analogues of GnRH, showing that the Glu^{301} and Arg^{8} residues are not required for the high affinity interactions of conformationally constrained peptides. This indicates that an interaction involving these two residues may induce changes in the conformation of GnRH after it has bound to the receptor.

INTRODUCTION

In mammalian GnRH there is a positively charged Arg residue in position 8, which is necessary for high affinity and specificity of binding to mammalian GnRH receptors (GnRHR). In contrast, GnRHs from most non-mammalian vertebrates contain uncharged residues in position 8 (King and Millar, 1992). Although these non-mammalian GnRHs are

fully active in the animals in which they occur naturally, they show diminished capacity to stimulate release of gonadotropins from mammalian pituitary cells (King and Millar, 1992; chapter 2) and to stimulate inositol phosphate (IP) production in cells transfected with mammalian GnRHRs (Illing *et al.*, 1993). Experiments using synthetic GnRH analogues have confirmed the importance of a positively-charged amino acid in position 8 for high-affinity interaction with mammalian GnRHRs (Sandow *et al.*, 1978; Karten and Rivier, 1986; Millar *et al.*, 1989; chapter 2).

It has been postulated that the Arg^8 sidechain may interact directly with the GnRHR via an electrostatic interaction with a negatively charged Asp or Glu residue (Hazum, 1987) or with a sialic acid residue in the carbohydrate moiety of this glycoprotein (Keinan and Hazum, 1985). A functional GnRHR was first cloned from the mouse αT3 gonadotrope cell line (Tsutsumi *et al.*, 1992). This, and the subsequent cloning of three other mammalian GnRHRs (Eidne *et al.*, 1992; Kaiser *et al.*, 1992; Kakar *et al.*, 1992; Brooks *et al.*, 1993; Illing *et al.*, 1993; Chi *et al.*, 1993; Kakar *et al.*, 1993; Perrin *et al.*, 1993; Weesner and Matteri, 1994), have allowed the application of site-directed mutagenesis in identifying amino acid residues which determine the specificity of mammalian GnRHRs for GnRHs which contain Arg in position 8.

The ligand binding sites of heptahelical, G-protein-coupled neurotransmitter receptors are contained within the transmembrane helical bundle (Strader *et al.*, 1989; Dohlman *et al.*, 1991; Strader *et al.*, 1994). However, the larger size of peptide hormones suggested that the extracellular loops of their receptors may also participate in ligand binding functions. To test the possibility that the high affinity of mammalian GnRH, which contains Arg^8, is dependent on an acidic residue, we systematically mutated conserved acidic residues in the extracellular and transmembrane domains of the mouse GnRHR. We show here that one of these mutant receptors, the [Gln^{301}]-GnRHR, displayed decreased ligand binding affinity for mammalian GnRH, but affinities for GnRH analogues with uncharged residues in position 8 were unchanged or increased.

MATERIALS AND METHODS

Peptides

GnRH, [Gln^8]-GnRH (chicken GnRH I), chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH), [D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH, [D-Trp^6]-GnRH, [D-Trp^6,Pro^9-NHEt]-GnRH and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH were prepared by conventional solid-phase methodology and purified by preparative C18 reversed-phase chromatography. [Glu^8]-GnRH and [His^8]-GnRH were gifts from R.W. Roeske and [Lys^8]-GnRH was a gift from J. Rivier.

Mutagenesis and transfection

The mouse GnRHR was cloned into pBluescript II SK + (Stratagene, La Jolla, CA) and site-directed mutagenesis was performed using Uracil-containing DNA (Kunkel *et al.*, 1991) for [Gln^{8}]-GnRHR, [Gln^{111}]-GnRHR, [Asn^{185}]-GnRHR, [Asn^{292}]-GnRHR, [Gln^{294}]-GnRHR and [Gln^{301}]-GnRHR. For [Gln^{90}]-GnRHR and [Asn^{98}]-GnRHR, the mouse GnRHR was cloned into the pALTER ^{TM}-1 vector and mutated using Altered Sites *in vitro* mutagenesis system (Promega). To confirm mutagenesis, DNA was sequenced manually, using a Sequenase Kit (USB, Cleveland, OH) or by automated sequencer (Biorad, Richmond, CA). The receptor was subcloned into the expression vector pcDNAI/Amp (Invitrogen Corp., San Diego, CA) and mutation sites were resequenced.

COS-1 cells were transiently transfected with pcDNAI/Amp-GnRHR constructs using a modification of the DEAE-dextran method (Keown *et al.*, 1990) as previously described (Chi *et al.*, 1993). 2.5μg of DNA construct was used per well in 12-well plates for IP assays and 15μg DNA per 9 cm dish for ligand binding assays. Receptor expression ranged from 150 to 600 fmol per 10^{6} cells for the wildtype GnRHR and from 35 to 150 fmol per 10^{6} cells for the [Gln^{301}]-GnRHR.

Inositol phosphate (IP) production

Transfected cells were labelled overnight with [^{3}H]-inositol (2 μCi/ml) and stimulated with GnRH or GnRH analogues for 1 h in the presence of LiCl (10mM). The reaction was terminated by addition of a perchloric acid solution and phytic acid. After neutralizing with KOH, IPs were separated on Dowex ion exchange columns and counted (Davidson *et al.*, 1990).

Radioligand binding assay

Ligand binding assays with the wildtype GnRHR and the screening ligand binding assay were performed as previously described (Chi *et al.*, 1993). Briefly, transfected COS-1 cells were detached from culture dishes in binding buffer (10mM HEPES, pH 7.4, 1mM EDTA, 0.1% BSA, fatty acid free), homogenized with a dounce homogenizer and centrifuged at 15 000 x g for 30 min at 4° C. The crude membrane pellet was resuspended in binding buffer and incubated (7.5 x 10^{5} cell equivalents/tube, ~200 fmol receptor) with 60 000 cpm ^{125}I-[D-Ala^{6},N-$MeLeu^{7}$,Pro^{9}-NHEt]-GnRH (~50pM) and varying concentrations of unlabelled test peptides in a final volume of 0.5 ml for 1 to 1.5 h on ice. The incubation was terminated by the addition of 3 ml phosphate-buffered saline (PBS, pH 7.5) containing 0.1% BSA and immediate filtration through glass-fibre filters (GF/C, Whatman) presoaked in PBS containing 1% BSA. The filters were washed twice with 0.1% BSA PBS and the retained radioactivity was counted. Non-specific binding was estimated in the presence of 10^{-7}M

unlabelled [D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH.

To compensate for the lower total binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH exhibited by the [Gln^{301}]-GnRHR, higher concentrations of membranes (1.2 x 10^6 cell equivalents/tube, ~75 fmol receptor) were used for subsequent experiments on the mutant receptor. Also, to avoid dissociation of the labelled ligand from the lower affinity receptor, the dilution step at the end of the assay was eliminated and the filters washed 4 times under vacuum with 0.1% BSA PBS to remove non-specifically bound ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH. Increasing amounts of the pcDNAI/Amp-[Gln^{301}]-GnRHR construct in the transfection reaction showed that maximal expression of the mutant receptor was achieved with 15µg DNA/9cm dish of cells, the same as with the wildtype GnRHR construct. Binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH was maximal after incubation for 75 min and remained stable for a further 75 min. Specific binding ranged from 2004 to 4518 cpm/tube (0.8 to 1.9 fmol, compared with 11700 to 20543 cpm/tube, 4.8 to 8.6 fmol, with wildtype GnRHR) while non-specific binding ranged from 2189 to 3228 cpm/tube (0.9 to 1.3 fmol).

Data analysis

Peptide concentrations required to half-maximally inhibit binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH (IC_{50}s) and to stimulate half-maximal IP production (EC_{50}s) were estimated by four-parameter non-linear curve fitting using Sigmaplot (Jandel Scientific, Corte Madera, CA). Binding assays were performed in triplicate and IC_{50}s were determined in three to five independent experiments. IC_{50} data in table 4.1 are means ± standard error of all experiments. Competitive binding curves for some GnRH peptides exhibited slopes which render Hill coefficients not equal to one. For this reason, we have reported IC_{50}s as indicators of apparent ligand binding affinity and supported our observations of changes in apparent affinity by measuring EC_{50}s for IP production in response to all GnRH peptides. Apparent dissociation constants (K_ds) were calculated from IC_{50}s using the Munson and Rodbard correction (Munson and Rodbard, 1988) to allow estimation of of changes in binding energy of receptor peptide complexes using the formula $\Delta G = -RT\ln(K_{d(mutant)}/K_{d(wildtype)})$. IP assays were performed in duplicate and EC_{50}s were determined in two or three independent experiments. EC_{50} data in table 4.1 are the mean ± standard error of all experiments. For figures 3 through 6, individual IP and binding data points from all experiments were averaged and curves were drawn using four-parameter non-linear curve-fitting as above.

RESULTS

Identification of a mutant GnRHR which did not discriminate GnRH and [Gln^8]-GnRH

We identified eight acidic amino acid residues (fig. 4.1) in the extracellular and superficial transmembrane domains of the mouse GnRHR which are conserved as acidic residues in all of the cloned GnRHRs, and which were therefore candidates for interaction with Arg^8 of GnRH. If the Arg^8 of GnRH were to interact directly with one of the acidic residues of its receptor, a mutant GnRHR in which this interaction is disrupted would be expected to have low affinity for the ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^9-NHEt]-GnRH used in the receptor binding assay. Because this decreased affinity would result in low specific binding of the labelled GnRH agonist, mutant GnRHRs were first screened for their ability to support GnRH-stimulated IP production. 10^{-8}M GnRH is just sufficient to stimulate maximal IP production in the wildtype GnRHR. A mutant GnRHR with decreased affinity for GnRH, but normal coupling to phospholipase C should exhibit decreased IP production in response to

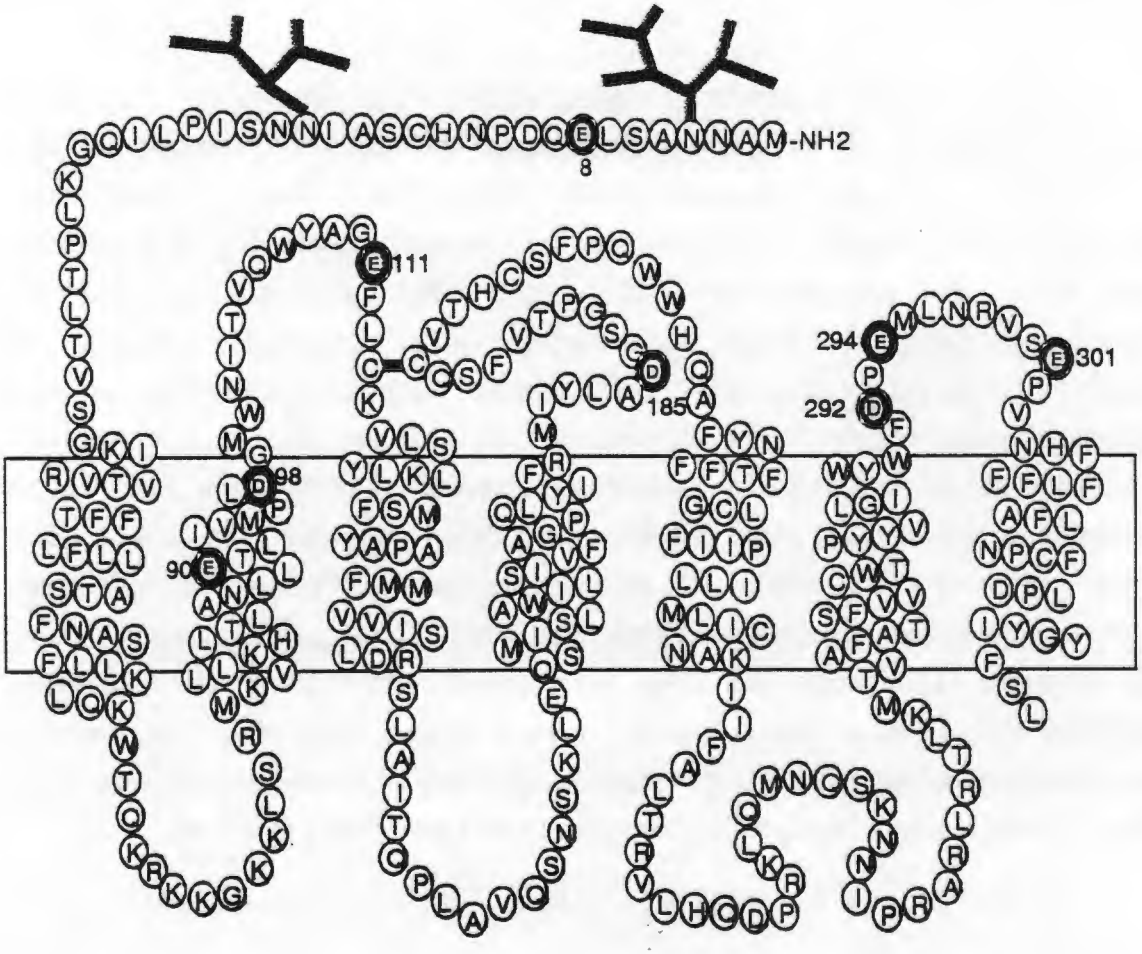


Figure 4.1: The amino acid sequence and proposed secondary structure of the mouse GnRHR. Conserved acidic residues in the extracellular and superficial transmembrane domains are indicated by their sequence numbers and bold typeface.

10^{-8}M GnRH, but a normal maximal response to 10^{-5}M GnRH.

Systematic substitution of six acidic residues Glu^{8}, Glu^{90}, Glu^{111}, Asp^{185}, Asp^{292} and Glu^{294} with their isosteric amides did not cause large changes in the ability of the mutant GnRHRs to support GnRH-stimulated IP production (fig. 4.2). Two GnRHR mutants, [Asn^{98}]-GnRHR and [Gln^{301}]-GnRHR, demonstrated reduced IP production in response to 10^{-8}M GnRH (fig. 4.2). Of these, only the [Gln^{301}]-GnRHR demonstrated a full response to 10^{-5}M GnRH (fig. 4.2). In a screening ligand binding assay, all mutant GnRHRs except [Asn^{98}]-GnRHR exhibited specific binding of ^{125}I-[D-Ala^{6},N-MeLeu^{7},Pro^{9}-NHEt]-GnRH (data not shown).

In competitive ligand binding assays the [Gln^{301}]-GnRHR showed decreased apparent affinity for GnRH, but the apparent affinity for [Gln^{8}]-GnRH was similar to that of the wildtype receptor (table 4.1, fig. 4.3). Both peptides showed low potency and very similar dose-response curves in stimulating IP production in COS-1 cells transfected with the [Gln^{301}]-GnRHR (table 4.1, fig. 4.3).

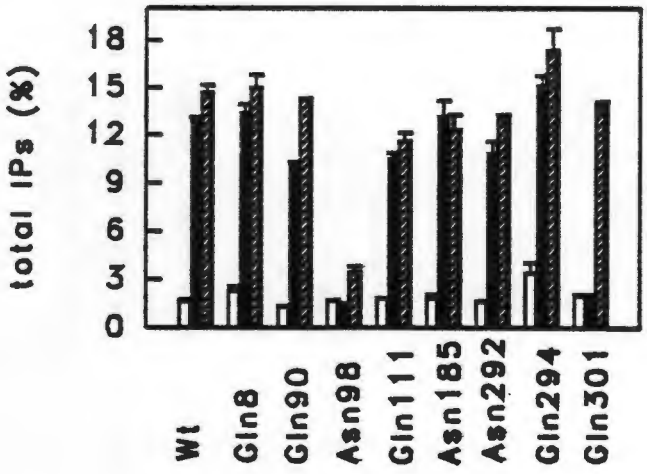


Figure 4.2: Screening assay for GnRH-stimulated IP production in COS-1 cells transfected with GnRHR mutants in which conserved acidic residues were mutated to Asn or Gln. Transfections were performed as described under Materials and Methods. Mutant receptors are identified by the name of the new residue (Gln or Asn) and its sequence number. Wt is the wildtype mouse GnRHR. IP production was measured in the absence of GnRH (open bars) and in the presence of 10^{-8}M (solid bars) and 10^{-5}M GnRH (cross-hatched bars).

Table 4.1 Summary of ligand binding and IP production data

	wildtype GnRHR		[Gln^{301}]-GnRHR		activity index[a]	
PEPTIDE	IC_{50} (nM)	EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	binding	IP
GnRH	20.9±7.9	1.14±0.02	1180±74	333±80	56.5	292
[Lys^{8}]-GnRH	1050±254	86.4±30.0	3290±494	1420±320	3.13	16.4
[Gln^{8}]-GnRH	2530±175	42.0±2.5	1870±498	369±67	0.74	8.78
[His^{8}]-GnRH	4630±1410	311±140	950±388	1010±410	0.21	3.25
[Glu^{8}]-GnRH	> 10^{5}	1160±180	22100±4410	1750±460	< 0.2	1.51
[His^{5},Trp^{7},Tyr^{8}]-GnRH (chicken GnRH II)	48±10	5.3±1.6	106±32.2	29.4±9.0	2.21	5.5
GnRHAg[b]	1.50±0.35	0.074±0.013	7.06±2.44	9.90±0.45	6.6	134
[D-Trp^{6}]-GnRH	2.08±0.42	0.095±0.032	1.18±0.37	15.9±1.4	0.57	167
[D-Trp^{6},Pro^{9}-NHEt]-GnRH	1.86±0.26	0.048±0.007	1.38±0.44	3.60±2.47	0.74	75
[D-Trp^{6},Gln^{8},Pro^{9}-NHEt]-GnRH	10.1±0.59	0.32±0.22	2.26±0.64	0.33±0.04	0.22	1.03

[a] activity index, which indicates the apparent decrease in potency of each peptide due to the mutation of Glu^{301}, was calculated as the ratio of the IC_{50}s (binding) or EC_{50}s (IP production) in the [Gln^{301}]-GnRHR and the wildtype GnRHR. An activity index of 1 indicates identical activity in both receptors.

[b] GnRHAg is [D-Ala^{6},N-MeLeu^{7},Pro^{9}-NHEt]-GnRH.

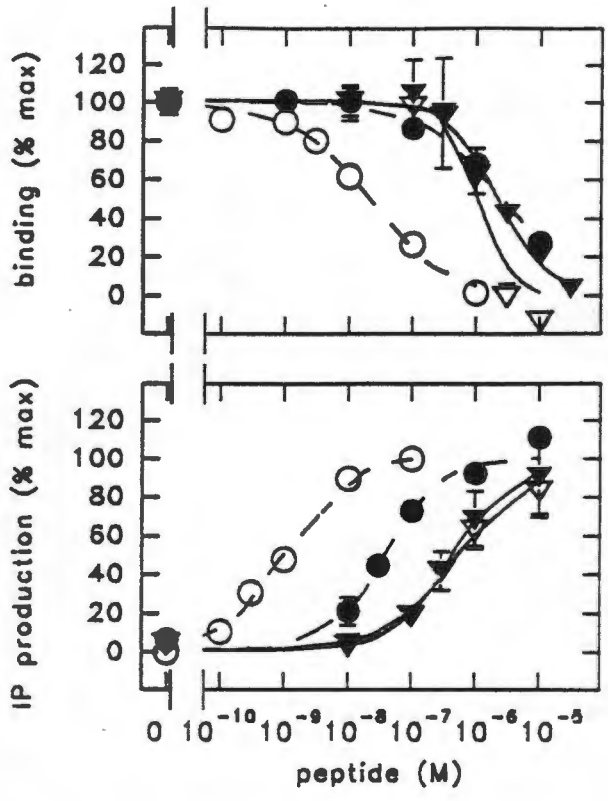


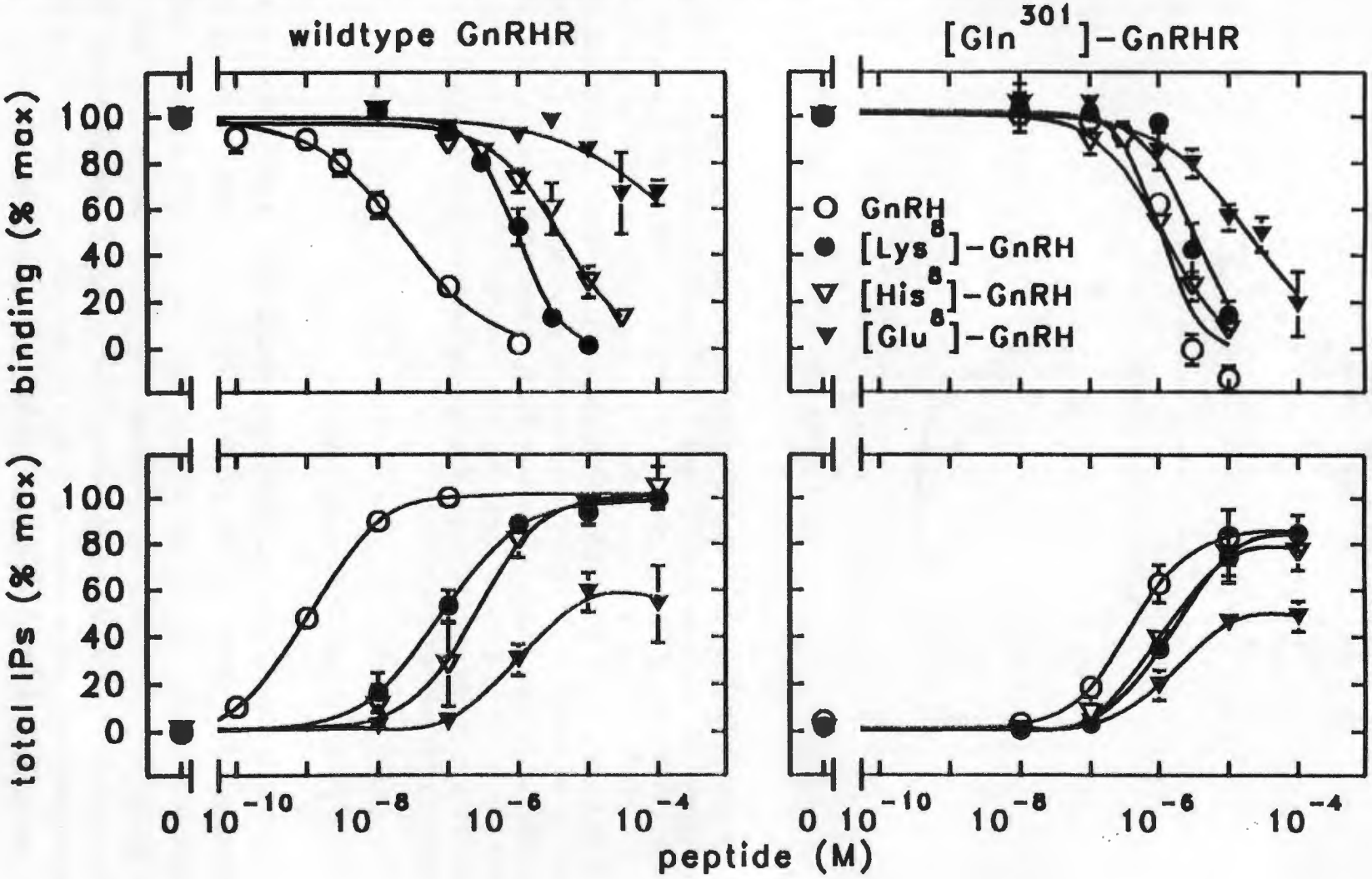
Figure 4.3: GnRH and [Gln^8]-GnRH ligand binding and IP production in COS-1 cells transfected with wildtype GnRHR and [Gln^{301}]-GnRHR. Binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH (top panel) in the presence of various concentrations of GnRH (○ , ▽) and [Gln^8]-GnRH (● , ▼) to membranes prepared from COS-1 cells transfected with wildtype GnRHR (--, ○ , ●) or [Gln^{301}]-GnRHR (—, ▽ , ▼) was measured as described under Materials and Methods. Data points are the mean ± standard error of 3 to 5 experiments performed in triplicate. IP production (lower panel) in response to GnRH (○ , ▽) and [Gln^8]-GnRH (● , ▼) in COS-1 cells transfected with wildtype GnRHR (--, ○ , ●) and [Gln^{301}]-GnRHR (—, ▽ , ▼) as described under Materials and Methods. Data points are the means of 2 or 3 experiments performed in triplicate.

Activities of other position 8-substituted GnRHs

GnRH analogues with Lys, His or Glu substituted for Arg^8 exhibited low apparent affinities for the wildtype GnRHR. [Lys^8]-GnRH was most potent, while the negatively-charged [Glu^8]-GnRH was least potent, being unable to cause 50% inhibition of binding of the labelled GnRH agonist at concentrations up to 10^{-4} M (table 4.1, fig. 4.4). Apparent affinities of these peptides for the mutant [Gln^{301}]-GnRHR were also low. The mutant receptor exhibited a 3-fold decreased affinity for the positively-charged [Lys^8]-GnRH and increased affinities for [His^8]-GnRH and [Glu^8]-GnRH (table 4.1, fig. 4.4).

Consistent with the ligand binding results, GnRH analogues with Lys and His residues in position 8 were less potent in stimulating IP production with the [Gln^{301}]-GnRHR than with wildtype, while the acidic [Glu^8]-GnRH showed only a small reduction in potency (table

Figure 4.4: Ligand binding and IP production of position 8-substituted GnRH analogues in COS-1 cells transfected with wildtype GnRHR and [Gln^{301}]-GnRHR. Binding of ^{125}I-[D-Ala^{6},N-MeLeu^{7},Pro^{9}NHEt]-GnRH (top panels) and IP production (lower panels) in the presence of various concentrations of GnRH, [Lys^{8}]-GnRH, [His^{8}]-GnRH, and [Glu^{8}]-GnRH in COS-1 cells transfected with wildtype GnRHR (left panels) or [Gln^{301}]-GnRHR (right panels) were measured as described under Materials and Methods. Binding data points are the mean ± standard error of 3 to 5 experiments performed in triplicate, and IP data points are the means of 2 or 3 experiments performed in triplicate.



4.1, fig. 4.4). [Glu^{8}]-GnRH did not stimulate maximal IP production in cells transfected with either wildtype or mutant receptors (fig. 4.4). Thus, mammalian GnRH and all position 8-substituted GnRH analogues showed low potency in activating the mutant GnRHR (table 4.1, fig. 4.4).

Other GnRH agonists

Low affinity binding interactions are generally less specific than high affinity interactions. Thus, a mutation which causes general disruption of the configuration of the ligand binding site, rather than eliminating a specific interaction, could generate a low affinity receptor which also does not discriminate different GnRH analogues, as was found for the [Gln^{301}]-GnRHR. To test whether this loss of discrimination was specific for modifications of GnRH at position 8, the activities of GnRH analogues which have high affinity due to modifications of other residues were tested. Chicken GnRH II ([His^{5},Trp^{7},Tyr^{8}]-GnRH) and two conformationally constrained GnRH superagonists, [D-Ala^{6},N-MeLeu^{7},Pro^{9}-NHEt]-GnRH and [D-Trp^{6}]-GnRH, showed higher apparent affinity for the [Gln^{301}]-GnRHR than did GnRH or analogues containing substitutions only at position 8 (table 4.1, fig. 4.5). These

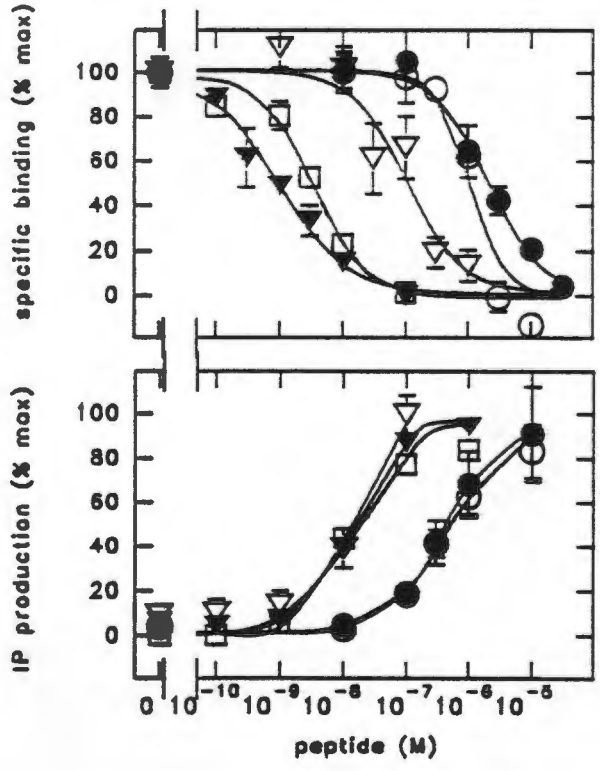


Figure 4.5: The mutant [Gln^{301}]-GnRHR exhibits wildtype specificity for GnRH analogues with substitutions in positions 5, 6, 7 and 10. Binding of ^{125}I-[D-Ala^{6},N-MeLeu^{7},Pro^{9}NHEt]-GnRH (top panel) and IP production (lower panel) in the presence of various concentrations of GnRH (○), [Gln^{8}]-GnRH (●), chicken GnRH II (▽), [D-Trp^{6}]-GnRH (▼) and [D-Ala^{6},N-MeLeu^{7},Pro^{9}-NHEt]-GnRH (□) in COS-1 cells transfected with [Gln^{301}]-GnRHR, as described under Materials and Methods.

peptides were also more active than GnRH and [Gln^8]-GnRH in stimulating IP production in cells transfected with the [Gln^{301}]-GnRHR (table 4.1, fig. 4.5). Thus, the [Gln^{301}]-GnRHR exhibited high affinity for GnRH agonists containing favourable substitutions in positions 5, 6, 7 and 10, and was able to discriminate between them and GnRH. This indicates specific loss of an interaction which requires Arg^8, rather than a generalized decrease in binding affinity.

Superagonists with- and without Arg^8

Having identified a residue (Glu^{301}) in the GnRHR which determines the specificity of the receptor for Arg^8 in GnRH, we addressed the question of whether this specificity depended on a direct interaction between the sidechains of the Arg^8 and Glu^{301} residues or on the intramolecular function of Arg^8 in stabilizing the preferred conformation of GnRH. To do

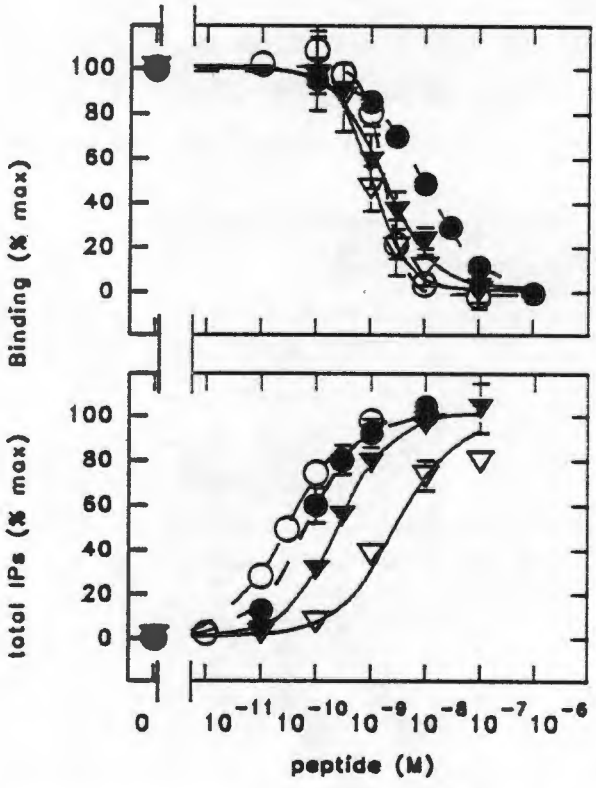


Figure 4.6: Two conformationally-constrained analogues of GnRH exhibit high affinity for both the wildtype GnRHR and the [Gln^{301}]-GnRHR mutant. Binding of ^{125}I-[D-Ala^6,N-MeLeu^7,Pro^{9}NHEt]-GnRH (top panel) in the presence of various concentrations of [D-Trp^6,Pro^9-NHEt]-GnRH (○ , ▽) and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (● , ▼) to membranes prepared from COS-1 cells transfected with wildtype GnRHR (–, ○ , ●) or [Gln^{301}]-GnRHR (—, ▽ , ▼) was measured as described under Materials and Methods. Data points are the mean ± standard error of 3 to 5 experiments performed in triplicate. IP production (lower panel) in response to [D-Trp^6,Pro^9-NHEt]-GnRH (○ , ▽) and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH (●, ▼) in COS-1 cells transfected with wildtype GnRHR (–, ○ , ●) and [Gln^{301}]-GnRHR (—, ▽ , ▼) as described under Materials and Methods. Data points are the means of 2 or 3 experiments performed in triplicate.

Table 4.2 Relative potencies of GnRH analogues containing D-amino acid substitutions in position 6. Peptide potencies were calculated relative to GnRH using the data in table 4.1.

	wildtype GnRHR		[Gln^{301}]-GnRHR	
peptide	binding	IP	binding	IP
GnRHAg[a]	13.9	15.4	167	33.6
[D-Trp^6]-GnRH	10.0	12.0	1000	20.9
[D-Trp^6,Pro^{9}NHEt]-GnRH	11.2	23.8	767	92.5
[D-Trp^6,Gln^8,Pro^{9}NHEt]-GnRH	2.1	3.6	525	1009

this we attempted to discount the intramolecular role of Arg^8 by comparing the activities of two high affinity GnRH agonists ([D-Trp^6,Pro^9-NHEt]-GnRH and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH) in which the preferred conformation is constrained by D-Trp in position 6, and which differ from each other only at position 8. Both peptides exhibited high affinity binding to both wildtype and mutant receptors. The Gln^8-containing peptide showed higher apparent affinity for the mutant [Gln^{301}]-GnRHR than for the wildtype receptor (table 4.1, fig. 4.6). The Gln^8-containing peptide also exhibited no loss of potency in stimulating IP production in the mutant receptor compared with wildtype (table 4.1, fig 4.6). The rank order of potency of the two peptides was reversed in the mutant [Gln^{301}]-GnRHR, with the Gln^8-containing peptide more potent than the Arg^8-containing peptide in stimulating IP production, although binding affinities were similar for both peptides (table 4.2, fig. 4.6). These results indicate that the mechanism by which the Glu^{301} residue confers specificity for Arg^8 is more complex than either a simple electrostatic interaction or simple stabilization of ligand conformation.

DISCUSSION

Eight GnRHR mutants were constructed, in which acidic residues in the superficial transmembrane and extracellular domains were exchanged for uncharged, hydrophilic, amide residues with equivalent sidechain lengths. Six mutant GnRHRs exhibited normal GnRH-stimulated IP production in a screening assay while two mutations caused changes in IP production. Mutation of Asp^{98} caused total loss of both agonist ligand binding and GnRH-stimulated IP production, suggesting that this mutation may affect expression or stability of the receptor or disrupt the configuration of the ligand binding site.

The [Gln^{301}]-GnRHR mutant had characteristics consistent with loss of specificity for Arg in position 8 of GnRH. It exhibited low apparent affinity for GnRH, similar to the low apparent affinity of the wildtype receptor for GnRH analogues which do not contain Arg in position 8. Its apparent affinity for [Gln^8]-GnRH was unchanged from that of the wildtype receptor for this ligand. However, its apparent affinity for [Glu^8]-GnRH was more than 10-

fold higher than that of the wildtype receptor. The mutant receptor did not clearly distinguish GnRH from analogues which have substitutions exclusively at position 8. Since low affinity interactions often lack specificity, it might be argued that the [Gln^{301}]-GnRHR is simply a low affinity receptor which has lost the ability to discriminate different ligands. However, the [Gln^{301}]-GnRHR retained appropriate affinities for GnRH analogues which contain activating substitutions in other positions. Thus, removal of the negatively-charged Glu^{301} residue of the GnRHR removed the preference of the receptor for Arg in position 8 of GnRH, but it did not remove the preference of the receptor for ligands with favorable substitutions in positions 5 and 7, as illustrated by chicken GnRH II, nor did it remove the preference for a D-amino acid in position 6. Thus, the Glu^{301} residue of the mouse GnRHR determines the ability of the receptor to recognize Arg^8 in GnRH.

It has been proposed that the Arg^8 of GnRH participates in a direct ionic interaction with one or more negatively charged residues in the receptor, either an amino acid sidechain (Hazum, 1987) or a polysaccharide sialic acid residue (Keinan and Hazum, 1985). The present results, combined with our recent demonstration that mutation of each of the putative glycosylation sites in the GnRHR does not affect ligand binding affinity (Davidson *et al.*, 1995), support the involvement of an amino acid sidechain, rather than a sialic acid residue, in the interaction of the GnRHR with Arg^8.

An electrostatic interaction is also supported by the experiments with [Glu^8]-GnRH, which has a negatively-charged residue in position 8. The very low binding affinity ($IC_{50} > 10^{-4}$ M) of the wildtype GnRHR for [Glu^8]-GnRH is consistent with repulsion between the negative charges of Glu^8 in the peptide and Glu^{301} in the GnRHR. Removing the negative charge of the amino acid in position 301 of the receptor improved the apparent binding affinity of this negatively-charged ligand more than ten-fold (IC_{50}, 2.21×10^{-5} M in the [Gln^{301}]-GnRHR). The apparent contribution of the Glu^{301} sidechain to the energy of binding between the GnRHR and mammalian GnRH was -2.3 kcal/mol. This is in agreement with the average free energies for enzyme-substrate ion-pair interactions reported for subtilisin (Wells *et al.*, 1987).

However, the mechanism by which specificity for Arg^8 is conferred by Glu^{301} appears more complex than a simple electrostatic interaction. Studies with enzyme substrate complexes have shown that binding affinity is higher where two uncharged hydrophilic sidechains are in contact with each other than where an uncharged hydrophilic sidechain is in contact with a charged residue (Wells *et al.*, 1987). From this it follows that, if there is contact between Arg^8 and Glu^{301}, the affinity of [Gln^8]-GnRH for the mutant [Gln^{301}]-GnRHR should be higher than the affinity of [Gln^8]-GnRH for the wildtype receptor (Gln^{301}---Gln^8 > Glu^{301}---Gln^8) and the mutant [Gln^{301}]-GnRHR should exhibit higher affinity for [Gln^8]-GnRH than for mammalian GnRH (Gln^{301}---Gln^8 > Gln^{301}---Arg^8). This was not the case,

[Gln^8]-GnRH had the same affinity for the mutant and wildtype receptors, and the mutant receptor exhibited similar affinities for GnRH and [Gln^8]-GnRH. A possible explanation for this is that Arg^8 plays an additional role in GnRH, affecting the structural conformation of the ligand. Like many peptides, GnRH is highly flexible in solution and exists as an equilibrium mixture of structural conformers. It has been proposed that the sidechain of Arg^8 stabilizes a preferred conformation of GnRH by forming a structural unit of hydrogen bonding with the sidechains of His^2 and Tyr^5 (Shinitzky and Fridkin, 1976; Shinitzky *et al.*, 1976; Hazum *et al.*, 1977). It has also been shown that while this type of structural unit is formed in GnRH, it is not formed in the neutral [Gln^8]-GnRH analogue (Milton *et al.*, 1983). Other studies have indicated that GnRH interacts with its receptor in a folded conformation with a β-turn which involves Gly^6. This conformation can be stabilized by substitution of Gly^6 with a D-amino acid, which increases GnRH activity (Monahan *et al.*, 1973; Momany, 1976a, 1976b; Freidinger *et al.*, 1980). Therefore, if Gly^6 in GnRH is substituted with D-Trp, the ligand can be constrained in the preferred conformation, independently of whether or not there is Arg in position 8. This constraint makes it possible to distinguish the role of Arg^8 in interacting with the receptor Glu^{301} residue from its contribution to ligand stabilization. Thus, we compared the apparent binding affinities, in wildtype and mutant receptors, of two conformationally constrained GnRH agonists ([D-Trp^6,Pro^9-NHEt]-GnRH and [D-Trp^6,Gln^8,Pro^9-NHEt]-GnRH) which are identical except for the substitution of Gln for Arg in position 8. The affinity of the [Gln^{301}]-GnRHR for the constrained Gln^8 agonist was higher than that of the wildtype receptor, consistent with contact between the two residues (Gln^{301}---Gln^8 > Glu^{301}---Gln^8, table 4.1). Also, the Gln^8 agonist was more potent than the Arg^8 agonist in stimulating IP production with the mutant [Gln^{301}]-GnRHR (Gln^{301}---Gln^8 > Gln^{301}---Arg^8, table 4.1). This supports the proposal of contact between the position 8 residue in GnRH and the position 301 residue of the receptor. However, in the presence of the conformational constraint, the apparent contribution of the Glu^{301} residue to the binding energy of the Arg^8 agonist is much lower (0.02 kcal/mol) than has been reported for catalytic site ion-pair interactions (Wells *et al.*, 1987). This could mean either, that there is no electrostatic interaction between these residues, or that the interaction occurs in an aqueous environment where hydrogen bonding of water plays a role in binding energies.

Thus, the Glu^{301} residue is necessary for high affinity binding of ligands which contain Arg^8, and which are not conformationally-constrained. However, conformationally-constrained ligands bind both the wildtype and the mutant GnRHR with high affinity, regardless of whether the residue in position 8 is Arg or Gln (table 4.1). The enhancement of potency due to introduction of conformational constraints of the ligand is much greater in the mutant [Gln^{301}]-GnRHR than in the wildtype receptor (table 4.2). The mutant receptor therefore prefers conformationally-constrained ligands, but cannot recognize the contribution of Arg^8 in conforming GnRH. This suggests that the wildtype GnRHR induces the preferred

conformation in unconstrained ligands which contain Arg^8. The [Gln^{301}]-GnRHR is not able to induce this preferred conformation, but it retains a preference for the conformation constrained by D-amino acids in position 6. Thus, the preferred conformation of GnRH is probably induced by an interaction which involves Glu^{301} and Arg^8 and this interaction is not required for binding of ligands which are constrained prior to their binding to the GnRHR.

The ligand binding sites of heptahelical receptors for small ligands such as catecholamines are contained within the hydrophobic transmembrane helices (Dixon *et al.*, 1987; Strader *et al.*, 1989; Dohlman *et al.*, 1991). In contrast, large amino-terminal domains form the high affinity ligand binding sites of receptors for glycoprotein hormones (Segaloff and Ascoli, 1993). Peptide receptors, including the GnRHR, do not contain large amino-terminal domains and the ligand binding sites in these receptors appear to involve residues both in the transmembrane helices (Zhu *et al.*, 1992) and in the extracellular domains (Fong *et al.*, 1992a, 1992c; Gether *et al.*, 1993). We have shown that the Glu^{301} residue, located in the third extracellular loop of the GnRHR, plays a major role in determining ligand specificity. This confirms the importance of extracellular domain of the GnRH receptor in ligand binding.

The Glu^{301} residue is conserved in the rat and mouse GnRHRs, but in sheep and human GnRHRs it is replaced with Asp (Illing *et al.*, 1993). This difference indicates that the length of the sidechain is probably not critical for the interaction with GnRH, although it may contribute to some of the subtle differences in pharmacologies of mammalian GnRHRs (Millar *et al.*, 1989; chapter 2).

The [Gln^{301}]-GnRHR shares some pharmacological characteristics with the chicken pituitary GnRHR in that GnRH and [Gln^8]-GnRH display equal activity, while chicken GnRH II is more active (Millar *et al.*, 1989; chapter 2). However, enhancement of activity resulting from D-amino acid substitutions in position 6 of GnRH is greater in the [Gln^{301}]-GnRHR (table 4.2) than in the chicken GnRHR, in agreement with suggestions that the chicken GnRHR does not require the same ligand conformation as is required by mammalian GnRHRs (Millar *et al.*, 1989; chapter 2). These results predict that while Glu^{301} is likely to be absent from the chicken GnRHR, the latter receptor may have other features which increase affinity in order to compensate for the absence of Arg^8 in chicken GnRHs.

Our findings may apply to other receptors. Vasopressin receptors possess an acidic residue in their third extracellular loop [Glu^{299} in the human V2 receptor (Birnbaumer *et al.*, 1992) and Glu^{323} in the rat V1 receptor (Morel *et al.*, 1992)], which is not present at the corresponding position in the oxytocin receptor (Kimura *et al.*, 1992). Since vasopressins possess positively charged residues (Arg or Lys) which are replaced by neutral Leu in oxytocin, it is possible that a similar mechanism may determine the specificity of vasopressin and oxytocin receptors for their respective ligands.

In conclusion, we have identified a residue, Glu^{301}, in a mammalian GnRHR which confers specificity for the Arg in position 8 of mammalian GnRH. This specificity may result from an electrostatic interaction between the two residues. Interactions which involve the Glu^{301} residue of the receptor and the Arg^{8} residue of the ligand appear to induce changes in the conformation of the ligand.

CHAPTER 5

CONCLUDING DISCUSSION

The work presented here has explored how GnRH, as a ligand, interacts with its receptors. At the initiation of the research, the structures of five naturally occurring forms of GnRH had been reported, but very little was known about the structures of the GnRH receptors. The different forms of GnRH were known to have different potencies in stimulating gonadotropin release in animals from different vertebrate classes (Millar *et al.*, 1986b; King and Millar, 1987). Therefore, we investigated the functional significance of each of the most common amino acid substitutions. Substitutions in each of the positions examined had significantly different consequences for GnRH potency in pituitary cells from two different classes of vertebrates, mammals and birds. In addition, smaller differences in receptor binding affinity were demonstrated between two species of mammals. To understand how changes in ligand structure effect changes in receptor binding affinity and receptor activation, it was necessary to know the structures of the GnRH receptors. Site-directed mutagenesis of the cloned mouse GnRH receptor, which became available during the course of this work, allowed identification of a receptor residue which determines the selective affinity of this mammalian receptor for mammalian GnRH. A continuing project in the laboratory is addressing the question of how a Glu or Asp residue in the third extracellular loop of the mammalian GnRH receptors determines this selectivity.

In order to study the functional significance of the amino acid substitutions which occur in GnRHs isolated from non-mammalian vertebrates, we synthesized chimaeric analogues consisting of various combinations of these substitutions. Substitution of Tyr^5 with His had subtle effects on GnRH activity in mammals, which became apparent only when receptor binding affinity and gonadotropin releasing potency were considered together. Analogues containing His^5 exhibited higher receptor binding potencies than gonadotropin releasing potencies. Substitution of Trp for Leu^7 also resulted in minimal effects on GnRH activity. However, the combination of His^5 and Trp^7 substitutions seems to account for the relatively high potency of chicken GnRH II ([His^5,Trp^7,Tyr^8]-GnRH) compared with other position 8-substituted GnRH analogues. Another study showed that [His^5,Trp^7,Leu^8]-GnRH had relatively high GnRH potency (Folkers *et al.*, 1986). The fact that His^5 and Trp^7 together increase GnRH activity in the presence of a hydrophobic residue in position 8, suggests that an interaction amongst the three sidechains accounts for the higher activity of chicken GnRH II in mammals.

In contrast, in chicken pituitary cells, His^5 incorporation in mammalian GnRH strongly decreased LH releasing potency. This substitution may cause GnRH to assume a

conformation which is not favoured by the chicken GnRH receptor, as the effect was reversed by the introduction of D-Trp in position 6. Trp substitution for Leu^7 increased LH releasing potency in chicken cells and could completely account for the high potency of chicken GnRH II compared with [Tyr^8]-GnRH. In the absence of a reliable chicken GnRH receptor binding assay it is not possible to speculate on the mechanisms of these potency changes.

Neutral substitutions for Arg^8 had opposite effects in chicken and mammalian pituitary cells. Analogues with uncharged residues in position 8 exhibited high potencies in stimulating chicken LH release, but had low potencies in stimulating release of LH and FSH from sheep cells and low affinity for sheep and rat GnRH receptors. The obvious explanation of this result was that mammalian receptors contain a negatively charged residue which forms a salt bridge with the positively charged Arg in mammalian GnRH (Keinan and Hazum, 1985; Hazum, 1987) and that chicken GnRH receptors do not (Hasegawa *et al.*, 1984). Testing this required information on the structures of the GnRH receptors.

Attempts to purify the sheep GnRH receptor by affinity chromatography resulted in the purification of a protein which, although it was similar to other proteins purified by similar protocols (Hazum *et al.*, 1986; Christiansen and Houen, 1994), was not the GnRH receptor. However, our collaboration with Dr. Sealfon's laboratory gave us access to the first GnRH receptor clone (Tsutsumi *et al.*, 1992), which allowed us to test the salt bridge hypothesis by site-directed mutagenesis.

We mutated acidic receptor residues located in the extracellular domain and in the extracellular half of the putative transmembrane domain, which could potentially be part of the ligand binding site. One mutant receptor exhibited decreased affinity for mammalian GnRH, unchanged affinity for [Gln^8]-GnRH, and increased affinity for the negatively charged analogue [Glu^8]-GnRH. Thus, substitution of the negatively charged Glu^{301} residue of the receptor removed the preference of the receptor for Arg^8 in GnRH. The loss of affinity was specific for Arg^8, since the mutant receptor retained the ability to distinguish favourable substitutions in positions 5, 6 and 7. These findings showed that the Glu^{301} residue of the mouse GnRH receptor plays a role in recognition of Arg^8 in the ligand and were consistent with an electrostatic interaction between these two residues.

These results led us to speculate that the Glu^{301} residue would not be conserved in the chicken GnRH receptor (Flanagan *et al.*, 1994; chapter 4). However, early PCR cloning experiments indicate that the Glu^{301} residue is conserved in GnRH receptors from several non-mammalian vertebrate species, including the chicken (B. Blackman, Y.-M. Sun, N. Illing, J. Hapgood, R. P. Millar, unpublished). While this was at first surprising, studies with other peptide receptors have shown that the residues which determine ligand selectivity are located at different positions in different receptor subtypes (Strader *et al.*, 1994; also see chapter 1,

The ligand binding domain). The differences in the sequences of the naturally occurring GnRHs are similar to the differences in the structures of families of peptides (e.g. neurokinins, endothelins) which are recognized by several receptor subtypes. Thus, the structural relationship of non-mammalian GnRH receptors to the mammalian receptor is likely to be similar to the relationship between subtypes of receptors for other peptides (60 - 80% amino acid conservation). Extending this analogy leads to the prediction that while the Glu^{301} residue determines the ligand selectivity of the mammalian GnRH receptor, it probably will not affect the ligand selectivity of the non-mammalian receptors. The positions of ligand contact sites vary among different peptide receptor subtypes. Within a single receptor, both conserved and divergent residues appear to interact with conserved ligand residues (Strader *et al.*, 1994). It has been proposed that each receptor subtype recognizes a different peptide conformation (Fong *et al.*, 1992a), and this may be the reason why ligand contact sites vary. Indeed, the Glu^{301} residue of the GnRH receptor appears to have a significant role in recognizing or modifying GnRH conformation.

The Glu^{301} residue is necessary for high affinity binding of ligands which contain Arg^8, and which are not conformationally constrained. However, ligands which were conformationally constrained by the incorporation of D-Trp^6 bound both the wildtype and the mutant [Gln^{301}]-GnRHR with high affinity, regardless of whether the residue in position 8 was Arg or Gln. The enhancement of potency due to introduction of conformational constraints of the ligand was greater in the mutant receptor than in the wildtype. The mutant receptor, therefore, preferred conformationally constrained ligands, but did not recognize the contribution of Arg^8 in conforming GnRH. This suggests that the wildtype GnRH receptor induces the preferred conformation in unconstrained ligands which contain Arg^8. The mutant [Gln^{301}]-GnRHR was not able to induce this preferred conformation, but it retained a preference for the conformation constrained by D-amino acids in position 6. Thus, the preferred conformation of GnRH may be induced by an interaction which involves the Glu^{301} and Arg^8 residues and this interaction does not occur during binding of ligands which are constrained prior to their binding to the GnRHR.

In conclusion, we have described the relationship between the known structures of GnRH and their function at three different GnRH receptors which were treated essentially as black boxes. A little light has been shone into the black boxes, in that the amino acid sequences of several mammalian GnRH receptors are now known and it is anticipated that the cloning of non-mammalian GnRH receptors will soon be reported. Several amino acid residues have been identified which are important determinants of ligand binding, the integrity of receptor structure and of receptor activation. We are continuing to investigate the nature of the interaction by which an acidic residue which we have identified in the third extracellular loop of the GnRH receptors determines ligand selectivity.

CHAPTER 6

OTHER STUDIES CONDUCTED DURING THE COURSE OF THIS THESIS AND LIST OF PUBLICATIONS

During the course of the work described here, I participated in several projects involving GnRH receptor ligand interactions, which have not been described in this thesis. We showed that GnRH antagonists, which have been developed in mammalian systems, are much less effective in cultured chicken pituitary cells and that some peptides which are antagonists in mammals behave as agonists in chickens (Jacobs *et al.*, 1995). I helped characterize several of the cloned GnRH receptors (Tsutsumi *et al.*, 1992; Chi *et al.*, 1993; Illing *et al.*, 1994) and also participated in studies which used the cloned receptors to examine the structure of the receptor and how it interacts with ligands. We showed that the mouse GnRH receptor is glycosylated at two of its three glycosylation consensus sequences when it is expressed in COS-1 cells and found that glycosylation affects expression of the receptor, but does not affect ligand binding affinity (Davidson *et al.*, 1995). Compared with other GPCRs, the GnRH receptor appears to have interchanged two highly conserved residues, an Asp which usually occurs in the second transmembrane helix and an Asn which usually occurs in the seventh. We used this natural reciprocal mutation to show that the sidechains of these two residues have complimentary roles in maintaining receptor structure and occupy the same microenvironment within the receptor (Zhou *et al.*, 1994). We are continuing to explore this interaction which is discussed in more detail in chapter 1. Comparison of the GnRH receptor with other GPCRs also allowed us to identify the Lys^{121} residue which has a role in binding of GnRH agonists but not GnRH antagonists.

LIST OF PUBLICATIONS

Publications based on the work described in this thesis and on related topics are listed below.

19. Zhou, W., Rodic, V., Kitanovic, S., Flanagan, C. A., Chi, L., Weinstein, H., Maayani, S., Millar, R. P. and Sealfon, S. C. (1995) A locus of the gonadotropin-releasing hormone receptor which differentiates agonist and antagonist binding sites, *J. Biol. Chem.* **270**: 18853-18857.

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CHAPTER 7

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