AN INVESTIGATION OF THE ROLE OF N102 OF THE GnRH RECEPTOR IN DETERMINING THE POTENCY OF C-TERMINALLY MODIFIED AGONIST ANALOGS

By

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ABSTRACT

BACKGROUND

G protein-coupled receptors (GPCR) form the largest family of receptors in the human genome. The gonadotropin releasing hormone (GnRH) receptor is a member of this family and plays a central role in the reproductive system. GnRH, a hypothalamic decapeptide, regulates the reproductive system by activating the GnRH receptor on pituitary gonadotrope cells, leading to inositol phosphate (IP) production, Ca^{2+} mobilisation, and secretion of gonadotropins.

There is substantial evidence that GnRH adopts a preferred folded conformation, which is stabilised by hydrogen bonding between the side chains of residues His^2 and Tyr^5 of the decapeptide, and interacts with its receptor through both the N and C termini of the ligand. The substitution of the GnRH residue Gly^6 to D-Ala has been found to stabilise the bent conformation and increase the potency of the peptide analog.

In common to all GPCRs, the GnRH receptor contains seven hydrophobic α-helical transmembrane domains, which are connected by extracellular and intracellular domains. Receptor residue N102 is situated at the extracellular end of transmembrane helix 2. A role for N102 in ligand binding was previously revealed fortuitously as a result of the investigation of the roles of consensus sequences for N-glycosylation. Using site directed mutagenesis, N102 was shown to be a critical determinant for the

potency of GnRH and analogs containing glycinamide at the C-terminus, possibly playing a direct role in the docking of the glycinamide C-terminus. The hypothesis that a hydrogen bond forms between the side chain of N102 and the glycinamide moiety of GnRH has been further investigated in the present study.

THE PRESENT THESIS

Three GnRH receptors with the mutations N102A, N102D and N102K, which were derived from the human GnRH receptor by site directed mutagenesis, were expressed in COS-1 cells. The effects of these mutations on the binding of GnRH analogs were first investigated. Secondly, the effects of these mutations on the potency of seven C-terminally modified GnRH agonist analogs for the stimulation of IP production were determined.

The N102A mutant receptor showed low binding of three iodinated GnRH radioligands. The addition of an extra glycosylated site that enhances receptor expression was introduced in an attempt to enhance binding of N102A mutant receptor. Although this increased ligand binding somewhat, it was not sufficient to enable measurement of receptor binding affinity. Since the measurement of binding affinity of the N102A GnRH receptor using radioligand methodology was shown not to be possible, the focus of the study was directed towards the agonist-stimulated IP response.

The N102A GnRH receptor was able to mediate a robust maximal IP response to agonists. The two GnRH analogs with glycinamide C-termini (GnRH-glycinamide

and D-Ala^6-GnRH-glycinamide) showed potency losses of 230 –470 fold for the N102A mutant receptor compared with the wild-type receptor, indicating a strong dependence on N102. Analogs exhibiting a potency loss, for the mutant receptor, similar to that of GnRH indicate that the analog retains the same interaction as does GnRH. The seven C-terminally modified analogs tested showed potency losses of different magnitudes indicating differences in their dependence on N102. Four of the seven analogs, which have alkyl amide C termini (D-Ala^6-GnRH-des-Gly^{10}-ethylamide, D-Ala^6-GnRH-des-Gly^{10}-propylamide, D-Ala^6-GnRH-des-Gly^{10}-trifluoroethylamide and D-Ala^6-GnRH-des-Gly^{10}-pentafluoropropylamide), showed smaller potency losses (3.2-17.9 fold) indicating a weaker dependence on N102. D-Ala^6-GnRH-des-Gly^{10}-hydrazide exhibited a similar potency loss to GnRH-glycinamide. GnRH-des-Gly^{10}-propargylamide and GnRH-des-Gly^{10}-dimethylamide for the N102A mutant receptor showed potency losses of intermediate magnitude.

These results are interpreted in terms of a proposed model explaining the docking of the C-terminal part of GnRH analogs. The model proposes that the C-terminal glycinamide H-bonds with N102, and that C-terminal alkyl amide moieties interact hydrophobically with the receptor at a different site. The model adequately explains the behaviour of seven of the eight different C-terminal moieties examined. The behaviour of the dimethylamide analog is anomalous, and possible reasons for this are discussed.

DEDICATION

This thesis is dedicated to my two children Robyn and James, and to the D'hondt Clan whose love and support have given me the courage to persevere.

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NOTE

Some figures are included with the text, for clarity of description, while figures 3.1.1 to 3.2.28 showing experimental data are in a separate chapter (6), for ease of comparison.

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LIST OF ABBREVIATIONS USED

B_{max}	Maximal binding
Bo	Radioligand bound in the absence of competing unlabelled ligand
DAG	Diacyl glycerol
DEAE	Diethylaminoethyl
DMEM	Dulbeccos's modified Eagles medium
DMSO	Dimethyl sulfoxide
EC	Extracellular domain
EC_{50}	Peptide concentration required to half-maximally stimulate production of inositol phosphates
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
G protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
GnRH	Gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HBS	Hepes buffered saline
HEPES	N-2-hydroxyethylpiperazine –N'-2-ethanesulfonic acid
hGnRHR	Human gonadotropin releasing hormone receptor

IC loop	Intracellular loop
IC_{50}	Peptide concentration required to half-maximally inhibit binding of labelled GnRH peptides
Ins-1,4,5P3	Inositol-1,4,5 triphosphate
Ins-1,4P2	Inositol-1,4 bisphosphate
IP	Inositol phosphate
IP_{max}	Maximal inositol phosphate response
LH	Luteinising hormone
mGnRH	Mammalian gonadotropin releasing hormone
OPTI-MEM	Reduced serum medium without sodium bicarbonate, modification of modified Eagles medium
PA	Phosphatidic acid
PCA	Perchloric acid
PIP_2	Phosphatidylinositol bisphosphate
PIP3	Phosphatidylinositol trisphosphate
PLA_2	Phospholipase A_2
SEM	Standard error of the mean
TM	Transmembrane domain
WT	Wild type

CHAPTER ONE

INTRODUCTION

1.1 GONADOTROPIN RELEASING HORMONE

1.1.1 ISOLATION AND EVOLUTION OF GnRH.

Gonadotropin-releasing hormone, a hypothalamic decapeptide, activates G protein-coupled receptors (GPCR) on pituitary gonadotrope cells, leading to Ca^{2+} mobilisation, and secretion of gonadotropins.

Since the report of the isolation of porcine GnRH by Schally (1971), twelve variants of the GnRH hormone have been characterised from mammalian and non-mammalian vertebrates (King and Millar, 1995 and 1997; Powell et al., 1996 a and b; Scalfon et al., 1997; Jimenez-Linan et al., 1997) as well as protochordates (Sherwood 1995; Powell et al., 1996b; Craig et al., 1997).

Two forms of GnRH are found to occur in most vertebrates, including some mammals. It was suggested by King and Millar (1992), that GnRH has undergone a gene duplication with the variants being distributed between two evolutionary arms. One arm contains the conserved Chicken GnRH II (or type II GnRH). The other variants (type I GnRHs) arose from evolution of the second arm. Type II GnRH, which is highly conserved between species has a neurotransmission and/or neuromodulation function; the other

form, which has several structural variant in different species, binds specifically to the pituitary receptors triggering the release of LH and FSH. At first only a single mammalian GnRH form (mGnRH) was believed to occur in higher eutharian mammals, until type II GnRH was found to occur in discrete neurones in monkey brain (Lescheid et al., 1987). However, a type II GnRH form has not yet been reported in human brain (Millar et al., 1997).

The type I GnRH form is secreted by the mediobasal hypothalamus in a pulsatile fashion into the hypophyseal portal blood, which facilitates delivery of effective concentrations to pituitary gonadotroph cells. These cells in turn release pulses of LH and FSH into the general circulation. The concentration of hypothalamic GnRH in portal blood is adequate to stimulate pituitary receptors, but after dilution into the general circulation is too low to activate the GnRH receptors present in other tissues. Gonadal and placental GnRH is secreted to affect adjacent target cells in a paracrine fashion (King and Millar 1992 and 1995). A similar system occurs when GnRH acts as neurotransmitter or autocrine regulator (King and Millar 1992 and 1995).

All forms of GnRH are decapeptides in which residues 1, 2, 4, 9 and 10 are highly conserved, while position 8 is the most variable (Table 1.1). A pyroglutamyl residue at the N terminus and a C-terminal amide group are also characteristics of all forms of GnRH. The amino-terminal modification is formed by the spontaneous cyclization of the side chain of the Glu residue with the NH_2 group of the amino acid (Jones G.H., 1974, Creighton, 1993). An N-terminal pyroglutamate residue is found in many proteins that operate in the extracellular space (Jones G.H., 1974; Schulz and Schirmer 1979).

The biological significance of modifying the N terminus, although not clearly understood, is believed to act as a protection against digestion by aminopeptidases. Another role for this modification could be the anchoring of the N-terminal part of the polypeptide in an apolar environment such as a receptor molecule (Schulz and Schirmer, 1979).

The C-terminal amide group is a characteristic feature of many peptide hormones. Amidating activity is present in almost every tissue including the hypothalamus (Bradbury and Smyth, 1991). The formation of the amide group is a two-step reaction catalysed by peptidylglycine α-amidating monooxygenase (PAM) and α-hydroxyglycine amidating dealkylase (HGAD) (Bradbury et al., 1982; Katopodis et al., 1990). The nitrogen atom of the amide group is derived from the nitrogen of a mandatory glycine residue (Bradbury et al., 1982). The C-terminal glycine is hydroxylated to an intermediate hydroxyglycine (Bateman et al., 1985; Takahashi et al., 1990) which is then converted to the peptide amide and glyoxylic acid (Katopodis et al., 1990; Bradbury and Smyth, 1991). Since amidating enzymes and amidated peptides are found in a wide variety of species ranging from anglerfish, insects and mammals, and an amidating mechanism similar to the mammalian mechanism was found in bees, it is believed that the hydroxylating mechanism is universally applicable (Suchanek et al., 1977; Bradbury and Smyth, 1991). This modification is often important for the biological activity of the hormone. It also contributes to the biostability of the peptide by protecting it from the attack of carboxypeptidases or by the accommodation of an otherwise negatively charged C terminus in a non-polar environment (Schultz and Schirmer 1979, Creighton 1993).

	1	2	3	4	5	6	7	8	9	10
MAMMAL	PGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly NH2
CATFISH	PGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly NH_2
SALMON	PGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly NH_2
DOGFISH	PGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly NH_2
CHICKEN I	PGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly NH_2
CHICKEN II	PGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly NH_2
SEA BREAM	PGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	GlyNH_2
GUINEA PIG	PGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	GlyNH_2
LAMPREY I	PGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly NH_2
LAMPREY III	PGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly NH_2
TUNICATE I	PGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly NH_2
TUNICATE III	PGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly NH_2

Table 1.1 Primary structures of GnRH isolated from vertebrate brain. Shaded residues represent those regions of the peptide, which have been conserved.

1.1.2 DEVELOPMENT OF GnRH ANALOGS.

Subsequent to the isolation of mGnRH and the elucidation of the amino acid sequence numerous studies evaluating the importance of the residues were undertaken. These led to the perfecting of the solid phase method for the synthesis of the peptide and analogs (Merrifield, 1963). The construction of a large variety of analogs with substitutions of various amino acids including the amino- and carboxy-termini led to the discovery of agonist and antagonist peptides of considerable potency (Karten and Rivier 1986, Sealfon et al. 1997).

1.1.2.1 Properties of amino acid residues in GnRH:

The amino acid sequence of the mGnRH decapeptide is pyro-Glu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2. GnRH is believed to adopt a folded conformation as shown in fig. 1.1 with a ß-II type bend involving the residues Ser^4, Tyr^5, Gly^6, Leu^7 (King and Millar 1995, Monahan et al. 1973, Momany F.A. 1976, Freidinger et al. 1980).

At physiological pH, Arg^8 is the only ionised amino acid that occurs in mGnRH. Acting as a cation it is believed to form a hydrogen bond system with the side chains of His^2 and Tyr^5 that stabilises the preferred bent conformation (Hazum and Fridkin, 1977; Shinitzky and Fridkin, 1976 (a) and 1976 (b)). The combined side-chains of His^2, Tyr^5 and Arg^8 make up one active region of GnRH. The side chain of Trp^3, essential for GnRH activity, is at a maximal distance from this region and may therefore act as a different activity locus. It has been hypothesised that the aromatic characteristics of the side chains of His^2, of Trp^3 and Tyr^5, acting as electron donors, may react with an electron acceptor site in the hormone receptor (Shinizky and Fridkin, 1975; Keinan and Hazum, 1985).

His^2-Trp^3 residues appear to be the most critical residues for receptor activation (Karten and Rivier, 1986; Millar et al., 1987). There is a relationship between Arg^8 and Trp^3 that contains the "LH releasing message" (Sieverton et al., 1971) and aromaticity at positions 2 and 3 was shown to be a requirement for ligand-induced receptor activation (Monahan et al. 1972, (a)). A site-directed mutagenesis study recently suggested that Arg^8 interacts electrostatically with E301 residue of the GnRH receptor (Flanagan et al., 1994).

In a study on the functional importance of His^2, Gly^2-GnRH and des-His^2-GnRH were the first examples of synthetic peptide analogs found to exhibit antagonism towards the release of LH and FSH hormones (Monahan et al. 1972(b)).

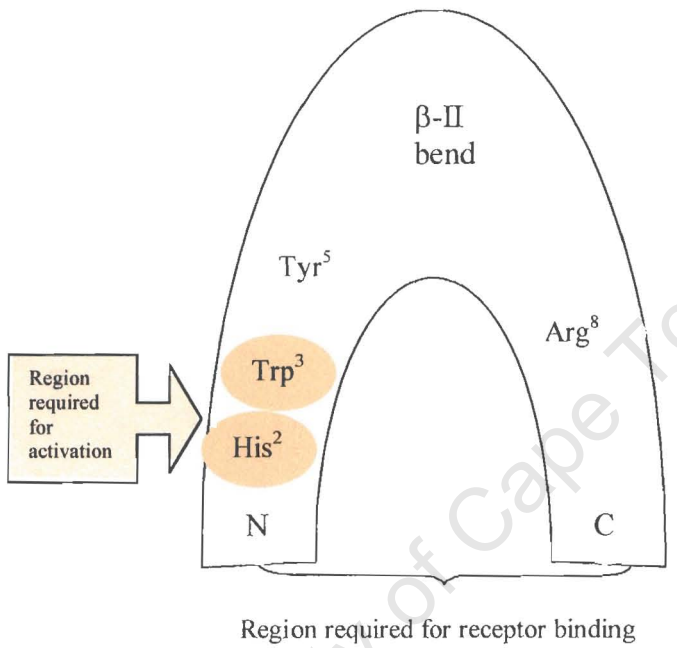


Fig 1.1 Schematic diagram showing the bent conformation of GnRH and regions believed to be involved in binding and activation

1.1.2.2. D-Ala^6-substitution:

A correlation of the active conformation with structure-activity of the peptide became necessary. The construction of D-Alanine analogs provided comparative information on the backbone structure of the peptide.

The D-Ala^6 substitution was shown to increase both agonist and antagonistic activities of GnRH analogs by enhancing binding affinity (Monahan et al., 1972). It led to a 3.5-4.5 fold increase in potency both in vivo and in vitro compared to mGnRH (Monahan et al., 1973), whilst Ala^6-GnRH was 25 times less potent.

The increased biological activity of D-Ala^6 GnRH can be explained as a result of a restriction in the conformational degrees of freedom of the peptide backbone when D-alanine is substituted for glycine. In a four-residue sequence, for example Ser^4-Tyr^5-Gly^6-Leu^7 of GnRH, a ß-II type bend, producing a reversal of direction of the peptide chain, is formed only when Gly or D-Ala occurs at the third position of the sequence. The structure is stabilised by a hydrogen bond formation between the COO group of the first residue (Ser^4) and the NH_2 group of the fourth residue (Leu^7) (Chandeasekaran et al., 1973).

Semi empirical conformational energy calculations on various types of turns in peptides showed that a ß-II type bend is stabilised by 4.7 kcal when a D-Ala is substituted for an L-Ala in the third residue whilst the ß-I and γ bends are destabilised (Nemethy and Printz, 1972). The restriction is believed to stabilise a conformation of GnRH favourable for receptor binding, resulting in the observed increase in biological activity (Monahan et al., 1973).

Bulky side chains at position 6 cause a decrease in hormonal activity (Arnold et al., 1973). However, with a D-amino acid at position six the spatial effect of the side chain is unimportant in peptide-receptor binding (Fujino et al., 1974). The activity of

the analog is higher when the hydrophobic character and size of the side chain of the D-amino acid are increased (Vilchez Martinez et al., 1974; Coy et al., 1975 and 1976).

1.1.2.3 Amino terminal modifications:

It is believed that in the bent conformation, GnRH interacts with its receptor through both the N and C termini (Millar et al., 1993). Studies of analogs involving substitution of pyroglutamic acid at position 1 showed very little bioactivity (Fujino et al., 1972; Yanaihara et al, 1972; Arnold et al., 1973). The work done by Arnold et al. (1973) suggests that it is the specific spatial structure of pyroglutamic acid that is important, rather than a protection function from N-terminal exopeptidases.

1.1.2.4 Carboxy terminal modifications:

The investigation of a large variety of analogs with carboxy terminal modifications has led to the following understanding of the C terminus:

a. Although the whole decapeptide is required for full biological activity, the C-terminal glycinamide residue is important for the binding of the peptide to the receptor rather than being involved in the hormone releasing activity (Fujino et al., 1972, Rivier et al., 1973, Coy et al. 1973). In a study of the effect of short-chain amide GnRH analogs, Rivier et al. (1973) demonstrated that the biological activity of Pro^{9}-NH_2-des-Gly^{10}-GnRH was decreased by a factor of 10. However, the active nonapeptide showed dose-response curves parallel to those of GnRH with similar maximum responses indicating that the decrease in biological activity resulted from a decrease in affinity of the analog for the GnRH receptor. The study on structure-activity relationships in the C-terminal part of GnRH conducted by Fujino et al. (1972) showed

that some analogs with a substituted C-terminus had increased biological activity relative to GnRH demonstrating that the C-terminal glycinamide residue of the GnRH is not essential for high levels of hormonal activity.

b. The peptide bond between residue 9 and 10 does not play an important role in binding affinity (Fujino et al., 1973). Fujino et al. demonstrated that [Des-Gly-NH_2^{10}, Pro-OEt^9]-GnRH analog, which has no peptide bond after the ninth residue, exhibited a relatively high potency compared to that of GnRH.

c. The substitutions with the closest conformational similarity to the N-C-C unit of the replaced glycinamide are most active, for example GnRH-des-Gly^{10}-ethylamide, GnRH-des-Gly^{10}-propylamide, GnRH-des-Gly^{10}-ethanolamide, GnRH-des-Gly^{10}-isopropylamide (Fujino et al., 1973).

d. A negative charge at the C terminus results in poor binding (Sieverton et al., 1971; Fujino et al., 1972). GnRH free acid (GnRH-OH) which has a free carboxyl group at its C terminus, is 325 fold less potent than GnRH (Davidson et al., 1996).

e. Certain alkyl amide substitutions at the C terminus resulted in an increase in activity, -ethylamide being the most potent (Fujino et al., 1972 and 1973). These modifications are shown in table 1.2.

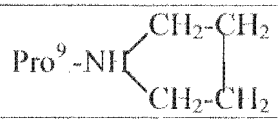
Name of C terminal substitution	*Chemical formula*	*Activity increase relative to GnRH*
GnRH-glycinamide	Pro^9--NH-CH_2-CO-NH_2	1
Pro^9-des-Gly^{10}-GnRH-ethylamide	Pro^9--NH-CH_2-CH_3	5
Pro^9-des-Gly^{10}-GnRH-propylamide	Pro^9--NH-CH_2-CH_2-CH_3	2 – 3
Pro^9-Des-Gly^{10}-GnRH-ethanolamide	Pro^9--NH-CH_2-CH_2-OH	1 – 1.5
Pro^9-des-Gly^{10}-GnRH-isopropylamide	Pro^9--NH-CH-$(CH_3)_2$	1.5
Pro^9-des-Gly^{10}-GnRH-pyrolidineamide		0.7 – 0.8

Table 1.2 Pro^9-des-Gly^{10}-alkyl amide C-terminal substitutions and their effects on GnRH potency.

The introduction of the D-Ala^6 substitution in addition to the ethylamide C-terminal modification had a multiplicative effect resulting in a fifteen fold increase of the potency of the peptide (Coy et al., 1974; Arimura et al., 1974; Karten and Rivier, 1986). Other D-amino acids at position 6 also increased binding affinity, e.g. D-Leu^6-GnRH-des-Gly^{10}-ethylamide had an activity increase 5 times that of GnRH (Vilchez-Maritinez et al., 1974), and D-Phe^6-GnRH-des-Gly^{10}-ethylamide and D-Trp^6-GnRH-des-Gly^{10}-ethylamide were 10 and 13 times more active than GnRH respectively (Coy et al., 1975, (b)).

Substituting fluorine for hydrogen in biologically active molecules can create intense biochemical effects due to the increased electronegativity (Coy et al., 1975 (a)). Experiments with Pro^9-des-Gly^{10}-GnRH-tifluoroethylamide showed an increase in activity when compared to Pro^9-des-Gly^{10}-GnRH-ethylamide (Coy et al., 1975 (a)).

This was attributed to the increased hydrophobicity and the effect of intense electron withdrawing properties on the peptide binding conformation.

The substitution of D-alanine in position 6 combined with the trifluoroethylamide terminus did not result in the anticipated multiplied effect but was of the same magnitude as that of D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide (Coy et al., 1975 (a) and 1976). The conformational constraint imposed by D-Ala^{6} was hypothesized to cause an overriding effect on the electronegativity of the trifluoromethyl group. Pro^{9}-GnRH-des-Gly^{10}-pentafluoropropylamide had a lower activity than Pro^{9}-GnRH-des-Gly^{10}-propylamide. D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide analog had been reported to be considerably less active than D-Ala^{6}-des-Gly^{10}-ethylamide, D-Ala^{6}-GnRH-des-Gly^{10}-trifluoroethylamide and D-Ala^{6}-GnRH-des-Gly^{10}-propylamide analogs (Coy et al., 1975 (a)).

1.2 THE GONADOTROPIN RELEASING HORMONE RECEPTOR

1.2.1 GENERAL FEATURES OF G PROTEIN-COUPLED RECEPTORS.

The GnRH receptor, cloned in 1992 (Tsutsumi et al. 1992), is a member of the superfamily of G protein-coupled receptors (GPCR) and is coupled via the $G_{q/11}$ protein to activation of phospholipase C, leading to calcium signalling and the release of gonadotropins by regulated exocytosis (Stojilkovic et al., 1994, Sealfon et al., 1997).

1.2.1.1 Structural features common to GPCRs:

The single polypeptide chain of GPCRs comprises an extracellular amino terminal domain, seven hydrophobic α-helical transmembrane domains (TM1-TM7) connected by hydrophilic extracellular (EC1, EC2, EC3) and intracellular (IC1, IC2, IC3) loops and an intracellular C terminus. The arrangement of the seven helices is suggested to be clockwise when viewed from the intracellular side and forming a closely packed structure at the intracellular surface where the G-protein interaction occurs (Baldwin, 1993). The extracellular surface is a more open structure forming a ligand-binding pocket. The depth of the helices within the membrane depends on the slope of the axes of helices TM5, TM6, TM7 and TM1. Helices TM2, TM3 and TM4 are approximately perpendicular in relation to the membrane (Baldwin, 1993).

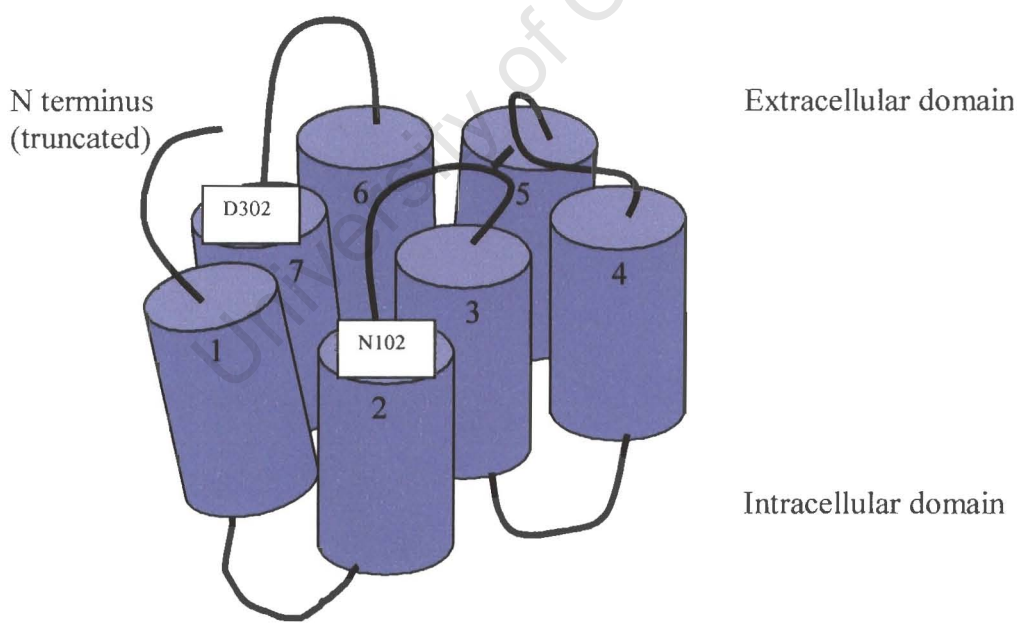


Fig 1.2 Schematic representation of the GnRH receptor: The numbered cylinders represent the transmembrane helices. The curved lines represent the extra- and intra-cellular domains. N102 and D302 are residues which markedly affect ligand binding affinity when mutated (see text).

Residues conserved throughout the GPCR family are thought to play a role in the formation and maintenance of the integral three-dimensional structure of the receptor. Residues conserved within subgroups of the GPCR superfamily are believed to be involved more specifically with ligand binding if situated at or near the extra-cellular surface or the TM segments (Hibert et al., 1991).

Of the conserved residues, most of those believed to be involved in maintaining the structure of the molecule so that it can bind to the G-protein, are situated on the side of the helices facing the hydrophilic pocket (Baldwin, 1993).

Transmembrane helices TM4, TM5, TM6 and TM7 each contain a proline residue that causes a kink in the helix. These residues are thought to act as molecular switches by possibly transferring energy of ligand binding to conformational changes in the intracellular loops implicated in the binding of G-proteins (Baldwin 1993; Millar et al., 1994).

In cationic neurotransmitter receptors, the cationic head group of the ligand interacts electrostatically with a conserved Asp of TM3 which acts as a counterion. In some of these receptors aromatic amino acids in TM3, TM5 and TM6 form π-bonds with the aromatic ring of the ligand (Hibert et al., 1991). The conservation of most of these aromatic amino acids in the GnRH receptor (F219, F275, W279, Y282, Y283, Y322), suggests that the same mechanism could possibly apply. These may form the electron acceptor site in the receptor that reacts with the electron donating side chains of aromatic residues of GnRH as was suggested by Shinizky and Fridkin (1976, (a)). Most

of these residues are positioned close to the putative proline molecular switches (Zhang and Weinstein, 1993).

There is a disulphide bridge between the highly conserved C114 residue in extra-cellular loop 1 and a Cys residue conserved in occurrence but not in position in extra-cellular loop 2 (Baldwin, 1993, Davidson et al., 1997).

Most GPCRs possess one or more consensus sequences (Asn-X-Ser/Thr) for N-glycosylation (Kornfeld and Kornfeld, 1985) in their extracellular domains. A highly conserved D/E-R-Y sequence at the intracellular end of TM3 is implicated in signal tansduction of G-proteins (Baldwin, 1993; Tsutsumi et al., 1992; Arora et al., 1995; Sealfon et al., 1997).

1.2.2 FEATURES SPECIFIC TO GNRH RECEPTORS.

The cloning of mammalian and non-mammalian vertebrate gonadotrope receptors has revealed features characteristic of GnRH receptors, the most striking being the absence of the C-terminal tail unique to mammalian GnRH receptors (Tsutsumi et al., 1992). Non-mammalian receptors have a high homology (70%) with each other, but only 40% homology with mammalian receptors (Millar et al., 1997; King et al., 2000). Both types exhibit high ligand selectivity, but differ in their requirement of ligand conformation indicating that the binding site is different. It has been suggested that mammalian GnRH receptors bind the ligand in the bent conformation whilst non-mammalian receptors bind the extended form of GnRH (Millar et al., 1997).

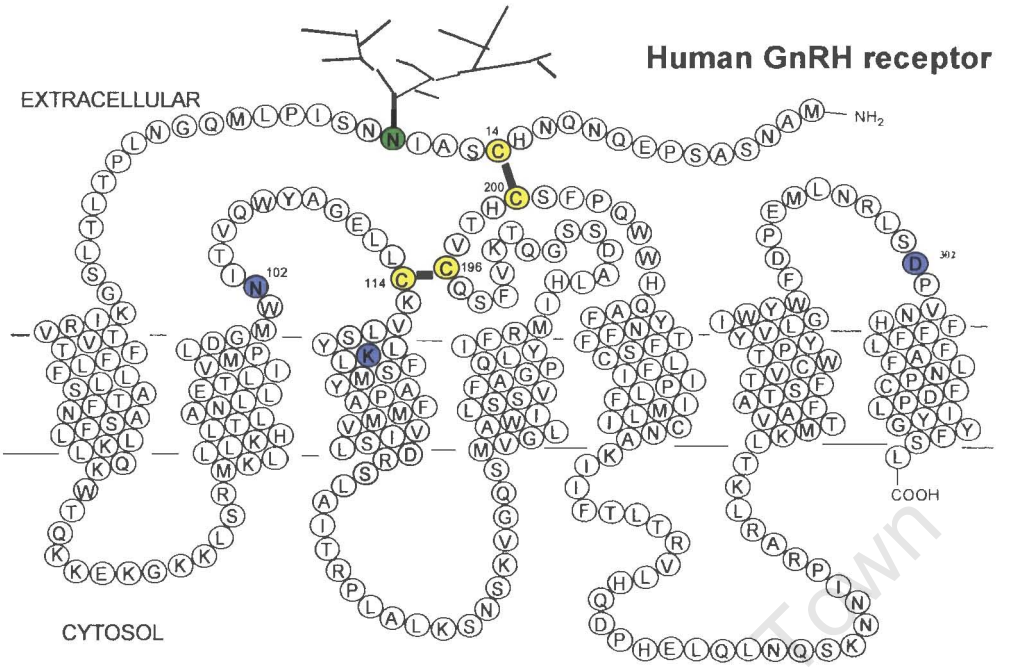


Fig 1.3 Amino acid sequence of the human GnRH receptor. Residues hypothesized to interact with GnRH residues are filled in blue. The branched structure represents glycosylation of N18 (green). Disulphide bridges are shown between cysteine residues (yellow).

A comparison of the sequences of the cloned receptors showed that the EC1 is the most conserved of the three extracellular loops, suggesting its possible role in ligand binding whilst EC3 may be involved in ligand selectivity. The highly conserved residues of the GPCR family (N53, R139, W164, P233, P282 and P320) are also conserved in GnRH receptors.

The mouse GnRH receptor has three consensus N-glycolsylation sites (N4, N18 and N102), while the human receptor has only the latter two sites. Site directed mutagenesis and photoaffinity labelling of the mouse GnRH receptor showed that glycosylation occurs only at N4 and N18 of the amino terminal domain, although N102 is also a potential glycosylation site (Davidson et al., 1995). In the human GnRH receptor only N18 is glycosylated. Mutation of either of the glycosylated sites led to a

decreased expression without a change in binding affinity. Incorporating an additional glycosylation site in the human GnRHR was shown to enhance expression in COS-1 cells (Davidson et al., 1996a)

The first intracellular loop in the mammalian GnRH receptor is longer by comparison to other GPCRs and is highly basic. The highly conserved DRY motif of GPCRs at the intracellular end of TM3 is mutated to DRS in the GnRH receptor, which creates a potential phosphorylation site (Tsutsumi et al. 1992). Residues I143 and the P146 – L147 motif of IC2 as well as A261 of IC3 are conserved and have been shown to be important in G-protein coupling (Arora et al., 1995; Millar et al., 1997; Ballesteros et al. 1998).

In mammalian GnRH receptors the N87 and D318 residues represent a reciprocal mutation of the highly conserved aspartate residue of TM2 and asparagine residue of TM7 of GPCRs. An interaction between these two residues, believed to have been inverted by a natural reciprocal mutation, has been suggested to contribute to the structural organisation of the GnRH receptor by maintaining TM2 and TM7 in close proximity (Zhou et al., 1994). Although D87 is present in non-mammalian GnRH receptors, the mutation of N318 to D318 has been found in frog, catfish, goldfish and chicken GnRH receptors. Zhou et al. (1994) demonstrated that the mammalian N87D mutant receptor showed a loss of ligand binding which was attributed to an abnormal conformation due to the charge repulsion between D87 and D318. It was suggested that protonation of one of the Asp side chains to restore hydrogen bonding or some other change in non-mammal receptors must occur to compensate for the D318 mutation (Sealfon et al., 1995, Blomenröhr et al., 1997).

In a study using photoaffinity labelling of the GnRH receptor, followed by protease digestion and the reduction of disulphide bonds, the presence of the –S-S- bond conserved throughout the GPCR family was directly confirmed in human and mouse GnRH receptors. The residues involved are C114 and C196 of EC1 and EC2 respectively (Davidson et al., 1997). Equivalent residues to C114 and C196 are conserved in non-mammalian GnRH receptors. This bond contributes to the positioning of TM3 and TM4. The same study revealed the presence of a second disulphide bond between C14 of the N-terminal domain and C200 of EC2 in the human GnRH receptor (Davidson et al. 1997).

The most striking and unique feature of mammalian GnRH receptors, is the absence of the C-terminal tail (Tsutsumi et al., 1992). Non-mammalian vertebrate receptors conform more closely to typical GPCRs in that they do have a C-terminal tail. The lack of a C-terminal tail is likely to be the reason for the absence of desensitisation of the mammalian receptor (Davidson et al., 1994). The chicken GnRH receptor (which has a tail) was shown to internalize 15-fold faster than the human receptor (Pawson et al., 1998). Truncation of the tail of the chicken GnRH receptor resulted in a markedly decreased internalization rate similar to that of the human receptor (Pawson et al., 1998). The addition of the C-terminal tail from the thyrotropin-releasing hormone receptor to the rat GnRH receptor resulted in the rapid desensitisation of IP production and significantly increased the internalization rate (Heding et al., 1998). Adding the C-terminal tail of the catfish GnRH receptor resulted in an increase in receptor expression, and an enhancement of receptor desensitisation (Lin et al., 1998).

A possible physiological role of the slow internalisation and lack of rapid desensitisation of the mammalian GnRH receptor has been proposed (Pawson et al., 1998). It is thought that it might be necessary to keep a large number of active receptors at the gonadotrope cell surface, particularly during the GnRH surge that triggers the preovulatory luteinizing hormone surge (Pawson et al., 1998; Heding et al., 1998). Desensitisation would limit the amplitude of the LH surge, and the loss of the C-terminal tail may have been advantageous on this basis.

1.2.3 SIGNAL TRANSDUCTION BY THE GnRH RECEPTOR.

The binding of GnRH to its receptor induces a conformational change, which in turn results in the GDP bound to the trimeric Gq protein to be exchanged for a GTP. GTP-bound Gq protein activates phosphoinositide specific phospholipase C (Berridge 1987 and 1993). Phospholipase C, in the presence of resting cellular Ca^{2+} level, cleaves phosphatidylinositol bisphosphate (PIP_2), which is situated mainly on the inner half of the phospholipid bilayer (Irvine et al., 1984; Naor et al., 1986). Inositol-1,4,5-trisphospate (Ins-1,4,5P3) and Diacylglycerol (DAG) are the products of PIP_2 cleavage (Fig. 1.4). Ins-1,4,5P3 diffuses through the cytosol and binds to a gated Ca^{2+} channel in the endoplasmic reticulum (ER) (Fig. 1.5), causing the release of intracellular Ca^{2+} (Berrige, 1993; Naor et al., 1988). The rapid formation of Ins-1,4,5P3 which causes the release of intracellular Ca^{2+} is responsible for a burst phase of LH release lasting approximately 100 seconds (Naor et al., 1988; Davidson et al., 1988).

DAG together with the increased Ca^{2+} concentration activates protein kinase C (Fig. 1.5) which is redistributed to the cell membrane (Hirota et al., 1985). Simultaneously, Ca^{2+} influx via nifedipine sensitive and insensitive channels further increases the Ca^{2+}

concentration (Davidson et al., 1988). This Ca^{2+} influx together with activated protein kinase C is thought to mediate the sustained phase of gonadotropin secretion by activating the exocytotic mechanism (Van der Merwe et al., 1989; Van der Merwe et al., 1990).

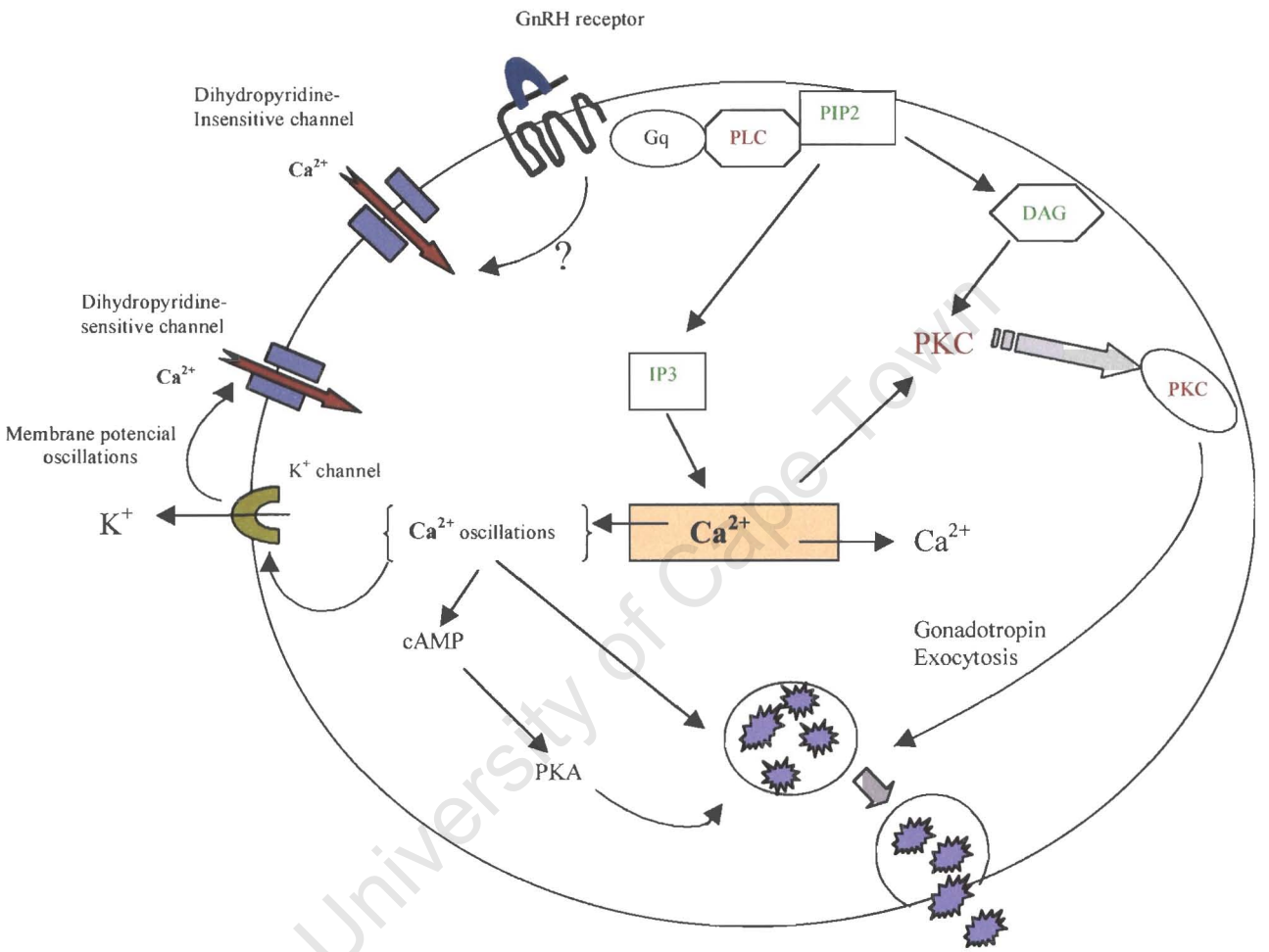


Fig 1.5 Schematic representation of the principal elements involved in signal transduction in the gonadotrope cell: Gq= Gq protein; PLC= phospholipase C; DAG= Diacyl glycerol; PKC= Protein Kinase C; IP3= Ins-1,4,5P3.

1.2.3.1 Inositol-1,4,5P3 metabolism:

The metabolism of Ins-1,4,5P3 takes place via two separate pathways. It can either be dephosphorylated to inositol or enter the Tetrakis pathway (Fig. 1.4).

1.2.3.1.1 Dephosphorylation of Ins-1,4,5P3 to inositol:

Ins-1,4,5P3 phosphatase dephosphorylates Ins-1,4,5P3 at position 5 to give Ins-1,4P2. This enzyme therefore is of importance in terminating the Ins-1,4,5P3 messenger action since Ins-1,4P2 is incapable of releasing Ca^{2+}. Studies on the disappearance of labelled PIP_2 revealed that the appearance of Ins-1,4P2 is from the dephosphorylation of Ins-1,4,5P3 rather than from that of PIP_2 (Naor et al., 1986). Inositol bisphosphatase hydrolyses Ins-1,4P2 to Ins-1P and Ins-4P. These in turn are dephosphorylated by inositol monophosphatase to myo-inositol (Berrige, 1987). Myo-inositol together with phosphatidic acid product from DAG metabolism can be reconverted to PIP_2 (Fig 2.2).

Lithium is an effective inhibitor (Ki value of approximately 1mM) of the inositol monophophatase that cleaves Ins-1P to Inositol (Martin T.F.J., 1986; Berridge M.J. 1987). Treatment of cells with LiCl causes the accumulation of labelled Ins-P in response to agonist stimulation of ^{3}H-myoinositol labelled cells, which facilitates measurement of receptor activation.

1.2.3.1.2 The inositol tetrakisphosphate pathway:

Ins-1,4,5P3 is further phosphorylated to Ins-1,3,4,5-tetrakisphosphate by Ins-1,4,5P3-3kinase. Ins-1,3,4,5-tetrakisphosphate may perform specific messenger functions within the cell, and is then dephosphorylated to Ins-1,3,4P3. Snyder et al. (1988) found that injection of Ins-1,3,4,5-tetrakisphosphate into *Xenopus laevis* oocytes produced an oscillatory release

of intracellular Ca^{2+}. Although this indicates a possible physiological role for this compound, it was found not to be sufficient or required for Ca^{2+} influx.

The isomer Ins-1,3,4P3 has no Ca^{2+} releasing activity but, in some systems, reaches greater concentrations than Ins-1,4,5P3 and remains elevated much longer, suggesting a possible role in the late response to cell stimulation (Burgess et al., 1985; Irvine et al., 1985). Ins-1,3,4P3 is eventually dephosphorylated to Ins-3P and Ins-4P and then to myo-inositol, which is recycled to PIP_2 (Fig 1.4).

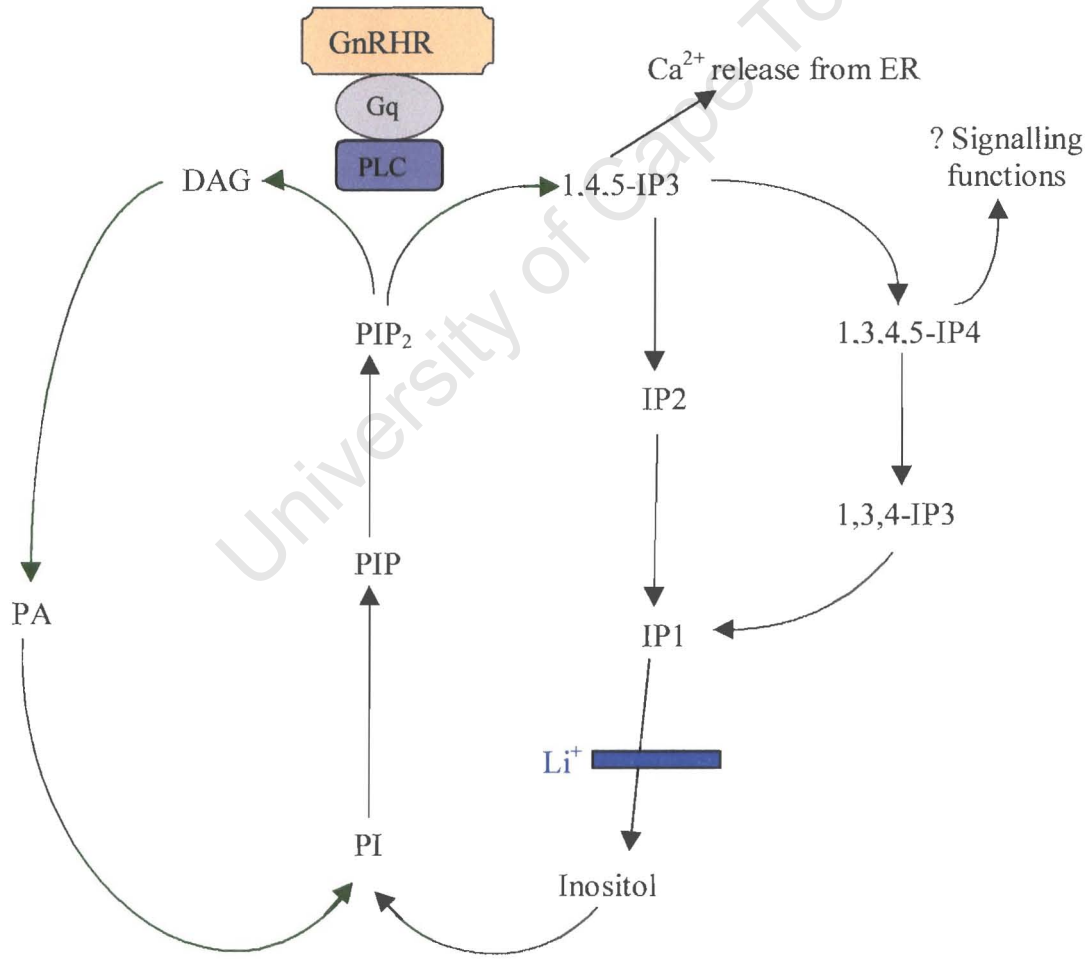


Fig 1.4 Illustration of the main receptor-mediated pathways of inositol phosphate metabolism. The site of Li^+ inhibition of the dephosphorylation of Ins-1P to Inositol is indicated.

1.2.3.2 Diacylglycerol (DAG) as second messenger:

After the hydrolysis of PIP_2, the transiently produced DAG remains closely associated with the cell membrane. Together with the Ca^{2+} released by Ins-1,4,5P3, it functions as a second messenger by activating Protein Kinase C. The activated Protein Kinase C phosphorylates proteins thought to contribute to the final exocytotic response (Van der Merwe et al., 1990; Berrige, 1993).

The metabolism of DAG may follow two separate pathways:

(a) Phosphorylation of DAG by DAG kinase to phosphatidic acid (PA), which eventually is recycled with myo-inositol, from the metabolism of Ins-1, 4,5P3, to form PIP_2.

(b) Hydrolysis of DAG by phospholipase A_2 (PLA_2) to arachidonic acid (Naor and Catt. 1981). Arachidonic acid is converted via the lypoxygenase and epoxygenase pathways to metabolites which may participate in the exocytotic response (Hulting et al., 1985; Snyder et al., 1983). Inhibitors of Phospholipase A_2 were shown to prevent the formation of arachidonic acid and to inhibit LH release. Although these results were interpreted as evidence that arachidonic acid was involved in regulating exocytosis, the possibility that the PLA_2 inhibitors inhibited exocytosis directly was not disproved. Arachidonic acid–induced LH release was later shown to be non-specific in nature, as its action did not require ATP and could not be blocked by N-ethyl-maleimide (Kaye et al., 1992). For these reasons doubt exists regarding the role of arachidonic acid in LH exocytosis.

1.2.3.3 Calcium signalling:

Extensive investigations have demonstrated a complex mechanism of Ca^{2+} signalling in the secretion of gonadotropins. Experiments with Ca^{2+} channel blockers and Ca^{2+} depletion showed that three sources of Ca^{2+} mobilisation contribute to the secretory response to GnRH stimulation (Davidson et al., 1988). LH release occurs in a spike and plateau pattern. The spike phase of LH secretion, lasting a few minutes, is supported partly by the intracellular Ca^{2+} release from the endoplasmic reticulum while Ca^{2+} influx via nifedipine-sensitive voltage channels contributes to the plateau phase of LH release (Davidson et al., 1988). Extracellular Ca^{2+} influx via nifedipine-insensitive Ca^{2+} channels is involved in both the spike and the plateau phases (Davidson et al., 1988).

1.2.4 SITE DIRECTED MUTAGENESIS EVIDENCE FOR RESIDUES INVOLVED IN BINDING OF GnRH.

Current evidence indicates that the binding sites of GPCRs for peptide ligands involve the extracellular domains as well as the interhelical pocket of the receptors. Mutational analysis has provided some insights into the location of the ligand-binding pocket of the GnRH receptor.

1.2.4.1 Ligand interaction with GnRH receptor residue K121:

The GnRH receptor counterpart of the conserved "counterion" D113 in TM3 of the cationic neurotransmitter receptor is K121. The possibility of a H bond formation, at physiological pH, between this residue and His^2 of the ligand was investigated by Zhou et al. (1995). The mutation of K121 resulted in a loss of agonist affinity whilst affinity

for antagonists was retained. This strongly suggests that one of the N-terminal residues of GnRH, which are required for agonist activity, interacts with K121 (Zhou et al., 1995).

1.2.4.2 Electrostatic interaction between GnRH receptor residue E301 and Arg^8 of the ligand:

Gln^8 Chicken GnRH I was found to be less potent than Arg^8 mammalian GnRH to stimulate the mouse GnRH receptor. It was postulated by Hazum (1987) that cationic Arg^8 of GnRH interacts directly with either a negatively charged receptor residue (Asp or Glu) or a Sialic Acid residue of the carbohydrate moiety of the extra-cellular N-terminal domain (Keinan and Hazum, 1985; Hazum, 1987). The carbohydrate moieties were shown not to be involved in binding by mutation of the Asn residues situated in consensus sequences for N-linked glycosylation (Davidson et al., 1995). This led to the investigation of the role of eight conserved acidic (Glu or Asp) residues of the mouse GnRH receptor. Mutating these residues to isosteric Asn or Gln resulted in the finding that E301 (D302 in human receptor) confers specificity for Arg^8 possibly as the result of an electrostatic interaction between the two residues (Flanagan et al., 1994).

1.2.4.3 A role for N102 receptor residue in ligand binding:

A role for N102 in ligand binding was revealed fortuitously as a result of the investigation of the roles of consensus sequences for N-glycosylation (N4, N8 and N102). Although present in a consensus N-glycosylation sequence, N102 is not glycosylated (Davidson et al., 1995). GnRH was found to have an increased potency for

the N102Q mutant receptor that was due to increased binding affinity (Davidson et al., 1995).

To investigate this effect, N102 was mutated to Ala, which resulted in a large decrease in potency for GnRH, and for analogs with glycinamide C-termini (Davidson et al., 1996(b)). In contrast, analogs with ethylamide C-termini were much less dependent on N102 for affinity. This indicated that although N102 is not glycosylated, this residue is a critical determinant for binding of GnRH and analogs containing glycinamide at the C-terminus, possibly playing a direct role in the docking of the glycinamide C-terminus (Davidson et al., 1996).

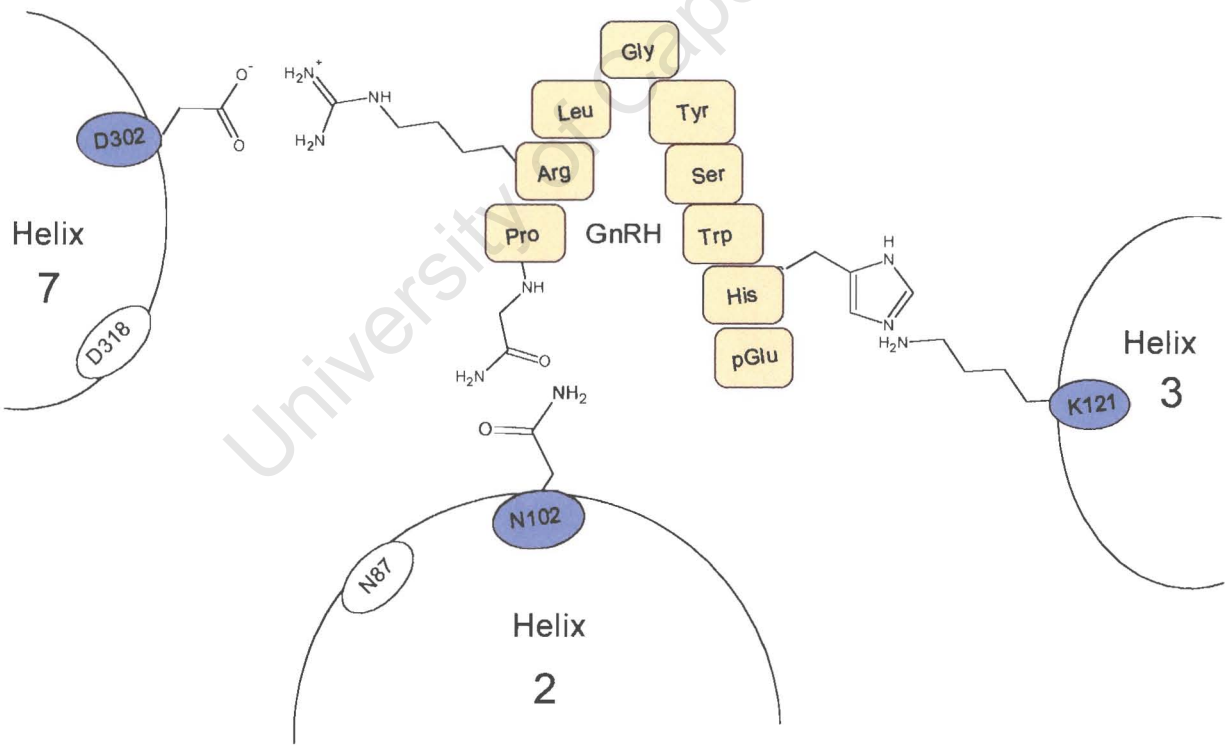


Fig 1.6 Schematic representation of the GnRH binding pocket in the GnRH receptor. Receptor residues hypothesized to be involved in the binding of GnRH are indicated in blue. N87 and D318 are postulated to interact (Zhou et al., 1994).

CHAPTER TWO

MATERIALS AND METHODS

2.1 GnRH ANALOGS.

Antagonist 26 (Batch DC12-119) (Table 2.2) and six agonist GnRH analogs (Table 2.1) with the C-termini modified were synthesised by Dr David Coy, Tulane University School of Medicine, New Orleans, Louisiana, USA, using conventional solid-phase methodology. In addition to the C-terminal changes, five of the six analogs also have the glycine residue at position 6 substituted by D-Alanine. D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide (Batch 7206) was obtained from Peninsula Laboratories, G11 Tailor Way, Belmont CA94002, USA. GnRH-glycinamide (mammalian GnRH) (Table 2.1), D-Ala^{6}-GnRH-glycinamide and GnRH-A (Table 2.2), were synthesised by Mr. R.C. deL. Milton, MRC, Regulatory Peptides Research Unit, University of Cape Town, Cape Town, South Africa.

Name of Peptide analog	*Carboxy terminal sequence*	*Source*
GnRH-glycinamide	-Pro^{9}-NH-CH_2-CO-NH_2	Mr R.C. deL. Milton
GnRH-des-Gly^{10}-propargylamide	-Pro^{9}-NH-CH_2-C≡CH	Dr David Coy
GnRH-des-Gly^{10}-dimethylamide	-Pro^{9}-N(CH_3)$_2$	Dr David Coy
D-Ala^{6}-GnRH-glycinamide	-Pro^{9}-NH-CH_2-CO-NH_2	Mr R.C. deL. Milton
D-Ala^{6}-GnRH-des-Gly^{10}-hydrazide	-Pro^{9}-NH-NH_2	Dr David Coy
D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide	-Pro^{9}-NH-CH_2-CH_3	Peninsula Laboratories
D-Ala^{6}-GnRH-des-Gly^{10}-propylamide	-Pro^{9}-NH-CH_2-CH_2-CH_3	Dr David Coy
D-Ala^{6}-GnRH-des-Gly^{10}-trifluroethylamide	-Pro^{9}-NH-CH_2-CF_3	Dr David Coy
D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide	-Pro^{9}-NH-CH_2-CF_2-CF_3	Dr David Coy

Table 2.1 List of GnRH agonist analogs and their carboxy terminal sequence used in the present study in IP production experiments: In addition to the carboxy terminal modification five of the analogs also have the glycine at position 6 substituted with D-Alanine.

Agonist analogs		
Name of Peptide analog	*Peptide modifications*	*Source*
GnRH-A	[D-Ala^{6}, *N*-methyl-Leu^{7}, Pro^{9}-ethylamide]-GnRH	Mr R.C. deL. Milton
D-Ala^{6}-GnRH	[D-Ala^{6}]-GnRH	Mr R.C. deL. Milton
Antagonist analog		
Name of Peptide analog	*Peptide modifications*	*Source*
Antagonist 26	[Ac-D-*p*-ClPhe^{1}, Ac-D-*p*-ClPhe^{2}, D-Trp^{3}, D-lys^{6}, D-Ala^{10}-NH2]-GnRH	Dr David Coy

Table 2.2 GnRH analogs used for binding experiments

2.2 MUTANT RECEPTORS.

NOMENCLATURE: Single-letter amino acid abbreviations are used for receptor mutations as in the following example: "N102A" refers to mutation of Asn^{102} to Ala.

The five mutant receptors N102A-GnRHR, N102D-GnRHR, N102K-GnRHR, S4NP7L-GnRHR and S4NP7L + N102A-GnRHR used for this work, were derived from the human GnRH receptor, by site directed mutagenesis by JS Davidson and A Pawson of the MRC Regulatory Peptides Unit, Department of Chemical Pathology, University of Cape Town, as described (Davidson et al., 1996 (a) and 1996 (b)).

Receptors N102D and N102K contain additional ECORV and NspI restriction sites respectively. The coding sequences of each of the receptors are inserted in the mammalian expression vector pcDNA/AMP (Invitrogen Corporation, 1600 Faraday Ave., Carlsbad, California, CA92008, USA).

2.3 PLASMID DNA PREPARATION.

Glycerol stock cultures of bacteria, containing the relevant plasmids, were plated on Ampicillin agar plates and incubated overnight at 37°C. Single colonies were inoculated into 10ml of sterile 2YT medium to which Ampicillin (50µg/ml) was added. The bacteria were allowed to grow for 12 hours in a rotating incubator (200 rpm), at 37°C. 0.5ml of the culture was then inoculated into 500ml 2YT sterile medium containing Ampicillin and incubated a further 13-14

hours under the same conditions. The bacteria were pelleted in a centrifuge at 6000-x g in a Beckman J-6B centrifuge for 20 minutes.

The plasmid DNA was purified, using the Wizard™ Maxipreps DNA purification system (PROMEGA Corporation, 2800 Wood Hollow Rd., Madison, Wisconsin, USA), according to the manufacturer's instructions. The purified DNA was eluted from the columns with pre-heated (65-70°C) sterile distilled H_2O. The DNA concentration was determined by measuring absorbance at 260nm. The quality of the DNA was monitored by measuring the A260/280 ratio and by visualising the DNA on 1% agarose/ethidium bromide gels (see Fig 2.3).

2.4 RESTRICTION ENZYME DIGESTION.

To confirm that inserts were of the correct size, plasmid DNA was incubated with appropriate restriction enzymes for 1-2 hours at the recommended temperature. The digest was stained with loading buffer (Section 2.10) and analysed by electrophoresis on a 1% Agarose gel containing ethidium bromide at 1µg/ml final concentration. The DNA fragments were visualised under UV light (see Fig 2.4).

2.5 CELL CULTURE.

COS-1 cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% heat inactivated foetal calf serum (FCS) (Highveld, Johannesburg, South Africa) in 10% CO_2 atmosphere at 37°C.

2.5.1 PREPARATION OF CELL CULTURE PLATES FOR ASSAYS.

2.5.1.1 Poly-D Lysine coating:

12-well plates were coated by adding 250µl of sterile aqueous solution of 120µg/L poly-D Lysine (Sigma) per well. The solution was then aspirated and the plates washed with 1ml of sterile double distilled water and allowed to dry under sterile conditions.

2.5.1.2 Seeding of plates:

COS-1 cells were lifted from culture flasks with 5ml Trypsin (BDH). Cells were pelleted at 1600rpm at room temperature, resuspended in 10ml DMEM/10%FCS containing a mixture of 0,15mg/ml Penicillin/0,05mg/ml Streptomycin (P/S) and counted in a haemocytometer. Cells were seeded in 12 well plates at a concentration of $2x10^5$ cells/well in DMEM/10%FCS/P/S medium, incubated overnight at 37°C under 10% CO_2.

2.6 TRANSFECTION OF CELLS.

2.6.1 DEAE-DEXTRAN METHOD (IP ASSAY).

Plasmid DNA, 2,5μg/well, was prepared in DMEM-Low Glucose medium solution containing 1:10 dilution HBS/DEAE dextran (Section 2.10). After washing twice with serum free DMEM containing 10mM HEPES, pH 7.4 (HEPES/DMEM), the cells were incubated with the DNA preparation, for four hours at 37°C in CO^2. The DNA solution was then replaced with a 100μM Chloroquin solution in DMEM-Low Glucose medium with 2%FCS and P/S, and incubated for a further one hour. The cells were washed in HEPES/DMEM medium. 0.5ml/well 10% Dimethyl Sulfoxide (DMSO) was added and incubated for exactly 2 minutes. The DMSO was removed and the cells were washed with HEPES/DMEM medium and cultured overnight in DMEM-Low-Glucose medium containing 10%FCS and P/S, under CO_2.

2.6.2 LIPOFECTIN METHOD (BINDING ASSAY).

The cells were washed with serum free HEPES/DMEM. A solution of 1.0 μg of plasmid DNA and 1.5 μl of Lipofectin (Gibco) in 0.5 ml of serum-free OPTI-MEM (Gibco) medium was added to each well. The cells were incubated for six hours at 37°C in CO_2. 0.5ml DMEM containing 20% FCS and P/S was then added to each well and the plates were incubated overnight under the same conditions.

2.7 INOSITOL PHOSPHATE PRODUCTION.

Twenty-four hours after transfection, cells were labelled overnight with 2μCi/ml myo-[2-^{3}H]inositol (Amersham) in 0,25ml/well Medium 199 (Gibco) containing 5% FCS. They then were washed twice with Buffer I (section 2.10), and stimulated with the various GnRH analogs, in Buffer I containing 10mM $LiCl_2$ for 60 minutes at 37°C. The stimulations were stopped by aspirating the GnRH-containing buffer and adding 0,5ml PCA stopping solution at 4°C and 0,05ml of 2.7mM Phytic Acid solution to each well.

After 5 minutes, the solution was transferred from each well to a test tube, and then neutralised with 0.5M KOH. After standing for 30 minutes at 4°C to allow precipitation of potassium perchlorate, the samples were chromatographed on Dowex-50W columns (1ml resin) as previously described (Davidson et al., 1990). After washing the columns with 10ml distilled water and 5ml of 5mM Myo-inositol/0.1M formic acid, the inositol phosphates were eluted with 3ml 1M Ammonium formate/0.1M formic acid. The eluent was mixed with 14 ml of scintillation fluid (Zinsser Analytic) and radioactivity counted in a Packard Scintillation Counter.

2.8 MEASUREMENT OF RADIOLIGAND BINDING.

The radioligand binding of the three mutant receptors N102A, N102D and N102K was investigated with homologous displacement of 3 iodinated ligands: agonist GnRH-A, agonist D-Ala^{6}-GnRH and Antagonist 26 (Table 2.2). Mr D Myburgh of the MRC Regulatory Peptides

Unit, Department of Chemical Pathology, University of Cape Town, performed the iodination of the radioligands.

COS-1 cells transfected 24 hours previously with relevant GnRH receptor plasmid DNA, as described above, were washed with 1ml/well buffer B (Section 2.10). Buffer B containing 10^5cpm/well radioligand and varying concentrations of the appropriate unlabelled peptides were added to the cells, and incubated on ice for 3 hours for the agonist radioligand and overnight for antagonist 26. Non-specific binding was measured in parallel using untransfected COS-1 cells. After incubation, the cells were washed rapidly three times with ice cold buffer B (Section 2.10). The cells were then solubilized in 0.5ml of 0.1M NaOH and the radioactivity was counted in a γ counter (RIAstar, Hewlett Packard).

2.9 DATA REDUCTION.

Peptide concentrations required to stimulate half-maximal IP production (EC_{50}) and half-maximally inhibit binding of radioligand (IC_{50}) were derived, using Graphpad Prism (Graphpad Software Incorporated).

Non-linear regression to the following equations were used:

a) Inositol Phosphate: $IP = IP_{max}/(1+EC_{50}/A)$, where IP is the inositol phosphate response to concentration A of agonist, and IP_{max} is the maximal response.

b) Binding: 1. One site competition

$Y = a + (b - a)/[1+10^{(X - Log\ IC_{50})}]$ where Y = Bound c.p.m.; X = log concentration of competing ligand; a = minimum c.p.m. bound and b = maximum c.p.m. bound.

2. Two sites competition

Part 1 = (b-a) x Fraction 1/(1+10^(X-Log IC_{50}_1)

Part 2 = (b-a) x (1-Fraction 1)/(1+10^(X-Log IC_{50}_2))

Y = a + Part1 + Part2

where Y = Bound c.p.m.; X = log concentration of competing ligand; a = minimum c.p.m. bound and b = maximum c.p.m. bound.; Fraction 1 is the fraction of all sites that have affinity 1; Log IC_{50}_1 and Log IC_{50}_2 are the Log IC_{50} values for the two sites.

2.10 BUFFERS AND SOLUTIONS.

Loading buffer:

0.25% Bromphenol blue; 0.25% Xylene cyanol; 30% Glycerol in distilled H_2O. Filter sterilise and aliquot.

HBS/DEAE dextran:

3mg DEAE dextran per ml HBS buffer (137mM NaCl, 5mM KCl, 0.7mM NaH_2PO_4, 21mM Hepes), pH7.1.

Buffer I:

140mM NaCl, 4mM KCl, 20mM Hepes, 0.1% Bovine albumin (fatty acid free, Pentex fraction V, Miles Laboratories), 8.3mM Glucose, 1mM $CaCl_2$, 1mM $MgCl_2$, 6mg/L Phenol red, pH 7.4.

Buffer B:

140mM NaCL, 4mM KCl, 20mM Hepes 0.5% Bovine albumin (fatty acid free, Pentex fraction V, Miles Laboratories), 8.3mM Glucose, 1mM $CaCl_2$, 1mM $MgCl_2$, 6mg/L Phenol red, pH 7.4.

PCA-stopping solution:

5mM EDTA (Ethylenediamine-tetra Acetic Acid, disodium salt), 1mM DTPA (Diethyltriamine Penta Acetic Acid), 5% (v/v) PCA (Perchloric Acid). Stored at 4°C.

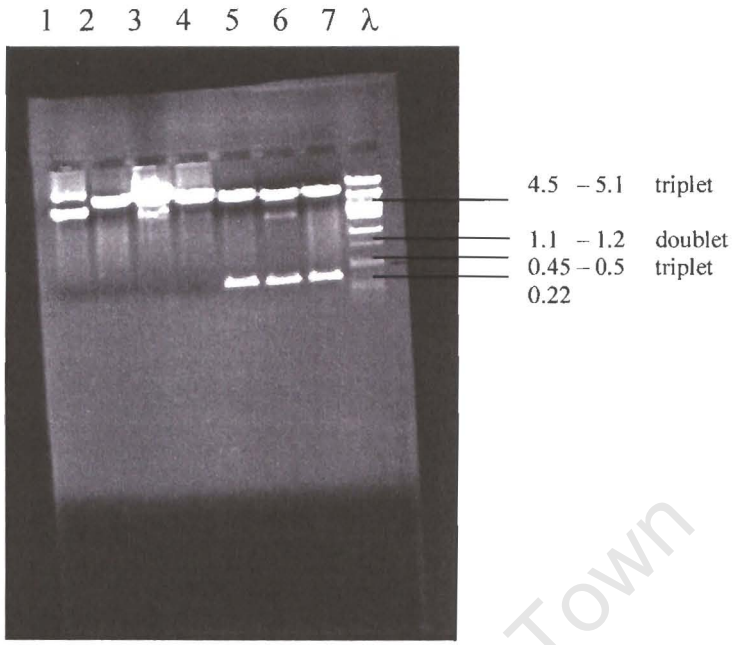


Fig 2.3 Example of electrophoresis, on a 1% agarose gel, of plasmid DNA preparations digested with restriction enzymes.

Lane legend: 1 = hGnRHR uncut (2 bands expected: supercoiled and nicked circular DNA)
2 – 4 = hGnRHR cut with ECORI and ECORV (1 band expected: 6.0Kb and a small piece of 0.012Kb too small to be visualised)
5 – 7 = N102D preparations cut with ECORI and ECORV (2 bands expected: 0.30Kb and 5.7Kb)
λ = control: λ DNA cut with PstI.

Kb = Kilobases.

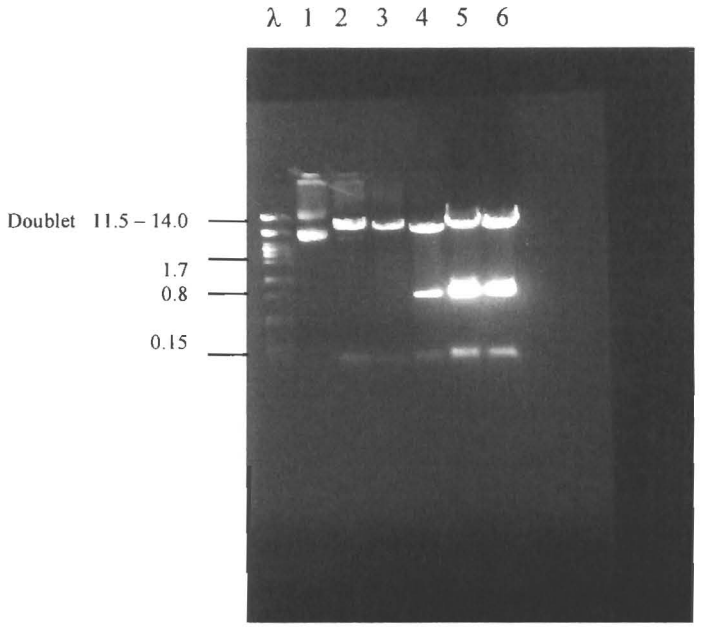


Fig 2.4 Example of a restriction enzyme digest of plasmid DNA confirming insert size

Lane legend: λ = control: λ DNA cut with PstI
1 = control: hGnRHR uncut (2 bands expected: supercoiled and nicked circular DNA)
2 = control: hGnRGR cut with NspI (2 bands expected: 0,15Kb and 5,8Kb)
3 = unknown DNA
4 – 6 = N102K cut with NspI (3 bands expected: 0,15Kb, 0,80Kb and 5.0Kb

Kb = Kilobases

CHAPTER THREE

RESULTS

In order to investigate the interaction of N102 side chain of the GnRH receptor with the GnRH glycinamide C terminus, the effects of receptor mutations N102A, N102D and N102K on ligand binding and IP production when stimulated with GnRH-glycinamide, were first investigated. Secondly, the effect of these mutations on the potency of seven GnRH agonist analogs with modified C termini was determined.

3.1 RADIOLIGAND BINDING OF GnRH RECEPTOR MUTANTS.

The effect of the mutations N102A, N102D and N102K on ligand binding was determined in four different binding experiments, using homologous displacement of the radioligands

a) Agonist GnRH-A: [D-Ala^6, *N*-methyl-Leu^7, Pro^9-ethylamide]-GnRH,

b) Agonist D-Ala^6-GnRH: [D-Ala^6]-GnRH and

c) Antagonist 26: [Ac-D-*p*-ClPhe^1, Ac-D-*p*-ClPhe^2, D-Trp^3, D-Lys^6, D-Ala^{10}-NH_2]-GnRH.

Maximal binding is affected by both receptor expression and binding affinity. Because binding was measured in intact whole cells, the values obtained represent those for receptors only expressed on the cell surface.

3.1.1 BINDING OF [^{125}I]-D-Ala^6-GnRH TO GnRH RECEPTOR MUTANTS N102A, N102D AND N102K.

The ability of the three mutant receptors N102A, N102D and N102K to bind agonist D-Ala^6-GnRH radioligand was determined in an experiment shown in Fig 3.1.1. The N102D and N102K receptors showed no detectable binding, while the N102A mutant showed only 10.1% of wild-type receptor binding. The absence of binding of mutant receptors N102D and N102K, in conjunction with the absence of the IP response (section 3.2.2), led to the conclusion that these two receptors are either unable to bind ligand, or are not expressed on the cell surface.

However, although only 10.1% of wild-type binding was obtained for the N102A mutant receptor, its mean maximal IP response, stimulated by GnRH-glycinamide, was 67.8% ±14.0 (SEM, n = 3 experiments) of the wild type receptor, indicating that this mutant receptor is active and expressed. Further binding experiments were undertaken to determine the binding affinity of the N102A receptor.

3.1.2 BINDING OF ANTAGONIST 26 RADIOLIGAND.

Binding of [^{125}I]Antagonist 26 to cells transfected with wild-type hGnRHR with homologous displacement showed a biphasic displacement, indicative of the presence of two binding sites with different affinities. The mean IC_{50}, for the two sites, obtained in two independent experiments, performed in triplicate, with hGnRHR were 1.42nM ±0.39 (SEM) and 304nM ± 39.6 (SEM) (Figs 3.1.2 and 3.1.3).

Homologous displacement of Antagonist 26 in untransfected cells showed displacement at high peptide concentration with an IC_{50} of 245nM (Fig. 3.1.2). This result was similar to that obtained at the second site of the hGnRHR receptor-transfected cells indicating that the low affinity binding is non-specific and not receptor related (Fig 3.1.2).

The mutant receptor N102A exhibited decreased binding of the Antagonist 26 radioligand. The mean % binding obtained in two individual experiments (Fig 3.1.3 and 3.1.6) was 26.9% ±7.0 (SEM) of the wild-type receptor. The binding affinity (IC_{50}) measured was 147nM for the mutant receptor. This corresponds to the low affinity binding site of the cells, which was demonstrated above to be non-specific binding. The binding affinity of the N102A receptor was therefore decreased to such an extent that it could not be measured (Fig 3.1.3).

3.1.3 BINDING OF GnRH-A RADIOLIGAND.

[^{125}I]GnRH-A bound to the wild-type hGnRHR with mean IC_{50} of 1.36nM ±0.34 SEM. in two independent experiments (Figs 3.1.4 and 3.1.5). Binding of this radioligand to the N102A mutant receptor was not measurable (Fig 3.1.5).

3.1.4 BINDING OF MUTANT RECEPTORS WITH EXTRA GLYCOSYLATION SITE.

In an attempt to improve the measurement of binding of the N102A receptor, an extra glycosylation site was used. The double mutation S4NP7L includes an extra glycosylated site which enhances receptor expression (Davidson et al., 1996 (a)). In addition to this mutation, a triple mutant receptor also contains the N102A mutation.

The binding (Bo) results obtained with both agonist and antagonist radioligands (Table 3.1) showed that the binding of the double mutant S4NP7L receptor was enhanced by about two fold. These results are in keeping with results previously reported by Davidson et al. (1996 (b)). Although the extra glycosylation site enhanced binding of the N102A mutant somewhat, this was not sufficient to permit accurate measurement of receptor affinity using radioligand binding methodology, due to the high non-specific binding (Fig. 3.1.6). For this reason, no further binding experiments were performed.

Agonist GnRH-A radioligand				
	Wt hGnRHR	N102A	S4NP7L mutant	S4NP7L+N102A mutant
Bo (% of wt)	100%	3.6%	267%	6.3%
Antagonist 26 radioligand				
Bo (% of wt)	100%	17.1%	172%	37.1%

Table 3.1 Effect of S4NP7L mutation on receptor binding. S4NP7L mutation codes for an additional glycosylated site that enhances receptor expression. This data is presented in Fig 3.1.6.

3.2 INOSITOL PHOSPHATE RESPONSE OF GnRH RECEPTOR MUTANTS.

3.2.1 CONTROL EXPERIMENTS TO TEST VARIOUS COMPONENTS OF THE IP EXPERIMENTAL PROTOCOL.

3.2.1.1 Untransfected cells:

In order to confirm that the IP response obtained in COS-1 cells transfected with receptor DNA was due solely to the stimulation of expressed receptors, the following experiment was performed. Duplicate wells of COS-1 cells transfected with hGnRHR as well as an equivalent set of wells of untransfected cells were stimulated with D-Ala^6-GnRH-ethylamide. The mean basal reading obtained for cells transfected with hGnRHR was 816 cpm. compared to a mean basal reading of 102 cpm. for the untransfected cells (Fig

3.2.1). The results show that no IP were produced in the untransfected cells. This confirms that only cells which have the GnRH receptors expressed at their surface are able to produce inositol phosphates.

3.2.1.2 Transfection with vector only:

This experiment was to confirm that the pc-DNA/AMP vector DNA has no effect on IP production. Two equivalent sets of duplicate wells containing COS-1 cells, one transfected with hGnRHR receptor and the other with pc-DNA/AMP vector DNA without insert were stimulated with GnRH-glycinamide and D-Ala^{6}-GnRH-glycinamide at the following dilutions: Basal, -9M and -6M.

No IP stimulation was obtained in the cells transfected with pc-DNA/AMP vector DNA only (Table 3.2 and Fig 3.2.2). The experiment confirmed that the vector DNA had no effect on the IP response.

Stimulation with GnRH-glycinamide				
Log [GnRH]	hGnRHR		PcDNA/amp vector only	
	Cpm			
Basal	744	1107	256	288
-9	13899	13005		
-6	16542	16584	266	256
Stimulation with D-Ala^6-GnRH-glycinamide				
Basal	464	373	262	228
-9	12469	12185		
-6	15269	14224	264	271

Table 3.2: Stimulation of IP response in COS-1 cells transfected with hGnRHR and pcDNA/amp vector only.

3.2.2 IP RESPONSE MEDIATED BY MUTANT RECEPTORS N102D AND N102K.

The N102D mutant receptor showed only a small IP response at high GnRH-glycinamide concentration (Table 3.3 and Figs. 3.2.3 - 3.2.4). There was no IP response when receptor N102K was stimulated with GnRH-glycinamide up to concentration of 10^{-8}M (Table 3.3 and Figs. 3.2.5 - 3.2.6).

Because of the charges at the side chain of residue 102 of the N102D and N102K mutant receptors, stimulation of these receptors with GnRH free acid (GnRH-OH), which has a free carboxyl group at the C terminus, was investigated. It was thought that a possible ionic interaction might form between the negatively charged terminus of the ligand and the positively charged side chain of the lysine residue of N102K mutant receptor, and possibly stimulate the receptor to mediate an IP response. The repulsion of the two negative charges between the carboxyl groups of the GnRH-OH C terminus and the D102 side chain was anticipated to cause a greater decrease in the EC_{50} than the decrease measured by Davidson et al. (1996 (b)) for wild-type and N102A mutant receptors.

The N102D mutant receptor failed to produce any inositol phosphate response when stimulated with GnRH-OH at concentration up to 10^{-5}M (Table 3.3 and Fig. 3.2.7) while N102K receptor produced a small response at 10^{-5}M (Table 3.3 and Fig. 3.2.8).

Stimulation of N102D receptor with D-Ala^{6}-GnRH-des-Gly^{10}-hydrazide (Table 3.3 and Fig. 3.2.9) failed to produce any inositol phosphate response.

The dose-response curve for IP production mediated by N102D mutant receptor when stimulated with D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide (Table 3.3 and Fig. 3.2.10) was markedly shifted to the right with an EC_{50} of 2207 nM compared with wild-type receptor. The ratio $EC_{50(mutant)}/EC_{50(wild\ type)}$ represents a potency loss of 4414 fold as a result of the N102D mutation. The results with the N102D and N102K receptors are summarised in Table 3.3.

Mutant receptor N102D				
Peptide	n	WT $EC_{50(wt)}$ (nM)	N102D $EC_{50(mut)}$ (nM)	Potency loss EC50mut/EC50wt
GnRH-glycinamide	3	0.13 ± 0.05	SR	-
GnRH-OH	1	19.7	NR	-
D-Ala^6-GnRH-des-Gly^{10}-hydrazide	1	0.65	NR	-
D-Ala^6-GnRH-des-Gly^{10}-pentafluoropropylamide	1	0.50	2207	4414
Mutant receptor N102K				
Peptide	n	WT $EC_{50(wt)}$ (nM)	N102K $EC_{50(mut)}$ (nM)	Potency loss EC50mut/EC50wt
GnRH-glycinamide	2	0.16 ± 0.07	NR	-
GnRH-OH	1	19.7	SR	-

Table 3.3 Potencies of agonist GnRH analogs for wild-type hGnRHR and mutant receptors N102D and N102K. EC_{50} values are the mean ± SEM of values from n independent experiments. SR indicates a small IP response. NR indicates the absence of IP response. The potency loss value shown for D-Ala^6-GnRH-des-Gly^{10}-pentafluoropropylamide is the ratio $EC_{50(mutant)}/EC_{50(wild-type)}$.

Due to the fact that binding could not be measured, and the absence of an IP response, no further investigations of the N102D and N102K mutant receptors were performed.

3.2.3 IP RESPONSE MEDIATED BY MUTANT RECEPTOR N102A.

Although ligand binding with the N102A mutant receptor was too low to be useful in affinity studies, a good IP response was obtained when the receptor was stimulated with

GnRH-glycinamide and other agonist analogs. The mean maximal IP responses, stimulated by GnRH-glycinamide and D-Ala^6-GnRH-glycinamide obtained from three independent experiments performed in duplicate, were 67.8% ±14.0 SEM and 91% ± 5.1 SEM respectively, of the wild-type receptor. However, the increased EC_{50} (23.1 nM) of the mutant receptor, from GnRH-glycinamide stimulation, represented a large (230 fold) potency loss relative to the wild-type hGnRHR (Table 3.4 and Figs. 3.2.11 – 3.2.12). There was a marked increase in the EC_{50} (14.8 nM) of the mutant receptor, stimulated with D-Ala^6-GnRH-glycinamide, which represented a 470-fold loss of potency relative to the wild-type receptor (Table 3.4 and Figs. 3.2.17 – 3.2.18). The good IP response made it possible to measure EC_{50} values with a useful degree of precision. This method was therefore used in further studies.

3.2.4 EFFECT OF THE N102A MUTATION ON THE POTENCY OF SEVEN GnRH AGONIST ANALOGS MODIFIED AT THE C TERMINUS.

Seven agonist GnRH analogs with a modified C terminus (Materials and Methods, Table 2.1) were used to stimulate the IP production in COS-1 cells expressing the human wild type and N102A GnRH receptors. In addition to the terminal modifications, five of the seven analogs have the glycine at position six substituted with D-Alanine. D-Ala^6-GnRH-glycinamide was therefore used as the reference peptide for the ligands containing this substitution.

Table 3.4 is a summary of the mean EC_{50} obtained when wild-type hGnRHR and N102A mutant receptor were stimulated with the seven GnRH agonist analogs. Each peptide was tested in 2-5 independent experiments performed in duplicate. The dose response curves of all peptides for stimulation of the N102A receptor were shifted to the right compared with the wild type receptor. The ratio $EC_{50(mutant)}/EC_{50(wt)}$ expresses the potency loss for the peptide analogs as a result of the N102A mutation. Figures 3.2.11 – 3.2.28 present the data of these experiments.

Peptide	n	WT $EC_{50(wt)}$ (nM)	N102A $EC_{50(mut)}$ (nM)	Potency loss $EC_{50(mut)}/EC_{50(wt)}$
1. GnRH-gycinamide	3	0.12 ± 0.03	23.1 ± 3.64	230 ± 54.1
2. GnRH-des-Gly^{10}-propargylamide	4	0.14 ± 0.04	8.03 ± 2.57	57.5 ± 4.50
3. GnRH-des-Gly^{10}-dimethylamide	3	3.17 ± 0.34	342 ± 72	106 ± 11.2
4. D-Ala^{6}-GnRH-glycinamide	3	0.03 ± 0.01	14.8 ± 3.34	470 ± 97
5. D-Ala^{6}-GnRH-des-Gly^{10}-hydrazide	4	0.38 ± 0.11	106 ± 45.0	260 ± 45.4
6. D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide	2	0.02 ± 0.002	0.41 ± 0.10	17.9 ± 5.25
7. D-Ala^{6}-GnRH-des-Gly^{10}-propylamide	4	0.06 ± 0.02	0.21 ± 0.04	4.60 ± 0.84
8. D-Ala^{6}-GnRH-des-Gly^{10}-tri-fluoroethylamide	5	0.07 ± 0.02	1.02 ± 0.17	17.9 ± 5.47
9. D-Ala^{6}-GnRH-des-Gly^{10}-penta-fluoropropylamide	3	0.43 ± 0.78	1.36 ± 0.57	3.1 ± 0.99

Table 3.4: Potencies of agonist GnRH analogs for wild-type hGnRHR and N102A mutant receptor. EC_{50} values are the mean ± SEM of values from n independent experiments. The potency loss values shown are the mean ± SEM of the ratio $EC_{50(mutant)}/EC_{50(wild\text{-}type)}$ from n independent experiments.

The effect of the C-terminal modifications on the potencies of the peptide analogs relative to the parent peptides (GnRH-glycinamide and D-Ala^6-GnRH-glycinamide) are set out in Table 3.5.

Peptide	WT $EC_{50(wt)}$ (nM)	Δ potency cf.Parent peptide	N102A $EC_{50(mut)}$ (nM)	Δ potency cf. Parent peptide
1. GnRH-gycinamide	**0.12**	**1**	**23.1**	**1**
2. GnRH-des-Gly^{10}-propargylamide	0.14	1.2 ↓	8.03	3.0 ↑
3. GnRH-des-Gly^{10}-dimethylamide	3.17	27.6 ↓	342	14.2 ↓
4. D-Ala^6-GnRH-glycinamide	**0.03**	**1**	**14.8**	**1**
5. D-Ala^6-GnRH-des-Gly^{10}-hydrazide	0.38	12.7 ↓	106	7.2 ↓
6. D-Ala^6-GnRH-des-Gly^{10}-ethylamide	0.02	1.5 ↑	0.41	36.1 ↑
7. D-Ala^6-GnRH-des-Gly^{10}-propylamide	0.06	1.9 ↓	0.21	70.4 ↑
8. D-Ala^6-GnRH-des-Gly^{10}-tri-fluoroethylamide	0.07	2.3 ↓	1.02	14.5 ↑
9. D-Ala^6-GnRH-des-Gly^{10}-penta-fluoropropylamide	0.43	14.3 ↓	1.36	10.9 ↑

Table 3.5: Potency change of GnRH agonist analogs relative to the parent peptides, GnRH-glycinamide and D-Ala^6-GnRH-glycinamide. ↑ Indicates an increase in potency relative to the parent peptide. ↓ Indicates a decrease in potency relative to the parent peptide.

The potency of D-Ala^6-GnRH-glycinamide was 4-fold higer than GnRH-glycinamide. The potency of D-Ala^6-GnRH-des-Gly^{10}-ethylamide was further increased by 1.5-fold. The multiplied increase in potency for both substitutions is 6-fold greater than wild type

GnRH. This is in keeping with previous reports that the introduction of both these substitutions resulted in a multiplicative effect on the activity of the peptide analog (Karten and Rivier 1986).

Four analogs with an alkylamide C-terminal modification exhibited potency losses for the N102A mutant receptor relative to wild-type hGnRHR, which were much smaller than those exhibited by the glycinamide analogs. Of these four analogs, D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide and D-Ala^{6}-GnRH-des-Gly^{10}-trifluoroethylamide have a 2-carbon C-terminal structure, while D-Ala^{6}-GnRH-des-Gly^{10}-propylamide and D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide have a 3-carbon structure. Interestingly, the two analogs with a 2-carbon C-terminal chain had a greater potency loss than those exhibited by the two analogs with a 3-carbon C-terminal chain (Table 3.4). The magnitude of the potency losses for the ligands with 2-carbon or 3-carbon C termini was the same irrespective of the introduction of fluorine atoms (Table 3.4 and Figs 3.2.21 to 3.2.28).

GnRH-des-Gly^{10}-hydrazide, which is potentially able to form a hydrogen bond with the N102 receptor residue, showed a potency loss for the N102A mutant receptor similar to that of GnRH-glycinamide (Figs. 3.2.19 and 3.2.20). The potency of this analog for the wild-type receptor was reduced by a factor of 12.7 when compared to that of the parent peptide, D-Ala^{6}-GnRH-glycinamide (Table 3.5).

GnRH-des-Gly^{10}-propargylamide and GnRH-des-Gly^{10}-dimethylamide exhibited potency losses for the N102A mutant receptor relative to the wild-type receptor which were of an

intermediate magnitude to those of the glycinamide analogs and the four alkylamide analogs, (Figs 3.2.13 – 3.2.16). The potency of GnRH-des-Gly^{10}-dimethylamide for the stimulation of the wild-type receptor was 27.6-fold lower relative to GnRH-glycinamide.

CHAPTER FOUR

DISCUSSION

Using site directed mutagenesis, Davidson et al (1996 (b)) demonstrated that residue N102 is involved in the interaction between the GnRH receptor and its ligand. A possible function of residue N102 of the GnRH receptor in the binding of the ligand was first revealed in a study which investigated the role of glycosylation in the GnRH receptor (Davidson et al., 1995). Although present in a consensus sequence for N-glycosylation, N102 was shown not to be glycosylated. However, its mutation to N102A markedly decreased the interaction with GnRH, suggesting that it might be involved in the ligand-binding site.

Subsequently, eight GnRH analogs with substitutions at positions 2,5,6,7,8,and 9 were used to identify the ligand residue involved in the interaction. These peptides all showed a similar large potency loss for the N102A receptor, suggesting that the interaction of N102 did not occur with the side chain of any of these residues (Davidson et al. 1996 (b)). Comparing the potencies of pairs of analogs differing only at the C terminus showed however, that C-terminal glycinamide analogs exhibited an 11- to 20-fold greater potency loss for the N102A GnRH mutant receptor than the corresponding C-terminal ethylamide derivatives. The results indicated that N102 is critical in determining high potency for peptides with a glycinamide C terminus and suggested that it is a contact

point for the glycinamide C-terminal moiety. The hypothesis that N102 is H-bonded with the C-terminal amide group of GnRH was formulated from this data.

In the present study, this hypothesis was further tested, and the role of the N102 side chain was further evaluated. Three GnRH receptors with the mutations N102A, N102D and N102K were derived from the human GnRH receptor by site directed mutagenesis and expressed in COS-1 cells. The effect of those mutations on binding and IP production stimulated by GnRH-glycinamide was first investigated. Secondly, the effect of the three mutations on the potency of several GnRH agonist analogs modified at the C terminus was determined.

4.1 BINDING EXPERIMENTS WITH N102A MUTANT GnRH RECEPTOR.

To characterise the ligand binding specificity of mutant receptotrs, binding studies were attempted. Davidson et al (1996 (b)) reported that ligand binding by mutant receptor N102A was undetectable. A similar result was obtained in the present study with ^{125}I-D-Ala^{6}-GnRH-glycinamide, where detectable, but low (10.1% of wild-type receptor) binding was shown (Fig 3.1.1).

Binding experiments performed by homologous displacement of radioligand Antagonist 26 showed that the low binding affinity of this ligand by the N102A mutant receptor was not distinguishable from the non-specific binding of untransfected cells (Figs. 3.1.2 and 3.1.3).

Binding of ^{125}I-GnRH-A by the wild-type hGnRH receptor was easily measured whilst binding of this ligand by N102A mutant receptor was undetectable (Figs. 3.1.4 and 3.1.5).

The incorporation of an extra N-glycosylation site in the double mutant S4NP7L receptor was shown previously to enhance expression of the hGnRHR without affecting the binding affinity (Davidson et al 1996 (a)). Therefore, we attempted to enhance the binding of the N102A mutant receptor by incorporating the extra glycosylation site. The extra glycosylation site increased the binding of both agonist and antagonist ligands, relative to the wild-type hGnRHR, by approximately 2-fold (Table 3.1 and Fig 3.1.6). Although the extra glycosylated site enhanced to some extent binding of Antagonist 26 by the N102A mutant receptor, it was not sufficient to enable accurate measurement of the binding affinity.

Thus, measurement of binding affinity of the N102A GnRH receptor using radioligand-binding methodology was shown not to be possible. For this reason, no further binding experiments were performed and the focus of this study was directed towards the IP response mediated by the N102A mutant receptor, which was stimulated with GnRH-glycinamide and various agonist analogs with C-terminal modifications.

4.2 CONTROL IP EXPERIMENTS.

Control experiments were performed to test components of the IP experimental protocol. The results confirmed that an inositol phosphate response to agonist was only seen in COS-1 cells transfected with hGnRH receptor cDNA. COS-1 cells either untransfected or transfected with vector DNA without insert did not generate an IP response when stimulated with agonist. It was noted with interest that in these experiments the basal (agonist-independent) results obtained in cells transfected with wild-type hGnRHR were significantly greater than in cells which were either untransfected (Fig. 3.2.1) or in cells which had been transfected with vector DNA only (Table 3.2). A brief discussion of a possible interpretation of these results is warranted.

A concept generally accepted is that GPCRs exist in an equilibrium between an inactive (R) and an active (R*) conformation (Kjelsberg et al., 1992; Pei et al., 1994). The wild type receptor sequence has been shown to be critical in maintaining the inactive conformation (Kjelsberg et al., 1992) in the absence of agonist via intramolecular interactions (Shenker et al., 1993; Samama et al., 1997). Binding of agonist to the receptor disrupts these interactions resulting in a conformational change (Shenker et al., 1993). According to the extended ternary complex model, stabilisation of R* requires agonist binding as well as G-protein interaction to form the high affinity ternary complex HR*G (Samama et al., 1993; Lefkowitz et al., 1993). The isomerization of R to R* is governed by an equilibrium constant J. Only R* can effectively interact with the G

protein. This interaction is defined by the equilibrium binding constant M. The efficiency of the ligand to facilitate the binding from R* to G leads to the formation of the ternary complex HR*G. Receptor activity in the absence of ligand (constitutive activity) is determined not only by the affinity of R* for G (M) which depends on receptor and G protein, but also by the fraction of receptors capable of interaction with G, which depends on the magnitude of the equilibrium constant J, an intrinsic property of the receptor (Lefkowitz et al., 1993). In a study on constitutive activity of the β_2-Adrenergic receptor (β_2-AR) as a result of mutation, Samama et al. (1993) reported that the basal (agonist-independent) cAMP levels were higher in cells transfected with the wild-type β_2-AR than in those transfected with vector alone. The basal results observed in the control experiments in the present study may be due to constitutive activity of the wild-type GnRH receptor.

4.3 IP RESPONSE MEDIATED BY N102A MUTANT RECEPTOR STIMULATED WITH GLYCINAMIDE ANALOGS.

Although ligand binding with N102A mutant receptor was too low to allow measurement of binding affinity, a good IP response was obtained when the receptor was stimulated with GnRH-glycinamide and other agonist analogs. The maximal inositol phosphate response mediated by the mutant N102A receptor stimulated by GnRH-glycinamide was 67.8% ± 14.0% (mean ± SEM, n = 3 experiments) of the wild type value. D-Ala^6-GnRH-glycinamide, which was used as the reference peptide for analogs containing the D-Ala^6 substitution, stimulated the N102A mutant receptor to a maximal IP response of

91% ± 5.1% (mean ± SEM, n = 3 experiments) of the wild type receptor. As the maximal IP response is dependent on receptor expression and receptor coupling, these results confirmed that mutant receptor is expressed at normal or near-normal levels.

GnRH-glycinamide and D-Ala^6-GnRH-glycinamide showed potency losses of 230- and 470-fold respectively for the N102A mutant receptor (Table 3.4). These results were similar to previous results obtained by Davidson et al (1996 (b)). Because the N102A mutant receptor is expressed normally, the loss of potency of the glycinamide analogs was attributed predominantly to a loss of receptor binding affinity caused by the mutation (Figs. 3.2.11, 3.2.12, 3.2.17 and 3.2.18). This indicates that the binding of analogs with a glycinamide C-terminus are strongly dependent on the presence of N102.

4.4 EFFECT OF N102A MUTATION ON THE POTENCY OF SEVEN AGONIST ANALOGS MODIFIED AT THE C TERMINUS.

If the side chain of a receptor residue interacts with a group on the ligand, then removal of the interacting side chain by its mutation to alanine should produce a decrease in affinity which is similar in magnitude to, but non-additive with, the removal of the interacting group from the ligand. This concept was used previously to investigate the interaction of N102 of the GnRH receptor with GnRH (Davidson et al. 1996 (b)).

A potency loss for a particular analog, of similar magnitude to that of GnRH, when used to stimulate the N102A mutant receptor, would indicate that the analog retains the same

interaction with N102 as does GnRH (Table 4.1). Conversely, a potency loss significantly less than that for GnRH would suggest that the interaction of the peptide with N102 is diminished or absent. GnRH analogs with ethylamide C-termini showed smaller potency losses than analogs with glycinamide C-termini, indicating that N102 is in some way important for the docking of the glycinamide C-terminus (Davidson et al, 1996). The studies discussed below, employing C-terminally modified GnRH analogs, are based on the same approach.

4.4.1 EFFECT OF ALKYLAMIDE C-TERMINAL MODIFICATIONS.

Analogs with -ethylamide, -propylamide, -trifluoroethylamide and -penta-fluoropropylamide C-termini showed much smaller potency losses (3.1 – 17.9 fold) than the -glycinamide parent peptides, for the N102A mutant receptor. These results indicated a weaker dependence on receptor residue N102 than was seen with glycinamide analogs. Davidson et al (1996 (b)) suggested that an alternative hydrophobic interaction occurs between the receptor and ligands with an alkylamide C-terminal moiety, that are unable to form H-bonds. This idea is shown in Fig 4.1, which presents a schematic model for the docking of the C terminus of GnRH analogs.

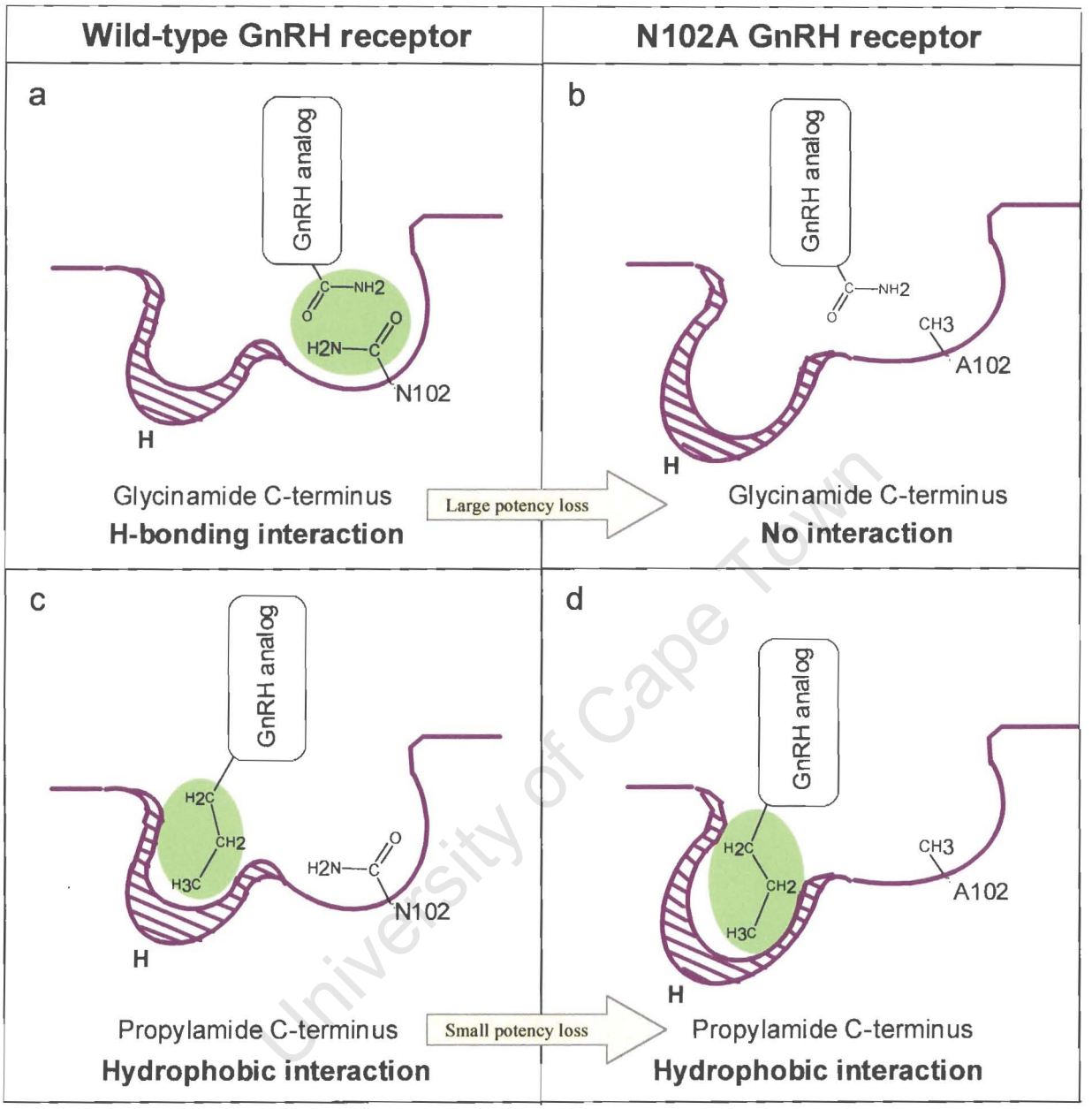


Fig 4.1 Schematic depiction of part of the ligand binding pocket of GnRH receptor showing proposed docking of the C-terminal part of GnRH analogs. H represents a putative hydrophobic site on the receptor. The green shaded areas represent interactions contributing to binding affinity.

Two types of ligand interaction are proposed to occur within the binding site depending on the analog C-terminal moiety. Analogs with a glycinamide C terminus are proposed

to interact with the N102 side chain through the formation of H-bonds between the two amide groups; whilst analogs with a hydrophobic C-terminus, which is unable to form H-bonds, are proposed to interact hydrophobically with another region of the binding pocket. The removal of the side chain by the N102A mutation not only removes the possibility of H-bond formation but also is thought to slightly alter the conformation of the binding pocket. The combined effect impairs the interaction with the glycinamide C-terminus to a large degree while the hydrophobic interaction, influenced only by the possible conformational change, is much less affected.

Interestingly, the two analogs containing a 2-carbon chain at the C terminus had a greater potency loss (17.9 fold), than the potency loss (3.1 - 4.6 fold) of the two analogs containing a 3-carbon chain at the C terminus.

The covalent bond length for a C-F group is 1.41 Å compared to 1.07 Å for a C-H bond (Ebbing, 1984). Thus, fluorinated C termini are bulkier than their hydrogenated counterparts. This however does not seem to have an effect on the interaction with N102 since the potency loss is the same whether fluorine atoms are inserted or not. This indicates that the binding pocket is large enough to accommodate the increase in bulk.

The observation, that analogs with an ethylamide (2-carbon chain) C terminus are more dependent on N102 than those with a propylamide (3-carbon chain) C terminal moiety reinforces the suggestion made by Fujino et al. (1972) that the total chain length of GnRH plays an important role in the binding affinity. Furthermore, the data shows that longer

C termini, containing three carbons, are less affected by the mutation to alanine. This could be explained by the proposed slight change in the conformation of the binding pocket caused by the mutation of N102 to alanine. Analogs with a 3-carbon chain at the C terminus seemed to fit better in the altered binding pocket. The loss of interaction caused by the mutation would thus be partially compensated by an increase in chain length.

Coy et al. (1979) reported that D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide, D-Ala^{6}-GnRH-des-Gly^{10}-propylamide and D-Ala^{6}-GnRH-des-Gly^{10}-trifluoroethylamide gave identical patterns of gonadotropin release and that although D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide contains an end group which is more hydrophobic than the other three analogs, the peptide was shown to be considerably less active than those three ligands. The potencies of the same analogs for the wild-type hGnRHR, measured in this study, are in keeping with the report of Coy et al. (1979) (Table 3.4). The potency of D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide for the wild-type receptor was considerably less than that of the other three analogs (Table3.4 and 3.5).

In summary, the results with GnRH agonists with –ethylamide, -trifluoroethylamide, -propylamide and –pentafluoropropylamide C termini are consistent with the proposed model (Fig. 4.1).

4.4.2 D-Ala^6-GnRH-des-Gly^{10}-HYDRAZIDE.

Of the five tested analogs that contain the D-Ala^6 mutation, D-Ala^6-GnRH-des-Gly^{10}-hydrazide is the only one that has a C-terminal moiety, which is able to form a hydrogen bond with the N102 receptor residue. The dependence of this analog on the interaction with the receptor residue N102, which was similar to the glycinamide analogs, was reflected by the large (260-fold) potency loss (Table 3.4 and Figs 3.2.19 and 3.2.20). This result is therefore consistent with the proposed model (Fig. 4.1).

Interestingly, the hydrazide analog was less potent than the parent peptide D-Ala^6-GnRH-glycinamide for the stimulation of both the wild type and N102A receptors (Table 3.5). The concept, suggested by Fujino et al. (1972 and 1973), that the total chain length of GnRH played an important role in the binding affinity, and that substitutions with the closest conformational similarity to the N-C-C unit of the replaced glycinamide are most active would explain why the shorter hydrazide group lost some potency with the wild type receptor. An interaction of the hydrazide C-terminus with the receptor residue N102 side chain seems to occur nevertheless as is indicated by the potency loss caused by the receptor mutation

4.4.3 GnRH-des-Gly^{10}-PROPARGYLAMIDE AND GnRH-des-Gly^{10}-DIMETHYL-AMIDE

GnRH-des-Gly^{10}-propargylamide and GnRH-des-Gly^{10}-dimethylamide exhibited intermediate potency losses (57.5- and 106-fold respectively) for N102A mutant receptor relative to the wild-type receptor, suggesting a weaker interaction with N102 than the glycinamide moiety.

The nature of the interaction of GnRH-des-Gly^{10}-propargylamide with receptor residue N102 could be explained in terms of the presence of the triple bond between the two C-terminal carbons of the GnRH-des-Gly^{10}-propargylamide (Table 2.1). The electrons of the two π bonds within the triple bond are sufficiently delocalised to impart a polarity to the molecule, which could enable it to interact with the polar amide moiety of the receptor residue N102 side chain. The loss of potency indicates a preference for this interaction over that with the totally apolar A102.

Unlike the other analogs, which have an alkylamide C-terminus, GnRH-des-Gly^{10}-dimethylamide exhibited a greater (106-fold) potency loss for the N102A mutant receptor, relative to the wild-type receptor (Table 3.4; Figs 3.2.15 and 3.2.16). Although this analog is unable to form a hydrogen bond with receptor residue N102 side chain, this result indicates that its binding is nevertheless strongly dependent on N102. This result is therefore not consistent with the proposed model (Fig. 4.1 and Table 4.1).

It must be noted, however, that the potency of this analog for the wild type receptor was decreased by a factor of 27.6 relative to that of GnRH-glycinamide (Table 3.5). This large potency loss for the wild type receptor suggests that this analog does not form a strong hydrophobic interaction as shown in fig. 4.1. In further studies on structure-activity relationships in the C-terminal part of LHRH, Fujino et al. (1973) reported that GnRH-des-Gly^{10}-isopropylamide, which also has a branched C-terminal structure (Fig.4.2), showed a 1.5 fold increase in activity relative to GnRH. By contrast GnRH-des-Gly^{10}-dimethylamide and GnRH-des-Gly10-isobutylamide were 6.7 and 33.3 times less active respectively. Once more, it is interesting to note that GnRH-des-Gly^{10}-isopropylamide is the closest to the N-C-C conformation of the glycinamide.

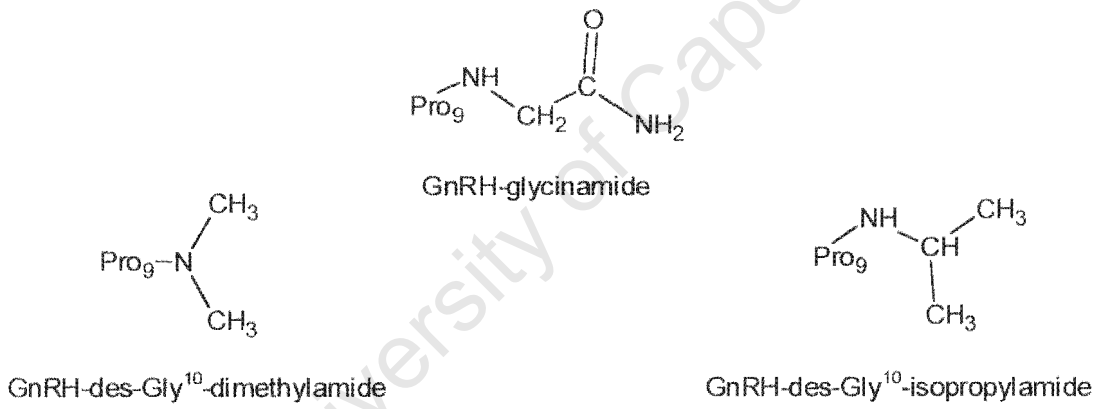


Fig 4.2 C-terminus of GnRH-glycinamide compared with the C-terminal modification of agonist analogs ending with dimethyl groups

The destabilisation of the interaction with the dimethyl C-terminal moiety could be interpreted in terms of the chain length, whereby this C-terminal alkyl amide modification is too short to interact properly with the hydrophobic region of the binding pocket. The dependence of this analog on N102, as shown by the potency loss for the

N102A mutant, would thus be indirect. Instead of being involved directly in an interaction with the ligand, the N102 residue, in this case, may be needed for the formation of a binding pocket conformation which is somewhat more suitable, for the docking of the dimethyl amide group, than the conformation created by the A102 mutation.

4.4.4 EFFECT OF D-Ala^6 SUBSTITUTION ON THE LIGAND C-TERMINAL INTERACTION WITH RECEPTOR RESIDUE N102.

The introduction of the D-Ala^6 substitution is known to enhance the potency of GnRH by a factor of 3-4 fold (Monahan et al. 1973; Coy et al. 1975; Coy et al. 1976). Our data (Table 3.5) obtained for the stimulation of the wild-type receptor by D-Ala^6-GnRH-glycinamide showed that it is four times more potent than GnRH-glycinamide for the stimulation of the wild-type hGnRHR, which is in keeping with these findings. The data also showed that the D-Ala^6 substitution increased the dependence of D-Ala^6-GnRH-glycinamide on N102 by a factor of two compared to GnRH-glycinamide.

In a previous study, Davidson et al. (1996 (b)) reported an 11.1-fold decrease of the potency of GnRH-des-Gly^{10}-ethylamide for the N102A mutant receptor relative to the wild-type receptor. The potency loss (17.9-fold) of D-Ala^6-GnRH-des-Gly^{10}-ethylamide, measured in this study, for the stimulation of the N102A mutant receptor, relative to wild-type hGnRHR, was 1.6 times that of GnRH-des-Gly^{10}-ethylamide.

These results indicate that the interaction between receptor residue N102 side chain and the C terminus of analogs that have a D-Ala at position six, is stronger than the interaction of ligands with a glycine residue at the same position.

The known enhancing effect of the D-Ala^{6} substitution on the potency of GnRH analogs could then be attributed more specifically to a direct enhancing effect on the interaction between the ligand C terminus and the receptor residue N102 side chain.

4.5 THE N102D MUTANT GnRH RECEPTOR:

Binding of D-Ala^{6}-GnRH-glycinamide by the N102D GnRH receptor was undetectable (Fig 3.1.1), and the IP response mediated by this mutant receptor stimulated with GnRH-glycinamide, showed only a small response at a high agonist concentration (10^{-5} M) (Figs. 3.2.3 and 3.2.4).

A fairly good IP response was obtained from the stimulation of the N102D mutant receptor by high concentrations of D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide (Fig. 3.2.10). Because the maximal IP response is dependent on receptor number and coupling efficiency, the maximal IP response from the stimulation of N102D mutant receptor with D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide (Fig 3.2.10) indicates some receptor expression at the cell surface and that the G protein was activated. Since it was shown that the receptor was expressed, the large increase in EC_{50} (2207 nM) compared to the wild-type hGnRHR, which represented a 4414-fold loss of potency, is

most likely due to loss of binding affinity for the ligand. It is possible that although the receptor is expressed at the cell surface, the negative charge at D102 destabilises its conformation, leading to an alteration of the binding pocket which results in the large loss of binding affinity.

In addition to the factors discussed above, the absence of an IP response with N102D mutant receptor stimulated with GnRH-OH (Fig. 3.2.7) could partially be attributed to a further loss of binding affinity for the analog as a result of the repulsion of the negative charges of the interacting groups. D-Ala^{6}-GnRH-des-Gly^{10}-hydrazide, although able to form a H-bond, failed to stimulate mutant receptor N102D to induce any inositol phosphate response.

4.6 THE N102K MUTANT GnRH RECEPTOR

Binding of D-Ala^{6}-GnRH-glycinamide by the N102K mutant receptor was also undetectable (Fig 3.1.1), and stimulation of this receptor with GnRH-glycinamide did not result in any IP response (Figs. 3.2.5 and 3.2.6).

The effect of a possible ionic interaction between the positive charge of K102 side chain with GnRH-OH C-terminal carboxyl group, which could enhance the potency of the ligand, was investigated. Only a very small IP response was measured at a concentration of 10^{-5}M (Fig. 3.2.8).

It was interpreted from these results that the N102K mutant receptor, as with the N102D mutant receptor, is destabilised by the charge at this position and is either unable to bind ligand or is not sufficiently expressed at the cell surface.

In summary, the experimental results of this thesis are consistent with the hypothesis that the residue N102 of the GnRH receptor interacts by forming H-bonds with the glycinamide C terminus of GnRH or with C-terminal groups (e.g. hydrazide) of analogs which are able to interact in this manner. Furthermore, the data showed that GnRH analogs with alkylamide C-terminal moieties have a weaker dependence on N102, and may have a hydrophobic interaction with the receptor. The relationship between proposed interactions with the GnRH receptor and potency losses (which reflect the dependence on N102) of GnRH analogs C-termini is shown in table 4.1. A model explaining the docking of the C-terminal part of GnRH analogs is proposed (Fig. 4.1). This model proposes a dual role of N102: a) to interact directly with ligands that have a C terminus able to H-bond with the amide group of its side chain; and b) to contribute to the conformation of the binding pocket. In agreement with the observations by Fujino et al. (1972 and 1973), the data also showed clearly that the optimal C-terminal length of GnRH analogs, which are accommodated in the binding pocket conformation in which N102 is involved, is the length closest to the glycinamide C-terminal moiety.

C terminus	Potency Loss (fold)	Proposed Interaction
Glycinamide	230-470	H-bond between glycinamide and N102
Hydrazide	260	H-bond between hydrazide and N102
Propargylamide	57.5	Polar interaction with N102
Dimethylamide	106	? (see Discussion)
Ethylamide	17.9	Hydrophobic Interaction
Trifluoroethylamide	17.9	
Propylamide	4.6	
Pentafluoropropylamide	3.2	

Table 4.1 Relationship between proposed interaction with the GnRH receptor and the loss of potencies of different GnRH analog C-termini. Large potency losses reflect a strong dependence on N102, as depicted in fig 4.1.

CHAPTER FIVE

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CHAPTER SIX

EXPERIMENTAL DATA FIGURES

Fig 3.1.1 Binding of [^{125}I]D-Ala^{6}-GnRH to N102A, N102D and N102K mutant GnRH receptors with homologous displacement with unlabelled ligand. Bo: no unlabelled ligand added. Error bars show SEM of triplicate wells

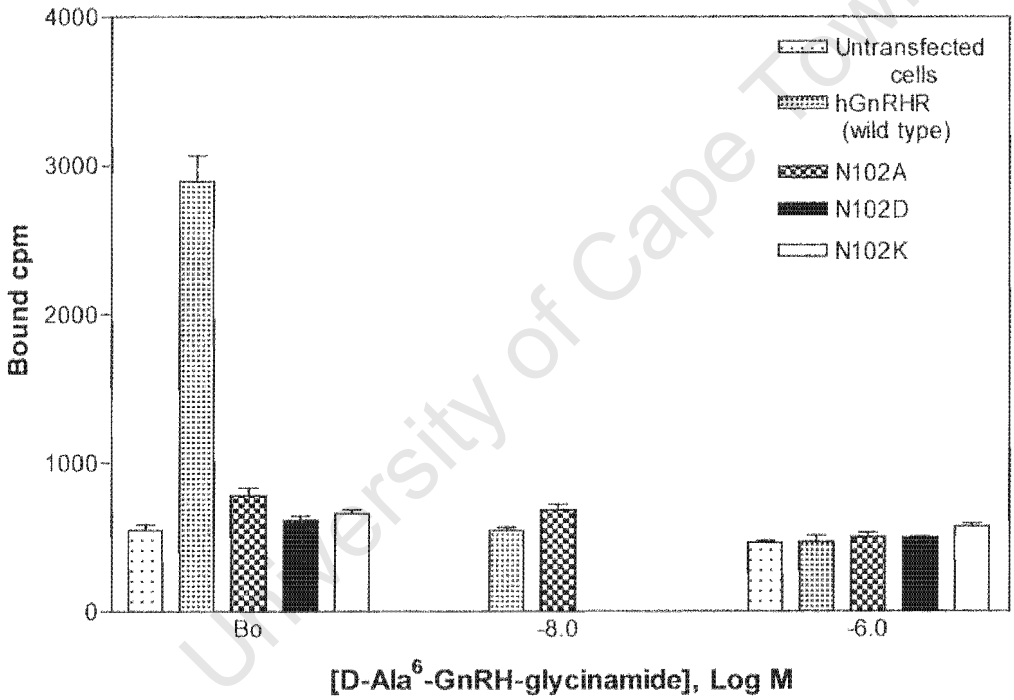


Fig 3.1.2 Binding of [^{125}I]Antagonist 26 to wild-type hGnRHR, with homologous displacement with unlabelled ligand. The hGnRHR transfected cells showed two binding sites with affinities of 1.81 nM and 264 nM. Binding affinity of untransfected cells was 245 nM. Data shown are from a single experiment. Error bars represent SEM of triplicate wells.

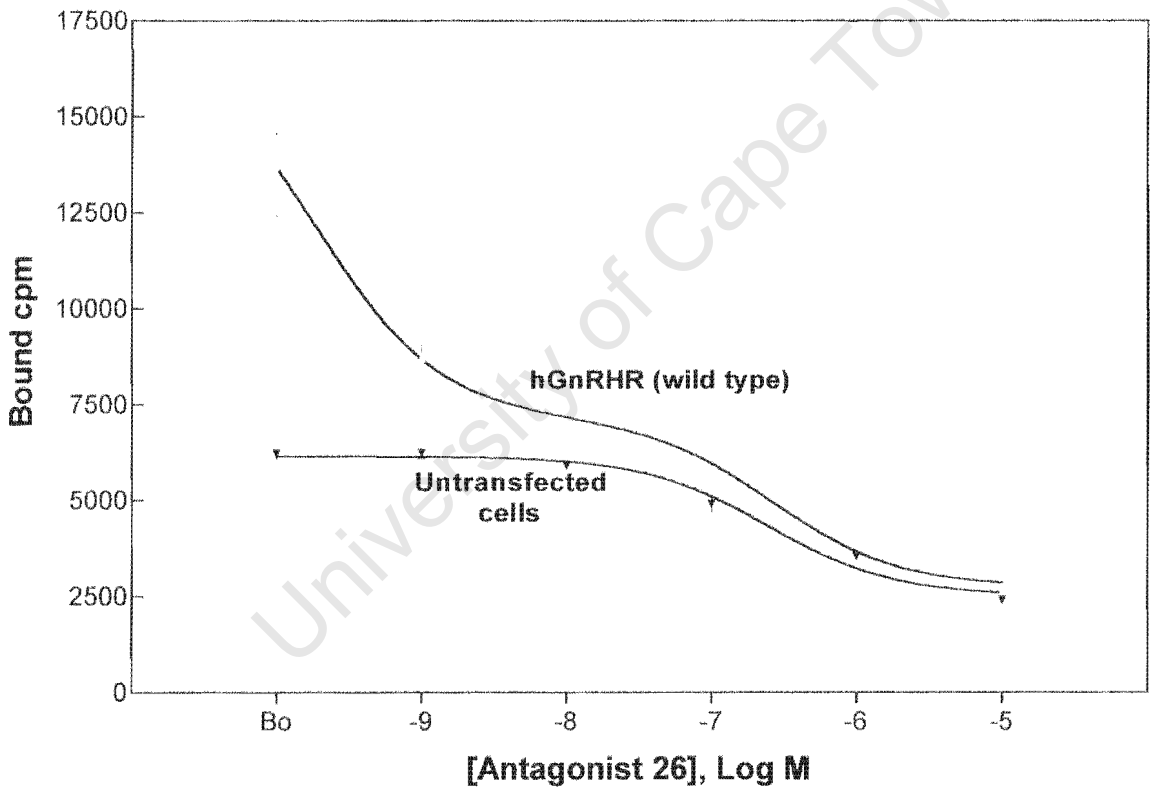


Fig 3.1.3 Binding of [^{125}I]Antagonist 26 to hGnRHR and N102A mutant receptorswith homologous displacement. Binding affinities for the two sites of hGnRH receptor transfected cells were 1.03 nM and 343 nM. Data shown are from a single experiment. Error bars represent SEM of triplicate wells.

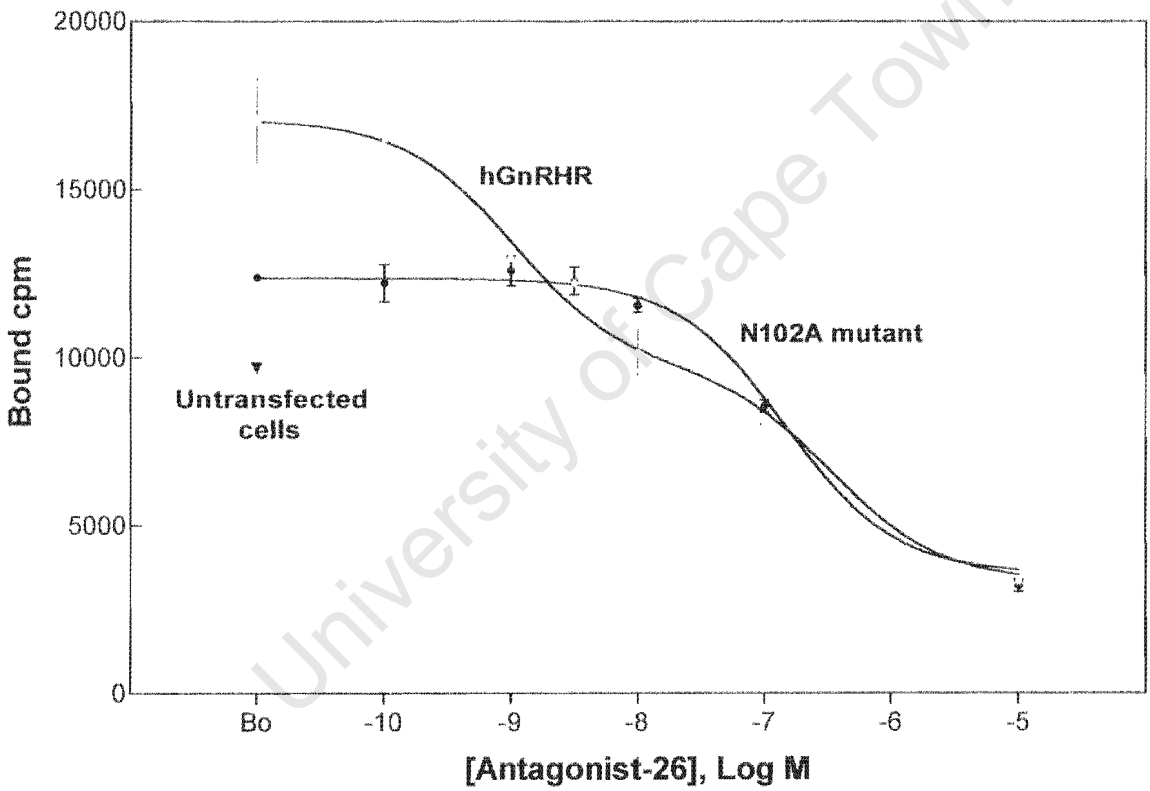


Fig 3.1.4 Binding of [^{125}I]-GnRH-A to wild-type hGnRHR with homologous displacement. The affinity of the binding site was 1.023 nM. Data is from a single experiment. Error bars represent SEM of triplicate wells.

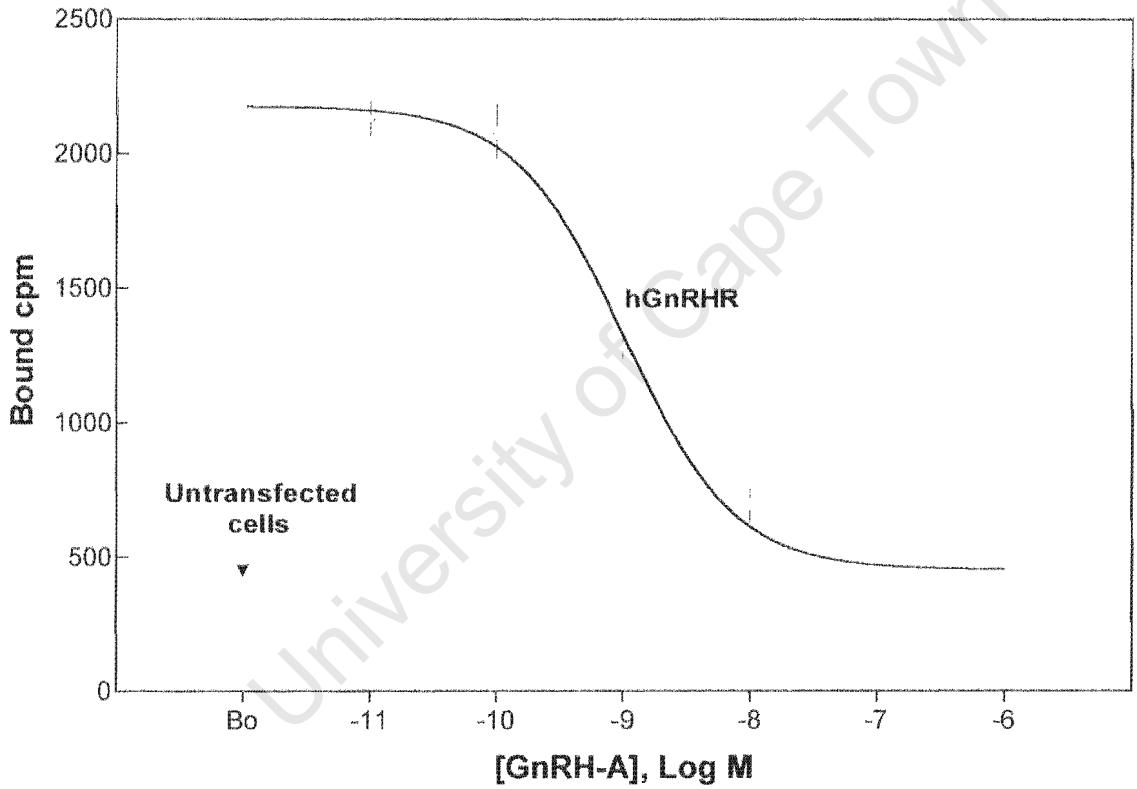


Fig 3.1.5 Binding of [^{125}I]-GnRH-A to wild-type hGnRHR and N102A mutant receptor with homologous displacement. Binding affinity of hGnRHR is 1.70 nM. Data is from a single experiment. Error bars represent SEM of triplicate wells

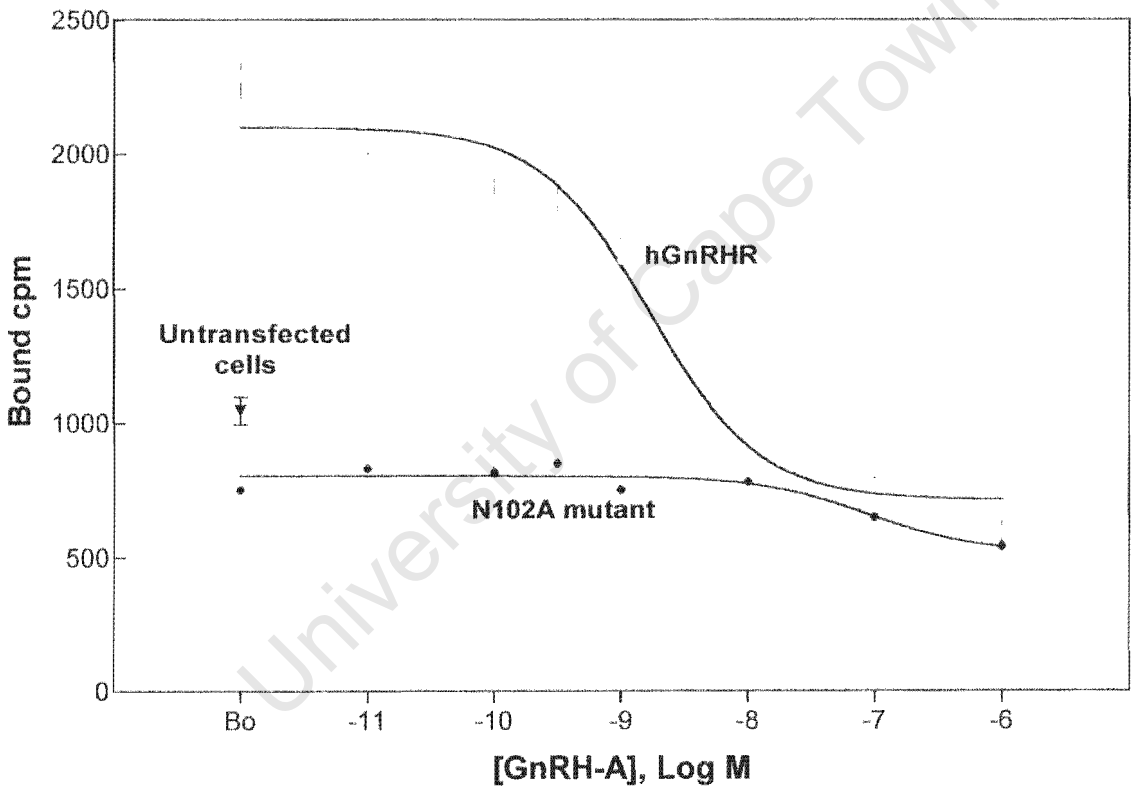
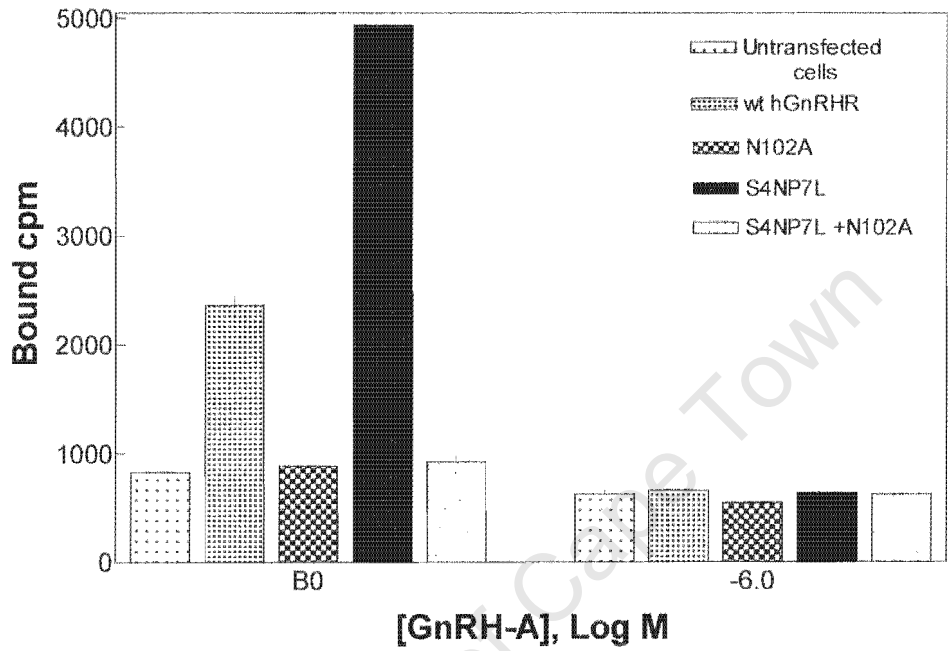


Fig 3.1.6 Effect of extra glycosylation site on binding of [^{125}I]-GnRH-A and [^{125}I]Antagonist 26. S4NP7L and S4NP7L+N102A receptors contain additional glycosylated site to enhance receptor expression. Data are from two independent experiments. Error bars represent SEM of triplicate wells.



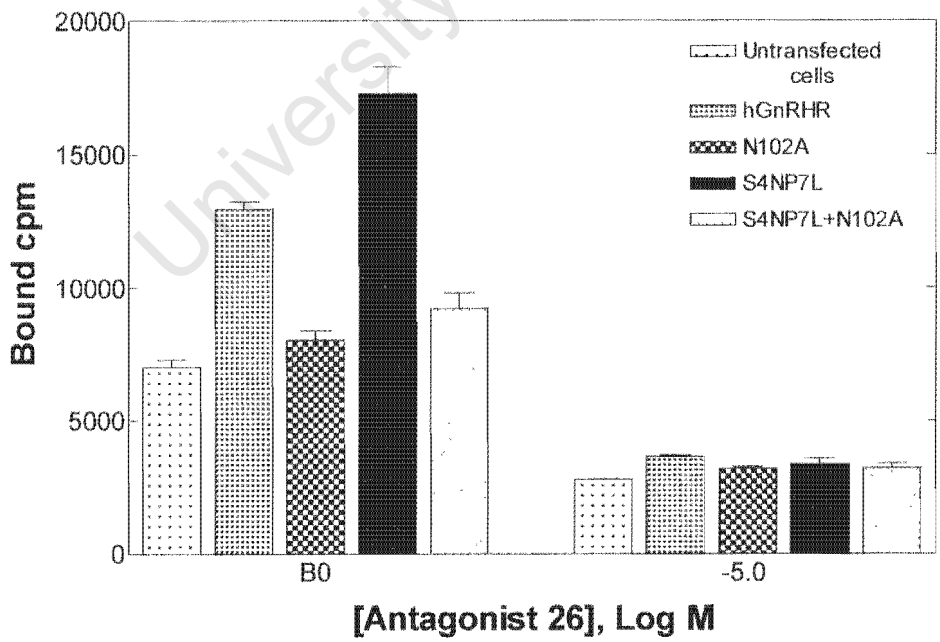


Fig 3.2.1 IP response in COS-1 cells transfected with hGnRH and N102A mutant receptors vs untransfected cells, stimulated with D-Ala^{6}-GnRH-des-Gly^{10}-ethylamide. Data shown are from a single experiment performed in duplicate. Error bars show the range of duplicate values

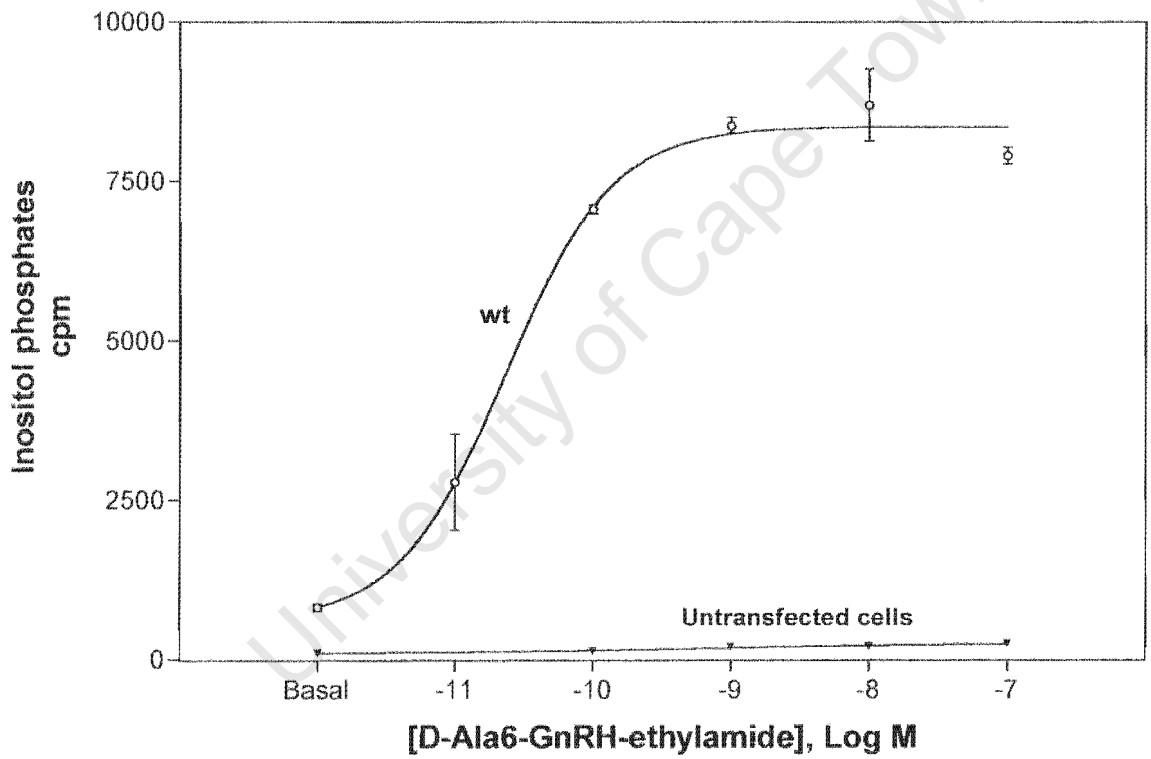
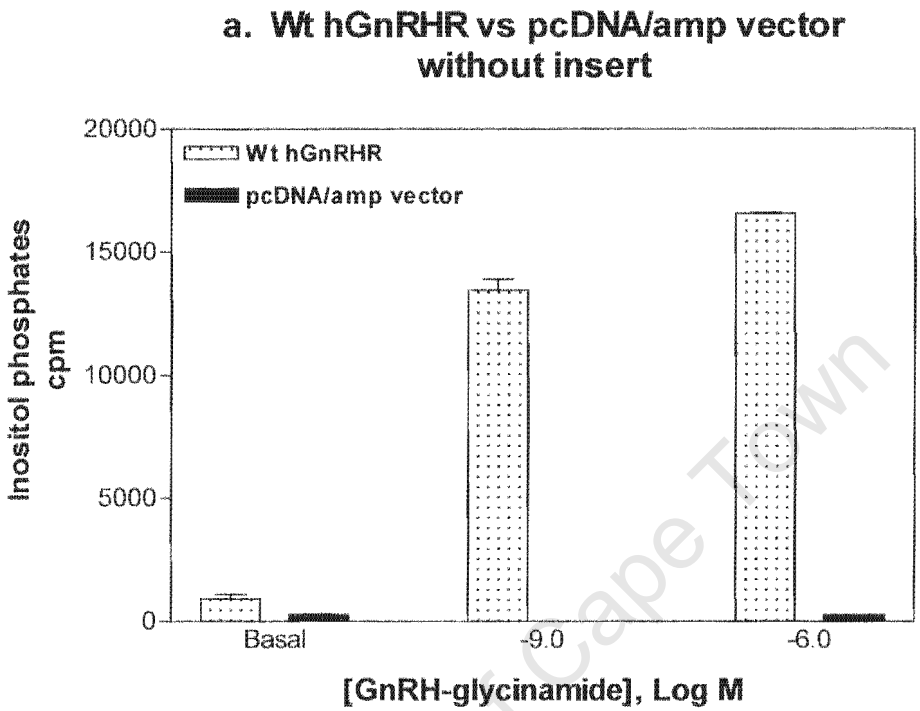


Fig 3.2.2 IP response in COS-1 cells transfected with wt hGnRHR and pcDNA/AMP vector DNA without insert, stimulated with GnRH-glycinamide (Graph a) and with D-Ala^6-GnRH-glycinamide (Graph b). Data are from two independent experiments each performed in duplicate. Error bars show the range of duplicate values.



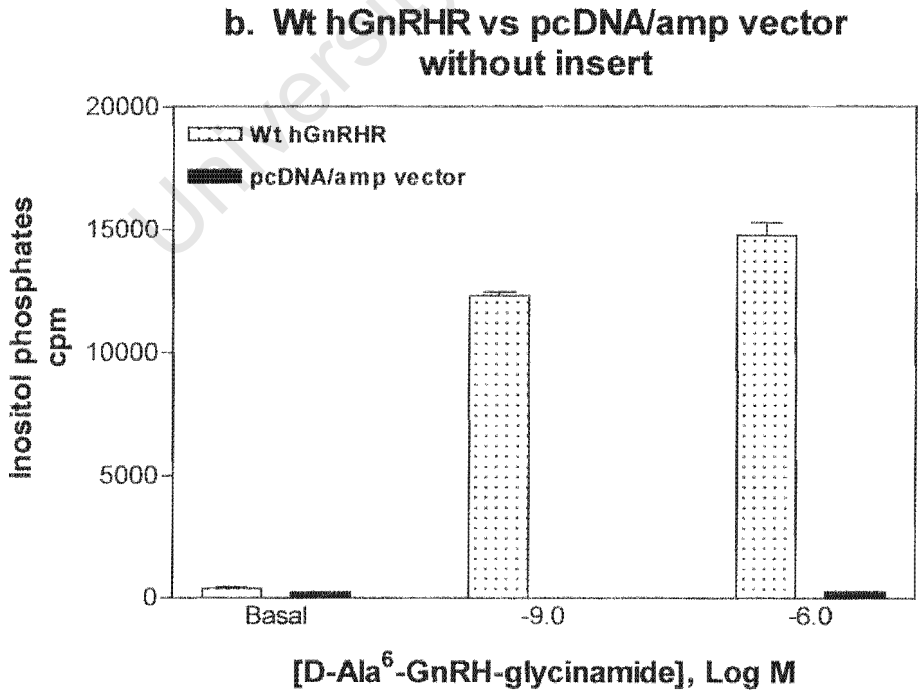


Fig 3.2.3 Inositol phosphate response mediated by wt hGnRH and N102D mutant receptors stimulated with GnRH-glycinamide. Data represent three independent experiments each performed in duplicate. Data has been normalized as a % of maximal wild-type response. Error bars show the range of duplicate values.

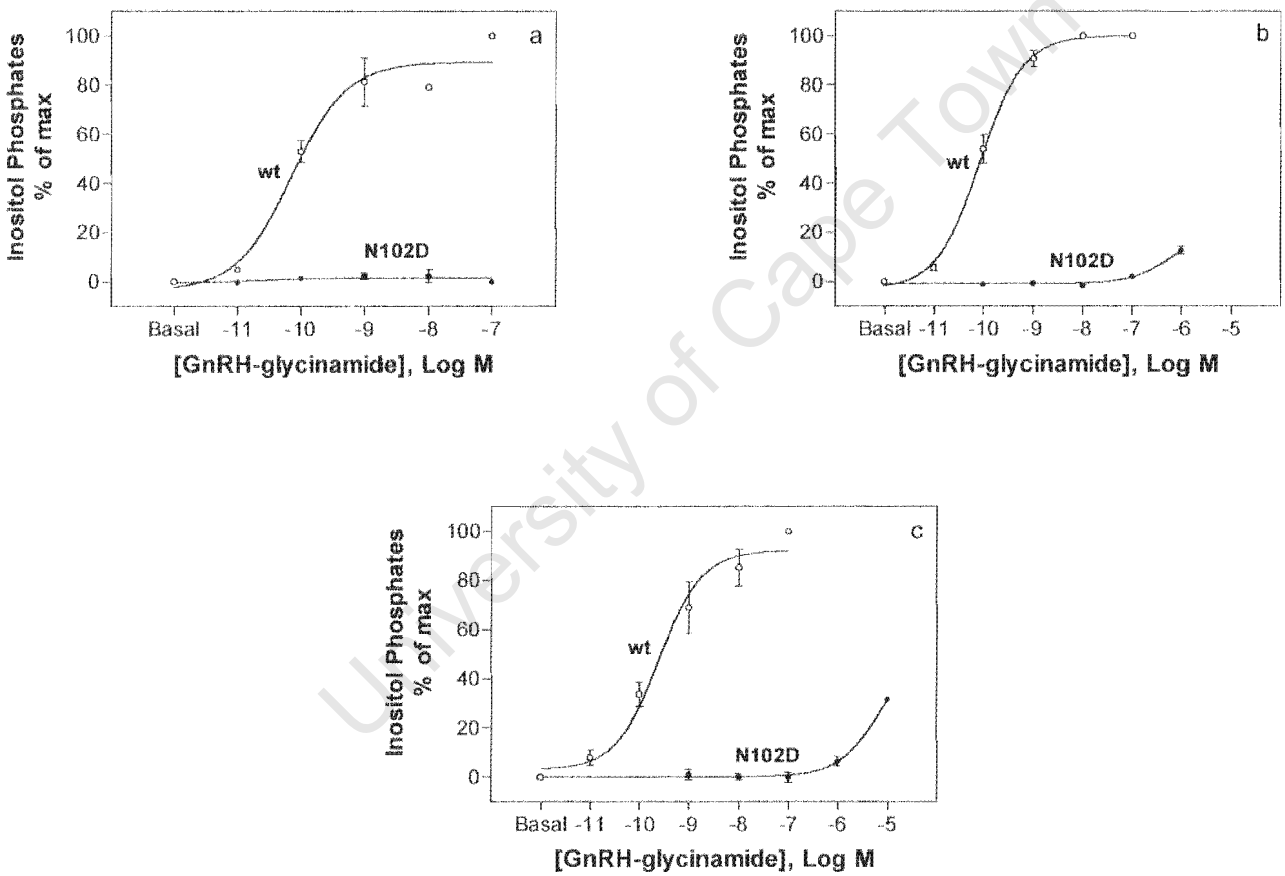


Fig 3.2.4 Inositol phosphate response mediated by wt hGnRH and N102D mutant receptors stimulated with GnRH-glycinamide. Data points represent the means ±SEM of the combined data of three independent experiments illustrated in Fig 3.2.3

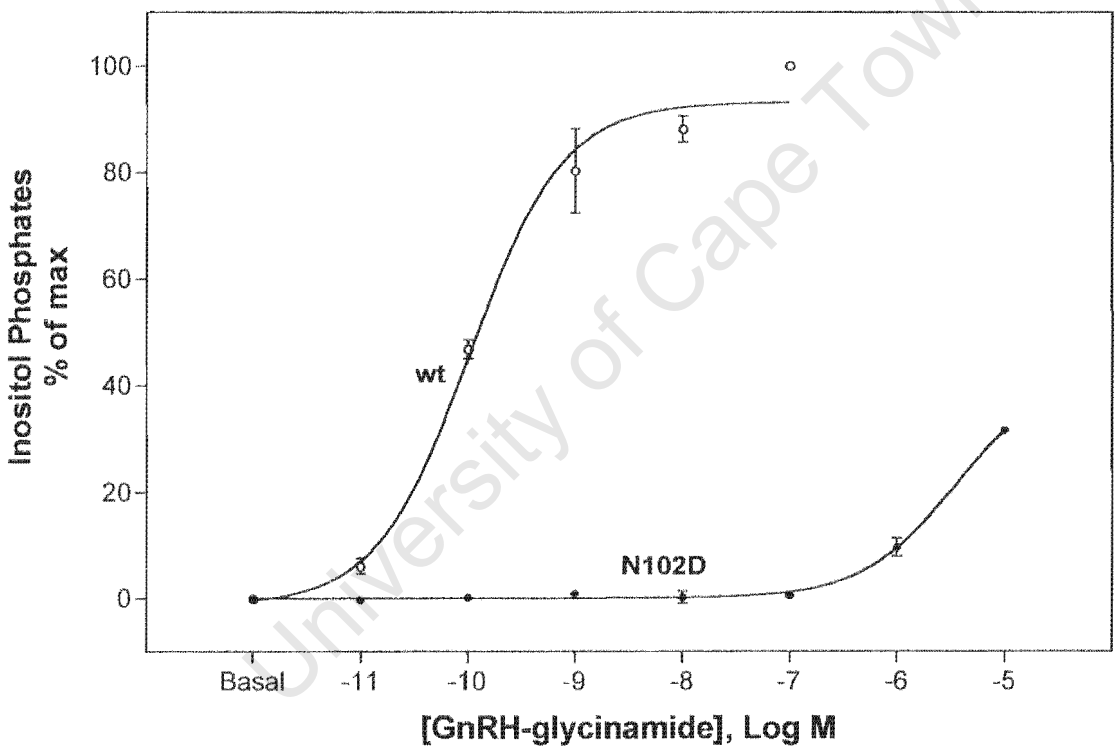


Fig 3.2.5 Inositol phosphate response mediated by hGnRH and N102K mutant receptors stimulated with GnRH-glycinamide. Data represent two independent experiments each performed in duplicate. Data has been normalized as a % of maximal wild-type receptor response. Error bars show the range of duplicate values.

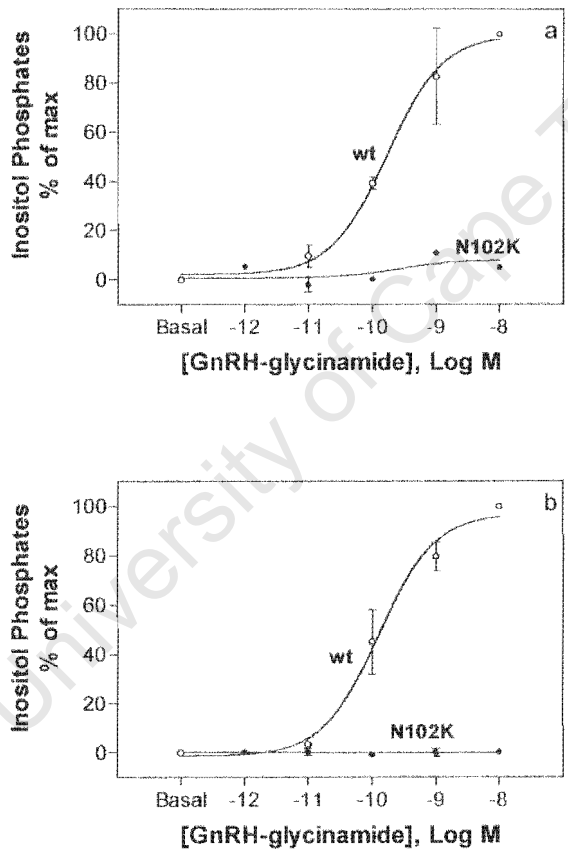


Fig 3.2.6 Inositol phosphate response mediated by wt hGnRH and N102K mutant receptors stimulated with GnRH-glycinamide. Data points represent the means ±SEM of the combined data of two independent experiments illustrated in Fig 3.2.5

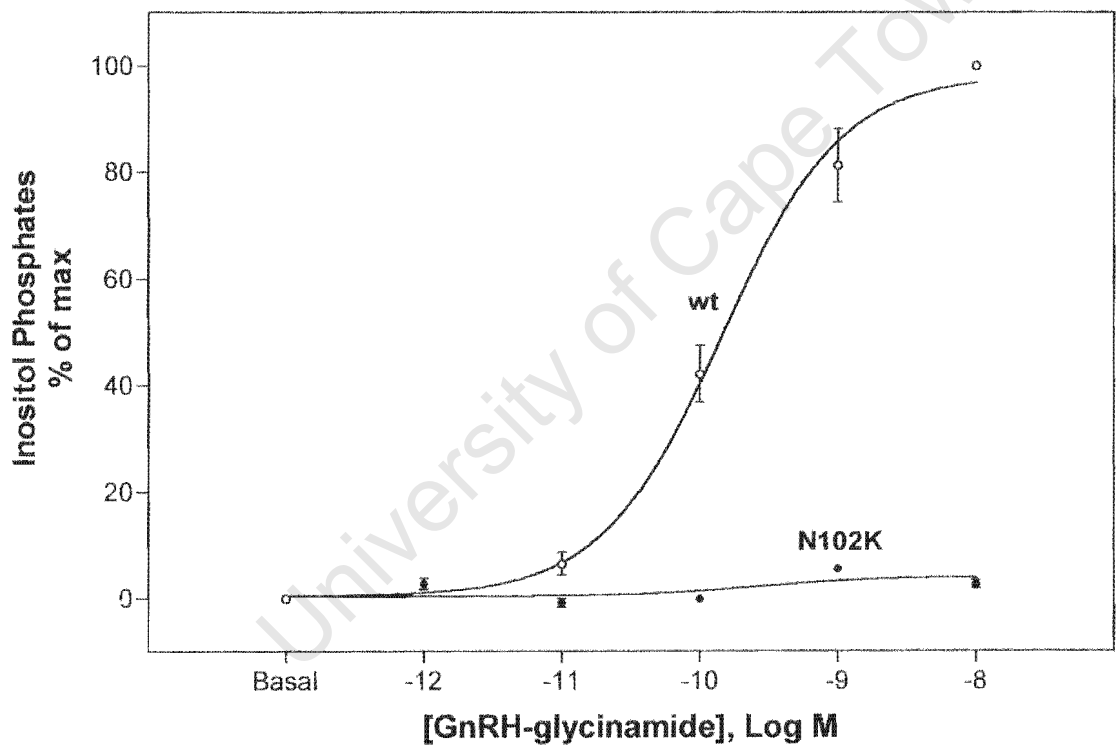


Fig 3.2.7 Inositol Phosphate response mediated by wt hGnRH and N102D mutant receptors stimulated with GnRH-OH. Data points are from a single experiment performed in duplicate. Error bars show the range of duplicate values.

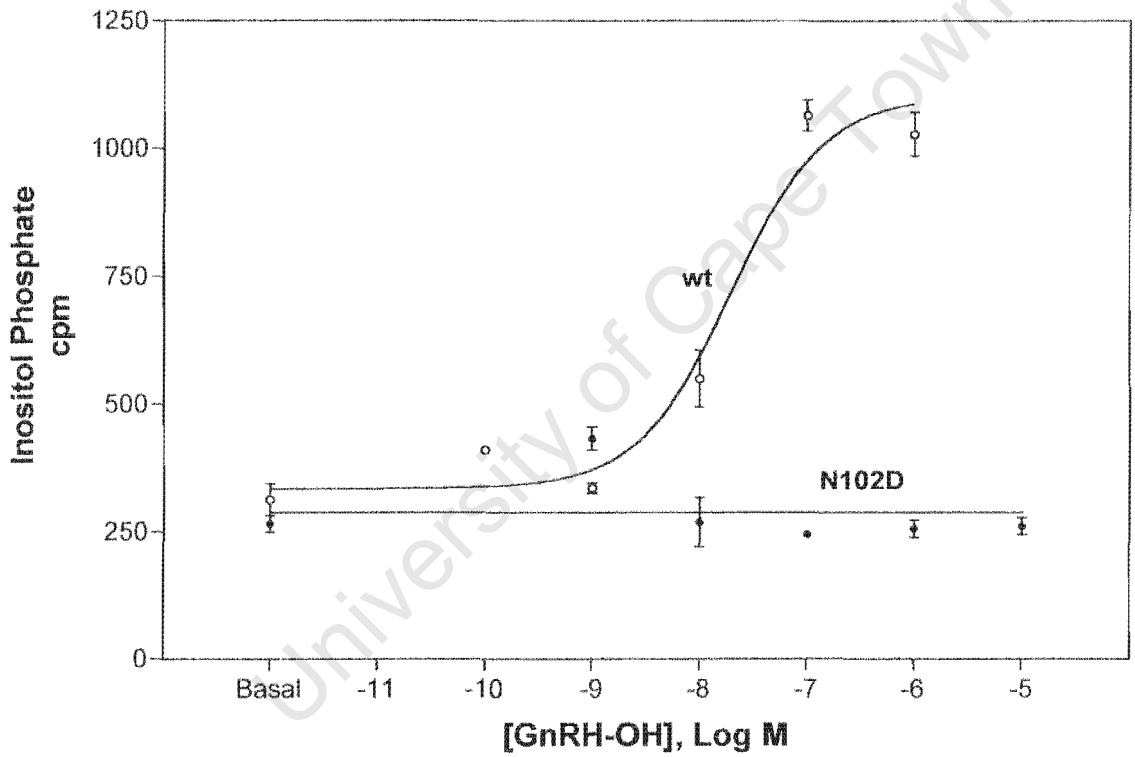


Fig 3.2.8 Inositol phosphate response mediated by wt hGnRH and N102K mutant receptors stimulated with GnRH-OH. Data points are from a single experiment performed in duplicate. Error bars show the range of duplicate values.

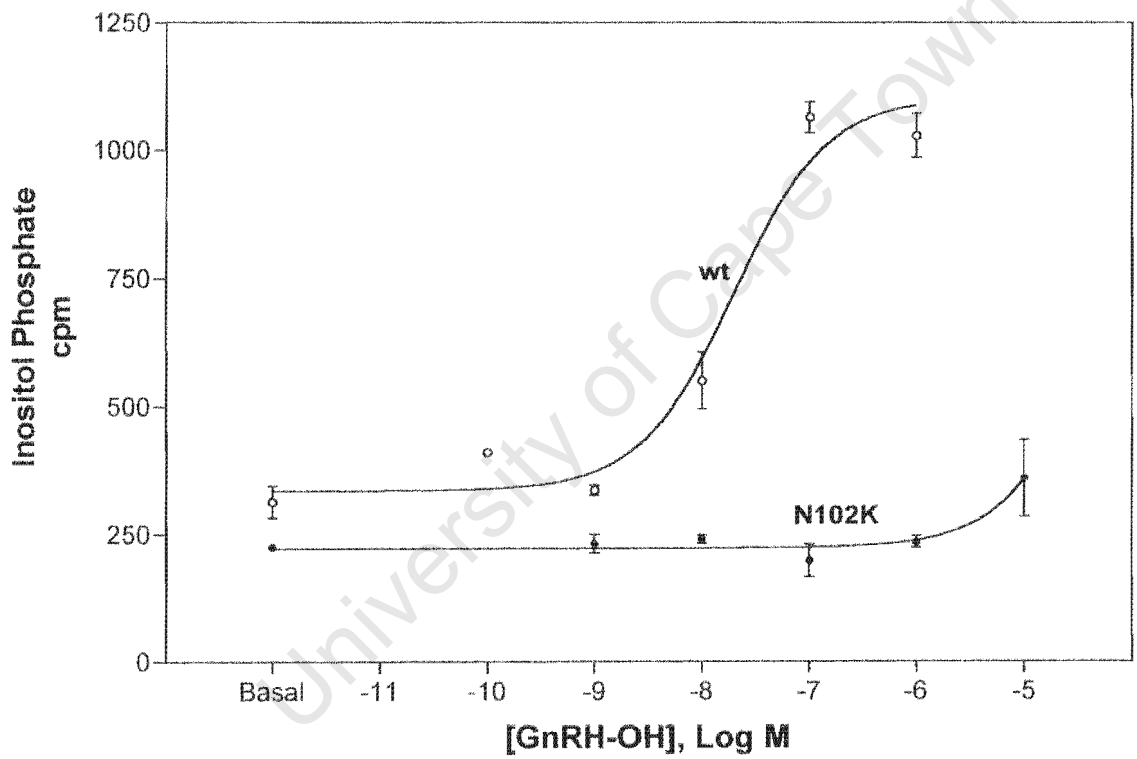


Fig 3.2.9 Inositol phosphate response mediated by wt hGnRH and N102D mutant receptors stimulated with D-Ala^{6}-GnRH-des-Gly^{10}-Hydrazide.
Data points are from one single experiment performed in duplicate. Error bars show the range of duplicate values.

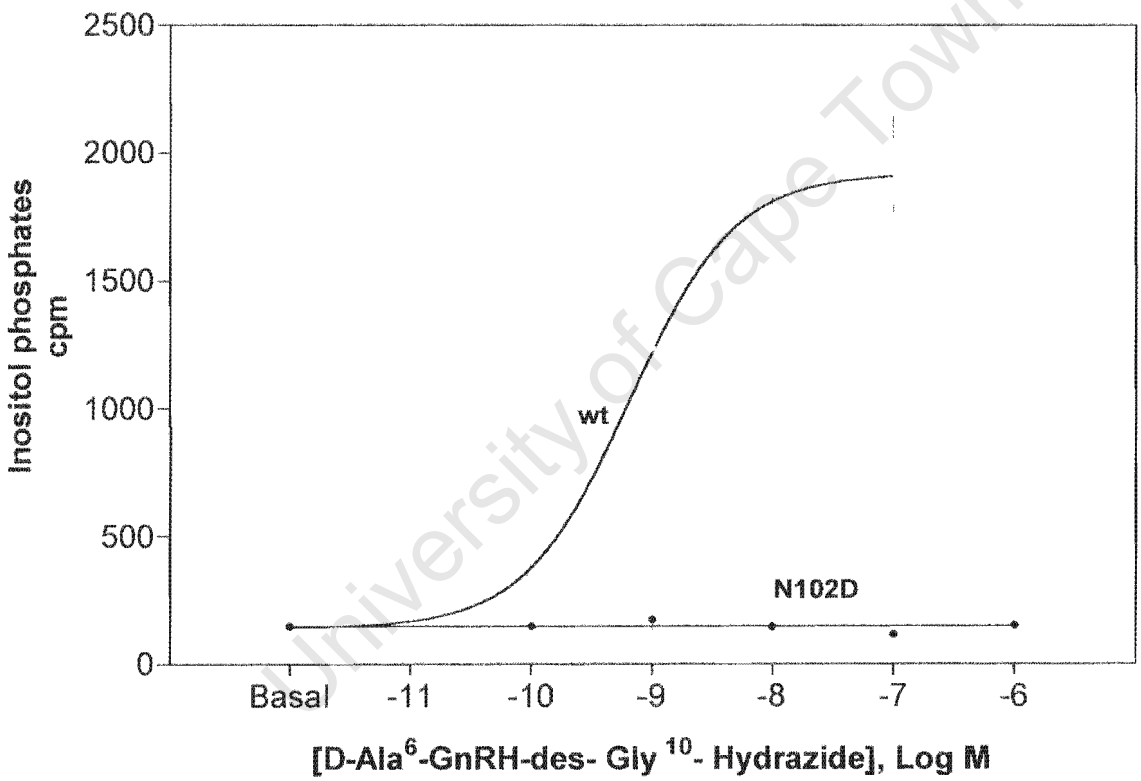


Fig 3.2.10 Inositol phosphate response mediated by wt hGnRH and N102D mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-pentafluoropropylamide. Data points are from one single experiment performed in duplicate. Error bars show the range of duplicate values. EC_{50} for hGnRHR is 0.50nM and EC_{50} for N102A is 2207nM.

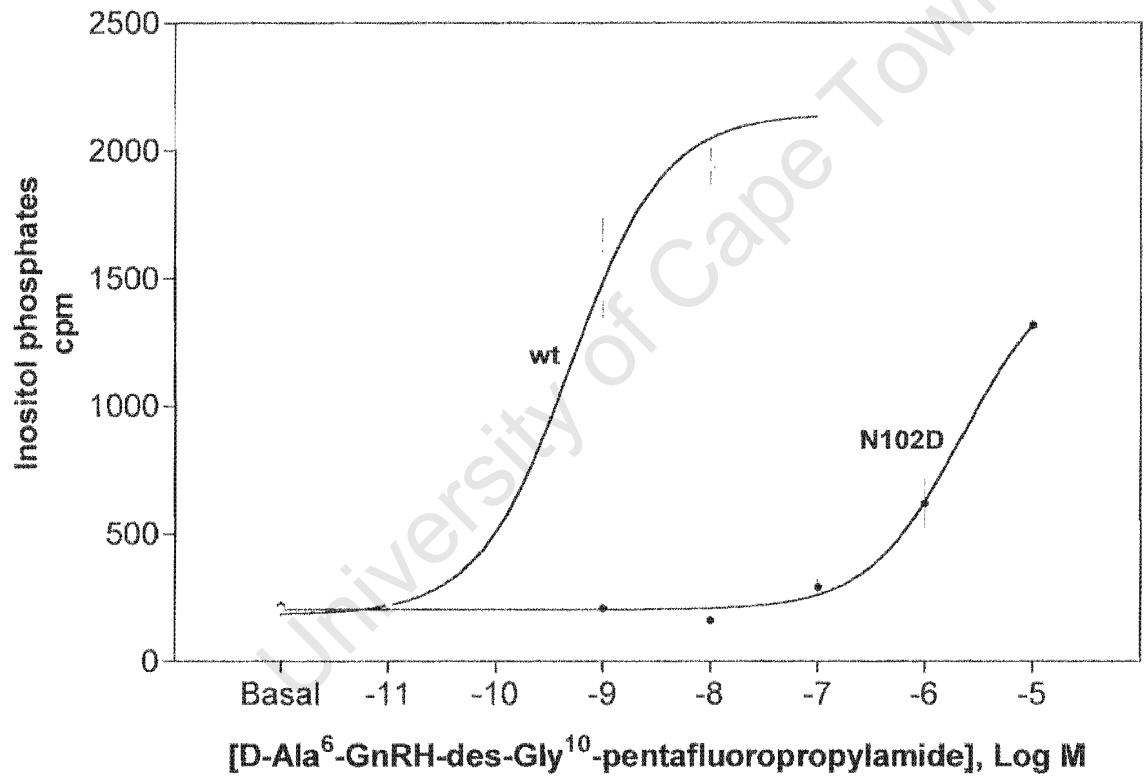


Fig 3.2.11 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with GnRH-glycinamide. Data represent three experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response. Data for graph c was obtained from Peter Davies of the Molecular Reproductive Endocrinology Unit, Department of Chemical Pathology, University of Cape Town.

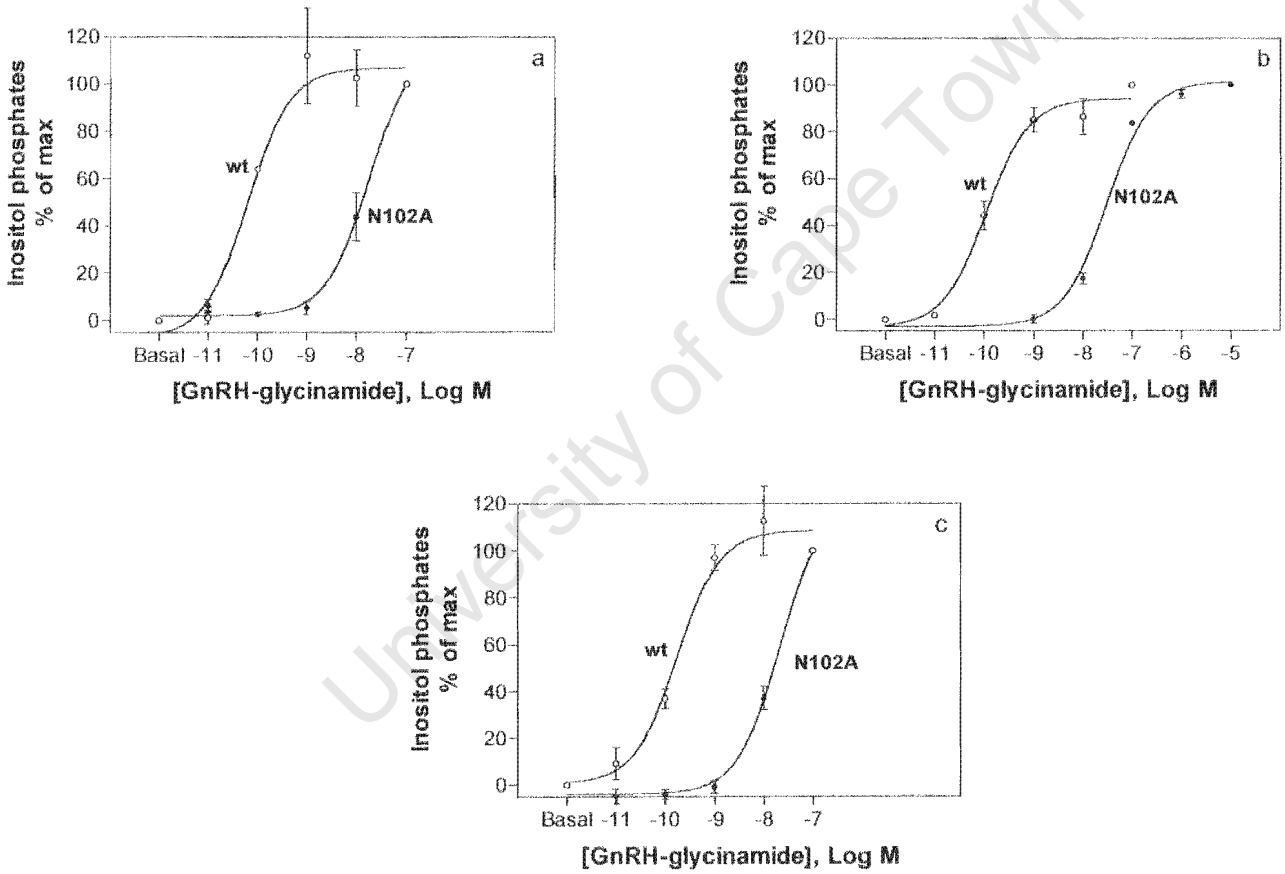


Fig 3.2.12 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with GnRH-glycinamide. The data points represent the means ±SEM of the combined data from three independent experiments illustrated in Fig 3.2.11.

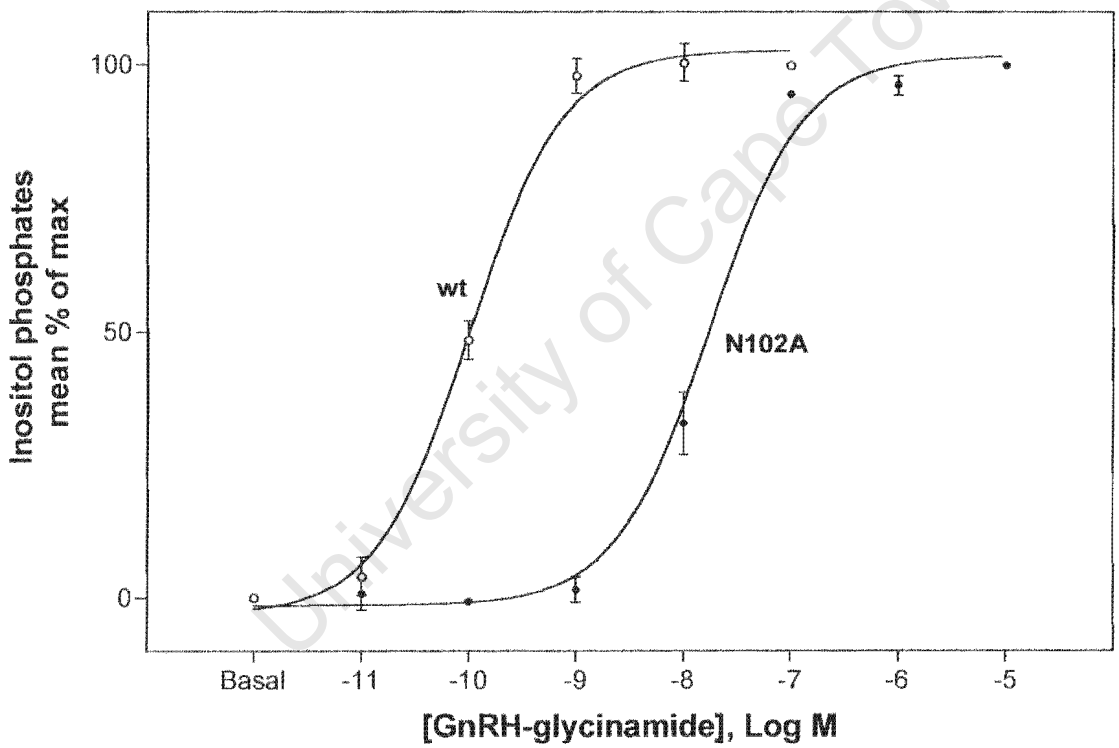


Fig 3.2.13 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with GnRH-propargylamide. Data represent four experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

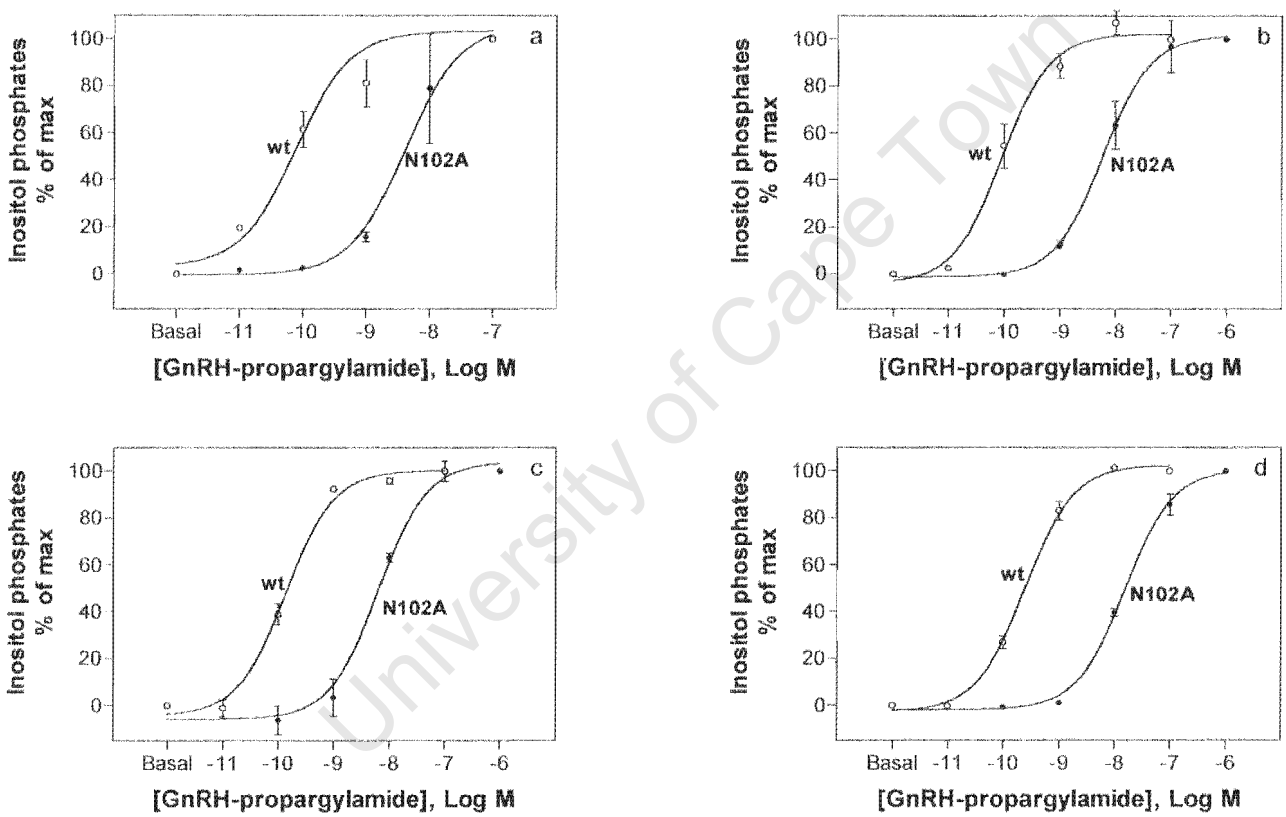


Fig 3.2.14 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with GnRH-propargylamide. The data points represent the means ±SEM of the combined data from four independent experiments illustrated in Fig 3.2.13.

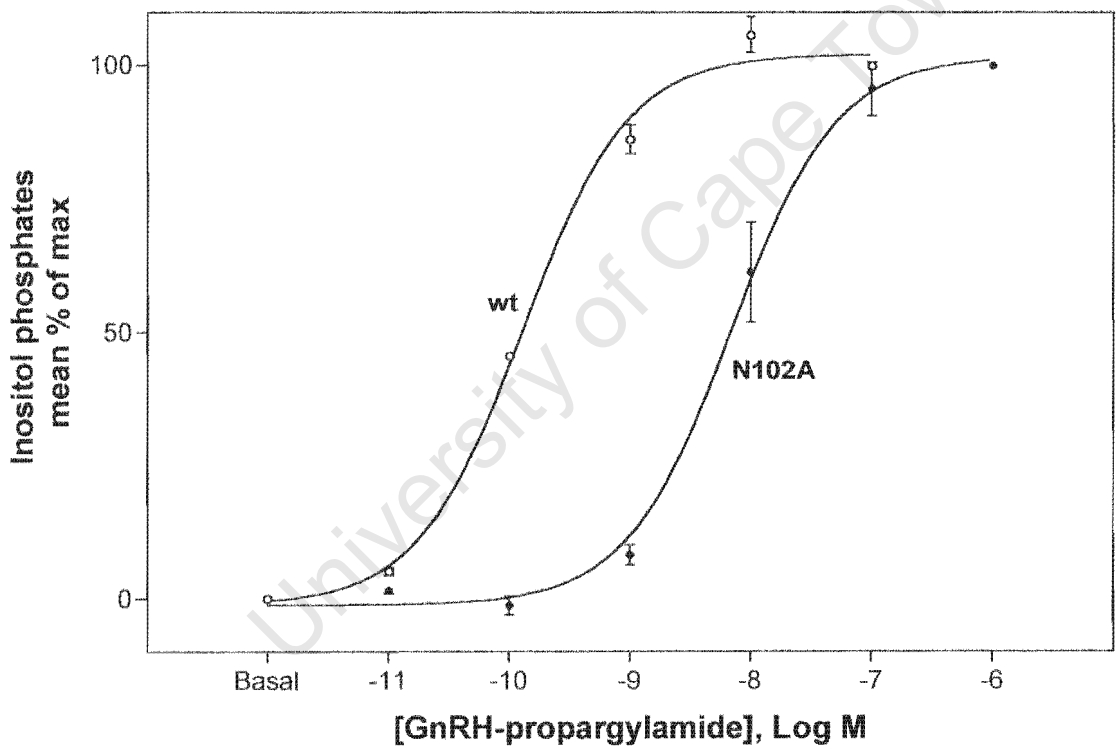


Fig 3.2.15 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with GnRH-dimethylamide. Data represent three experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

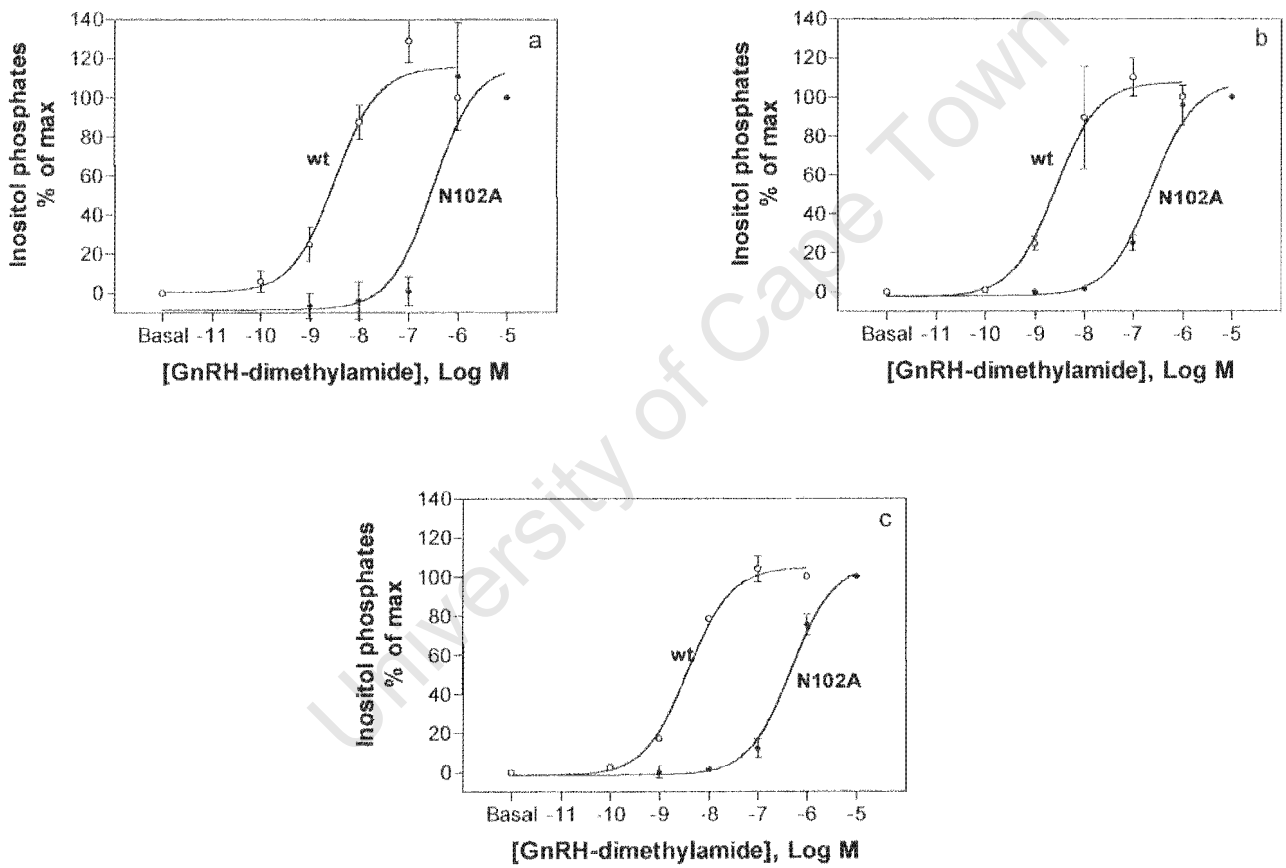


Fig 3.2.16 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with GnRH-dimethylamide. The data points represent the means ±SEM of the combined data from three independent experiments illustrated in Fig 3.2.15.

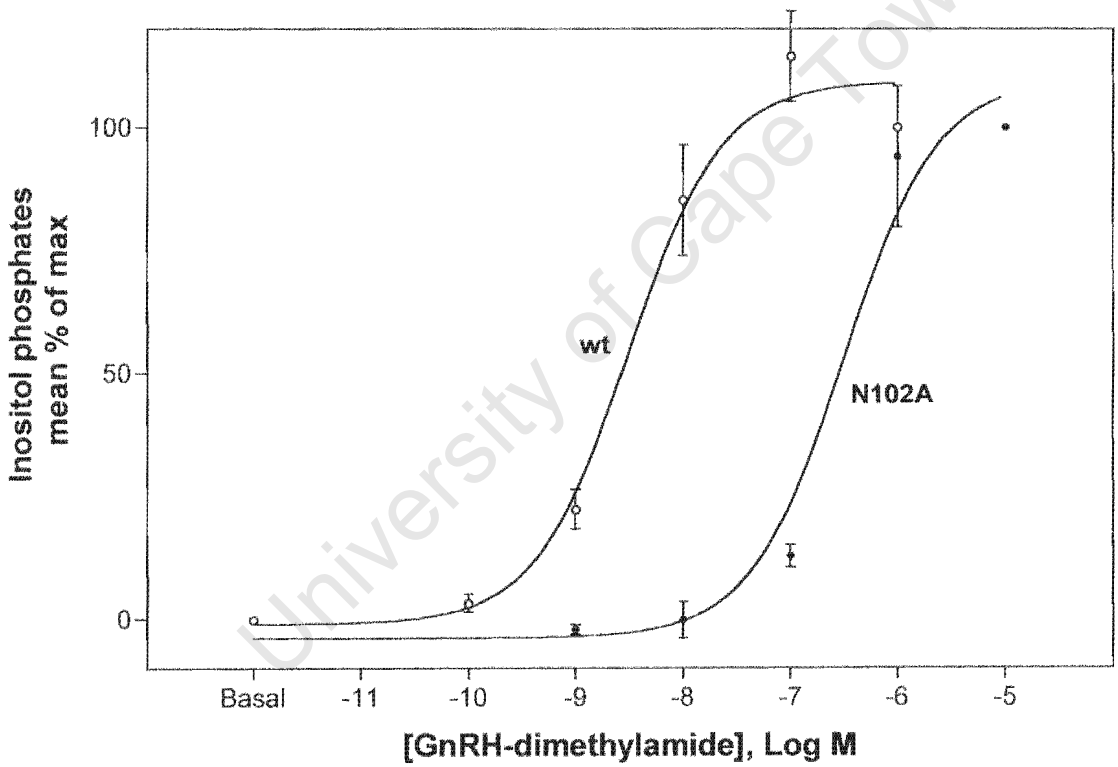


Fig 3.2.17 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-glycinamide. Data represent three experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

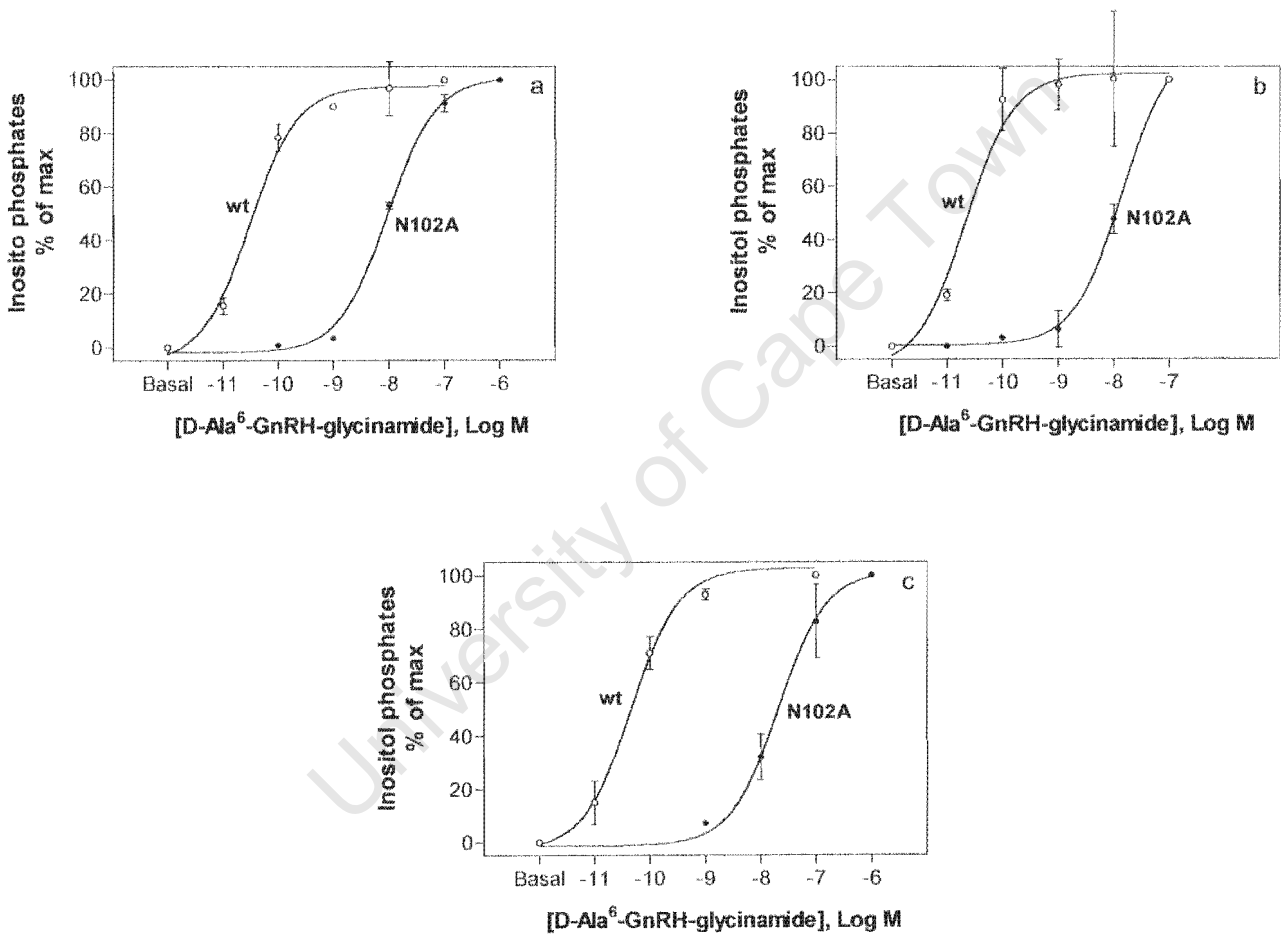


Fig 3.2.18 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6 -GnRH-glycinamide. The data points represent the means ±SEM of the combined data from three independent experiments illustrated in Fig 3.2.17.

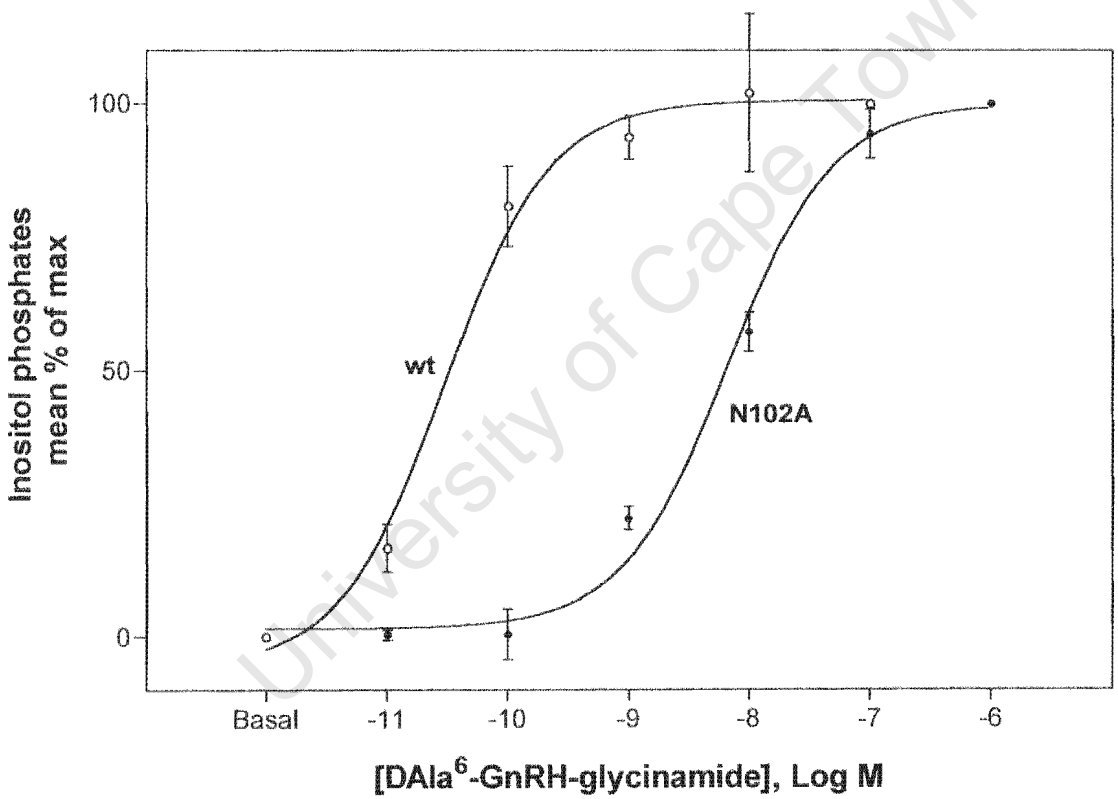


Fig 3.2.19 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-hydrazide. Data represent four experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

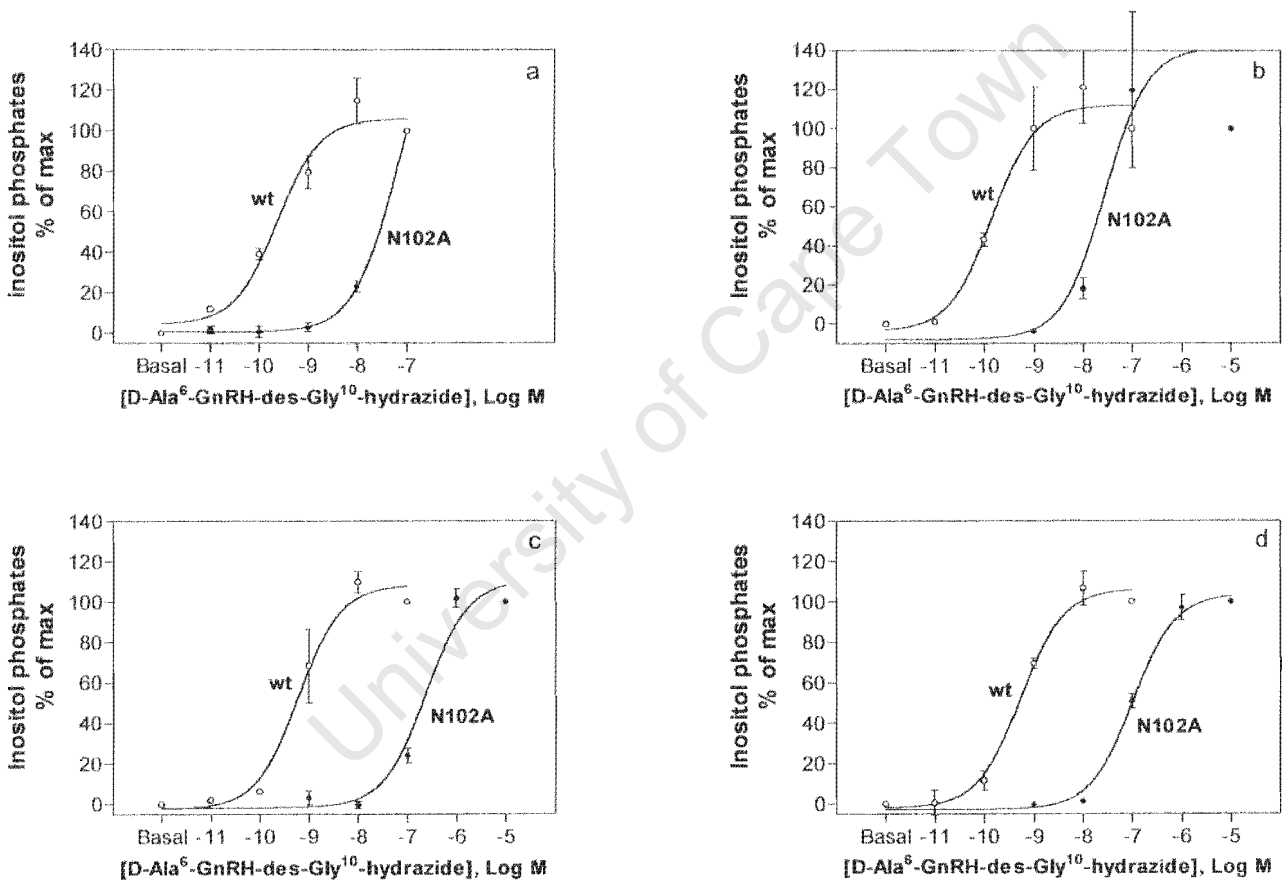


Fig 3.2.20 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-hydrazide. The data points represent the means ±SEM of the combined data from four independent experiments illustrated in Fig 3.2.19.

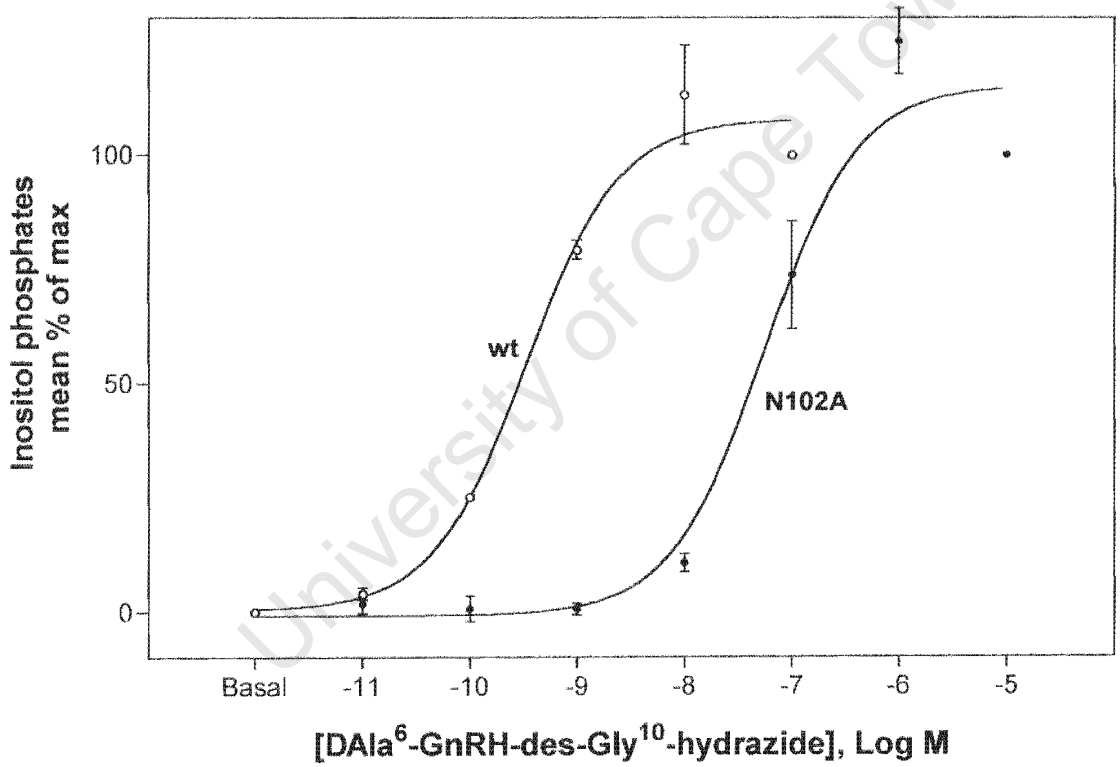


Fig 3.2.21 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-ethylamide. Data represent two experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

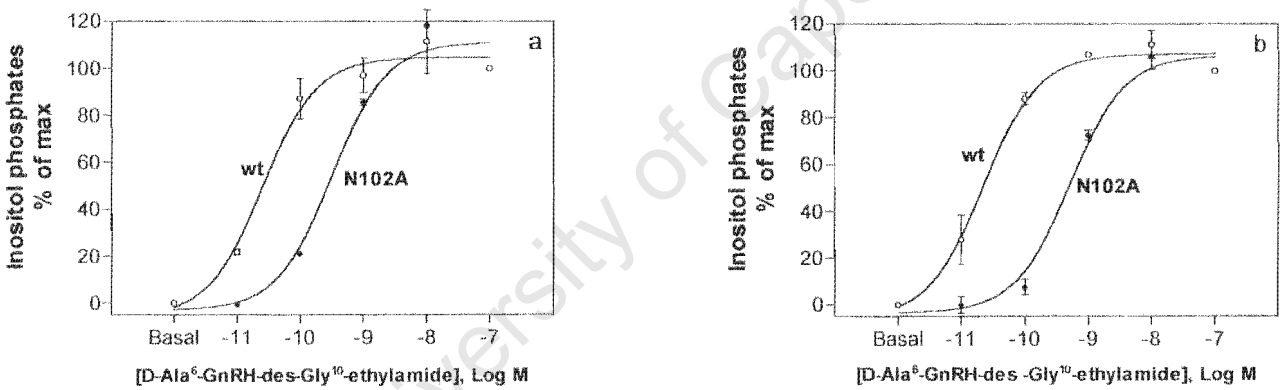


Fig 3.2.22 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-ethylamide. The data points represent the means ±SEM of the combined data from two independent experiments illustrated in Fig 3.2.21.

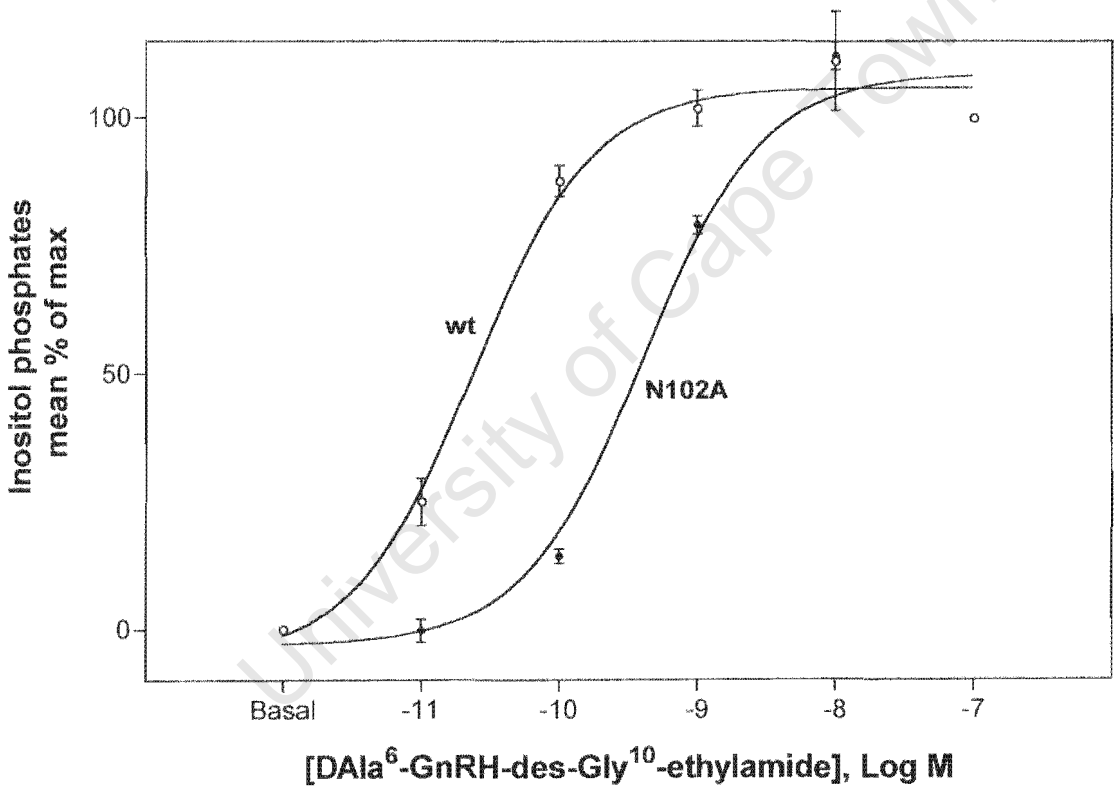


Fig 3.2.23 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-propylamide. Data represent four experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

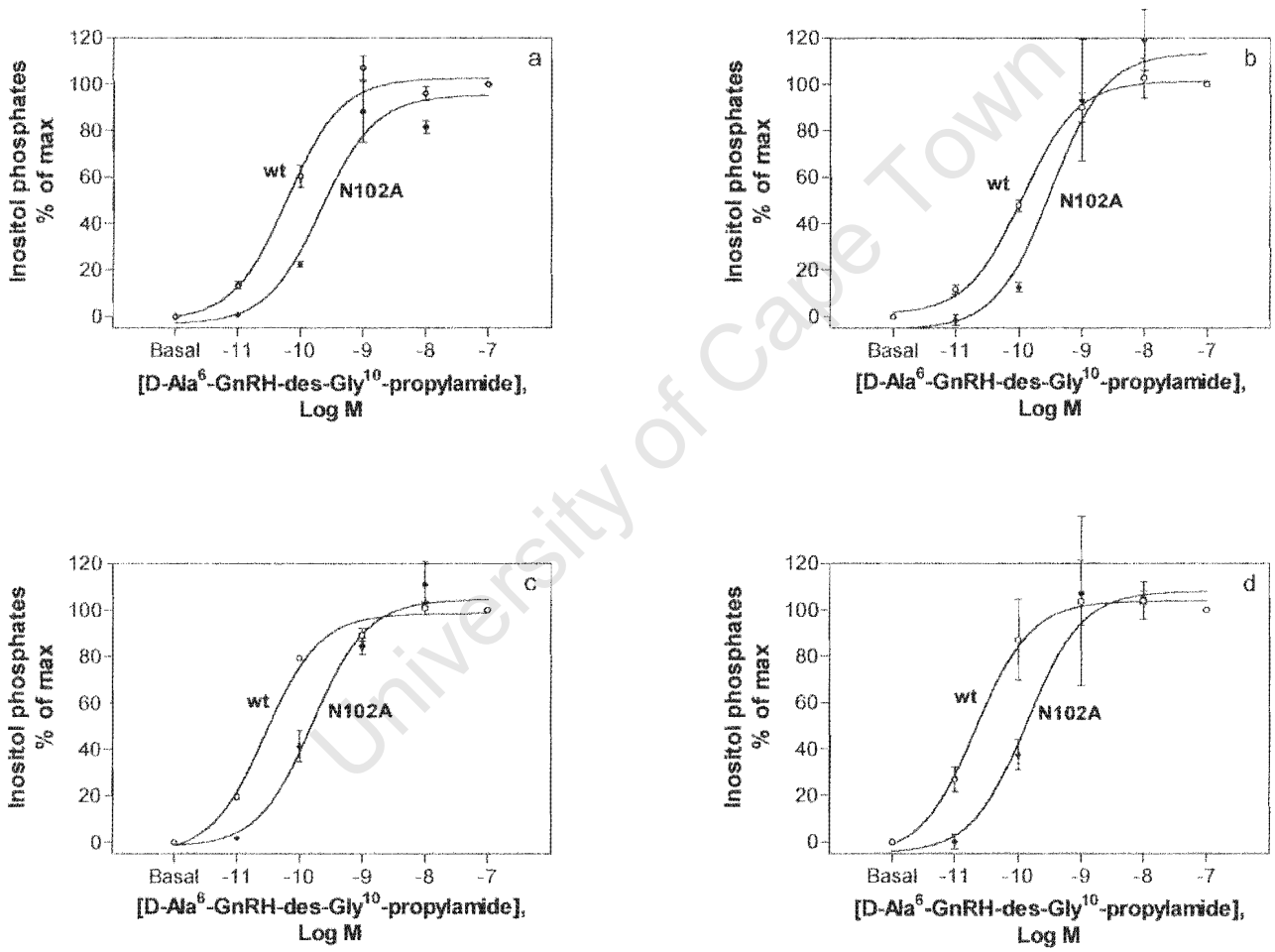


Fig 3.2.24 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-propylamide. The data points represent the means ±SEM of the combined data from four independent experiments illustrated in Fig 3.2.23.

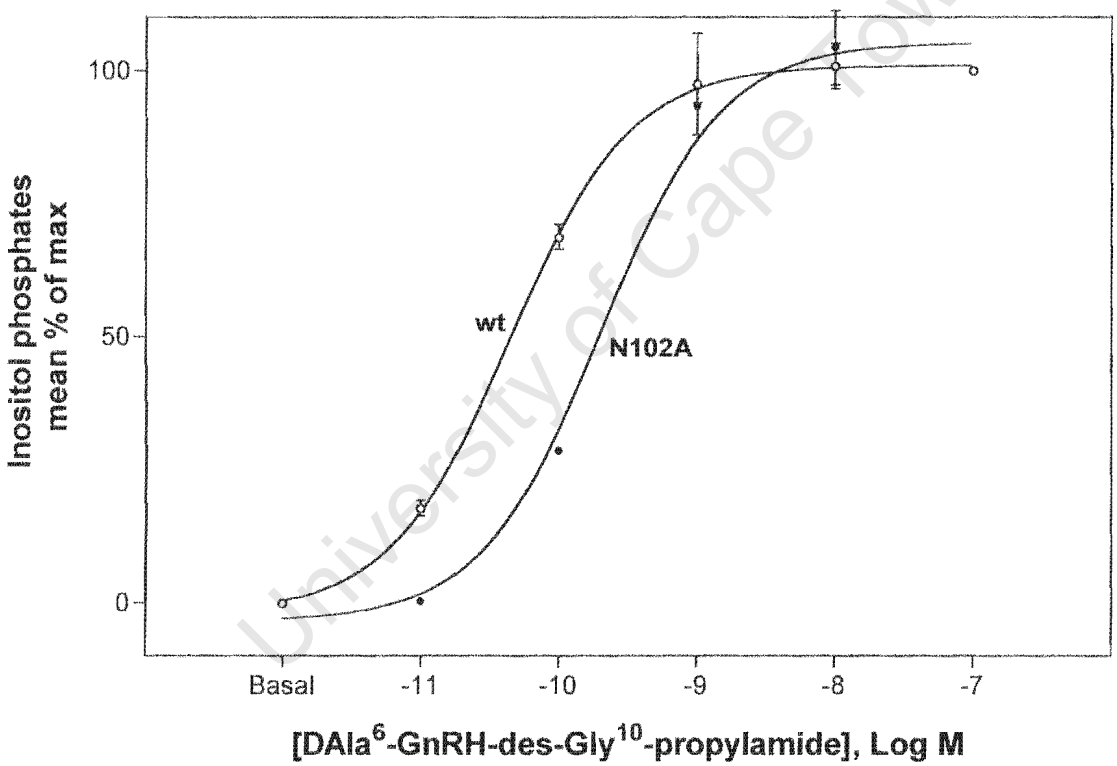


Fig 3.2.25 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-trifluoroethylamide. Data represent five experiments each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

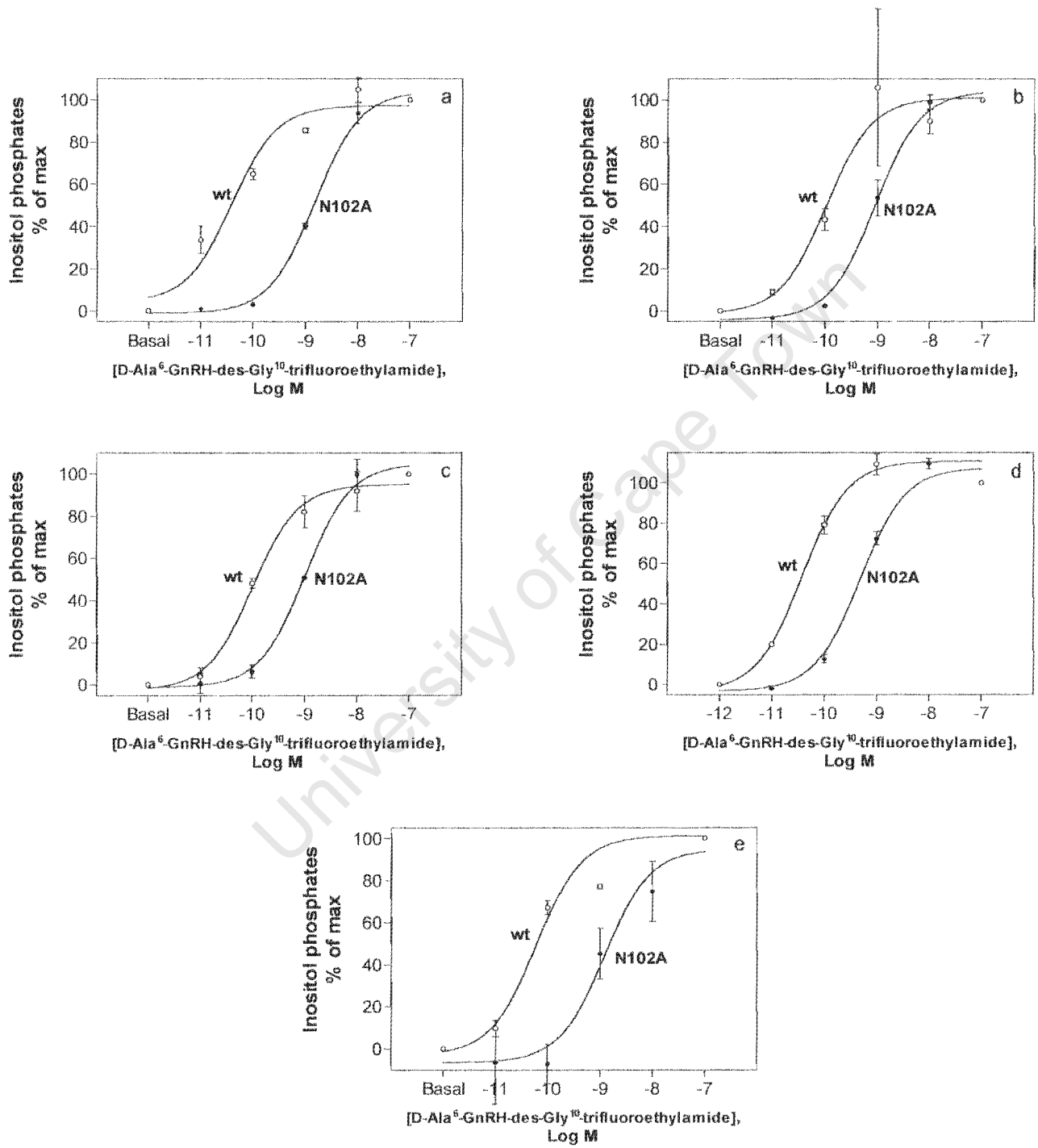


Fig 3.2.26 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-trifluoroethylamide. The data points represent the means ±SEM of the combined data from five indepedent experiments illustrated in Fig 3.2.25.

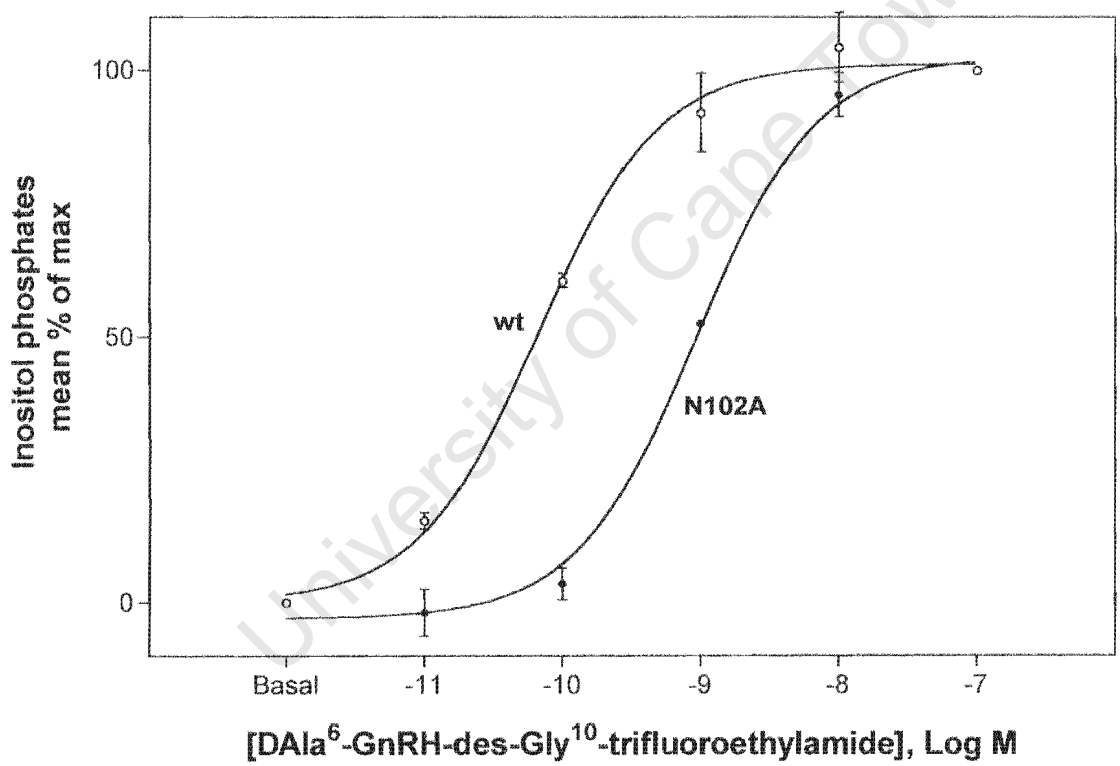


Fig 3.2.27 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^6-GnRH-des-Gly^{10}-pentafluoropropylamide. Data represent three experiment each performed in duplicate. Error bars show the range of duplicate values. Data has been normalized as a % of maximal response.

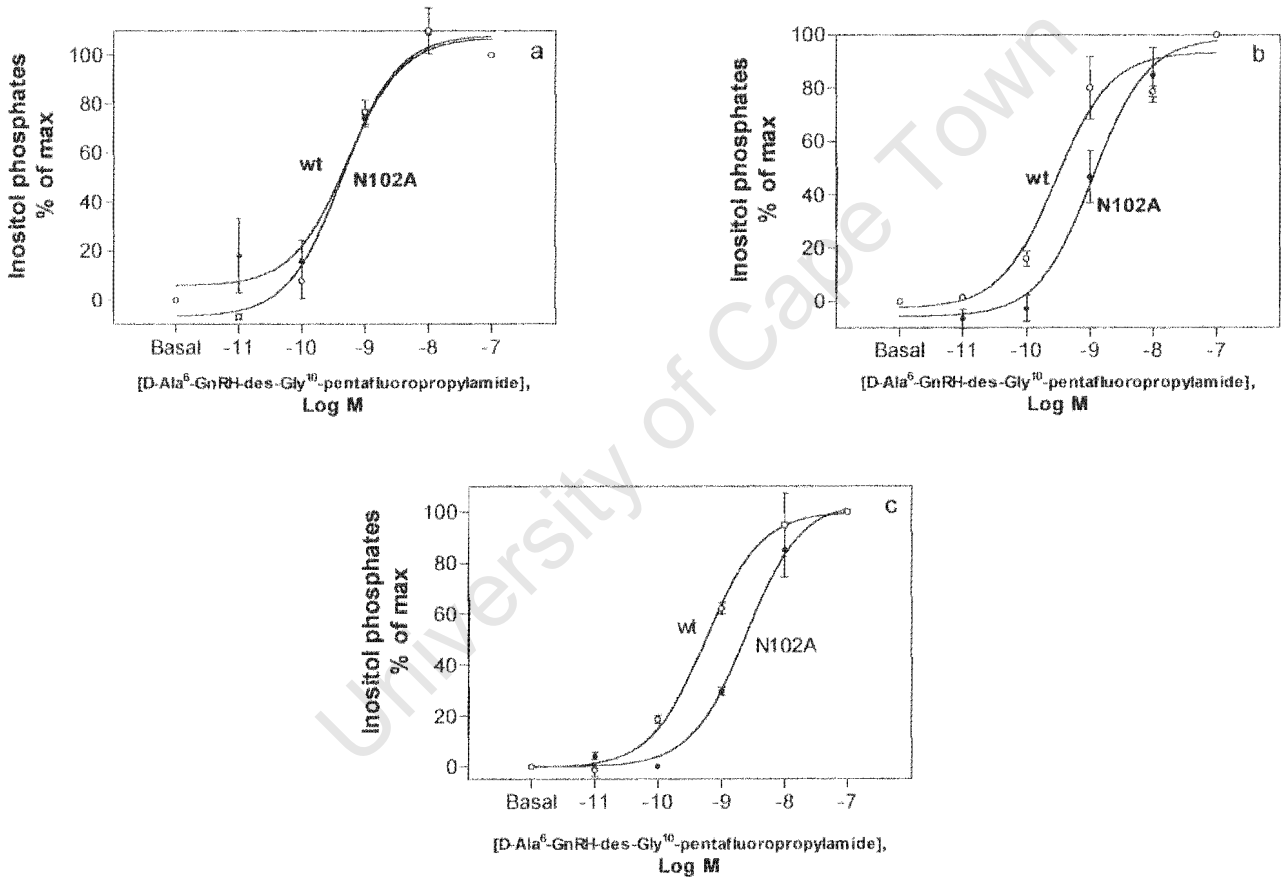


Fig 3.2.28 Inositol phosphate response mediated by wt hGnRH and N102A mutant receptors stimulated with D-Ala^{6}-GnRH-des-Gly^{10}-pentafluoropropylamide. The data points represent the means ±SEM of the combined data from three independent experiments illustrated in Fig 3.2.27.