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CELLULAR AND MOLECULAR CHARACTERIZATION OF THE TYPE I GONADOTROPIN RELEASING HORMONE RECEPTOR

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CELLULAR AND MOLECULAR CHARACTERIZATION OF THE TYPE I GONADOTROPIN RELEASING HORMONE RECEPTOR

(Spine title: Cellular and Molecular Characterization of GnRH-RI)

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ABSTRACT

The receptor for gonadotropin releasing hormone (GnRH-RI) is a G protein-coupled receptor (GPCR) that plays a key role in reproduction. Despite the clinical importance of the human GnRH-RI, studies defining basic properties of this receptor were lacking and this led me to characterize (i) the spatial characteristics of hGnRH-RI in a homologous (HTR-8/SVneo) and heterologous (HEK 293) cell system, and (ii) the early signaling pathway to which GnRH-RI is coupled in HEK 293 cells. The first study revealed GnRH-RI is weakly expressed at the plasma membrane but strongly expressed on the nuclear membrane of both cell types, indicating that it is a nuclear GPCR. In the second study, I demonstrate that the plasma membrane-bound GnRH-RI/PKC-coupled signaling pathway regulates several PKC isoforms. Based on the responses of various isoforms following receptor activation, it appears that the isoforms may have both unique and redundant roles in regulating GnRH-RI signaling.

KEY WORDS

gonadotropin releasing hormone receptor, GPCR, nuclear membrane, localization, hypogonadotropic hypogonadism, Gq/11, protein kinase C, HEK 293, HTR-8/SVneo

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α	alpha
αT3-1	mouse pituitary gonadotrope cell line
β	beta
β2AR	β_2-adrenergic receptor
γ	gamma
δ	delta
ε	epsilon
ζ	zeta
η	eta
θ	theta
ι	iota
λ	lambda
μ	micro
μCi	microCuries
μg	micrograms
μl	microliter
μM	micromolar
μm	micrometer
Ala	alanine
Arg	arginine
ART	assisted reproductive technology
Asn	asparagine
Asp	aspartic acid
ATI-R	angiotensin II type I receptor
B2-R	bradykinin receptor
BSA	bovine serum albumin
°C	degrees centrigrade
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate

cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary-KI cell line
CO_2	carbon dioxide
COS-1	African green monkey kidney cell line
COS-7	African green monkey kidney cell line
cPKC	conventional PKC
CystLT1-R	leuknotriene D4 receptor
DAG	diacylglycerol
DNA	deoxyribonucleic acid
E	glutamic acid
EP1, 2, 4	prostaglandin E_2 receptor 1, 2 or 4
ER	endoplasmic reticulum
ERK	extracellular signal-related kinase
ETA-R	endothelin A receptor
ETB-R	endothelin B receptor
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GAP	GTPase-accelerating protein
GDP	guanosine-5'-diphosphate
GFP	green fluorescent protein
Gln	glutamine
GnRH	gonadotropin releasing hormone
GnRH-I	gonadotropin releasing hormone I
GnRH-II	gonadotropin releasing hormone II
GnRH-R	gonadotropin releasing hormone receptor
GnRH-RI	gonadotropin releasing hormone receptor type I
GnRH-RII	gonadotropin releasing hormone receptor type II
GnRH-RIII	gonadotropin releasing hormone receptor type III
G protein	guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase

GTP	guanosine-5'-triphosphate
^3H	tritium
h	hour
HA	influenza hemagglutin
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotropin
HEK 293	human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGnRH-RI	human gonadotropin releasing hormone receptor type II
HH	hypogonadotropic hypogonadism
HPG axis	hypothalamic-pituitary-gonadal axis
HTR-8/SVneo	human cytotrophoblast cell line
IP_3	inositol 1,4,5-trisphosphate
JNK	jun-N-terminal kinase
K	lysine
KCl	potassium chloride
$KHCO_3$	potassium bicarbonate
KH_2PO_4	monopotassium phosphate
KOH	potassium hydroxide
Leu	leucine
LH	luteinizing hormone
LHRHR	luteinizing hormone releasing hormone receptor
LiCl	lithium chloride
LPA	lysophosphatidic acid
LPA1R	lysophosphatidic acid type I receptor
Lys	lysine
M	molar
MAPK	mitogen-activated protein kinase
mGluR1a	metabotropic glutamate receptor 1a
mGluR5a	metabotropic glutamate receptor 5a
$MgSO_4$	magnesium sulfate

min	minute
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor κB
NK3-R	tachykinin substance K receptor
NLS	nuclear localization signal
nM	nanomolar
NPC	nuclear pore complex
nPKC	novel PKC
NTF2	nuclear transport factor 2
ORF	open reading frame
PAF	platelet-activating factor
PAFR	platelet-activating factor receptor
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK-1	PIP2-dependent kianse 1
pH	hydrogen ion concentration
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLA_2	phospholipase A_2
PLC-β	phospholipase C-β
PLD	phospholipase D
PME	plasma membrane expression
PS	phosphatidylserine

QCS	quality control system
RGS	regulators of G protein signaling
RNA	ribonucleic acid
RT-PCR	reverse transcrition polymerase chain reaction
s	second
Ser	serine
S.E.	standard error
snRNP	small nuclear riboprotein
TIMPs	tissue inhibitors of matrix metalloproteinases
Tyr	tyrosine
uPA	urokinase-type plasminogen activator

Chapter 1- Introduction

1.1 G Protein-Coupled Receptors

The mammalian Type I Gonadotropin releasing hormone receptor (GnRH-RI), also know as luteinizing hormone releasing hormone receptor (LHRH-R), is a member of the rhodopsin-like, or Class A, guanine nucleotide-binding protein-coupled receptor (GPCR) family (Fig 1.1).

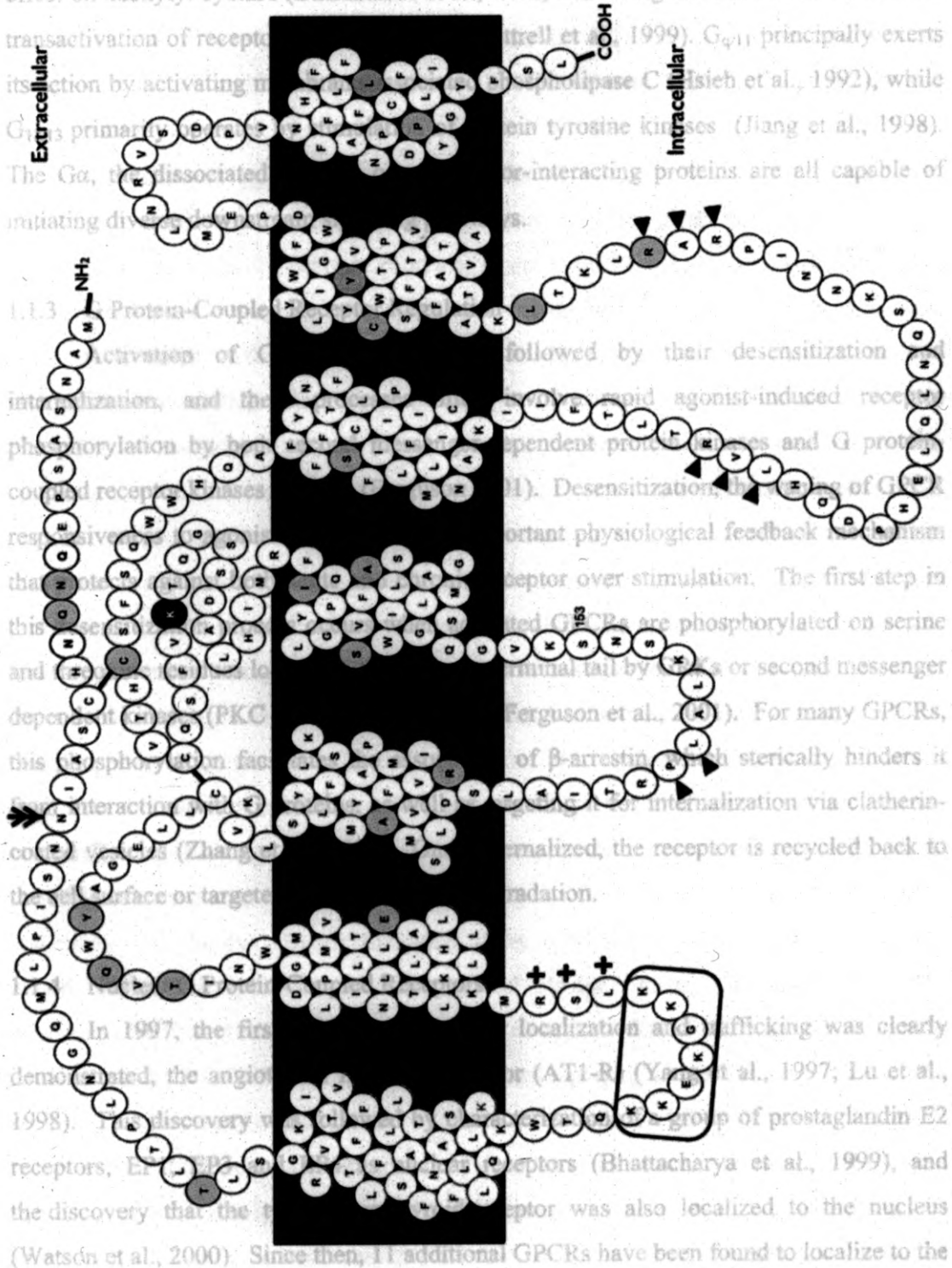
1.1.1 G Protein-Coupled Receptor Structure

GPCRs are a superfamily of cell-surface receptors that transduce extracellular stimuli such as light, odorants, neurotransmitters, hormones and chemokines into intracellular signals, primarily via guanine nucleotide-binding proteins (G proteins) (Ferguson, 2001). Since the cloning of the first GPCR in 1983 (Nathans et al., 1983), 800 members of this receptor superfamily have been discovered. GPCRs share a common molecular structure, including seven transmembrane-spanning domains that make up the hydrophobic core, three extracellular loops and three intracellular loops, an extracellular N-terminus and an intracellular C-terminus. GPCRs can be categorized into three classes: the rhodopsin family, the glucagon receptor family, and the metabotropic glutamate receptor family. Each class represents heptahelical receptors of related gene and amino acid sequence; however, no sequence relationship across classes is apparent (Ferguson, 2001).

1.1.2 G Protein-Coupled Receptor Function

As previously mentioned, GPCRs signal primarily through interaction with G proteins. G proteins are heterotrimeric proteins composed of an α subunit (Gα) that binds guanine nucleotides and a dimer that consists of a β and γ subunit (Gβγ) (Lodish et al., 2003). Ligand activated GPCRs cause Gα to dissociate from the Gβγ dimer rendering it to its active GTP-bound form that influences effector molecules. The βγ dimer remains attached to the plasma membrane and can by itself initiate several signaling events (Lodish et al, 2003). G proteins are classified according to the subtype of their α subunit into the four groups: G_s, G_i, $G_{q/11}$, and $G_{12/13}$. G_s mainly exerts its downstream effects via stimulation of adenylyl cyclase, which induces the production of high levels of the second messenger cAMP (Ulloa-Aguirre et al., 1999). Unlike G_s, the G_i protein has an inhibitory

Figure 1.1 Schematic of the human GnRH-RI showing the positions of its naturally occurring mutations and other residues of interest. Human GnRH-RI depicting the location of the 21 known natural mutations identified to date (grey circles), position of the lysine 191 residue (black circle) and the location of the putative NLS (square). Other residues of interest include a glycosylation site at Asp18 (branching symbol), $G_{q/11}$ coupling sites (arrowheads), G_s coupling sites (crosses) and the Ser153 that has been indicating in receptor desensitization.


Extracellular
Intracellular
COOH
NH2
153

effect on adenylyl cyclase (Birnbaumer et al., 1992) and its signals can be transmitted via transactivation of receptor tyrosine kinases (Luttrell et al., 1999). $G_{q/11}$ principally exerts its action by activating membrane-associated phospholipase C (Hsieh et al., 1992), while $G_{12/13}$ primarily operates by stimulation of protein tyrosine kinases (Jiang et al., 1998). The Gα, the dissociated βγ, and other receptor-interacting proteins are all capable of initiating diverse downstream signaling pathways.

1.1.3 G Protein-Coupled Receptor Regulation

Activation of GPCRs is typically followed by their desensitization and internalization, and these processes often involve rapid agonist-induced receptor phosphorylation by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs) (Ferguson 2001). Desensitization, the waning of GPCR responsiveness to agonist with time, is an important physiological feedback mechanism that protects against both acute and chronic receptor over stimulation. The first step in this desensitization process occurs when activated GPCRs are phosphorylated on serine and threonine residues located in the carboxy-terminal tail by GRKs or second messenger dependent kinases (PKC or PKA for example)(Ferguson et al., 2001). For many GPCRs, this phosphorylation facilitates the association of β-arrestin, which sterically hinders it from interaction with G proteins, as well as targeting it for internalization via clatherin-coated vesicles (Zhang et al., 1997). Once internalized, the receptor is recycled back to the cell surface or targeted to lysosomes for degradation.

1.1.4 Nuclear G Protein-Coupled Receptors

In 1997, the first GPCR with nuclear localization and trafficking was clearly demonstrated, the angiotensin II type I receptor (AT1-R) (Yang et al., 1997; Lu et al., 1998). This discovery was followed by characterization of a group of prostaglandin E2 receptors, EP1, EP3 and EP4 as nuclear receptors (Bhattacharya et al., 1999), and the discovery that the type I parathyroid receptor was also localized to the nucleus (Watson et al., 2000). Since then, 11 additional GPCRs have been found to localize to the nucleus: the apelin receptor, bradykinin receptor (B2-R), the endothelin A and B receptors (ETA-R, ETB-R), the leukotriene D4 receptor (CystLT1-R), the

lysophosphatidic acid type I receptor (LPA1R), the metabotropic glutamate receptor 5 (mGluR5a), the platelet-activating factor receptor (PAFR), the tachykinin substance K receptor (NK3-R), and the orphan receptor GPCR6A (see Table 1.1 for details and references).

Transport of proteins into the nucleus is normally directed by a nuclear localization signal (NLS) sequence. NLS sequences were first discovered when the observation was made that certain proteins, larger than the passive diffusion limit (~50 kDa) for the nuclear pore complex (NPC) can accumulate within the nucleus (Hanover, 1992). The first NLS sequence was found in the simian virus 40 large-T antigen (Dingwall et al., 1982). Since then, a variety of NLS sequences have been found in thousands of nuclear localized proteins. NLS sequences come in many varieties, including the classical monopartite or bipartite varieties, where monopartite consists of a string of basic amino acid residues and bipartite consists of two stretches of basic amino acid residues separated by a spacer region. Nuclear localization of a protein occurs when a free importin in the cytoplasm binds to the NLS of a cargo protein, forming a bimolecular cargo complex. The adapter protein importin α bridges the NLS and importin β, forming a trimolecular cargo complex which then diffuses through the NPC by interacting with successive FG-nucleoporins. In the nucleoplasm, interaction of Ran-GTP with the importin causes a conformational change that decreases its affinity for the NLS, releasing the cargo. To support another cycle of import, the importin-Ran-GTP complex is transported back to the cytoplasm. A GTPase-accelerating protein (GAP) associated with the cytoplasmic filaments of the NPC stimulates Ran to hydrolyze the bound GTP. This generates a conformational change causing dissociation from the importin, which can then initiate another round of import. Ran-GDP is bound by nuclear transport factor 2 (NTF2) and returned to the nucleoplasm, where a guanine nucleotide-exchange factor (GEF) causes release of GDP and rebinding of GTP (Lodish et al., 2003; Macara, 2001). Of the 13 GPCRs that have demonstrated nuclear localization, 9 of these contain a consensus sequence for an NLS, although only 3 of them, AT1-R, the apelin receptor and CystLT1-R have shown NLS functionality. It is interesting to note that while AT1-R and the apelin receptor are both found in the nucleoplasm, which would fit the well-known model of NLS trafficking, CystLT1-R is found on the outer nuclear

Table 1.1 Summary table of nuclear GPCRs described in the literature. Receptor abbreviation, GPCR class, requirement of agonist status, specific nuclear localization, NLS sequence and references are described. Adapted from BW Pickard. Used with permission.Figure 1.2 Species expressing GnRH receptors, organized by type I, II and III receptor classes. The type I and II GnRH-R classes are divided into mammalian and non-mammalian species.

	Class	Agonist Activated	Nuclear Distribution	NLS Motif	References
AT_1-R	A	Yes/No	Nucleoplasm	KKFKR	Yang et.al. 1997, Lu et.al. 1998, Lee et.al. 2004
EP_1, EP_3, EP_4	A	No	Nuclear Membrane	Unknown	Bhattacharya et.al. 1999
ETA-R	A	Yes	Nuclear Membrane	KKFK	Boivin et.al. 2003
ETB-R	A	Yes	Nuclear Membrane	KRFK	Boivin et.al. 2003
Apelin R	A	No	Nucleoplasm	RKRRR	Lee et.al. 2004
B2-R	A	No	Nucleoplasm	KRFRK	Lee et.al. 2004
$CysLT_1$-R	A	Yes	Nuclear Membrane	RKHSLSSV TYVPRKK	Nielsen et.al. 2005
PAFR	A	Yes	Nuclear Membrane	KKFRK	Marrache et.al. 2005
LPA_1 R	A	Yes	Nuclear Membrane	Unknown	Marrache et.al. 2005
NK3-R	A	Yes/No	Nucleoplasm	KRFR	Howe et.al. 2004
PTH1R	B	Unknown	Nucleoplasm	KKSWSRWL ALDFKRKAR	Watson et.al. 2000a/b, Pickard et.al. 2006
mGlu5	C	Yes	Nuclear Membrane	Unknown	O'Malley et.al. 2003, Jong et.al. 2005
GPCR6A	C	Unknown	Nuclear Membrane	Unknown	Kuang et.al. 2005

membrane and yet its nuclear localization is disturbed if its NLS sequence is truncated (Nielsen et al., 2005).

1.2 Gonadotropin Releasing Hormone Receptor

In 1992, Tsutsumi et al. cloned and sequenced the mouse GnRH receptor from the mouse pituitary cell line αT3-1. This sequence showed over 80% amino acid identity with GnRH receptors later found in rat, human, sheep, cow, and pig (Reinhart et al., 1992; Kaiser et al., 1992; Perrin et al., 1993; Eidne et al., 1992; Chi et al., 1993; Kakar et al., 1992; Illing et al., 1993; Brooks et al., 1993; Kakar, 1997; Weesner et al., 1994). Three forms of GnRH-R have been described and are found in a wide range of vertebrates (Fig 1.2). The type I GnRH-R regulates the hypothalamic-pituitary-gonadal HPG axis and is considered the mammalian GnRH-R, although some primates produce the full-length type II GnRH-R (Figs 1.2, 1.3). The hGnRH-RI gene is composed of three exons separated by two introns and spans more than 15 kb along chromosome 4q21.2 (Fan et al., 1995; Kakar et al., 1997). The predicted size of the mRNA transcript is 2160bp and encodes a 328 amino acid protein (Chi et al., 1993).

Non-mammalian vertebrates possess the type II receptor, which, unlike the type I receptor, has a carboxy-terminal tail of varying length. Non-mammalian GnRH-RII does not possess the premature stop codon of the human type II receptor. Types I and II GnRH-R show considerable sequence homology, including conservation of amino acids important for ligand binding and effector coupling (Troskie et al., 2000) but have important structural differences, such as the aforementioned lack of a carboxy-terminal tail in the type I receptor. A putative human type II GnRH receptor (GnRH-RII) gene has been reported on chromosome 1q12 (Millar et al., 1999; Neill et al., 2002; Morgan et al., 2003). This gene shares 40% sequence homology with the type I receptor, however a -1 frameshift changes the codon for Arg179 to a UGA premature stop codon (Morgan et al., 2003). This would generate a receptor with 5 instead of 7 transmembrane domains. There is evidence suggesting that the truncated protein is sufficient for normal GPCR functioning. In a study by Ling et al. (1999) it was demonstrated that truncated chemokine receptors, which also only contain 5 transmembrane domains, behave as the wild-type receptor. Coexpression of the truncated GnRH-RII with GnRH-RI in COS-7

Figure 1.2 Species expressing GnRH receptors, organized by type I, II and III receptor classes. The type I and II GnRH-R classes are divided into mammalian and non-mammalian species.

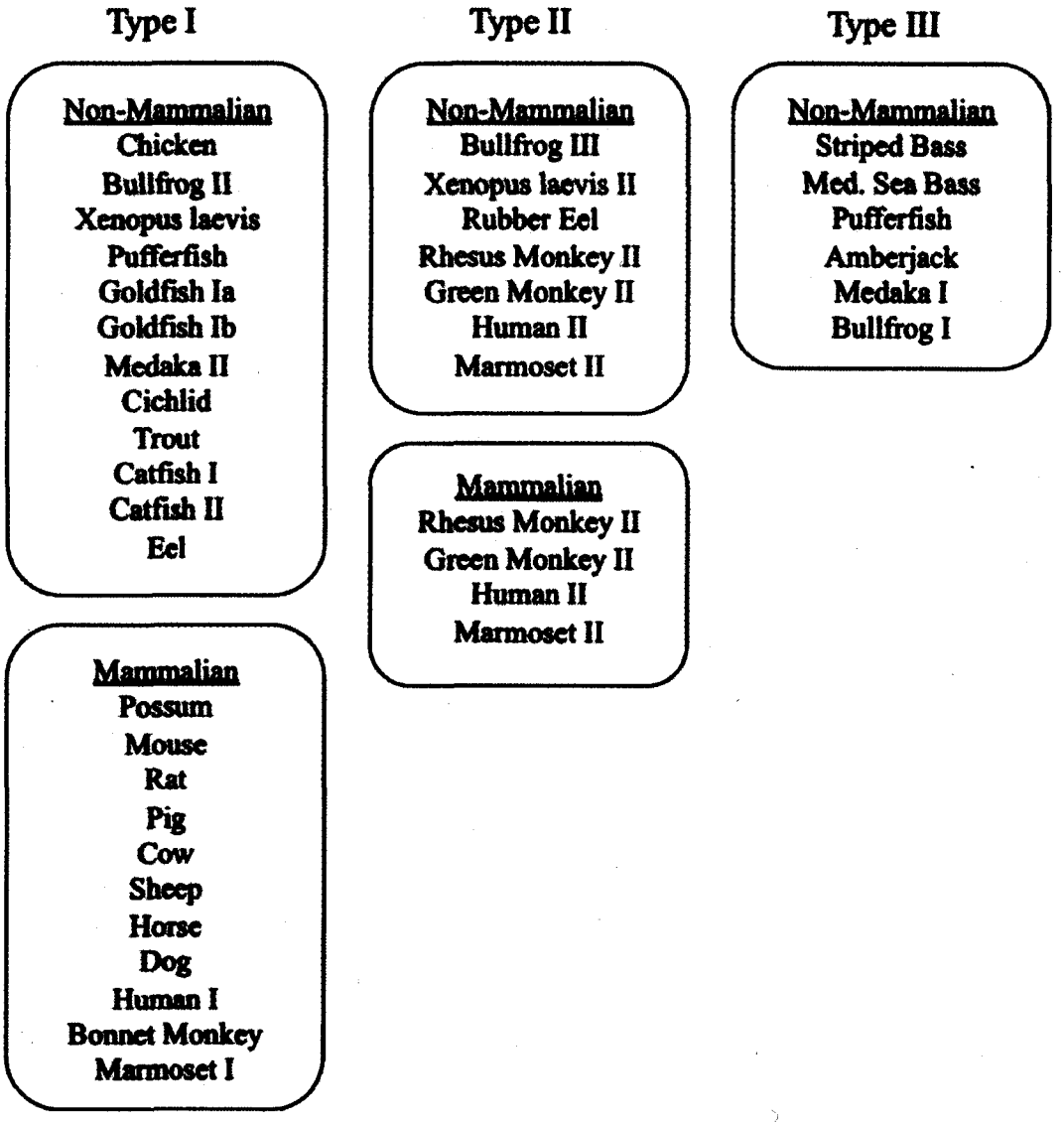
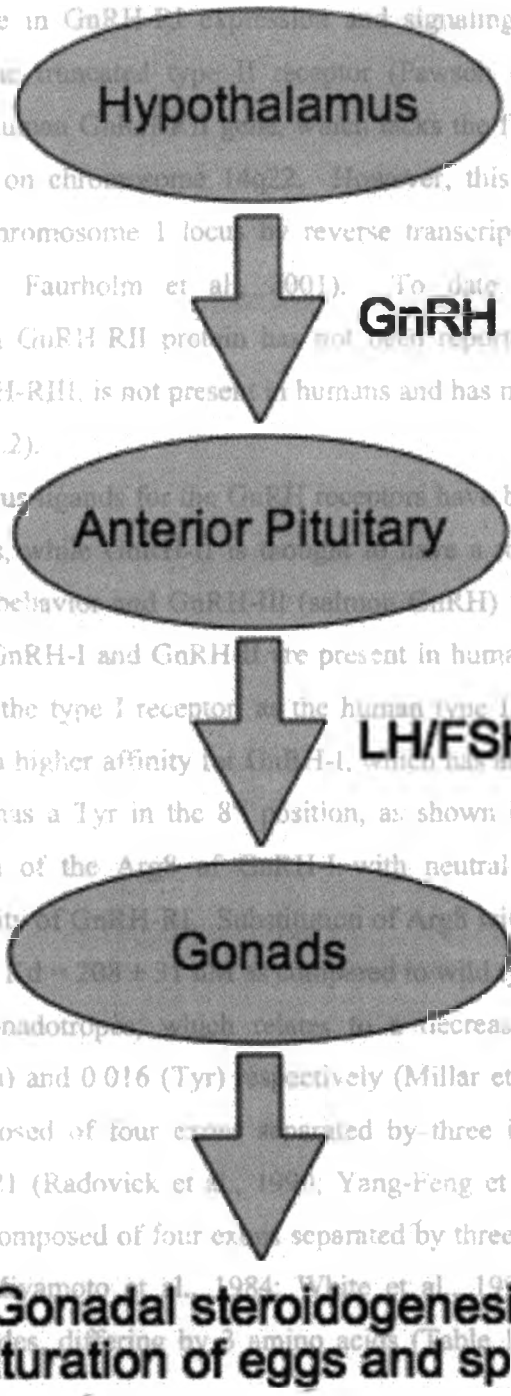

Type I
Non-Mammalian
Chicken
Bullfrog II
Xenopus laevis
Pufferfish
Goldfish Ia
Goldfish Ib
Medaka II
Cichlid
Trout
Catfish I
Catfish II
Eel
Mammalian
Possum
Mouse
Rat
Pig
Cow
Sheep
Horse
Dog
Human I
Bonnet Monkey
Marmoset I
Type II
Non-Mammalian
Bullfrog III
Xenopus laevis II
Rubber Eel
Rhesus Monkey II
Green Monkey II
Human II
Marmoset II
Mammalian
Rhesus Monkey II
Green Monkey II
Human II
Marmoset II
Type III
Non-Mammalian
Striped Bass
Med. Sea Bass
Pufferfish
Amberjack
Medaka I
Bullfrog I

Figure 1.3 Schematic of the hypothalamic-pituitary-gonadal (HPG) axis. The gonadotropin releasing hormone receptor plays a crucial role in reproduction. GnRH is released from the hypothalamus into the portal circulation and binds its receptor on the anterior pituitary. This causes the release of FSH and LH into the systemic blood where they act on the gonads to regulate gonadal steroidogenesis as well as the maturation of sperm and eggs.


Hypothalamus
GnRH
Anterior Pituitary
LH/FSH
Gonads
Gonadal steroidogenesis
Maturation of eggs and sperm

cells causes a decrease in GnRH-RI expression and signaling, suggesting a possible modulatory role for the truncated type II receptor (Pawson et al., 2005). Another truncated copy of the human GnRH-RII gene, which lacks the first exon and part of the first intron, is present on chromosome 14q22. However, this is likely a pseudogene originating from the chromosome 1 locus by reverse transcription (Neill et al., 2002; Morgan et al., 2003; Faurholm et al., 2001). To date, a conventional seven transmembrane domain GnRH-RII protein has not been reported in humans. A third receptor subclass, GnRH-RIII, is not present in humans and has not been identified in any mammals to date (Fig 1.2).

Three endogenous ligands for the GnRH receptors have been described. GnRH-I regulates the HPG axis, while GnRH-II is thought to have a role as a neuromodulator affecting reproductive behavior and GnRH-III (salmon GnRH) functions only in teleost (Millar et al., 2004). GnRH-I and GnRH-II are present in humans (Table 1.2) and both ligands signal through the type I receptor, as the human type II receptor is a truncated protein. GnRH-RI has a higher affinity for GnRH-I, which has an Arg in the 8th position, than GnRH-II, which has a Tyr in the 8th position, as shown by previous studies that found that substitution of the Arg8 of GnRH-I with neutral amino acids markedly decreased peptide affinity of GnRH-RI. Substitution of Arg8 with Leu or Tyr resulted in a Kd = 590±80 nM and Kd = 208 ± 31 nM as compared to wild type GnRH-I with a Kd = 3.4±0.2 nM in rat gonadotropes, which relates to a decrease in potency from 1.0 (wildtype) to 0.06 (Leu) and 0.016 (Tyr) respectively (Millar et al., 1989). The human GnRH-I gene is composed of four exons separated by three introns and is found on chromosome 8p11.2-p21 (Radovick et al., 1990; Yang-Feng et al., 1986). The human GnRH-II gene is also composed of four exons separated by three introns, but is found on chromosome 20p13 (Miyamoto et al., 1984; White et al., 1998). Both GnRH-I and GnRH-II are decapeptides, differing by 3 amino acids (Table 1.2) (White et al., 1998; Millar, 2003).

1.3 GnRH-RI Structure

Human GnRH-RI belongs to the rhodopsin family of GPCRs and contains many amino acids residues important for GPCR functioning. These include Ala261 in the third

Table 1.2 Protein sequences and sites of expression of GnRH-I and GnRH-II in the human body.

GnRH-I: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

GnRH-II: pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2

Area	GnRH-I	GnRH-II
Brain	Preoptic area, basal hypothalamus Median eminence and infundibular stalk Adenohypophysis Septal region, anterior olfactory area	Periaqueductal region of the midbrain Caudate nucleus (basal ganglia) Amygdala (medial temporal lobe)
Placenta	+	+
Uterus	All endometrial types	Stromal and epithelial cells
Ovary	Granulosa-luteal cells Ovarian surface epithelial cells Ovarian carcinoma Fallopian tube epithelium (luteal phase)	Granulosa-luteal cells Ovarian surface epithelial cells Ovarian carcinoma
Jurkat leukemic T cells	+	+

intracellular loop, which is necessary for G protein coupling and receptor internalization (Myburgh et al., 1998), as well as residues Asp98, Trp101, Asn102, Lys121, Asn212, and Asp302 which are important for ligand binding (Millar et al., 2004). Although the human GnRH-RI belongs to the rhodopsin class of GPCRs, they are structurally unique among GPCRs in that the mammalian GnRH-RIs do not have a cytoplasmic tail (Fig 1.1), making them the smallest known GPCRs. The lack of a cytoplasmic tail sequence bestows some very unique characteristics upon the mammalian GnRH-RI, including its inability to undergo rapid β-arrestin-dependent desensitization as well as an unusually high intracellular expression, which are detailed further below. GnRH-RI, like many other receptors, functions as a dimer at the plasma membrane of pituitary cells (Hazum et al., 1985) as well as in the African green monkey kidney cell line COS-7 (Brothers et al., 2004).

1.3.1 Cellular Localization of Mammalian GnRH-RI

The loss of cytoplasmic tail sequences during evolution is believed to be the reason why GnRH-RI displays reduced plasma membrane expression (PME) (Janovick et al., 2006). When the carboxy-terminal tail from the catfish GnRH-RI was cloned onto the human GnRH-RI, PME was increased as shown by the ability of the receptor to produce inositol trisphosphate (IP_3) in response to the GnRH-RI agonist Buserelin (Janovick et al., 2003). The carboxy terminal tail of the catfish GnRH-R is serine-rich (9 Ser of 51 amino acids) and contains a single consensus site for palmitoylation (Bouvier et al., 1995), the covalent attachment of fatty acids, such as palmitic acid, to cysteine residues of membrane proteins that enhances the hydrophobicity of proteins and contributes to their membrane association. The PME of the GnRH-RI decreases with higher stages of evolution, as demonstrated by the differences in PME levels among fish, rodents, and primates (Conn et al., 2002).

1.3.2 Effect of Lysine 191 on hGnRH-RI Localization

Another portion of the receptor that has been indicated as important for the PME of the human GnRH-RI is the lysine 191 residue present in the second extracellular loop of the receptor (Fig 1.1). This residue is primate-specific and contributes to reduced

cellular expression and increased internalization kinetics of the receptor leading to an overall reduction in PME (Arora et al., 1999; Maya-Nunez et al., 2000). It is believed that the presence of this primate-specific lysine 191 causes disruption of a sulfhydryl bridge, causing formation of misfolded receptors, which lead to the retention of the receptor in the endoplasmic reticulum (ER) and eventual degradation by the cell's quality control system (QCS) (Janovick et al., 2006). The QCL 'proof-reads' newly synthesized proteins, so that only native conformers reach their final destinations (Ellgaard and Helenius, 2003). Removal of the lysine 191 residue from the hGnRH-RI increased the PME as shown by IP_3 and radioligand binding assays (Janovick et al., 2006). Interestingly, in the hGnRH-RI deletion of the lysine 191 residue in combination with addition of the catfish carboxy-terminal tail leads to PME greater than the additive effect (Maya-Nunez et al., 2000). The misfolding of the human GnRH-RI, as for any other protein, results in the nascent protein being retained by the QCS of the ER. This system recognizes misfolded proteins by the exposure of hidden hydrophobic domains, unpaired cysteine residues and the tendency to aggregate (Ellgaard et al., 2003). If molecular chaperones are unable to stabilize a protein, then it will either lead to accumulation in the ER or be targeted to a proteasome for degradation (Werner et al., 1996). Both the lack of the carboxy-terminal tail and the presence of the lysine 191 residue cause hGnRH-RI to be primarily located in the intracellular compartment as shown by biochemical assays. Mutation of hGnRH-RI, discussed below, also leads to the rentention of this receptor within the QCS and decreases PME.

1.4 GnRH-RI Function

As a GPCR, hGnRH-RI transduces extracellular stimuli into an intracellular signal via interactions with G-proteins. The nature of the G protein-coupling depends largely on the cellular context. The most thoroughly studied signaling pathway of hGnRH-RI is its $G_{q/11}$-coupled pathway in pituitary gonadotropes, where it plays a key role in the process of reproduction through regulation of the hypothalamic-pituitary-gonadal (HPG) axis (Fig 1.3). GnRH-RI is located on gonadotropes of the anterior pituitary where it binds its cognate ligand, gonadotropin releasing hormone type I (GnRH-I). GnRH-I is released from the hypothalamic neurosecretory cells in a pulsatile manner into the hypothalamo-

hypophyseal portal circulation (Conn and Crowley, 1994). Ligand-activated hGnRH-RI couples preferentially to the $G_{q/11}$ protein and stimulates phospholipase C-β-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) leading to the formation of second messengers, IP_3 and diacylglycerol (Fig 1.4). IP_3 subsequently stimulates the release of intracellular Ca^{2+} from the endoplasmic reticulum (Streb et al., 1983). The Ca^{2+} and DAG produced downstream of $G_{q/11}$ activate the conventional protein kinase C (cPKC) isoforms, which are part of a ubiquitously-expressed family of serine/threoine kinases that will be discussed in further detail below. After a short lag, GnRH-RI activates phospholipase A_2 (PLA_2) and phospholipase D (PLD) and provide late DAG $Ca^{2+}$and arachidonic acid (AA) for activation of Ca^{2+}-independent PKC isoform (Naor et al., 1990; Naor et al., 1995; Shacham et al., 2001). Additional products of PLA_2 such as arachidonate and oleate also activate several PKC isoforms (Bell et al., 1991). The activation of PKC leads to the activation of members of the mitogen-activated protein kinase (MAPK) superfamily, specifically extracellular signal-regulated kinase (ERK), jun-N-terminal kinase (JNK) and p38MAPK (Naor et al., 2000; Benard et al., 2001; Bonfil et al., 2004; Kraus et al., 2001; Levi et al., 1998; Mulvaney et al., 1999; Mulvaney et al., 2000). These pathways are responsible for gonadotropin regulation. The biosyntheis of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are primarily due to PKC and MAPK signaling, while Ca^{2+} is responsible for their exocytosis from pituitary gonadotropes into the systemic blood (Naor et al., 1998). The culminating effect of hGnRH-RI stimulation in pituitary gonadotropes is the production of LH and FSH, which regulate gonadal steroidogenesis and gametogenesis in both sexes (Fig 1.3) (Conn and Crowley, 1994).

In addition to this well-known endocrine function, it has become evident that GnRH-RI and its ligands may play an important role in autocrine and/or paracrine regulation in some extra-pituitary compartments such as the ovary, placenta, uterus, immune cells, olfactory neurons, sperm as well as in many cancerous cells (Cheng et al., 2005). These sites are summarized in Table 1.3. Some of the roles of GnRH-RI at these sites have been identified. In brief, activated GnRH-RI decreases proliferation of a number of cancerous cells, including melanoma (Moretti et al., 2003), ovarian (Choi et al., 2001), endometrial (Grundker et al., 2002), breast (Miller et al., 1985) and prostate

Figure 1.4 $G_{q/11}$-coupled pathway. GnRHR is a G protein-coupled receptor, or GPCR belonging to the rhodopsin family and couples predominantly to Gq, in the human pituitary and placenta. Upon ligand activation, GnRH-RI causes the dissociation of $G\alpha_{q/11}$ from the $\beta\gamma$ subunit, allowing it to activate phospholipase C. Phospholipase C leaves the membrane phospholipid PIP_2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3) for short. IP_3 diffuses into the cytosol and opens ligand-gated calcium channels on the endoplasmic reticulum, releasing calcium into the cytosol. Calcium, along with membrane-bound diacylglcerol activate protein kinase C or PKC which phosphorylates proteins involved in downstreams effects such as secretion, cell proliferation, differentiation and apoptosis. Figure created by Natasha Camuso. Used with permission.

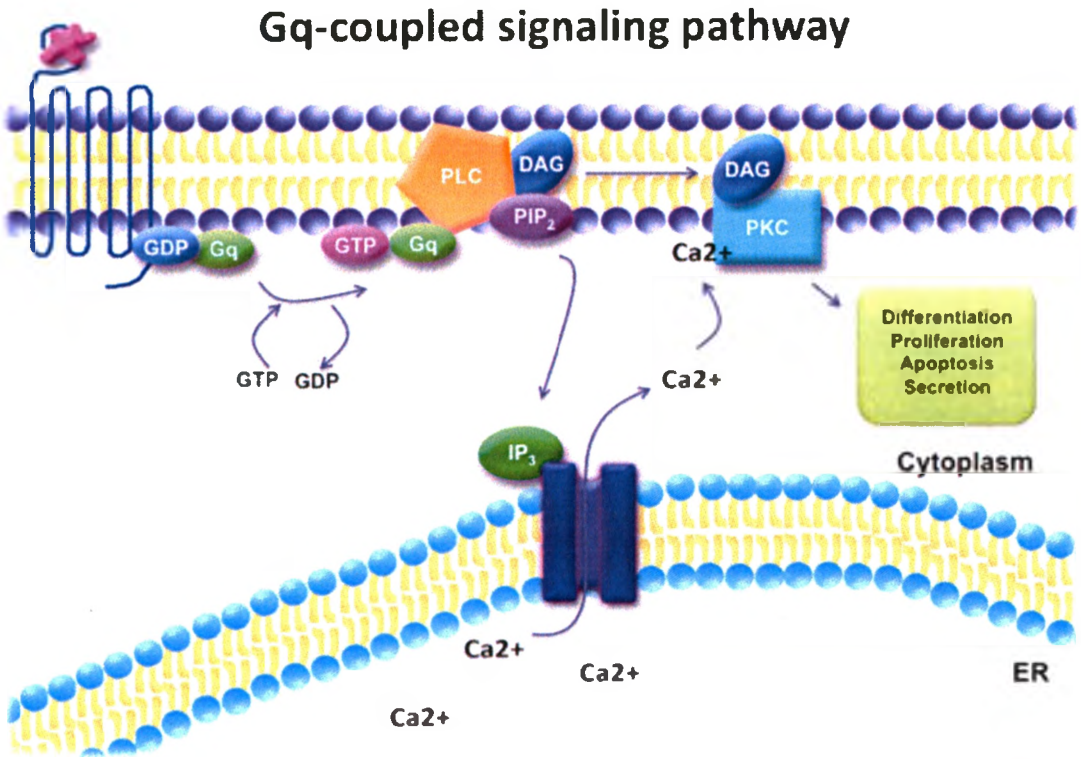

Gq-coupled signaling pathway
PLC
DAG
PIP2
DAG
PKC
GDP
Gq
GTP
Gq
Ca2+
GTP
GDP
Ca2+
Differentiation
Proliferation
Apoptosis
Secretion
IP3
Cytoplasm
Ca2+
Ca2+
ER
Ca2+

Table 1.3 Sites of expression and functions of GnRH-RI in the human body. Studies reviewed in Cheng and Leung, 2005.

Target Site	Action
Anterior pituitary gonadotropes	Biosynthesis and secretion of LH and FSH
Ovarian cancer cell	Decreases proliferation
Endometrial cancer cell	Decreases proliferation
Prostate cancer cell	Decreases proliferation
Melanoma cell	Decreases proliferation
Epidermoid cancer cell	Decreases proliferation
Uterine leiomyoma cell	Decreases proliferation
Ovarian OSE cell	Decreases proliferation
Endometriotic cell	Increases apoptosis
Ovarian GL cell	Increases apoptosis, decreases steroidogenesis
Placenta	hCG release
Cytotrophoblast	Increases uPA, MMP-2 and MMP-9 expression Decreases PAI-I and TIMP-1 expression
Decidual stromal cell	Increases uPA, MMP-2 and MMP-9 expression Decreases PAI-I
T cell	Increases proliferation, adhesion, chemotaxis, homing and laminin receptor expression
Olfactory neuron	Increases migration
Sperm	Increases zona pellucida binding

cancers (Wells et al., 2002). In T cells it increases proliferation, adhesion, chemotaxis and homing (Azad et al., 1997; Enomoto et al., 2001). In the placenta it upregulates the expression of human chorionic gonadotropin (hCG), urokinase-type plasminogen activator (uPA) and the matrix metalloproteinases-2 and -9 (MMP-2, MMP-9), downregulating plasminogen activator inhibitor (PAI-1) and tissue inhibitors of MMPs (TIMPs) in extra-villous trophoblasts and decidual stromal cells, which play an important role in implantation (Islami et al., 2001; Siler-Khodr et al., 2001; Chou et al., 2002; Chou et al., 2003). Activated GnRH-RI causes increased migration of olfactory neurons (Romanelli et al., 2004) and increases zona pellucida binding of sperm (Morales et al., 2000).

The signaling mechanisms through which GnRH-RI has its effects in extra-pituitary compartments are not fully defined at this point in time, however some preliminary data exists. Human GnRH-RI coupling to the $G_{q/11}$ protein has been demonstrated in heterologous Chinese hamster ovary-K1 (CHO) and COS-7 cells (Grosse et al., 2000). In the human placenta hGnRH-RI is coupled to both $G_{q/11}$ and Gs, activating both the second messenger pathways for PLC-β and adenylate cyclase respectively (Millar et al., 2004; Cheng and Leung, 2005). It has also been reported that hGnRH-RI couples selectively to G_i in some reproductive tract tumors and their derived cell lines, inhibiting cAMP production and mediating the anti-proliferative actions of GnRH analogues previously discussed (Grundker et al., 2002; Limonta et al., 2003). Again, G protein coupling depends largely on cell type and the signalling pathways of hGnRH-RI are yet to be fully elucidated. To exemplify the complicated nature of this signalling pathway, there is evidence showing that the rodent GnRH receptor couples to multiple G proteins in a single cell type (Liu et al., 2002; Stanislaus et al., 1998). It may also be that G protein coupling is a more dynamic process than originally thought, as in GT1–7 neurons, high GnRH-I analog concentrations induce a ligand-dependent switch of G protein coupling from G_s to G_i (Krsmanovic et al., 2003).

1.4.1 Naturally-Occuring Mutation of Human GnRH-RI

GnRH-RI and its ligands play a crucial role in the regulation of the human reproductive system, so it is reasonable that mutation of this receptor that causes

disruption of its normal signaling can lead to disease (Bhagavath et al., 2005). Hypogonadotropic hypogonadism (HH) is the umbrella term applied to a disease state where there is absent or decreased function of the gonads. Individuals present with delayed sexual development and inappropriately low gonadotropin and sex steroid level in the absence of anatomical or functional abnormalities of the hypothalamic-pituitary axis, although in some cases HH can be diagnosed in male infants where there is cryptorchidism and/or microphallus (Bhagavath et al., 2005). There are many subtypes of HH, a subset which results from a defect in the signaling of GnRH. Kallmann's syndrome is characterized by the association of HH and anosmia (or hyposmia) and is caused by a migration defect that involves the GnRH neuronal system (Hardelin et al., 1993). HH associated with congenital adrenal hypoplasia has also been described (Zanaria et al., 1994). This disease, as well as the X-linked form of Kallmann's syndrome, have been mapped to mutations within the X chromosome, p22.3 (Meitinger et al., 1990; Bick et al., 1992; Zanaria et al., 1994). Although a mutation in the GnRH gene has been reported in the hypogonadotropic *hpg/hpg* mouse (Mason et al., 1986), no such mutation has been detected in humans.

Mutation of hGnRH-RI is a rare cause of HH, accounting for only 2-5% of all HH patients (Bhagavath et al., 2005). GnRH-RI was the first gene shown to have mutations in autosomal recessive IHH. To date there are 21 known naturally-occuring mutations of hGnRH-RI, which all lead to partial or complete (60%) HH (Bhagavath et al., 2005). Most cases of HH caused by GnRH-RI mutation are due to compound heterozygous missense mutations (Kim et al., 2008). Nearly all hGnRH-RI mutations occur in exons 1 or 3, but they are dispersed throughout different domains of the receptor (extracellular, transmembrane, and intracellular). Two mutations comprise nearly half of the reported alleles, even among different ethnic groups – Gln106Arg mutation (32.6%) and Arg262Gln (15.2%)(Bhagavath et al., 2005). Interestingly, studies utilizing site-directed mutagenesis and *in vitro* analysis on 3 hGnRH-RI mutants, E90K, L266R and S168R, which are representative of fully, partially and non-rescuable mutants respectively, have shown that hGnRH-RI mutations appear to act as dominant negative mutations (Brothers et al., 2004). The clinical significance of this is unclear, since reported HH patients have

two mutant alleles while heterozygotes do not have any noticeable response (Kim et al., 2008).

It was originally thought that many of the hGnRH-RI mutations interfered with receptor binding and signal transduction, however identification of membrane-permeable pharmalogical chaperones that could rescue the majority of HH mutants (14 of the 17 mutants tested to date) (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006). This suggested that the mutants did not lose the intrinsic ability to bind ligand or activate effector, but rather, were misrouted proteins that were retained by the QCS (Leaños-Miranda et al., 2002). These pharmacoperones bind at or near the ligand binding site and alter the molecular structure to such that the receptor can pass through the QCS and route to the plasma membrane where they function similarly to WT hGnRH-RI. The results for the pharmacoperone studies were obtained biochemically using IP_3 and binding assays.

1.4.2 GnRH-RI as a pharmaceutical target

GnRH-RI has been a target for many synthetic GnRH-I analogues as a treatment for a variety of endocrinopathies, such as hypogonadotropic hypogonadism (HH) (Conn and Crowley, 1994). Low doses of synthetic GnRH analogs administered in a pulsatile fashion are able to restore fertility by restoring the HPG axis. In contrast, high doses of GnRH agonists desensitize the HPG axis, resulting in a decline in the production of estrogen and testosterone (Conn and Crowley, 1994). This protocol is often used clinically in the treatment of hormone-dependent cancers, such as prostate and breast cancers. Other treatments that currently take advantage of the ability of GnRH-I to cause homologous desensitization of GnRH-RI include treatments for benign prostatic hypertrophy, endometriosis, uterine fibroids, premenstrual syndrome, polycystic ovarian syndrome, hirsutism, acne vulgaris and precocious puberty (Conn et al., 1994). GnRH antagonists are a new line of treatment that is increasing in popularity due to the decreased side effects and lack of an initial stimulation and disease flare up caused by agonists. (Millar et al., 2000; Schultze-Mosgau et al., 2005).

A third major use of GnRH-I analogues is ovarian hyperstimulation regimens for assisted reproductive technology (ART). In humans this protocol uses a synthetic FSH

analog to cause maturation of follicles in conjunction with a GnRH-RI antagonist that blocks endogenous FSH and LH release, effectively blocking ovulation. When follicles are fully mature (17 mm), women are treated with human chorionic gonadotropin (hCG), which would cause ovulation within 36 h. However, eggs are retrieved from multiple mature follicles by ultrasound-guided needle prior to ovulation (Antagon FDA product sheet). Novel clinical applications of GnRH analogs are also being developed, such as their use as a new class of male and female contraceptives (Amory et al., 2006; Griesinger et al., 2000; Anderson et al., 2002; Fraser, 1993). The large number of drugs targeting the GnRH receptor is not surprising, as over 200 major prescription drugs target GPCRs, representing over 30% of the total drugs on the market (Wise et al., 2002).

1.5 GnRH-RI Regulation

The carboxy-terminal tail is uniquely absent in human GnRH-RI (Millar et al., 2004). GnRH-RI does not demonstrate an abilitiy to undergo rapid homologous desensitization and does not exhibit agonist-induced receptor phosphorylation, which may be due to the lack of key tail sequences needed to mediate desensitization (Davidson et al., 1994; Heding et al., 1998; Vrecl et al., 1998; Willars et al., 1999; McArdle et al., 2002). GnRH-RII, which has a carboxy-terminal tail, is rapidly internalized upon agonist binding. As well, the addition of the carboxy-terminal tail from the catfish GnRH-R to the human GnRH-RI conferred rapid desensitization to the receptor upon agonist binding (Willars et al., 1999). This supports the hypothesis that the unusual resistance of the mammalian GnRH-RI to receptor desensitization is due to the lack of key sequences needed to mediate desensitization.

Although GnRH-RI is not desensitized through rapid internalization, a relatively rapid desensitization of GnRH-stimulated Ca^{2+} mobilization does occur (McArdle et al., 1996). In the absence of receptor desensitization, it has been proposed that this reflects a reduction in the efficiency with which IP_3 mobilizes Ca^{2+} from the intracellular pool (McArdle et al., 1996). Studies later revealed this effect to be dependent on a reduction in the number of IP_3 receptors (Willars et al., 2001). It should be mentioned that agonist-activated GnRH-RI will internalize slowly via clathrin-coated vesicles, and this process occurs independently of β-arrestin and dynamin (Vrecl et al., 1998; McArdle et al., 2002;

Hislop et al., 2001). A recent study by Pawson et al. (2008) has shown that GnRH-RI undergoes agonist-independent constituitive internalization (Pawson et al., 2008). Decreased expression of GnRH-RI, $G_{q/11}$, PKC and cAMP proteins after long term GnRH stimulation, as well as the attenuation of PLD activity and arachadonic acid release, could all play a role in desensitization (McArdle et al., 2002; Liu et al., 2003). A study by Shacham et al. (2005) employed sythetic peptides mimicking the intracellular loops of GnRH-RI in combination with inositol phosphate assays to identify domains important in desensitization. This study was performed on the premise that a synthetic peptide that contained residues important for desensitization would acts as decoys for desensitizing accessory proteins, allowing the receptor to continue $G_{q/11}$ activation and thus IP_3 production longer, thus identifying this portion of the receptor. They identified a serine residue in position 153 within the first intracellular loop of hGnRH-RI that appears to play a role in desensitization of hGnRH-RI. The signaling inhibition at this site is phosphorylation-independent and may involve accessory proteins such as RGS proteins, GRKs, Src homology 2 domain-containing proteins, small G proteins, polyproline-binding proteins, receptor activity-modifying proteins, and members of the scaffolding family of proteins such as PDZ domain-containing proteins, which are all known to regulate GPCR signaling (Millar et al., 2004). The balance between regulated endocytic and exocytic receptor trafficking dictates the level of GPCR expression at the plasma membrane and thus the magnitude of cellular response elicited by a given signal. This supports the first objective of my study: to investigate the unique localization of hGnRH-RI, which biochemical data has suggested includes a decreased plasma membrane expression and evolutionary trend towards an intracellular receptor.

1.6 Objective 1: Cellular Localization Study of human GnRH-RI

Our current understanding of GPCR signaling and the mechanisms that regulate it are perhaps best understood for the β_2-adrenergic receptor and accordingly this has served as the prototypical receptor for subsequent studies (Kroeze et al., 2003). However, it is now very clear that there are significant variations in the mechanisms that regulate GPCR signaling. GnRH-RI is a structurally unique GPCR that differs greatly from the prototypical GPCR in its structure, cellular localization as well as its regulation

(Ferguson, 2001). Recent research suggests that the mammalian GnRH-RI is one of the most evolutionary advanced members within the GPCR superfamily and its mechanism of action may be representative of numerous other GPCRs (Janovick et al., 2006). Strong biochemical data suggesting that hGnRH-RI is primarily an intracellular receptor with weak surface expression led me to consider that this receptor is evolving towards greater intracrine signaling. Since none of the prior reports that described the intracellular retention of GnRH-RI actually performed image-based cellular localization studies of the receptor, I undertook a detailed spatial characterization of the receptor in two human cell lines, HEK 293 and HTR-8/SVneo. The centrality of GnRH-R1 in the reproduction process renders this and further molecular research both warranted and imperative.

1.7 Protein Kinase C

1.7.1 Discovery of PKC

The study of how protein phosphorylation mediates hormone action first began in the 1950s, with the first protein kinase, PKA, being described in 1960 (Walsh et al., 1968) followed by the discovery of PKG in 1970 (Kuo et al., 1970). In 1977, a very active protein kinase was isolated from rat brain that was independent of any cyclic nucleotide (Inoue et al., 1977). This enzyme could be activated by diacylglycerol in the presence of phospholipids and very low concentrations of Ca^{2+}, and so this protein was named protein kinase C (C for its requirement of Ca^{2+}) (Takai et al., 1979). Protein kinase C research grew in popularity with the discovery that it acts as the receptor of tumor-promoting phorbol esters, which contains a diacylglycerol-like structure in its molecule, allowing it to directly activate PKC and elicit a cellular response (Castagna et al., 1982).

1.7.2 PKC classes and structure

In 1986 the first papers describing PKC as a group of distinct isoforms, rather than a single identity were published (Coussens et al., 1986; Ono et al., 1986). Today, we know that the mammalian PKC is a large family of serine/threonine protein kinases consisting of 10 isotypes encoded by 9 genes (Ohno et al., 2002). The isoforms can be classified into three groups: the conventional, novel and atypical PKCs. The conventional PKCs α, βI, βII and γ are activated by DAG, Ca^{2+} and phosphatidylserine (PS), the novel

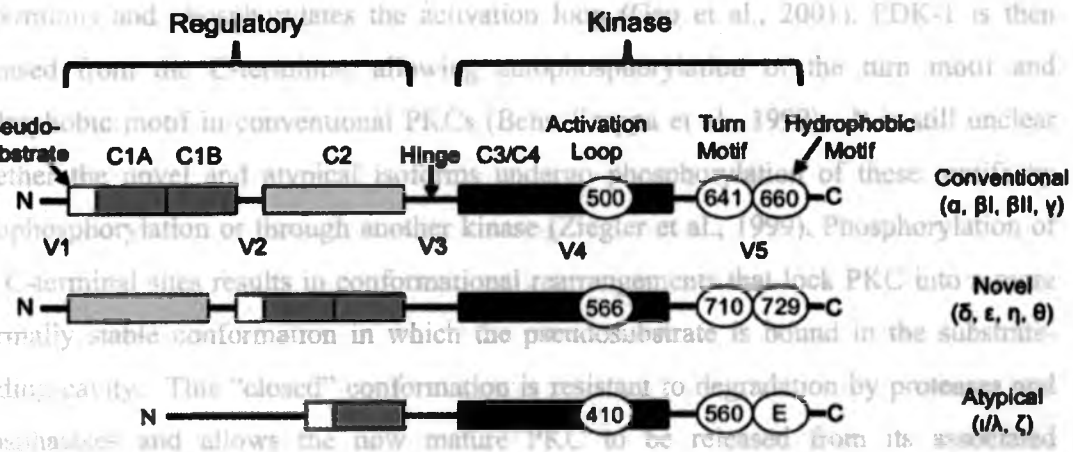
PKCs δ, ε, η/L, and θ are activated by DAG and PS, and the atypical PKC ι/λ and ζ are only activated by PS (Newton et al., 2003). The PKC isoforms share a conserved serine/threonine protein kinase domain located in the C-terminal half of each isoform, but differ in the N-terminal domain which house the membrane-targeting modules (Newton et al., 2003). Figure 1.5 depicts the differences in primary structure between PKC isoforms. PKCs contain two types of membrane-targeting domains, C1 and C2 (Newton et al., 2003). The C1 domain functions as a diacylglycerol sensor and is a tandem repeat in the conventional and novel PKCs. The atypical PKCs have an impaired C1 domain that lacks critical residues required for interaction with diacylglycerol or phorbol esters. The C2 domain acts as a Ca^{2+}-regulated phospholipid binding module in conventional PKCs that shows specificity to acidic phospholipids such as phosphatidylserine (Ohno et al., 2002). The C2 domain in novel PKCs does not bind Ca^{2+} or phospholipid and this domain is entirely absent in the atypical PKCs. One portion of the regulatory domain, the autoinhibitory pseudosubstrate, is conserved in all isoforms. The pseudosubstrate is found N-terminal to the C1 domain in all isoforms.

PKC isoforms are widely expressed, from *Drosophila* and *C. elegans* to mammals (Ohno et al., 2002). Although they do not express all three classes of PKCs, even yeast displays one or two closely related PKCs depending on the species (Ohno et al., 2002). The yeast PKCs contain domains common to multiple protein kinases, suggesting that yeast retains an ancestral form of the protein that evolved into many of the protein kinases that we have in multicellular organisms today (Mellor et al., 1998). The mammalian PKCs, specifically the human and mouse PKC isoforms, which will be the focus of this dissertation, are found on multiple chromosomes range from 592 (PKC ζ) to 737 (PKC ε) amino acids in length (Ohno et al., 2002).

1.7.3 Processing and activation of PKC

Newly synthesized PKC associates with a membrane compartment in the cell (Sonnenburg et al., 2001) in an "open" conformation in which the autoinhibitory pseudosubstrate in removed from the substrate binding cavity (Dutil et al., 2000). This open conformation exposes the activation loop and the C terminus, which is critical for the next steps of PKC processing in which PIP_2-dependent kinase 1 (PDK-1) docks on the

Figure 1.5 Primary structure of the three classes of PKC isoforms: conventional, novel and atypical. All kinases have a conserved kinase core (black rectangle) made up of the C3-V4-C4 domains. The kinase core contains a phosphorylation site called the activation loop (circle). The C-terminal extension (V5 domain) contains two phosphorylation sites: the turn motif and the hydrophobic motif (circles). The isoforms differ in their regulatory N-terminal sequences, which contains the membrane-targeting modules. All isoforms contain an autoinhibitory pseudosubstrate sequence (white square) that is N-terminal to the C1 domain (dark grey rectangle). The C1 domain is a tandem repeat for conventional and novel PKCs (C1A and C1B) and functions as a diacylglycerol sensor. Atypical PKCs have an impaired C1 domain that does not respond to diacylglycerol (or phorbol esters) and lacks a C2 domain (light grey rectangle) that acts as a calcium-regulated phospholipid binding module in conventional PKCs. The C2 domain in the novel PKCs binds neither calcium nor membrane phospholipids. Modified after Newton et al., 2003.


Regulatory
Kinase
Pseudo-substrate
C1A
C1B
C2
Hinge
C3/C4
Activation Loop
Turn Motif
Hydrophobic Motif
N
500
641
660
C
Conventional (α, βI, βII, γ)
V1
V2
V3
V4
V5
N
566
710
729
C
Novel (δ, ε, η, θ)
N
410
560
E
C
Atypical (ι/λ, ζ)

C terminus and phosphorylates the activation loop (Gao et al., 2001). PDK-1 is then released from the C-terminus, allowing autophosphorylation of the turn motif and hydrophobic motif in conventional PKCs (Behn-Krappa et al., 1999). It is still unclear whether the novel and atypical isoforms undergo phosphorylation of these motifs by autophosphorylation or through another kinase (Ziegler et al., 1999). Phosphorylation of the C-terminal sites results in conformational rearrangements that lock PKC into a more thermally stable conformation in which the pseudosubstrate is bound in the substrate-binding cavity. This "closed" conformation is resistant to degradation by proteases and phosphatases and allows the now mature PKC to be released from its associated membrane to diffuse into the cytosol. The bulk of the mature enzyme is localized to the cytosol, however, isoform-specific binding partners may localize a fraction of the PKC to specific intracellular sites (Mochly-Rosen et al., 1998; Jaken et al., 2000).

Activation of PKCs into an active form with full kinase ability occurs differently for each class of isoform. Both the conventional and novel isoforms benefit from $G_{q/11}$ activation, which produces IP_3, DAG and Ca^{2+}, whereas the atypical PKCs do not use either of these substrates, but may utilize other products downstream of $G_{q/11}$ signaling such as arachadonic acid (Muller et al., 1995). The conventional PKC isoforms bind Ca^{2+} on their C2 domain, causing a conformational change, which exposes a hydrophobic plate (Newton et al., 2003) that will tether PKC to the plasma membrane upon collision (Nalefski et al., 2001; Schafer et al., 2001). The tethered PKC will diffuse in the plane of the membrane until in comes in contact with membrane-bound diacylglycerol. The C1 domain will then bind DAG, causing a high-affinity interaction between the membrane and PKC and giving the PKC enough energy to release its pseudosubstrate from the substrate-binding cavity. This allows PKC to bind and phosphorylate its substrate, leading to downstream signaling (Newton et al., 1998; Sakai et al., 1997; Oancea et al., 1998).

Novel PKCs are not pre-targeted to the membrane by Ca^{2+} like the conventional isoforms, decreasing the probability of encountering membrane-bound DAG (Schaefer et al., 2001). When random contact with the plasma membrane brings the novel PKCs in contact with DAG, the high-affinity membrane binding, release of pseudosubstrate and activation of the kinase is identical as that seen for the conventional PKCs (Newton et al.,

2003). The atypical PKCs do not bind DAG or Ca^{2+} and what regulates their activation is not well defined, although evidence has shown that lipid components, such as phosphatidylinositols (PIs) (Nakanishi et al., 1993), phosphatidic acid (Limatola et al., 1994), arachidonic acid and ceramide (Muller et al., 1995) all activate atypical PKCs.

The membrane-bound active PKC is highly susceptible to dephosphorylation by phosphatases (Dutil et al., 1994) and is rapidly dephosphorylated. The dephosphorylated PKC becomes associated with the detergent-insoluble cell fraction where it will eventually be degraded. It is proposed that dephosphorylated PKC may be targeted for degradation by ubiquination (Lee et al., 1997; Lu et al., 1998) and are targeted to endosomes in a caveolin-dependent manner (Prevostel et al., 2000). A fraction of dephosphorylated PKC will bind the molecular chaperone Hsp70, which stabilizes the protein and allows it to be rephosphorylated (Gao et al., 2002) and become catalytically competent, but inactive, in the cytosol. Binding of Hsp70 prevents PKC degradation and is energetically favourable for the cell (Gao et al., 2002).

1.7.4 PKC isoforms activated by GnRH-RI signaling

As previously mentioned, in both the placenta and the pituitary, GnRH-RI is coupled to the $G_{q/11}$ pathway, producing secondary messengers with the ability to activate PKC isoforms. However, it is still unclear as to which specific PKC isoforms are activated downstream of GnRH-RI in these human tissues. A mouse pituitary gonadotrope cell line, αT3-1, has been used as a model to address this question, however, the possibility remains that this mouse model does not recapitulate what happens in the human cell. The mouse and human GnRH-RI differ significantly in a few key residues, as discussed in section 1.3.2, further calling into question the validity of these studies. The PKC isoforms also contain species-specific differences, which may also confound attempts to elucidate the human signaling pathway using this mouse model.

A variety of methods, some of which are described below, have been developed to study PKC activation. Gene expression studies determine which isoforms have been activated in response to GnRH stimulation by looking for an upregulation of gene product, working under the assumption that a fraction of activated PKC is degraded and must be replaced (Shraga-Levine et al., 1994). Protein studies of PKC activation look for

the localization of the PKC within the cell, using cell fractionation following western blot, immunofluorescene in fixed cells or performing live cell imaging using GFP-tagged PKC isoforms (Kratzmeier et al., 1996; Sakai et al., 1997; Babwah et al., 2003; Farshori et al., 2003; Maccario et al., 2004). In 2003, Farshori et al. undertook a comprehensive study of all of the mouse PKC isoforms activated by the mouse GnRH-RI in HEK 293 cells. Using immunofluorescene with anti-PKC antibodies at fixed time points post-stimulation, they determined which isoforms were activated by looking for PKC at the plasma membrane. They reported that PKC α and δ both translocated to the plasma membrane, whereas PKC ε, ι and λ did not (Farshori et al., 2003). Western blot analysis on subcellular fractions of GnRH-RI agonist-stimulated αT3-1 gonadotrope cell lines showed than PKC δ, but not PKC ε translocated to the plasma membrane (Maccario et al., 2004), whereas Kratzmeier et al (1996) found that GnRH stimulation caused translocation of PKC ε and ζ but not PKCα using the same technique and the same cell line. The evidence surrounding which PKC isoforms are activated in response to mouse GnRH-RI is inconclusive and in some cases contradictory. Once again, these studies were all performed using the mouse GnRH-RI and mouse PKC isozymes and cannot necessarily explain the signaling in a human system.

1.8 Objective 2: Molecular characterization of early GnRH-RI signaling

To complement my cellular characterization of hGnRH-RI, I investigated the early signaling pathway of the $G_{q/11}$-coupled hGnRH-RI; specifically the activation of PKC isoforms. The crucial role that PKC plays in the synthesis and release of the gonadotropins from the anterior pituitary downstream of GnRH binding has been well studied, however there is no literature describing which PKC isoforms in the human cell are activated downstream of hGnRH-RI. Previous studies that investigate this question using mouse pituitary cells, which endogenously express the mouse PKC isoforms and mouse GnRH-RI, may not recapitulate what happens in the human cell. For example, the rat metabotropic glutamate receptor isoforms 1a and 5a differ in several key residues (Dale et al., 2001). One of them is Asp854 (mGluR1a) and Thr840 (mGluR5a) and it has been shown that this single residue regulates PKC βII oscillatory frequency (Dale et al., 2001). Additionally PKC isoform protein sequences are not fully conserved across

species. For example, a study by Aris et al. (1993) showed that human PKC δ had about 88% nucleotide sequence identity to the rat and mouse PKC δ sequence, however biochemical differences, such as substrate and lipid dependencies, were observed between the species. Given the fact that just single amino acid changes in an isoform can significantly alter PKC responses upon receptor activation there is little support to suggest that the relationships between the rodent GnRH-RI and PKC isoforms can be extrapolated to the human cell. Therefore, I proposed there is a need for a direct study looking at the hGnRH-RI stimulated hPKC isoform responses.

A second focus of my study was to compare the ability of known naturally-occurring mutants of hGnRH-RI to stimulate PKC translocation to the plasma membrane in a similar fashion to their wild type counterpart. There are currently 21 known naturally occurring mutations of hGnRH-RI, which cause partial to complete hypogonadotropic hypogonadism, a disease state in which there is absent or decreased function of the gonads, presenting as a delay in sexual development and inappropriately low gonadotropin and sex steroid levels (Bhagavath et al., 2005). Previous studies performed on some of the known mutants in COS-7 cells have shown that many of these mutants are incapable of normal signaling, as shown by their inability to produce IP_3 in response to agonist stimulation (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006). Since the naturally-occuring GnRH-RI mutants have reduced DAG and Ca^{2+} generating cofactors relative to the WT receptor, I was interested in determining whether this diminished capacity significantly affected the spatial and temporal characteristics of the downstream PKC response. I chose to look at 17 of the known mutants in HEK 293 cells coexpressed with GFP-PKC βII. I chose PKC βII since I previously found that this isoform gives a robust oscillatory response to the agonist activated WT receptor and felt that subtle changes in its response to mutant receptor activation cold be best detected relative to the other isoforms. Although previous studies have reported on the ability of the GnRH-RI mutants to produce IP_3, I thought it pertinent to perform IP_3 assays to gain confidence, as well as avoid confounding factors, such as the fact that I have used a FLAG-tagged receptor and HEK 293 cells as opposed to the COS-7 cells used in previous studies.

Although the GnRH-RI is a GPCR and shares many structural and signaling properties with other GPCRs, it is also a very unique GPCR. As a result of the many unique properties and the clinical importance of GnRH-RI, it is important to characterize this receptor in detail. Surprisingly, I found that while there is a plethora of detailed and complex studies performed on this receptor, such as the effect of GnRH pulse frequency on GnRH-RI-mediated gene transcription (Ciccone et al., 2008) some very basic studies are lacking. Two such studies are the characterization of the spatial and temporal properties of the receptor in a homologous and heterologous cell system and a clearer picture of the early signaling pathway the receptor couples to following activation. It was therefore the primary focus of my research to better characterize basic cellular and molecular properties of GnRH-RI. In the following chapter, I report largely on the spatial characteristics of GnRH-RI in the heterologous and homologous cell lines, HEK 293 and Human Trophoblast (HTR-8/SVneo), respectively. This study revealed that while GnRH-RI is weakly expressed at the plasma membrane of both cell types, it is strongly expressed in the cytoplasm and on the nuclear membrane. In the next chapter, I then characterized the plasma membrane-bound GnRH-RI/PKC-coupled signaling pathway in HEK 293 cells. Based on the spatial and temporal responses of these PKC isoforms following receptor activation, it appears that various isoforms may have both unique and redundant roles in regulating GnRH-RI signaling. While this was only a preliminary study, it is nevertheless the most detailed and direct study to date to examine which PKC isoforms are involved in regulating GnRH-RI signaling in human cells.

1.9 References

Amory JK 2006 Male hormonal contraceptives. Minerva Ginecol. 58(3):215-226.

Anderson RA, Baird DT 2002 Male contraception. Endocr Rev. 2002 6:735-62.

Antagon FDA product sheet www.fda.gov/Cder/foi/label/1999/21057lbl.pdf

Aris JP, Basta PV, Holmes WD, Ballas LM, Moomaw C, Rankl NB, Blobel G, Loomis CR, Burns DJ 1993 Molecular and biochemical characterization of a recombinant human PKC-delta family member. Biochim Biophys Acta. 1174(2):171-181.

Arora KK, Sakai A, Catt KJ 1995 Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. J Biol Chem 270:22820-22826.

Azad N, LaPaglia N, Kirsteins L, Uddin S, Steiner J, Williams DW, Lawrence AM, Emanuele NV 1997 Jurkat cell proliferative activity is increased by luteinizing hormone-releasing hormone. J Endocrinol 153:241–249

Behn-Krappa A, Newton AC 1999 The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation. Curr. Biol. 9:728-737.

Bell RM, Burns DJ 1991 Lipid activation of protein kinase C. J Biol. Chem. 266:4661-4664.

Benard O, Naor Z, Seger R 2001 Role of dynamin, Src, and Ras in the protein kinase C-mediated activation of ERK by gonadotropin-releasing hormone. J Biol Chem. 276(7):4554-63.

Bhagavath B, Ozata M, Ozdemir IC, Bolu E, Bick DP, Sherins RJ, Layman LC 2005 The prevalence of gonadotropin-releasing hormone receptor mutations in a large cohort of patients with hypogonadotropic hypogonadism. Fertil. Steril. 84: 951–957.

Bhattacharya M, Peri KG, Almazan G, Ribeiro-da-Silva A, Shichi H, Durocher Y, Abramovitz M, Hou X, Varma DR, Chemtob S 1998 Nuclear localization of prostaglandin E2 receptors. Proc Natl Acad Sci (U S A) 95:15792-15797.

Bick D, Franco B, Sherins RJ, Heye B, Pike L, Crawford J, Maddalena A, Incerti B, Pragliola A, Meitinger T 1992 Brief report: intragenic deletion of the KALIG-1 gene in Kallmann's syndrome. N Engl J Med. 326(26):1752-1755.

Birnbaumer L 1992 Receptor-to-effector signaling through G proteins: roles for beta gamma dimers as well as alpha subunits. Cell 71:1069-1072.

Bonfil 2004 Bonfil D, Chuderland D, Kraus S, Shahbazian D, Friedberg I, Seger R, Naor Z 2004 Extracellular signal-regulated kinase, Jun N-terminal kinase, p38, and c-Src are involved in gonadotropin-releasing hormone-stimulated activity of the glycoprotein hormone follicle-stimulating hormone beta-subunit promoter. Endocrinology. 145(5):2228-2244.

Brothers SP, Cornea A, Janovick JA, Conn PM 2004 Human loss-of-function gonadotropin-releasing hormone receptor mutants retain wild-type receptors in the endoplasmic reticulum: molecular basis of the dominant- negative effect. Mol Endocrinol 18:1787–1797.

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa, U, Nishizuka Y 1982 Direct activation of Ca^{2+}-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. 257:7847–7851.

Cheng KW, Nathwani PS, Leung PC 2000 Regulation of human gonadotropin-releasing hormone receptor gene expression in placental cells. Endocrinology. 141(7):2340-2349.

Cheng CK, Leung PC. 2005 Molecular biology of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and their receptors in humans. Endocr Rev. 26(2):283-306.

Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP, Sealfon SC 1993 Cloning and characterization of the human GnRH receptor. Mol Cell Endocrinol. 91(1-2):R1-R6.

Choi KC, Auersperg N, Leung PC 2001 Expression and antiproliferative effect of a second form of gonadotropin-releasing hormone in normal and neoplastic ovarian surface epithelialcells.J Clin Endocrinol Metab 86:5075–5078.

Chou CS, Zhu H, Shalev E, MacCalman CD, Leung PC 2002 The effects of gonadotropin-releasing hormone (GnRH) I and GnRH II on the urokinase-type plasminogen activator/plasminogen activator inhibitor system in human extravillous cytotrophoblasts *in vitro*. J Clin Endocrinol Metab 87:5594 –5603

Chou CS, Zhu H, MacCalman CD, Leung PC 2003 Regulatory effects of gonadotropin-releasing hormone (GnRH) I and GnRH II on the levels of matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinases-1 in primary cultures of human extravillous cytotrophoblasts. J Clin Endocrinol Metab 88: 4781– 4790.

Ciccone NA, Lacza CT, Hou MY, Gregory SJ, Kam KY, Xu S, Kaiser UB 2008 A Composite Element that Binds Basic Helix Loop Helix and Basic Leucine Zipper Transcription Factors Is Important for GnRH Regulation of the FSH{beta} Gene. Mol Endocrinol. 2008 Jun 11. [Epub ahead of print]

Conn PM, Crowley WF Jr 1994 Gonadotropin-releasing hormone and its analogs. Annu Rev Med 45:391-405.

Conn PM, Leaños-Miranda A, Janovick JA 2002 Protein origami: therapeutic rescue of misfolded gene products. Mol Interv. 2(5):308-316.

Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke U, Ullrich A 1986 Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 233:859–866.

Dale LB, Babwah AV, Bhattacharya M, Kelvin DJ, Ferguson SS 2001 Spatial-temporal patterning of metabotropic glutamate receptor-mediated inositol 1,4,5-triphosphate, calcium, and protein kinase C oscillations: protein kinase C-dependent receptor phosphorylation is not required.J Biol Chem. 276(38):35900-35908.

Davidson JS, Wakefield IK, Millar RP 1994 Absence of rapid desensitization of the mouse gonadotropin-releasing hormone receptor. Biochem J. 300 (2):299-302.

Dingwall C, Sharnick SV, Laskey RA 1982 A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell. 30(2):449-458.

Dutil EM, Keranen LM, DePaoli-Roach AA, Newton AC 1994 In vivo regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. J. Biol. Chem. 269:29359–29362.

Dutil EM, Newton AC 2000 Dual role of pseudosubstrate in the coordinated regulation of protein kinase C by phosphorylation and diacylglycerol. J. Biol. Chem. 275:10697-10701.

Eidne KA, Sellar RE, Couper G, Anderson L, Taylor PL 1992 Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Mol Cell Endocrinol. 90(1):R5-R9.

Ellgaard L, Helenius A 2003 Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol. 4(3):181-191.

Enomoto M, Mori T, Park MK 2001 GnRH agonist Buserelin affects colony-forming efficiency of HHUA and Jurkat cells. Biochem Biophys Res Commun 289:1180 –1187

Fan NC, Peng C, Krisinger J, Leung PC 1995 The human gonadotropin-releasing hormone receptor gene: complete structure including multiple promoters, transcription initiation sites, and polyadenylation signals. Mol Cell Endocrinol. 107(2):R1-R8.

Farshori PQ, Shah BH, Arora KK, Martinez-Fuentes A, Catt KJ 2003 Activation and nuclear translocation of PKCdelta, Pyk2 and ERK1/2 by gonadotropin releasing hormone in HEK293 cells. J Steroid Biochem Mol Biol. 2003 Jun;85(2-5):337-47.

Faurholm 2001 Faurholm B, Millar RP, Katz AA 2001 The genes encoding the type II gonadotropin-releasing hormone receptor and the ribonucleoprotein RBM8A in humans overlap in two genomic loci. Genomics. 78(1-2):15-18.

Ferguson SG 2001 Evolving concepts in g protein-coupled receptor endocytosis: The role in Receptor desensitization and signaling. Pharmacol Rev 53:1-24.

Fraser HM 1993 GnRH analogues for contraception. Br Med Bull. 1:62-72.

Gao T, Toker A, Newton, AC 2001 The carboxyl terminus of protein kinase C provides a switch to regulate its interaction with the phosphoinositide-dependent kinase, PDK-1. J. Biol. Chem. 276:19588-19596.

Gao T, Newton AC 2002 The turn motif is a phosphorylation switch that regulates the binding of hsp70 to protein kinase C. J. Biol. Chem. 277:31585–31592.

Griesinger G, Felberbaum R, Diedrich K 2000 GnRH-antagonists in reproductive medicine. Arch Gynecol Obstet. 2:71-78.

Grosse R, Schmid A, Schöneberg T, Herrlich A, Muhn P, Schultz G, Gudermann T. 2000 Gonadotropin-releasing hormone receptor initiates multiple signaling pathways by exclusively coupling to G(q/11) proteins. J Biol Chem. 13:9193-9200.

Grundker C, Gunthert AR, Millar RP, Emons G 2002 Expression of gonadotropin-releasing hormone II (GnRH-II) receptor in human endometrial and ovarian cells and effects of GnRH-II on tumor cell proliferation. J Clin Endocrinol Metab 87:1427–1430 tropes. Science 260:82– 84.

Hanover JA 1992 The nuclear pore: at the crossroads. FASEB J. 6(6):2288-2295.

Hardelin JP, Levilliers J, Blanchard S, Carel JC, Leutenegger M, Pinard-Bertelletto JP, Bouloux P, Petit C 1993 Heterogeneity in the mutations responsible for X chromosome-linked Kallmann syndrome. Hum Mol Genet. 2(4):373-377.

Hazum E, Keinan D. 1985 Gonadotropin releasing hormone activation is mediated by dimerization of occupied receptors. Biochem Biophys Res Commun. 1985 Dec 17;133(2):449-456.

Heding A, Vrecl M, Bogerd J, McGregor A, Sellar R, Taylor PL, Eidne KA 1998 Gonadotropin-releasing hormone receptors with intracellular carboxyl-terminal tails undergo acute desensitization of total inositol phosphate production and exhibit accelerated internalization kinetics. J Biol Chem. 273(19):11472-7.

Hemmings BA, Parker PJ 1999 Rapamycin-sensitive phosphorylation of PKC on a carboxy-terminal site by an atypical PKC complex. Curr. Biol. 9:522-529.

Hislop JN, Everest HM, Flynn A, Harding T, Uney JB, Troskie BE, Millar RP, McArdle CA 2001 Differential internalization of mammalian and non-mammalian gonadotropin-releasing hormone receptors. Uncoupling of dynamin-dependent internalization from mitogen-activated protein kinase signaling. J Biol Chem. 276(43):39685-39694.

Hsieh KP, Martin TF 1992 Thyrotropin-releasing hormone and gonadotropin-releasing

hormone receptors activate phospholipase C by coupling to the guanosine triphosphate-binding proteins Gq and G11. Mol Endocrinol 6:1673-1681.

Illing N, Jacobs GF, Becker II, Flanagan CA, Davidson JS, Eales A, Zhou W, Sealfon SC, Millar RP 1993 Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors.Biochem Biophys Res Commun. 196(2):745-751.

Inoue M, Kishimoto A, Takai Y, Nishizuka Y 1977 Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by Ca^{2+}-dependent protease from rat brain. J. Biol. Chem. 252:7610–7616.

Islami D, Chardonnens D, Campana A, Bischof P 2001 Compar- son of the effects of GnRH-I and GnRH-II on HCG synthesis and secretion by first trimester trophoblast. Mol Hum Reprod 7:3–9.

Jaken S, Parker PJ 2000 Protein kinase C binding partners. Bioessays 22, 245-254.

Janovick JA, Goulet M, Bush E, Greer J, Wettlaufer DG, Conn PM 2003a Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. J Pharmacol Exp Ther 305:608-614.

Janovick JA, Ulloa-Aguirre A, Conn PM 2003b Evolved regulation of gonadotropin-releasing hormone receptor cell surface expression. Endocrine 22:317-327.

Janovick JA, Knollman PE, Brothers SP, Ayala-Yanez R, Aziz AS, Conn PM 2006 Regulation of G protein-coupled receptor trafficking by inefficient plasma membrane expression: molecular basis of an evolved strategy. J. Biol. Chem. 281:8417-8425

Jiang Y, Ma W, Wan Y, Kozasa T, Hattori S, Huang XY 1998 The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. Nature 395:808-813.

Kakar SS, Rahe CH, Neill JD. 1993 Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH). Domest Anim Endocrinol. 4:335-342.

Kakar SS 1997 Molecular structure of the human gonadotropin-releasing hormone receptor gene. Eur J Endocrinol. 2:183-192.

Kaiser UB, Zhao D, Cardona GR, Chin WW 1992 Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. Biochem Biophys Res Commun. 189(3):1645-1652.

Kim HG, Bhagavath B, Layman LC 2008 Clinical Manifestations of Impaired GnRH Neuron Development and Function. Neurosignals 2008;16:165–182

Knollman PE, Conn PM 2008 Multiple G proteins compete for binding with the human gonadotropin releasing hormone receptor. Arch Biochem Biophys. 2008 May 27. [Epub ahead of print]

Kratzmeier M, Poch A, Mukhopadhyay AK, McArdle CA 1996 Selective translocation of non-conventional protein kinase C isoenzymes by gonadotropin-releasing hormone (GnRH) in the gonadotrope-derived alpha T3-1 cell line. Mol Cell Endocrinol. 118(1-2):103-11.

Kraus S, Naor Z, Seger R 2001 Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor. Arch Med Res. 32(6):499-509.

Kroeze WK, Sheffler DJ, Roth BL 2003 G-protein-coupled receptors at a glance. J Cell Sci. 116(Pt 24):4867-4869.

Krsmanovic LZ, Mores N, Navarro CE, Arora KK, Catt KJ 2003 An agonist-induced switch in G protein coupling of the gonadotropin-releasing hormone receptor regulates pulsatile neuropeptide secretion. Proc Natl Acad Sci U S A. 100(5):2969-74.

Kuo JF, Greengard P 1970 Cyclic nucleotide-dependent protein kinases. VI. Isolation and partial purification of a protein kinase activated by guanosine 3',5'-monophosphate. J Biol Chem. 245(10):2493-2498.

Leaños-Miranda A, Janovick JA, Conn PM 2002 Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 87(10):4825-4828.

Lee HW, Smith L, Pettit GR, Smith JB 1997 Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts. Mol. Pharmacol. 51:439–447.

Levi NL, Hanoch T, Benard O, Rozenblat M, Harris D, Reiss N, Naor Z, Seger R. 1998 Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary alpha T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42. Mol Endocrinol. 12(6):815-824.

Limatola C, Schaap D, Moolenaar WH, van Blitterswijk WJ 1994 Phosphatidic acid activation of protein kinase C-z overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. Biochem. J. 304:1001–1008.

Limonta P, Moretti RM, Marelli MM, Motta M. 2003 The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans. Front Neuroendocrinol. 2003 Dec;24(4):279-295.

Ling K, Wang P, Zhao J, Wu YL, Cheng ZJ, Wu GX, Hu W, Ma L, Pei G. 1999 Five-transmembrane domains appear sufficient for a G protein-coupled receptor: functional five-transmembrane domain chemokine receptors. Proc Natl Acad Sci U S A. 96(14):7922-7927.

Liu F, Usui I, Evans LG, Austin DA, Mellon PL, Olefsky JM, Webster NJ 2002 Involvement of both G(q/11) and G(s) proteins in gonadotropin-releasing hormone receptor-mediated signaling in L beta T2 cells. J Biol Chem. 277(35):32099-32108.

Liu F, Austin DA, Webster NJ 2003 Gonadotropin-releasing hormone-desensitized LbetaT2 gonadotrope cells are refractory to acute protein kinase C, cyclic AMP, and calcium-dependent signaling. Endocrinology. 144(10):4354-4365.

Lodish H, Berk A, Matsudaira P, Kaiser CA, Krieger M, Scott MP, Zipursky L, Darnell J 2003 Molecular Cell Biology, 5e. Freeman & Co., New York, NY.

Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA 1998 Activation of protein kinase C triggers its ubiquitination and degradation. Mol. Cell. Biol. 18:839–845.

Luttrell LM, Daaka Y, Lefkowitz RJ 1999 Regulation of tyrosine kinase cascades by G protein-coupled receptors. Curr Opin Cell Biol. 11:177-183.

Macara IG 2001 Transport into and out of the nucleus. Microbiol Mol Biol Rev. 65(4):570-594.

Maccario H, Junoy B, Poulin B, Boyer B, Enjalbert A, Drouva SV 2004 Protein kinase Cdelta as gonadotropin-releasing hormone target isoenzyme in the alphaT3-1 gonadotrope cell line. Neuroendocrinology. 79(4):204-220.

Mason AJ, Hayflick JS, Zoeller RT, Young 3rd WS, Phillips HS, Nikolics K, Seeburg PH 1986 A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the *hpg* mouse. Science 234:1366–1371.

Maya-Núñez G, Janovick JA, Conn PM 2000 Combined modification of intracellular and extracellular loci on human gonadotropin-releasing hormone receptor provides a mechanism for enhanced expression. Endocrine 13:401-407

McArdle CA, Willars GB, Fowkes RC, Nahorski SR, Davidson JS, Forrest-Owen W 1996 Desensitization of gonadotropin-releasing hormone action in alphaT3-1 cells due to uncoupling of inositol 1,4,5-trisphosphate generation and Ca2+ mobilization. J Biol Chem. 271(39):23711-23717.

McArdle CA, Franklin J, Green L, Hislop JN 2002 The gonadotrophin-releasing hormone receptor: signalling, cycling and desensitisation. Arch Physiol Biochem. 110(1-2):113-122.

Meitinger T, Heye B, Petit C, Levilliers J, Golla A, Moraine C, Dalla Piccola B, Sippell WG, Murken J, Ballabio A 1990 Definitive localization of X-linked Kallman syndrome (hypogonadotropic hypogonadism and anosmia) to Xp22.3: close linkage to the hypervariable repeat sequence CRI-S232. Am J Hum Genet. 47(4):664-669.

Mellor H, Parker PJ 1998 The extended protein kinase C superfamily. Biochem. J. 332:281-292.

Millar RP, Flanagan CA, Milton RC, King JA 1989 Chimaeric analogues of vertebrate gonadotropin-releasing hormones (GnRHs) comprising substitutions of the variant amino acids in positions 5, 7 and 8: characterization of requirements for receptor binding and gonadotropin release in mammalian and chicken pituitary gonadotropes. J Biol Chem 264:21007–21013.

Millar R, Conklin D, Lofton-Day C, Hutchinson E, Troskie B, Illing N, Sealfon SC, Hapgood J 1999 A novel human GnRH receptor homolog gene: abundant and wide tissue distribution of the antisense transcript. J Endocrinol. 162(1):117-126.

Millar RP, Zhu YF, Chen C, Struthers RS 2000 Progress towards the development of non-peptide orally-active gonadotropin-releasing hormone (GnRH) antagonists: therapeutic implications. Br Med Bull. 56(3):761-772.

Millar RP 2003 GnRH II and type II GnRH receptors. Trends Endocrinol Metab. 14(1):35-43.

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR 2004 Gonadotropin-Releasing Hormone Receptors. Endocr Rev 25:235-275.

Miller WR, Scott WN, Morris R, Fraser HM, Sharpe RM 1985 Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist. Nature 313:231–233.

Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H 1984 Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc Natl Acad Sci U S A. 81(12):3874-3878.

Mochly-Rosen D, Gordon AS 1998 Anchoring proteins for protein kinase C: a means for isozyme selectivity. FASEB J. 12:35-42.

Morales P, Pizarro E, Kong M, Kerr B, Ceric F, Vigil P 2000 Gonadotropin-releasing hormone-stimulated sperm binding to the human zona is mediated by a calcium influx. Biol Reprod 63:635– 642.

Moretti RM, Marelli MM, van Groeninghen JC, Motta M, Limonta P 2003 Inhibitory activity of luteinizing hormone-releasing
hormone on tumor growth and progression. Endocr Relat Cancer

10:161–167

Morgan K, Conklin D, Pawson AJ, Sellar R, Ott TR, Millar RP 2003 A transcriptionally active human type II gonadotropin-releasing hormone receptor gene homolog overlaps two genes in the antisense orientation on chromosome 1q.12. Endocrinology. 144(2):423-436.

Muller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K 1995 PKCz is a molecular switch in signal transduction of TNF-a, bifunctionally regulated by ceramide and arachidonic acid. EMBO J. 14:1961–1969.

Mulvaney JM, Zhang T, Fewtrell C, Roberson MS 1999 Calcium influx through L-type channels is required for selective activation of extracellular signal-regulated kinase by gonadotropin-releasing hormone. J Biol Chem. 274(42):29796-804.

Mulvaney JM, Roberson MS 2000 Divergent signaling pathways requiring discrete calcium signals mediate concurrent activation of two mitogen-activated protein kinases by gonadotropin-releasing hormone. J Biol Chem. 275(19):14182-9.

Myburgh DB, Millar RP, Hapgood JP 1998 Alanine-261 in intracellular loop III of the human gonadotropin-releasing hormone receptor is crucial for G-protein coupling and receptor internalization. Biochem J. 331:(3):893-896.

Nakanishi H, Brewer KA, Exton JH 1993 Activation of the z isozyme of protein kinase C by phosphatidylinositol 3, 4, 5-trisphosphate. J. Biol. Chem. 268:13–16.

Nalefski EA, Newton AC 2001 Membrane binding kinetics of protein kinase CbII mediated by the C2 domain. Biochemistry 40:13216-13229.

Naor Z 1990 Signal transduction mechanisms of Ca2+ mobilizing hormones: the case of gonadotropin-releasing hormone. Endocr Rev. 11(2):326-353.

Naor Z, Shacham S, Harris D, Seger R, Reiss N 1995 Signal transduction of the gonadotropin releasing hormone (GnRH) receptor: cross-talk of calcium, protein kinase C (PKC), and arachidonic acid. Cell Mol Neurobiol. 15(5):527-44.

Naor Z, Harris D, Shacham S 1998 Mechanism of GnRH Receptor Signaling: Combinatorial Cross-Talk of Ca^{2+} and Protein Kinase C Front Neuroendocrinol 19:1–19.

Naor Z, Benard O, Seger R 2000 Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends Endocrinol Metab.11(3):91-99.

Nathans J, Hogness DS. 1983 Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. Cell. 34(3):807-814.

Neill JD 2002 GnRH and GnRH receptor genes in the human genome. Endocrinology. 143(3):737-743.

Nielsen CK, Campbell JI, Ohd JF, Mörgelin M, Riesbeck K, Landberg G, Sjölander A 2005 A novel localization of the G-protein-coupled CysLT1 receptor in the nucleus of colorectal adenocarcinoma cells. Cancer Res. 65(3):732-742.

Newton AC, Johnson JE 1998 Protein kinase C: a paradigm for regulation of protein function by two membrane-targeting modules. Biochim. Biophys. Acta 1376:155-172.

Newton AC 2003 Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem J. 370(Pt 2):361-371.

Oancea E, Meyer T 1998 Protein kinase C as a molecular machine for decoding Ca^{2+} and diacylglycerol signals. Cell 95:307-318.

Ono Y, Kurokawa T, Fujii T, Kawahara K, Igarashi K, Kikkawa U, Ogita K, Nishizuka Y 1986 Two types of complementary DNAs of rat brain protein kinase C. Heterogeneity determined by alternative splicing. FEBS Lett. 206:347–352.

Ohno S, Nishizuka Y 2002 Protein kinase C isotypes and their specific functions: prologue. J. Biochem. 132:509–511.

Pawson AJ, Maudsley S, Morgan K, Davidson L, Naor Z, Millar RP 2005 Inhibition of human type i gonadotropin-releasing hormone receptor (GnRHR) function by expression of a human type II GnRHR gene fragment. Endocrinology. 146(6):2639-2649.

Pawson AJ, Faccenda E, Maudsley S, Lu ZL, Naor Z, Millar RP 2008 Mammalian type I gonadotropin-releasing hormone receptors undergo slow, constitutive, agonist-independent internalization. Endocrinology 149(3):1415-22.

Perrin MH, Bilezikjian LM, Hoeger C, Donaldson CJ, Rivier J, Haas Y, Vale WW 1993 Molecular and functional characterization of GnRH receptors cloned from rat pituitary and a mouse pituitary tumor cell line. Biochem Biophys Res Commun. 191(3):1139-1144.

Prevostel C, Alice V, Joubert D, Parker PJ 2000 Protein kinase C! actively downregulates through caveolae-dependent traffic to an endosomal compartment. J. Cell Sci. 113, 2575–2584.

Radovick S, Wondisford FE, Nakayama Y, Yamada M, Cutler GB Jr, Weintraub BD 1990 Isolation and characterization of the human gonadotropin-releasing hormone gene in the hypothalamus and placenta. Mol Endocrinol. 4(3):476-480.

Reinhart J, Mertz LM, Catt KJ 1992 Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor.J Biol Chem. 267(30):21281-21284.

Romanelli RG, Barni T, Maggi M, Luconi M, Failli P, Pezzatini A, Pelo E, Torricelli F, Crescioli C, Ferruzzi P, Salerno R, Marini M, Rotella CM, Vannelli GB 2004 Expression and function of gonadotropin-releasing hormone (GnRH) receptor in human olfactory GnRH-secreting neurons. An autocrine GnRH loop underlies neuronal migration. J Biol Chem 279:117–126

Sakai N, Sasaki K, Ikegaki N, Shirai Y, Ono Y, Saito N 1997 Direct visualization of the translocation of the c-subspecies of protein kinase C in living cells using fusion proteins with green fluorescent protein. J. Cell Biol. 139:1465-1476.

Schaefer, M., Albrecht, N., Hofmann, T., Gudermann, T. and Schultz, G. (2001) Diffusion-limited translocation mechanism of protein kinase C isotypes. FASEB J. *15*, 1634±1636

Schultze-Mosgau A, Griesinger G, Altgassen C, von Otte S, Hornung D, Diedrich K. 2005 New developments in the use of peptide gonadotropin-releasing hormone antagonists versus agonists. Expert Opin Investig Drugs. 2005 Sep;14(9):1085-1097.

Shacham S, Harris D, Ben-Shlomo H, Cohen I, Bonfil D, Przedecki F, Lewy H, Ashkenazi IE, Seger R, Naor Z 2001 Mechanism of GnRH receptor signaling on gonadotropin release and gene expression in pituitary gonadotrophs.Vitam Horm. 63:63-90.

Shacham S, Cheifetz MN, Fridkin M, Pawson AJ, Millar RP, Naor Z 2005 Identification of Ser153 in ICL2 of the gonadotropin-releasing hormone (GnRH) receptor as a phosphorylation-independent site for inhibition of Gq coupling. J Biol Chem. 280(32):28981-28988.

Shraga-Levine Z, Ben-Menahem D, Naor Z 1994 Activation of protein kinase C beta gene expression by gonadotropin-releasing hormone in alpha T3-1 cell line. Role of Ca2+ and autoregulation by protein kinase C. J Biol Chem. 269(49):31028-31033.

Siler-Khodr TM, Grayson M 2001 Action of chicken II GnRH on the human placenta. J Clin Endocrinol Metab 86:804 – 810.

Sonnenburg ED, Gao T, Newton AC 2001 The phosphoinositide dependent kinase, PDK-1, phosphorylates conventional protein kinase C isozymes by a mechanism that is independent of phosphoinositide-3-kinase. J. Biol. Chem. 276:45289-45297.

Stanislaus D, Ponder S, Ji TH, Conn PM 1998 Gonadotropin-releasing hormone receptor couples to multiple G proteins in rat gonadotrophs and in GGH3 cells: evidence from palmitoylation and overexpression of G proteins. Biol Reprod. 59(3):579-586.

Streb H, Irvine RF, Berridge MJ, Schulz I 1983 Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1, 4, 5-trisphosphate. Nature 306:67–69.

Takai Y, Kishimoto A, Kikkawa U, Mori T, Nishizuka Y 1979 Unsaturated diacylglycerol as a possible messenger for the activation of Ca^{2+}-activated, phospholipid-dependent protein kinase system. Biochem. Biophys. Res. Commun. 91, 1218–1224.

Topaloglu AK, Lu ZL, Farooqi IS, Mungan NO, Yuksel B, O'Rahilly S, Millar RP. 2006 Molecular genetic analysis of normosmic hypogonadotropic hypogonadism in a Turkish population: identification and detailed functional characterization of a novel mutation in the gonadotropin-releasing hormone receptor gene. Neuroendocrinology. 2006;84(5):301-8.

Troskie BE, Hapgood JP, Millar RP, Illing N 2000 Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor expressed in the pituitary and midbrain of Xenopus laevis. Endocrinology.141(5):1764-71.

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol Endocrinol. 1992 Jul;6(7):1163-1169.

Ulloa-Aguirre A, Stanislaus D, Janovick JA, Conn PM 1999 Structure-activity relationships of g protein-coupled receptors. Arch Med Res. 30:420-435.

Vrecl 1998 recl M, Anderson L, Hanyaloglu A, McGregor AM, Groarke AD, Milligan G, Taylor PL, Eidne KA 1998 Agonist-induced endocytosis and recycling of the gonadotropin-releasing hormone receptor: effect of beta-arrestin on internalization kinetics. Mol Endocrinol. 12(12):1818-29.

Walsh, D.A., Perkins, J.P., and Krebs, E.G. (1968) An adenosine 3', 5'-monophosphate-dependent protein kinase from rabbit skeletal muscle. J. Biol. Chem. 243, 3763–3765

Watson PH, Fraher LJ, Hendy GN, Chung UI, Kisiel M, Natale BV, Hodsman AB 2000 Nuclear localization of the type 1 PTH/PTHrP receptor in rat tissues. J Bone Miner Res. 2000 Jun;15(6):1033-1044.

Weesner GD, Matteri RL 1994 Rapid communication: nucleotide sequence of luteinizing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary. J Anim Sci. 72(7):1911.

Wells A, Souto JC, Solava J, Kassis J, Bailey KJ, Turner T 2002 Luteinizing hormone-releasing hormone agonist limits DU-145 prostate cancer growth by attenuating epidermal growth factor signaling. Clin Cancer Res 8:1251–1257

Werner ED, Brodsky JL, McCracken AA 1996 Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proc Natl Acad Sci U S A. 1996 Nov 26;93(24):13797-137801.

White RB, Eisen JA, Kasten TL, Fernald RD 1998 Second gene for gonadotropin-releasing hormone in humans. Proc Natl Acad Sci U S A. 1998 Jan 6;95(1):305-9.

Willars GB, Heding A, Vrecl M, Sellar R, Blomenröhr M, Nahorski SR, Eidne KA 1999 Lack of a C-terminal tail in the mammalian gonadotropin-releasing hormone receptor confers resistance to agonist-dependent phosphorylation and rapid desensitization. J Biol Chem. 1999 Oct 15;274(42):30146-53.

Willars GB, Royall JE, Nahorski SR, El-Gehani F, Everest H, McArdle CA 2001 Rapid down-regulation of the type I inositol 1,4,5-trisphosphate receptor and desensitization of gonadotropin-releasing hormone-mediated Ca2+ responses in alpha T3-1 gonadotropes. J Biol Chem. 276(5):3123-3129.

Wise A, Gearing K, Rees S 2002 Target validation of G-protein coupled receptors. Drug Discov Today. 7(4):235-246.

Yang-Feng TL, Seeburg PH, Francke U 1986 Human luteinizing hormone-releasing hormone gene (LHRH) is located on short arm of chromosome 8 (region 8p11.2----p21). Somat Cell Mol Genet. 12(1):95-100.

Yang H, Lu D, Raizada MK 1997 Angiotensin II-induced phosphorylation of the AT1 receptor from rat brain neurons. Hypertension 30:351-357

Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, Moser C, Walker AP, McCabe ER 1994 An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature. 372(6507):635-41.

Zhang J, Barak LS, Winkler KE, Caron MG, Ferguson SS 1997 A central role for beta-arrestins and clathrin-coated vesicle-mediated endocytosis in beta2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types. J Biol Chem. 1997 Oct 24;272(43):27005-14.

Ziegler WH, Parekh DB, Le Good JA, Whelan RD, Kelly JJ, Frech M, Hemmings BA, Parker PJ 1999 Rapamycin-sensitive phosphorylation of PKC on a carboxy-terminal site by an atypical PKC complex. Curr Biol. 9(10):522-529.

Chapter 2- The Human Gonadotropin Releasing Hormone Type I Receptor is an Intracellular GPCR Expressed on the Nuclear Membrane

Co-Authorship

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2.1 Introduction

The gonadotropin releasing hormone receptor (GnRH-R) is a G protein-coupled receptor (GPCR) belonging to the rhodopsin family (Millar et al., 2004). In mammals, the type I receptor (GnRH-RI) is expressed in a variety of cell-types including pituitary gonadotropes, T-cells and placental cytotrophoblasts (Cheng et al., 2000). In these cells, ligand-activated GnRH-RI couples predominantly to $G_{q/11}$ but in other cell-types also couples to other G proteins such as Gs and G_i (Millar et al., 2004; Cheng and Leung, 2005). In humans, GnRH-RI is located at 4q13.2–3 and consists of three exons and two introns that encode a 328 amino acid protein (Ulloa-Aguirre et al., 2004).

The mammalian type I GnRH-R is structurally unique among GPCRs, including other GnRH-Rs expressed in non-mammalian and some primate species, in that it does not possess an intracellular tail (Millar et al., 2004; Cheng and Leung, 2005). The absence of COOH-tail sequences may have resulted in a receptor that lacks sequences necessary for promoting rapid desensitization and internalization as well as strong plasma membrane expression (PME) (Janovick et al., 2006). In addition, based on biochemical studies (radioligand binding and inositol phosphate formation assays) performed in African Green Monkey Kidney Fibroblast Cells (COS-1) and Human Embryonic Kidney (HEK 293) cells, it has been demonstrated that the presence of a primate-specific lysine residue at position 191 (K191) in the human GnRH-RI also contributes to reduced cellular expression and increased internalization kinetics of the receptor, leading to an overall reduction in PME compared to its rat and mouse counterparts (Arora et al., 1995, Maya-N Núñez et al., 2000). The presence of this primate-specific K191 causes the apparent disruption of a sulfhydryl bridge and subsequent formation of misfolded receptors resulting in the retention of the protein in the endoplasmic reticulum (ER) (Janovick et al., 2006). The loss of cytoplasmic tail sequences and presence of the K191, which have led to decreased PME and greater intracellular expression of GnRH-RI, are believed to be the result of a recently evolved strategy. This strategy provides the cell a very effective mechanism for rapidly regulating cell surface receptor number and hence GnRH-RI-mediated cell signaling events (Janovick et al., 2006), while at the same time being able to dispense away with the cellular machinery required for rapid desensitization and internalization.

It is believed that the ER-retained misfolded receptors are eventually degraded by the cell's QCS (Janovick et al., 2006). It has also been suggested that part of this retained intracellular pool of receptor provides a source of GnRH-RI needed for rapid availability to the cell without the need for transcription or translation (Janovick et al., 2006). In addition to these suggestions, we propose that greater intracellular expression of a receptor, which is normally expressed at the cell surface, is perhaps an indication that the cell may be evolving towards greater intracrine signalling, with respect to that receptor. Since none of the prior reports that described the intracellular retention of GnRH-RI actually performed image-based cellular localization studies of the receptor, we undertook a detailed spatial characterization of the receptor with the aim of providing evidence to support our idea that the mammalian GnRH-RI is evolving towards greater intracrine signalling. The following provides a brief introduction to this study.

N-terminal FLAG-epitope tagged human GnRH-RI (hGnRH-RI) was expressed in the heterologous and homologous cell lines, HEK 293 and Human Trophoblast (HTR-8/SVneo), respectively (Graham et al., 1993). In addition, the N-terminal FLAG-epitope tagged mouse GnRH-RI (mGnRH-RI) was also expressed in these cell lines. An epitope tagged receptor was used so that the cellular distribution of GnRH-RI could be determined in immunocytochemical studies through the use of an antibody against the epitope. Ideally, it would have been preferable to conduct these studies with an untagged receptor, however, anti-GnRH-RI antibodies that are effective in detecting GnRH-RI immunocytochemically are not commercially available. The FLAG epitope was chosen for two reasons. The first is because the anti-FLAG antibodies have proven their utility in providing strong spatial data on FLAG-tagged proteins in a variety of cells (Baroudi et al., 2002; Panyi et al., 2003; Bhattacharya et al., 2004; Han et al., 2008). The second is because a study by Brothers et al. (Brothers et al., 2003) demonstrated that the use of other epitopes, such as the HA epitope can promote the non-specific association of the receptor with the plasma membrane while the GFP tag can significantly affect protein conformation. The study by Brothers et al. (Brothers et al., 2003) did not report on the effects of the FLAG epitope, but experiments conducted in our laboratory, and described within this report, demonstrate that the human untagged and FLAG-tagged GnRH-RI have similar biochemical properties and these are different from that of the HA-tagged

GnRH-RI. This led us to conclude that the untagged and FLAG-tagged GnRH-RI may be used interchangeably in our study.

Here we report, based on spatial imaging data, that in both human cell lines the human and mouse FLAG-GnRH receptors were expressed at the nuclear membrane. Additionally, both receptors were expressed in the cytoplasm, however, the human receptor was highly associated with the ER, while the mouse receptor was distributed more widely in the cytoplasm. These findings reveal that GnRH-RI is a member of a small but growing class of nuclear GPCRs and this suggests the existence of an entirely new facet to GnRH-RI signaling within the cell.

2.2 Materials and Methods

2.2.1 Materials

Restriction enzymes were obtained from Promega (Madison, WI, USA) and New England Biolabs Inc. (Pickering, ON, Canada). HEK 293 cells were from American Type Culture Collection (ATCC, Manassas, VA, USA). HTR-8/SVneo cells were a gift from Dr. Peeyush Lala from the University of Western Ontario. Fetal bovine serum (FBS), collagen, rabbit anti-FLAG antibody and Buserelin were purchased from Sigma Aldrich Inc. (Oakville, ON, Canada). Mouse anti-lamin A/C, and mouse anti-calnexin were purchased from AbCam (Cambridge, MA, USA). Mouse anti-GM130 was purchased from Transduction Laboratories (BD Biosciences, Mississauga, ON, Canada). All primary antibodies detect the human and mouse homologues. Secondary antibodies conjugated to Alexa Fluors, Hoechst dye, pcDNA3.1/Hygro(+) vector, media and media supplements were acquired from Invitrogen (Burlington, ON, Canada). Quisqualate was from Tocris Biosciences (Avonmouth, Bristol, UK). All other biochemical reagents and culture products were purchased from BioShop, Fisher Scientific and VWR.

2.2.2 Plasmids expressing human GnRH-RI and its mutants

A 987-bp cDNA fragment containing the complete coding sequence of human GnRH-RI (accession number NM_000406.2) was obtained by RT-PCR from QUICK-CloneTM cDNA library (Clontech Laboratories Inc., Mountain View, CA, USA). The FLAG tag at the amino terminus of hGnRH-RI was obtained by inserting the cDNA

between the HindIII and EcoRI sites of pFLAG/CMV-II vector. FLAG-hGnRH-RI was then cloned into the pcDNA3.1/Hygro(+) vector using Nhe1 and Not1 sites.

To create the HA tagged hGnRH-RI construct, hGnRH-RI was subcloned from the FLAG-GnRH-RI vector using Nhe1 and Not1 into a pcDNA 3 vector that had been modified to include the HA tag sequence upstream of Nhe1.

The untagged receptor was created by inserting an EcoRI site with a Kozak sequence between the FLAG tag and hGnRH-RI by PCR and then subcloning hGnRH-RI into the pcDNA 3.1 Neo vector using EcoRI and Not1.

To create the NLS deletion mutant, K191deletion mutant, K191E mutant and 3 naturally occuring mutants, FLAG-hGnRH-RI was subjected to site-directed mutagenesis using the QuickChange kit following manufacturer's instructions (Stratagene, Santa Clara, CA, USA). Oligonucleotides from Sigma Genosys (Oakville, ON, Canada) were used. The putative NLS deletion mutant was created by performing two sequential deletions; first removing K66 and K67, followed by the deletion of E68, K69, G70, K71 and K72. The following oligonucleotides were used. The mutated codon is highlighted in grey and the altered nucleotides are underlined. In the case of deletion mutants, a Δ signifies deleted codons.

Putative NLS deletion mutant

K66del/K67del FWD CTTCAGAAGTGGACACAGΔGAGAAAGGGAAAAAGCTC

K66del/K67del REV GAGCTTTTTCCCTTTCTCΔCTGTGTCCACTTCTGAAG

E68del/K69del/G70del/K71del/K72del FWD

GTTGAAACTTCAGAAGTGGACACAGΔCTCTCAAGAATGAAGC

E68del/K69del/G70del/K71del/K72del REV

GCTTCATTCTTGAGAGΔCTGTGTCCACTTCTGAAGTTTCAAC

The lysine 191 mutants and naturally occurring mutants were created in a single mutagenesis reaction using the following oligonucleotides:

Lysine 191 mutants

K191E FWD GCAGACAGCTCTGGACAGACAGAAGTTTTCTCTCAATGTG

K191E REV CACATTGAGAGAAAACTTCTGTCTGTCCAGAGCTGTCTGC

K191deletion FWD GCTCTGGACAGACAΔGTTTTCTCTCAATGTGTAACACAC

K191deletion REV GTGTGTTACACATTGAGAGAAAACΔTGTCTGTCCAGAGC

Naturally Occuring Mutants

A129D GnRH-RI FWD CCATGTATGCCCCAGACTTCATGATGGTGG

A129D GnRH-RI REV CCACCATCATGAAGTCTGGGGCATACATGG

A171T GnRH-RI FWD

CTGGATCCTCAGTAGTGTCTTTACAGGACCACAGTTATACATCTTCAGG

A171T GnRH-RI REV

CCTGAAGATGTATAACTGTGGTCCTGTAAAGACACTACTGAGGATCCAG

R262Q GnRH-RI FWD

CAATATACCAAGAGCACAGCTGAAGACTCTAAAAATGACGGTTG

R262Q GnRH-RI REV

CAACCGTCATTTTTAGAGTCTTCAGCTGTGCTCTTGGTATATTG

A 984-bp cDNA fragment containing the complete coding sequence of mouse GnRH-RI (accession number NM_010323.1) was isolated by RT-PCR using RNA from the pituitary of a female 129/Sv mouse (Charles River). Bam HI and Not I sites were cloned onto the 3' and 5' end of the cDNA construct, respectively. To create the FLAG-tagged mouse GnRH-RI construct, the human GnRH-RI was removed from the FLAG-pcDNA 3.1 Hygro+ vector using BamHI and NotI and the mouse GnRH-RI ORF was ligated in. The sequence integrity of each of the constructs were verified by sequencing conducted by Robarts Research Institute (London, ON, Canada).

2.2.3 Cell culture and Immunofluorescence

HEK 293 cells were cultured in MEM supplemented with 10% (v/v), FBS, 1% (v/v) non-essential amino acids and gentamicin (5 μg/ml) (Babwah et al., 2003). HTR-8/SVneo cells were maintained in RPMI supplemented with 10% FBS, 1% non-essential amino acids, 1% glutamax, 1% sodium pyruvate and 1% penicillin/streptomycin (v/v) (Graham et al., 1993). Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. HEK 293 cells were transiently transfected with 5 μg of DNA using a modified Ca^{2+} phosphate method as previously described (Babwah et al., 2003). After transfection (18 h), the cells were incubated with fresh medium, allowed to recover for 6 hours and reseeded onto 18 mm collagen-coated glass coverslips. Cells were allowed to grow an additional 18 h prior to experimentation. HTR-8/SVneo cells

were transiently transfected with 10 μg of DNA by electroporation using the Bio-Rad Gene Pulser Xcell System with 0.2 mm electroporation cuvettes (BioRad) and the CHO preset protocol. Post transfection, cells were allowed to recover for 24 h before being plated on 18 mm collagen coated glass coverslips in 12 well plates. 42-44 h post-transfection, both HTR-8/SVneo and HEK 293 cells were washed twice with Hanks' balanced salt solution (HBSS: 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) and either stimulated [100 nM Buserelin for GnRH-RI and 100 μM Quisqualate for mGluR5a] for two hours or left untreated, based on previous studies (Leaños-Miranda et al., 2005). Cells were fixed and permeabilized using 4% formaldehyde and 0.2% Triton-X in HBSS for 20 minutes, washed 4 times with HBSS and blocked with HBSS-3%BSA for 30 minutes before the addition of primary antibody. Cells were incubated with primary antibody overnight at 4 °C at the following concentrations: polyclonal anti-FLAG 1:2000, monoclonal anti-calnexin 1:100 (to detect endoplasmic reticulum), monoclonal anti-GM130 1:100 (to detect the Golgi apparatus), monoclonal anti-lamin A/C 1:10 (to detect the inner nuclear membrane). Coverslips were then washed 4 times with HBSS and blocked for an additional 30 min in HBSS-3%BSA before incubation with secondary antibody for 45 min at room temperature. Goat anti-mouse antibody conjugated to AlexaFluor 488 was used at a dilution of 1:250, while goat anti-rabbit antibody conjugated to AlexaFluor 568 was used at 1:1200. Cells were washed 4 times with HBSS and then counter-stained using Hoechst at 1:50000 (v/v) for 7 min to detect DNA. After 4 additional washes with HBSS, cells were mounted onto glass slides and allowed to dry overnight at room temperature. Confocal analysis was performed on an Olympus Fluoview 1000 laser scanning confocal microscope using either the 60X Plan Apochromat 1.42 oil objective or the 100x Plan superapochromat 1.4 Oil objective. Colocalization studies were performed using multiple excitation (405, 488, 559) and emission (band pass 425-475, 500-545 nm and 575-675 nm for Hoechst, AlexaFluor 488 and AlexaFluor 568 respectively) filter sets. Multi-colour images were acquired in the sequential acquisition mode to avoid cross-excitation

2.2.4 Nuclei isolation

Nuclei were isolated using the Sigma Nuclei EZ Prep Nuclei Isolation Kit. In brief, nuclei were isolated from HEK 293 cells expressing FLAG-GnRH-RI or FLAG-mGluR5a approximately 42-44 hours post-transfection. Cells were stimulated with agonist for 2 hours or left untreated, washed twice with ice cold PBS and then lysed with the provided buffer. Cells were collected and lysed using 15 strokes of a 15 mL Dounce homogenizer using the tight pestle. The lysate was mixed with a 1.8 M sucrose solution and then layered over 10 mL of 1.8 M sucrose solution in a 40 mL Beckman centrifuge tube (Beckman Coulter Inc., Fullerton, CA, USA). Tubes were spun at 12900 rpm in a swinging bucket rotor for 85 min to pellet the nuclei. The supernatant was removed and nuclei were washed and resuspended in storage buffer (provided). A sample of nuclei were plated on 18 mm collagen-coated coverslips in 12 well plates and fixed and immunostained as described above.

2.2.5 Inositol Phosphate Formation

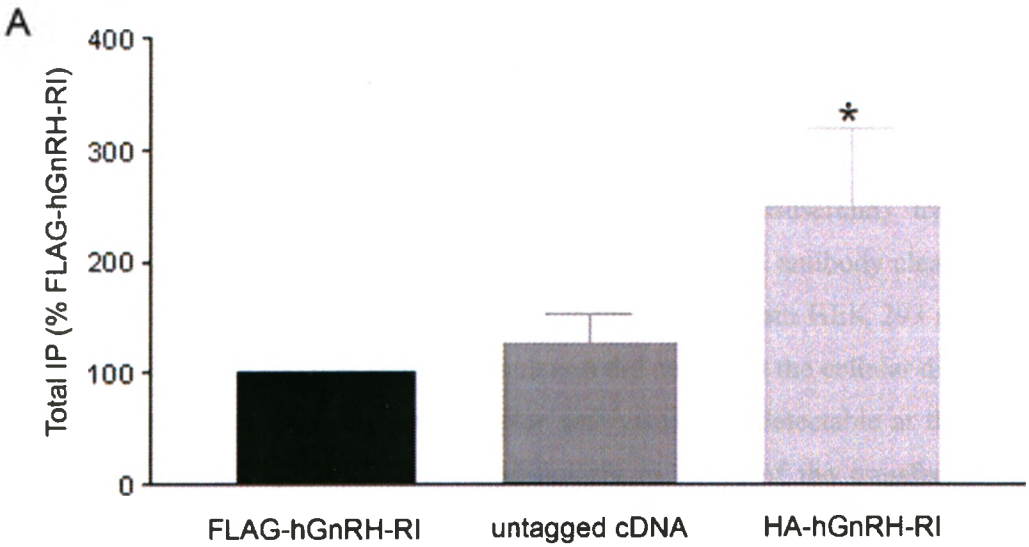
Inositol lipids were radiolabeled by incubating the cells overnight with 1 μCi/ml [^{3}H]myo-inositol in Dulbecco's modified Eagle's medium. Unincorporated [^{3}H]myo-inositol was removed by washing the cells with HBSS. Cells were preincubated for 1 h in HBSS at 37 °C and then preincubated in 500 μl of the same buffer containing 10 mM LiCl for an additional 10 min at 37 °C. Next, the cells were incubated in either the absence or the presence of 100 nM Buserelin for 2 hs at 37 °C. The reaction was stopped on ice by adding 500 μl of 0.8 M perchloric acid and then neutralized with 400 μl of 0.72 M KOH, 0.6 M $KHCO_3$. The total [^{3}H]inositol incorporated into the cells was determined by counting the radioactivity present in 50 μl of the cell lysate. Total inositol phosphate was purified from the cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin. [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a Wallac LKB 1211 RackBeta liquid scintillation counter. The means ± S.E. are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data for statistical significance. Statistical significance was determined by

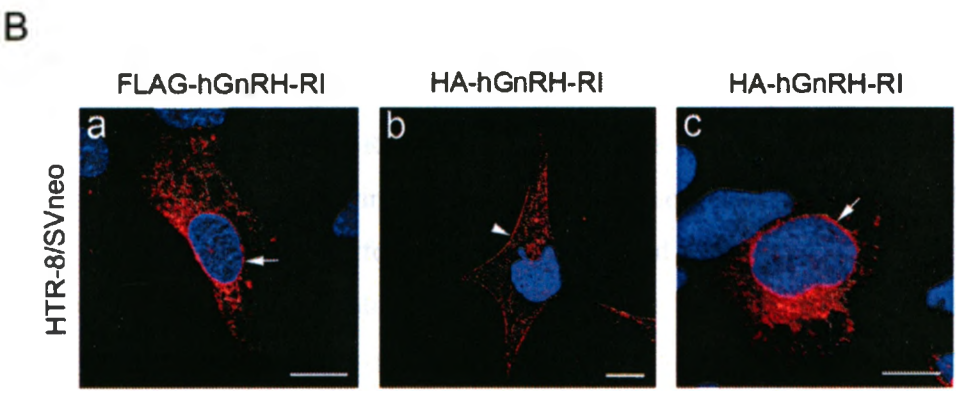
Student's t-test or one-way analysis of variance with Dunn's post hoc multiple comparison test.

2.3 Results

This study employed the use of the human embryonic kidney (HEK 293) and human trophoblast (HTR-8/SVneo) cell lines as models of heterologous and homologous cell systems, respectively. The HEK 293 cell line is an established cell system for studying regulatory, temporal and spatial characteristics of GPCRs (Babwah et al., 2003; Gardner et al., 2006, Wang et al., 2007), while HTR-8 SV/Neo is a trophoblast cell line that is an established system for studying human placentation (Graham et al., 1993). We were unable to detect the expression of GnRH-RI in HEK 293 cells by RT-PCR (data not shown) and therefore used this as a heterologous cell system for studying GnRH-RI expression. A previous study demonstrated that trophoblasts, including HTR-8/SVneo cells, express GnRH-RI (Cheng et al., 2000) and based on this we used HTR-8/SVneo as a homologous cell system for studying GnRH-RI expression. As previously stated, in the absence of effective anti-GnRH-RI antibodies for conducting immunofluorescene, FLAG and HA amino terminus epitope-tagged receptors (open-reading frame only) were constructed and their utility in conducting this study was tested. Using inositol phosphate (IP) formation as an indication of signaling capacity, the FLAG and HA-tagged hGnRH-RI were compared to an untagged hGnRH-RI cDNA (accession number NM_000406.2) for their ability to stimulate agonist-dependent IP formation. Our data revealed that the GnRH agonist Buserelin stimulated FLAG-hGnRH-RI and cDNA expressing HEK 293 cells produced similar levels of IP, while the HA-hGnRH-RI produced significantly higher levels (Fig 2.1A). Additionally, immunocytochemical analysis of the HA- and FLAG-tagged receptors revealed that while both receptors were expressed and strongly localized intracellularly, the HA-tagged receptor was also readily detectable at the plasma membrane while the FLAG-tagged receptor was not (Fig 2.1B) and this plasma membrane expression may have accounted for the higher IP levels associated with the HA-tagged receptor. Based on these findings, and taken together with those reported by Brothers et al. (Brothers et al., 2003), we selected the FLAG-tagged GnRH-RI (human and mouse) for all subsequent studies. The FLAG-GnRH-RI DNA was introduced into

Figure 2.1 Effect of epitope tags on hGnRH-RI signaling and spatial localization. (A) Inositol phosphate (IP) production of HEK cells expressing hGnRH-RI constructs in response to 100 nM Buserelin was assessed as described in "Materials and Methods". Data represent three to six independent experiments performed in triplicate and normalized to FLAG-hGnRHRI ± S.E. *, $p < 0.05$ versus IP formation of FLAG-hGnRH-RI. (B) HTR-8/SVneo cells transfected with either FLAG-hGnRH-RI (a) or HA-hGnRH-RI (b,c) subjected to indirect immunofluorescent staining using affinity purified rabbit anti-FLAG antibody followed by Alexa Fluor 568-conjugated anti-rabbit IgG (*red*) and counterstained with the nuclear dye, Hoechst (*blue*). Note the perinuclear localization of the FLAG-tagged hGnRH-RI (*a, arrow*) and the plasma membrane localization of HA-hGnRH-RI (*b, arrowhead*). Perinuclear localization of the HA-hGnRH-RI can also be seen in some cells (*c, arrow*). Scale bar = 10 μm.


A
400
300
200
100
0
Total IP (% FLAG-hGnRH-RI)
*
FLAG-hGnRH-RI
untagged cDNA
HA-hGnRH-RI


B
FLAG-hGnRH-RI
HA-hGnRH-RI
HA-hGnRH-RI
HTR-8/SVneo
a
b
c

HEK 293 cells by Ca^{2+}-phosphate transfection, while it was introduced into HTR-8/SVneo cells by electroporation. Transfection efficiency of the HEK 293 cells was always greater than 75%, while that for HTR-8/SVneo cells was closer to 50% (data not shown).

Immunocytochemical studies performed on agonist (Buserelin) treated and untreated Triton X 100-permeabilized cells, using the anti-FLAG antibody clearly reveal that the spatial expression of FLAG-hGnRH-RI is identical in both HEK 293 and HTR-8/SVneo cells (Fig. 2.2) and that agonist stimulation did not affect the cellular distribution (data not shown). Expression of the receptor was visually undetectable at the plasma membrane but was strongly localized intracellularly in 100% of the transfected cells. While the receptor was distributed intracellularly throughout the cell there were two intracellular regions in which expression was very high. In 100% of all transfected cells, one region corresponded to a large part of the cytoplasm, generally located to one side of the nucleus only, while in 75-95% of these cells, the receptor was also strongly observed at the perinuclear region (Fig 2.2). This perinuclear localization was also observed with the HA epitope-tagged hGnRH-RI (Fig 2.1). Since the cellular distribution of FLAG-hGnRH-RI was the same both in the absence and presence of agonist, all subsequent studies with hGnRH-RI were performed in agonist-treated cells.

To characterize the spatial localization of the receptor in greater detail we examined the location of the receptor relative to calnexin, GM130 and lamin A/C; these are organellar markers for the ER, Golgi and the nuclear membrane, respectively, in both HEK 293 and HTR-8 SV/Neo cells. Our results revealed that a large fraction of the cytoplasmic pool of receptors localized to the ER (Fig 2.3) while the perinuclear pool localized to the nuclear membrane (Fig 2.3). Receptor localization to the Golgi was detectable but not as high as in the ER (Fig 2.3). In all cases no differences were observed between the HEK 293 and HTR-8 SV/Neo cells (Fig 2.3). Since we consistently showed no differences in the localization of the receptor in the two cell lines all other localization studies were conducted in the HTR-8 SV/neo cell line only, except for data presented in Fig 2.5.

Next, we compared the distribution pattern of FLAG-hGnRH-RI in HTR-8 SV/neo cells to that of FLAG-mGluR5a (metabotropic glutamate receptor 5a) and FLAG-

Figure 2.2 Spatial localization of FLAG-hGnRH-RI in agonist treated and untreated HTR-8/SVneo and HEK 293 cells. HTR-8/SVneo and HEK 293 cells transfected with FLAG-hGnRH-RI were treated with 100 nM Buserelin or left untreated and subjected to indirect immunofluorescent staining using affinity purified rabbit anti-FLAG antibody followed by Alexa Fluor 568-conjugated anti-rabbit IgG (*red*) and counterstained with Hoechst (*blue*). Note the perinuclear localization of the FLAG-tagged hGnRH-RI seen in both cell lines (*arrows*). Scale bar = 10 μm.

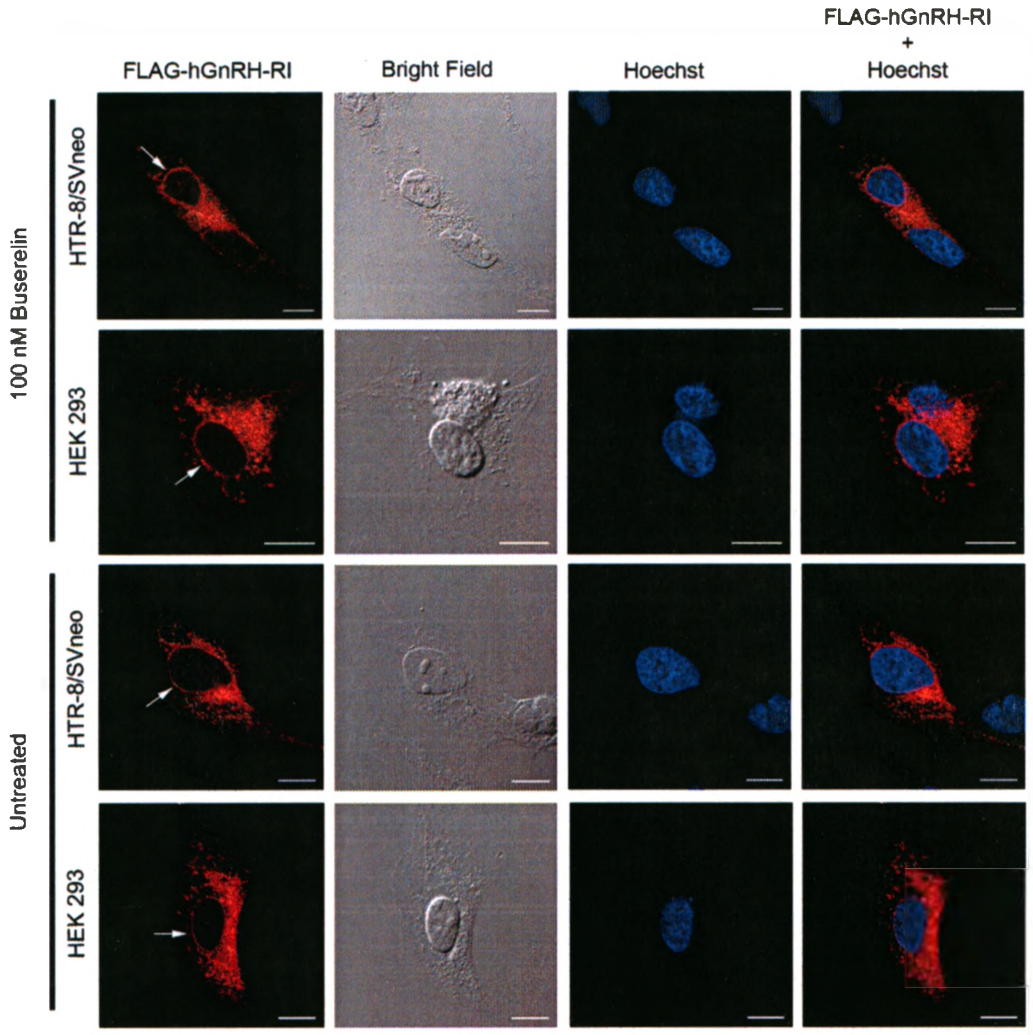
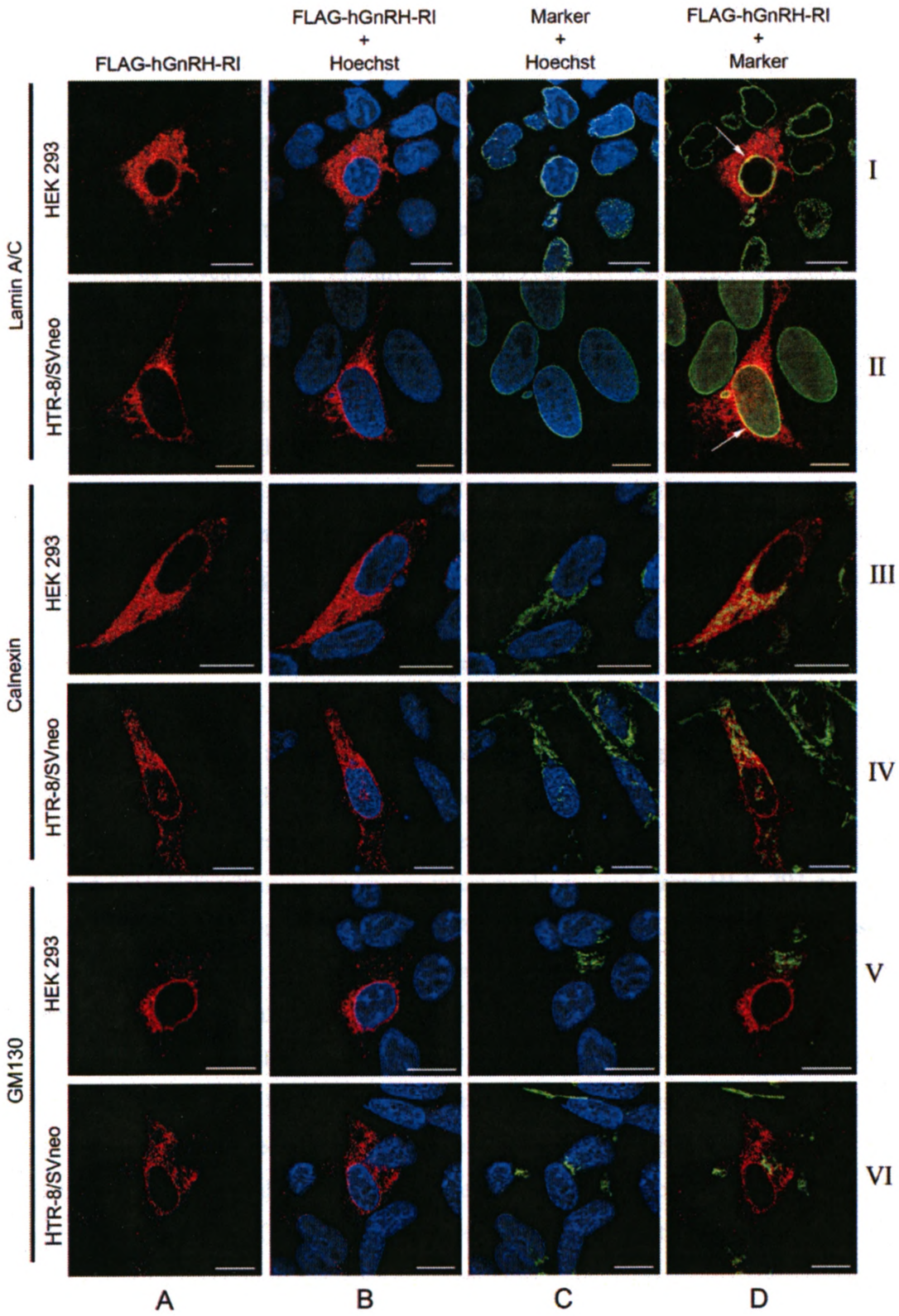

FLAG-hGnRH-RI
Bright Field
Hoechst
FLAG-hGnRH-RI
+
Hoechst
100 nM Buserelin
Untreated
HTR-8/SVneo
HEK 293
HTR-8/SVneo
HEK 293

Figure 2.3 Spatial localization of FLAG-hGnRH-RI in HEK 293 and HTR-8/SVneo cell lines using organellar markers. HTR-8/SVneo cells expressing FLAG-hGnRH-RI were subjected to indirect immunofluorescent staining for the receptor (red) as well as the nuclear membrane, endoplasmic reticulum and Golgi (*all shown in green*). Colocalization is seen as yellow staining. Column A: receptor alone; Column B: receptor + Hoescht; Column C: organelle marker (lamin A/C, calnexin or GM130) + Hoescht; Column D: receptor + organelle marker. Column letters and roman numerals used as a coordinate system. hGnRH-RI immunoreactivity co-localized with the nuclear marker, lamin A/C, as seen by the yellow staining (ID, IID, *arrows*) as well as colocalizing with the endoplasmic reticulum marker, calnexin (IIID, IVD). Less colocalization was seen between the receptor and the Golgi, as shown by less yellow staining (VD, VID). Scale bar = 10 μm.


FLAG-hGnRH-RI
FLAG-hGnRH-RI + Hoechst
Marker + Hoechst
FLAG-hGnRH-RI + Marker
Lamin A/C
Calnexin
GM130
HEK 293
HTR-8/SVneo
I
II
III
IV
V
VI
A
B
C
D

β2-AR (β2-adrenergic receptor) in HTR-8/SVneo cells (Fig 2.4). Both receptors are GPCRs and mGluR5a has been well characterized as a nuclear GPCR that is expressed on the nuclear membrane (O'Malley et al., 2003; Jong et al., 2005) while the β2-AR has only been detected on the plasma membrane and in the cytoplasm (Salahpour et al., 2004; Cao et al., 2005). Immunocytochemical studies on agonist (quisqualate for mGluR5a and isoproterenol for β2-AR) treated cells permeabilized prior to the incubation of the anti-FLAG antibody revealed that mGluR5a colocalized with lamin A/C at the nuclear membrane in a pattern identical to that of hGnRH-RI, while as expected, β2-AR was expressed at the plasma membrane (Fig 2.4). β2-AR and mGluR5a were also seen intracellularly, in vesicles and almost evenly distributed throughout the rest of the cytoplasm. However, unlike mGluR5a, β2-AR was completely absent at the nuclear membrane. Interestingly, unlike FLAG-mGluR5a and FLAG- β2-AR, the cytoplasmic distribution of FLAG-hGnRH-RI was often more strongly localized to one side of the nucleus, rather than throughout the cell, and enrichment on vesicles was never detected (Fig 2.4). To test whether the differences in the spatial distribution were in part due to relative expression levels between the receptors, we performed SYBR green real-time PCR analysis of receptor gene expression in HEK 293 cells. We found that all the receptors were expressed at similar levels (data not shown), suggesting that differential expression was not the underlying cause for these observations.

In addition to investigating the localization of GnRH-RI in whole cells, we also performed immunolocalization studies on intact nuclei isolated from HEK 293 cells that were previously transfected with FLAG-hGnRH-RI or FLAG-mGluR5a. The purpose of these studies was to verify the nuclear localization of hGnRH-RI was real or was a visual aberration due to the result of hGnRH-RI. It was possible that lamin A/C might have only appeared to colocalize with the pool of receptor molecules in the adjoining perinuclear region. Thus by stripping away the cytoplasmic pool of receptor, we could more confidently assess receptor expression on the nuclear membrane. The results from these experiments revealed that in the absence of the cytosolic components, both FLAG-hGnRH-RI and FLAG-mGluR5a were still readily detectable at the nuclear membrane and colocalized with lamin A/C (Fig 2.5). These results demonstrate that the nuclear

Figure 2.4 Spatial localization of FLAG-mGluR5a and FLAG-β_2AR in HTR-8/SVneo cells. HTR-8/SVneo cells transfected with either FLAG-mGluR5a (*top row*) or FLAG-β_2AR (*bottom row*) were subjected to indirect immunofluorescent staining for the receptor (*red*) as well as the nuclear membrane marker lamin A/C (*green*). Cells were counterstained with Hoechst (*blue*). Note the perinuclear localization of the FLAG-tagged mGluR5a (*top row, arrow*) and the plasma membrane localization of both FLAG mGluR5a and FLAG-β_2AR (*arrowheads*). Scale bar = 10 μm.

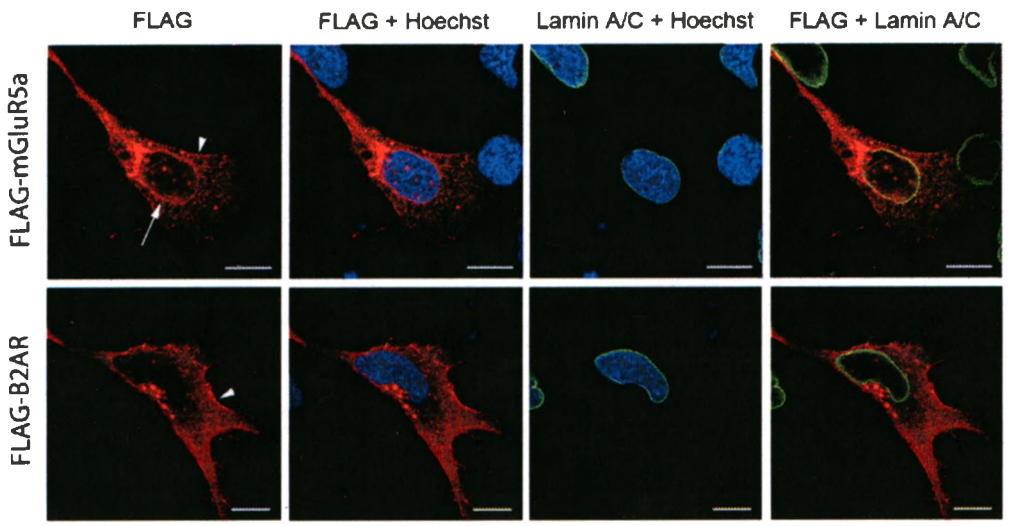
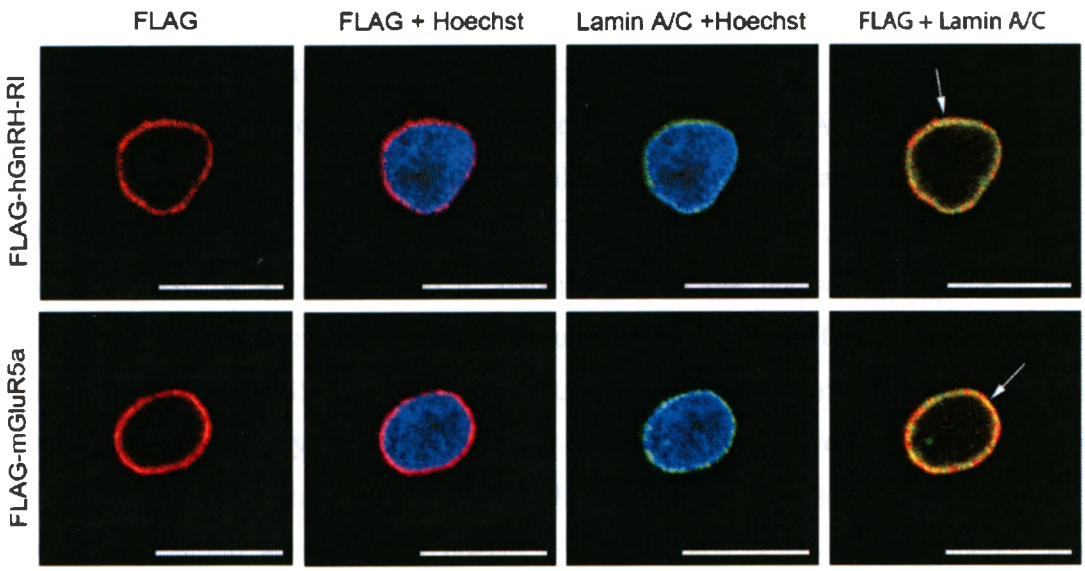

FLAG
FLAG + Hoechst
Lamin A/C + Hoechst
FLAG + Lamin A/C
FLAG-mGluR5a
FLAG-B2AR

Figure 2.5 Confocal microscopy of nuclei isolated from HEK 293 cells expressing FLAG-hGnRH-RI or FLAG-mGluR5a. Isolated nuclei from HEK293 cells expresing FLAG-hGnRH-RI or FLAG-mGluR5a were subjected to indirect immunofluorescent staining for the receptor (*red*) as well as the nuclear membrane marker lamin A/C (*green*). Cells were counterstained with Hoechst (*blue*). Note the colocalization of both FLAG-hGnRH-RI and FLAG-mGluR5a with the inner nuclear membrane marker lamin A/C (*yellow, arrows*). Scale bar = 10 μm.


FLAG
FLAG + Hoechst
Lamin A/C +Hoechst
FLAG + Lamin A/C
FLAG-hGnRH-RI
FLAG-mGluR5a

membrane localization of hGnRH-RI is real and not the result of a visual aberration due to overexpression of the receptor in the perinuclear region.

Both the strong colocalization of hGnRH-RI and lamin A/C in HEK 293 and HTR-8/SVneo cells (Fig 2.3) and the identical pattern of colocalization observed to that of the nuclear mGluR5a and lamin A/C in HTR-8/SVneo cells (Fig 2.4) suggested that hGnRH-RI may be a nuclear GPCR associated with the nuclear membrane. This possibility led us to examine the protein sequence of hGnRH-RI for a putative nuclear localization signal (NLS). Using the Basic Local Alignment Search Tool (BLAST) to search for short, nearly exact matches in the National Centre for Biotechnology Information protein database, we identified a putative monopartite NLS sequence, KKEKGKK (amino acid position 66-72) (Fig 2.6, Table 2.1). This sequence was conserved in other proteins characterized as nuclear localized proteins (Table 2.1). These include DNA topoisomerase II from Candida spp., pleiotrophic factor α2 (transcriptional regulator) from Xenopus laevis, HAI-2 related small protein (transcriptional regulator) from humans, and a number of other proteins from various species with putative nuclear functions such as the putative U3 small nuclear ribonucleoprotein (snRNP) from Leishmania major. Next, using the DNASTAR Lasergene MegAlign program, we performed protein sequence alignments and observed that this sequence is fully conserved in the GnRH-RI protein expressed in the chimpanzee while in the mouse and rat, a sequence (KRKKGKK) with very high identity (71.4%) to the primate sequence is found at the equivalent site (Table 2.1). This murine sequence, however, is still a fully conserved monopartite NLS. Interestingly, the chicken GnRH receptor which is only stimulated by the type II hormone also contains a highly basic stretch of residues (RKRRK) at the equivalent region in the first intracellular loop (Fig 2.6, Table 2.1), yet similar sequences are not located in any part of the mammalian type II GnRH-R (data not shown), a receptor that also appears to be activated only by the type II GnRH.

To test whether the putative NLS located in the first intracellular loop of hGnRH-RI was required for the nuclear localization of the receptor, we performed single and multiple amino acid substitutions (mutating K and E to G) and deletions as well as a full deletion of the KKEKGKK sequence in FLAG-hGnRH-RI and expressed the mutant receptor in HEK 293 cells. Surprisingly, we found that none of the mutations, including

Figure 2.6 Schematic of the human GnRH-RI showing the positions of its naturally occurring mutations and other residues of interest. Human GnRH-RI depicting the location of 3 of the known natural mutations (denoted by grey shading), position of the lysine 191 residue (triangle) and the location of the putative NLS (square).

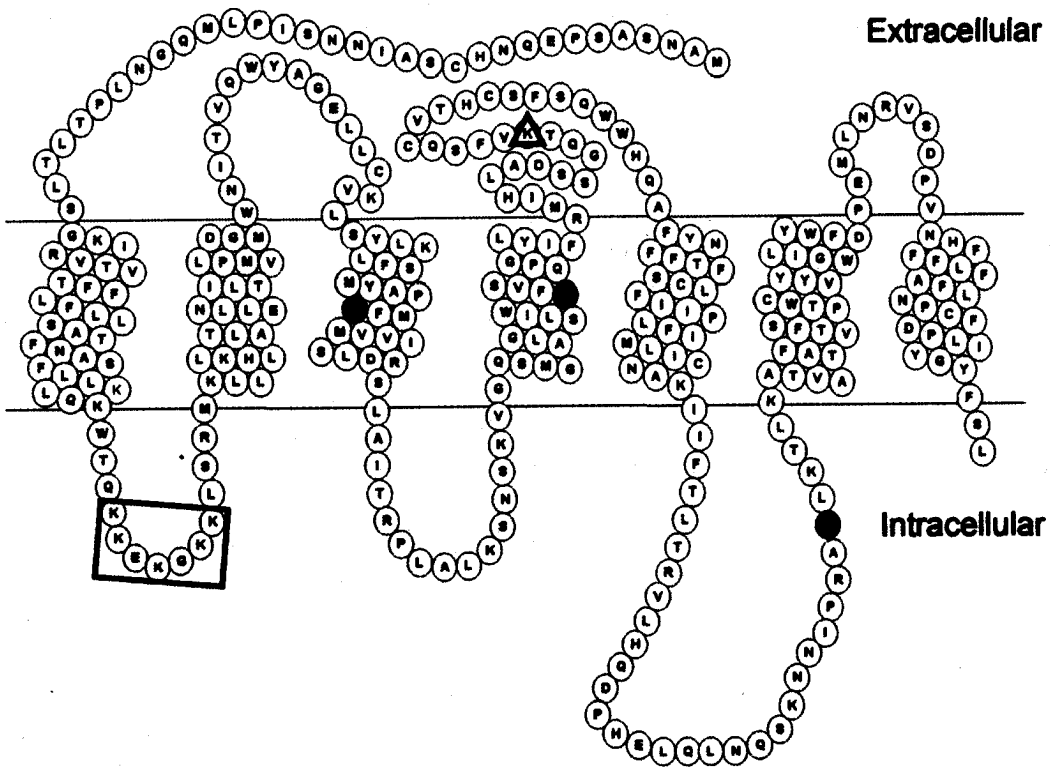

Extracellular
Intracellular

Table 2.1 Proteins containing a putative monopartite NLS related to that found in the human gonadotropin releasing hormone receptor isoform 1

Definition	Accession	Sequence	Amino Acid Position
Gonadotropin releasing hormone receptor isoform 1 [*Homo sapiens*]	NP_000397	KKEKGKK	66-72
U3 small nuclear ribonucleoprotein (snRNP) [*Leishmania infantum* JPCM5]	XP_001466980	KKEKGKK	235-241
Type II DNA topoisomerase [*Candida tropicalis*]	BAB13749	KKEKGKK	288-294
Pleiotrophic factor-alpha1 [*Xenopus laevis*]	BAA07658	KKEKGKK	25-31
Alkylbase DNA N-glycosylase [*Cryptococcus neoformans* var. neoformans JEC21]	XP_570311	KKEKGKK	723-729
HAI-2 related small protein [*Homo sapiens*]	AAG43574	KKEKGKK	95-101
Gonadotropin releasing hormone receptor isoform 1 [*Pan troglodytes*]	XP_001163813	KKEKGKK	66-72
Gonadotropin releasing hormone receptor [*Mus musculus*]	NP_034453	KRKKGKK	66-72
Gonadotropin releasing hormone receptor [*Rattus norvegicus*]	NP_112300	KRKKGKK	66-72
Gonadotropin releasing hormone receptor [*Gallus gallus*]	NP_989984	RKRRK	69-73

the full KKEKGKK deletion mutant, altered the spatial distribution of the receptor or frequency of cells expressing the receptor on the nuclear membrane (as assessed by lamin A/C colocalization) relative to the non-mutated receptor (Fig 2.7A, data shown for the full putative NLS deletion mutant only). Interestingly, we found that although the full putative NLS deletion mutant still localized to the nuclear membrane, it showed a significantly diminished capacity to stimulate IP formation following agonist treatment relative to its wild-type counterpart (Fig 2.7B). This therefore suggested that nuclear localization is independent of GnRH-RI/$G_{q/11}$-coupling, which in turn is consistent with the earlier observation that the nuclear localization of hGnRH-RI is not an agonist-dependent event.

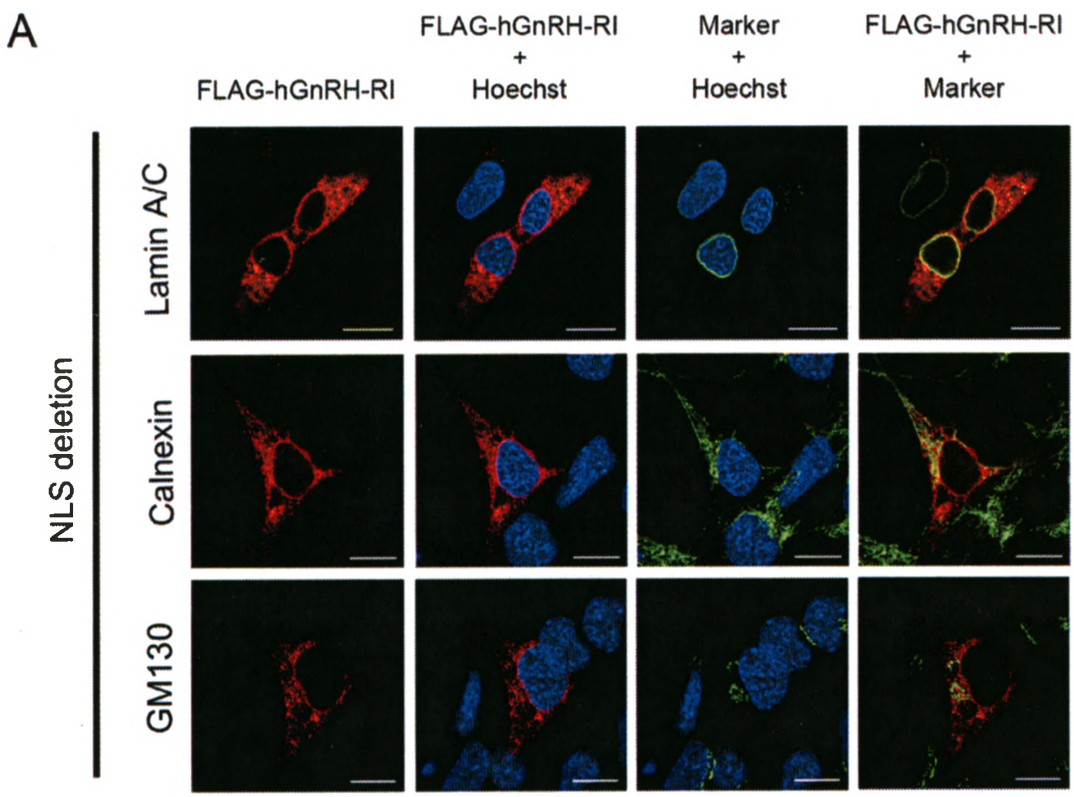
Next, based on previous data which revealed that the presence of a primate specific-residue, K191, in the hGnRH-RI (Fig 2.6) contributed to an overall reduced PME, we investigated whether this residue also regulated nuclear membrane localization of hGnRH-RI. Our studies revealed that neither mutating the basic lysine residue to the acidic glutamic residue (E) nor deleting K191 led to any visual change on the cellular distribution of the receptor, particularly with respect to its nuclear membrane localization (Fig 2.8A). This observation was further supported by IP formation data that revealed that there was no significant difference between the mutants, K191E and K191 deletion, compared to the wild-type FLAG-tagged human receptor. Nevertheless, both mutants did show a trend towards an increase in IP formation relative to the non-mutated receptor (Fig 2.8B).

Since the murine receptor lacks the K191 residue but contains a fully conserved monopartite NLS in the homologous position to the human NLS, we looked at the expression of the mouse FLAG-GnRH-RI in HEK 293 and HTR-8/SVneo cells. Our studies revealed that in both cell types, the mouse receptor is also expressed on the nuclear membrane (Fig. 2.9, HEK 293 data not shown) like its hGnRH-RI counterpart (Fig 2.3). However, unlike the hGnRH-RI, the mGnRH-R is localized throughout the cytoplasm, and is detected at the plasma membrane (Fig 2.9). Additionally, it was not as strongly localized to the ER as seen with the hGnRH-RI (Fig 2.9 vs. Fig 2.3).

Finally, based on the discovery of this novel localization for hGnRH-RI at the nuclear membrane, we visually examined the spatial properties of three naturally

Figure 2.7 Effect of deleting putative NLS on hGnRH-RI localization and signaling. (A) HTR-8/SVneo cells expressing FLAG-hGnRH-RI in which the putative NLS was deleted were subjected to indirect immunofluorescent staining for the receptor (*red*) as well as the markers for the nuclear membrane, endoplasmic reticulum and Golgi (*shown in green*). Cells were counterstained with Hoechst (*blue*). The NLS deletion mutant showed the same phenotype as the wild-type receptor, showing strong colocalization with lamin A/C and calnexin and less colocalization with GM130. Scale bar = 10 μm.

(B) IP formation data represent seven independent experiments performed in triplicate and normalized to FLAG-hGnRHRI ± S.E. ***, $p < 0.001$ versus IP formation of FLAG-hGnRH-RI.


A
FLAG-hGnRH-RI
FLAG-hGnRH-RI + Hoechst
Marker + Hoechst
FLAG-hGnRH-RI + Marker
NLS deletion
Lamin A/C
Calnexin
GM130

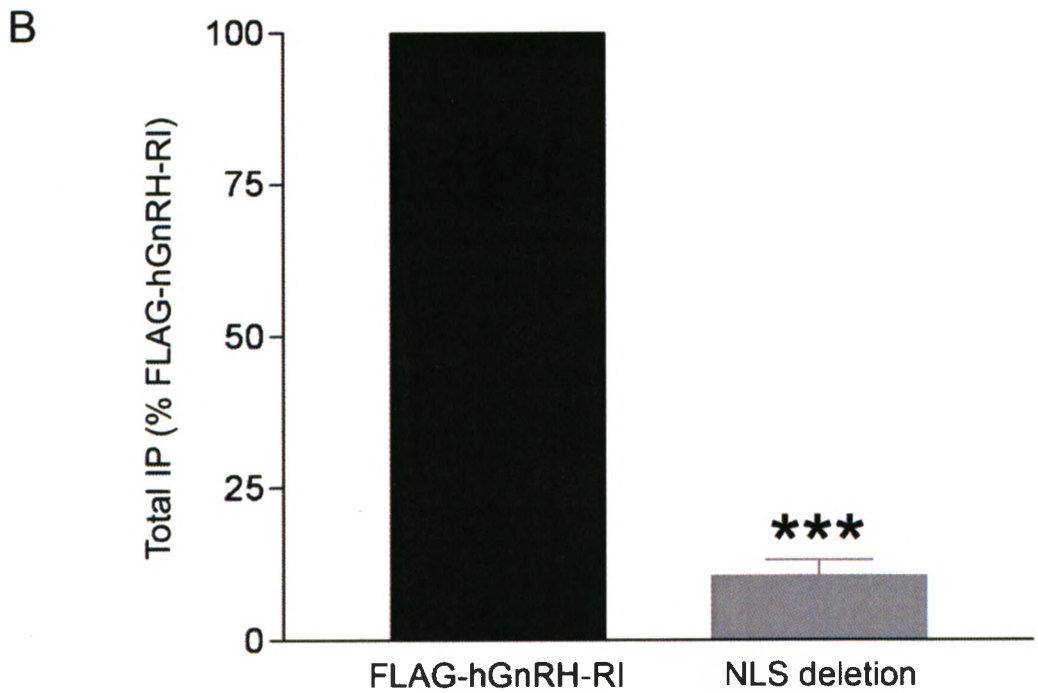

B
Total IP (% FLAG-hGnRH-RI)
100
75
50
25
0
***
FLAG-hGnRH-RI
NLS deletion

Figure 2.8 Effect of lysine 191 hGnRH-RI mutants on receptor localization and signaling. (A) HTR-8/SVneo cells expressing FLAG-hGnRH-RI in which lysine 191 was deleted or mutated to a glutamic acid residue (K191E) were subjected to indirect immunofluorescent staining for the receptor (*red*) as well as the markers for the nuclear membrane, endoplasmic reticulum and Golgi (*shown in green*). Cells were counterstained with Hoechst (*blue*). K191 deletion and K191E mutants showed the same phenotype as the wild-type receptor, showing strong colocalization with lamin A/C and calnexin and less colocalization with GM130. Scale bar = 10 μm.

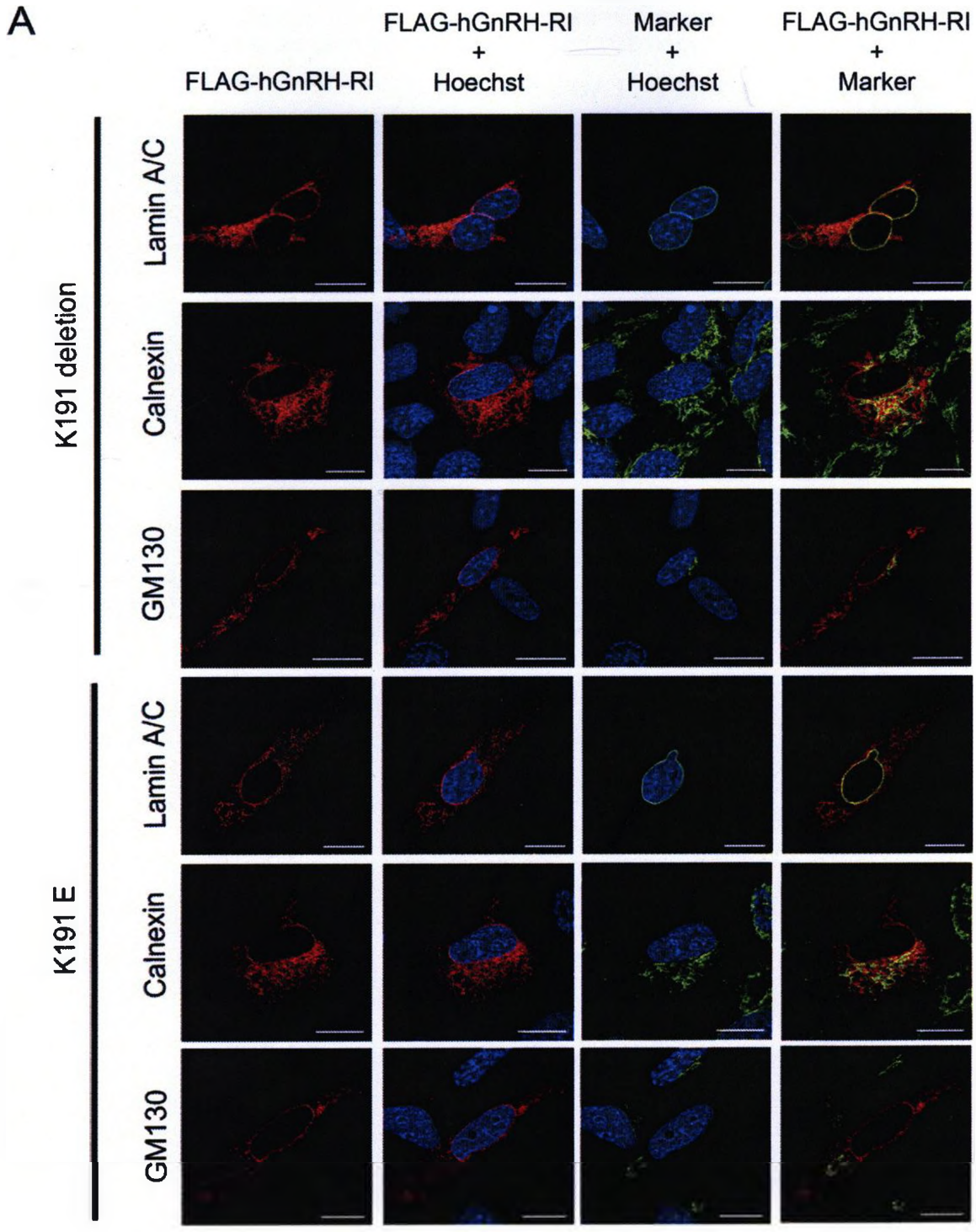

A
FLAG-hGnRH-RI
FLAG-hGnRH-RI + Hoechst
Marker + Hoechst
FLAG-hGnRH-RI + Marker
K191 deletion
K191 E
Lamin A/C
Calnexin
GM130
Lamin A/C
Calnexin
GM130

Figure 2.8 (B) IP formation data represent 6-7 independent experiments performed in triplicate and normalized to FLAG-hGnRHRI $\pm$ S.E.

B

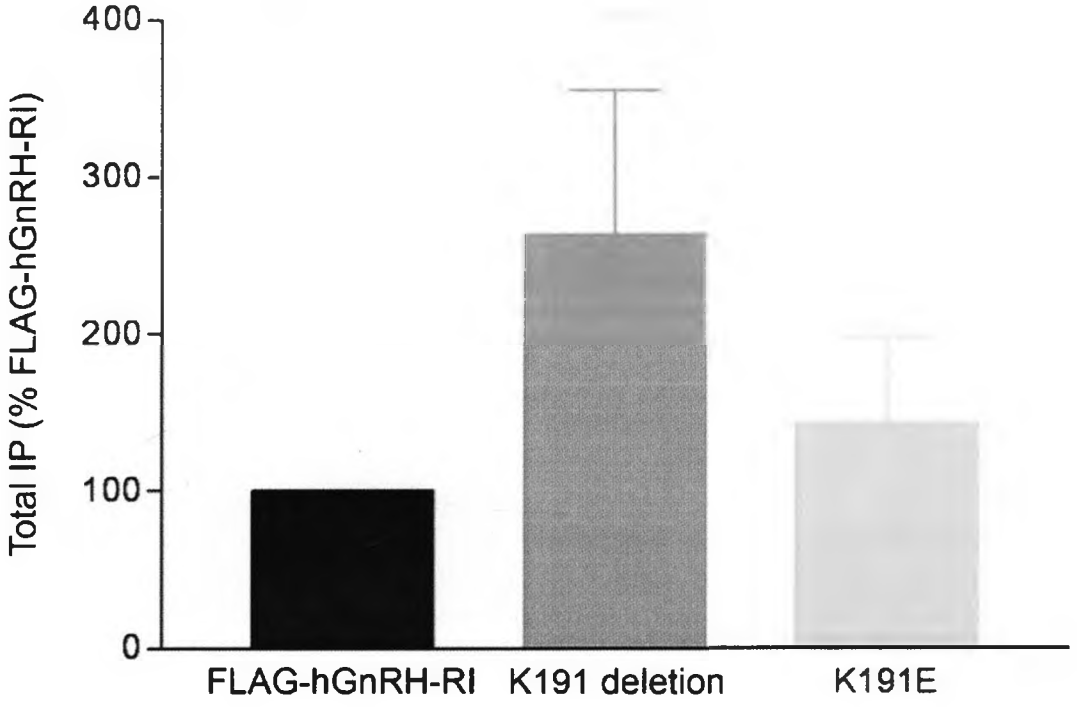
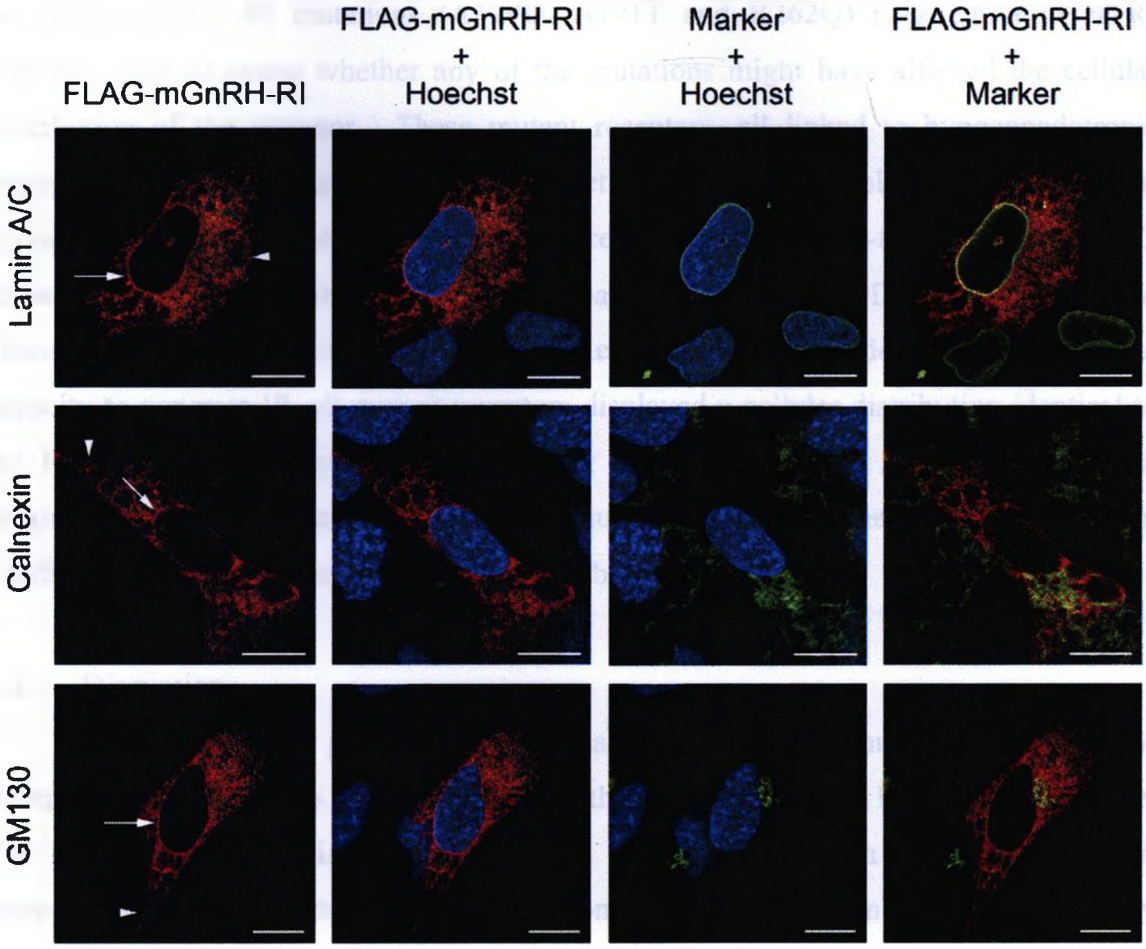

400
300
200
100
0
Total IP (% FLAG-hGnRH-RI)
FLAG-hGnRH-RI
K191 deletion
K191E

Figure 2.9 Spatial localization of FLAG-mGnRH-RI in HTR-8/SVneo cells. HTR-8/SVneo cells expressing either FLAG-hGnRH-RI or mGnRH-RI were subjected to indirect immunofluorescent staining for the receptor (*red*) as well as the markers for the nuclear membrane, endoplasmic reticulum and Golgi (*shown in green*). Cells were counterstained with Hoechst (*blue*). FLAG-mGnRH-RI showed a similar perinuclear phenotype (arrows) to FLAG-hGnRH-RI with strong colocalization with lamin A/C and less colocalization with GM130. However, less colocalization was seen between the FLAG-mGnRH-RI and calnexin than was seen with the FLAG-hGnRH-RI. As well, there was evidence of plasma membrane localization (arrowheads) with FLAG-mGnRH-RI that was not seen with FLAG-hGnRH-RI. Scale bar = 10 μm.


FLAG-mGnRH-RI
FLAG-mGnRH-RI + Hoechst
Marker + Hoechst
FLAG-mGnRH-RI + Marker
Lamin A/C
Calnexin
GM130

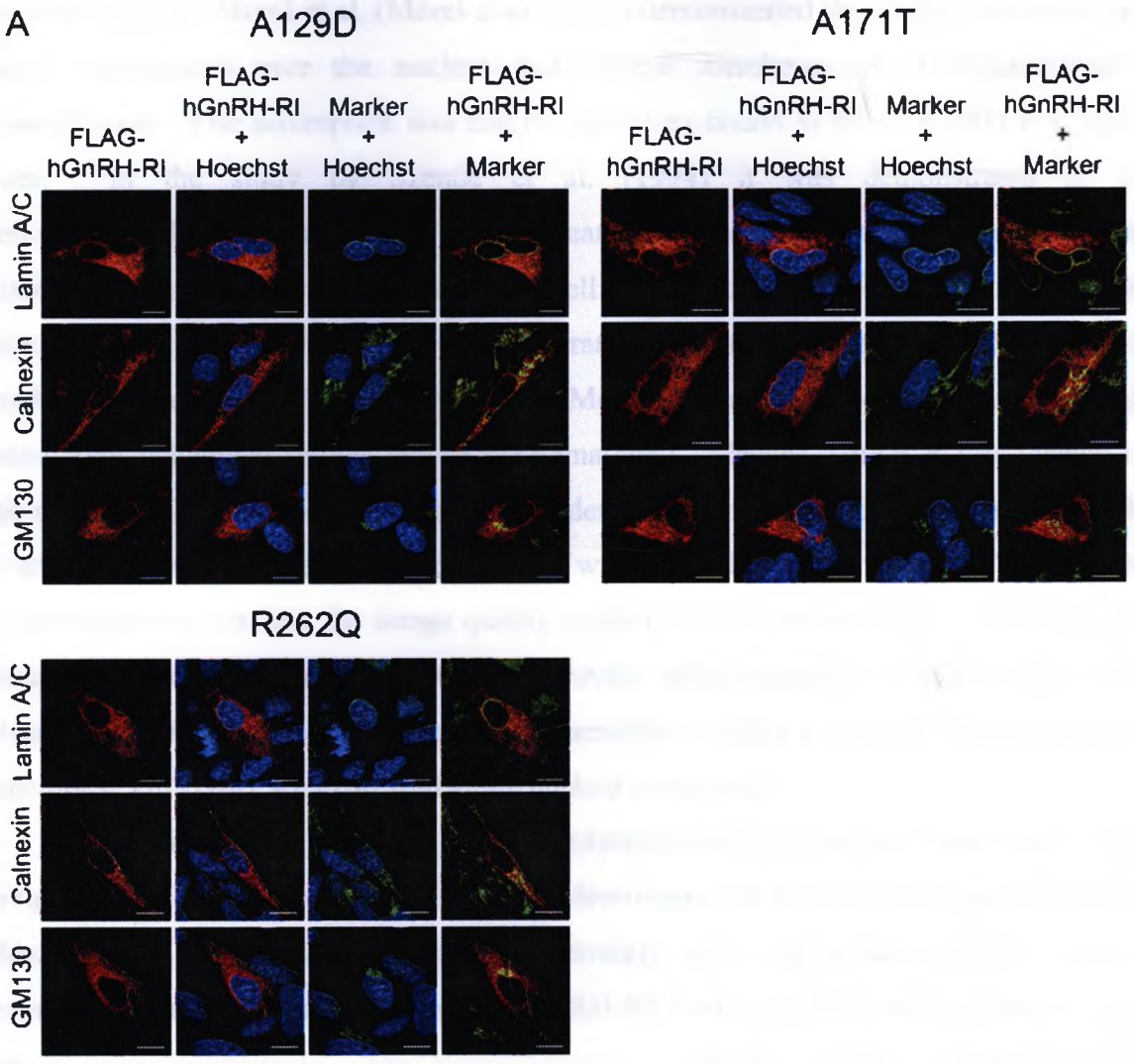
occurring hGnRH-RI mutations (A129D, A171T and R262Q) (Fig 2.6A) in HTR-8/SVneo cells to assess whether any of the mutations might have affected the cellular distribution of the receptor. These mutant receptors, all linked to hypogonadotropic hypogonadism in humans (Leanos-Miranda et al., 2005), were selected based on their agonist-stimulated IP generating capacity where relative to the wild-type receptor, R262Q showed about 25% diminished IP forming capacity while the A129D and A171T showed almost no IP forming capacity (Fig 2.10B). Results revealed that despite the differential capacity to generate IP, all mutant receptors displayed a cellular distribution identical to the FLAG-tagged wild-type hGnRH-RI (Fig 2.10A). This was consistent with data obtained with the full putative NLS receptor mutant (Fig 2.7) and the agonist-independent localization of the receptor to the nuclear membrane.

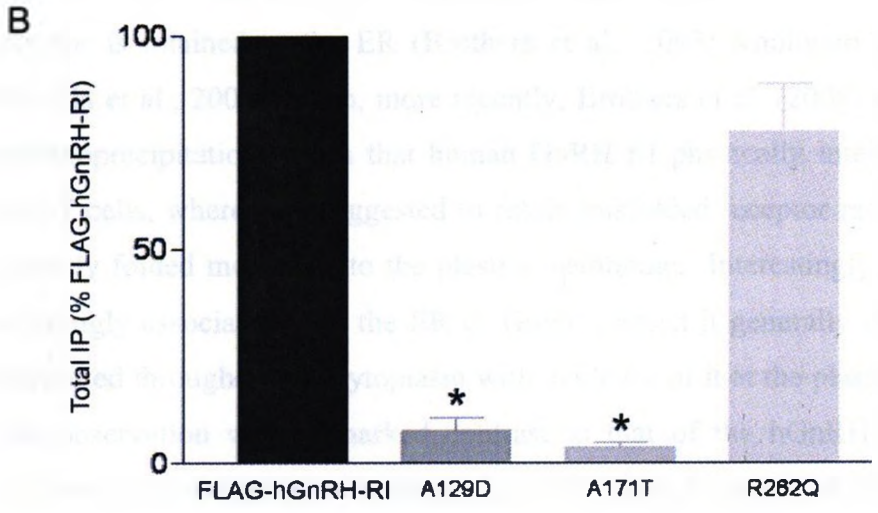
2.4 Discussion

The present study provides strong visual evidence that the human GnRH receptor is expressed at high levels intracellularly in both a heterologous and homologous cell line. The mouse homologue is also expressed at high levels in both cell lines and both receptors additionally show marked expression at the nuclear membrane as revealed by colocalization with lamin A/C. Lamin A/C, a type V nuclear lamin, is an Intermediate Filament protein that is a component of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane which is suggested to provide a framework for the nuclear envelope and may interact with chromatin (Verstraeten et al., 2007). Our visual observations are consistent with previous findings (Leanos-Miranda et al., 2002; Brothers et al., 2003; Janovick et al., 2003a; Janovick et al., 2003b; Leanos-Miranda et al., 2005; Janovick et al., 2006; Janovick et al., 2007) that suggest, based on biochemical evidence, that both the wild-type and naturally occurring mutant human GnRH-RI are expressed weakly at the plasma membrane and are retained intracellularly. In addition, our spatial data also support previous biochemical data that state, unlike the mouse receptor, a large fraction of the human receptor is retained in the ER (Arora et al., 1995; Maya-Núñez et al., 2000; Brothers et al., 2003).

To date, there have been at least four studies that provide some evidence that GnRH-R is detectable at the nucleus. Through the use of radiolabeled GnRH and

Figure 2.10 Effect of naturally occurring hGnRH-RI mutations on spatial localization and signaling. (A) HTR-8/SVneo cells expressing FLAG-hGnRH-RI containing naturally occurring mutations (A129D, A171T and R262Q) were subjected to indirect immunofluorescent staining for the receptor (*red*) as well as the markers for the nuclear membrane, endoplasmic reticulum and Golgi (*shown in green*). Cells were counterstained with Hoechst (*blue*). All three naturally occurring mutants showed the same phenotype as the wild-type receptor, showing strong colocalization with lamin A/C and calnexin and less colocalization with GM130. Scale bar = 10 μm. (B) IP formation data represent three to 3-5 independent experiments performed in triplicate and normalized to FLAG-hGnRH-RI cDNA $\pm$ S.E. *, $p < 0.05$ versus IP formation of FLAG-hGnRH-RI.


A
A129D
A171T
FLAG-hGnRH-RI
FLAG-hGnRH-RI + Hoechst
Marker + Hoechst
FLAG-hGnRH-RI + Marker
Lamin A/C
Calnexin
GM130
R262Q


B
Total IP (% FLAG-hGnRH-RI)
100
50
0
*
*
FLAG-hGnRH-RI
A129D
A171T
R262Q

autoradiography, Morel et al. (Morel et al., 1987) demonstrated that in rat cells extensive label accumulated over the nucleus and nuclear membrane of radioligand-treated gonadotrophs. The assumption was that the label was bound to the rat GnRH-R at these sites. In the study by Szende et al. (1994) it was demonstrated in an immunohistochemical analysis of ligand-treated hamster pancreatic cancer cells that GnRH-R accumulated in the nucleus. In cell fractionation studies using human breast cancer tissue, Mangia et al. (2002) demonstrated that GnRH-R was associated with the nuclear compartment; while more recently, Meethal et al. (2006) demonstrated through immunohistochemical studies that in the nematode C. elegans, GnRH-R was located in the nucleus. These four studies provided evidence that endogenously expressed GnRH-R from the rat, hamster, nematode and human was detectable at the nucleus. While these were informative studies, the image quality could not allow for conclusive proof that the receptor is nuclear. Nevertheless, they do provide initial support for our findings which clearly show through the use of the nuclear membrane marker lamin A/C, that the human and mouse GnRH-RI are expressed at the nuclear membrane.

In addition, both receptors are also expressed at other intracellular sites. In the Golgi hGnRH-RI was weakly detected, as determined by colocalization with GM130. However, the receptor was detected very strongly in the ER as assessed by calnexin immunostaining. This latter finding that GnRH-RI interacts with calnexin, a member of the cell's quality control system (Vassilakos et al., 1998; Ellgaard et al., 2003), was not surprising given the wealth of biochemical data suggesting that a large quantity of this receptor is retained in the ER (Brothers et al., 2003; Knollman et al., 2005; Leanos-Miranda et al., 2005). Also, more recently, Brothers et al. (2006) demonstrated through immunoprecipitation assays that human GnRH-RI physically interacts with calnexin in COS-7 cells, where it is suggested to retain misfolded receptor molecules while routing correctly folded molecules to the plasma membrane. Interestingly, mGnRH-RI was not as strongly associated with the ER or Golgi; instead it generally appeared more widely distributed throughout the cytoplasm with evidence of it at the plasma membrane as well. This observation was in marked contrast to that of the hGnRH-RII. Consistent with previous biochemical data (Arora et al., 1995; Maya-Núñez et al., 2000) this observation suggests that the absence of the K191 residue in the murine receptor prevents protein

retention in the ER and normal traffic to final destination sites throughout the cell, including the plasma membrane. This would therefore account for the higher IP formation others have observed for the mGnRH-RI compared to hGnRH-RI (Arora et al., 1995; Maya-Núñez et al., 2000).

The HTR-8/SVneo cell line is an established model of the extra-villous trophoblast and used extensively for studying human placentation (Graham et al., 1993). GnRH-RI expression in this cell line (renamed IEVT for invasive extravillous trophoblast) was first described by Cheng et al. (2000) and the sequence analysis of the cloned cDNA revealed a 100% similarity to its pituitary counterpart. Additional characterization of the receptor in this cell line revealed that the receptor was coupled to both $G_{q/11}$ and G_s (Cheng et al., 2001).

At the outset of this study, we were aware of the findings that epitope-tagged and chimeric GnRH-R molecules can display significantly altered signal transduction properties compared to the untagged receptor (Brothers et al., 2003). However, upon testing a number of commercially available human anti-GnRH-R antibodies to examine the localization of the untagged human receptor in dissociated cell cultures, we found that they were ineffective (data not shown) and therefore we would not have been able to conduct this study with the untagged receptor. We therefore constructed and compared the signaling capacity (at the level of IP formation) of a FLAG and HA amino terminus epitope-tagged receptor (open reading frame only) to the untagged receptor cDNA. Our data revealed that cells expressing the FLAG-hGnRH-RI and untagged cDNA produced similar levels of IP, however, the HA-tagged receptor produced significantly higher levels. The data derived for the untagged and HA-tagged receptors were consistent with the findings of Brothers et al. (2003) which suggested that the HA-tagged receptor may have been inappropriately expressed at the plasma membrane, a conclusion that we were able to confirm visually in this study. This finding would suggest that the HA-tagged receptor is not suitable for all investigations involving this receptor. However, despite the strong PME of the receptor with this tag, we did observe the HA-tagged receptor localized to all other regions of the cells in which the FLAG-tagged receptor is seen; these include the ER and nuclear membrane.

The observation that hGnRH-RI is weakly expressed at the plasma membrane and strongly expressed intracellularly is proposed to be the result of a progressive and convergent evolutionary trend of the GnRH-R (Janovick et al., 2006). PME necessitates a metabolic cost to create more, yet unused receptors that in turn create an environment which becomes more sensitive to receptor-borne mutations. Potentially, intracellular retention of the receptor creates and provides a source of GnRH-RI needed for rapid availability without transcription or translation. A similar mechanism might also regulate the human δ opioid receptor since a study by Petäjä-Repo et al. (Petäjä-Repo et al., 2002) demonstrated that permeable agonists and antagonists of the receptor allowed post-translational processing and increased export of the ligand-stabilized receptor from the ER to the cell surface. Other reports indicate that receptors such as mGluR1a, α1D adrenoreceptor, odorant and luteinizing hormone receptors are also inefficiently expressed at the plasma membrane (Saito et al., 2004; Petrovska et al., 2005; Pietila et al., 2005; Uberti et al., 2005; Vandenberghe et al., 2005), thus suggesting that reduced receptor trafficking may represent a more widespread mechanism for regulating protein availability (Janovick et al., 2006). Interestingly, the prostaglandin E2 receptors (EP) were also reported as being barely detectable at the plasma membrane of cells in the newborn brain and cerebral microvasculature (Li et al., 1996; Dumont et al., 1998) but instead were expressed at high levels intracellularly particularly at the nuclear membrane; this nuclear pool was also demonstrated to be functional (Bhattacharya et al., 1998; Bhattacharya et al., 1999).

In this study. we proposed that the mammalian GnRH-R has been evolving towards greater intracrine signalling. In cells that express both the receptor and ligand, such as the HTR-8/SVneo, intracrine signalling would appear to offer the cell more efficient and rapid control of a signalling pathway. This is in contrast to autocrine signalling which emanates at the plasma membrane, incorporates additional signalling steps and requires more energy only to eventually culminate in the same final cellular response as a pathway that was initiated intracellularly.

To date, there is an increasing number of GPCRs identified as nuclear GPCRs and perhaps the best characterized nuclear localized GPCR is the angiotensin (AT1) receptor. The nuclear localization of the AT1 receptor is induced by angiotensin II (Lu et al., 1998,

Chen et al., 2000) and studies have uncovered angiotensin II-binding sites in the nucleus and have also demonstrated the angiotensin II-induced transcription of renin and angiotensinogen mRNA (Eggena et al., 1993). Other GPCRs that are localized to the nucleus include the prostaglandin EP1 (Bhattacharya et al., 1998), EP3, and EP4 receptors (Bhattacharya et al., 1999), parathyroid hormone receptor (Watson et al., 2000a, Watson et al., 2000b), metabotropic glutamate mGluR1a and 5 receptors (O'Malley et al., 2003; Jong et al., 2005), endothelin ETA and ETB receptors (Boivin et al., 2003), apelin and bradykinin (B2) receptors (Lee et al., 2004). Some of these receptors are located in the nucleoplasm and/or at the nuclear membrane (Jong et al., 2005). Our results indicate that GnRH-RI is strongly expressed at the nuclear membrane, however, given the technical limitations of our study we cannot rule out the possibility that it may also be nucleoplasmic. The EP and mGluR1a and 5a receptors are expressed at the nuclear membrane (O'Malley et al., 2003; Jong et al., 2005; Bhattacharya et al., 1998; Bhattacharya et al., 1999). In the case of the EP1 receptor, the authors show by transmission electron microscopy that the receptor is located at both the inner and outer nuclear membranes (Bhattacharya et al., 1998). In both locations, the receptor would have easy access to its ligand since the prostanoid synthesis enzymes are located at both the inner and outer nuclear membranes (Spencer et al., 1998). In the case of mGluR1a and 5a, it is reported that the receptors are oriented with their N-terminal, ligand-binding domain within the lumen of the nuclear envelope; hence, agonists must cross both the plasma and nuclear membranes to access binding domains (Jong et al., 2005; Jong et al., 2007). In addition to other mechanisms that may exist, ligand activation of nuclear mGluRs expressed in HEK 293 cells is achieved via endogenous sodium-dependent transporters and cystine glutamate exchangers that mediate the rapid delivery of quisqualate or glutamate into the cell and into the nuclear lumen (Jong et al., 2005; Jong et al., 2007). It has been demonstrated that cytotrophoblasts isolated from the human placenta express GnRH-RI as well as the ligands, GnRH-I and -II (Cheng et al., 2000; Chou et al., 2004). Thus it remains a formal possibility that ligands produced intracellularly can somehow be routed to the nuclear membrane to cause an activation of GnRH-RI and thus trigger an intracrine signaling pathway. Energetically, compared to autocrine signaling which requires secretion of the ligand followed by that ligand acting

back upon the cell, the ability to bypass the secretion step can be viewed as a preferred mechanism.

In addition to the AT1 receptor (Eggena et al., 1993), some additional nuclear GPCRs are signaling competent, for example, stimulation of prostaglandin EP receptors on isolated myometrium nuclei modulated nuclear Ca^{2+} and gene transcription (Bhattacharya et al., 1998; Bhattacharya et al., 1999) while mGluR1a and 5a receptors on isolated nuclei from receptor-overexpressing HEK 293 cells and mGluR1a from isolated cortical neurons mediated oscillatory patterns of Ca^{2+} flux following agonist stimulation of the receptor (O'Malley et al., 2003; Jong et al., 2007). Thus, intracellular G protein-coupled receptors may not just represent proteins waiting to go on or having just come off the plasma membrane, rather they may represent a new in situ mode of signaling.

The AT1 receptor was reported to traffic to cell nuclei by the presence of an NLS sequence in its 8th helix (membrane proximal C-terminal sequences) (Lu et al., 1998). Subsequently NLS sequences have been identified in the majority of GPCRs observed to have nuclear localizations. These include the prostanoid, endothelin, bradykinin, metabotropic and apelin receptors. To date, several NLS sequences identified in GPCRs are found in the 8th helix, however, in the apelin receptor it is located in the third intracellular loop (Lee et al., 2004). The human (KKEKGKK) and mouse (KRKKGKK) GnRH-RI putative NLS sequences are located in the first intracellular loop and form a stretch of basic amino acid residues that contains the monopartite consensus sequence K-K/R-X-K/R (Chelsky et al., 1989) which strongly resembles both well established and putative NLS sequences identified in nuclear localized proteins like DNA topoisomerase II from Candida spp., pleiotrophic factor α2 (transcriptional regulator) from Xenopus laevis, HAI-2 related small protein (transcriptional regulator) from humans, and a number of other proteins from various species with putative nuclear functions such as the putative U3 small nuclear ribonucleoprotein (snRNP) from Leishmania major. Based on the presence of this sequence in proteins with established nuclear function, we hypothesized that the human and mouse GnRH-RI contained a functional NLS. However, mutagenesis studies revealed that in both HEK 293 and HTR-8/SVneo cells, a hGnRH-RI mutant lacking the KKEKGKK sequence was still strongly expressed at the nuclear membrane. Similarly, mutation or deletion of the K191 residue from the hGnRH-RI, also did not lead

to a visually discernible difference in receptor expression compared to the wild-type receptor.

Some reports have indicated that the localization of the GPCR to the nucleus is an agonist-dependent event. A close examination of the literature would also suggest that the ability of the receptor to be localized to the nucleus in an agonist-dependent or independent manner in part depends on whether the receptor is expressed at the nuclear membrane or in the nucleoplasm. For example, EP1, 3-4 (Bhattacharya et al., 1998; Bhattacharya et al., 1999) and mGluR5a (O'Malley et al., 2003; Jong et al., 2007) localization to the nuclear membrane requires exogenous treatment with agonist while trafficking of the AT1-R, Apelin and NK3-R to the nucleoplasm does not require it (Yang et al., 1997; Lu et al., 1998; Howe et al., 2004; Lee et al., 2004). Given the trend observed thus far with other nuclear GPCRs, it was therefore surprising to find that the localization of the FLAG-hGnRH-RI to the nuclear membrane was not dependent on an exogenous source of agonist, even when expressed in heterologous HEK 293 cells which do not produce GnRH. Thus it appears that GnRH-RI is localized constitutively at the nuclear membrane.

Finally, we were interested in determining whether or not the naturally occurring folding-defective hGnRH-RI mutants linked to hypogonadotropic hypogonadism (HH), would show an altered spatial distribution in HTR-8/SVneo cells. If they did, it would be plausible that HH may be linked to an inability of the receptors to traffic to the plasma membrane as well as the nuclear membrane. However, for each of the three mutants selected for this study, A129D, A171T and R262Q, we were unable to detect differences in the cellular distribution between the mutants and the wild-type receptor. If there was greater ER retention and/or reduced PME, it was below the level of detection using confocal imaging.

Our finding that the human GnRH-RI receptor is a nuclear GPCR opens up many exciting possibilities on novel signaling mechanisms associated with this unique GPCR. Evidence exists that this receptor has undergone recent and rapid evolution that has resulted in a molecule whose signaling potential is more efficiently regulated. It is also possible that these recent evolutionary changes resulted in a receptor that can bypass most of the plasma membrane/cytoplasmic signaling cascade and signal directly at the nucleus.

We would suggest that nuclear GnRH-RI (intracrine) signaling does not replace signalling emanating at the cell surface, instead, it may simply represent a specific type, or sub-class of GnRH-RI-mediated signaling, at least within human trophoblasts.

2.5 References

Arora KK, Sakai A, Catt KJ 1995 Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. J Biol Chem 270:22820-22826

Babwah AV, Dale LB, Ferguson SS 2003 Protein kinase C isoform-specific differences in the spatial-temporal regulation and decoding of metabotropic glutamate receptor 1a-stimulated second messenger responses. J Biol Chem 278:5419-5426

Baroudi G, Acharfi S, Larouche C, Chahine M 2002 Expression and Intracellular Localization of an SCN5A Double Mutant R1232W/T1620M Implicated in Brugada Syndrome. Circ Res 90:e11-16

Bhattacharya M, Peri KG, Almazan G, Ribeiro-da-Silva A, Shichi H, Durocher Y, Abramovitz M, Hou X, Varma DR, Chemtob S 1998 Nuclear localization of prostaglandin E2 receptors. Proc Natl Acad Sci (U S A) 95:15792-15797

Bhattacharya M, Peri K, Ribeiro-da-Silva A, Almazan G, Shichi H, Hou X, Varma DR, Chemtob S 1999 Localization of functional prostaglandin E2 receptors EP3 and EP4 in the nuclear envelope. J Biol Chem 274:15719-15724

Bhattacharya M, Babwah AV, Godin C, Anborgh PH, Dale LB, Poulter MO, Ferguson SS 2004 Ral and phospholipase D2-dependent pathway for constitutive metabotropic glutamate receptor endocytosis. J Neurosci 24:8752-8761

Boivin B, Chevalier D, Villeneuve LR, Rousseau E, Allen BG 2003 Functional endothelin receptors are present on nuclei in cardiac ventricular myocytes. J Biol Chem 278:29153-29163

Brothers SP, Janovick JA, Conn PM 2003 Unexpected Effects of Epitope and Chimeric Tags on Gonadotropin-Releasing Hormone Receptors: Implications for Understanding the Molecular Etiology of Hypogonadotropic Hypogonadism. J Clin Endocrinol Metab 88:6107-6112

Brothers SP, Janovick JA, Conn PM 2006 Calnexin regulated gonadotropin-releasing hormone receptor plasma membrane expression. J Mol Endocrinol 37:479-488

Cao TT, Brelot A, von Zastrow M 2005 The Composition of the β2 Adrenergic Receptor Oligomer Affects Its Membrane Trafficking after Ligand-Induced Endocytosis. Mol Pharmacol 67:288-297

Chelsky D, Ralph R, Jonak G 1989 Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol. Cell. Biol. 9:2487-2492

Chen R, Mukhin YV, Garnovskaya MN, Thielen TE, Iijima Y, Huang C, Raymond JR, Ullian ME, Paul RV 2000 A functional angiotensin II receptor-GFP fusion protein: evidence for agonist-dependent nuclear translocation. Am J Physiol 279:F440-F448

Cheng KW, Nathwani PS, Leung PC 2000 Regulation of human gonadotropin-releasing hormone receptor gene expression in placental cells. Endocrinology 141:2340-2349

Cheng CK, Leung PC 2005 Molecular Biology of Gonadotropin-Releasing Hormone (GnRH)-I, GnRH-II, and Their Receptors in Humans. Endocr Rev. 26:283-306

Chou CS, Beristain AG, MacCalman CD, Leung PC 2004 Cellular localization of gonadotropin-releasing hormone (GnRH) I and GnRH II in first-trimester human placenta and decidua. J Clin Endocrinol Metab 89:1459-1466

Dumont I, Peri KG, Hardy P, Hou X, Martinez-Bermudez AK, Molotchnikoff S, Varma DR, Chemtob S 1998 PGE2, via EP3 receptors, regulates brain nitric oxide synthase in the perinatal period. Am J Physiol Regul Integr Comp Physiol 275:R1812-1821

Eggena P, Zhu JH, Clegg K, Barrett JD 1993 Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA. Hypertension 22:496-501

Ellgaard L, Frickel EM 2003 Calnexin, calreticulin, and ERp57: teammates in glycoprotein folding. Cell Biochem Biophys. 39:223-47

Gardner LA, Tavalin SJ, Goehring AS, Scott JD, Bahouth SW 2006 AKAP79-mediated Targeting of the Cyclic AMP-dependent Protein Kinase to the β1-Adrenergic Receptor Promotes Recycling and Functional Resensitization of the Receptor. J. Biol. Chem. 281:33537-33553

Graham CH, Hawley TS, Hawley RG, MacDougall JR, Kerbel RS, Khoo N, Lala PK 1993 Establishment and Characterization of First Trimester Human Trophoblast Cells with Extended Lifespan. Exp Cell Res. 206:204-211

Han X, Saito H, Miki Y, Nakanishi A 2008 A CRM1-mediated nuclear export signal governs cytoplasmic localization of BRCA2 and is essential for centrosomal localization of BRCA2. Oncogene 27:2969-2977

Howe HE, Somponpun SJ, Sladek CD 2004 Role of Neurokinin 3 Receptors in Supraoptic Vasopressin and Oxytocin Neurons. J. Neurosci. 24:10103-10110

Janovick JA, Goulet M, Bush E, Greer J, Wettlaufer DG, Conn PM 2003a Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. J Pharmacol Exp Ther 305:608-614

Janovick JA, Ulloa-Aguirre A, Conn PM 2003b Evolved regulation of gonadotropin-releasing hormone receptor cell surface expression. Endocrine 22:317-327

Janovick JA, Knollman PE, Brothers SP, Ayala-Yanez R, Aziz AS, Conn PM 2006 Regulation of G protein-coupled receptor trafficking by inefficient plasma membrane expression: molecular basis of an evolved strategy. J. Biol. Chem. 281:8417-8425

Janovick JA, Brothers SP, Cornea A, Bush E, Goulet MT, Ashton WT, Sauer DR, Haviv F, Greer J, Conn PM 2007 Refolding of misfolded mutant GPCR: Post-translational pharmacoperone action in vitro. Mol Cell Endocrinol. 272:77-85

Jong YJ, Kumar V, Kingston AE, Romano C, O'Malley KL 2005 Functional metabotropic glutamate receptors on nuclei from brain and primary cultured striatal neurons. Role of transporters in delivering ligand. J Biol Chem 280:30469-30480

Jong YJ, Schwetye KE, O'Malley KL 2007 Nuclear localization of functional metabotropic glutamate receptor mGlu1 in HEK293 cells and cortical neurons: role in nuclear Ca^{2+} mobilization and development. J Neurochem. 101:458-469

Knollman PE, Janovick JA, Brothers SP, Conn PM 2005 Parallel Regulation of Membrane Trafficking and Dominant-negative Effects by Misrouted Gonadotropin-releasing Hormone Receptor Mutants. J. Biol. Chem. 280:24506-24514

Leanos-Miranda A, Janovick JA, Conn PM 2002 Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism. J Clin Endocrinol Metab 87:4825-4828

Leanos-Miranda A, Ulloa-Aguirre A, Janovick JA, Conn PM 2005 In vitro coexpression and pharmacological rescue of mutant gonadotropin-releasing hormone receptors causing hypogonadotropic hypogonadism in humans expressing compound heterozygous alleles. J.Clin.Endocrinol.Metab. 90:3001-3008

Lee DK, Lanca AJ, Cheng R, Nguyen T, Ji XD, Gobeil F Jr, Chemtob S, George SR, O'Dowd BF 2004 Agonist-independent nuclear localization of the Apelin, angiotensin AT1, and bradykinin B2 receptors. J Biol Chem 279:7901-7908

Li DY, Abran D, Peri KG, Varma DR, Chemtob S 1996 Inhibition of prostaglandin synthesis in newborn pigs increases cerebral microvessel prostaglandin F2 alpha and prostaglandin E2 receptors, their second messengers and vasoconstrictor response to adult levels. J Pharmacol Exp Ther 278:370-377

Lu D, Yang H, Shaw G, Raizada MK 1998 Angiotensin II-induced nuclear targeting of the angiotensin type 1 (AT1) receptor in brain neurons. Endocrinology 139:365-375

Mangia A, Tommasi S, Reshkin SJ, Simone G, Stea B, Schittulli F, Paradiso A 2002 Gonadotropin releasing hormone receptor expression in primary breast cancer:

comparison of immunohistochemical, radioligand and Western blot analyses. Oncology Reports 9:1127-1132

Maya-Núñez G, Janovick JA, Conn PM 2000 Combined modification of intracellular and extracellular loci on human gonadotropin-releasing hormone receptor provides a mechanism for enhanced expression. Endocrine 13:401-407

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR 2004 Gonadotropin-Releasing Hormone Receptors. Endocr Rev 25:235-275

Morel G, Dihl F, Aubert ML, Dubois PM 1987 Binding and internalization of native gonadoliberin (GnRH) by anterior pituitary gonadotrophs of the rat. A quantitative autoradiographic study after cryoultramicrotomy. Cell Tissue Res. 248:541-550

O'Malley KL, Jong YJ, Gonchar Y, Burkhalter A, Romano C 2003 Activation of metabotropic glutamate receptor mGlu5 on nuclear membranes mediates intranuclear Ca^{2+} changes in heterologous cell types and neurons. J Biol Chem 278:28210-28219

Panyi G, Bagdany M, Bodnar A, Vámosi G, Szentesi G, Jenei A, Mátyus L, Varga S, Waldmann TA, Gáspar R, Damjanovich S 2003 Colocalization and nonrandom distribution of Kv1.3 potassium channels and CD3 molecules in the plasma membrane of human T lymphocytes. Proceedings of the National Academy of Sciences 100:2592-2597

Petäjä-Repo UE, Hogue M, Bhalla S, Laperrière A, Morello JP, Bouvier M. 2002 Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. EMBO Journal 21:1628-1637

Petrovska R, Kapa I, Klovins J, Schiöth HB, Uhlén S 2005 Addition of a signal peptide sequence to the α1D-adrenoceptor gene increases the density of receptors, as determined by [3H]-prazosin binding in the membranes. Br J Pharmacol. 144:651-659

Pietila EM, Tuusa JT, Apaja PM, Aatsinki JT, Hakalahti AE, Rajaniemi HJ, Petäjä-Repo UE 2005 Inefficient Maturation of the Rat Luteinizing Hormone Receptor: A putative way to regulate receptor numbers at the cell surface. J. Biol. Chem. 280:26622-26629

Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H 2004 RTP family members induce functional expression of mammalian odorant receptors. Cell 119:679-691

Salahpour A, Angers S, Mercier JF, Lagace M, Marullo S, Bouvier M 2004 Homodimerization of the β2-Adrenergic Receptor as a Prerequisite for Cell Surface Targeting. J. Biol. Chem. 279:33390-33397

Spencer AG, Woods JW, Arakawa T, Singer II, Smith WL 1998 Subcellular Localization of Prostaglandin Endoperoxide H Synthases-1 and -2 by Immunoelectron Microscopy. J. Biol. Chem. 273:9886-9893

Szende B, Csikós A, Szepeshazi K, Neill JD, Mulchahey JJ, Halmos G, Lapis K, Schally AV 1994 The concentration of LH-RH receptors in the nuclei of pancreatic cancer cells. Effect of (D-Trp6)LH-RH on tumor-bearing Syrian golden hamsters. Receptor 4:201-207

Uberti MA, Hague C, Oller H, Minneman KP, Hall RA 2005 Heterodimerization with β2-Adrenergic Receptors Promotes Surface Expression and Functional Activity of α1D-Adrenergic Receptors. J Pharmacol Exp Ther 313:16-23

Ulloa-Aguirre A, Janovick JA, Brothers SP, Conn PM 2004 Pharmacologic rescue of conformationally-defective proteins: implications for the treatment of human disease (Review). Traffic 5:821-837

Vadakkadath Meethal S, Gallego MJ, Haasl RJ, Petras SJ 3rd, Sgro JY, Atwood CS. 2006 Identification of a gonadotropin-releasing hormone receptor orthologue in Caenorhabditis elegans. BMC Evolutionary Biology 29:103-119

Vandenberghe W, Nicoll RA, Bredt DS 2005 Interaction with the Unfolded Protein Response Reveals a Role for Stargazin in Biosynthetic AMPA Receptor Transport. J. Neurosci. 25:1095-1102

Vassilakos A, Michalak M, Lehrman MA, Williams DB 1998 Oligosaccharide Binding Characteristics of the Molecular Chaperones Calnexin and Calreticulin. Biochemistry 37:3480-3490

Verstraeten VL, Broers JL, Ramaekers FC, van Steensel MA 2007 The nuclear envelope, a key structure in cellular integrity and gene expression. Curr Med Chem.14(11):1231-1248.

Wang B, Bisello A, Yang Y, Romero GG, Friedman PA 2007 NHERF1 Regulates Parathyroid Hormone Receptor Membrane Retention without Affecting Recycling. J. Biol. Chem. 282:36214-36222

Watson PH, Fraher LJ, Hendy GN, Chung UI, Kisiel M, Natale BV, Hodsman AB 2000a Nuclear localization of the type 1 PTH/PTHrP receptor in rat tissues. J Bone Miner Res 15:1033-1044

Watson PH, Fraher LJ, Natale BV, Kisiel M, Hendy GN, Hodsman AB 2000b Nuclear localization of the type 1 parathyroid hormone/parathyroid hormone-related peptide receptor in MC3T3-E1 cells: association with serum-induced cell proliferation. Bone (NY) 26:221-225

Yang H, Lu D, Raizada MK 1997 Angiotensin II-induced phosphorylation of the AT1 receptor from rat brain neurons. Hypertension 30:351-357

Chapter 3- Spatial and Temporal Characterization of Protein Kinase C Isoforms Following Activation of Human Gonadotropin Releasing Hormone Receptor Type I

3.1 Introduction

The human type I gonadotropin releasing hormone receptor (hGnRH-RI) is a member of the G protein-coupled receptor superfamily (GPCRs) that is activated by the decapeptide GnRH and plays an essential role in controlling the release of the gonadotropins, which regulate gonadal steroidogenesis and gametogenesis. As a GPCR, hGnRH-RI transduces extracellular stimuli into an intracellular signal via interactions with G proteins. The nature of the G protein coupling depends largely on the cellular context. In the pituitary gonadotropes, ligand-activated hGnRH-RI is coupled to the $G_{q/11}$ protein and stimulates phospholipase C-β-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate leading to the formation of second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Fig 1.4). IP_3 subsequently stimulates the release of intracellular Ca^{2+} from the endoplasmic reticulum (Ulloa-Aguirre et al., 2004; Babwah et al., 2003). These secondary messengers in turn activate a large number of downstream proteins, including protein kinase C (PKC), a serine/threonine kinase that regulates a number of processes such as cell secretion, proliferation, differentiation, and apoptosis. Using the classification system of Alexandra Newton (Newton et al., 2003), there are 10 human PKC isoforms that can be divided into three groups based on structural properties and cofactor requirements. The activation and plasma membrane localization of the conventional PKC isoforms (α, βI, βII and γ) is regulated by intracellular Ca^{2+}, DAG and phospholipid, such as phosphatidylserine (PS). The novel PKC isoforms (δ, ε, η and θ) are regulated by DAG and PS and the atypical PKCs (ι/λ and ζ) are only regulated by PS (reviewed in Newton et al., 2003).

Although a great deal of research has been conducted concerning the regulation of PKC at the protein level including cofactor requirements, translocation to the membrane, substrate phosphorylation and degradation, relatively little is know about what causes specific isoforms to be activated downstream of a given receptor. The crucial role that PKC plays in the synthesis and release of the gonadotropins from the anterior pituitary downstream of GnRH binding has been well studied, however there is no literature describing which PKC isoforms in the human cell are activated downstream of hGnRH-RI. Previous studies on murine gonadotropes have shown that gene expression of PKCδ and PKCε are upregulated by GnRH stimulation (Harris et al., 1997). The use of a mouse

pituitary gonadotrope cell line, αT3-1, which endogenously expresses GnRH-RI, has been used as a model to address this question (Kratzmeier et al., 1996; Farshori et al., 2003; Maccario et al., 2004). However, the possibility remains that this mouse model does not recapitulate what happens in the human cell.

Since the mouse and human GnRH-RI differ in a few key residues (see Section 1.3.2), there is a possibility that these differences may differentially modulate PKC responses. For example, the rat metabotropic glutamate receptor isoforms 1a and 5a differ in several key residues (Dale et al., 2001). One of them is Asp854 (mGluR1a) and Thr840 (mGluR5a) and it has been shown that this single residue regulates PKC βII oscillatory frequency. Additionally, PKC isoform protein sequences are not fully conserved across species. For example, a study by Aris et al. (1993) showed that human PKC δ has about 88% nucleotide sequence identity to the rat and mouse PKC δ sequence, however biochemical differences, such as substrate and lipid dependencies, are observed between the species. Given the fact that PKC isoform function is not fully conserved across species, the relationship between the rodent GnRH-RI and PKC isoforms can not be extrapolated to the human cell. Therefore, there is a need for a direct study investigating the hGnRH-RI stimulated hPKC isoform responses and this is the first of two aims of the study described in this chapter. In this first section, I describe the spatial and temporal responses of eight human PKC isoforms representing the three different PKC classes following the activation of hGnRH-RI in HEK 293 cells.

A second focus of this study was to characterize PKC responses following the activation of known naturally-occurring mutants of hGnRH-RI and determine how these compared to the wild type hGnRH-RI. There are currently 21 known naturally occurring mutations of hGnRH-RI, which cause partial to complete hypogonadotropic hypogonadism, a disease state in which there is absent or decreased function of the gonads, presenting as a delay in sexual development and inappropriately low gonadotropin and sex steroid levels (Bhagavath et al., 2005). Previous studies performed on some of the known mutants in COS-7 cells have shown that many of these mutants are incapable of normal signaling, as shown by their inability to produce IP_3 in response to agonist stimulation. Since the naturally-occuring GnRH-RI mutants have reduced ability to generate DAG and Ca^{2+} relative to the WT receptor, I was interested in determining

whether this diminished capacity affected the spatial and temporal characteristics of the downstream PKC response. I chose to look at 17 of the known mutants in HEK 293 cells coexpressed with GFP-PKC βII. I chose PKC βII, since I found that this isoform gives a robust response to the agonist-activated WT receptor and felt that subtle changes in its response to mutant receptor activation cold be best detected relative to the other isoforms. Although previous studies have reported on the IP_3 generating capacity of GnRH-RI mutants, I thought it pertinent to recharacterize their ability to generate IP_3 since the cell system and the GnRH-RI constructs used in this study were not identical to those used in the previously published reports (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006). Specifically, previous studies characterized the IP_3 generating capacity of untagged receptors in COS-7 cells. However, I perform my studies with a FLAG-tagged receptor in HEK 293 cells.

3.2 Materials and methods

3.2.1 Plasmid constructs

A 987-bp cDNA fragment containing the complete coding sequence of human GnRH-RI (accession number NM_000406.2) was obtained by RT-PCR from QUICK-CloneTM cDNA library (Clontech Laboratories Inc., Mountain View, CA, USA). The FLAG tag at the amino terminus of hGnRH-RI was obtained by inserting the cDNA between the HindIII and EcoRI sites of pFLAG/CMV-II vector. FLAG-hGnRH-RI was then cloned into the pcDNA3.1/Hygro(+) vector using Nhe1 and Not1 sites. EGFP-tagged PKC constructs were previously created as described (Feng 1998, Babwah 2003). To create the naturally-occuring hGnRH-RI mutants, FLAG-hGnRH-RI was subjected to site-directed mutagenesis using the QuickChange kit following manufacturer's instructions (Stratagene, Santa Clara, CA, USA). Oligonucleotides from Sigma Genosys (Oakville, ON, Canada) were used.

3.2.2 Cell culture

HEK 293 cells were cultured in MEM supplemented with FBS [10% (v/v)], non-essential amino acids [1% (v/v)] and gentamicin (5 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. HEK 293 cells were transiently

transfected with 5 μg of DNA using a modified calcium phosphate method as previously described (Babwah et al., 2003). After transfection (18 h), the cells were incubated with fresh medium, allowed to recover for 6 h and reseeded onto 18 mm collagen-coated glass coverslips. Cells were allowed to grow an additional 18 h prior to experimentation.

3.2.3 Confocal Microscopy

Following transfection (42-44 h) with plasmid constructs encoding EGFP-PKC constructs and FLAG-hGnRH-RI, cells were reseeded on collagen-coated 15 mm glass-cover slips. All experiments were conducted at 37 °C and, prior to visualization, cells were washed twice with Hanks' balanced salt solution (HBSS: 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) and were maintained in this solution for the duration of the experiment. Cells were stimulated with 100 nM Buserelin by adding 10 μL of 10 μM Buserelin to 1 mL of HBSS in the confocal dish, or left untreated. Enhanced GFP fluorescence was visualized with excitation at 488 nm and 500-545 nm emission filter set.

3.2.4 Confocal Data Analysis

PKC translocation time courses were recorded as a time series of 500-700 confocal images for each experiment. Image analysis was performed using the Olympus Fluoview 1000 analysis software and was plotted as the relative change in cytoplasmic fluorescence intensity over time in a region of interest. All time course data were plotted using GraphPad Prism.

3.2.5 Inositol Phosphate Formation

Inositol lipids were radiolabeled by incubating the cells overnight with 1 μCi/ml [^{3}H]myo-inositol in Dulbecco's modified Eagle's medium. Unincorporated [^{3}H]myo-inositol was removed by washing the cells with HBSS. Cells were preincubated for 1 h in HBSS at 37 °C and then preincubated in 500 μl of the same buffer containing 10 mM LiCl for an additional 10 min at 37 °C. Next, the cells were incubated in either the absence or the presence of 100 nM Buserelin for 2 hs at 37 °C. The reaction was stopped on ice by adding 500 μl of 0.8 M perchloric acid and then neutralized with 400 μl of 0.72

M KOH, 0.6 M $KHCO_3$. The total [^{3}H]inositol incorporated into the cells was determined by counting the radioactivity present in 50 µl of the cell lysate. Total inositol phosphate was purified from the cell extracts by anion exchange chromatography using Dowex 1-X8 (formate form) 200-400 mesh anion exchange resin. [^{3}H]Inositol phosphate formation was determined by liquid scintillation using a Wallac LKB 1211 RackBeta liquid scintillation counter. The means ± S.E. are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data for statistical significance. Statistical significance was determined by Student's t-test or one-way analysis of variance with Dunn's post hoc multiple comparison test.

3.3 Results

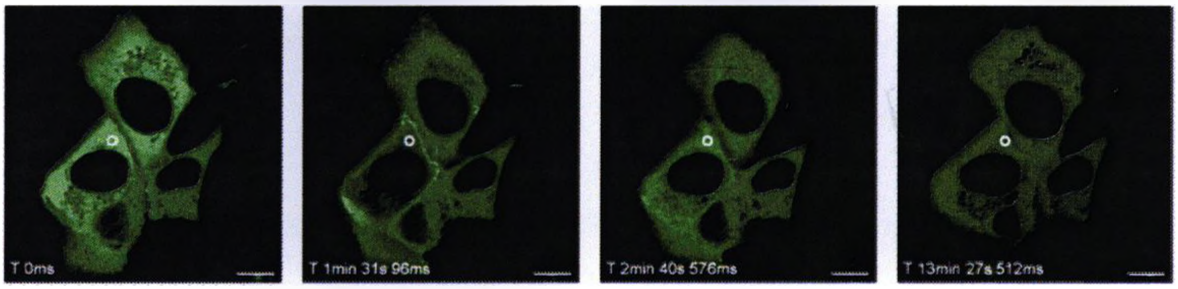
3.3.1 PKC Isozyme-specific Plasma Membrane Translocation Profiles in HEK 293 cells

Differences in receptor-activated translocation profiles of individual conventional (α, βI, βII and γ), novel (δ, ε and θ) and atypical (ι/λ) GFP-tagged PKC isoforms were examined in HEK 293 cells following hGnRH-RI agonist activation. The results are summarized in Table 3.1 and indicate the number of cells observed as well as the number of separate experiments formed. In HEK 293 cells, each of the conventional PKC isoforms displayed two activation responses: a single translocation response where PKC moves from the cytosol to the plasma membrane and back to the cytosol and an oscillation response, where PKC repeatedly translocates from the cytosol to the plasma membrane. These responses are depicted in Fig 3.1 and 3.2, respectively. The initial translocation of the conventional isoforms to the plasma membrane occurred between 17 seconds and 15 minutes following addition of agonist. The novel and atypical PKCs showed similar variability in the time to respond. An agonist-stimulated oscillatory response pattern that represents the response exhibited by all four conventional PKC isoforms is shown in Fig 3.2. In cells that showed an oscillatory response, 6/17 cells for GFP-PKCα, 20/35 cells for GFP-PKCβI and 28/71 for GFP-PKCβII, the frequency of

Table 3.1 Summary of PKC isoforms activated by 100 nM Buserelin stimulation in HEK 293 expressing FLAG-GnRH-RI and GFP-PKC isoforms. Single translocation versus oscillation of an isoform is described. The number of cells observed and percentage of cells giving a specific response are described. The number of separate experiments is given (*n*).

	Isoform	Single Translocation	Oscillation	n
Conventional	α	Yes (8/17, 47.1%)	Yes (6/17, 35.3%)	1
	βI	Yes (8/35, 22.9%)	Yes (20/35, 57.1%)	4
	βII	Yes (26/71, 36.6%)	Yes (28/71, 39.4%)	9
	γ	Yes (19/34, 55.8%)	Yes (2/34, 5.9%)	2
Novel	δ	Yes (7/15, 46.7%)	No (0/15)	1
	ε	Yes (15/17, 88.2%)	No (0/17)	2
	θ	Yes (6/24, 25.0%)	No (0/24)	3
Atypical	ι/λ	No (0/21)	No (0/21)	2

Figure 3.1 Conventional GFP-tagged PKC isoform single translocation response to FLAG-hGnRH-RI activation in HEK 293 cells. HEK cells were transfected with cDNA encoding FLAG-GnRH-RI and GFP-PKC βII. Shown are representative images selected from a time series of 500-700 laser scanning confocal microscopic images collected at 1.1 s intervals. The images demonstrate the single translocation of the conventional GFP-PKC proteins from the cytosol to the plasma membrane and back in response to persistant FLAG-hGnRH-RI activation with 100 nM Buserelin in HEK 293 cells. Drug was added 10 s after the live imaging began. Scale bar = 10 μm.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T 13min 27s 512ms

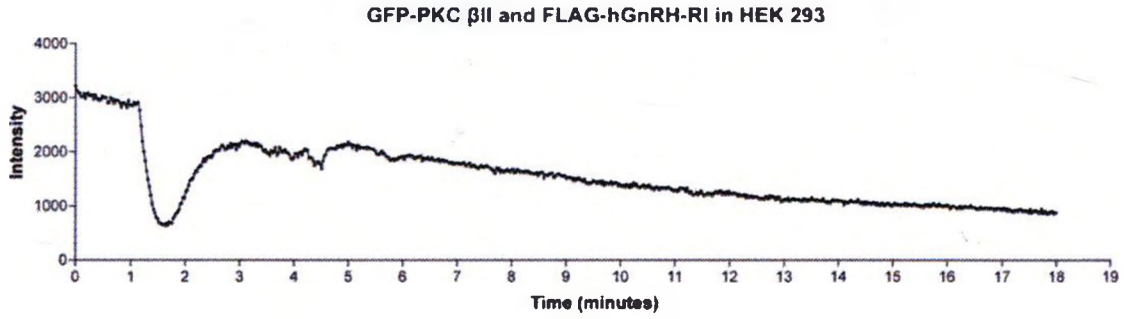
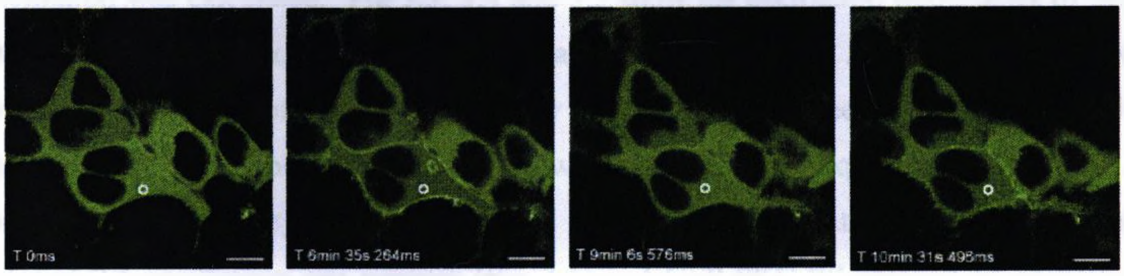

GFP-PKC βII and FLAG-hGnRH-RI in HEK 293
Intensity
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Time (minutes)

Figure 3.2 Conventional GFP-tagged PKC isoform oscillation response to FLAG-hGnRH-RI activation in HEK 293 cells. HEK 293 cells were transfected with cDNA encoding FLAG-GnRH-RI and GFP-PKC βII. Shown are representative images selected from a time series of 500-700 laser scanning confocal microscopic images collected at 1.1 s intervals. The images demonstrate the repetitive translocation of the conventional GFP-PKC proteins between the cytosol and the plasma membrane in response to persistant FLAG-hGnRH-RI activation with 100 nM Buserelin in HEK 293 cells. Drug was added 10 s after the live imaging began. Scale bar = 10 μm.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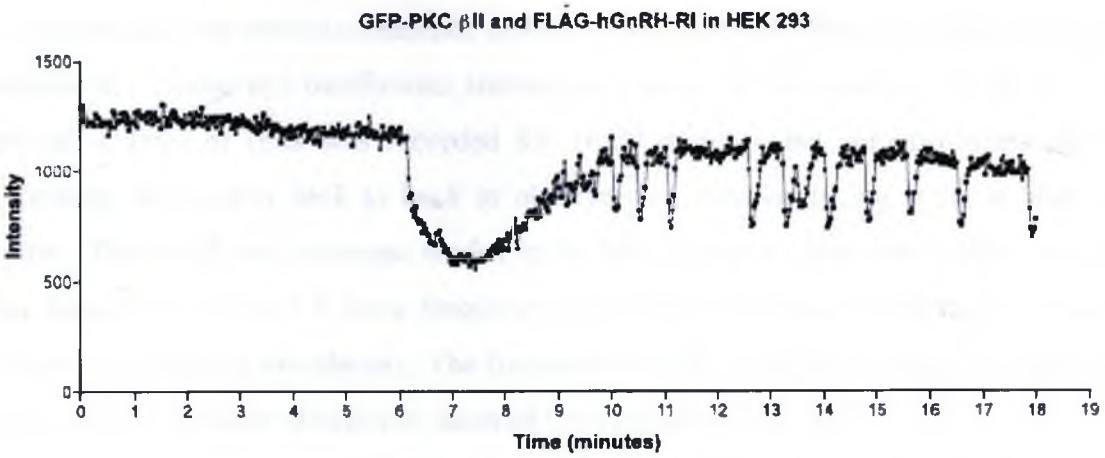
T 9min 6s 576ms
T 10min 31s 498ms


GFP-PKC βII and FLAG-hGnRH-RI in HEK 293
Intensity
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Time (minutes)

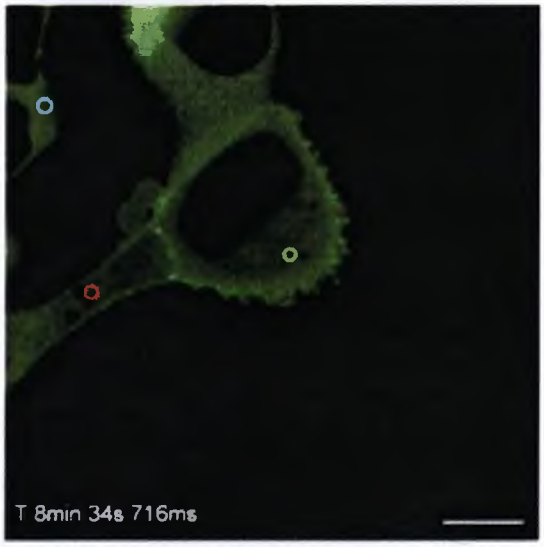
oscillation was calculated. Fig 3.3, 3.4 and 3.5 show the oscillation frequencies of cells expressing GFP-PKCα, βI, βII in representative fields of view. As these figures show, within a given field of view the frequency of oscillation between cells expressing the same isoform is variable. GFP-PKC α showed an oscillation frequency between 0.4-1.2 oscillations per minute (Fig 3.3), GFP-PKC βI showed an oscillation frequency between 0.1 and 2.5 oscillations per minutes (Fig 3.4) and GFP-PKC βII showed an oscillation frequency of 0.4 and 2.3 oscillations per minute (Fig 3.5). It should be noted that PKC oscillations did not always commence directly after the initial translocation to the plasma membrane. I observed oscillations starting as late as 38 minutes post stimulation. In general, a field of cells was recorded for 16-20 minutes, but sometimes consecutive recordings were taken back to back to observe what was occurring at these later time points. The oscillatory response tended to be more robust at later time points, which is why Figs 3.3, 3.4 and 3.5 show frequency data from recordings which began 27 to 29 minutes post-agonist stimulation. The frequency of PKC γ oscillation is not reported here due to the low number of cells that showed this response (2/34 cells).

Unlike the conventional PKC isoforms, which all showed an oscillatory response, the novel PKCs that were tested, δ, ε and θ showed a single translocation response, as depicted in Figure 3.1. It should be noted that in some cells expressing either the conventional or novel PKCs, after the initial translocation from the cytosol to the plasma membrane, the PKC isoform often remained at the plasma membrane for the duration of the recording. An example of this is shown in Fig 3.6 where it is observed that PKCθ remained at the plasma membrane from its initial translocation until the end of the recording. One cell was observed where PKC βI remained at the plasma membrane after 46 minutes post-agonist stimulation.

The only atypical PKC isoform tested in these experiments, PKC ι/λ, was not activated by hGnRH-RI stimulation in either cell line and remained in the cytosol throughout the experiments. Cells expressing GFP alone showed a similar distribution to unstimulated GFP-PKC isoforms, with slightly higher nuclear expression, but did not show any response to GnRH stimulation.

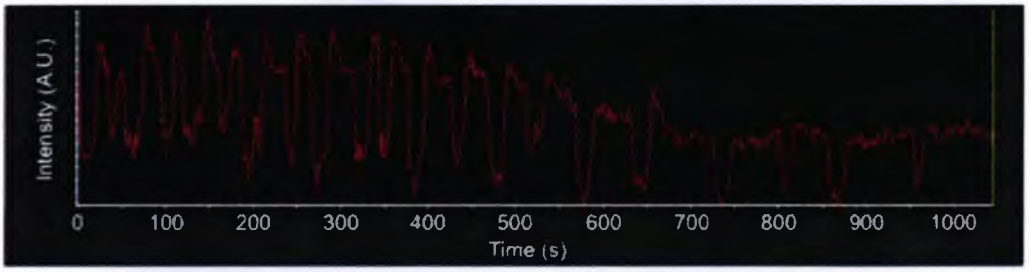
Figure 3.3 Frequency of oscillation of GFP-PKC α in HEK 293 cells. Representative cells (top right) in which regions of interest are shown (circles). Fluorescence intensity within the region of interest was measured over time and plotted (top 3 graphs). Bottom graph shows oscillation frequencies of all regions of interest measured in the field of view. Data shown was recorded 27 minutes post oscillation. Note the large variation of oscillation frequency between cells. Scale bar = 10 μm.

GFP-PKC α and FLAG-hGnRH-RI
in HEK 293 cells
(Recording started 27 minutes post
agonist stimulation)

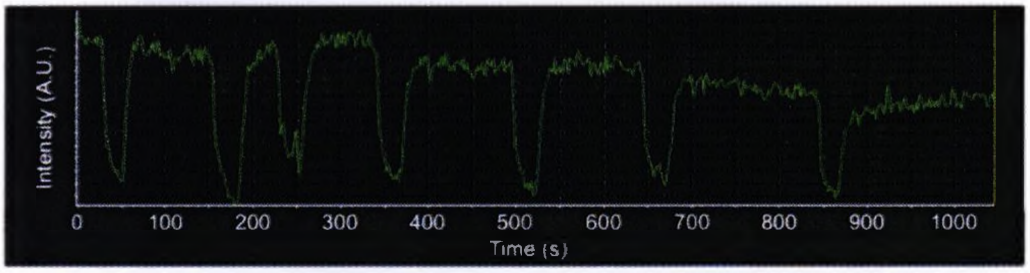


Oscillations
per minute

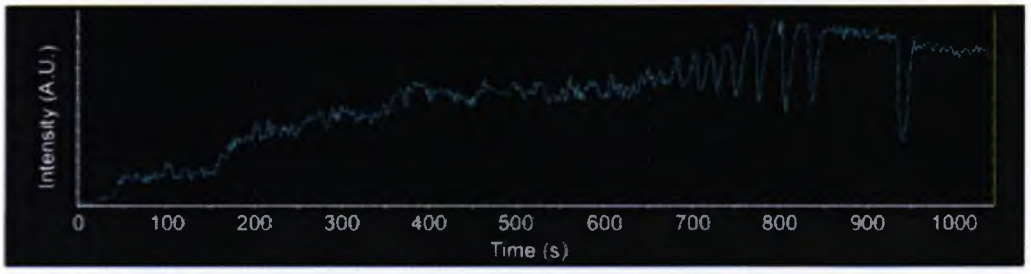
1.2



0.4



1.2



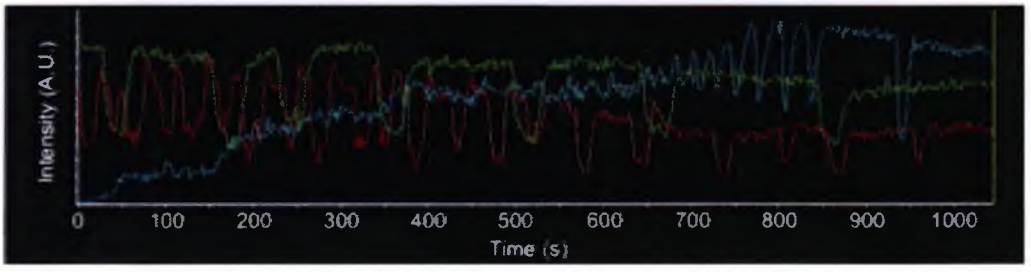
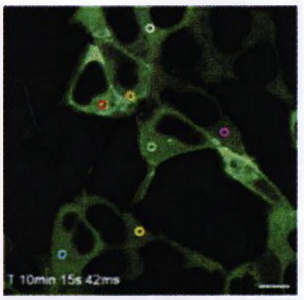


Figure 3.4 Frequency of oscillation of GFP-PKC βI in HEK 293 cells. Representative cells (top right) in which regions of interest are shown (circles). Fluorescence intensity within the region of interest was measured over time and plotted. Data shown was recorded 27 minutes post oscillation. Note the large variation of oscillation frequency between cells. Scale bar = 10 μm.

GFP-PKC βI and FLAG-hGnRH-RI in HEK 293

(Recording started 29 minutes post agonist stimulation)



Oscillations per minute

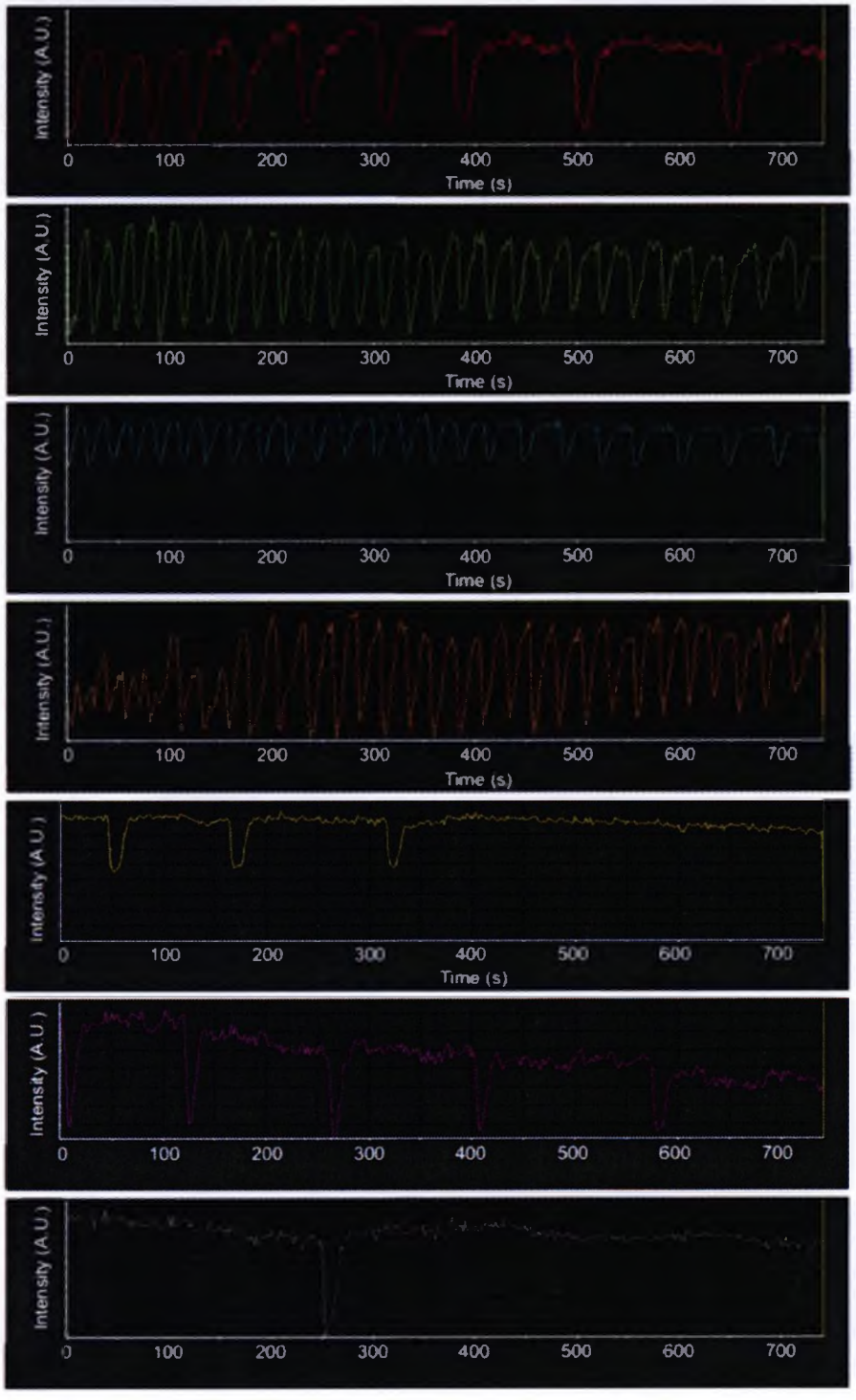
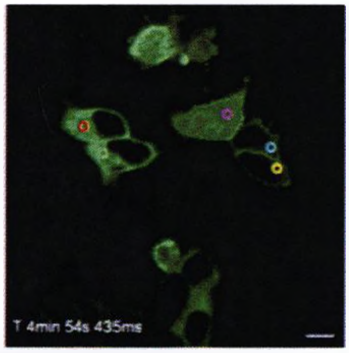


Figure 3.5 Frequency of oscillation of GFP-PKC βII in HEK 293 cells. Representative cells (top right) in which regions of interest are shown (circles). Fluorescence intensity within the region of interest was measured over time and plotted (top 5 graphs). Bottom graph shows oscillation frequencies of all regions of interest measured in the field of view. Data shown was recorded 29 minutes post oscillation. Note the large variation of oscillation frequency between cells. Scale bar = 10 μm.

GFP-PKC βII and FLAG-hGnRH-RI in HEK 293

(Recording started 27 minutes post agonist stimulation)



Oscillations per minute

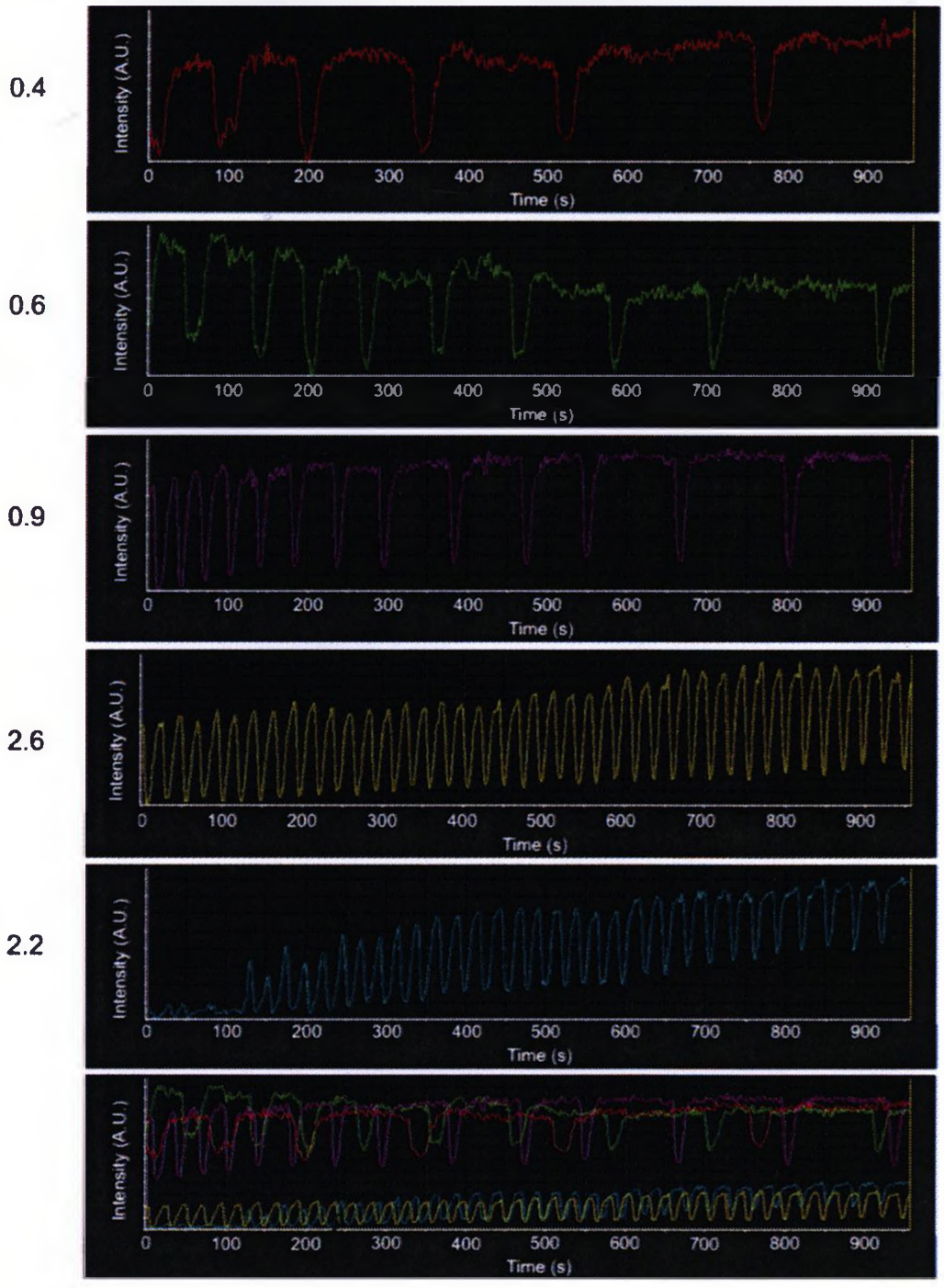
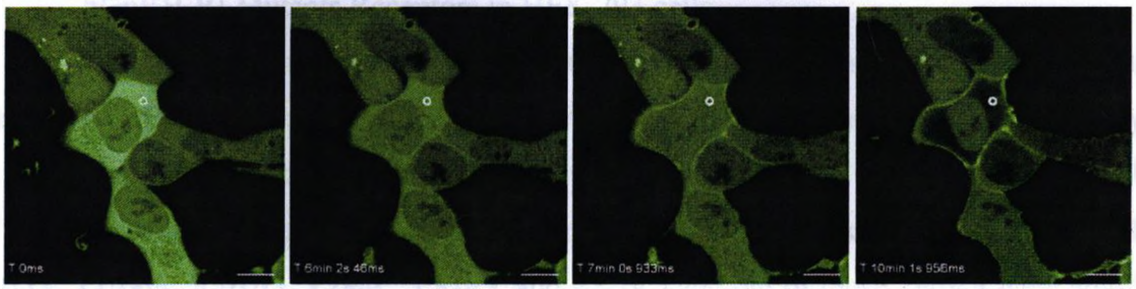


Figure 3.6 Single translocation response of PKC, where PKC remains at the plasma membrane for the duration of the recording. This response was observed in some cells from each isoform. Show here is a representative image of GFP-PKC θ in HEK 293 cells. Drug was added 10 s after the live imaging began. Scale bar = 10 μm.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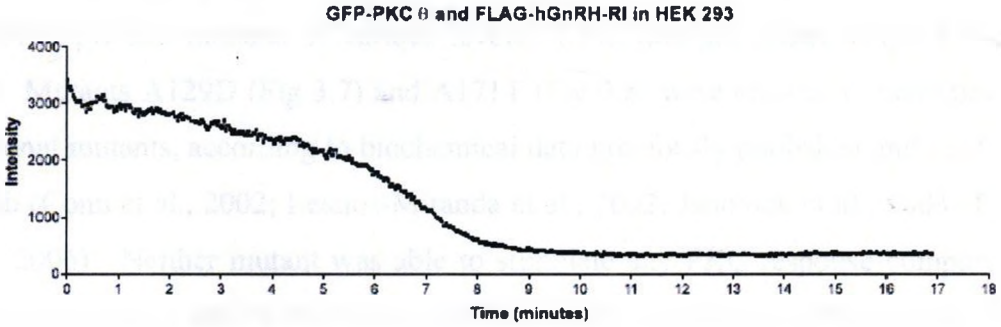

GFP-PKC θ and FLAG-hGnRH-RI in HEK 293
Intensity
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Time (minutes)

3.3.2 PKC βII Plasma Membrane Translocation Profiles in Response to FLAG-hGnRH-RI Mutants Receptors in HEK 293 cells

The ability of the naturally-occuring GnRH-RI mutants to stimulate GFP-PKC βII response in HEK 293 cells was assessed. PKC βII was the isoform chosen, as it displays a robust oscillation pattern in HEK 293 cells (Fig 3.2). Out of the 17 mutants tested, only 7 of them were able to stimulate PKC translocation to the plasma membrane (N10K, Q11K, Q106R, R262Q, Y284C, L314X and P320L) and out of those, only 4 were able to stimulate an oscillatory response pattern (N10K, Q11K, Q106R and Y284C) (data summarized in Table 3.2). Representative micrographs of HEK 293 cells expressing GFP-PKC βII and mutants of various levels of function are shown (Figs 3.7, 3.8, 3.9, 3.10). Mutants A129D (Fig 3.7) and A171T (Fig 3.8) were chosen as examples of non-functional mutants, according to biochemical data previously published and confirmed by our lab (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006). Neither mutant was able to stimulate any PKC response compared to the wild type receptor and in both cases GFP-PKC βII remained in the cytosol. Mutants R262Q and Q106R both retain greater than 50% IP_3 generating capacity of the wild type receptor (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006). Only 1 of 24 cells expressing GFP-PKC βII and mutant R262Q showed any response to agonist stimulation; this was a weak GFP-PKC βII translocation event, as shown in Fig. 3.9, in which we were unable to perform a trace due to cell ruffling and the weak response. This is representative of L314X and P320L, which also stimulated a single GFP-PKC βII translocation upon stimulation with agonist. Mutant Q106R stimulated a more robust response to agonist stimulation, displaying an oscillation pattern resembling that stimulated by the wild type receptor (Fig. 3.10). This oscillation pattern is comparable to mutants N10K, Q11K and Y284C.

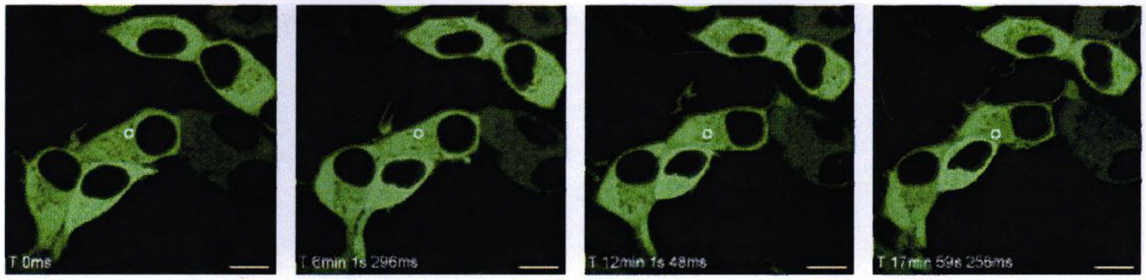
3.4 Discussion

In the present study using the HEK 293 cell system I determined that each of the conventional PKC isoforms (α, βI, βII and γ) show two different responses to FLAG-GnRH-RI activation: a single translocation to the plasma membrane and a repetitive translocation (oscillation) between the cytosol and the plasma membrane. I propose that

Table 3.2 Summary of PKC βII activation and IP_3 production of cells expressing naturally-occuring FLAG-GnRH-RI mutants following 100 nM Buserelin stimulation. Single translocation versus oscillation of an isoform is described. The number of cells observed and percentage of cells giving a specific response are described. IP_3 formation of mutants is expressed as percentage of wild type receptor response. + describes IP_3 formation below 15% of wild type response, ++ describes IP_3 formation between 16-70% of wild type response, +++ describes IP_3 formation between 71-100% of wild type response.

Mutant	Single Translocation	Oscillation	IP_3 Formation (n = 3-5)
N10K	Yes (3/15, 20.0%)	Yes (7/15, 46.7%)	74.9% +++
Q11K	Yes (6/14, 42.9%)	Yes (2/14, 14.3%)	92.8% +++
E90K	No (0/11)	No (0/11)	9.6% +
Q106R	Yes (2/24, 8.3%)	Yes (8/24, 33.3%)	91.9% +++
A129D	No (0/40)	No (0/40)	7.6% +
R139H	No (0/36)	No (0/36)	15.7% +
S168R	No (0/7)	No (0/7)	3.8% +
A171T	No (0/23)	No (0/23)	3.8% +
I177X	No (0/24)	No (0/24)	12.0% +
C200Y	No (0/14)	No (0/14)	32.3% ++
S217R	No (0/18)	No (0/18)	10.3% +
R262Q	Yes (1/24, 4.17%)	No (0/24)	77.6% +++
L266R	No (0/16)	No (0/16)	4.7% +
C279Y	No (0/52)	No (0/52)	4.3% +
Y284C	No (0/18)	Yes (2/18, 11.11%)	10.9% +
L314X	Yes (1/41, 2.44%)	No (0/41)	13.8% +
P320L	Yes (2/21, 9.52%)	No (0/21)	14.8% +

Figure 3.7 PKC βII translocation responses to FLAG-hGnRH-RI mutant A129D activation in HEK 293 cells. HEK 293 cells were transfected with cDNA encoding FLAG-GnRH-RI mutant A129D and GFP-PKC βII. Shown are representative images selected from a time series of 500-700 laser scanning confocal microscopic images collected at 1.1s intervals. The images demonstrate the lack of PKC βII response to agonist activation of this non-functional mutant receptor in HEK 293 cells. Drug was added 10 s after the live imaging began. Scale bar = 10 μm.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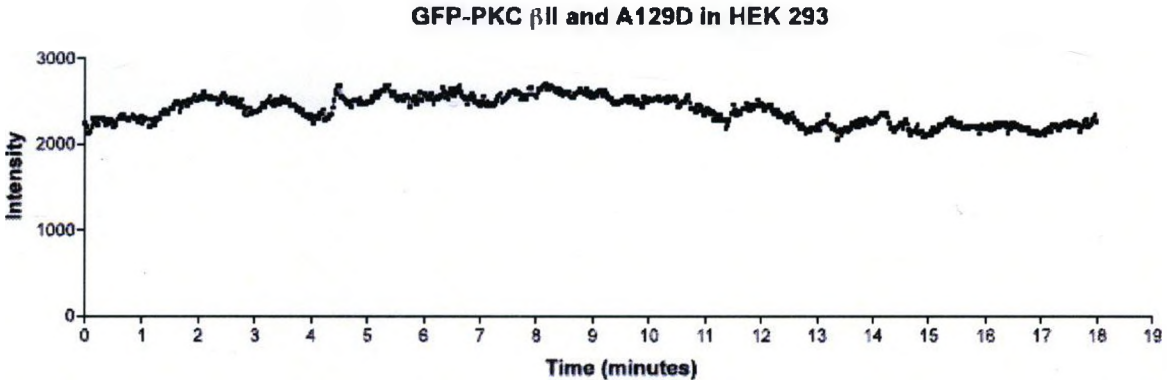
GFP-PKC βII and A129D in HEK 293
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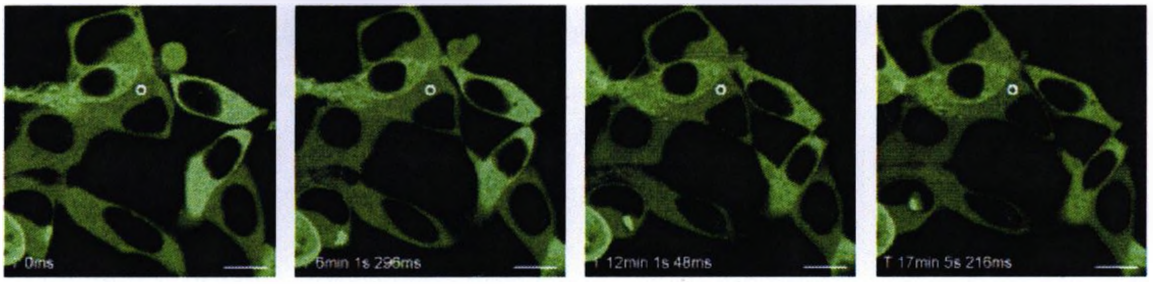
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Time (minutes)

Figure 3.8 PKC βII translocation responses to FLAG-hGnRH-RI mutant A171T activation in HEK 293 cells. HEK 293 cells were transfected with cDNA encoding FLAG-GnRH-RI mutant A171T and GFP-PKC βII. Shown are representative images selected from a time series of 500-700 laser scanning confocal microscopic images collected at 1.1s intervals. The images demonstrate the lack of PKC βII response to agonist activation of this non-functional mutant receptor in HEK 293 cells. Drug was added 10 s after the live imaging began. Scale bar = 10 μm.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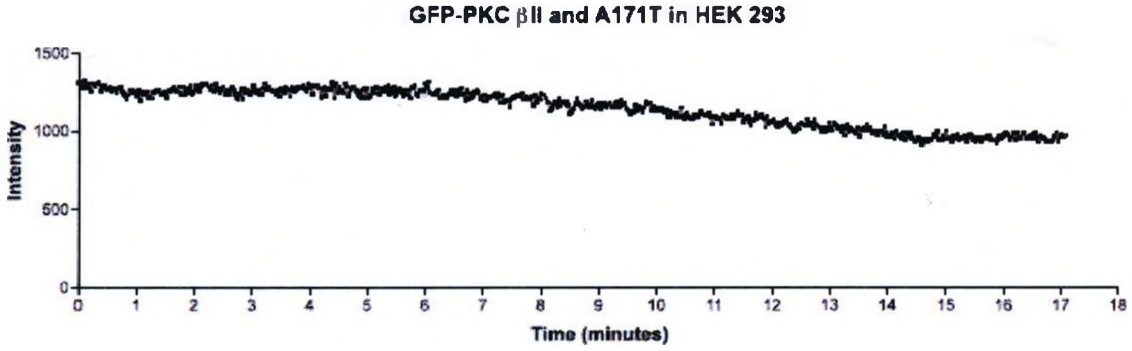
GFP-PKC βII and A171T in HEK 293
Intensity
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1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Time (minutes)

Figure 3.9 PKC βII translocation responses to FLAG-hGnRH-RI mutant R262Q activation in HEK 293 cells. HEK 293 cells were transfected with cDNA encoding FLAG-GnRH-RI mutant R262Q and GFP-PKC βII. Shown are representative images selected from a time series of 500-700 laser scanning confocal microscopic images collected at 1.1s intervals. The images demonstrate the weak translocation of PKC βII in response to agonist activation of this partially-functional mutant receptor in HEK 293 cells. A trace of PKC βII translocation was not created due to the cell ruffling and weak translocation seen by this cell (*arrows*). Drug was added 10 s after the live imaging began. Scale bar = 10 μm.

R262Q

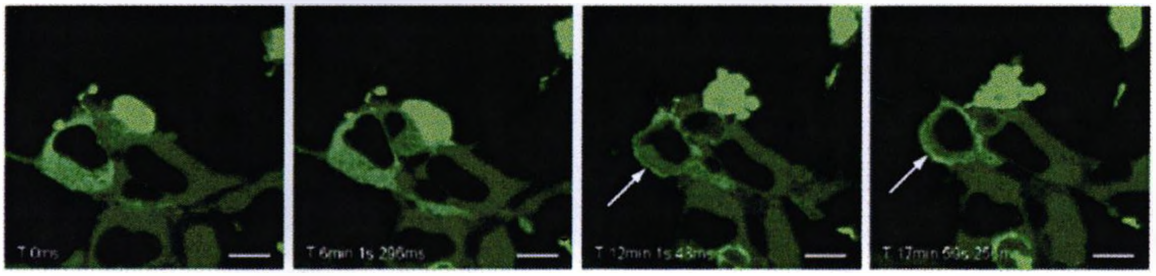
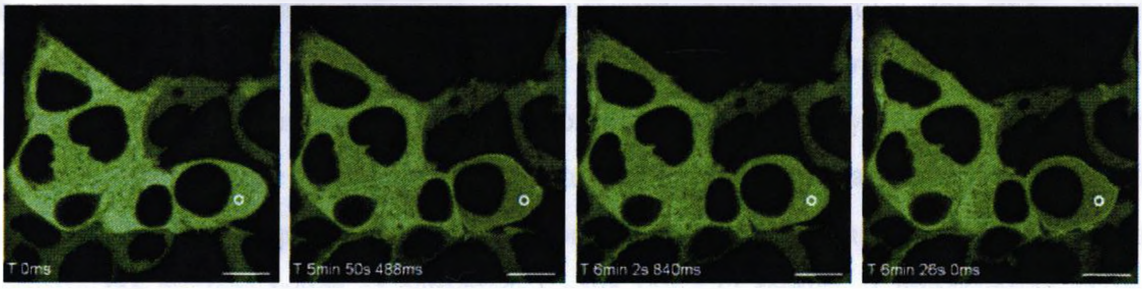


Figure 3.10 PKC βII translocation responses to FLAG-hGnRH-RI mutant Q106R activation in HEK 293 cells. HEK 293 cells were transfected with cDNA encoding FLAG-GnRH-RI mutant Q106R and GFP-PKC βII. Shown are representative images selected from a time series of 500-700 laser scanning confocal microscopic images collected at 1.1s intervals. The images demonstrate the robust oscillation of PKC βII in response to agonist activation of this partially-functional mutant receptor in HEK 293 cells. Drug was added 10 s after the live imaging began. Scale bar = 10 μm.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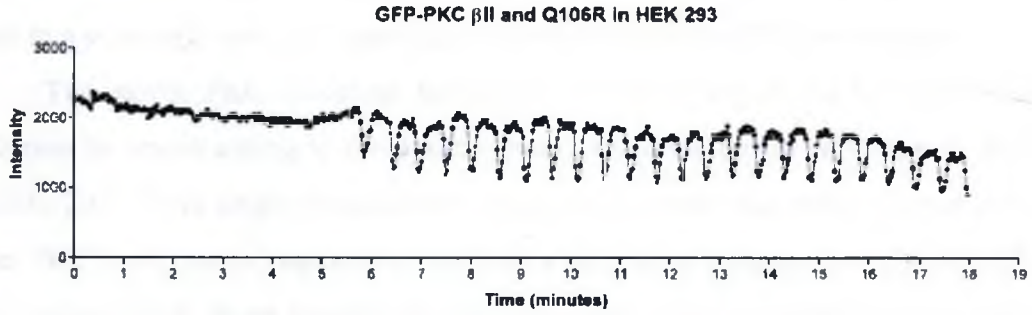
T 6min 26s 0ms


GFP-PKC βII and Q106R in HEK 293
Intensity
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2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Time (minutes)

the two responses may represent two different regulatory roles for PKC. Subtle differences in second messengers have the ability to direct very different outcomes in cells. For example, the frequency of Ca^{2+} oscillations is critically important for efficacy and specificity of gene transcription. Gene transcription via nuclear factor κ B (NF-κB) is enhanced by low frequency Ca^{2+} oscillation, whereas high frequency Ca^{2+} oscillations activate the nuclear factor of activated T-cells (NFAT) family of transcription factors (Dolmetsch et al., 1998). Although the effects of PKC isoform oscillation are wide-ranging and are yet to be well-defined, I propose that single PKC translocations will result in a very different cell signaling outcome compared to PKC oscillations.

The novel PKC isoforms tested (δ, ε and θ) responded to FLAG-GnRH-RI activation by translocating to the plasma membrane once before returning to the cytosol in HEK 293. This single translocation event is different than what is observed for the novel PKC isoforms in response to mGluR1a activation in agonist-stimulated HEK 293 cells, where there is an oscillatory response pattern in both conventional and novel isoforms (Babwah et al., 2003). However, it is not surprising to see differences in activation patterns between isoforms downstream of the same activated receptor. The angiotensin II type 1 receptor expressed in HEK 293 cells causes a single translocation of GFP-PKC βII, while causing GFP-PKC βI to oscillate in 4% of cells in response to stimulation with 100 nM Angiotensin II (Policha et al., 2006). In this study the only PKC isoform that failed to show a response to FLAG-hGnRH-RI activation was the atypical PKC ι/λ. It is important to note, however, that I only tested one of the two atypical isoforms and therefore the possibility remains that PKC ζ might be activated by agonist stimulated FLAG-hGnRH-RI in HEK 293 cells.

A study by Babwah et al. in 2003, demonstrated that in cells co-expressing DsRed-tagged PKC βI, GFP-PKC βII and mGluR1a displayed different PKC isoform translocation response patterns following receptor activation. Based on this study, it seems unlikely that differences between PKC isoform activation patterns can be attributed to differences in receptor expression levels or G protein coupling efficiency (Babwah et al., 2003). To address this issue using our my assay, a receptor dose-response experiment was performed where I co-expressed 2 μg GFP-PKC βII with various levels of receptor (5, 2.5, 1.25, 0.625 or 0.3125 μg) into HEK 293 cells. I found no difference in PKC

response based on level of receptor transfected (data not shown), ruling this out as the cause of the multiple responses that I saw within a single isoform.

The second aim of the study was to characterize PKC responses following the activation of known naturally-occurring mutants of hGnRH-RI and determine how these compared to the wild type hGnRH-RI. Since the naturally-occuring GnRH-RI mutants have a diminished capacity to produce the PKC cofactors DAG and Ca^{2+} relative to the WT receptor, I was interested in determining whether this diminished capacity affected the spatial and temporal characteristics of the downstream PKC response. I chose PKC βII since I had previously found that this isoform gives a robust oscillatory response to the agonist activated WT receptor and felt that subtle changes in its response to mutant receptor activation could be best detected relative to the other isoforms. We also performed IP_3 assays in HEK 293 cells in order to look for a correlation between the ability of the mutants to produce IP_3 and their ability to cause an oscillatory response of GFP-PKC βII similar to the wild type receptor. Based on my results I observed that a correlation does exist (Table 3.1). For example, mutants N10K, Q11K and Q106R all show IP_3 production of at least 74% of wild type receptor, and all three of these mutants are able to cause GFP-PKCβII oscillation upon agonist stimulation (7/15 cells, 2/14 cells and 8/24 cells respectively). Some unexpected results were also noted. Based on an IP_3 production of 77.6% of wild type GnRH-RI, I would have expected mutant R262Q to stimulate a robust PKC oscillation. However, no oscillation was seen (0/24 cells) and only 1 of 24 cells showed a single translocation response (1/24). On the other hand, mutant Y284C showed only 10.9% of IP_3 forming ability compared to wild type, but was able to stimulate GFP-PKCβII oscillation (2/18 cells). All of the mutants that showed IP_3 production of 12% or below, compared to wild type, which included E90K, A129D, R139H, S168R, A171T, I177X, S217R, L266R and C279Y, failed to activate GFP-PKCβII, as seen by their inability to stimulate either a single translocation or oscillation response. This did not include mutant Y284C, which was previously discussed.

This preliminary study was the first to visually document which PKC isoforms respond to hGnRH-RI activation in human cells, although some isoforms have low n values and further studies are required. This was also the first study to explore the signaling potential of the mutant receptors downstream of IP_3 production. Overall I have

demonstrated that a number of PKC isoforms are regulated by hGnRH-RI mediated signaling events. I also show that many mutant receptors may in fact have a greater signaling potential than was previously anticipated based on their ability to produce IP_3 only.

3.5 References

Amory JK, Page ST, Bremner WJ. Drug Drug Insight: recent advances in male hormonal contraception. Nature Clinical Practice Endocrinology & Metabolism (2006) 2, 32-41

Aris JP, Basta PV, Holmes WD, Ballas LM, Moomaw C, Rankl NB, Blobel G, Loomis CR, Burns DJ 1993 Molecular and biochemical characterization of a recombinant human PKC-delta family member. Biochim Biophys Acta. 1174(2):171-181.

Babwah AV, Dale LB, Ferguson SS 2003 Protein kinase C isoform-specific differences in the spatial-temporal regulation and decoding of metabotropic glutamate receptor1a-stimulated second messenger responses. J Biol Chem. 2003 Feb 14;278(7):5419-26. Epub 2002 Nov 25.

Bhagavath B, Ozata M, Ozdemir IC, Bolu E, Bick DP, Sherins RJ, Layman LC 2005 The prevalence of gonadotropin-releasing hormone receptor mutations in a large cohort of patients with hypogonadotropic hypogonadism. Fertil. Steril. 84: 951–957.

Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ, McNeilly AS 1993 Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. Mol Cell Endocrinol 94:R23–R27

Chi L, Zhou W, Prikhozhan A, Flanagan C, Davidson JS, Golembo M, Illing N, Millar RP, Sealfon SC 1993 Cloning and characterization of the human GnRH receptor. Mol Cell Endocrinol 91:R1–R6

Conn PM, Leaños-Miranda A, Janovick JA 2002 Protein origami: therapeutic rescue of misfolded gene products. Mol Interv. 2(5):308-316.

Dale LB, Babwah AV, Bhattacharya M, Kelvin DJ, Ferguson SS. 2001 Spatial-temporal patterning of metabotropic glutamate receptor-mediated inositol 1,4,5-triphosphate, calcium, and protein kinase C oscillations: protein kinase C-dependent receptor phosphorylation is not required. J Biol Chem. 276(38):35900-35908.

Dolmetsch RE, Xu K, Lewis RS 1998 Calcium oscillations increase the efficiency and specificity of gene expression. Nature. 392(6679):933-936.

Eidne KA, Sellar RE, Couper G, Anderson L, Taylor PL 1992 Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. Mol Cell Endocrinol 90:R5–R9

Feng X, Zhang J, Barak LS, Meyer T, Caron MG and Hannun YA 1998 Visualization of dynamic trafficking of a protein kinase C betaII/green fluorescent protein conjugate reveals differences in G protein-coupled receptor activation and desensitization. J. Biol. Chem. 273:10755-10762.

Griesinger G, Felberbaum R, Diedrich K. GnRH-antagonists in reproductive medicine. Arch Gynecol Obstet. 2005 Dec;273(2):71-8.

Harris D, Reiss N, Naor Z 1997 Differential activation of protein kinase C delta and epsilon gene expression by gonadotropin-releasing hormone in alphaT3-1 cells. Autoregulation by protein kinase C. J Biol Chem. 1997 May 23;272(21):13534-40.

Janovicj 2003
Kaiser UB, Zhao D, Cardona GR, Chin WW 1992 Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. Biochem Biophys Res Commun 189:1645–1652[CrossRef][Medline]

Janovick JA, Goulet M, Bush E, Greer J, Wettlaufer DG, Conn PM 2003a Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. J Pharmacol Exp Ther 305:608-614.

Kakar SS, Musgrove LC, Devor DC, Sellers JC, Neill JD 1992 Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. Biochem Biophys Res Commun 189:289–295[CrossRef][Medline]

Kakar SS, Rahe CH, Neill JD 1993 Molecular cloning, sequencing and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH). Domest Anim Endocrinol 10:335–342[CrossRef][Medline]

Leaños-Miranda A, Janovick JA, Conn PM 2002 Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 87(10):4825-4828.

Newton AC 2003 Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem J. 370(Pt 2):361-371.

Perrin MH, Bilezikjian LM, Hoeger C, Donaldson CJ, Rivier J, Hass Y, Vale WW 1993 Molecular and functional characterization of GnRH receptors cloned from rat pituitary and a mouse pituitary tumor cell line. Biochem Biophys Res Commun 191:1139–1144[CrossRef][Medline]

Policha A, Daneshtalab N, Chen L, Dale LB, Altier C, Khosravani H, Thomas WG, Zamponi GW, Ferguson SS 2006 Role of angiotensin II type 1A receptor phosphorylation, phospholipase D, and extracellular calcium in isoform-specific protein kinase C membrane translocation responses. J Biol Chem. 281(36):26340-26349.

Reinhart J, Mertz LM, Catt KJ 1992 Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing receptor. J Biol Chem 267:21281–21284[Abstract/Free Full Text]

Schultze-Mosgau A, Griesinger G, Altgassen C, von Otte S, Hornung D, Diedrich K. New developments in the use of peptide gonadotropin-releasing hormone antagonists versus agonists. Expert Opin Investig Drugs. 2005 Sep;14(9):1085-97. Links

Topaloglu AK, Lu ZL, Farooqi IS, Mungan NO, Yuksel B, O'Rahilly S, Millar RP. 2006 Molecular genetic analysis of normosmic hypogonadotropic hypogonadism in a Turkish population: identification and detailed functional characterization of a novel mutation in the gonadotropin-releasing hormone receptor gene. Neuroendocrinology. 2006;84(5):301-308.

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B, Sealfon SC 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol Endocrinol 6:1163–1169[Abstract]

Ulloa-Aguirre A, Janovick JA, Brothers SP, Conn PM 2004 Pharmacologic rescue of conformationally-defective proteins: implications for the treatment of human disease (Review). Traffic 5:821-837.

Weesner GD, Matteri RL 1994 Rapid communication: nucleotide sequence of luteinzing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary. J Anim Sci 72:1911.

Chapter 4: Summary of results and general discussion

At the start of this project, the main objective was to perform a cellular and molecular characterization of the human gonadotropin releasing hormone receptor in order to understand the molecular biology of this important receptor. My focus was to characterize the spatial properties of hGnRH-RI as well as study the early signaling events downstream of the $G_{q/11}$-coupled hGnRH-RI. I chose to perform a visual assay to investigate the cellular localization of hGnRH-RI because of previous biochemical data that suggested that this was primarily an intracellular receptor, an interesting attribute for a GPCR which plays a pivotal role in transmitting extracellular stimuli into intracellular signals. Using a FLAG-GnRH-RI construct expressed in HEK 293 and HTR-8/SVneo cells, I observed through the use of organellar markers that this receptor is primarily intracellular with strong localization on the nuclear membrane.

My studies uncovered a putative NLS sequence in the first intracellular loop of hGnRH-RI that is fully conserved in many proteins with known nuclear functions. Mutation as well as full deletion of this NLS sequence however, did not affect the nuclear localization of hGnRH-RI in HEK 293 or HTR-8/SVneo cells. It is possible that since hGnRH-RI is found on the nuclear membrane, as opposed to within the nucleoplasm that it does not localize to the nucleus through the use of an NLS. There is evidence suggesting that the endothelin receptor subtypes A and B, which have a perinuclear distribution, localize to the nuclear membrane via *de novo* synthesis and retrograde transport (Boivin et al., 2003). Further research is required to see if hGnRH-RI localizes to the nuclear membrane in a similar fashion. It is also a possibility that the cell systems used in my experiments do not express the combination of importins and accessory proteins required for nuclear import of hGnRH-RI. It is evident that a number of possible mechanisms exist for nuclear translocation of GPCRs and further research is required to elucidate these mechanisms.

The nuclear localization of hGnRH-RI was not dependent on the presence of agonist, unlike many other nuclear GPCRs such as the lysophosphatidic acid type 1 receptor and the endothelin receptors (Marrache et al., 2005; Boivin et al., 2005). However, the question still remains, if hGnRH-RI is a nuclear GPCR, what is its function at the nucleus and how does it see agonist? Our IP_3 studies of hGnRH-RI in HEK 293 cells show basal activity of the receptor equivalent to background, suggesting hGnRH-RI

is not constitutively active and requires agonist for activity. Multiple mechanisms for nuclear GPCR activation have been discovered. One mechanism includes GPCRs that are originally localized to the plasma membrane. Upon ligand binding, the activated receptor is transported to the nucleus. AT1-R and CystLT1-R undergo this agonist-dependent nuclear translocation (Lu et al., 1998; Nielsen et al., 2005). There is evidence that some receptors are constitutively present on the nuclear membrane, waiting to respond to ligand like their plasma membrane-localized counterparts. In this case, for the GPCR to be functional, ligand must either be produced intracellularly or transported from the extracellular environment into the cell in order to bind the receptor. There is evidence to support both theories. Platelet-activating factor (PAF), lysophosphatidic acid (LPA) and prostaglandins are all synthesized within the cell and would have access to nuclear receptors on the nuclear membrane. The HTR-8/SVneo cell line produces both GnRH-I and GnRH-II, opening up the possibility of an intracrine signaling pathway in these cells. However, unlike PAF, LPA and prostaglandins, which are phospholipids and eicosanoids and are free to diffuse within the cell, GnRH-I and II are peptides and would require a carrier to reach their nuclear receptor. The mGluR5a receptor is also constitutively present on the nuclear membrane, but its ligand, glutamate, is not synthesized within the cell. The mGluR5a ligand binding domains were found to be within the lumen of the nuclear envelope and sodium-dependent transporters and xCT exchangers are involved in moving glutamate across both the plasma and outer nuclear membrane to access the receptor (Jong et al., 2005). At this point, transporters for GnRH have not been discovered, but it remains a possibility. As the list of nuclear GPCRs increases, new mechanisms of agonist activation will undoubtedly arise.

As the field of nuclear GPCRs expands to include more GPCRs, new mechanisms of nuclear localization as well as agonist activation are sure to become apparent. The role that these receptors play in a broader cellular context have yet to be elucidated, but evidence that nuclear GPCRs may represent a forward step in GPCR evolution makes this an exciting field of study. In the case of hGnRH-RI in placental cells such as HTR-8/SVneo cells, which produce both the receptor and its ligands, this evolutionary step forward may result in a receptor that can bypass most of the plasma membrane/cytoplasmic signaling cascade and signal directly to the nucleus. I would

suggest however, that this intracrine signaling may represent a subclass of GnRH-RI-mediated signaling and does not replace signaling emanating from the plasma membrane.

If hGnRH-RI has a functional role at the nucleus, it will drastically change our ideas of cellular regulation and change the way we approach hGnRH-RI as a drug target. At the present time, treatment regimes assume that GnRH agonists and antagonists are binding their receptor at the surface of a cell. My discovery that human type I GnRH-R is a nuclear G protein-coupled receptor opens up the possibility of a functional nuclear receptor and a subclass of GnRH-mediated signaling. Transcriptional studies on agonist-stimulated isolated nuclei from cells expressing nuclear hGnRH-RI are a potential future study to determine if GnRH signaling at the nucleus activates the same genes as the plasma membrane hGnRH-RI. If this is in fact the case, it could change the way clinicians choose to administer GnRH, whether in a cell permeant or non-permeant form.

The molecular characterization portion of this study focused on early signaling events downstream of the $G_{q/11}$-coupled hGnRH-RI. Specifically, I examined the effect of the activated receptor to stimulate a PKC response. My studies were performed using GFP-tagged PKC isoforms from the conventional, novel and atypical classes expressed in HEK 293 cells. Live cell imaging performed on agonist-stimulated cells identified an obvious difference in translocation pattern between PKC isoforms. In HEK cells, conventional PKCs showed the ability to oscillate between the cytosol and plasma membrane as well as undergo a single translocation to the plasma membrane, whereas the novel PKCs could showed a single translocation response. The atypical PKC isoform tested, PKC ι/λ, was not activated by agonist stimulation of hGnRH-RI. Several reports have demonstrated that PKC translocation is subtype- and stimulus-specific (Shirai et al., 1998; Ohmori et al., 1998), supporting my studies.

Subtle differences in second messengers have the ability to direct very different outcomes in cells. For example, the frequency of Ca^{2+} oscillations is critically important for efficacy and specificity of gene transcription. Gene transcription via nuclear factor κ B (NF-κB) is enhanced by low frequency Ca^{2+} oscillation, whereas high frequency Ca^{2+} oscillations activate the nuclear factor of activated T-cells (NFAT) family of transcription factors (Dolmetsch et al., 1998). Similarly, different PKC responses may reflect different regulatory roles. Although the effects of PKC isoform oscillation are wide and are yet to

be well-defined, I propose that a single PKC translocation will result in a very different cell signaling outcome than PKC oscillations.

The second focus of the molecular characterization study was to look at the signaling capacity of 17 of the naturally occurring mutants, using GFP-tagged PKC βII, which gave a robust oscillation pattern in HEK 293 cells in response to wild type receptor activation. In general, my findings supported previous biochemical data (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006), as well as IP_3 data produced in my lab, which identified 9 of the mutants as non-functional (E90K, A129D, R139H, S168R, A171T, C200Y, S217R, L266R and C279Y). The residues E90 and R139 are thought to be involved with receptor activation and the E90K and R139H receptor mutants have been shown to be inactive in patients (Zhou et al., 1995). Only 4 of the mutants tested (N10K, Q11K, Q106R andY 284C) were able to induce oscillation of GFP-PKC βII in response to agonist. All of these mutants have shown partial activity in previous studies (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006) as well as in our own IP_3 assays. As previous studies suggest, mutation of hGnRH-RI may result in a receptor which is retained within the ER (Leanos-Miranda et al., 2002), which would result in diminished second messenger formation upon agonist stimulation, attenuating or ablating PKC response. The ability of IN3 to rescue 14 of the 17 mutants (Conn et al., 2002; Leanos-Miranda et al., 2002; Janovick et al., 2003; Topaloglu et al., 2006) as shown by IP_3 assay, would support this theory. It would be interesting to look at the ability of IN3 to rescue hGnRH-RI mutants by PKC assay in future studies.

In conclusion, these studies have revealed several novel findings regarding the spatial, temporal and signaling properties of the hGnRH-RI. Specifically, I showed that the hGnRH-RI is weakly expressed at the plasma membrane in homologous and heterologous cells, but strongly expressed in the cytoplasm and on the nuclear membrane. These findings have led me to hypothesize that this receptor is currently evolving towards greater intracrine signaling, a conclusion that may be more energetically favourable to a cell that expresses both the receptor and its ligands. Next I showed that while several PKC isoforms regulate plasma membrane-bound hGnRH-RI signaling, it does not appear that they have redundant roles based on very distinct spatial and temporal responses of the

isoforms following receptor activation. Overall, these studies have provided a more in-depth understanding of the biology of this clinically important GPCR.

4.1 References

Boivin B, Chevalier D, Villeneuve LR, Rousseau E, Allen BG 2003 Functional endothelin receptors are present on nuclei in cardiac ventricular myocytes. J Biol Chem 278:29153–29163[Abstract/Free Full Text]

Dolmetsch RE, Xu K, Lewis RS 1998 Calcium oscillations increase the efficiency and specificity of gene expression. Nature. 392(6679):933-936.

Janovick JA, Goulet M, Bush E, Greer J, Wettlaufer DG, Conn PM 2003a Structure-activity relations of successful pharmacologic chaperones for rescue of naturally occurring and manufactured mutants of the gonadotropin-releasing hormone receptor. J Pharmacol Exp Ther 305:608-614.

Jong YJ, Kumar V, Kingston AE, Romano C, O'Malley KL 2005 Functional metabotropic glutamate receptors on nuclei from brain and primary cultured striatal neurons. Role of transporters in delivering ligand. J Biol Chem. 280(34):30469-30480.

Leaños-Miranda A, Janovick JA, Conn PM 2002 Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropic hypogonadism. J Clin Endocrinol Metab. 87(10):4825-4828.

Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA 1998 Activation of protein kinase C triggers its ubiquitination and degradation. Mol. Cell. Biol. 18:839–845.

Nielsen CK, Campbell JI, Ohd JF, Mörgelin M, Riesbeck K, Landberg G, Sjölander A 2005 A novel localization of the G-protein-coupled CysLT1 receptor in the nucleus of colorectal adenocarcinoma cells. Cancer Res. 65(3):732-742.

Marrache AM, Gobeil F, Zhu T, Chemtob S 2005 Intracellular signaling of lipid mediators via cognate nuclear G protein-coupled receptors. Endothelium. 2005 Jan-Apr;12(1-2):63-72.

Ohmori S, Shirai Y, Sakai N, Fujii M, Konishi H, Kikkawa U, Saito N 1998 Three distinct mechanisms for translocation and activation of the delta subspecies of protein kinase C. Mol. Cell. Biol. 18, 5263–5271.

Shirai Y, Kashiwagi K, Yagi K, Sakai N, Saito N 1998 Distinct effects of fatty acids on translocation of gamma- and epsilon-subspecies of protein kinase C.J. Cell Biol. 143, 511–521.

Topaloglu AK, Lu ZL, Farooqi IS, Mungan NO, Yuksel B, O'Rahilly S, Millar RP. 2006 Molecular genetic analysis of normosmic hypogonadotropic hypogonadism in a Turkish population: identification and detailed functional characterization of a novel mutation in the gonadotropin-releasing hormone receptor gene. Neuroendocrinology. 2006;84(5):301-8.

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