Investigating crosstalk in lipid rafts between the glucocorticoid receptor and gonadotropin-releasing hormone receptor signaling pathways in a gonadotrope cell line

by

Lance Wehmeyer

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Supervisor: Professor Janet P. Hapgood

Co-supervisor: Doctor Andrea Kotitschke

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously submitted any part of it at any university for a degree.

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Signature

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Date

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Abstract

It is becoming increasingly evident that cell signaling is much more complex than originally perceived and involves crosstalk of different signaling pathways to define the cellular response to multiple simultaneous signals. The gonadotropin-releasing hormone (GnRH) receptor (GnRHR), a plasma membrane G protein-coupled receptor, is the primary regulator of mammalian reproduction. The glucocorticoid receptor (GR), a cytosolic or nuclear steroid receptor, plays a central role in various processes of mammalian physiology, by maintaining homeostasis of basal and stress-related conditions through modulation of a vast array of metabolic and immune functions. Some of the effects of stress on reproduction are mediated via glucocorticoids (GCs) from the adrenal gland as part of a crosstalk mechanism between the hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-gonadal (HPG) axis. A recent study from the Hapgood laboratory demonstrated the presence of a novel crosstalk mechanism between the GR and GnRHR, indicating an additional direct mechanism for the effects of stress on reproduction. The present study investigated whether this crosstalk between the GR and GnRHR involves the co-localization of these receptors to lipid rafts, providing a specialized distinct region where the receptors can be in close proximity and reciprocally modulate each other's signaling pathways. Specific aims of the present study included confirming the GnRH and synergistic transcriptional responses with dexamethasone (Dex) and GnRH induced by crosstalk between the GR and GnRHR. This study investigated whether this reciprocal modulation involves co-localization and an interaction of these receptors in lipid rafts, while also investigating a functional role for lipid rafts in GR and GnRHR signaling. Using promoter-reporter assays, the results showed that GnRH was able to activate the unliganded GR, to a similar extent as Dex to transactivate via a GRE-reporter gene in LβT2 cells. Co-stimulation with Dex and GnRH synergistically enhanced the transcriptional activity of the GRE-reporter gene in these cells. Modulations of the GnRHR signaling pathway by the GR on other *cis*-elements were also investigated. Dex enhanced the GnRH-induced transcriptional activity of a minimal activator protein-1 (AP-1)-reporter gene, but had no effect on the GnRH-induced activation of a minimal nuclear factor-kappa B (NF-κB)-reporter gene in LβT2 cells. The novel finding that GnRH induced GR protein degradation is supporting evidence for the activation of the GR with GnRH in LβT2 cells. Both the GR and GnRHR appeared to co-localize to lipid rafts containing Flot-1 at the plasma membrane of LβT2

cells, as shown by immunoflorescence and density-gradient analysis. Interestingly, the lipid raft association of the GR was found to be independent of 30 min treatment with Dex, GnRH or both together. Co-immunoprecipitation assays showed that the GR interacts with Flot-1 in a complex that is independent of ligands, supporting the results of the immunoflorescence and density-gradient experiments. Although results using cholesterol depletion agents suggest that lipid rafts are not required for the hormone-induced transcriptional response on a GRE or the GC-independent phosphorylation of the GR in LβT2 cells, they were inconclusive. However, siRNA knock down experiments revealed that Flot-1 appeared to be required for the Dex-induced transcriptional response and the synergistic transcriptional response with Dex and GnRH on a GRE in LβT2 cells. The results suggests a novel mechanism for the crosstalk between the GR and GnRHR signaling pathways involving co-localization of both receptors to lipid rafts containing Flot-1, which is required for signaling of both receptors.

List of abbreviations

ACTH	-	adrenocorticotropin hormone
AP-1	-	activator protein-1
AF	-	activation function
AR	-	androgen receptor
p300	-	adenovirus E1A binding protein 300
APP	-	amyloid precursor protein
ANOVA	-	analysis of variance
BMK1/ERK-5	-	big mitogen-activated protein kinase
BRG1	-	brahma-related gene 1
BACE	-	β-secretase
bHLH	-	basic-helix-loop-helix
BHK	-	baby hamster kidney
CoCoA	-	coiled-coil co-activator
CRH	-	corticotrophin-releasing hormone
cAMP	-	cyclic adenosine monophosphate
CREB	-	cAMP response element binding protein
CBP	-	CREB-binding protein
CDK5	-	cyclin-dependent kinase 5
CAP	-	Cbl-associated protein
PrP^c	-	cellular prion protein
ChIP	-	chromatin immunoprecipitation
CHIP	-	C-terminal of heat shock 70-interacting protein
c-src	-	c-src tyrosine kinase
CHO	-	chinese hamster ovary
Cav	-	caveolin
DRM	-	detergent-resistant membranes
Dex	-	dexamethasone
DBD	-	DNA-binding domain
DMEM	-	Dulbecco's Modified Eagles Medium

ERK-1/2	-	extracellular-regulated kinase 1/2
EGF	-	epidermal growth factor
EGFR	-	epidermal growth factor receptor
eNOS	-	endothelial nitric oxide synthase
ER	-	estrogen receptor
ERE	-	estrogen-response-element
ELB	-	extraction lysis buffer
Flot	-	flotillin
FAK	-	focal adhesion kinase
FRET	-	fluorescence resonance energy transfer
FCS	-	fetal calf serum
FSH	-	follicle-stimulating hormone
FKBP	-	FK506 binding protein
FACS	-	fluorescent-activated cell sorting
GR	-	glucocorticoid receptor
GC	-	glucocorticoid
GRE	-	glucocorticoid-response-element
GRIP-1	-	glucocorticoid receptor interacting protein-1
GSK3β	-	glycogen synthase kinase 3β
GPI	-	glycosylphosphatidylinositol
GPCR	-	G-protein-coupled receptor
GnRH	-	gonadotropin-releasing hormone
GnRHR	-	gonadotropin-releasing hormone receptor
GILZ	-	glucocorticoid-inducible leucine zipper
Grb2	-	growth factor receptor-bound protein 2
GAPDH	-	glyceraldehyde 3-phosphate dehydrogenase
αGSU	-	glycoprotein hormone α-subunit
HDAC2	-	histone deacetylase 2
HB	-	homogenization buffer
HSP	-	heat-shock protein
HPA	-	hypothalamic-pituitary-adrenal

HPG	-	hypothalamic-pituitary-gonadal
HAT	-	histone acetyltransferase
HIV-1	-	human immunodeficiency virus 1
HMG-CoA	-	3-hydroxy-3-methylglutaryl-coenzyme A
IL	-	interleukin
IP3	-	inositol-1, 4, 5-triphosphate
JNK	-	c-Jun N-terminal kinase
LBD	-	ligand-binding domain
LH	-	luteinizing hormone
LCK	-	leukocyte-specific protein tyrosine kinase
MAPK	-	mitogen-activated protein kinase
MEK1/2	-	MAPK/ERK kinase 1/2
MR	-	mineralocorticoid receptor
mGR	-	membrane glucocorticoid receptor
MβCD	-	methyl-β-cyclodextrin
NTD	-	N-terminal domain
NGRE	-	negative glucocorticoid-response-element
NF-κB	-	nuclear factor-kappa B
NGF	-	nerve growth factor
NSC	-	non-silencing control
PR	-	progesterone receptor
PRE	-	progesterone-response-element
PP5	-	protein phosphatase 5
PACAP	-	pituitary adenylate cyclase-activating polypeptide
p/CAF	-	p300/CBP associated factor
PEPCK	-	phosphoenolpyruvate carboxykinase
PKC	-	protein kinase C
Pyk2	-	proline-rich tyrosine kinase 2
PG	-	prostaglandin
PDGF	-	platelet-derived growth factor
PTOV-1	-	prostate tumor overexpressed gene-1

PHB	-	prohibitin homology domain
PI3K	-	phosphatidyl inositol 3-kinase
PBS	-	phosphate-buffered saline
RTK	-	receptor tyrosine kinase
RIPA	-	radio-immunoprecipitation assay
SWI/SNF	-	switch/sucrose non-fermentable
SRC	-	steroid receptor co-activator
STAT	-	signal transducer and activator of transcription
SUMO-1	-	small-ubiquitin-related modifier-1
SGK	-	serum- and glucocorticoid-induced protein kinase
SPFH	-	Stomatin/Prohibitin/Flotillin/HflK/C
SH3	-	src homology 3
SALM4	-	synaptic adhesion-like molecule 4
SB	-	solubilization buffer
TFIID	-	transcription factor II D
TBP	-	TATA-box binding protein
TAT	-	tyrosine aminotransferase
TCR	-	T-cell receptor
TM	-	transmembrane
TNFα	-	tumor necrosis factor α
TBS	-	Tris-buffered saline
ZAP-70	-	zeta-chain-associated protein kinase 70

Table of contents

List of abbreviations vi

Chapter 1: Literature review 1

1.1 Glucocorticoid receptor 1

1.1.1 Physiological function in the pituitary 1

1.1.2 Structural organization 2

1.1.3 The classical ligand-dependent GR mechanism of action 4

1.1.3.1 Ligand binding and activation 4

1.1.3.2 Dimerization and nuclear translocation 4

1.1.3.3 Transcriptional regulation of gene expression 6

1.1.3.3.1 Direct DNA binding 6

1.1.3.3.2 Tethering 8

1.1.4 Post-translational modifications 10

1.1.5 Protein degradation 13

1.1.6 Rapid glucocorticoid-mediated non-genomic actions 15

1.1.6.1 Crosstalk of the classical GR with intracellular signaling pathways 16

1.1.6.2 Crosstalk of the membrane-associated GR with intracellular signaling pathways 18

1.2 Gonadotropin-releasing hormone receptor 20

1.2.1 Physiological function 21

1.2.2 Protein structure 22

1.2.3 Signaling pathways 23

1.2.4 Crosstalk with other receptor signaling pathways 25

1.3 Lipid rafts 27

1.3.1 Properties and structure 27

1.3.1.1 Caveolae 28

1.3.2 Physiological function 30

1.3.2.1 Cell signaling 30

1.3.2.2 Role in health and disease 34

1.3.3 The reggie/flotillin family of proteins 36

1.3.3.1 Structural features 37

1.3.3.2 Tissue distribution and expression 38

1.3.3.3 Subcellular localization 38

1.3.3.4 Cellular function 40

1.4 Hypothesis, aims and strategies 43

Chapter 2: Materials and Methods 45

2.1 Compounds and antibodies 45

2.2 Plasmids 46

2.3 Plasmid transformation and preparation 46

2.4 Cell culture 47

2.5 Transient transfection of cells **47**

2.6 SDS-PAGE and Western blotting **48**

2.7 Confocal immunoflorescence **50**

2.7.1 Live cell GnRHR staining **50**

2.7.2 Live cell cholera toxin staining **50**

2.7.3 Confocal imaging **51**

2.8 Lipid raft isolation **52**

2.9 Co-immunoprecipitation assays **54**

2.10 Incubation with cholesterol depletion agents **55**

2.10.1 Luciferase gene reporter assays **55**

2.10.2 Phosphorylation assays **55**

2.11 RNA interference **56**

2.12 Statistical analysis **57**

Chapter 3: Results **58**

3.1 Confirmation of the GnRH and the synergistic transcriptional responses on a GRE in LβT2 cells **58**

3.1.1 GnRH activates the endogenous unliganded GR to induce a transcriptional response on a GRE in LβT2 cells **58**

3.1.2 Co-stimulation with Dex and GnRH induces a synergistic transcriptional response on a GRE in LβT2 cells **58**

3.2 Dex increases the GnRH-induced transcriptional response on an AP-1 *cis*-element in LβT2 cells **60**

3.3 Crosstalk does not occur between the Dex and GnRH signaling pathways on an NF-κB *cis*-element in LβT2 cells **61**

3.4 Similar to Dex, GnRH appears to induce GR protein degradation in a dose-dependent manner **62**

3.5 Subcellular localization of the GnRHR and GR with lipid raft markers by immunoflorescence analysis in LβT2 cells **64**

3.5.1 LβT2 cells express the lipid raft marker protein Flot-1 and not Cav-1 **64**

3.5.2 Immunoflorescence analysis shows that the endogenous GnRHR co-localizes with endogenous Flot-1 at the plasma membrane of LβT2 cells, independent of ligand **65**

3.5.3 Immunoflorescence analysis shows that a small population of endogenous GR appears to co-localize with endogenous Flot-1 to lipid rafts independent of ligand in LβT2 cells **68**

3.6 Density-gradient analysis shows that the endogenous GR localizes with the endogenous GnRHR to Flot-1 containing lipid rafts independent of ligand in LβT2 cells **74**

3.7 Co-immunoprecipitation assays show the *in vitro* interaction between the endogenous GR and endogenous Flot-1 in a ligand-independent manner in LβT2 cells **79**

3.8 Although results using cholesterol depletion agents suggest that lipid rafts are not required for the hormone-induced transcriptional response on a GRE or the GC-independent phosphorylation of the GR in LβT2 cells, they were inconclusive **82**

3.9 Flot-1 is required for Dex-induced transcriptional response and the synergistic transcriptional response with Dex and GnRH on a GRE in LβT2 cells **86**

Chapter 4: Discussion and Conclusions **91**

4.1 Evidence for the crosstalk mechanism between GR and GnRHR signaling pathways in LβT2 cells **91**

4.2 Co-localization of the GR and GnRHR to lipid rafts in LβT2 cells **96**

4.3 Functional role of lipid rafts and Flot-1 in GR and GnRHR signaling **103**

4.4 Physiological implications in the pituitary **108**

4.5 Conclusions **109**

4.6 Future perspectives **110**

Addendum **115**

Bibliography **121**

Chapter 1: Literature review

1.1 Glucocorticoid receptor

1.1.1 Physiological function in the pituitary

The endogenous ligand for the glucocorticoid receptor (GR) in humans, namely cortisol, is synthesized in the adrenal cortex and its release is under the control of the hypothalamic-pituitary-adrenal axis. Corticotrophin-releasing hormone (CRH) secretion from the hypothalamus regulates glucocorticoid (GC) synthesis by controlling the release of adrenocorticotropin hormone (ACTH) (Chrousos, 1995). The GR plays a vital role in the control of homeostasis and adaptive processes including immune, metabolic, neural and behavioral systems (Bloom *et al*., 1982; Wiegers and Reul, 1998). Given the fundamental role of GCs, they are extensively utilized as therapeutical drugs in many inflammatory and immune diseases as well as in certain cancer treatments (Rhen and Cidlowski, 2005).

Chronic and acute stress have been implicated in the regulation of mammalian reproduction although the underlying mechanisms are not well understood. While chronic stress primarily reduces gonadotropin secretion and reproduction, the effects of acute stress are variable with both positive and negative effects reported in the literature (Brann and Mahesh, 1991; Tilbrook *et al*., 2000). The effects of stress on reproduction are mediated via GCs from the adrenal gland as part of a crosstalk mechanism between the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axis (Rivier and Rivest, 1991). The pulsatile administration of gonadotropin-releasing hormone (GnRH) to rat pituitary cells was shown to increase GnRH receptor (GnRHR) mRNA levels, an effect that was further increased with dexamethasone (Dex) (Rosen *et al*., 1991). Furthermore, in cultured rat pituitary cells (Baldwin *et al*., 1991; Brann and Mahesh, 1991; Kilen *et al*., 1996; McAndrews *et al*., 1994; D'Agostino *et al*., 1990), GCs increased the secretion of follicle-stimulating hormone (FSH), while having no effect or decreasing luteinizing hormone (LH) secretion. In addition, the activated GR was shown to

enhance expression of the glycoprotein hormone α-subunit gene in the immortalized gonadotrope cell line, LβT2 (Sasson *et al*., 2008). In contrast, some of the effects of stress on reproduction have been shown to involve a GC-dependent decrease in LH secretion in primary rat pituitary cultures (Kamel and Kubajak, 1987), resulting from a decrease in pituitary responsiveness to GnRH (Breen and Karsch, 2004). Furthermore, cortisol was shown to inhibit the estrogen-induced increase of GnRHR mRNA, suggesting a repressive role for GCs in GnRHR expression (Adams *et al*., 1999). The results from pituitary cells strongly support a mechanism of GCs affecting mammalian reproduction through either increasing GnRHR levels or directly influencing gonadotropin levels. The physiological role of the GC-dependent increase in FSHβ secretion was proposed to protect and maintain the health of the follicle for the next reproductive cycle (Kilen *et al*., 1996). The expression of functional GR protein has previously been reported in primary mouse pituitary cells and conditional knockout mice with a deletion of the GR in the pituitary impaired the GC-mediated negative feedback on the HPA axis (Schmidt *et al*., 2009).

1.1.2 Structural organization

Steroid hormone receptors such as the GR, progesterone receptor (PR), androgen receptor (AR), mineralocorticoid receptor (MR) and the estrogen receptor (ER) belong to the nuclear receptor subfamily 3 (Nuclear Receptors Nomenclature Committee, 1999). The human GR gene comprises of 10 exons spanning a region of 110 kb while alternative splicing of exons 9α and 9β results in two GR isoforms, namely GRα and GRβ, respectively (Zhou and Cidlowski, 2005). These two proteins contain the same functional domains, except GRβ has a truncated C-terminal domain that prevents it from binding to ligands. In the absence of ligand, the GRα is mainly cytoplasmic, while GRβ is nuclear and represses the expression of certain cytokine genes (Kelly *et al*., 2008). In addition, the GRβ isoform can form heterodimers with GRα, acting as a dominant-negative for the transcriptional activity of GRα (Oakley *et al*., 1999; Bamberger *et al*., 1995). Furthermore, a recent study was the first to report GRβ mRNA and protein expression in mice, which displayed similar properties to the human GRβ (Hinds *et al*., 2010). Increased GRβ expression with its dominant negative effect on the transcriptional activity of GRα could result in GC resistance that is associated with various diseases (Hamilos *et al*., 2001). Three additional GR splice variants have been identified in various tissue types, namely GRγ, GR-A and GR-P.

Besides the above mentioned isoforms, alternative translation initiation of GRα and GRβ results in multiple GRα and GRβ isoforms. All of the translational GR isoforms are expressed in mouse and rat tissues, although they were differentially expressed compared to human tissues (Lu and Cidlowski, 2005). The existence of various GR isoforms can be a mechanism for the differential cellular responsiveness to GC.

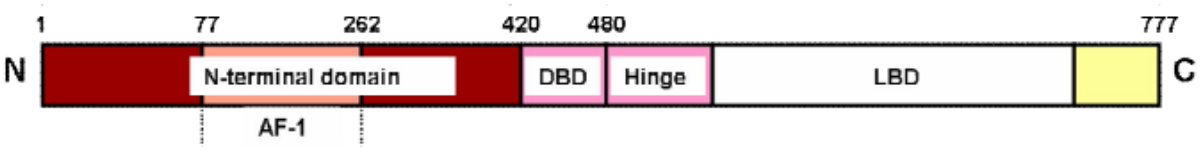


Figure 1.1.1: Structural organization of the human GRα protein. AF-1, activation function 1; DBD, DNA-binding domain; LBD, ligand-binding domain. Taken and adapted from (Avenant, 2009).

The GRα protein consists of 777 amino acids and can be divided into three independent functional domains, namely a variable N-terminal domain (NTD), a central highly conserved DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Griekspoor *et al*., 2007) (Figure 1.1.1). The NTD is the most variable region with respect to sequence homology and size between different species of GR, while containing a region required for maximal transcriptional activity, known as the transcriptional activation function-1 (AF-1). The AF-1 region has been shown to interact directly with components of the basal transcription machinery and many co-factors involved in the regulation of transcription (Kumar and Thompson, 2005; Heitzer *et al*., 2007). Mutational studies have shown that the NTD is a constitutive activator of transcription in the absence of the LBD (Godowski *et al*., 1987). Furthermore, the NTD is a major target for post-translational modifications such as phosphorylation and sumoylation (Faus and Haendler, 2006).

The central DBD is the most conserved region consisting of two zinc-finger motifs where each zinc atom is coordinated by four cysteine residues in a tetrahedral conformation (Freedman *et al*., 1998). The amino acids from the first zinc-finger interact with specific DNA sequences in the promoter of target genes while the second zinc-finger stabilizes the DNA-protein interaction and is important for receptor homodimerization (Dahlman-Wright *et al*., 1991). The DBD also interacts directly with other proteins modulating the transcriptional activity of the GR. The DBD

is connected to the LBD by a flexible hinge region, allowing the receptor to change conformation and it contains a nuclear localization signal (Picard and Yamamoto, 1987). The LBD is located at the C-terminal end of the receptor and is responsible for recognition and binding of hormone ligands. The LBD consists of twelve amino acid residues and four β-strands that form a central hydrophobic ligand-binding pocket. Mutational studies have revealed that two residues within the LBD are very important for receptor homodimerization (Bledshoe *et al*., 2002). The LBD contains a second nuclear localization signal and the transcriptional AF-2 region, which plays a role in binding of heat shock proteins and recruitment of co-factors (Savory *et al*., 1999; Bledshoe *et al*., 2004).

1.1.3 The classical ligand-dependent GR mechanism of action

1.1.3.1 Ligand binding and activation

In the absence of ligand, the GR exists in a multi-protein complex that is mainly cytoplasmic. The multi-protein complex maintains the GR in an inactive state and includes chaperones such as heat-shock protein 90 (HSP90), HSP70, HSP23, phosphatases such as protein phosphatase 5 (PP5) and immunophilins like FK506 binding protein 51 (FKBP51) (Pratt and Toft, 1997; Wang *et al*., 2007; Kumar and Thompson, 2005). GCs are lipophillic hormones that enter the cell by passive diffusion across the plasma membrane (Lu *et al*., 2006). Upon ligand binding, the GR changes conformation and is released from the cytoplasmic chaperone proteins. The resulting change in conformation of the GR is accompanied by hyperphosphorylation of the receptor and exposure of the nuclear localization signals (Zhou and Cidlowski, 2005).

1.1.3.2 Dimerization and nuclear translocation

In the classical model of GR activation, after ligand binding the receptor homodimerizes through distinct hydrophobic regions in the LBD, followed by nuclear translocation where it regulates transcription of target genes (Luisi *et al*., 1991). A study by Savory *et al*. suggests that receptor dimerization occurs in the cytoplasm (Savory *et al*., 1999). Mutations of key residues in the DBD

and LBD that is important for homodimerization, still results in nuclear import and transrepression of target genes, but not transactivation (Bledshoe *et al*., 2002; Reichardt *et al*., 1998). This suggests that receptor dimerization is not required for nuclear import or transrepression of GR target genes, but is important for transactivation.

The GR contains two nuclear localization signals, one in the hinge region and one in the LBD, that are uncovered during the conformational change upon ligand-binding (Savory *et al*., 1999). Not all chaperone proteins are released upon ligand-binding and the resulting conformational change of the GR, such as HSP90, which has been shown to play an important role in the mobility of the GR within the nucleus (Elbi *et al*., 2004). Furthermore, a recent finding demonstrated that FKBP51 and FKBP52 are involved in GR nuclear localization through direct binding of HSP90 (Banerjee *et al*., 2008; Zhang *et al*., 2008). The same study showed that when the unliganded GR exists in the same complex with FKBP52, the GR is mostly nuclear, but in contrast the GR is mostly cytoplasmic when in the same complex with FKBP51 (Banerjee *et al*., 2008). Additionally, it was previously shown that ligand-binding induces a switch from an inactive GR protein complex with FKBP51 to an active GR protein complex with FKBP52 and subsequent nuclear translocation (Davies *et al*., 2002). The mechanism of nuclear import involves FKBP52 interacting with dynein, which can move along the microtubule network to the nuclear-pore (Silverstein *et al*., 1999). The nuclear translocation signals of the GR are recognized by proteins involved in nuclear import known as importins, which are responsible for translocation of the GR through nuclear pores into the nucleus (Freedman and Yanamoto, 2004).

In contrast to the important role FKBP52 appears to play in nuclear import of the GR, no defects in GR-regulated physiology were detected in FKBP52 knockout mice. However, embryonic fibroblast cells from these animals had a 70% reduction in the transcriptional activity of the GR, suggesting that FKBP52 is important for the ability of the GR to regulate expression of target genes. In addition, the same study reported different effects on the expression of endogenous GR target genes, with reduced expression of the glucocorticoid-inducible leucine zipper (GILZ) gene, but no effect on the Dex-induced expression of serum- and glucocorticoid-induced protein kinase (SGK) gene (Wolf *et al*., 2009). The results from Wolf *et al*. suggest that FKBP52 is a gene-

specific modulator of GR activity and that alternative pathways of nuclear import could exist for the GR.

1.1.3.3 Transcriptional regulation of gene expression

1.1.3.3.1 Direct DNA binding

In the nucleus, the GR can enhance transcription by binding to regulatory elements within the promoters of target genes, a process called transactivation. The activated GR binds as a homodimer to specific DNA sequences located in the regulatory elements of GC-responsive genes, called glucocorticoid-response-elements (GRE), as shown in Figure 1.1.2. The GRE consists of two conserved six-base pair half sites separated by a non-conserved three-base pair spacer: 5'-GGTACAnnnTGTTCT-3' (Comings *et al*., 1995; Nordeen *et al*., 1990). The 15 base pair consensus sequence has been found to vary slightly between different GR target genes, but the specific GRE located in each gene is highly conserved between species (So *et al*., 2007).

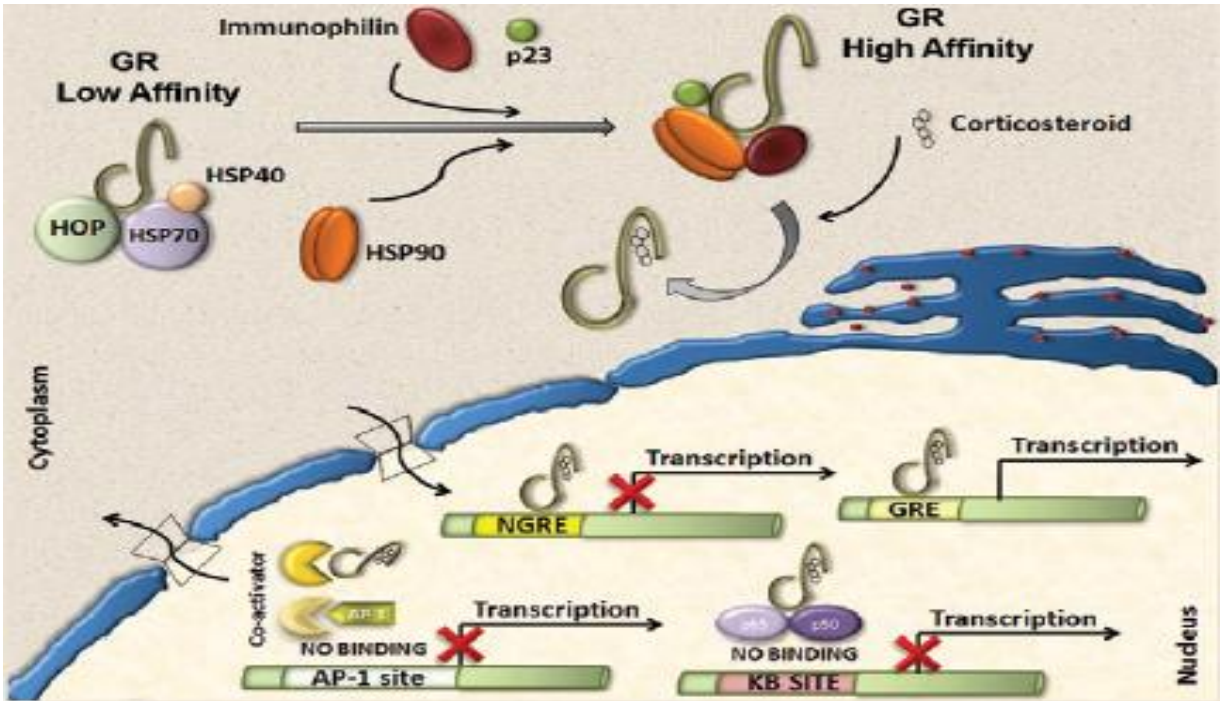


Figure 1.1.2: GR mechanism of action. Diverse mechanisms for GR transactivation or transrepression are demonstrated. HOP, heat shock protein 90/70 organizing protein; NGRE, negative glucocorticoid-response-element; KB, nuclear factor-kappa B; AP-1, activator protein-1. Taken from (Japiassu *et al*., 2009).

When bound to the DNA, activated GR serves as a platform for the recruitment of transcription factors and the basal transcription machinery to induce transcription (Ford *et al*., 1997). The NTD of ligand-activated GR has been shown to interact with the transcription factor II D (TFIID) and TATA-box binding protein (TBP), resulting in recruitment of chromatin-remodeling complexes (Ford *et al*., 1997). The GR has been shown to interact with brahma-related gene 1 (BRG1), a component of the human switch/sucrose non-fermentable (SWI/SNF), ATP-dependent chromatin-remodeling complex, which leads to relaxation of heterochromatin allowing access for the basal transcription machinery and transcription factors to the regulatory regions within the promoter (Fryer and Archer, 1998). Furthermore, the AF domains of the GR interact in a ligand-dependent manner with many co-factors involved in regulation of transcription (Gronemeyer *et al*., 2004), which in turn recruit additional co-factors such as cAMP response element binding protein (CREB)-binding protein (CBP), adenovirus E1A binding protein 300 (p300) and p300/CBP associated factor (p/CAF). These co-factors possess histone acetyltransferase (HAT) activity (McKenna *et al*., 1999). The acetylation of lysine residues within histone tails involves neutralization of the charges, resulting in dissociation of the DNA from the histones allowing binding of transcription factors and increasing the efficiency of transcription (Eberharter and Becker, 2002).

The most well known and extensively studied GR co-factors are members of the steroid receptor co-activator (SRC) family, also known as the p160 family. Members of this family include SRC-1 (also known as NcoA-1), SRC-2 (also known as GRIP-1 or TIF-2) and SRC-3 (also known as p/CIP, RAC3, ACTR or AIB1) (Carapeti *et al*., 1998; Ning *et al*., 1999; Anzick *et al*., 1997). The SRC family of proteins interact with steroid receptors in a ligand-dependent manner through a LXXLL (L = leucine, X = any amino acid) motif that is present within the nuclear receptor interaction domains (Heery *et al*., 1997; Voegel *et al*., 1998). The N-termini of the SRC proteins contain a basic-helix-loop-helix (bHLH) domain that has been shown to interact with the coiled-coil co-activator (CoCoA) (Kim *et al*., 2003), while the C-termini has been shown to recruit the arginine methyltransferase CARM1 as well as HATs like p300/CBP and p/CAF (Lee *et al*., 2002; Lee *et al*., 2005; Vottero *et al*., 2002). Furthermore, all the members of the SRC family of co-factors possess weak HAT activity themselves, resulting in decondensation of chromatin providing access on the DNA for additional co-factors (Chen *et al*., 1997; Goel and Janknecht,

2004; Spencer *et al*., 1997). It is clear that GC-induced transcriptional regulation of gene expression by the GR involves multiple mechanisms to achieve the desired responses. A few examples of well-studied genes that contain GREs in their promoters and are induced by GCs to result in transactivation by the GR are the tyrosine aminotransferase (TAT) and phosphoenolpyruvate carboxykinase (PEPCK) genes (Schmid *et al*., 1987; Hanson and Reshef, 1997).

In some promoters the activated GR can bind to an undefined non-consensus negative GRE (NGRE) resulting in transrepression, of which the osteocalcin gene is a good example (Meyer *et al*., 1997). Another well-studied example is the CRH gene that contains three NGRE half sites with an adjacent activator protein-1 (AP-1) site, of which both are required for GR-mediated repression in mouse corticotroph AtT-20 cells (Malkoski and Dorin, 1999; Drouin *et al*., 1998). Additionally, the GR can inhibit transcription by competing with transcription factors for DNA binding, which involves a GRE overlapping a transcription factor binding site in the promoter (Kassell and Herrlich, 2007; Schoneveld *et al*., 2004). The GR has also been shown to bind as a monomer to GRE half sites in the promoter of certain genes resulting in transactivation, such as the relaxin gene that contains multiple GRE half sites and encodes for the insulin-related peptide in HeLa and THP-1 cells (Dschietzig *et al*., 2009).

1.1.3.3.2 Tethering

The GR can regulate gene transcription without direct binding of DNA, but rather through a tethering mechanism and modulation of the activity of several transcription factors, such as AP-1, nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 5 (STAT-5) (De Bosscher and Haegeman, 2009; Stoecklin *et al*., 1997; Doppler *et al*., 2001). The effects of this crosstalk can either be positive or have a suppressive role on transcription (Kassel and Herrlich, 2007). A study by Heck *et al*. showed that the DBD of the GR is required for this modulation in transcription factor activity by a mechanism involving a direct physical interaction with AP-1 (Heck *et al*., 1994). The same study showed that homodimerization of the GR was not required for the repression of AP-1 regulated genes in CV-1 and COS-7 cells (Heck *et al*., 1994). Therefore, it appears that the activated GR functions as a GR monomer forming hetero-

complexes with other transcription factors to regulate transcription. Target genes that are negatively regulated by this tethering mechanism of the GR have been shown to involve transcription factors such as AP-1 and NF-κB, involved in regulating the pro-inflammatory response (De Bosscher and Haegeman, 2009). It has been reported that tethering of GR to AP-1 proteins does not alter the composition or decrease the binding of c-Jun and c-Fos to the AP-1 site in the promoter of the collegenase gene in U2-OS cells (Kassel *et al*., 2004; Rogatsky *et al*., 2001). Distinct domains of the NF-κB protein, namely p65, are required for binding of the GR and the resulting transrepression in COS-1 cells (Wissink *et al*., 1997). Only a few examples of transactivation by the GR through a tethering mechanism have been described, including an interaction with STAT-5 on the promoter of the β-casein gene in CV-1 and COS-7 cells (Stoecklin *et al*., 1997; Doppler *et al*., 2001), and c-Jun on the promoter of the α-2 macroglobulin gene in H35 cells (Lerner *et al*., 2003).

Both positive and negative effects have been reported for the tethering of GR to AP-1 proteins (De Bosscher and Haegeman, 2009). In successive chromatin immunoprecipitation (ChIP) experiments the GR and AP-1 were shown to be recruited to the same collegenase-1 promoter, resulting in transrepression in HeLa cells (Kassel *et al*., 2004). c-Fos was reported to be the major target of GR tethering in the inhibition of AP-1 target genes *in vitro* (Kerppola *et al*., 1993). In contrast to the inhibition of AP-1-regulated genes by the GR such as interleukin (IL) 6, 8 and the collegenase (Lerner *et al*., 2003; Kassell and Herrlich, 2007), some studies have reported an increase in transcription by tethering of the GR to an AP-1-containing promoter. Rani *et al*. showed that Dex treatment resulted in increased expression of the rat tyrosine hydrolase gene via an AP-1 site located in the promoter in PC12 cells (Rani *et al*., 2009). Dex also increased expression via an AP-1 site in the GnRHR gene in a GR-dependent manner by recruitment of GRIP-1, although not via a minimal AP-1-reporter gene, indicating that additional *cis*-elements in the GnRHR gene are required for the Dex-mediated increase in GnRHR gene expression in LβT2 cells (Kotitschke *et al*., 2009). Transactivation via the ligand-activated GR by tethering to AP-1 is also supported by Rogatsky *et al*. showing that GRIP-1, recruited by the ligand-activated GR, could activate or repress transcription via an AP-1 site, depending on the composition of the c-Jun/c-Fos dimer in U2-OS cells (Rogatsky *et al*., 2001 and 2002). Thus, transcriptional regulation by the GR through tethering to transcription factors involves

recruitment of co-factors, although the precise mechanisms that result in a positive or negative response remain to be determined.

1.1.4 Post-translational modifications

Several reports in the literature have shown that post-translational modifications of the GR (Figure 1.1.3) play an important role in modulating its biological function, including transcriptional regulation, protein-protein interactions, receptor degradation and sub-cellular localization (Faus and Haendler, 2006). The process of sumoylation is similar to ubiquitination and involves the covalent attachment of a small-ubiquitin-related modifier-1 (SUMO-1) peptide to lysine residues by the SUMO conjugase Ubc9 (Le Drean *et al*., 2002). Sumoylation of the GR appears to play a role in protein stability as over-expression of SUMO-1 enhanced GR degradation by the proteasome pathway (Tian *et al*., 2002). Three sumoylation sites (K277, K293 and K703) have been identified in the human GR and if mutated simultaneously can enhance Dex-mediated transcription of simple GRE promoters (2 X GRE), but not complex GRE promoters (4 X GRE with additional *cis*-elements) like that of the TAT and mouse mammary tumor virus (MMTV) promoters, respectively (Holmstrom *et al*., 2003).

Besides the well-described acetylation of lysine residues within histone tails, the acetylation of various transcription factors has been reported (Kouzarides, 2000). The lysine residues that serve as possible acetylation acceptor sites are conserved amongst related steroid receptors (Fu *et al*., 2003). An acetylation motif (KXKK/RXKK) in the DBD of the GR corresponding to amino acids 492 – 495 in the human GR has been reported. Ito *et al*. identified two residues, K495 and K496, within the DNA binding domain of the human GR that are acetylated after Dex-binding (Ito *et al*., 2006). They reported a decrease in the level of acetylated GR upon mutation of these two residues to alanine (Ito *et al*., 2006), although it did not result in a complete loss of acetylation detected, suggesting the presence of other acetylation sites not yet identified. They also established that acetylated GR is a substrate for histone deacetylase 2 (HDAC2) and deacetylation of the GR is required for its interaction with p65 and the resulting transrepression of inflammatory genes through a tethering mechanism (Ito *et al*., 2006). A previous study showed that the over-expression of p300 resulted in increased GR-mediated transactivation of the MMTV

promoter (Li *et al*., 2002). The same study showed that this mechanism involved GR binding to p300 with the increase in transcriptional activity dependent on the HAT activity of p300 (Li *et al*., 2002). However, a more recent study showed that over-expression of p300 in astrocytes decreased the transcriptional activity of GR on a simple GRE, while over-expression of CBP increased GR-mediated transactivation (Fonte *et al*., 2007). The results indicate that acetylation of the GR increases or decreases transcription of target genes in a cell- or promoter-specific manner. Taken together, the above findings suggest that the acetylation status of the GR plays an important role in regulating the protein interactions and transcriptional activity of the GR.

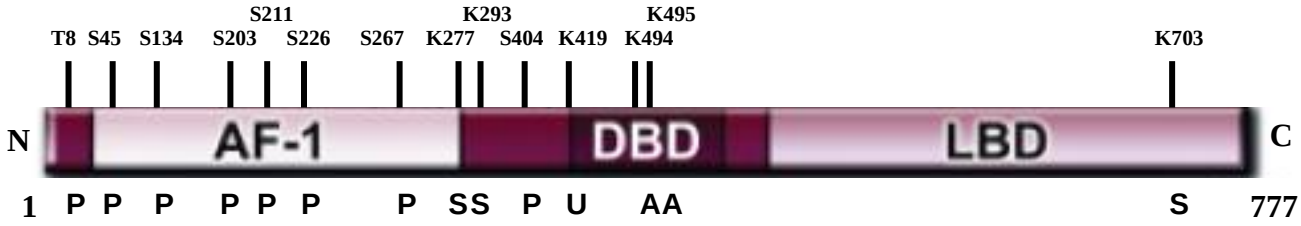


Figure 1.1.3: Post-translational modification of the human GR. P, phosphorylation; A, acetylation; S, sumoylation; U, ubiquitination. Taken and modified from (Galliher-Beckley and Cidlowski, 2009) and (Faus and Haendler, 2006).

Early findings have shown that the GR exists as a phospho-protein in intact cells and becomes hyperphosphorylated at multiple residues upon hormone treatment (Singh and Moudgil, 1985; Orti *et al*., 1989; Housley and Pratt, 1983). Eight phosphorylation sites that are highly conserved between species have been identified in the mouse GR, most of which are serine residues located in the NTD (Galliher-Beckley and Cidlowski, 2009). Interestingly, three of the residues namely S212, S220 and S234 (corresponding to S203, S211 and S226 in the human GR) located in the AF-1 domain of the mouse GR, become hyperphosphorylated with Dex treatment, suggesting that phosphorylation of the GR may function to regulate transcription of target genes (Wang *et al*., 2002). The functional significance of the phosphorylation sites in the mouse GR was investigated by individual or simultaneous mutations of the phosphorylation residues to alanine. These unphosphorylated mutant receptors were compared to the wild type receptor for the ability to transactivate a MMTV reporter gene. The results showed that the mutated receptors had a similar ability compared to the wild type receptor for activating a GRE in COS-1 cells (Mason

and Housley, 1993). More recent studies report an increase in the transcriptional activity of the mutated GR in HCT116 cells (Kino *et al*., 2007), while the combination of S212A, S220A and S234A corresponding mutations in the human GR decreased transactivation of a MMTV reporter gene in COS-1 cells (Avenant, 2009). These two findings suggest that phosphorylation of the GR regulates the transcriptional activity in a species- or cell-specific manner.

Other studies have shown that the phosphorylation status of the GR plays a critical role in regulating various other GR properties. For instance, it was shown that phosphorylation of the mouse GR at all the abovementioned phosphorylation sites affects the stability of the GR protein by abolishing the ligand-induced GR protein degradation (Webster *et al*., 1997). However, a more recent study showed that single or a combination of mutations of the phosphorylation sites in the human GR has no effect on the ligand-induced degradation of the GR (Avenant, 2009). Additionally, it was shown that the phosphorylation status of the GR controls protein-protein interactions with co-factors such as GRIP-1 (Avenant, 2009) and p300/CBP (Galliher-Beckley *et al*., 2008) to regulate the transcriptional activity of the GR.

Phosphorylation of the GR at a specific residue has been shown to affect the phosphorylation status at other sites, with an inverse relationship between the phosphorylation level at S203 and S226 of the human GR (Wang *et al*., 2007). The S203 phosphorylated GR is contained solely in the cytoplasm and the GRE-binding ability was abolished in the S203A mutant GR (Wang *et al*., 2002; Blind and Garabedian, 2008). The inhibition of the extracellular-regulated kinase 1/2 (ERK-1/2) reduces the level of GR phosphorylation at S203 and results in enhanced nuclear import with increased Dex-mediated regulation of GLUT5 mRNA expression, supporting a role for phosphorylation in regulating GR transcription (Takabe *et al*., 2008). Together, these results suggest that when the GR is phosphorylated at S203, the receptor localizes to the cytoplasm and is transcriptionally inactive, while the level of phosphorylation at S211 of the GR directly correlates with the transcriptional activity of the GR as measured by gene reporter assays (Wang *et al*., 2002). In support, the S211 phosphorylated GR has been shown to be recruited to many GRE promoters and is thus considered a marker for GR activation (Blind and Garabedian, 2008). Although GR phosphorylated at S211 localized to both the nucleus and the cytoplasm of cells, the majority of the GR phosphorylated at S211 was found in the nucleus (Wang *et al*., 2002). The

amount of GR localizing to the nucleus is directly proportional to the level of S211 phosphorylation (Chen *et al*., 2008), which has been shown to be mediated by the mitogen-activated protein kinase (MAPK) p38 in 3T3-L1 cells (Miller *et al*., 2005; Nader *et al*., 2010). Taken together, the ligand-induced S211 phosphorylation of the GR is associated with nuclear import and transcriptional regulation of target genes and can be considered as a marker for GR activation.

The human GR has been shown to be phosphorylated at S226 *in vitro* by the MAPKs, c-Jun N-terminal kinase (JNK) and cyclin-dependent kinase 5 (CDK5) in a cell-specific manner, resulting in the reduced transcriptional activity of a GRE reporter gene (Avenant *et al*., 2010a; Itoh *et al*., 2002; Kino *et al*., 2007; Rogatsky *et al*., 1998). The attenuation of GC signaling by JNK-mediated phosphorylation of the GR at S226 resulted from enhanced nuclear export of the GR (Itoh *et al*., 2002). However, a more recent study showed that the S226 phosphorylated GR strongly associated with two different endogenous GRE-containing promoters, the TAT and sulfonyl transferase 1A1 genes (Blind and Garabedian, 2008), suggesting it is still transcriptionally active. In another report, Galliher-Beckley *et al*. showed that phosphorylation at S404 of the human GR occurs in response to Dex and is mediated by glycogen synthase kinase 3β (GSK3β) in human osteosarcoma cells (Galliher-Beckley *et al*., 2008). Furthermore, constitutive phosphorylation at S404 decreased the stability of the GR protein and resulted in altered recruitment of co-factors (Galliher-Beckley *et al*., 2008). Taken together, the studies above suggest that the ligand-induced phosphorylation of the GR plays an important role in regulating GR transcription in a species-, cell- and promoter-specific manner.

1.1.5 Protein degradation

It has previously been shown that both GR protein and mRNA levels are down regulated in response to ligand (Okret *et al*., 1986; Dong *et al*., 1988; Hoeck *et al*., 1989; Burnstein *et al*., 1994). The decrease in GR protein expression is a mechanism that may limit cellular responsiveness to GCs. The down regulation of GR protein levels with ligand is similar to other steroid receptors and involves degradation by the proteasome-ubiquitination pathway (Sheflin *et al*., 2000; Alarid *et al*., 1999; Lange *et al*., 2000). The post-translational modification of a

protein by the covalent attachment of an ubiquitin protein is a process that involved three-steps. First, an E1 enzyme activates the 76-amino acid protein, ubiquitin, followed by transfer to a specific E3 ubiquitin ligase by E2 conjugating enzymes. Subsequently, the activated ubiquitin is covalently linked to lysine residues located within special degradation motifs of target proteins (Wallace and Cidlowski, 2001). As mentioned above, the human GR is ubiquitinated at K419, which is located in a PEST (amino acid sequence Pro (P) - Glu (E) - Ser (S) –Thr (T)) degradation motif (Hershko and Ciechanover, 1998; Dvorak *et al*., 2005). The importance of the E3 ubiquitin ligase CHIP (C-terminal of heat shock 70-interacting protein) for GR degradation was shown in HT22 hippocampal cells, which is naturally deficient in the expression of the CHIP E3 ligase. It was shown that over-expression of the CHIP E3 ligase in these cells restored the ligand-dependent degradation of the GR, resulting in reduced transactivation of a MMTV promoter (Wang and DeFranco, 2005). A recent study identified the calcium-activated cysteine protease, namely calpain, to be a protease involved in degradation of the ligand-activated GR (Kim *et al*., 2008).

An early study by Dong *et al*. showed that the unliganded GR is gradually degraded over time with a half life of about 25 h, while stimulation with Dex significantly reduced the half-life to about 11 h in rat hepatoma cells (Dong *et al*., 1988). Interestingly, Hoeck *et al*. showed the Dex-induced degradation of the GR to be a dose-dependent effect in NIH3T3 cells. Furthermore, the same study showed the unliganded GR having a half-life of about 8 h and that Dex stimulation decreased the half-life to about 3 h (Hoeck *et al*., 1989). The mutation of the ubiquitination site K419 in the PEST degradation motif of the human GR abolished the ligand-induced degradation, while increasing the transcriptional activity of the GR (Wallace *et al*., 2010). Furthermore, a few studies have shown proteasome inhibitors to have a positive effect on GR-mediated transactivation of various GRE promoters (Wallace and Cidlowski, 2001; Deroo *et al*., 2002; Garside *et al*., 2006). Taken together, the above studies suggest that the GR protein is targeted for degradation via the proteasome-ubiquitin pathway, while the ligand-induced degradation of the GR is a dose-dependent effect with the half-life varying in a cell-specific manner. The ligand-dependent degradation of the GR could be a mechanism to prevent over-stimulation of the GR signaling pathway.

1.1.6 Rapid glucocorticoid-mediated non-genomic actions

Since the early studies that investigated the mechanism of GC action, it became apparent that these steroid hormones could induce effects within a very short time (≤ 30 min) that occur in both *in vitro*, as well as *in vivo* systems. These rapid GC effects are not mediated by the delayed responses (hours) of the classical genomic model for steroid action, which involves nuclear translocation of the GR with regulation of gene expression and protein synthesis (Falkenstein *et al*., 2000). Rapid GC-mediated (non-genomic) effects are characterized by responses that do not directly influence gene expression initially, but result in the activation of signaling cascades that are not sensitive to protein synthesis inhibitors (Losel and Wehling, 2003). Therefore, the non-genomic actions of GCs suggest that the GR has an additional mechanism to regulate gene transcription, which involves the reciprocal modulation of various receptors and signaling pathways (Tasker *et al*., 2006).

Some of the non-genomic GC effects are mediated in a GR-independent manner through altering the physiochemical properties of the plasma- and mitochondrial membranes. These non-specific effects only occur at high concentrations of GCs, leading to the lipophillic ligands intercalating into the membranes, which affects the function of membrane-associated proteins (Buttgereit and Scheffold, 2002; Buttgereit *et al*., 2004). A few of these effects have previously been reported such as reduced transport of calcium (Ca^{+2}) and sodium across the plasma membranes of immune cells and increased proton leakage in the mitochondrial membrane resulting in reduced levels of cellular ATP (Buttgereit and Scheffold, 2002; Stahn *et al*., 2007). However, the *in vivo* relevance of these membrane effects of GCs remains doubtful as the effects required high concentrations of GC (> 10 μM) *in vitro*, which are above the physiological and therapeutic range (Buttgereit and Scheffold, 2002). Another study showed that treatment of human primary bronchial epithelial cells with physiological concentrations (0.1 – 1 μM) of Dex rapidly reduced the level of intra-cellular Ca^{+2} resulting in a decrease of ATP-induced secretion of Cl^-. Furthermore, experiments with specific antagonists suggested that the Dex-induced non-genomic mechanism was independent of the classical GR, but rather through activation of the protein kinase A (PKA) signaling pathway (Urbach *et al*., 2002). However, showing that the rapid GC signaling is insensitive to the GR/PR antagonist RU486, is not evidence that the classical GR is not involved.

This is supported by the study of Kotitschke *et al*. showing that the rapid GnRH-induced activation of the GR was insensitive to RU486, but the classical GR was involved as determined by siRNA-mediated knockdown experiments in LβT2 cells (Kotitschke *et al*., 2009). This is supported by studies showing that the GnRH-induced activation of a progesterone-response-element (PRE)-containing gene was insensitive to the PR antagonist RU486, but the PR was shown to be involved as determined by siRNA-mediated knockdown experiments in αT3-1 and LβT2 cells, respectively (An *et al*., 2006, 2009). Rapid GC effects can be mediated by the classical GR or a membrane-associated GR (Falkenstein *et al*., 2000; Buttgereit and Scheffold, 2002; Lowenberg *et al*., 2008) as described below.

1.1.6.1 Crosstalk of the classical GR with intracellular signaling pathways

The majority of rapid GC-induced effects appear to be mediated via a crosstalk mechanism between the classical GR and other signaling pathways. Croxtall *et al*. reported that Dex could inhibit the rapid epidermal growth factor (EGF)-stimulated activation of phospholipase A2 (PLA2) activation and subsequent release of arachidonic acid in A549 cells (Croxtall *et al*., 2000). Additionally, the Dex-induced decrease of phosphorylation was shown to be unaffected by actinomycin D, an inhibitor of transcription, while specific antagonists showed the mechanism required the GR, JNK and ERK-1 (Croxtall *et al*., 2000). Interestingly, the liganded GR was shown to directly interact with JNK, reducing its activity and the resulting phosphorylation of c-Jun (Caelles *et al*., 1997). Furthermore, GCs have been reported to rapidly activate p38 and JNK in PC12 cells and hippocampal neurons (Li *et al*., 2001; Qi *et al*., 2005). Taken together, these findings suggest that the GR signaling pathway can regulate transcription via a non-genomic crosstalk mechanism with members of the MAPK family.

In addition to the MAPK signaling pathways, the GR has been shown to crosstalk with the Akt (protein kinase B) kinase signaling pathway. A recent study reported rapid Dex-induced phosphorylation and activation of Akt by c-src tyrosine kinase (c-src) in a GR-dependent manner in A549 cells (Matthews *et al*., 2008). Dex has been shown to rapidly activate the endothelial nitric oxide synthase (eNOS) enzyme in a GR-dependent and transcription-independent manner

(Hafezi-Moghadam *et al*., 2002), which was significantly reduced with specific inhibitors of phosphatidyl inositol 3-kinase (PI3K) and eNOS. Activation of the PI3K signaling pathway with Dex results in the activation of Akt, which phosphorylates and activates eNOS (Dimmeler *et al*., 1999). Furthermore, a study by Solito *et al*. showed rapid GC-induced phosphorylation and membrane translocation of annexin-1, via a non-genomic GR-dependent mechanism involving the PI3K/Akt pathway and protein kinase C (PKC) in human folliculostellate cells (Solito *et al*., 2003). Thus, these findings suggest that the GR can crosstalk with the PI3K signaling pathway via a non-genomic mechanism in different cells, resulting in the rapid activation of Akt that modulates the activity of various signaling molecules.

Several studies have reported rapid GC effects can be mediated via the activation of various receptor tyrosine kinase (RTK) signaling pathways. As mentioned above, GCs have been shown to reduce the EGF stimulation of PLA2 activity by inhibiting the phosphorylation of MAPK/ERK kinase 1/2 (MEK1/2) in A549 cells (Malcher-Lopes *et al*., 2008). Another study showed GCs inhibiting insulin signaling in a GR-dependent non-genomic mechanism in T-lymphocytes and adipocytes (Lowenberg *et al*., 2006a). A few studies reported that the GR modulates T-cell signaling through a non-genomic crosstalk mechanism with members of the T-cell receptor (TCR) pathway (Lowenberg *et al*., 2005; Lowenberg *et al*., 2006b). The unliganded GR was shown to localize with the TCR at the plasma membrane in a protein complex that included HSP90, zeta-chain-associated protein kinase 70 (ZAP-70) and non-receptor tyrosine kinases, such as FYN kinase and leukocyte-specific protein tyrosine kinase (LCK). Short exposure of T-cells to Dex impaired signaling by disruption of the membrane protein complex resulting in reduced activity of LCK and FYN kinases (Lowenberg *et al*., 2006b). Furthermore, the inhibition of LCK leads to a decrease in the activity of inositol-1, 4, 5-triphosphate (IP3) receptors, attenuating the signaling of intracellular Ca^{+2} (Harr *et al*., 2009). In addition, Bartis *et al*. showed rapid Dex-induced GR-dependent tyrosine phosphorylation of ZAP-70 in Jurkat cells (Bartis *et al*., 2007). These studies indicate that by inhibiting LCK through disruption of the TCR protein complex with the subsequent down regulation of IP3 receptors, GCs are able to suppress immune function by decreasing the response of the TCR. Besides modulating MAPK and PI3K/Akt kinase pathways, the GR appears to also modulate RTK signaling pathways upstream of MAPKs, such as those of the EGF, insulin and T-cell receptors.

Several reports in the literature suggest GR crosstalk with G-protein-coupled receptors (GPCRs) and G-proteins via rapid-non-genomic mechanisms (Tasker *et al*., 2006). Iwasaki *et al*. suggested the involvement of G_i proteins in the rapid GC-mediated inhibition of ACTH secretion (Iwasaki *et al*., 1997). Interestingly, Kino *et al*. reported that the activated GR directly interacts with G_β proteins and co-migrates to the nucleus resulting in the repression of GR-mediated transactivation. In addition, the authors reported that stimulation of the somatostatin receptor, a GPCR, results in co-localization of the GR and G_β at the plasma membrane in HTC116 cells (Kino *et al*., 2005). A recent study showed the involvement of a G_s-coupled receptor in the GC-induced synthesis and release of encannabinoids from neuroendocrine cells (Malcher-Lopes *et al*., 2008). The reports described above indicate that the association of the GR with G-proteins could explain some of the rapid GC-mediated effects. A recent study performed by Kotitschke *et al*. demonstrated a crosstalk mechanism between the GR and the GnRHR that requires both receptors. The authors showed that GnRH rapidly induced site-specific phosphorylation at S234 of the unliganded mouse GR, resulting in nuclear translocation and transactivation of a GRE reporter gene in LβT2 cells (Kotitschke *et al*., 2009). Furthermore, co-stimulation of the cells with Dex and GnRH resulted in a synergistic transcriptional activity of the GRE reporter gene (Kotitschke *et al*., 2009).

1.1.6.2 Crosstalk of membrane-associated GR with intracellular signaling pathways

It is possible that rapid non-genomic GC-induced effects are mediated via classical or novel GRs associated with the plasma membrane (mGR). A novel mGR may have several distinct differences from the classical receptor (Falkenstein *et al*., 2000; Levin, 2008). The existence of a 63-kDa mGR was shown for the first time in amphibian neuronal membranes (Gametchu *et al*., 1993), followed by identification in rat lymphoma and human leukemia cells (Gametchu *et al*., 1999). Several studies have reported the mGR to be distinct from the classical cytoplasmic GR in several ways, including cell localization, molecular size and ligand-binding characteristics. However, the mGR has also been reported to have certain similarities with the cytoplasmic GR such as shared epitope recognition for antibodies, phosphorylation status and interactions with heat shock proteins (Gametchu *et al*., 1999; Powell *et al*., 1999). Furthermore, Barthalome *et al*.

showed the presence of a mGR, which was similar to the classical GR, in primary human monocytes and B cells with a high-sensitivity immunofluorescent staining technique (Barthalome *et al*., 2004). However, further work is required, such as cloning and functional domain analysis, to characterize and prove the identity of a mGR. Furthermore, whether such a cloned and expressed receptor binds to GCs and can mediate a non-genomic GC response remains to be determined.

As mentioned above, rapid GC effects may be mediated via the classical GR acting at the plasma membrane. There have been several reports in the literature suggesting that the GR associates with lipid rafts, which are specialized plasma membrane microdomains that recruit various signaling proteins involved in coordinating the cellular response. Jain *et al*. showed that the classical GR localizes with HSP90 and STAT3 in caveolin-1-containing membrane microdomains, known as caveolae, in human liver Hep3B cells (Jain *et al*., 2005). This study further provided evidence for a functional role of the membrane-associated GR in Dex-mediated transcription, since Dex-induced GRE transactivation was significantly repressed in the presence of a lipid raft disrupter (Jain *et al*., 2005). A more recent study showed the unliganded GR localizing with c-src to caveolae to facilitate the rapid Dex-induced phosphorylation of Akt and caveolin-1 in A549 cells. Furthermore, the same study showed the loss of Dex-induced phosphorylation of Akt and caveolin-1 in the presence of a lipid raft disruptor, while the knockdown of caveolin-1 protein reduced the Dex-induced activation of Akt, but had no effect on GRE transactivation (Matthews *et al*., 2008). Taken together, since the existence of a novel mGR remains inconclusive, this suggests that the rapid non-genomic effects of GCs are most likely to be mediated via the classical GR localizing at the plasma membrane. The membrane-associated appears to crosstalk with several intracellular signaling pathways via a mechanism involving its association with a multi-protein signaling complex in membrane microdomains.

1.2 Gonadotropin-releasing hormone receptor

The hypothalamic neurohormone, gonadotropin-releasing hormone (GnRH), is released in a pulsatile manner and acts through a specific GPCR, the GnRHR, to activate a variety of intracellular signaling cascades regulating the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from gonadotrope cells in the anterior pituitary (Fink, 1988). The FSH and LH multi-subunit proteins form heterodimers and their synthesis requires the expression of three genes, *Cga, Lhb and Fshb*. *Cga* encodes an α-subunit common to both hormones, while *Lhb* and *Fshb* encode β-subunits unique to each hormone (Gharib *et al*., 1990; Jorgensen *et al*., 2004). Once LH and FSH are secreted from the gonadotropes, they bind to distinct GPCRs in both male and female gonads stimulating gametogenesis and steroidogenesis (Segaloff and Ascoli, 1993; Simoni *et al*., 1997).

The pituitary responsiveness to GnRH is dependent on the GnRHR numbers expressed on the cell surface of the gonadotropes (Kaiser *et al*., 1993; Norwitz *et al*., 1999). GnRH has been identified as a positive regulator of GnRHR gene expression *in vivo* and *in vitro* (Kaiser *et al*., 1993; Young *et al*., 1984; Wu *et al*., 1994), thereby generating a feedback mechanism controlling reproduction. At least two forms of the decapeptide hormone (GnRH I and GnRH II), as well as the receptor (GnRHR I and GnRHR II), have been found in most vertebrates, including mammals (Millar, 2003; Neill *et al*., 2004; Pawson *et al*., 2003). However, both GnRH peptides can bind to and activate both receptors, while GnRH I has a greater affinity for GnRHR I and vice versa (Millar *et al*., 2001). The regulation of GnRHR expression has been shown to occur at the transcriptional, translational and post-translational level, but the underlying molecular mechanisms involved have not been fully investigated (Hapgood *et al*., 2005; Ciechanowska *et al*., 2010; Naor, 2009).

The amplification of the genomic DNA from several different vertebrates with a highly conserved domain of the GnRHR identified novel GnRHR sequences. The type II receptor was found in many mammalian and non-mammalian species, while this approach also identified type III receptors in several amphibian species (Troskie *et al*., 1998; Millar *et al*., 2004). The existence of several other GnRHRs suggests an early evolution of the three GnRH and GnRHR subtypes in

vertebrates. However, a full-length functional transcript of type II receptor has not been found in human or mouse cells, although it is present in monkey cells (Ronacher *et al*., 2004; Millar *et al*., 2001; Hapgood *et al*., 2005). The absence of the full-length GnRHR type II transcript results from the presence of a frame-shift and premature stop codon in the human and mouse type II receptor genes (van Biljon *et al*., 2002; Faurholm *et al*., 2001; Morgan *et al*., 2003; Gault *et al*., 2004). It is now becoming apparent that the vast effects of GnRH I and II are both mediated by the human type I GnRHR via coupling to different signaling pathways (Hawes *et al*., 1993).

1.2.1 Physiological function

The physiological importance of the GnRHR in mammalian reproduction is evident from the findings that several naturally-occurring mutations in the human receptor result in the disease named hypogonadotropic hypogonadism, with symptoms of delayed sexual development and low gonadotropin and steroid hormone levels (Seminara *et al*., 1998; Millar *et al*., 2004). The majority of these mutated GnRHR are incorrectly folded proteins that are retained in the endoplasmic reticulum or display attenuated trafficking to plasma membrane, which together result in low levels of functional GnRHR (Knollmann *et al*., 2005; Brothers *et al*., 2004). GnRHR agonists and antagonists have been extensively used in clinical treatments for fertility-related disorders, various reproductive diseases and hormone-dependent cancers (Conn and Crowley, 1994; Neill, 2002).

In addition to its important function in regulating gonadotropin expression in the pituitary, there is convincing evidence in the literature showing the expression of GnRHR I in female reproductive tissues, including the breast, ovary, endometrium and the placenta (Hapgood *et al*., 2005; Cheng and Leung, 2005). In these tissues, GnRH-activation of GnRHR I plays a role in the establishment and maintenance of pregnancy, regulation of the menstrual cycle, regulation of breast and ovary development and inhibition of steroidogenesis in the ovary (Kottler *et al*., 1997; Kogo *et al*., 1999; Raga *et al*., 1998; Guerrero *et al*., 1993). Several studies have shown the expression of GnRH and GnRHR I in the human testis and prostate, where they play a role in regulating testicular development, sperm motility and sperm-oocyte interactions (van Biljon *et al*., 2002; Kakar *et al*., 1992; Cheung and Hearn, 2003; Morales, 1998).

Besides the wide distribution of the GnRHR in the pituitary and reproductive systems, the receptor has been found in other cell types. Hypothalamic GnRH neurons have been found to express GnRHR I, while both GnRHR I and II has been detected in many mammalian brain tissues, supporting a role for GnRH as a neurotransmitter (Hapgood *et al*., 2005; Martinez-Fuentes *et al*., 2004). In addition, a few studies have indicated a role for GnRH in regulating immune responses. From this point onwards, the type I GnRHR will be referred to as the GnRHR. The expression of GnRH and GnRHR has been detected in T-cells, where GnRH was shown to stimulate the adhesion, migration and homing of T-cells into specific organs (Chen *et al*., 2002; Jacobson *et al*., 1998). The expression of functional GnRHR has been detected in a wide range of carcinomas originating from the endometrium, ovary and breast tissues (Imai *et al*., 1994; Imai and Tamaya, 2000; Limonta *et al*., 2003). It is well known that continuous administration of GnRH analogues can inhibit proliferation of human malignant tumors (Cheng and Leung, 2005; Grundker *et al*., 2002). Another study found GnRH analogs to have anti-proliferative effects in human melanoma cells, suggesting that the GnRHR-mediated inhibition of growth effects are not unique to reproductive tissue carcinomas (Limonta *et al*., 2003). The anti-tumor effects appear to be mediated via inhibition of gonadal steroids and modulation of growth factor expression (Cheng and Leung, 2005). However, the signaling pathways and mechanisms mediating the anti-proliferative effects of GnRH are unknown and further work is required (Pawson *et al*., 2003). Taken together, the above findings indicate that the GnRHR is expressed in a wide variety of mammalian tissues and appears to be involved in a wide range of functions, including proliferation of several types of carcinomas by modulating growth factor expression and steroid synthesis in the gonads.

1.2.2 Protein structure

The GnRHR was first identified in the mouse by cloning the receptor from the pituitary αT3-1 gonadotrope cell line (Tsutsumi *et al*., 1992), which was confirmed by another study (Reinhart *et al*., 1992). Subsequently, the GnRHR sequence was cloned from pituitary tissues of various mammalian species including human (Chi *et al*., 1993; Kakar *et al*., 1992), rat (Kaiser *et al*., 1992; Eidne *et al*., 1992), sheep (Illing *et al*., 1993; Brooks *et al*., 1993) and pig (Weesner *et al*., 1994). In contrast to the genes of many other members of the GPCR family, which are without

introns, the human and mouse GnRHR genes span more than 15 kb of DNA and contain two introns (Fan *et al*., 1994; Zhou and Sealfon, 1994). The introns are located at similar regions in the human and mouse GnRHR genes, although they appear to vary in size. Both the human and the mouse genome only have a single copy of the GnRHR gene, as determined by Southern blot analysis (Zhou and Sealfon, 1994).

The mammalian GnRHRs share a conserved amino acid sequence with over 80% homology across species (Stojilkovic *et al*., 1994). Homologs of the mammalian GnRHR have also been found in various other non-mammalian species, sharing 42 – 47% amino acid sequence identity with mammalian receptors and 58 – 67 % homology among each other (Millar *et al*., 1994; Troskie *et al*., 1998). The GnRHR belongs to the rhodopsin-like GPCR superfamily and consists of a single polypeptide chain of 327 or 328 amino acids for the mouse and the human GnRHR, respectively (Sealfon *et al*., 1997). The mammalian GnRHR consist of a N-terminal domain and seven transmembrane helix (TM) domains, which are connected by three extracellular loop domains and three intracellular loop domains. A unique feature of the mammalian type I GnRHR is the absence of a carboxyl-terminal tail, which is present in all other GPCRs and in type II GnRHR (Millar *et al*., 2004; Sealfon *et al*., 1997). The extracellular domains are involved in the binding of ligands and the TM domains are believed to be involved in conformational changes associated with signal propagation of the activated receptor. These conformational changes are thought to be crucial for the receptor to interact with G-proteins and other proteins involved in signal transduction (Millar *et al*., 2004).

1.2.3 Signaling pathways

When GnRH binds to its receptor in the plasma membrane of gonadotrope cells, it induces a conformational change in the receptor that stimulates the activation of heterotrimeric G-proteins. GnRHR may couple to multiple G-proteins to achieve the various biological effects of GnRH (Stanislaus *et al*., 1998; Liu *et al*., 2003). The members of the $G\alpha_{q/11}$ protein family are the main group of G-proteins that are activated by GnRH in a GnRHR-dependent manner, while the activation of $G\alpha_{i/o}$ and $G\alpha_{s}$ has also been reported (Naor, 2009). However, the activation of $G\alpha_{i/o}$ and $G\alpha_{s}$ proteins by the GnRHR was not found in all experimental systems, suggesting that the

nature of G-protein coupling to the GnRHR depends largely on the cellular context (Ruf *et al*., 2003). Two immortalized mouse pituitary gonadotrope cell lines have mainly been used to investigate GnRHR signaling. These are αT3-1 cells, that represent precursor gonadotrope cells and do not express the β-subunit of gonadotropins, and LβT2 cells that represent more mature gonadotrope cells expressing fully functional gonadotropin mRNA and proteins (Mellon *et al*., 1990; Turgeon *et al*., 1996). The GnRHR has been shown to couple to $G\alpha_{q/11}$ in αT3-1 cells, while both $G\alpha_{q/11}$ and $G\alpha_{s}$ can couple to the receptor in LβT2 cells (Grosse *et al*., 2000; Liu *et al*., 2002a). In primary pituitary cultures, GnRHR can couple to several different G-proteins, including G_{i}, G_{q11} and G_{s} (Hawes *et al*., 1992; Stanislaus *et al*., 1998; Stanislaus *et al*., 1997). In addition, the GnRHR can couple to different G-proteins, as would potentially be required from the significant variation in the expression levels of G-proteins in the pituitary during the different phases of the reproduction cycle (Bouvier *et al*., 1991).

The GnRHR activates many intracellular signaling pathways, including phospholipase A_2, Cβ and D, which result in the release of various secondary signaling molecules such as IP3, diacylglycerol and arachidonic acid (Ando *et al*., 2001; Kraus *et al*., 2001). The release of IP3 mobilizes intracellular Ca^{+2} stores and induces Ca^{+2}-influx by voltage-gated ion channels, which activates PKC isoforms expressed in gonadotrope cells (Liu *et al*., 2002b; Kraus *et al*., 2001). In addition, GnRH-stimulation was shown to increase intracellular cAMP levels via a Ca^{+2}-independent mechanism involving the activation of adenylyl cyclase 5 and 7 by PKC in LβT2 cells (Lariviere *et al*., 2007). Furthermore, GnRH has been reported to activate all four of the known mammalian MAPK cascades, namely JNK, p38, ERK-1/2 and the big MAPK (BMK1/ERK-5) in pituitary gonadotrope cell lines (Naor *et al*., 2000; Kraus *et al*., 2001). Additionally, GnRH activates all of the MAPKs in both αT3-1 and LβT2 cells in a PKC-dependent manner (Bonfil *et al*., 2004; Roberson *et al*., 1999, Liu *et al*., 2002b; Liu *et al*., 2003; Mulvaney *et al*., 1999; Sundaresan *et al*., 1996). PI3K and c-src have also been reported to be activated in a GnRHR-dependent manner and to be involved in the regulation of the FSHβ gene in LβT2 cells (Bonfil *et al*., 2004; Kanasaki *et al*., 2006). Taken together, the above results suggest that the GnRHR can couple to and activate various G-proteins leading to the activation of many different intracellular signaling pathways, such as MAPKs and other protein kinases, to regulate mammalian reproduction.

1.2.4 Crosstalk with other receptor signaling pathways

The GnRHR can crosstalk with several other receptor signaling pathways as a mechanism for generating signaling diversity. Crosstalk of GnRH with the EGF receptor (EGFR) has been reported in the literature, with studies reporting the GnRH-induced transactivation or inhibition of the EGFR (Cheung and Wong, 2008). The negative crosstalk of GnRH with the EGFR appears to be accountable for the anti-proliferative effects of GnRH on various reproductive tumor cells (Gunthert *et al*., 2005; Yates *et al*., 2005; Eicke *et al*., 2006; Moretti *et al*., 1996; Grundker *et al*., 2001). The mechanism appears to be mediated by the GnRHR via activation of a tyrosine-phosphatase, which reduces the EGF-induced autophosphorylation of the EGFR (Moretti *et al*., 1996; Grundker *et al*., 2001; Lamharzi *et al*., 1998a). Similarly, GnRH inhibits expression of the insulin-like growth factor receptor, tyrosine phosphorylation of the receptor and the subsequent downstream activation of the PI3K/Akt pathway (Marelli *et al*., 1999; Lamharzi *et al*., 1998b; Montagnami Marelli *et al*., 2007). In addition to its inhibitory role in mitogenic signaling, the GnRHR has also been shown to crosstalk with several non-receptor tyrosine kinases such as focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2), suggesting a role for the GnRHR in regulating integrin signaling and cytoskeletal reorganization (Choi *et al*., 2006).

The GnRHR has also been shown to modulate NF-κB signaling. A study by Sakamoto *et al*. showed that GnRH reduced the expression of IL-8 through attenuating the tumor necrosis factor α (TNFα)-induced signaling of NF-κB in endometriotic stromal cells (Sakamoto *et al*., 2003). A more recent study showed GnRH stimulates the phosphorylation of p65 to induce expression of the cyclooxygenase-2 gene by binding to composite NF-κB elements in the promoter in LβT2 cells (Naidich *et al*., 2010). These findings suggest cell-specific differences in the modulation of NF-κB signaling by the GnRHR. In a recent study, Naor *et al*. demonstrated a reciprocal genomic crosstalk mechanism between the GnRHR and prostaglandin (PG) receptors in LβT2 cells (Naor *et al*., 2007). The authors showed that GnRH stimulates PG synthesis through induction of the cyclooxygenase-2 gene, which in turn inhibited the GnRH-induced expression of the GnRHR and LH genes (Naor *et al*., 2007).

The GnRHR has also been reported to crosstalk with several steroid hormone receptors. GnRH was shown to induce nuclear translocation of the AR by a mechanism involving c-src, however the receptor was not transcriptionally active (Maudsley *et al*., 2006). In addition, GnRH was shown to activate the PR through PKC-dependent phosphorylation in a ligand-independent manner, resulting in induction of PR-responsive genes, such as glycoprotein hormone α-subunit and FSHβ in αT3-1 and LβT2 cells, respectively (An *et al*., 2006; An *et al*., 2009). A recent study by Chen *et al*. demonstrated that GnRH stimulates the ligand-independent activation of the estrogen receptor by phosphorylation in a GnRHR-dependent manner to induce transactivation of ER-responsive genes, such as FosB, in LβT2 cells (Chen *et al*., 2009). Kotitschke *et al*. demonstrated a reciprocal crosstalk mechanism between the GnRHR and the GR (Kotitschke *et al*., 2009). The authors showed that GnRH induced the rapid phosphorylation and activation of the GR resulting in transactivation of a transient GRE reporter gene or the induction of the endogenous GnRHR gene in a GR- and GnRHR-dependent manner. In addition, co-stimulation with Dex and GnRH resulted in a synergistic transcriptional response on both the transient GRE reporter and the endogenous GnRHR gene (Kotitschke *et al*., 2009). It is important to note that the underlying mechanisms involved for GnRHR crosstalk with other signalling pathways are not fully understood. There is no report in literature indicating a direct reciprocal modulation of GnRHR activity with any other receptors, while most of the above-mentioned effects of GnRH appear to be mediated via downstream signalling molecules, such as kinases or transcription factors. An important question that remains to be determined is if the mechanism of GnRHR crosstalk with other receptor signalling pathways could be mediated by co-localization and perhaps even an interaction of these receptors. In summary, the above results suggest that the GnRHR can crosstalk with several signalling pathways, including tyrosine kinases, MAPKs, transcription factors and steroid receptors, resulting in transcriptional regulation of target genes in the pituitary.

1.3 Lipid rafts

1.3.1 Properties and structure

The plasma membrane of eukaryotic cells defines the cell boundary and is involved in transport, cell signaling, cellular contact and many other cellular events to maintain their physiological state. For a long time, the fluid mosaic model, postulated by Singer and Nicholson in 1972, has provided the foundation for the understanding of the structure of cellular membranes (Singer and Nicholson, 1972). In this model, membranes are described as a sea of lipids that exists in a liquid-disordered state with membrane proteins randomly associated within the membrane. However, numerous studies over the past 15 years have indicated that it is possible for liquid-ordered domains to exist that allow the organization of proteins within membranes (Brown and London, 1998; Pike, 2006). These ordered domains in membranes are called lipid rafts, which are localized regions of elevated cellular cholesterol and glycosphingolipid content. The fatty acid side-chains of the phospholipids in lipid rafts tend to be more saturated than those in the surrounding membrane, allowing the close packing of the lipids leading to phase separation (Lingwood *et al*., 2009). Furthermore, the high cholesterol content decreases the membrane fluidity, which further promotes the phase separation and results in the formation of lipid rafts (Pike, 2006). Simons and Ikonen have formulated a hypothesis regarding lipid rafts postulating the existence of lateral assemblies of glycosphingolipids and cholesterol, which associate with specific proteins while excluding others. The differential separation and co-localization of membrane proteins to a distinct phase of the cell membrane is the fundamental principle by which lipid rafts are thought to exert their physiological function (Simons and Ikonen, 1997).

The first definition of lipid rafts was suggested by Brown and Rose, who reported that domains enriched in glycosylphosphatidylinositol (GPI)-anchored proteins and spingholipids from cellular membranes were insoluble in Triton X-100 and floated to a characteristic density with equilibrium density gradient centrifugation (Brown and Rose, 1992). Together with the observation that this detergent-resistance was dependent on cholesterol and mainly enriched for constituents of the liquid-ordered phase, these preparations, known as detergent-resistant

membranes (DRM), became the method for assigning the affinity of various proteins for lipid rafts (Schroeder *et al*., 1994a). The presence of lipid rafts in intact cell membranes is supported by several lines of evidence, including studies involving fluorescence resonance energy transfer (FRET), single-particle tracking, photonic force microscopy and the biochemical cross-linking of lipid raft proteins (Simons and Toomre, 2000; Pike, 2006; Brown and London, 1997; Mayor and Rao, 2004; Pike, 2004). A few recent studies have suggested that lipid rafts are small structures (10 – 200 nm), while rafts have also been reported to coalesce into larger platforms through protein-protein and protein-lipid interactions (Kusumi *et al*., 2004; Pike, 2006; Mayor and Rao, 2004).

1.3.1.1 Caveolae

Caveolae are characterized by flask-shaped invaginations of the plasma membrane that comprise a special subpopulation of lipid rafts and can be distinguished by the presence of caveolin-1 (Figure 1.3.1) (Harder and Simons, 1997). They were first identified based on their morphology in mouse gall bladder epithelial cells by electron microscopy over 50 years ago (Yamada, 1955). Caveolae are found in many different types of cells, but they are most numerous in well-differentiated cells, such as smooth-muscle cells, fibroblasts, endothelial cells and adipocytes (Volonte *et al*., 1999; Lisanti *et al*., 1995; Parton, 1996). Caveolin proteins (21 – 24 kDa) are the fundamental components of caveolae and there are three known proteins, caveolin-1 (Cav-1) with splice variants α and β, Cav-2 and Cav-3 (Tang *et al*., 1996; Scherer *et al*., 1996; Glenney, 1992). Human Cav-1 and Cav-2 have overlapping expression patterns in a variety of cell types, including endothelial, epithelial, glia and neurons (Galbiati *et al*., 1998a; Ikezu *et al*., 1998; Lisanti *et al*., 1994; Vogel *et al*., 1998), whereas Cav-3 is found in skeletal and smooth muscle cells (Tang *et al*., 1996; Way and Parton, 1995). Cav-1 has been shown to bind cholesterol, which is required for attaining a proper morphology and cellular function (Murata *et al*., 1995). This sequestration of cholesterol by Cav-1, together with the polymerization of caveolin proteins, which are hairpin-like palmitoylated integral membrane proteins, drives the invagination of caveolae into the cell membrane (Cohen *et al*., 2004). The discovery that caveolin proteins are highly enriched in DRMs led to the interpretation that caveolae and lipid rafts are equivalent membrane structures. However, lipid rafts could also be isolated from cells that do not have

caveolae (Fra *et al*., 1994; Gorodinsky *et al*., 1995). In addition, caveolae from isolated membrane fractions can be separated from the bulk lipid raft-associated GPI-anchored proteins (Schnitzer *et al*., 1995). These findings indicated that lipid rafts are distinct from caveolae and could exist inside or outside caveolae (Parton and Simons, 1995).

It was previously shown that the over-expression of Cav-1 in cells lacking caveolae, such as lymphocytes, was sufficient to induce the formation of caveolae (Fra *et al*., 1995). Furthermore, the knockdown of Cav-1 and Cav-3 proteins results in the loss of caveolae formation in the specific cell type in which they are expressed (Galbiati *et al*., 2001; Razani *et al*., 2001; Cohen *et al*., 2004). In contrast, the knockdown of Cav-2 has no affect on caveolae formation *in vivo* (Razani *et al*., 2002), but it might contribute to stabilization of caveolae through oligomerization with Cav-1 in certain cell types (Lahtinen *et al*., 2003; Sowa *et al*., 2003). In summary, caveolae are a sub-population of lipid rafts that form omega-shape invaginations in the plasma membrane and are stabilized by caveolin proteins. The structure of caveolae provides a functional domain where specific proteins can associate for efficient activation of signaling pathways at the plasma membrane.

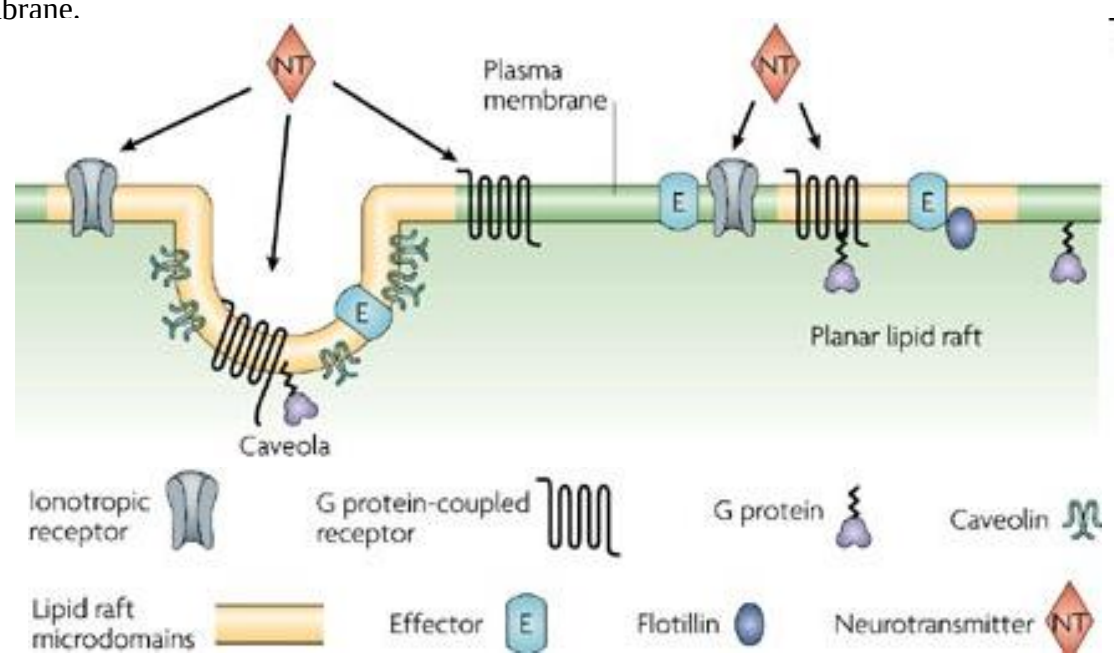


Figure 1.3.1: Plasma membrane organization of caveolae and lipid rafts. Several commonly associated molecules and proteins are shown, as well as the regions considered lipid raft microdomains, which are present in a more liquid-ordered state as compared to the bulk plasma membrane. Taken from (Allen *et al*., 2007).

1.3.2 Physiological function

1.3.2.1 Cell signaling

A great variety of proteins, especially those involved in cell signaling, have been shown to associate with lipid rafts via different mechanisms (Simons and Toomre, 2000; Pike, 2004; Zajchowski and Robbins, 2002). The mechanisms that dictate the lipid raft association of proteins are variable and not well understood. For some membrane proteins, lipid raft association is mediated via the transmembrane domain (Scheiffele *et al*., 1997), while other proteins can associate with lipid rafts through direct binding of cholesterol (Murata *et al*., 1995). However, the majority of proteins, including GPI-anchored proteins, members of the src kinase family and eNOS, associate with lipid rafts through the modification of reversible lipid modifications (Shenoy-Scaria *et al*., 1994; Shaul *et al*., 1996). Some membrane receptors have a weak affinity for lipid rafts in the unliganded state. After binding to a ligand, some membrane receptors changes conformation and oligomerize, which increases the affinity of association with lipid rafts (Harder *et al*., 1998). Taken together, the above findings suggest that a variety of mechanisms is employed to regulate the localization of proteins to lipid rafts.

Many RTKs, including the EGFR, insulin receptor and the platelet-derived growth factor (PDGF) receptor, have been shown to localize to lipid rafts (Mineo *et al*., 1996, Gustavsson *et al*., 1999; Liu *et al*., 1996; Wu *et al*., 1997). The effect of ligand binding on this association of RTKs with lipid rafts is highly variable (summarized in Table 1.3.1). The unliganded EGFR exists in lipid rafts and partially moves out of lipid rafts upon agonist-binding and activation (Mineo *et al*., 1999), a feature that is unique among RTKs. Consistent with its movement out of lipid rafts, the autophosphorylated EGFR localizes to intra-cellular compartments (Wang *et al*., 2009; Balbis *et al*., 2007). Interestingly, Puri *et al*. showed that several endocytic proteins are recruited to lipid rafts containing the activated EGFR (Puri *et al*., 2005). Therefore, lipid rafts have the ability to assemble both a signaling complex for the EGFR and proteins required for receptor internalization (Balbis *et al*., 2007). The insulin receptor is constitutively localized to caveolae, while ligand-binding is required for the receptor to localize to lipid rafts in cells that lack caveolae (Vainio *et al*., 2002). Furthermore, the constitutive localization of the PDGF and nerve

growth factor (NGF) receptors to lipid rafts appears to be unaffected by ligands (Huang *et al*., 1999; Liu *et al*., 1996). Interestingly, PDGF and NGF receptors were shown to be autophosphorylated within lipid rafts after hormone treatment, which is required for these receptors to associate with their downstream signaling cascades (Liu *et al*., 1996; Huang *et al*., 1999; Liu *et al*., 1997).

Table 1.3.1: Effects of agonist-binding on receptor localization

Receptor	Moves into lipid rafts	Moves out of lipid rafts	Unaffected	Reference
Receptor tyrosine kinase				
EGF		X		Mineo *et al*., 1999
Insulin	X		X	Gustavsson *et al*., 1999 Vainio *et al*., 2002
NGF			X	Liu *et al*., 1996
G-protein-coupled receptor				
GnRH			X	Navratil *et al*., 2003
β_2-adrenergic		X		Rybin *et al*., 2000
Endothelin			X	Chun *et al*., 1994
Rhodopsin			X	Seno *et al*., 2001
Angiotensin II type I	X			Ishizaka *et al*., 1998

A large number of GPCRs have been shown to be enriched in lipid rafts, including the type I GnRHR, β1 and β2-adrenergic receptors, acetylcholine receptor, muscarinic cholinergic receptor, rhodopsin receptor, angiotensin II type I receptor and endothelin receptor (Navratil *et al*., 2003; Rybin *et al*., 2000; Xiang *et al*., 2002; Chun *et al*., 1994; Feron *et al*., 1997; Seno *et al*., 2001; Ishizaka *et al*., 1998; Dessy *et al*., 2000). Like the RTKs, the localization of GPCRs to lipid rafts appears to be modulated by ligand (summary in Table 1.3.1). The unliganded β-adrenergic receptor is present in lipid rafts and moves out upon ligand treatment, while the acetylcholine, muscarinic cholinergic and angiotensin II type I receptors are targeted to lipid rafts upon activation with ligand (Ishizaka *et al*., 1998; Feron *et al*., 1997; Dessy *et al*., 2000). In contrast, the GnRHR, endothelin receptor and rhodopsin receptor are constitutively localized to lipid rafts, and this localization is unaffected by ligand (Navratil *et al*., 2003; Chun *et al*., 1994). In addition,

various G-proteins have been reported to localize to lipid rafts through a mechanism involving lipid modifications (Seno *et al*., 2001; Pike and Miller, 1998; Huang *et al*., 1997; Li *et al*., 1995; Lisanti *et al*., 1994; Moffett *et al*., 2000).

In addition to receptor localization with lipid rafts in the presence of ligand, many downstream signaling molecules can be recruited to the activated receptor in lipid rafts. For instance, stimulation of Rat1 cells with EGF resulted in the recruitment of raf-1 to lipid rafts within 30 seconds (Mineo *et al*., 1996). Raf-1 recruited to lipid rafts is activated through phosphorylation of the lipid raft-associated protein Ras, which results in activation of MEK1/2 and the subsequent MAPK pathways (Zhong *et al*., 2001; Simons and Toomre, 2000). Furthermore, several different types of adenylate cyclase, including type III, IV, V and VI, have been found to localize to lipid rafts (Rybin *et al*., 2000; Ostrom *et al*., 2001; Ostrom *et al*., 2000; Ostrom *et al*., 2002). Several other types of signaling molecules have also been found in lipid rafts, including src family kinases, the phosphotyrosine phosphatase syp, growth factor receptor-bound protein 2 (Grb2), the p85 subunit of PI3K and CD44. (Sargiacoma *et al*., 1993; Lisanti *et al*., 1994; Gorodinsky and Harris, 1995; Bickel *et al*., 1997; Gomez-Mouton *et al*., 2001).

In order to study the function of lipid rafts in signaling, several researchers have employed an approach that involves the cholesterol depletion of cells to disrupt the integrity of lipid raft structures (Table 1.3.2). The integrity of lipid rafts can be disrupted by treating cells with agent such as methyl-β-cyclodextrin (MβCD) and filipin-III, which extract and sequester cellular cholesterol (Ohtani *et al*., 1989; Kilsdonk *et al*., 1995; Pike and Miller, 1998). Treatment of cells with MβCD attenuated the insulin-induced autophosphorylation of its receptor and the insulin receptor substrate 1, while reducing glucose uptake and oxidation in adipocytes, suggesting a role for lipid rafts in insulin signaling and metabolism (Parpal *et al*., 2001; Gustavsson *et al*., 1999; Vainio *et al*., 2002; Le Lay *et al*., 2001). Furthermore, cholesterol depletion also decreased the insulin-induced activation of Akt, while having no effect on the activation of ERK-1/2 in adipocytes, suggesting that Akt rather than MAPK signaling is involved (Parpal *et al*., 2001). Similarly, disruption of lipid rafts inhibited the PDGF-induced activation of PI3K and decreased the tyrosine kinase activity of the PDGF receptor in endothelial cells (Liu *et al*., 1997; McGuire *et al*., 1993). In contrast, lipid rafts seem to play an inhibitory role in EGFR signaling, since

disruption of lipid rafts resulted in an increased affinity for ligand binding of the EGFR and enhanced receptor dimerization, autophosphorylation and activation of the ERK-1/2 signaling pathway (Furuchi and Anderson, 1998; Pike and Casey, 2002; Ringerike *et al*., 2002; Roepstorff *et al*., 2002). Therefore, the unique feature of the EGFR to move out of lipid rafts seems to be coupled to activation of the receptor.

Table 1.3.2: Summary of the effects of cholesterol depletion on the signaling of RTKs and GPCRs

Function	Receptor	Effect of cholesterol depletion	Reference(s)
Receptor tyrosine kinase			
Ligand-binding	EGF	+	Pike and Casey, 2002
Dimerization	EGF	+	Chen and Resh, 2002
Autophosphorylation	EGF	+	Ringerike *et al*., 2002
MAPK activation	EGF	+	Furuchi and Anderson, 1998
Ligand-binding	Insulin	0	Parpal *et al*., 2001
Autophosphorylation	Insulin	0 or -	Vainio *et al*., 2002; Parpal *et al*., 2001
IRS-1 phosphorylation	Insulin	-	Parpal *et al*., 2001
Glucose uptake	Insulin	-	Gustavsson *et al*., 1999
MAPK activation	Insulin	0	Parpal *et al*., 2001
Akt activation	Insulin	-	Parpal *et al*., 2001
G-protein-coupled receptor			
MAPK activation	GnRH	-	Navratil *et al*., 2003
Adenylate cyclase activation	β_2-Adrenergic	+	Rybin *et al*., 2000
MAPK activation	Endothelin	-	Teixeira *et al*., 1999
FAK activation	Endothelin	-	Teixeira *et al*., 1999
EGFR transactivation	Angiotensin II	-	Ushio-Fukai *et al*., 2001
Activation	Rhodopsin	+	Niu *et al*., 2002

Cholesterol extraction with MβCD prevented the GnRHR from activating the ERK-1/2 pathway resulting in decreased gene expression of FosB in αT3-1 cells (Navratil *et al*., 2003; Bliss *et al*., 2007). Similarly, the thrombin-stimulated phosphatidic acid generation and IP3 production was inhibited when lipid rafts were disrupted by cholesterol extraction, resulting in decreased activation of human platelets (Bodin *et al*., 2001). Likewise, the endothelin-stimulated tyrosine phosphorylation of ERK-1/2 and FAK was inhibited when primary astrocytes were treated with

filipin-III (Teixeira *et al*., 1999). In contrast, cholesterol extraction increased both adenylate cyclase activation and myocyte contraction mediated by the β-adrenergic receptor in cardiac myocytes (Rybin *et al*., 2000; Xiang *et al*., 2002). A very interesting observation is that both the β-adrenergic receptor and EGFR move out of lipid rafts upon ligand-binding and the signaling pathways of both receptors are activated with depletion of cholesterol. It is possible that the activation of the EGF and β-adrenergic receptors are inhibited by associating with specific inhibitory proteins in lipid rafts, while ligand-binding changes the conformation of the receptor with release of these inhibitory proteins. Thus, cholesterol depletion assays showed that lipid rafts are important for GPCR, RTK and MAPK signaling pathways as summarized in Table 1.3.2.

1.3.2.2 Role in health and disease

Lipid rafts regulate a wide variety of signaling pathways that are responsible for controlling crucial cellular functions. Therefore, lipid rafts have been implicated in a great variety of diseases, including atherosclerosis, diabetes, cancer, muscular dystrophy and neurodegenerative disorders (Frank *et al*., 2004; Inokuchi, 2006; Patra, 2008; Cohen *et al*., 2004; Simons and Ehehalt, 2002; Wolozin, 2001). Furthermore, there is increasing data in the literature showing that lipid rafts function as the entry and budding sites in host cells for various bacteria, viruses and pathogens (summarized in Table 1.3.3).

Alzheimer's disease is characterized by the formation of senile plaques containing the amyloid-β-peptide (Aβ). The sequential enzymatic cleavage of the large type I transmembrane protein, amyloid precursor protein (APP), by β-secretase (BACE) and γ-secretase results in the formation of the peptide Aβ. Alternative enzymatic cleavage of APP in the middle of the Aβ domain by α-secretase results in the release of the non-amyloidogenic α-domain (Mattson, 2004; Selkoe, 2001). Importantly, the α- and β-secretase enzymes directly compete for their common substrate APP, suggesting that Aβ production could be regulated by controlling availability of APP to these enzymes. Interestingly, it has been reported that the β-, γ-secretases and Aβ partially localize to lipid rafts (Lee *et al*., 1998; Riddell *et al*., 2001; Wada *et al*., 2003), while APP appears to be largely excluded from lipid rafts in CHO cells (Kaether and Haass, 2004).

Furthermore, it was shown that cholesterol depletion inhibited the formation of Aβ by decreasing the activity of β-secretase, while enhancing α-cleavage (Simons *et al*., 1998; Kojro *et al*., 2001). Taken together, the above results suggest that the association of BACE with lipid rafts, which restricts access to APP, could be a potential mechanism regulating the formation of Aβ.

Table 1.3.3: Diseases in which lipid rafts are involved

Disease	Reference
Alzheimer's disease	Cecchi *et al*., 2009
Atherosclerosis	Yu *et al*., 2010
Pulmonary hypertension	Mathew *et al*., 2004
Systemic lupus	Deng and Tsokos, 2008
Asthma	Karman *et al*., 2010
Niemann-Pick disease	Vainio *et al*., 2005
Muscular dystrophy	Moral-Naranjo *et al*., 2010
Viral infections	
Enveloped viruses	
Influenza virus	Parpal *et al*., 2001
HIV-1	Kilsdonk *et al*., 1995
Herpes simplex virus	Koshizuka *et al*., 2007
Ebola virus	Freitas *et al*., 2007
Non-enveloped viruses	
SV-40	Pike and Miller, 1998
Rotavirus	Cuadras and Greenberg, 2003
Bacterial infections	
Escherichia coli	Clark *et al*., 2005
Vibrio cholerae	Deng and Tsokos, 2008
Mycobacterium tuberculosis	Shin *et al*., 2008
Other pathogens	
Shiga toxin	Huang *et al*., 2010
Lipopolysaccharide	Dhungana *et al*., 2009
Prion (Creutzfeldt-Jakob disease)	Taylor *et al*., 2009
Plasmodium falciparum	Garcia *et al*., 2009

Several bacteria and viruses utilize lipid raft-associated proteins to infect host cells (Table 1.3.3). The influenza virus was the first to be characterized and contains two integral membrane spike proteins, namely neuraminidase and hemagglutinin, which have been shown to localize to lipid rafts, based on studies of cholesterol depletion in MDCK cells (Scheiffele *et al*., 1999; Barman and Nayak, 2000). Influenza viruses preferentially include lipid raft-containing lipids in its envelope during budding from the apical membrane of MDCK cells (Zhang *et al*., 2000). Similarly, the envelope of the human immunodeficiency virus 1 (HIV-1) was shown to be derived from lipid rafts, and to include the host ganglioside GM1, as well as proteins such as

Thy-1 and CD59 (Brugger *et al*., 2006; Tritel and Resh, 2001; Chazal and Gerlier, 2003; Nguyen and Hildreth, 2000), suggesting that budding from lipid rafts may be a general mechanism amongst envelope viruses. HIV-1 mediates entry into host cells by binding to the CD4 receptor through the viral envelope glycoprotein gp120. The binding to CD4 receptors induces a conformational change in gp120, allowing the virus to interact with the cell-surface chemokine receptors, namely CXCR4 and CCR5, resulting in fusion of the virus with the plasma membrane and intracellular release of the genetic material (Manes *et al*., 2000). Interestingly, the CD4, CXCR4 and CCR5 receptors have been localized to lipid rafts and cholesterol depletion decreased the efficiency of infection of HIV-1 in T-lymphocytes (Manes *et al*., 2000). Similarly, a recent study by Carter *et al*. showed that the CD4 and CCR5 receptors partitioned into DRMs with the lipid raft-associated protein flotillin-1 and early virus particles co-localized with GM1 in macrophages (Carter *et al*., 2009). Taken together, the above results suggest that lipid rafts play an important role in the infection and budding of envelope viruses.

1.3.3 The reggie/flotillin family of proteins

A family of proteins, which is found in lipid rafts in cells that lack caveolae, is the reggie/flotillin family of proteins (Figure 1.3.1). To identify key proteins that are upregulated in retinal ganglion cells during axon regeneration after optic nerve lesion in the goldfish, two 47-kDa proteins called reggie-1 and -2 were discovered (Schulte *et al*., 1997). In the same year, another group identified two proteins associated with the low-density floating lipid raft fraction from mouse lung tissue, which were called flotillin-1 and -2 (Bickel *et al*., 1997). A study by Lang *et al*. reported that the molecular cloning of reggie-1 and -2 revealed 80% homology to the goldfish reggie proteins, while reggie-2 is practically identical to the mouse flotillin-1 (Flot-1) (Lang *et al*., 1998). Flotillin proteins or their homologs have been found in almost all species, with orthologs demonstrated in plants, bacteria and fungi (Rivera-Milla *et al*., 2006; Edgar and Polak, 2001; Borner *et al*., 2005). Flotillin proteins are highly conserved amongst species, with amino acid identity of 99% between human and mouse, whereas the mouse Flot-1 shares 61% homology with *Drosophila* (Galbiati *et al*., 1998b). In vertebrates, flotillin proteins show a similarity of about 90%, while invertebrates have 64% homology (Rivera-Milla *et al*., 2006). The high evolutionary conservation of flotillin proteins within vertebrates, especially within mammals, suggests that these proteins are likely to

be involved in fundamental cellular processes, which could be characteristic of their structural conformation.

1.3.3.1 Structural features

There are two closely related flotillin proteins that differ only in structure by a second hydrophobic region of the Flot-1 protein and the precise function of these two proteins remains to be determined (Babuke and Tikkanen, 2007; Langhorst *et al*., 2005). The human gene encoding for Flot-1 is located on chromosome 6 and contains 13 exons extending over 15 kb (Edgar and Polak, 2001), while the human Flot-2 gene is located on chromosome 17 and consists of 11 exons (Cho *et al*., 1995). Both genes are single copy genes that encode for proteins with 428 amino acids. Flotillin proteins belong to the Stomatin/Prohibitin/Flotillin/HflK/C (SPFH) protein family that shares a common SPFH domain in the N-termini (Tavernarakis *et al*., 1999). This domain was discovered by another group that called it the prohibitin homology domain (PHB) (Schultz *et al*., 1998). The three-dimensional structure of the SPFH domain of mouse Flot-2 has been solved, which indicated that the domain is a compact ellipsoidal-globular structure of four to five α-helices and six β-strands (Miyamoto *et al*., 2004). Flotillin proteins contain a unique flotillin domain in the C-terminal region that is not present in other members of the SPFH family. This flotillin domain is characterized by several repeats of glutamic acid and alanine (EA repeats), which have been predicted to form coiled-coil structures (Schroeder *et al*., 1994b; Bickel *et al*., 1997).

The C-terminal end of human Flot-2 has been shown to be important for the formation of homo-oligomers in HeLa cells (Neumann-Giesen *et al*., 2004). In addition, chemical cross-linking experiments showed that the smallest oligomer of flotillin proteins that could be detected was a tetramer (Langhorst *et al*., 2005). Furthermore, a recent study by Babuke *et al*. showed that Flot-1 and -2 could form hetero-oligomers, which require the tyrosine residue 163 of Flot-2 in HeLa cells (Babuke *et al*., 2009).

1.3.3.2 Tissue distribution and expression

Flot-2 is ubiquitously expressed in almost all mammalian tissues, while Flot-1 has a more restrictive expression pattern than Flot-2 (Schroeder *et al*., 1994b; Volonte *et al*., 1999). The stability of Flot-1 proteins is strongly dependent on the presence of Flot-2, as knockdown of Flot-2 protein expression reduces Flot-1 protein levels in mammalian cells and *Drosophila* (Langhorst *et al*., 2005; Chintagari *et al*., 2008; Hoehne *et al*., 2005). In contrast, the knockdown of Flot-1 has a much weaker effect on the protein stability of Flot-2 (Langhorst *et al*., 2005, Chintagari *et al*., 2008), suggesting that the stability of Flot-1 proteins is dependent on the presence of Flot-2.

The expression patterns of flotillin proteins during differentiation have been investigated in several types of cells. The expression of Flot-2 is enhanced during the *in vitro* differentiation of C2C12 skeletal myoblasts (Volonte *et al*., 1999). Similarly, differentiation of osteoclasts strongly induces the expression of Flot-1 (Ha *et al*., 2003). During differentiation of 3T3 fibroblasts to adipocytes, the expression of Flot-1 is increased and Flot-1 translocates from intracellular compartments to the plasma membrane (Liu *et al*., 2005). In addition, the expression of Flot-1 appears to be increased during the formation of cell-cell interactions in 3T3 fibroblasts (Lopez-Casas and del Mazo, 2003). In contrast, the expression of flotillin proteins is unaffected during differentiation of PC12 cells (Volonte *et al*., 1999). In summary, the expression and sub-cellular localization of Flot-1 and -2 is regulated during differentiation of cells in a cell-specific manner.

1.3.3.3 Subcellular localization

The plasma membrane association of flotillin proteins has been shown in many cell types, including neurons and lymphocytes (Lang *et al*., 1998; Stuermer *et al*., 2001). Interestingly, flotillin proteins do not contain a transmembrane domain, but seem to interact with membranes through their hydrophobic region that results in both the N- and C-termini facing the cytoplasm (Gkantiragas *et al*., 2001; Morrow *et al*., 2002). Anchoring of flotillin proteins to the inner leaflet of the plasma membrane is mediated by lipid modifications (Neumann-Giesen *et al*., 2004). Although Flot-1 and -2 shares a high degree of homology between them, they have been shown to associate with the membrane via different mechanisms. Mouse Flot-1 has been shown to be

palmitoylated at Cys34, which was required for the plasma membrane localization in baby hamster kidney (BHK) cells (Morrow *et al*., 2002). In contrast, Liu *et al*. reported that mutation of Cys34 had no effect on the localization of mouse Flot-1 in mouse 3T3-L1 adipocytes, suggesting cell-specific differences (Liu *et al*., 2005). It has also been reported that a hydrophobic region within the SPFH domain of Flot-1 might be imbedded in the membrane without traversing it (Morrow *et al*., 2002), similar to other proteins of the SPFH family (Roselli *et al*., 2002; Snyers *et al*., 1998). In addition, Flot-1 contains two hydrophobic regions (amino acids 10 - 36 and 134 - 151), both of which are important for membrane association. The first hydrophobic region of Flot-1 was shown to be important for the association with lipid rafts, while the second hydrophobic domain was required for plasma membrane localization (Liu *et al*., 2005). The Flot-2 protein has been shown to be myristoylated at Gly2 and palmitoylated at multiple residues, including Cys4, 19 and 20. Both post-translational modifications were shown to be required for plasma membrane localization in mouse 3T3-L1 adipocytes (Neumann-Giesen *et al*., 2004). Interestingly, myristoylation of Flot-2 has been shown to be a requirement for palmitoylation in HeLa cells. The authors also reported that a mutant Gly2Ala protein, which is neither myristoylated nor palmitoylated, shows increased solubility with decreased lipid raft association, supporting a role for lipid modifications in targeting Flot-2 to lipid rafts. In addition, the homo-oligomerization of Flot-2 has been reported to play a role in targeting Flot-2 to lipid rafts (Neumann-Giesen *et al*., 2004, 2007). In contrast to Flot-1, there is only one short hydrophobic region present in Flot-2, which is not continuous and therefore unlikely to act as a membrane insertion (Babuke *et al*., 2007). Taken together, lipid modifications regulate the association of flotillin proteins with lipid rafts and the plasma membrane.

Unlike other lipid raft-associated proteins, flotillins form stable cluster-size hetero-oligomers at the plasma membrane that are readily detectable by electron microscopy and immunogold staining of rat neurons and brain tissue (Lang *et al*., 1998; Stuermer *et al*., 2001; Kokubo *et al*., 2003). The flotillin clusters were found to be uniform in size with an estimated diameter of 100 nm (Stuermer *et al*., 2001; Kokubo *et al*., 2003). Flotillin clusters appear to be quite widely spaced along the plasma membrane with increased clustering at cell-cell contact points and after crosslinking of lipid raft-associated proteins, such as the GPI-anchored Thy-1 and the cellular prion protein (PrP^c) (Stuermer *et al*., 2001, 2004).

Flotillin proteins have also been localized to various intracellular vesicular compartments, including endosomal compartments in rat neurons and astrocytes, phagosomes in mouse J774 macrophages and Golgi compartments in CHO cells (Dermine *et al*., 2001; Stuermer *et al*., 2001; Gkantiragas *et al*., 2001). In addition, Flot-1 has been shown to associate with the phosphatase prostate tumor overexpressed gene 1 protein (PTOV-1), followed by translocation to the nucleus in a cell cycle-dependent manner in PC-3 cells (Santamaria *et al*., 2005). Taken together, these findings indicate a wide subcellular distribution of flotillin proteins that appears to be a highly dynamic process, which is regulated by lipid modifications in a cell-specific manner (Glebov *et al*., 2006; Neumann-Giesen *et al*., 2007).

1.3.3.4 Cellular function

Although, they are ubiquitously expressed and evolutionary well conserved, the exact molecular function of flotillin proteins is still unclear. Nonetheless, several studies have indicated a role for flotillin in signaling processes through membrane receptors, endocytosis and regulation of cytoskeleton signaling (Baumann *et al*., 2000; Dermine *et al*., 2001; Glebov *et al*., 2006; Hazarika *et al*., 2004; Lang *et al*., 1998; Neumann-Giesen *et al*., 2004, 2007; Schulte *et al*., 1997). In addition, a variety of proteins has been shown to co-localize and interact with flotillin proteins as described below (Langhorst *et al*., 2005; Babuke *et al*., 2007; Morrow and Parton, 2005).

The GPI-anchored protein Thy-1 was shown to co-localize and interact with flotillin proteins in PC12 cells and lymphocytes (Stuermer *et al*., 2001). Interestingly, flotillins also co-localize and can be co-immunoprecipitated with other GPI-anchored proteins, including F3/contactin and PrP^c (Stuermer *et al*., 2001, 2004). In addition, flotillin proteins interact with the src kinases LCK and FYN in T-cells and adipocytes, as shown by co-immunoprecipitation, suggesting a role for flotillins in GPI-anchored proteins and tyrosine-kinase signaling (Liu *et al*., 2005; Slaughter *et al*., 2003). Flot-2 has been shown to be co-immunoprecipitated with the thrombin GPCR receptor PAR-1 in melanoma cells (Hazarika *et al*., 2004), while Flot-1 was identified to interact with neuroglobin in a yeast two-hybrid assay (Wakasugi *et al*., 2004), suggesting a role for flotillins in GPCR and neuronal signaling, respectively. Taken together, these findings suggest that flotillins

are involved in the signal transduction by GPI-anchored proteins, RTKs and GPCRs across the plasma membrane.

The most well described examples of a functional role for flotillin proteins are in glucose uptake and cytoskeletal remodeling. An early study by Baumann *et al*. described a novel insulin-signaling pathway mediated via lipid rafts (Baumann *et al*., 2000). In this study it was shown that in the absence of insulin, a complex between a proto-oncogene, called c-Cbl, which is recruited by an adaptor protein with multiple functions called Cbl-associated protein (CAP, also known as ponsin), associates with the insulin receptor in adipocytes. Stimulation of these cells with insulin induced the phosphorylation of c-Cbl and resulted in dissociation of the CAP-Cbl complex from the insulin receptor. Subsequently, the complex translocates to lipid rafts where it forms a ternary structure with Flot-1, resulting in glucose uptake by the glucose transporter GLUT4 (Baumann *et al*., 2000). Therefore, localization of the CAP-Cbl complex to lipid rafts enriched with Flot-1 appears to generate a pathway that is important in the regulation of glucose uptake. The interaction between Flot-1 and CAP is mediated by a sorbin homology (SoHo) domain in the N-terminus of CAP and the first hydrophobic region of Flot-1 (Baumann *et al*., 2000; Liu *et al*., 2005). Several isoforms of CAP have been found with some containing more than one SoHo domain (Mandai *et al*., 1999; Ribon *et al*., 1998). However, Flot-1 seems to interact specifically with an isoform called CAP4 in adipocytes, which contains only one SoHo domain (Liu *et al*., 2005). CAP has been shown to recruit c-Cbl and the tyrosine kinase Pyk-2 to lipid rafts via three src homology 3 (SH3) domains in the carboxy terminus, a process that is crucial for neuritogenesis, a process involved in development of the adult nervous system and spinal cord, in differentiating PC12 cells (Haglund *et al*., 2004). In addition, both flotillin proteins have been shown to be involved in the process of differentiation in primary rat hippocampal neurons. Flot-2 was shown to be crucial for differentiation by mediating the recruitment of CAP and c-Cbl to lipid rafts in primary rat hippocampal neurons (Langhorst *et al*., 2008), while Flot-1 was shown to be a molecular mediator of neurite outgrowth by the synaptic adhesion-like molecule 4 (SALM4) (Swanwick *et al*., 2010). Interestingly, the SoHo domain is also present in two other proteins, namely ArgBP2 and vinexin-α (Kimura *et al*., 2001). Vinexin-α has been shown to interact with Flot-1 directly, indicating a role for actin remodeling (Kioka *et al*., 2002; Kimura *et al*., 2001). Taken together, the above findings support a role for flotillins in regulating

metabolism by enhancing glucose uptake by GLUT4 and a role in cytoskeletal remodeling. The latter would explain the increased expression of flotillins during differentiation of various cell types.

A previous study by Neumann-Giesen *et al*. indicated that Flot-2 plays a role in growth factor signaling by showing EGF-induced tyrosine 163 phosphorylation of Flot-2 by src kinases in HeLa cells (Neumann-Giesen *et al*., 2007). It was also shown that stimulation of cells with EGF promoted tyrosine phosphorylation at Y163 and endocytosis of Flot-2 from the plasma membrane to late endosomes, where it partially co-localizes with the EGFR. Similarly, a recent study by Riento *et al*. showed with phospho-specific antibodies that EGF-induced the tyrosine phosphorylation of both Flot-1 and -2 at Y160 and Y163, respectively, which was required for their internalization from the plasma membrane (Riento *et al*., 2009). The same study showed that the EGF-induced internalization of flotillins was inhibited in SYF cells that lack expression of LYN kinase, which was restored by overexpression of LYN kinase (Riento *et al*., 2009). Taken together, the above findings suggest a novel function of flotillin lipid rafts as a tyrosine-kinase-regulated endocytic pathway.

The flotillin proteins have also been implicated to have a role G-protein signaling. Sugawara *et al*. showed that $G_{\alpha q}$ proteins interact with both Flot-1 and -2 and knockdown of Flot-2 attenuated the UTP-induced activation of p38 by src-kinases, but not the activation of ERK-1/2 in HeLa cells (Sugawara *et al*., 2007). A more recent study reported that knockdown of Flot-2 reduced the insulin-like growth factor 1-induced activation of ERK-1/2, p38 and FAK, while having no effect on the activation of Akt, PKC or JNK in mouse N2a neuroblastoma cells (Munderloh *et al*., 2009), suggesting cell- or species-specific differences. Taken together, these above findings support a role for flotillins in the signal transduction of GPCRs, RTKs, MAPKs and other signaling pathways in a species- and cell-specific manner, but the main functions of flotillins remain unknown.

1.4 Hypothesis, aims and strategies

The project was based on the following hypothesis:

Crosstalk between the glucocorticoid receptor (GR) and the gonadotropin-releasing hormone receptor (GnRHR) involves the co-localization of these receptors to lipid rafts in LβT2 cells.

This project consists of three parts. The aim of the first part (sections 1 – 4) was to confirm the GnRH and synergistic response with Dex and GnRH on a GRE-reporter gene in LβT2 cells. Additional evidence for the crosstalk mechanism between the GR and GnRHR signaling pathways on other *cis*-elements and the effect of GnRH on the stability of GR protein was also investigated.

The second part (sections 5 – 7) of this study focused on the sub-cellular localization of the GR and GnRHR in LβT2 cells. Firstly, the lipid raft marker proteins that are expressed in LβT2 cells had to be identified and several techniques were set up and optimized. These included optimization of LβT2 cell growth conditions on different coating substrates for immunoflorescence experiments, sucrose-density gradient fractionation and co-immunoprecipitation assays. Specific aims of the second part were to investigate whether the GR localized to lipid rafts containing flotillin-1 (Flot-1) in LβT2 cells, under various hormonal conditions, by immunoflorescence analysis. In addition, it was investigated whether the GnRHR localized to lipid rafts containing Flot-1 in LβT2 cells, by immunoflorescence analysis. To provide additional evidence for the localization of the GR and GnRHR to lipid rafts and the effects of ligands thereon, the aims included to set up and optimize isolation of lipid rafts by density-gradient fractionation. Thereafter, the aim was to investigate whether the GR and the GnRHR were present in the lipid raft fraction, under various hormonal conditions. Whether the GR occurs in a complex with Flot-1 under the various hormonal conditions was also investigated, after optimization of the method, by co-immunoprecipitation assays using extracts from LβT2 cells.

The third part (sections 8 and 9) of this study was to investigate if lipid rafts or Flot-1 have a functional role in GR and GnRHR signaling in LβT2 cells. Specifically, the aim was to investigate whether lipid raft disruption, with cholesterol depletion agents, affects the ability of the GR to transactivate a GRE-reporter gene in response to the various hormonal conditions. In addition, whether lipid raft disruption affects GnRH-induced phosphorylation of the GR was also investigated. Finally, a requirement for Flot-1 in the transactivation of a GRE-reporter gene by Dex, GnRH or by co-stimulation with both was investigated by siRNA-mediated knockdown of Flot-1 in LβT2 cells, after optimization of this method.

Chapter 2: Materials and methods

2.1 Compounds and antibodies

Dex, GnRH, TNFα (T7539), Hoechst (86140-5), methyl-β-cyclodextrin (MβCD) (C4555), Filipin-III (F4767), phenylmethanesulfonyl fluoride (PMSF), poly-L-ornithine (P3655) and all other general reagents and chemicals were purchased from Sigma-Aldrich, South Africa. Dithiothreitol (DTT) was obtained from Promega Corp., USA. Bovine-serum albumin (BSA), leupeptin, aprotinin and FuGENE 6 were purchased from Roche Diagnostics, South Africa.

Laminin (354239) and antibodies to caveolin-1 (610406) and GM130 (610822) were purchased from BD Transduction Laboratories, USA. The non-specific rabbit IgG antibody (R1131),was obtained from Sigma-Aldrich, South Africa, while the anti-mouse HRP (sc-2005) and anti-rabbit HRP (sc-2313) secondary antibodies were purchased from Santa Cruz Biotechnology, USA. Antibodies to GAPDH (14C10), p44/42 MAPK (9102) and phospho-p44/42 MAPK (Thr202/Tyr204) (9101) were obtained from Cell Signaling Technology, USA. The anti-histone-H3 antibody (ab1791) was obtained from Abcam, UK. The rabbit anti-GnRHR antibody, raised against amino acids 193 – 212 in the extracellular loop of the ovine receptor, was a generous gift from D.C. Skinner (University of Wyoming, Department of Zoology and Physiology & Neurobiology Program, USA) and has been described before (Bliss *et al*., 2007). The rabbit polyclonal anti-GR H300 (sc-8992) antibody, raised against amino acids 121 – 420 of the human receptor, was purchased from Santa Cruz Biotechnology, USA, while the anti-phospho-serine 226 GR antibody (corresponding to pSer234 in the mouse) was a generous gift from M.J. Garabedian (New York University, School of Medicine, USA). The rabbit polyclonal anti-flotillin-1 antibody, raised against the full-length goldfish flotillin-1 protein, was a generous gift from D.M. Lang (University of Cape Town, Medical School, South Africa), while the monoclonal anti-flotillin-1 antibody (610820), raised against amino acids 312 – 428 of mouse flotillin-1, was purchased from BD Transduction Laboratories, USA. The anti-rabbit AlexaFluor488 (A21206) and Cholera Toxin subunit B AlexaFluor647 (C-34778) were

purchased from Invitrogen, USA, while the donkey anti-mouse Cy3 antibody (715-166-150) was obtained from Jackson Immuno Research, USA.

2.2 Plasmids

The pTAT-GRE-E1b-luc plasmid was a kind gift from G. Jenster (University of Rotterdam, Netherlands) and has been described before (Sui *et al*., 1999). The minimal luciferase reporter construct (IL-6κB)$_3$-50hu.IL6P that contains three repeats of the NF-κB responsive element from the human IL-6 promoter, was kindly provided by G. Haegeman (University of Ghent, Belgium) and has been described before (Plaisance *et al*., 1997). The pRK7-Flotillin-1 plasmid that encodes a FLAG-tagged mouse Flot-1 protein was a generous gift from A.R. Saltiel (University of Michigan Medical School, USA). The pAP-1 luciferase plasmid containing seven copies of a consensus AP-1 site was purchased from Stratagene, USA. The pSV-β-galactosidase expression vector was obtained from Promega Corp., USA.

2.3 Plasmid transformation and preparation

Plasmids were transformed into competent *Escherichia coli* DH5α cells by the heat-shock procedure described by Sambrook *et al*. (Sambrook *et al*., 1989). Briefly, 50 μL of competent cells were incubated on ice with 10 ng of plasmid DNA for 30 min. The cells were heat-shocked for 2 min at 42°C, followed by 2 min incubation on ice. Thereafter, outgrowth was stimulated by the addition of 500 μL Luria Broth (LB, 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl) and incubation for 1 h at 37°C shaking. Cells were plated onto LB-agar (LB with 1.5% agar) plates-containing 100 μg/mL ampicillin and incubated over-night at 37°C. For plasmid purification, a single colony was picked to inoculate LB cultures containing 100 μg/mL ampicillin and incubated over-night at 37°C shaking. The following day, plasmid DNA was purified with the Promega Pureyield Plasmid Midi-prep kit (Promega Corp., USA), according to the manufacturer's instructions. The integrity and purity of the plasmids were analyzed by restriction enzyme digestion and agarose-gel electrophoresis (Sambrook *et al*., 1989).

2.4 Cell culture

P.L. Mellon at the University of California, USA, kindly provided the immortalized mouse LβT2 pituitary gonadotrope cells (Turgeon *et al*., 1996). The COS-7 monkey kidney fibroblast cells were a generous gift from S. Prince at the University of Cape Town, South Africa. Both cell lines were grown in high glucose (1 g/mL) Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, South Africa), 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen, UK). The cells were maintained in 75-cm^2 culture flasks (Greiner Bio-one International, Austria) at 37°C in an environment of 5% CO_2 and 90% humidity up to a confluency of 85% before sub-culturing. Cells were sub-cultured with 0.25% trypsin/0.1% EDTA in calcium- and magnesium-free PBS. The LβT2 cells proved very sensitive to the trypsinization procedure and were therefore never incubated with the trypsin solution for longer than three min. LβT2 cells were cultured up to a maximum of 25 passages. Cells were routinely tested for mycoplasma infection by Hoechst staining and suspicious cell cultures were further analyzed by a luminescent-based assay (Glo-Max, Promega Corp., USA). Only mycoplasma-negative cells were used in experiments.

2.5 Transient transfection of cells

To generate a positive control of the Flot-1 protein for the experiment in Figure 3.5.1, COS-7 cells were seeded into 12-well plates at a density of 1 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described above. Twenty-four hours after plating the medium was replaced and the cells were transfected with 250 ng pRK7-Flotillin-1 using 0.5 µL FuGENE 6 in 100 µL serum free DMEM, according to the manufacturer's instructions. Forty-eight hours after transfection, cells were washed twice with ice-cold PBS and harvested in 50 µL 2 X SDS sample buffer (5 X SDS sample buffer: 100 mM Tris-Cl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% ß-mercaptoethanol and 0.1% (w/v) bromophenol-blue). The samples were boiled for 10 min at 100°C before storage at -20°C or equal amounts of cell lysates were analyzed by Western blotting as described below, probing with specific anti-GR and anti-Flot-1 antibodies (see section 2.6 for dilutions of antibodies used).

For the reporter gene assays in Figures 3.1, 3.2, 3.3 and 3.8, LβT2 cells were seeded in 24-well plates at a density of 1 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. Forty-eight hours after plating, medium was replaced with fresh medium and the cells were co-transfected with 250 ng of pTAT-GRE-E1b-luc plasmid (Figure 3.1, 3.8) or 250 ng of pAP-1-luc plasmid (Figure 3.2) or 400 ng of $(IL\text{-}6\kappa B)_3$-50hu.IL6P-luc plasmid (Figure 3.3) and 25 ng pSV-β-galactosidase plasmid to correct for transfection efficiency. Twenty-four hours after transfection, cells were washed once with PBS before being stimulated in serum-free medium as indicated in the figure legends. The cells were harvested in 50 µl reporter lysis buffer (Promega Corp., USA) per well. Both the luciferase assays (Luciferase Assay System, Promega Corp., USA) and the β-galactosidase assays (Galacto-Star, Tropix Inc, USA) were performed with 10 µL of cell extracts in white 96-well plates in a Modulus microplate reader (Turner Biosystems, USA). The luciferase values were normalized for transfection efficiency with β-galactosidase values and the fold induction expressed relative to vehicle (control).

2.6 SDS-PAGE and Western blotting

LβT2 cell lysates were prepared and adjusted to a final concentration of 1 X SDS sample buffer before separating on an 8% SDS-polyacrylamide gel at 120 V in 1 X SDS running buffer (25 mM Tris-Cl (pH 8.4), 250 mM glycine and 0.1% SDS) (Sambrook et al., 1989), using a BioRad Mini Protean II electrophoresis cell chamber. Proteins were transferred onto a HyBond ECL nitrocellulose membrane (AEC-Amersham, South Africa) for 1 h at 180 mA in a Tris/glycine buffer (25 mM Tris, 250 mM glycine and 20% (v/v) methanol), using a Mini Protean II blotting system (BioRad, South Africa). The membranes were blocked for 1 h at room temperature in 4% ECL blocking solution (4% (w/v)) ECL advance blocking powder, (AEC-Amersham, South Africa) and Tris-buffered saline (TBS: 50 mM Tris-Cl (pH 7.5) and 150 mM NaCl) containing 0.1% Tween-20 (TBST). After blocking, membranes were incubated with primary antibodies (dilutions in Table 2.1) in 4% ECL blocking solution (all except pERK-1/2 and ERK-1/2, which were diluted in TBST) at 4°C over-night. The following day, the membranes were washed with TBST for 15 min, followed by 2 X 5 min washes at room temperature before incubation with secondary HRP-conjugated antibodies for 1 h at room temperature in 5% non-fat milk powder (w/v) in TBST. Membranes were washed for 1 X 15 min and 2 X 5 min with TBST, followed by

1 X 5 min wash at room temperature with TBS and visualized by autoradiography. The membranes were stripped for 30 min at 60°C in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) SDS and 62.5 mM Tris-Cl (pH 6.8)), washed twice for 10 min with TBST, blocked for 1 h at room temperature in 4% ECL blocking solution before incubating with antibody again as described above. The proteins were visualized with ECL Western blotting detection reagents (AEC-Amersham, South Africa) and Hyperfilm MP high performance autoradiography film (AEC-Amersham, South Africa), according to the manufacturer's instructions. Bands on the autoradiography film were scanned and quantification was performed with AlphaEaseFC FluorChem 5500 (Alpha Innotech).

Table 2.1: Dilutions of antibodies used in Western blot analysis

Antibody	**Dilution**
GR	1:4000
Flot-1	1:5000
Cav-1	1:1000
GnRHR	1:4000
Histone-H3	1:4000
GAPDH	1:2000
ERK-1/2	1:1000
GM130	1:1000
pSer226-GR	1:5000
pERK-1/2	1:1000
rabbit-HRP	1:10 000
mouse-HRP	1:5000

2.7 Confocal immunoflorescence

2.7.1 Live cell GnRHR staining

Glass cover slips were washed twice for 30 min in 3.2% HCL and rinsed with distilled water and absolute ethanol. Cover slips were sterilized by flaming and placed in 6-well plates followed by seeding LβT2 cells at a density of 3 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. After 48 h, cells were washed twice with ice-cold PBS, followed by live-cell staining for 1 h with rabbit anti-GnRHR (1:400) in ice-cold PBS, on ice water. Thereafter, cells were washed with ice-cold PBS for 3 X 5 min, fixed and permeabilized with methanol at -20°C for 10 min and washed with PBS for 3 X 5 min. Cells were blocked with 5% BSA in PBS for 1 h at room temperature, followed by staining with mouse anti-Flot-1 (1:50) antibody in PBS with 5% BSA for 1 h at room temperature. Subsequently, the cells were washed with 1% BSA in PBS for 3 X 5 min before incubating with anti-rabbit labeled Alexa488 (1:500) and anti-mouse labeled Cy3 (1:1000) antibodies in PBS with 5% BSA for 1 h at room temperature in the dark. The cells were washed with 1% BSA in PBS for 3 X 5 min, followed by incubation with Hoechst (100 µg/mL) in PBS for 5 min. Slides were mounted in Mowiol (475904, Calbiochem, USA) containing n-propyl gallate (Sigma-Aldrich, South Africa) as anti-fading agent and allowed to set over-night at room temperature in the dark, followed by storage at 4°C in the dark until visualization.

2.7.2 Live cell cholera toxin staining

Glass cover slips were washed twice for 30 min in 3.2% HCL and rinsed with distilled water and absolute ethanol. Cover slips were sterilized by flaming and placed in 6-well plates followed by seeding LβT2 cells at a density of 3 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described previously. The following day cells were washed once with PBS before replacing the media with DMEM containing 10% charcoal stripped serum and antibiotics as described above. The following day the cells were stimulated with 100 nM Dex, 100 nM GnRH or a combination of both for 30 min followed by two washes with ice-cold PBS. To minimize internalization of the cholera toxin during the live-cell staining procedure, until after the fixation process, the cells were maintained on ice water. The live cells were stained with 10 µg/mL cholera toxin subunit B conjugated to Alexa647 in PBS with 1% BSA for 30 min at 4°C in a

humidified environment. Following the live-cell stain, the cells were washed for 3 X 5 min with ice-cold PBS followed by fixation with freshly prepared 2% para-formaldehyde in PBS (pH 7.0) for 10 min at room temperature. Optimal fixation conditions for the lipid raft stain were investigated and it was found that 2% para-formaldehyde was appropriate, while methanol fixation reduced the amount of lipid raft staining significantly. The cells were washed for 2 X 5 min with ice-cold PBS, followed by permeabilization with 1% Triton X-100 in ice-cold PBS for 10 min on ice water. Cells were washed with PBS for 5 min, blocked with 5% BSA in PBS for 1 h at room temperature, before staining with rabbit anti-GR (1:250) and mouse anti-Flot-1 (1:50) antibodies in PBS with 5% BSA for 1 h at room temperature in a humidified environment. Thereafter, cells were washed for 3 X 5 min with 1% BSA in PBS, followed by incubation with anti-rabbit labeled Alexa488 (1:500) and anti-mouse labeled Cy3 (1:1000) antibodies in PBS with 5% BSA for 1 h at room temperature in a lightproof humidified environment. Slides were washed for 3 X 5 min with 1% BSA in PBS and incubated with Hoechst (100 μg/mL) in PBS for 5 min, followed by mounting in Mowiol at room temperature over-night in the dark. Slides were stored at 4°C in the dark until visualization.

2.7.3 Confocal imaging

Confocal microscopy was performed with a Zeiss Axiovert 200M LSM 510 Meta NLO Confocal Microscope using the 40X water immersion objective. A multi-track scanning configuration using the 488nm (HeNe gas laser), 561nm (solid state laser), 633nm (argon laser) and 800nm (Ti:Sa femtosecond infrared laser) excitation lines was employed to minimize bleed through between the fluorophores. The photomultiplier gain and offset were adjusted to exclude any background fluorescence emitted by the cells and fluorophores. At least three different fields of view from three independent experiments were collected. The GnRHR images were analyzed with ImageJ software with the Pearson correlation co-efficient (Manders *et al*., 1993) between the two fluorophores. The cholera toxin images were analyzed with the LSM 510 Meta software by demarcating the region of interest, setting the channel intensities to zero, adjusting the amount of co-localization with the appropriate thresholds intensities of each channel, followed by determination of the Pearson correlation co-efficient (Manders *et al*., 1993) between the two relevant fluorophores. The Pearson correlation co-efficient values range from -1 to 1 and a value near 1 suggests true co-localization. Similarly, the Mander's overlap co-efficient values range

from 0 to 1 and a value near 1 suggests true co-localization, whereas the intensity correlation analysis values range from -0.5 to 0.5 and a value near 0.5 suggests true co-localization (Manders *et al*., 1993).

2.8 Lipid raft isolation

The non-detergent based method for lipid raft isolation as shown in Addendum Bi has been described before (Gagescu *et al*., 2000) and was performed as follows. LβT2 cells were seeded in 150-mm^2 dishes at a density of 8 X 10^6 cells per dish in DMEM with 10% FCS with antibiotics as described elsewhere. After three days of growth, LβT2 cells were washed with PBS and scraped on ice in 1 mL PBS. Thereafter, the cells were collected by centrifugation for 5 min at 500 X g, washed once with 4 mL of homogenization buffer (HB, 250 mM sucrose, 3 mM imidazole (pH 7.4), 2 mM EDTA and 1 mM PMSF) and resuspended in 2.2 mL HB. Thereafter, the cells were lysed with 8 strokes in a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 4°C for 10 min at 3000 X g, followed by adjusting the sample to 40.6% sucrose with the addition of 2.4 mL HB containing 60% sucrose. Subsequently, the sample was laid at the bottom of a SW40 centrifuge tube (13 mL) and overlaid with a discontinuous density-gradient of 4 mL of 35% sucrose in HB, 3 mL of 25% sucrose in HB and 0.6 mL HB. Thereafter, the sample was subjected to equilibrium flotation at 4°C for 16 h at 30 000 rpm in a SW40Ti rotor. Twelve 1 mL fractions were collected from the top of the gradient and equal amounts of fractions were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with a specific antibody against Flot-1.

The detergent based method for lipid raft isolation as shown in Addendum Bii and Biii has been described before (Lafont and Simons, 2001) and was performed with a few modifications as follows. LβT2 cells were seeded in 150-mm^2 dishes at a density of 8 X 10^6 cells per dish in DMEM with 10% FCS with antibiotics as described elsewhere. After three days of growth, LβT2 cells were washed with PBS and scraped on ice in 1 mL PBS. Thereafter, the cells collected by centrifugation for 5 min at 500 X g, washed once with 1 mL of extraction lysis buffer (ELB, 10 mM Hepes (pH 7.9), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin) and resuspended in 1 mL ELB. Thereafter, cells were lysed with 12

strokes in a Dounce homogenizer and unbroken cells and nuclei were removed by centrifugation at 4°C for 3 min at 350 X g. The crude membrane fraction was obtained by centrifugation at 4°C for 15 min at 15 000 X g, washed once with 1 mL ELB and resuspended in 0.4 mL solubilization buffer (SB) (25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin) containing 1% or 0.05% Triton X-100. After incubation for 45 min on ice, the crude membrane fraction was adjusted to 60% sucrose with the addition of 0.8 mL SB containing 90% sucrose. Subsequently, the sample was laid at the bottom of a SW65 (5 mL) centrifuge tube and overlaid with a discontinuous density-gradient with 2 mL of 43% sucrose in ELB, 1 mL of 13% sucrose in ELB and 0.8 mL ELB. Thereafter, the sample was subjected to equilibrium flotation at 4°C for 18 h at 40 000 rpm in a SW65Ti rotor. Ten fractions (0.5 mL) were collected from the top of the gradient and equal amounts of fractions were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with a specific antibody against Flot-1 (ii) or Flot-1 and GR (iii).

Plasma membrane lipid rafts as shown in Figure 3.6 were prepared using the Triton X-100 procedure as described by Lafont and Simons with some modifications (Lafont and Simons, 2001). This method was optimized as described in Addendum B. LβT2 cells were seeded in 150-mm^2 dishes at a density of 8 X 10^6 cells per dish in DMEM with 10% FCS containing antibiotics as described elsewhere. The cells were washed twice with PBS and serum starved over-night in serum-free DMEM. The following day the cells were stimulated with 100 nM Dex, 100 nM GnRH or a combination of both for 30 min before being washed twice with ice-cold PBS. The cells were scraped on ice in 1 mL PBS containing 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin per dish. Thereafter, the cells were centrifuged at 500 X g for 5 min and each cell pellet (10 dishes) was resuspended in 1 mL SB containing 0.05% Triton X-100 and incubated on ice water for 45 min. The lysates was adjusted to 60% sucrose in SB and layered at the bottom of SW40 Ultraclear centrifuge tubes (Beckman, South Africa). A discontinuous sucrose gradient was prepared consisting of 2 mL extraction lysis buffer (ELB), 10 mM Hepes (pH 7.9), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin), 4 mL of 13% sucrose in ELB, 4 mL of 43% sucrose in ELB and 4 mL of 60% sucrose containing the sample. Thereafter, the samples were subjected to equilibrium flotation in a SW40Ti rotor (38 000 rpm for 18 h at 4°C). Flocculent material could be seen at the interfaces and fractions (1 mL)

were collected as follows: 1) Top of the gradient, 2) ELB/13% interface, 3) 13%/43% interface (lipid raft fraction), 4) middle of 43% sucrose, 5) 43%/60% interface, 6) middle of 60% sucrose (loading fraction) and fraction 7) the pellet. All fractions were sonicated for 30 sec pulses in a water bath at room temperature until a homogenous solution was obtained. Fractions were aliquoted and stored at -80°C. For analysis, sample preparation was optimized and performed as follows. Fractions were adjusted to a final concentration of 1 X SDS sample buffer without β-mercaptoethanol, sonicated again for 2 X 30 sec and incubated at 100°C for 10 min. The samples were prepared without β-mercaptoethanol as previous studies have reported that when membrane proteins are boiled in the presence of reducing reagents, it can potentially result in aggregate formation, which could make Western blot analysis difficult. After boiling, the samples were briefly incubated on ice before analyzing equal amounts of fractions by Western blotting as described elsewhere. The membranes were probed with specific antibodies against the GR, GnRHR, Flot-1 and histone H3.

2.9 Co-immunoprecipitation assays

LβT2 cells were seeded in 100-mm^2 dishes at a density of 3 X 10^6 cells per dish in DMEM with 10% FCS and antibiotics as described elsewhere. Seventy-two hours after plating, cells were washed twice with PBS and incubated for 2 h in serum-free DMEM, before being stimulating with 100 nM Dex, 100 nM GnRH or a combination of both for 30 min as indicated in the figure legends. The cells were washed twice with ice-cold PBS and scraped on ice in 1 mL RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 2 μg/mL aprotinin, 5 μg/mL leupeptin and 2.5% (w/v) casein). The lysates were briefly vortexed before incubating on ice for 10 min and centrifugation at 5000 X g for 10 min at 4°C, after removing 50 μL aliquot to represent inputs. The supernatants were collected and incubated with 1 μg rabbit anti-GR, 4 μL rabbit anti-Flot-1 or non-specific rabbit IgG antibodies by rotating at 4°C over-night. The following day the Protein A/G-Agarose beads (Santa Cruz, USA) were prepared by blocking them for 30 min with saturated casein in PBS, while rotating at 4°C, followed by 30 min incubation with 5% BSA in PBS, while rotating at 4°C. The beads were washed once with PBS after which a 25% slurry was prepared with PBS. The antibodies and bound protein complexes were incubated with 20 μL of

the Protein A/G-Agarose bead slurry and rotated for 1 h at 4°C. The protein-immune complexes were collected by centrifugation at 1 000 X g for 5 min at 4°C, followed by two washes with 1 mL PBS. The proteins were eluted from the beads by addition of 24 µL of 2 X SDS sample buffer and incubated at 100°C for 5 min. The samples were centrifuged at 20 000 X g at room temperature and the supernatants collected and resolved on an 8% SDS-PAGE followed by Western Blotting as described elsewhere, probing with antibodies as indicated in the figure legends.

2.10 Incubation with cholesterol depletion agents

2.10.1 Luciferase gene reporter assays

LβT2 cells were seeded in 24-well plates at a density of 1 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. Forty-eight hours after plating, medium was replaced with fresh medium and the cells were transfected with 250 ng of pTAT-GRE-E1b-luc plasmid and 25 ng pSV-β-galactosidase plasmid to correct for transfection efficiency. Twenty-four hours after transfection, the cells were treated with cholesterol depletion agents. For Figure 3.8A, the cells were incubated for 45 min with 10 mM MβCD in serum-free medium, followed by a P BS wash before being stimulated for 8 h in serum-free medium as indicated in the figure legend. For Figure 3.8D, cells were pre-treated for 30 min with 5 ng/mL Filipin-III in serum-free medium, followed by stimulation for 8 h in the presence Filipin-III as indicated in the figure legend. The cells were harvested in 50 µl reporter lysis buffer (Promega Corp., USA) per well. Both the luciferase assays (Luciferase Assay System, Promega Corp., USA) and the β-galactosidase assays (Galacto-Star, Tropix Inc, USA) were performed with 10 µL of cell extracts in white 96-well plates in a Modulus microplate reader (Turner Biosystems, USA). The luciferase values were normalized for transfection efficiency with β-galactosidase values and the fold induction expressed relative to vehicle (control).

2.10.2 Phosphorylation assays

LβT2 cells were seeded in 24-well plates at a density of 1 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. Forty-eight hours after plating, medium was replaced with fresh medium and cells pre-treated for 30 min with 5 ng/mL Filipin-III in serum-

free medium, followed by stimulation for 1 h with 100 nM GnRH in the presence Filipin-III. Cells were washed twice with ice-cold PBS and harvested in 25 μL 2 X SDS sample buffer. The samples were incubated for 10 min at 100°C before analyzing equal amounts of cell lysates by Western blotting as described elsewhere. The membranes were probed with specific antibodies against pSer234 GR, total GR and GM130 (Figure 3.8B) or pERK-1/2 and total ERK-1/2 (Figure 3.8C).

2.11 RNA interference

Optimization of Flot-1 siRNA-mediated knockdown experiments as shown in Addendum D1 was performed as follows. LβT2 cells were seeded in 24-well plates at a density of 1.5 X 10^5 cells per well in 500 μL DMEM with 10% FCS and antibiotics as described elsewhere. Twenty-four hours after plating, medium was replaced with fresh medium and the cells were transfected with siRNA using HiPerfect transfection reagent (Qiagen, USA), according to the manufacturer's instructions. Briefly, either mouse Flot-1 siRNA (Mm_FLot1_1 (SI01003583), Mm_FLot1_2 (SI01003590), Mm_FLot1_3 (SI01003597), or Mm_FLot1_4 (SI01003604), (FlexiTube siRNA (1027415), Qiagen, USA)) or non-silencing scrambled (NSC) siRNA (Negative control siRNA (1027310), Qiagen, USA) was diluted in 25 μL Opti-MEM+GlutaMAX-I (Gibco-BRL Life Technologies, UK) with 1.75 μL HiPerfect. The mixture was incubated for 10 min at room temperature and added drop-wise to the cells to obtain a final concentration of 20 nM siRNA per well. Either 48 h or 72 h after transfection, the cells were washed twice with ice-cold PBS and harvested in 25 μL 2 X SDS sample buffer, followed by boiling at 100°C for 10 min. Equal amounts of cell lysates were analyzed by Western blotting as described elsewhere, probing with specific anti-Flot-1 and anti-GM130 antibodies. The optimization of siRNA concentration experiment shown in Addendum D2 was performed as in D1 with a few modifications. Briefly, an equimolar combination of all four Flot-1 siRNA oligonucleotides was diluted in either 25 μL Opti-MEM+GlutaMAX-I with 1.75 μL HiPerfect or diluted in 50 μL Opti-MEM+GlutaMAX-I with 3.5 μL HiPerfect and added dropwise to the cells to achieve a final concentration per well of 20 nM or 40 nM, respectively. After incubation with siRNA for 72 h, the cells were washed twice with ice-cold PBS and harvested in 25 μL 2 X SDS sample buffer, followed by boiling at 100°C

for 10 min. Equal amounts of cell lysates were analyzed by Western blotting as described elsewhere, probing with specific anti-Flot-1 and anti-GM130 antibodies.

For the optimized method as reported in Figure 3.9, LβT2 cells were seeded in 24-well plates at a density of 1.5 X 10^5 cells per well in 500 µL DMEM with 10% FCS and antibiotics as described elsewhere. Twenty-four hours after plating, medium was replaced with fresh medium and the cells were transfected with siRNA using HiPerfect transfection reagent (Qiagen, USA), according to the manufacturer's instructions. Briefly, mouse Flot-1 siRNA (Mm_FLot1_1, Mm_FLot1_2, Mm_FLot1_3, Mm_FLot1_4, (FlexiTube siRNA, Qiagen, USA)) or non-silencing scrambled (NSC) siRNA (Negative control siRNA (1027310), Qiagen, USA) were diluted in 50 µL Opti-MEM+GlutaMAX-I (Gibco-BRL Life Technologies, UK) with 3.5 µL HiPerfect. The mixture was incubated for 10 min at room temperature and added drop-wise to the cells to obtain a final concentration of 40 nM siRNA per well. After incubation with siRNA for 48 h, the medium was replaced with fresh medium, followed by transfection of the cells with 250 ng of pTAT-GRE-E1b-luc plasmid. After 24 h, the cells were stimulated for 8 h in serum-free medium as indicated in the figure legends. The cells were harvested in 50 µl reporter lysis buffer (Promega Corp., USA) per well. The luciferase assay (Luciferase Assay System, Promega Corp., USA) was performed with 10 µL of cell extracts in white 96-well plates in a Modulus microplate reader (Turner Biosystems, USA). Luciferase values were normalized to total protein content per well as determined by standard Bradford assay (Bradford, 1976). Equal amounts of cell lysates were analyzed by Western blotting as described elsewhere, probing with specific anti-Flot-1, anti-GR and anti-GAPDH antibodies.

2.12 Statistical analysis

Statistical analysis were performed with GraphPad Prism software (version 5) using the one-way ANOVA analysis of variance with either a Dunnett (when comparing all values to a single control) or Tukey (when comparing all values to each other) post-test. Statistical significance is denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively. The statistical tests performed for each experiment are indicated in the respective figure legends.

Chapter 3: Results

3.1 Confirmation of the GnRH and the synergistic transcriptional responses on a GRE in LβT2 cells

The present study was to further investigate the mechanisms of ligand-independent activation of the endogenous GR by GnRH and explore the synergistic transcriptional responses observed with co-stimulation of GnRH and Dex. Firstly, it was necessary to establish that GnRH induces transactivation and that co-stimulation with Dex and GnRH induces a synergistic transcriptional response of a transient GRE reporter gene in LβT2 cells in the hands of the present author.

3.1.1 GnRH activates the endogenous unliganded GR to induce a transcriptional response on a GRE in LβT2 cells

It has previously been shown that GnRH activates the unliganded GR inducing a transcriptional response on a GRE-luciferase reporter construct in LβT2 cells (Kotitschke *et al* 2009). To confirm this result, LβT2 cells were transfected with a GRE-luciferase plasmid, followed by stimulation for 8 h with 100 nM GnRH or 100 nM Dex in serum-free medium. As shown in Figure 3.1A, Dex treatment significantly increased the transcriptional activity of the luciferase reporter gene by about 4-fold (Figure 3.1A, bar 2). This result is a positive control required for the GRE assay and indicates LβT2 cells express functional endogenous GR. Treatment of the cells with GnRH significantly increased the transcriptional activity of the luciferase reporter gene by about 4-fold (Figure 3.1A, bar 3). The GnRH response was similar to the response induced with Dex treatment (Figure 3.1A), confirming the result from Kotitschke *et al*. (Kotitschke *et al*., 2009).

3.1.2 Co-stimulation with Dex and GnRH induces a synergistic transcriptional response on a GRE in LβT2 cells

Kotitschke *et al*. also reported that co-treatment with Dex and GnRH results in a synergistic transcriptional response on a GRE-reporter in LβT2 cells. To confirm this result, LβT2 cells were transfected with a GRE-luciferase plasmid, followed by co-stimulation for 8 h with 100 nM

GnRH and 100 nM Dex in serum-free medium. From the results presented in Figure 3.1A, it is clear that Dex and GnRH have an equivalent ability to increase transcription of the GRE-reporter gene by about 4-fold (Figure 3.1A, compare bar 2 and 3). The results presented in Figure 3.1B show that the presence of both Dex and GnRH results in a significant 12-fold increase in the transcriptional activity of a GRE-reporter gene in LβT2 cells (Figure 3.1B), indicating that the hormones act synergistically. The above data indicate that crosstalk occurs between the GR and GnRHR signaling pathways, resulting in the ligand-independent activation of the GR by GnRH and a synergistic transcriptional response on a GRE-promoter in LβT2 cells.

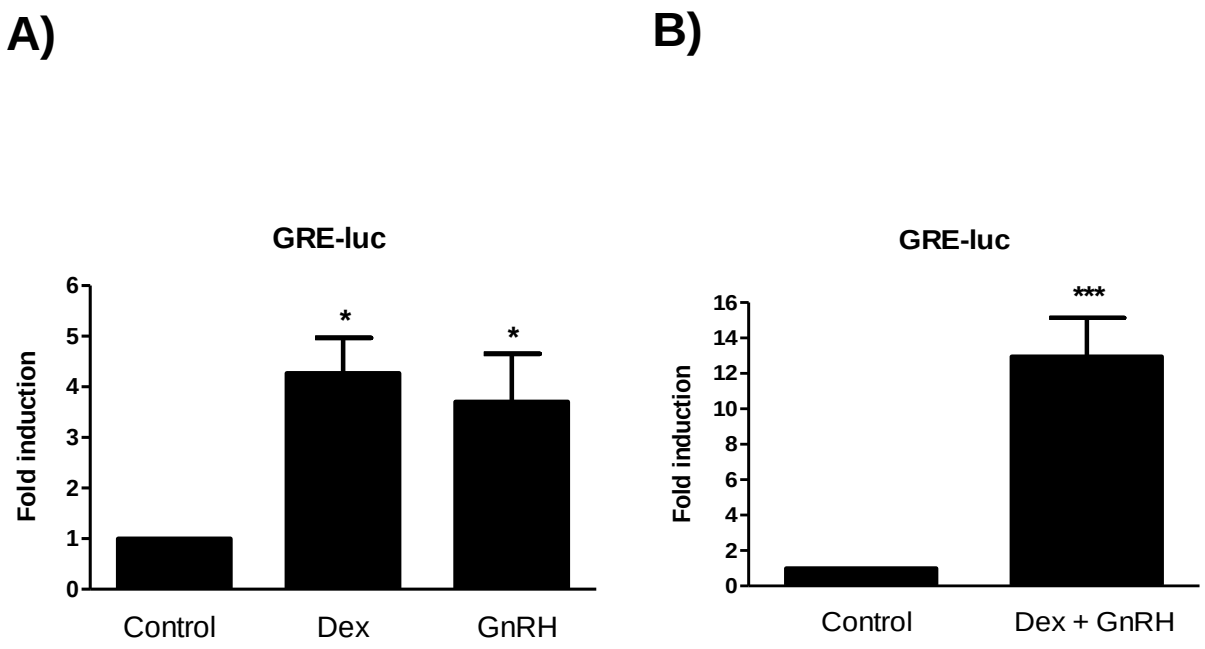


Figure 3.1: Ligand-independent activation of the GR by GnRH occurs in LβT2 cells and Dex and GnRH have a synergistic effect on the transcriptional activity of a GRE-reporter gene

LβT2 cells were transfected with 250 ng of TAT-GRE-luciferase construct and 25 ng pSV β-galactosidase. After 24 h cells were incubated for 8 h in serum-free medium with 100 nM Dex and 100 nM GnRH **(A)** or a combination of both **(B)**. Results were normalized for transfection efficiency and expressed relative to vehicle (control). The graphs show combined results from four independent experiments, each performed in triplicate. A one-way ANOVA with Dunnett's

post-test was used for statistical analysis and denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

3.2 Dex increases the GnRH induced transcriptional response on an AP-1 *cis*-element in LβT2 cells

To investigate whether crosstalk between the GR and GnRHR signaling pathways occurs on a minimal AP-1 reporter construct in LβT2 cells, the cells were transfected with an AP-1-luciferase plasmid. The ability of GnRH to activate the AP-1 reporter gene was compared to that of TNFα, a cytokine previously shown to upregulate AP-1-containing genes in other cell lines (Kassel *et al*., 2004; Park *et al*., 2004; De Bosscher and Haegeman, 2009; Van Bogaert *et al*., 2010). TNFα was unable to induce a transcriptional response after 24 h stimulation (Figure 3.2A, bars 2 and 4), while GnRH induced a significant increase in AP-1 reporter activity in a dose-dependent manner (Figure 3.2A, bars 5 and 6). Interestingly, co-stimulation of LβT2 cells with 10 nM GnRH and 100 nM or 1 μM Dex appeared to further enhance transcription, as compared to 10 nM GnRH alone (Figure 3.2B). The results show that Dex significantly increased the GnRH-induced transcriptional response by about 1.6-fold (Figure 3.2B). The above results provide evidence for crosstalk between Dex and GnRH signaling pathways on a minimal synthetic AP-1 *cis*-element in LβT2 cells.

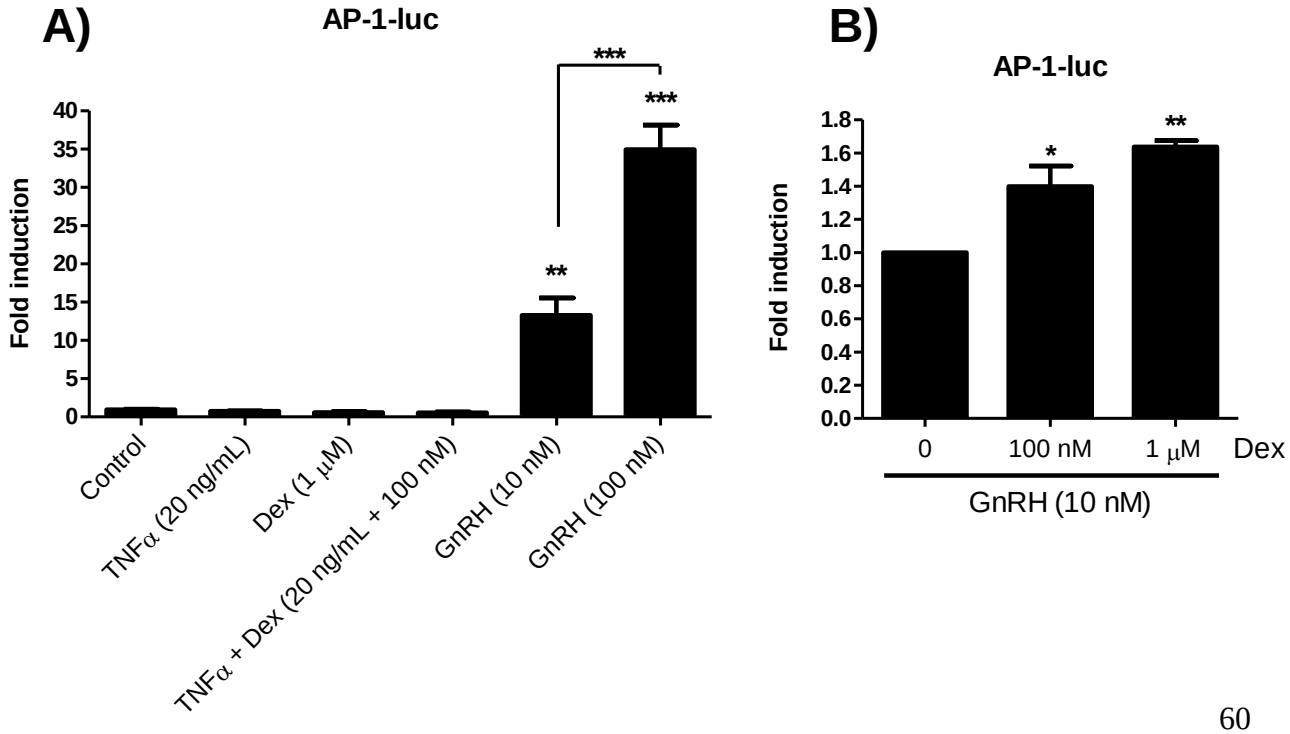


Figure 3.2: Dex increases the GnRH-induced transcriptional response on an AP-1 *cis*-element in LβT2 cells

LβT2 cells were transfected with 250 ng of a pAP-1-luciferase construct and 25 ng pSV β-galactosidase. After 24 h, cells were incubated for 24 h in serum-free medium with hormones, as indicated. Results were normalized for transfection efficiency and expressed relative to vehicle (control). The graphs show combined results from three independent experiments, each performed in triplicate. A one-way ANOVA with Dunnett's and Tukey's (Figure 3.2A, bars 5 and 6) post-tests were used for statistical analysis and denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

3.3 Crosstalk does not occur between the Dex and GnRH signaling pathways on an NF-κB *cis*-element in LβT2 cells

To investigate if crosstalk between the GR and GnRHR signaling pathways occurs on a minimal NF-κB reporter construct in LβT2 cells, the cells were transfected with an NF-κB luciferase plasmid. The results presented in Figure 3.3 show a significant increase of approximately 3-fold in the transcriptional activity after stimulating the cells for 24 h with 20 ng/mL TNFα (Figure 3.3, bar 5), as compared to vehicle (control). Interestingly, treatment with 100 nM GnRH resulted in a similar and statistically significant transcriptional response of about 3-fold relative to vehicle (control) (Figure 3.3, compare bars 3 and 5). Thus, TNFα selectively increased the transcriptional activity of an NF-κB (Figure 3.3, bar 5) but not an AP-1 (Figure 3.2A, bar 2) reporter gene, while both were induced by GnRH. In contrast, addition of 100 nM Dex repressed basal reporter activity (Figure 3.3, compare bars 1 and 2) as well as TNFα-induced transcriptional activity (Figure 3.3, compare bars 5 and 6), while having no effect on the GnRH response (Figure 3.3, compare bars 3 and 4). Co-stimulation with GnRH and TNFα resulted in an additive transcriptional response of the NF-κB reporter gene (Figure 3.3, compare bars 3 and 5 with 7). Furthermore, the additive response of GnRH and TNFα on NF-κB transcription remained unaffected by addition of 100 nM Dex (Figure 3.3, compare bars 7 and 8).

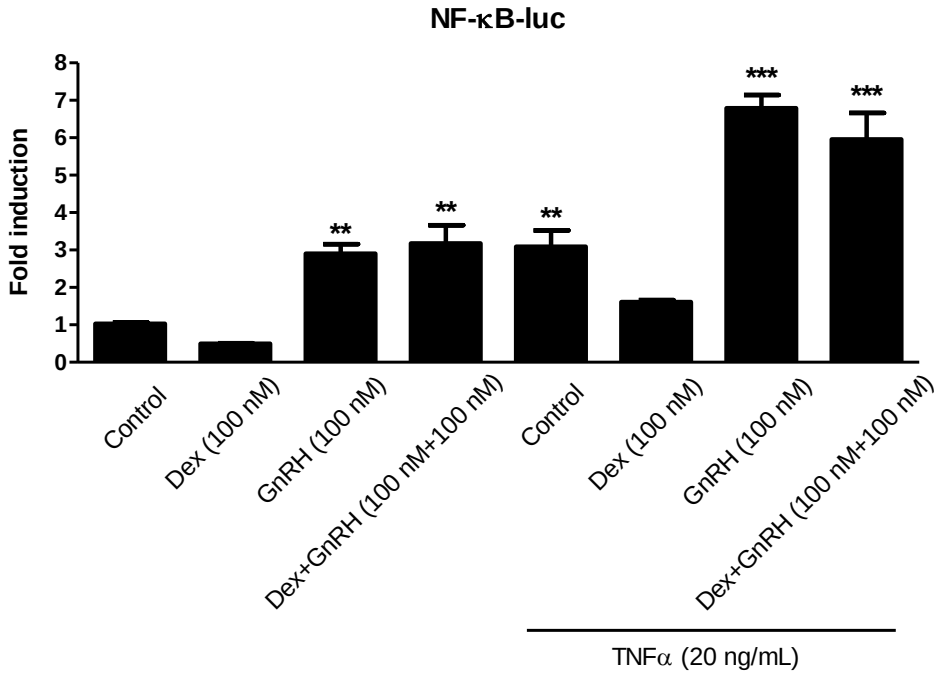


Figure 3.3: Crosstalk does not occur between the Dex and GnRH signaling pathways acting on an NF-κB *cis*-element in LβT2 cells

LβT2 cells were transfected with 400 ng of $(IL\text{-}6\kappa B)_3$-50hu.IL6P-luciferase construct and 25 ng pSV β-galactosidase. After 24 h, cells were incubated for 24 h in serum-free medium with hormones, as indicated. Results were normalized for transfection efficiency and expressed relative to vehicle (control) in the absence of TNFα. The graph shows combined results from three independent experiments, each performed in triplicate. A one-way ANOVA with Dunnett's post-test was used for statistical analysis and denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

3.4 Similar to Dex, GnRH appears to induce GR protein degradation

The GR protein levels are down regulated after ligand binding and transcriptional activation of target genes (Dong *et al*., 1988; Webster *et al*., 1997). To assess if GnRH results in protein degradation of the GR and whether the transcriptional responses observed in Figure 3.2 could result in changes of GR protein expression levels, the cell lysates of the AP-1 gene reporter assay were analyzed by Western blotting. The results shown in Figure 3.4 are from cell lysates analyzed in Figure 3.2A and B, after stimulation for 24 h with 1 μM Dex, 10 or 100 nM GnRH, a

combination of 10 nM GnRH with 100 nM or 1 μM Dex. Stimulation of LβT2 cells with 1 μM Dex for 24 h, appeared to result in a 40% decrease in GR protein expression (Figure 3.4, compare bars 1 and 2). Interestingly, treatment of LβT2 cells with increasing concentrations of GnRH resulted in a decrease of GR protein levels (Figure 3.4, compare bars 1, 3 and 4). Co-stimulation with 10 nM GnRH and varying amounts of Dex appeared to increase the GR protein turnover (Figure 3.4, compare bars 3 with 5 and 6). No statistical significance could be established from two independent experiments. The decrease in GR protein levels with GnRH co-treatment provides further support for crosstalk between the GR and GnRHR signaling pathways in LβT2 cells.

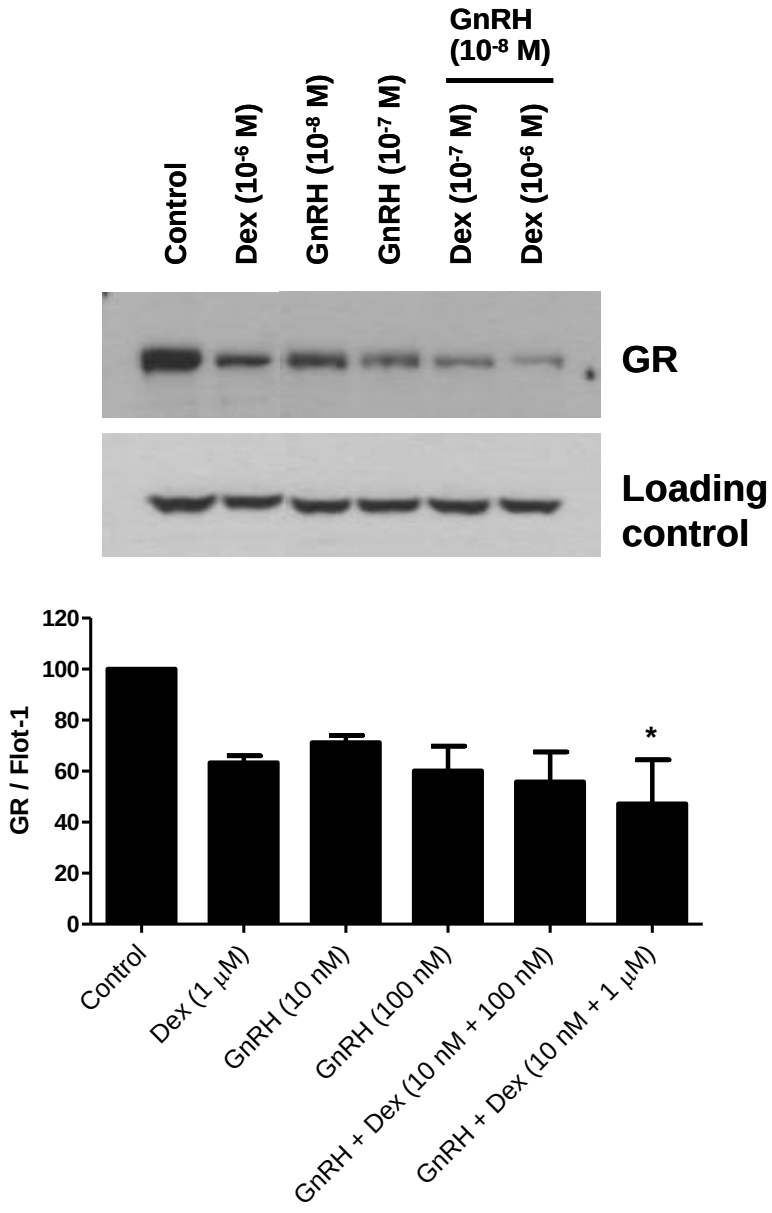


Figure 3.4: Similar to Dex, GnRH appears to induce GR protein degradation

LβT2 cells were incubated with hormone for 24 h in serum-free medium as indicated in the figure. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with antibodies to GR and Flot-1 (loading control). The results on the left panel shows a representative Western blot and the graph shows combined results of two independent experiments. A one-way ANOVA with Dunnett's post-test was used for statistical analysis and denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

3.5 Subcellular localization of the GnRHR and GR with lipid raft markers by immunoflorescence analysis in LβT2 cells

3.5.1 LβT2 cells express the lipid raft marker protein Flot-1 and not Cav-1

Recently, lipid rafts have been implicated in rapid GC signaling and the GR was shown to localize to caveolae in A549 cells (Matthews *et al*., 2008). The GnRHR has been shown to localize exclusively to lipid rafts and to be necessary for activation of the ERK signaling pathway by GnRH in αT3-1 cells (Navratil *et al*., 2003; Bliss *et al*., 2007). It was hypothesized in the present study that the crosstalk between the GR and GnRHR signaling pathways could involve a co-localization of the receptors to lipid rafts in LβT2 cells. To investigate whether LβT2 cells express the lipid raft proteins Flot-1 and Cav-1, Western blot analysis was performed with whole cell LβT2 lysates using specific Flot-1 and Cav-1 antibodies. As shown in Figure 3.5.1, Flot-1 protein expression was detected in LβT2 cells. To confirm the identity of the band, COS-7 cells were transfected with a Flot-1 expression plasmid as a positive control (Figure 3.5.1). In contrast, Cav-1 protein expression was not detected in LβT2 cells while a band of the right size was detected in COS-7 cells (Figure 3.5.1). This result shows that LβT2 cells express a detectable level of endogenous Flot-1 and not Cav-1 protein.

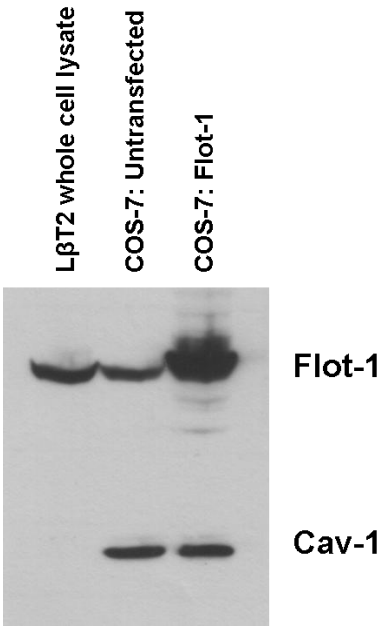


Figure 3.5.1: LβT2 cells express a detectable level of the lipid raft marker protein Flot-1, but not Cav-1

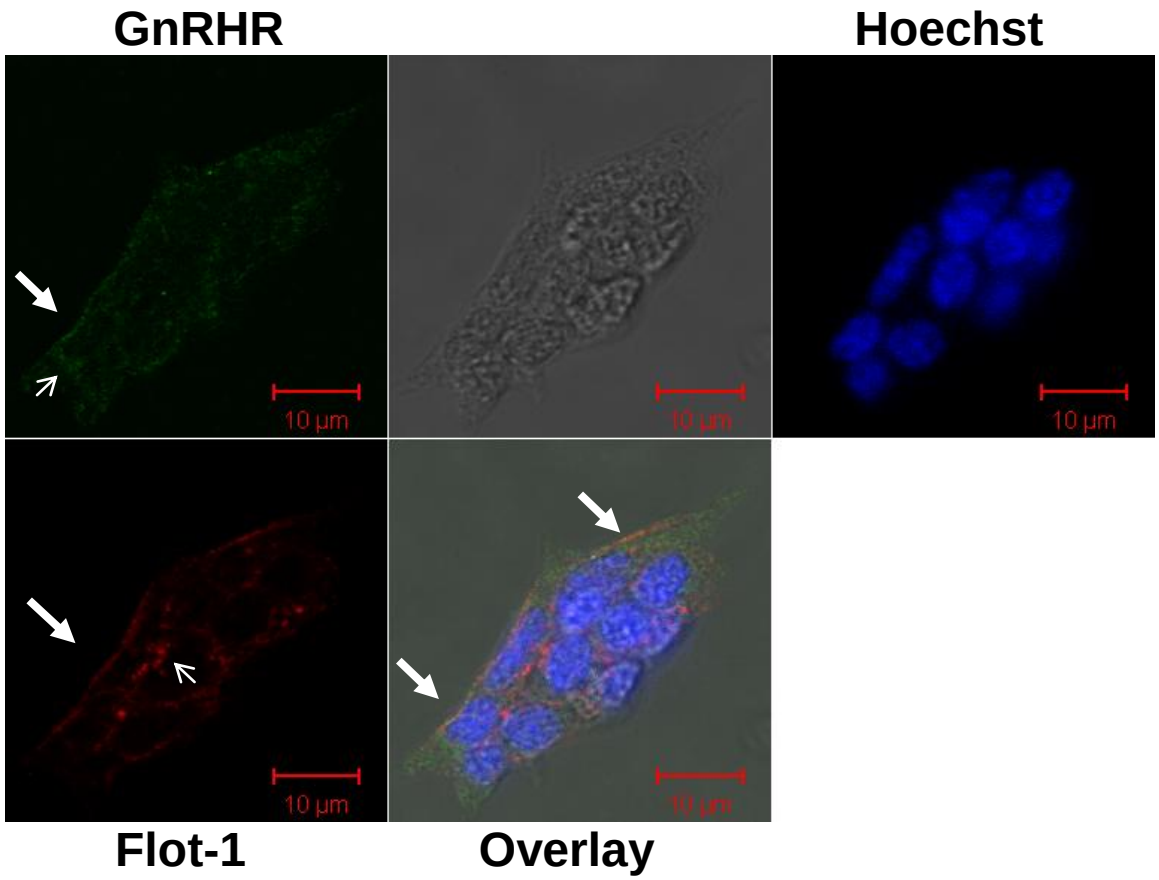
Whole LβT2, untransfected COS-7 and Flot-1 transfected COS-7 cell lysates were loaded on a 10% SDS-PAGE gel, followed by transfer onto nitrocellulose membrane and probing with specific antibodies to Flot-1 and Cav-1.

3.5.2 Immunoflorescence analysis shows that the endogenous GnRHR co-localizes with endogenous Flot-1 at the plasma membrane of LβT2 cells, independent of ligand

In order to investigate protein co-localization of endogenous GnRHR and Flot-1 in LβT2 cells by immunoflorescence using specific primary antibodies and fluorescently labeled secondary antibodies, optimum growth conditions for slide preparation needed to be established. In order to detect expression of proteins in the plasma membrane by immunoflorescence, cells needed to grow as a monolayer. LβT2 cells have a strong tendency to grow in clusters tightly together that makes membrane co-localization difficult to detect. It has previously been shown that many cell lines and primary cells have an increased rate of proliferation and grow in a monolayer when certain coating substrates have been applied to the growth surface (Beck *et al*., 1990; Calof and Lander, 1991). Therefore, the effect on morphology of LβT2 cells growing on uncoated, laminin-

or poly-L-ornithine-coated glass cover slips for two days was investigated. The results from Addendum A show that there was no difference in proliferation or cell morphology between uncoated or laminin coated slides. It also appears that the cells growing on poly-L-ornithine have significant changes in cell morphology with a decreased rate of proliferation, as compared to cells in normal culture conditions. Therefore, LβT2 cells were grown on uncoated glass cover slips for two days for immunoflorescence experiments.

The GnRHR has been shown to localize exclusively to lipid rafts in αT3-1 cells (Bliss *et al*., 2007) and Flot-1 has been shown to interact with the G-protein $G_{\alpha q}$ in HeLa cells (Sugawara *et al*., 2007). The αT3-1 cell line represents precursor gonadotrope cells, whereas the LβT2 cell line represents more mature and differentiated gonadotrope cells (Fernandez-Vazquez *et al*., 1996; Turgeon *et al*., 1996). To investigate whether the endogenous GnRHR co-localizes with endogenous Flot-1 in LβT2 cells, live-cell staining for the receptor was performed followed by fixation and staining for Flot-1. The cells were visualized with a confocal microscope and it was found that Flot-1 was mainly localized to the plasma membrane (large arrow) with some distribution in vesicle-like intracellular compartments (Figure 3.5.2, small arrow). The GnRHR was found to mainly localize to the plasma membrane (large arrow), but a small percentage was also detected in the cytoplasm (Figure 3.5.2, small arrow). This could be due to internalization of the receptor upon antibody binding that could not be avoided. The data was analyzed by over-laying the GnRHR (green) and Flot-1 (red) channels and a significant amount of GnRHR was found to co-localize with Flot-1 at the plasma membrane of LβT2 cells (Figure 3.5.2). Furthermore, the Pearson's and Mander's co-localization coefficient values were close to 1 and the intensity correlation value was near to 0.5, suggesting true co-localization (Figure 3.5.2i). The fluorogram showing the intensity correlation analysis of the co-localization between the GnRHR and Flot-1 displays a diagonal relationship (Figure 3.5.2ii), also suggesting true co-localization. This novel result demonstrating the endogenous GnRHR co-localizing with endogenous Flot-1 in the plasma membrane of LβT2 cells supports a role for Flot-1 in GnRHR signaling.



i)

Pearson's co-efficient	Mander's co-efficient	Intensity correlation analysis
0.979	0.989	0.489

ii) Fluorogram

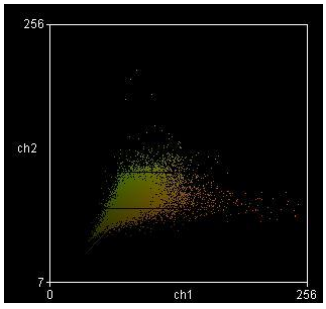


Figure 3.5.2: Immunoflorescence analysis shows that the endogenous GnRHR co-localizes with endogenous Flot-1 at the plasma membrane of LβT2 cells, independent of ligand

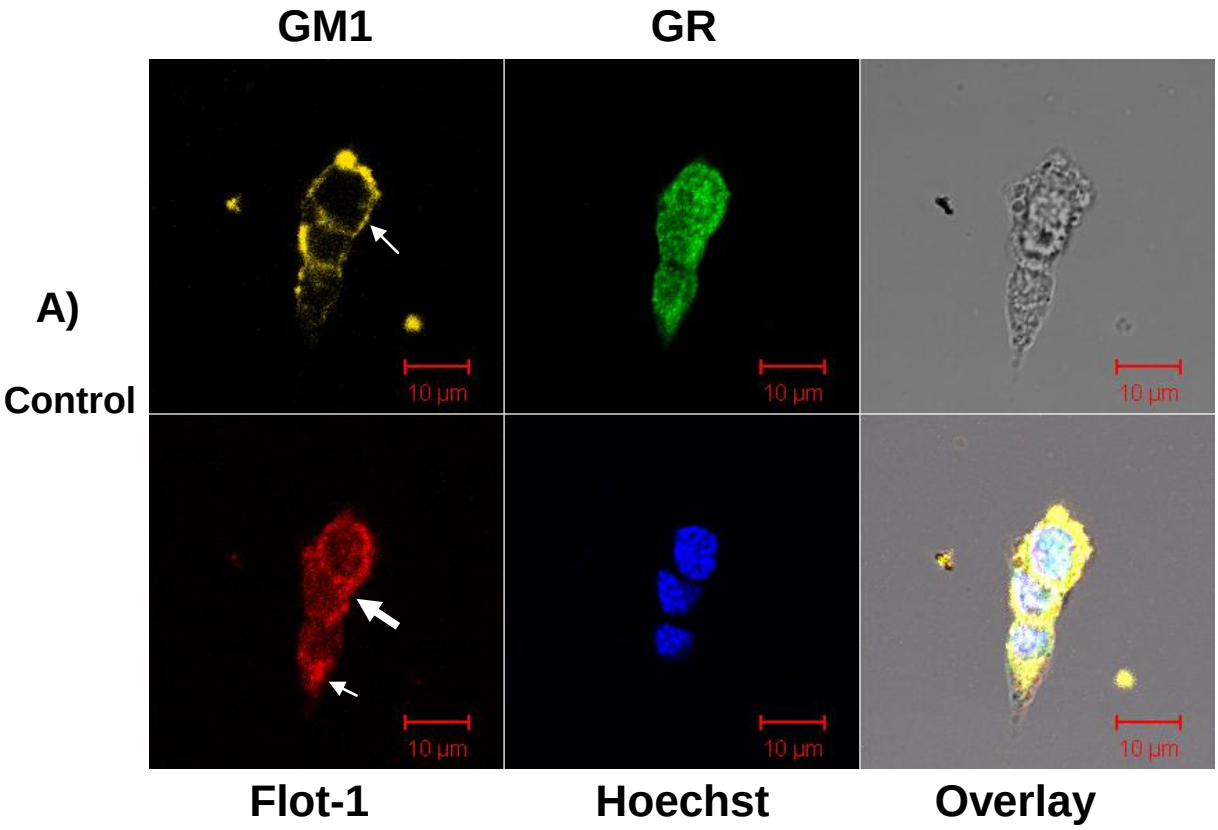
LβT2 cells were grown on glass cover slips and were stained live with rabbit anti-GnRHR (Green) for 1 h, followed by fixation. Slides were stained with mouse anti-Flot-1 (Red) antibody, followed by incubation with anti-rabbit Alexa488 and anti-mouse Cy3 antibody. Nuclei were stained with Hoechst before mounting in Mowiol. Staining was visualized with a Zeiss LSM510 Meta Confocal microscope using the 40X objective and a representative image is shown for a

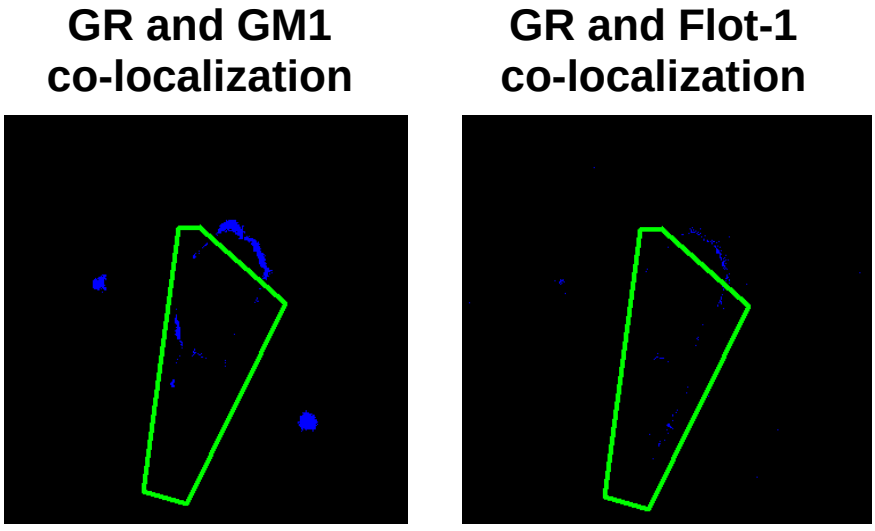
group of cells, as indicated by the multiple nuclei visible in the Hoechst stain. Co-localization analysis was performed as described in Materials and Methods. A representative analysis of co-localization co-efficients **(i)** and a fluorogram **(ii)** is shown. The results shown are representative of three independent experiments. Large and small arrows indicate a localization to the plasma membrane and intra-cellular vesicles, respectively.

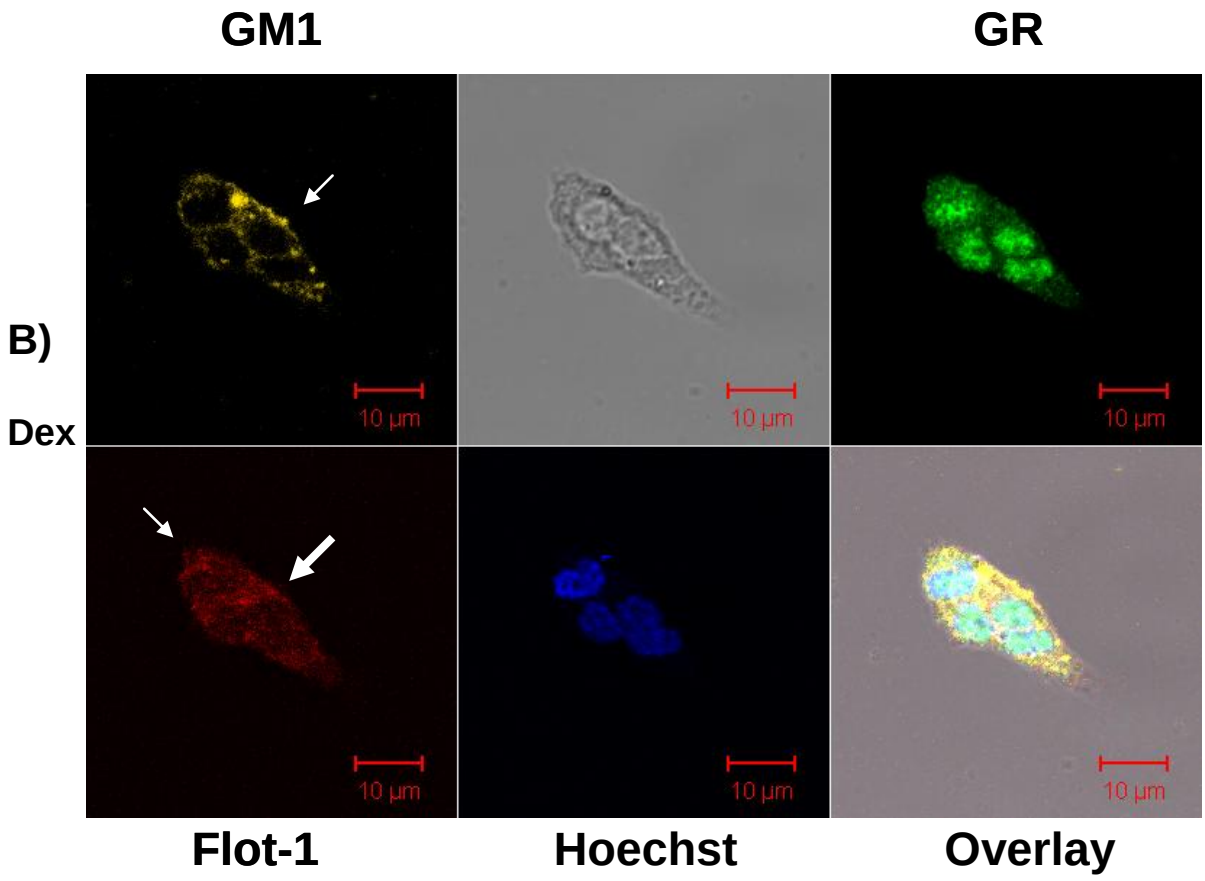
3.5.3 Immunoflorescence analysis shows that a small population of endogenous GR appears to co-localize with endogenous Flot-1 to lipid rafts independent of ligand in LβT2 cells

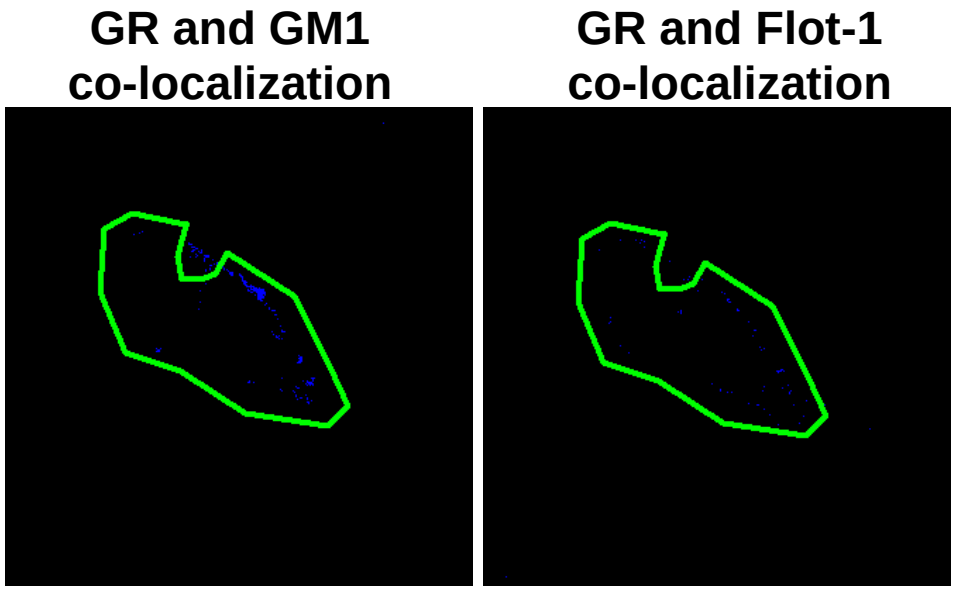
The previous result showed the GnRHR localizes to Flot-1-containing lipid rafts. There is no report in the literature indicating GR localization to lipid rafts in the pituitary, while two studies have indicated its association with caveolae in Hep3B and A549 cells (Jain *et al*., 2005; Matthews *et al*., 2008). This could be a potential mechanism enabling crosstalk between the GR and GnRHR signaling pathways in LβT2 cells shown in Figures 3.1, 3.2 and 3.4. To explore this possibility, LβT2 cells were grown on glass cover slips, stimulated for 30 min with 100 nM Dex, 100 nM GnRH or a combination of both, followed by live-cell staining with cholera toxin B subunit conjugated to Alexa647. The toxin binds specifically to GM1, a ganglioside that is highly enriched in lipid rafts (Janes *et al*., 1999; Harder, 1998). GnRH has previously been reported to induce maximal GR phosphorylation after 30 min in LβT2 cells (Kotitschke *et al*., 2009). Therefore, a time point of 30 min was chosen for all ligand stimulations of LβT2 cells to investigate if lipid rafts are involved in this non-genomic crosstalk mechanism between the GR and the GnRHR. Subsequently, cells were fixed and permeabilized, followed by staining with anti-GR- and anti-Flot-1-specific antibodies. The results presented in Figure 3.5.3 panels A to D show that the GM1 stain is exclusively localized to the plasma membrane (small arrow) and displays a clustering pattern, characteristic of lipid rafts. The stain for Flot-1 displays a partial membrane localization (large arrow) together with intra-cellular compartments (small arrow) (Figure 3.5.3, panels A to D). In untreated cells, the GR appears evenly distributed throughout the cell, with a small percentage co-localizing with GM1 and Flot-1 (Figure 3.5.3A). Treatment with Dex resulted in distinct nuclear translocation of the GR, but a significant amount of GR remained in the cytoplasm with a small percentage co-localizing with GM1 and Flot-1 (Figure 3.5.3, compare panel A with B). When cells were treated with GnRH, there was no clear difference in

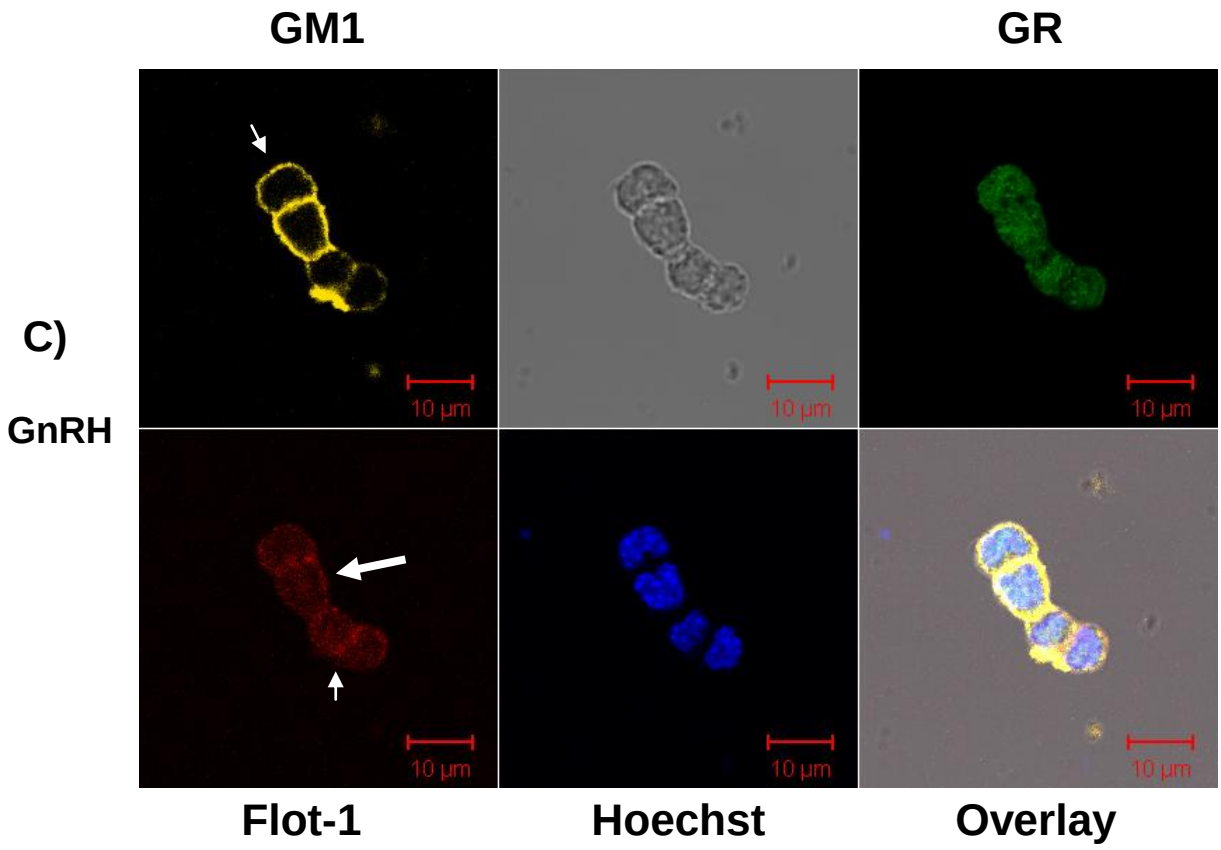
localization of the GR compared to untreated cells after 30 min (Figure 3.5.3, compare panels A and C). Co-stimulation with GnRH had no detectable effect on the amount of Dex-induced nuclear import of the GR (Figure 3.5.3, compare panel A with B and D). However, regardless of the large percentage of nuclear import of the GR, a small population co-localizes with GM1 and Flot-1 (Figure 3.5.3D). Interestingly, co-stimulation with Dex and GnRH resulted in the most GR co-localizing with Flot-1 under all stimulated conditions (Figure 3.5.3, panels A to D). The amount of GR that co-localizes with Flot-1 relative to the remaining GR proved insufficient to perform statistical analysis from three independent experiments, although it is clear that the GR appears to co-localize with Flot-1 in LβT2 cells.

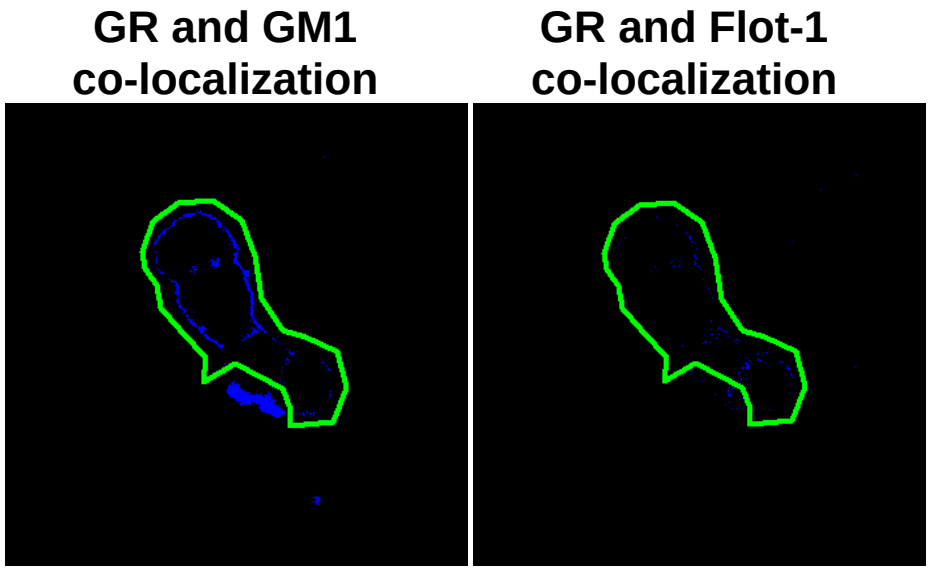

GM1
GR
A)
Control
10 µm
Flot-1
Hoechst
Overlay

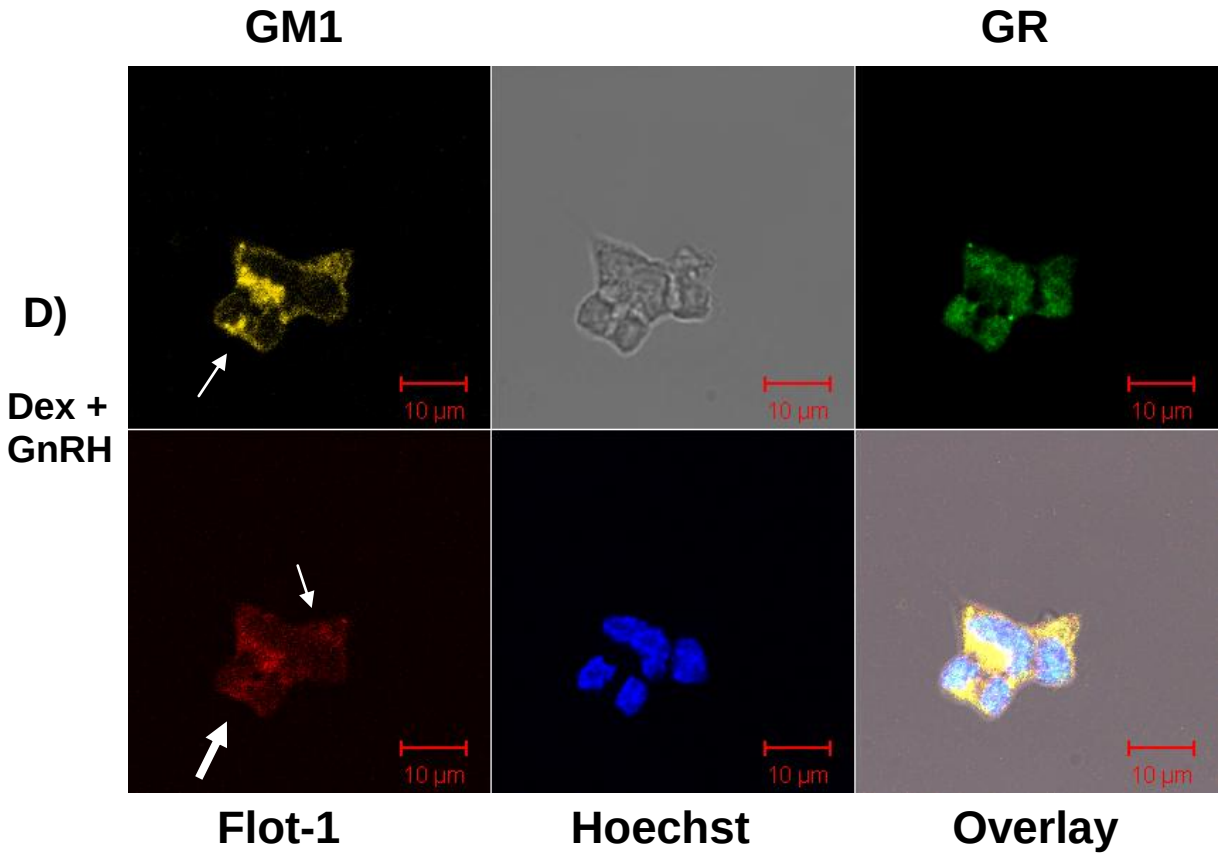

GR and GM1
co-localization
GR and Flot-1
co-localization


GM1
GR
B)
Dex
10 µm
Flot-1
Hoechst
Overlay


GR and GM1
co-localization
GR and Flot-1
co-localization


GM1
GR
C)
GnRH
10 µm
Flot-1
Hoechst
Overlay


GR and GM1
co-localization
GR and Flot-1
co-localization


GM1
GR
D)
Dex +
GnRH
10 µm
Flot-1
Hoechst
Overlay

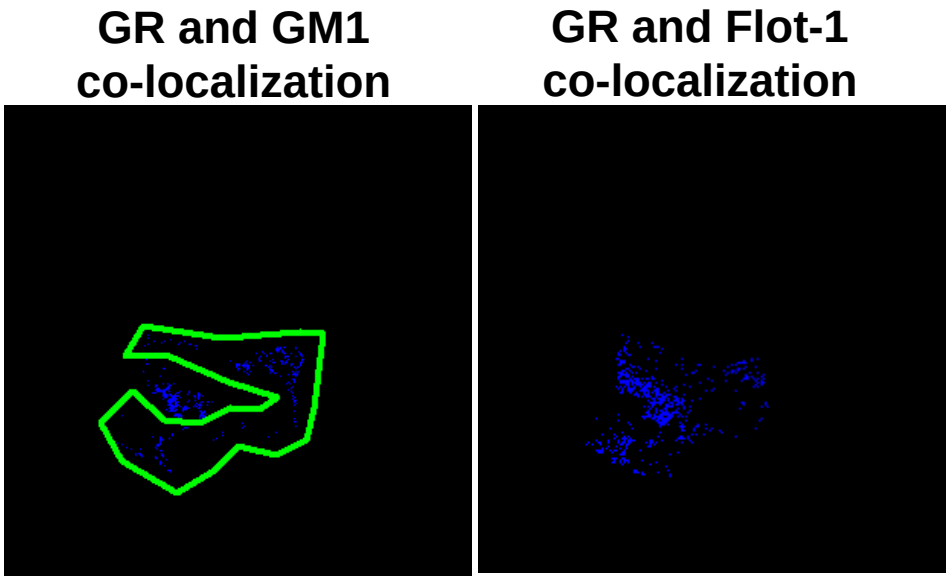

GR and GM1
co-localization
GR and Flot-1
co-localization

Figure 3.5.3: Immunoflorescence analysis shows that the endogenous GR co-localizes with endogenous Flot-1 at the plasma membrane of LβT2 cells, independent of ligand

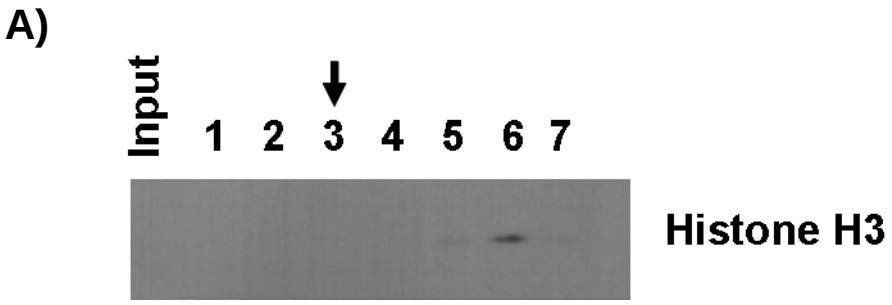
LβT2 cells were grown on glass cover slips and incubated for 30 min in medium containing charcoal-stripped serum (A), or including 100 nM Dex (B), 100 nM GnRH (C) or a combination of both (D). Cells were stained live for GM1 (Yellow) with Cholera Toxin B subunit conjugated to Alexa647 for 30 min before being fixed and permeabilized. Slides were stained with rabbit anti-GR (Green) and mouse anti-Flot-1 (Red) antibodies followed by incubation with anti-rabbit Alexa488 and anti-mouse Cy3 antibodies. Nuclei were stained with Hoechst before mounting in Mowiol. Staining was visualized with a Zeiss LSM510 Meta Confocal microscope using the 40X objective and a representative image is shown for a group of cells, as indicated by the multiple nuclei visible in the Hoechst stain. Co-localization analysis was performed with the LSM510 Meta software as described in Materials and Methods with the area of interest demarcated in green if needed to exclude artifacts resulting from cellular debris. The images in blue show the regions of co-localization between the GR and GM1, or Flot-1, respectively. The results shown are representative of three independent experiments.

3.6 Density-gradient analysis shows that the endogenous GR localizes with the endogenous GnRHR to Flot-1 containing lipid rafts independent of ligand in LβT2 cells

Having shown by immunoflorescence that the GnRHR co-localizes with Flot-1 and the GR appears to localize to lipid rafts at the plasma membrane, a different strategy was persued to provide additional evidence that these receptors localize to lipid rafts in LβT2 cells. Initial experiments were performed to set up a method for the biochemical isolation of lipid rafts in LβT2 cells. The following parameters were investigated to optimize the fractionation of lipid rafts. The use of a non-detergent method (Gagescu *et al*., 2000) and a detergent-resistant method (Lafont and Simons, 2001), different concentrations of Triton X-100 and the use of a crude membrane fraction or whole cell lysates as starting material, were compared. The search criteria were based on the detectable isolation of lipid rafts into minimal fractions with a low-density, as analyzed by Western blotting for the lipid raft marker protein, Flot-1. The results from Addendum B show that the use of a non-detergent-based method did not result in adequate

fractionation of Flot-1, while the detergent-resistant method showed a better degree of fractionation at 0.05% and not 1% Triton X-100. Using whole cell lysates as compared to a crude membrane fraction as starting material in the 0.05% Triton X-100 method dramatically decreased the amount of degraded GR in the lipid raft fraction (compare Addendum B with Figure 3.6). Therefore, it was decided to use whole cell lysates as starting material with the 0.05% Triton X-100 method for the isolation of lipid rafts from LβT2 cells.

To investigate if the GR localizes to lipid rafts in LβT2 cells, whole cells were incubated with 0.05% Triton X-100 before fractionation on a discontinuous sucrose gradient. First, it was important to establish that adequate fractionation was achieved by analyzing the lipid raft fraction for nuclear material. Therefore, the nuclear fraction was identified with the nuclear marker, histone H3. The results from Figure 3.6A show that histone H3 localized only to fractions 5, 6 and 7, which are the most-dense fractions where the starting material was applied. Importantly, no histone H3 could be detected in the lipid raft fraction (Figure 3.6A), indicating the absence of nuclear protein histone H3 in the lipid raft fraction.

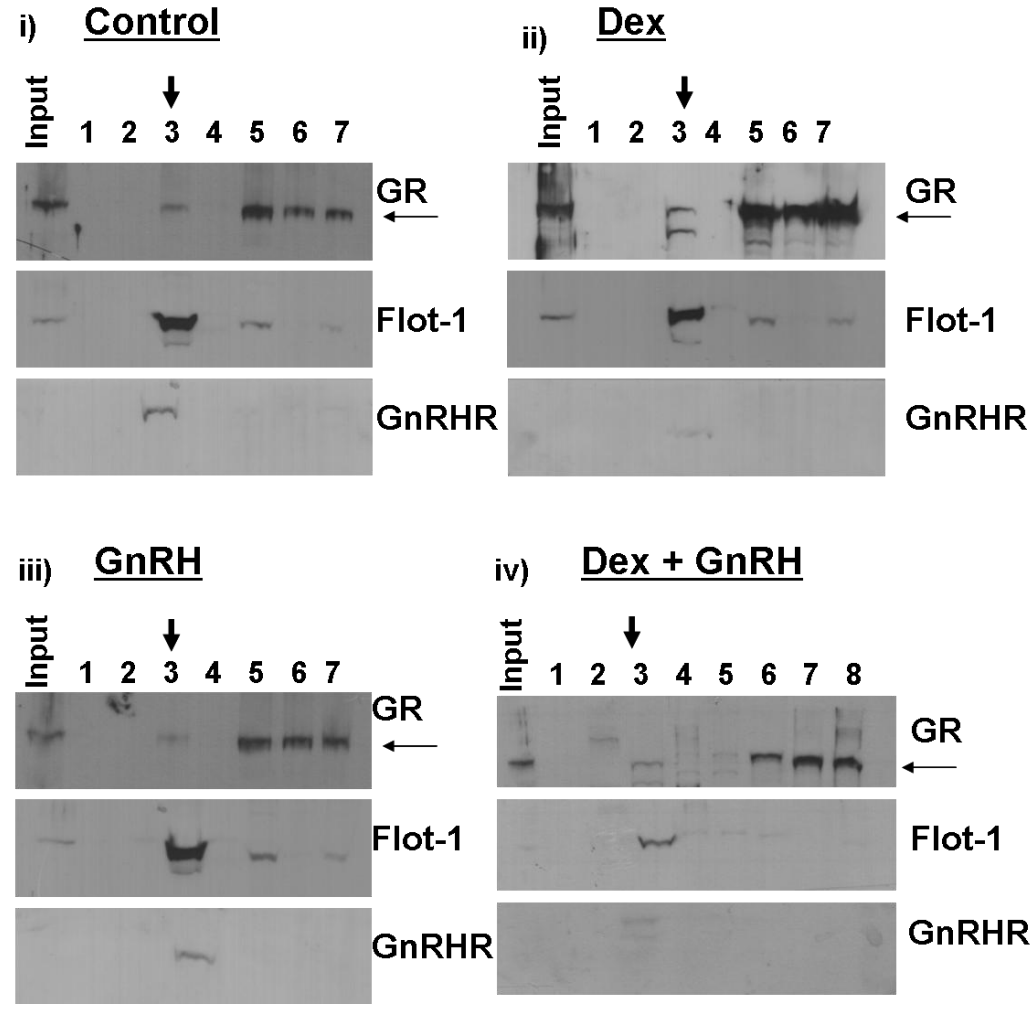


The results from Figure 3.6B show that the majority of Flot-1 is located in fraction 3 (lipid rafts) under basal and all stimulated conditions. A small amount of Flot-1 was detected in fraction 5 under all treated conditions (Figure 3.6B, panels i to iv), which represents the interface of the 60%/43% interface. No Flot-1 could be detected in fraction 6 under all tested conditions (representing a sampling of the region where the cell lysate was made up to 60% and loaded), while a small amount of Flot-1 were detected in fraction 7 under all tested conditions (unsuspended cells and unbroken nuclei) (Figure 3.6B, panels i to iv).

Interestingly, the results suggest that the GnRHR localizes exclusively to Fraction 3 in untreated and under all stimulated conditions (Figure 3.6B, panels i to iv). The results indicate that all of the detectable endogenous GnRHR is located in the lipid raft fraction in LβT2 cells. This is supported by the result from Figure 3.5.2 showing significant co-localization of the GnRHR with Flot-1 in the plasma membrane of LβT2 cells.

A small percentage of GR was detected in the lipid raft fraction in untreated cells (Figure 3.6B, panel i). Despite the resulting nuclear import of the GR with Dex, a small amount of GR was detected in the lipid raft fraction after 30 min treatment with Dex (Figure 3.6B, panel ii). Similarly, a small amount of GR was detected in lipid rafts after 30 min stimulation with either GnRH or co-stimulation with Dex and GnRH (Figure 3.6B, panels iii and iv). No GR was detected in Fraction 4, indicating specific localization of the GR to lipid rafts rather than incomplete separation of the density-gradients (Figure 3.6B, panels i to iv). The remaining percentage of GR fractionated into fractions 5, 6 and 7 in unstimulated cells and under all stimulated conditions (Figure 3.6B, panels i to iv), which is the 60% sucrose fraction of the gradient where the cell lysate was applied and contains all cellular material excluding the low-density membrane fractions. To quantify the relative extent of GR recruitment to lipid rafts under hormonal stimulation, the lipid raft GR protein levels were expressed relative to lipid raft Flot-1 protein levels (Figure 3.6B, panel v). The results show the level of GR localized to lipid rafts is independent of short exposures to Dex, GnRH or a combination of both (Figure 3.6B). This is in agreement with the result from Figure 3.5.3 showing a small percentage of GR co-localizing with Flot-1 and GM1 at the plasma membrane of LβT2 cells independent of hormone treatment (Figure 3.5.3, panels A to D).

Figure 3.6 B


i) Control
Input
1 2 3 4 5 6 7
GR
Flot-1
GnRHR
ii) Dex
Input
1 2 3 4 5 6 7
GR
Flot-1
GnRHR
iii) GnRH
Input
1 2 3 4 5 6 7
GR
Flot-1
GnRHR
iv) Dex + GnRH
Input
1 2 3 4 5 6 7 8
GR
Flot-1
GnRHR

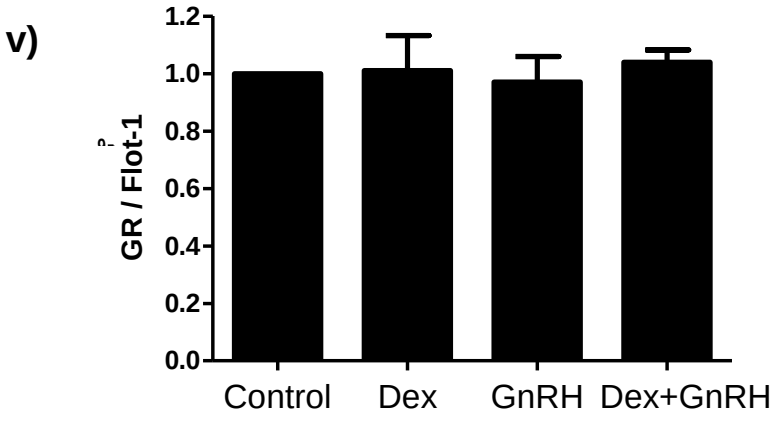

v)
GR / Flot-1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Dex
GnRH
Dex+GnRH

Figure 3.6: Density-gradient analysis shows the endogenous GR localizes with the endogenous GnRHR to Flot-1 containing lipid rafts independent of ligand in LβT2 cells

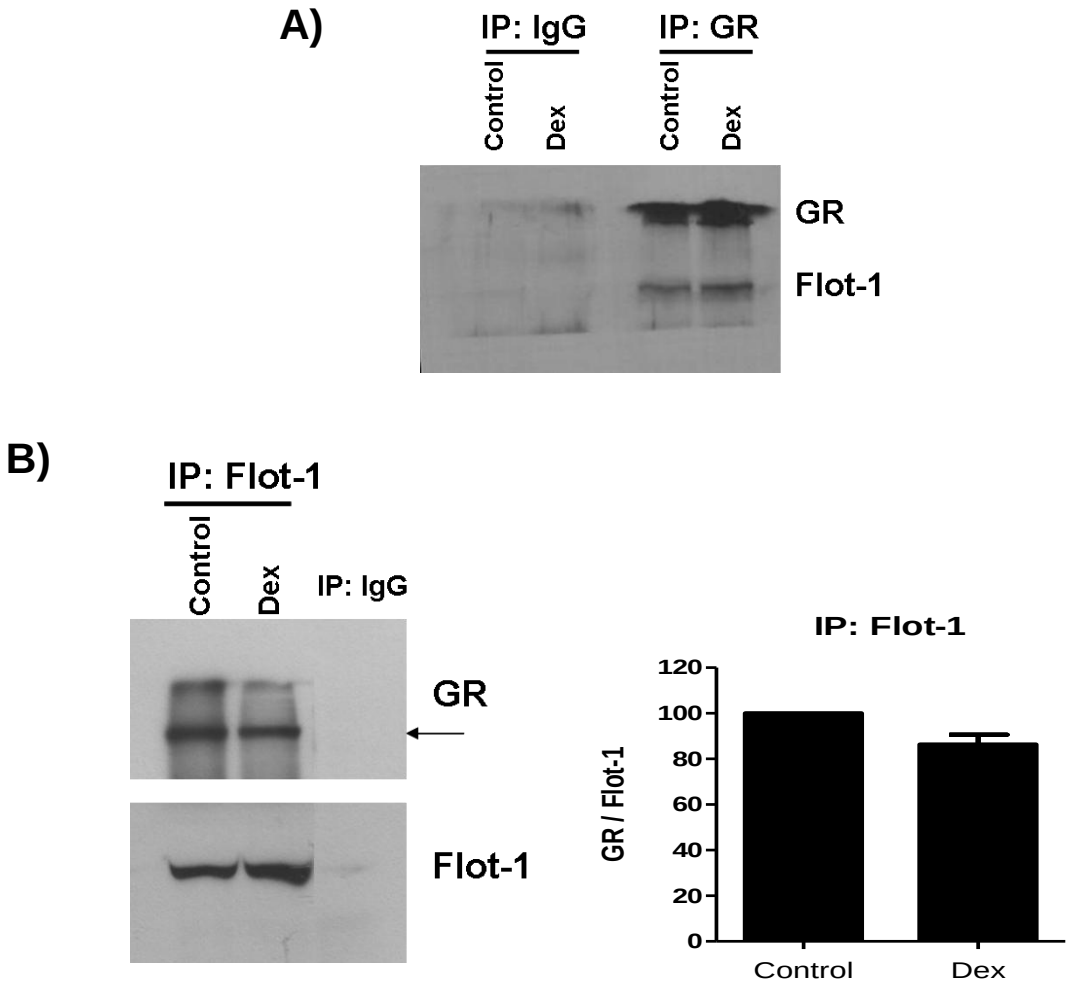
A. Lipid rafts were isolated by ultracentrifugation employing the detergent resistant flotation strategy in a discontinuous sucrose density gradient consisting of 2 mL ELB, 4 mL of 13% sucrose in ELB, 4 mL of 43% sucrose in ELB and 4 mL of 60% (the sample). Seven 1 mL fractions were collected: 1) Top of the gradient, 2) ELB/13% interface, 3) 13%/43% interface (lipid raft fraction), 4) middle of 43% sucrose, 5) 43%/60% interface, 6) middle of 60% sucrose (middle of loading fraction containing unbroken nuclei and unsuspended cells) and fraction 7) the pellet. Samples were prepared as described in Materials and Methods. Equal amounts of fraction samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with an antibody against histone H3. The Western blot shown is representative of three independent experiments. Fraction 3 is considered the lipid raft fraction as indicated with an arrow. **B. i - iv.** LβT2 cells were serum-starved over night and incubated for 30 min in serum-free medium (panel i), including 100 nM Dex (panel ii), 100 nM GnRH (panel iii) or a combination of both (panel iv). Lipid rafts were isolated as described in A. Equal amounts of fraction samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed successively with antibodies against GR, Flot-1 and GnRHR. Results shown in panels (i) to (iv) are single Western blots that are representative of three independent experiments. The lower band detected in panel (ii) from the GR Western blot resulted from partial degradation of the receptor. The detectable band in fraction 2 of panel (iv) in the GR Western blot has a larger size than the GR and is most likely a non-specific band. The GR protein levels were normalized against Flot-1 protein levels for each stimulated condition from a single gel and expressed relative to vehicle (control) (iv). The graph shows combined results of three independent experiments as performed in B, starting with a completely new batch of cells for each experiment.

3.7 Co-immunoprecipitation assays show the *in vitro* interaction between the endogenous GR and endogenous Flot-1 in a ligand-independent manner in LβT2 cells

The results from Figure 3.6B show that endogenous GR localizes with endogenous GnRHR in Flot-1 containing lipid rafts. Furthermore, the localization appears to be independent of short exposures to 100 nM Dex, 100 nM GnRH or a combination thereof. The GR has been shown to localize to caveolae through a physical interaction with Cav-1 in A549 cells (Matthews *et al*., 2008). To investigate if a similar mechanism underlies the GR localization to lipid rafts in LβT2 cells, co-immunoprecipitation assays were performed with whole LβT2 cell lysates and an anti-GR antibody. Remarkably, the results show that Flot-1 was co-immunoprecipitated with the unliganded GR (Figure 3.7A). Furthermore, treating the cells with 100 nM Dex for 30 min had no effect on the interaction of Flot-1 with the endogenous GR (Figure 3.7A). Importantly, there was no GR or Flot-1 that co-immunoprecipitated with the non-specific IgG under basal or Dex-treated cells (Figure 3.7A), suggesting that the interaction of Flot-1 with the GR is specific.

It was important to confirm this association of the GR with Flot-1 in LβT2 cells by reciprocal immunoprecipitation with a Flot-1 antibody. When Flot-1 was precipitated from whole-cell lysates with anti rabbit polyclonal antibody, co-immunoprecipitation of the GR occurred independent of Dex treatment (Figure 3.7B). There was no statistically significant differential interaction of Flot-1 with the GR in the presence of Dex for 30 min as compared to unstimulated cells from pooled results of two independent experiments (Figure 3.7B). It is important to note that no precipitated GR proteins were detected when a mouse monoclonal Flot-1 antibody was used in the co-immunoprecipitation assay (data not shown). A possible explanation could be that the immunogen used to raise the monoclonal anti-Flot-1 antibody contains the domain of Flot-1 required for an interaction with the GR. The monoclonal antibody was raised to the last 116 C-terminal residues of the 428 amino acid Flot-1 protein. Therefore, the antibody recognizes a significant region of the protein, which could be masked by a GR-Flot-1 complex allowing no access for that specific antibody. The result suggests that the GR possibly interacts with the C-terminal domain of Flot-1.

In order to investigate if treatment with GnRH alone or with Dex plus GnRH could result in a differential interaction of Flot-1 with the GR in LβT2 cells, the GR was immunoprecipitated from whole cell lysates with an anti-GR antibody. In agreement with the results from Figure 3.7A, a similar amount of Flot-1 co-immunoprecipitated with the GR under basal and Dex-treated conditions (Figure 3.7C). Stimulation with GnRH or co-stimulation with Dex and GnRH did not result in a differential interaction of Flot-1 with the GR from three independent experiments (Figure 3.7C). The results from Figure 3.7 together with results shown in Figure 3.5 and 3.6 strongly suggest that the co-localization of the endogenous GR with Flot-1 in lipid rafts at the plasma membrane of LβT2 cells could be mediated by a mechanism involving a physical interaction that is independent of ligand.



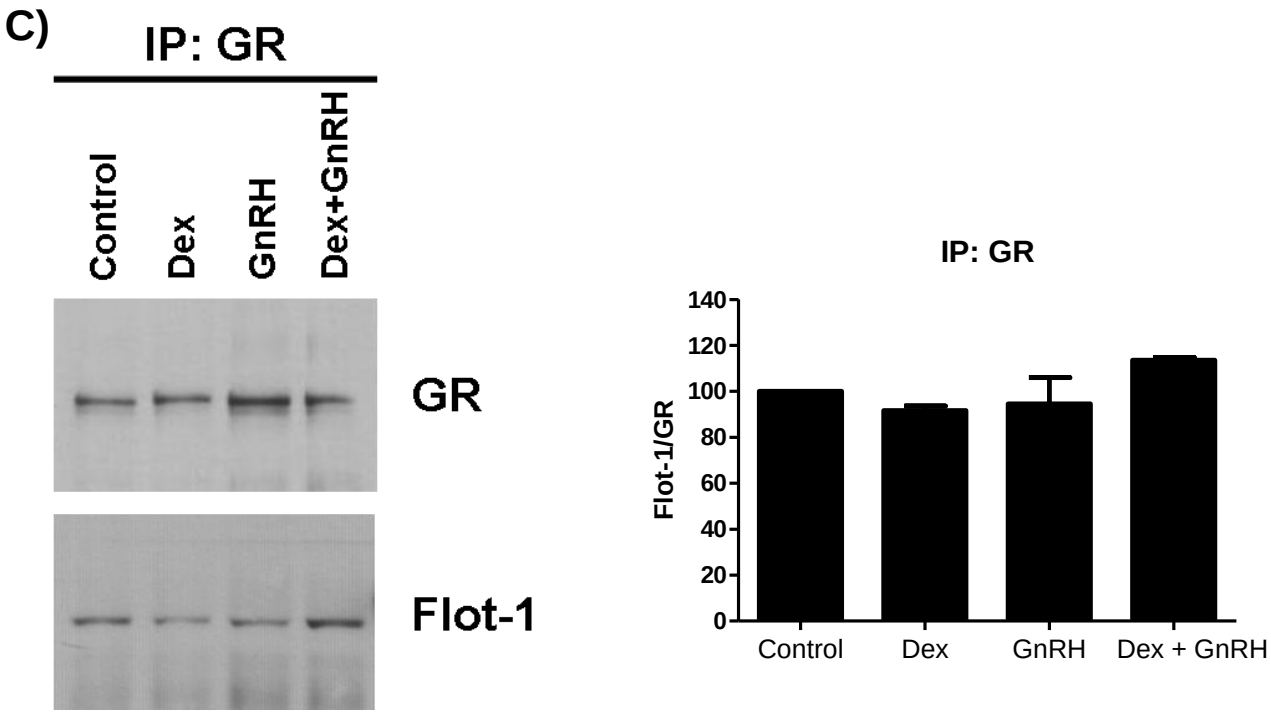
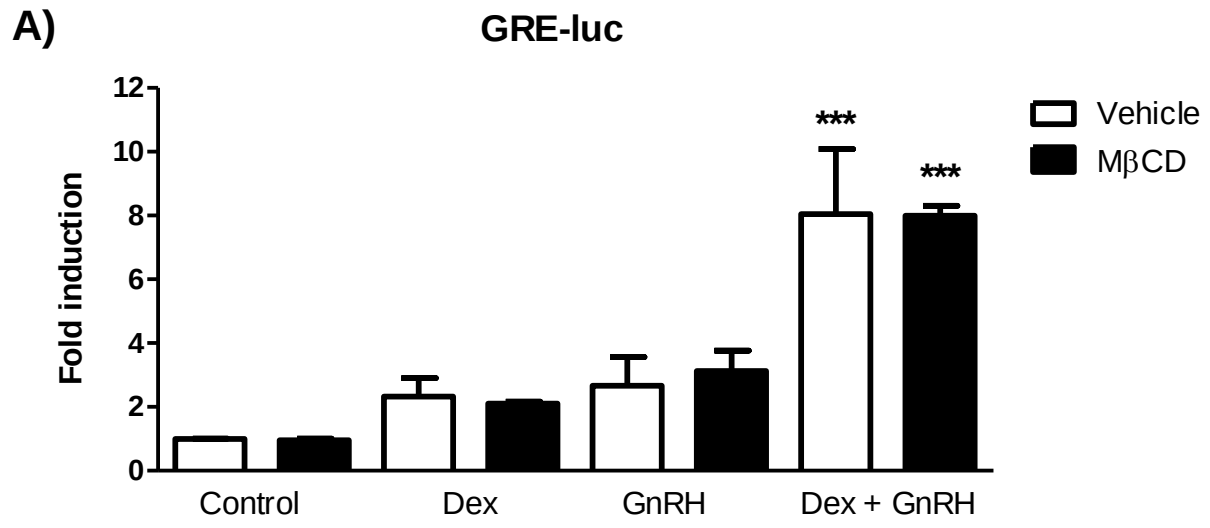


Figure 3.7: Co-immunoprecipitation assays show the *in vitro* interaction between the endogenous GR and endogenous Flot-1 in a ligand-independent manner in LβT2 cells

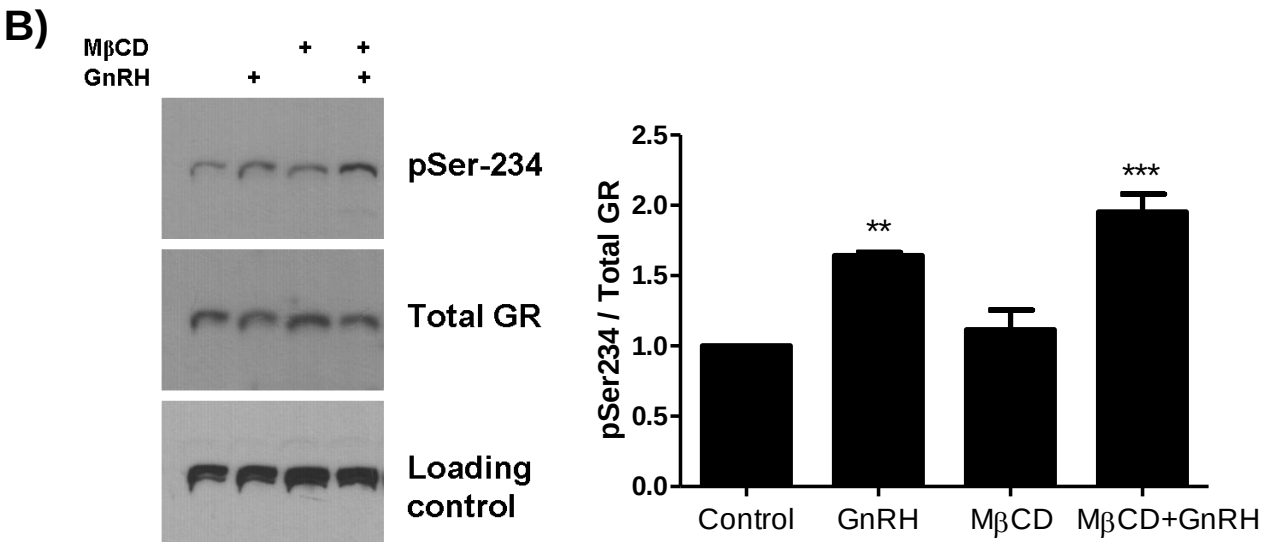
A. LβT2 cells were incubated in serum-free medium for 2 h before addition of 100 nM Dex for 30 min. Equal amounts of cell lysates were incubated with GR or non-specific IgG antibody followed by precipitation with Protein A/G beads. The samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed separately with anti-GR- and anti-Flot-1-specific antibodies. **B.** LβT2 cells were incubated in serum-free medium for 2 h before addition of 100 nM Dex for 30 min. Equal amounts of cell lysates were incubated with a rabbit anti-Flot-1 or non-specific IgG antibody followed by precipitation with Protein A/G beads. The samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with anti-GR- and anti-Flot-1-specific antibodies. The panel on the left shows a single representative Western blot and the graph shows combined results of two independent experiments where vehicle (control) was set to 100%. **C.** LβT2 cells were incubated in serum-free medium for 2 h before addition of 100 nM Dex, 100 nM GnRH or both together for 30 min. Co-immunoprecipitation was performed as in A. The panel on the left shows a single representative Western blot and the graph shows combined results of three independent experiments where vehicle (control) was set to 100%.

3.8 Results using cholesterol depletion agents suggest that lipid rafts are not required for the hormone-induced transcriptional response on a GRE or the GC-independent phosphorylation of the GR in LβT2 cells

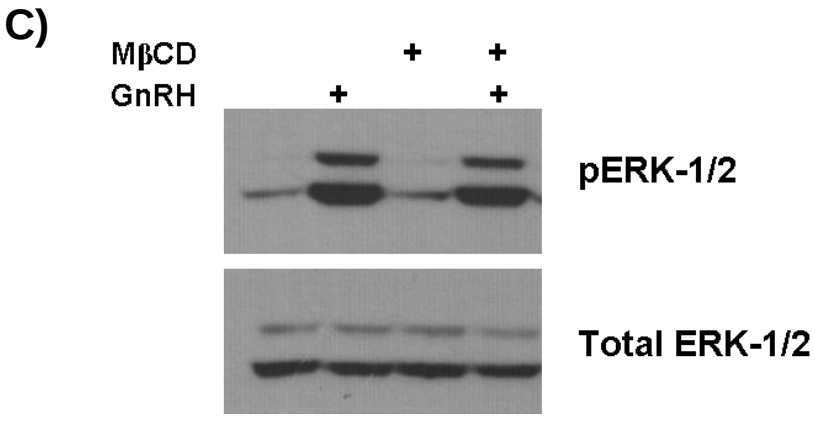
Having established that a small amount of GR localizes at the plasma membrane in a complex with Flot-1, it was next investigated whetheher lipid rafts play a functional role in GR signaling. LβT2 cells were transfected with a GRE-luciferase plasmid and incubated continuously for 8 h with MβCD in the presence of 100 nM Dex or 100 nM GnRH. The results indicate that the continuous treatment with 2% MβCD (approximately 15 mM) for 8 h dramatically decreased the basal, Dex- and GnRH-induced transcriptional activity of the GRE-reporter gene in LβT2 cells, while also dramatically decreasing the expression of the constitutive β-galactosidase gene (Addendum C). In addition, the 8 h incubation with MβCD resulted in significant changes in the morphology of LβT2 cells (data not shown). Taken together, these findings suggests that continuous treatment with MβCD results in cell death, which prevents the use of this method for the investigation of a role for lipid rafts in GR transactivation in LβT2 cells. Therefore, a different strategy involving incubation of cells for 45 min with MβCD, followed by washing of the cells with PBS before incubation with ligand, was investigated (Navratil *et al*., 2003). LβT2 cells were pretreated with 10 mM MβCD for 45 min and the cholesterol extraction drug was washed out, followed by stimulation for 8 h with 100 nM Dex, 100 nM GnRH or a combination of both. Importantly, the constitutive expression of the β-galactosidase gene was unaffected in cells that were incubated with MβCD, as compared to cells not incubated with MβCD (data not shown). The results from Figure 3.8A show that Dex increased the transcriptional activity of the reporter gene by about 2-fold and the increased GRE reporter activity was unaffected by MβCD (Figure 3.8A). Similarly, GnRH increased the transcriptional activity of the reporter gene by about 2-fold, which remained unchanged by MβCD treatment (Figure 3.8A). Co-stimulation of the cells with Dex and GnRH resulted in a synergistic increase of the GRE reporter gene of about 8-fold (Figure 3.8A). However, the synergistic transcriptional response with Dex and GnRH also remained unaffected in the presence of MβCD (Figure 3.8A). The results suggest that lipid rafts are not required for GR-mediated transactivation of a GRE by Dex, GnRH or both together.



Having established that the disruption of lipid rafts with MβCD appeared to have no effect on the GnRH-induced transcriptional response on a GRE, a possible role for lipid rafts in site-specific phosphorylation of the GR by GnRH was investigated. Therefore, LβT2 cells were treated with 10 mM MßCD for 45 min, after which the cholesterol extraction drug was washed out, followed by incubation with 100 nM GnRH for 1 h. Equal amounts of cell lysates were separated on SDS-PAGE, followed by Western blotting with an antibody specific for phospho-Ser234 GR. The results from Figure 3.8B show that GnRH significantly increased the phosphorylation status of the endogenous GR at Ser234 by about 1.5-fold (Figure 3.8B). Furthermore, no difference was detected in the basal phosphorylation status of the GR in the presence of MßCD (Figure 3.8B). Importantly, treatment with MßCD had no effect on the phosphorylation status of the GR in the presence of GnRH at Ser234 (Figure 3.8B). Therefore, the results from Figure 3.8B suggest that disruption of lipid rafts with MßCD has no effect on the ligand-independent phosphorylation of the GR with GnRH at Ser234.



It must be noted that there is no evidence to prove that the lipid rafts were disrupted in the above experiments. It has previously been shown that the GnRH-induced activation of ERK-1/2 was abolished when αT3-1 cells were treated with MβCD (Navratil *et al*., 2003; Bliss *et al*., 2007). In the same study it was shown that GnRH-induced activation of ERK-1/2 requires intact lipid rafts, since it was established that MβCD extracted cellular cholesterol and the GnRH-induced activation of ERK-1/2 was restored by cholesterol repletion (Navratil *et al*., 2003). Therefore, the LβT2 cell lysates from Figure 3.8B were analyzed by Western blotting for ERK-1/2 activation. However, the results in Figure 3.8C show MβCD had no effect on the GnRH-induced phosphorylation of ERK-1/2 (Figure 3.8C). Thus, this result suggests that lipid rafts were not disrupted with MβCD in LβT2 cells and therefore, the results from Figure 3.8A and B are inconclusive.



There is one report in the literature suggesting inhibition of Dex-induced transactivation of a GRE-reporter mediated via the GR with continuous treatment of Hep3B cells with Filipin-III (Jain *et al*., 2005). Filipin-III is a potentially less toxic cholesterol-binding drug that can be continuously included during stimulation and is a lipid raft disrupting agent. Thus, to investigate a role for lipid rafts in Dex-induced transactivation of a GRE via the GR, LβT2 cells were transfected with a GRE-luciferase plasmid and pre-treated for 30 min with 5 ng/mL Filipin-III, followed by stimulation for 8 h with 100 nM Dex in the presence or absence of Filipin-III. Similar to results obtained previously, stimulation with Dex resulted in a significant 6-fold increase in the transcriptional activity of the reporter gene compared to vehicle treated cells (Figure 3.8D). However, treatment with Filipin-III had no effect on the basal or Dex-induced transcriptional response (Figure 3.8D). Taken together the results from Figure 3.8 suggest that the disruption of lipid rafts has no effect on the Dex- or GnRH-induced transcriptional responses on a GRE-reporter gene in LβT2 cells. However, no direct evidence exists for the disruption of lipid rafts with the cholesterol depletion agents used in the present study. In addition, the finding that MβCD treatment did not inhibit GnRH-induced phosphorylation of ERK-1/2 in LβT2 cells, suggests that the integrity of lipid rafts was not disrupted with MβCD in LβT2 cells. Thus, the role of lipid rafts in GR signaling could not be established using these cholesterol depletion agents.

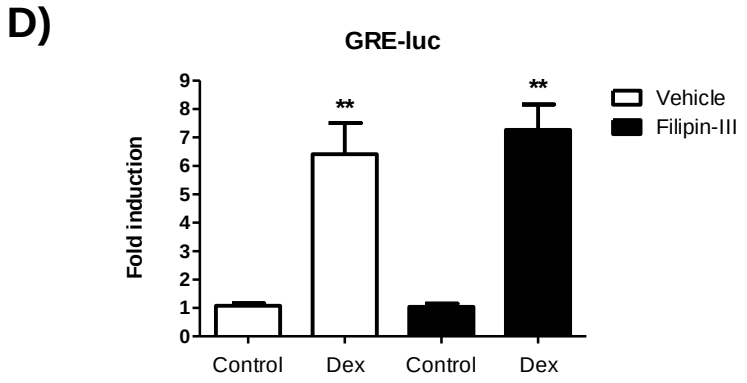


Figure 3.8: Cholesterol depletion of cells suggest that lipid rafts are not required for the hormone-induced transcriptional response on a GRE or the GC-independent phosphorylation of the GR in LβT2 cells.

A. LβT2 cells were transfected with 250 ng of TAT-GRE-luciferase construct and 25 ng pSV β-galactosidase. After 24 h cells were incubated for 45 min with 10 mM MβCD in serum-free medium, followed by one PBS wash and incubation in serum-free medium for 8 h with 100 nM

Dex or 100 nM GnRH or a combination of both. Results were normalized for transfection efficiency and expressed relative to vehicle (control) without MβCD. The graph shows combined results from three independent experiments, each performed in triplicate. **B.** LβT2 cells were incubated for 45 min with 10 mM MβCD in serum-free medium, followed by one wash with PBS and incubated in serum-free medium for 1 h with 100 nM GnRH. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel and transferred to nitrocellulose membrane and probed with specific antibodies against pSer234 GR, total GR and GM130 (loading control). The pSer234 GR levels were normalized to total GR and expressed relative to vehicle (control). The panel on the left shows a single representative Western blot and the graph shows combined results of three independent experiments expressed relative to vehicle (control). **C.** As in B, except the membrane was probed with specific antibodies against pERK-1/2 and total ERK. The Western blot shown is representative of two independent experiments. **D.** LβT2 cells were transfected with 250 ng of TAT-GRE-luciferase construct and 25 ng pSV β-galactosidase. After 24 h, cells were pre-treated with 5 ng/mL Filipin-III for 30 min in serum-free medium, followed by incubation for 8 h with addition of 100 nM Dex. Results were normalized for transfection efficiency and expressed relative to vehicle control without Filipin-III. The graph shows combined results from three independent experiments, each performed in triplicate. A one-way ANOVA with Dunnett's post-test was used for statistical analysis and denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

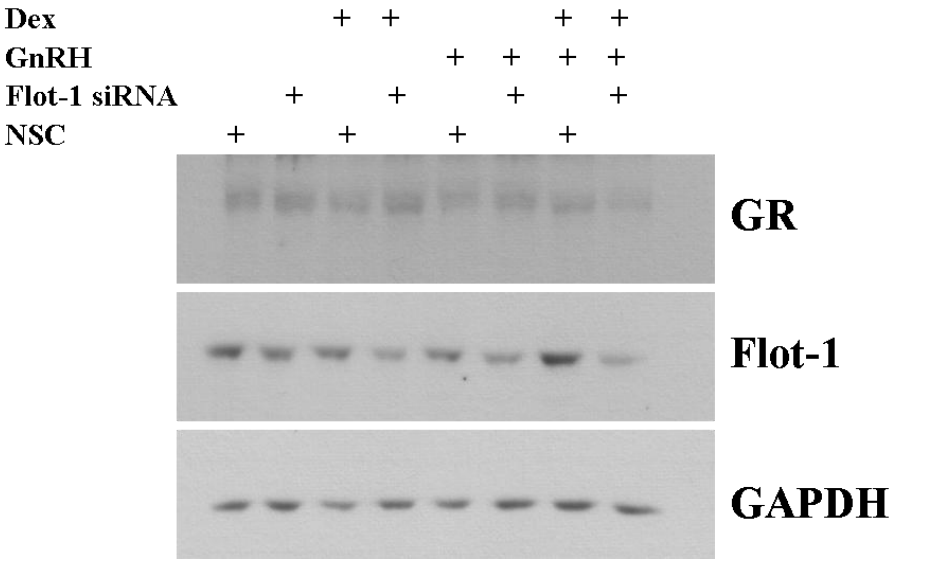
3.9 Flot-1 is required for Dex-induced transcriptional response and the synergistic transcriptional response with Dex and GnRH on a GRE in LβT2 cells

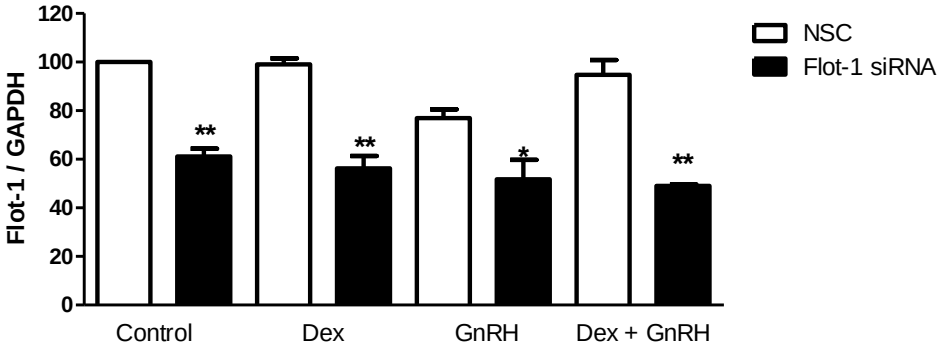
The results from the cholesterol depletion assays in Figure 3.8 were inconclusive, hence another approach was needed to investigate a potential role for lipid rafts in the transcriptional response of the GR on a GRE-reporter gene in LβT2 cells. Therefore, a role for Flot-1 in transcriptional activity of the GR on a GRE-reporter was investigated by specific knockdown of Flot-1 protein expression. To decrease Flot-1 protein expression in LβT2 cells, cells were transfected with siRNA specific for mouse Flot-1 or non-silencing scrambled siRNA (NSC) as control. To obtain an approximate 50% decrease in Flot-1 protein levels, the siRNA transfection conditions was

optimized for LβT2 cells, including different siRNA oligonucleotides sequences, time of incubation with siRNA and concentration of siRNA required. The results showed that there was no distinct difference between the four different oligonucleotides investigated with respect to the amount of Flot-1 knockdown (Addendum, Figure D1). However, a greater level of Flot-1 knockdown was achieved after 72 h incubation compared to 48 h incubation with a combination of all four siRNA oligonucleotides (Addendum, Figure D1). In addition, incubating the cells for 72 h with a combination of all four siRNA oligonucleotides at final concentration of 40 nM appeared to result in more Flot-1 knockdown as compared to using a final concentration of 20 nM siRNA (Addendum, Figure D2). Thus, for knockdown experiments of Flot-1 in LβT2 cells, an equimolar combination of all four siRNA oligonucleotides was used for 72 h at a final concentration of 40 nM.

To investigate if Flot-1 is required for the transactivation of a GRE-reporter gene in LβT2 cells, cells were transfected with Flot-1 siRNA as described above, followed by transfection with a GRE-luciferase plasmid and stimulation for 8 h with 100 nM Dex, 100 nM GnRH or both together. To validate the efficiency of Flot-1 knockdown in the siRNA-transfected cells, an aliquot of the GRE-reporter cell lysates was analyzed by Western blotting. The results from Figure 3.9A show that Flot-1 protein expression was decreased by approximately 50% for all stimulated conditions in the siRNA-transfected cells as compared to NSC in several repeat experiments (Figure 3.9A). In addition, the effect of decreased Flot-1 protein levels on the stability of the GR protein was investigated for all stimulated conditions. The results from Figure 3.9B show that knockdown of Flot-1 protein expression appears to have no effect on the degradation of the GR under basal and all stimulated conditions, suggesting that Flot-1 is not involved in the degradation of unliganded- or liganded-GR.

A)


Dex + + + +
GnRH + + + +
Flot-1 siRNA + + + +
NSC + + + +
GR
Flot-1
GAPDH


Flot-1 / GAPDH
120
100
80
60
40
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0
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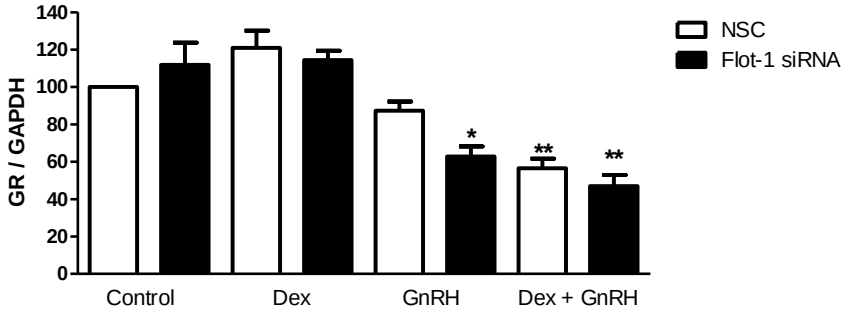
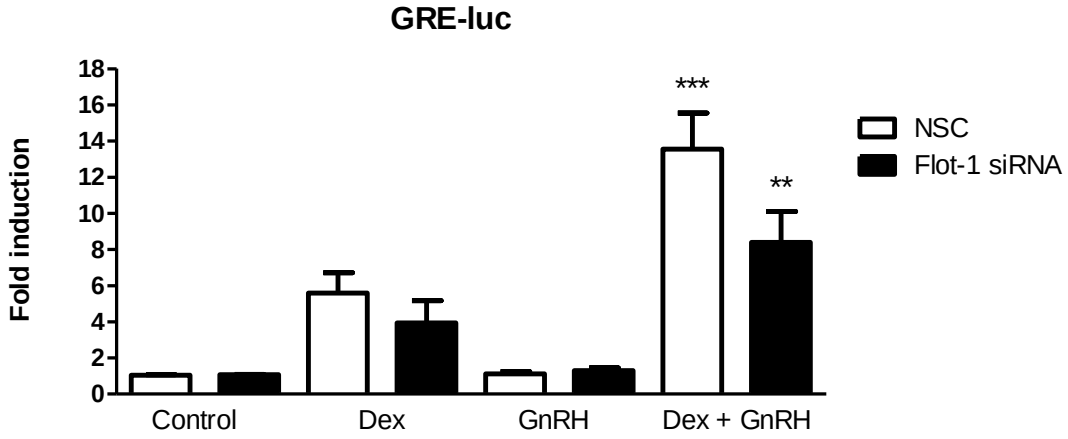
Control
Dex
GnRH
Dex + GnRH
NSC
Flot-1 siRNA

B)


GR / GAPDH
140
120
100
80
60
40
20
0
*
**
**
Control
Dex
GnRH
Dex + GnRH
NSC
Flot-1 siRNA

Having confirmed that the Flot-1 siRNA-transfected cells have statistically significant decreased levels of Flot-1 protein expression (Figure 3.9A), the effect of decreased Flot-1 protein levels on GRE transactivation was analyzed. It was found that Dex increased the transcriptional activity of the reporter gene by about 5.5-fold, while knockdown of Flot-1 protein levels with siRNA reduced the activity slightly relative to NSC (Figure 3.9Ci). The decrease in the Dex-induced transcriptional response of the reporter gene with Flot-1 siRNA was not statistically significant. However, GnRH-induced GRE-reporter activity as shown previously could not be established in the NSC or siRNA-transfected cells (Figure 3.9Ci). Therefore, the requirement for Flot-1 in the GnRH-induced transcriptional response on a GRE in LβT2 cells could not be investigated. However, it was found that the synergistic transcriptional response mediated by co-stimulation with Dex and GnRH was decreased by about 40% in the presence of Flot-1 siRNA (Figure 3.9Ci).

Ci)



To illustrate that decreased Flot-1 protein levels result in decreased GR-mediated transactivation, the transcriptional responses in the presence of Flot-1 siRNA were expressed as a percentage relative to each NSC (Figure 3.9Cii). Interestingly, the transformed results from Figure 3.9C show that knockdown of Flot-1 significantly repressed the Dex-induced transcriptional response of the GRE-reporter gene by about 30% in LβT2 cells (Figure 3.9Cii). Similarly, the synergistic transcriptional response with both Dex plus GnRH was significantly repressed when Flot-1 protein expression was decreased in LβT2 cells (Figure 3.9Cii). These results from Figure 3.9 strongly suggest a requirement for Flot-1 in the Dex-mediated response as well as the synergistic transcriptional response on a GRE in LβT2 cells.

Cii)

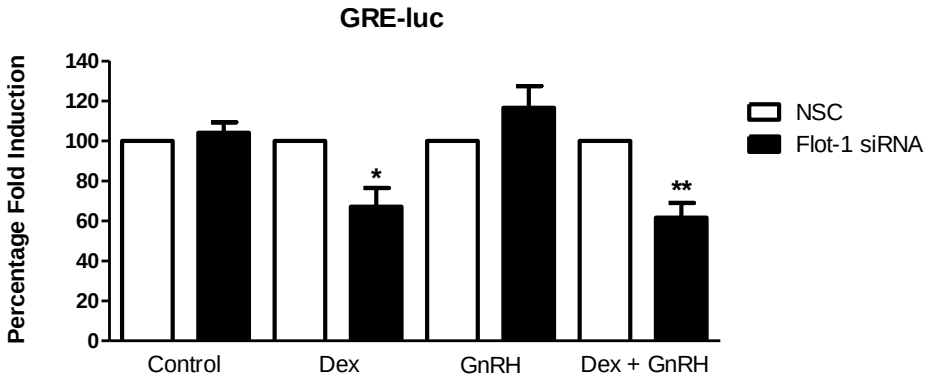


Figure 3.9: Flot-1 is required for the synergistic transcriptional response with Dex and GnRH on a GRE promoter in LβT2 cells

A. LβT2 cells were transfected with non-silencing control (NSC) or specific mouse Flot-1 siRNA at a final concentration of 40 nM and incubated for 72 h. The medium was replaced with fresh medium and cells were transfected with 250 ng TAT-GRE-luciferase construct. After 24 h, cells were incubated for 8 h in serum-free medium with 100 nM Dex or 100 nM GnRH or a combination of both. Equal amounts of samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with specific antibodies against GR, Flot-1 and GAPDH. The Flot-1 protein levels were normalized to GAPDH protein levels and expressed relative to NSC vehicle (control). The panel on left shows a Western blot that is representative of two independent experiments and the graph shows combined results from the two independent experiments. One-way ANOVA with Tukey's post-tests was used for statistical analysis between NSC and Flot-1 siRNA for each condition respectively. **B.** As in A. The graph shown is representative of three independent experiments. A one-way ANOVA with Dunnett's post-tests was used for statistical analysis. **C.** As in A. Results were normalized to total protein and expressed relative to NSC vehicle (control) (i) or the percentage of repression expressed relative to NSC for each condition (ii). The graph shows combined results from three independent experiments, each performed in triplicate. One-way ANOVA with Dunnett's (Figure 3.9Ci) and Tukey's (Figure 3.9Cii) post-tests were used for statistical analysis and denoted as *, ** or *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

Chapter 4: Discussion and Conclusions

4.1 Evidence for the crosstalk mechanism between GR and GnRHR signaling pathways in LβT2 cells

Some of the effects of stress on reproduction are mediated via glucocorticoids (GCs) from the adrenal gland as part of a cross talk mechanism between the hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-gonadal (HPG) axis (Rivier and Rivest, 1991). A recent study from the Hapgood laboratory demonstrated a novel crosstalk mechanism between the GR and GnRHR (Kotitschke *et al*., 2009), indicating an additional direct mechanism for the effects of stress on reproduction. The study showed that GnRH activates the unliganded GR through site-specific phosphorylation, resulting in nuclear translocation and transactivation of a transient GRE-reporter gene and the endogenous mouse GnRHR gene in LβT2 cells in a GR- and GnRHR-dependent manner (Kotitschke *et al*., 2009). In addition, Kotitschke *et al*. reported a synergistic transcriptional response on both the GRE-reporter gene and the endogenous GnRHR gene upon co-stimulation for 8 h with Dex and GnRH in LβT2 cells (Kotitschke *et al*., 2009). However, the exact mechanisms underlying the crosstalk between the GR and GnRHR are unknown. The findings are intriguing considering the difference in sub-cellular localization of the receptors involved. For instance, the GR is cytoplasmic in the absence and nuclear in the presence of GCs, while the GnRHR is a 7-transmembrane receptor found on the cell surface that has previously been shown to localize exclusively to lipid rafts in another gonadotrope cell line, αT3-1 (Navratil *et al*., 2003; Bliss *et al*., 2007). Interestingly, a recent study showed that the GR could also associate with caveolae, which is required for the rapid Dex-induced phosphorylation of the GR in A549 cells (Matthews *et al*., 2008). Thus, it is possible that the crosstalk mechanism between the GR and GnRHR could involve their co-localization to lipid rafts, whereby activation of the one receptor modulates the activity of the other. These findings lead to the formulation of the following hypothesis in the current thesis i.e. that the crosstalk mechanism between the GR and GnRHR involves the co-localization and interaction of these receptors in lipid rafts in LβT2 cells. Several strategies were pursued in the present study to investigate this hypothesis. Co-localization of both receptors with markers for lipid rafts was investigated by confocal

immunflorescence. Isolation of lipid rafts with density-gradients followed by Western blotting for both receptors and the lipid raft marker protein Flot-1 was performed. In addition, the interaction between the GR and Flot-1 was investigated by co-immunoprecipitation assays. In the last part of the study a functional role for the co-localization of both the GnRHR and the GR to lipid rafts were investigated by disruption with cholesterol depletion agents and by Flot-1 siRNA-mediated knockdown.

First, it needed to be established in the hands of the present author that GnRH induces transactivation and that co-stimulation with Dex and GnRH induces a synergistic transcriptional response on a transfected GRE-reporter gene in LβT2 cells. In Figure 3.1A it is clear that stimulation of LβT2 cells for 8 h with GnRH increased the transcriptional activity of the GRE-reporter gene by about 4-fold, while co-stimulation with Dex and GnRH synergistically increased the transcriptional activity by about 12-fold (Figure 3.1B). These results confirmed the study of Kotitschke *et al.* and enabled the present investigation into the mechanism of ligand-independent activation of the GR by GnRH in these cells. Interestingly, GnRH and Dex treatment exhibited a similar fold response in inducing transactivation of the GRE-reporter gene (Figure 3.1A), indicating that GnRH activates the GR to an equal extent as compared to Dex.

GnRH has recently been shown to induce the phosphorylation and activation of other steroid receptors in mouse gonadotrope cells, including the PR and ER. A study by An *et al*. reported that GnRH treatment induced the nuclear translocation of the PR within 1 h in αT3-1 cells (An *et al*., 2006). A more recent study showed that GnRH induced the phosphorylation of the PR at S249, resulting in nuclear translocation and transactivation of a progesterone-response-element (PRE)-reporter gene and the endogenous FSHβ gene in LβT2 cells (An *et al*., 2009). Similarly, Chen *et al*. reported that treatment with GnRH induced phosphorylation of the ER in the cytoplasm and the nucleus, resulting in transactivation of an estrogen-response-element (ERE)-reporter gene and the endogenous FosB gene in LβT2 cells (Chen *et al*., 2009). Therefore, the ligand-independent activation of steroid receptors by GnRH through phosphorylation appears to be a common mechanism for regulating target gene expression in murine gonadotrope cell lines.

The study by Kotitschke *et al*. was the first to demonstrate the ligand-independent activation of the GR by GnRH as well as the synergistic transcriptional effect with Dex and GnRH on a GRE-containing promoter in the pituitary (Kotitschke *et al*., 2009). The only other report of the synergistic behavior between Dex and GnRH was by Sasson *et al*. on a glycoprotein hormone α-subunit-reporter gene in LβT2 cells (Sasson *et al*., 2008). However, there have been several reports of the synergistic transcriptional effect of GnRH with other sex hormones in gonadotrope cell lines. An *et al*. reported that the GnRH-induced PRE-reporter activity was synergistically enhanced with progesterone in αT3-1 cells (An *et al*., 2006). Furthermore, the LHβ and glycoprotein α-subunit gene expression is synergistically increased in the presence of estrogen and GnRH in LβT2 cells (Kowase *et al*., 2007), while estrogen has no effect on the GnRH-induced ERE-reporter activity in LβT2 cells (Chen *et al*., 2009). Taken together, these findings indicate that GnRHR can act synergistically with several steroid receptors to increase expression of target genes in gonadotrope cell lines. However, there is no study in the literature investigating the synergistic behavior of GnRHR with GCs on AP-1 or NF-κB minimal promoters in gonadotrope cell lines.

To investigate if the GR and GnRHR can crosstalk on other *cis*-elements, LβT2 cells were transfected with a minimal AP-1-reporter gene in the present study. The continuous treatment of cells with 10 nM or 100 nM GnRH for 24 h resulted in a dose-dependent increase in the transcriptional activity of the AP-1-reporter gene (Figure 3.2A). The finding that GnRH increased the transcriptional activity of the AP-1-reporter gene in a dose-dependent manner is consistent with a recent study showing the importance of the AP-1 site in mediating the GnRH-induction of the pituitary adenylate cyclase-activating polypeptide (PACAP) gene in LβT2 cells (Grafer *et al*., 2009). In addition, a recent study by Tsutsumi *et al.* reported that both continuous and pulsatile stimulation with GnRH increased transcription via an AP-1-reporter gene in LβT2 cells (Tsutsumi *et al*., 2010). The further increase in the GnRH-induced transcriptional activity of the AP-1-reporter gene in the presence of Dex is interesting (Figure 3.2B), as other reports have shown a decrease via transrepression by the GR on AP-1 regulated genes (Heck *et al*., 1994; Kassel *et al*., 2004). However, the ligand-activated GR has also been shown to enhance transcription of AP-1 regulated genes, depending on the nature of the dimeric AP-1 complex (Teurich and Angel, 1995). GnRH stimulation most likely results in differential phosphorylation

of the GR that favors interactions with co-activators, rather than co-repressors. This is supported by a recent study by Avenant *et al*. demonstrating the requirement of GR phosphorylation for the recruitment of co-activators in COS-1 cells (Avenant *et al*., 2010a). The increase of the GnRH-induced response of the AP-1-reporter gene in the presence of Dex suggests that the synergistic transcriptional response of the endogenous GnRHR with Dex and GnRH reported by Kotitschke *et al*. might be partly mediated via the AP-1 site in LβT2 cells (Kotitschke *et al*., 2009). However, Dex alone was unable to induce a transcriptional response of the minimal AP-1 reporter gene, indicating that additional *cis*-elements are required for the Dex response of the GnRHR gene. The increase of the GnRH-induced AP-1 response with Dex also indicates that in addition to effects on a GRE containing promoter, the GR and GnRHR signaling pathways can positively crosstalk on an AP-1 *cis*-element in LβT2 cells.

The above results provide strong support for the ligand-activated GR to modulate the GnRH-induced transcriptional response on an AP-1-containing promoter in LβT2 cells. To investigate the effects of the crosstalk mechanism between these signaling pathways on a well-characterized transrepression model for the GR (De Bosscher and Haegeman, 2009), a minimal NF-κB-reporter gene was transfected into LβT2 cells. Interestingly, GnRH was found to activate pathways targeting both AP-1- and NF-κB-regulated genes (Figure 3.2A and Figure 3.3), while TNFα was only able to activate a pathway targeting NF-κB. This finding suggests that different signaling pathways regulate these *cis*-elements in LβT2 cells. In contrast, it has previously been shown that TNFα induces expression of both AP-1- and NF-κB-regulated genes in other cell types (Kassel *et al*., 2004; Park *et al*., 2004; De Bosscher and Haegeman, 2009; Van Bogaert *et al*., 2010). Although the AP-1 and NF-κB *cis*-elements appear to be regulated by different signaling pathways in LβT2 cells, the TNFα response was similar to the GnRH-induced response of the NF-κB-reporter gene. The TNFα-induction was repressed by addition of Dex, while the GnRH-induction remained unaffected by the addition of Dex (Figure 3.3, bar 5 and vs 3 and 4). Co-stimulation of LβT2 cells with GnRH and TNFα resulted in an additive transcriptional response of the NF-κB-reporter gene, which remained unaffected by addition of Dex (Figure 3.3, bar 7 vs 8). Taken together, the above results show that the crosstalk mechanism between the GR and GnRHR signaling pathways has no effect on a minimal NF-κB regulated gene.

The GnRH-induced activation of the NF-κB-reporter gene is consistent with a recent study by Naidich *et al*. showing that this mechanism involves hyper-phosphorylation of p65 in LβT2 cells (Naidich *et al*., 2010). The finding that the additive effect of the NF-κB transcriptional activity with TNFα and GnRH co-stimulation was unaffected by Dex treatment is surprising as Dex stimulation repressed the TNFα-induced response (Figure 3.3, bar 5 vs 6). The inability of the ligand-activated GR to repress the GnRH-mediated increase of an NF-κB as well as the additive response of GnRH with TNFα on the NF-κB-reporter gene, suggests that the GnRH activation of the GR interferes with the Dex-induced transrepression ability of the GR. This finding is consistent with the result in the present study showing the partial increase of the GnRH-induced transcriptional activity with Dex of the AP-1-reporter gene. This abolishment of transrepression by Dex-activated GR on both AP-1- and NF-κB-reporter genes in the presence of GnRH is interesting, as this indicates a mechanism that selects for the transactivation and not the transrepression activity of the GR. Although the precise mechanisms regulating this finding are unknown, this could involve specific GnRH-induced post-translational modifications of the GR that could favor an interaction with co-activators rather than co-repressors. The study by Kotitschke *et al*. showed that co-stimulation with Dex and GnRH did not result in a different GR phosphorylation pattern at Ser220 and 234 as compared with Dex alone (Kotitschke *et al*., 2009). However, it is possible that co-stimulation with Dex and GnRH could induce different patterns of post-translational modifications of the GR as compared to Dex alone, at sites not yet investigated.

It is well established that activation of steroid receptors by their respective steroid ligands induces receptor degradation (Berry *et al*., 2008; Lee and Chang, 2003). To investigate whether the observed increase with Dex of the GnRH-induced AP-1-reporter transcriptional activity was mediated by increased GR protein expression levels, cell lysates were analyzed by Western blotting. The results from Figure 3.4 show that 1 μM Dex appeared to decrease GR protein levels after 24 h by about 40% (Figure 3.4, bar 2). This finding is similar to a recent report showing the human GR having a half-life of about 12 h with 100 nM Dex stimulation (Wallace *et al*., 2010). Interestingly, stimulation of cells with 10 nM or 100 nM GnRH for 24 h appeared to increase GR protein degradation also resulting in about 40% degradation compared to vehicle control (Figure 3.4, bars 3 and 4). The GnRH-induced degradation of the GR is a novel finding and the mechanism probably involves post-translational modifications of the GR that decreases its

stability. Furthermore, the addition of 100 nM or 1 μM Dex appeared to partially enhance the GnRH-induced degradation of GR protein (Figure 3.4, bar 3 vs 5 and 6), with the addition of 1 μM Dex showing approximately 50% receptor turnover (Figure 3.4, bar 6). Therefore, the increase in the GnRH-induced transcriptional activity of the AP-1 reporter gene by Dex appeared not to be mediated via increased GR protein expression levels. Taken together the above findings suggest that GnRH alone induces degradation of GR protein, which provides further evidence that GnRH can activate the GR in LβT2 cells.

4.2 Co-localization of the GR and GnRHR to lipid rafts in LβT2 cells

Having established functional evidence for the GnRHR to modulate the activity of the GR in the hands of the present author in LβT2 cells, the next aim of the present study was to demonstrate co-localization in lipid rafts, which would suggest a mechanism whereby the GR and GnRHR could interact. It first needed to be established which lipid raft marker proteins are expressed in LβT2 cells by Western blotting of whole-cell lysates. The results from Figure 3.5.1 show that LβT2 cells express the lipid raft protein Flot-1 but not Cav-1, which is consistent with reports from other studies in gonadotrope cells (Navratil *et al*., 2003, Bliss *et al*., 2007), but in contrast to a recent report in LβT2 cells (Dobkin-Bekman *et al*., 2009). In order to investigate the sub-cellular localization and lipid raft association of the GnRHR, LβT2 cells were stained and visualized with a confocal microscope. The results from Figure 3.5.2 show the endogenous GnRHR (green) co-localizing with endogenous Flot-1 (red) in the absence of ligand at the plasma membrane (large arrow) and at intracellular vesicles (small arrow) in LβT2 cells (Figure 3.5.2). The finding that the GnRHR localizes to lipid rafts in LβT2 cells suggests that lipid rafts play a key role in regulating GnRHR function, most likely by the assembly of a pre-formed signaling complex that efficiently fine-tunes GnRHR signaling. In addition to the plasma membrane localization of the GnRHR, there seems to be a small percentage of GnRHR localized to the cytoplasm in LβT2 cells (Figure 3.5.2, small arrow).

Previous studies in the literature have reported that the GnRHR localizes exclusively to lipid rafts containing Flot-1 in αT3-1 cells, as determined by density-gradient fractionation experiments (Navratil *et al*., 2003). Similarly, a more recent study by Bliss *et al*. showed that the transiently

transfected GnRHR-GFP fusion protein co-localized exclusively with GM1, a ganglioside that is highly enriched in lipid rafts, in αT3-1 cells (Bliss *et al*., 2007). However, the present study is the first to demonstrate the co-localization of the endogenous GnRHR with endogenous Flot-1 in any cell type of pituitary-origin by immunoflorescence analysis. In contrast to results in αT3-1 cells, a small amount of GnRHR localized to the cytoplasm in LβT2 cells. This could be due to the experimental design in the live-cell staining process, resulting in receptor internalization upon antibody-binding. However, some intra-cellular localization of the GnRHR is not unexpected since Finch *et al*. showed that exogenous human GnRHR displays intra-cellular and plasma membrane localization in HeLa cells (Finch *et al*., 2010). In the present study, the small amount of intra-cellular GnRHR co-localized with intra-cellular Flot-1. Flot-1 has also previously been reported by others to reside within intra-cellular vesicles (Lang *et al*., 1998). The intra-cellular co-localization of some of the GnRHR and Flot-1 suggests that Flot-1 could be involved in the internalization of the GnRHR to result in its degradation or recycling to the plasma membrane. The mechanisms that regulate the sub-cellular trafficking and localization of the GnRHR are not well understood and further investigations are necessary.

Having established that the endogenous GnRHR co-localizes with the lipid raft marker protein Flot-1 at the plasma membrane, the sub-cellular localization of the endogenous GR upon stimulation with Dex, GnRH or both together for 30 min was investigated in LβT2 cells. The results show that the unliganded GR is almost evenly distributed throughout the cell (Figure 3.5.3A), while Dex stimulated distinct nuclear translocation of the GR (Figure 3.5.3B). However, 30 min GnRH treatment did not induce any observable difference in the localization of the GR (Figure 3.5.3C). Co-stimulation with GnRH and Dex increased the amount of Dex-induced nuclear import of the GR (Figure 3.5.3D). Furthermore, it is very interesting that a small amount of GR did not translocate to the nucleus with Dex or with Dex and GnRH co-stimulation, but remained in the cytoplasm with a small percentage appearing to localize at the plasma membrane in LβT2 cells (Figure 3.5.3, panel B and D). A possible explanation for the remaining GR that did not translocate to the nucleus could be that if associated with lipid rafts.

Having established that a small amount of GR localizes to the plasma membrane under all stimulated conditions, whether this membrane-localized GR was associated with lipid rafts was

subsequently investigated. This involved staining for the general lipid raft marker GM1, a ganglioside that is highly enriched in lipid rafts (Janes *et al*., 1999; Harder, 1998). The results from Figure 3.5.3 show that GM1 is localized exclusively to the plasma membrane under all conditions investigated (Figure 3.5.3). In addition, a small percentage of GR co-localizes with GM1 for all stimulated conditions at the plasma membrane (Figure 3.5.3). The amount of GR that co-localizes with GM1 relative to the remaining GR proved insufficient to perform statistical analysis from three independent experiments. Taken together, the above results suggest that a small population of GR localizes at the plasma membrane, which appears to co-localize with GM1, under all stimulated conditions. The finding that both the GR and GnRHR localize to lipid rafts at the plasma membrane suggests that they could interact to modulate each other's activity or signaling pathways.

Whether the GR co-localizing with GM1-containing lipid rafts also localizes with a more specific class of lipid rafts containing Flot-1 was next investigated. Similar to that of GM1, the sub-cellular localization of Flot-1 appeared to be largely localized to the plasma membrane under all stimulated conditions (Figure 3.5.3). A small amount of Flot-1 displayed a clustering pattern with a cytoplasmic localization under all stimulated conditions (Figure 3.5.3). Similar to GM1, there appears to be a small population of GR co-localizing with Flot-1 at the plasma membrane under all stimulated conditions (Figure 3.5.3). However, the amount of GR co-localizing with Flot-1 at the plasma membrane was little and was found not to be statistically significant with four independent experiments. Taken together, the above findings suggest that endogenous GR and Flot-1 appears to co-localize to lipid rafts at the plasma membrane of LβT2 cells. This finding provides a possible mechanism for GnRHR-mediated modulation of GR activity within lipid rafts in LβT2 cells and supports the hypothesis of this study.

The nuclear translocation of the GR with Dex is consistent with other reports (Nishi, 2010; Sarabdjitsingh *et al*., 2010). However, the finding that 30 min treatment with GnRH did not induce nuclear translocation of the GR was surprising as Kotitschke *et al.* demonstrated that 1 h treatment with 100 nM GnRH resulted in approximately 50% nuclear translocation of the GR, as compared with Dex (Kotitschke *et al*., 2009). A possible explanation for not detecting any significant change in the localization of the GR with 30 min GnRH treatment could be that the

GR displays a lag-phase in its nuclear import within the first 30 min, after which the rate is increased making nuclear localization detectable after 1 h stimulation with GnRH.

The present study investigated the effects of Dex, GnRH or both together on the localization of the GR to lipid rafts containing Flot-1. A small population of GR co-localizes with Flot-1 at the plasma membrane under all stimulated conditions. Similar to the co-localization with GM1, no statistical significance could be established, most like resulting from the small percentage of GR. Another interesting finding is that the localization of Flot-1 with co-stimulation of Dex and GnRH appears to be largely intra-cellular (Figure 3.5.3D), potentially indicating a role for the GR and GnRHR in regulating Flot-1 internalization. There appears to be more GR that co-localizes with Flot-1 after co-stimulation with Dex and GnRH compared to the other stimulated conditions (Figure 3.5.3, compare panels A, B and C with D). It is surprising that there was no statistically significant differential recruitment to the plasma membrane with the ligands investigated. However, this is supported by a recent study of Grossmann *et al*. showing that the MR localizes to the plasma membrane independent of short exposures to ligand, while 24 h stimulation with aldosterone resulted in the disappearance of MR at the plasma membrane in HEK-293 cells (Grossmann *et al*., 2010). Therefore, the plasma membrane localization of steroid receptors independent of ligand could potentially be a mechanism to ensure the sensitivity for the rapid signaling of steroid hormones. This could be achieved by the constitutive localization of the receptor at the membrane leading to the modulation of non-genomic signaling pathways upon hormone exposure. This is the first study investigating the localization of GR to lipid rafts by immunoflorescence.

Having shown GnRHR and GR co-localization with Flot-1, it was important to determine co-localization of the GR with GnRHR in LβT2 cells. However, the experiment investigating direct co-localization of these receptors could not be performed due to the lack of suitable commercially available GnRHR antibodies. The present author obtained two different GnRHR antibodies (sc-8681 and sc-13944, Santa Cruz, USA), which proved to be unsuccessful in this experiment after several attempts. Therefore, a non-commercial antibody obtained from D.C. Skinner that was raised in a rabbit against the ovine GnRHR, was used to perform the co-localization experiments of the GnRHR with Flot-1 (Figure 3.5.2). This antibody has been shown to recognize the murine

GnRHR in LβT2 cells (Albertson *et al*., 2008) and in whole mouse pituitaries (Bliss *et al*., 2007). In order to investigate the possible co-localization of the endogenous GR with the endogenous GnRHR, a GR antibody raised in a species other than rabbit was required. The mouse anti-GR antibody did not detect the GR on a Western blot and failed to show any specific staining, that was above the background level, in immunoflorescence analysis. Therefore, due to time constraints of the present study, the experiment could not be performed. However, it is likely that the GR and GnRHR co-localize in LβT2 cells, as the present study showed the co-localization of the GnRHR with Flot-1 and the co-localization of the GR with Flot-1.

Having shown by immunoflorescence analysis that both the endogenous GR and GnRHR localize to lipid rafts at the plasma membrane of LβT2 cells, a different strategy was employed to provide further support. This strategy involved the isolation of lipid rafts by biochemical fractionation with a discontinuous density-gradient to obtain adequate separation of lipid rafts in LβT2 cells. First, a method for the isolation of lipid rafts from LβT2 cells needed to be established. A few previously described methods were investigated, including a non-detergent method (Gagescu *et al*., 2000) and a detergent-resistant method (Lafont and Simons, 2001), as shown in Addendum B. The criteria dictating selection of a particular method were based on the sufficient isolation of lipid rafts into one or two fractions, as analyzed by Western blotting for the lipid raft marker protein Flot-1. The gradient in the method chosen consisted of five different phases and employed the use of 0.05% Triton X-100, which is a more stringent test compared to non-detergent methods for association of proteins with lipid rafts. The initial investigation into localization of the GR to lipid rafts was performed with overexpressed GR and used a crude membrane fraction as the starting material for isolation of lipid rafts from LβT2 cells. Although the advantage of using a crude membrane fraction as the starting material is ensuring the isolation of only plasma membrane-derived lipid rafts, this proved to be problematic owing to GR degradation problems, despite including protease inhibitors (Addendum B). The degradation appeared to be specific for the GR, as the stability of Flot-1 was unaffected (Addendum B). In order to minimize this degradation problem, the use of whole-cell lysates as starting material was investigated. The results from Addendum B show that starting with whole-cell lysates decreased the amount of GR degradation compared to the use of a crude membrane fraction for starting material in the isolation of lipid rafts from LβT2 cells (compare Addendum B with Figure 3.6).

This decrease in the amount of degradation of the GR is most likely a result of increased substrate availability for the proteases when starting with whole-cell lysates, because this degradation probably occurs during the 45 min incubation with Triton X-100 before layering of the gradients in the isolation process.

The results from Figure 3.6B show the novel finding that the unliganded GR appears to localize with GnRHR to lipid rafts containing Flot-1 in LβT2 cells. The localization of the endogenous unliganded GnRHR with Flot-1 to lipid rafts supports the immunoflorescent result from Figure 3.5.2 showing co-localization of GnRHR with Flot-1 at the plasma membrane of LβT2 cells. In order to determine if the GR was differentially recruited to lipid rafts with Dex, GnRH or a combination of both for 30 min, the GR protein levels in the lipid raft fraction were normalized to Flot-1 protein levels. The results from Figure 3.6Bv show that there is no difference in the levels of GR localized to lipid rafts with treatment of Dex, GnRH or a combination of both (Figure 3.6B, panel v). The present study is the first to report the localization of GR to lipid rafts under different hormonal stimulation conditions in the pituitary and the first report localizing the GR with Flot-1 in any cell type. Furthermore, the ligand-independent localization of endogenous GR to lipid rafts further supports the immunoflorescence data of Figure 3.5.3 showing a small percentage of GR localizing with Flot-1 at the plasma membrane of LβT2 cells under all stimulated conditions. The above results and the small percentage of GR co-localizing with Flot-1 at the plasma membrane in Figure 3.5.3 together with the significant co-localization of GnRHR with Flot-1 at the plasma membranes, suggests that it is likely that the GR and GnRHR are both present in lipid rafts at the plasma membrane of LβT2 cells.

The density-gradient results from Figure 3.6A show that the nuclear marker histone H3 localized only to fractions 6 and 7, which is the high-density fractions where the starting material was applied (Figure 3.6A). Importantly, Flot-1 localizes to a single fraction under all tested conditions, indicating specific fractionation of lipid rafts. The GnRHR is still present in lipid rafts after 30 min of treatment with GnRH, consistent with the study by Navratil *et al*. showing that localization of the GnRHR to lipid rafts is independent of ligand in αT3-1 cells (Navratil *et al*., 2003). The finding that there is no differential recruitment of the GR to lipid rafts with these hormones is similar to a recent study indicating that the MR localizes to lipid rafts in a ligand-

independent manner in HEK-293 cells (Grossmann *et al*., 2010). It is possible that the GR is differentially recruited to lipid rafts in response to hormone, at times other than the 30 min time-point investigated in this study. In support, a study by Jain *et al.* reported that Dex treatment (time not stated) increased the association of GR with caveolae in Hep3B cells (Jain *et al*., 2005). However, the levels of GR in lipid rafts were not normalized to Cav-1 levels and were judged by analyzing equal amounts of the lipid raft fraction with only one experiment shown (Jain *et al*., 2005).

The GR and GnRHR co-localize with Flot-1-containing lipid rafts at the plasma membrane of LβT2 cells in a ligand-independent manner as shown in Figure 3.5 and 3.6. This finding suggests that the GR and GnRHR exist in a pre-formed signaling complex at the plasma membrane. However, the co-localization of these receptors to lipid rafts provides no proof of a multi-protein complex. To investigate if the GR exists in a complex with Flot-1 in a ligand-dependent manner in LβT2 cells, co-immunoprecipitation with an anti-GR and non-specific IgG antibody were performed on whole LβT2 cell lysates with or without 30 min Dex treatment. The results from Figure 3.7A show that Flot-1 co-immunoprecipitated with the unliganded GR, while the addition of Dex had no effect on this interaction (Figure 3.7A). Furthermore, no detectable Flot-1 protein co-immunoprecipitated with the IgG antibody in the absence or presence of Dex (Figure 3.7A), suggesting that the GR interaction with Flot-1 is specific. In addition, the reciprocal co-immunoprecipitation assay with a rabbit Flot-1 antibody confirms the interaction of Flot-1 with GR (Figure 3.7B). Therefore, the above results suggest that the GR is found in a complex with Flot-1 in LβT2 cells. Since multi-protein signaling complexes including the GR and Cav-1 have been shown to occur in caveolae in Hep3B and A549 cells (Jain *et al*., 2005; Matthews *et al*., 2008), the association of GR with Flot-1 suggest that the GR may be part of such a complex. This suggests that the GR could be activated by lipid raft-associated proteins, such as other receptors and kinases, providing a mechanism for the reciprocal modulation of receptor activity. Thus, the reciprocal modulation of GR and GnRHR signaling pathways could involve a physical interaction between these receptors.

To further investigate if the GR interacts with Flot-1 in a ligand-dependent manner, co-immunoprecipitation assays with an anti-GR antibody were performed on whole LβT2 cell

lysates stimulated for 30 min with Dex, GnRH or a combination of both. The results from Figure 3.7C show that there was no difference in the amount of Flot-1 that co-immunoprecipitated with the GR in the presence of Dex, GnRH or both together (Figure 3.7C). This finding that the interaction of Flot-1 with the GR was ligand-independent, is consistent with the immunofluorescence results showing ligand-independent co-localization of the GR with Flot-1 in lipid rafts, as shown in Figure 3.5.3 and 3.6B.

Several attempts were made to identify the GnRHR in the immunoprecipitated complex. However, the co-immunoprecipitation assay significantly enriched several non-specific proteins that resulted in the detection of several non-specific bands at a similar size to the GnRHR in Western blots. In addition, the limitation of commercially available GnRHR antibodies resulted in the author being unable to provide evidence for the presence of the GnRHR in the novel GR-Flot-1 complex precipitated from LβT2 cell extracts. A potentially important finding that could give insight on the nature of the complex formed between the GR and Flot-1 is the finding that no GR could be detected in the co-immunoprecipitation assay using the mouse monoclonal anti-Flot-1 antibody. As suggested before, a possible reason for this could be that the GR interacts with the epitope recognized by the antibody, i.e. the C-terminal domain of mouse Flot-1. Therefore, the C-terminal domain of Flot-1, which excludes the SPFH and hydrophobic domains, but includes the EA repeats, could be important for the interaction with GR. Further work is required to characterize this novel interaction of Flot-1 with the GR in LβT2 cells.

4.3 Functional role of lipid rafts and Flot-1 in GR and GnRHR signaling

The results of the present study showed that a crosstalk mechanism exists between the GR and GnRHR, supported with immunoflorescence data and density-gradient analysis showing that the GR and GnRHR co-localize with Flot-1 to lipid rafts at the plasma membrane of LβT2 cells. Furthermore, co-immunoprecipitation assays showed that the GR exists in a complex with Flot-1, which provides a mechanism for the GR to associate with lipid rafts through a ligand-independent interaction with Flot-1. A possible explanation for the crosstalk mechanism observed between the GR and GnRHR signaling pathways could result from the following theory, i.e. GnRH activates the GnRHR and since the GR is in close proximity and most likely in a complex

with kinases, these kinases get activated and results in the direct or indirect modulation of GR in the complex. This is supported by the GnRH-induced ligand-independent phosphorylation of the GR, which could then result in activation and nuclear translocation of the GR to regulate transcription of target genes.

The last part of the present study was to investigate if lipid rafts have a functional role in GR and GnRHR signaling in LβT2 cells. A well-established method for investigating the role of lipid rafts in mediating the signaling of various receptors involves disrupting the integrity of lipid rafts by incubating cells with cholesterol depletion agents (Literature review, Table 1.3.2). In order to investigate if lipid rafts have a functional role in the transcriptional activity of the GR on GRE transactivation, LβT2 cells were incubated continuously for 8 h with MβCD in the presence of Dex, GnRH or both together. The results from Addendum C indicate that the continuous treatment with 2% MβCD (approximately 15 mM) for 8 h dramatically decreased the basal, Dex- and GnRH-induced transcriptional activity of the GRE-reporter gene in LβT2 cells, while also dramatically repressing the expression of the constitutive β-galactosidase gene (Addendum C). In addition, the 8 h incubation with MβCD resulted in significant changes in the morphology of LβT2 cells (data not shown). Taken together, these findings suggests that continuous treatment with MβCD results in cell death of LβT2 cells, which prevents the use of this method for the investigation of a role for lipid rafts in GR transactivation in LβT2 cells.

A similar strategy to disrupt lipid rafts previously described by Navratil *et al*. in αT3-1 cells was employed to investigate the role of lipid rafts in GR and GnRHR signaling. This method involves incubating cells for 45 min with MβCD, followed by washing of the cells with PBS before incubation with ligand (Navratil *et al*., 2003). The results from Figure 3.8A show incubation of cells with MβCD for 8 h had no effect on the GRE-reporter gene activity mediated by Dex, GnRH or both together in LβT2 cells. Similarly, the results from Figure 3.8B show that there was no effect on the GnRH-induced phosphorylation at S226 of the GR when cells were treated with MβCD (Figure 3.8B). Taken together, the above results suggest that the disruption of lipid rafts had no effect on GR or GnRHR signaling in LβT2 cells.

The finding that the transactivation of a GRE-containing promoter was unaffected in the presence of MβCD in LβT2 cells is surprising (Figure 3.8A). It is possible that this could have resulted

from the reformation of lipid rafts during the 8 h stimulation period. The incubation time with ligand could not be reduced due to the nature of the assay, which requires the synthesis of the luciferase protein. The finding that disruption of lipid rafts with MβCD had no effect on the GnRH-induced phosphorylation at Ser234 of the GR in LβT2 cells is inconsistent with a recent report of Matthews *et al*. showing that the knockdown of Cav-1 protein expression, which decreases caveolae formation, increased the basal and prevented the Dex-induced phosphorylation of the human GR at S211 in A549 cells (Matthews *et al*., 2008). Thus, the above results suggest that either cholesterol depletion has no effect on GR and GnRHR signaling or cholesterol depletion has no effect on the integrity of lipid rafts in LβT2 cells.

Previous studies reported that cholesterol depletion with MβCD disrupted lipid rafts in another gonadotrope cell line, αT3-1, which prevented the GnRH-induced activation of ERK-1/2 (Navratil *et al*., 2003; Bliss *et al*., 2007). Therefore, to investigate if MβCD treatment of LβT2 cells had a similar effect on the activation of ERK-1/2, cells were treated with MβCD and the GnRH-induced activation of ERK-1/2 was investigated by Western blotting and probing with a phospho-specific ERK-1/2 antibody. In contrast to the previous reports in αT3-1 cells, the results from Figure 3.8C show that MβCD had no effect on the GnRH-induced ERK-1/2 phosphorylation in LβT2 cells (Figure 3.8C). This finding suggests that cholesterol depletion with MβCD had no effect on the integrity of lipid rafts in LβT2 cells. Thus, the results of Figure 3.8A and B are inconclusive and the role of lipid rafts in GR and GnRHR signaling could not be established using this strategy in LβT2 cells.

There is one report in the literature showing that the Dex-induced transactivation of a GRE-reporter gene by the GR is inhibited with continuous treatment of Hep3B cells with the cholesterol depletion agent, Filipin-III (Jain *et al*., 2005). Therefore, to investigate a role for lipid rafts in transactivation of a GRE-reporter by the GR, the effect of cholesterol depletion with Filipin-III was investigated in LβT2 cells. The results from Figure 3.8D show that Filipin-III had no effect on the Dex-induced transcriptional activity of a GRE-reporter gene in LβT2 cells. Therefore, neither MβCD nor Filipin-III treatment yielded evidence for a role of lipid rafts in GR and GnRHR signaling in LβT2 cells.

Since the results using cholesterol depletion agents to disrupt lipid rafts in LβT2 cells were inconclusive, a more specific approach was employed to investigate a functional role of Flot-1 in GR and GnRHR signaling. This was investigated by knockdown of Flot-1 protein expression with specific siRNA in LβT2 cells. To achieve sufficient knockdown of Flot-1 protein, initial optimization experiments of the knockdown conditions were performed. This involved testing four different Flot-1 siRNA oligonucleotides, varying the incubation time and the final concentration of siRNA in LβT2 cells. The results from Addendum D show that there was no significant difference with respect to knockdown efficiency between the four different siRNA sequences investigated for Flot-1 knockdown. However, the incubation of the cells with siRNA for 72 h compared to 48 h appeared to result in more Flot-1 protein knockdown in LβT2 cells (Addendum D). Therefore, all four siRNA oligonucleotides were combined in the attempt to increase the amount of Flot-1 knockdown. The results from Addendum D show that there appears to be more Flot-1 knockdown with 40 nM siRNA compared to 20 nM siRNA after 72 h incubation in LβT2 cells. Therefore, all four oligonucleotides were used at a combined concentration of 40 nM for 72 h in all further Flot-1 knockdown experiments in LβT2 cells.

To investigate a functional role for Flot-1 in GR and GnRHR signaling, Flot-1 protein levels were decreased by siRNA-mediated knockdown and the transcriptional activity of the GR on a GRE-promoter in response to Dex, GnRH or both in combination was investigated in LβT2 cells. The results from Figure 3.9A show that Flot-1 protein levels were significantly reduced when cells were incubated with specific mouse Flot-1 siRNA, as compared to non-silencing scrambled control siRNA (NSC) for all tested conditions. Furthermore, the levels of GR protein appears unchanged in the presence of Flot-1 siRNA compared to NSC-transfected cells under all treatment conditions (Figure 3.9B). Knockdown of Flot-1 significantly repressed the Dex-mediated transcriptional response on a GRE-reporter gene in LβT2 cells (Figure 3.9C). In addition, the synergistic response with Dex and GnRH was significantly repressed in cells with decreased Flot-1 protein levels (Figure 3.9C). The present study is the first report showing Flot-1 to have a role in the GR-mediated transactivation of a simple GRE-reporter gene. Taken together, the results from Figure 3.9 indicate that Flot-1 is required for the Dex and synergistic transcriptional responses on a GRE-reporter gene, which supports a role for lipid rafts in GR and GnRHR signaling in LβT2 cells.

The decrease of the Dex-induced transcriptional activity of the GRE-promoter in Flot-1 siRNA-transfected cells appears to be responsible for the decrease in the synergistic transcriptional response observed in the presence of Flot-1 siRNA. However, a previous study by Sugawara *et al.* reported that Flot-1 and -2 interact with $G_{\alpha q}$, which is important for the UTP-induced activation of p38 and not ERK-1/2, as shown by knockdown of Flot-1 and -2 in HeLa cells (Sugawara *et al*., 2007). Thus, this finding suggests that attenuation of GnRHR signaling through Flot-1 knockdown could potentially play a part in the repression of the synergistic transcriptional response in LβT2 cells.

The effect of decreased Flot-1 protein levels on the GnRH-induced transactivation of a GRE-reporter gene could not be investigated, because GnRH failed to induce a significant transcriptional response in NSC- or siRNA-transfected cells (Figure 3.9C). However, co-stimulation with Dex and GnRH still resulted in a synergistic transcriptional response on a GRE-reporter gene, even though there was no response with GnRH alone (Figure 3.9C). This finding did come as a surprise and resulted in numerous attempts to investigate the source behind it. The first experiment towards solving the problem was to confirm that the cells still responded to GnRH. Therefore, the ability of GnRH to induce a transcriptional response on an AP-1 reporter gene was investigated. The results showed a significant increase in the transcriptional activity of the AP-1 reporter gene in LβT2 cells in response to GnRH (data not shown), which was similar to results shown in Figure 3.2. In addition, the GnRH-induced transcriptional response on a GRE-reporter gene was also confirmed (data not shown). However, all of these investigations were performed under normal growth conditions, whereas in the Flot-1 knockdown GRE-reporter experiments the cells grew for two extra days, as this was shown to be optimal for Flot-1 knockdown. In addition, Dex and GnRH co-stimulation still induced a synergistic transcriptional response (Figure 3.9C), suggesting that the reagents and the oligonucleotides do not interfere with GnRH signaling. Therefore, the exact source of the problem is still unknown, but it appears that cell culture conditions are involved. This suggests that the GnRHR signaling pathway is sensitive to the cellular conditions. It is possible that expression of the GnRHR is decreased upon increased cell densities and cell-cell contacts, which would result in the slightly weaker GnRH

response. This would result in decreased activation of the GR by this weaker GnRH signaling as compared to normal cell culture conditions, which could result in the lack of a GnRH response.

4.4 Physiological implications in the pituitary

The localization of the GnRHR with Flot-1 at the plasma membrane of LβT2 cells could indicate that Flot-1 is involved in the intra-cellular trafficking and degradation of the GnRHR. This could suggest that Flot-1 is directly involved in maintaining gonadotropin-sensitivity for GnRH and regulation of reproduction through controlling gonadotropin-release indirectly. Furthermore, the finding that Flot-1 appeared to be mostly cytoplasmic upon co-stimulation with Dex and GnRH suggests the presence of a novel Flot-1 internalization pathway regulated by GR and GnRHR crosstalk. This finding is similar to a recent report showing the EGF-induced internalization of Flot-1 and -2 in HeLa cells (Riento *et al*., 2009), and supports a role for Flot-1 in trafficking of the GnRHR in LβT2 cells.

The novel finding that the GR localizes to lipid rafts through a physical interaction with Flot-1, indicates that the GR could exist in a multi-protein complex at the plasma membrane of LβT2 cells. This is similar to a previous study showing the unliganded GR associating with caveolae via a physical interaction with Cav-1 in A549 cells (Matthews *et al*., 2008). The important finding of GR and Flot-1 existing in the same complex suggests a mechanism for rapid effects of GCs, resulting in the activation of various intra-cellular signaling pathways. This finding also suggests a mechanism for the GnRHR to modulate GR activity and GR-responsive genes via a direct or indirect interaction with GR in a complex, possibly through the activation of specific signaling molecules like kinases, which could modulate GR activity. The finding that lipid raft association of the GR is independent of ligands could be mechanism that maintains cellular sensitivity to hormones, by ensuring the localization of the unliganded GR at the plasma membrane, which upon hormone stimulation could result in the reciprocal modulation of the GR and other signaling pathways. In addition, the localization of the GR with the GnRHR to lipid rafts could indicate a possible mechanism for fine-tuning the interplay between stress and reproductive processes by the reciprocal modulation of signaling pathways in the pituitary.

Another important aspect will be to investigate the function of the GR in reproduction with the aid of knockout mice. In the literature there are a few references that reported effects of dimerization of the GR in mice and show that the GR is still able to translocate to the nucleus and affect transcription (Reichardt *et al*., 1998). A more recent study used the same system to show that lactation, milk protein synthesis and fertility were unaffected in the GR knockout mice (Reichardt *et al*., 2001). The latter effect on fertility suggests that dimerisation of the GR is not a requirement for fertility. This could be relevant to cross talk between the GR and GnRHR, and may be consistent with a model whereby the modulation of GnRH signaling in pituitary gonadotropes by the GR does not require transactivation by the GR, but rather a tethering model. However, none of these studies looked at the direct effect of GR knockout on gonadotropin expression and thus requires future work.

4.5 Conclusions

The present study demonstrated the GnRH-induced activation of the GR resulting in transactivation of a GRE-reporter gene and the synergistic transcriptional response when LβT2 cells were co-stimulated with Dex and GnRH. It was established that GR and GnRHR signaling pathways crosstalk on a minimal AP-1- but not on an NF-κB- reporter gene in these cells, indicating that the crosstalk mechanisms between the GR and GnRHR is promoter-specific. In addition, the study reported the novel finding that GnRH induced degradation of the GR after 24 h stimulation in LβT2 cells, further supporting the crosstalk mechanism of GnRH activating the unliganded GR. The present study is the first report demonstrating that the endogenous GnRHR co-localizes with Flot-1 at the plasma membrane of LβT2 cells by immunoflorescence, while a small population of the GR appeared to localize with Flot-1 at the plasma membrane, independent of hormone treatment. This is the first report demonstrating that both the GR and the GnRHR appear to co-localize to lipid rafts containing Flot-1, and that this co-localization is unaffected by 30 min treatment with Dex, GnRH or both together. These findings are important and could suggest a possible mechanism for the crosstalk between the GR and GnRHR in LβT2 cells. In addition, the present study is the first report showing by co-immunoprecipitation assays that Flot-1 and GR interact in a complex independent of ligand, further supporting the lipid raft association of the GR. The results suggest that the GR exists in a pre-formed multi-protein

signaling complex in LβT2 cells, which includes the GnRHR. This novel finding suggests that the lipid raft-associated GR could mediate the rapid non-genomic actions of GCs reported in other cell types, i.e. the activation of Akt in A549 cells. A functional role for lipid rafts in GR and GnRHR signaling could not be established using the cholesterol depletion experiments, which proved to be inconclusive. However, siRNA-mediated knockdown of Flot-1 protein expression suggests Flot-1 is required for transactivation of a GRE-reporter gene via the GR in response to Dex and of Dex with GnRH in LβT2 cells. This finding suggests that the association of the GR and GnRHR with Flot-1 is important for signaling by these receptors to result in the transactivation of a GRE in LβT2 cells. In conclusion, the mechanism of crosstalk between the GR and GnRHR involves the co-localization of both receptors with Flot-1 to lipid rafts at the plasma membrane of LβT2 cells and Flot-1 is required for transactivation of a GRE-reporter gene in response to Dex as well as Dex and GnRH in combination in LβT2 cells.

4.6 Future perspectives

Using a reporter gene to elucidate molecular mechanisms of transcription is a valuable approach, but the DNA is not integrated into the genome making it is possible for the results to be different from an endogenous GRE-containing gene, which is in the context of chromatin and histones. Thus, it is important to verify the result of GnRH-induced activation of the GR to transactivate via a GRE on an endogenous GRE-containing gene in LβT2 cells. Similarly, investigation of the synergistic transcriptional response with co-stimulation by Dex and GnRH on an endogenous GRE-containing gene in these cells would also be of importance. After confirmation of the results on an endogenous gene in LβT2 cells, verification of the above results in primary gonadotrope cells will provide physiologically relevant evidence. This is required because it is possible that immortalized gonadotrope cell lines could behave slightly different to primary gonadotrope cells. Confirmation of these results in primary cells could be performed by the isolation of the anterior pituitaries from mature mice, followed by enrichment for gonadotrope cells by fluorescent-activated cell sorting (FACS). The isolated gonadotrope cells could then be incubated with ligands as described for the GRE-reporter assays in the present study, followed by RNA isolation and real-time PCR to confirm the responses on an endogenous GRE-containing gene in primary cells.

An important question is how GnRH activates the GR to induce transactivation of a GRE-reporter gene in LβT2 cells. One possible mechanism that could be investigated is that GnRH activation of the GnRHR results in activation of enzymes within the lipid raft complex that are in close proximity to the GR, resulting in post-translational modifications of the GR. This modification could then activate the GR in the absence of GCs, i.e. induce a conformational change in the GR, similar in some respects to that induced by the GC ligand. Such post-translational modifications could include phosphorylation by kinases, acetylation by acetylases or even addition of lipid modifications such as palmitoylation, myristoylation or GPI-anchoring (Neumann-Giesen *et al*., 2004; Faus *et al*., 2006; Zhou and Cidlowski, 2005; Kumar and Thompson, 2005; Lee *et al*., 2005; Allen *et al*., 2007). The study by Kotitschke *et al.* demonstrated that GnRH treatment resulted in a differential phosphorylation pattern of the GR compared to Dex (Kotitschke *et al*., 2009). The effect of these phosphorylation sites on the transcriptional activity of the GR could be investigated with a GRE-reporter assay and over-expression of phosphorylation deficient GR mutants in cells that have a low level of endogenous GR, such as COS-7 cells. It would be interesting to investigate other serine phosphorylation sites of the mouse GR in response to GnRH as several reports have indicated the importance of receptor phosphorylation in regulating transcription (Avenant *et al*., 2010a, b; Webster *et al*., 1997; Chen *et al*., 2008; Kino *et al*., 2007; Galliher-Beckley and Cidlowski, 2009). In addition, other post-translational modifications of the GR could be investigated, including the acetylation status of the GR and several lipid modifications, by using the same strategy as for the phosphorylation GR mutants. Furthermore, to determine if GR dimerization is required for the GnRH response on a GRE-reporter gene, a dimerization-deficient GR mutant can be used as above in COS-7.

The Dex-mediated increase of the GnRH-induced transcriptional activity of the AP-1-reporter gene was interesting as usually AP-1-containing genes are repressed by the GC-activated GR (De Bosscher and Haegeman, 2009). To explore if this increase is also mediated by a genomic mechanism, the co-factor recruitment of the GR in the presence of Dex and GnRH could be investigated by ChIP-assays in LβT2 cells. It is possible that the GR has a differential phosphorylation pattern in the presence of Dex and GnRH on residues not yet investigated, which

favors interaction of the receptor with co-activators over co-repressors. This could be investigated by using different GR phosphorylation mutants and ChIP-assays on AP-1 regulated genes in COS-7 cells.

In addition to a genomic mechanism described above, it is also possible that the Dex-mediated increase of the GnRH-induced transcriptional activity of the AP-1-reporter gene involves a non-genomic mechanism. This could be mediated via modulation of signaling proteins involved in the GnRH-induced activation of the GnRHR signaling pathway that specifically targets AP-1. Furthermore, it could be investigated whether specific proteins such as various G- proteins, ERK-1/2 and protein kinase A and C are involved in the synergistic response with Dex and GnRH on an AP-1-reporter gene (Grundker and Emons, 2003; Fink *et al*., 2010). This could be achieved by siRNA-mediated knockdown experiments.

It is well established that the GC-activated GR is down-regulated at the protein level (Wallace *et al*., 2010; Avenant *et al*., 2010b). The increased degradation of the GR that occurs to the same extent in response to GnRH as compared to Dex supports the finding that GnRH activates the GR. The GnRH-induced degradation of the GR could possibly be mediated via increased ubiquitination of the receptor. Ubiquitination of the human GR at S419A was previously shown to be involved in the GC-dependent degradation of the receptor (Wallace *et al*., 2010). To investigate if the mechanism underlying the GnRH-induced degradation of the GR is similar to that for Dex-mediated degradation, the ability of GnRH to induce degradation of wild type and S419A mutant GR proteins could be investigated in COS-7 cells. The proteases involved in the GnRH-induced degradation could be identified with specific inhibitors, such as MG132 for the ubiquitin-proteasome pathway. Alternatively, the expression of specific protease proteins, such as the calpain family, could be reduced by specific siRNA-mediated knockdown.

The second part of the present study demonstrated the co-localization of the GR and GnRHR to lipid rafts containing Flot-1 under all hormonal conditions investigated. An additional control would be helpful to evaluate the purity of the lipid rafts isolated. The localization of the transferrin receptor (which localizes to plasma membrane, but is excluded from lipid rafts) by immunoflorescence would distinguish the bulk plasma membrane from the lipid rafts.

Furthermore, probing for the transferrin receptor on the Western blot of the lipid raft gradient fractions would indicate the degree of plasma membrane contaminants.

A time-course experiment to identify the time of stimulation that results in maximum lipid raft association of the GR would be interesting, as this could indicate the optimal time-point for investigating the co-localization of the GR and GnRHR in LβT2 cells. It would be important to further characterize the lipid rafts for the presence of other signaling molecules such as G-proteins, MAPKs, RTKs, src kinase family members and Akt, which have previously been shown to localize to lipid rafts (Ha *et al*., 2003; Slaughter *et al*., 2003; Moffett *et al*., 2000; Zajchowski and Robbins, 2002). Although there is no differential localization of the GR to lipid rafts after 30 min treatment with Dex, GnRH, or both together, it is possible that other signaling molecules in lipid rafts are activated by hormone treatment. This could result in activation of specific signaling pathways initiated by either dissociation or formation of a specific signaling complex within the lipid rafts of the activated receptors.

The present study established by immunoflorescence analysis and Western blotting of lipid rafts isolated with density-gradients in LβT2 cells that both the GnRHR and a small amount of GR co-localize with Flot-1. These findings suggest that the GnRHR and GR also co-localize, but is not conclusive evidence. Thus, an important experiment would be to investigate if the GR co-localize with the GnRHR at the plasma membrane of LβT2 cells by immunoflorescence analysis. Furthermore, an important experiment would be to determine if the GnRHR is also present in the novel GR-Flot-1 complex in LβT2 cells by immunoprecipitation assays.

It would be interesting to investigate the mechanism of lipid raft association for the GR, as this would give insight into the proteins responsible for targeting the GR to lipid rafts. A recent report suggested that a highly conserved nine amino acid motif in the LBD of ERα and β, PR-A and B, and the AR is involved in plasma membrane targeting of these receptors. This domain was shown to contain palmitoylation sites that are important for membrane localization of these receptors (Pedram *et al*., 2007). Since members of the steroid receptor family share a highly conserved structure, it is likely that the GR could also contain this highly conserved nine amino acid domain. Thus, it is possible that palmitoylation could play a role in targeting the GR to lipid rafts.

The importance of this nine amino acid domain and the palmitoylation sites in the lipid raft association of the GR could be investigated by mutational studies of this domain. In addition, specific inhibitors of palmitoylation or myristoylation could be used to investigate the importance of these post-translational modifications on GR membrane-association.

The present study reported a mechanism for the lipid raft association of the GR through a physical interaction in a complex with Flot-1 under all conditions investigated. This suggests that the GR already exists in a signaling complex in the absence of ligand, which could fine-tune hormonal responses by modulating the resulting downstream signaling cascades. Thus, to understand the complex signaling pathways required to fine-tune the hormonal responses in LβT2 cells, characterization of this signaling complex, in terms of kinases and other signaling molecules, is important. It would be interesting to investigate the domains of the GR that are important for the Flot-1 interaction, as this could potentially give insight into the functional role of Flot-1 in GR signaling. An important experiment will be to investigate if the endogenous GnRHR is also present in the complex and if this association might be dependent on ligand.

The cholesterol depletion experiments were inconclusive since MβCD treatment had no effect on the GnRH-induced activation of ERK-1/2. These results suggested that the lipid rafts were not disrupted in LβT2 cells. Thus, it would be important to investigate more directly if the lipid rafts were disrupted by the MβCD and Filipin-III treatments. This could be performed by live-cell imaging where the cells are stained with GM1, followed by addition of cholesterol depletion agents to the live cells. If the agents disrupted lipid rafts, it would be evident by the dissociation of GM1 from the plasma membrane. An alternative strategy that could be used to prevent the formation of lipid rafts in cells, involves reducing the synthesis of cholesterol by inhibiting the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme by siRNA-mediated knockdown or specific inhibitors.

The Flot-1 knockdown experiment showed that Flot-1 is required for the Dex and the synergistic transcriptional responses on a GRE-reporter gene in LβT2 cells. It would be important to identify the mechanism involved for this repression of GR transactivation in the absence of Flot-1, as this could give insight into the mechanism of transcriptional regulation by the GR. It would be

interesting to examine if GR phosphorylation is altered by decreased Flot-1 protein levels, as phosphorylation has previously been shown to be important in the transcriptional activity of the GR (Avenant *et al*., 2010a, b). In addition, a very important experiment would be to determine what effect decreased Flot-1 protein levels will have on the GnRH response on a GRE-containing gene in LβT2 cells. This is a difficult experiment to perform and should perhaps rather be performed on an endogenous GRE-containing gene. Showing the effect on endogenous gene expression does not require the additional transfection of cells with a GRE-reporter plasmid, which could have contributed to GnRH failing to induce a transcriptional response in Figure 3.9.

Addendum

Supplementary optimization data

A)

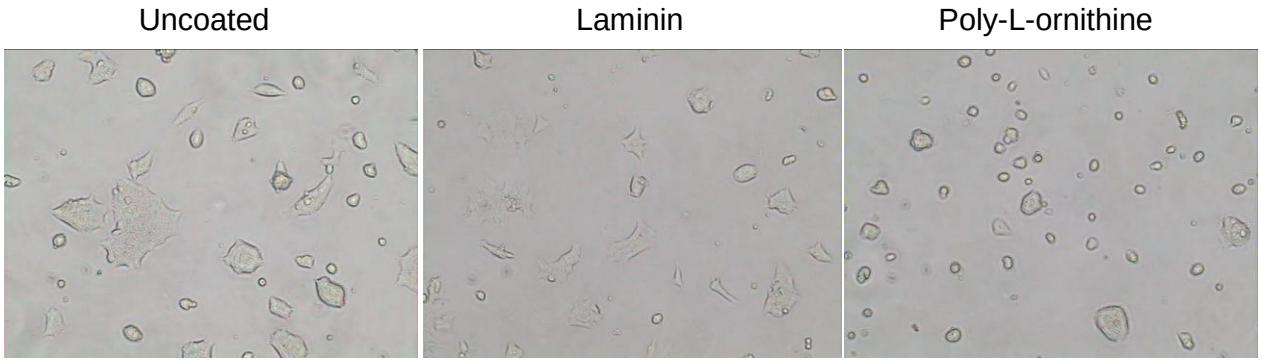


Figure A: No difference in proliferation or morphology of LβT2 cells between uncoated or laminin coated glass slides, while cells growing on poly-L-ornithine have significant changes in cell morphology with a decreased rate of proliferation

Cover slips were coated with 5 μg laminin or 2.5 μg poly-L-ornithine and incubated for 1 h at room temperature. Both cover slips were washed with PBS and only the poly-L-ornithine-coated cover slip was allowed to dry. LβT2 cells were seeded at a density of 3 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. Cells were cultured for two days before visualization with an inverted light microscope.

B)

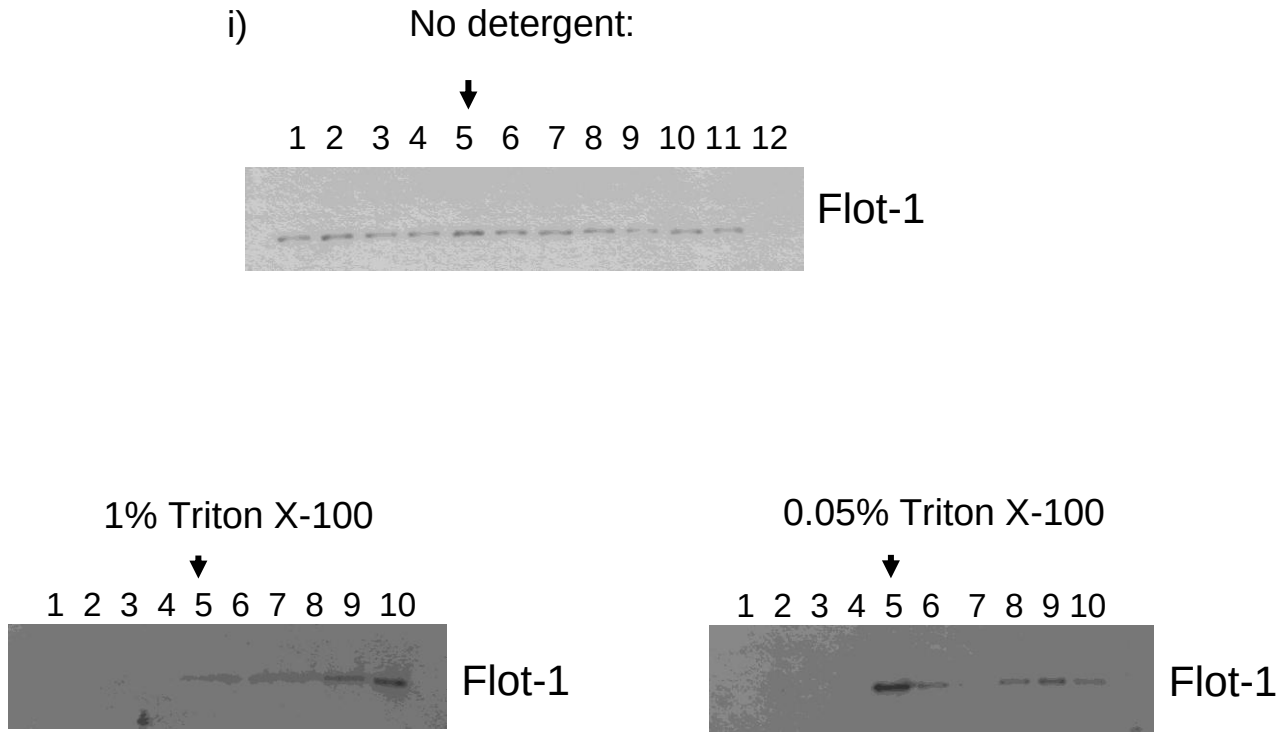
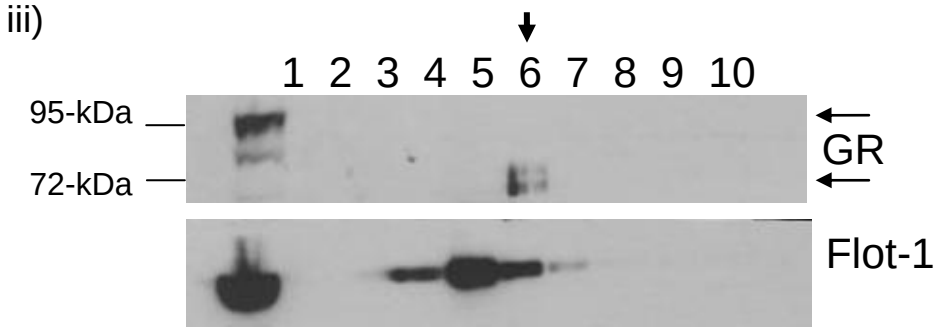


Figure B: Isolation of lipid rafts from LβT2 cells with a non-detergent method resulted in ineffective fractionation of Flot-1, whereas a method employing 0.05% Triton X-100 resulted in more effective fractionation of Flot-1 as compared to 1% Triton X-100, which increased the solubility of Flot-1 resulting in its fractionation into the higher density fractions

i. LβT2 cells were washed with PBS and the cells collected and resuspended in homogenization buffer (HB, 250 mM sucrose, 3 mM imidazole (pH 7.4), 2 mM EDTA and 1 mM PMSF), followed by lysis in a Dounce homogenizer. Unbroken cells and nuclei were removed by low-speed centrifugation, followed by equilibrium flotation through a discontinuous sucrose-density gradient. Twelve fractions were collected from the top of the gradient and equal amounts of fractions were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with a specific antibody against Flot-1. The arrow indicates the expected position of the lipid raft fraction. **ii.** LβT2 cells were washed with PBS and the cells collected and resuspended in extraction lysis buffer (ELB) (10 mM Hepes (pH 7.9), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM

DTT and 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin), followed by lysis in a Dounce homogenizer. Unbroken cells and nuclei were removed by low-speed centrifugation and a crude membrane fraction was obtained by centrifugation. The membrane pellet was washed with ELB and resuspended in solubilization buffer (SB) (25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin) containing 1% or 0.05% Triton X-100. After incubation for 45 min on ice, the crude membrane fraction was subjected to equilibrium flotation through a discontinuous sucrose-density gradient. Ten fractions were collected from the top of the gradient and equal amounts of fractions were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with a specific antibody against Flot-1. The arrow indicates the expected position of the lipid raft fraction.



Biii: Lipid raft-associated GR appears to be degraded when using a crude membrane fraction as the starting material for the isolation of lipid rafts from LβT2 cells

LβT2 cells were washed with PBS and the cells collected and resuspended in extraction lysis buffer (ELB) (10 mM Hepes (pH 7.9), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin), followed by lysis in a Dounce homogenizer. Unbroken cells and nuclei were removed by low-speed centrifugation and a crude membrane fraction was obtained by centrifugation. The membrane pellet was washed with ELB and resuspended in solubilization buffer (SB), 25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin and 2 μg/mL aprotinin) containing 0.05% Triton X-100. After incubation for 45 min on ice, the crude membrane fraction was subjected to equilibrium flotation through a discontinuous sucrose-density gradient. Ten fractions were collected from the top of the gradient and equal amounts of fractions were loaded on an 8% SDS-PAGE gel, transferred

onto nitrocellulose membrane and probed with specific antibodies against Flot-1 and GR. The vertical arrow indicates the lipid raft fraction.

C)

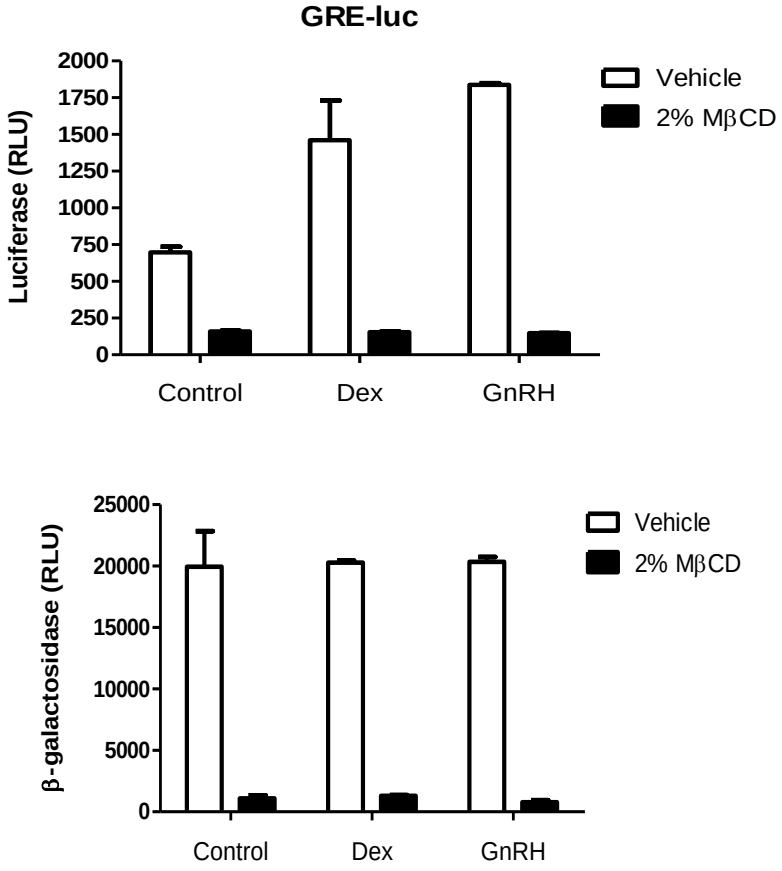


Figure C: Cholesterol depletion of cells with the continuous treatment with MβCD abolishes luciferase and constitutive β-galactosidase expression and results in death of LβT2 cells

LβT2 were seeded in 24-well plates at a density of 1 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. The cells were cultured for two days and transfected with 250 ng of TAT-GRE-luciferase construct and 25 ng pSV β-galactosidase plasmid. After 24 h, the cells were incubated for 8 h with MβCD in serum-free medium in combination with 100 nM Dex or 100 nM GnRH. The cells were harvested in 50 μL reporter lysis buffer per well and both the luciferase and β-galactosidase assays were performed with 10 μL of cell lysates. The results are representative of two independent experiments.

D)

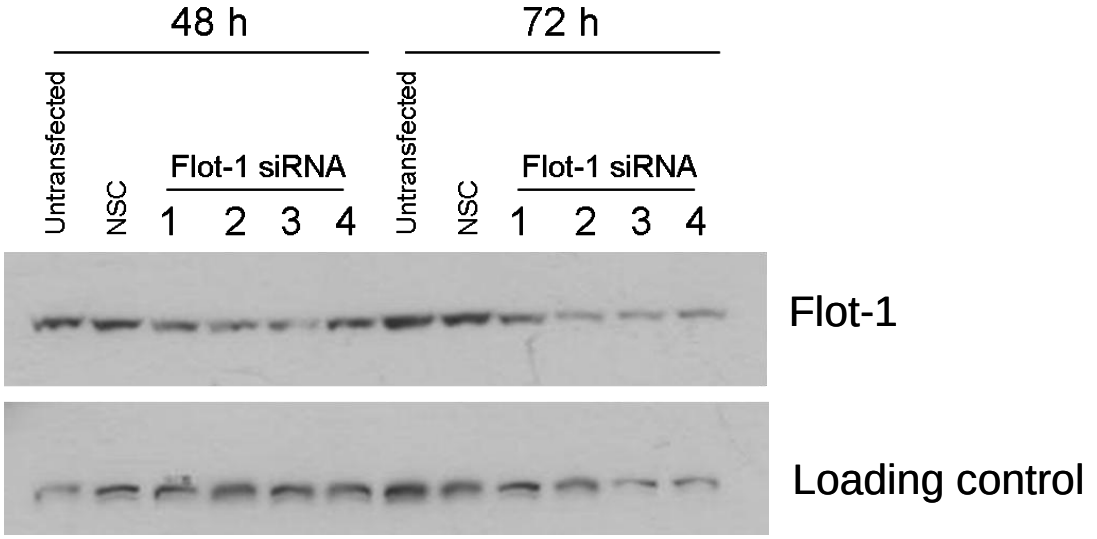


Figure D1: No reproducible differences were detected in the amount of Flot-1 knockdown between four different siRNA oligonucleotides tested, while 72 h incubation with siRNA appears to result in a greater decrease in the expression of Flot-1 protein compared to 48 h in LβT2 cells

LβT2 cells were transfected with non-silencing control or four different specific mouse Flot-1 siRNA oligonucleotides at a final concentration of 20 nM and incubated for 48 h or 72 h. Equal amounts of samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with specific antibodies against Flot-1 and GM130 (loading control). The Western blot shown is representative of at least two independent experiments.

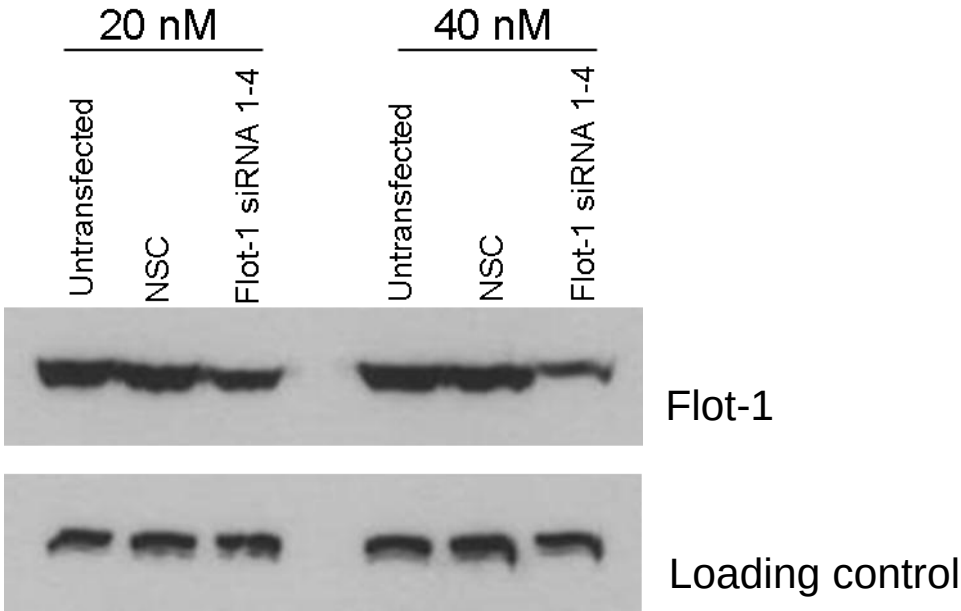


Figure D2: A combination of all four Flot-1 siRNA oligonucleotides at a final concentration of 40 nM appears to induce a greater amount of Flot-1 knockdown in LβT2 cells as compared to 20 nM

LβT2 cells were transfected with non-silencing control or a combination of four different specific mouse Flot-1 siRNA oligonucleotides at a final concentration of 20 nM or 40 nM and incubated for 72 h. Equal amounts of samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with specific antibodies against Flot-1 and GM130 (loading control). The Western blot shown is representative of two independent experiments.

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