The glucocorticoid receptor plays a central role in mammalian reproduction and signal integration in pituitary gonadotropes

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

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UNIVERSITY OF CAPE TOWN

Supervisor: Professor Janet P. Hapgood

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work (unless acknowledged otherwise) and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature....................................................

Date..........................................................
Acknowledgements

Every single step in life leads you down an exciting or daring pathway with the occasional appearance of unanticipated challenges where you have a simple or challenging life-changing decision to make. The successful deciphering of such problems would not have been possible without the support, guidance and friendship of many people throughout my PhD career. It is to them that I owe my greatest gratitude. I would like to thank all of you, no matter what the part was you played in helping me to choose correctly and putting me back on the narrow road towards my objective.

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My academic career and this project would not have been possible without the financial support provided by NRF, DAAD, UCT, Boehringer Ingelheim Fonds, Ernst & Ethel Ericksen Trust, and for this I am truly grateful.
Reciprocal modulation between the glucocorticoid receptor (GR) and gonadotropin-releasing hormone (GnRH) signalling pathways is a potential mechanism for integrating cellular responses to stress with reproductive function. This study investigated if membrane rafts play a role in GR and GnRH receptor (GnRHR) crosstalk and explored the mechanism involved in the mouse pituitary gonadotrope LβT2 cell line by dexamethasone (Dex), GnRH and both together. Immunofluorescence showed GnRHR and a small population of the GR co-localize with the membrane raft protein flotillin-1 (Flot-1) at the plasma membrane. Real-time qPCR showed the serum/glucocorticoid regulated kinase 1 (SGK-1) gene is upregulated by Dex and GnRH, whereas the combination synergistically increases SGK-1 mRNA levels. Protein knockdown, antagonist and inhibitor strategies showed the synergistic increase of SGK-1 mRNA requires the GR, GnRHR, Flot-1 and the protein kinase C (PKC) pathway. Nuclear translocation as well as the SGK-1 promoter occupancy of the GR induced by Dex remain unchanged in the presence of GnRH, while several cofactors are differentially recruited, suggesting a mechanism of crosstalk involving changes in promoter occupancy by cofactors. Dex and GnRH co-stimulation synergistically attenuated gonadotrope cell proliferation in a SGK-1, Flot-1 and PKC dependent manner. Towards investigating the role of the GR and the presence of crosstalk in primary cells and tissue, mouse pituitaries and primary mouse gonadotrope cells isolated from the tissue were investigated. Strong support for a physiologically relevant role for the GR in mammalian reproduction was obtained in mouse pituitary tissue and primary gonadotrope cells in vitro. Immunohistochemistry of GRIC/R26-YFP mouse pituitary sections showed that the GR protein is expressed in the majority of the cells present in the pituitary, including the gonadotrope cells. Real-Time PCR revealed that Dex upregulated mRNA levels in pituitary tissue of the SGK-1 and GILZ genes, in both the tissue and gonadotropes, the effect of which was reversed by the GR antagonist RU486 in the tissue. Furthermore, Dex upregulated mRNA levels of key reproductive genes including the GnRHR, the glycoprotein hormone α subunit (αGSU) and β subunits of luteinizing hormone (LHβ) and follicle-stimulating hormone (FSHβ) in pituitary tissue, and GnRHR mRNA levels in the primary gonadotropes, suggesting multiple levels of upregulation of reproduction by glucocorticoids directly at the level of the pituitary in response to acute stress. Upregulation of GnRHR and FSHβ mRNA levels in the pituitary tissue is consistent with at least in part the occurrence of transcriptional mechanisms for GnRH upregulation of reproductive function directly at the pituitary level. Interestingly, both Dex and GnRH alone decreased GRα mRNA levels in the primary gonadotropes, suggesting a mechanism for negative
feedback of glucocorticoids at the gonadotrope level, which may contribute to differential effects of acute versus chronic stress. Comparison of the results in gonadotrope versus pituitary tissue provides evidence for extensive paracrine effects on direct modulation of expression of reproductive genes at the level of the whole pituitary tissue. Comparison of results in pituitary tissue from female mice at different stages of the estrous cycle supports a model whereby reproduction is regulated by fine-tuning of gene expression during different stages of the estrous cycle and as a function of time. The genes investigated appeared to be more sensitive to GnRH in the LβT2 cell line than the primary gonadotrope cells but similar qualitative effects were observed on mRNA levels with GnRH or Dex for some genes, while other genes showed different effects, suggesting only partial conservation of mechanisms in the cell line. Towards understanding the role of membrane rafts in GR and GnRHR signalling in the pituitary tissue and primary gonadotropes, it was shown by immunohistochemistry in mouse pituitary sections that Flot-1 protein is expressed in the majority of the pituitary cells including gonadotrope cells, although the functional significance remains to be determined in the primary models. Although synergism was not observed with Dex + GnRH co-treatment for any gene, the responses were reciprocally modulated upon co-stimulation in both pituitaries and primary gonadotrope cells. The primary cell and tissue findings are consistent with crosstalk of the GR and GnRHR signalling pathways directly at the level of the pituitary where GR signaling can genespecifically both increase and decrease reproductive function, depending on GnRH levels.
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<th>Definition</th>
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic-releasing hormone</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BIM</td>
<td>bisindolylmaleimide</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>Cav</td>
<td>caveolin</td>
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<td>CBP</td>
<td>CREB-binding protein</td>
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<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
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<tr>
<td>Dex</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DRM</td>
<td>detergent-resistant membranes</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELB</td>
<td>extraction lysis buffer</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<tr>
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<td>estrogen-response-element</td>
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<td>ERK-1/2</td>
<td>extracellular-regulated kinase 1/2</td>
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<td>E₂</td>
<td>estrogen</td>
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<tr>
<td>FACS</td>
<td>fluorescent-activated cell sorting</td>
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<td>fetal calf serum</td>
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<td>FK506 binding protein</td>
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<td>flotillin</td>
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<td>follicle-stimulating hormone</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GC</td>
<td>glucocorticoid</td>
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<tr>
<td>GILZ</td>
<td>glucocorticoid-inducible leucine zipper</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
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</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
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<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<td>histone acetyltransferase</td>
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<td>HPA</td>
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<td>HPG</td>
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<td>HSP</td>
<td>heat-shock protein</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>IP3</td>
<td>inositol-1, 4, 5-triphosphate</td>
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<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>mGR</td>
<td>membrane glucocorticoid receptor</td>
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<tr>
<td>MKP-1 AKA DUSP</td>
<td>MAPK phosphatase 1 AKA dual specificity phosphatase</td>
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<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
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<tr>
<td>MTT</td>
<td>thiazolyl blue tetrazolium bromide</td>
</tr>
<tr>
<td>MβCD</td>
<td>methyl-β-cyclodextrin</td>
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<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
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<tr>
<td>NSC</td>
<td>non-silencing control</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
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<tr>
<td>P₄</td>
<td>progesterone</td>
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<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase-activating polypeptide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PI3K</td>
<td>phosphatidyl inositol 3-kinase</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>POMC</td>
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<td>progesterone receptor</td>
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<td>adenovirus E1A binding protein 300</td>
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<td>radio-immunoprecipitation assay</td>
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<td>receptor tyrosine kinase</td>
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<td>SB</td>
<td>solubilization buffer</td>
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<td>SGK</td>
<td>serum/glucocorticoid regulated kinase</td>
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<td>SPFH</td>
<td>Stomatin/Prohibitin/Flotillin/HflK/C</td>
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<tr>
<td>SRC</td>
<td>steroid receptor co-activator</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>SWI/SNF</td>
<td>switch/sucrose non-fermentable protein</td>
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<td>TAT</td>
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<td>YFP</td>
<td>yellow fluorescent protein</td>
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<tr>
<td>αGSU</td>
<td>glycoprotein hormone alpha subunit</td>
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THESIS OUTLINE

Please note that several sections in chapter 3 of this study have been published in the Journal of Biological Chemistry. (Wehmeyer et al., 2014) (Wehmeyer et al., 2014) (Wehmeyer et al 2014) (Wehmeyer et al 2014) (Wehmeyer et al 2014) (Wehmeyer et al 2014) (Wehmeyer et al 2014) (Wehmeyer et al 2014)


All the experimental work in this manuscript was performed by the present author and a copy of the above published research article is shown in the addendum.

Animal ethics clearance, **2012/V40/JH**, was obtained from the University of Cape Town for the research of chapter 4, which was performed in the laboratory and under the supervision of Professor Ulrich Boehm in the Department of Pharmacology and Toxicology, University of Saarland, School of Medicine, Homburg, Germany.

This thesis contains the following sections:

1. A summary of the general Hypotheses and Aims of the study.

2. Chapter 1: Literature Review. This chapter provides a brief overview of the current literature, with a focus on GR crosstalk, pituitary tissue and gonadotropes, GnRHR crosstalk, membrane rafts, Flot-1 and SGK-1.
3. Chapter 2: **Materials and Methods.** This chapter provides detailed information about the experimental procedures used to obtain the results presented in chapters 3 and 4.

4. Chapter 3: **Results.** In the first part of this study the mechanism of **crosstalk between the GR and GnRHR** signalling pathways and of the **synergistic responses** including the **physiological function** was investigated in LβT2 cells. A brief introduction, the specific aims and the results are presented in this chapter.

5. Chapter 4: **Results.** In the second part of this study, the regulation by **GCs and the GR** in the **pituitary** of **model GRE-containing** and **reproductive genes** was investigated, as well as whether **responses** for the **mRNA expression of several genes** observed in LβT2 cells could be **validated** in **primary gonadotrope cells.** This chapter contains a short introduction, strategy with the aims and the results.

6. Chapter 5: **Discussion and Conclusions.** In the final chapter the results of the previous two chapters are discussed and some perspectives of future research with strategies are provided.

7. The **Bibliography** consisting of an alphabetical list of all the references cited in this thesis.

8. **Addendum:** A copy of the research article by **Wehmeyer et al. 2014** that is published in the Journal of Biological Chemistry.
AIMS AND HYPOTHESES

This project was based on the following hypotheses:

I) That crosstalk between the GR and GnRHR induces phosphorylation and activation of the GR and regulates the expression of some endogenous glucocorticoid response element (GRE)-containing genes in the absence and presence of a GR ligand;

II) Crosstalk between the GR and GnRHR signalling pathways results in a synergistic response on some endogenous GRE-containing genes and requires the localization of the receptors to membrane rafts in immortalized mouse gonadotrope and primary mouse gonadotrope cells;

III) The GR and GnRHR co-localization forms a signal transduction platform with other signalling molecules to modulate downstream genomic signalling in a gene-specific manner in LβT2 cells;

IV) The Dex + GnRH-mediated synergism is involved in regulating cellular growth via membrane rafts and some intracellular signalling pathways in LβT2 cells;

V) The Dex and GnRH induced mRNA regulation of several model genes is reciprocally modulated upon co-stimulation in primary mouse gonadotrope cells;

The aim of the first part of the study was to explore the mechanism of GR and GnRHR crosstalk on endogenous GRE-containing genes and to investigate the physiological significance of this regulation in LβT2 cells. In particular the project focussed on the functional role of membrane rafts in GR and GnRHR crosstalk, in particular the role in non-genomic and genomic actions as well as in cell proliferation. Firstly, the membrane raft localization of GR, GnRHR and other signalling molecules was investigated as well as the activation of the GR and kinases present in a pre-formed signalling complex. Furthermore, the signalling pathways that are involved in synergistic mRNA expression response were determined. Nuclear import of the GR, expression of different GRE-
containing genes and the genomic mechanism of synergism on a selected model gene were investigated. The final section of the first part explored whether the physiological function of synergism involved in the regulation of cellular growth, which was determined through cell viability and proliferation assays and also included the siRNA-mediated knockdown and pharmacological inhibitor strategies. The last part of the study aimed to determine the role played by the GR in the pituitary on the expression of key mammalian reproductive genes as well as whether GC- and/or GnRH-induced effects on selected mRNA expression levels observed in the gonadotrope cell line also occur in mouse pituitary tissue and primary gonadotrope cells. In addition, this part also investigated whether paracrine effects and the estrous cycle are involved in modulating the ligand-induced regulation of the model genes in the pituitary by comparing the gene expression responses of the pituitary with primary gonadotrope cells as well as comparing the responses for all four stages of the estrous cycle. The final section investigated whether GR and Flot-1 proteins are expressed in these primary tissue and cell systems. More specific aims are presented in the relevant chapters.
CHAPTER 1
LITERATURE REVIEW

1.1 Glucocorticoid receptor

1.1.1 The hypothalamic-pituitary-adrenal axis

All living organisms exist by maintaining a complex dynamic equilibrium known as homeostasis, which is constantly challenged by intrinsic or extrinsic adverse forces called the stressors. To maintain homeostasis in response to stressors, living organisms have developed a regulatory stress system, which upon activation leads to a collection of physiologic and behavioral central nervous system (CNS) and peripheral adaptive responses (Charmandari et al., 2005, Chrousos and Gold 1992, Chrousos 2009). If the stressor is chronic or prolonged it may negatively influence personality development, behavior and disrupt fundamental physiologic functions, like development, reproduction, metabolism and the immune response. The hypothalamic-pituitary-adrenal (HPA) axis is one of the two major limbs of the stress system that regulates homeostasis by acting synergistically with the locus caeruleus/norepinephrine-autonomic nervous system. This neuroendocrine axis consist of three components, which are the paraventricular nuclei (PVN) in the hypothalamus, corticotrope cells located in the pituitary gland and the zona fasciculata located in the adrenal cortices (Nicolaides et al., 2015).

The HPA axis is activated in response to stress and regulates the synthesis of the endogenous glucocorticoid (GC) cortisol that is secreted from the adrenal cortex. In humans cortisol, or in rodents corticosterone, is the natural ligand for the glucocorticoid receptor (GR). During a period of inflammation, lymphocytes and macrophages are activated which lead to the production of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin (IL) 6 and IL-1β. These cytokines induce the expression of pro-opiomelanocortin (POMC), the pre-cursor of corticotrophin-releasing hormone (CRH). CRH from the hypothalamus induces the synthesis and secretion of adrenocorticotropin-releasing hormone (ACTH) in pituitary corticotrope cells in the anterior pituitary. ACTH enters into the blood circulation system, and stimulates the adrenal cortex, resulting in the synthesis and secretion of GCs (Chrousos 1995, Smoak and Cidlowski 2004). GCs are anti-inflammatory hormones that affect the function of various cell types including the hypothalamus, corticotropes, gonadotropes, T-cells, macrophages, eosinophils, neutrophils, mast
cells, endothelial and epithelial cells (Kotitschke et al., 2009, Nicolaides et al., 2015, Smoak and Cidlowski 2004).

1.1.2 Physiological function of GCs and GR in the pituitary

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, Breen and Mellon 2014, Tilbrook et al., 2000). Some of the effects of stress on reproduction are mediated via GCs from the adrenal gland as part of a crosstalk mechanism between the 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 a synthetic GC called Dex (Rosen et al., 1991). Furthermore, in cultured rat pituitary cells, GCs increased the secretion of follicle-stimulating hormone (FSH), while having no effect or decreasing luteinizing hormone (LH) secretion (Baldwin et al., 1991, Brann and Mahesh 1991, D'Agostino et al., 1990, Kilen et al., 1996, McAndrews et al., 1994). 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 FSH 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).
Figure 1.1: Structural organization of the human GRα protein and its splice variants and alternative translation initiation sites.

The GR is composed of three major functional domains, the N-terminal domain (NTD); the central DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) which is linked to the DBD by a hinge region (H). Each domain consists of a specific function, like the NTD is involved in the recruitment of the basal transcriptional machinery. The NTD has a transcriptional activation function (AF)-1 domain which contains the majority of residues that receive post-translational modifications (PTM). The DBD consists of two zinc-fingers that are involved in DNA binding, GR dimerization and nuclear translocation. The LBD contains an AF-2 domain that interacts with co-factors in a ligand-dependent manner. The initiation of translation from eight different methionine residues in one GRα mRNA transcript generates isoforms with progressively shorter NTDs (Cruz-Topete and Cidlowski 2015, Oakley and Cidlowski 2011).
1.1.3 GR isoforms and functional domains

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 is comprised 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 (Bamberger et al., 1995, Oakley et al., 1999). 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, like GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, GRα-D3 and the same for GRβ (Lu and Cidlowski 2005, Nicolaides et al., 2010, Nicolaides et al., 2014, Oakley and Cidlowski 2011, Zhou and Cidlowski 2005). The existence of various GR isoforms can be a mechanism for the differential cellular responsiveness to GCs.

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) (Cruz-Topete and Cidlowski 2015, Griekspoor et al., 2007, Oakley and Cidlowski 2013) (Figure 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 (Heitzer et al., 2007, Kumar and Thompson 2005). 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., 1988). 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. However, a recent study provided evidence for the underlying mechanism of glucocorticoid resistance in patients with a rare genetic condition. This condition was associated with a natural mutation of V423A located in the DBD of the human GR that resulted in structural alterations of the receptor. The altered structural conformation of the GR primarily result in a decreased ability to bind target GRE sites and attenuated translocation into the nucleus (Roberts et al., 2013). 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 (Bledsoe et al., 2002). The LBD contains a second nuclear localization signal and the transcriptional AF-2 region, which plays a role in the binding of heat-shock proteins (HSP)s and recruitment of co-factors (Beck et al., 2011, Bledsoe et al., 2004, Busillo and Cidlowski 2013, Cruz-Topete and Cidlowski 2015, Nicolaides et al., 2010, Oakley and Cidlowski 2011, Oakley and Cidlowski 2013, Savory et al., 1999, Schena et al., 1989).

1.1.4 Ligand-dependent genomic GR mechanism of action

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 HSP90, HSP70, HSP23, phosphatases such as protein phosphatase 5 (PP5) and immunophilins like FK506 binding protein (FKBP) 51 (Kumar and Thompson 2005, Pratt and Toft 1997, Wang et al., 2007). The majority of circulating GCs (90%) is bound to corticosteroid-binding globulin (CBG), whereas the free or loosely-bound GCs are lipophillic and enter the cell by diffusing across the plasma membrane (Lu et al., 2006, Torpy and Ho 2007). Upon ligand binding, the GR changes conformation
and is released from some of the cytoplasmic chaperone proteins. The resulting change in conformation of the GR is accompanied by hyper-phosphorylation of the receptor and exposure of the nuclear localization signals (Zhou and Cidlowski 2005).

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 are important for homodimerization, still results in nuclear import and transrepression of target genes, but not transactivation (Bledsoe et al., 2002, Reichardt et al., 1998). These findings suggest that receptor dimerization is not required for nuclear import or transrepression of GR target genes, but is important for transactivation. The transcriptional activity of the GR has been correlated with its oligomerization status (De Bosscher and Haegeman 2009, Kleiman and Tuckermann 2007). However, a recent study by Presman et al. showed that there is no correlation between monomeric or dimeric GR and transcriptional activity (Presman et al., 2014).

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, Vandevyver et al., 2012). Not all chaperone proteins are released upon ligand-binding, 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, it has been 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 is present in a complex with FKBP52, the GR is mostly nuclear, while the GR is mostly cytoplasmic when in present in a 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 Yamamoto 2004). Recent ChIP-seq experiments have identified various genomic GR binding regions which differ in underlying DNA sequence motifs and GR functional surfaces for gene regulation. The identification of GR binding
regions that specifically utilize certain DNA surfaces may discriminate motifs and half sites utilized (Schiller et al., 2014).

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/glucocorticoid regulated 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.

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 1.2. The GRE consists of two conserved six-base pair half sites separated by a non-conserved three-base pair spacer: 5’T-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). When bound to the DNA, activated GR serves as a platform for the recruitment of transcription factors and the basal transcription machinery to induce transcriptional activation of target genes (Ford et al., 1997, Newton and Holden 2007). 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, King et al., 2012). 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 cyclic-AMP (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 results in neutralization of the charges and dissociation of DNA from the histones allowing the binding of transcription factors to increase 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, Rollins et al., 2015). 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), GILZ, SGK-1 and phosphoenolpyruvate carboxykinase (PEPCK) genes.

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 activator protein 1 (AP-1), nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription 5 (STAT-5) (De Bosscher and Haegeman 2009, Doppler et al., 2001, Kassel and Herrlich 2007, Stoelcklin et al., 1997). 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. 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). Many of the target genes that are negatively regulated by this tethering mechanism have been shown to contain cis-elements in the DNA regulatory regions for transcription factors such as AP-1 and NF-κB, which are well-known to be involved in mediating pro-inflammatory responses (De Bosscher
and Haegeman 2009). It has been reported that tethering of the 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 collagenase 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 to mediate 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 (Doppler et al., 2001, Stoecklin et al., 1997), 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) assays the GR and AP-1 were shown to be recruited to the same collagenase-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 IL-6, 8 and collagenase (Kassel and Herrlich 2007, Lerner et al., 2003), 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 of GnRHR mRNA expression in LβT2 cells (Kotitschke et al., 2009). Transactivation mediated by the ligand-activated GR through tethering to AP-1 is 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, Rogatsky et al., 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.
1.1.5 Post-translational modifications

Several reports in the literature have shown that post-translational modifications of the GR (Figure 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). 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). The authors reported a decrease in the level of acetylated GR upon mutation of these two residues to alanine (Ito et al., 2006), although acetylation could still be detected, suggesting the

**Figure 1.2: GR mechanism of action.**

After hormone binding the GR undergoes a conformational change and translocates to the nucleus where it binds to a GRE site in DNA that results in transactivation or interacts with transcription factors, like NF-κB to regulate transcription. **GRE**: glucocorticoid-response-element; **NF-κB**: nuclear factor-kappa B; **HAT**: histone acetyltransferase. Taken from (Faus and Haendler 2006, Holgate and Polosa 2008).
presence of other as yet unidentified acetylation sites. The same study 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 overexpression of p300 increased the GR-mediated transactivation of a mouse mammary tumor virus (MMTV) reporter gene in HeLa cells (Li et al., 2002). The same study showed that the mechanism involved GR and p300 association in a complex that required the HAT activity of p300 (Li et al., 2002). A different study showed that overexpression of p300 in primary rat astrocytes decreased the transcriptional activity of the GR on a simple GRE, while overexpression of CBP increased GR-mediated transactivation (Fonte et al., 2007). These results indicate that acetylation of the GR increases or decreases the 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. Importantly, the differential effects of p300 and CBP on GR transactivation require further experiments for verification on endogenous GRE-containing genes, such as GILZ or SGK-1.

Early findings showed that the GR exists as a phospho-protein within whole cells and becomes hyperphosphorylated at multiple residues upon hormone treatment (Housley and Pratt 1983, Orti et al., 1989, Singh and Moudgil 1985). Ten phosphorylation sites that are highly conserved between species have been identified in the GR, most of which are serine residues located in the NTD (Galliher-Beckley and Cidlowski 2009, Lambert et al., 2013). Interestingly, three of the mouse residues, namely Ser-212, Ser-220 and Ser-234 (corresponding to Ser-203, Ser-211 and Ser-226 in the human GR), located in the AF-1 domain of the GR, become hyperphosphorylated in response to

Figure 1.3: Post-translational modifications of the human GR.

P: phosphorylation; A: acetylation; S: sumoylation; U: ubiquitination. The bold and underlined residues (S203, S211 and S226) are investigated in the present study.

Obtained and modified from (Faus and Haendler 2006, Galliher-Beckley and Cidlowski...
GC 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 human GR was investigated by individual or simultaneous mutations of the phosphorylation residues to alanine. These unphosphorylated mutant receptors were compared with the wild-type receptor for the ability to transactivate an 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). A study by Kino et al. also reported an increase in the transcriptional activity of the mutated GR in HCT116 cells (Kino et al., 2007), while the combination of Ser-212A, Ser-220A and Ser-234A corresponding mutations in the human GR decreased transactivation of a MMTV reporter gene in COS-1 cells (Avenant et al., 2010a). These two findings suggest that phosphorylation of the GR regulates the transcriptional activity in a species- or cell-specific manner. Two more human GR phosphorylation sites were discovered in the past five years, Ser-134 and Ser-267 (Galliher-Beckley et al., 2011, Lambert et al., 2013). Galliher-Beckley et al. reported that Ser-134 phosphorylation is ligand-independently induced by a variety of stress-activating stimuli, like energy starvation, UVC irradiation and oxidative stress responses. The authors proposed that phosphorylation at Ser-134 may act as a molecular sensor on the GR that monitors the level of cellular stress to redirect GC-regulated transcriptional signalling (Galliher-Beckley et al., 2011). Lambert et al. investigated the impact of neurotrophic signalling on GR-dependent gene expression to obtain more insight into numerous psychiatric disorders implicated by abnormal GC signalling. The author’s reported that brain-derived neurotrophic factor (BDNF) stimulation enhanced the Dex-induced transcriptional activity of the GR on a TAT-GRE reporter gene. The same study also found a novel GR phosphorylation site at Ser-267, which is induced by Dex and BDNF in primary rat cortical neurons (Lambert et al., 2013). However, the role of this residue in GR function remains to be determined.

Previous studies have reported that the phosphorylation status of the GR also plays a critical role in various other GR properties. For instance, phosphorylation of the mouse GR at the sites located in the AF-1 domain affects the stability of the GR protein by abolishing the ligand-induced GR protein degradation (Wallace and Cidlowski 2001, Wallace et al., 2010, Webster et al., 1997). However, another study showed that mutating a single or combination of these phosphorylation sites has no effect on the ligand-induced GR degradation (Avenant et al., 2010b). Additionally, it was shown that the phosphorylation status of the GR controls protein-protein interactions with co-factors such as GRIP-1 (Avenant et al., 2010a) 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 Ser-203 and Ser-226 of the human GR (Wang et al., 2007). The Ser-203 phosphorylated GR is contained solely in the cytoplasm and the GRE-binding ability was abolished in the Ser-203A mutant GR (Blind and Garabedian 2008, Wang et al., 2002). The inhibition of extracellular-regulated kinase 1 and 2 (ERK-1/2) reduces the level of GR phosphorylation at Ser-203 and results in enhanced nuclear import with increased Dex-mediated regulation of GLUT-5 mRNA expression, which suggests a role for phosphorylation in regulating GR transcription (Takabe et al., 2008). Together, these results suggest that when the GR is phosphorylated at Ser-203, the receptor localizes to the cytoplasm and is transcriptionally inactive.

The GR phosphorylated at Ser-211 localizes to both the nucleus and the cytoplasm of cells with the quantity in the nucleus found to be directly proportional to the phosphorylation level, which directly correlates with the transcriptional activity of the GR as measured by gene reporter assays (Chen et al., 2008, Wang et al., 2002). The Ser-211 phosphorylation of the GR appears to be mediated by the mitogen-activated protein kinase (MAPK) p38 in 3T3-L1 cells (Miller et al., 2005, Nader et al., 2010). The Ser-211 phosphorylated GR was also shown to occupy the promoters of many GRE-containing genes including SGK-1, TAT and GILZ (Blind and Garabedian 2008, Peffer et al., 2014). Taken together, the ligand-induced Ser-211 phosphorylation of the GR is associated with nuclear import and transcriptional regulation of target genes and may be considered as a bio-marker for GR activation.

The human GR has been shown to be phosphorylated at Ser-226 in vitro by the MAPK, c-Jun N-terminal kinase (JNK) and cyclin-dependent kinase 5 (CDK5) in a cell-specific manner, which is implicated in the reduced transcriptional activity of a GRE reporter gene (Avenant et al., 2010a, Galliher-Beckley and Cidlowski 2009, Itoh et al., 2002, Kino et al., 2007, Rogatsky et al., 1998). The attenuation of GC signalling by JNK-mediated phosphorylation of the GR at Ser-226 is implicated in enhanced nuclear export of the GR (Itoh et al., 2002). However, a more recent study showed that the Ser-226 phosphorylated GR strongly associated with two different endogenous GRE-containing promoters, the TAT and sulfonil transferase 1A1 genes (Blind and Garabedian 2008), suggesting it is still transcriptionally active. In another report, Galliher-Beckley et al. showed that phosphorylation at Ser-404 of the human GR induced with Dex is mediated by glycogen synthase kinase 3 (GSK3) in human osteosarcoma cells (Galliher-Beckley et al., 2008). Furthermore,
constitutive phosphorylation at Ser-404 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 the transcriptional activity of the GR in a species-, cell- and promoter-specific manner.

1.1.6 Rapid glucocorticoid-mediated non-genomic actions

Besides transcriptional genomic effects, steroid hormones can induce effects within a very short time (≤ 30 min) both in vitro, as well as in vivo. Rapid GC effects are not due to delayed responses (hours) via the classical genomic model for steroid action, which involves nuclear translocation of the GR followed by transcription and protein synthesis (Falkenstein et al., 2000). Rapid GC-mediated (non-genomic) effects are characterized by responses that do not directly influence transcription initially, but result in the activation of signalling cascades that are not sensitive to protein synthesis inhibitors (Hammes and Levin 2007, Losel and Wehling 2003, Losel et al., 2003).

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 uncertain 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 intracellular 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) signalling pathway (Urbach et al., 2002). However, showing that the rapid GC signalling is insensitive to the GR/PR antagonist RU486, is not conclusive 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
This is supported by studies showing that the GnRH-induced activation of a progesterone-response-element (PRE) containing gene was insensitive to the 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, An et al., 2009). Rapid GC effects can be mediated by the classical GR or a membrane-associated GR (Buttgereit and Scheffold 2002, Finch et al., 2010, Lowenberg et al., 2008, Strehl and Buttgereit 2014) as described below.

1.1.6.1 Crosstalk of classical GR with intracellular signalling pathways

Several rapid GC-induced effects appear to be mediated via crosstalk mechanisms between the classical GR and other signalling pathways. The liganded GR has previously been 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).

In addition to the MAPK signalling pathways, the GR has been shown to crosstalk with the protein kinase B (Akt) signalling pathway. A study by Matthews et al. reported the rapid Dex-induced phosphorylation and activation of Akt by c-src tyrosine kinase 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 phosphatidylinositol 3-kinase (PI3K) and eNOS. Activation of the PI3K signalling 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 and protein kinase C (PKC) pathways in human folliculostellate cells (Solito et al., 2003). Thus, these findings suggest that the GR can crosstalk with the PI3K signalling pathway via a non-genomic mechanism in different cells, resulting in the rapid activation of Akt that modulates the activity of various signalling molecules.

Previous studies have reported that some rapid GC effects could modulate the responses of tyrosine kinase signalling pathways. GCs have been shown to decrease the epidermal growth factor (EGF) stimulation of phospholipase A2 (PLA2) activity by inhibiting the phosphorylation of MAPK/ERK kinase 1/2 (MEK1/2) in A549 cells (Malcher-Lopes et al., 2008). GCs have also been shown to
diminish the signalling of insulin by 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 signalling 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 exposures of T-cells to Dex decreased the TCR signalling by disruption of the membrane protein complex that attenuated the activity of LCK and FYN kinases (Lowenberg et al., 2006b). Furthermore, the dampening of LCK leads to a decrease in the activity of inositol-1, 4, 5-triphosphate (IP3) receptors, attenuating the signalling 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 attenuating 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. Thus besides modulating MAPK and PI3K/Akt kinase pathways, the GR appears to also modulate the signalling of non-receptor tyrosine kinase pathways and receptor tyrosine kinases upstream of MAPKs.

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 Gi 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\(_{\beta}\) proteins and co-migrates to the nucleus resulting in the repression of GR-mediated transactivation. In addition, the author’s reported that stimulation of the somatostatin receptor, a GPCR, results in co-localization of the GR and G\(_{\beta}\) at the plasma membrane in HTC116 cells (Kino et al., 2005). Another study showed the involvement of a G\(_{\alpha}\)-coupled receptor in the GC-induced synthesis and release of endocannabinoids from neuroendocrine cells (Malcher-Lopes et al., 2008). A study by Schmidt et al. demonstrated that epinephrine, acting via the \(\beta_2\)-adrenergic receptor, enhances GR-mediated transactivation of a GRE reporter gene in a strictly glucocorticoid-dependent fashion in a hippocampus-derived cell line (Schmidt et al., 2001). The crosstalk between the GR and the adrenergic receptor was shown to involve increased \textit{in vitro} DNA binding of the GR as well as signalling via G\(_{\beta\gamma}\), PI3K and Akt (Schmidt et al., 2001). The reports described above indicate that the association of the GR with G-proteins could explain some of the rapid GC-mediated effects. A 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 Ser-234 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 the synergistic transcriptional activation of a GRE reporter gene (Kotitschke et al., 2009).

1.1.6.2 Crosstalk of membrane-associated GR with intracellular signalling 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 GR-like molecule was shown for the first time in mouse lymphoma membranes in 1987 (Gametchu 1987). The presence of a 63-kDa κ opioid-like receptor that had a high affinity for GCs and similar functions as the GR was shown in amphibian neuronal membranes (Evans et al., 1998, Evans et al., 2000a, Evans et al., 2000b, Orchinik et al., 1991). In the past 25 years, since the mGR was discovered by Gametchu, several studies have reported detection of mGR in different types of tissues, cells and species (Strehl and Buttgereit 2014). Some studies have reported the mGR to be distinct from the classical cytoplasmic GR in several ways, including cell localization, molecular weight and ligand-binding characteristics, although 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 HSPs (Gametchu et al., 1999, Powell et al., 1999). A recent study by Vernocchi et al. used three different monoclonal antibodies to detect the mGR on the cell surface and determined the amino acid sequence of the epitopes in MCF-7 and U2-OS cells as well as in CCRF-CEM and Jurkat human T-cell lymphoblast-like cell lines. The results showed high-sequence homology between the classical cytoplasmic GR and mGR (Vernocchi et al., 2013). Bartholome et al. also previously showed that the mGR was similar to the classical GR in primary human monocytes and B cells with a high-sensitivity immunofluorescent staining technique (Bartholome et al., 2004). Since the mGR and classical GR appear to share a great level of sequence homology, the authors hypothesized that both these GR’s are most likely encoded by the same gene. However, overexpression of human GRα was not sufficient to enhance mGR expression in CHO cells (Bartholome et al., 2004). Interestingly, Strehl et al. verified this hypothesis about the origin of classical GR and mGR by performing human GR knockdown experiments with RNA-interference technology in HEK-293T cells. Their results showing a reduction in the expression levels of mGR,
classical cytoplasmic GR and GR mRNA, strongly support the one-gene hypothesis (Strehl et al., 2011).

As mentioned above, rapid GC effects may be mediated via the classical GR acting at the plasma membrane, but the low number of mGRs present in cells relative to the number of classical GRs has hindered the analysis of this receptor. There have been several reports in the literature suggesting that the GR associates with membrane rafts, which are specialized plasma membrane microdomains that recruit various signalling 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 membrane raft disrupter (Jain et al., 2005). A more recent study showed the unliganded GR localizing with c-src in caveolae to facilitate the rapid Dex-induced phosphorylation of Akt and caveolin-1 (Cav-1) in A549 cells. Furthermore, the same study showed the loss of Dex-induced phosphorylation of Akt and Cav-1 in the presence of a membrane raft disruptor, while the knockdown of Cav-1 protein reduced the Dex-induced activation of Akt, but had no effect on GRE transactivation (Matthews et al., 2008). A study performed by Samarasinghe et al. showed that the GR localizes to caveolae and is together in a complex with Cav-1 in a ligand-independent manner in embryonic mouse neural progenitor cells (Samarasinghe et al., 2011). The same study further provided physiologically relevant evidence for the membrane-associated GR by showing that Cav-1-dependent activation of ERK-1/2 by GCs inhibited gap junction intercellular communication, which resulted in decreased cell proliferation (Samarasinghe et al., 2011). 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 GR appears to crosstalk with several intracellular signalling pathways via a mechanism involving its association with a multi-protein signalling complex in membrane microdomains (Samarasinghe et al., 2012, Strehl and Buttgereit 2014).
1.2 Hypothalamic pituitary signalling

1.2.1 The hypothalamic-pituitary-gonadal axis

Mammalian reproduction is tightly regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which consists of the hypothalamus, pituitary and gonads that all produce hormones mediating positive and/or negative feedback mechanisms (Burns and Matzuk 2002, Coss et al., 2010, Thackray et al., 2010). The HPG axis controls all levels of reproduction, which include sexual development, puberty, gametogenesis and pregnancy. The decapeptide GnRH is the central regulator of the HPG axis and is synthesized and secreted from hypothalamic GnRH neurons in a pulsatile manner. GnRH acts through a specific GPCR, the GnRHR, to activate a variety of intracellular signalling cascades regulating the synthesis and secretion of LH and FSH from gonadotrope cells in the anterior pituitary (Conn and Crowley 1991, Fink 1988, Kaiser et al., 1997, Seeburg and Adelman 1984, Tsutsumi and Webster 2009). The FSH and LH multi-subunit proteins form heterodimers and their synthesis requires the expression of three genes, Cga, Lhb and Fshb. Cga encodes the glycoprotein hormone α-subunit (αGSU), which is common to both LH and FSH hormones through forming heterodimers with unique β-subunits encoded by Lhb and Fshb (Gharib et al., 1990, Jorgensen et al., 2004). After LH and FSH are secreted from gonadotrope cells they bind to distinct GPCRs in both male and female gonads, which induces gametogenesis and steroidogenesis (Conn and Crowley 1991, Segaloff and Ascoli 1993, Simoni et al., 1997).

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 (HHG), with symptoms of delayed sexual development and low gonadotropin and steroid hormone levels (Fraietta et al., 2013, Millar et al., 2004, Seminara et al., 1998). The majority of these mutated GnRHR proteins are incorrectly folded and are retained in the endoplasmic reticulum or display attenuated trafficking to the plasma membrane, which together results in a reduced level of functional GnRHR (Brothers et al., 2004, Knollman et al., 2005). GnRHR agonists and antagonists have been extensively used in clinical treatments for fertility-related disorders, various reproductive diseases and hormone-dependent sarcomas (Conn and Crowley 1994, Neill 2002).
1.2.2 HPG and HPA crosstalk

In times of stress, the nervous system and HPA axis allows an organism to mobilize all of the body’s resources. Chronic and acute stress have been implicated in regulating reproduction in mammals (Breen and Mellon 2014). Crosstalk between the HPG and HPA endocrine signalling pathways has been shown to modulate GnRH gene expression in the hypothalamus (Berga et al., 1989, Chandran et al., 1994, De Kloet et al., 1998, Dubey and Plant 1985, Ferin 1999). Administering a stress level of GCs to female mice has been shown to severely reduce the GnRH-induced synthesis of LHβ mRNA and LH secretion in the pituitary (Baldwin et al., 1991, Berga et al., 1989, Breen et al., 2012). These findings indicate that stress has a negative influence on mammalian reproduction. However, HPG and HPA crosstalk has also been shown to positively influence GnRHR gene expression levels in mouse gonadotrope cells, through a mechanism mediated by the GR and GnRHR (Kotitschke et al., 2009). These findings reported by Kotitschke et al. exposes the gonadotrope as an important neuroendocrine site where the HPA axis modulates the HPG axis to regulate reproductive function in mammals. Furthermore, these reports suggest that stress and immune function can influence HPG signalling through receptor crosstalk (Breen et al., 2012, Kotitschke et al., 2009, Navratil et al., 2010), which supports the concept that all hypothalamic-pituitary signalling axes are functionally integrated to maintain homeostasis in mammals (Chrousos 2010, Da Silva et al., 1993, Da Silva 1995).

1.2.3 Cell types of the pituitary

The pituitary gland is a small organ involved in endocrine physiology located at the bottom of the brain. It is comprised of three different lobes, the posterior pituitary, anterior pituitary and the intermediate lobe in most mammals (de Moraes et al., 2012, Kelberman et al., 2009, Rizzoti 2015). The anterior pituitary lobe consists of a heterogeneous mixture of five differentiated endocrine hormone producing and secreting cell types. The corticotropes are the first cell type to differentiate during pituitary development and produce a precursor peptide POMC, which is cleaved into several products including CRH, endorphin and encephalin (Horn et al., 1992). Other cell types that differentiate later are thyrotopes, which produce thyroid-stimulating hormone (TSH), somatotropes, which produce growth hormone (GH), lactotropes, which produce prolactin (PRL), and the gonadotropes, which produces LH and FSH (Horn et al., 1992).

The posterior pituitary lobe is much smaller and consists of axonal projections extending from the hypothalamus and pituicytes. It predominantly serves as a storage site for vasopressin and oxytocin.
hormones, which are both produced in the hypothalamus. The intermediate lobe forms a sub-division of the anterior lobe and is composed of melanocytes that produce melanocyte-stimulating hormone (de Moraes et al., 2012, Hadley and Levine 2007).

1.2.4 Model gonadotrope cell systems

In the anterior pituitary the gonadotropes are the last cell type that reaches maturation and express their specific hormones. Gonadotropes form a heterogeneous cell population consisting of monohormonal (~18% LH and ~22% FSH containing cells) as well as multihormonal gonadotropes containing both LH and FSH (~60% of the gonadotropes) (Childs 2006). In the pituitary, gonadotropes comprise about ~5-15% of the cells (Hyde et al., 1982, Ibrahim et al., 1986, Ooi et al., 2004). In the past, studies investigating the physiological function of gonadotropes in reproduction have been performed either in vivo or in primary pituitary cultures. However, the heterogeneity of the pituitary cell population limits the interpretation of results derived from these studies. Furthermore, primary pituitary tissue and cells can only be cultured for a limited period of time which restricted experimental possibilities. Therefore, to overcome these limitations the immortalized αT3-1 and LβT2 gonadotrope cell lines were developed and characterized by the Mellon laboratory (Alarid et al., 1996, Turgeon et al., 1996, Windle et al., 1990).

The αT3-1 and LβT2 clonal cell lines were created by targeted tumourigenesis in transgenic mice, using the promoters of the human αGSU and rat LHβ genes, respectively (Alarid et al., 1996, Turgeon et al., 1996, Windle et al., 1990). The αT3-1 cells represent precursor gonadotrope cells with some gonadotrope functions, like the expression of functional GnRHR and responsiveness to GnRH treatments. Additionally, they express several activin receptor subunits, as well as the β-subunits, but not the α-subunit of inhibin (Fernandez-Vazquez et al., 1996). Furthermore, αT3-1 cells also express, synthesise and secrete αGSU, but not the LHβ or FSHβ gonadotropin subunits (Windle et al., 1990). The LβT2 cell line represent more mature and differentiated gonadotrope cells. These cells express functional GnRHR, LHβ, FSHβ (Pernasetti et al., 2001, Turgeon et al., 1996) and have been reported to express functional steroid receptors including the PR, ER and GR (An et al., 2009, Chen et al., 2009, Kotitschke et al., 2009). In addition, these cells also expresses activin, inhibin, follistatin and the activin receptors, therefore displaying all of these investigated hallmarks of fully differentiated gonadotrope cells (Pernasetti et al., 2001). Various studies investigating gonadotrope signalling and gene expression have been carried out in the αT3-1 (Bliss et al., 2007, Duval et al., 1997, Navratil et al., 2003, Norwitz et al., 1999, White et al., 1999), and the LβT2 cell lines (An et
The development of immortalized gonadotrope cell lines has been an important breakthrough for studying gonadotrope signalling, like the GnRHR pathway for which Fink et al. created a web-accessible knowledgebase (Fink et al., 2010). Gonadotropes in the anterior pituitary are comprised of a scattered population of cells. Accurate biological experiments on live primary gonadotropes requires them to be identified and isolated from the pituitary cell population. This has been accomplished in previous studies by either performing density-gradient centrifugation of a pituitary cell suspension (Heyward et al., 1995) or by performing a reverse hemolytic plaque assay (RHPA) with cultured pituitary cells to detect the secretion of LH and FSH (Tse and Hille 1992). Primary gonadotrope cells possibly only represent a sub-population since not all gonadotropes have similar densities, which could result in their migration evading the Percoll fraction, whereas some may not secrete LH/FSH or respond to GnRH, and thus may evade detection with the RHPA. This assessment is supported by morphological criteria and gonadotropin immunocytochemical studies that suggested the existence of considerable heterogeneity within gonadotrope populations (Childs 2006). To overcome this inadequacy, U. Boehm devised a binary genetic strategy to generate a new mouse model that expresses yellow fluorescent protein (YFP) specifically in gonadotrope cells (Wen et al., 2008). The authors validated the GRIC/R26-YFP mouse model with antibodies against LH and FSH and found that 99.9% of the positively stained pituitary cells expressed the YFP. This tagging of the gonadotropes with a fluorescent marker provides a simple versatile tool for their identification and isolation with fluorescence-activated cell sorting (FACS), which enables the accurate profiling of gene expression in primary gonadotrope cells.

1.2.5 The estrous cycle

Sexual reproduction in mammals requires the menstrual cycle that induces natural changes in the uterus and ovaries. The menstrual cycle is essential for the ovary to produce oocytes and preparation of the uterus for pregnancy. The menstrual cycle can be defined by the ovarian and uterine cycle. The ovarian cycle is described by modifications that occur in the follicles of the ovary and the uterine cycle is described by changes occurring in the endometrial lining of the uterus. The ovarian
and uterine cycles can each be divided into three stages, the ovarian consists of the follicular, ovulatory and luteal stages whereas the uterine consists of proliferative and secretory stages as well as menstruation (Mihm et al., 2011).

The menstrual cycle in rodents is called the estrous cycle and the major physical difference in the cycle between humans and rodents is that menstruation, the physical shedding of the endometrium, is not a feature of the rodents’ estrous cycle. Instead of shedding they reabsorb the endometrium (Owens and Ashby 2002). The mouse estrous cycle usually only last four to five-days and can be separated into four different stages called proestrous, estrous, metestrous and diestrous. During these four stages the endogenous levels of progesterone (P_4) and estradiol (E_2) produced by the ovaries vary in a cyclical pattern. One of the major features that has been observed in mice is the peak of E_2 levels in the proestrous stage prior to ovulation that occurs in the estrous stage (Fata et al., 2001, Walmer et al., 1992). In the human menstrual cycle the hormone levels also vary in a cyclical pattern with P_4 being highest in luteal and E_2 highest in follicular stages (Figure 1.4) (Pant et al., 1977). It is well-known that changes in the levels of these sex hormones results in extensive remodeling of the endometrium during each reproductive cycle. This involves a synchronized reoccurrence of cellular proliferation, apoptosis, differentiation, angiogenesis, leukocyte infiltration and breakdown of extracellular matrix, which happens in both humans and rodents (Evans et al., 1990, Rugh 1990).

Similar to the E_2 and P_4 hormone levels, LH and FSH levels also fluctuate throughout the reproductive cycle (Figure 1.4). As shown in Figure 1.4, LH and FSH levels significantly increase during the proestrous stage of the cycle. In the metestrous stage the ovarian follicles undergoes several waves of development and reach maturity in the proestrous stage influenced by FSH. In the proestrous stage the high E_2 hormone level increases the secretion of GnRH from hypothalamic neurons. This surge of GnRH released into the hypophyseal portal blood acts on pituitary gonadotropes to increase the synthesis and secretion of LH (Knobil 1974, Legan and Karsch 1975). The high level of LH induced by E_2 is known as the LH surge, which matures the oocyte and weakens the wall of the follicle present in the ovary. The weakening of the wall results in the completion of the first meiotic division within the oocyte and ovulation of a secondary oocyte that matures into an ovum. Following ovulation, LH induces the remaining parts of the follicle to transform into the corpus luteum, which produces P_4 and leads to reorganization of the uterine lining for the next cycle (Caligioni and Franci 2002, Caligioni 2009, Evans and Ganjam 2011).
1.3 Gonadotropin-releasing hormone receptor

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, Wu et al., 1994, Young et al., 1984), 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 et al., 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 (Ciechanowska et al., 2010, Hapgood et al., 2005, 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 (Millar et al., 2004, Troskie et al., 1998). The existence of several other

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**Figure 1.4: Comparison of the human menstrual cycle and the murine estrous cycle.**

Circulating levels of the sex hormones, progesterone and estrogen, which are produced by the ovaries, and circulating levels of the gonadotropins, LH: luteinizing hormone; FSH: follicle-stimulating hormone, fluctuate during the reproductive cycle. D: Diestrous; P: Proestrous; E: Estrous; M: Metestrous. Ovulation is shown with the arrow. Obtained and modified from (Lebron-Milad and Milad 2012, Staley and Scharfman 2005).
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 (Hapgood et al., 2005, Millar et al., 2004, Ronacher et al., 2004). 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 (Faurholm et al., 2001, Gault et al., 2004, Morgan et al., 2003, van Biljon et al., 2002).

1.3.1 Physiological function of GnRHR

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 (Cheng and Leung 2005, Hapgood et al., 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 (Kogo et al., 1999, Kottler et al., 1997, Raga et al., 1998). 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 (Cheung and Hearn 2003, Kakar et al., 1992, Morales 1998, van Biljon et al., 2002).

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. Unless stated otherwise, 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 and Resh 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 analogues 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 signalling pathways and mechanisms mediating the anti-proliferative effects of GnRH are unknown and further work is required (Pawson et al., 2003). Taken together, these findings suggest 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.

1.3.2 Protein structure

The GnRHR was first identified in the mouse by cloning the receptor from the pituitary aT3-1 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 (Eidne et al., 1992, Kaiser et al., 1992), sheep (Brooks et al., 1993, Illing et al., 1993) and pig (Weesner and Matteri 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 (Fan et al., 1994, 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., 2004, 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 an 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).

### 1.3.3 Signalling 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 (Liu et al., 2003, Stanislaus et al., 1998). The members of the Ga_{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 Ga_{i/o} and Ga_{s} has also been reported (Naor 2009). However, the activation of Ga_{i/o} and Ga_{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, Ruf and Sealfon 2004). The GnRHR has been shown to couple to Ga_{q/11} in αT3-1 cells, while both Ga_{q/11} and Ga_{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., 1997, Stanislaus et al., 1998). 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 signalling pathways, including phospholipase A_{2}, Cβ and D, which result in the release of various secondary signalling 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 (Kraus et al., 2001, Liu et al., 2002b). In addition, GnRH-stimulation was shown to increase intracellular cAMP levels via a Ca^{2+}-independent mechanism involving the activation of adenyl 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 (Kraus et al., 2001, Naor et al., 2000). Additionally, GnRH activates all of the MAPKs in both αT3-1 and LβT2 cells in a PKC-dependent manner (Bonfil et al., 2004, Liu et al., 2002b, Liu et al., 2003, Mulvaney et al., 1999, Roberson 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 regulation of the
FSHβ gene in LβT2 cells (Bonfil et al., 2004, Kanasaki and Miyazaki 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 signalling pathways, such as MAPKs and other protein kinases, to regulate mammalian reproduction.

1.3.4 Crosstalk with other receptor signalling pathways

The GnRHR can crosstalk with several other receptor signalling pathways as a mechanism for generating signalling 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 (Eicke et al., 2006, Grundker et al., 2001, Gunthert et al., 2005, Moretti et al., 1996, Yates et al., 2005). The mechanism appears to be mediated by the GnRHR via activation of a tyrosine-phosphatase, which reduces the EGF-induced autophosphorylation of the EGF receptor (EGFR) (Grundker et al., 2001, Lamharzi et al., 1998a, Moretti et al., 1996). 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 (Lamharzi et al., 1998b, Marelli et al., 1999, Montagnani Marelli et al., 2007). Interestingly, it has been shown previously that insulin potentiates the GnRH-induced activation of ERK-1/2, which results in enhanced cap-dependent translation in LβT2 cells (Navratil et al., 2009). In addition to its role in mitogenic signalling, 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 signalling and cytoskeletal reorganization (Choi et al., 2006).

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 αGSU and FSHβ in αT3-1 and LβT2 cells, respectively (An et al., 2006; An et al., 2009). A study by Chen et al. demonstrated that GnRH stimulates the ligand-independent activation of the ER 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 in 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 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. 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.

1.4 Membrane rafts

1.4.1 Properties and structure

The plasma membrane of eukaryotic cells defines the cell boundary and is involved in transport, cell signalling, cellular contact and many other cellular events to maintain their physiological state. For a long time, the fluid mosaic model, postulated by Singer and Nicolson in 1972, has provided the foundation for the understanding of the structure of cellular membranes (Singer and Nicolson 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 20 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, Simons and Ikonen 1997). These ordered domains in membranes are called membrane rafts, which are localized regions of elevated cellular cholesterol and glycosphingolipid content. The fatty acid side-chains of the phospholipids in membrane 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 membrane rafts (Pike 2006). Simons and Ikonen have formulated a hypothesis regarding membrane 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 membrane rafts are thought to exert their physiological function (Simons and Ikonen 1997).

The first definition of membrane rafts was suggested by Brown and Rose, who reported that domains enriched in glycosylphosphatidylinositol (GPI)-anchored proteins and sphingolipids 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 membrane rafts (Schroeder et al., 1994a). The presence of membrane 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 membrane raft proteins (Brown and London 1997, Mayor and Rao 2004, Pike 2004, Pike 2006, Simons and Toomre 2000). A few studies have suggested that membrane 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, Mayor and Rao 2004, Pike 2006).

1.4.1.1 Caveolae
Caveolae are characterized by flask-shaped invaginations of the plasma membrane that comprise a special subpopulation of membrane rafts and can be distinguished by the presence of Cav-1 (Figure 1.5) (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 (Lisanti et al., 1995, Parton 1996, Volonte et al., 1999). Caveolin proteins (21-24 kDa) are the fundamental components of caveolae and there are three known proteins, Cav-1 with splice variants α and β, Cav-2 and Cav-3 (Glenney 1992, Scherer et al., 1996, Tang et al., 1996). 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 membrane rafts are equivalent membrane structures. However, membrane rafts could also be isolated from cells that do not have caveolae (Fra et al., 1994, Gorodinsky and Harris 1995). In addition, caveolae from isolated membrane fractions can be separated from the bulk membrane raft-associated GPI-anchored proteins (Schnitzer et al., 1995). These findings indicated that membrane rafts are distinct from caveolae and could exist inside or outside caveolae (Parton and Simons 1995).

It was previously shown that the overexpression 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 (Cohen et al., 2004, Galbiati et al., 2001, Razani et al., 2001). In contrast, the knockdown of Cav-2 has no effect on caveolae formation in vivo (Razani et al., 2002), but it may 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 membrane 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 signalling pathways at the plasma membrane.

1.4.2 Cellular function

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

Figure 1.5: Plasma membrane organization of caveolae and membrane rafts.

Several commonly associated molecules and proteins are shown, as well as the regions considered membrane raft microdomains, which are present in a more liquid-ordered

Many receptor tyrosine kinase (RTK)s, including the EGFR, insulin receptor and the platelet-derived growth factor (PDGF) receptor, have been shown to localize to membrane rafts (Gustavsson et al., 1999, Liu et al., 1996, Mineo et al., 1996, Wu et al., 1997). The effect of ligand binding on this association of RTKs with membrane rafts is highly variable (summary in Table 1.4.1). The unliganded EGFR exists in membrane rafts and partially moves out of membrane rafts upon agonist-binding and activation (Mineo et al., 1999), a feature that is unique among RTKs. Consistent with its movement out of membrane rafts, the autophosphorylated EGFR localizes to intracellular compartments (Balbis et al., 2007, Wang et al., 2009). Interestingly, Puri et al. showed that several endocytic proteins are recruited to membrane rafts containing the activated EGFR (Puri et al., 2005). Therefore, membrane rafts have the ability to assemble both a signalling 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 membrane rafts in cells that lack caveolae (Vainio et al., 2002). The constitutive localization of the PDGF and nerve growth factor (NGF) receptors to membrane 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 membrane rafts after hormone treatment, which is required for these receptors to associate with their downstream signalling cascades (Huang et al., 1999, Liu et al., 1997, Liu et al., 1996).
A large number of GPCRs have been shown to be enriched in membrane 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 (Chun et al., 1994, Dessy et al., 2000, Feron et al., 1997, Ishizaka et al., 1998, Navratil et al., 2003, Rybin et al., 2000, Seno et al., 2001, Xiang et al., 2002). Like the RTKs, the localization of some GPCRs to membrane rafts appears to be modulated by ligand (summary in Table 1.4.1). The unliganded β-adrenergic receptor is present in membrane rafts and moves out upon ligand treatment, while the acetylcholine, muscarinic cholinergic and angiotensin II type I receptors are targeted to membrane rafts upon activation with ligand (Dessy et al., 2000, Feron et al., 1997, Ishizaka et al., 1998). In contrast, the GnRHR, endothelin receptor and rhodopsin receptor are constitutively localized to membrane rafts, and this localization is unaffected by ligand (Chun et al., 1994, Navratil et al., 2003). In addition, various G-proteins have been reported to localize to membrane rafts through a mechanism involving lipid modifications (Huang et al., 1997, Li et al., 1995, Lisanti et al., 1994, Moffett et al., 2000, Pike and Miller 1998, Seno et al., 2001).

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

To study the function of membrane rafts in signalling pathways, several studies have employed a strategy that involves the depletion of cellular cholesterol to disrupt the integrity of membrane raft structures (Table 1.4.2). The integrity of membrane rafts can be disrupted by treating cells with chemicals such as methyl-β-cyclodextrin (MβCD) and filipin-III, which extract and sequester cellular cholesterol (Kilsdonk et al., 1995, Ohtani et al., 1989, 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 membrane rafts in insulin signalling and metabolism (Gustavsson et al., 1999, Le Lay et al., 2001, Parpal et al., 2001, Vainio et al., 2002). 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 signalling is involved (Parpal et al., 2001). Similarly, disruption of membrane 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, membrane rafts seem to play an inhibitory role in EGFR signalling, since disruption of membrane rafts resulted in an increased affinity for ligand binding of the EGFR and enhanced receptor dimerization, autophosphorylation and activation of the ERK-1/2 signalling 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 membrane rafts seems to be coupled to activation of the receptor.
### Table 1.4.2: Effects of cholesterol depletion on the signalling of RTKs and GPCRs

<table>
<thead>
<tr>
<th>Function</th>
<th>Receptor</th>
<th>Effect of cholesterol depletion</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptor tyrosine kinase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligand-binding</td>
<td>EGF</td>
<td>+</td>
<td>(Pike and Casey 2002)</td>
</tr>
<tr>
<td>Dimerization</td>
<td>EGF</td>
<td>+</td>
<td>(Chen and Resh 2002)</td>
</tr>
<tr>
<td>Autophosphorylation</td>
<td>EGF</td>
<td>+</td>
<td>(Ringerike et al., 2002)</td>
</tr>
<tr>
<td>MAPK activation</td>
<td>EGF</td>
<td>+</td>
<td>(Furuchi and Anderson 1998)</td>
</tr>
<tr>
<td>Ligand-binding</td>
<td>Insulin</td>
<td>0</td>
<td>(Parpal et al., 2001)</td>
</tr>
<tr>
<td>Autophosphorylation</td>
<td>Insulin</td>
<td>0 or -</td>
<td>(Parpal et al., 2001, Vainio et al., 2002)</td>
</tr>
<tr>
<td>IRS-1 phosphorylation</td>
<td>Insulin</td>
<td>-</td>
<td>(Parpal et al., 2001)</td>
</tr>
<tr>
<td>Glucose uptake</td>
<td>Insulin</td>
<td>-</td>
<td>(Gustavsson et al., 1999)</td>
</tr>
<tr>
<td>MAPK activation</td>
<td>Insulin</td>
<td>0</td>
<td>(Parpal et al., 2001)</td>
</tr>
<tr>
<td>Akt activation</td>
<td>Insulin</td>
<td>-</td>
<td>(Parpal et al., 2001)</td>
</tr>
<tr>
<td><strong>G-protein-coupled receptor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK activation</td>
<td>GnRH</td>
<td>-</td>
<td>(Navratil et al., 2003)</td>
</tr>
<tr>
<td>Adenylate cyclase activation</td>
<td>β2-Adrenergic</td>
<td>+</td>
<td>(Rybin et al., 2000)</td>
</tr>
<tr>
<td>MAPK activation</td>
<td>Endothelin</td>
<td>-</td>
<td>(Teixeira et al., 1999)</td>
</tr>
<tr>
<td>FAK activation</td>
<td>Endothelin</td>
<td>-</td>
<td>(Teixeira et al., 1999)</td>
</tr>
<tr>
<td>EGFR transactivation</td>
<td>Angiotensin II</td>
<td>-</td>
<td>(Ushio-Fukai et al., 2001)</td>
</tr>
<tr>
<td>Activation</td>
<td>Rhodopsin</td>
<td>+</td>
<td>(Niu et al., 2002)</td>
</tr>
</tbody>
</table>

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 (Bliss et al., 2007, Navratil et al., 2003). Similarly, the thrombin-stimulated phosphatidic acid generation and IP3 production was inhibited when membrane 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 membrane rafts upon ligand-binding and the signalling 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 membrane rafts, while ligand-binding changes the conformation of the receptor with release of these inhibitory
proteins. Thus, cholesterol depletion assays showed that membrane rafts are important for GPCR, RTK and MAPK signalling pathways as summarized in Table 1.4.2.

1.4.3 The reggie/flotillin family of proteins
A family of proteins, which is found in membrane rafts in cells that do not contain caveolae, is the reggie/flotillin family of proteins. 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 membrane 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 orthologues demonstrated in plants, bacteria and fungi (Borner et al., 2005, Edgar and Polak 2001, Rivera-Milla et al., 2006). 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.

1.4.3.1 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. 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). 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 (Bickel et al., 1997, Schroeder et al., 1994b). 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).

1.4.3.2 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 (Chintagari et al., 2008, Langhorst et al., 2005). In contrast, the knockdown of Flot-1 has a much weaker effect on the protein stability of Flot-2 (Chintagari et al., 2008, Langhorst et al., 2005).

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.

1.4.3.3 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
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 share 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 Cys-34, 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 Cys-34 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 membrane 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 Gly-2 and palmitoylated at multiple residues, including Cys-4, 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 Gly-2-Ala protein, which is neither myristoylated nor palmitoylated, shows increased solubility with decreased membrane raft association, supporting a role for lipid modifications in targeting Flot-2 to membrane rafts. In addition, the homo-oligomerization of Flot-2 has been reported to play a role in targeting Flot-2 to membrane rafts (Neumann-Giesen et al., 2004, Neumann-Giesen et al., 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 domain (Babuke and Tikkanen 2007). Taken together, lipid modifications regulate the association of flotillin proteins with membrane rafts and the plasma membrane.

Unlike other membrane 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 (Kokubo et al., 2003, Lang et al., 2009, Stuermer et al., 2001). The flotillin clusters were found to be uniform in size with an estimated diameter of 100 nm (Kokubo et al., 2003, Stuermer et al., 2001). Flotillin clusters appear to be quite widely spaced along the plasma membrane with increased clustering at cell-cell contact points and after cross-linking of membrane
raft-associated proteins, such as the GPI-anchored Thy-1 and the cellular prion protein (PrP$^c$) (Stuermer et al., 2001, Stuermer et al., 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 (Derminé et al., 2001, Gkantiragas et al., 2001, Stuermer 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 (Derminé et al., 2001, Gkantiragas et al., 2001, Santamaria et al., 2005, Stuermer et al., 2001). 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).

1.4.3.4 Cellular function

Although, they are ubiquitously expressed and evolutionary well conserved, the precise molecular function of flotillin proteins is still unclear. Nonetheless, several studies have indicated a role for flotillin in signalling processes through membrane receptors, endocytosis and regulation of cytoskeleton signalling (Baumann et al., 2000, Derminé et al., 2001, Glebov et al., 2006, Hazarika et al., 2004, Lang et al., 1998, Neumann-Giesen et al., 2004, Neumann-Giesen et al., 2007, Schultz et al., 1997). In addition, a variety of proteins has been shown to co-localize and interact with flotillin proteins as described below (Babuke and Tikkanen 2007, Langhorst et al., 2005, 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$^c$ (Liu et al., 2005, Slaughter et al., 2003, Stuermer et al., 2001, Stuermer et al., 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 signalling (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 signalling,
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-signalling pathway mediated via membrane 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 membrane 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 membrane 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 membrane 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 membrane 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 (Kimura et al., 2001, Kioka et al., 2002). Taken together, these 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 signalling 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 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 membrane rafts as a tyrosine-kinase-regulated endocytic pathway.

The flotillin proteins have also been implicated to have a role G-protein signalling. Sugawara et al. showed that Gαq 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 signalling pathways in a species- and cell-specific manner, but the main functions of flotillins remain unknown.

1.5 Serum/glucocorticoid regulated kinase

The serum/glucocorticoid regulated kinase (SGK) is a member of the serine/threonine protein kinase family that was first identified as an immediate early gene transcriptionally induced by serum and glucocorticoids and was cloned from the Con8.hd6 rat mammary tumor cell line (Firestone et al., 2003, Webster et al., 1993). The gene encoding the human SGK-1 isoform has been localized to chromosome 6q23 (Waldegger et al., 1998). Two translational isoforms of SGK-1 have been discovered, termed SGK-2 and 3, which are products from distinct genes (Kobayashi et al., 1999).

The SGK isoforms share 80% amino acid sequence identity in their catalytic domains and have the same phosphorylation consensus (Kobayashi et al., 1999). Despite the high degree of sequence similarity between the three SGK isoforms, the mechanisms regulating their expression and activation vary significantly (Arteaga et al., 2007, Kobayashi et al., 1999, Raikwar et al., 2008).
SGK forms dimers between two intermolecular disulfide bonds at Cys-258 and Cys-193 in its activation loop (A-loop) (Zhao et al., 2007) and has been detected in a variety of species (Lang et al., 2006).

### 1.5.1 Expression and regulation of SGK-1 activity

SGK-1 is virtually ubiquitously expressed in almost all mammalian tissues (Waldegger et al., 1997), but the level of expression appears to vary between different cell types like the brain, liver, kidney, pancreas and the ovary (Keller-Wood et al., 2006, Lang et al., 2006, Stichel and Luebbert 2007). Besides serum and glucocorticoids, transcriptional upregulation of the SGK-1 gene has been reported to be induced by a wide variety of stimuli (Table 1.5.1) (Lang et al., 2009, Loffing et al., 2006). Several consensus sequences for transcription factors have been reported to be present in the SGK-1 gene, such as a functional GRE, Sp1, p53, NF-xB and CRE (de Seigneux et al., 2008, Firestone et al., 2003, Itani et al., 2002, Lambert et al., 2013, Lang and Cohen 2001, Maiyar et al., 1997). It has previously been reported that Dex induced the expression of the SGK-1 gene, to about 3-fold, within 15 min in *Xenopus laevis* kidney A6 cells. Furthermore, the authors reported that aldosterone also increased the expression of the SGK-1 gene in primary rat kidney cells (Chen et al., 1999). These findings by Chen et al. suggests that expression of the SGK-1 gene is regulated by both the GR and the MR (Chen et al., 1999). Interestingly, Alliston et al. reported that FSH increased the expression of the SGK-1 gene in primary rat ovarian granulosa cells. However, the authors treated the cells with FSH in combination with the natural ligand for the AR, testosterone (Alliston et al., 1997). Therefore it is possible that the AR could have played a major role in the upregulation of the SGK-1 gene and more experiments have to be performed to obtain conclusive evidence that FSH induces expression of this gene.

Expression of the SGK-1 gene has been shown to be induced by Dex in a GR- and dose-dependent manner in A549 cells (Itani et al., 2002). A recent study by Lambert et al. showed that Dex treatment resulted in recruitment of the GR to the GRE in the promoter of the SGK-1 gene. Furthermore, the same study showed that Dex also induced recruitment of GRIP-1 to the promoter that formed a classical GR co-activator complex and resulted in transactivation of the SGK-1 gene in 293TrkB cells, which are HEK-293 cells stably expressing the receptor for BDNF, tyrosine kinase transmembrane receptor B (TrkB), and called 293TrkB cells by the authors (Lambert et al., 2013). Co-stimulation with Dex + BDNF resulted in the recruitment of CREB to the complex occupying the GRE region in the promoter of the SGK-1 gene in HEK 293 cells. Furthermore, co-stimulation also
induced p300 recruitment, which interacted with CREB in this complex (Lambert et al., 2013). The authors identified a CRE cis-element that is in close proximity to the GRE-site, which suggests that Dex + BDNF regulation could be mediated through a composite transcription factor mechanism. Since the above-mentioned report showed p300 recruitment it would have been interesting to know whether CBP is also recruited, potentially in a time- or dose-dependent manner. Taken together, these findings indicate that the SGK-1 gene is regulated by Dex in a classical GR-mediated transactivation manner and synergistically in a composite GRE and CRE transcriptional mechanism in response to a combination of hormones that induces activation of both transcription factors.

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoids</td>
<td>(Firestone et al., 2003, Kim et al., 2009, Leong et al., 2003, Pondugula et al., 2006, van Gemert et al., 2006, Webster et al., 1993, Yaylaoglu et al., 2006)</td>
</tr>
<tr>
<td>Serum</td>
<td>(Webster et al., 1993)</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>(Chen et al., 1999, Naray-Fejes-Toth et al., 1999)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>(Fillon et al., 2002, Khan et al., 2005, Lang et al., 2000, Waldegger et al., 1999)</td>
</tr>
<tr>
<td>Glucose</td>
<td>(Khan et al., 2005)</td>
</tr>
<tr>
<td>FGF</td>
<td>(Mizuno and Nishida 2001)</td>
</tr>
<tr>
<td>PDGF</td>
<td>(Mizuno and Nishida 2001)</td>
</tr>
<tr>
<td>FSH</td>
<td>(Alliston et al., 1997)</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>(Leong et al., 2003)</td>
</tr>
<tr>
<td>PRARγ</td>
<td>(Hong et al., 2003)</td>
</tr>
<tr>
<td>cAMP</td>
<td>(Feroze-Zaidi et al., 2007)</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>(Turpaev et al., 2005)</td>
</tr>
<tr>
<td>UV radiation</td>
<td>(Kim et al., 2007, Shibata et al., 2007)</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>(Pfau et al., 2007)</td>
</tr>
<tr>
<td>Iron</td>
<td>(Marzullo et al., 2004)</td>
</tr>
<tr>
<td>IL-6</td>
<td>(Meng et al., 2005)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>(BelAiba et al., 2006)</td>
</tr>
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</table>

SGK-1 forms part of the family of AGC kinases, which consists of PKA, PKB, PKC and PKG. The catalytic domain of SGK-1 shares 54% identity with that of Akt, 48% with that of PKC-β and 45% with that of PKA (Lang et al., 2006; Webster et al., 1993). Most AGC kinases contain conserved phosphorylation motifs located in their catalytic domains and the C-terminal region. The motif in the catalytic domain is situated in the A-loop and was originally discovered in Akt and shown to be a target of 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (Alessi et al., 1997, Stokoe et al., 1997). SGK-1 is activated by the PI3K pathway through phosphorylation at Ser-422 in the C-terminal region, although the kinase responsible for this is still unknown. This phosphorylation at Ser-422 induces the PDK-1 phosphorylation at Thr-256 in the A-loop and results in activation of SGK-1 (Biondi et al., 2001). It has previously been reported that SGK-1 is also phosphorylated by ERK-5 and p38 at Ser-78, which is not situated within the catalytic domain, but increases the activity of SGK-1 independent of the phosphorylation status of Thr-256 in the A-loop (Hayashi et al., 2001,
Meng et al., 2005). Taken together, these findings indicate that the activity of SGK-1 is regulated through phosphorylation by various signalling pathways.

1.5.2 Cellular function

The consensus phosphorylation sequence R-X-R-X-X-S/T (X = any amino acid) for SGK-1 was determined in vitro with peptide kinase assays and is shared by Akt (Kobayashi and Cohen 1999, Park et al., 1999). SGK-1 has been reported to phosphorylate a variety of proteins (Table 1.5.2). The first substrate found was GSK3 in HEK-293 cells, a kinase that is involved in the regulation of glycogen and insulin protein synthesis (Kobayashi and Cohen, 1999). Phosphorylation of GSK3 has been reported to increase glycogen synthesis in 3T3-L1 cells (Sakoda et al., 2003). However, Akt has also been shown to phosphorylate GSK3 and the respective contributions of SGK-1 and Akt in GSK3 phosphorylation have not yet been clearly established (Collins et al., 2003). Two important kinases, Raf and mitogen-activated protein kinase/ERK kinase 3 (MEKK3) that are involved in the MAPK signalling pathway have been reported to be phosphorylated and inactivated by SGK-1 (Chun et al., 2003, Zhang et al., 2001). Raf plays a fundamental role in the transmission of signals from Ras to the MAPK signalling pathway. It is interesting that Raf and MEKK3 become inactivated by SGK-1 since p38 and ERK-5, which are downstream kinases in the MAPK pathway, increases the activity of SGK-1. These findings indicate that there is a feedback control mechanism regulating the SGK-1 and MAPK signalling pathways.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cell or tissue type</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK3</td>
<td>HEK-293 cells</td>
<td>(Kobayashi and Cohen, 1999)</td>
</tr>
<tr>
<td>FOXO3</td>
<td>Primary rat cerebellar granule neurons, HEK-293T cells and CCL39 fibroblasts</td>
<td>(Brunet et al., 2001)</td>
</tr>
<tr>
<td>Raf</td>
<td>HEK-293 cells</td>
<td>(Zhang et al., 2001)</td>
</tr>
<tr>
<td>MEKK3</td>
<td>COS-1 cells</td>
<td>(Chun et al., 2003)</td>
</tr>
<tr>
<td>Nedd4</td>
<td><em>Xenopus laevis</em> oocytes, COS-7 cells and mpkCCD&lt;sub&gt;di&lt;/sub&gt; cells</td>
<td>(Debonneville et al., 2001, Flores et al., 2005, Palmada et al., 2004, Snyder et al., 2002)</td>
</tr>
<tr>
<td>NDRG-1 and 2</td>
<td>Mouse skeletal muscle</td>
<td>(Murray et al., 2004)</td>
</tr>
<tr>
<td>NHE3</td>
<td>IEC6 cells</td>
<td>(Wang et al., 2005)</td>
</tr>
<tr>
<td>ROMK</td>
<td><em>Xenopus laevis</em> oocytes</td>
<td>(Yoo et al., 2003)</td>
</tr>
<tr>
<td>α-ENaC</td>
<td><em>Xenopus laevis</em> oocytes</td>
<td>(Diakov and Korbmacher 2004)</td>
</tr>
</tbody>
</table>

SGK-1 has been shown to increase the apical plasma membrane expression and activity of the apical epithelial sodium channel (ENaC) by phosphorylating the NEDD4 ubiquitin ligase, which decreases the internalization of ENaC in collecting duct cells (Bhalla et al., 2006, Debonneville et al., 2001,
SGK-1 has also been reported to phosphorylate the transcription factor FOXO3, which results in its export from the nucleus and leads to a decrease in apoptosis in HEK-293 and CCL39 cells (Brunet et al., 2001). A recent study by Anacker et al. showed that SGK-1 potentiated and maintained the activation of the GR in the presence of cortisol and after cortisol withdrawal by increasing GR phosphorylation at Ser-203 and Ser-211 and GR nuclear translocation. Furthermore, this study also showed that SGK-1 is involved in the cortisol-induced decrease of proliferation in human hippocampal progenitor cells (Anacker et al., 2013). In addition, a more recent study by Peffer et al. reported that SGK-1 is required for the anti-proliferative effects of GCs in embryonic mouse neural progenitor cells (Peffer et al., 2014). Taken together, these findings suggest that SGK-1 could be involved in a mechanism that also involves the GR and GC-induced effects on cellular proliferation.
CHAPTER 2
MATERIALS AND METHODS

2.1 Compounds and antibodies
Gonadotropin-releasing hormone (GnRH), thiazolyl blue tetrazolium bromide (MTT), dexamethasone (Dex), phorbol 12-myristate 13-acetate (PMA), progesterone (P4), R5020, Antide, Mifepristone (RU486), 8-bromo cyclic-AMP, Hoechst, paraformaldehyde, n-propyl gallate and bisindolylmaleimide (BIM) were purchased from Sigma-Aldrich, South Africa. Bovine serum albumin (BSA), Complete Mini protease inhibitor tablets, leupeptin, aprotinin, FuGENE 6 and the Cell proliferation BrdU colorimetric ELISA kit (#1164729001) were purchased from Roche Applied Sciences, South Africa. The anesthetics ketamine and xylazin were purchased from Bayer, Germany and Isoflurane was purchased from Abbott, Germany. The anti-rabbit AlexaFluor488 (#A21206) antibody was purchased from Invitrogen, USA, whereas the donkey anti-mouse Cy3 (#715-166-150) and the donkey anti-rabbit Cy5 (#711-175-152) antibodies were obtained from Jackson ImmunoResearch, USA. Fluoromount-G and Mowiol was purchased from Southern Biotechnology, USA and Calbiochem, Merck, South Africa, respectively.

Antibodies for GR (sc-8992), SRC-1 (sc-8995), SRC-3 (sc-25742), CBP (sc-369), p300 (sc-32244), anti-mouse HRP (sc-2005) and anti-rabbit HRP (sc-2313) were purchased from Santa Cruz Biotechnology, USA. Antibodies to Akt (#610876) and flotillin-1 (#610822) were purchased from BD Transduction Laboratories, USA. Antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14C10) (#2118), phospho-p38 MAPK (Thr180/Tyr182) (#9211), p38 MAPK (#9212), phospho-p44/42 MAPK (Thr202/Tyr204) (#9101), p44/42 MAPK (#9102) and JNK (#9252) were obtained from Cell Signaling Technology, USA. The GRIP-1 antibody (#G8970-10) was obtained from United States Biological, USA. The PR antibody (#PA0312) was purchased from Leica Biosystems, USA. The non-specific rabbit IgG antibody (#R1131) was purchased from Sigma-Aldrich, South Africa. The rabbit anti-GnRHR antibody, raised against amino acids 193-212 in the extracellular loop of the ovine receptor, was a generous gift from Prof. D. C. Skinner (University of Wyoming, Department of Zoology and Physiology) and has been described before (Bliss et al., 2007). The human anti-phospho-Ser-203 GR, anti-phospho-Ser-211 GR and anti-phospho-Ser-226 GR antibodies (corresponding to Ser-212, Ser-220 and Ser-234 in mouse, respectively) were a
generous gift from Prof. M. J. Garabedian (New York University, School of Medicine, USA). Refer to table 2.3 for dilutions of the primary antibodies used in Western blot analysis.

2.2 Treatment with test compounds
Dex and RU486 were dissolved in 100% ethanol (EtOH) to a concentration of 100 µM, whereas P₄ and R5020 were dissolved in 100% EtOH to a concentration of 1 mM and PMA was dissolved in 100% EtOH to a concentration of 20 µg/mL. GnRH, Antide and 8-bromo cyclic-AMP were dissolved in dH₂O to a concentrations of 100 µM, 100 µM and 100 mM, respectively. The PKC inhibitor BIM was dissolved in DMSO to a concentration of 100 µM. All test compounds were added to cells or tissue at 1:1 000 dilution in culture medium unless stated otherwise in the Figure legend. Vehicle controls were either culture medium alone, or culture medium containing 0,1% DMSO or 0,1% EtOH depending on the test compounds added.

2.3 Plasmid transformation and preparation
The pMT-PR-B (encoding the human PR-B) plasmid was a generous gift from Prof. S. Okret (Karolinka Institute, Sweden) (Cairns et al., 1993). The plasmid was transformed into competent Escherichia coli DH5α cells with the heat-shock procedure as 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 1h at 37°C shaking. Cells were plated onto LB-agar (LB with 1.5% agar) plates-containing 100 µg/mL ampicillin and incubated overnight at 37°C. For plasmid purification, a single colony was selected to inoculate LB cultures containing 100 µg/mL ampicillin and incubated overnight 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
The immortalized mouse LβT2 pituitary gonadotrope cell line was kindly provided by Prof. P. L. Mellon at the University of California, San Diego, USA (Alarid et al., 1996, Turgeon et al., 1996). COS-1 monkey kidney fibroblast cells were purchased from American Type Culture Collection
Both cell lines were grown in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, South Africa), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Gibco-BRL Life Technologies, UK). The cells were maintained in 75-cm² culture flasks (Greiner Bio-one International, Austria) at 37°C in an environment of 5% CO₂ and 90% humidity. LβT2 cells and COS-1 cells were passaged with 0.25% trypsin/0.1% EDTA in calcium- and magnesium-free PBS (Highveld Biologicals, South Africa) once or twice a week, respectively. Cells were routinely tested for mycoplasma infection by Hoechst staining (Freshney 1987), and only mycoplasma-negative cells were used in experiments.

2.5 Transient transfection of cells
To generate a positive control of the PR protein for the Western blot experiment in Figure 3.10A, COS-1 cells were seeded in 12-well plates at a density of 1 X 10⁵ 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 1 µg pMT-PR-B using 2 µL FuGENE 6 (Roche Applied Sciences, South Africa) 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% β-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 volumes of cell lysates were analyzed by Western blotting as described below.

2.6 RNA interference
LβT2 cells were seeded in 12-well plates at a density of 3.5 X 10⁵ cells per well in 1 mL of 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 (#301705), Qiagen, USA) or DharmaFECT 1 transfection reagent (#T-2001-01), Dharmacon, USA) according to the manufacturer’s instructions. Briefly, either a combination of mouse Flot-1 siRNA (Mm_Flot1_1, Mm_Flot1_2, Mm_Flot1_3, and Mm_Flot1_4 FlexiTube siRNA (#1027415), Qiagen, USA) or SMARTpool (ON-TARGETplus mouse Flot-1 siRNA (#L-045422-01-0005), Dharmacon, USA) or GR siRNA (Mn_Nr3c_3, Mn_Nr3c_4, Mn_Nr3c_5, and Mn_Nr3c_6, FlexiTube siRNA (#1027416) or SGK-1 siRNA (#01416555), Qiagen, USA) or just non-silencing scrambled (NSC) siRNA (Negative control siRNA (#1027310), Qiagen, USA) was diluted in 50 µL of Opti-MEM+GlutaMAX-I (Invitrogen, USA) with either 3.5 µL of HiPerfect or 5
µL of DharmaFECT 1. 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. Three-days after transfection, the cells were washed once with PBS followed by stimulation for 8h with 100 nM Dex, 100 nM GnRH, or a combination of both in serum-free DMEM for gene expression assays, unless stated otherwise in the Figure legend. Either increasing or decreasing the amount of Flot-1 siRNA oligonucleotides, transfection reagent, incubation times or cell densities did not increase the knockdown efficiency of Flot-1 protein, without cell death.

2.7 Isolation of total RNA
LβT2 cells were seeded in 12-well plates at a density of 4.5 X 10^5 cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. Forty-eight hours after plating the cells were washed with PBS followed by stimulation in serum-free medium as indicated in the respective Figure legends. Total RNA was isolated with the Trizol reagent (Sigma-Aldrich, South Africa) according to the manufacturer’s instructions. Briefly, 400 µL of Trizol reagent was added per well and incubated at RT for 5 min before being subjected to a -80°C freeze-thaw cycle. Thereafter the cell lysates were transferred into micro-centrifuge tubes and centrifuged for 10 min at 12 000 X g at 4°C. The supernatant was transferred into new micro-centrifuge tubes and 80 µL chloroform was added to each sample and vortexed vigorously for 15 sec, followed by centrifugation for 15 min at 12 200 X g at 4°C. The top aqueous phase was transferred into new micro-centrifuge tubes and 200 µL of isopropanol was added. Subsequently, the samples were incubated at RT for 10 min followed by centrifugation at 4°C for 10 min at 20 000 X g to precipitate the RNA. The RNA precipitates were washed twice with 400 µL 75% EtOH and centrifuged for 5 min at 20 000 X g at 4°C. The RNA was allowed to air dry for 5 min and resuspended in 10 µL diethyl pyrocarbonate (DEPC)-treated water and incubated at 55°C for 5 min, where after the samples were stored at -80°C.

To confirm the integrity of the isolated RNA denaturing formaldehyde agarose gel electrophoresis was performed (Sambrook et al., 1989). Briefly, 15 µL sample loading buffer [12% (v/v) DEPC-treated water, 0.1% (w/v) bromophenol blue, 7% (v/v) glycerol, 10% (v/v) 10 X MOPS buffer (0.2 M MOPS in DEPC water, 0.05 M sodium acetate and 0.01 M EDTA), 17% (v/v) 12.3 M formaldehyde and 49% formamide] with 20 µg/mL ethidium bromide (EtBr) was added to 0.5 µg of each RNA sample, incubated at 65°C for 10 min to denature secondary structures and cooled on ice. The samples were analyzed by gel electrophoresis on a 1% formaldehyde agarose gel in 1 X MOPS buffer at 65 V for approximately 1h (Sambrook et al., 1989).
2.8 cDNA synthesis
Total RNA was reverse-transcribed using the Transcriptor First Strand cDNA Kit (Roche Applied Sciences, South Africa) according to the manufacturer’s protocol for cDNA synthesis using anchored oligo (dT) priming. Each RNA sample (0.5 μg) was mixed with 0.5 μL oligo (dT) primers (final concentration 2.5 μM) and DEPC-treated water to make up a final volume of 6.5 μL. The mixture was incubated for 10 min at 65°C. Samples were put on ice and allowed to cool down. Subsequently, 2 μL Transcriptor Reverse Transcriptase Reaction Buffer, 0.25 μL Protector RNase Inhibitor (10 units), 1 μL dNTP mix (final concentration 1 mM of each dNTP) and 0.25 μL Transcriptor Reverse Transcriptase (5 units) were added to each sample, mixed carefully and incubated at 50°C for 1h. To terminate the Transcriptor Reverse Transcriptase the samples were incubated for 5 min at 85°C. Thereafter the samples were used for PCR or stored at -80°C.

2.9 Conventional PCR
Conventional PCR was performed using GoTaq buffer and GoTaq Flexi DNA polymerase (Promega Corp., USA). The PCR reaction contained the following:

1 X GoTaq Buffer
0.2 mM of each dNTP
0.3 μM PR-B sense primer: GGTCCCCCTTGCTTGCA
0.3 μM PR-B anti-sense primer: CAGGACCAGGAAAAAGCAG
1.5 mM MgCl₂
1.25 U GoTaq Flexi DNA Polymerase
1 μL Template cDNA
PCR-grade H₂O, Make up to final volume of 25 μL

The PCR protocol was as follows: Initial denaturing 95°C for 2 min followed by 35 cycles of denaturing at 95°C for 30 sec, annealing at 60°C for 45 sec, extending at 72°C for 45 sec, followed by a final extension at 72°C for 5 min.

2.10 Quantitative Real-Time PCR
Quantitative Real-Time PCR was performed using SensiMix™ dT kit (Quantace, UK) and the Corbett Real-Time PCR machine and reaction tubes according to the manufacture’s protocols (Quantace, UK). The following reaction mix was prepared: 10 μL Sensi-MixdT, sense and anti-sense
primers (refer to table 2.1 for final concentration of primers), 1 μL cDNA and PCR-grade water to make up a total volume of 20 μL. The PCR protocol was as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, annealing for 10 sec (refer to table 2.1 for annealing temperatures) and 72°C for 10 sec. Melting curve analysis and gel electrophoresis were performed to confirm the expected size of the generated amplicon for each sample. Standard curves were used to determine the efficiency of each primer pair, and the relative expression of the genes of interest in each sample was calculated with the “Fit Points” equation described by Pfaffl et al. (Pfaffl 2001) and were normalized to relative GAPDH transcript levels used as the reference gene.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Final concentration of primer (µM)</th>
<th>Annealing temperature (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGK-1 Fwd</td>
<td>ATCTCCAGAGGGAGGC</td>
<td>0.25</td>
</tr>
<tr>
<td>SGK-1 Rev</td>
<td>TCAATGAGGACGCTGTCGC</td>
<td>58</td>
</tr>
<tr>
<td>GnRHR Fwd</td>
<td>CCAAGTCTGGTCCGAGCTTGC</td>
<td>0.125</td>
</tr>
<tr>
<td>GnRHR Rev</td>
<td>TACGCTTCAGGCGAGCTTGC</td>
<td>58</td>
</tr>
<tr>
<td>GAPDH Fwd</td>
<td>TTGACCCCATGGAGACTGTA</td>
<td>0.25</td>
</tr>
<tr>
<td>GAPDH Rev</td>
<td>CGCATGGAAGCTGAGCTCA</td>
<td>58</td>
</tr>
<tr>
<td>GILZ Fwd</td>
<td>CCCTAGACAAACAGATTGAGC</td>
<td>0.2</td>
</tr>
<tr>
<td>GILZ Rev</td>
<td>CAGAGGACTTTACACCGCC</td>
<td>48</td>
</tr>
<tr>
<td>GRα Fwd</td>
<td>TGGTATGCTTGCCTCGATCTG</td>
<td>0.3</td>
</tr>
<tr>
<td>GRα Rev</td>
<td>TTGACGGATATACACCGGC</td>
<td>52</td>
</tr>
<tr>
<td>LHβ Fwd</td>
<td>GGCCCGAGAATAGATTGTCC</td>
<td>0.25</td>
</tr>
<tr>
<td>LHβ Rev</td>
<td>CTGAGCATTGACTGAGATGTA</td>
<td>56</td>
</tr>
<tr>
<td>FSHβ Fwd</td>
<td>GGCACCAGGAATACAGTGTG</td>
<td>0.25</td>
</tr>
<tr>
<td>FSHβ Rev</td>
<td>GACATTACCTCGAGGTC</td>
<td>50</td>
</tr>
<tr>
<td>αGSU Fwd</td>
<td>GAGATATACCTCGAGGTC</td>
<td>0.25</td>
</tr>
<tr>
<td>αGSU Rev</td>
<td>CGTACGGAGAGACTG</td>
<td>45</td>
</tr>
<tr>
<td>MKP-1 Fwd</td>
<td>ACTACCTCTCTCTACCTGCAG</td>
<td>0.25</td>
</tr>
<tr>
<td>MKP-1 Rev</td>
<td>TCTGATGCTGAAATGTCATATGA</td>
<td>55</td>
</tr>
<tr>
<td>FKB5 Fwd</td>
<td>GGCCAGACAGAATACAGTGA</td>
<td>0.25</td>
</tr>
<tr>
<td>FKB5 Rev</td>
<td>GGACACAGGCTTCTCTTCTGG</td>
<td>60</td>
</tr>
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</table>

Table 2.1: Sequences, concentrations and annealing temperatures of primers used for quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Efficiency (%)</th>
<th>Product size (bp)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGK-1</td>
<td>96</td>
<td>160</td>
<td>Dr. A. Kotitschke</td>
</tr>
<tr>
<td>GnRHR</td>
<td>95</td>
<td>192</td>
<td>(Sadie 2006)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>98</td>
<td>263</td>
<td>(Overbergh et al., 1999)</td>
</tr>
<tr>
<td>GILZ</td>
<td>84</td>
<td>281</td>
<td>Dr. A. Kotitschke</td>
</tr>
<tr>
<td>GRα</td>
<td>100</td>
<td>299</td>
<td>(Thackray et al., 2006)</td>
</tr>
<tr>
<td>LHβ</td>
<td>70</td>
<td>83</td>
<td>(Chen et al., 2009)</td>
</tr>
<tr>
<td>FSHβ</td>
<td>80</td>
<td>280</td>
<td>(Coss et al., 2004)</td>
</tr>
<tr>
<td>αGSU</td>
<td>90</td>
<td>298</td>
<td>Dr. A. Kotitschke</td>
</tr>
<tr>
<td>MKP-1</td>
<td>65</td>
<td>250</td>
<td>(Rauhala et al., 2005)</td>
</tr>
<tr>
<td>FKB5</td>
<td>75</td>
<td>220</td>
<td>(Nuber et al., 2005)</td>
</tr>
</tbody>
</table>

Table 2.2: Primer pair efficiencies, product sizes and reference(s) of primers used for quantitative Real-Time PCR
2.11 SDS-PAGE and Western blot analysis

LβT2 cell lysates were separated on 8-10% SDS-polyacrylamide gels at 120 V in 1 X SDS running buffer (25 mM Tris-Cl (pH 8.4), 250 mM glycine and 0.1% SDS) using a Bio-Rad Mini Protean II electrophoresis cell chamber. Proteins were transferred onto a HyBond ECL nitrocellulose membrane (Amersham Biosciences) for 1h at 180 mA in a Tris/glycine buffer (25 mM Tris, 250 mM glycine and 10% (v/v) methanol) using a Mini Protean II blotting system (Bio-Rad). The membranes were blocked for 1h at room temperature in 4% ECL blocking solution [4% (w/v)] ECL advance blocking powder, (Amersham Biosciences) 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 in 4% ECL blocking solution diluted in TBST at 4°C overnight. The following day the membranes were washed with TBST for 3 X 5 min at room temperature before incubation with secondary HRP-conjugated antibodies for 1h at room temperature in 5% non-fat milk powder (w/v) in TBST. Membranes were washed for 3 X 5 min with TBST followed by a 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 1h 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 (Amersham Biosciences, USA) and Hyperfilm MP high performance autoradiography film (Amersham Biosciences, USA) according to the manufacturer’s instructions. Bands on the autoradiography film were scanned and quantification was performed with AlphaEaseFC FluorChem 5500 software (Alpha Innotech. USA).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>1:4 000</td>
</tr>
<tr>
<td>PR</td>
<td>1:1 000</td>
</tr>
<tr>
<td>Flotillin-1</td>
<td>1:5 000</td>
</tr>
<tr>
<td>phospho-ERK-1/2</td>
<td>1:1 000</td>
</tr>
<tr>
<td>ERK-1/2</td>
<td>1:1 000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:5 000</td>
</tr>
<tr>
<td>phospho-p38</td>
<td>1:1 000</td>
</tr>
<tr>
<td>p38</td>
<td>1:1 000</td>
</tr>
<tr>
<td>Akt</td>
<td>1:1 000</td>
</tr>
<tr>
<td>JNK</td>
<td>1:1 000</td>
</tr>
<tr>
<td>phospho Ser-212 GR</td>
<td>1:5 000</td>
</tr>
<tr>
<td>phospho Ser-220 GR</td>
<td>1:5 000</td>
</tr>
<tr>
<td>phospho Ser-234 GR</td>
<td>1:5 000</td>
</tr>
</tbody>
</table>

Table 2.3: Dilutions of primary antibodies used in Western blot analysis
2.12 Immunofluorescence staining and confocal microscopy
Coverslips 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 above. After 48h, cells were washed twice with ice-cold PBS and live cell-stained for 1h at 4°C with rabbit anti-GnRHR (1:400) antibody in PBS with 5% BSA followed by 2 washes with ice-cold PBS. Subsequently, the cells were 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 1h at room temperature followed by staining with mouse anti-Flot-1 (1:50) antibody in PBS with 5% BSA for 1h at room temperature. For the GR and Flot-1 immunofluorescence the cells were fixed, permeabilized, blocked and stained with rabbit anti-GR (1:250) and Flot-1 as mentioned above. Subsequently, the cells were washed with 1% BSA in PBS for 3 X 5 min before incubating with anti-rabbit-labelled Alexa488 (1:500) (to detect GnRHR and GR) and anti-mouse-labelled Cy3 (1:1 000) (to detect Flot-1) antibodies in PBS with 5% BSA for 1h 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 (Calbiochem, Merck, South Africa) containing n-propyl gallate (Sigma-Aldrich, South Africa) as the anti-fading agent and allowed to set overnight at room temperature in the dark followed by storage at 4°C in the dark until visualization. Confocal microscopy was performed with a Zeiss Axiovert 200M LSM 510 Meta NLO Confocal Microscope using the 40 X water-immersion objective and the 63 X oil-immersion objective. A multi-track scanning configuration using the 488 nm (HeNe gas laser), 561 nm (solid state laser), 633 nm (argon laser), and 800 nm (titanium:sapphire 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 images were analyzed for co-localization with the Carl Zeiss ZEN software (Version 2009) using Manders correlation and overlap coefficients (Manders et al., 1993) for the fluorophores.

2.13 Membrane raft isolation
Plasma membrane membrane rafts were isolated using the Triton X-100 procedure as described by Lafont et al. with some minor modifications (Lafont and Simons 2001). LβT2 cells were seeded in 150-mm² dishes at a density of 8 X 10^6 cells per dish in DMEM with 10% FCS containing antibiotics as described above. The cells were washed twice with PBS and stimulated with 100 nM Dex, 100 nM GnRH, or a combination of both for 30 min in serum-free medium before being washed twice
with ice-cold PBS. The cells were scraped on ice in 1 mL of 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 was resuspended in 1 mL of 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 and incubated on ice-water for 45 min. The lysates were adjusted to 60% sucrose in SB and layered at the bottom of SW40 Ultraclear centrifuge tubes (Beckman Coulter). A discontinuous sucrose gradient was prepared consisting of 2 mL of extraction lysis buffer (ELB), 10 mM Hepes (pH 7.9), 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 5 µg/mL leupeptin, and 2 µg/mL aprotinin), 4 mL of 13% sucrose in ELB, 0 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 18h at 4°C). Flocculent material could be seen at the interfaces, and fractions (1.5 mL) were collected as follows: 1) top of the gradient, 2) ELB/13% interface, 3) 13%/43% interface, 4) remaining 13%/43% interface, 5) middle of 43% sucrose, 6) 43%/60% interface, 7) middle of 60% sucrose (loading fraction), and 8) 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, equal volumes of membrane raft fractions from the stimulated conditions were analyzed by Western blotting and quantified as described elsewhere. The protein of interest levels were normalized against Flot-1 protein levels for each experiment and expressed relative to vehicle-treated (control).

2.14 Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described (Ma et al., 2003), with some minor modifications. LβT2 cells were seeded in 150-mm² dishes at a density of 8 X 10⁶ cells per dish in DMEM with 10% charcoal-stripped FCS and antibiotics as described above. Seventy-two hours after plating the medium was replaced before the cells were stimulated with 100 nM Dex, 100 nM GnRH, or a combination of both for 1h. Thereafter, proteins were cross-linked with 1% formaldehyde for 40 min before the reaction was quenched with 125 mM glycine for 10 min. The cells were washed and scraped in PBS containing Complete Mini protease inhibitor mixture (Roche Applied Sciences, South Africa). Cells were centrifuged and resuspended in 0.5 mL of nuclear lysis buffer (1% SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and protease inhibitor mixture). Cross-linked DNA was sheared by sonication to obtain DNA fragments between 200-400 bp. After sonication the samples were centrifuged at 15 000 X g for 10 min at 4°C and the supernatant was collected. To normalize for equal amounts of DNA per immunoprecipitation, the chromatin was diluted (1:20) in dH₂O and
quantified after measuring the optical density at 260 nm. For the input a 30 µg aliquot of chromatin was used, whereas 100 µg of chromatin was diluted in immunoprecipitation dilution buffer (0.01% SDS, 20 mM Tris-HCl (pH 8.0), 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, and protease mixture inhibitors) before being pre-cleared for 1h with BSA/salmon sperm DNA-pre-blocked Protein A/G PLUS agarose beads rotating at 4°C. The pre-cleared samples were then incubated with 5 µg of anti-GR or 5 µg of non-specific rabbit IgG antibodies or 2 µg of anti-SRC-1, anti-GRIP-1, anti-SRC-3, anti-p300 or anti-CBP antibodies, by rotating at 4°C overnight. The following day, 40 µL of Protein A/G PLUS agarose beads was added to the mixture for 6h at 4°C. The beads were collected by centrifugation and washed sequentially with 1 mL of each wash buffer I (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl), II (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), and III (1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA, and 10 mM Tris, pH 8.0) followed by three washes with 1 mL of Tris-EDTA. Complexes were eluted from the beads with 300 µL of immunoprecipitation elution buffer (1% (w/v) SDS and 100 mM NaHCO₃) and the cross-links were reversed by the addition of 300 mM NaCl and incubation at 65°C overnight. The following day each sample was adjusted to 150 mM EDTA, 125 mM Tris-HCl pH 6.5, and 20 µg of proteinase K followed by incubation for 1h at 45°C. The DNA was purified with the QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer’s instructions. Quantitative Real-Time PCR was performed using specific primers (Wong et al., 2010) that span the GRE region in the promoter of the SGK-1 gene and generate a product with an expected size of 239 bp, forward and reverse 5’- CTAACCTCGCCACCTCCTCAC -3’, 5’-TCCCAGAACTTGGAAGAGGA -3’, respectively. The PCR was performed with 0.25 µM of primers, 2 µL and 4 µL of the purified DNA from the input and immunoprecipitated samples, respectively, and under the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 56°C for 10 sec, and 72°C for 10 sec.

2.15 Cell growth and MTT assays
LβT2 cells were seeded in 12-well plates at a density of 3.5 X 10⁵ cells per well in 1 mL of DMEM with 10% FCS and antibiotics as described elsewhere. Twenty-four hours after plating the cell number was determined (Day-0) and the cells were stimulated for 24h or 48h with 100 nM Dex, 100 nM GnRH, or a combination of both in charcoal-stripped full medium. After 24h (Day-1) and 48h (Day-2) the cell numbers were determined. For thiazolyl blue tetrazolium bromide (MTT) assays, LβT2 cells were seeded in 24-well plates at a density of 1.75 X 10⁵ cells per well in 0.45 mL of DMEM with 10% FCS and antibiotics as described elsewhere. After 24h, cells were stimulated with
100 nM Dex, 100 nM GnRH, or a combination of both in charcoal-stripped full medium for 3, 5 or 7 days and at each time-point, 50 μL MTT solution (5 mg/mL in medium) was added to give a final concentration of 0.5 mg/mL. Cells were incubated for 4h, after which 500 μL of the solubilization reagent (2-propanol with 0.01 M HCl) was added to resuspend the formazan metabolic product and measurement of the optical density at 560 nm with subtraction of the blank control, which consisted of the entire procedure without cells.

2.16 Bromodeoxyuridine (BrdU) incorporation cell proliferation ELISA

The proliferation assay without prior siRNA-mediated SGK-1 or Flot-1 protein knockdown (Figure 3.11 C and 3.12 A) was performed as follows: LβT2 cells were seeded in 48-well plates at 7.5 X 10^4 cells per well in 200 μL of DMEM with 10% charcoal-stripped serum and antibiotics as described elsewhere. After 24h the cells were stimulated for 48h with 100 nM Dex, 100 nM GnRH, or a combination of both (Figure 3.11 C), in the presence or absence of 100 nM BIM (Figure 3.12 A), as indicated in the Figure legends. Thereafter, the cell proliferation was determined with a cell proliferation BrdU colorimetric kit (11647229001) (Roche Applied Sciences, South Africa) according to the manufacturer’s instructions.

The proliferation assay with prior siRNA-mediated SGK-1 or Flot-1 protein knockdown (Figure 3.12 C) was performed as follows: LβT2 cells were seeded in 12-well plates at a density of 3.5 X 10^5 cells per well in 1 mL of 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 40 nM Flot-1 siRNA, 40 nM SGK-1 siRNA, or 40 nM NSC using HiPerfect transfection reagent (Qiagen, USA) as described elsewhere. After 72h the cells were incubated with trypsin as described in Section 2.4 and re-seeded in 48-well plates at 7.5 X 10^4 cells per well in 200 μL of DMEM with 10% charcoal-stripped serum and antibiotics as described elsewhere. Twenty-four hours after plating the medium was replaced with fresh medium and the cells (~70% confluency) were stimulated for 48h with 100 nM Dex, 100 nM GnRH, or a combination of both. Thereafter, the cell proliferation was determined with a cell proliferation BrdU colorimetric kit (#11647229001) (Roche Applied Sciences, South Africa) according to the manufacturer’s instructions.
2.17 Mice
Female C57BL/6 (WT) mice (12-weeks old) and female GRIC/R26-YFP mice (12-weeks old) were maintained on a standard 12h light, 12h dark cycle with access to food and water \textit{ad libitum}. Animal care and experimental procedures were conducted in accordance with the guidelines established by the Animal Welfare Committee of the University of Saarland.

The GRIC/R26-YFP mouse model was developed by Prof U. Boehm with a binary genetic strategy to express YFP in gonadotrope cells. The strategic approach taken by the Boehm laboratory to generate this mouse model is briefly summarized below (Wen \textit{et al.}, 2008).

With a gene targeting approach a novel knock-in mouse model was generated in which Cre recombinase is co-expressed with the GnRHR gene. The Cre recombinase was knocked into the GnRHR gene locus along with an internal ribosome entry site (IRES) to facilitate expression of the Cre recombinase in cells that endogenously express the GnRHR. These GnRHR-IRES-Cre (GRIC) mice were bred with ROSA26-YFP mice which have a targeted insertion of an YFP gene in the ubiquitously expressed ROSA26 (R26-YFP) locus. The Cre-mediated homologous recombination of the R26-YFP allele results in constitutive YFP expression in gonadotropes of GRIC/R26-YFP double-heterozygous mice with a C57BL/6 background (Wen \textit{et al.}, 2008).

2.18 Estrous cycle stage determination by vaginal cytology
Vaginal smears were collected by flushing the vagina with distilled H\textsubscript{2}O and the smears were mounted on glass slides. The cells were examined by light microscopy and three different types were identified; 1: epithelial cells were round and nucleated; 2: cornified squamous epithelial cells was irregularly shaped without a nucleus and 3: leukocytes was small and round. The proportion among them was used for the determination of the estrous cycle phases as follows, estrous stage consisted of cell type 2, diestrous stage consisted predominantly of cell type 3, metestrous stage consisted of all three cell types and the proestrous stage consisted predominantly of cell type 1 (Caligioni 2009).
For each time-point, i.e. 30 min:

- **Pro-Est**
- **Met-Est**
- **Di-Est**
- **Est**

**Pro-Est**: Proestrous; **Met-Est**: Metestrous; **Di-Est**: Diestrous; **Est**: Estrous.

Figure 2.1: Experimental procedure followed to generate estrous cycle stages mouse pituitary tissue RNA samples for gene expression

The estrous cycle stage of mice was determined as described in Section 2.18, followed by administering anesthesia with a saturated atmosphere of Isoflurane and sacrificed by cervical dislocation. Thereafter, the pituitary was quickly isolated and divided into four equal sections followed by a 1h incubation in serum-free medium before stimulation. After stimulation, the total RNA was isolated, cDNA synthesized and qPCR performed. Stimulation time-points used were 30 min, 1h, 2h, 4h and 8h. Three independent repeat experiments for each stage of the estrous cycle were performed. **Pro-Est**: Proestrous; **Met-Est**: Metestrous; **Di-Est**: Diestrous; **Est**: Estrous.

### 2.19 Mouse perfusion, pituitary isolation and immunohistochemistry

GRIC/R26-YFP mice were anesthetized by an intraperitoneal injection with ketamine/xylazine (Bayer, Germany). Thereafter, the mice were perfused transcardially with 37°C PBS, followed by ice-cold 4% paraformaldehyde. Fifteen minutes later the pituitary was removed and incubated in 30% sucrose at 4°C for 3h. Subsequently, the pituitary was frozen in tissue-freezing medium OCT (Leica Microsystems, Germany) and 10 µm thick sections were generated with a cryostat (Leica Microsystems, Germany). The sections were collected on SuperFrost Plus glass-slides (Roth, Germany) and blocked for 1h at room temperature in PBS containing 5% normal goat serum, 0.2% Triton X-100, and 0.02% sodium azide. The slides were stained with rabbit anti-GR (1:200) and mouse anti-Flot-1 (1:50) antibodies at 4°C overnight in PBS containing 0.5% λ-carrageenan (Sigma-
Aldrich, Germany) and 0.02% sodium azide. The following day, the slides were washed with PBS for 3 X 20 min at room temperature before incubating with anti-rabbit-labelled Cy5 (1:1 000) (to detect GR) and anti-mouse-labelled Cy3 (1:1 000) (to detect Flot-1) antibodies in PBS with 0.5% λ-carrageenan and 0.02% sodium azide for 2h at room temperature in the dark. Thereafter, the slides were washed with PBS for 3 X 10 min followed by incubation with Hoechst (100 µg/mL) in PBS for 5 min. Slides were mounted in Fluoromount-G (Southern Biotechnology, USA) and allowed to set overnight at room temperature in the dark followed by storage at 4°C in the dark until visualization. Confocal microscopy was performed with a Zeiss Axiovert 200M LSM 510 Meta NLO Confocal Microscope using the 40 X water-immersion and 63 X oil-immersion objectives. A multi-track scanning configuration using the 488 nm (HeNe gas laser), 561 nm (solid-state laser), 633 nm (argon laser), and 800 nm (titanium:sapphire 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 as determined with the negative controls in which the slides were stained without GR and Flot-1 primary antibodies. At least three different fields of view from three independent experiments were collected.

For the isolation of RNA from WT mouse pituitary tissue the mice were anesthetized with a saturated atmosphere of Isoflurane and sacrificed by cervical dislocation. Thereafter, the pituitary was quickly isolated and divided into four equal sections followed by a 1h incubation in 1 mL of serum- and phenol red-free DMEM (Invitrogen, Germany) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Germany) in 24-well plates at 37°C in an environment of 5% CO₂ and 90% humidity. Thereafter, the pituitary tissue sections were stimulated as indicated in the Figure legends. After stimulation the tissue was washed once with PBS and the total RNA was isolated with 400 µL Trizol reagent as described in Section 2.7. Each pituitary was divided into four equal size sections (by size only and not by weight). The four sections were randomly chosen for treatment with the ligand. This random selection of sections for each independent experiment), makes it unlikely that changes in the integrity and levels of cell surface receptors would occur between conditions.

2.20 Isolation of primary mouse gonadotrope cells and RNA isolation

GRIC/R26-YFP mice were anesthetized with a saturated atmosphere of Isoflurane and sacrificed by cervical dislocation. The pituitaries were quickly removed and transferred into DMEM-F12 (Invitrogen, Germany) followed by cutting the tissue into small pieces with a sterile scalpel.
Thereafter, the tissue was digested with 0.5% Trypsin (Invitrogen, Germany) at 37°C for 25 min followed by additional digestion at 37°C in Hanks balanced salt solution (Invitrogen, Germany) with 2 mM EDTA or 1 mM EDTA for 5 min and 15 min, respectively. The cell clumps were gently tritutated with glass pipettes and centrifuged at 300 X g for 15 min at 4°C. Thereafter, the cell pellet was resuspended in phenol red-free DMEM with 10% charcoal-stripped serum and antibiotics as described elsewhere. The gonadotrope cells were isolated from the mouse pituitary cells mixture by FACS with a MoFlo cell sorter (Beckman Coulter) and settings for maximum purity. The average percentage enrichment of gonadotrope cells from the FACS sorting method was approximately five percent. Importantly, αGSU is expressed in both gonadotrope and thyrotrope cells, which indicates a potential caveat. Therefore, the results obtained for the αGSU gene in purified gonadotrope cells should be cautiously interpreted. An 100 µm nozzle, photomultiplier tube, 488 nm laser, 525 nm dichroic, and a 543 nm excitation filters were used in the sorting procedure. Cells were collected and seeded at 7 500 cells/well in a 96-well plate pre-coated with laminin and poly-L-lysine (Sigma-Aldrich, Germany) in phenol red-free DMEM with 10% charcoal-stripped serum and antibiotics as described elsewhere. After 24h the cells were stimulated for 8h with 100 nM Dex, 100 nM GnRH, or a combination of both. After stimulation the cells were harvested with RNAprotect Cell Reagent (Qiagen, Germany) and total RNA was isolated with the NucleoSpin RNA XS kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions.

2.21 mRNA amplification

The RNA isolated from the primary mouse gonadotrope cells was amplified with a TargetAmp™ 1-Round aRNA Amplification Kit 103 (#TAU1R5124) (Epicentre Biotechnologies, USA) according to the manufacturer’s instructions. Briefly, one µL of TargetAmp T7-Oligo (dT) Primer A was added to 25 ng of RNA and annealed at 65ºC for 5 min before incubating on ice for 1 min. First-strand cDNA synthesis was performed by adding 2 µL of reaction mixture consisting of 1.5 µL TargetAmp Reverse Transcription PreMix-SS, 0.25 µL dithiothreitol and 50 U of SuperScript III (Invitrogen, Germany) and incubating for 30 min at 50ºC. Thereafter, second-strand cDNA synthesis was performed by adding 5 µL of reaction mixture consisting of 4.5 µL TargetAmp DNA Polymerase PreMix-SS 1 and 0.5 µL TargetAmp DNA Polymerase-SS 1 and incubating for 10 min at 65ºC, followed by stopping the reaction by incubating the sample for 3 min at 80ºC. The aRNA generated up to now was subjected to an in vitro transcription reaction by incubating the sample for 4h at 42ºC after adding 40 µL of a master mix that consisted of the following, (13.6 µL RNase-free H2O, 4 µL TargetAmp T7 transcription buffer, 3.6 µL of 100 mM ATP, 3.6 µL of 100 mM CTP, 3.6 µL of 100
mM UTP 3.6 µL of 100 mM GTP, 4 µL DTT and 4 µL TargetAmp T7 RNA Polymerase). The DNA was digested by adding 2 µL DNase I and incubating at 37°C for 15 min. Thereafter, the aRNA was purified with an RNeasy MinElute Cleanup kit (Qiagen, Germany). cDNA was synthesized and quantitative Real-Time PCR was performed as described elsewhere.

### 2.22 Statistical analysis

Statistical analysis was performed with GraphPad Prism software (Version 5) using the one-way ANOVA analysis of variance with a Dunnett’s (comparing all samples to a single control sample) or Tukey (comparing all samples with each other) post-test or a two-tailed paired t-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

MEMBRANE RAFT-ASSOCIATED GR AND GnRHR SIGNALLING PATHWAY CROSSTALK AND UPREGULATE SGK-1 mRNA EXPRESSION SYNERGISTICALLY TO DECREASE THE PROLIFERATION OF LβT2 CELLS

3.1 BACKGROUND

Several lines of evidence suggest that rapid glucocorticoid-dependent and GC-independent signalling via classical GRs may occur in specialized membrane membrane raft or caveolae microdomains that are proposed to function as platforms for the assembly of multi-protein signalling complexes (Brown and London 1998, Matthews et al., 2008, Strehl and Buttgereit 2014). The GR and other steroid receptors have been shown to localize to the plasma membrane in the presence and absence of ligands (Gametchu et al., 1993, Liposits and Bohn 1993, Razandi et al., 2002, Razandi et al., 2010). The GR has been shown to localize to caveolae membrane rafts and mediate the phosphorylation of Akt, but the membrane-association appeared to have no effect on the transactivation of a GRE reporter gene in A549 cells (Matthews et al., 2008). However, a study performed by Bartholome et al. reported that GR localized in caveolae is involved in regulating the GC-induced transactivation of a GRE reporter gene in a human liver cell line (Bartholome et al., 2004). Caveolin-1 has previously been reported to be involved in the GC-mediated inhibition of gap junction intercellular communication of coupled embryonic neural progenitor cells. Furthermore, the same study also reported caveolae-associated GR was required to mediate the anti-proliferative effects of GCs in neural progenitor cells (Samarasinghe et al., 2011). The GnRHR has been shown to exclusively localize to membrane rafts in αT3-1 cells and the GnRH-induced activation of ERK-1/2 was abolished when the cells were treated with the cholesterol extraction reagent MβCD, which disrupts the structural formation of membranes and membrane rafts (Bliss et al., 2007, Navratil et al., 2003). The authors established the GnRH-induced activation of ERK-1/2 appeared to require intact membrane rafts (Navratil et al., 2003). It has previously been shown that crosstalk between the GR and GnRHR signalling pathways modulates the expression of an endogenous AP-1-containing gene
and an exogenous GRE reporter gene in a synergistic manner (Kotitschke et al., 2009). Taken together, membrane rafts have been reported to be involved in the function of both the GR and GnRHR signalling pathways. Furthermore, the GR and GnRHR have both been localized to membrane rafts although in different cell types. Since the study by Kotitschke et al. showed the presence of crosstalk between the GR and GnRHR signalling pathways in LβT2 cells (Kotitschke et al., 2009), additional studies are required to further unravel the detailed molecular mechanisms and determine the physiological significance in LβT2 gonadotrope cells.

3.2 AIMS

The aim of the present part of the project was to investigate whether the GR and GnRHR signalling pathways regulates the mRNA expression of endogenous GRE-containing genes in a synergistic manner and to determine the role of membrane rafts in the mechanism as well as the physiological significance of GR and GnRHR crosstalk. The membrane raft localization of GR and GnRHR have been shown in separate reports and Dex + GnRH co-stimulation was reported to induce a synergistic transcriptional response on a GRE reporter gene in LβT2 cells. In the present study the experiments were performed with Dex or GnRH alone, or the combination, to identify a model endogenous GRE-containing gene that responded to each ligand in isolation and synergistically to the combination. Thereafter the mechanism was further explored with various strategies in LβT2 cells.

The detailed aims for this part of the study were the following:

- Explore endogenous GRE-containing genes to establish a model gene that is regulated by Dex, GnRH and synergistically by the combination in LβT2 cells;
- Examine whether GR and GnRHR co-localize with the membrane raft protein Flot-1 and establish if membrane rafts have a pre-formed signalling complex containing kinases that have been reported to modulate the activity of the GR in LβT2 cells;
- Determine whether membrane rafts are required for phosphorylation of the GR or kinases in LβT2 cells;
- Investigate whether Dex + GnRH-mediated synergism may be a result of enhanced GR or GnRHR protein expression levels, enhanced GR nuclear import or differential GR promoter occupancy as well as differential GR co-factor recruitment to the promoter in LβT2 cells;
- Explore if synergism involves the PKA or PKC signalling pathways since GnRH has previously been reported to induce activation of both pathways in LβT2 cells;
- Determine if Dex + GnRH synergism requires the GR, GnRHR and Flot-1 in LβT2 cells;
- Explore whether the physiological significance of GR and GnRHR crosstalk may be in regulating cell proliferation.

3.3 RESULTS

3.3.1 GnRHR and GR co-localize with Flot-1-containing membrane rafts independent of their ligands in LβT2 cells

To investigate if the GnRHR co-localizes with Flot-1 at the plasma membrane in LβT2 cells, live cells were stained for GnRHR followed by fixation, permeabilization and staining for Flot-1. The stained cells were visualized with a confocal microscope and the result showed that a large portion of Flot-1 was localized in the vicinity of the plasma membrane with some distributed in intracellular vesicle compartments (Figure 3.1.1). To detect membrane-associated GnRHR the cells were stained with the antibody before fixation and to detect all membrane rafts (intra cellular and membrane-associated) the cells were fixed and permeabilized followed by staining for Flot-1. The specificity of the GnRHR antibody has previously been determined by Donal C. Skinner (Albertson et al., 2008a, Albertson et al., 2008b). The GnRHR was also found to mainly localize at the plasma membrane region, but a small percentage was detected in the cytoplasm, which could be due to some internalization (Figure 3.1.1). The panel on the right shows the co-localized pixels between the green (0.94 at marked region of interest) and red (0.84 at marked region of interest) channels. The data was analyzed for co-localization with Manders correlation and overlap coefficients and the values indicate that in the marked plasma membrane region that 94% of GnRHR co-localizes with Flot-1 and 84% of Flot-1 co-localizes with GnRHR in LβT2 cells (Figure 3.1.1). Furthermore, the co-localization map and overlap coefficient of the GnRHR and Flot-1 signals indicate 50% co-localization of both channels across the entire cell, indicating that these two proteins occur together in signalling complexes.

Figure 3.1.1: Immunofluorescence analysis shows the GnRHR co-localizes with Flot-1 in LβT2 cells
LβT2 cells were grown on glass coverslips and live cell-stained with rabbit anti-GnRHR followed by fixation and staining with a mouse anti-Flot-1 antibody. Thereafter, the coverslips were incubated with anti-rabbit Alexa488 (green) and anti-mouse Cy3 (red) antibodies. Nuclei were stained with Hoechst (blue) before mounting in Mowiol. Staining was visualized with a Zeiss LSM510 Meta confocal microscope using the 63X objective, and a representative image is shown for a group of cells, as indicated by the multiple nuclei visible in the Hoechst stain. Manders co-localization coefficients (M) are shown for the red and green channels in regions of interest in the vicinity of the cell membrane. The panel on the right marked Co-localization displays a map of co-localized pixels for the green and red channels across the entire group of cells. The degree of overlap between the two channels is indicated by the overlap coefficient (O). The scale bar represents 10 µm. The results shown are representative of three independent experiments.

Having established that the GnRHR co-localizes with Flot-1 at the plasma membrane, it was investigated if the GR is associated with Flot-1 at the membrane and whether this potential association is affected by its activation. Cells were stimulated for 30 min with 100 nM Dex, 100 nM GnRH or a combination of both followed by fixation and permeabilization. Subsequently, the cells were stained with anti-GR- and anti-Flot-1-specific antibodies, followed by visualization with a confocal microscope. The results presented in Figure 3.1.2A-D show that a population of Flot-1 localizes to the plasma membrane, while there was also some detected in intracellular compartments under all treatment conditions. In vehicle control treated cells the GR appears evenly distributed throughout the entire cell, while 84% of the plasma membrane-associated GR co-localizes with Flot-1 (Figure 3.1.2 A) (0.84 and 0.98 at marked region of interest). To identify which intracellular compartments Flot-1 localizes to, the same experiment could have been repeated with an additional staining for specific markers proteins like GM130 for the Golgi or Bip/GRP78 for the endoplasmic reticulum or EEA1 for endosomes or Bcl-2 for mitochondria or Lamp-1 for lysosomes. However this was not done due to time constraints.

Treating the cells with Dex resulted in substantial nuclear translocation of the GR, but a small fraction remained in the cytoplasm with 75% of the plasma membrane-associated GR co-localizing with Flot-1 (Figure 3.1.2 B) (0.75 and 0.83). Treatment with GnRH did not result in detectable nuclear import of the GR, while 82% of the plasma membrane-associated GR co-localizes with Flot-1 (Figure 3.1.2 C) (0.82 and 0.74 at marked region of interest). Co-stimulation with Dex + GnRH showed a similar distribution of the GR as Dex, while 97% of the plasma membrane-associated GR co-localizes with Flot-1 (Figure 3.1.2 D) (0.97 and 0.84 at marked region of interest). The overlap
coefficients for panels A-D were 0.74; 0.47; 0.66 and 0.51 respectively (Figure 3.1.2). These values indicate that there appear to be differences for co-localization between the conditions, although statistical significance could not be established. Determination of the percentage of GR co-localizing with Flot-1 was performed on one experiment.

Figure 3.1.2: Immunofluorescence analysis shows the GR co-localizes with Flot-1 in LβT2 cells ligand independently

LβT2 cells were grown on glass coverslips and incubated for 30 min in medium containing charcoal-stripped serum and vehicle-treated control (Ctrl) (A), or including 100 nM Dex (B), 100 nM GnRH (C), or a combination of both (D). Cells were stained with rabbit anti-GR and mouse anti-Flot-1 antibodies followed by incubation with anti-rabbit Alexa488 (green) and anti-mouse Cy3 (red) antibodies. Nuclei were stained with Hoechst (blue) before mounting in Mowiol and visualization were performed as for Figure 3.1.1. Manders co-localization coefficients (M) are shown for the red and green channels in regions of interest in the vicinity of the cell membrane. The panels on the right
marked Co-localization display maps of co-localized pixels for the green and red channels across the entire group of cells. The degree of overlap between the two channels is indicated by the overlap coefficient (O). The scale bar represents 10 µm. The results shown are representative of three independent experiments.

3.3.2 GnRH has no effect on the Dex-induced nuclear import of the GR

Kotitschke et al. have previously reported that co-stimulation with Dex + GnRH results in a synergistic transcriptional response on a GRE reporter gene and synergistically increased the mRNA levels of the GnRHR gene expression in LβT2 cells (Kotitschke et al., 2009). It may be possible that this synergism is a result of enhanced nuclear GR levels. Therefore, to investigate whether treatment with Dex + GnRH for 30 min induces a greater percentage of GR nuclear translocation than Dex alone, the results shown in Figure 3.1.2 were analyzed by quantifying the intensity of nuclear GR. The 30-min time point was chosen because this is the same time-point previously shown to result in maximal GR phosphorylation by Kotitschke et al. (Kotitschke et al., 2009).

The results presented in Figure 3.2 show that treatment with Dex induced ~2.5-fold nuclear import of the GR. Although GnRH treatment resulted in a small amount of GR nuclear localization, there appeared to be no additive or synergistic effect on GR nuclear import as the amount induced by Dex + GnRH was similar to Dex alone (Figure 3.2). This result suggests that the synergistic responses reported with co-stimulation of Dex + GnRH are not dependent on the level of GR nuclear translocation. However, it is possible that Dex + GnRH induces a greater level of GR nuclear translocation compared to Dex alone at different time-points not investigated in this study.
Figure 3.2: GnRH do not increase the Dex-mediated nuclear import of the GR in LβT2 cells
The results presented in Figure 3.1.2 were analyzed with Carl Zeiss ZEN software (Version 2009) to
determine the intensity of the GR in the nucleus. The intensity of the GR signal was normalized to
the intensity of the Hoechst signal and were calculated relative to vehicle-treated control (Ctrl)
samples. The graph shows the combined results of three independent experiments and the nuclear
import of the GR was analyzed for at least 20 different cells.

3.3.3 ERK-1/2 localizes to the membrane raft fraction independent of ligands and
this localization is not required for its activity in LβT2 cells
Previous work by the present author for an MSc project showed that the GR and GnRHR are both
located in the membrane raft fraction after biochemical density-gradient fractionation of whole cell
lysates. Both the GR and GnRHR were present in the membrane raft fraction and this localization
appeared to not be influenced by ligand treatment. Co-immunoprecipitation assays were also
performed and the results showed that the GR interacts directly or indirectly with Flot-1 in a complex
that is independent of ligands (Wehmeyer 2010). Previous findings by others in the Hapgood
laboratory showed that the activation of ERK-1/2, p38 and PKC is required for the GnRH-induced
transactivation of a GRE reporter construct in LβT2 cells (Kotitschke et al., 2009). The study also
reported ERK-1/2 and PKC to be involved in the GnRH-mediated Ser-234 phosphorylation of the
mouse GR (Kotitschke et al., 2009). Furthermore, previous studies by others reported that the
transactivation of GR target genes requires phosphorylation of the human GR at Ser-203, Ser-211
and Ser-226 via the MAPK proteins ERK-1/2, p38 and JNK, respectively (Blind and Garabedian
2008, Kino et al., 2007, Takabe et al., 2008). It has previously been reported that the Akt signalling
pathway is involved in decreasing the stability of the GR through mediating its phosphorylation at
Ser-404 in human osteosarcoma cells (Galliher-Beckley et al., 2008). Interestingly, Matthews et al.
reported that GCs induced the activation of Akt via phosphorylation in A549 cells in a membrane
raft-dependent manner (Matthews et al., 2008). These findings suggest that the GR and Akt are
involved in a GC-mediated feedback mechanism that requires the presence of membrane rafts.
Besides the membrane raft-dependent GC-mediated modulation of the Akt signalling pathway, Akt
has also been reported to localize to membrane rafts in human mantle lymphoma cells (Reis-Sobreiro
et al., 2013). In addition, the membrane raft localization of ERK-1/2 has previously been reported in
αT3-1 gonadotrope cells (Bliss et al., 2007, Navratil et al., 2003). These findings, taken together
with the results shown in Figure 3.1.1 and 3.1.2, indicate that the GR, GnRHR, and MAPKs and/or
Akt are localized to similar sub-cellular compartments within gonadotrope cells. This could form a
signalling platform in membrane rafts where specific kinases could modulate the activity of the GR through phosphorylation.

To investigate the above, whether MAPK proteins and Akt localize to membrane rafts was investigated in LβT2 cells. Cells were stimulated for 30 min with 100 nM Dex, 100 nM GnRH or a combination of both followed by incubation with 0.05% Triton X-100 before fractionation on a discontinuous sucrose density-gradient. The membrane raft fractions were collected and analyzed by Western blotting with specific antibodies for ERK-1/2, JNK, p38, Akt and Flot-1. The results from Figure 3.3.1A show that ERK-1/2 localized to Flot-1-containing membrane rafts and this localization appears to be independent of 30 min treatment with Dex, GnRH or both together. Furthermore, the presence of p38, JNK and Akt in the membrane raft fraction was not detected in the presence or absence of ligands in LβT2 cells (Figure 3.3.1 B). It was not possible in the present study to investigate whether the PKC protein localize to the Flot-1 containing membrane rafts in the mouse LβT2 gonadotrope cells since the PKC protein has numerous isoforms and to the present author’s knowledge there is not a commercial mouse-specific antibody available that will detect all isoforms. These results show that ERK-1/2, and not p38, JNK or Akt, localizes to membrane rafts in a ligand-independent manner.
Figure 3.3.1: Fractionation shows that ERK-1/2 localizes to Flot-1-containing membrane rafts ligand independently in LβT2 cells

LβT2 cells were serum-starved overnight and incubated for 30 min in serum-free medium with 100 nM Dex, 100 nM GnRH, or a combination of both. Membrane rafts were isolated by ultracentrifugation employing the detergent-resistant flotation strategy in a discontinuous sucrose density gradient.

A. Fifteen-µl of the membrane raft fraction samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane, and probed successively with antibodies against ERK-1/2 and Flot-1. The result shown in the left panel is a single Western blot that is representative of three independent experiments. The results were quantified with AlphaEaseFC software and the membrane raft ERK-1/2 protein levels were normalized to membrane raft Flot-1 protein levels for each experiment and the pooled results are expressed relative to vehicle-treated control (Ctrl). The graph on the right shows the combined results of three independent experiments for the combination of ERK-1 and ERK-2. B. As in A, except that the membrane was successively probed with antibodies against Akt and Flot-1.

The finding that ERK-1/2 localizes to membrane rafts in a ligand-independent manner is consistent with previous studies that showed a similar localization of ERK-1/2 and also reported that the integrity of membrane rafts were required for GnRH-induced activation of ERK-1/2 in αT3-1 cells (Navratil et al., 2003; Bliss et al., 2007). Therefore, it was investigated if membrane rafts plays a similar functional role in the activation of ERK-1/2 in LβT2 cells. The expression of the membrane raft structural protein Flot-1 was decreased by siRNA-mediated knockdown, followed by stimulation with a combination of 100 nM Dex and 100 nM GnRH for 30 min and the activation of ERK-1/2 and p38 were analyzed by Western blotting with phospho-specific ERK-1/2 and p38 antibodies. The results of Figure 3.3.2A show that Flot-1 siRNA decreased Flot-1 protein expression levels by ~45% and the short exposure to Dex + GnRH did not affect the Flot-1 protein levels in the presence or absence of Flot-1 siRNA. Treatment with Dex + GnRH significantly increased the phosphorylation status of ERK-1/2, whereas decreased Flot-1 protein levels appeared to have no detectable effect on the phosphorylation status of ERK-1/2 in the absence or presence of Dex + GnRH (Figure 3.3.2 B). Similar to the basal and Dex + GnRH-induced phosphorylation of ERK-1/2 with decreased Flot-1 protein levels, the result of Figure 3.3.2C show that decreasing the expression levels of Flot-1 also appeared to have no effect on the activation of p38 induced with stimulation of Dex + GnRH in LβT2 cells. However, the activation of ERK-1/2 and p38 may have been affected if a greater level of
Flot-1 protein knockdown was obtained. Although different methods and a different type of siRNA for Flot-1 protein knockdown were investigated, these did not result in a greater level of knockdown efficiency.

Figure 3.3.2: The Dex + GnRH-induced activation of ERK-1/2 and p38 does not appear to require the presence of Flot-1

A and B. 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 72h. The cells were incubated for 30 min in serum-free medium with a combination of 100 nM Dex and 100 nM GnRH, followed by harvesting the proteins. Equal volumes of samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with a specific antibody against phospho-ERK-1/2. The membrane was stripped and probed with specific antibodies against ERK-1/2, Flot-1 and GAPDH. Panel A shows the combined percentage decrease of Flot-1 protein levels of panel B and C for the
control and Dex + GnRH samples in the presence of Flot-1 siRNA. The panel on the left shows a single representative Western blot and the graph shows the combined results of three independent experiments for the combination of ERK-1 and ERK-2 where NSC vehicle-treated control (Ctrl) was set to 1. C. As in B, except that the membrane was probed with a specific antibody against phospho-p38, followed by stripping and probing with specific antibodies against p38, Flot-1 and GAPDH. p-values for comparing a sample against the NSC Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.4 Flot-1 appears to not be required for site-specific GR phosphorylation

It has previously been shown that intact membrane rafts are required for Dex-induced GR phosphorylation at Ser-211 in A549 cells (Matthews et al., 2008). Additionally, Kotitschke et al. previously showed that Dex induces phosphorylation of the GR at Ser-220 and Ser-234 of the mouse GR, whereas GnRH induces Ser-234 and not Ser-220 phosphorylation of the mouse GR in LβT2 cells (Kotitschke et al., 2009). Therefore, to investigate if Flot-1 is required for the site-specific phosphorylation of the GR in LβT2 cells, Flot-1 protein levels were decreased with siRNA-mediated knockdown followed by treatment with 100 nM Dex, 100 nM GnRH or both together for 30 min. The results from Figure 3.4A show that Flot-1 protein levels were decreased by ~60% with this strategy when NSC control is compared with Flot-1 siRNA control (Figure 3.4A). The stimulation conditions had no effect on the reduction of Flot-1 protein levels obtained with the siRNA-mediated knockdown strategy. The results from Figure 3.4B and C show that neither Dex, GnRH nor the combination of both induced a significant increase in phosphorylation of the GR at Ser-212 in the NSC or Flot-1 siRNA conditions. However, a trend was observed where all the stimulation conditions appeared to slightly increase the Ser-212 phosphorylation in the NSC samples and this small increase appeared to be lost in the Flot-1 siRNA condition, which suggests that Ser-212 phosphorylation of the GR may require membrane raft association (Figure 3.4B and C). In the NSC condition, treatment with Dex resulted in a significant increase in GR phosphorylation at Ser-220, while GnRH did not increase phosphorylation and the combination of Dex + GnRH resulted in a similar level of phosphorylation at Ser-220 as Dex in isolation (Figure 3.4D and E). The decrease in Flot-1 protein levels had no detectable significant effect on Ser-220 phosphorylation of the GR when compared to the NSC condition in the absence or presence of ligands (Figure 3.4D and E). In the NSC condition, treatment with Dex resulted in a significant increase in Ser-234 phosphorylation of the GR (Figure 3.4F and G). Interestingly, treatment with Dex + GnRH appeared to slightly increase Ser-234 phosphorylation of the GR compared to Dex in isolation (Figure 3.4F and G). However,
statistical significance between Dex and Dex + GnRH could not be established from the combination of four independent repeat experiments (Figure 3.4 F and G). Treatment with GnRH resulted in a similar level of Ser-234 phosphorylation of the GR as Dex alone (Figure 3.4 F and G). Similarly to Ser-220, decreasing the Flot-1 protein levels had no detectable significant effect on Ser-234 phosphorylation of the GR in the absence or presence of ligands (Figure 3.4 F and G). Taken together, these results indicate that decreasing the Flot-1 protein levels by ~60% does not have a detectable significant effect on Ser-212, Ser-220 or Ser-234 phosphorylation of the GR in the absence or presence of ligands, which suggests that membrane raft association is not required for phosphorylation of the GR. However, there could have been a significant affect observed on GR phosphorylation if a greater level of Flot-1 protein knockdown has been achieved.
3.4 Flot-1 appears not to be required for site-specific GR phosphorylation

LβT2 cells were transfected with NSC or specific mouse Flot-1 siRNA at a final concentration of 40 nM and incubated for 72h. The cells were incubated for 30 min in serum-free medium containing 100 nM Dex or 100 nM GnRH or a combination of both, and the proteins were harvested. Equal volumes of the samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane, and probed with anti-phospho-Ser-212 (P-S212) GR-, anti-phospho-Ser-220 (P-S220) GR-, and anti-phospho-Ser-234 (P-S234) GR-specific antibodies. The membranes were stripped and probed with specific antibodies to GR, Flot-1 and GAPDH. Panel A shows the average percentage decrease of Flot-1 protein levels for the control samples in the presence of Flot-1 siRNA. The panels on the left (B, D, and F) show a single representative Western blot and the graphs (C, E, and G) show the combined results of four independent experiments where vehicle-treated control (Ctrl) was set to 1. One-way ANOVA with Dunnett’s post-test was used for statistical analysis and is denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.5 Co-treatment with Dex + GnRH synergistically and selectively enhances SGK-1 mRNA levels

It has previously been shown that crosstalk between the GR and GnRHR signalling pathways modulates the expression of an endogenous AP-1-containing gene and an exogenous GRE reporter gene in a synergistic manner (Kotitschke et al., 2009). To investigate whether Dex + GnRH treatment results in synergism on endogenous genes containing GRE response elements in their promoters, LβT2 cells were incubated for 8h with 100 nM Dex, 100 nM GnRH and both together, followed by RNA extraction and cDNA synthesis. The results of Figure 3.5A-E show that Dex significantly increased mRNA expression levels of the GILZ, MAPK phosphatase 1 (MKP-1), FKBP5, FSHβ and SGK-1 genes (Figure 3.5 A-E), which is consistent with previous reports in the
literature (Arteaga et al., 2007, Bruscoli et al., 2006, D'Agostino et al., 1990, Kassel et al., 2001, Vermeer et al., 2003). Treatment with GnRH in isolation had no effect on the mRNA expression levels of the GILZ, MKP-1 and FKBP5 genes, but significantly increased expression of the FSHβ and SGK-1 genes (Figure 3.5 A-E). Although Dex + GnRH stimulation did not result in a significant change in mRNA expression for the GILZ, MKP-1, FKBP5 and FSHβ genes compared with Dex alone, a statistically significant synergistic response was observed for the SGK-1 gene (Figure 3.5 E). Even though Dex and GnRH in isolation increased SGK-1 mRNA levels by ~8- and ~3.5-fold, respectively, treatment with Dex + GnRH together increased SGK-1 mRNA levels by ~17-fold, which is not merely additive but synergistic (Figure 3.5 E). Taken together these results suggest that crosstalk between the GR and GnRHR signalling pathways synergistically enhances expression of selective GRE-containing genes.
Figure 3.5: Co-treatment of cells with Dex + GnRH synergistically increases SGK-1 mRNA levels

LβT2 cells were incubated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative mRNA levels of several endogenous GRE-containing genes, including GILZ (A), MKP-1 (B), FKBP5 (C), FSHβ (D) and SGK-1 (E) were determined by quantitative Real-Time PCR. Fold changes in the gene of interest mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graphs shows the combined results of three independent
experiments. One-way ANOVA with Dunnett’s (stars above bars) and Tukey (stars above lines joining bars) post-tests were used for statistical analysis and denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.6 Flot-1, GR, and the GnRHR are required for the synergistic increase of SGK-1 mRNA levels

To investigate whether Flot-1 is required for the observed synergistic increase in SGK-1 mRNA levels in response to Dex + GnRH, Flot-1 protein levels were decreased with siRNA-mediated knockdown and the cells stimulated for 8h with 100 nM Dex, 100 nM GnRH or both together. The results in Figure 3.6A show that decreased Flot-1 protein levels did not affect the Dex- or GnRH-induced increase of SGK-1 mRNA expression. The Flot-1 protein levels were decreased by ~60% using this strategy (Figure 3.6 B). In the absence of Flot-1 siRNA it was found that the GnRH-induced increase in SGK-1 mRNA was not statistically significant (Figure 3.6 A). Interestingly, reducing the Flot-1 protein levels by ~60% decreased the Dex + GnRH-induced synergistic response by ~50%, which was similar to the level induced by Dex alone (Figure 3.6 A). These results suggest that the association of the GR with Flot-1-containing membrane rafts is not required for the Dex-mediated regulation of SGK-1 mRNA levels, but is required for the synergistic increase of SGK-1 mRNA levels mediated by co-treatment with Dex + GnRH. To obtain evidence that this synergistic response observed for the SGK-1 gene requires the GR, the GR protein levels were decreased by ~55% with siRNA-mediated knockdown (Figure 3.6 C). The results from Figure 3.6D show that the Dex-induced increase in SGK-1 mRNA levels requires the GR, as GR knockdown completely ablated the response. Similarly, the results suggest that the Dex + GnRH-induced response also requires the GR, although in this experiment the synergism was not as pronounced (Figure 3.6 D). More support that the GR is required for this synergistic response was obtained using the GR antagonist RU486, which attenuated the increase in mRNA levels observed when cells were treated with Dex alone and the synergistic response with Dex + GnRH (Figure 3.6 E). To obtain evidence that the synergism requires the activity of the GnRHR, cells were incubated with ligands in the presence or absence of the GnRHR antagonist Antide. The results from Figure 3.6E show that the GnRHR antagonist had no effect on the Dex-induced increase in SGK-1 mRNA levels. The GnRHR antagonist attenuated the small GnRH-induced increase in SGK-1 mRNA levels and it dramatically decreased the synergistic response induced by co-stimulation with Dex + GnRH (Figure 3.6 E). Taken together, these results indicate that the synergistic increase of SGK-1 mRNA levels requires membrane-associated Flot-1, GR and GnRHR.
Figure 3.6: Flot-1, GR and the GnRHR are required for the synergistic increase of SGK-1 mRNA levels.
A. LβT2 cells were transfected with NSC or specific mouse Flot-1 siRNA at a final concentration of 40 nM and incubated for 72h. The cells were incubated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH or a combination of both. Total RNA was isolated, reverse-transcribed and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. The panels (B and C) show Western blots that are representative images showing the extent of Flot-1 and GR protein knockdown, respectively. The histograms in B and C show the quantitative analysis of the percentage decrease in Flot-1 and GR protein levels, respectively. D. As in A, except that the cells were transfected with 20 nM GR siRNA for 96h. E. LβT2 cells were incubated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH or a combination of both in the presence and absence of 100 nM Antide. Total RNA was isolated, reverse-transcribed and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. One-way ANOVA with Dunnett’s (stars above bars) and Tukey (stars above lines joining bars) post-tests were used for statistical analysis and denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.7 Synergism involves differential recruitment of SRC co-factors to the SGK-1 promoter compared with Dex and GnRH alone, whereas GR recruitment remains unchanged

Since the previous results showed no detectable difference in GR nuclear localization between Dex and Dex + GnRH treatment, it was investigated whether increased GR recruitment to the GRE present in the promoter of the SGK-1 gene could explain the augmented mRNA expression response. Therefore, cells were incubated with 100 nM Dex, 100 nM GnRH or both together for 1h followed by a ChIP assay with a specific anti-GR antibody. The results presented in Figure 3.7A show that Dex treatment resulted in a significant ~2.5-fold recruitment of GR to the SGK-1 promoter (Figure 3.7 A), which is consistent with the GR knockdown results from Figure 3.6D. Interestingly, GnRH treatment resulted in a significant ~1.5-fold recruitment of the GR to the SGK-1 promoter compared with control treated cells (Figure 3.7 A). However, treatment with Dex + GnRH resulted in a similar amount of GR recruitment to the SGK-1 promoter as compared with Dex in isolation (Figure 3.7 A).
These results suggest that the synergistic mRNA expression induced by Dex + GnRH on the SGK-1 gene is not due to increased promoter occupancy by the GR.

Since an additive or synergistic level of GR recruitment to the SGK-1 gene was not detected in response to Dex + GnRH co-stimulation and a study performed by Kotitschke et al. reported SRC-1 to be involved in the synergism of the GnRHR gene, it was investigated whether well-known GR co-factors are differentially recruited to the SGK-1 promoter. Dex, GnRH and Dex + GnRH treated cells were immunoprecipitated with specific antibodies to SRC-1, GRIP-1, SRC-3, p300 and CBP. As shown in Figure 3.7B, treatment with Dex and GnRH resulted in a significant ~2- and ~2.25-fold recruitment of SRC-1 to the SGK-1 promoter, respectively. Interestingly, co-stimulation with Dex and GnRH resulted in significantly less recruitment of SRC-1 to the SGK-1 promoter compared to treatment with the ligands in isolation (Figure 3.7 B). Recruitment of GRIP-1 to the promoter was only detected when cells were stimulated with Dex (Figure 3.7 C), whereas SRC-3, p300 and CBP recruitment to the SGK-1 promoter were not differentially recruited in response to Dex, GnRH or Dex + GnRH treatment compared to control-treated cells (Figure 3.7 D-F). It is possible that SRC-1 and GRIP-1 move away from the promoter and allows access for another cofactor that is still to be determined or it may also that GRIP-1 and SRC-1 are recruited, but at a different time-point.

Importantly, a control non-specific antibody showed that all the primary antibodies produced specific results (data not shown). In summary, the results indicate that increased SGK-1 mRNA levels induced by Dex, GnRH and a combination of both are mediated by differential recruitment of co-factors, suggesting that synergistic effects initiated in membrane rafts exert downstream differences in nuclear promoter occupancy compared to the classical response of Dex in isolation.
Figure 3.7: Synergism involves differential recruitment of SRC co-factors compared with Dex and GnRH alone without an increase in GR recruitment to the SGK-1 promoter

ChIP assays were performed in LβT2 cells that were treated for 1h with 100 nM Dex, 100 nM GnRH or both together using anti-GR (A), anti-SRC-1 (B), anti-GRIP-1 (C), anti-SRC-3 (D), anti-CBP (E) and anti-p300 (F) antibodies. Precipitated complexes that were bound to the SGK-1 promoter were detected with quantitative Real-Time PCR using primers that span the GRE region. Results were normalized against the input samples and are represented relative to vehicle-treated control (Ctrl) samples. The graphs show the combined results of three independent experiments. One-way ANOVA with Dunnett’s post-test was used for statistical analysis and is denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.8 GnRH acting via the PKC but not the PKA pathway augments the Dex-mediated increase in SGK-1 mRNA levels

It has previously been shown that GnRHR activates both the PKA and PKC signalling pathways in LβT2 cells (Grafer et al., 2009) and the PKC pathway has also been implicated in mediating Dex + GnRH synergy (Kotitschke et al., 2009). Therefore, the possible involvement of the PKA and PKC signalling pathways in the synergistic increase of SGK-1 mRNA levels induced by co-stimulation with Dex + GnRH was investigated. An inducer of the PKA signalling pathway, 8-Bromo-cAMP was used to stimulate the cells in the presence and absence of Dex. The result presented in Figure 3.8A show that 8-Bromo-cAMP had no effect on the mRNA expression levels of the SGK-1 gene. In
addition, treatment with Dex + 8-Bromo-cAMP did not result in a synergistic response as seen for co-stimulation with Dex + GnRH (Figure 3.8 A). This result indicates that the PKA signalling pathway is not involved in regulating the mRNA expression of the SGK-1 gene in LβT2 cells. To investigate a potential role for the PKC signalling pathway, the cells were incubated with PMA, which is a diacylglycerol analogue known to activate PKC. The result showed that PMA resulted in a significant ~4-fold induction of SGK-1 mRNA levels (Figure 3.8 B). Interestingly, co-stimulation with Dex + PMA resulted in a response that had a similar magnitude as co-stimulation with Dex + GnRH (Figure 3.8 B). This similar level of gene expression strongly suggests that the PKC pathway is involved in mediating the synergistic upregulation of SGK-1 mRNA levels in response to Dex + GnRH. To obtain more evidence that the PKC signalling pathway is involved in, cells were stimulated in the presence and absence of the PKC inhibitor BIM. The result in Figure 3.8C show that, as expected, the PMA-induced mRNA expression of the SGK-1 gene was strongly decreased with BIM. Interestingly, the PKC inhibitor also decreased the synergistic Dex + GnRH-induced response of the SGK-1 gene (Figure 3.8 C). Taken together, the results suggests that the PKC signalling pathway is involved in mediating the synergism induced by treatment with Dex + GnRH of the SGK-1 gene in LβT2 cells.
Figure 3.8: GnRH acting via the PKC but not the PKA pathway synergistically increases the Dex-induced upregulation of SGK-1 mRNA levels

A. LβT2 cells were incubated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, a combination of Dex + GnRH, 2 mM 8-Bromo-cAMP or a combination of Dex + 8-Bromo-cAMP. Total RNA was isolated, reverse-transcribed and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. B. As in A, except that 8-Bromo-cAMP was replaced with 20 ng/mL PMA. C. As in B, except the cells were stimulated in the presence or absence of 100 nM PKC inhibitor. One-way ANOVA with Dunnett’s post-test and two-tailed paired t-tests (stars above lines joining bars) were used for statistical analysis and is denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.9 Protein expression levels of the GR and GnRHR are not enhanced by co-stimulation with Dex + GnRH

Increased GR or GnRHR protein levels could explain the Dex + GnRH-mediated synergistic response observed for the SGK-1 gene. However, it was previously shown that treatment with Dex + GnRH for 8h did not increase GR protein levels (Kotitschke 2009). To investigate whether treatment with Dex + GnRH results in upregulation of GnRHR protein levels, two different GnRHR antibodies (sc-8681 and sc-13944, Santa Cruz, USA) were obtained. However, both proved to be unsuccessful in Western blot experiments after several attempts. Therefore, a non-commercial antibody obtained from D.C. Skinner that was raised in a rabbit against the ovine GnRHR that was used to perform the co-localization experiments of the GnRHR with Flot-1 shown in Figure 3.1.1, was explored for use in Western blotting. However, using this antibody in Western blot experiments with whole cell lysates was also unsuccessful. Therefore, a different and indirect strategy to investigate the expression of the GnRHR protein in LβT2 cells was utilized. Briefly, this strategy investigated the mRNA expression level of the αGSU gene, which is only upregulated by treatment with GnRH and not with Dex, under the present experimental conditions in LβT2 cells. Furthermore, increasing GnRHR protein levels has previously been reported to increase the expression of a human αGSU reporter gene after treatment with GnRH for 8h in HeLa cells (Armstrong et al., 2010). Therefore, if 8h stimulation with Dex + GnRH increases GnRHR protein levels the αGSU mRNA levels would most likely be greater with Dex + GnRH co-treatment compared to GnRH treatment in isolation. Thus, cells were treated with 100 nM Dex, 100 nM GnRH and both together for 8h, followed by
RNA extraction, cDNA synthesis and Real-Time qPCR. The result presented in Figure 3.9 show that treatment with Dex had no effect on expression of the αGSU gene. Stimulation with GnRH increased αGSU mRNA levels by ~2.5-fold, whereas co-treatment with Dex + GnRH increased αGSU mRNA levels by ~2-fold (Figure 3.9). The result suggests that 8h treatment with Dex + GnRH does not increase the expression of GnRHR protein levels in LβT2 cells.

![αGSU gene expression](image)

Figure 3.9: GnRHR protein levels do not appear to be increased with co-treatment of Dex + GnRH

LβT2 cells were incubated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated, reverse-transcribed and relative levels of αGSU transcripts were determined by quantitative Real-Time PCR. Fold changes in αGSU mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. One-way ANOVA with Dunnett’s post-test was used for statistical analysis and is denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

3.3.10 SGK-1 mRNA levels are not regulated by the PR in LβT2 cells

It has previously been shown that the PR protein is expressed in LβT2 cells and that GnRH increases its activity to induce the expression of a PRE reporter gene (An et al., 2009). Since the promoter of the SGK-1 gene contains a GRE cis-element it could be possible that the PR is involved in the GnRH- or Dex + GnRH-induced upregulation of SGK-1 mRNA levels. Therefore, to investigate whether PR protein is detectable in this clone of the LβT2 cell line, Western blot experiments were performed with unstimulated LβT2 whole cell lysates using a specific anti-PR antibody. To confirm the specificity of the antibody a positive control was prepared by transfecting a PR cDNA expression vector into COS-1 cells. The result from Figure 3.10A shows that the PR protein was strongly...
detectable in the positive control sample, but could not be detected in LβT2 cells. To investigate if PR protein is expressed but at too low a level to be detected with Western blot analysis, cells were incubated with 100 nM Dex, 100 nM GnRH or both together in the presence and absence of 1 µM of the PR agonists P4 or R5020. The result from Figure 3.10B shows that treatment with P4 and R5020 did not increase SGK-1 mRNA levels. Furthermore, the PR agonists had no effect on the Dex- or GnRH-induced increase of SGK-1 mRNA levels (Figure 3.10 B). In addition, the synergistic increase of SGK-1 mRNA expression was unaffected by co-treatment with either P4 or R5020 (Figure 3.10 B). Taken together, these results suggest that active PR protein is not expressed in the LβT2 cells used in these experiments.

Figure 3.10: The PR does not appear to regulate SGK-1 mRNA levels in response to progesterone receptor agonists, or GnRH in LβT2 cells

A. Whole cell lysates from LβT2 and COS-1 cells exogenously expressing the PR (+ ve Ctrl) were loaded on an 8% SDS-PAGE gel followed by transfer onto nitrocellulose membrane and probing with a specific antibody against PR. B. LβT2 cells were incubated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH or a combination of both in the presence and absence of 1 µM progesterone (P4) or 1 µM R5020. Total RNA was isolated, reverse-transcribed and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments.
3.3.11 Co-stimulation with Dex + GnRH synergistically decreases proliferation of LβT2 cells

Membrane raft-associated GR has previously been implicated in playing a role in cell proliferation of mouse neural progenitor cells (Samarasinghe et al., 2011) and GnRH has previously been shown to decrease proliferation of LβT2 cells (Feng et al., 2008). In addition, treatment with Dex has previously been shown to induce cell growth arrest that required membrane rafts in A549 cells (Matthews et al., 2008). Therefore, it was investigated whether the membrane raft-mediated synergistic effect on SGK-1 mRNA by Dex + GnRH plays a role in LβT2 cell growth. Cells were stimulated with 100 nM Dex, 100 nM GnRH or both together for 3, 5 and 7 days after which an MTT cell viability assay was performed. An increased MTT activity could be due to increased cell proliferation, whereas a negative value would indicate a lower number of viable cells that could be a result of cell apoptosis or cell growth arrest. The results in Figure 3.11A show that stimulation with Dex slightly decreased the number of viable cells after 3 and 7 days of treatment and significantly after 5 days of treatment. Consistent with previous reports in the literature, GnRH treatment significantly reduced the number of metabolically active cells and to a significantly greater extent after 7 days compared to 3 days, which suggests that GnRH decreases cell viability in a time-dependent manner (Figure 3.11 A). Interestingly, co-stimulation with Dex + GnRH synergistically decreased the cell viability at all time-points investigated (Figure 3.11 A).

The decrease in metabolic activity of the cells could be due to a reduction in cell proliferation or apoptosis. Cells were treated for 24h and 48h with 100 nM Dex, 100 nM GnRH and both together,
followed by counting the cell number at each time-point. The result presented in Figure 3.11B shows that the cell number did consistently increase over time for each treatment condition. Surprisingly, stimulation with Dex did not reduce the cell number after 24h or 48h treatment compared to control (Figure 3.11 B). Stimulation with GnRH resulted in a significant decrease in cell density compared to control treated cells (Figure 3.11 B). Interestingly, co-stimulation with Dex + GnRH significantly decreased the cell number in a synergistic manner (Figure 3.11 B). These results indicate that stimulation with GnRH and Dex + GnRH decreases cell proliferation instead of inducing apoptosis.

To obtain further evidence that stimulation with GnRH and Dex + GnRH affects cell proliferation, cells were stimulated for 48h with 100 nM Dex, 100 nM GnRH or both together, followed by a cell proliferation ELISA assay. The result in Figure 3.11C show that treatment with Dex had no significant effect on cell proliferation, whereas stimulation with GnRH decreased cell proliferation by ~25%. Interestingly, co-stimulation with Dex + GnRH synergistically attenuated cell proliferation by ~45% (Figure 3.11 C).
3.3.12 SGK-1, Flot-1 and PKC are required for the synergistic Dex + GnRH decrease in LβT2 cell proliferation

As the previous results showed the PKC signalling pathway is involved in mediating the synergistic upregulation of SGK-1 mRNA levels in response to co-stimulation with Dex + GnRH it was
investigated if this pathway is involved in the synergistic attenuation of cell proliferation. Therefore, LβT2 cells were treated for 48h with 100 nM Dex, 100 nM GnRH or both together in the presence and absence of the PKC inhibitor, BIM. The result presented in Figure 3.12A show that Dex did not affect cell proliferation, while GnRH significantly decreased proliferation to a small extent and the combination of Dex + GnRH synergistically attenuated cellular proliferation. Although there was no effect detected with BIM in the absence of ligands, Dex or GnRH stimulation, inhibiting PKC did significantly attenuate the repressive effect induced by co-stimulation with Dex + GnRH (Figure 3.12 A). This result indicates that the PKC signalling pathway is involved in mediating the synergistic repressive response of cell proliferation induced by Dex + GnRH.

The previous results have shown that Flot-1 and PKC are required for the synergistic upregulation of SGK-1 mRNA expression induced by co-treatment with Dex + GnRH and that PKC is involved in the repressive effect of cell proliferation, therefore it was investigated whether Flot-1 and SGK-1 played a role in proliferation of LβT2 cells. Flot-1 and SGK-1 expression was decreased by siRNA-mediated knockdown and the cells were replated and stimulated for 48h with 100 nM Dex, 100 nM GnRH or a combination of both. To determine that the SGK-1 protein expression was decreased, two different antibodies were used, which both unfortunately resulted in many non-specific bands that made interpretation difficult. Therefore, SGK-1 mRNA levels were determined with quantitative Real-Time PCR to establish that gene expression was decreased with siRNA-mediated knockdown and the results from Figure 3.12B show that SGK-1 mRNA was decreased by ~60%. The results obtained with the cell proliferation assay show that in the absence of knockdown, GnRH decreased proliferation by ~20%, whereas treatment with Dex + GnRH together induced a synergistic attenuation in proliferation (Figure 3.12 C), which is consistent with results shown in Figure 3.11C and 3.12A. Decreasing Flot-1 protein levels by ~60% did not significantly change the effects of Dex or GnRH treatment alone on proliferation of LβT2 cells (Figure 3.12 C). However, decreased Flot-1 protein levels significantly attenuated the repressive effect of Dex + GnRH to a small extent (Figure 3.12 C). Decreased SGK-1 mRNA expression levels did not appear to change the effects of Dex or GnRH treatment in isolation on cell proliferation (Figure 3.12 C). Interestingly, decreasing SGK-1 mRNA levels completely lifted the synergistic repression of cell proliferation in response to co-stimulation with Dex + GnRH (Figure 3.12 C). Taken together the results suggest that synergistic upregulation of SGK-1 mRNA levels by Dex + GnRH as well as the presence of Flot-1 is required for a synergistic repression of LβT2 cell proliferation.
Figure 3.12: Dex + GnRH synergistically decreases cell proliferation via the PKC pathway and by upregulating SGK-1

A. LβT2 cells were stimulated in the presence and absence of 100 nM PKC inhibitor for 48h with 100 nM Dex, 100 nM GnRH, or a combination of both in charcoal-stripped full medium. During the last hour of stimulation, the thymidine analogue, BrdU, was added to the cells, and newly synthesized DNA was quantified with a colorimetric immunoassay. The graph shows the combined results of three independent experiments.

B. Flot-1 and SGK-1 expression levels were decreased with 40 nM siRNA-mediated knockdown for 72h, after which the cells were harvested for RNA isolation. Total RNA was isolated, reverse-transcribed and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments.

C. The cells from B were reseeded and stimulated for 48h with 100 nM Dex, 100 nM GnRH, or a combination of both in medium containing charcoal-stripped serum. During the last hour of stimulation, the thymidine analogue, BrdU, was added to the cells, and newly synthesized DNA was quantified with a colorimetric immunoassay. The graph shows the combined results of three independent experiments. One-way ANOVA with Dunnett’s (B) or both Dunnett’s and Tukey (A and C) 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
RESULTS

GR REGULATES THE mRNA EXPRESSION OF GRE-CONTAINING AND KEY REPRODUCTIVE GENES IN THE PITUITARY AND PRIMARY GONADOTROPE CELLS

4.1 BACKGROUND

Stress has been reported to strongly influence reproductive function. The effects of stress on reproduction appear to be variable, with positive and negative findings published in the current literature. The physiological response to stress is activation of the HPA axis, which increases the level of circulating GCs released from the adrenal cortex (Tsigos and Chrousos 2002). The expression of GR protein has previously been detected in mouse and rat primary gonadotrope cells (Breen et al., 2012, Kononen et al., 1993). This finding indicates that the effects of stress, which induces the release of GCs, could influence reproductive function directly in the gonadotrope through activation of the GR to regulate expression of the gonadotropins and the receptor of the central reproductive hormone GnRH (Breen and Mellon 2014, Sapolsky et al., 2000).

The decreased reproductive function effects of stress have been shown in mammals to be mediated by a decline in the levels of circulating gonadotropins (Ferin 1999, Rivier and Rivest 1991, Tilbrook et al., 2000, Tilbrook et al., 2002). GCs have been shown to reduce the frequency of LH pulses in sheep, rats and women (Breen et al., 2005, Li et al., 2004, Saketos et al., 1993), which was shown to be mediated by GC-induced suppression in the frequency of GnRH pulses in sheep (Oakley et al., 2009). GCs could also decrease reproductive function by regulating the expression of gonadotropin genes in the gonadotrope. A recent study by Breen et al. showed that the GnRH-induced LHβ mRNA expression and secretion of LH were acutely reduced by exogenous GCs in the pituitaries of WT mice. The authors also showed that mice subjected to restraint stress had diminished levels of circulating LH and pituitary LHβ mRNA expression in response to exogenous GnRH (Breen et al., 2012). Furthermore, the same study reported that the GC-mediated attenuation of the GnRH-induced upregulation of the LHβ gene expression was mediated via the GR binding to the early growth
response factor 1 (Egr-1) and disrupts the formation of the transcriptional complex (Breen et al., 2012).

Contrary to the negative effects of GCs on reproductive function by decreasing LHβ expression, GCs have been reported to increase expression of the FSHβ gene in mouse and rat pituitary cell cultures and in LβT2 cells (Bohnsack et al., 2000, Kilen et al., 1996, McAndrews et al., 1994, Ringstrom et al., 1991, Thackray et al., 2006). It has previously been shown that the GC-induced upregulation of FSHβ mRNA levels is mediated by the GR binding as a homodimer directly to a highly conserved hormone-response-element in the proximal promoter in LβT2 cells (Thackray et al., 2006).

The expression of the αGSU gene produces a common α-subunit that heterodimerizes with the specific β-subunits of the gonadotropins to form functional LH and FSH glycoprotein hormones in the pituitary gonadotrope cells. The central role that the gonadotropins have in reproductive function is exemplified by the two functionally active glycoprotein hormones that are composed as heterodimers requiring the expression of three genes, which provides multiple levels of regulation. Furthermore, the αGSU gene is also expressed in pituitary thyrotrope cells in all mammalian species, which forms the α-subunit for the TSH hormone as well (Gharib et al., 1990, Simmons et al., 1990). The expression of the αGSU gene in two of the pituitary’s hormone producing cell types indicate that there could be additional levels of regulation between pituitary gonadotrope and thyrotrope cells, such as paracrine effects mediating intercellular communication.

Previous studies have reported that GCs increased, decreased or had no effect on αGSU mRNA levels in rat pituitary cell cultures, these differences were dependent on the treatment times (Kilen et al., 1996, McAndrews et al., 1994, Ringstrom et al., 1991). Sasson et al. reported that 24h treatment with Dex and GnRH increased the activity of a human αGSU reporter gene, although GnRH induced a much greater response than Dex in LβT2 cells. However, co-stimulation with Dex + GnRH resulted in a synergistic transcriptional increase of the human αGSU reporter gene in LβT2 cells (Sasson et al., 2008). Furthermore, the authors reported that the Dex-, GnRH- and Dex + GnRH-induced activation of the reporter gene was greatly enhanced by the addition of exogenous GR protein, which suggests that the transcriptional activation of the αGSU reporter gene induced by the ligands is at least in part mediated via the GR in LβT2 cells.

Besides the above-mentioned effects of GCs on the expression of the gonadotropin genes, the most investigated and well characterized essential reproductive gene is the GnRHR gene. The
responsiveness of the pituitary to GnRH is dependent on the GnRHR protein numbers present on the cell surface of the gonadotropes which is regulated by several steroid hormones as well as GnRH itself (Kaiser et al., 1993, Norwitz et al., 1999, Wu et al., 1994, Yasin et al., 1995). GCs have been reported to regulate the expression of the GnRHR gene in the pituitary of various mammals, but the underlying mechanisms are not well established. GCs have been shown to attenuate the GnRH-induced mRNA expression of the GnRHR gene in rats and cortisol was also reported to decrease GnRHR protein levels without affecting the mRNA levels (Breen and Karsch 2004, Daley et al., 1999, Rosen et al., 1991, Tilbrook et al., 2000). The most well established effects of GCs on GnRHR gene expression were reported by Kotitschke et al., showing Dex increased the expression of the GnRHR gene via the AP-1 cis-element in LβT2 cells. The authors also showed that co-stimulation with Dex + GnRH induced a synergistically transcriptional response that involved the recruitment of SRC-1 and formed a transcriptional cofactor complex on the AP-1 site of the GnRHR gene in LβT2 cells (Kotitschke et al., 2009).

In summary, the responsiveness of the LHβ gene to GnRH is decreased by GCs through GR binding to Egr-1 on the promoter of the LHβ gene and disrupting the structure of the transcriptional complex or preventing transcription factors to occupy the promoter. GCs increases FSHβ mRNA levels via a classical GR homodimer transactivation mechanism. GCs increases the expression of the αGSU reporter and endogenous GnRHR genes that is enhanced in a synergistic manner by the addition of GnRH. These findings indicate that GCs strongly modulate female reproductive function by regulating the expression of the gonadotropin and GnRHR genes in the pituitary. However, the literature appears to contain some contradictory results, most likely due to differences in the experimental conditions or models, i.e. species, tissue and cell types, endogenous and reporter genes, variations in hormonal stimulation conditions. While some of the reports provide physiologically relevant evidence for the effects of stress on reproduction, like the LHβ gene in mouse pituitaries, more studies performed in primary pituitary tissue and gonadotrope cells are needed to establish the physiological relevance and mechanisms of the effects that GCs have on expression of the GnRHR, FSHβ and αGSU genes.

4.2 AIMS

The central aim of the following part of the present study was to investigate whether mammalian reproduction is influenced by stress. Firstly, the expression of well-established GRE-containing genes was investigated for determining responsiveness to Dex in the primary tissue and cell model
systems. Whether Dex, GnRH or both together regulate the mRNA levels of key reproductive genes in mouse pituitary tissue and primary mouse gonadotrope cells was investigated. The reciprocal modulation between the GR and GnRHR signalling pathways was also explored by investigating whether the ligands regulate mRNA levels of the GRα and GnRHR genes.

The detailed aims of this part of the study were the following:
- Investigate whether Dex, GnRH or both together regulate mRNA levels of model GRE-containing, GRα, GnRHR and other key reproductive genes in vitro in mouse pituitary tissue and primary mouse gonadotrope cells, in vitro, in a time- and estrous cycle-dependent manner;
- Investigate if Dex + GnRH co-stimulation in vitro induces synergistic regulation of mRNA levels of the selected genes in mouse pituitaries or primary mouse gonadotrope cells;
- Determine whether paracrine effects in the pituitary are involved in this regulation;
- Establish the physiological significance of the immortalized LβT2 gonadotrope cell line for the selected responses;
- Determine if the GR and PR are expressed in mouse pituitaries and primary mouse gonadotrope cells;
- Establish if the activity of the GR is required for the selected mRNA responses.

4.3 RESULTS

4.3.1 Regulation of mRNA expression by Dex and GnRH of GRE-containing genes in mouse pituitaries and primary mouse gonadotrope cells

4.3.1.1 SGK-1 mRNA expression is upregulated by Dex and GnRH in mouse pituitaries and primary mouse gonadotrope cells under some conditions

Pituitary glands were isolated from twenty twelve-week old mice. Vaginal smears were also collected to determine the stage of the estrous cycle. Each pituitary was divided into four equal portions and subjected to treatment with vehicle-control (Ctrl), 100 nM Dex, 100 nM GnRH or both together for the following times: 30 min, 1h, 2h, 4h and 8h. Following treatment, total RNA was extracted and cDNA was synthesized to perform qPCR. The result in Figure 4.1.1 show that treatment with Dex induced a ~3- and ~4-fold increase in mRNA expression of the SGK-1 gene at 4h
and 8h, respectively. Treatment with GnRH for 1h and 8h appeared to increase SGK-1 mRNA levels, although significance could not be established. Furthermore, co-treatment with Dex and GnRH induced a significant level of upregulation after 2h and 8h (Figure 4.1.1). Synergistic mRNA expression responses of the SGK-1 gene upon co-stimulation with Dex and GnRH were not observed in the mouse pituitary tissue samples (Figure 4.1.1). The mRNA expression responses induced with Dex + GnRH co-treatment were similar to the responses induced with Dex treatment alone (Figure 4.1.1). Although the SGK-1 mRNA expression with Dex + GnRH appeared greater than the Dex-induced response at 8h, statistical significance could not be established (Figure 4.1.1).

Figure 4.1.1: Dex and GnRH upregulate the expression of SGK-1 mRNA levels in mouse pituitaries

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

To investigate whether the estrous cycle plays a role in regulation of SGK-1 mRNA expression, the pooled results from Figure 4.1.1 were re-plotted according to the phase of the estrous cycle and shown in Figure 4.1.2A-D. The results in Figure 4.1.2A for the estrous phase show that treatment
with Dex for 2h and 8h significantly increased SGK-1 mRNA levels by ~3-fold. Co-stimulation with Dex + GnRH for 30 min appeared to slightly induce mRNA expression of the SGK-1 gene, although statistical significance could not be established. Interestingly, co-treatment with Dex + GnRH for 1h significantly upregulated SGK-1 mRNA levels by ~2-fold during the estrous phase of the cycle, while no effect observed for Dex or GnRH alone (Figure 4.1.2 A). Co-treatment with Dex and GnRH increased mRNA expression of the SGK-1 gene to a similar level as treatment with Dex alone at 2h and 8h (Figure 4.1.2 A). There appeared to be no induction of SGK-1 mRNA expression by GnRH at any of the time-points investigated. In the diestrous phase of the cycle the SGK-1 mRNA expression appeared to not be induced by stimulation of Dex, GnRH or both together at any time-points investigated, although after 8h stimulation there appeared to be some regulation induced by Dex with a high amount of error (Figure 4.1.2 B). The result presented in Figure 4.1.2C show the mRNA expression of the SGK-1 gene in the metestrus phase of the cycle. Treatment with Dex significantly induced SGK-1 mRNA levels by ~6- and ~8-fold at 4h and 8h, respectively, while GnRH appeared to have no effect at any of the time-points investigated (Figure 4.1.2 C). Co-treatment with Dex and GnRH increased mRNA expression of the SGK-1 gene to a similar level as treatment with Dex alone at 4h and 8h (Figure 4.1.2 C). Figure 4.1.2D show that treatment with Dex for 1h and 8h significantly increased SGK-1 mRNA levels by ~3- and ~4.5-fold, respectively, during the proestrous phase. Interestingly, 8h treatment with GnRH significantly increased mRNA expression of the SGK-1 gene by ~3-fold (Figure 4.1.2 D). Furthermore, co-treatment with Dex and GnRH for 1h and 8h induced expression of the SGK-1 to a similar level as treatment with Dex alone (Figure 4.1.2 D).
The estrous cycle stages of the twenty mice used in Figure 4.1.1 were determined by vaginal cytology and the results obtained for SGK-1 gene expression were re-plotted according to the four stages of the cycle, A: Estrous, B: Diestrous, C: Metestrous, D: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1). p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

The results presented in chapter 3 showed that Dex + GnRH co-stimulation for 8h induces a synergistic increase of SGK-1 mRNA levels in immortalized gonadotrope cells (Figure 3.5 E). However, no synergistic effects were observed with Dex + GnRH co-treatment in pituitary tissue experiments, although the 1h Dex + GnRH-induced response in the estrous stage may possibly be synergistic (Figure 4.1.2 A). The results presented in Figure 4.1.1 and 4.1.2 did not display conclusive synergistic responses in WT mouse pituitaries.

To investigate if SGK-1 mRNA expression is regulated by Dex, GnRH or both together in primary mouse gonadotrope cells, pituitaries from six twelve-week old GRIC/R26-YFP mice were homogenized and subjected to FACS. The purified gonadotrope cells were treated for 8h with 100
nM Dex, 100 nM GnRH or both together. Following treatment the RNA was extracted, mRNA was amplified and mRNA expression of the SGK-1 gene was investigated by RT-qPCR. The result in Figure 4.1.3 show that Dex treatment significantly increased mRNA expression of the SGK-1 gene by ~5-fold and GnRH induced the expression by ~3-fold. Interestingly, co-stimulation with Dex + GnRH appeared to decrease the Dex-induced upregulation of SGK-1 mRNA (Figure 4.1.3). The Dex- and GnRH-induced increase in SGK-1 mRNA levels is consistent with results previously obtained in the LβT2 cell line. However, co-stimulation with Dex + GnRH did not induce a synergistic upregulation of SGK-1 mRNA as previously shown in the cell line (Figure 3.5 E).

Taken together, the results from pituitary tissue and gonadotrope cells show that Dex and GnRH in isolation and in combination increases mRNA expression of the SGK-1 gene in a time- and estrous cycle-dependent manner. Interestingly, the SGK-1 mRNA expression was upregulated the most at the 8h time-point for most of the treatments and stages of the cycle. GnRH appears to regulate mRNA expression of the SGK-1 gene only in the proestrous stage, while for Dex the greatest fold-induction was observed during the metestrous stage. Furthermore, co-treatment with Dex + GnRH appeared to result in synergistic upregulation of SGK-1 mRNA levels only after 1h treatment in the estrous stage.

**Figure 4.1.3: GnRH attenuates the Dex-mediated increase of SGK-1 mRNA levels in primary mouse gonadotrope cells**

Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1
mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

4.3.1.2 Dex, but not GnRH increases GILZ mRNA expression in mouse pituitaries and primary mouse gonadotrope cells

To investigate whether Dex, GnRH or both together regulate the mRNA expression of the GILZ gene in pituitary tissue in a time-dependent manner, the RNA isolated as described for Figure 4.1.1 was analyzed for the expression of the GILZ gene by RT-qPCR. The results presented in Figure 4.2.1 show that GnRH treatment in isolation appeared to have no effect on the mRNA expression of the GILZ gene at any time-point investigated. Treatment with Dex for 2h, 4h and 8h significantly increased GILZ mRNA levels by ~3-, ~8- and ~10-fold, respectively (Figure 4.2.1). Co-stimulation with Dex + GnRH showed similar levels of GILZ mRNA expression as treatment with Dex alone (Figure 4.2.1). However, there appeared to be slight attenuation of the Dex-induced response by GnRH at 4h, although statistical significance could not be established (Figure 4.2.1). Interestingly, the mRNA expression of the GILZ gene with Dex and Dex + GnRH was greater at longer stimulation times (Figure 4.2.1).

Figure 4.2.1: Dex upregulates GILZ mRNA levels in a time-dependent manner in mouse pituitaries

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a
combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of GILZ transcripts were determined by quantitative Real-Time PCR. Fold changes in GILZ mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

To determine if the estrous cycle plays a role in the regulation of GILZ mRNA expression induced by Dex, GnRH or both together, the pooled results from Figure 4.2.1 were re-plotted according to the stage of the estrous cycle and shown in Figure 4.2.2A-D. The results presented in Figure 4.2.2 show that GnRH treatment did not appear to regulate the mRNA expression of the GILZ gene at any stage of the estrous cycle. In the estrous stage, treatment with Dex induced a ~9-fold increase in GILZ mRNA levels at both 4h and 8h (Figure 4.2.2 A). Co-stimulation with Dex + GnRH resulted in a ~6- and ~11-fold upregulation of GILZ mRNA, respectively (Figure 4.2.2 A). In the diestrous stage the mRNA expression of the GILZ gene by Dex + GnRH were generally similar to the Dex-induced expression, although it appeared that GnRH decreased the Dex-induced response at the 8h time-point, but statistical significance could not be established (Figure 4.2.2 B). Dex treatment for 4h and 8h increased GILZ mRNA levels by ~6- and ~21-fold, respectively (Figure 4.2.2 B). In the metestrous stage of the cycle, the GILZ gene was significantly upregulated after treatment with Dex for 2h and 8h by ~5- and ~12-fold, respectively (Figure 4.2.2 C). Although treatment with Dex for 4h appeared to increase expression of the GILZ gene, statistical significance could not be established. Co-treatment with Dex + GnRH induced a similar increase in GILZ mRNA as treatment with Dex in isolation for the 2h and 4h time-points, while 8h co-treatment appeared to result in a smaller increase in GILZ mRNA than treatment with Dex in isolation (Figure 4.2.2 C). In the proestrous stage, treatment with Dex and co-treatment with Dex + GnRH for 2h showed a similar level of upregulation of GILZ mRNA (Figure 4.2.2 D). Although statistical significance could not be established, treatment with Dex and co-treatment with Dex + GnRH for 4h and 8h appeared to increase GILZ mRNA expression (Figure 4.2.2 D). It is interesting to note that treatment with Dex for 8h in the diestrous stage resulted in the greatest GILZ mRNA expression.

The result presented in Chapter 3 showed that GILZ mRNA expression was significantly upregulated by treatment with Dex and co-treatment with Dex + GnRH in the immortalized gonadotrope cell line LβT2. In addition, GnRH alone had no effect and did not appear to modulate the effect of Dex on
GILZ mRNA levels (Figure 3.5A). To investigate if the GILZ gene regulation with Dex, GnRH and Dex + GnRH in primary mouse gonadotrope cells is similar to results obtained in the LβT2 cell line and pituitary tissue, the RNA isolated as described for Figure 4.1.3 was analyzed for the expression of the GILZ gene by RT-qPCR. The result presented in Figure 4.2.3 show that treatment of primary gonadotrope cells with Dex or GnRH alone for 8h appeared to slightly increase GILZ mRNA expression by ~2- and ~1.4-fold, respectively. Interestingly, unlike for the pituitary tissue, co-treatment with Dex + GnRH significantly induced the mRNA expression of the GILZ gene by ~2.5-fold (Figure 4.2.3), although this effect appeared to be an additive and not a synergistic response.

![Graphs showing GILZ mRNA expression](image)

**Figure 4.2.2:** The estrous cycle influences the expression of GILZ mRNA levels in mouse pituitaries

The estrous cycle stages of the twenty mice used in Figure 4.2.1 were determined by vaginal cytology and the results obtained for GILZ gene expression were re-plotted according to the four stages of the cycle, A: Estrous, B: Diestrous, C: Metestrous, D: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1). p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.
Taken together, the results in pituitary tissue and gonadotrope cells show that Dex, but not GnRH increases the mRNA expression of the GILZ gene and GnRH appears to modulate the Dex-induced response differently in pituitary tissue compared with primary gonadotrope cells. Dex appeared to increase GILZ mRNA levels by ~2-fold and by ~2.5-fold in combination with GnRH in primary mouse gonadotrope cells (Figure 4.2.3). In pituitary tissue the treatment with Dex did not result in large fold-inductions in the proestrous stage, but greatly increased GILZ mRNA levels after 8h in the other three stages. Treatment with Dex for 8h increased GILZ mRNA levels by ~9-fold and by ~11-fold in the presence of GnRH during the estrous stage in pituitary tissue (Figure 4.2.2 A) while treatment with Dex for 8h increased GILZ mRNA levels by ~21-fold, which appeared to be attenuated by the addition of GnRH to ~12-fold during the diestrous stage in pituitary tissue (Figure 4.2.2 B), but statistical significance could not be established. During the metestrous stage the treatment with Dex for 8h increased GILZ mRNA levels by ~12-fold and the addition of GnRH appeared to decrease this response to ~8-fold in mouse pituitary tissue (figure 4.2.2 C). The Dex-induced upregulation of GILZ mRNA levels did not appear to be modulated by GnRH in mouse pituitary tissue during the proestrous stage (Figure 4.2.2 D). The finding that the most frequent and greatest increase of GILZ mRNA expression occurred at the longer stimulation times suggests the involvement of indirect effects. Furthermore, GnRH appeared to slightly attenuate the Dex-induced upregulation of GILZ mRNA levels, although not in a time- or stage-dependent manner.

Figure 4.2.3: Co-stimulation with Dex + GnRH upregulates GILZ mRNA levels in primary mouse gonadotrope cells

Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels
of GILZ transcripts were determined by quantitative Real-Time PCR. Fold changes in GILZ mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

### 4.3.2 Regulation of mRNA expression by Dex and GnRH of the GnRHR and GRα genes in mouse pituitaries and primary mouse gonadotrope cells

#### 4.3.2.1 GnRHR mRNA expression is upregulated by Dex and GnRH in mouse pituitaries and primary mouse gonadotrope cells under some conditions

To investigate whether Dex, GnRH or both together regulate the expression of the GnRHR gene in the pituitary in a time-dependent manner, the RNA isolated as described for Figure 4.1.1 was analyzed for the expression of the GnRHR gene by RT-qPCR. The results presented in Figure 4.3.1 show that treatment for 8h with Dex, GnRH or the combination of both, significantly increased GnRHR mRNA levels by ~4-, ~3.5- and ~3-fold, respectively. Treatment with Dex or GnRH for 2h, and Dex alone for 4h appeared to slightly increase GnRHR mRNA levels, although statistical significance could not be established (Figure 4.3.1). Furthermore, no evidence of synergistic upregulation in GnRHR mRNA levels with co-treatment of Dex + GnRH could be detected, while GnRH appeared to slightly decrease the Dex-induced upregulation (Figure 4.3.1).
Figure 4.3.1: Dex and GnRH upregulates GnRHR mRNA levels in mouse pituitaries

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of GnRHR transcripts were determined by quantitative Real-Time PCR. Fold changes in GnRHR mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

To determine if the estrous cycle plays a role in the regulation of GnRHR mRNA expression induced by Dex, GnRH or both together, the pooled results from Figure 4.3.1 were re-plotted according to the stage of the estrous cycle and shown in Figure 4.3.2A-D. During the estrous phase treatment with Dex for 4h and 8h appeared to increase the mRNA expression of the GnRHR gene by ~2-fold, although statistical significance could not be established (Figure 4.3.2 A). GnRH treatment did not regulate the mRNA levels of the GnRHR gene at any of the early time-points, but appeared to increase the expression by ~4-fold after 8h treatment (Figure 4.3.2 A). Treatment with the combination of Dex + GnRH for 8h significantly increased GnRHR mRNA levels to a similar extent as GnRH alone (Figure 4.3.2 A). During the diestrous stage treatment with Dex or GnRH alone for 2h resulted in a significant ~2.5- and ~3-fold increase in GnRHR mRNA levels, respectively, which was attenuated with co-stimulation (Figure 4.3.2 B). During the metestrous phase, Dex treatment resulted in significant upregulation after 4h and 8h by ~2- and ~4.5-fold, respectively (Figure 4.3.2 C). However, GnRH treatment did not appear to regulate expression of the GnRHR gene at any time-point investigated, while co-stimulation with both Dex and GnRH induced a similar increase in GnRHR mRNA levels as treatment with Dex alone for 4h and 8h (Figure 4.3.2 C). Figure 4.3.2D show that treatment with Dex, GnRH and both together for 8h significantly increased GnRHR mRNA levels by ~3-fold in the proestrous stage.
The estrous cycle stages of the twenty mice used in Figure 4.3.1 were determined by vaginal cytology and the results obtained for GnRHR gene expression were re-plotted according to the four stages of the cycle, A: Estrous, B: Diestrous, C: Metestrous, D: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1). p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

It has previously been reported that Dex and GnRH induce mRNA expression of the GnRHR gene in the immortalized gonadotrope cell line LβT2 (Kotitschke et al., 2009). This study also reported that co-stimulation with Dex + GnRH synergistically increased mRNA expression of the GnRHR gene. The results of Figures 4.3.1 and 4.3.2 showed that the GnRHR mRNA is regulated by treatment with Dex, GnRH and both together in wild-type mouse pituitaries, but no synergistic responses were observed. Therefore, to investigate if the GnRHR mRNA expression is regulated by these ligands and if co-stimulation with Dex + GnRH induces a synergistic response in primary gonadotrope cells, the RNA isolated as described for Figure 4.1.3 was analyzed for the expression of the GnRHR gene by RT-qPCR. The result presented in Figure 4.3.3 show that 8h treatment with Dex induced a
significant ~2.5-fold increase in GnRHR mRNA levels, while GnRH treatment appeared to have no effect on GnRHR mRNA expression. Co-stimulation with Dex + GnRH had no significant effect on GnRHR mRNA levels compared to Dex alone (Figure 4.3.3). Interestingly, treatment with Dex and GnRH in isolation showed remarkably reproducible responses, while co-treatment with both together resulted in much greater error (Figure 4.3.3).

Figure 4.3.3: Dex increases GnRHR mRNA levels in primary mouse gonadotrope cells
Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels of GnRHR transcripts were determined by quantitative Real-Time PCR. Fold changes in GnRHR mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

Taken together, the results show that Dex upregulated expression of the GnRHR gene more frequently than GnRH and in each stage of the estrous cycle and induced the greatest fold-induction of ~4.5-fold in the metestrous stage compared to all other treatment conditions. Dex also appeared to increase GnRHR mRNA in a time-dependent manner at 4h and 8h in the metestrous stage. GnRH significantly increased GnRHR mRNA levels only in the diestrous and proestrous stages at different time-points. At some time-points and stages of the estrous cycle the GnRHR mRNA expression appeared to be regulated or controlled more by either Dex or GnRH. For example, co-stimulation for
8h of tissue from mice in the estrous or metestrous stages gave results similar to those with GnRH or Dex alone. However, Dex and GnRH also appeared to mediate GnRHR mRNA similarly after 2h and 8h in the diestrous and proestrous stages, respectively. Co-stimulation with both ligands appeared to slightly attenuate the upregulation of GnRHR mRNA expression induced by treatment alone in pituitary tissue and GnRH decreased the Dex-induced increase in GnRHR mRNA in primary mouse gonadotrope cells.

4.3.2.2 GRα mRNA expression is decreased by Dex and GnRH in primary mouse gonadotrope cells

To investigate whether Dex, GnRH or both together regulate the mRNA expression of the GRα gene in the pituitary in a time-dependent manner, the RNA isolated as described for Figure 4.1.1 was analyzed for the expression of the GRα gene by RT-qPCR. The results presented in Figure 4.4.1 show that the GRα mRNA appears to be upregulated with Dex, GnRH and both together at the 4h time-point, although statistical significance could not be established. This result indicates that these hormones potentially regulate the GRα gene, but more experiments are required to obtain statistically significant evidence.

![GRα gene - Mouse pituitary](image)

**Figure 4.4.1: Dex and GnRH appear to upregulate GRα mRNA expression in mouse pituitaries**

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of GRα
transcripts were determined by quantitative Real-Time PCR. Fold changes in GRα mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments.

To investigate if the estrous cycle plays a role in the Dex-, GnRH- and Dex + GnRH-mediated regulation of the GRα gene in mouse pituitaries, the data from the previous result were re-plotted according to the phase of the estrous cycle. The results presented in Figure 4.4.2 did not reveal statistically significant responses, since stimulation with the ligands resulted in responses which were within the range of technical error and biological variation. However, the results presented in Figure 4.4.2A show that treatment with either Dex or GnRH for 4h both appeared to induce a ~2-fold increase in GRα mRNA levels, while co-stimulation appeared to upregulate GRα mRNA by ~4-fold in the estrous stage. In the diestrous phase, treatment with either Dex or GnRH alone for 30 min appeared to induce a ~2.5-fold increase and co-treatment a ~4-fold increase in GRα mRNA levels (Figure 4.4.2 B). Interestingly, four-hour treatment with both Dex and GnRH alone appeared to induce a ~2.5-fold increase in GRα mRNA, which was similar to the effect observed with these two ligands in the estrous phase (Figure 4.4.2 B). In the metestrous phase of the cycle there was very little regulation observed with the ligands at the time-points investigated, although treatment with Dex for 1h did appear to induce a ~1.5-fold increase in GRα mRNA levels (Figure 4.4.2 C). Similarly to the estrous and diestrous stages, treatment with Dex and GnRH for 4h in the proestrous phase both appeared to increase GRα mRNA expression by ~3-fold, although co-stimulation with Dex + GnRH had no effect on GRα mRNA expression (Figure 4.4.2 D). Treatment with Dex for 8h appeared to increase GRα mRNA expression by ~5-fold, but a large degree of error was obtained (Figure 4.4.2 D).
Figure 4.4.2: GRα mRNA levels are regulated by the estrous cycle in mouse pituitaries

The estrous cycle stages of the twenty mice used in Figure 4.4.1 were determined by vaginal cytology and the results obtained for GRα gene expression were re-plotted according to the four stages of the cycle, **A**: Estrous, **B**: Diestrous, **C**: Metestrous, **D**: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1).

To investigate if mRNA expression of the GRα gene is regulated by Dex, GnRH or both together in primary mouse gonadotrope cells, the RNA isolated as described for Figure 4.1.3 was analyzed for the expression of the GRα gene by RT-qPCR. Figure 4.4.3 show that stimulation with Dex, GnRH and both together significantly repressed mRNA expression of the GRα gene to ~0.3-, ~0.4- and ~0.25-fold, respectively. This result was surprising as the results obtained from the pituitaries did not show any repression.
Figure 4.4.3: Dex, GnRH and both together decreases the expression of GRα mRNA levels in primary mouse gonadotrope cells

Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels of GRα transcripts were determined by quantitative Real-Time PCR. Fold changes in GRα mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

Taken together, the results indicate that Dex, GnRH and both together regulate GRα mRNA expression differentially in pituitary tissue compared to primary gonadotrope cells. There was no consistent trend observed in the ligand-induced responses on the GRα mRNA expression levels in pituitary tissue, except that the majority of different treatment conditions appeared to increase instead of repressing GRα mRNA levels. In contrast, in primary mouse gonadotrope cells each ligand significantly repressed GRα mRNA levels alone and in combination. These findings suggest that either paracrine effects in the pituitary cell types influence regulation of the GRα gene or that the GRα gene is differentially regulated in the cell types of the pituitary gland, with an increase induced in other cell types masking the repression in the gonadotrope cells.
4.3.3 Regulation of mRNA expression by Dex and GnRH of gonadotropin genes in mouse pituitaries and primary mouse gonadotrope cells

4.3.3.1 LHβ mRNA levels appear to be differentially regulated by Dex and GnRH in mouse pituitaries and primary mouse gonadotrope cells

To investigate whether Dex, GnRH or both together regulate the mRNA expression of the LHβ gene in the pituitary in a time-dependent manner, the RNA isolated as described for Figure 4.1.1 was analyzed for the expression of the LHβ gene by RT-qPCR. The results presented in Figure 4.5.1 show that treatment with Dex for 2h appeared to increase expression of LHβ mRNA levels by ~2.5-fold, whereas GnRH and co-treatment with Dex + GnRH both appeared to result in a ~2-fold increase. Treatment with Dex for 4h appeared to increase LHβ mRNA levels by ~3.5-fold (Figure 4.5.1). However, statistical significance could not be established for any of the conditions investigated.

Figure 4.5.1: Dex and GnRH appear to upregulate LHβ mRNA levels in mouse pituitaries

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of LHβ transcripts were determined by quantitative Real-Time PCR. Fold changes in LHβ mRNA levels
were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments.

To investigate if the estrous cycle plays a role in the Dex-, GnRH- or Dex + GnRH-mediated regulation of the LHβ gene in mouse pituitaries, the result shown in Figure 4.5.1 were re-plotted according to the stage of the estrous cycle. The results presented in Figure 4.5.2 did not show statistically significant responses, although some trends were observed. The result presented in Figure 4.5.2A show that in the estrous stage, treatment with Dex, GnRH and both together for 30 min appeared to increase expression of LHβ mRNA levels by ~2-fold with a large degree of variability. Dex treatment for 1h, 2h and 8h appeared to upregulate mRNA expression of the LHβ gene by ~2-, ~3- and ~4-fold, respectively, while 2h treatment with GnRH and Dex + GnRH both appeared to increase mRNA levels by ~2-fold (Figure 4.5.2 A). During the diestrous phase treatment with Dex, GnRH and both together for 30 min appeared to increase the mRNA expression of LHβ by ~2-fold, whereas two-hour treatment with Dex, GnRH and Dex + GnRH appeared to increase LHβ mRNA levels by ~3-, ~2.5- and ~2-fold, respectively (Figure 4.5.2 B). Treatment with GnRH and Dex + GnRH for 4h appeared to increase LHβ mRNA levels by ~3- and ~2-fold respectively, although a large degree of error was present in these responses (Figure 4.5.2 B). Furthermore, eight-hour incubation with Dex, GnRH and Dex + GnRH appeared to increase the expression of the LHβ gene by ~4-, ~3- and ~2-fold respectively (Figure 4.5.2 B). Unlike the estrous and diestrous stages, treatment of pituitaries with Dex, GnRH and Dex + GnRH for 30 min in the metestrous stage appeared to slightly repress LHβ mRNA expression (Figure 4.5.2 C). Interestingly, four-hour treatment with Dex appeared to increase LHβ mRNA levels by ~3-fold, while GnRH treatment alone had no effect on LHβ mRNA levels, although GnRH appeared to strongly decrease the Dex-induced response (Figure 4.5.2 C). This result suggests that GnRH attenuates the Dex-mediated induction of LHβ mRNA levels at this time-point. Treatment with Dex and Dex + GnRH for 8h both appeared to increase LHβ mRNA levels by ~2.5-fold (Figure 4.5.2 D). In the proestrous stage, treatment with Dex for 30 min and GnRH for 4h appeared to upregulate expression of LHβ mRNA by ~1.5- and ~2.5-fold, respectively (Figure 4.5.2 D).
Figure 4.5.2: The estrous cycle appears to modulate LHβ mRNA expression levels in mouse pituitaries

The estrous cycle stages of the twenty mice used in Figure 4.5.1 were determined by vaginal cytology and the results obtained for LHβ gene expression were re-plotted according to the four stages of the cycle, A: Estrous, B: Diestrous, C: Metestrous, D: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1).

To investigate whether expression of the LHβ gene is regulated by Dex, GnRH or both together in primary mouse gonadotrope cells, the RNA isolated as described for Figure 4.1.3 was analyzed for the expression of the LHβ gene by RT-qPCR. The result presented in Figure 4.5.3 shows that treatment with Dex for 8h significantly repressed the mRNA expression of the LHβ gene by ~20%. GnRH appeared to slightly increase LHβ mRNA levels, while co-treatment with Dex + GnRH appeared to result in a similar level of repression as Dex alone (Figure 4.5.3).

Taken together, the results suggest that 8h treatment with Dex induces the greatest increase of about ~4-fold in LHβ mRNA levels in the diestrous stage. Dex and GnRH alone upregulate LHβ mRNA
expression at most time-points in the estrous and diestrous stages. During the metestrous and proestrous phases, no general consistent trend for LHβ mRNA expression was observed for the ligands alone or in combination. Co-stimulation with Dex and GnRH did not result in synergistic responses in the pituitary tissue or gonadotrope cells.

Figure 4.5.3: Dex decreases LHβ mRNA levels in primary mouse gonadotrope cells
Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels of LHβ transcripts were determined by quantitative Real-Time PCR. Fold changes in LHβ mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

4.3.3.2 FSHβ mRNA levels are upregulated by Dex and GnRH in mouse pituitaries
To investigate whether Dex, GnRH or both together regulate expression of the FSHβ gene in WT mouse pituitaries in a time-dependent manner, the RNA isolated as described for Figure 4.1.1 was analyzed for the expression of the FSHβ gene by RT-qPCR. The results presented in Figure 4.6.1 show GnRH treatment for 30 min appeared to increase expression of the FSHβ gene by ~3-fold while 1h stimulation with Dex appeared to result in a ~2-fold increase. Four-hour treatment with Dex and GnRH both significantly increased FSHβ mRNA expression by ~4-fold, whereas co-treatment
with Dex + GnRH resulted in a slightly lower significant ~3.5-fold response (Figure 4.6.1). Treatment with Dex for 8h appeared to increase FSHβ mRNA levels by ~2.5-fold, while treatment with GnRH and Dex + GnRH both did not appear to regulate expression of the FSHβ gene (Figure 4.6.1).

Figure 4.6.1: Dex and GnRH upregulates FSHβ mRNA expression in mouse pituitaries

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of FSHβ transcripts were determined by quantitative Real-Time PCR. Fold changes in FSHβ mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments. p-values for comparing a sample against the Ctrl sample were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

To determine if the estrous cycle plays a role in the regulation of the FSHβ gene expression induced by Dex, GnRH or both together, the result shown in Figure 4.6.1 were re-plotted according to the stage of the estrous cycle. The result presented in Figure 4.6.2A show that in the estrous stage, treatment with GnRH for 30 min appeared to increase FSHβ mRNA levels by ~3-fold. Interestingly, co-treatment with Dex + GnRH for 30 min resulted in great attenuation of the GnRH-induced response (Figure 4.6.2 A). Stimulation with Dex and GnRH for 1h both appeared to result in a ~2-fold increase and co-treatment with Dex + GnRH did not appear to regulate FSHβ mRNA levels,
which was similar to the 30 min time-point (Figure 4.6.2 A). Dex and Dex + GnRH incubation for 2h both appeared to upregulate the expression of the FSHβ gene by ~2.5- and 3.5-fold respectively (Figure 4.6.2 A). Treatment for 4h with Dex appeared to induce a ~2-fold increase in FSHβ mRNA levels, whereas GnRH and Dex + GnRH both appeared to increase mRNA expression by ~3.5-fold (Figure 4.6.2 A). Interestingly, as seen with the 30 min and 1h time-points, 8h treatment with Dex + GnRH also appeared to reduce the ~2-fold increase of FSHβ mRNA levels obtained with Dex alone (Figure 4.6.2 A). In the diestrous stage, 30 min treatment with Dex, GnRH and Dex + GnRH all appeared to increase the expression of the FSHβ gene by ~2-fold (Figure 4.6.2 B). Stimulation with Dex for 4h significantly upregulated the expression of the FSHβ gene by ~10-fold, while Dex and Dex + GnRH both resulted in a statistically insignificant ~6-fold response (Figure 4.6.2 B). In the metestrous stage, treatment with GnRH for 30 min appeared to induce a ~4-fold increase in FSHβ mRNA levels and co-treatment with Dex + GnRH resulted in attenuation of the Dex-induced response (Figure 4.6.2 C). One-hour stimulation with Dex and GnRH appeared to increase expression by ~3- and ~2-fold respectively, while co-stimulation with both ligands showed no effect (Figure 4.6.2 C). Interestingly, two-hour treatment with Dex + GnRH appeared to result in a synergistic ~7-fold increase in FSHβ mRNA levels as Dex alone had no effect and GnRH appeared to induce a ~3-fold increase (Figure 4.6.2 C). Treatment with GnRH and Dex + GnRH for 4h appeared to increase FSHβ mRNA levels by ~4- and ~3-fold respectively (Figure 4.6.2 C). In the proestrous stage, treatment with Dex for 1h appeared to increase expression of the FSHβ gene by ~6-fold and co-treatment with Dex + GnRH did not increase the expression relative to Ctrl (Figure 4.6.2 D). Two-hour stimulation with Dex and GnRH appeared to upregulate FSHβ mRNA levels by ~3- and ~4-fold respectively (Figure 4.6.2 D). Four-hour treatment with Dex significantly increased FSHβ mRNA levels by ~10-fold and GnRH appeared to induce a ~4-fold response, while co-treatment with Dex + GnRH had no effect (Figure 4.6.2 D). GnRH treatment for 8h appeared to increase FSHβ mRNA levels by ~4-fold (Figure 4.6.2 D).

To investigate if expression of the FSHβ gene is regulated by Dex, GnRH or both together in primary mouse gonadotrope cells, the RNA isolated as described for Figure 4.1.3 was analyzed for the expression of the FSHβ gene by RT-qPCR. The result presented in Figure 4.6.3 show that treatment with Dex for 8h appeared to slightly repress the expression of the FSHβ mRNA in primary mouse gonadotrope cells. GnRH treatment and co-treatment with Dex + GnRH appeared to have no effect on the mRNA expression of the FSHβ gene in primary mouse (Figure 4.6.3).
Figure 4.6.2: Dex increases FSHβ mRNA levels in the diestrous and proestrous stages of the estrous cycle in mouse pituitaries

The estrous cycle stages of the twenty mice used in Figure 4.6.1 were determined by vaginal cytology and the results obtained for FSHβ gene expression were re-plotted according to the four stages of the cycle, A: Estrous, B: Diestrous, C: Metestrous, D: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1).
Figure 4.6.3: Dex and GnRH appear to differentially regulate FSHβ mRNA expression levels in primary mouse gonadotrope cells

Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels of FSHβ transcripts were determined by quantitative Real-Time PCR. Fold changes in FSHβ mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments.

Taken together, the results suggest that the greatest increase in FSHβ mRNA levels of ~10-fold is induced by Dex treatment for 4h in both the diestrous and proestrous stages. Throughout all four stages and time-points it appears that GnRH upregulates the expression of FSHβ mRNA under most of the conditions. Interestingly, upon co-stimulation the Dex and GnRH signalling pathways appeared to attenuate or enhance each other’s responses. These results suggest that the FSHβ gene is highly regulated by both Dex and GnRH signalling pathways and there appears to be some crosstalk between the two pathways in a phase- and time-dependent manner to fine-tune the mRNA expression of the FSHβ gene.

4.3.3.3 Dex attenuates the GnRH-induced upregulation of αGSU mRNA expression in primary mouse gonadotrope cells

To investigate whether Dex, GnRH or both together regulate expression of the αGSU gene in WT mouse pituitaries in a time-dependent manner, the RNA isolated as described for Figure 4.1.1 was analyzed for the expression of the αGSU gene by RT-qPCR. The result presented in Figure 4.7.1 did not show any statistically significant responses, but some interesting trends were observed. Treatment for 30 min with Dex, GnRH and both together appeared to slightly increase mRNA expression of the αGSU gene by ~1.5-fold. Two-hour treatment with the ligands appeared to increase αGSU mRNA levels by ~2-fold (Figure 4.7.1). Treatment with Dex and GnRH for 4h appeared to increase αGSU mRNA levels to the same extent of ~1.5-fold and co-treatment with Dex + GnRH appeared to have no effect (Figure 4.7.1). Treatment with Dex and Dex + GnRH co-treatment for 8h both appeared to increase αGSU mRNA levels by ~1.5-fold (Figure 4.7.1).
Figure 4.7.1: Dex and GnRH appear to differentially regulate αGSU mRNA expression levels in mouse pituitaries

Pituitaries were isolated from twenty WT mice and each pituitary was divided into four equal portions followed by stimulation with vehicle control (Ctrl), 100 nM Dex, 100 nM GnRH or a combination of both per pituitary in serum-free medium for the time-points indicated in the Figure (refer to Figure 2.1). Total RNA was isolated and reverse-transcribed, and relative levels of αGSU transcripts were determined by quantitative Real-Time PCR. Fold changes in αGSU mRNA levels were normalized to GAPDH transcripts and calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of twelve independent experiments.

To investigate if the estrous cycle plays a role in the Dex-, GnRH- or Dex + GnRH-mediated regulation of the αGSU gene in mouse pituitaries, the result shown in Figure 4.7.1 were re-plotted according to the stage of the estrous cycle. In the estrous stage, treatment with Dex, GnRH and Dex + GnRH for 2h appeared to increase αGSU mRNA levels by ~2.5-, ~2- and ~3-fold respectively (Figure 4.7.2 A). During the diestrous stage, 30 min treatment with Dex, GnRH and Dex + GnRH appeared to similarly increase αGSU mRNA levels by ~2.5-fold (Figure 4.7.2 B). Four-hour treatment with Dex, GnRH and Dex + GnRH appeared to increase mRNA expression of the αGSU gene by ~2-, 1.5- and 1.5-fold, respectively, while 8h treatment with Dex appeared to increase mRNA expression by ~1.5-fold (Figure 4.7.2 B). In the metestrous stage, mRNA expression of the αGSU gene appeared to be increased by ~2-fold with 30 min GnRH treatment and 4h treatment with Dex and GnRH (Figure 4.7.2 C). Eight-hour treatment with Dex appeared to increase αGSU mRNA levels by ~2-fold and GnRH appeared to slightly repress the basal expression, while co-stimulation with Dex + GnRH appeared to result in a synergistic ~3-fold response (Figure 4.7.2 C). In the proestrous stage, treatment with Dex and GnRH for 1h both appeared to increase αGSU mRNA
levels by ~2-fold (Figure 4.7.2 D). Treatment with GnRH for 4h and 8h both appeared to increase the αGSU mRNA levels by ~2-fold (Figure 4.7.2 D).

![Graphs showing estrogenic and GnRH treatment effects on αGSU mRNA expression](image)

**Figure 4.7.2: Dex and GnRH appear to differentially regulate αGSU mRNA expression levels in mouse pituitaries during the different stages of the estrous cycle**

The estrous cycle stages of the twenty mice used in Figure 4.7.1 were determined by vaginal cytology and the results obtained for αGSU gene expression were re-plotted according to the four stages of the cycle, **A**: Estrous, **B**: Diestrous, **C**: Metestrous, **D**: Proestrous. Each graph shows the combined results of three independent experiments (refer to Figure 2.1).

To investigate if expression of the αGSU gene is regulated by Dex, GnRH or both together in primary mouse gonadotrope cells, the RNA isolated as described for Figure 4.1.3 was analyzed for the expression of the αGSU gene by RT-qPCR. The result presented in Figure 4.7.3 show that 8h treatment with Dex did not regulate the mRNA expression of the αGSU gene. However, treatment with GnRH significantly upregulated αGSU mRNA levels by ~2-fold, which was significantly repressed to ~1.5-fold by the addition of Dex (Figure 4.7.3).
Figure 4.7.3: Dex attenuates the GnRH-mediated increase of αGSU mRNA expression in primary mouse gonadotrope cells

Primary mouse gonadotrope cells were purified with FACS from pituitaries of six GRIC/R26-YFP mice and the cells were stimulated for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH, or a combination of both. Total RNA was isolated and reverse-transcribed, and relative levels of αGSU transcripts were determined by quantitative Real-Time PCR. Fold changes in αGSU mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. p-values for comparing samples against each other were obtained by using two-tailed paired t-tests and are denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

Taken together, the results suggests that αGSU mRNA expression is regulated similarly by the ligands in isolation and in combination at the shortest stimulation time in the diestrous stage in pituitary tissue. Throughout all four stages and time-points it appears that Dex regulated the expression of αGSU mRNA under more conditions than GnRH. Dex + GnRH co-treatment for 8h appeared to synergistically increase αGSU mRNA expression in pituitary tissue whereas Dex attenuated the GnRH-induced upregulation of αGSU mRNA levels in primary gonadotrope cells after 8h.
4.3.4 The GR and Flot-1 are both expressed in mouse pituitary tissue and primary mouse gonadotrope cells

The gene expression results obtained in the primary model systems thus far do not provide evidence for the mechanism through which these ligands induce the responses. However it is highly likely that the Dex responses are mediated via the GR since Dex is a selective GR agonist.

The results from Figure 4.4.1 and 4.4.2 show that GRα mRNA was detected in mouse pituitaries and Figure 4.4.3 shows that GR mRNA was also detected in primary mouse gonadotrope cells. In addition, the results of Chapter 3 showed that the Dex + GnRH synergistic increase of SGK-1 mRNA levels required the presence of Flot-1 in LβT2 cells (Figure 3.5 E and 3.6 A). A possible reason for not detecting synergistic responses in mouse pituitary tissue or primary gonadotrope cells could be due to the lack of Flot-1 protein expression in mouse pituitaries. Therefore, to investigate whether GR and Flot-1 protein expression could be detected in these models, pituitaries from GRIC/R26-YFP mice were cryosectioned, followed by fixation, permeabilization and staining with anti-GR and anti-Flot-1 antibodies. The results in Figure 4.8A show that the GR protein is expressed in the majority of the cells present in the pituitary. Furthermore, quantification analysis showed that ~7% of the pituitary cells were positive for the YFP signal, which is the marker for gonadotrope cells in the GRIC/R26-YFP mouse strain (Figure 4.8). To determine the percentage of gonadotrope cells that express the GR protein the YFP-positive cells of three different fields of view were quantified and the analysis showed that ~85% of gonadotrope cells express a detectable level of GR protein (Figure 4.8 A). Interestingly, the GR is predominantly localized to the nucleus within all the cells of the pituitary (Figure 4.8 A).

The results in Figure 4.8B show that Flot-1 is expressed in the majority of the pituitary cells, including the gonadotrope cells. Flot-1 appears distinctly localized to the plasma membrane region and intracellular compartments (Figure 4.8 B), which is similar to the results of Chapter 3 obtained in LβT2 cells (Figure 3.1). There appears to be a small amount of GR and Flot-1 that may potentially co-localize in primary gonadotrope cells (Figure 4.8 C). However, co-localization analysis was not performed since localization of the GR was distinctly nuclear and Flot-1 appeared to be over-stained. Taken together, the results strongly suggest that the GR is highly expressed in mouse pituitary tissue and in primary mouse gonadotrope cells.
Figure 4.8: GR and Flot-1 protein expression are detectable in primary mouse gonadotrope cells

Immunohistochemistry of 10 µm pituitary sections prepared from female GRIC/R26-YFP mice with intrinsic YFP fluorescence within the gonadotrope cells (green). Slides were blocked before staining with rabbit anti-GR and mouse anti-Flot-1 antibodies. Thereafter, the slides were incubated with anti-rabbit Cy5 and anti-mouse Cy3 (red) antibodies. Nuclei were stained with Hoechst (blue) before mounting in Fluoromount-G and visualization were performed as described for Figure 3.1.1 with a Zeiss LSM510 Meta confocal microscope using the 63 X objective. Quantification analysis to determine the percentage of GR positive gonadotrope cells was performed on three different fields of view from each independent repeat experiment with ImageJ software. Panel A shows the GR-Cy5 stain in red and the arrow indicates GR-positive gonadotrope cells, while the arrow in panel B indicates Flot-1-positive gonadotrope cells. Panel C shows the GR-Cy5 stain in green and the arrow indicates the possible co-localization of the GR and Flot-1 in a gonadotrope cell, the scale bar represents 10 µm. The results shown are representative of three independent experiments.
The finding that GR protein expression is detectable in mouse pituitary tissue and primary gonadotrope cells and that Dex is a GR-specific agonist strongly suggests that the Dex-induced responses observed for the genes investigated in Figures 4.1-4.7 are mediated through the GR. GnRH has previously been shown to activate the GR and the PR in LβT2 cells (Anne et al., 2009; Kotitschke et al., 2009). Therefore, to investigate if the PR mRNA is expressed at a detectable level in mouse pituitary tissue and primary gonadotrope cells, the RNA isolated as described for Figure 4.1.1 and 4.1.3 was used in conventional RT-PCR with PR-B-specific primers. The results presented in Figure 4.9 show that PR-B mRNA expression was detected in LβT2 cells, with detection of a single PCR product of ~121 bp (Kotitschke 2009). No PCR products were detected in the mouse pituitary RNA sample, but RT-PCR of primary gonadotrope cell RNA did result in detection of a single product that was the expected size (Figure 4.9). The amplified PCR products were sequenced and it was confirmed that the products were generated from mouse PR mRNA, showing that PR-B mRNA is expressed in both mouse pituitaries and primary mouse gonadotrope cells.

Figure 4.9: Endogenous PR-B mRNA is expressed in primary mouse gonadotrope cells
Total RNA was isolated from LβT2 cells, pituitary tissue and primary gonadotrope cells, followed by a reverse transcriptase reaction to generate cDNA. Signals were amplified using conventional PCR with PR-B-specific primers for 35 PCR cycles and the products were separated on a 2% agarose gel using electrophoreses and visualized with ethidium bromide staining. The LβT2 cells were used as a positive Ctrl and the arrow indicates the 121 bp PR-B product. NTC defines the no template control. The result shown is representative of three independent experiments.
The detection of PR-B mRNA in primary mouse gonadotrope cells, but not in mouse pituitary tissue was surprising. However, it may be that within the pituitary the PR is exclusively expressed in gonadotrope cells, but at a level below the limit of detection with RT-PCR in total pituitary RNA. Due to the limited availability of mice it was not possible to investigate whether PR protein is expressed in pituitary tissue by Western blot analysis. In addition, since a suitable commercially available anti-PR antibody could not be obtained it was not possible to investigate if PR protein is expressed in primary mouse gonadotrope cells with immunohistochemistry analysis. Taken together, the result indicates that PR mRNA is expressed in primary mouse pituitary gonadotrope cells, but whether the protein is also expressed is not established.

4.3.5 The Dex- and Dex + GnRH-induced upregulation of the SGK-1, GILZ and GnRHR mRNA expression requires the activity of the GR in mouse pituitary tissue

The results from the previous section showed that GR protein is expressed in mouse pituitaries and PR mRNA was detected in primary gonadotrope cells. To investigate whether the Dex-, GnRH- and Dex + GnRH-induced regulation of the SGK-1, GILZ and GnRHR mRNA requires the activity of the GR, WT mouse pituitaries were divided into eight equal portions and subjected to 8h stimulation with 100 nM Dex, 100 nM GnRH or both together in the absence or presence of the GR antagonist RU486. These experiments could only be performed in pituitary tissue and not in primary gonadotrope cells due to their limited availability. Furthermore, the stages of the estrous cycle of the mice used in this pituitary tissue experiment were unknown due to the limited number of mice available for this part of the project. Thus the expression of only a few genes could be investigated. This selection was based on genes that have previously been reported to exhibit synergistic responses, like SGK-1 in Chapter 3 and GnRHR in the study by Kotitschke et al., 2009. The classical GRE-regulated gene, GILZ, was selected as a control for validation of the experimental technique. The result presented in Figure 4.10 shows that treatment with Dex increased SGK-1 mRNA levels by ~4-fold and that the upregulation was significantly reduced in the presence of RU486. GnRH treatment appeared to induce a ~3-fold increase in SGK-1 mRNA levels, which was slightly attenuated in the presence of the GR antagonist (Figure 4.10). Furthermore, co-treatment with Dex + GnRH increased SGK-1 mRNA levels to a similar extent as treatment with Dex alone and the Dex + GnRH-induced response was significantly reduced in the presence of the antagonist (Figure 4.10). Taken together, the results suggest that the activity of the GR is required for the Dex- and Dex + GnRH-mediated upregulation of SGK-1 mRNA levels in pituitary tissue. However, whether the
activity of the GR is required for the GnRH-mediated increase in SGK-1 mRNA levels remains to be determined.

**Figure 4.10: The antagonist RU486 inhibits the Dex-mediated increase of SGK-1 mRNA levels in mouse pituitaries**

Pituitaries were isolated from WT mice and each divided into eight equal portions followed by stimulation for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH or a combination of both in the presence and absence of 100 nM RU486. Total RNA was isolated and reverse-transcribed, and relative levels of SGK-1 transcripts were determined by quantitative Real-Time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. A two-tailed paired t-test to compare the effect of RU486 for each treatment condition was used for statistical analysis and denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

To investigate whether the activity of the GR is required for the Dex-, GnRH- and Dex + GnRH-mediated mRNA expression of the GILZ gene, the RNA isolated as described for Figure 4.10 was analyzed for the expression of the GILZ gene by RT-qPCR. The result presented in Figure 4.11 show that the Dex- and the Dex + GnRH-mediated upregulation of the GILZ gene was significantly decreased in the presence of RU486, which suggests that the GR is involved in the regulation of this gene. Furthermore, the finding that GnRH treatment did not increase GILZ mRNA levels is
consistent with results shown in Figures 4.2.1 and 4.2.2. Taken together, it appears that GILZ mRNA expression is regulated by Dex, but not by GnRH, and requires the activity of the GR.

Figure 4.11: The antagonist RU486 inhibits the Dex-induced expression of GILZ mRNA levels in mouse pituitaries

Pituitaries were isolated from WT mice and each divided into eight equal portions followed by stimulation for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH or a combination of both in the presence and absence of 100 nM RU486. Total RNA was isolated and reverse-transcribed, and relative levels of GILZ transcripts were determined by quantitative Real-Time PCR. Fold changes in GILZ mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. A two-tailed paired t-test to compare the effect of RU486 for each treatment condition was used for statistical analysis and denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.

To investigate if the activity of the GR is required for the Dex-, GnRH- and Dex + GnRH-mediated upregulation of the GnRHR gene, the RNA isolated as described for Figure 4.10 was analyzed for the expression of the GnRHR gene by RT-qPCR. The result presented in Figure 4.12 show that treatment with Dex increased GnRHR mRNA levels by ~3-fold and this response was significantly decreased by the addition of RU486. Treatment with GnRH resulted in a ~2.5-fold increase in mRNA expression of the GnRHR gene, which was slightly attenuated in the presence of RU486 (Figure 4.12). The increase in GnRHR mRNA levels induced by co-treatment with Dex + GnRH was
completely abolished by the antagonist RU486 (Figure 4.12). This result suggests that the activity of the GR is required for the Dex- and Dex + GnRH-mediated upregulation of the GnRHR gene in mouse pituitaries. Furthermore, it appears that the GR is involved in the GnRH-mediated regulation of the GnRHR gene in primary mouse gonadotrope cells.

**Figure 4.12: The antagonist RU486 inhibits the Dex-, but not the GnRH-mediated increase of GnRHR mRNA levels in mouse pituitaries**

Pituitaries were isolated from WT mice and each divided into eight equal portions followed by stimulation for 8h in serum-free medium containing 100 nM Dex, 100 nM GnRH or a combination of both in the presence and absence of 100 nM RU486. Total RNA was isolated and reverse-transcribed, and relative levels of GnRHR transcripts were determined by quantitative Real-Time PCR. Fold changes in GnRHR mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated control (Ctrl) samples. The graph shows the combined results of three independent experiments. A two-tailed paired t-test to compare the effect of RU486 for each treatment condition was used for statistical analysis and denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.
CHAPTER 5
DISCUSSION AND CONCLUSIONS

5.1 Exploring the membrane raft-associated crosstalk mechanism between the GR and GnRHR signalling pathways in LβT2 cells

Some of the effects of stress on reproduction are mediated via GCs from the adrenal cortex as part of a crosstalk mechanism between the HPA and HPG axis (Rivier and Rivest, 1991). A study performed by Kotitschke et al. demonstrated a novel crosstalk mechanism between the GR and GnRHR (Kotitschke et al., 2009), indicating an additional mechanism for the effects of stress on reproduction, acting directly on pituitary gonadotropes. The study showed that GnRH activates the unliganded GR through site-specific phosphorylation, resulting in transactivation of a GRE reporter and endogenous mouse GnRHR genes in LβT2 cells in a GR- and GnRHR-dependent manner. In addition, the authors reported a synergistic transcriptional response on the GnRHR gene upon co-stimulation 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 membrane rafts in another gonadotrope cell line, αT3-1 (Bliss et al., 2007; Navratil et al., 2003). Interestingly, a study by Matthews et al. showed that the GR could also associate with caveolae, which is required for the rapid Dex-induced phosphorylation of the GR at Ser-211 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 membrane rafts, whereby activation of the one receptor modulates the activity of the other. Previous results from the Hapgood laboratory suggest that both the GR and GnRHR localize to membrane rafts and are together in a complex (Wehmeyer, 2010). The same study showed that Dex and GnRH induced similar transcriptional responses on a GRE-containing reporter gene, while both together induced a synergistic transcriptional response on a GRE-reporter gene in LβT2 cells and was shown to require the presence of Flot-1-containing membrane rafts. The study also investigated the functional role of the Dex + GnRH synergistic transcriptional response using the cholesterol extraction reagent, MβCD, which resulted in cellular death. In addition, that study also investigated whether the Dex + GnRH-mediated synergistic effects of GR transactivation could also be playing a role in transrepression of the GR. The results obtained
with a NF-κB reporter gene suggested that the GnRHR and GR crosstalk is not involved in GR-mediated transrepression of NF-κB-containing genes (Wehmeyer 2010).

In the present study, more conclusive evidence was obtained for the membrane raft localization of the GR and GnRHR (Figure 3.1). It is possible that other proteins involved in cellular signalling could localize to this complex to form a signal transduction platform that modulates downstream genomic signalling by the cytoplasmic GR. The hypothesis that the GR and GnRHR form such a platform with other signalling proteins was investigated in the present study. Results obtained showed that ERK-1/2 localizes to Flot-1-containing membrane rafts in a ligand-independent manner (Figure 3.3.1), consistent with previous reports that showed ERK-1/2 localizing to membrane rafts in αT3-1 cells and mouse pituitaries (Bliss et al., 2007). In addition, Bliss et al. reported that the GnRH-induced phosphorylation of ERK-1/2 requires intact membrane rafts in αT3-1 cells (Bliss et al., 2007). In the present study it was found that ERK-1/2 phosphorylation is not affected by decreased Flot-1 protein levels (Figure 3.3.2 A). This is different to the result reported by Bliss et al., but the difference could be because the current study was performed in a more mature gonadotrope cell line. Furthermore, in the present study the strategy to disrupt the integrity of membrane rafts was performed by decreasing the protein expression level of the structural protein Flot-1 with siRNA-mediated knockdown, whereas the study by Bliss et al. used MβCD. In the present study it is possible that Flot-1 knockdown did not result in disruption of all the membrane rafts, possibly unlike the raft extraction reagent or that the raft extraction reagent had other non-specific effects besides raft disruption like disruption of the plasma membrane or the nuclear envelope (Bliss et al., 2007). Therefore, results obtained through the depletion of cellular cholesterol should be assessed in a cautious manner since the reagent contains various caveats.

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 1h in αT3-1 cells (An et al., 2006). A more recent study showed that GnRH induced the phosphorylation of the PR at Ser-249, resulting in nuclear translocation and transactivation of a 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 mouse gonadotrope cell lines.

It has previously been shown that intact membrane rafts are required for Dex-induced GR phosphorylation at Ser-211 in A549 cells (Matthews et al., 2008). Additionally, Kotitschke et al. previously showed that Dex induces phosphorylation of the GR at Ser-220 and Ser-234 of the mouse GR, whereas GnRH induces Ser-234 and not Ser-220 phosphorylation of the mouse GR in LβT2 cells (Kotitschke et al., 2009). Therefore, in the present study it was investigated if Flot-1-containing membrane rafts are involved in phosphorylation of the GR. The results presented in this study suggest that Flot-1-associated membrane rafts are not required for phosphorylation of the GR at Ser-212, Ser-220 or Ser-234 (Figure 3.4). However, it is possible that if a greater level of Flot-1 knockdown had been achieved that an effect on GR phosphorylation could have been detected. It should be stated that two different methods to knockdown Flot-1 protein levels were investigated, but ~60% knockdown was the greatest level that could be achieved. Furthermore, it is possible that membrane rafts may have a role in GR phosphorylation at different stimulation time-points. In addition, Ser-212 phosphorylation of the GR appeared to be slightly diminished with decreased Flot-1 protein levels. The finding that disruption of membrane rafts with Flot-1 siRNA-mediated knockdown had no effect on the Dex-induced phosphorylation at Ser-220 of the GR in LβT2 cells is different to a 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 Ser-211 in A549 cells (Matthews et al., 2008). However, besides the level of protein knockdown another possible explanation for this difference could be that Cav-1- and Flot-1-containing membrane rafts have different cellular functions or that this requirement for membrane rafts in GR phosphorylation is cell- or species-specific.

5.2 Co-treatment with Dex + GnRH synergistically and selectively enhances SGK-1 mRNA levels

It has previously been showed that crosstalk between the GR and GnRHR signalling pathways modulates the expression of the endogenous AP-1-containing GnRHR gene synergistically (Kotitschke et al., 2009). Whether Dex + GnRH synergism occurs on endogenous GRE-containing genes was investigated in the present study. These genes were firstly screened with Dex to determine if they are responsive in this cell line. Dex increased the mRNA expression of the GILZ, FKB5, FSHβ, MKP-1 and SGK-1 genes (Figure 3.5), which was consistent with previous reports in the
literature (Arteaga et al., 2007, Ayroldi and Riccardi 2009, Bruscoli et al., 2006, D'Agostino et al., 1990, Kassel et al., 2001, Klengel et al., 2013, Ringstrom et al., 1991, Vermeer et al., 2003). FSHβ mRNA levels were also increased with GnRH treatment, as previously reported in literature (Ringstrom et al., 1991). Interesting novel findings were that GnRH stimulation significantly increased SGK-1 mRNA levels and co-stimulation with Dex + GnRH resulted in a synergistic response (Figure 3.5 E). The present study is the first to show that GnRH increases SGK-1 mRNA and to the present author’s knowledge it is the first study showing that crosstalk between the GR and GnRHR results in synergism on an endogenous GRE-containing gene.

The finding that Dex synergistically enhances the GnRH-induced increase in endogenous SGK-1 mRNA expression in the LβT2 gonadotrope cell line is consistent with other reports of synergistic action of these hormones in the pituitary. Kotitschke et al. reported previously that Dex enhanced the GnRH-induced upregulation of GnRHR mRNA levels synergistically in LβT2 cells (Kotitschke et al., 2009). Co-stimulation with Dex + GnRH has previously been shown to synergistically upregulate GnRHR mRNA levels in rat pituitary cultures (Rosen et al., 1991), as well as FSHβ secretion in rat pituitary fragments (D'Agostino et al., 1990). An important physiological action of the GR in reproductive function in rat gonadotrope cells is further supported by a study that reported GCs synergistically enhanced the activin-induced increase in FSHβ gene expression in both primary rodent pituitary cells and in LβT2 cells (Leal et al., 2003, McGillivray et al., 2007). The synergistic upregulation of SGK-1 mRNA levels is consistent with a previous report that showed Dex synergistically increased the BDNF-induced expression of the SGK-1 gene in 293TrkB cells, which are HEK-293 cells stably expressing the receptor for BDNF, TrkB (Lambert et al., 2013). Furthermore, the authors also reported that BDNF did not modulate the Dex-mediated expression of GILZ mRNA levels (Lambert et al., 2013), consistent with results of the present study (Figure 3.5). However, the authors showed that Dex did synergistically enhance the BDNF-induced upregulation of FKBP5 mRNA levels. These data collectively suggests that the mechanism of GR-mediated synergism via crosstalk with other signalling pathways requires the gene to be responsive to both hormones in isolation. Taken together, it appears that only certain select GRE-containing genes are synergistically upregulated upon treatment with Dex in the presence of another hormone.
5.3 Mechanism of Dex + GnRH-induced synergism of SGK-1 gene expression in LβT2 cells

The next part of the present study was to investigate if membrane rafts have a functional role in GR and GnRHR signalling in LβT2 cells. Many findings in the present study suggest that the membrane raft-associated GR-GnRHR complex acts as a signal transduction platform that modulates downstream genomic signalling by the cytoplasmic GR. By a combination of protein knockdown, pharmacological inhibitor/activator and ChIP strategies, and incubation of cells with Dex, GnRH or both in combination several insights into the role of membrane raft-associated GR on gene expression for classical Dex-mediated transactivation, GnRH induced ligand-independent GR responses, and responses to the combination of Dex + GnRH, for an endogenous GRE-containing gene in LβT2 cells were obtained. It is possible that Flot-2 plays a redundant role with Flot-1 although further studies are required to investigate this scenario. The aim of the experiments in the present study was not to investigate the role Flot-2 in the integrity of membrane rafts. However, as mentioned in the literature review Flot-1 and Flot-2 form hetero-oligomers and it is therefore likely that if the expression of either one is reduced that the formation of membrane rafts could also be affected (Meister and Tikkanen 2014).

The results showed that membrane raft-associated GR does not have a role in mediating the classical Dex-dependent GR transactivation response on the SGK-1 gene and that the PKA and PKC pathways are not required for this response. Most of the GR translocated to the nucleus in the presence of Dex, whereas the small subpopulation of GR associated with membrane rafts did not appear to change location. Moreover, the GR was recruited to the GRE region of the SGK-1 promoter. These data are consistent with a classical mechanism of GR activation as well as with a previous report showing GR recruitment to the SGK-1 GRE in 10T1/2 and 293TrkB cells (Lambert et al., 2013, Wu and Bresnick 2007). In contrast, others have shown a functional role for caveolae-associated GR in Dex-mediated GRE reporter gene transactivation (Jain et al., 2005). The estrogen receptor has also been found in the membrane and was shown to be required for the early membrane estrogenic effects to potentiate the slower genomic actions, as measured by the transactivation of a ERE-reporter gene in a nerve cell line (Vasudevan et al., 2001). The results in the present study suggest that Flot-1-associated GR may act differently to caveolae-associated GR or that the dependence of genomic transactivation on membrane-associated GR responses is cell-specific. The ChIP results showing increased recruitment of GRIP-1 to the GRE in response to Dex alone are in agreement with a report for the SGK-1 gene in 1470.2 cells (Barr et al., 2009) as well as published
data for other GRE-containing genes (Ronacher et al., 2009). Interestingly, the results show that SRC-1 is also recruited by this classical GR transactivation mechanism in response to Dex on the SGK-1 promoter in LβT2 cells. Although it has been reported that the GR preferentially recruits GRIP-1 rather than SRC-1 to a GRE reporter gene (Li et al., 2010a), others have shown that SRC-1 can act as a GR co-activator (Ronacher et al., 2009), suggesting that the recruitment of SRC-1, GRIP-1, or both may be promoter- or cell-specific. It is possible that SRC-1 is important in the synergistic mRNA increase of the SGK-1 gene induced with Dex + GnRH treatment. This could be investigated by reducing SRC-1 protein levels with siRNA, followed by real-time qPCR to detect changes in SGK-1 mRNA levels.

In the next part of the present study the results were scrutinized to assess whether Flot-1-associated GR is involved in regulating the GnRH-induced ligand-independent GR mediated expression of the SGK-1 gene. The result showing that the GR is significantly recruited to the SGK-1 promoter in response to GnRH are novel and suggest that ligand-independently activated GR contributes to the GnRH-induced upregulation of the endogenous GRE-containing gene. Furthermore, the absence of a response to GnRH for the GRE-containing GILZ, MKP1, and FKBP5 genes suggests that GnRH-activated GR exerts promoter-specific downstream effects. It could not be established conclusively whether Flot-1 is required for the GnRH-induced response on the SGK-1 gene. This was due to the relatively small but statistically significant response on the SGK-1 gene with GnRH, which was even further reduced with the NSC knockdown conditions. Due to the small response it was also not possible to establish whether PKC activation is required for the GnRH response on the SGK-1 gene, although the apparent slight reduction with a PKC inhibitor and mimicking of the response with PMA, but not the cAMP analogue, suggest that PKC and not PKA is involved in regulating GnRH-induced SGK-1 expression, consistent with previous published data (Kotitschke et al., 2009). Besides recruitment of the GR to the SGK-1 promoter, albeit less than with Dex alone, treatment with GnRH also resulted in the recruitment of GRIP-1, but not SRC-1 or SRC-3 to the promoter. By contrast, in Dex-treated cells it was found that both GRIP-1 and SRC-1, but not SRC-3, are associated with the promoter. Interestingly, this result for GnRH is different to that previously reported for the GnRHR promoter (Kotitschke et al., 2009), where neither GRIP-1 nor SRC-1 was recruited. These findings highlight gene-specific effects of GnRH-activated GR signalling at responsive promoters. It is likely that promoter occupancy of GR co-factors is determined by both the promoter gene architecture as well as the ligands activating the GR, which may result in distinct conformational changes in the GR itself, thus allowing differential interactions with other factors.
Thirdly, the results were assessed to determine whether Flot-1-associated GR is involved in mediating the combined effects of Dex + GnRH-induced upregulation of the SGK-1 gene. The findings provide several novel insights into the mechanism of synergy between the GR and GnRHR signalling pathways. This present study presents evidence for the first time that the GR and GnRHR synergistically upregulate the SGK-1 gene and this effect, in contrast to the effects elicited by the hormones alone, requires Flot-1-containing membrane rafts. Furthermore, the results show that this synergism only occurs on a subset of GRE-containing endogenous genes. This gene-specific effect supports a model whereby both receptors act in concert to selectively activate only a distinct set of genes to fine-tune the response of a cell to changes in the hormonal environment.

It was established that the synergistic effect on SGK-1 gene expression is not due to detectable changes in localization of the GR to the membrane or increased GR nuclear import or GR recruitment to the promoter. The findings led to the hypothesis that the underlying mechanism of synergistic upregulation of the SGK-1 gene by Dex + GnRH involves the activation of a cytoplasmic signalling pathway by the membrane raft associated GnRHR-GR complex. Consistent with this, the results show a requirement for the PKC pathway in the combined effect of Dex and GnRH and not for the response observed for Dex treatment alone. The observed PMA-dependent and cAMP-independent increase in SGK-1 mRNA levels is consistent with a previous report showing that PKC activates transcription of the SGK-1 gene in rat neonatal cardiomyocytes (Lister et al., 2006). The same study showed that PMA enhanced the corticosterone-induced mRNA expression of the SGK-1 gene, similar to the effects observed with PMA + Dex in the present study, suggesting that the PKC pathway augments GC responses in other cells and species as well.

The data obtained in the present study suggest that in LβT2 cells, crosstalk between the GnRHR and GR involves the activation of the PKC pathway and converges on the SGK-1 gene. As a requirement for Flot-1-containing membrane rafts in the synergistic transcriptional response and a direct interaction of Flot-1 and the GR was previously reported (Wehmeyer, 2010), it is tempting to speculate that these membrane domains provide a platform where GnRHR and GR constitutively assemble and crosstalk to modulate downstream signalling from the membrane. The finding that Dex + GnRH did not alter the membrane localization of the GR together with the result that GR levels at the SGK-1 promoter were similar to single-hormone treatment suggests that other factors mediate the combined response, which results in synergism. Similarly, others have found that co-treatment of cells with cortisol + FGF-2 did not alter the cortisol-induced GR recruitment to the SGK-1 promoter.
(Wu and Bresnick, 2007). In contrast, it has been shown that Dex + BDNF co-treatment resulted in enhanced GR promoter occupancy of the SGK-1 gene compared with Dex alone in 293TrkB cells (Lambert et al., 2013). This present study show that the relative levels of members of the SRC family of co-activators are different for Dex + GnRH compared with Dex alone, which suggests that another co-factor or an entirely different complex might be recruited to the SGK-1 promoter. Although, a possible role for other co-activators in mediating the synergistic response on the SGK-1 gene was investigated, but such a positively acting co-factor was not identified. In retrospect, it would have been interesting to investigate whether GnRH or Dex + GnRH induced the recruitment of CREB to the GRE region in the promoter of the SGK-1 gene, since Lambert et al. showed the synergism with BDNF + Dex co-treatment involved GR, CREB, GRIP-1 and p300 recruitment to the GRE region of the SGK-1 gene (Lambert et al., 2013). Although in the present study the results showed that neither p300 nor the CREB-binding protein (CBP) were recruited in response to Dex, GnRH or Dex + GnRH to the SGK-1 gene, which suggest that the Dex + GnRH synergism effect involves a different mechanism. The present study did not establish that the effect of Dex + GnRH on synergistic upregulation of SGK-1 mRNA levels in LβT2 cells is due solely to a direct transcriptional effect of the GR acting on the SGK-1 promoter. However, the ChIP results showing GR recruited to the SGK-1 promoter, as well as the results on a GRE-reporter gene (Wehmeyer, 2010; Kotitschke et al., 2009) do support a predominantly transcriptional mechanism. It is possible that additional mechanisms are involved such as post-transcriptional effects on SGK-1 mRNA stability, post-translational modifications such as non-genomic modification of proteins, and/or indirect effects involving a requirement for synthesis of other proteins.

In the present study the results showed that Dex and Dex + GnRH induce phosphorylation of the GR at Ser-234 and Ser-220 of the GR, but that GnRH only induces phosphorylation of the GR at Ser-234, similar to the report by Kotitschke et al. (Kotitschke et al., 2009). The present study’s novel results suggest that membrane raft association of the GR is not required for GR phosphorylation at Ser-212, Ser-220 or Ser-234 in response to GnRH, Dex, or the combination thereof. Furthermore, the SGK-1 response to Dex was unaffected, whereas the response to GnRH was slightly but not significantly reduced, and the synergistic response was significantly inhibited in the presence of the PKC inhibitor. These results suggest that ligand-induced phosphorylation of the GR does not require membrane raft association. Arguably, a greater reduction in Flot-1 protein levels may have led to detectable changes in GR phosphorylation levels. However, the findings that a 60% reduction of Flot-1 levels results in a significant decrease in the synergistic upregulation of the SGK-1 gene together with the finding that membrane raft association is not involved in GR phosphorylation.
supports the notion that the Flot-1-dependent synergistic response does not require GR phosphorylation at Ser-234 or Ser-220.

5.4 Membrane-associated GR is involved in mediating LβT2 cell proliferation

Both the GR and GnRHR signalling pathways have previously been reported to be involved in the regulation of cellular growth and the following part of the study investigated if these receptors are involved in this physiological function in gonadotrope cells. Signalling by both the GR and the GnRHR has been previously shown to affect cell proliferation in several different cell types (Abel et al., 2002, Chen et al., 2008, Hsu et al., 1992, Hsu and DeFranco 1995, Matthews et al., 2008, Matthews et al., 2011, Rogatsky et al., 1997, Wang et al., 2007). The finding in the present study that GnRH alone decreases cell proliferation in LβT2 cells is consistent with a previous report (Feng et al., 2008). The insensitivity of this effect to both Flot-1 and SGK-1 knockdown taken together with the gene expression and ChIP results suggest that the mechanism of this GnRH anti-proliferative effect does not involve membrane raft-associated GR or SGK-1 expression. Since the GnRHR activates several downstream signalling pathways in LβT2 cells (Lariviere et al., 2007, Naor 2009), which could potentially target several mediators of cell proliferation effects, factors other than the GR and SGK-1 expression are likely to predominate in mediating the anti-proliferative response to GnRH in these cells. The finding that synergism between the GR and the GnRHR regulates proliferation in LβT2 cells via upregulating the SGK-1 gene is novel and suggests a mechanism for crosstalk between the HPA and HPG axes to regulate proliferation of gonadotrope cells in the pituitary. The cell proliferation effects were significantly ablated by reduction of Flot-1 protein levels, consistent with a role for Flot-1 in the synergistic mechanism.

Membrane-associated GR has previously been linked to anti-proliferative effects in A549 and mouse neural/progenitor stem cells (Matthews et al., 2008, Peffer et al., 2014, Samarasinghe et al., 2011). However unlike these reports showing anti-proliferative effects with Dex alone, this present study show that in LβT2 cells, Dex alone does not affect cell proliferation but only in combination with GnRH, which is consistent with cell-specific effects. It is tempting to speculate that membrane raft-associated GR modulates GnRHR signalling to enhance the GnRH-induced anti-proliferative response by direct effects on the GnRHR. The finding for a role of SGK-1 in cell proliferation is reminiscent of a previous report showing that SGK-1 activity is required for cortisol-induced reduction of cell proliferation in a hippocampal progenitor cell line (Anacker et al., 2013). Since increases in pituitary cell proliferation have previously been linked to some pathologies such as pituitary tumors, Cushing’s disease, pituitary hyperplasia, and pituitary developmental dysregulation
(Jayakody et al., 2012, Langlais et al., 2013, Rees et al., 2002), synergy between Dex and GnRH may be a mechanism to prevent such negative physiological consequences. Synergy between membrane-associated GR and GnRHR represents an attractive mechanism for fine-tuning responses and the interplay between signalling pathways and may be representative of a more widespread mechanism that awaits further discovery.

5.5 Glucocorticoids regulate the mRNA expression of model GRE-containing and key reproductive genes in pituitary tissue and primary gonadotrope cells via the GR

The next part of the present study was to investigate whether Dex, GnRH or both together regulate the expression of selective GRE-containing and reproductive genes in physiologically relevant primary mouse pituitary tissue and gonadotrope cell models. The selection of primary model systems was based on the following criteria. Primary gonadotrope cells were selected to determine whether mechanisms found to occur in the transformed LβT2 gonadotrope cell line are maintained in primary untransformed gonadotrope cells. Since gonadotrope cells function within the context of the pituitary, which consists of several types of endocrine hormone producing cells, it was deemed important to investigate effects in this primary tissue. To determine whether paracrine effects in the pituitary influence the expression of selected genes in the gonadotropes.

The genes chosen to investigate in the primary model systems were based on four different criteria. Firstly, genes that have previously been shown to exhibit synergy with Dex + GnRH were selected, which are SGK-1 (Figure 3.5) and GnRHR (Kotitschke et al., 2009). Secondly, well-established GRE-containing genes were selected to establish the activity and responsiveness of the GR in these primary model systems. These include the SGK-1 the GILZ genes. Thirdly, to investigate the effects of GCs and GnRH in mammalian reproduction, in addition to the central reproduction gene, GnRHR, the gonadotropin genes were selected. These include LHβ, FSHβ and the common alpha subunit for both of the gonadotropins, the αGSU gene. Together, these four genes are vital for functional mammalian reproduction (Childs 2006). Finally, another goal was to determine whether the responsiveness of the GR and GnRHR signalling pathways in the primary model systems is regulated by the reciprocal modulation of the expression level of their receptors. Therefore in addition to GnRHR the expression of the GRα was investigated.
A central aim of the present study was to investigate the role of GR in regulating expression of target genes. Therefore, experiments performed in the present study used the synthetic glucocorticoid (GC) Dex, which is a GR-specific agonist with a similar efficacy as the natural GC cortisol that is an agonist for both the GR (Ronacher et al., 2004, Ronacher et al., 2009) and mineralocorticoid receptor (MR) (Reul et al., 1990). Although the synthetic agonist Dex is a full agonist with similar efficacy to cortisol, but greater potency, it is possible that different responses could be obtained for cortisol compared to Dex. Thus future experiments should be performed with the more physiologically relevant ligand cortisol. Firstly, it was determined whether the activity of the GR could be detected in the primary mouse pituitary and primary mouse gonadotrope cell model systems. Therefore, whether Dex regulated the mRNA levels of the well-established GRE-containing SGK-1 and GILZ genes was determined. The results showed that Dex increased SGK-1 and GILZ mRNA levels in WT mouse pituitaries to the greatest extent after 8h treatment for the time-points investigated (Figure 4.1.1 and 4.2.1). Interestingly, the Dex-induced increase of GILZ mRNA expression was more rapid compared to the SGK-1 gene. In addition, Dex increased GILZ mRNA levels to a greater extent than SGK-1 mRNA levels (Figure 4.1.1 and 4.2.1). SGK-1 has previously been shown to co-localize with ACTH in human pituitary tissue, which indicates that SGK-1 is expressed in corticotropes (Reiter et al., 2011). The same study reported that Dex increased SGK-1 mRNA levels in the AtT-20 mouse corticotrope cell line, which is consistent with results obtained in the present study. The increase in GILZ mRNA levels in the pituitary with Dex treatment is similar to a previous report that showed that corticosterone increased the expression of GILZ mRNA levels in the pituitary of chicken embryos (Ellestad et al., 2009), suggesting that pituitary GILZ mRNA regulation by Dex is conserved in mammals and birds. Taken together, these findings suggest that Dex-mediated regulation of the GRE-containing SGK-1 and GILZ genes in pituitary tissue is conserved between different animal kingdoms which indicates that this response is most likely highly physiologically relevant. It is tempting to speculate that the GC-mediated expression of these genes in the pituitary has a role in cell growth, possibly the inflammatory responses and to control endocrine function (Anacker et al., 2013, Eddleston et al., 2007).

Similarly to the primary WT mouse pituitary tissue model, it was determined whether GR activity could be detected in primary mouse gonadotrope cells. Due to limited availability of genetically modified GRIC/R26-YFP mice and the low occurrence and yield of gonadotrope cells the number of experiments that could be performed with purified gonadotrope cells was limited. The stimulation time of 8h was selected for the experiments performed in primary gonadotrope cells since it showed the greatest increase in mRNA expression levels of SGK-1 and GILZ genes in WT mouse pituitary...
tissue (Figure 4.1.1 and 4.2.1). The 8h stimulation time-point also enables a direct comparison of results between the primary gonadotrope cells and the immortalized LβT2 mouse gonadotrope cell line. Figure 4.1.3 and 4.2.3 show that Dex significantly increased SGK-1 and GILZ mRNA levels in primary mouse gonadotrope cells, consistent with the results obtained in the gonadotrope cell line shown in Chapter 3 (Figure 3.5 A and E). To the current author’s knowledge, this is the first report showing that Dex increases the mRNA expression levels of the SGK-1 and GILZ genes in primary mouse gonadotrope cells.

The finding that Dex increased the mRNA expression of the GRE-containing SGK-1 and GILZ genes in WT mouse pituitary tissue and primary gonadotrope cells strongly supports a role for the GR in the pituitary. In addition, Dex is a well-known GR-specific agonist and it was shown in Chapter 3 that the Dex-mediated regulation of SGK-1 required the presence and activity of the GR (Figure 3.6 D and E). The finding that GR protein is detected in pituitary tissue and in most primary gonadotrope cells is consistent with a previous study showing that the GR co-localizes with LHβ in WT mouse pituitary tissue (Breen et al., 2012). The finding that Flot-1 is expressed in mouse pituitary tissue is consistent with a previous study that reported that Flot-1 is exclusively localized to membrane rafts in mouse pituitary cells (Bliss et al., 2007). The current result showing that Flot-1 is also expressed in primary mouse gonadotrope cells is consistent with unpublished results from the Roberson lab reporting Flot-1 and 2 are expressed in anterior pituitary gonadotropes as discussed in a review (Navratil et al., 2010). Bliss et al. also reported that both the GnRHR and ERK-1/2 localizes to Flot-1-containing membrane rafts in mouse pituitary cells (Bliss et al., 2007), which is consistent with results obtained in LβT2 gonadotrope cells showed in Chapter 3 (Figure 3.1.1 and 3.3.1 A).

Having established that the GR is expressed in the primary model systems, it was investigated whether the activity of the GR is required for the Dex-induced regulation of the SGK-1 and GILZ genes. The available quantity of GRIC/R26-YFP mice allowed this experiment to only be performed in WT mouse pituitary tissue from mice of mixed stages of the estrous cycle. The results presented in Figure 4.10 and 4.11 showed that the upregulation of SGK-1 and GILZ mRNA by Dex was significantly reduced in the presence of the antagonist RU486. RU486 is a GR- and PR-specific antagonist and was used since there is not a commercially available GR-only specific antagonist. Since Dex is a GR-specific agonist and the GR is highly expressed in most of the cells in the pituitary it is highly unlikely that the PR has any role in the Dex-induced responses on the SGK-1 and GILZ genes.
The results showed that PR mRNA was detectable in primary gonadotrope cells, but not in the pituitary tissue (Figure 4.9). Since the pituitary consists of multiple cell types of which only 5-15% are gonadotropes, it is possible that the PR mRNA expression in gonadotrope cells was below the limits of detection in the context of pituitary tissue RNA. However, it is also possible that the cDNA sample could have been degraded since it was used as the template in the Real-Time qPCR experiments before performing the conventional PCR with PR-B primers that is shown in Figure 4.9. In retrospect, it would have been helpful to perform another PCR reaction with a different set of primers to determine whether the cDNA was degraded or not, and a housekeeping/reference gene control PCR reaction. Due to the limited availability of mice and the absence of a suitable commercially available PR-specific antibody it was not possible to determine whether PR protein is expressed by immunofluorescence or Western blot analysis in pituitary tissue or primary gonadotrope cells. Although PR protein may be expressed in primary gonadotrope cells, since Dex does not act as a PR agonist at the concentration of 100 nM, collectively the findings strongly support a role for the GR in mediating upregulation of the SGK-1 and GILZ mRNA levels in response to Dex via transactivation of these GRE-containing genes in mouse pituitary tissue and primary gonadotrope cells. Dex increased SGK-1 mRNA levels in both pituitary tissue and primary gonadotrope cells to a similar extent, which suggests that this response is not influenced by paracrine effects within the pituitary. However, the Dex-induced upregulation of GILZ mRNA levels was much greater in pituitary tissue compared to primary gonadotrope cells, which suggests that the Dex-induced upregulation of GILZ mRNA is greater in the other cell types within the pituitary. Taken together, the findings indicate that Dex-induced expression of most of these GRE-containg genes in primary gonadotropes is not modulated by paracrine effects within the pituitary.

Having established that the activity of the GR is detectable in the model primary tissue and cell systems, it was investigated whether Dex regulates GnRHR mRNA expression levels in the primary model systems. The finding that 8h Dex treatment significantly upregulated the mRNA levels of the GnRHR gene in primary gonadotrope cells (Figure 4.3.3) is similar to previous studies performed in the immortalized mouse gonadotrope cell line LβT2 (Kotitschke et al., 2009; McGillivray et al., 2007). Interestingly, treatment of WT pituitary tissue with Dex for 8h also increased GnRHR mRNA levels significantly (Figure 4.3.1). Dex treatment for 8h regulated GnRHR mRNA levels to a similar extent in proestrous and metestrous stages, but not in the estrous and diestrous stages of the cycle, which suggest that the Dex-mediated response is affected by circulating E2 and P4 hormone levels (Figure 4.3.2). Importantly, it has previously been established that in the pituitary the GnRHR is expressed only in gonadotrope cells (Ehlers and Halvorson 2013, La Rosa et al., 2000). Taking these
findings together suggests the Dex-mediated regulation of GnRHR mRNA levels in gonadotrope cells is not modulated by paracrine responses of other cell types in the pituitary.

The result showing that the antagonist RU486 decreased the Dex-induced expression of the GnRHR mRNA levels in WT mouse pituitaries provides strong support that the response is mediated by the GR. This is consistent with a requirement for the GR in the Dex-mediated upregulation of the endogenous GnRHR gene and exogenously transfected reporter gene in LβT2 cells (Kotitschke et al., 2009, McGillivray et al., 2007, von Boetticher 2008). In the rat GGH3 cell line Dex was found to upregulate a transfected mouse GnRHR promoter reporter gene (Maya-Nunez and Conn 2003). Although the detailed mechanism of Dex regulation in the primary models could not be further investigated, it is likely to be similar to that established in LβT2 cells and involves the GR, GnRHR and GRIP-1 (Kotitschke et al., 2009). It has previously been reported that the responsiveness of the pituitary to GnRH is dependent on the GnRHR numbers expressed on the cell surface of the gonadotropes (Kaiser et al., 1993; Norwitz et al., 1999). Taken together, these findings indicate that Dex modulates GnRHR mRNA expression through direct effects on the pituitary and gonadotrope cells, which suggests that an elevated stress level increases pituitary gonadotrope function. This Dex-induced upregulation of GnRHR mRNA levels in the pituitary and gonadotrope cells indicates that acute stress could be beneficial for reproduction. It would have been informative to determine if Dex increases GnRHR protein levels as well. This was unfortunately not possible to assess due to the limited availability of mice and GnRHR primary antibody. To the present author’s knowledge, this is the first report showing that GCs increase GnRHR mRNA levels in primary gonadotrope cells.

Having shown that the GR protein is detectable in mouse pituitary tissue and primary gonadotrope cells, it was investigated whether Dex treatment regulates GRα mRNA expression in the primary model systems. It has previously been shown that GRα mRNA levels are down-regulated by GCs in COS-1 cells and rat liver tissue (Burnstein et al., 1990, Freeman et al., 2004). The present study showed that treatment of mouse pituitary tissue with Dex did not result in repression of GRα mRNA levels (Figure 4.4.1). Although this finding is different to previous studies in the literature that reported the GC-induced down-regulation of GRα mRNA levels in the anterior pituitary of rats (Makino et al., 2001), this could be due to species-specific differences in the regulation of GRα mRNA expression in the pituitary. Interestingly, Dex did significantly directly repress GRα mRNA levels in primary gonadotrope cells (Figure 4.4.3). This finding suggests that the GRα mRNA levels are differentially regulated in the cell types of the pituitary.
Taking together the Dex-induced upregulation of GnRHR mRNA levels with the Dex-induced repression of GRα mRNA expression in primary gonadotrope cells is interesting as it shows a direct role for the GR in reproduction in the gonadotrope cells. The Dex-mediated decrease of GRα mRNA levels indicates the existence of a feedback mechanism to regulate the effects of GCs in gonadotrope cells. Furthermore, this could also be a mechanism whereby GCs mediates a transient effect on GnRHR mRNA levels to regulate reproductive function, although further investigation is required to obtain conclusive evidence for the physiological significance of these effects in vivo.

To obtain more insight into the role of GCs in mammalian reproduction, other key genes involved in reproduction, like the gonadotropins, were investigated. The GR has previously been reported to attenuate the secretion of LH in male rats and ovariectomized ewes undergoing elevated circulating levels of GCs induced by immobilization or psychosocial stress (Breen et al., 2007, Briski et al., 1995), although the underlying neuroendocrine mechanism of this effect is not well understood. Similar to the effect of GCs on LH secretion, GCs have previously been shown to decrease LHβ reporter gene expression by 30% in LβT2 cells (Thackray et al., 2006). In the present study the results in WT mouse pituitary tissue showed that the mRNA levels of the LHβ gene with 2h and 4h Dex treatment appeared to be increased (Figure 4.5.1), while Dex repressed LHβ mRNA expression in primary mouse gonadotrope cells (Figure 4.5.3), consistent with findings reported in LβT2 cells (Thackray et al., 2006). The difference in these results suggest that paracrine effects within the pituitary can modulate the outcome of effects in the gonadotrope cells. To the author’s knowledge, the present study is the first to report repression of LHβ gene expression with in vitro Dex treatment in primary mouse gonadotrope cells. Taken together, the findings suggest a possible mechanism for GCs to directly result in repression of gonadotrope and reproductive function.

Having shown that Dex represses the mRNA expression of the LHβ gonadotropin gene in primary gonadotrope cells, it was investigated whether Dex regulate FSHβ gonadotropin mRNA expression in the primary model systems. GCs have been shown to upregulate FSHβ mRNA in LβT2 cells (Thackray et al., 2006) and in mouse and rat pituitary cultures (Bohnsack et al., 2000, Kilen et al., 1996, McAndrews et al., 1994, Ringstrom et al., 1991). In the present study a general trend was observed for most time-points in all stages of the estrous cycle where Dex increased FSHβ mRNA levels in WT mouse pituitary tissue, similar to results obtained for the LHβ gene, although the latter showed lower fold apparent inductions. However, no effect with Dex was observed in primary gonadotrope cells, suggesting that upregulation of FSHβ mRNA levels is not mediated by direct effects of GCs alone on gonadotrope cells but is due to the modulation of paracrine effects in the
pituitary. Additionally, the different findings in the LβT2 cell line compared to primary gonadotrope cells (Figure 3.5.D and 4.6.3) (Thackray et al., 2006), suggests that the cell line exhibits different regulatory mechanisms for FSHβ compared to primary gonadotrope cells. Taken together, the findings suggest that the Dex-induced increase of FSHβ gene expression in WT pituitary tissue may be strongly influenced by paracrine effects that modulates gonadotrope cells and results in the upregulation of FSHβ mRNA levels.

To further investigate the role of GCs in mammalian reproductive function the GC-mediated mRNA expression of the αGSU gene was investigated. The αGSU gene expresses the common α-subunit for both the gonadotropins, LH and FSH. Previous studies have reported that corticosterone increased, decreased or had no effect on αGSU mRNA levels in rat pituitary cell cultures (Kilen et al., 1996, McAndrews et al., 1994, Ringstrom et al., 1991), while Dex increased expression of a human αGSU reporter gene in LβT2 cells (Sasson et al., 2008). In the present study Dex treatment appeared to upregulate αGSU mRNA levels at all the time-points investigated in pituitary tissue (Figure 4.7.1), although statistical significance could not be established. Dex did not induce mRNA expression of the αGSU gene in primary gonadotrope cells, consistent with the results of Chapter 3 (Figure 3.9).

To the author’s knowledge, the present study is the first to investigate expression of the endogenous αGSU gene in primary mouse gonadotrope cells. The results of the present study, taken together with the literature, collectively suggest that the mRNA expression of the αGSU gene is not regulated by Dex in pituitary tissue or gonadotrope cells.

Taken together these findings indicate that GCs regulate the mRNA expression of the gonadotropins in a gene-specific manner either in pituitary tissue or primary gonadotrope cells. It should be noted that a lack of detection of regulation of mRNA levels does not preclude regulation of expression of a gene, since this may occur at the level of translation, protein synthesis or cellular secretion, which was not investigated in the current study, and is known to be involved in the regulation of gonadotropin genes.

5.6 The GnRH signalling pathway regulates the expression of well-established GR-target and reproductive genes in pituitary tissue and primary gonadotrope cells

Having shown in the previous section that the mRNA levels of GRE-containing and key reproductive genes are regulated by Dex via the GR, in pituitary tissue, whether GnRH regulates mRNA levels of
these genes in WT mouse pituitary tissue and primary gonadotrope cells was subsequently investigated. GnRH treatment did not increase mRNA levels of the GILZ gene in WT mouse pituitary tissue or primary gonadotrope cells, consistent with the result in LβT2 cells (Figure 3.5 of Chapter 3). However, treatment with GnRH for 8h resulted in significant upregulation of SGK-1 mRNA levels in WT mouse pituitary tissue and primary gonadotrope cells (Figure 4.1.1 and 4.1.3). This novel finding is similar to the result obtained in Chapter 3 that showed that GnRH-induced mRNA levels of the SGK-1 gene in immortalized gonadotrope cells through a GR-dependent mechanism. The antagonist RU486 did not significantly repress the GnRH-induced mRNA levels of the SGK-1 gene in WT mouse pituitaries, although a smaller fold induction in the presence of RU486 was obtained, which suggests that the GR plays a role in the GnRH-mediated upregulation of the SGK-1 gene (Figure 4.10). The finding of RU486 not significantly repressing the GnRH-induced mRNA expression of the SGK-1 gene in pituitary tissue (Figure 4.10) is similar to the result of Chapter 3, which is consistent with a previous study performed by Kotitschke et al. which reported that ligand-independent activation of the GR by GnRH is insensitive to RU486 in LβT2 cells (Kotitschke et al., 2009). In addition, Chapter 3 also provided evidence that GnRH induced GR recruitment to the GRE region of the SGK-1 promoter. Therefore, it is likely that the response is mediated by a similar mechanism in both primary gonadotrope cells and pituitary tissue.

The mechanism of gene-specific regulation by GnRH of GRE-containing genes has not previously been characterized. Although the present study suggests that both the GR and PKC are required for the effect of GnRH on SGK-1 expression in LβT2 cells (Figure 3.6 and 3.8). It is tempting to speculate that activation of the GR by GnRH results in distinct conformational changes within the GR to allow differential association with other co-factors on some promoters such as that of the SGK-1 gene, but not others like the GILZ gene. It may also be possible that the GnRH-induced activation of PKC results in post-translational modifications of proteins involved in the regulation of the SGK-1 gene, but not the GILZ gene. Taken together, the findings suggest that GnRH mediate ligand-independent activation of the GR to induce the upregulation of GRE-containing genes in a gene-specific manner in pituitary tissue and primary gonadotrope cells.

The transcriptional regulation of the GnRHR gene by GnRH has been the focus of several groups. Studies performed in αT3-1 cells and the more mature LβT2 gonadotrope cells described the upregulation of GnRHR mRNA levels (Norwitz et al., 1999, Sadie et al., 2003, White et al., 1999). Kotitschke et al. reported that in LβT2 cells the GnRH-induced increase in GnRHR mRNA levels requires the AP-1 cis-element and Bedecarrats et al. showed that GnRH slightly increases the
activity of a GnRHR reporter construct (Bedecarrats and Kaiser 2003, Kotitschke et al., 2009), which suggests that the mRNA increase is mediated via a transcriptional response. In the current study, treatment with GnRH for 8h increased GnRHR mRNA levels in WT pituitary tissue of mice in the proestrous stage, but not the other stages of the estrous cycle (Figure 4.3.1 and 4.3.2), which suggests that the GnRH-induced response is sensitive to the fluctuating E$_2$ and P$_4$ hormone levels. However, due to the variation observed for the Dex- GnRH- and Dex + GnRH-induced GnRHR mRNA levels throughout the time-points investigated for each stage of the cycle, it is not possible to conclusively interpret the general effect of the fluctuating E$_2$ and P$_4$ hormone levels.

In the present study, GnRH significantly upregulated GnRHR mRNA levels after 8h in pituitary tissue (Figure 4.3.1). Similarly to the result obtained for the SGK-1 gene, RU486 did not significantly repress the GnRH-mediated upregulation of GnRHR mRNA levels in pituitary tissue (Figure 4.12). This suggests that this effect is not mediated via the GR or the PR, although it is likely that the GR is involved as this has previously been reported in LβT2 cells (Kotitschke et al., 2009). The finding that GnRH did not increase GnRHR mRNA levels in primary mouse gonadotrope cells was surprising as it is different to other reports in the literature (Kotitschke et al., 2009; Sadie et al., 2003; White et al., 1999). The studies by Sadie et al. and White et al. were both performed in αT3-1 cells with a GnRHR reporter construct, whereas the study of Kotitschke et al. was performed on the endogenous GnRHR gene in LβT2 cells, which suggests that the GnRHR gene is regulated differently in primary gonadotrope cells compared with immortalized gonadotrope cell lines. Furthermore, the finding in the present study that continuous stimulation with GnRH significantly increased GnRHR mRNA levels in pituitary tissue is also different to a previous study that reported continuous stimulation with GnRH of rat pituitary cells had no effect on GnRHR mRNA levels (Yasin et al., 1995). It is possible that the GnRH-mediated regulation of GnRHR mRNA expression in primary gonadotrope cells is modulated by paracrine effects from the other cell types within the pituitary. Furthermore, it is possible that the experimental procedure performed to isolate the primary gonadotrope cells could have affected the integrity of their membrane proteins. However, this is unlikely since other genes like SGK-1 are still regulated by GnRH. Taken together, these findings indicate that mRNA expression of the GnRHR gene is increased by GnRH in pituitary tissue through a conserved mechanism in mammals. They suggest that modulation by paracrine effects are required for the GnRH-induced upregulation of gonadotrope GnRHR mRNA levels in pituitary tissue.

Regulation of the GRα gene by GnRH in pituitary tissue has to the present author’s knowledge not been investigated to date. GnRH treatment in WT mouse pituitary tissue did not appear to result in
significant regulation of GRα mRNA levels (Figure 4.4.1). However, both GnRH and Dex alone significantly reduced the expression of GRα mRNA expression to a similar level in primary mouse gonadotrope cells (Figure 4.4.3). This finding is consistent with previous data obtained in the Hapgood laboratory that showed GnRH significantly repressed the mRNA levels of the GRα gene by 50% in LβT2 cells (Hills 2011). This finding suggests that GnRH, just like Dex, appears to be involved in a signalling feedback mechanism that controls the level of GRα mRNA expression in gonadotrope cells. The Dex- and GnRH-induced decrease in GRα mRNA levels may lead to a decreased stress-induced response over a longer period of time, such as during chronic stress. Taken together, these findings indicate that GnRH is playing a similar role as Dex in GR mRNA regulation and is consistent with the finding that GnRH induces a ligand-independent activation of the GR in gonadotrope cells.

The aim of the following part of the present study was to investigate whether GnRH regulates the mRNA expression of the gonadotropin genes in the primary model systems. Continuous treatment of anestrous cows with GnRH has previously been reported to decrease the mRNA expression levels of LHβ in the pituitary (Vizcarra et al., 1997). However, continuous GnRH treatment of mice has been reported to increase LHβ mRNA levels in pituitary tissue and immortalized LβT2 gonadotrope cells (Breen et al., 2012, Sharma et al., 2013). Therefore, it is surprising that treatment with GnRH did not appear to regulate the mRNA expression of the LHβ gene in WT mouse pituitaries (Figure 4.5.1). However, GnRH administration by Breen et al. was through subcutaneous injection of the mice (Breen et al., 2012), while Vizcarra et al. infused the anestrous cows with GnRH (Vizcarra et al., 1997). Taken together, this indicates that the method of GnRH administration influences the regulation of the LHβ gene or may be a result of indirect in vivo effects. The current findings suggest that the mRNA expression of the LHβ gene in mouse pituitary tissue is unresponsive to direct continuous in vitro GnRH stimulation, which is supported by a previous study that reported continuous GnRH stimulation of rat pituitary cultures had no effect on LHβ mRNA expression levels (Weiss et al., 1989). The finding that GnRH did not regulate the mRNA expression of the LHβ gene in primary gonadotrope cells is different to a previous study performed by Breen et al. in LβT2 cells that reported GnRH induced a small increase in LHβ mRNA levels (Breen et al., 2012), which suggests that the GnRH-induced regulation of LHβ mRNA levels in the gonadotrope cell line is different than the primary gonadotrope cells. It is also possible that the GnRH-induced regulation of the LHβ gene occurs predominantly at the protein level, either via protein synthesis or release and secretion of pre-synthesized LH contained in intracellular storage granules (McNeilly et al., 2003).
In the present study, treatment of WT mouse pituitary tissue for 4h with GnRH significantly upregulated the mRNA expression of the FSHβ gene (Figure 4.6.1). This finding indicates that the FSHβ gene is responsive to continuous and direct GnRH treatment, unlike the LHβ gene. However, this result is different to a previous study that reported GnRH had no effect on FSHβ mRNA expression levels and decreased the protein expression levels of FSHβ in the pituitary of anestrous cows (Vizcarra et al., 1997). However, that study was performed in cows and the pituitaries were stimulated with GnRH for 13-days (Vizcarra et al., 1997). This indicates that the decrease in FSHβ mRNA levels in cows could be a result of indirect effects. The finding that 8h GnRH treatment did not induce the mRNA expression of the FSHβ gene in primary gonadotrope cells (Figure 4.6.3) is similar to a previous study by Bédécarrats and Kaiser that reported GnRH did not affect the transcriptional activity of an FSHβ reporter gene in LβT2 cells (Bédécarrats and Kaiser 2003). This finding is different to the result of Chapter 3 shown in Figure 3.5D, indicating that this gene is regulated differently in primary gonadotrope cells compared to the gonadotrope cell line. The present study is the first to investigate the GnRH-mediated regulation of the FSHβ gene in primary gonadotrope cells. Taken together, the mRNA levels of FSHβ and LHβ genes are both repressed by continuous GnRH stimulation in primary mouse gonadotrope cells. It is interesting that the regulation of these genes with GnRH is different between pituitary tissue and primary gonadotrope cells, which indicates that paracrine effects are involved in regulating their expression.

It has previously been reported that GnRH increases the mRNA expression of the αGSU gene in primary rat pituitary cell cultures (Kaiser et al., 1997). Consistent with this effect, and arguing against paracrine effects, GnRH treatment in the present study resulted in a significant increase in αGSU mRNA levels in primary mouse gonadotrope cells. This result is also consistent with the result obtained in Chapter 3 shown in Figure 3.9 and a previous study that reported GnRH to increase the activity of a αGSU reporter gene in LβT2 cells (Sasson et al., 2008).

Taking together the results obtained for the gonadotropin genes suggest that mRNA expression levels are regulated by GnRH in a gene-specific manner and selectively in either pituitary tissue or primary gonadotrope cells. Treatment of the primary model systems with GnRH increased the mRNA expression levels of the FSHβ gene only in pituitary tissue and the αGSU gene only in primary gonadotrope cells, which suggests that the GnRH-induced regulation of the gonadotropin genes is modulated by paracrine effects within the pituitary in a gene-specific manner. Although it appears that the mRNA expression of the LHβ gene is not regulated by GnRH in pituitary tissue, but it is possible that the GnRH-induced regulation may only occur at the protein level.
5.7 Reciprocal modulation between Dex- and GnRH-induced gene expression responses upon co-stimulation in pituitary tissue and primary gonadotrope cells

The following part of the present study investigated whether co-stimulation with Dex + GnRH results in reciprocal modulation in the primary model systems. Interestingly, for each gene investigated in Chapter 4, a general trend was observed whereby co-stimulation with Dex + GnRH appeared to result in either potentiation or attenuation of the Dex- or GnRH-induced response. Furthermore, co-stimulation with Dex + GnRH appeared to result in attenuation of mRNA levels more than potentiation. There was no evidence for a synergistic increase or decrease in mRNA expression levels observed for any of the genes investigated in the primary mouse gonadotrope cells. These results are summarized in Table 5.1. However, statistical significance could not be established for most of these apparent effects. This is most likely since the responses obtained were within the range of experimental error and biological variation, with the number of variables and repeat experiments giving insufficient statistical power to establish significance. Attenuation of Dex- or GnRH-induced mRNA expression responses by co-stimulation may be mediated by the recruitment of different co-factors in a promoter-specific manner compared to the hormones in isolation. Similarly, attenuation versus potentiation effects may be time- and promoter-specific and also depend on the combination of other hormones present, like the variation in E2 and P4 levels during the estrous cycle. The finding that the GnRH-induced increase of αGSU mRNA levels was statistically significantly attenuated in the presence of Dex in primary gonadotrope cells is novel and provides evidence that Dex and GnRH reciprocally modulate mRNA levels directly in primary gonadotrope cells. The trend shown in Table 5.1 whereby co-stimulation with Dex + GnRH results in attenuation of mRNA levels of key reproductive genes, compared to each hormone alone, supports a role for the GR in repressing reproductive function directly in the pituitary and in gonadotropes during acute stress conditions. Since this attenuation was more consistently observed in pituitary tissue than in gonadotrope cells, and since the GnRHR and gonadotropin genes are predominantly expressed in gonadotrope cells within the pituitary, the results suggest that the GR mediated effects are partly due to direct effects of Dex and GnRH on gonadotrope cells, and partly due to paracrine effects from other cell types modulating the responses of gonadotrope cells.

It was surprising that conclusive synergistic responses with Dex + GnRH co-stimulation were not detected in pituitary tissue at any stage of the estrous cycle and any time-point investigated for the SGK-1 gene. The pituitary consists of many different cell types which secrete a mixture of different
hormones that exert paracrine effects which could affect the Dex + GnRH-induced regulation of SGK-1 mRNA expression. For instance, a specific cell type in the pituitary could secrete a hormone that partially inhibits the activity of the GR or other proteins involved in SGK-1 gene expression within gonadotrope cells at a stimulation time-point other than 8h, or at 8h in a different cell type. The lack of synergism could also be due to some of the differences that were observed between the primary and immortalized gonadotrope cells, as discussed in more detail in section 5.9. Nonetheless, although evidence for synergism was not obtained in the primary model systems, the Dex + GnRH modulation effects observed, as mentioned above, suggest crosstalk between GR and GnRHR signalling pathways do occur in the primary systems.

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Table 5.1: Modulation of Dex- or GnRH-induced changes in mRNA levels upon co-stimulation in pituitary tissue or primary gonadotrope cells.

Co-treatment with Dex + GnRH results in potentiation or attenuation of the response induced by Dex or GnRH treatment in isolation for the genes shown in the Table. The greatest increase or decrease with the lowest amount of error in mRNA levels is shown for both or just one primary model systems. Fold-inductions are shown for each ligand, -: no detectable modulation effect, Time: stimulation time, Stage: stage of the estrous cycle.

Comparison of the results in primary gonadotropes and in pituitary tissue revealed similar responses for some genes that are predominantly expressed in gonadotrope cells, but not others, consistent with paracrine effects. This was observed for both Dex and GnRH treatment in isolation. For example, while both LHβ and FSHβ mRNA levels were increased with Dex in the tissue, a decrease or no effect was obtained with Dex for LHβ and FSHβ mRNA levels, respectively, in the primary gonadotrope cells. Similarly, while GnRH alone increased FSHβ mRNA levels and had no effect on αGSU mRNA levels in the tissue, it had no effect on FSHβ mRNA levels but increased αGSU
mRNA levels in the gonadotropes. The GnRHR mRNA levels were increased by both Dex and GnRH in the tissue but not in the gonadotropes, where only Dex had this effect. Continuous stimulation of pituitary tissue and primary gonadotrope cells with GnRH for up to 8 hours in vitro did not appear to result in regulation of mRNA levels for many of the genes investigated, with the exception of upregulation in mRNA levels for the SGK-1, FSHβ and GnRHR genes in the pituitary tissue and upregulation of the SGK-1 and αGSU genes and the attenuation of GRα mRNA in the gonadotrope cells. Taken together, these findings indicate that paracrine effects in the pituitary strongly influence the Dex- and GnRH-induced mRNA expression levels in a gene- and primary model system-specific manner.

5.8 The estrous cycle appears to modulate the Dex-, GnRH- or Dex + GnRH-induced mRNA expression levels of selected genes in the pituitary

Whether the estrous cycle plays a role in regulation of the gene expression responses induced by the ligands alone or in combination in the pituitary was also investigated. Unfortunately, due to the large number of variables and small responses it was not possible to establish statistical significance for most of the differences observed with Dex-, GnRH- or Dex + GnRH-induced responses in the mRNA expression levels for the selected genes. However, a trend was observed whereby responses for some target genes showed the greatest fold changes in response to Dex as compared to GnRH and Dex + GnRH, between stages and time points. SGK-1 showed significant upregulation with Dex and the greatest fold-induction was observed during the metestrous stage at the 8h time-point. GnRH appears to regulate mRNA expression of the SGK-1 gene only in the proestrous stage. Furthermore, co-treatment with Dex + GnRH appeared to result in synergistic upregulation of SGK-1 mRNA levels only after 1h treatment in the estrous stage. Dex treatment increased GILZ mRNA levels after 8h treatment by ~8- to 16-fold in the estrous, diestrous and metestrous phases, but only by ~3- to 4-fold in the proestrous phase. Furthermore, GnRH appeared to slightly attenuate the Dex-induced upregulation of GILZ mRNA levels, although not in a time- or stage-dependent manner. Dex regulated expression of the GnRHR gene in each stage of the estrous cycle and induced the greatest fold-induction in the metestrous stage, which also appeared to increase in a time-dependent manner, while GnRH significantly increased GnRHR mRNA levels only in the diestrous and proestrous stages, but at different time-points. Dex induced the greatest apparent increase in LHβ mRNA levels in the diestrous stage, while Dex appeared to increase FSHβ mRNA levels by ~4- to 8-fold for most time-points at most stages of the estrous cycle, but only by ~3-fold in the estrous stage. The GRα, LHβ and FSHβ mRNA levels appeared to be less subjected to estrous cycle stage or time-dependent
differences in regulation for all stimulation conditions. Even though these apparent estrous cycle stage- and time-mediated effects on the ligand-induced responses for most genes warrants confirmation with more repeat experiments, the results provide support in a primary tissue model for a mechanism whereby reproduction is regulated by fine-tuning of gene expression during different stages of the estrous cycle.

5.9 Comparison of Dex-, GnRH- and Dex + GnRH-induced regulation of gene expression between primary gonadotrope cells and the LβT2 cell line

Towards investigating the physiological relevance of the immortalized mouse LβT2 gonadotrope cell line, responses to hormones were compared in the cell line with those obtained in the primary mouse gonadotrope cells, and summarized in Table 5.2. To simplify the comparison between primary and immortalized gonadotrope cells, symbols are shown to distinguish between positive (Δ) and negative (ǂ) findings. Similar responses were observed between the immortalized and primary gonadotrope cells for the Dex- and GnRH-induced upregulation of SGK-1 mRNA levels, although co-treatment of the primary cells did not result in a synergistic response (Figure 3.5 E and 4.1.3). This indicates that mRNA expression of the SGK-1 gene is regulated differently in the primary and immortalized gonadotrope cells. Possible reasons for co-treatment with Dex + GnRH not synergistically increasing SGK-1 mRNA levels in primary gonadotrope cells may be due to a time-dependent effect, which would suggest a difference in the mechanism or a difference in the activity of proteins involved in the mechanism from a post-translational modification or different expression levels of proteins involved in the synergistic mechanism. In addition, regulation of the model genes investigated appeared to be more responsive to GnRH in the LβT2 cell line than the primary gonadotrope cells. Similar effects were observed on mRNA levels with GnRH treatment for the SGK-1, GILZ, GR, αGSU genes, but the effects on GnRHR, LHβ, and FSHβ genes were different between the primary cells and the cell line. Responsiveness to GnRH and the combination with Dex in the primary models may require mechanisms and signalling proteins for GnRH responsiveness on some genes that are different to the cell line. One noticeable difference observed between the gonadotropes in the pituitary tissue and the gonadotrope cell line is that the GR is predominantly nuclear in the former and predominantly cytoplasmic in the latter, in the absence of Dex. Thus, it is possible that there is less membrane-associated GR in the primary models, which may be required for the synergistic responses, as suggested by the results in the cell line. Additionally, there may be a greater expression level of GnRHR protein in the cell line.
However, it could also be possible that Dex + GnRH-mediated synergism is an effect that is only observed in immortalized transformed cells, and not present in physiologically relevant primary cells. The finding that Dex + GnRH treatment also did not result synergistic mRNA regulation of the GnRHR gene in primary gonadotrope cells is similar to the result observed for the SGK-1 gene and provides more evidence that a different mechanism regulates the Dex + GnRH-mediated responses in LβT2 cells compared to primary gonadotrope cells (Hills 2011, Kotitschke et al., 2009). However, it is possible that mRNA expression of the GnRHR gene in primary gonadotrope cells is dependent on the administration method of GnRH, such that it may only be responsive to pulsatile GnRH treatment.

Similar responses were obtained with the ligands for the mRNA expression levels of the GILZ gene in primary gonadotrope cells compared to the immortalized gonadotrope cell line. Although GnRH appeared to slightly increase the mRNA expression in primary gonadotrope cells, but significance could not be established. However, Dex + GnRH co-treatment appeared to result in an additive response in primary gonadotrope cells, unlike the Dex + GnRH-induced response that was similar to the Dex-induced response in LβT2 cells. The co-treatment of Dex + GnRH resulted in lower fold-induction responses for the GnRHR, LHβ and FSHβ genes in primary gonadotrope cells compared to the responses in LβT2 cells. The Dex-mediated upregulation of GnRHR mRNA levels was similar to previously reported findings in LβT2 cells (Hills 2011), whereas the continuous GnRH treatment did not increase mRNA expression levels of the GnRHR, LHβ or FSHβ genes in primary gonadotrope cells, like it did in LβT2 cells (Figure 3.5 D) (Breen et al., 2012, Hills 2011, Kotitschke et al., 2009). Interestingly, the most consistent ligand-induced responses between the primary and immortalized gonadotrope cells were observed for the GRα and αGSU genes, although the mRNA levels of both genes displayed slight variations in the fold-inductions. The Dex-induced response of GRα mRNA levels for primary gonadotrope cells was greater as in LβT2 cells, and αGSU mRNA levels displayed the same trend but in the LβT2 cells the basal level was slightly repressed with Dex and Dex did not attenuate the GnRH-induced increase of αGSU mRNA as greatly as in the primary cells (Table 5.2).

Taken together, these findings suggest that the LβT2 cell line is a physiologically relevant gonadotrope cell system to characterize the mechanisms involved in the Dex-, GnRH- and Dex + GnRH-induced mRNA expression for some of the genes investigated in the present study, like the GRα and αGSU genes under the same experimental conditions.
Primary gonadotrope cells | LβT2 cells
---|---
| | Dex (8h) | GnRH (8h) | D + G (8h) | Dex (8h) | GnRH (8h) | D + G (8h) |
SGK-1 | ~5.5 Δ | ~3 Δ | ~4 † | ~8 | ~4 | ~16 |
GILZ | ~2 Δ | ~1.5 Δ | ~2.5 † | ~1.5 | ~1 | ~1.5 |
GnRHR | ~2.5 Δ | ~0.75 † | ~1.75 † | ~4 * | ~3 * | ~9 * |
GRα | ~0.25 † Δ | ~0.5 Δ | ~0.25 Δ | ~0.5 * | ~0.5 * | ~0.25 * |
LHβ | ~0.75 † | ~1 † | ~0.75 † | ~1 # | ~2 # | ~1.5 # |
FSHβ | ~0.75 † | ~1.25 † | ~1 † | ~5 | ~3 | ~4 |
αGSU | ~1 Δ | ~2.25 Δ | ~1.5 Δ | ~0.75 | ~2.5 | ~2 |

Table 5.2: Comparison of fold changes in mRNA levels with Dex, GnRH and Dex + GnRH in primary gonadotrope cells and the LβT2 cell line.

Summary showing the fold-inductions obtained for the genes shown in the Table when primary gonadotrope cells and LβT2 cells were stimulated for 8h with Dex, GnRH or Dex + GnRH.

*: Results obtained from (Hills 2011) or #: (Breen et al., 2012), respectively. Δ: shows a similarity and †: shows a difference.

5.10 Conclusions

This is the first report demonstrating that in the LβT2 mouse gonadotrope cell line, both the GR and the GnRHR appear to co-localize to membrane rafts containing Flot-1, independent of treatment with Dex, GnRH or both together. It is also shown for the first time that in this cell line, crosstalk between the GR and GnRHR signalling pathways results in gene-selective and synergistic upregulation of mRNA levels of the endogenous GRE-containing SGK-1, but not the GILZ, FKBP5, MKP-1, αGSU or FSHβ genes. Several insights into the membrane-mediated mechanism were obtained, including the requirement for Flot-1-containing membrane rafts, PKC and SGK-1 to synergistically attenuate proliferation of gonadotropes. These findings suggest a novel mechanism whereby the effects of stress modulate gonadotrope function. Strong support for a physiologically relevant role for the GR in mammalian reproduction was obtained in mouse pituitary tissue and primary gonadotrope cells in vitrō. Immunohistochemistry of mouse pituitary sections showed that the GR protein is expressed in the majority of the cells present in the pituitary, including the gonadotrope cells. Dex upregulated mRNA levels in pituitary tissue of the SGK-1 and GILZ genes, in both the tissue and gonadotropes, the effect of which was reversed by the GR antagonist RU486 in the pituitaries. Furthermore, Dex upregulated mRNA levels of the key GnRHR, LHβ, FSHβ and αGSU reproductive genes in pituitary tissue, and GnRHR mRNA levels in the primary gonadotropes, suggesting multiple levels of
upregulation of reproduction by glucocorticoids directly at the level of the pituitary in response to acute stress. Interestingly, both Dex and GnRH alone decreased GRα mRNA levels in the primary gonadotropes, suggesting a mechanism for negative feedback of glucocorticoids at the gonadotrope level, which may contribute to differential effects of acute versus chronic stress. Comparison of the results in gonadotrope versus pituitary tissue revealed similar responses for some genes predominantly expressed in gonadotrope cells, but not others, consistent with paracrine effects. Taken together the results provide evidence for extensive paracrine effects on direct modulation of expression of gonadotropin genes at the level of the whole pituitary.

Given the established role of GnRH in mammalian reproduction at the pituitary level, the gene expression results suggest that the primary tissue and cells are not as responsive to direct in vitro continuous stimulation with GnRH as they would be to pulsatile GnRH stimulation, and/or that most of the direct effects of GnRH occur at the post-transcriptional level and/or that effects of GnRH on the pituitary are modulated significantly by other hormones in the blood in vivo. Nevertheless, the upregulation of GnRHR and FSHβ levels in the pituitary tissue would be consistent with at least in part the occurrence of transcriptional mechanisms for GnRH upregulation of reproductive function directly at the pituitary level.

No evidence was obtained for synergism on SGK-1 mRNA levels or for any other gene investigated in pituitary tissue at any stage of the estrous cycle, or in primary gonadotrope cells incubated under similar conditions with Dex + GnRH as the cell line. This suggests that the synergism may not be physiologically relevant in primary cells, or that the conditions under which it occurs may differ in the primary models, or that the primary models have other confounding factors that preclude direct comparison. Although synergism was not observed in the primary models, a trend was observed whereby Dex and GnRH did exhibit reciprocal modulation of mRNA levels, mostly causing attenuation in both pituitaries and primary gonadotrope cells, suggesting crosstalk of the GR and GnRHR signalling pathways directly at the level of the pituitary. The finding also suggest that mechanisms exist whereby Dex can gene-specifically both increase and decrease reproductive function, depending on GnRH levels. Preliminary data was obtained for a role of membrane rafts in GR and GnRHR signalling in the pituitary tissue and in gonadotropes, it was shown for the first time by immunohistochemistry in mouse pituitary sections that Flot-1 protein is expressed in the majority of the pituitary cells including gonadotrope cells. The distribution of Flot-1 within all the pituitary cells was predominantly localized to the plasma membrane region as well as intracellular compartments, which is similar to findings obtained in the gonadotrope cell line. However, whether
the GR and Flot-1 co-localize in primary mouse gonadotrope cells, as well as its functional role in
the primary models could unfortunately not be determined due to technical and logistical constraints.

Towards investigating whether the LβT2 cell line is a physiologically relevant model for primary
gonadotrope cells, it was noted that regulation of the model genes investigated appeared to be more
responsive to GnRH in the LβT2 cell line than the primary gonadotrope cells. In addition, although
similar effects were observed on mRNA levels with GnRH or Dex for some genes, other genes
showed different effects when comparing results between the primary cells and the cell line. Most
strikingly, as mentioned above, the synergistic responses observed in this study and in previous work
on the SGK-1 and GnRHR genes for Dex + GnRH in the cell line were not observed under the same
apparent conditions for the primary gonadotropes. Taken together, the findings suggest that although
the LβT2 cell may be used to investigate mechanisms of gene regulation for some genes via Dex and
GnRH, the results need to be verified in primary cells. It is, however, possible that other confounding
factors may have affected the comparison, such as a loss of some functions during primary
gonadotrope cell purification.

Differential regulation of the selected genes during different stages of the estrous cycle would
suggest modulation of responses due to changes in circulating levels of E$_2$ and P$_4$ and possibly other
cycle-related factors. Apparent stage-specific and time-dependent differences in mRNA levels were
observed for all the genes investigated after stimulation with Dex, GnRH or the combination.
However due to the large number of variables and small fold changes for some of the responses, it
was not possible to establish statistical significance for some of the differences. Thus while the
results indicated different patterns of gene-specific, stage-specific and time-dependent regulation of
mRNA levels for most of the genes, many of the responses need to be confirmed with more repeat
experiments and with fewer variables to establish significance in most cases. However the results
certainly provide strong support in a primary tissue model for mechanism whereby reproduction is
regulated by fine-tuning of gene expression during different stages of the estrous cycle and as a
function of time.

The finding that Dex increased GnRHR mRNA levels but attenuated the GnRH-induced
upregulation of αGSU mRNA levels in primary gonadotrope cells suggests a mechanism whereby
Dex could gene-specifically either increase or decrease reproductive function, depending on GnRH
levels. Additional evidence supporting the central role of the GR in pituitary tissue was determined
with immunohistochemistry showing that localization of the GR in the majority of pituitary cells is
exclusively nuclear in the absence of ligands which could be one of the mechanism that maintains cellular sensitivity to effects of acute and chronic stress, by ensuring the localization of the unliganded GR primed for mediating reciprocal modulation with other signalling pathways.

5.11 Future perspectives

The results of the present study show that crosstalk between the GR and GnRHR involves the localization of these receptors to membrane rafts to mediate an apparently synergistic increase of SGK-1 mRNA levels in a PKC- and Flot-1 -dependent manner in the LβT2 gonadotrope cell line. However, many questions remain unanswered.

As briefly mentioned in the discussion, the present study did not investigate whether the Dex + GnRH induced synergistic increase of SGK-1 mRNA levels is solely due to a direct transcriptional effect of the GR on the SGK-1 promoter, or whether indirect mechanisms such as de novo protein synthesis or effects on mRNA stability are involved. The former can be investigated by inhibiting protein synthesis with cycloheximide and determining whether synergistic SGK-1 mRNA levels are still detectable. Effects on mRNA stability could be investigated in future using strategies involving the general transcriptional inhibitor actinomycin D.

The present study suggests that the GR mediates the GnRH response on the SGK-1 gene in the cell line and primary models. However, knock down of the GR in the cell line did not result in conclusive evidence to answer this question, due to the small fold increase with GnRH. In addition, using the GR/PR antagonist RU486 is not diagnostic since it was previously shown to not inhibit ligand-independent GR activation (Kotitschke et al., 2009). Additional experiments to investigate this question could involve overexpression of the GnRHR to increase the GnRH response in the cell line, or work with conditional gonadotrope-specific GR knockout mice. Involvement of the PR in the GnRH response on the SGK-1 gene in the cell line is unlikely since saturating concentrations of PR agonists also did not appear to induce transcription of SGK-1 or synergize with GnRH. However, PR mRNA was detected in the primary gonadotrope cells and the involvement of the PR in primary cells cannot be excluded based on the results of this study. Further experiments to confirm that the PR is not involved in the GnRH- or Dex + GnRH-induced responses in the cell line could include siRNA knock down of PR mRNA. For the primary cells a Western blot could be performed to detect PR protein, or experiments using a PR agonist similar to those done in the cell line, could be performed on the tissue and primary gonadotrope cells.
The present study showed that expression of the membrane raft structural protein Flot-1 is required for the synergistic effect on SGK-1 mRNA in the cell line. Whether Flot-1-containing membrane rafts are required for phosphorylation of the GR, and whether GR phosphorylation is required for synergism has not been conclusively established. Decreasing Flot-1 protein levels by 60% had no effect on the basal or ligand-induced GR phosphorylation at any of the residues investigated. However, it is possible that a greater percentage knockdown of Flot-1 protein levels may be required to affect GR phosphorylation. Thus other strategies for increasing transient or stable Flot-1 knockdown could be pursued, such as using lentivirus. Additionally, a gonadotrope-specific conditional Flot-1 knockout mouse model could be explored, but the SGK-1 synergism and GR phosphorylation should firstly be established in this primary model system. Whether the selected GR phosphorylation residues are required for the SGK-1 synergistic response could also be investigated using overexpression of GR phosphorylation mutants after knock down of endogenous receptor, using a published approach (Matthews et al., 2015). Whether GR phosphorylation plays a role in the synergism could be investigated by determining if the phosphorylated GR is recruited to the promoter of the SGK-1 gene in LβT2 cells, using established phospho-specific anti-GR antibodies. Besides the GR phosphorylation residues investigated here, whether other GR phosphorylation sites are involved in the SGK-1 synergism could be investigated by the same strategies. Such residues include Ser-134, Ser-404 and Ser-267 (Galliher-Beckley et al., 2008, Galliher-Beckley et al., 2011, Lambert et al., 2013).

It would be interesting to investigate the mechanism of membrane raft association for the GR, as this would give insight into the proteins responsible for targeting the GR to membrane rafts. A few studies have shown that a highly conserved nine amino acid motif in the LBD of ERα and β, PR-A and B and AR are involved in plasma membrane targeting of these receptors. This domain was shown to contain a palmitoylation site that is important for membrane localization of these receptors (Groeneweg et al., 2011, Pedram et al., 2006, Strehl and Buttgereit 2014). These residues are conserved in the GR (Groeneweg et al., 2011, Samarasinghe et al., 2012). Thus, it is possible that palmitoylation could play a role in targeting the GR to membrane rafts. However, a previous study showed that mutation of this palmitoylation residue in the human GR did not alter its membrane localization in CHO cells (Samarasinghe et al., 2011), suggesting that the mechanim may be different for the GR. Nevertheless, specific inhibitors of palmitoylation (fluoropalmitic acid) or myristoylation (hydroxymyristic acid) could be used to investigate this for the GR in LβT2 cells (Strehl and Buttgereit 2014), but it should first be determined whether the GR in these cells is
palmityollated or myristoylated. This can be determined with Western blot experiments after incubation of the cells with radioactively-labelled fatty acids, like tritiated ($^3$H)-palmitic acid or ($^3$H)-myristic acid (Galluzzo et al., 2007, Rokaw et al., 1996) or by performing mass spectrometry.

Synergism on the SGK-1 gene has previously been shown to be induced by co-treatment with Dex and brain-derived neurotrophic factor (BDNF) and is mediated via a composite transcriptional mechanism that involves the recruitment of the GR and GRIP-1 to the GRE site as well as CREB and p300 to the CRE site. The role of the CRE and other cis-elements in the promoter of the SGK-1 gene in mediating the Dex + GnRH synergism could be further investigated with ChIP assays. It is possible that the CRE and NF-κB sites may recruit complexes containing CREB, p50 or p65 transcription factors. However, the results of the present study did investigate the recruitment of CBP and determined that it was not recruited to the promoter with Dex + GnRH. However, this was only investigated at one stimulation time-point of 1h and the ChIP primers did not span the CRE or NF-κB sites but only the GRE cis-element. Thus, ChIP assays could be performed to further explore the recruitment of CREB to the CRE, and p50 and p65 to the NF-κB sites. The mechanism of synergism could involve enhanced recruitment of the basal transcriptional machinery or enhanced decondensation of the chromatin structure to form euchromatin. These mechanisms could be investigated by performing ChIP assays with antibodies to RNA Pol II for the former and modified histone residues for the latter.

The SGK-1 mRNA expression levels induced with co-stimulation of Dex + GnRH were reported as synergism, but true tests of synergism were not performed. Even though the effects in the present study were more than additive, dose-response and isobologram curves need to be performed in future studies to test whether true synergism was achieved (Chou 2006).

It is still not known whether synergistic transcriptional responses occur in the primary model systems under conditions other than used in this study. This could be investigated in both mouse pituitary tissue and primary gonadotrope cells under varying experimental conditions, such as different treatment times, different hormone concentrations or pulsatile administration of GnRH. It should also be investigated what the in vivo effects of the hormones used in this study are on the expression of the model genes in the pituitary. This can be performed by intraperitoneal injection of the mice and the quick removal of the pituitary after anesthetization.
Although the results on a few genes suggest that the Dex + GnRH-induced synergistic transcriptional response observed for SGK-1 expression is a gene-specific effect, a broader screen of all GRE-containing genes would establish the specificity more broadly. This could be investigated with a transcriptome-array or by RNA-Seq of RNA isolated from LβT2 cells and primary mouse gonadotrope cells, which would reveal more insights into the mechanism and signalling pathways involved in the synergistic Dex + GnRH effects for both gene expression and the physiological significance.

In the present study the results obtained in pituitary tissue during the four different stages showed variations in the mRNA expression levels for all the genes, but these variations were not consistently detected across all the time-points for all the ligands within every stage of the cycle. Additional repeat experiments with fewer variables are required to establish with more confidence that stage- and time-dependent differences for some of these effects. Furthermore, variations in intracellular protein expression and secreted protein levels, where appropriate, could be investigated to determine the involvement of post-transcriptional effects (Stellato 2004). Protein levels could be determined by Western blot or ELISA.

To investigate whether the E$_2$ and P$_4$ hormone levels present in the estrous cycle stages influence the mRNA expression levels of some genes in the mouse pituitary tissue a key experiment will be to treat LβT2 cells with the same E$_2$ and P$_4$ concentrations during the four different estrous cycle stages and determine the mRNA expression levels of key genes of interest.

The present study showed that GR and Flot-1 proteins are both expressed in the pituitary and primary mouse gonadotrope cells, but did not obtain conclusive evidence for co-localization in these primary systems. This could be further investigated with immunohistochemistry of GRIC/R26-YFP mouse pituitary sections using different Flot-1 antibody concentrations or with different Flot-1 antibodies. The co-localization could also be performed in a mono-layer of primary gonadotrope cells isolated with FACS from GRIC/R26-YFP mouse pituitaries. These strategies could result in a greater level of specific signal intensity with less non-specific background fluorescence. It should also be investigated whether the GR and Flot-1 are together in a complex in mouse pituitary tissue and primary gonadotrope cells. This could be performed with reciprocal immunoprecipitation assays using GR and Flot-1 antibodies in whole cell lysates of pituitary tissue or primary gonadotropes or from density-gradient fractions isolated from the primary tissue and cells.


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Lipid Raft- and Protein Kinase C-mediated Synergism between Glucocorticoid- and Gonadotropin-releasing Hormone Signaling Results in Decreased Cell Proliferation*

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Cross-talk between the glucocorticoid receptor (GR) and other receptors is emerging as a mechanism for fine-tuning cellular responses. We have previously shown that gonadotropin-releasing hormone (GnRH) ligand-independently activates the GR and synergistically modulates glucocorticoid-induced transcription of an endogenous gene in LBT2 pituitary gonadotrope precursor cells. Here, we investigated GR and GnRH receptor (GnRHR) cross-talk that involves co-localization with lipid rafts in LBT2 cells. We report that the GnRHR and a small population of the GR co-localize with the lipid raft protein flotillin-1 (Flot-1) at the plasma membrane and that the GR is present in a complex with Flot-1, independent of the presence of ligands. We found that the SGK-1 gene is up-regulated by Dex and GnRH alone, whereas a combination of both ligands resulted in a synergistic increase in SGK-1 mRNA levels. Using siRNA-mediated knockdown and antagonist strategies, we show that the gene-specific synergistic transcriptional response requires the GR, GnRHR, and Flot-1 as well as the protein kinase C pathway. Interestingly, although several GR cofactors are differentially recruited to the SGK-1 promoter in the presence of Dex and GnRH, GR levels remain unchanged compared with Dex treatment alone, suggesting that lipid raft association of the GR has a role in enhancing its transcriptional output in the nucleus. Finally, we show that Dex plus GnRH synergistically inhibit cell proliferation in a manner dependent on SGK-1 and Flot-1. Collectively, the results support a mechanism whereby GR and GnRHR cross-talk within Flot-1-containing lipid rafts modulates cell proliferation via PKC activation and SGK-1 up-regulation.

Glucocorticoids (GCs) are essential for life and regulate a wide array of physiological functions, including homeostasis, tissue remodeling and repair, immune function, cell cycle, and apoptosis (1–3). GCs mediate their transcriptional genomic effects by binding to the ubiquitous classical cytoplasmic glucocorticoid receptor (GR) followed by nuclear translocation and modulation of transcription of target genes by direct DNA binding of the GR or its tethering to other transcription factors (1–3). Recent evidence suggests, however, that the cellular responses mediated by the classical GR are much more complex and involve multiple parallel mechanisms integrating simultaneous signals from several different hormones involving cross-talk with intracellular signaling proteins. These include rapid, non-genomic, cytoplasmic GC-dependent effects (4–9). The GR has also been shown to cross-talk with both G-protein-coupled receptors, the T-cell receptor, cytokine, and receptor-tyrosine kinase pathways (10–19). Furthermore, several reports show that the GR can be activated by hormones, cytokines as well as by cellular stress and the cell cycle, in the absence of GCs, which modulates the activity of the unliganded GR (10, 11, 19–22). Several lines of evidence suggest that rapid glucocorticoid-dependent and glucocorticoid-independent signaling via classical GRs may occur in specialized membrane lipid raft or caveolae microdomains that are proposed to function as platforms for the assembly of multiprotein signaling complexes (23). Steroid receptors, including the GR, have been localized to the plasma membrane in both the absence and presence of steroid ligands (24–27). These may represent only a small population of the total pool of steroid receptor present in a cell, as suggested by the finding that a subset of the GR resides in the plasma membrane of human leukemic cells (25, 28).
mouse monocytes, and B-cells (29). The GR has been shown to localize in caveolae in a human liver cell line and to be involved in GC-induced transactivation (30). Another study showed that the unliganded GR localizes with caveolae to facilitate the rapid Dex-induced phosphorylation of Akt and caveolin-1 (Cav-1) in A549 cells but with no effect on glucocorticoid-response element (GRE) transactivation (27). Caveola-associated GR has been implicated in a role for Dex-mediated intracellular communication and cell proliferation in mouse neural progenitor cells (31). Collectively these findings suggest that the localization of the GR in lipid rafts or caveolae may regulate several GR-dependent responses in a cell- and/or promoter-specific manner. The association of the GR with the membrane could provide a potential mechanism allowing the reciprocal modulation of downstream signaling pathways between the GR and other membrane-associated receptors. Interestingly, synergistic responses involving GCs and other ligands such as gonadotropin-releasing hormone (GnRH), interleukin-2 (IL-2), IL-13, activin, and tumor necrosis factor α (TNF-α) have been reported (10, 19, 32–34). However, the mechanisms of these synergistic responses and whether they involve membrane-associated GR in complex with other receptors has not been previously reported.

Our previous results in LβT2 pituitary gonadotrope precursor cells, which express the endogenous GnRH receptor (GnRHR) (35), suggest that this is an ideal model to investigate the role of membrane-associated GR in multiple parallel classical and non-classical actions. We reported for the first time that the endogenous GR can be ligand-independent activated by GnRH to result in site-specific phosphorylation and transactivation of an endogenous gene in these cells (10). GnRH, in addition to ligand-independently activating the GR, also induces a synergistic transcriptional response on a GRE reporter gene and an endogenous gene in the presence of GCs (10). The GnRHR is a seven-transmembrane receptor found on the cell surface that has previously been shown to localize exclusively to endogenous flotillin-1 (Flot-1)-enriched lipid rafts in the αT3-1 gonadotrope cell line (36, 37). In the present study we identify Flot-1-associated GR in LβT2 cells and investigate its role in mediating genomic transactivation, ligand-independent GR activation by GnRH, and synergy with the GnRHR signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—P. L. Mellon at the University of California kindly provided the immortalized mouse LβT2 pituitary gonadotrope cell line (38). 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 DMEM supplemented with 10% fetal calf serum (Sigma), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The cells were maintained in 75-cm² culture flasks (Greiner Bio-one International) at 37 °C in an environment of 5% CO₂ and 90% humidity. Cells were subcultured with 0.25% trypsin, 0.1% EDTA in calcium- and magnesium-free PBS. Cells were routinely tested for mycoplasma infection by Hoechst staining, and only mycoplasma-negative cells were used in experiments.

**Antibodies and Materials**—GnRH, Dex, phorbol 12-myristate 13-acetate (PMA), Antibodies, RU486, 8-bromo cyclic-AMP, bisindoylmaleimide (BIM) and the nonspecific rabbit IgG antibody (R1131) were purchased from Sigma. Antibodies for GR (sc-8992), SRC-1 (sc-8995), SRC-3 (sc-25742), CBP (sc-369), p300 (sc-32244), anti-mouse HRP (sc-2005), and anti-rabbit HRP (sc-2313) secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz). Antibodies to caveolin-1 (610406) and flotillin-1 (610822) were purchased from BD Transduction Laboratories. Bovine serum albumin (BSA), Complete protease inhibitor tablets, leupeptin, aprotonin, and FuGENE 6 were purchased from Roche Diagnostics. The anti-histone-H3 antibody (ab1791) was obtained from Abcam. The GR-1 antibody (G970-10) was obtained from United States Biological. 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 and Neurobiology Program) and has been described before (37). The anti-rabbit AlexaFluor488 (A21206) was purchased from Invitrogen, whereas the donkey anti-mouse Cy3 antibody (715-166-150) was obtained from Jackson ImmunoResearch. 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).

**Transient Transfection of Cells**—To generate a positive control of the Flot-1 protein, COS-7 cells were seeded into 12-well plates at a density of 1 X 10⁵ 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 of pRK7-flotillin-1 plasmid using FuGENE 6 according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were washed twice with ice-cold PBS and harvested in 50 μl of SDS sample buffer (5 X SDS sample buffer: 100 μM Tris-Cl (pH 6.8), 5% (w/v) SDS, 20% (v/v) glycerol, 2% β-mercaptoethanol, and 0.1% (w/v) bromphenol blue). The samples were boiled for 10 min at 100°C before equal amounts of cell lysates were analyzed by Western blotting.

**SDS-PAGE and Western Blotting**—LβT2 cell lysates were separated on 8 –10% SDS-polyacrylamide gels at 120 V in 1 X SDS running buffer (25 mM Tris-Cl (pH 8.4), 250 mM glycine, and 0.1% SDS) using a Bio-Rad Mini Protean II electrophoresis cell chamber. Proteins were transferred onto a HyBond ECL nitrocellulose membrane (Amersham Biosciences) 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 (Bio-Rad). The membranes were blocked for 1 h at room temperature in 4% ECL blocking solution (4% (w/v)) ECL advance blocking powder, (Amersham Biosciences) 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 in 4% ECL blocking solution diluted in TBST at 4 °C overnight. The following day the membranes were washed with TBST for 3 X 5 min at room temperature before incubation with secondary HRP-conjugated antibodies for 1 h at room temperature in 5% nonfat milk powder (w/v) in TBST. Membranes were washed for 3 X 5 min with TBST followed by a 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 (Amer sham Biosciences) and Hyperfilm MP high performance autoradiography film (Amer sham Biosciences) according to the manufacturer’s instructions. Bands on the autoradiography film were scanned, and quantification was performed with AlphaEaseFC FluorChem 5500 (Alpha Innotech).

**Immunofluorescence Staining and Confocal Microscopy**—Coverslips were sterilized by flaming and placed in 6-well plates followed by seeding LBT2 cells at a density of 3 × 10⁴ cells per well in DMEM with 10% FCS and antibiotics as described above. After 48 h, cells were washed twice with ice-cold PBS and live cell-stained for 1 h at 4 °C with rabbit anti-GnRHR (1:400) followed by 2 washes with ice-cold PBS. Subsequently, the cells were fixed and permeabilized with methanol at −20 °C for 10 min and washed with PBS for 3 × 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. For the GR and Flot-1 immunofluorescence the cells were stained after fixation with rabbit anti-GR (1:250) and Flot-1 as mentioned above. Subsequently, the cells were washed with 1% BSA in PBS for 3 × 5 min before incubating with anti-rabbit-labeled Alexa488 (1:500) (to detect GnRHR and GR) and anti-mouse-labeled Cy3 (1:1000) (to detect Flot-1) 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 × 5 min followed by incubation with Hoechst (100 μg/ml) in PBS for 5 min. Slides were mounted in Mowiol (475904, Calbiochem) containing n-propyl gallate (Sigma) as the anti-fading agent and allowed to set overnight at room temperature in the dark followed by storage at 4 °C in the dark until visualization. Confocal microscopy was performed with a Zeiss Axiovert 200M LSM 510 Meta NLO Confocal Microscope using the 40× water immersion objective and the 63× oil-immersion objective. A multi-track scanning configuration using the 488-nm (HeNe gas laser), 561-nm (solid state laser), 633-nm (argon laser), and 800-nm (titanium:sapphire 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 images were analyzed for co-localization with the Carl Zeiss ZEN software (Version 2009) Manders correlation and overlap coefficients (39) for the two fluorophores.

**Lipid Raft Isolation**—Plasma membrane lipid rafts were prepared using the Triton X-100 procedure as described by Lafont and Simons with some modifications (40). LBT2 cells were seeded in 150-mm² dishes at a density of 8 × 10⁶ cells per dish in DMEM with 10% FCS containing antibiotics as described above. The cells were washed twice with PBS and stimulated with 100 nM Dex, 100 nM GnRH, or a combination of both for 30 min in serum-free medium before being washed twice with ice-cold PBS. The cells were scraped on ice in 1 ml of PBS containing 1 mM PMSE, 5 μg/ml leupeptin, and 2 μg/ml aprotinin per dish. Thereafter the cells were centrifuged at 500 × g for 5 min, and each cell pellet was resuspended in 1 ml of solubilization buffer (SB) (25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSE, 5 μg/ml leupeptin, and 2 μg/ml aprotinin) containing 0.05% Triton X-100 and incubated on ice for 45 min. The lysates were adjusted to 60% sucrose in SB and layered at the bottom of SW40 Ultraclear centrifuge tubes (Beckman). A discontinuous sucrose gradient was prepared consisting of 2 ml of extraction lysis buffer (ELB), 10 mM HEPES (pH 7.9), 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, 1 mM PMSE, 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 (1.5 ml) were collected as follows: 1) top of the gradient, 2) ELB/13% interface, 3) 13%/43% interface, 4) remaining 13%/43% interface, 5) middle of 43% sucrose, 6) 43%/60% interface, 7) middle of 60% sucrose (loading fraction), and 8) the pellet. All fractions were sonicated for 30-s pulses in a water bath at room temperature until a homogenous solution was obtained. Fractions were aliquoted and stored at −80 °C. For analysis, equal amounts of fractions were analyzed by Western blotting as described elsewhere. The membranes were probed with specific antibodies against the GR, GnRHR, Flot-1, and histone H3. The results were quantified by scanning the Western blots and determining the intensity of the protein bands with AlphaEaseFC, whereby the GR protein levels were normalized against Flot-1 protein levels for each experiment and expressed relative to vehicle (control).

**Co-immunoprecipitation Assays**—LBT2 cells were seeded in 100-mm² dishes at a density of 3 × 10⁴ cells per dish in DMEM with 10% FCS and antibiotics as described above. Seventy-two hours after plating, cells were washed twice with PBS and incubated for 2 h in serum-free DMEM before being stimulated 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 of radioimmuno precipitation assay lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA, 1 mM PMSE, 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 × g for 10 min at 4 °C after removing 50 μl aliquots to represent inputs. The supernatants were collected and incubated with 1 μg of rabbit anti-GR, 1 μg of rabbit anti-Flot-1, or nonspecific rabbit IgG antibodies and rotation at 4 °C overnight. The following day the antibodies–protein complexes were incubated with 20 μl of the protein A/G-agarose bead (Santa Cruz) slurry and rotated for 1 h at 4 °C. The protein-immune complexes were collected by centrifugation at 1000 × g for 5 min at 4 °C followed by 2 washes with 1 ml of radioimmuno precipitation assay buffer and 1 ml of PBS. The proteins were eluted from the beads by the addition of 24 μl of 2 × SDS sample buffer and incubated at 100 °C for 5 min. The...
samples were centrifuged at 20,000 × g at room temperature, and the supernatants were collected and resolved on an 8% SDS-PAGE followed by Western blotting as described elsewhere, probing with antibodies as indicated in the figure legends.

Quantitative Real-time PCR—LßT2 cells were seeded in 12-well plates at a density of 4.5 × 10³ cells per well in DMEM with 10% FCS and antibiotics as described elsewhere. Forty-eight hours after plating cells were washed with PBS before being stimulated for 8 h in serum-free medium as indicated in the figure legend. After stimulation, total RNA was isolated with the TRizol reagent (Sigma) according to the manufacturer’s instructions. A total of 0.5 µg of RNA was reverse-transcribed using the Transcripter first-strand cDNA synthesis kit (Roche Applied Science) according to the manufacturer’s instructions. Quantitative real-time PCR was performed with the SensiMix dT kit using the primers serum/glucocorticoid-phosphatase-1 (MKP-1) forward and MKP-1 reverse primers (5'-AGTACCCCTCCTACGGTACGG-3', 5'-TGATGGAG-TCTATGAAGTGCAATATG-3', respectively), follicle-stimulating hormone β (FSHβ) forward and FSHβ reverse primers (5'-GGTGTTGCTGCCATATCAGATTCCG-3', 5'-GACATCA-AGTCTGACTACTACCTTG-3', respectively), glucocorticioid-induced leucine zipper (GILZ) forward and GILZ reverse primers (5'-CCCTAGAACAACAGATGAGACG-3', 5'-CAGAGGCCAATCCACCACG-3', respectively), FK506-binding protein 5 (FKBP5) forward and FKBP5 reverse primers (5'-GGCGACCAGTAAATGAGGCGAG-3', 5'-GGCAATGGCTTCTTCCTTG-3', respectively), and GAPDH forward and reverse primers (5'-TTCCACCATGAGAAGGCG-3', 5'-GCCGATGGACTGTGGTCATCA-3', respectively) under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s. Melt-curve analysis and gel-electrophoresis were performed to confirm that there was a single product amplified in the PCR reactions. Relative SGK-1, MKP-1, FSHβ, GILZ, and FKBP5 transcript levels were calculated with the Fit Points method (41) and were normalized to relative GAPDH transcript levels.

RNA Interference—LßT2 cells were seeded in 12-well plates at a density of 3.5 × 10³ cells per well in 1 ml of 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) according to the manufacturer’s instructions. Briefly, either a combination of mouse Flot-1 siRNA (Mm_FLot1-1, Mm_FLot1-2, Mm_FLot1-3, and Mm_FLot1-4 (FlexiTube siRNA (1027415), Qiagen) or GR siRNA (Mn_Nr3c_3, Mn_Nr3c_4, Mn_Nr3c_5, and Mn_Nr3c_6, FlexiTube siRNA (1027416) or SGK-1 siRNA (01416555) (Qiagen) or just non-silencing scrambled (NSC) siRNA (Negative control siRNA (1027310), Qiagen) was diluted in 50 µl of OptiMEM+GlutaMAX-I (Invitrogen) with 3.5 µl of HiPerfect. The mixture was incubated for 10 min at room temperature and added dropwise to the cells to obtain a final concentration of 40 nM siRNA per well. Three days after transfection, the cells were washed once with PBS followed by stimulation for 8 h with 100 nM Dex, 100 nM GnRH, and a combination of both in serum-free DMEM for gene expression assays.

ChIP Assay—LßT2 cells were seeded in 150-mm² dishes at a density of 8 × 10⁵ cells per dish in DMEM with 10% charcoal-stripped FCS and antibiotics as described above. Seventy-two hours after plating the medium was replaced before the cells were stimulated with 100 nM Dex, 100 nM GnRH, or a combination of both for 1 h. Thereafter, proteins were cross-linked with 1% formaldehyde for 40 min before the reaction was quenched with 125 mM glycine for 10 min. The cells were washed and scraped in PBS containing Complete Mini protease inhibitor mixture (Roche Applied Science). Cells were centrifuged and resuspended in 0.5 ml of nuclear lysis buffer (1% SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and protease inhibitor mixture). Cross-linked DNA was sheared by sonication. For the input a 30-µg aliquot of chromatin was used, whereas 100 µg of chromatin was diluted in immunoprecipitation dilution buffer (0.01% SDS, 20 mM Tris-HCl (pH 8.0), 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, and protease mixture inhibitors) before being pre cleared for 1 h with Protein A/G PLUS beads rotating at 4 °C. The precleared samples were then incubated with 5 µg of anti-GR antibody or 2 µg of anti-SRC-1, anti-GRIP-1 or anti-SRC-3, anti-p300, anti-CBP antibodies rotating at 4 °C overnight. The next day, 40 µl of Protein A/G PLUS-agarose beads were added to the mixture for 6 h at 4 °C. The beads were collected by centrifugation and washed sequentially with 1 ml of each wash buffer I (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 20 mM EDTA, 2 mM DTT, 500 mM LiCl), II (0.1% (v/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), and III (1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA, and 10 mM Tris, pH 8.0) followed by three washes with 1 ml of Tris-EDTA. Complexes were eluted from the beads with 300 µl of immunoprecipitation elution buffer (1% (w/v) SDS and 100 mM NaHCO₃). Cross-links were reversed by the addition of 300 mM sodium chloride and incubation at 65 °C overnight. The following day each sample was adjusted to 150 mM EDTA, 125 mM Tris-HCl pH 6.5, and 20 µg of proteinase K followed by incubation for 1 h at 45 °C. The DNA was purified with the QIAquick PCR purification kit according to the manufacturer’s instructions. Quantitative real-time PCR were performed using specific primers SGK-1 forward and reverse (5'-CTAACGTGGCCACTCTCTCAC-3', 5'-TCCCCAGACTTGGAG-GAGGA-3', respectively), which span the GRE in the promoter of the SGK-1 gene. Quantitative real-time PCR were performed under the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 10 s.

Cell Proliferation Assay—LßT2 cells were seeded in 12-well plates at a density of 3.5 × 10³ cells per well in 1 ml of 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 40 nM Flot-1 siRNA, 40 nM SGK-1 siRNA, or 40 nM NSC using HiPerfect transfection reagent (Qiagen) as described elsewhere. After 72 h the cells were seeded in 48-well plates at 7.5 × 10⁴ cells per well in 200 µl of DMEM with 10% charcoal-stripped serum and antibiotics as described elsewhere. Twenty-four hours after
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FIGURE 1. Immunofluorescence shows the GnRHR co-localizes with Flot-1 in LβT2 cells. A, whole LβT2, untransfected COS-7 (+ve Ctrl) and Flot-1 transfected COS-7 (+ve Ctrl) 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. B, LβT2 cells were grown on glass coverslips and live cell-stained with rabbit anti-GnRHR followed by fixation and staining with a mouse anti-Flot-1 antibody. Thereafter, the coverslips were incubated with anti-rabbit Alexa488 (green) and anti-mouse Cy3 (red) antibodies. Nuclei were stained with Hoechst (blue) before mounting in Mowiol. Staining was visualized with a Zeiss LSM510 Meta confocal microscope using the 63× objective, and a representative image is shown for a group of cells, as indicated by the multiple nuclei visible in the Hoechst stain. Manders co-localization coefficients (M) are shown for the red and green channels in regions of interest in the vicinity of the cell membrane. The panel on the right marked Co-localization displays a map of co-localized pixels for the green and red channels across the entire group of cells. The degree of overlap between the two patterns is indicated by the overlap coefficient (O). The scale bar represents 10 μm. The results shown are representative of three independent experiments.

plating medium was replaced with fresh medium, and the cells (~70% confluency) were stimulated for 48 h with 100 nM Dex, 100 nM GnRH, or a combination of both for 48 h. Thereafter the cell proliferation was determined with a Cell Proliferation BrdU colorimetric kit (Roche Applied Science, 11647229001) according to the manufacturer’s instructions.

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.

RESULTS

GnRHR and GR Co-localize with Flot-1-containing Lipid Rafts Independent of Ligands in LβT2 Cells—To investigate whether LβT2 cells express the lipid raft marker 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 Fig. 1A, Flot-1, but not Cav-1 protein expression, was detected in LβT2 cells, whereas endogenous Cav-1 was detected in COS-7 cells as previously reported (42). To investigate whether the GnRHR co-localizes with Flot-1 in LβT2 cells, live cells were stained for GnRHR followed by cell fixation, permeabilization, and staining for Flot-1. The cells were visualized with a confocal microscope, and it was found that a substantial portion of Flot-1 was localized at or in the vicinity of the plasma membrane with some distribution in vesicle-like intracellular compartments (Fig. 1B). The GnRHR was also found to mainly localize to the plasma membrane, but a small percentage was also detected in the cytoplasm, possibly due to some internalization (Fig. 1B). The panel on the right shows the co-localized pixels between the green (0.94 at marked region of interest) and red (0.84 at marked region of interest) channels. The data were analyzed using Manders co-localization coefficients, and the values indicate a substantial amount of GnRHR co-localization with Flot-1 at the plasma membrane and intracellular regions of LβT2 cells (Fig. 1B). Furthermore, the co-localization map and overlap coefficients of the GnRHR and Flot-1 signals also indicate a high degree of co-localization of both channels across the entire cell, indicative of potential interactions of both proteins in cellular compartments other than the cell membrane. Having established the co-localization of the GnRHR and Flot-1 at the plasma membrane, we next investigated whether the GR is associated with Flot-1 at the membrane and whether this potential association is affected by the presence of GR agonist. Cells were stimulated for 30 min with saturating concentrations of Dex or GnRH (100 nM each) or a combination of both. Subsequently, cells were fixed, permeabilized, and stained with anti-GR- and anti-Flot-1-specific antibodies. The results presented in Fig. 2, panels A–D, show that a population of Flot-1 is localized to the plasma membrane, whereas some Flot-1 was also detected in intracellular compartments under all treatment conditions. In untreated cells, the GR appears evenly distributed throughout the cell, with a small percentage co-localizing with Flot-1 at the plasma membrane (Fig. 2, panel A) (0.84 and 0.98, at region of interest). Treatment with Dex resulted in dis-
tinct nuclear translocation of the GR, but a small fraction remained scattered in the cytoplasm. Interestingly, we found that a small percentage of the GR co-localized with Flot-1 at the membrane even in the presence of the GR agonist (Fig. 2, compare panel A with B) (0.75 and 0.83, at marked region of interest). By contrast, we could not detect GR nuclear localization after GnRH treatment (Fig. 2, compare panels A and C). Co-stimulation with GnRH had no detectable effect on the amount of Dex-induced nuclear import of the GR (Fig. 2, compare stimulation with GnRH had no detectable effect on the amount of Dex and GnRH (Fig. 3, panel D). The percentage nuclear translocation of the GR was quantified, and no difference between the amount of GR that translocated into the nucleus in response to Dex and Dex + GnRH was detected (data not shown). Co-localization of GR and Flot-1 was still detectable after stimulation with GnRH and Dex + GnRH (Fig. 2, panel C and D) (0.82 and 0.74 (C) and 0.97 and 0.84 (D), respectively, at marked region of interest). However, significant differences between co-localization for different conditions could not be established.

Having shown by immunofluorescence that both the GnRHR and a small population of the GR co-localize with Flot-1 at the plasma membrane, a biochemical strategy was pursued to provide additional evidence that these receptors localize to lipid rafts in LβT2 cells. Cells were incubated with 0.05% Triton X-100 before fractionation on a discontinuous sucrose density gradient. The results from Fig. 3A show that histone H3 localized only to fractions 6, 7, and 8, which are the most-dense fractions where the starting material was applied. Importantly, no histone H3 could be detected in the lipid raft fraction (Fig. 3A), indicating the absence of chromatin in the lipid raft fraction. The results from Fig. 3B show that the majority of Flot-1 is located in fraction 4 (lipid rafts) under basal and all stimulated conditions. The results show that the GnRHR localizes exclusively to the lipid raft fraction (fraction 4) in untreated cells and under all stimulated conditions (Fig. 3B, panels i–iv). This together with results presented in Fig. 1B shows that the GnRHR co-localizes with Flot-1 in lipid rafts at the plasma membrane of LβT2 cells. A small percentage of GR was detected in the lipid raft fraction in untreated cells (Fig. 3B, panel i). Despite the resulting nuclear import of the GR with Dex, a small amount of GR was still detected in the lipid raft fraction after 30 min of treatment with Dex (Fig. 3B, panel ii), which is in agreement with results shown in Fig. 2. Similarly, a small amount of GR was detected in the lipid raft fraction after 30 min of stimulation with either GnRH or co-stimulation with Dex and GnRH (Fig. 3B, panels iii and iv). Almost no GR was detected in fraction 5, indicating specific localization of the GR to lipid rafts rather than incomplete separation of the density-gradients (Fig. 3B, panels i–iv). The remaining GR was present in fractions 6, 7, and 8 in unstimulated cells and under all stimulated conditions (Fig. 3B, panels i–iv). These fractions contain all cellular material excluding the low density membrane fractions. To quantify the relative amount of GR associated with lipid rafts in the absence and presence of ligands, the lipid raft GR protein levels were expressed relative to lipid raft Flot-1 protein levels (Fig. 3C). The results show that the extent of GR localized to lipid rafts is independent of short exposures to Dex, GnRH, or a combination of both. This is in agreement with the result from Fig. 2 showing a small percentage of GR co-localizing with Flot-1 in LβT2 cells independent of hormone treatment (Fig. 2, panels A–D). Taken together, the results from Fig. 3B show that GR co-localizes with 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.

FIGURE 3. Fractionation shows the GR and GnRHR localize to Flot-1-containing lipid rafts ligand independently. A, lipid rafts were isolated by ultracentrifugation employing the detergent-resistant flotation strategy in a discontinuous sucrose density gradient consisting of 2 ml of ELB, 4 ml of 13% sucrose in ELB, 4 ml of 43% sucrose in ELB, and 4 ml of 60% (containing the sample). Eight fractions were collected: 1, top of the gradient; 2, ELB/13% interface; 3, 13%/43% interface; 4, remaining 13%/43% interface; 5, middle of 43% sucrose; 6, 43%/60% interface; 7, middle of 60% sucrose (loading fraction); 8, the pellet. 15 μl 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–iv are single Western blots that are representative of three independent experiments. B, i–iv, LβT2 cells were serum-starved overnight and incubated for 30 min in serum-free medium (panel i), 100 nM Dex (panel ii), 100 nM GnRH (panel iii), or a combination of both (panel iv). Lipid rafts were isolated as described for A. 15 μl 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–iv are single Western blots that are representative of three independent experiments. Fractions 3 and 4 contain the lipid raft material, indicated with a star. The detectable band in fraction 3 of panel iv in the Western blot has a larger size than the GR and is most likely a nonspecific band. The shift of the lipid raft material from fraction 3 (i) to fraction 4 (ii–iv) is most likely due to technical variation between experiments arising from manual collection of the fractions. The variable detection of a small % of total Flot-1 in fractions 6–8, containing unsuspended cells and unbroken nuclei, most likely reflects variations in sensitivity of the blotting procedure for these independent Western blots. C, to determine the ratio of GR lipid raft protein compared with Flot-1 lipid raft protein, only the lipid raft fractions of each condition were re-analyzed on one Western blot, and results were quantified by αEaseFC software. The lipid raft GR protein levels were normalized to lipid raft Flot-1 protein levels for each experiment, and pooled results are expressed relative to vehicle (Control). The graph shows the combined results of three independent experiments.

**Flot-1- and PKC-mediated GR and GnRHR Cross-talk**
Flot-1 and PKC-mediated GR and GnRHR Cross-talk

suggest that the co-localization of the GR with Flot-1 in lipid rafts in LβT2 cells involves a physical direct or indirect interaction that is independent of ligand.

Flot-1 Appears Not to Be Required for Site-specific GR Phosphorylation—It has previously been shown that intact lipid rafts are required for Dex-induced GR phosphorylation at Ser-211 in A549 cells (27). Additionally, we have previously shown that Dex induces rapid phosphorylation of Ser-220 and Ser-234 of the mouse GR (mGR), whereas GnRH induces rapid Ser-234 but not Ser-220 mGR phosphorylation in LβT2 cells (10). The human GR residues 203, 211, and 226 are equivalent to the mGR residues 212, 220, and 234, respectively. Therefore, we investigated if the presence of Flot-1, and thus lipid rafts, is required for the GR phosphorylation in LβT2 cells. Flot-1 protein levels were decreased with siRNA-mediated knockdown followed by treatment of these Flot-1-depleted cells with 100 nM Dex, 100 nM GnRH, or both together for 30 min. The results from Fig. 5 show that Flot-1 levels were reduced by ~60% using this strategy when NSC control is compared with Flot-1 siRNA Ctrl (Fig. 5G). The stimulation also had no effect on the decrease of Flot-1 protein obtained with the siRNA-mediated knockdown strategy. Further reduction in Flot-1 levels was accompanied by cell death (data not shown). The results from Fig. 5, A and B, show that neither Dex, GnRH, or the combination of both resulted in a significant increase in Ser-212 phosphorylation of the mGR in the NSC or Flot-1 siRNA conditions. However, a trend was apparent whereby Dex, GnRH, and Dex plus GnRH all appeared to slightly increase Ser-212 phosphorylation in the NSC, and this increase appeared to be lost when Flot-1 protein levels were reduced, suggesting that phosphorylation of the GR at Ser-212 may require membrane association. However, given the small responses, it was not possible to establish significance above technical error and/or biological variation. In the absence of Flot-1 siRNA, treatment with Dex resulted in a significant increase in GR phosphorylation at Ser-220, whereas GnRH did not increase phosphorylation at that residue, and the combination of Dex plus GnRH all appeared to slightly increase Ser-212 phosphorylation in the NSC, and this increase appeared to be lost when Flot-1 protein levels were reduced, suggesting that phosphorylation of the GR at Ser-212 may require membrane association. However, given the small responses, it was not possible to establish significance above technical error and/or biological variation. In the absence of Flot-1 siRNA, treatment with Dex resulted in a significant increase in GR phosphorylation at Ser-220, whereas GnRH did not increase phosphorylation at that residue, and the combination of Dex plus GnRH increased Ser-220 phosphorylation to the same extent as Dex alone (Fig. 5, C and D). Decreasing Flot-1 protein levels by 60% had no detectable significant effect on Ser-220 phosphorylation of the mGR (Fig. 5, C and D) in the absence or presence of ligands. In the absence of Flot-1 siRNA, treatment with Dex resulted in a significant increase in GR phosphorylation at Ser-234 (Fig. 5, E and F). Although the extent of Ser-234 phosphorylation for Dex plus GnRH appeared to be slightly greater than for Dex alone, statistical significance could not be established (Fig. 5, E and F). As previously reported, GnRH resulted in a similar level of Ser-234 phosphorylation of the mGR as Dex (Fig. 5, E and F) (10). Decreasing Flot-1 protein levels had no detectable significant effect on Ser-234 phosphorylation of the mGR (Fig. 5, E and F) in the absence or presence of ligands. Taken together, decreasing the Flot-1 protein levels by 60% had no detectable significant effect on Ser-212, Ser-220, or Ser-234 phosphorylation of the mGR (basal and ligand-induced), suggesting that lipid raft association is not required for phosphorylation of the GR. However, we cannot discount the possibility that a greater percentage knockdown of Flot-1 may have significantly affected ligand-induced mGR phosphorylation.
Co-treatment with Dex plus GnRH Synergistically and Selectively Enhances SGK-1 mRNA Levels

We previously showed that cross-talk between the GR and GnRHR signaling pathways modulates expression of an endogenous AP-1-containing gene, most likely via a GR tethering mechanism involving GR phosphorylation. To investigate whether Dex plus GnRH synergism occurs on endogenous genes containing GREs in their promoters, LβT2 cells were incubated for 8 h with 100 nM Dex, 100 nM

![Diagram](https://example.com/diagram.png)

**FIGURE 5. Flot-1 appears not to be required for site-specific GR phosphorylation.** LβT2 cells were transfected with NSC or specific mouse Flot-1 siRNA at a final concentration of 40 nM and incubated for 72 h. The cells were incubated for 30 min in serum-free medium with 100 nM Dex or 100 nM GnRH or a combination of both, and the proteins were harvested. The samples were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane, and probed with anti-phospho-Ser-212 (P-S212) GR-, anti-phospho-Ser-220 (P-S220) GR-, and anti-phospho Ser-234 (P-S234)-specific antibodies. The membranes were stripped and probed with specific antibodies to GR, Flot-1, and GAPDH. The panels on the left (A, C, and E) show a single representative Western blot, and the graphs (B, D, and F) show the combined results of four independent experiments where vehicle (Ctrl, control) was set to 1. Panel G shows the average percentage decrease of Flot-1 protein for the control samples in the presence of Flot-1 siRNA. One-way ANOVA with Dunnett’s post test was used for statistical analysis and is denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001 respectively.
GnRH, and both together followed by RNA extraction and cDNA synthesis and real-time PCR. The results from Fig. 6, A–E, show that Dex increased mRNA levels of the GILZ, MKP-1, FKBPs, FSHβ, and SGK-1 genes, consistent with previous reports in the literature (43–47). Treatment with GnRH alone had no effect on mRNA levels of the GILZ, MKP-1, and FKBPs genes but increased expression of the FSHβ gene (Fig. 6D), as previously reported (43). Interestingly, we found that GnRH alone also significantly increased mRNA levels of the SGK-1 gene (Fig. 6E). Although Dex plus GnRH treatment did not result in a significant change in mRNA expression for the GILZ, MKP-1, FKBPs, and FSHβ genes compared with Dex alone, a statistically significant synergistic response was apparent for the SGK-1 gene. Although Dex and GnRH alone increased SGK-1 mRNA by 8- and 3.5-fold, respectively, the combination increased SGK-1 mRNA levels by 17-fold (Fig. 6E). These results collectively suggest that cross-talk between the GR and GnRHR signaling pathways resulting in synergistic effects on gene expression is selective for only some GRE-containing genes.

**Flot-1, GR, and the GnRHR Are Required for the Synergistic Increase in SGK-1 Gene Expression Levels**—To investigate whether Flot-1 is required for the observed synergistic increase in SGK-1 mRNA in response to Dex plus GnRH, Flot-1 protein levels were decreased using an siRNA-mediated protein knockdown approach, and the cells were stimulated for 8 h with 100 nM Dex, 100 nM GnRH, or both together. The results from Fig. 7A show that decreased Flot-1 protein levels did not affect the extent of Dex or GnRH-induced increase of SGK-1 mRNA. Fig. 7B shows that Flot-1 levels were reduced by ~60% using this strategy. It was found that in the NSC, the GnRH-induced increase in SGK-1 expression was not statistical significant. Interestingly, reducing the Flot-1 protein levels by ~60% decreased the synergistic Dex plus GnRH transcriptional response by ~50% (Fig. 7A), to a level similar to that of Dex alone. These results suggest that the association of the GR with
Flot-1 is not required for the SGK-1 response to Dex alone but is required for the synergistic response to Dex plus GnRH. To obtain more evidence that this synergistic response observed for the SGK-1 gene also requires the GR, the GR protein levels were decreased by ~55% with siRNA (Fig. 7D). The results from Fig. 7C show that, as expected, the Dex-induced increase in SGK-1 mRNA levels requires the presence of the GR, as GR knockdown completely ablates the response. Similarly, the results are consistent with a requirement for the GR in the Dex plus GnRH response (Fig. 7C), although in these experiments the synergism was not as pronounced. Further support for a requirement for the GR for the synergistic response was obtained using the GR antagonist RU486, which attenuated the transcriptional increase seen when cells were treated with Dex.
alone and the synergistic response in the presence of both Dex and GnRH (Fig. 7E). To obtain evidence that the synergism is also GnRHR-dependent, cells were incubated with ligands in the absence or presence of the GnRHR antagonist Antide. The results from Fig. 7E show that the GnRHR antagonist had no effect on the Dex-induced increase in SGK-1 mRNA levels. The GnRHR antagonist attenuated the small GnRH-induced increase in SGK-1 mRNA levels, and it abolished the synergistic response induced by co-stimulation with Dex plus GnRH (Fig. 7E). Taken together, these results indicate that the synergistic transcriptional up-regulation of SGK-1 requires membrane-associated Flot-1, GR and the GnRHR.

Synergism Involves Differential Recruitment of SRC Cofactors to the SGK-1 Promoter Compared with Dex and GnRH Alone, Whereas GR Recruitment Remains Unchanged—As we could not detect increased GR phosphorylation in the presence of Dex and GnRH compared with single-hormone treatment, we next investigated whether increased GR recruitment to the GRE present in SGK-1 could explain the augmented transcriptional response. Cells were incubated with 100 nM Dex, 100 nM GnRH, and both together for 1 h followed by immunoprecipitation using an anti-GR antibody. The results in Fig. 8A show that Dex treatment resulted in a significant 2.5-fold recruitment of the GR. However, treatment of cells with both Dex and GnRH together resulted in similar amounts of GR being recruited to the SGK-1 promoter as compared with Dex alone (Fig. 8A). These results suggest that the synergistic transcriptional response induced by Dex plus GnRH on the SGK-1 gene is not due to increased GR promoter occupancy. On the basis of these results, we next determined whether known GR cofactors are differentially recruited to the promoter. As shown in Fig. 8B, treatment with Dex or GnRH alone resulted in a significant recruitment of SRC-1 to the SGK-1 promoter (2- and 2.25-fold, respectively) (Fig. 8B). Interestingly, co-treatment with Dex and GnRH resulted in significantly less SRC-1 recruitment to the SGK-1 promoter compared with both ligands alone (Fig. 8B). GRIP-1 seemed to only be recruited to the SGK-1 promoter in response to Dex (Fig. 8B), whereas SRC-3 (Fig. 8D), p300, CBP, and p65 (data not shown) were not detected at the gene promoter under any treatment conditions. In summary, the results suggest that increased SGK-1 transcription by Dex, GnRH, or a combination of both is mediated by differential recruitment of cofactors, suggesting that synergistic effects initiated in membrane lipid rafts exert downstream differences in nuclear promoter occupancy compared with the classical response to Dex alone.

GnRH Acting via the PKC but Not the PKA Pathway Augments the Dex-mediated Increase in SGK-1 mRNA Levels—Because the GnRHR has been shown to activate both the PKA and PKC signaling pathways in LβT2 cells (48) and our previous results implicated PKC in mediating Dex plus GnRH synergy
(10), we investigated the possible involvement of the PKA and PKC signaling pathways in the SGK-1 response. 8-Bromo-cAMP, which is an inducer of the PKA signaling pathway, was used to stimulate the cells in the absence and presence of Dex. We found that 8-bromo-cAMP alone had no effect on the mRNA expression levels of the SGK-1 gene (Fig. 9A). In addition, stimulating cells with Dex and cAMP did not result in a synergistic response, as seen for co-treatment with Dex and GnRH. This result indicates that the PKA signaling pathway is not involved in regulating the expression of the SGK-1 gene in LβT2 cells. To investigate a potential role for the PKC signaling pathway, we incubated cells with PMA, a diacylglycerol analog known to activate PKC. The results show that PMA resulted in a significant 4-fold induction of the SGK-1 mRNA expression (Fig. 9B). Interestingly, stimulating cells with both Dex and PMA resulted in a transcriptional response with a magnitude comparable with treatment with both Dex plus GnRH (Fig. 9B).

This similar gene induction strongly suggests that the PKC pathway is involved in mediating the synergistic up-regulation of SGK-1 mRNA levels in response to Dex plus GnRH (Fig. 9B). In support of this notion, the PKC inhibitor BIM not only decreased PMA-induced SGK-1 gene expression but also the synergistic Dex plus GnRH response as well as the synergistic response induced by Dex plus PMA (Fig. 9C).

**FIGURE 9.** GnRH acting via the PKC but not the PKA pathway synergistically increases the Dex-mediated up-regulation of the SGK-1 gene. A, LβT2 cells were incubated for 8 h in serum-free medium with 100 nM Dex, 100 nM GnRH, a combination of Dex plus GnRH, 2 mM 8-bromo-cAMP, and a combination of Dex plus 8-bromo-cAMP. Total RNA was isolated and reverse-transcribed, and relative levels of SGK-1 transcripts were determined by quantitative real-time PCR. Fold changes in SGK-1 mRNA levels were normalized to GAPDH transcripts and were calculated relative to vehicle-treated (control (Ctrl)) samples. The graph is representative of three independent experiments. B, as in A, except that 8-bromo-cAMP was replaced with 20 ng/ml PMA. C, as in B, except the cells were stimulated in the presence and absence of 100 nM PKC inhibitor. One-way ANOVA with Dunnett’s post-test was used for statistical analysis and is denoted as *, **, or *** to indicate p < 0.05, p < 0.01, or p < 0.001, respectively.
GnRH, or both together. Two different SGK-1 antibodies were used to detect the protein, but unfortunately both resulted in many nonspecific bands (data not shown). To establish that SGK-1 gene expression was decreased with the siRNA, SGK-1 mRNA levels were determined with real-time PCR. The results from Fig. 10A show that SGK-1 mRNA was decreased by 60%. A cell proliferation assay was performed, and the results from Fig. 10B show that Dex had no effect on cell proliferation in the absence or presence of Flot-1 or SGK-1 knockdown. Interestingly, in the absence of knockdown, GnRH decreased proliferation by 20%, whereas treatment with Dex and GnRH together showed a synergistic attenuation in cell proliferation (Fig. 10B). Decreasing the Flot-1 protein levels by 60% did not significantly change the effects of Dex or GnRH alone on proliferation of LβT2 cells (Fig. 10B). Decreased Flot-1 protein levels, however, significantly attenuated the repressive effect of Dex plus GnRH to a small extent (Fig. 10B). Interestingly, siRNA-mediated knockdown of SGK-1 lifted the synergistic repression of cell proliferation in response to co-stimulation with Dex and GnRH. Taken together the results suggest that synergistic up-regulation of SGK-1 mRNA levels by Dex plus GnRH as well as the presence of Flot-1 is required for a synergistic repression of LβT2 cell proliferation.

DISCUSSION

Cross-talk between the GR and other receptors is emerging as a potential mechanism for fine-tuning cellular responses via several different genomic and non-genomic mechanisms. We have previously shown that in LβT2 cells the GR is ligand-independently activated by GnRH and that a combination of Dex plus GnRH synergistically increases transcription of a GRE reporter gene in a manner dependent on the presence of the GR and the GnRHR. In the present study we investigated the hypothesis that the localization of the GR to lipid rafts or caveolae is required for some of these genomic or non-genomic GR-mediated effects in LβT2 cells. We show here for the first time that both the GnRHR and a small population of the GR co-localize with the lipid raft protein Flot-1 at the plasma membrane, independent of the presence of ligands. Furthermore, the GR is present in a complex with Flot-1, independent of the presence of ligands. These results are consistent with previous studies showing that localization of the GnRHR to lipid rafts in αT3-1 cells (36) and the mineralocorticoid receptor to lipid rafts in HEK-293 cells (49) is independent of their respective ligands. Our findings suggest that the lipid raft-associated GR-GnRHR complex acts as a signal transduction platform that
modulates downstream genomic signaling by the cytoplasmic GR. By a combination of protein knockdown, pharmacological inhibitor/activator and ChIP strategies, and incubation of cells with 1) Dex or 2) GnRH alone or 3) in combination, we have obtained insights into the role of lipid raft-associated GR on gene expression for 1) classical Dex-mediated transactivation, 2) GnRH induced ligand-independent GR responses, and 3) responses to the combination of Dex plus GnRH, for an endogenous GRE-containing gene in LβT2 cells.

Results were first assessed to determine whether Flot-1-associated GR is involved in regulating classical genomic GR agonist-dependent transcription of GRE-containing genes. Because we did not previously investigate endogenous GRE-containing genes (10), we wished to identify such genes in the present study for further mechanistic investigation. We show that Dex increased mRNA levels, as expected, of several GRE-agonist-dependent transcription of GRE-containing genes. In LβT2 cells, activation mechanism in response to Dex on the SGK-1 promoter showed that SRC-1 is also recruited by this classical GR transactivation of the endogenous GRE-containing gene in 1470.2 cells (53) as well as published data for ChIP results showing increased recruitment of GRIP-1 to the membrane-associated GR responses is cell-specific. Our findings led us to hypothesize that the underlying mechanism of synergistic up-regulation of the SGK-1 gene by Dex plus GnRH involves the activation of a cytoplasmic signaling pathway by the lipid raft-associated GnRHR-GR complex. Consistent with this, our results show a requirement for the PKC pathway in the combined effect of Dex and GnRH and not for the response observed for Dex treatment alone. The observed PMA-dependent and cAMP-independent increase in SGK-1 mRNA levels is in contrast to a previous report showing that PKA and not PKC activates the SGK-1 gene in rat neonatal cardiomyocytes (56), suggesting cell-specific effects. However, the same study showed that PMA enhanced the cortisol-induced mRNA expression of the SGK-1 gene, similar to the effects observed

The presence of a response to GnRH for the GRE-containing GILZ, MKP1, and FKBP5 genes suggests that GnRH-activated GR exerts promoter-specific downstream effects. We could not, however, establish conclusively whether Flot-1 is required for the GnRH-induced response on the SGK-1 gene. This was due to the relatively small but statistically significant response on the SGK-1 gene with GnRH, which was even further reduced under NSC knockdown conditions. Note that due to the small response we were also unable to establish whether PKC activation is required for the GnRH response on the SGK gene, although the apparent slight reduction with a PKC inhibitor and mimicking of the response with PMA, but not the cAMP analog, suggest that PKC but not PKA is involved regulating GnRH-induced SGK-1 expression, consistent with our previously published data (10). Besides recruitment of the GR to the SGK-1 promoter, albeit less than with Dex alone, treatment with GnRH also resulted in the recruitment of GRIP-1 but not SRC-1 or SRC-3 to the promoter. By contrast, in Dex-treated cells we found that both GRIP-1 and SRC-1, but not SRC-3, are associated with the promoter. Interestingly, this result for GnRH is different to that obtained for the GnRHR promoter (10), where neither GRIP-1 nor SRC-1 is recruited. These data highlight gene-specific effects of GnRH-activated GR signaling at responsive promoters. It is likely that promoter occupancy of GR cofactors is determined by both the promoter gene architecture as well as the ligands activating the GR, which may result in distinct conformational changes in the GR itself, thus allowing differential interactions with other factors.

Thirdly, we assessed whether Flot-1-associated GR is involved in mediating the combined effects of Dex plus GnRH on transcription of the SGK-1 gene. Our findings provide several novel insights into the mechanism of synergy between the GR and GnRHR signaling pathways. We present evidence for the first time that the GR and GnRHR synergistically up-regulate the SGK-1 gene and, importantly, that this effect, in contrast to the effects elicited by the hormones alone, requires Flot-1-containing lipid rafts. Furthermore, our results show that this synergism only occurs on a subset of GRE-containing endogenous genes. This gene-specific effect supports a model whereby both receptors act in concert to selectively activate only a distinct set of genes to fine-tune the response of a cell to changes in the hormonal environment. We established that the synergistic effect on SGK-1 gene expression is not due to detectable changes in localization of the GR to the membrane or increased GR recruitment to the promoter. Our findings led us to hypothesize that the underlying mechanism of synergistic up-regulation of the SGK-1 gene by Dex plus GnRH involves the activation of a cytoplasmic signaling pathway by the lipid raft-associated GnRHR-GR complex. Consistent with this, our results show a requirement for the PKC pathway in the combined effect of Dex and GnRH and not for the response observed for Dex treatment alone. The observed PMA-dependent and cAMP-independent increase in SGK-1 mRNA levels is in contrast to a previous report showing that PKA and not PKC activates the SGK-1 gene in rat neonatal cardiomyocytes (56), suggesting cell-specific effects. However, the same study showed that PMA enhanced the cortisol-induced mRNA expression of the SGK-1 gene, similar to the effects observed
with PMA plus Dex in the current study, suggesting that the PKC pathway augments GC responses in other cells. The data obtained here suggest that in LβT2 cells, cross-talk between the GnRHR and GR involves the activation of the PKC pathway and converges on the SGK-1 gene. As we found a requirement for Flot-1-containing lipid rafts in the synergistic transcriptional response and a direct interaction of Flot-1 and the GR, it is tempting to speculate that these membrane domains provide a platform where GnRHR and GR constitutively assemble and cross-talk to modulate downstream signaling from the membrane.

The finding that Dex plus GnRH did not alter the membrane localization of either receptor together with the result that GR levels at the SGK-1 promoter were similar to single-hormone treatment suggests that other factors mediate the combined transcriptional effect. Similarly, others have found that co-treatment of cells with cortisol plus FGF-2 did not alter the cortisol-induced GR recruitment to the SGK-1 promoter (51). We show that the relative levels of members of the SRC family of co-activators are different for Dex plus GnRH compared with Dex alone, which suggests that another cofactor or an entirely different complex might be recruited to the SGK-1 promoter. Although we investigated a possible role for other co-activators in mediating the synergistic response on the SGK-1 gene, we were unable to identify such a positively acting cofactor. However, in the future it will be important to determine whether other cofactors assemble on the promoter and enhance SGK-1 transactivation compared with Dex and GnRH alone. The genomic synergistic effect is likely to involve non-genomic-driven changes in the phosphorylation status and nuclear translocation of cofactors and/or other transcription factors. Thus it is possible that other factors are not involved but, rather, the same factors that are differentially phosphorylated. On the basis of our results we hypothesize that such phosphorylation and translocation events could be mediated by the PKC pathway.

Previous results from our group suggested a role for PKC in the GnRH-induced but not the Dex-induced transactivation of a GRE reporter and ligand-induced Ser-234 phosphorylation of the GR (10). In the present study we show that Dex and Dex plus GnRH induce phosphorylation of the GR at Ser-234 and Ser-220 of the mGR but that GnRH only induces phosphorylation of the mGR at Ser-234, similar to our previous report (10). Our current novel results suggest that lipid raft association of mouse GR is not required for GR phosphorylation at Ser-212, Ser-220, and Ser-234 in response to GnRH, Dex, or the combination thereof. Furthermore, the SGK-1 response to Dex was unaffected, whereas the response to GnRH was slightly but not significantly reduced, and the synergistic response was significantly inhibited in the presence of the PKC inhibitor. Our results suggest that ligand-induced phosphorylation of the GR does not require lipid raft association. Arguably, a greater reduction in Flot-1 protein levels >60% may have led to detectable changes in GR phosphorylation levels. However, the findings that a 60% reduction of Flot-1 levels results in a significant decrease in the synergistic up-regulation of the SGK-1 gene and a significant reduction of the proliferative effect strongly support the notion that the Flot-1-dependent synergistic response does not require GR phosphorylation at Ser-234 or Ser-220. Furthermore, when taken together with our previous results, we cannot as yet discount the possibility that membrane-associated GR is phosphorylated directly or indirectly by PKC or SGK-1 via a GnRH-induced pathway.

Signaling by both the GR and the GnRHR has been previously shown to affect cell proliferation in several different cell types (27, 57–63). Our finding that GnRH alone decreases cell proliferation in LβT2 cells is consistent with a previous report (64). The insensitivity of this effect to both Flot-1 and SGK-1 knockdown taken together with the gene expression and ChIP results suggests that the mechanism of repression of cell proliferation by GnRH does not involve lipid raft-associated GR or SGK-1 expression. Because the GnRHR activates several downstream pathways in LβT2 cells (65), which could potentially target several mediators of cell proliferation effects, factors other than the GR and SGK-1 expression are likely to predominate in mediating the anti-proliferative response to GnRH in these cells. Our finding that synergism between the GR and the GnRHR regulates cell proliferation in LβT2 via up-regulating the SGK-1 gene is novel and suggests a mechanism for cross-talk between the hypothalamic pituitary adrenal and hypothalamic pituitary gonadal axes to regulate proliferation of gonadotrope cells in the pituitary. The cell proliferation effects were significantly ablated by reduction of Flot-1 protein levels, consistent with a role for Flot-1 in the synergistic mechanism. Membrane-associated GR has previously been linked to anti-proliferative effects in A549 cells (27). However unlike these reports showing anti-proliferative effects with Dex alone, we show that in LβT2 cells, Dex alone does not affect cell proliferation but only does so in combination with GnRH, consistent with cell-specific effects. It is tempting to speculate that lipid raft-associated GR modulates GnRH signaling to enhance the GnRH-induced anti-proliferative response by direct effects on the GnRHR. However, we did not detect any changes in tyrosine phosphorylation of the GnRHR in the presence of Dex in LβT2 cells (data not shown). Our finding for a role for SGK-1 in cell proliferation is reminiscent of a previous report showing that SGK-1 activity is required for cortisol-induced reduction of cell proliferation in a hippocampal progenitor cell line (66). Because increases in pituitary cell proliferation have been linked to pathologies such as pituitary tumors, Cushing disease, pituitary hyperplasia, and pituitary developmental dysregulation (67–69), synergy between Dex and GnRH may be a mechanism to prevent such negative physiological consequences. Synergy between membrane-associated GR and GnRHR represents an attractive mechanism for fine-tuning responses and interplay between signaling pathways and may be representative of a more widespread mechanism that awaits further discovery.

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Flot-1- and PKC-mediated GR and GnRHR Cross-talk
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