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**GENOMIC AND NON-GENOMIC CROSS TALK BETWEEN THE
GONADOTROPIN-RELEASING HORMONE RECEPTOR AND
GLUCOCORTICOID RECEPTOR SIGNALLING PATHWAYS**

by

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DECLARATION

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

Signature.....

Date.....

University of Cape Town

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ABSTRACT

The gonadotropin-releasing hormone receptor (GnRHR), a member of the G protein-coupled receptor (GPCR) family, is a central regulator of reproductive function in all vertebrates. A central question in the field is to identify which hormones regulate the GnRHR gene, the signalling pathways that are activated, the transcription factors involved in mediating responses to various signals, and the complex interplay between all these factors. This study investigated the mechanisms of regulation of transcription of the mouse GnRHR gene in the mouse pituitary gonadotrope L β T2 cell line by GnRH and dexamethasone (dex). Real-time PCR showed that both dex and GnRH increase transcription of the endogenous mouse GnRHR gene. Chromatin immunoprecipitation (ChIP) assays revealed the presence of the activating protein-1 (AP-1) proteins c-Jun and c-Fos on the mouse GnRHR promoter under basal as well dex- and GnRH-stimulated conditions. Furthermore, recruitment of the glucocorticoid receptor (GR) to the AP-1 element in response to dex was established by ChIP and interestingly, treatment with GnRH also resulted in recruitment of the GR in the absence of steroid. Knockdown of GR levels by small interference RNA decreased the dex- and GnRH-induced upregulation of the GnRHR gene revealing a requirement for the GR for both the dex and GnRH response. ChIP and immunofluorescence assays provided evidence that both GnRH and dex upregulate the GnRHR gene via nuclear translocation and interaction of the GR with the AP-1 region on the mouse GnRHR promoter. It was also shown that GnRH activates the unliganded GR by rapid phosphorylation of the GR at Ser-234 in a GnRHR-dependent fashion to transactivate a GRE-reporter gene in L β T2 and COS-1 cells. Using kinase inhibitors a direct link was established between GnRH-induced protein kinase C and mitogen-activated protein kinase activation, leading to unliganded GR phosphorylation at Ser-234 and transactivation of the glucocorticoid-response-element (GRE). Furthermore it was shown that GnRH and dex synergistically activate the endogenous GnRHR promoter in L β T2 cells, via a mechanism involving SRC-1 recruitment to the GnRHR AP-1 region. The results suggest a novel mechanism of rapid non-genomic cross talk between the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes via GnRHR-dependent phosphorylation and activation of the unliganded GR in response to GnRH.

TABLE OF CONTENTS

| | |
|---|-------------|
| LIST OF ABBREVIATIONS | xii |
| THEISIS OUTLINE | xv |
| INTRODUCTION | xvii |
| AIMS AND HYPOTHESIS | xix |
| CHAPTER 1 | |
| LITERATURE REVIEW | |
| 1.1 G-PROTEIN COUPLED RECEPTOR FAMILY | 1 |
| 1.1.1 G-protein coupled receptors | 1 |
| 1.1.2 The gonadotropin-releasing hormone receptor | 3 |
| 1.1.3 Tissue distribution and physiological function of the GnRHR | 4 |
| 1.1.4 GnRHR signalling pathways | 5 |
| 1.1.5 Mammalian GnRHR gene structure | 6 |
| 1.1.6 Mouse GnRHR promoter characterization | 7 |
| 1.2 TRANSCRIPTIONAL REGULATION OF THE GnRHR GENE | 9 |
| 1.2.1 Homologous regulation of GnRHR gene expression | 9 |
| 1.2.2 Regulation of GnRHR gene expression by gonadal steroid hormones | 10 |
| 1.2.3 Regulation GnRHR gene expression by glucocorticoids | 11 |
| 1.3 EFFECTS OF STRESS ON REPRODUCTION | 12 |
| 1.4 STEROID HORMONE RECEPTORS | 13 |
| 1.4.1 The glucocorticoid receptor | 13 |
| 1.4.2 GR mechanism of action | 14 |
| 1.4.2.1 Control of gene expression by DNA binding of the GR | 16 |
| 1.4.2.2 Modulation of transcription factor function by the GR | 18 |
| 1.4.4.3 Modulation of activator protein-1 function by the GR | 20 |
| 1.4.3 Post-translational modifications of the GR | 21 |

| | |
|--|-----------|
| 1.5 RAPID STEROID ACTION | 23 |
| 1.5.1 Rapid non-genomic effects of steroid receptors | 23 |
| 1.5.2 Rapid GC-mediated effects | 25 |
| 1.5.2.1 Cross talk of the membrane-bound GR | 25 |
| 1.5.2.2 Cross talk of the classical GR with intracellular signalling proteins | 26 |
| 1.5.2.3 Cross talk of the classical GR with GPCRs | 27 |
| 1.6 GPCR CROSS TALK: FINE-TUNING OF MULTIPLE RECEPTOR SIGNALLING PATHWAYS | 28 |
| 1.6.1 Microdomains as a functional complex to facilitate signalling cross talk | 28 |
| 1.6.2 Scaffolding proteins as mediators of GPCR cross talk | 29 |
| 1.6.3 Cross talk between GPCRs by heterodimerization | 30 |
| 1.6.4 Cross talk of the GnRHR with other receptors | 31 |
| CHAPTER 2 | |
| MATERIALS AND METHODS | |
| 2.1 Compounds and antibodies | 33 |
| 2.2 Incubation with test compounds | 33 |
| 2.3 Plasmids | 34 |
| 2.4 Transformation of bacterial cells and plasmid preparation | 35 |
| 2.5 Cell culture | 35 |
| 2.6 Transient transfections | 36 |
| 2.7 RNA interference | 36 |
| 2.8 Isolation of total RNA | 37 |
| 2.9 cDNA synthesis | 38 |
| 2.10 Quantitative real-time PCR | 38 |
| 2.11 Western blot analysis | 39 |
| 2.12 Autoradiography and quantification | 40 |
| 2.13 Immunofluorescence | 40 |
| 2.14 Chromatin immunoprecipitation assay | 41 |

| | |
|--|-----------|
| 2.14.1 Chromatin preparation | 41 |
| 2.14.2 Immunoprecipitation | 42 |
| 2.14.3 Reversing formaldehyde crosslinks, DNA purification and analysis of precipitated DNA | 43 |
| 2.15 Statistical analysis | 44 |
| CHAPTER 3 | |
| AN INVESTIGATION INTO HOMOLOGOUS AND GC REGULATION OF THE MOUSE GnRHR GENE IN THE LβT2 GONADOTROPE CELL LINE | |
| 3.1 BACKGROUND | 45 |
| 3.2 AIMS | 47 |
| 3.3 RESULTS | 48 |
| 3.3.1 Both GnRH and dex increase expression of the endogenous mouse GnRHR gene in L β T2 cells | 48 |
| 3.3.2 GnRH but not dex induce a minimal AP-1 reporter construct in L β T2 cells | 49 |
| 3.3.3 GnRH but not dex increases AP-1 protein expression levels | 50 |
| 3.3.4 Both c-Jun and c-Fos proteins are bound under basal as well as dex- and GnRH-stimulated conditions to the AP-1 region of the endogenous GnRHR promoter in intact cells | 53 |
| 3.3.5 Steroid receptor expression in L β T2 cells | 55 |
| 3.3.6 Both dex and GnRH induce recruitment of the GR to the AP-1 region on the endogenous GnRHR promoter in intact cells | 58 |
| 3.3.7 Protein expression levels of the GR remain unchanged in response to dex or GnRH | 59 |
| 3.3.8 The GR is required for transcriptional regulation of the endogenous GnRHR by GnRH and dex | 61 |

CHAPTER 4

INVESTIGATION OF LIGAND-INDEPENDENT ACTIVATION OF THE GLUCOCORTICOID RECEPTOR BY GnRH AND SIGNALLING PATHWAYS IN THE L β T2 GONADOTROPE CELL LINE

| | |
|--|-----------|
| 4.1 BACKGROUND | 63 |
| 4.2 AIMS | 65 |
| 4.3 RESULTS | 66 |
| 4.3.1 GnRH mediates nuclear translocation of the unliganded endogenous GR | 66 |
| 4.3.2 Activation of a GRE by GnRH is GR- and GnRHR-dependent in L β T2 cells | 67 |
| 4.3.3 Ligand-independent activation of a GRE is dependent on both the GR and the GnRHR in COS-1 cells | 70 |
| 4.3.4 The GnRHR mediates site-specific phosphorylation of the unliganded GR by GnRH in L β T2 cells | 72 |
| 4.3.5 Activation of MAPKs by GnRH in L β T2 cells | 75 |
| 4.3.5.1 GnRH but not dex rapidly activates c-Jun-N-terminal kinase (JNK) | 75 |
| 4.3.5.2 p38 is rapidly induced by GnRH and not dex in L β T2 cells | 77 |
| 4.3.5.3 ERK is activated by short-term GnRH treatment while only long-term dex stimulation results in ERK activation in L β T2 cells | 78 |
| 4.3.6 Role of Kinases in dex- and GnRH-mediated transactivation and GR phosphorylation | 80 |
| 4.3.6.1 MAPKs are involved in GnRH- and dex-mediated transactivation of the GR in L β T2 cells | 80 |
| 4.3.6.2 MAPK are involved in GnRH-mediated AP-1 transactivation | 82 |
| 4.3.6.3 GnRH-stimulated phosphorylation of the unliganded GR at Ser-234 is mediated by a combination of JNK, p38 and ERK | 83 |
| 4.3.6.4 PKC and c-src are involved in GnRH-mediated transactivation | 87 |
| 4.3.6.5 PKC and c-src are involved in AP-1 transactivation by GnRH | 89 |
| 4.3.6.6 PKC is involved in GnRH-mediated GR phosphorylation of Ser-234 | 89 |

CHAPTER 5

AN INVESTIGATION INTO THE SYNERGISTIC EFFECT OF DEX AND GnRH IN L β T2 CELLS

| | |
|---|------------|
| 5.1 BACKGROUND | 92 |
| 5.2 AIMS | 93 |
| 5.3 RESULTS | |
| 5.3.1 GnRH and dex synergistically increase gene expression | 94 |
| 5.3.1.1 GnRH and dex synergistically increase expression of the endogenous mouse GnRHR gene and a transfected GRE in a GR- and GnRHR-dependent fashion | 94 |
| 5.3.1.2 The GR and GnRHR are essential for the GnRH- and dex-induced synergistical increase in GRE-transactivation in COS-1 cells | 96 |
| 5.3.2 Co-stimulation with dex plus GnRH does not increase GR phosphorylation at Ser-220 or Ser-234 LβT2 cells | 99 |
| 5.3.3 GnRH plus dex-stimulated phosphorylation of the GR is mediated by a combination of JNK, p38 and ERK | 100 |
| 5.3.4 Transcription factors involved in synergism | 102 |
| 5.3.4.1 AP-1 protein levels bound to the GnRHR remain unchanged in response to dex plus GnRH in intact L β T2 cells | 102 |
| 5.3.4.2 GR recruitment to the endogenous GnRHR promoter in response to dex plus GnRH is similar to that observed for dex alone in intact L β T2 cells | 104 |
| 5.3.4.3 Synergism between dex and GnRH involves recruitment of SRC-1 to the GnRHR promoter in L β T2 cells | 105 |

CHAPTER 6

DISCUSSION AND CONCLUSION

| | |
|---|------------|
| 6.1 Both GnRH and dex upregulate the GnRHR gene via the GR by a genomic cross talk mechanism converging on the AP-1 site on the mGnRHR promoter in LβT2 cells | 108 |
| 6.2 GnRH activates the unliganded GR by a rapid non-genomic cross talk mechanism | |

| | |
|---|------------|
| involving GnRHR-dependent GR phosphorylation at Ser-234 | 113 |
| 6.3 Synergism occurs between the dex and GnRH response | 118 |
| 6.4 Ligand-independent activation of the GR and other steroid receptors: physiological implications | 120 |
| 6.5 Proposed model for genomic and non-genomic cross talk of the GnRHR and GR signalling pathways in L β T2 cells | 122 |
| 6.6 Conclusions | 131 |
| 6.7 Future perspectives | 131 |
| ADDENDUM A | |
| SUPPLEMENTARY DATA | 136 |
| ADDENDUM B | |
| RESULTS RELEVANT TO THE PRESENT STUDY OBTAINED BY OTHER'S IN PROF. HAPGOOD'S LABORATORY | 149 |
| ADDENDUM C | |
| PRIMER SEQUENCES | 151 |
| C1: Primers used for real-time RT PCR | 151 |
| C2: Primers used in CHIP assays | 151 |
| ADDENDUM D | |
| BUFFERS AND SOLUTIONS | 152 |
| D1. Growth medium | 152 |
| D2: RNA isolation | 152 |
| D3: Western blot analysis | 153 |
| D4: Chromatin Immunoprecipitation (ChIP) buffers | 154 |
| BIBLIOGRAPHY | 156 |

LIST OF ABBREVIATIONS

| | |
|-------|---------------------------------------|
| ANOVA | analysis of variance |
| AP-1 | activator protein-1 |
| AC | adenyly cyclase |
| AF | activation function |
| BIM | bisindolylmaleimide |
| bp | base-pair |
| cAMP | cyclic adenosine monophosphate |
| cDNA | complementary DNA |
| c-Fos | cellular Fos |
| ChIP | chromatin immunoprecipitation |
| c-Jun | cellular Jun |
| CRE | cAMP response element |
| CREB | cAMP response element binding protein |
| CBP | CREB-binding protein |
| DAG | diacylglycerol |
| DEPC | diethylpyrocarbonate |
| dex | dexamethasone |
| DMEM | dulbecco's modified eagle medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediaminetetra-acetic acid |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| eNO | endothelial nitric oxide |
| eNOS | endothelial nitric oxide synthase |
| ER | estrogen receptor |
| ERK | extracellular signal-regulated kinase |

| | |
|-----------------|---|
| FCS | fetal calf serum |
| FSH | follicle-stimulating hormone |
| GC | glucocorticoid |
| GR | glucocorticoid receptor |
| GRAS | GnRHR-activating sequence |
| GRE | glucocorticoid response element |
| GRIP-1 | glucocorticoid receptor interacting protein-1 |
| GnRH | gonadotropin-releasing hormone |
| GnRHR | GnRH receptor |
| GPCR | G-protein coupled receptor |
| HAT | histone acetyltransferase |
| HPA | hypothalamic-pituitary-adrenal |
| HPG | hypothalamic-pituitary-gonadal |
| HRE | hormone response element |
| IgG | immunoglobulin G |
| IP ₃ | inositol-(1,4,5)-triphosphate |
| JNK | jun N-terminal kinase |
| kb | kilobasepair |
| LB | Luria-Bertani |
| LH | luteinizing hormone |
| MAPK | mitogen-activated protein kinase |
| MMTV | mouse mammalian tumor virus |
| MOPS | 4-morpholine-propanesulfonic acid |
| MR | mineralocorticoid receptor |
| mRNA | messenger ribonucleic acid |
| NF- κ B | nuclear factor-kappa B |
| PAGE | polyacrylamide gel electrophoresis |
| PR | progesterone receptor |
| PRE | progesterone response element |
| PBS | phosphate-buffered saline |

| | |
|---------|--|
| PKA | protein kinase A |
| PKC | protein kinase C |
| RT | room temperature |
| RTK | receptor tyrosine kinase |
| SDS | sodium dodecyl sulphate |
| SF-1 | steroidogenic factor-1 |
| SRC-1 | steroid receptor co-activator-1 |
| SRC-2 | steroid receptor co-activator-2 |
| STAT | signal transducer and activator of transcription |
| SURG | sequence underlying responsiveness to GnRH |
| SWI/SNF | switch/sucrose nonfermentable protein |
| TAE | Tris-acetate EDTA |
| TBS | Tris-buffered saline |
| TE | Tris-EDTA |
| UTR | untranslated region |
| v/v | volume per volume |
| w/v | weight per volume |

THESIS OUTLINE

Please note that sections of this study have been accepted for publication in Molecular Endocrinology (MS # ME-08-0464):

GENOMIC AND NON-GENOMIC CROSS TALK BETWEEN THE GONADOTROPIN-RELEASING HORMONE RECEPTOR AND GLUCOCORTICOID RECEPTOR SIGNALLING PATHWAYS

Andrea Kotitschke, Hanél Sadie-Van Gijzen, Chanel Avenant, Sandra Fernandes, Janet P. Hapgood

This thesis contains the following sections:

1. A short **introduction** will outline the main field of the present study.
2. A summary of the general **hypotheses and aims** of the study.
3. Chapter 1: **Literature review**. This chapter gives a detailed overview of the current literature, with a focus on the expression and transcriptional regulation of the mouse GnRHR, the GR mechanism of action, non-classical GR action and GPCR cross talk.
4. Chapter 2: **Materials and Methods**. This chapter gives details regarding the experimental protocols used to obtain the results presented in chapters 3 to 5.
5. Chapter 3: **Results**. In the first part of this study **homologous- and GC-mediated transcriptional regulation of the mouse GnRHR gene** was investigated in the mouse gonadotrope LBT2 cell line. A brief introduction the specific aims and the results will be presented in this chapter.
6. Chapter 4: **Results**. The second part of the study was further investigating the **ligand-independent activation of the GR by GnRH** which was found in Chapter 3, as well as the **signalling pathways** involved in dex- and GnRH-mediated transactivation. A short introduction will give an overview on the current knowledge in the field and specific aims are formulated.
7. Chapter 5: **Results**. The 3rd results chapter contains results of an extensive study on the **synergistic effect of dex plus GnRH on gene transcription**. The chapter includes a brief introduction, aims and all the results obtained.

8. Chapter 6: **Discussion and Conclusions**. In this final chapter, the results presented in previous chapters 3 to 5 are discussed. This chapter also contains proposed models which will be discussed in context with the results obtained. In addition, some perspectives about future research are included.
9. **Addendum A**: This addendum contains additional results from the study not presented in chapters 3 to 5.
10. **Addendum B**: Results obtained by previous students in Prof. Hapgood's laboratory are shown, which are relevant for the present study.
11. **Addendum C**: This addendum contains sequences of all primers used in this study.
12. **Addendum D**: This addendum contains information regarding buffers and solutions used in the experimental protocols.
13. The **Bibliography** contains a list of all the references used throughout the thesis in alphabetical order.
14. Thereafter follows a copy of the **publication** "Genomic and non-genomic cross talk between the gonadotropin-releasing hormone receptor and glucocorticoid receptor signalling pathways" (2009) Kotitschke A, Sadie-Van Gijsen H, Avenant C, Fernandes S, Hapgood JP. Mol Endocrinol 23(11):1726-1745.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the central endocrine regulator of reproduction. GnRH is a hypothalamic decapeptide synthesized by hypothalamic neurosecretory cells and released in a pulsatile fashion into the portal circulation (Fink, 1988). After binding to its cognate receptor (GnRHR) on pituitary gonadotropes, the hormone stimulates the biosynthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn regulate gonadal steroidogenesis and gametogenesis in both sexes (Fink, 1988). The GnRHR is a member of the plasma membrane G-protein coupled receptor (GPCR) family (Bockaert and Pin, 1999) and is primarily expressed in the anterior pituitary in gonadotrope cells (Hyde *et al.*, 1982). The expression of the GnRHR in various extra-pituitary tissues including the ovary, breast, placenta as well as hormone dependent tumors has been reported (Emons *et al.*, 1989). Thus it has become evident that GnRH is a potentially important autocrine and/or paracrine regulator of local cellular functions in some extra-pituitary tissues (Cheng and Leung, 2005; Hapgood *et al.*, 2005).

The responsiveness of the pituitary to GnRH is dependent on receptor numbers expressed on the cell surface which is in part regulated by GnRHR gene expression (Kaiser *et al.*, 1993; Marian *et al.*, 1981; Norwitz *et al.*, 1999a; Savoy-Moore *et al.*, 1980; Yasin *et al.*, 1995). GnRH has been identified as a potent regulator of GnRHR gene expression *in vivo* (Kaiser *et al.*, 1993; Wu *et al.*, 1994; Yasin *et al.*, 1995) and *in vitro*. (Kaiser *et al.*, 1993; Loumaye and Catt, 1982; Young *et al.*, 1984). Although regulation of expression of GnRHR has been shown to occur at the transcriptional, translational and post-translational level, the underlying molecular mechanisms including signalling pathways and transcription factors involved have not been fully investigated.

In addition to GnRH, other hormones have been shown to regulate GnRHR gene expression, including gonadal steroids such as estradiol and progesterone (Cheng *et al.*, 2001), activin (Fernandez-Vazquez *et al.*, 1996; Pernasetti *et al.*, 2001) and pituitary adenylate cyclase-activating peptide (PACAP) (Cheng and Leung, 2001; Pincas *et al.*, 2001). Furthermore, glucocorticoids (GCs) have also been shown in some cases to regulate GnRHR gene expression in vertebrates, although the underlying mechanisms are not well defined. Although chronic stress and GCs appear to

predominantly suppress gonadotropin secretion and reproduction, the effects of transient or acute stress are variable and the effects may depend on species and physiological status (Brann and Mahesh, 1991; Tilbrook *et al.*, 2000). Clearly further work is required to determine the target cells, target genes, GC-receptors involved and mechanism of action of GC-mediated regulation of GnRHR gene expression. It is possible that a combination of one or all of these mechanisms may be involved, resulting in different responses.

It is becoming increasingly clear that multiple signalling pathways are simultaneously activated in the same cell and these pathways cross talk to affect biological responses thereby possibly fine-tuning biological responses. The GnRHR may be a potential target facilitating cross talk between neuroendocrine-, stress- and immune-responses. Unravelling the molecular mechanism of this complex interplay between several hormones might add another level towards understanding receptor physiology and may be of great relevance to drug design since both GCs and GnRH are administered as therapeutic drugs.

AIMS AND HYPOTHESES

The project was based of the following hypotheses:

- I) That the mouse GnRHR gene is transcriptionally upregulated by dexamethasone (dex) and GnRH in L β T2 cells;
- II) That the activator-protein 1 (AP-1) regulatory element in the GnRHR promoter plays an important role in GnRH- and dex-mediated regulation of the GnRHR due to binding of the AP-1 proteins c-Jun and c-Fos to the promoter in intact L β T2 cells;
- III) That the glucocorticoid receptor (GR) is recruited to the AP-1 site in the GnRHR promoter as an essential transcriptional factor mediating transcriptional regulation of the GnRHR gene expression in response to dex in L β T2 cells;
- IV) That cross talk between the GnRHR and the GR results in rapid post-transcriptional modifications of the GR, nuclear translocation of the receptor and transcriptional activation of a glucocorticoid response element (GRE) in the absence of a GR ligand;
- V) That mitogen-activated-protein kinases (MAPKs), protein kinase C (PKC), protein kinase A (PKA) and tyrosine kinase (c-src) are important signalling pathways mediating transcriptional effects of dex and GnRH in L β T2 cells;
- VI) That dex synergistically enhances the GnRH-induced GnRHR gene expression in L β T2 cells;

The aim of the first part of this study was to unravell the molecular mechanism of homologous and dex-mediated GnRHR gene regulation in L β T2 cells. In particular the project focused on the involvement of the AP-1 proteins c-Jun and c-Fos as well as the glucocorticoid receptor (GR) in mediating basal, GnRH-, and dex-mediated transcriptional regulation of the GnRHR gene. Therefore the protein expression levels as well as *in vivo* binding of these proteins to the AP-1 regulatory element in the GnRHR promoter were investigated. Furthermore a requirement for the GR in GnRHR gene expression was established by using GR specific siRNA. The second part of the study aimed to follow up and further investigate ligand independent activation of the GR by GnRH. Several steps of the classical GR mechanism of action were investigated including nuclear translocation, phosphorylation and transactivation. Furthermore, the signalling pathways activated in response to

dex and GnRH and their potential involvement in mediating transcriptional effect were investigated in this part of the study. In particular the three major MAPKs namely c-Jun N-terminal Kinase (JNK), extracellular regulated Kinase (ERK) and p38 as well as PKC, PKA and c-src were investigated in L β T2 cells. The last part of the study investigated the effect of dex plus GnRH on GnRHR gene expression. The underlying mechanism was investigated. More specific aims are presented in the relevant chapters.

University of Cape Town

CHAPTER 1

LITERATURE REVIEW

1.1 G-PROTEIN COUPLED RECEPTOR FAMILY

1.1.1 G-protein coupled receptors

Among membrane bound receptors G-protein coupled receptors (GPCR) form the largest and most diverse family of cell surface molecules that detect extracellular stimuli and activate intracellular signal transduction pathways and, ultimately, cellular responses (Bockaert and Pin, 1999). The wide array of stimuli that activate these receptors includes hormones, light, pheromones, peptides, neurotransmitters, lipids and ions. GPCRs share a common central core domain comprised of 7 membrane spanning α -helices which are connected by 3 intracellular and 3 extracellular loops, as well as a carboxyl (C)-terminal intracellular domain and an amino (N)-terminal extracellular domain (Bockaert and Pin, 1999). These distinct domains confer specific properties to the GPCRs and contribute to ligand recognition, activation of the receptor, effector molecule binding and initiation of downstream signalling events (Bockaert and Pin, 1999; Millar *et al.*, 2004). GPCRs are desensitized by sustained stimulation with ligand followed by internalization of the receptor. These processes require serine/threonine phosphorylation in the C-terminal tail (Carman *et al.*, 1998; Liggett *et al.*, 1989; McArdle *et al.*, 2002).

GPCRs mediate signal transduction of extracellular stimuli through interaction of their intracellular domains with heterotrimeric G-protein. They consist of the G_{α} -subunit associated with GDP and the tightly associated $G_{\beta\gamma}$ -subunits. More than 20 G_{α} -subunits are known and can be divided into four major classes of G_{α} -subunits: G_{α_s} , G_{α_i} , $G_{\alpha_q/11}$, and $G_{\alpha_{12/13}}$ (Kraus *et al.*, 2001). Agonist binding to the GPCR induces a conformational change in the receptor that allows the exchanges of GDP for GTP on the G_{α} subunit. This exchange triggers the dissociation of the G_{α} -subunit, now bound to GTP, from the receptor and the $G_{\beta\gamma}$ dimer. Both G_{α} -GTP and $G_{\beta\gamma}$ can then activate different signalling cascades to regulate key biological functions (Ostrom *et al.*, 2000). Depending on the class of G-protein to which the GPCR is coupled, a wide variety of downstream effector proteins and signalling pathways can be activated (Marinissen and Gutkind, 2001; Neves *et al.*, 2002). The G_{α_s} -subunit exerts its action by

activating adenylyl cyclase (AC) which in turn increases intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP) and results in activation of protein kinase A (PKA) (Beebe, 1994; Birnbaumer, 1992). In contrast, the G_{α_i} -subunit inhibits adenylyl cyclase but has been shown to be involved in activation of ion channels and phospholipases (PLs) (Birnbaumer, 1992; Naor *et al.*, 2000). The α_q -subunit of G_q activates primarily PLC which increases the second messengers diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP3) leading to Ca^{2+} mobilization and activation of protein kinase C (PKC) (Naor *et al.*, 2000; Ostrom *et al.*, 2000). In addition it has become clear that $\beta\gamma$ -subunit dimers can mediate signal transduction pathways including activation of phosphoinositide-3 kinase (PI3k), molecules in the MAPK pathways and PLC (Hur and Kim, 2002; Luttrell *et al.*, 1995; Naor *et al.*, 2000; Stephens *et al.*, 1997). The combination of interactions of a GPCR with several G-proteins may confer increased diversity and specificity of complex signalling pathways (Hur and Kim, 2002). Because of the diverse array of ligands, the diversity of G-proteins and effector molecules, GPCRs regulate most biological functions including cell proliferation and growth, metabolism, cell survival and differentiation (Marinissen and Gutkind, 2001).

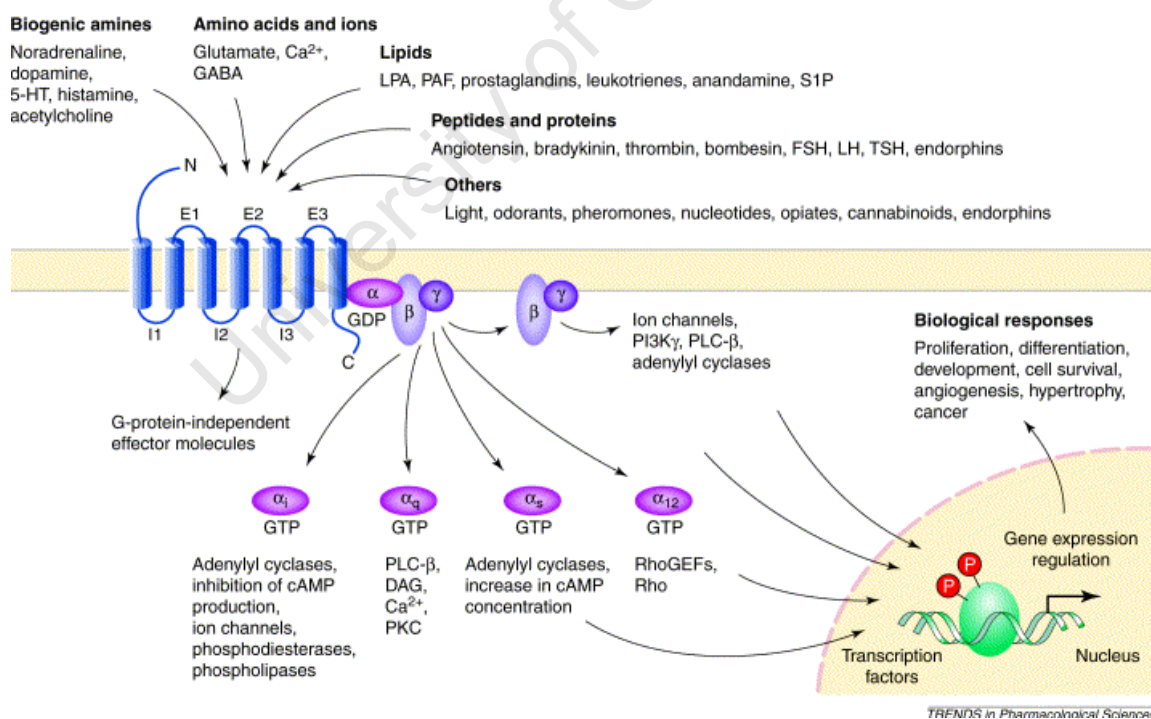


Figure 1.1: The G-protein coupled receptor (GPCR) signaling network. Activation of the GPCR by ligand leads to the dissociation of the GDP-bound G_{α} -subunit and the tightly associated $G_{\beta\gamma}$ -subunit

which are able to interact with a diverse array of effector molecules involved in signal transduction pathways to regulate key biological responses (Marinissen and Gutkind, 2001).

1.1.2 The gonadotropin-releasing hormone receptor

The gonadotropin-releasing hormone receptor (GnRHR) is a member of the plasma membrane G-protein-coupled receptor (GPCR) family (Bockaert and Pin, 1999) which is primarily expressed in gonadotrope cells in the anterior pituitary (Hyde *et al.*, 1982). The interaction of gonadotropin-releasing hormone (GnRH) and its receptor initiates transduction of hormone signals across the cell membrane which is a crucial event in endocrine signalling. GnRH is a decapeptide secreted in a pulsatile fashion from the hypothalamus from where it travels through the hypophysial portal to the anterior pituitary (Fink, 1988). Upon binding of GnRH to its cognate receptor (GnRHR) on pituitary gonadotropes, a broad range of signalling cascades is activated leading to synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fink, 1988). The release of these two hormones stimulates gametogenesis and steroidogenesis in both sexes (Cheng and Leung, 2005). Changes in frequency and amplitude of GnRH pulses have differential effects on the synthesis rate of LH β and FSH β in the gonadotropes (Shupnik, 1996) and result in a differential secretion pattern of these hormones thereby regulating the synthesis and release of sex hormones in the gonads (Fink, 1988; Millar *et al.*, 2004). In turn, sex hormones such as androgens and estrogens regulate GnRH responsiveness via positive and negative feedback loops to the hypothalamus and the anterior pituitary (Gharib *et al.*, 1990).

The first primary structure of the mammalian GnRHR was determined by sequencing of the receptor cDNA isolated from an immortalized murine gonadotrope cell line (α T3-1 cells) (Reinhart *et al.*, 1992; Tsutsumi *et al.*, 1992). Subsequently, the sequences of GnRHRs from several mammalian species including rat (Eidne *et al.*, 1992), sheep (Brooks *et al.*, 1993), human (Chi *et al.*, 1993), bovine (Kakar *et al.*, 1993), pig (Weesner and Matteri, 1994) as well as non-mammalian vertebrates have been described (Millar *et al.*, 2004). These receptors were all designated as type I GnRHRs. The mammalian type I GnRHR lacks a C-terminal tail setting it apart from non-mammalian GnRHRs and other GPCRs, and appears not to undergo agonist-induced receptor desensitisation and thus internalizes slowly (Kaiser *et al.*, 1997; McArdle *et al.*, 2002).

At least two isoforms of the peptide hormone GnRH (GnRH I and GnRH II) have been found in most vertebrates, however only type I GnRHR proteins have been identified in pituitary as well as extra-pituitary tissues and cell lines from several mammalian species (Millar, 2003; Pawson *et al.*, 2003; van Biljon *et al.*, 2002). Although type II GnRHR transcripts have been detected in most vertebrates, expression of a functional type II GnRHR protein has not been conclusively shown and its precise role remains to be elucidated (Millar *et al.*, 2004; Morgan *et al.*, 2003; Neill, 2002; van Biljon *et al.*, 2002)

1.1.3 Tissue distribution and physiological function of the GnRHR

The GnRHR was first identified exclusively in pituitary gonadotropes (Fink, 1988; Hyde *et al.*, 1982; Kaiser *et al.*, 1997). Here, binding of GnRH to its receptor activates intracellular signal transduction pathways to affect the synthesis of the heterodimeric glycoprotein hormones composed of the common subunit α and the specific β -subunits LH β and FSH β (Gharib *et al.*, 1990; Hamernik, 1995). The pulsatile release of these gonadotropins regulates development, growth, pubertal maturation, and reproductive processes by the release of sex steroids. Naturally-occurring mutations of GnRHR have been associated with a condition named hypogonadotropic hypogonadism with symptoms such as the absence of secondary sexual characteristics, delayed onset of puberty or low sex steroid levels (Millar *et al.*, 2004; Seminara *et al.*, 1998) demonstrating a crucial role of the receptor in the physiology of reproduction (Naor, 2009). GnRHR agonists and antagonists have been widely used in clinical applications such as treatment of infertility, hormone-dependent diseases and cancers (Conn and Crowley, 1994; Neill, 2002).

In addition to its established endocrine role in gonadotropin regulation in the pituitary, there is accumulating evidence in the literature showing that the GnRHR is widely expressed in various extra-pituitary mammalian tissues and cells including reproductive tissues and non-reproductive tissues suggesting a functional role for GnRH and its receptor as an important autocrine and/or paracrine regulator in diverse extra-pituitary tissues (Cheng and Leung, 2005; Hapgood *et al.*, 2005). In female reproductive tissues GnRH, acting via the GnRHR, has been shown to be involved in regulation of the menstrual cycle (Raga *et al.*, 1998), ovarian steroidogenesis (Guerrero *et al.*, 1993), embryo implantation and maintenance of pregnancy (Rama and Rao, 2001). In male reproductive tissues

GnRH and its cognate receptor have been shown to play a role in testis and sperm development (Cheung and Hearn, 2003) as well as sperm oocyte interaction (Morales, 1998).

Functional GnRHR has been detected in a wide range of the carcinomas and tissues originating from the endometrium, ovary, breast and in tumors not related to the reproductive system such as melanoma (Imai *et al.*, 1994; Imai and Tamaya, 2000; Limonta *et al.*, 2003). The expression of the receptor in cancer tissues implies that GnRH may have a direct regulatory role in cell proliferation (Imai and Tamaya, 2000). It is well known that GnRH analogues can inhibit proliferation in human malignant tumors (Cheng and Leung, 2005; Grundker *et al.*, 2002). The anti-tumor effects appear to be mediated via inhibition of gonadotropins and gonadal steroids and modulation of growth factor or cytokine expression (Cheng and Leung, 2005). However the intracellular mechanisms mediating the antiproliferative effects are not yet fully understood (Pawson *et al.*, 2003).

A few studies support an active role for GnRH in regulating immune responses. GnRH has been found to interact directly with T-cells and stimulate adhesion, migration and homing of normal T-cells into specific organs (Chen *et al.*, 2002). In addition GnRHRs have been identified in the spleen and thymus in mice and the expression of the receptor was increased in response to GnRH in immune cells (Jacobson *et al.*, 1998).

1.1.4 GnRHR signalling pathways

The nature of the intracellular signalling pathways initiated by the GnRHR has been shown to vary between cell types. It was proposed that the GnRHR may exert its various effects by interacting with several G-proteins (Liu *et al.*, 2002b; Stanislaus *et al.*, 1998). By contrast, GnRHR may interact with a single G-protein leading to activation of different downstream signalling cascades (Grosse *et al.*, 2000; Naor, 2009). It was reported that the GnRHR interacts mainly with $G_{q/11}$ in α T3-1 pituitary gonadotrope cell lines (Grosse *et al.*, 2000; Hsieh and Martin, 1992; Naor *et al.*, 1986) while other investigators show that the GnRHR can couple to $G_{q/11}$, G_i and G_s in rat pituitary cell cultures (Hawes *et al.*, 1993; Stanislaus *et al.*, 1998) and L β T2 cells (Knollman and Conn, 2008; Kraus *et al.*, 2001; Liu *et al.*, 2002b; Yokoi *et al.*, 2000).

Several reports in the literature have further unravelled the downstream kinase pathways in gonadotrope cell lines. In pituitary derived α T3-1 cells GnRH activates PLC β which enhances the turnover of phosphoinositide, leading to increased levels in DAG and IP3 and activation of various PKC subspecies (Harris *et al.*, 1997). GnRH can induce mobilization of intracellular Ca²⁺ and influx of extracellular Ca²⁺ which are important triggers for GnRH-mediated secretion of LH and FSH (Naor, 1990; Stojilkovic and Catt, 1995). In addition GnRH stimulation was shown to elevate intracellular cAMP level in rat pituitary cultures (Borgeat *et al.*, 1972) and L β T2 cells (Lariviere *et al.*, 2007), but not in α T3-1 cells (Horn *et al.*, 1992; Kanasaki *et al.*, 2002) which is consistent with the finding that the GnRHR couples exclusively to G_{q/11} in α T3-1 cells (Grosse *et al.*, 2000) whereas the receptor couples to both G_s and G_q in L β T2 cells (Liu *et al.*, 2002b). Activation of a number of mitogen-activated protein kinases (MAPK) by GnRH in pituitary cells has been demonstrated and appears to depend on the cell-context (Dobkin-Bekman *et al.*, 2006; Kraus *et al.*, 2001; Naor, 2009; Yokoi *et al.*, 2000). MAPK kinases (MKKs) catalyze the phosphorylation of MAPK and thereby increase their activity (Johnson and Lapadat, 2002). Inversely, phosphatases dephosphorylate MAPKs and return them in an inactive state. The MAPK family is composed of four major subfamilies of kinases: extracellular-regulated kinases (ERK1,2) also known as p42/p44; ERK5 (big MAPK), the c-Jun-N-terminal kinases (JNK1/2/3), and p38 (α , β , β 2, γ , δ) (Harris *et al.*, 2002; Johnson and Lapadat, 2002; Liu *et al.*, 2002a; Liu *et al.*, 2002b; Pearson *et al.*, 2001; Yokoi *et al.*, 2000). An important characteristic of MAPKs is their ability to translocate to the nucleus and activate gene transcription (Naor *et al.*, 2000; Pearson *et al.*, 2001). Upon stimulation of the GnRHR extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK) and p38 are activated in both α T3-1 and L β T2 cells, by both protein kinase A (PKA) - and protein kinase C (PKC) -dependent and -independent pathways (Bonfil *et al.*, 2004; Liu *et al.*, 2002a; Liu *et al.*, 2003; Mulvaney *et al.*, 1999; Roberson *et al.*, 1999; Sundaresan *et al.*, 1996). In addition, GnRH activates phosphoinositide-3-kinase (PI3K) and src-tyrosine kinase (c-src) in L β T2 cells (Bonfil *et al.*, 2004; Kanasaki H, 2006).

1.1.5 Mammalian GnRHR gene structure

Cloning of the GnRHR gene from mouse (Zhou and Sealfon, 1994), human (Fan *et al.*, 1995), pig (Jiang *et al.*, 2001), sheep (Campion *et al.*, 1996) and rat (Reinhart *et al.*, 1997) reveals that the gene contains two introns and three exons (Fan *et al.*, 1994; Kakar, 1997). Exon 1 encodes the 5'-

untranslated region (UTR), the transmembrane (TM) domains I to III and a portion of TMD IV. Exon 1 is separated by a 4.2 kb intron from exon 2 which encodes the remainder of the TMD IV, TMD V and part of the intracellular loop 3. Exon 3 is followed by a 5 kb intron and encodes the remaining sequence of the open reading frame which includes TMD VI, VII and the 3'-UTR (Chi *et al.*, 1993; Kakar, 1997; Kakar *et al.*, 1992). Location of all exon-intron boundaries are perfectly conserved amongst the different species (Cheng and Leung, 2005). The GnRHR gene exists as a single copy gene and encodes a 327 to 328-amino acid protein (Cheng and Leung, 2005; Fan *et al.*, 1994).

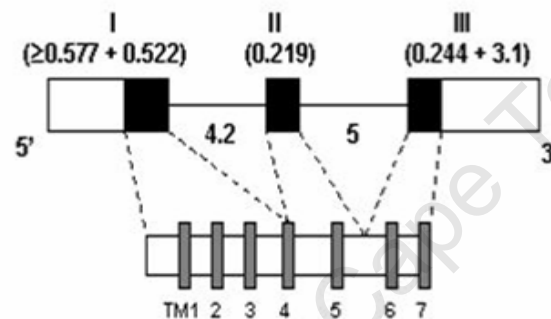


Figure 1.2: Structural organization of the human GnRHR I gene

Exons (I, II and III) are illustrated as boxes, with portions of exons containing coding sequences shown in black and untranslated regions in white. Sizes of coding portions are indicated above in kilobasepairs. The sizes of the introns separating the exons are indicated below. (Hapgood *et al.*, 2005)

1.1.6 Mouse GnRHR promoter characterization

The 5'-flanking region of the mouse GnRHR gene has been cloned and the major transcriptional start site has been identified 62 nucleotides upstream of the translational start site (Albarracin *et al.*, 1994). While several minor transcriptional start sites were found to be present further upstream in the promoter, TATA or CAAT sequences were found to be absent from the promoter region (Albarracin *et al.*, 1994). Albarracin *et al.* further demonstrated in transient transfection studies that a 1.2 kb mouse GnRHR genomic fragment fused to luciferase reporter gene appears to be sufficient to direct high levels of transcription in α T3-1 cells. This region appears to be pituitary- and gonadotrope-specific since considerably lower reporter expression occurred in transfected rat somatolactotrophic GH₃ cells

and only very low expression levels were found in the transfected placental cell line JEG-3 and the kidney fibroblast cell line CV-1 (Albarracin *et al.*, 1994; Kaiser *et al.*, 1997). Sequencing analysis revealed a consensus activator protein-1 (AP-1) element at position -336/-330 relative to the translational start site as well as a gonadotrope-specific element (GSE)-like sequence at position -15/-7 (Albarracin *et al.*, 1994; Kaiser *et al.*, 1997). The latter has been identified as a binding site for the orphan nuclear receptor steroidogenic factor-1 (SF-1) (Duval *et al.*, 1997a). Clay *et al.* showed that elements residing between -500 and -400 bp relative to translational start site of mouse GnRHR promoter confer cell-specific expression (Clay *et al.*, 1995) and other investigators identified an tripartite enhancer element that regulates cell specific expression comprised of a binding site for SF-1 at -244/-236 bp (Duval *et al.*, 1997a), a consensus AP-1 site at -336/-330 bp and an element referred to as GnRHR activating sequences (GRAS) at -391/-380 bp relative to translational start site (Duval *et al.*, 1997b). GRAS was found to contain overlapping functional elements and binding sites for SMAD, AP-1 and FoxL2 proteins (Ellsworth *et al.*, 2003a; Norwitz *et al.*, 2002b). In addition a region designated Sequences underlying Responsiveness to GnRH-1 (SURG-1) has been identified including binding sites for Oct-1 and nuclear factor Y (NF-Y) (Kam *et al.*, 2005). Furthermore a region termed DARE (down-stream activin regulatory element) was found to contain binding sites for LHX2 a member of the LIM homeodomain family (Cherrington *et al.*, 2005), as well as an ATTA element located at -360 bp in the proximal GnRHR promoter where LHX3 was shown to bind *in vitro* and *in vivo* (McGillivray *et al.*, 2005).

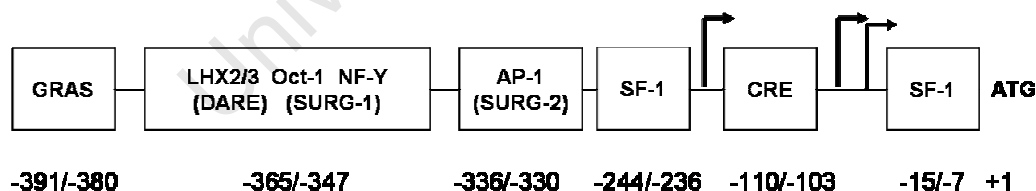


Figure 1.3: Functional elements in the mouse GnRHR promoter

Functional *cis*-elements that have been characterized are shown as black boxes. Transcription start sites (arrows) and the translation start site (ATG) are indicated. Abbreviations: SF-1 = steroidogenic factor-1 binding site; CRE = cAMP response element; AP-1 = activator protein 1 binding site; Oct-1 = octamer transcription factor-1 binding site; GRAS = GnRH receptor activating sequence; DARE = downstream activin response element; SURG = sequence underlying responsiveness to GnRH; NF-Y = nuclear factor-Y binding site (Hapgood *et al.*, 2005).

1.2 TRANSCRIPTIONAL REGULATION OF THE GnRHR GENE

1.2.1 Homologous regulation of GnRHR gene expression

The responsiveness of the pituitary to GnRH is dependent on receptor numbers expressed on the cell surface which is in part regulated by GnRHR gene expression (Kaiser *et al.*, 1993; Marian *et al.*, 1981; Norwitz *et al.*, 1999a; Savoy-Moore *et al.*, 1980; Yasin *et al.*, 1995). Several hormones including GnRH regulate GnRHR gene expression *in vivo* (Kaiser *et al.*, 1993; Wu *et al.*, 1994; Yasin *et al.*, 1995). Studies in rats showed that pulsatile administration of GnRH upregulated GnRHR expression while continuous stimulation with GnRH did not increase pituitary GnRHR mRNA levels (Yasin *et al.*, 1995). In contrast, continuous GnRH administration in sheep reduced GnRHR mRNA and protein levels (Wu *et al.*, 1994).

Early reports indicate that continuous administration of low doses of GnRH increased GnRHR numbers in cultured rat pituitary cells (Loumaye and Catt, 1982; Loumaye and Catt, 1983; Young *et al.*, 1984). However, another study in rat pituitary primary cultures showed that continuous GnRH treatment had no effect on GnRHR mRNA levels, but that pulsatile GnRH administration increased GnRHR mRNA levels (Kaiser *et al.*, 1993).

In the incompletely differentiated α T3-1 cell line changes in receptor numbers without changes in mRNA levels have been reported after short term (20 min) stimulation with 0,1 nM or 1 nM GnRH (Tsutsumi *et al.*, 1993). In a later study by the same group it was found that continuous stimulation with 1 μ M GnRH over 24 h decreased GnRHR numbers without any effect on GnRHR mRNA levels (Tsutsumi *et al.*, 1995). In contrast to these findings, GnRHR mRNA levels were found to increase after 4 h of stimulation with 100 nM GnRH and only decreased after longer incubations in α T3-1 cells (Norwitz *et al.*, 1999a). Consistent with these results, mouse GnRHR promoter-reporter constructs transfected into α T3-1 cells responded significantly after 4 h to continuous treatment with 100 nM GnRH (Norwitz *et al.*, 1999a; Sadie *et al.*, 2003; White *et al.*, 1999). Responsiveness to GnRH was localized to two sequences namely SURG-1 and SURG 2 (Norwitz *et al.*, 1999a). SURG-2 as described earlier contains the consensus AP-1 binding site which is a critical component in regulation of the mouse GnRHR gene by GnRH in α T3-1 cells (Norwitz *et al.*, 1999a). Homologous regulation via SURG-2 appears to be mediated by increased binding of AP-1 proteins in a PKC- and JNK-dependent

fashion in α T3-1 cells (Ellsworth *et al.*, 2003b; Norwitz *et al.*, 1999a; White *et al.*, 1999). A later study by Norwitz *et al.* demonstrated that GnRH-mediated transcriptional activation of the mouse GnRHR is in part mediated by increased binding of SMAD and AP-1 protein complexes to an overlapping GRAS/SBE (SMAD-binding-element) element of the promoter (Norwitz *et al.*, 2002b). In addition GnRH-mediated increase in binding of the nuclear proteins Oct-1 and NF-Y to SURG-1 *in vivo* has been proposed to direct expression of the mouse GnRHR gene (Kam *et al.*, 2005).

Very few reports have been published on homologous regulation of GnRHR gene expression in the more differentiated gonadotrope cell line L β T2. Turgeon *et al.* found that GnRHR mRNA levels are upregulated in response to pulsatile GnRH administration after 3 days of treatment (Turgeon *et al.*, 1996), and another study demonstrated that pulsatile GnRH stimulation for 10 h increased transcriptional activity of a transfected mouse GnRHR promoter construct as well as receptor levels on the cell surface (Bedecarrats and Kaiser, 2003). By contrast, in the same study Bedecarrats *et al.* found that continuous GnRH treatment had little effect on GnRHR-reporter activity in L β T2 cells and receptor levels appeared to be downregulated after 10 h of continuous stimulation (Bedecarrats and Kaiser, 2003).

1.2.2 Regulation of GnRHR gene expression by gonadal steroid hormones

In early studies estradiol was found to increase GnRHR numbers *in vivo* (Crowder and Nett, 1984; Savoy-Moore *et al.*, 1980; Wu *et al.*, 1994) and in primary pituitary cultures (Gregg *et al.*, 1990; Laws *et al.*, 1990) suggesting that augmented receptor numbers may contribute to increased pituitary sensitivity to GnRH during preovulatory LH surge. Although it has been proposed that the estradiol-mediated increase in receptor numbers is mediated at the transcriptional level (Duval *et al.*, 2000; Wu *et al.*, 1994), in pituitary derived cell lines it was found that estradiol either downregulates GnRHR numbers in α T3-1 cells (McArdle *et al.*, 1992) or had little effect on endogenous GnRHR mRNA in L β T2 cells (Turgeon *et al.*, 1996).

Several studies suggest a negative regulatory effect of progesterone on GnRHR expression in the pituitary since high levels of progesterone were found to correlate with reduced GnRH sensitivity of the pituitary (Marrs *et al.*, 1981; Reyes *et al.*, 1976; Rubinstein *et al.*, 1978; Sowers *et al.*, 1978). Consistent with this hypothesis GnRHR numbers were found to decrease during the luteal phase of the estrous cycle (Crowder and Nett, 1984). In addition further studies revealed that GnRHR mRNA

levels were decreased in the presence of progesterone in primary ovine pituitary cultures (Sakurai *et al.*, 1997; Wu *et al.*, 1994), further supporting a direct effect of progesterone on GnRHR expression in the pituitary. Recent results with the transfected human GnRHR promoter in α T3-1 cells show that progesterone decreased GnRHR promoter activity via direct transcriptional effects through binding of the PR to a putative GRE-PRE in the human GnRHR promoter (Cheng *et al.*, 2001).

Similarly, testosterone was found to exhibit a negative effect on GnRHR transcription since mRNA levels appeared to decrease in the presence of the steroid hormone (Kaiser *et al.*, 1993; Zapatero-Caballero *et al.*, 2003). Consistence with these findings GnRHR numbers were found to decrease in primary pituitary cultures from male rats after treatment with α -dihydrotestosterone (DHT) (Tibolt and Childs, 1985). However, a study by Curtin *et al.* demonstrated that stimulation with DHT resulted in upregulation of GnRHR mRNA levels in L β T2 cells (Curtin *et al.*, 2001).

1.2.3 Regulation of GnRHR gene expression by glucocorticoids

In addition to sex steroids, glucocorticoids (GCs) have also been shown to regulate GnRHR gene expression in mammals, although the underlying mechanisms are not well defined. It has been reported that GCs play an important role in modulating GnRHR levels as part of a feedback mechanism from the adrenal to the pituitary (Breen and Karsch, 2004; Daley *et al.*, 1999; Rosen *et al.*, 1991; Tilbrook *et al.*, 2000). Rosen *et al.* have shown that GCs augmented GnRH-induced increase in GnRHR numbers in rats (Rosen *et al.*, 1991). In contrast another study in sheep showed that cortisol resulted in decreased GnRHR protein levels while the mRNA levels remained unchanged (Daley *et al.*, 1999). Similarly, cortisol was found to decrease responsiveness to GnRH in the pituitary independent of changes in GnRHR gene expression (Breen *et al.*, 2008). There is evidence for direct effects of GCs on GnRHR transcription in gonadotrope cells of the pituitary, with dexamethasone (dex) upregulating expression of both the endogenous GnRHR gene and a transfected mouse GnRHR promoter-reporter construct in L β T2 cells (McGillivray *et al.*, 2007; Turgeon *et al.*, 1996). These results are consistent with those of Maya-Nunez *et al.* showing that GCs can upregulate activity of the mouse GnRHR promoter in the somatolactotrope GGH₃ cell line expressing exogenous GnRHR (Maya-Nunez and Conn, 2003). Furthermore, the authors suggest that the GC-activated glucocorticoid receptor (GR) interacts with AP-1 proteins to increase GnRHR transcription, although they do not provide experimental evidence for this mechanism (Maya-Nunez and Conn, 2003).

1.3 EFFECTS OF STRESS ON REPRODUCTION

Chronic and acute stress have been implicated in regulation of reproduction in mammals although the underlying mechanisms are not well defined and the results are contradicting. While chronic stress appears to predominantly suppress gonadotropin secretion and reproduction, the effects of acute stress have been found to either inhibit or enhance reproductive function (Brann and Mahesh, 1991; Tilbrook *et al.*, 2000). The effects of stress on reproduction are mediated by GCs from the adrenals as part of a cross talk mechanism between the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes (Rivier and Rivest, 1991).

The inhibitory effects of stress on reproduction were shown to be mediated by corticosterone- or cortisol-mediated inhibition of LH secretion in rat primary pituitary cultures (Kamel and Kubajak, 1987) or ovariectomized ewe respectively (Breen and Karsch, 2004). The latter study suggested that cortisol suppresses pulsatile LH secretion by inhibiting pituitary responsiveness to GnRH rather than by suppressing hypothalamic GnRH release (Breen and Karsch, 2004). Evidence for direct effects of GCs on the pituitary comes from reports in the literature showing that cortisol inhibits GnRH-induced LH release from bovine and porcine pituitary cultures (Li, 1994; Padmanabhan *et al.*, 1983). In addition, cortisol was found to inhibit the estrogen-induced increase in GnRHR mRNA and protein suggesting a suppressive effect of GCs on GnRHR gene expression (Adams *et al.*, 1999). In contrast, an early study found that pulsatile administration of GnRH increased GnRHR mRNA levels in rat pituitary cells, and the effect was further increased by dex (Rosen *et al.*, 1991). These results suggest a stimulatory effect on reproduction of GCs via increasing GnRHR (Rosen *et al.*, 1991). Furthermore, in cultured rat pituitary cells (Baldwin *et al.*, 1991; Brann and Mahesh, 1991; Kilen *et al.*, 1996; McAndrews *et al.*, 1994) and rat pituitary fragments (D'Agostino *et al.*, 1990) GC stimulation increased FSH β synthesis and secretion, while having no effect or decreasing LH levels. The GnRH-stimulated secretion of FSH was also enhanced in pituitaries from both male and female rats after *in vitro* incubation with either corticosterone or cortisol (D'Agostino *et al.*, 1990).

1.4 STEROID HORMONE RECEPTORS

1.4.1 The glucocorticoid receptor

Steroid hormone receptors such as the glucocorticoid receptor (GR), the androgen receptor (AR), the mineralocorticoid receptor (MR), the progesterone receptor (PR) and the estrogen receptor (ER) are members of the nuclear receptor subfamily 3 (Evans, 1988; Griekspoor *et al.*, 2007; Gronemeyer *et al.*, 2004; Mangelsdorf *et al.*, 1995). These receptors are modulator proteins sharing a common structural organization that contains distinct functional regions including a divergent N-terminal transactivation domain (NTD), a highly conserved central zinc-finger DNA-binding domain (DBD) and a less conserved C-terminal ligand-binding domain (LBD). The NTD of the GR harbours a region known as the activation function-1 (AF-1) domain which has been shown to interact directly with components of the basal transcription machinery and many cofactors involved in transcriptional regulation (Dahlman-Wright *et al.*, 1995; Heitzer *et al.*, 2007; McEwan *et al.*, 1993). The central DBD contains two zinc-finger motifs where each zinc atom is coordinated in a tetrahedral arrangement by four cysteines. The amino acids of the first zinc-finger enable the receptor to bind specific DNA sequences in target promoters and those of the second zinc-finger are important for receptor dimerization with another GR monomer (Dahlman-Wright *et al.*, 1991). A variable hinge region is adjacent to the DBD. This region enables the receptor to change conformation and contains a nuclear localization sequence (NLS) (Picard and Yamamoto, 1987). A second activation function domain (AF-2) is present at the LBD at the C-terminal end of the receptor.

| | | | | | | | |
|-----------------|----------|-------------|------------|------------|------------|----------------|------------|
| rat GR | 1 | 98 | 282 | 440 | 500 | 543 | 795 |
| mouse GR | 1 | 86 | 270 | 228 | 488 | 531 | 783 |
| human GR | 1 | 77 | 262 | 421 | 481 | 525 | 777 |
| N | | AF-1 | | DBD | h | AF/2LBD | C |

Figure 1.4: Functional domains of the glucocorticoid receptor

GR functional domains include the DNA-binding domain (DBD), ligand-binding domain (LBD), the hinge region (h) and two transactivation domains (AF1 and AF2) and are indicated in the boxes (Kassel and Herrlich, 2007).

The human GR was first cloned in 1985 (Hollenberg *et al.*, 1985) and is a ubiquitously expressed protein found in almost all cell types and tissues (Gross *et al.*, 2009). Since then, five GR protein subtypes arising from alternative splicing have been identified, namely GR α , GR β , GR γ , GR-A and GR-P. GR α is known as the classic, functional receptor and consists of 777 amino acids. The shorter GR isoform GR β , made up of 742 amino acids, shares an identical NTD with GR α and can be distinguished from GR α by a differential C-terminal end. In addition alternative translation initiation results in more diverse GR proteins, i.e. GR α -A, GR α -B, GR α -C1, C2, D1, D2 and D, respectively. All the translational GR isoforms were detected in mouse and rat; however the expression levels were found to be different to human tissues (Lu and Cidlowski, 2005). The existence of several GR isoforms may represent one mechanism for modulating differential cellular responsiveness to glucocorticoids (GCs).

The endogenous ligands of the GR, cortisol and corticosterone in humans and rodents respectively, are synthesized in the adrenal cortex (Lieberman *et al.*, 2007). The release of GCs is under the control of the hypothalamic-pituitary-adrenal axis. Corticotropin-releasing hormone (CRH) is secreted in the hypothalamus triggering the release of adrenocorticotropin hormone (ACTH) which in turn stimulates the synthesis of steroids in the adrenal cortex (Chrousos, 1995). The GR and GCs play a crucial role in control of homeostatic and adaptive processes including metabolic, immune, neural, and behavioural mechanisms (Bloom *et al.*, 1982; De Kloet *et al.*, 1998; Wiegers and Reul, 1998). Given the essential role of GCs they are extensively applied as therapeutics in many immune and inflammatory diseases including acute and chronic asthma, rheumatoid arthritis as well as in cancer treatment (Barnes, 2006; Rhen and Cidlowski, 2005).

1.4.2 GR mechanism of action

GCs are highly lipophilic and are either transported bound to corticosteroid-binding-globulin (CBG) or diffuse across the plasma membrane (Hammes *et al.*, 2005). In the absence of hormone the GR is localized mostly in the cytoplasm in a multiprotein complex associated with multiple chaperones such as heat-shock proteins (hsp) 90, 70 or 40 and co-chaperones like the immunophilins FKBP 51 and FKBP 52, cyclophilin 40 or protein-phosphatase 5 (Cheung and Smith, 2000; Pratt *et al.*, 1996; Pratt and Toft, 1997). The LBD of the GR contains a sequence to facilitate protein-protein interaction with hsp90. This interaction enables proper folding of the receptor and maintains the GR in a conformation

that prevents DNA binding in the absence of hormone (Griekspoor *et al.*, 2007). In the classical cascade of events ligand binding induces rearrangements of the GR heteroprotein complex resulting in dissociation from the cytoplasmic chaperones and the exposure of its nuclear localization sequences (NLS) (Zhou and Cidlowski, 2005). These signals are recognized by nuclear translocation proteins such as importins (Freedman and Yamamoto, 2004) which actively shuttle the GR into the nucleus where the ligand-activated GR interacts at regulatory promoter regions of target genes to either enhance/initiate transcription (transactivation) or down regulate transcription (transrepression) (Newton and Holden, 2007).

It has become apparent from many studies that the GR can modulate gene expression by interacting with other regulatory transcription factors (Kassel and Herrlich, 2007). The activated GR initiates the assembly of distinct multiprotein complexes on the promoter that include co-activator proteins, chromatin remodelling factors and other transcription factors involved in transcriptional regulation to enhance or repress transcription according to the composition of the complex (Hayashi *et al.*, 2004; Newton and Holden, 2007). In fact many factors determine the outcome of the transcriptional response mediated by the GR. It has been proposed that modulation of the receptor through specific ligands, post-transcriptional modifications of the receptor, the specificity of the promoter architecture in the target promoter as well as the availability of specific cofactors and other transcription factors significantly contribute to the diverse effects of the GR (Chinenov and Rogatsky, 2007).

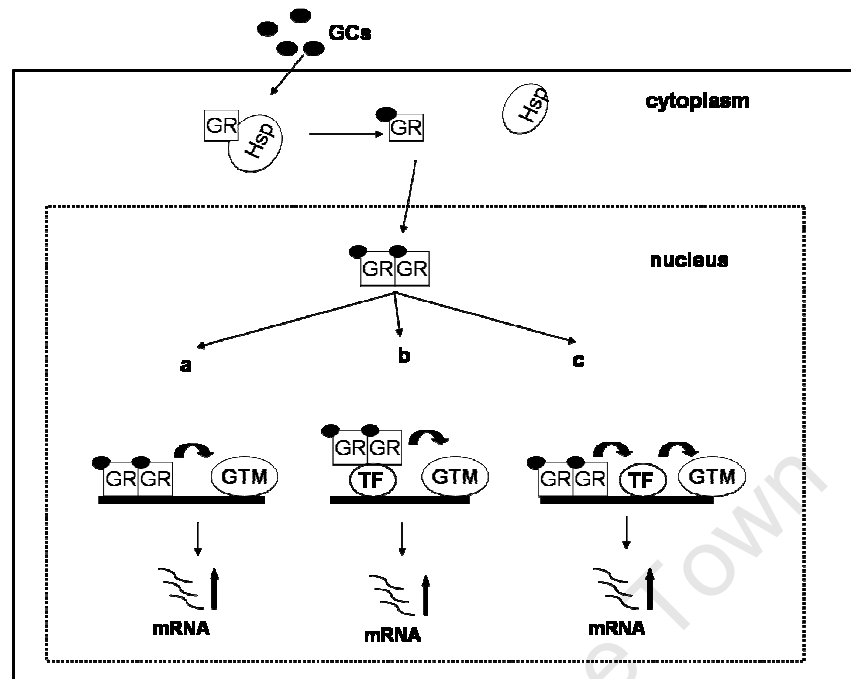


Figure 1.5: GR-mediated transcriptional regulation

Upon ligand binding the cytosolic GR dissociates from the multiprotein complex and translocates to the nucleus where the receptor modulates gene transcription via direct interaction with specific DNA-elements (a), cross talk at the promoter elements without GR binding sites such as AP-1 or NF- κ B (b) or at composite sites (c). GCs = Glucocorticoids, GR = glucocorticoid receptor, Hsp = heat shock protein, GTM = general transcription machinery, TF = transcription factor.

1.4.2.1 Control of gene expression by DNA binding of the GR

The classical model of GR function involves a number of steps including transformation of the receptor to an active form upon ligand binding, homodimerization (Griekspoor *et al.*, 2007) followed by binding of the liganded receptor to specific palindromic DNA consensus sequence (Nordeen *et al.*, 1990; Strahle *et al.*, 1987) termed the glucocorticoid response elements (GRE) located within regulatory promoter regions of GC-responsive gene and modulation of gene expression of target genes. Genes positively regulated by the GR contain GREs consisting of two conserved 6-base pair half-sites separated by three nonconserved bases: 5'-GGTACAnnnTGTTCT-3' (Nordeen *et al.*, 1990; Strahle *et al.*, 1987). However, gene sequences recognized by the GR have been found to deviate from the 15 bp consensus sequence but maintain specific functional groups on critical nucleotides within each half site (Heitzer *et al.*, 2007; So *et al.*, 2007). The GR has been shown to bind to the GRE as a

homodimer whereby the dimerization occurs via distinct hydrophobic motifs within the ligand binding domain and is stabilized by interactions in the DNA binding domain of each GR molecule (Bledsoe *et al.*, 2004; Luisi *et al.*, 1991). When bound to the DNA the GR serves as a platform for recruitment of transcription factors and in addition the GR itself was found to interact with several members of the transcriptional machinery like the transcription factor TFIIB or TATA-box binding protein (TBP) to induce transcription (Ford *et al.*, 1997; Horwitz *et al.*, 1996). Another group of co-activators are components of large complexes such as thyroid receptor associated proteins (TRAP) and vitamin D interacting proteins (DRIPS) (Heitzer *et al.*, 2007). The GR has been shown to interact with BRG1, a component of the SWI-SNF chromatin remodelling complex, and this interaction leads to improved accessibility of the promoter region for several cofactors and the basal transcription machinery (BTM) (Fryer and Archer, 1998). Furthermore, the GR can interact with other co-regulatory factors at both activation domains of the GR in a ligand-dependent fashion (Gronemeyer *et al.*, 2004; Rosenfeld *et al.*, 2006). CREB-binding protein (CBP) and p300, which serve as co-activators for a wide variety of transcription factors including the GR, possess histone acetyltransferase activities and are able to remodel chromatin structure and promote transcription (Heitzer *et al.*, 2007; McKenna *et al.*, 1999). Chromatin modification is considered to be an essential mechanism of regulating gene expression to generate coordinated responses to environmental changes (Eberharter and Becker, 2002). Some co-activators are implicated in assisting in the assembly of the pre-initiation complex formation allowing access of RNA-polymerase (Spiegelman and Heinrich, 2004). Acetylation of histones relaxes the chromatin structure which facilitates easier binding of transcription factors and increases efficiency in transcription (Eberharter and Becker, 2002).

The most well studied co-activators are members of the steroid receptor co-activator (SRC) family, also known as the p160 family of co-activators, which includes SRC-1 (or NcoA-1), SRC-2 (also known as TIF-2 or GRIP-1, NcoA-2) and SRC-3 (also known as RAC3, ACTR, AIB1, P/CIP and TRAM) (Griekspoor *et al.*, 2007; Perissi and Rosenfeld, 2005; Rosenfeld *et al.*, 2006; Xu and O'Malley, 2002). All members of the p160 family interact with nuclear receptors through an LxxLL motif in the nuclear receptor interaction domain (NID) (Heery *et al.*, 1997; Le Douarin *et al.*, 1996; Savkur and Burris, 2004; Torchia *et al.*, 1997). The N-terminus harbours a bHLH/PAS domain, which was shown to interact with several secondary co-activators (Kim *et al.*, 2003; Lee *et al.*, 2004) while the C-terminus was found to recruit arginine methyltransferases CARM1 and PRT1 and histone acetyltransferases

CBP/p300 and pCAF (Chen *et al.*, 1999a; Lee *et al.*, 2005; Lee *et al.*, 2002; Voegel *et al.*, 1998). Furthermore, SRC-1 (Spencer *et al.*, 1997) and SRC-3 (Chen *et al.*, 1997) are known to possess histone acetylase (HAT) activity, leading to disruption of nucleosomes (Edwards, 2000; Kleiman and Tuckermann, 2007). In addition GRIP-1 has been shown to possess HAT-activity *in vitro* (Goel and Janknecht, 2004). Unlike other members of the p160 family GRIP-1 possesses a repressor domain which mediates ligand-dependent repression of AP-1 and NF- κ B (Rogatsky *et al.*, 2002; Rogatsky *et al.*, 2003).

The mechanism of transcriptional regulation by the GR has only been fully described for a few promoters such as the phosphoenolpyruvate carboxykinase (Hanson and Reshef, 1997) and a recent study by Ronacher *et al.* demonstrates a direct correlation between GRE-transactivation mediated by the GR and GR binding of GRIP-1 (Ronacher *et al.*, 2009). However for most GC-regulated genes the precise mechanism remains unknown.

1.4.2.2 Modulation of transcription factor function by the GR

The GR can interfere with the activities of other transcription factors through cross talk with several transcription factors (Kassel and Herrlich, 2007). Often the modulation of the transcriptional activity is bidirectional and can be enhancing or inhibitory. Depending on the composition of the target promoter cross talk can occur at composite response elements, at overlapping response elements or at response elements lacking binding sites for the modulating transcription factor (Kassel and Herrlich, 2007).

In the first mode of cross talk both GR and other transcription factors can bind to the promoter in target genes containing binding sites for both transcription factors and modulate the transcriptional response either positively or negatively (Diamond *et al.*, 1990). Composite binding sites do not contain conventional binding sites for either factor and transcriptional regulation involves protein-protein interactions as well as protein-DNA interactions (Kassel and Herrlich, 2007). For example the GR may function as a monomer at these composite sites, forming heterocomplexes with other transcription factors (Chinenov and Rogatsky, 2007). An interaction between the GR and these other transcription factors can either result in enhancement or repression of gene transcription. For example, GCs enhance phosphoenolpyruvate carboxykinase (PEPCK) (Imai *et al.*, 1993) and somatostatin gene transcription (Liu *et al.*, 1994) via protein-protein interactions of the GR with the cAMP response

element (CRE) binding protein (CREB). Similarly, the GR and the octamer-binding transcription factor (Oct-1) activate transcription at a composite response element in the mouse mammary tumor virus (MMTV) promoter (Bruggemeier *et al.*, 1991; Miksicik *et al.*, 1987) while binding of both the GR and c-Jun/c-Fos heterodimers to a composite site in the promoter results in repression of mouse proliferin gene transcription (Diamond *et al.*, 1990).

At overlapping response elements the GR interferes with binding of other transcription factors thereby blocking transcription (Kassel and Herrlich, 2007). In the human osteocalcin gene the GRE overlaps with the TATA box in the promoter. GR binding to the GRE prevents binding of transcription factor IID (TFIID) to the promoter and results in repression of gene transcription (Stromstedt *et al.*, 1991).

Another mode of cross talk between the GR and other transcription factors occurs at response elements without binding sites for the GR mostly resulting in transcriptional repression (Kassel and Herrlich, 2007; Yamamoto *et al.*, 1998). In this case the GR can interfere with transcriptional function of other transcription factors by physically interacting directly or indirectly with other proteins. This mode of cross talk is described as a tethering mechanism and has been shown to occur for pro-inflammatory transcription factors such as AP-1 (Jonat *et al.*, 1990; Schule *et al.*, 1990; Yang-Yen *et al.*, 1990), NF- κ B (Mukaida *et al.*, 1994; Ray *et al.*, 1991; Scheinman *et al.*, 1995b), SMAD3/4 (Song *et al.*, 1999), Oct-1/2 (Kutok *et al.*, 1992; Wieland *et al.*, 1991) and STAT6 (Biola *et al.*, 2001). The DNA-binding domain but not the dimerization-domain of the receptor appears to be essential for the interaction suggesting that transcriptional repression is a function of GR monomers (Heck *et al.*, 1994). The physiological importance of the dimerization-independent repressor function of the GR was demonstrated in a few reports showing that transcriptional regulation via GREs was impaired while AP-1 and NF- κ B repression was fully active in GR^{dim} knockout mice containing a dimerization deficient receptor (Reichardt *et al.*, 1998; Reichardt *et al.*, 2001; Tuckermann *et al.*, 1999). Numerous models have been proposed for a transrepression mechanism via tethering of the monomeric GR to other transcription factors including prevention of co-activator (De Bosscher *et al.*, 1997) or corepressor binding or interaction with RNA-polymerase II (Nissen and Yamamoto, 2000). In addition the GR can indirectly antagonise transcription factor activity by inducing the synthesis of inhibitory factors such as I κ B (Auphan *et al.*, 1995; Scheinman *et al.*, 1995a). It was proposed that GCs induce the synthesis of I κ B which binds to NF- κ B in the cytoplasm and thereby preventing NF- κ B-mediated gene transcription.

There are only a few reports demonstrating enhanced transcriptional activity when GR interacts with transcription factors at promoters lacking GR binding sites. Stoecklin *et al.* showed STAT5 response elements in the promoter are sufficient for GR to enhance STAT5-induced transcriptional activity (Engblom *et al.*, 2007; Stoecklin *et al.*, 1997). Lerner *et al.* reported that the ligand-activated GR can associate with an enhanceosome that constitutively contains c-Jun and OCT-1 as well as IL-6 induced STAT3 on the α 2-macroglobulin (α 2-M) promoter to enhance transcription (Lerner *et al.*, 2003). While all of these factors contain transcription activation domains only STAT3 was shown to be required for the observed transcriptional increase of the α 2-macroglobulin gene (Lerner *et al.*, 2003). Another study demonstrated that transactivation of COUP-TFII regulated genes containing only a response element for COUP-TFII could be synergistically increased by the ligand-activated GR (De Martino *et al.*, 2004).

1.4.2.3 Modulation of activator protein-1 function by the GR

AP-1 is a sequence-specific transcription factor containing a basic region required for dimerization and a basic region involved in recognition of DNA motifs (Angel and Karin, 1991). AP-1 is composed of members of the Jun family (c-Jun, JunB, and JunD), the Fos family (c-Fos, FosB, Fra1, and Fra2) (Angel and Karin, 1991) or the CREB/ATF family (Cohen *et al.*, 1989; Zerial *et al.*, 1989). It has been reported that AP-1 plays an important role in several cellular functions such as development, differentiation and cell growth (Angel and Karin, 1991). Activation of AP-1 is induced by mitogens, cytokines, and stress agents such as ultraviolet (UV) light. JNK was shown to mediate phosphorylation of c-Jun at Ser-63 and Ser-73 resulting in enhanced transcriptional activity (Smeal *et al.*, 1991). While Jun family members can form homodimers or heterodimers with other AP-1 proteins, the Fos leucine zipper is incapable of forming homodimers (Turner and Tjian, 1989). Depending on the composition of the AP-1 dimer, different sequence elements are recognized; Jun/Fos dimer preferentially bind to heptameric TPA responsive elements (TRE) (De Cesare *et al.*, 1995) while Jun/ATF dimer bind to the octameric cAMP responsive elements (CRE) (Hai and Curran, 1991).

Cross talk between AP-1 and the GR appears to be mutual and involves complex formation of both transcription factors (Jonat *et al.*, 1990; Wissink *et al.*, 1997). It was suggested that GCs interfere with AP-1 via direct or indirect interaction of the GR with c-Fos and c-Jun (Jonat *et al.*, 1990; Schule *et al.*, 1990; Touray *et al.*, 1991; Yang-Yen *et al.*, 1990) while a study by Kerppola *et al.* demonstrated that c-

Fos is the primary target for GR inhibition of AP-1 *in vitro* (Kerppola *et al.*, 1993). GR binding to AP-1 does not depend on the presence of a GR binding site in the promoter (Teurich and Angel, 1995) and was shown not to alter the occupancy at the AP-1 site at the collagenase-1 promoter by *in vivo* footprinting experiments (Konig *et al.*, 1992) and ChIP assays (Kassel *et al.*, 2004). Similarly, Rogatsky *et al.* showed that GCs do not decrease binding of c-Jun and c-Fos proteins to the collagenase-3 promoter *in vivo* (Rogatsky *et al.*, 2001). Furthermore, for genes containing a composite element, such as the proliferin gene, the composition of the AP-1 dimer appears to determine whether cross talk with the GR is positive or negative whereby it can be inhibitory for the Jun/Fos heterodimer, but stimulatory for the Jun homodimer (Diamond *et al.*, 1990; Hoepfner *et al.*, 1995; Miner and Yamamoto, 1992; Teurich and Angel, 1995). A recent study by Rani *et al.* in PC12 cells identified a 7 bp region of the rat tyrosine hydrolase (TH) gene promoter which has no similarities with any known GRE but resembles an AP-1 binding site as the sole regulatory region for the dex-mediated increase in transactivation (Rani *et al.*, 2009). In addition, the authors found that the GR as well as several AP-1 proteins including c-Fos, FosB, JunD and JunB but not c-Jun bind to that region designated as a novel TH-GRE/AP1 sequence in intact cells. In addition cross talk between AP-1 and the GR occurs indirectly. For example, GCs were shown to inhibit JNK resulting in failure to phosphorylate c-Jun and thereby reducing AP-1 activity (Caelles *et al.*, 1997). Furthermore GCs induce the synthesis of the transcription factor glucocorticoid-inducible leucine zipper (GILZ) which inhibits AP-1-driven reporter constructs via a mechanism involving direct interference with AP-1 DNA binding to its target promoters (Mittelstadt and Ashwell, 2001).

1.4.3 Post-translational modifications of the GR

Several reports in the literature have postulated that post-translational modifications of the GR play an important role in GR transcriptional activity, receptor stability, subcellular localization and protein-protein interactions (Faus and Haendler, 2006).

Besides the well described acetylation of lysine residues in the N-terminus of histones (Kouzarides, 2000) a variety of transcription factors are known to be directly acetylated resulting in enhanced or decreased transcriptional activity. Ligand-dependent acetylation of the GR has recently been shown to occur at several lysine residues within the DBD (Ito *et al.*, 2006). Ito *et al.* further report that deacetylation of the GR by histone deacetylase-2 (HDAC-2) enables NF- κ B repression of target

genes. Ubiquitylation, another post-translational modification usually targets proteins for degradation via the proteasome pathway, while ubiquitylation has also been shown to affect subcellular localization or protein-protein interaction (Faus and Haendler, 2006). A potential target residue for ubiquitylation has been identified in the degradation motif of the human GR and the authors further suggested a potential role of the ubiquitin-proteasome pathway in regulating GR protein degradation (Wallace and Cidlowski, 2001). Sumoylation leads to attachment of small ubiquitin-modifier (SUMO) chains onto lysine residues and has been implicated in fine tuning steroid receptor function (Faus and Haendler, 2006). Recent *in vitro* studies have identified the GR as a potential target protein since 3 different lysine residues have been found to be acceptor residues for SUMO (Zhou and Cidlowski, 2005). Sumoylation of the GR has been reported to affect protein stability and transcriptional function, however reports in the literature are controversial and it has yet to be established if the endogenous GR is a target for sumoylation.

Early findings suggest that the GR is constitutively phosphorylated under physiological conditions and undergoes agonist-induced hyperphosphorylation (Housley and Pratt, 1983; Orti *et al.*, 1989; Singh and Moudgil, 1984). Several phosphorylation sites have been identified, most of which are serine residues located at the N-terminal end of the receptor, and have been shown to be highly conserved amongst species (Bodwell *et al.*, 1991; Ismaili and Garabedian, 2004). Solid phase sequencing of the mouse GR demonstrated that Ser-212, Ser-220, and Ser-234 are situated in the AF-1 domain of the receptor and that phosphorylation at these specific residues is required for full transcriptional activity and thus a role of phosphorylation in GR-mediated transactivation has been suggested (Bodwell *et al.*, 1991). While Ser-203 and Ser-226 of the human GR were found to be phosphorylated in the absence and presence of hormone, Ser-211 phosphorylation was only observed in the presence of a GR agonist in human U2O2 osteocarcinoma cells (Wang *et al.*, 2002) and rat hepatoma cells (Blind and Garabedian, 2008). The role of GR phosphorylation in receptor function is controversial. While single or multiple phosphorylation site mutations of the mouse GR at Ser-203, Ser-211 and Ser-226 (equivalent to the human GR Ser-203, Ser-211 and Ser-226) were found to result in a modest reduction in transcriptional activity of a mouse mammalian tumor virus (MMTV) promoter in COS-1 cells (Mason and Housley, 1993; Webster *et al.*, 1997) others reported an increase in transcription in HCT116 cells (Kino *et al.*, 2007) or a significant decrease of the MMTV-promoter activity in COS-1 cells with the equivalent human GR mutant constructs (Avenant, 2009). Ligand-induced

transactivation of a GRE-reporter was shown to be abolished when these specific phospho-sites were mutated in the mouse GR (Webster *et al.*, 1997) or the human GR (Avenant, 2009) in COS-1 cells. Furthermore, phosphorylation has been implied to affect cellular distribution of the GR. In U2OS cells the ligand-activated human GR, phosphorylated at Ser-211 was predominately found in the nucleus while GR isoforms phosphorylated at Ser-203 remain mainly in the cytoplasm (Wang *et al.*, 2002). However, other groups reported that mutation of various phosphorylation sites of the mouse GR (Webster *et al.*, 1997) or the human GR (Avenant, 2009) had no effect on ligand induced nuclear translocation in COS-1 cells (Avenant, 2009; Webster *et al.*, 1997).

Webster *et al.* demonstrated that ligand-induced GR protein degradation was abolished when several serine residues of the mouse GR were mutated and thus proposed that phosphorylation plays a role in receptor turnover (Webster *et al.*, 1997). However, a recent study showed that single mutations or combinations of serine mutations had no effect on agonist-induced degradation of human GR (Avenant, 2009). In addition it has been shown in the literature that the phosphorylation status of the GR dictates protein-protein interaction with other transcription factors like components of the mediator complex such as TSG101 (Ismaili *et al.*, 2005), or the vitamin D receptor interacting protein 150 (DRIP150) (Hittelman *et al.*, 1999), or cofactors such as p300/CBP (Galliher-Beckley *et al.*, 2008), or GRIP-1 (Avenant, 2009) thereby regulating GR transcriptional activity.

Taken together the studies above suggest that agonist-induced phosphorylation modulates GR transcription at various levels. Although ligand-induced phosphorylation has been linked to subcellular localization, protein degradation, protein-protein interaction and modulatory effects on target gene expression, it appears that cell-, promoter- and possibly even species-specific effects play a role in the mechanism.

1.5 RAPID STEROID ACTION

1.5.1 Rapid non-genomic effects of steroid receptors

According to the classical model for genomic steroid action, steroid hormones bind to specific receptors which interact at the regulatory regions of their primary target genes to either enhance or repress transcription (Beato, 1989). These effects are characterized by a specific delay and sensitivity towards inhibitors of transcription and translation since gene transcription and protein synthesis are required (Falkenstein *et al.*, 2000). In contrast to delayed genomic steroid actions, rapid non-genomic

effects are characterized by their rapid onset of action (usually within minutes), their insensitivity towards transcriptional or translational inhibitors as well as rapid activation of signalling cascades to ensure the fast effects (Stellato, 2004). Rapid actions of steroids do not fit in the classical genomic model of steroid action and might reflect that the steroid receptors have an alternative mechanism of transcriptional regulation that runs in parallel to the classical genomic pathway via reciprocal cross talk between various receptors and signalling pathways (Tasker *et al.*, 2006). Rapid signalling has been found for virtually all groups of steroids and their receptors including the ER, AR, PR and the GR (Falkenstein *et al.*, 2000). The existence of plasma membrane-associated receptors for steroid hormones such as estrogen, GCs and aldosterone has been proposed (Falkenstein *et al.*, 2000). However, the functional role and identity of these membrane-associated receptors is under intense investigation and both genomic- and non-genomic effects appear to be involved in activation of such receptors.

17 β -estradiol has been demonstrated to rapidly stimulate endothelial nitric-oxide synthase (eNOS) and the effect was blocked with the specific ER antagonists tamoxifen but not with the transcription inhibitor actinomycin D, indicating that the nuclear ER is capable to activate eNOS in a novel, nongenomic manner (Chen *et al.*, 1999d; Kim *et al.*, 1999; Lantin-Hermoso *et al.*, 1997). Furthermore, another study linked MAPK pathways to the rapid activation of eNOS by 17 β -estradiol (Shaul, 1999). Several groups have reported evidence for the existence of a membrane ER structurally similar to the intracellular ER (Falkenstein *et al.*, 2000). Plasma membrane-impermeable BSA-conjugated estradiol was found to stimulate eNO release and the ER antagonist ICI 182,780 completely blocked this effect, indicating that the site of estradiol action is on the plasma membrane. The binding of estradiol to the membrane was confirmed using FITC-labelled estradiol coupled to BSA (Kim *et al.*, 1999). Additionally the cytoplasmic ER has been shown to associate with the plasma membrane of endothelial cells via caveolae further supporting the proposal that rapid non-genomic 17 β -estradiol effects are mediated by a membrane form of the ER which is structurally similar to the cytoplasmic ER (Razandi *et al.*, 2002; Razandi *et al.*, 1999). Rapid non-genomic effects of progesterone have been found in spermatozoa (Baldi *et al.*, 1998; Blackmore, 1998) oocytes (Morrill and Kostellow, 1999) as well as in rat hepatocytes (Waldegger *et al.*, 1995) and natural killer cells (Mandler *et al.*, 1993) involving increase in Ca²⁺ influx and activation of phospholipase C (PLC) and phospholipase A (PLA) (Falkenstein *et al.*, 2000). Recently the existence of a membrane-bound PR has been identified in seatrout (Zhu *et al.*,

2003b) and humans (Thomas *et al.*, 2007; Zhu *et al.*, 2003a) with characteristics of a GPCR possibly mediating non-genomic effects in various tissues including T-lymphocytes and Jurkat cells (Dosiou *et al.*, 2008; Thomas *et al.*, 2007). Similarly, rapid non-genomic signalling induced by androgens mostly involves Ca^{2+} signalling and phosphorylation and activation of MAPK (Falkenstein *et al.*, 2000).

1.5.2 Rapid GC-mediated effects

In the literature there is accumulating evidence for rapid non-genomic GC actions including modulating general cellular functions, brain and neurophysiological functions as well as behavioural responses (Makara and Haller, 2001; Orchinik *et al.*, 1994). Some rapid non-genomic effects of GCs have been shown to be mediated by alteration of the physicochemical property of cell membranes (Song and Buttgereit, 2006). It has been proposed that GCs interact with cellular membranes such as the plasma or mitochondrial membrane and ultimately altering cellular function by modifying cation transport through the plasma membrane or by increasing the proton leak of the mitochondria (Song and Buttgereit, 2006). In human bronchial epithelial cells it was shown that treatment with low concentrations of dex (0,1 nM to 1 μM) results in decreased Cl^- secretion and Ca^{2+} levels via a non-genomic mechanism that is independent of the classical GR and involves a ATPase, adenylate cyclase and PKA signalling (Urbach *et al.*, 2002). In addition, rapid GC effects can be mediated via the classical cytoplasmic GR or a membrane-bound GR (Gross and Cidlowski, 2008; Lowenberg *et al.*, 2008; Moutsatsou and Papavassiliou, 2008; Song and Buttgereit, 2006) as described below in detail.

1.5.2.1 Cross talk of the membrane-bound GR

In early studies membrane receptors for corticosterone were characterized in neuronal plasma membranes of the amphibian *Taricha granulosa* (Moore and Orchinik, 1994; Moore *et al.*, 1995). The corticosterone-binding receptor was found to be enriched in neuronal plasma fractions with an estimated molecular weight of 63 kDa and appears to be distinct from the classical cytosolic GR (Breuner and Orchinik, 2002; Evans *et al.*, 2000; Falkenstein *et al.*, 2000). Consistent with this report other groups showed that the membrane-associated GR exhibits distinct differences in cell localization, molecular size, and binding characteristics from the cytosolic GR (Gametchu *et al.*, 1999; Powell *et al.*, 1999). On the other hand similarities between the membrane-associated GR and the cytosolic GR such as shared epitope recognition for antibodies directed against the cytosolic GR, a

similar phosphorylation pattern, and similar heat shock protein binding properties, have also been described, to support the hypothesis that the membrane GR is a modified form of the cytosolic GR (Stellato, 2004).

The presence of the GR in several types of immune cells provides evidence for the hypothesis that non-genomic GC signalling is involved in modulating immune function (Lowenberg *et al.*, 2008). It was shown that a subset of the GR population resides in the plasma membrane of human leukemic cells (Gametchu *et al.*, 1999; Gametchu *et al.*, 1993) mouse monocytes and B-cells (Bartholome *et al.*, 2004) and S-49 mouse lymphoma cells (Chen *et al.*, 1999b; Chen *et al.*, 1999c; Powell *et al.*, 1999). Recent studies provide molecular insight into the functional role of non-genomic GC action in T-cells (Lowenberg *et al.*, 2005; Lowenberg *et al.*, 2006b). The unliganded GR was shown to be an essential part of a plasma-membrane T-cell receptor-linked multiprotein complex containing hsp90, LCK, FYN and ZAP-70 (Bartis *et al.*, 2007; Bartis *et al.*, 2006; Lowenberg *et al.*, 2006b). Short-term GC treatment induced disruption of that protein complex resulting in impaired T-cell receptor signalling as a consequence of cellular redistribution and abrogated activation of LCK and FYN (Lowenberg *et al.*, 2006b). Similarly, Bartis *et al.* reported rapid GC-induced GR-dependent phosphorylation of ZAP-70 in Jurkat cells thereby facilitating non-genomic GC action in T-cells (Bartis *et al.*, 2007; Bartis *et al.*, 2006; Lowenberg *et al.*, 2006b) and Löwenberg *et al.* show that short-term GC treatment inhibited insulin signalling through a GR-dependent and transcription-independent mechanism in adipocytes and T-lymphocytes (Lowenberg *et al.*, 2006a).

1.5.2.2 Cross talk of the classical GR with intracellular signalling proteins

The classical cytosolic GR appears to cross talk with various intracellular signalling pathways to mediate rapid effects. Jain *et al.* presented a study showing the presence of the classical cytosolic GR, hsp90 and STAT3 in caveolae-containing low-density detergent-resistant membranes (DRM) derived from liver cells (Jain *et al.*, 2005). This study further provides evidence for a functional contribution of the GR associated with these membrane rafts towards transcription since the presence of a raft disrupter, filipin III, significantly inhibited dex-induced GRE-transactivation (Jain *et al.*, 2005). In addition, it has been reported that liganded GR can directly interact with a member of the MAPK family, namely JNK (Bruna *et al.*, 2003). This protein-protein interaction results in repression of AP-1 regulated genes since GC-mediated inhibition of JNK activity decreases the phosphorylation of c-Jun

(Caelles *et al.*, 1997). Croxtall *et al.* demonstrated that epidermal-growth factor (EGF) stimulated PLA activation and subsequent release of arachidonic acid (AA) can be inhibited by dex in a GR- and MAPK-dependent, but transcription independent fashion in A549 cells supporting the hypothesis of non-genomic GR-mediated mechanism (Croxtall *et al.*, 2000). A study by Solito *et al.* further suggests that GCs induce rapid serine phosphorylation and membrane translocation of ANXA1 via a non-genomic GR-dependent mechanism that requires MAPK, phosphatidylinositol 3-kinase (PI3K), and PKC pathways in folliculostellate cells (Solito *et al.*, 2003).

Interestingly, GCs have also been reported to rapidly activate p38, JNK and ERK in PC12 cells and cultured hippocampal neurons (Di and Tasker, 2004; Li *et al.*, 2001; Qi *et al.*, 2005; Tasker *et al.*, 2006). The activation of these downstream messengers might provide a novel mechanism whereby GCs signal rapidly from the plasma membrane to the nucleus via the cytosolic GR (Tasker *et al.*, 2006). Similarly, rapid activation (phosphorylation) of MAPK and PKB/Akt by GCs has been shown in ovarian follicular cells (Sasson *et al.*, 2003). The authors propose that rapid GC effects may either involve a non-genomic mechanism via interaction with a putative membrane-bound receptor or alternatively specific interaction of GC with the cytosolic GR resulting in the release of associated proteins from the multi-protein complex (Sasson *et al.*, 2003). In support of the latter, proteins from the GR-associated multi-protein complex consisting of heat shock proteins, chaperones and co-chaperone as well as kinases may have a potential role in rapid modulation of intracellular signalling after dissociation from the ligand activated GR. Evidence for this hypothesis comes from a study by Croxtall *et al.* showing that GC-induced activation of lipocortin I and inhibition of AA release is in fact independent of GR nuclear translocation and appears to be mediated via release of src-kinase from the cytoplasmic GR multi-protein complex (Croxtall *et al.*, 2000). This implies that GCs may orchestrate a broad and complex signalling cascade beyond the GR-dependent genomic pathways.

1.5.2.3 Cross talk of the classical GR with GPCRs

There is increasing evidence that rapid GC effects are in part mediated by membrane-bound GPCRs (Tasker *et al.*, 2006). Iwasaki *et al.* proposed a possible involvement of G_i in rapid GC-mediated inhibition of ACTH in AT20 cells (Iwasaki *et al.*, 1997) and Malcher-Lopez *et al.* found that a G_s -coupled receptor is involved in rapid GC-induced synthesis and release of endocannabinoids from neuroendocrine cells (Malcher-Lopes *et al.*, 2008). Another study reported an inhibitory effect of

corticosterone on nicotine-mediated Ca^{2+} influx involving a G-protein-PKC-dependent pathway in PC12 cells (Qiu *et al.*, 1998). In addition these effects were also observed in the presence of BSA-conjugated corticosterone, implying the possible contribution of a membrane GC receptor (Qiu *et al.*, 1998).

Interestingly, Kino *et al.* demonstrated that the liganded cytosolic GR directly interacts with G-protein- β and the proteins comigrated to the plasma membrane after activation of the somatostatin receptor, a GPCR, by somatostatin in HCT116 cells. Thus, it is likely that G-protein associated GR might explain some of the non-genomic effects of GCs at the plasma membrane (Kino *et al.*, 2005).

1.6 GPCR CROSS TALK: FINE-TUNING OF MULTIPLE RECEPTOR SIGNALLING PATHWAYS

GPCR's utilize complex signalling pathways to transmit signals from the plasma membrane to the cytoplasm and the nucleus. The complexity is highlighted by the fact that several G-proteins, interacting receptors, and effector proteins are usually simultaneously expressed in the same cell. In addition, GPCRs can affect signalling of members of another receptor family via pathways interacting at mutual cellular regulatory proteins resulting in numerous points of cross talk (Hur and Kim, 2002).

1.6.1 Microdomains as a functional complex to facilitate cross talk

The classical theory of GPCR signalling is based on random collisions between proteins in the plasma membrane (Hur and Kim, 2002). However, there is accumulating evidence in the literature suggesting that receptors, G-proteins and effector molecules are much less diffuse than expected and rather organized in distinct specialized microdomains. Caveolae and lipid rafts are such microdomains and have been shown to contain many proteins including GPCRs (Bliss *et al.*, 2007; Dobkin-Bekman *et al.*, 2009; Haasemann *et al.*, 1998; Navratil *et al.*, 2003; Roberson *et al.*, 2005), G-proteins (Moffett *et al.*, 2000), adenylyl cyclases, nitric oxidase synthase and protein kinase C (Dobkin-Bekman *et al.*, 2009; Hur and Kim, 2002; Lisanti *et al.*, 1994; Shaul and Anderson, 1998). The significance of the association of signalling molecules in microdomains is not clear and it needs to be determined whether they are required to facilitate cross talk of the downstream signalling cascade or if the specialized domains rather reduce the functional activity of the molecules through interactions with caveolin (Hur and Kim, 2002). De Weerd *et al.* showed that treatment with B_2 -receptor agonist

bradykinin resulted in the recruitment of the B₂-receptor and the receptor-coupled G α -subunits to caveolae in smooth muscle cells (de Weerd and Leeb-Lundberg, 1997). The results suggest that GPCR agonists enable the association of their receptors and coupled G-proteins with caveolae (de Weerd and Leeb-Lundberg, 1997). In contrast, another study demonstrated that the β ₂-adrenergic receptor was restricted to caveolae structures in its basal inactive state in rat cardiomyocytes since the presence of receptor was found to be decreased in caveolae upon agonist stimulation (Rybin *et al.*, 2000). Similarly, Engelman *et al.* show that caveolin-1 expression can inhibit signal transduction from the p42/44 MAPK cascade both *in vitro* and *in vivo*, suggesting an inhibitory role for caveolae in signal transduction (Engelman *et al.*, 1998). This is in agreement with a recent study by Dobkin-Bekman *et al.* demonstrating that a signalling complex including the GnRHR, c-src, FAK, caveolin-1, PKC and ERK is pre-formed in the absence of ligand while GnRH stimulation results in re-structuring of that complex and the release of ERK in L β T2 cells (Dobkin-Bekman *et al.*, 2009). The authors suggest that the signalling complex has a restrictive role for ERK nuclear translocation and signalling in these cells.

1.6.2 Scaffolding proteins as mediators of GPCR cross talk

Scaffolding proteins can facilitate the association of different proteins to enhance or ensure specificity of cellular responses (Hall and Lefkowitz, 2002). GPCRs can associate with scaffolding proteins via intracellular loops or via their intracellular C-termini thereby bridging the GPCR directly to effector molecules in the cytoplasm and enabling cross talk with other pathways (Hall and Lefkowitz, 2002). In support of this, activation of the AT₁ receptor has been shown to trigger the Jak/STAT signalling pathway which is usually activated by cytokine or growth factor receptors but not by GPCRs (Bhat *et al.*, 1994; Marrero *et al.*, 1996; Sayeski *et al.*, 2001). The activation of the Jak/STAT pathway has been found to be dependent on direct interaction between the C-terminal tail of AT₁ and Jak, resulting in Jak-induced recruitment, phosphorylation and activation of STAT1. A recent study by Maudsley *et al.* demonstrates that a calcium-dependent proline-rich tyrosine kinase 2 (Pyk2) is a scaffold for a focal adhesion/cytoskeleton-dependent complex containing c-src, Grb2, and mSos. The complex was further shown to be involved in GnRH-mediated ERK translocation to the nucleus in the gonadotrope cells α T3-1 and L β T2 (Maudsley *et al.*, 2007).

β -arrestins were originally discovered as cytosolic regulator, adaptor and scaffolding proteins in the context of GPCR desensitization, internalization and signalling (Lefkowitz *et al.*, 2006). More recently these proteins have been implicated in regulating non-seven-membrane-spanning receptor pathways and facilitating protein-protein interactions of various proteins enabling a broad variety of cross talk from the plasma membrane to the cytoplasm. In addition, β -arrestins have been shown to link GPCRs to signalling pathways such as MAPKs (Lefkowitz *et al.*, 2006). For example, Ahn *et al.* demonstrate that β -arrestin-dependent activation of ERK results in accumulation of the kinase in the cytoplasm in HEK-293 cells (Ahn *et al.*, 2004a; Ahn *et al.*, 2004b). The authors suggest that β -arrestin-activated ERK mediates phosphorylation of cytosolic proteins in contrast to nuclear ERK which is known to phosphorylate transcription factors (Yoon and Seger, 2006). Furthermore, reports in the literature identified β -arrestin 2 as a MAPK scaffold for the activation of JNK3 (McDonald *et al.*, 2000) or p38 (Sun *et al.*, 2002) or as a mediator in assembling a RAF/MEK/ERK multiprotein complex to enable receptor-specific signalling (Luttrell *et al.*, 2001).

Moreover, β -arrestin was implicated to be involved in immune responses since it was shown to inhibit NF- κ B-mediated gene expression by interacting directly with the NF- κ B-inhibitor I κ B (Witherow *et al.*, 2004). Similarly, β -arrestins directly interact with TNF receptor-associated factor 6 (TRAF6) after activation of TLR-IL-1R thereby preventing activation of NF- κ B and AP-1 (Wang *et al.*, 2006).

Interestingly, Kang *et al.* proposed a novel role for β -arrestin as a messenger in GPCR signalling from the cell membrane to the nucleus through histone modification (Kang *et al.*, 2005). Stimulation of the GPCR delta-opioid receptor induces nuclear translocation of β -arrestin 1 followed by enrichment of β -arrestin 1 at specific promoters such as that of p27 and c-fos, where it facilitates the recruitment of p300, resulting in enhanced histone acetylation and transcription of these genes (Kang *et al.*, 2005).

1.6.3 Cross talk between GPCRs by heterodimerization

In contrast to other families of cell surface receptors GPCRs were thought to function as monomers (Hur and Kim, 2002). However, it has been proposed that receptor dimerization and oligomerization is required for GPCR signal transduction in a similar fashion to that known for RTKs and growth hormone receptors (Bockaert and Pin, 1999; Bouvier, 2001; Milligan, 2001; Salahpour *et al.*, 2000). Furthermore it has been postulated that activation of one particular signalling pathway by one GPCR can reduce or augment the effects of another GPCR by homo-and hetero-oligomerization of these

receptors (Hur and Kim, 2002). A study by Suh *et al.* demonstrated that β -adrenergic receptor-mediated elevated cAMP levels were found to decrease upon activation of the P2Y₂ receptor via G_i in PGT-3 cells (Suh *et al.*, 2001). In contrast another study proposed that formation of a heterodimer consisting of the AT₁ receptor and the B2 receptor resulted in enhanced activation of G α_q and G α_i proteins, the two major G α -proteins coupled to the AT₁ receptor (AbdAlla *et al.*, 2000). Furthermore, Rocheville *et al.* reported that formation of a functional heterodimer of the dopamine D₂ receptor and the somatostatin SSTR₅ receptor results in enhanced dopaminergic and somatostatinergic effects (Rocheville *et al.*, 2000a). In addition the authors show in a subsequent study that functional hetero-oligomer D2R/SSTR5 receptor units are formed in cells expressing both receptor types (Rocheville *et al.*, 2000b). The authors suggest that hetero-oligomerization generates a novel receptor with enhanced functional activity. It is likely that similar functional hetero-oligomers could be formed between many potential pairs of GPCR family members when they are expressed in the same cell thereby adding a further level of complexity to the concept of receptor cross talk.

1.6.4 Cross talk of the GnRHR with other receptors

As mentioned earlier cross talk between cell surface receptors including the GnRHR is a mechanism of generating signal diversity. Cross talk between the GnRHR and the epidermal growth factor (EGF) receptor (EGFR) has been described in the literature and can either result in GnRH-induced transactivation of the EGFR or attenuation of EGFR signalling (Cheung and Wong, 2008). It has been demonstrated that the GnRHR antagonizes EGF-induced mitogenic signalling and cell proliferation by interfering with the stimulatory effects of the EGFR in human prostate (Moretti *et al.*, 1996), ovarian and endometrial cancer cells (Grundker *et al.*, 2001). The ligand activated GnRHR was shown to mediate this negative mode of cross talk via activation of phosphotyrosine phosphatases thereby reducing the EGF-induced autophosphorylation of the EGFR (Grundker *et al.*, 2001; Moretti *et al.*, 1996). Similarly, negative cross talk has been reported for the GnRHR and the insulin-like growth factor (IGF) receptor (IGFR) in human prostate cancer cells (Marelli *et al.*, 1999; Montagnani Marelli *et al.*, 2007). These studies show that GnRHR agonists interfere with the IGF-signalling by inhibiting IGFR protein expression and tyrosine-phosphorylation thereby inhibiting the downstream IGF-mediated phosphorylation and activation of Akt (Montagnani Marelli *et al.*, 2007). The ligand-activated GnRHR has also been shown to interfere with other signalling pathways such as cytokine signalling

via cross talk with intracellular non-receptor targets. Grunker *et al.* demonstrated GnRHR-induced activation and nuclear translocation of NF- κ B via G α i thereby inhibiting apoptosis in ovarian cancer cells (Grundker *et al.*, 2000) while another study showed that GnRH suppressed IL-8 expression by inhibiting TNF α -mediated NF- κ B activation in endometrial stromal cells (Sakamoto *et al.*, 2003). Furthermore, in a recent study Naor *et al.* describe reciprocal cross talk between the GnRHR and the prostaglandin receptor in L β T2 cells (Naor *et al.*, 2007). GnRH was shown to induce synthesis of prostanoids which in turn inhibit GnRH-induced GnRHR gene expression. The authors suggest that inhibition of the GnRHR by PGF $_{2\alpha}$ and PGI $_2$ is mediated by inhibition of phosphoinositide turnover possibly resulting in altered protein assembly at the AP-1 site in the GnRHR promoter (Naor *et al.*, 2007).

The GnRHR has further been shown to affect sex steroid hormone receptor function. In HEK293 cells GnRH induced nuclear translocation of the AR mediated by c-src and Hic5 but in contrast to testosterone GnRH did not transcriptionally activate the receptor (Maudsley *et al.*, 2006). In addition it was found that GnRH can activate the PR in the absence of progesterone in rat anterior pituitary cells (Turgeon and Waring, 1994) resulting in transcription of a transfected progesterone-response element (PRE)-reporter constructs as well as the gonadotropin-alpha subunit gene and FSH β gene in α T3-1 cells (An *et al.*, 2006) and L β T2 cells (An *et al.*, 2009), respectively. Similarly, a recent study by Chen *et al.* demonstrated that GnRH activates the ER in L β T2 cells in a GnRHR-dependent pathway leading to transactivation of ERE-containing genes (Chen *et al.*, 2009).

CHAPTER 2

MATERIALS AND METHODS

2.1 Compounds and antibodies

GnRH, dexamethasone (dex) and Mifepristone (RU486) were purchased from Sigma-Aldrich, South Africa. Antibodies to GR (H-300, sc-8992), c-Jun (sc-44 and sc-1694), c-Fos (sc-253), PR (sc-539), AR (sc-13062), MR (sc 14112), p300 (sc-32244) and SRC-1 (sc-8995) were obtained from Santa Cruz Biotechnology, USA. Antibodies to β -actin (#4967), SAP/JNK (#9252), phospho-SAPK/JNK (#9251), p44/42 (#9102), phospho-p44/42 (9101), p38 (#9212) and phospho-p38 (#9211) were purchased from Cell Signaling, South Africa and the rabbit HRP conjugate (NA934VS) from AEC Amersham, South Africa. The GRIP-1 antibody (G8970-10) was obtained from United States Biological, USA. The fluorescent labeled secondary antibody AlexaFluor[®]488 (#A21206) was obtained from Molecular Probes, USA. The anti-phospho-serine 226-GR and anti-phospho-serine 211-GR antibodies (corresponding to Ser-220 and Ser-234 in mouse, respectively) were a generous gift from Dr. M. J. Garabedian (New York University, School of Medicine, USA). See table 2.2 for dilutions of all the primary antibodies.

The GnRHR antagonist Antide (A8802) and the JNK (SP600125), PKA (Dihydrochloride, H89), ERK (PD98059) and p38 (SB230580) inhibitors were purchased from Merck chemicals, South Africa. The c-src inhibitor PP2 was purchased from Calbiochem, UK, and the PKC inhibitor Bbisindolylmaleimide x Hydrochloride (# B 3931) (synonym Ro 31-8425, from now on referred to as BIM) was obtained from Sigma-Aldrich, South Africa.

2.2 Incubation with test compounds

Dex and RU486 were diluted in 100% EtOH to a final concentration of 100 μ M. GnRH was purchased as lyophilized salt and dissolved in H₂O to a final concentration of 100 μ M. Antide was obtained as a lyophilized product and dissolved in H₂O to a final concentration of 100 μ M. All kinase inhibitors were dissolved in DMSO to a final concentration of: 10 mM for SP600125 (from now on referred to as JNK inhibitor), SB230580 (from now on referred to as p38 inhibitor) and PP2 (c-src inhibitor), 50 mM for PD98059 (from now on referred to as ERK pathway inhibitor), 1 mM for H89 (PKA inhibitor) and 100

μM for BIM (PKC inhibitor). All test compounds were added to cells as 1/1000 dilution in culture medium unless stated otherwise in the figure legends. Vehicle controls were either culture medium alone, or culture medium containing 0,1% DMSO or 0,1% EtOH depending on test compounds added. For kinase inhibitor studies cells were pre-incubated for 30 min with the c-src inhibitor PP2 or for 15 min with the p38, JNK or ERK pathway inhibitor before adding the test compound.

2.3 Plasmids

Wild type HA-tagged human GR (pCMV-HA-human GR) expression vector was obtained from Prof. M. J. Garabedian. The pTAT-GRE-E1b-luc plasmid was a gift from Dr G. Jenster at Erasmus University of Rotterdam, Netherlands and the pCMV- β -galactosidase plasmid was a gift from Dr. G. Haegeman (University of Gent, Belgium). The pSV-hAR α was a kind gift from Frank Classens Catholic University of Leuven, Belgium, the pRS-hMR expression plasmid was obtained from Prof. R.M. Evans (University of California, USA) and the pMT-PR-B was kind gift from Prof. S. Okret (Karolinka Institute, Sweden). The pAP-1 luc plasmid (containing seven copies of a consensus AP-1 site) was obtained from Stratagene, USA. The expression construct R10, containing the full-length (1.2 kb) mouse GnRHR cDNA (Tsutsumi 1992) in the pcDNA1 expression vector, was obtained from Dr. S.C. Sealfon, Mt Sinai Medical School, New York, USA. The MMTV-luciferase plasmid was a kind gift from Gordon L. Hager (National Cancer Institute, USA). The expression plasmid pSG5-hER α were obtained gift from F. Gannon (EMBL, Germany) and the ERE.vit2-luc was a kind gift from K. Korach (National Institute of Environmental Health Science, USA). The promoterless luciferase expression vector pGL2-basic was purchased from Promega (Promega Corp., USA). The pLG luciferase reporter plasmid containing 580 bp of the mouse GnRHR gene (-579 to +1 relative to the translation start site) has been previously described (Sadie *et al.*, 2003). The pLGmAP1 expression plasmid was constructed by Dr. Hanel Sadie by site-directed PCR mutagenesis using the pLG expression vector as template, as described (Sadie *et al.*, 2003), using primers containing the underlined mutated AP-1 promoter sequence in the anti-sense (PCRmAP1a 5'CTGATGTCGAAAGgaAtTCcTAATATCATCCAATT3') and sense (PCRmAP1s 5'AATTGGATGATATTAgGAaTtcCTTTCGACATCAG 3') orientation (mutated bases are indicated in lowercase). Isolated plasmids were sequenced to confirm the presence of the correct insert.

2.4 Transformation of bacterial cells and plasmid preparation

Plasmids were transformed into *Escherichia coli* DH5 α cells by heat shock transformations according to Sambrook *et al.* (Sambrook *et al.*, 1989). Briefly, 100 μ l competent cells were mixed with 10 ng DNA. The mixture was put on ice for 30 min, followed by 2 min incubation at 42°C and 2 min incubation on ice (Sambrook *et al.*, 1989). Immediately after transformation, cells were mixed with 900 μ l SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 20 mM glucose; (Sambrook *et al.*, 1989)) and incubated for 1 h at 37°C while shaking at 200 rpm. Cells were subsequently plated out on LB agar plates (1% (w/v) tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar, (Sambrook *et al.*, 1989)) containing 50 μ g/ml ampicillin (Sigma-Aldrich, South Africa) and allowed to grow overnight at 37°C. The following day, colonies were picked and grown in 50 ml LB medium (1% (w/v) tryptone, 0.5% yeast extract and 1% NaCl; (Sambrook *et al.*, 1989)) containing 50 μ g/mL ampicillin for at least 8 h at 37°C while shaking. For glycerol stocks 500 μ l 80% (v/v) glycerol was mixed with 500 μ l of the cell suspension and stored at -80°C. For plasmid preparations, LB medium containing ampicillin 50 μ g/mL ampicillin was inoculated and allowed to grow overnight at 37°C while shaking. The next day the plasmid DNA was purified with the PUREYIELD plasmid maxiprep kit from Promega (Promega, Madison, WI, USA), according to the manufacturer's protocol. The integrity and purity of the plasmids were tested by restriction digestion and agarose gel electrophoresis.

2.5 Cell culture

L β T2 mouse pituitary gonadotrope cells were kindly provided by Dr. P.Mellon from the University of California, San Diego, USA. COS-1 monkey kidney fibroblast cells were purchased from American Type Culture Collection (ATCC, USA). The cells were maintained in high glucose Dulbeccos's Modified Eagles Medium (DMEM, Sigma-Aldrich, South Africa) supplemented with 10% fetal calf serum (Delta Bioproducts, South Africa), 50 IU/ml penicillin and 50 μ g/ml streptomycin (Gibco-BRL Life Technologies, UK), unless stated otherwise. Cultures were maintained in 75 cm² culture flasks (Greiner Bio-one International, Austria) at 37°C, in an atmosphere of 90% humidity and 5% CO₂. L β T2 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. L β T2 cells were very sensitive to overtrypsinizing, and were therefore not incubated in the trypsin solution for longer than 3

min. Both cell lines were regularly tested for mycoplasma infection by means of Hoechst staining (Freshney, 1987), and only mycoplasma-negative cells were used in experiments.

2.6 Transient transfections

Cells were plated in 24-well culture plates (Nunc, Denmark) at a density of 1×10^5 or 5×10^4 cells per well for L β T2 cells or COS-1 cells, respectively, in 500 μ l DMEM supplemented with 10% fetal calf serum and antibiotics as described above. Twenty-four h after plating, medium was replaced with fresh medium and cells were transfected with 250 ng pTAT-GRE-E1b-luc, MMTV-luciferase construct or pAP-1-luc promoter-reporter constructs, or cotransfected with 125 ng pCMV-HA-human GR and 6 ng R10 GnRHR expression vector. In order to correct for transfection efficiency, 25 ng pCMV- β -galactosidase were co-transfected. All transfections were performed according to the FuGENE™ 6 (Roche Applied Science, South Africa) product protocol, at a ratio of 1 μ g DNA : 2 μ l FuGENE™ 6. At least 24 h after transfection, the medium was replaced with serum-free medium. Incubations were performed with test compounds as indicated in the figure legends. Cells were harvested in 50 μ l reporter lysis buffer (Promega Corp., USA) per well. The luciferase assays (Luciferase Assay System, Promega, USA) and β -galactosidase assays (Galacto-Star, Tropix Inc, USA) were carried out with 10 μ l cell extract in white 96-well plates in a Modulus microplate reader (Turner Biosystems, USA). Luciferase values were normalized to corresponding β -galactosidase values and expressed relative to vehicle control values. The constitutive CMV promoter of the pCMV- β -galactosidase expression vesctor was found not to be regulated under experimental conditions and therefore chosen in the present study.

2.7 RNA interference

L β T2 cells were plated in 12-well plates (Nunc, Denmark) at a density of 2×10^5 cells per well in 1 ml DMEM supplemented with 10% fetal calf serum and antibiotics as described above. After 24 h cells were transfected with siRNA using HiPerfect transfection reagent (Qiagen, USA) according to the manufacturer's protocol. Briefly, GR siRNA (Mn_Nr3c_3, Mn_Nr3c_4, Mn_Nr3c_5, Mn_Nr3c_6, GeneSolution siRNA Cat no.1027416, Qiagen, USA) or scrambled siRNA as non-silencing control (Negative Control siRNA Cat no 1027310, Qiagen, USA) were diluted in 50 μ l Opti-MEM+GlutaMAX™-I (Gibco-BRL Life Technologies, UK) and 3,5 μ l transfection reagent was added to

the diluted siRNA. The mixture was incubated for 10 min at RT and added drop-wise to the cells to obtain a final concentration of 20 nM for the GR siRNA or the scrambled siRNA per well. After 96 h, transfected cells were either harvested and the efficiency of the gene silencing determined by Western blotting, or treated with compounds for a further 8 h in serum-free medium for real-time PCR analysis. Using increased amounts of siRNA oligos or transfection reagent, longer incubation times, or transfecting twice with siRNA oligos did not further decrease the GR protein levels. In addition, the use of 3 different GR siRNA oligos resulted in the same extent of GR knockdown.

2.8 Isolation of total RNA

L β T2 cells were plated in 12-well culture plates (Nunc, Denmark) at a density of $4,5 \times 10^5$ cells per well in 1 ml medium. Forty-eight h after plating, medium was replaced with fresh medium containing test compounds. Total RNA was isolated with Trizol reagent (Sigma-Aldrich, South Africa) according to the manufacturer's protocol. Briefly, 400 μ l Trizol reagent was added per well and incubated at RT for 5 min. Thereafter cell lysates were transferred into microcentrifuge tubes and centrifuged for 10 min at 12 000 g (4°C). The supernatant was transferred into new microcentrifuge tubes and 80 μ l chloroform was added to each sample and the samples were vortexed vigorously for 15 sec. Samples were centrifuged for 15 min at 12 200 g (4°C). The top aqueous phase was transferred into new microcentrifuge tubes and an equal volume of isopropanol was added. Subsequently, the samples were incubated at RT for 10 min followed by centrifugation for 10 min at 20 000 g to pellet the RNA (4°C). The RNA precipitates were washed twice with 400 μ l 70% EtOH and centrifuged for 5 min at 20 000 g (4°C). The RNA was allowed to air dry for 5 min and resuspended in 15 μ l DEPC-treated-water. The samples were stored at -20°C.

To confirm the integrity of the isolated RNA denaturing formaldehyde agarose gel electrophoresis was performed (Addendum D2 (Sambrook *et al.*, 1989)). Sample loading buffer (15 μ l, Addendum C2) was added to 0,5 μ g 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 and 1% formaldehyde agarose gel in RNA electrophoresis buffer (Addendum D2) at 65 V for approximately 1.5 h (Sambrook *et al.*, 1989).

2.9 cDNA synthesis

Total RNA was reverse-transcribed using the Transcriptor First Strand cDNA Kit (Roche Applied Science, South Africa) according to the manufacturer's protocol for c-DNA synthesis using anchored oligo (dT) priming. Each RNA sample (1 µg) was mixed with 1 µl oligo(dT) primers (final concentration 2,5 µM) and DEPC-treated-water added to make up a final volume of 13 µl. The mixture was incubated for 10 min at 65°C. Samples were put on ice and allowed to cool down. Subsequently, 4 µl Transcriptor Reverse Transcriptase Reaction Buffer, 0,5 µl Protector RNase Inhibitor (20 units), 2 µl dNTP mix (final concentration 1 mM of each dNTP) and 0,5 µl Transcriptor Reverse Transcriptase (10 units) were added to each sample, mixed carefully and incubated at 50°C for 1 h. To stop the Transcriptor Reverse Transcriptase samples were incubated for 5 min at 85°C.

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: 25 µl SensiMixdT, 1 µl 50x SYBR®Green I solution, sense and anti-sense primers (see table 2.1 for final concentration of primers), 2 µl cDNA and PCR-grade H₂O to make up a total volume of 50 µl. The PCR protocol was as follows: 95°C for 10 min followed by 40 cycles of 95°C for 10 sec, 58°C for 10 sec and 72°C for 10 sec. Melting curve analysis and gel electrophoresis were performed to confirm the generated amplicon in each sample. Relative GnRHR transcript levels were calculated with the "Fit Points" method described by Pfaffl *et al.* (Pfaffl, 2001) and were normalized to relative GAPDH transcript levels.

| | Primer Sequence 5' – 3' | Stock concentration of primer | Final concentration of primer | Product size |
|---------------------------|------------------------------------|--|--|-------------------------|
| mGnRHR forward | CCACAGTGGTGGCATCAGGCCTTC | 5 µM | 0,125 µM | 192 bp |
| mGnRHR reverse | TAGCGTTCTCAGCCGAGCTCTTGG | | | |
| mGAPDH forward | TTCACCACCATGGAGAAGGC | 5 µM | 0,25 µM | 263 bp |
| mGAPDH reverse | GGCATGGACTGTGGTCATCA | | | |

Table 2.1: Sequences and concentrations of primers used for quantitative real-time PCR

2.11 Western blot analysis

L β T2 cells were plated at a density of 9×10^6 cells per well in 6-well plates (Nunc, Denmark) in 2 ml DMEM supplemented with 10% fetal calf serum and antibiotics as described above. Twenty four h after plating, medium was replaced with serum-free medium and cells were serum-starved overnight. The following day cells were incubated with test compounds as indicated in figure legends. Cells were washed twice with cold PBS and harvested in 100 μ l 1 x SDS sample buffer (5X SDS sample buffer: 100 mM Tris-HCL pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% β -mercaptoethanol and 0.1% (w/v) bromophenolblue) and incubated at 95°C for 10 min. Protein samples were separated by SDS-PAGE at 150 V in 25 mM Tris-HCL, 250 mM glycine and 0.1% SDS, pH 8.4 (Sambrook *et al.*, 1989) using a BioRad Mini Protean® II electrophoresis cell. Proteins were electroblotted onto Hybond™-ECL™ (AEC Amersham Biosciences, South Africa) nitrocellulose membrane for 1 h at 180 mA (Sambrook *et al.*, 1989) using a BioRad Mini Trans-Blot® cell in transfer buffer (25 mM Tris, 200 mM glycine, 10% (v/v) methanol). Membranes were blocked in 4% blocking solution (4% (w/v) ECL Advance blocking powder, AEC Amersham Biosciences, South Africa) in Tris buffered saline (50 mM Tris, 150 mM NaCl) (TBS) containing 0.1% (v/v) Tween (TBS-Tween) for 1 h at RT and subsequently incubated with primary antibody (see table 2.2 for specific dilutions) in blocking solution at 4°C overnight. The following day membranes were incubated at RT with an anti-rabbit HRP conjugate (1:10000) for 1 h at RT in 5% milkpowder (w/v) in TBS-Tween. After antibody incubation, membranes were washed for 15 min and 2 X 5 min in TBS-Tween at RT. For consecutive detection steps, membranes were stripped in stripping buffer (100 mM β -mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-Cl, pH 6.7, (Sambrook *et al.*, 1989)) for 30 min at 65°C, washed 2 X 10 min with TBS-Tween and blocked for 1 h in 4% blocking solution before repeating antibody incubations as described above.

| Antibody | Dilution |
|-----------------|----------|
| GR | 1:4000 |
| PR | 1:1000 |
| MR | 1:1000 |
| AR | 1:1000 |
| β -actin | 1:5000 |
| phospho JNK | 1:1000 |
| JNK | 1:1000 |
| phospho p38 | 1:1000 |
| p38 | 1:1000 |
| phospho ERK | 1:1000 |
| ERK | 1:1000 |
| c-Jun | 1:3000 |
| c-Fos | 1:3000 |
| phospho-Ser-234 | 1:10000 |
| phospho-Ser-220 | 1:10000 |

Table 2.2: Dilutions of primary antibodies used in Western blot analysis

2.12 Autoradiography and quantification

Autoradiography and ECL visualization were performed with Amersham Hyperfilm™ MP high performance autoradiography film. Autoradiograms were photographed using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290, and bands were quantified with Kodak 1D Image Analysis Software. Autoradiograms were quantified using AlphaEaseFC™ Software (AlphaInnotech, USA).

2.13 Immunofluorescence

L β T2 cells were grown on glass cover slips in 6 well plates (Nunc, Denmark) at a density of 2×10^5 cells per well. Twenty four h after plating, medium was changed to serum-free medium and cells were serum-starved overnight. The next day cells were incubated with test compounds for 1 h as indicated in figure legends. After stimulation, cells were washed twice with PBS, fixed and permeabilised with methanol at -20°C for 10 min and washed again 3 X with cold PBS. Cells were stained with anti-GR H-300 antibody (1:100 dilution) in PBS-5% BSA for 1 h, washed 3 x 5 min with PBS and subsequently

incubated with a fluorescent labeled secondary antibody (AlexaFluor488 labeled donkey anti-rabbit IgG, Molecular Probes, USA) in PBS-5%BSA (1:500) for 1 h. After 3 X 10 min washes with PBS, the slides were incubated with Hoechst DNA stain (1:1000) (Sigma-Aldrich, South Africa) in PBS for 10 min. The slides were washed again and mounted in fluorescent mounting medium (KPL, USA). Staining was visualized with an Olympus IX 81 microscope and analyzed with the Cell^R software (Olympus, Germany). For each treatment approximately 80 individual and randomly chosen cells were subjected to intensity analysis whereby the intensity of the fluorescent signal was measured in the region of interest (ROI).

2.14 Chromatin immunoprecipitation assay (ChIP assay)

ChIP assay was performed based on (Ma *et al.*, 2003) with some modifications.

2.14.1 Chromatin preparation

Cells were grown in 15 cm dishes (Nunc, Denmark) to 80% confluency and treated with compounds for 1 h in the absence of serum as indicated in the figure legends. Proteins were cross-linked with 1% formaldehyde for 10 min at 37°C and the reaction stopped with 0,125 M glycine for 5 min at RT. Cells were washed twice with cold PBS and scraped in PBS containing 1 x Complete Mini Protease Inhibitor Cocktail (1 tablet per 10 ml) (Roche Applied Science, South Africa). Subsequently, the cells were pelleted by centrifugation at 5000 g (4°C) and resuspended in 500 µl nuclear lysis buffer (1% SDS, 50 mM Tris-HCL, pH 8.0, 10 mM EDTA, 1 tablet 1 x Complete Mini Protease Inhibitor Cocktail per 10 ml). Cross-linked samples were fragmented by sonication to obtain DNA fragments between 100 – 400 bp using the Misonix Sonicator[®] 3000 with a microtip at the following conditions: 20 pulses at setting 4 for 20 sec each with resting phases in between each pulse for 40 sec, on ice. After sonication the samples were centrifuged at 15000 g for 10 min (4°C) and the supernatant was transferred into a new microcentrifuge tube. To normalize for equal amounts of DNA per immunoprecipitation, the chromatin was quantified by means of optical density readings. Therefore, the sonicated chromatin was diluted 1:20 in dH₂O and the optical density measured at 260 nm. The sonicated chromatin was stored at -80°C for short terms or used immediately for immunoprecipitation. To confirm the size of the chromatin fragments generated by sonication, the DNA was purified as described below (2.14.3) and analyzed on a 1% agarose gel by means of gel electrophoresis.

2.14.2 Immunoprecipitation

An aliquot of 100 µg of chromatin in 100 µl nuclear lysis buffer was diluted with 900 µl IP dilution buffer (0.01% SDS, 20 mM Tris-HCL, pH 8.0, 1.1% Triton X-100, 167 mM NaCl, 1.2 mM EDTA, 1 x Complete Mini Protease Inhibitor Cocktail, 1 tablet per 10 ml). To reduce non-specific binding, the chromatin was pre-cleared for 1 h with 20 µl pre-blocked Protein A/G PLUS beads (sc-2003, Santa Cruz Biotechnology, USA) on a rotating wheel at 4°C. Pre-blocked Protein A/G PLUS beads were prepared by incubating 500 µl of pure beads with 2 ml IP dilution buffer, salmon sperm DNA (final concentration 0,2 mg/ml) and BSA (final concentration 1 mg/ml) for 1 h on a rotating wheel at 4°C. After centrifugation the beads are resuspended as 50% slurry in IP dilution buffer. After pre-clearing the chromatin was centrifuged at 15000 g for 10 min to pellet the beads and the supernatant transferred into a new microcentrifuge tube followed by incubation with 5 µg of anti-GR (H-300, sc 8992), anti-c-Jun (D, Sc-44), anti-c-Fos (K-25, sc-253), or 2 µg anti-p300 (NM11, sc-32244), anti-SRC-1 (M-341, sc8995, Santa Cruz Biotechnology, USA), anti-GRIP-1 (G8970-10, United States Biological, USA) or anti-IgG antibody (sc-2350, Santa Cruz Biotechnology, USA) overnight on a rotating wheel at 4°C. The next day 40 µl of pre-blocked Protein A/G PLUS beads were added to the mixture and incubated for further 6 h rotating at 4°C to allow complexes to form. The complexes were collected by centrifugation at 5500 g for 1 min (4°C) and the pellet 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), wash buffer 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 TE (10 mM Tris-HCL pH 8.0 and 0.1 mM EDTA). The DNA-protein complexes were eluted from the Protein A/G PLUS beads by incubating the beads twice with 150 µl IP elution buffer (1% (w/v) SDS and 100 mM NaHCO₃) at RT on a rotating wheel. An aliquot of 30 µg sonicated chromatin was set aside as the input, to which 90 µl IP elution buffer was added and the volume was adjusted to a total of 120 µl with nuclear lysis buffer. The cross-links were reversed and input DNA was purified as described below (2.14.3).

2.14.3 Reversing formaldehyde crosslinks, DNA purification and analysis of precipitated DNA

To reverse cross-links NaCl was added to the input or immunoprecipitated samples to a final concentration of 300 nM followed by an overnight incubation at 65°C. The next day EDTA (final concentration 15 nM EDTA), Tris-HCL pH 6,5 (final concentration 125 nM) and proteinase K to a final concentration of 60 ng/μl (Roche Applied Science, South Africa) were added. The mixture was incubated for 1 h at 45°C and immunoprecipitated and input DNA was purified using the QIAquick® PCR Purification Kit (Qiagen, USA) according to manufacturer's protocol.

Conventional PCR and real-time RT-PCR were performed using specific primers spanning the AP-1 region of the mouse GnRHR-promoter (forward 5' GTATCTGTCTAGTCACAACAG 3' and reverse 5' TCCTGAAGGCCAAGTGTAACC 3' (Sadie, 2006) under the following conditions:

| GnRHR | Denaturation | | Annealing | | Extension | | cycling |
|-------------------------|--------------|------|-----------|------|-----------|------|---------|
| Conventional PCR | 45 sec | 95°C | 45 sec | 72°C | 45 sec | 51°C | 35 |
| real-time PCR | 8 sec | 95°C | 8 sec | 72°C | 10 sec | 51°C | 40 |

Primers used for CHIP AP-1 positive control were forward 5'GGTGTGCTGCCATATCAGATTCGG 3' and reverse 5' GCATCAAGTGCTGCTACTCACCTGTG 3', spanning the AP-1 site of the mouse FSHβ-promoter (Coss *et al.*, 2004) under the following conditions:

| FSHβ | Denaturation | | Annealing | | Extension | | cycling |
|----------------------|--------------|------|-----------|------|-----------|------|---------|
| real-time PCR | 8 sec | 95°C | 8 sec | 72°C | 10 sec | 56°C | 40 |

Each conventional PCR reaction contained the following:

| | |
|--------------------------------------|--|
| Input DNA / precipitated DNA | 2 μl / 4 μl |
| sense primer | final 0,2 μM |
| antisense primer | final 0,2 μM |
| dNTP Mix | 0,05 μM of each dNTP final (0,2 μM dNTP mix final) |
| MgCL ₂ | 1 mM final |
| 5xGoTaq® buffer (Promega) | 1 x |
| GoTaq®Flexi DNA Polymerase (Promega) | 1,25U |
| PCR H ₂ O | to make up a final volume of 50 μl |

Each real-time PCR reaction contained the following:

| | |
|------------------------------|---|
| Input DNA / precipitated DNA | 2 μ l / 4 μ l |
| forward primer | final 0,5 μ M |
| revers primer | final 0,5 μ M |
| SensiMixdT (Quantace) | 12,5 μ l |
| PCR H ₂ O | to make up a final volume of 25 μ l |

PCR amplicons were analyzed on a 1,5 % Agarose gel by means of gel electrophoresis.

Real-time PCR was used to calculate the relative protein recruitment to the gene of interest with the "Fit Points" method described by Pfaffl *et al.* (Pfaffl, 2001) after normalization to the inputs.

2.15 Statistical analysis

Results were analysed with GraphPad PRISM™ (version 4) software from GraphPad Software Inc, using One-way ANOVA, with either Dunnett's post-test (when comparing all values to a single control) or Bonferroni's post-test (when comparing all values to each other). P values for comparison of 2 samples were obtained by using the paired t-test. Where statistical significance of difference was determined relative to a single control statistical significance is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively. Where all values were compared to each other, statistical significance of difference is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

CHAPTER 3

RESULTS

AN INVESTIGATION INTO HOMOLOGOUS AND GC REGULATION OF THE MOUSE GnRHR GENE IN THE L β T2 GONADOTROPE CELL LINE

3.1 BACKGROUND

At present, the most utilized model systems for investigating expression of gonadotrope-specific genes in cell culture are the α T3-1 and L β T2 immortalized mouse gonadotrope cell lines. The α T3-1 cell line is an immature precursor gonadotrope cell line, which expresses a functional GnRHR, as well as the glycoprotein hormone α -subunit, but neither of the glycoprotein hormone-specific β -subunits, LH β and FSH β (Windle *et al.*, 1990). The L β T2 cell line is a more mature and differentiated gonadotrope cell line since these cells express functional GnRHR, as well as the glycoprotein hormone α -subunit, LH β and FSH β , thus displaying functional characteristics of normal fully differentiated gonadotropes (Pernasetti *et al.*, 2001; Turgeon *et al.*, 1996).

In the past, homologous regulation of the mouse GnRHR gene has been studied predominantly in the α T3-1 cell line (Duval *et al.*, 1997b; Norwitz *et al.*, 1999a; White *et al.*, 1999) and very few reports have been published on the regulation of the GnRHR gene in L β T2 cells. The synthetic GC dex has been shown to upregulate expression of both the endogenous GnRHR gene and a transfected mouse GnRHR promoter-reporter construct in somatolactotrope GGH₃ cells expressing exogenous GnRHR and L β T2 cells (Maya-Nunez and Conn, 2003; McGillivray *et al.*, 2007; Turgeon *et al.*, 1996). The transcriptional regulation of the proximal mouse GnRHR gene by GnRH and dex has previously been investigated by others in Prof. Hapgood's laboratory in L β T2 cells (Addendum B figure B1) (Fernandes, 2007; Sadie, 2006). It was found that continuous stimulation for 8 h with 100 nM GnRH significantly increased the transcriptional activity of the transiently transfected GnRHR promoter-reporter construct (pLG wild-type, containing 580 bp of the mouse GnRHR gene) by approximately 2,5-fold compared to the vehicle control (Addendum B figure B2 A and B) (Sadie, 2006). A study by Bedecarrats *et al.* found only weak activation (about 1,5 fold) of a GnRHR-promoter construct by GnRH in L β T2 cells (Bedecarrats and Kaiser, 2003). However, in that particular study the cells were

stimulated continuously for 10 h with low concentration (10 nM) of GnRH. In addition, it was previously found that continuous stimulation of L β T2 cells with 100 nM dex results in a statistically significant increase in pLG promoter activity by about 2,5-fold similarly to the response observed with GnRH (Addendum B figure B1 C) (Fernandes, 2007). This is in agreement with a recent study showing that a 1,2 kb mouse GnRHR-promoter construct responds to continuous (24 h) stimulation with 100 nM dex by approximately 2-fold (McGillivray *et al.*, 2007). Furthermore, preliminary results from Prof. Hapgood's laboratory show that the endogenous GnRHR gene is transcriptionally regulated by GnRH and dex in L β T2 cells (Sadie, 2006; von Boetticher, 2008).

Previously it has been reported that the activating protein-1 (AP-1) binding site is a critical component in regulation of both basal and GnRH-stimulated mouse GnRHR gene expression in α T3-1 cells (Kam *et al.*, 2005; Norwitz *et al.*, 1999a; White *et al.*, 1999). The role of the AP-1 site in basal, as well as GnRH- and dex-induced expression of the GnRHR has recently been investigated by others in this laboratory in L β T2 cells (Addendum B figure B2) (Fernandes, 2007; Sadie, 2006). Basal promoter activity of a mutant construct containing a mutation in the AP-1 site within the context of the pLG GnRHR promoter-reporter construct (pLGmAP1), was significantly decreased by about 50% to that of the wild-type pLG construct (Addendum B figure B2 A) (Fernandes, 2007). This is consistent with findings by other groups showing that AP-1 is part of a tripartite basal transcriptional enhancer, since the loss of the AP-1 site decreased basal promoter activity in α T3-1 cells (Duval *et al.*, 1997b; White *et al.*, 1999). These reports together with previous result presented in Addendum B figure B2 A indicate an important role for the AP-1 *cis*-element in maintaining basal expression in gonadotrope cells. Furthermore the loss of the AP-1 site was found to significantly reduce the GnRH-mediated response (Addendum B figure B2 B) (Sadie, 2006) which is in agreement with other reports showing the involvement of this *cis*-element in responsiveness to GnRH in transgenic mice (Ellsworth *et al.*, 2003b) and in transfected α T3-1 cells (Norwitz *et al.*, 1999a; White *et al.*, 1999). Similarly, mutation of the AP-1 site has been shown to result in a complete loss of the dex-induced transcriptional activation of the promoter (Addendum B figure B2 C) (Fernandes, 2007), as was found by Maya-Nunez and Conn in GGH₃ cells (Maya-Nunez and Conn, 2003). These data provide evidence that a functional AP-1 site is a requirement for the full transcriptional response of the GnRHR gene to GnRH and dex in L β T2 cells. While homologous and dex-mediated regulation of endogenous GnRHR levels and a synthetic mouse GnRHR promoter occurs in L β T2 cells (Bedecarrats and Kaiser, 2003; Fernandes, 2007; McGillivray

et al., 2007; Sadie, 2006; Turgeon *et al.*, 1996; von Boetticher, 2008), more studies are needed to unravel the detailed molecular mechanisms of transcriptional regulation of the GnRHR in this more differentiated gonadotrope cell line.

3.2 AIMS

The aim of the present part of the project was to investigate transcriptional regulation of the GnRHR gene by GnRH and dex in L β T2 cells and to determine *cis*-regulatory elements as well as transcription factors involved.

Since both GCs and GnRH have been proposed to regulate GnRHR gene expression in separate reports, in the present study each experiment was carried out with dex and GnRH in parallel to directly compare the magnitude of the response and the mechanisms involved in L β T2 cells.

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

- To establish whether the endogenous GnRHR gene is regulated by dex and GnRH in L β T2 cells;
- To investigate the role of the AP-1 site in the GnRHR promoter region in the transcriptional response under basal, dex-, and GnRH-stimulated conditions;
- To examine expression levels of the AP-1 proteins c-Jun and c-Fos as well as binding of these transcription factors to the endogenous GnRHR gene in under basal, dex-, or GnRH-stimulated conditions in intact L β T2 cells;
- To investigate expression of functional steroid receptors in L β T2 cells;
- To investigate recruitment of the GR to the endogenous GnRHR gene in response to hormone stimulation *in vivo*;
- To establish a requirement for the GR in GnRHR gene expression in L β T2 cells

3.3 RESULTS

3.3.1 Both GnRH and dex increase expression of the endogenous mouse GnRHR gene in L β T2 cells

Previously it has been shown that GnRH and dex increase endogenous GnRHR gene expression in L β T2 cells although statistical significance could not be established (Sadie, 2006; von Boetticher, 2008). Thus in the present study it was thought to confirm these results. Therefore GnRHR mRNA levels were measured by means of quantitative real-time PCR. RNA was isolated from cells stimulated continuously for 8 h with 100 nM dex or 100 nM GnRH in serum-free medium. It was found that both GnRH and dex increased endogenous GnRHR mRNA levels significantly after 8 h of treatment, compared to vehicle control in several repeat experiments (figure 3.1). The magnitude of the response varied between individual experiments probably due to the high sensitivity of gonadotrope cells to their environment and biological variation. However it could be established that both GnRH and dex increased GnRHR mRNA levels (figure 3.1) suggesting a direct transcriptional effect on GnRHR expression in L β T2 cells.

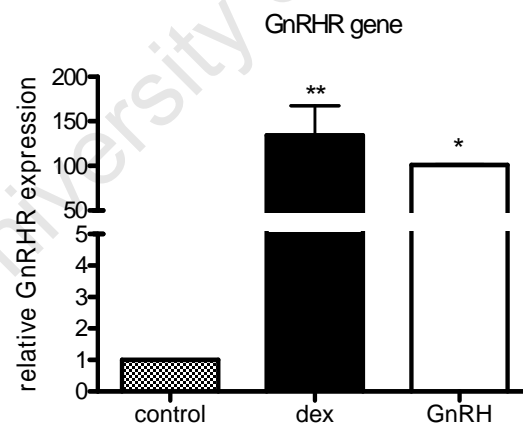


Figure 3.1: Both GnRH and dex increase expression of the endogenous mouse GnRHR gene in L β T2 cells

Total RNA was isolated from cells treated with test compounds for 8 h. RNA was reverse-transcribed, and relative levels of GnRHR transcripts were determined by quantitative real-time PCR. Relative levels of GnRHR mRNA were normalized to GAPDH mRNA levels transcripts were determined to serve as internal control. Fold changes in GnRHR mRNA levels were calculated relative to vehicle-treated (control) samples. The graph shows combined results of 3 independent experiments.

3.3.2 GnRH but not dex induces a minimal AP-1 reporter construct in L β T2 cells

Since it has previously been found that a functional AP-1 site within the GnRHR promoter is a requirement for the full transcriptional dex- and GnRH-mediated response of the GnRHR gene in L β T2 cells (Addendum B figure B2) (Fernandes, 2007; Sadie, 2006) it was determined in the present study whether the AP-1 site within the GnRHR promoter is sufficient for the response to dex and GnRH. Therefore L β T2 cells were transfected with a minimal synthetic AP-1-luciferase construct (pAP1-luc) and stimulated for 8 h with dex or GnRH. The result presented in figure 3.2 shows a significant increase (approximately 50-fold) in the activity of the pAP1-luc construct when cells were treated with GnRH, supporting the hypothesis that, in L β T2 cells, GnRH can upregulate transcription from an AP-1 site without a requirement for other *cis*-elements. The higher magnitude of the GnRH response of pAP1-luc, compared to that of pLG (Addendum B figure B2), may be due to the presence of seven AP-1 sites in the pAP1-luc construct, as opposed to only one in the GnRHR promoter. The result together with previous results from Prof. Hapgood's laboratory, showing that mutation of the AP-1 site results in decreased GnRH-mediated transcriptional activity of a transfected GnRHR promoter construct, suggest that this *cis*-element is an essential requirement for GnRH-induced transcriptional activation of the GnRHR promoter in L β T2 cells.

In contrast to the results obtained with GnRH, treatment with dex did not increase the transcriptional activity of a pAP1-luc construct, compared to vehicle-treated cells. The result suggests that the AP-1 site alone is not sufficient to mediate the dex-induced upregulation of the GnRHR promoter activity and that other *cis*-elements present within - 569 to +1 of the GnRHR promoter construct are most likely to be involved in the dex-mediated upregulation of this gene.

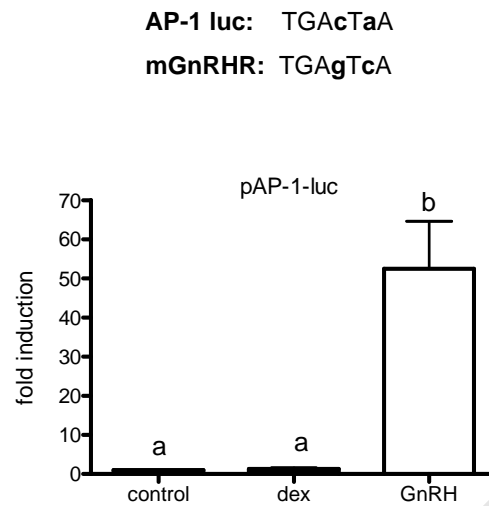


Figure 3.2: GnRH but not dex induces a minimal AP-1 reporter construct in LβT2 cells

LβT2 cells were transfected with 250 ng of the pAP-1 luc construct and after approximately 24 h cells were incubated for 8 h in serum-free medium in the absence or presence of 100 nM GnRH or 100 nM dex (A and B). The luciferase activity was normalized to β-galactosidase activity and the hormone-stimulated increase in promoter activity was calculated relative to the control. Graphs show combined results of at least 3 independent experiments, each performed in triplicate. The sequences of the consensus AP-1 site and the AP-1 element in the mouse GnRHR promoter are shown.

3.3.3 GnRH but not dex increases AP-1 protein expression levels

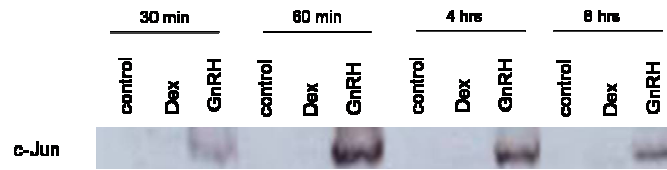
Since it was previously established in this laboratory that the AP-1 site is an essential regulatory promoter element for basal as well as dex- and GnRH-stimulated gene expression of the GnRHR in LβT2 cells (Addendum B figure B2) (Fernandes, 2007; Sadie, 2006) it was of interest to further investigate the role of this *cis*-element in these cells. Various stimuli increase expression or activity of several members of the AP-1 protein family such as Fos and Jun in gonadotrope cell lines (Cesnjaj *et al.*, 1994; Coss *et al.*, 2004; Ellsworth *et al.*, 2003b; Kakar *et al.*, 2003; Liu *et al.*, 2002a; Wurmbach *et al.*, 2001). In particular GnRH was found to rapidly induce protein expression of c-Jun and c-Fos in LβT2 cells (Coss *et al.*, 2004; Liu *et al.*, 2002a; Sadie, 2006) and therefore it is likely that increased

expression of c-Jun or c-Fos might present a potential mechanism by which GnRH and/or dex regulate GnRHR expression via the AP-1 site.

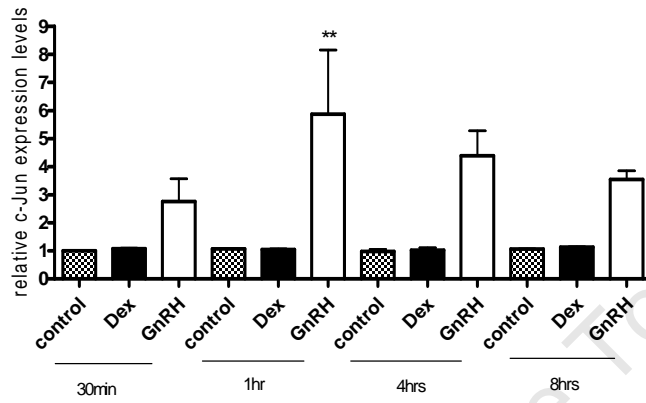
In order to investigate the effect of dex and GnRH on AP-1 proteins expression levels, Western blot analysis was performed on whole cell extracts of L β T2 cells stimulated with 100 nM dex or 100 nM GnRH as indicated in the figure legend (figure 3.3). Immunodetection was performed using specific antibodies for c-Jun (figure 3.3 A) and c-Fos (figure 3.3 C). Importantly, since it has been stressed in the literature that serum, present in culture medium, results in increased Jun and Fos mRNA levels (Chen *et al.*, 1995; Jaffey *et al.*, 1992), in the present study L β T2 cells were serum-starved over night. As shown in figure 3.3 A, c-Jun proteins were not detectable in unstimulated or dex-treated samples. In contrast GnRH stimulation resulted in a rapid increase in c-Jun protein expression after 30 min, reaching its peak at 1 h of treatment (figure 3.3 A and B) which is consistent with reports in the literature (Coss *et al.*, 2004; Liu *et al.*, 2002a; Sadie, 2006). Similarly, GnRH strongly induced expression of c-Fos proteins in L β T2 cells after 30 min of treatment and was decrease after 4 h while c-Fos remained undetectable in untreated as well as dex-stimulated cells at any time point investigated (figure 3.3 C and D). Note that in figure 3.3 C the mobility of the bands differs at different time points. Since the anti-Fos antibody used in this experiment does detect c-Fos as well as other family members, it is likely that these bands represent different Fos proteins.

Western blotting is a measure of the relative abundance of a protein in whole cell lysates, strongly depending on the sensitivity of the specific antibody. Thus, the absence of c-Jun and c-Fos proteins in unstimulated or dex-treated samples could be due to very low expression of these proteins in L β T2 cells. Other reports in the literature show low detectable amounts of c-Jun and c-Fos proteins in unstimulated L β T2 cells in the absence of serum (Coss *et al.*, 2004; Liu *et al.*, 2002a). Taken together, it appears that both c-Jun and c-Fos proteins are strongly and transiently increased by GnRH while dex treatment appeared to have no effect on the expression levels of these proteins.

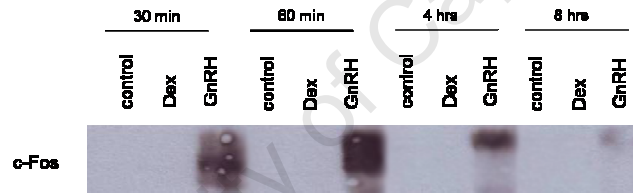
A



B



C



D

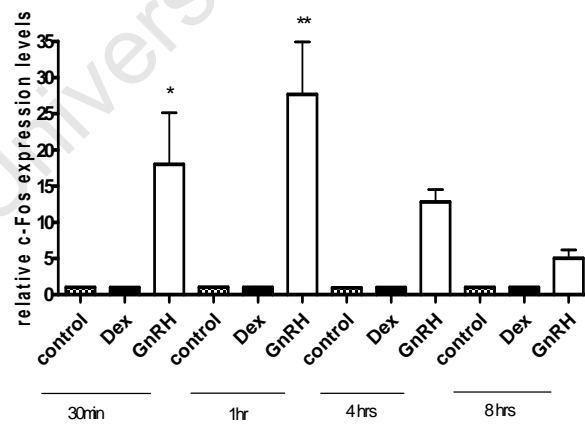


Figure 3.3: Effect of GnRH treatment on AP-1 protein expression in LβT2 cells (legend to follow on next page)

LβT2 cells were grown and serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex for the indicated times. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific antibodies for c-Jun (A) or c-Fos (C). The graphs in B and D show combined results of at least 2 independent experiments. The relative amount of protein was quantified with the value for control (vehicle) set as 1.

3.3.4 Both c-Jun and c-Fos proteins are bound under basal as well as dex- and GnRH-stimulated conditions to the AP-1 region of the endogenous GnRHR promoter in intact cells

Specific *in vitro* binding of the AP-1 proteins Jun and Fos to the AP-1 region of the GnRHR gene in response to GnRH has been shown in α T3-1 cells (Norwitz *et al.*, 1999a; White *et al.*, 1999) as well as in LβT2 cells (Sadie, 2006). Furthermore a study by Jeong *et al.* demonstrated by ChIP the binding of c-Jun to the GnRHR gene in the absence of hormone in LβT2 cells (Jeong *et al.*, 2004). However, binding of c-Jun and c-Fos proteins to the endogenous GnRHR promoter in response to dex or GnRH in intact cells has not been reported.

Therefore, the proteins interacting with the AP-1 site on the endogenous GnRHR promoter in response to GnRH or dex were investigated in intact LβT2 cells by ChIP. When anti-c-Jun or anti-c-Fos antibodies were used for immunoprecipitation, PCR products could be detected in samples from untreated cells (figure 3.4 A), suggesting that both AP-1 proteins are occupying the AP-1 site under basal conditions and are thus likely to be involved in maintaining basal promoter activity. This is in agreement with results presented in figure 3.2 A where the pLGmAP-1 construct exhibited decreased basal activity, compared to wild type pLG construct. The Western blot results presented in figure 3.3 did not detect any AP-1 proteins from untreated whole cell lysates which could be due to the low affinity of the AP-1 antibodies in conjunction with very low abundance of the AP-1 proteins investigated. However, the specificity of the AP-1 antibodies was confirmed using a control IgG antibody (figure 3.4 A).

Stimulation with GnRH or dex did not result in increased binding of either c-Jun or c-Fos proteins, suggesting that neither the amounts nor the relative composition of c-Jun or c-Fos proteins at the AP-1 site were modulated upon hormone stimulation (figure 3.4 A and B). These data further indicate that c-Jun and c-Fos are bound to the AP-1 site of the GnRHR promoter under basal -, and hormone-

stimulated conditions, possibly forming a platform for other transcription factors to bind in response to hormone. Since the c-Jun antibody used in this experiment detects Jun-B and Jun-D while the c-Fos antibody shows cross reactivity with Fos-B, Fra-1 and Fra-2, it is likely that these AP-1 family members can be potential occupants on the endogenous GnRHR promoter in the absence and presence of hormone. Amplification of the AP-1 region of the FSH β gene promoter from the same control and GnRH-treated sample immunoprecipitates revealed that, unlike for the GnRHR AP-1 region, GnRH stimulation results in an greater than 15-fold recruitment of both c-Jun and c-Fos to this promoter (figure 3.4 C and D), as shown previously by others (Coss *et al.*, 2004). This result shows that the similar levels of AP-1 proteins observed on the GnRHR promoter in the absence and presence of GnRH (figure 3.4 A and B) are not due to a deficiency in the AP-1 ChIP procedure. This is in contrast to a report showing that GnRH induces an increase in AP-1 proteins, specifically c-Jun, binding to the promoter in intact α T3-1 cells (Kam *et al.*, 2005), as well as *in vitro* results showing that c-Jun and not c-Fos proteins are able to bind to the AP-1 element in response to dex in GGH₃ cells (Maya-Nunez and Conn, 2003). The discrepancy in the results obtained is probably due to cell specific differences as well as differences in culture conditions.

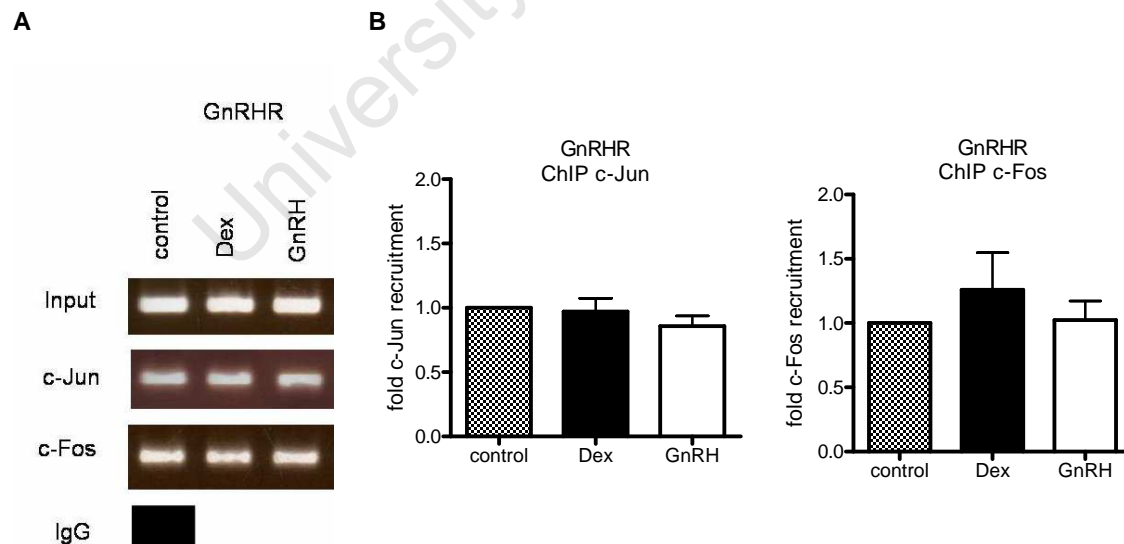


Figure 3.4: (continued on next page)

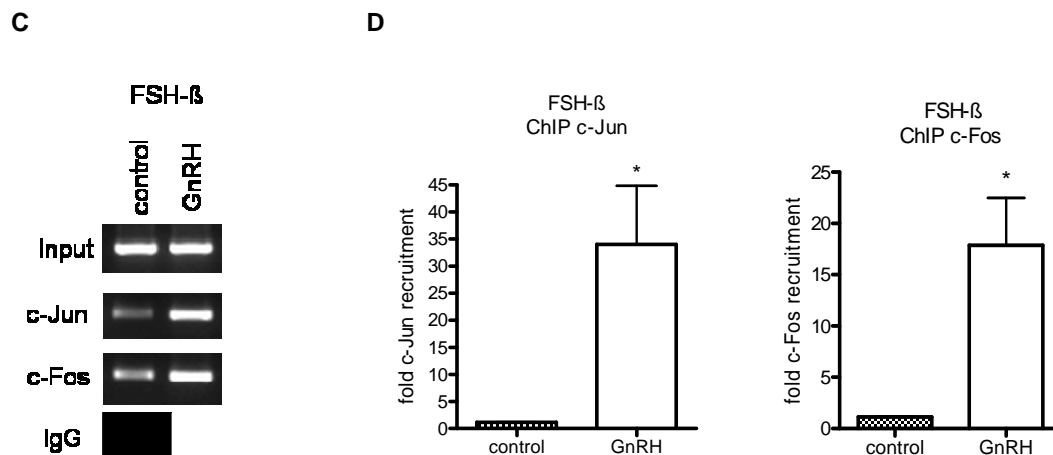


Figure 3.4: Both c-Jun and c-Fos proteins are bound under basal as well as dex- and GnRH-stimulated conditions to the AP-1 region of the endogenous GnRHR promoter in intact cells

Chromatin immunoprecipitation (ChIP) assays were performed in LβT2 cells treated with vehicle (control), 100 nM GnRH or 100 nM dex for 1 h. ChIP was performed with anti-c-Jun or anti-c-Fos (A, B). Precipitated complexes bound to the endogenous GnRHR (A) or FSH-β promoter (C) were detected using PCR primers encompassing the AP-1 region of the promoters. The FSH-β promoter was analyzed using the same samples as a positive control for GnRH-stimulated recruitment of the AP-1 proteins (Coss *et al.*, 2004). The co-immunoprecipitated DNA fragments and input DNA were analyzed by conventional PCR and by real-time PCR. Pooled and quantified results of three independent experiments are shown normalized to input and expressed as fold-response relative to control values (B,D).

3.3.5 Steroid receptor expression in LβT2 cells

There are a few reports in the literature showing expression of mRNA transcripts of the glucocorticoid receptor (GR), the progesterone receptor (PR), the estrogen receptor (ER) as well as the androgen receptor (AR) in LβT2 cells (Eertmans *et al.*, 2007; Lawson *et al.*, 2001; Schreihofner *et al.*, 2000; Thackray *et al.*, 2006). While dex- (Eertmans *et al.*, 2007; Sasson *et al.*, 2008; Turgeon *et al.*, 1996), estrogen- (Turgeon *et al.*, 1996) and androgen-mediated induction of gene transcription was shown (Eertmans *et al.*, 2007; Lawson *et al.*, 2001), protein expression has only been reported for the PR (An *et al.*, 2009) and the ERα (Chen *et al.*, 2009; Schreihofner *et al.*, 2000) in LβT2 cells. However, a study by Eertmans *et al.* reported a lack of ERα protein expression in LβT2 cells and moreover they reported that estrogenic responses were absent following co-transfection with an ERα expression vector

(Eertmans *et al.*, 2007). In view of these inconsistencies it was of interest to investigate the expression of functional GR, ER, PR, MR and AR in the specific L β T2 subclone used in the present study.

Whole cell lysates from L β T2 cells were analyzed by Western blotting using specific antibodies for the GR, PR, MR and AR to determine the expression of these steroid receptors in these cells. As shown in figure 3.5 A GR protein expression was detected in the L β T2 cell lysates. The specificity of the band was confirmed by using COS-1 cells transfected with a GR expression plasmid as positive control (figure 3.5 A). By contrast the PR, MR or AR could not be detected in these cells while specific bands with the right molecular weight were detected in COS-1 positive control cell lysates (figure 3.5 A). Note that in figure 3.5 A two protein bands can be seen when probing with the MR antibody in COS-1 cells. It could be established that the upper band presents a non-specific band while the band with lower molecular weight was shown to present the MR since this band was only detectable in COS-1 cells expressing exogenous MR and it was absent in untransfected COS-1 cells and L β T2 cells. Due to failure of the ER specific antibody, protein expression of this steroid receptor could not be determined in L β T2 cells.

As an alternative strategy towards detecting the presence of a particular receptor in L β T2 cells steroid-receptor mediated gene transcription was investigated in L β T2 cells using a GRE-luciferase construct or an estrogen-responsive-element (ERE) reporter-construct. Treatment of the transfected cells with 100 nM dex (a potent GR agonist) increased the transcriptional response of the GRE-reporter construct by approximately 3-fold which is in agreement with the Western blot result showing protein expression of the GR in L β T2 cells. By contrast, treatment with steroid-receptor specific agonists for the AR (mibolerone Mib), MR (aldosterone, Ald) or PR (progesterone, Prog and R5020) did not result in a significant increase in transcriptional activity of the steroid receptor-driven reporter construct (figure 3.5 B). In addition estrogen stimulation failed to activate the ERE-reporter as shown in figure 3.5 C suggesting that no functional ER is expressed in L β T2 cells. As a positive control assay, COS-1 cells were cotransfected with a GRE- or a ERE-luciferase construct and expression plasmids coding for the full length MR, PR, AR or ER and stimulated with the specific ligands as described above (figure 3.5 D and E). The results show that all the specific ligands are functional. Attempts to overexpress the steroid receptors in L β T2 cells were unsuccessful possibly due to the low transfection efficiency of the cell line. Taken together the results show that the L β T2 cells used for this study express functional endogenous GR but not ER, AR, PR or MR.

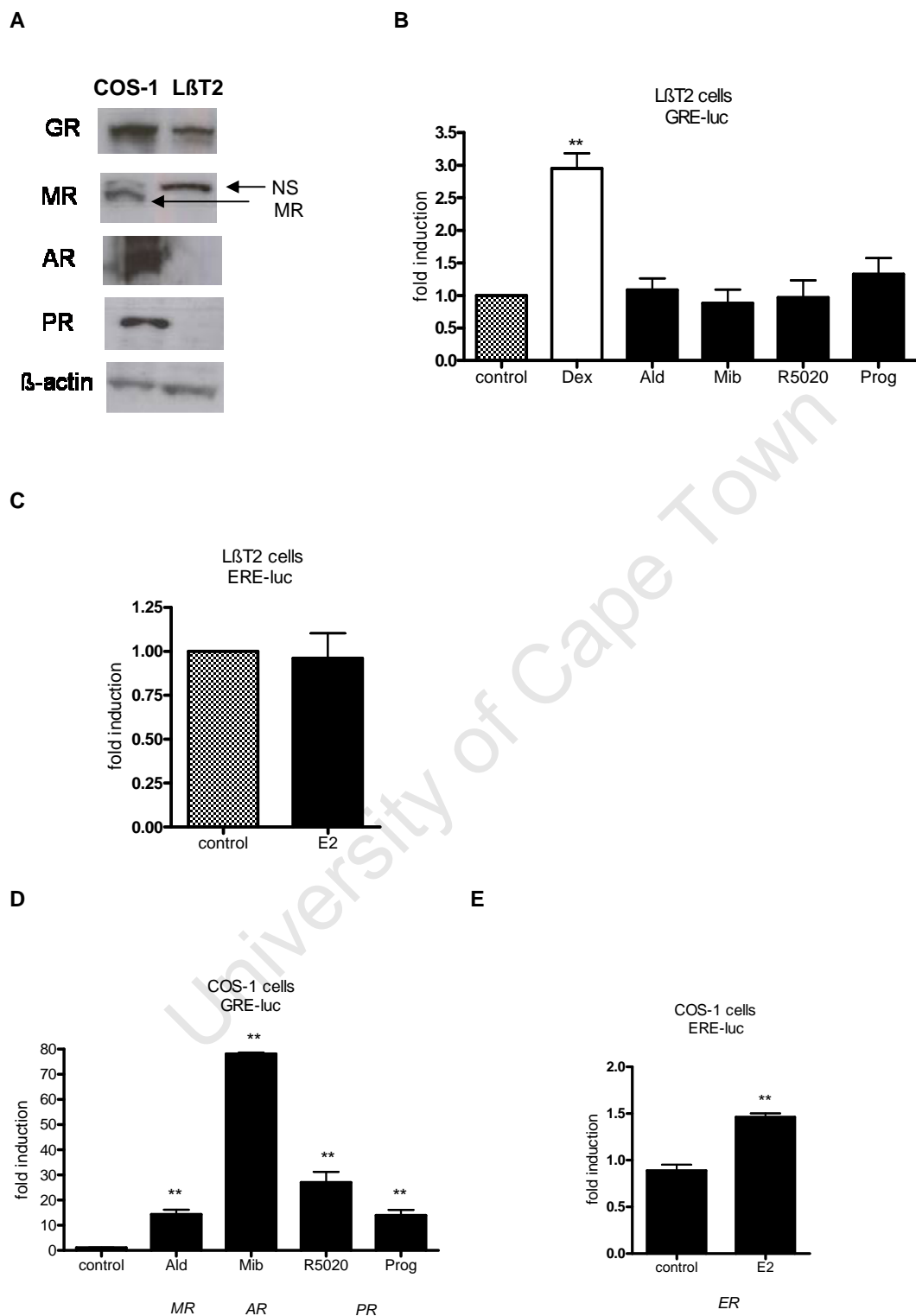


Figure 3.5: LBT2 cells express functional GR (figure legend to follow on next page)

A: L β T2 cells were grown and whole cell lysates prepared. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific antibodies for GR, MR, AR, PR or β -actin as a loading control. **B-E:** L β T2 cells (B and C) or COS-1 cells (D and E) were transfected with 250 ng TAT-GRE-luciferase construct (B and D) or with 250 ng pSG5-ERE-luciferase construct (C and E) in the absence (B and C) or presence of expression plasmids for the MR, AR, PR (D) or ER (E). After approximately 24 h cells were incubated for 8 h in serum-free medium with vehicle (control), 100 nM dex, 100 nM aldosterone, 100 nM mibolerone, 100 nM R5020, 100 nM progesterone (B and D) or 100 nM estradiol (E2) (C and E) as indicated below the relevant bars. The luciferase activity was normalized to β -galactosidase and the hormone-stimulated increase in promoter activity was calculated relative to the vehicle control. The graphs show the promoter activity relative to vehicle (control) of combined results of three (B,C,E) or one (D) independent experiment(s), each performed in triplicate, where the luciferase activity was normalized to β -galactosidase. NS: non-specific band

3.3.6 Both dex and GnRH induce recruitment of the GR to the AP-1 region on the endogenous GnRHR promoter in intact cells

As established in section 3.3.4, AP-1 proteins, specifically c-Jun and c-Fos, appear to be bound to the GnRHR promoter under basal and stimulated conditions possibly providing a platform for other transcription factors to bind. Thus, the same samples (section 3.3.4) were analyzed by ChIP to explore whether the GR is recruited to the AP-1 promoter element in response to dex. The results presented in figure 3.6 A and B show that treatment with dex promotes a rapid and strong recruitment of the GR to the AP-1 region in the GnRHR promoter by approximately 3,5 fold compared to vehicle control treated cells (figure 3.6 A and B). Immunoprecipitation with a non-specific IgG antibody confirmed the specificity of the GR antibody. This is the first report showing dex-mediated recruitment of the GR to the AP-1 region of the GnRHR promoter in intact L β T2 cells. Although it can be concluded that the GR is recruited to the endogenous promoter in a hormone dependent fashion, the particular mechanism of GR binding to the DNA is unknown. The PCR primers span a region of 168 bp of the proximal GnRHR promoter, encompassing the AP-1 site at -336/-330 relative to the translational start site. Thus, the possibility of GR binding to proteins on adjacent DNA motifs occupied with other transcription factors besides AP-1 cannot be excluded. This hypothesis is in agreement with the result presented in figure 3.2 showing that dex cannot induce transactivation of a minimal promoter containing only an AP-1 sites. Attempts to investigate whether GR and AP-1 proteins occur in the same complex by co-immunoprecipitation and re-ChIP experiments were unsuccessful, possibly due to the low abundance

of the endogenous complexes. Interestingly, when chromatin from GnRH-treated samples was analyzed, the GR was also found to be significantly recruited to the AP-1 region of the endogenous promoter although to a slightly lesser extent than dex (~3,5fold) by approximately 2 fold (figure 3.6 B). This result was surprising and unexpected and indicates that GnRH results in activation of the GR in the absence of a GR ligand in L β T2 cells.

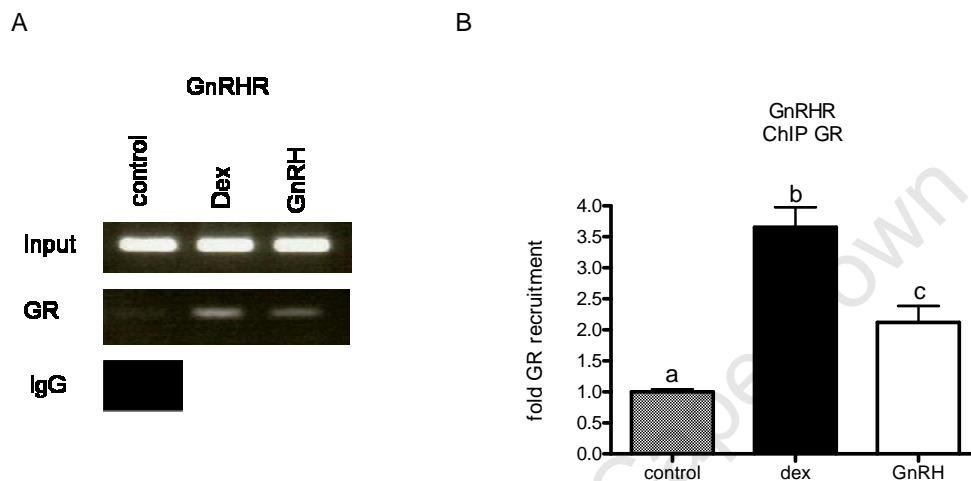


Figure 3.6: Both dex and GnRH induce recruitment of the GR to the AP-1 region on the endogenous GnRHR promoter in intact cells

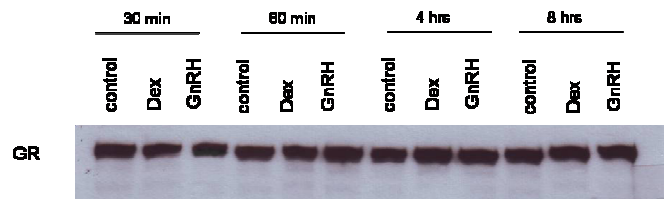
Chromatin immunoprecipitation assays (ChIP) in L β T2 cells treated with vehicle (control), 100 nM GnRH or 100 nM dex for 1 h. ChIP was performed with an anti-GR specific antibody. Precipitated complexes bound to the endogenous GnRHR promoter were detected using PCR primers encompassing the AP-1 region of the promoter. The co-immunoprecipitated DNA fragments and input DNA were analyzed by conventional PCR (A) and by real-time PCR (B). Pooled and quantified results of 3 independent experiments are shown normalized to input and expressed as fold-response relative to control values (B).

3.3.7 Protein expression levels of the GR remain unchanged in response to dex or GnRH

To investigate whether the dex- and GnRH-mediated increase in GR recruitment to the endogenous GnRHR promoter (shown in figure 3.6) was the result of increased GR protein levels, Western blot analysis was performed on whole cell lysates of L β T2 cells treated with 100 nM dex or 100 nM GnRH for the indicated times. In figure 3.7 it can be seen that GR protein expression levels remained unchanged over an 8 h time course in cells treated with dex or GnRH compared to vehicle control

suggesting that altered GR protein expression levels are not involved in increased GR recruitment to the AP-1 region of the GnRHR in response to dex or GnRH.

A



B

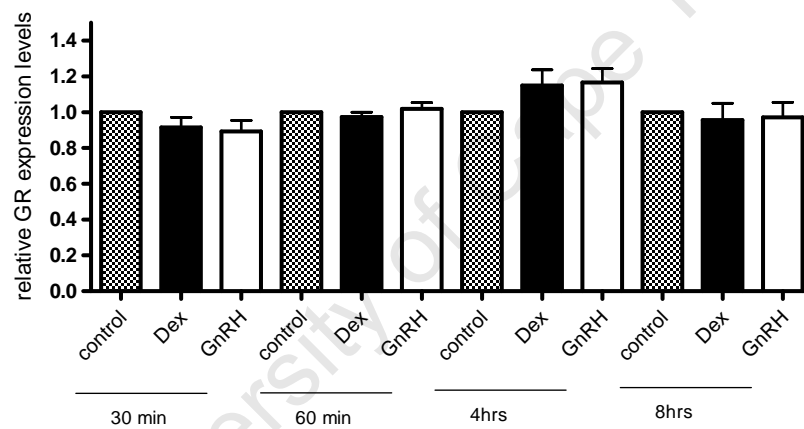


Figure 3.7: GR expression levels in response to dex or GnRH in LβT2 cells remain unchanged

LβT2 cells were grown and serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex for the indicated times. 1/10 of a whole cell lysate was loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific anti-GR antibody. The graph in B shows combined results of at least 4 independent experiments. Relative GR expression levels were plotted with the value control (vehicle) for each time-point set as 1.

3.3.8 The GR is required for transcriptional regulation of the endogenous GnRHR by GnRH and dex

To investigate the requirement for the GR in transcriptional regulation of the GnRHR gene in response to both dex and GnRH, small interfering RNA (siRNA) was used to decrease the GR protein levels in L β T2 cells. The cells were transfected with siRNA for the mouse GR or with a scrambled non-silencing siRNA, to demonstrate the specificity of the GR oligos. The efficiency of the siRNA was determined by Western blotting and in repeated experiments the GR protein levels were decreased by about 50% as compared to the non-silencing control (figure 3.8 A and B). Using increased amounts of siRNA oligos or transfection reagent as well as longer incubation times or transfecting twice with GR siRNA did not further decrease the GR protein levels. In addition, the use of 3 different GR siRNA oligos resulted in the same extent of GR knockdown (approximately 50%).

The siRNA-transfected cells were stimulated with GnRH or dex for 8 h and quantitative real-time PCR was performed to determine the effect of reduced GR protein levels on dex- and GnRH-mediated GnRHR gene expression (figure 3.8 C). GnRHR mRNA levels were found to be upregulated by dex as shown earlier (figure 3.1), and the response was significantly decreased by about 50% in the presence of the GR siRNA. The result demonstrates that the GR is required for the dex-mediated upregulation of the endogenous GnRHR gene. Moreover, when GR protein levels were diminished by siRNA, the GnRH-mediated increase in GnRHR transcripts was also found to be decreased by about 50% relative to the non-silencing control. Since the magnitude of the responses varied, significance could not be established. However reduced GnRH induction of GnRHR gene expression in the presence of GR siRNA was observed in several repeat experiments. These results strongly support the conclusion that the GR is required for both dex- and GnRH-mediated transcription of the endogenous GnRHR gene in L β T2 cells.

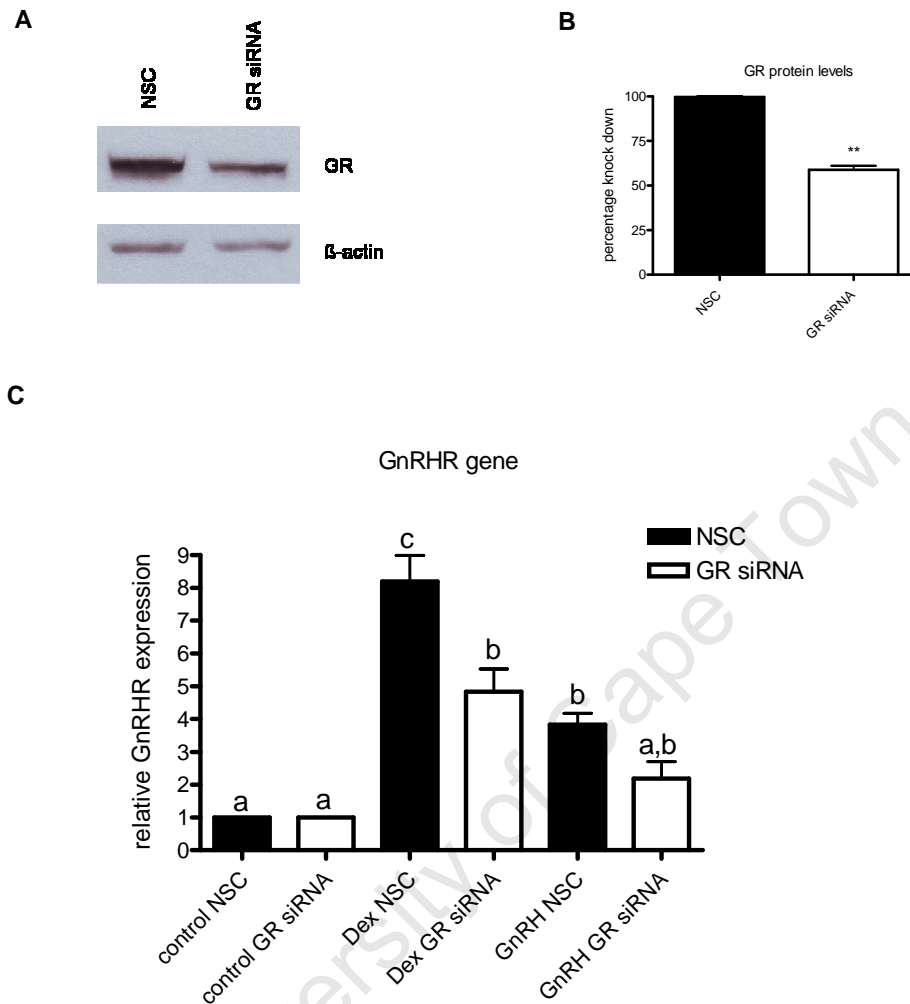


Figure 3.8: The GR is required for transcriptional regulation of the endogenous GnRHR by GnRH and dex

A,B: LBT2 cells were transfected either with scrambled siRNA (non silencing control, NSC, black bars) or with siRNA for the mouse GR (GR siRNA, white bars). After 96 h, whole cell lysates were analysed by Western blotting. The Western blot result of a single experiment is shown in A. The pooled results from three independent Western blot experiments are shown in B, where quantification was performed using AlphaEaseFC™ Software and the relative GR levels were normalized to β -actin protein levels.

C: Cells were transfected as described for A and B. After 96 h cells were stimulated with either vehicle, 100 nM GnRH or 100 nM dex and incubated for further 8 h in serum-free medium. Total RNA was isolated, reverse-transcribed, and relative levels of GnRHR transcripts were determined by quantitative real-time PCR, normalized to GAPDH. The graph shows combined results of at least 3 experiments where the response is expressed relative to vehicle control with NSC siRNA or GR siRNA, respectively.

CHAPTER 4

RESULTS

INVESTIGATION OF LIGAND-INDEPENDENT ACTIVATION OF THE GLUCOCORTICOID RECEPTOR BY GnRH AND SIGNALLING PATHWAYS IN THE L β T2 GONADOTROPE CELL LINE

4.1 BACKGROUND

In the classical mechanism of action, the GR resides in the cytoplasm in a multiprotein complex containing heat shock proteins and chaperones. Upon ligand binding the GR dissociates from the complex and the liganded receptor translocates to the nucleus to regulate expression of specific target genes (Griekspoor *et al.*, 2007). In addition to the conventional hormone-dependent activation of steroid receptors, there is accumulating evidence for substantial cross talk between signal transduction pathways and steroid receptors which is often referred to as non-classical or ligand-independent activation of steroid receptors. The estrogen receptor (ER) (Bunone *et al.*, 1996; El-Tanani and Green, 1997), progesterone receptor (PR) (Pierson-Mullany and Lange, 2004) and the androgen receptor (AR) (Maudsley *et al.*, 2006) all exhibit ligand-independent activation under appropriate conditions. There are a few reports in the literature suggesting that ligand-independent activation of the GR occurs in cells other than L β T2 cells. Tanaka *et al.* found that in hamster ovary cells ursodeoxycholic acid (UDCA) promotes nuclear translocation of the GR and transactivation of a GRE (Tanaka *et al.*, 1996). Consistent with these findings, ligand-independent activation of the GR has been described in primary human lung fibroblasts and vascular smooth muscle cells by beta-adrenergic receptor agonists salmeterol and salbutamol resulting in rapid nuclear translocation of the receptor and activation of a GR-inducible luciferase reporter gene (Eickelberg *et al.*, 1999). However, whether the above examples of ligand-independent activation of the GR occur by rapid non-genomic mechanisms such as GR phosphorylation, or cross talk with other receptors is not known.

Several reports have unravelled the downstream kinase pathways in gonadotrope cell lines, showing that GnRH can activate extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)

and p38 in both α T3-1 (Levi *et al.*, 1998; Reiss *et al.*, 1997; Roberson *et al.*, 1999; Sim *et al.*, 1995) and L β T2 cells (Harris *et al.*, 2002; Liu *et al.*, 2002a; Liu *et al.*, 2002b; Yokoi *et al.*, 2000). The role of MAPKs pathways in regulation of gonadotropin subunit gene expression is somewhat controversial. While some investigators have shown a role for JNK, but not for PKC and ERK, in LH β gene expression L β T2 cells, others have shown that PKC, ERK and JNK are involved in GnRH regulation of LH β in these cells. In α T3-1 cells it has been suggested that PKC-dependent activation of JNK but not ERK plays a critical role in GnRH regulation of the GnRHR (Ellsworth *et al.*, 2003b). In L β T2 cells both PKC- and PKA-pathways have been shown to be involved in the transcriptional regulation of a transfected GnRHR-promoter construct and the endogenous GnRHR gene (Sadie, 2006). However, a possible role for MAPKs in GnRHR gene expression in L β T2 cells has not been reported.

In rat granulosa cells dex induced rapid activation of ERK and not p38 within 1 min of stimulation (Sasson *et al.*, 2003). Other reports show that dex stimulates p38 MAPK phosphorylation in lymphoid cells (Miller *et al.*, 2005) and activates p38 and JNK but not ERK in a time-dependent manner in human eosinophils (Zhang *et al.*, 2000). By contrast dex appears to prevent proinflammatory stimuli induced MAPK activation (Hulley *et al.*, 1998; Lasa *et al.*, 2001; Pelaia *et al.*, 2001). In A549 cells dex was shown to attenuate IL-1 β -mediated activation of p38 and JNK (Jang *et al.*, 2007) while another group demonstrated that dex significantly inhibited LPS-induced activation of JNK but not p38 or ERK in RAW264.7 cells (Swantek *et al.*, 1997). It appears that the opposing results above may be due to different cell types, species and culturing condition. To date there are no reports in the literature describing signalling pathways induced by GCs in L β T2 cells.

4.2 AIMS

In chapter 3 it was found that GnRH results in recruitment of the GR to the endogenous GnRHR in intact L β T2 cells in the absence of a GR ligand. In addition, it could be established that the GR plays an essential role in transcriptional regulation of the GnRHR gene in these cells. Thus, it was decided in the present part of the study to further investigate the mechanism of ligand-independent activation of the GR by GnRH. All experiments were carried out with dex and GnRH to explore both differences and similarities in ligand-dependent versus ligand-independent activation of the GR. Furthermore it was of great interest to unravel the signalling pathways involved in GnRH- and dex-mediated gene expression in L β T2 cells.

The specific aims of this study were as follows:

- To investigate cellular distribution of the endogenous GR under basal, dex- and GnRH-stimulated conditions in L β T2 cells;
- To determine whether the GR can transactivate a minimal GRE reporter-promoter construct;
- To establish a requirement for the GR and GnRHR signalling pathways for GnRH-mediated transcriptional activation of a GRE in L β T2 cells;
- To determine whether ligand independent activation of exogenously expressed GR can occur in COS-1 cells dependent on the GnRHR;
- To investigate the phosphorylation status of the GR at two specific serine-residues, Ser-220 and Ser-234, respectively and examine the role of GnRHR signalling in GnRH-mediated GR phosphorylation;
- To determine whether dex or GnRH can activate the MAPKs JNK, p38 and ERK in L β T2 cells;
- To investigate the role of JNK, p38 and ERK in dex- and GnRH-mediated GRE and AP-1 transactivation;
- To determine the role of MAPK in GnRH- and dex-induced GR phosphorylation at Ser-220 and Ser-234 in L β T2 cells;
- To explore whether PKA, PKC and src-tyrosine kinase are involved in dex- and GnRH-induced transactivation and GR phosphorylation

4.3 RESULTS

4.3.1 GnRH mediates nuclear translocation of the unliganded endogenous GR

The GR resides in the cytoplasm in an inactive state and classical hormone-dependent activation of the GR results in nuclear translocation and its interaction with regulatory regions in primary target genes (Griekspoor *et al.*, 2007). Therefore it was explored whether GnRH can cause nuclear translocation of the GR in the absence of a GR ligand. L β T2 cells grown on cover slips were serum starved over night and stimulated for 1 h with 100 nM dex or 100 nM GnRH followed by fixing and staining of the cells with a GR specific antibody. As shown in figure 4.1, the GR was predominantly found in the cytoplasm in vehicle-treated control cells. Treatment with dex promoted translocation of about 100% of the GR to the nucleus as expected. This is consistent with a study by Webster *et al.* showing that the over expressed mouse GR is predominantly cytoplasmic in the absence of ligand, while dex stimulation resulted in rapid nuclear translocation of the receptor (Webster *et al.*, 1997). Importantly, treatment with GnRH-induced accumulation of about 50% of the GR in the nucleus in the absence of a GR ligand (figure 4.1). The result presented here provides strong evidence for ligand-independent activation of the GR in the absence of GCs.

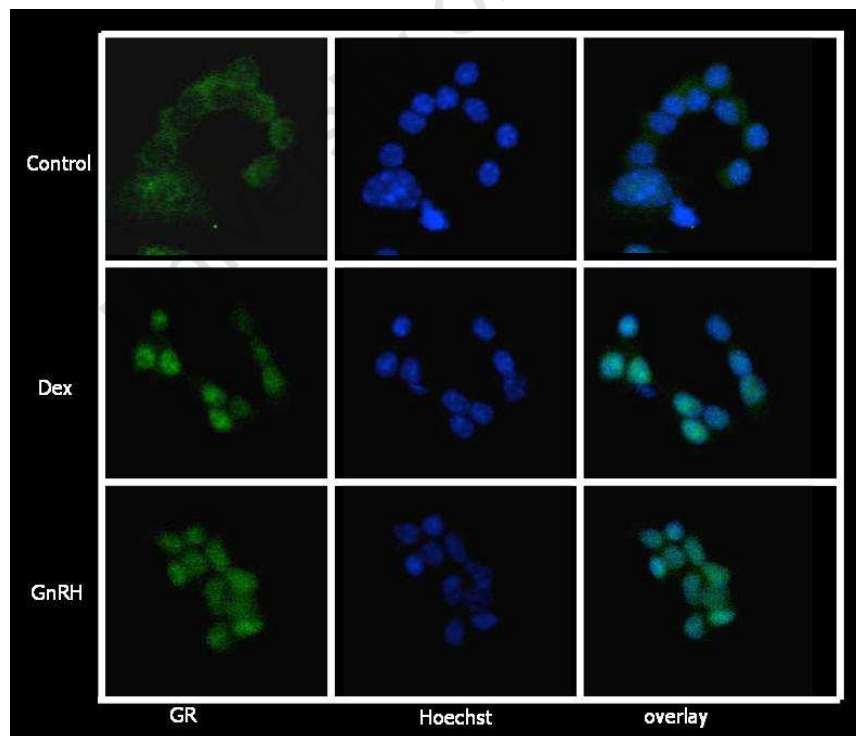


Figure 4.1: (continued on next page)

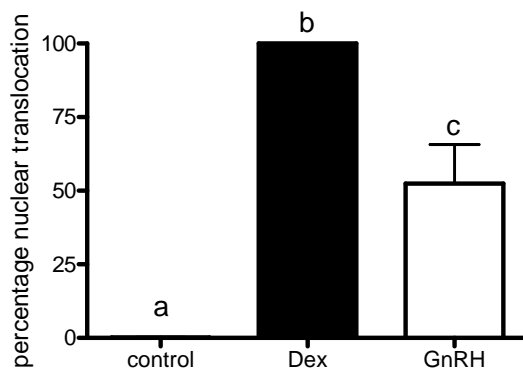


Figure 4.1: Both dex and GnRH mediate nuclear translocation of the endogenous GR

L β T2 cells were grown on glass cover slips and serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex for 1 h. Samples were fixed and stained with a GR-specific primary antibody and an anti-rabbit secondary fluorescent labelled antibody followed by counterstaining with the DNA dye Hoechst. Staining was visualized with an Olympus IX 81 microscope and analyzed with Cell^R software. The graph shows combined results of 3 individual experiments.

4.3.2 Activation of a GRE by GnRH is GR- and GnRHR-dependent in L β T2 cells

Having established that GnRH induces nuclear import of the GR it was of interest to investigate whether the ligand-independent activated GR can activate transcription via glucocorticoid-response-elements (GREs). Therefore, L β T2 cells were transiently transfected with a GRE-luciferase reporter construct (containing 2 copies of the rat GRE) and stimulated with 100 nM dex or 100 nM GnRH for 8 h in serum-free medium. As shown in figure 4.2 A, dex treatment increased the transcriptional activity of a GRE-luciferase construct by approximately 4 fold. Importantly, GnRH treatment resulted in a similar increase in promoter activity of the GRE-reporter in the absence of dex. Similarly, both dex and GnRH significantly increased the transcriptional activity of a MMTV-luciferase reporter construct (containing 4 repeats of the GRE) but to a slightly lesser extent (~2,5 fold) compared to the GRE-reporter construct (figure 4.2 B). The results indicate that GnRH-mediated transactivation is not promoter specific.

Next it was investigated whether the GnRH-induced increase in GRE-promoter activity shown in figure 4.2 A is mediated via the GR. Therefore, L β T2 cells were transfected with a GRE-reporter construct in

the absence and presence of overexpressed GR. Exogenously expressed GR resulted in a significant increase in dex-induced GRE-luciferase activity, as compared to in cells expressing only endogenous GR (figure 4.2 C). Furthermore exogenously expressed GR significantly increased GnRH-mediated transactivation (figure 4.2 C). The result strongly supports the hypothesis that the GnRH-induced increase in transcriptional activity of a GRE is mediated via the GR.

Since it cannot be excluded that GnRH directly or indirectly increase endogenous GC concentration by increasing the levels or the activity of steroidogenic enzymes in L β T2 cells which in turn could activate the GR, it was of interest to determine whether GnRH signalling to GR is GC independent. Thus, cells were transfected with a GRE-reporter plasmid and treated with dex and GnRH in the absence or presence of the anti-glucocorticoid RU486. As can be seen in figure 4.2 D the dex-induced increase in transcription was significantly decreased in the presence of RU486. In contrast the GnRH-induced transactivation was unaffected by RU486. The result provides evidence that the GnRH-mediated transactivation is independent of ligand binding to the GR in L β T2 cells.

Having established that GnRH mediates transactivation of a GRE by the endogenous unliganded GR it was next determined whether the response is dependent on endogenous GnRHR signalling. Therefore L β T2 cells were stimulated with dex or GnRH in the absence and presence of the GnRHR antagonist Antide. As shown in figure 4.2 E the dex-mediated upregulation of GRE activity was unaffected by the GnRHR antagonist. In contrast, GnRH-induced transactivation was completely abolished in the presence of Antide.

Taken together the results provide strong evidence for GnRH-mediated transactivation of a GRE by the endogenous unliganded GR and via the GnRHR in L β T2 cells.

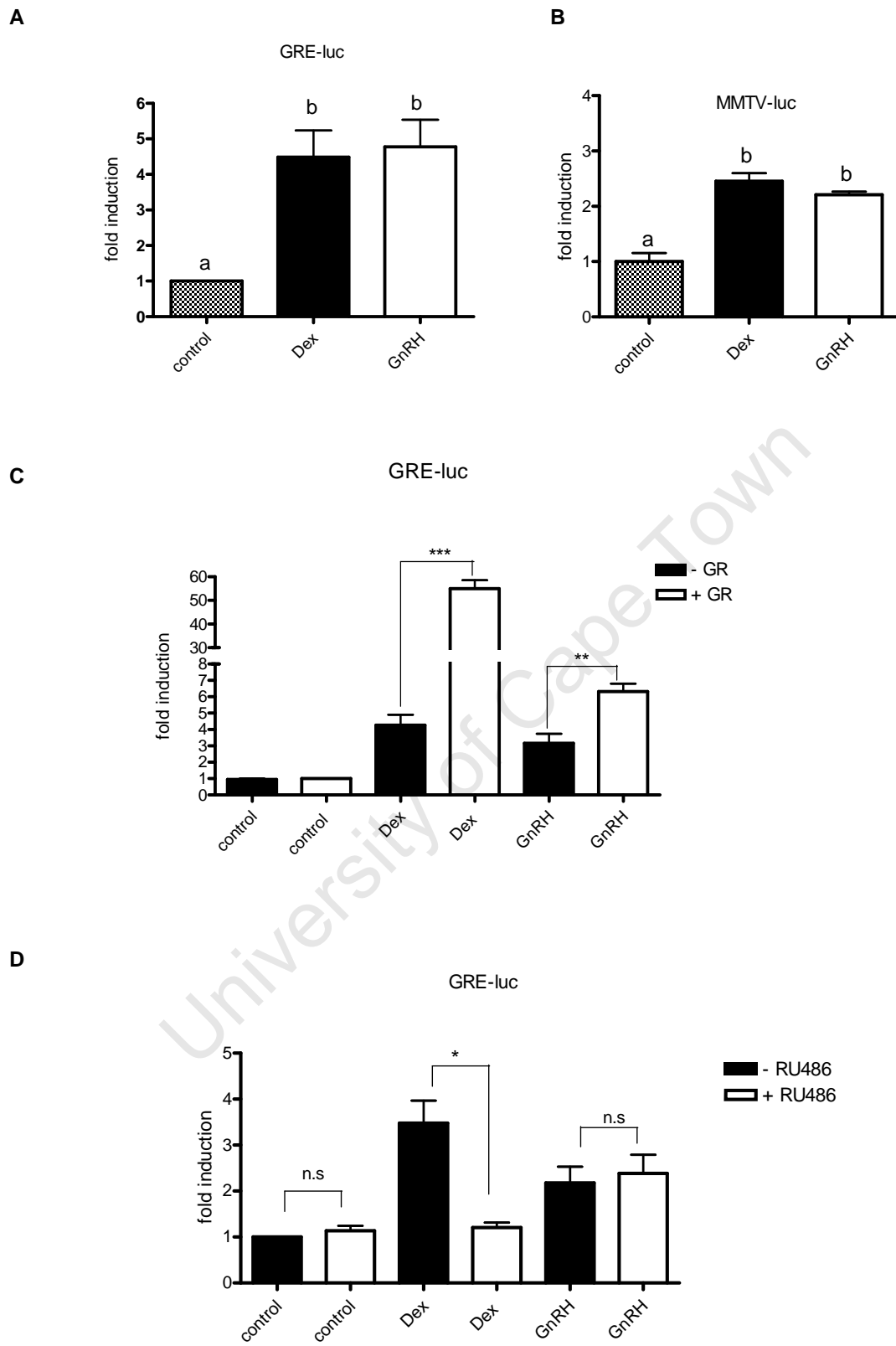


Figure 4.2: (continued on next page)

E

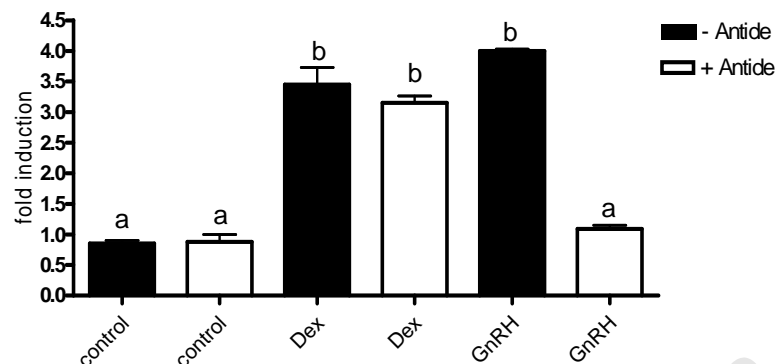


Figure 4.2: Activation of a GRE by GnRH is GR- and GnRHR-dependent

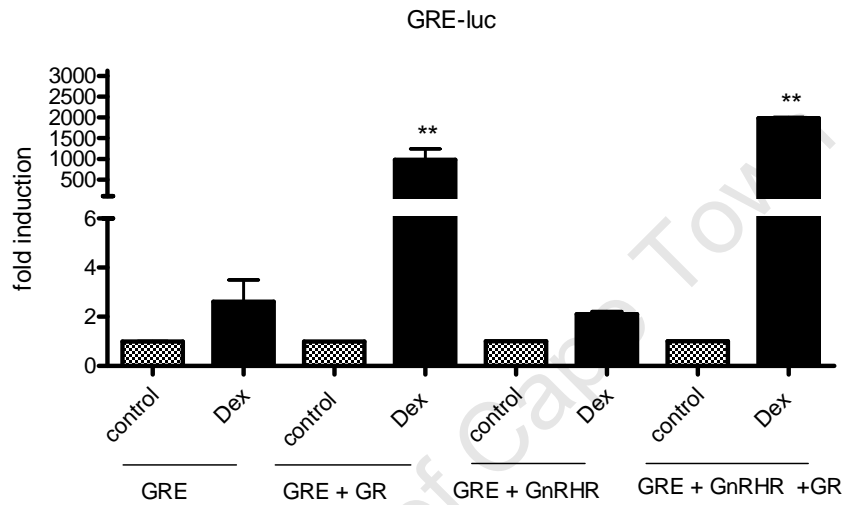
L β T2 cells were transfected with 250 ng TAT-GRE-luciferase construct (A,C-E) or with 250 ng MMTV-luciferase construct (B) in the absence (-GR) or presence (+GR) of cotransfection with 125 ng HA-human GR expression vector (C). After approximately 24 h cells were incubated for 8 h in serum-free medium with vehicle (control) or 100 nM GnRH or 100 nM dex, in the absence or presence of 100 nM RU486 (D) or 100 nM Antide (E). The luciferase activity was normalized to β -galactosidase and the hormone-stimulated increase in promoter activity was calculated relative to the vehicle control. The graphs show the promoter activity relative to vehicle (control) of combined results of at least 2 independent experiments, each performed in triplicate.

4.3.3 Ligand-independent activation of a GRE is dependent on both the GR and the GnRHR in COS-1 cells

To investigate whether ligand-independent activation of the GR is cell specific and to further establish a requirement for the GR and the GnRHR, the experiments in section 4.3.2 were repeated in COS-1 cells that do not express detectable levels of both GR and GnRHR (van Biljon, 2006). These cells were transfected with the GRE-luciferase construct in the absence and presence of expression vectors for the mouse GnRHR and the human GR. Over expression of these constructs was found not to affect basal luciferase activity. Significant dex-induced transactivation was only observed when COS-1 cells were co-transfected with a human GR expression construct (figure 4.3 A). Importantly, GnRH treatment only resulted in a significant increase in promoter activity in the presence of exogenous GR and GnRHR, further establishing that GnRH, via the GnRHR, is able to mediate GR-regulated gene expression (figure 4.3 B). Additionally, since ligand independent activation was found

to occur with exogenously expressed human GR and mouse GnRHR in COS-1 cells the result suggests that GnRH-mediated GR activation is not cell- or species-specific. Increased transactivation by GnRH was not due to changes in GR levels, since GnRH treatment had no effect on the total GR levels compared to vehicle control as determined by Western blotting in COS-1 cells (figure 4.3 C and D).

A



B

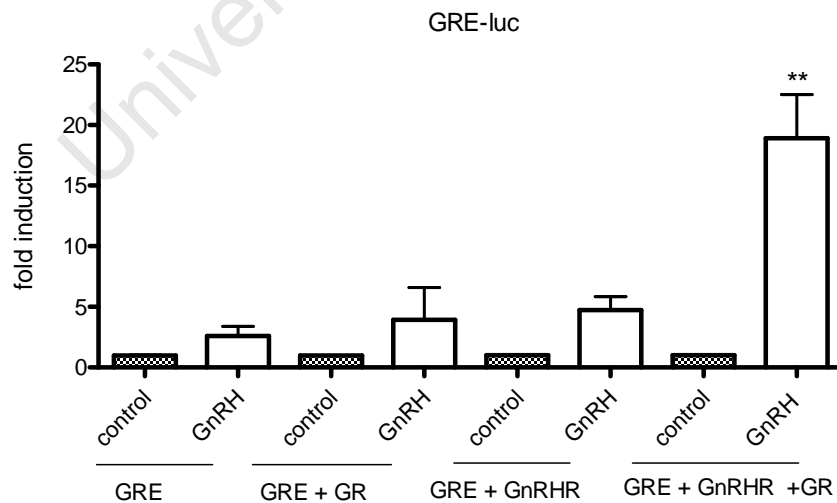


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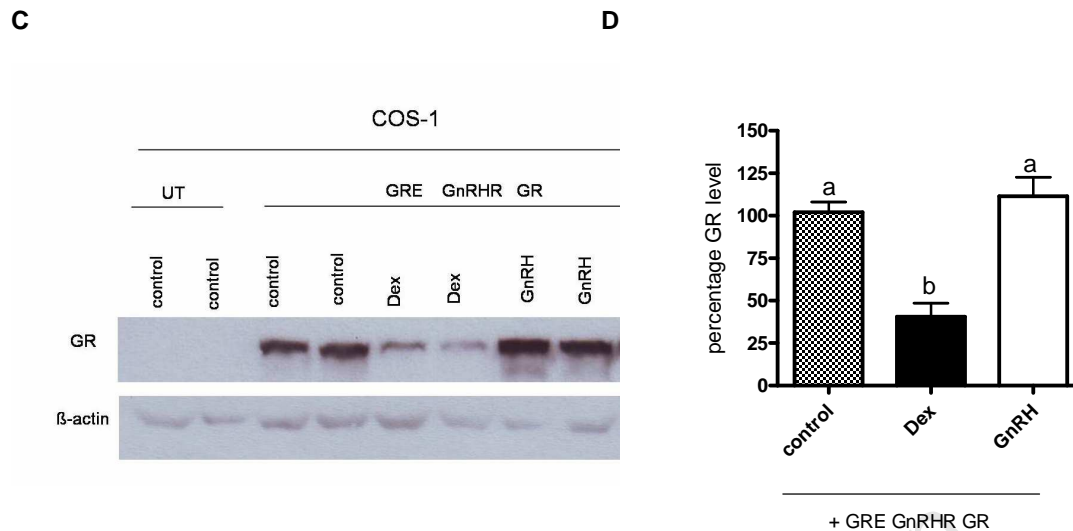


Figure 4.3 Ligand-independent activation of a GRE is dependent on both the GR and the GnRHR in COS-1 cells

A,B: COS-1 cells were transfected with 250 ng TAT-GRE-luciferase construct (GRE) in the absence or presence of cotransfection with 6 ng mouse GnRHR expression vector (GnRHR) and/or 125 ng HA-human GR expression vector (GR), as indicated below relevant bars. Transfected cells were incubated for 8 h in serum-free medium with vehicle (control) or 100 nM dex (A) or 100 nM GnRH (B). The luciferase activity was normalized to β -galactosidase and the hormone-stimulated increase in promoter activity was calculated relative to vehicle control. The graphs show the promoter activity relative to vehicle (control) of combined results of at least 2 independent experiments, each performed in triplicate. **C,D:** COS-1 cells were transfected with 250 ng TAT-GRE-luciferase construct (GRE) in the absence (UT) or presence of cotransfection with 6 ng mouse GnRHR expression vector (GnRHR) and/or 125 ng HA-human GR expression vector (GR) and serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex for 8 h. Equal amounts of cell lysates were loaded on a 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific anti-GR or anti- β -actin antibodies (C) The graph in D shows combined results of 3 independent experiments. The relative amounts of protein was normalized the β -actin and quantified with the value for control (vehicle) set to 100%.

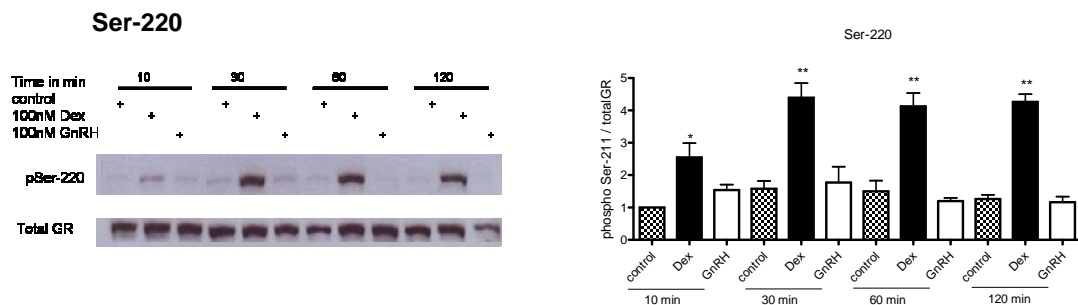
4.3.4 The GnRHR mediates site-specific phosphorylation of the unliganded GR by GnRH

Both Ser-220 and Ser-234 of the mouse GR (corresponding to Ser-211 and Ser-226, respectively, in the human GR) are highly conserved and hyperphosphorylated in response to GR agonist, suggesting a link between GR phosphorylation and GR transcriptional activity (Hoeck and Groner, 1990).

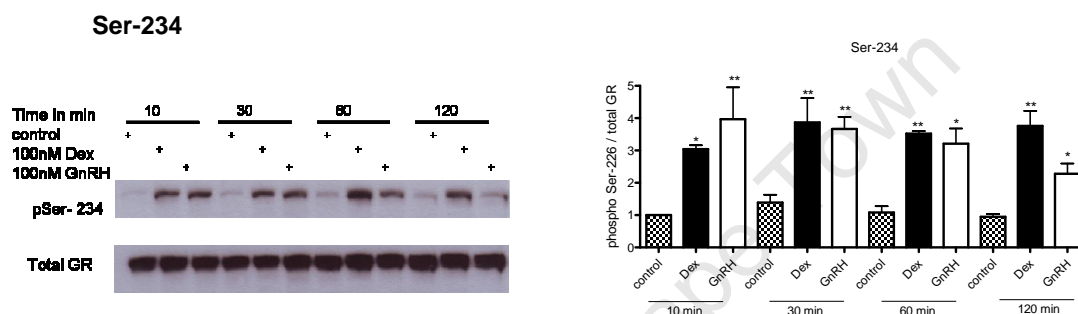
Furthermore, it has recently been shown that in COS-1 cells, dex-induced phosphorylation of the human GR at one or more of residues Ser-203/Ser-211/Ser-226 is required for maximal GR transactivation of a GRE and to facilitate GRIP-1 co-activator recruitment (Avenant, 2009). Thus, GnRH-induced phosphorylation of the GR in the absence of GCs may be a mechanism whereby GnRH activates the GR. To investigate this hypothesis, the phosphorylation status of endogenous GR was examined in LBT2 cells, in the absence or presence of either GnRH or dex, using specific anti-P-Ser-220-GR and anti-P-Ser-234-GR antibodies in Western blotting. The results demonstrate a rapid and sustained increase in phosphorylation at Ser-220 and Ser-234 in response to dex (figure 4.4 A and B). In contrast, GnRH treatment induced rapid and sustained phosphorylation at Ser-234 but not at Ser-220 (figure 4.4 A and B). Phosphorylation at Ser-220 or Ser-234 was not detectable in vehicle-treated cells, indicating that the observed phosphorylation is strictly hormone-dependent (figure 4.4 A and B). These results clearly demonstrate that GnRH selectively modifies the phosphorylation status of endogenous GR in LBT2 cells at a specific serine residue in the absence of a GR ligand.

In order to establish the involvement of the GnRHR in the GnRH-induced phosphorylation of the GR at Ser-234, cells were treated with the GnRHR antagonist Antide in the presence of both dex and GnRH. The results in figure 4.4 C clearly show that the GnRH-induced phosphorylation at Ser-234 was abolished in the presence of the GnRHR antagonist, indicating that the GnRHR is required for the hyperphosphorylation at that specific serine residue by GnRH. Furthermore, the dex-induced phosphorylation remained unchanged in the presence of Antide, demonstrating the specificity of the antagonist.

A



B



C

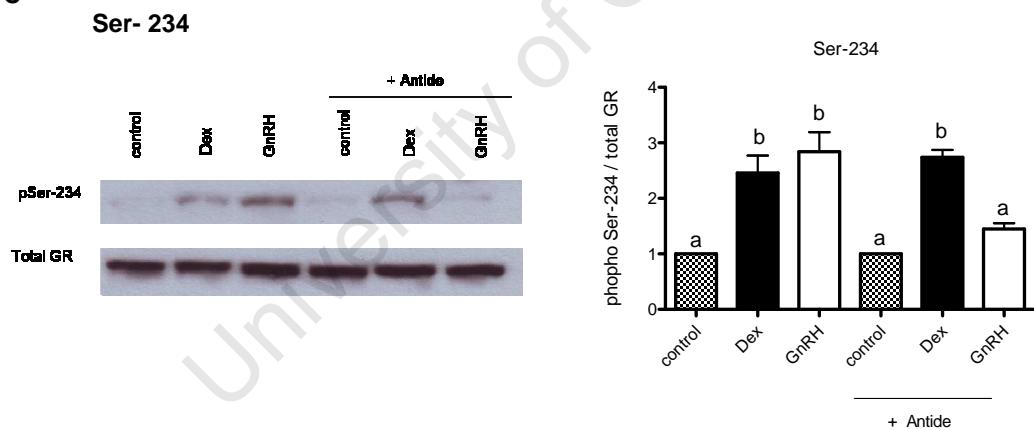


Figure 4.4: The GnRHR mediates site-specific phosphorylation of the unliganded GR by GnRH
 L β T2 cells were grown and serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex for the indicated times. Equal amounts of cell lysates were loaded on a 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific antibodies for GR phosphorylated at Ser-220 (A) or Ser-234 (B). The blots were stripped and re probed with an anti-GR antibody as control (Total GR). C: L β T2 were treated with hormone for 30 min in the presence or absence of 100 nM of the GnRHR antagonist Antide. The graphs show combined results of at least 3 independent experiments. The relative amounts of phosphorylation at the specific serine residues were quantified and expressed as phosphorylated GR content relative to total GR, with the value for control (vehicle) set as 1.

4.3.5 Activation of MAPKs by GnRH

4.3.5.1 GnRH but not dex rapidly activates c-Jun-N-terminal kinase (JNK) in L β T2 cells

In order to determine if JNK is activated by GnRH or dex, L β T2 cells were serum-starved over night and stimulated for the indicated times with 100 nM dex or 100 nM GnRH. The activity of the kinase was determined by using a phospho-specific JNK antibody detecting endogenous levels of phospho JNK1 (p46) and phospho JNK2/3 (p54) dually phosphorylated at threonine 183 and tyrosine 185. Western blot analysis revealed that treatment with 100 nM GnRH resulted in a rapid activation of endogenous JNK within 10 min, reaching its peak at 30 min and declining after 1 h of treatment (figure 4.5 A and B). This is in agreement with reports in the literature showing maximal induction of JNK with 100 nM GnRH at 30 min in L β T2 cells (Bonfil *et al.*, 2004; Liu *et al.*, 2002a). In addition when L β T2 cells were treated continuously for 8 h with 100 nM GnRH JNK was found to be in its inactive, unphosphorylated form as compared to the active, phosphorylated form found at 30 min (Addendum A figure A1). In contrast, phosphorylated JNK could not be detected at any time point investigated in dex-treated cells (up to 2 h figure 4.5 A and B or 8 h in Addendum A figure A1) indicating that dex stimulation does not activate the JNK signalling pathway in L β T2 cells. Furthermore as a control the total JNK protein levels were measured by Western blotting using a JNK specific antibody detecting JNK1 and JNK2/3. As shown in figure 4.5 C, neither GnRH nor dex treatment modulated the relative total JNK protein expression levels in L β T2 cells. This further underscores the specific activation of JNK by GnRH since only the phosphorylation status of the kinase and not the total protein levels were found to be affected.

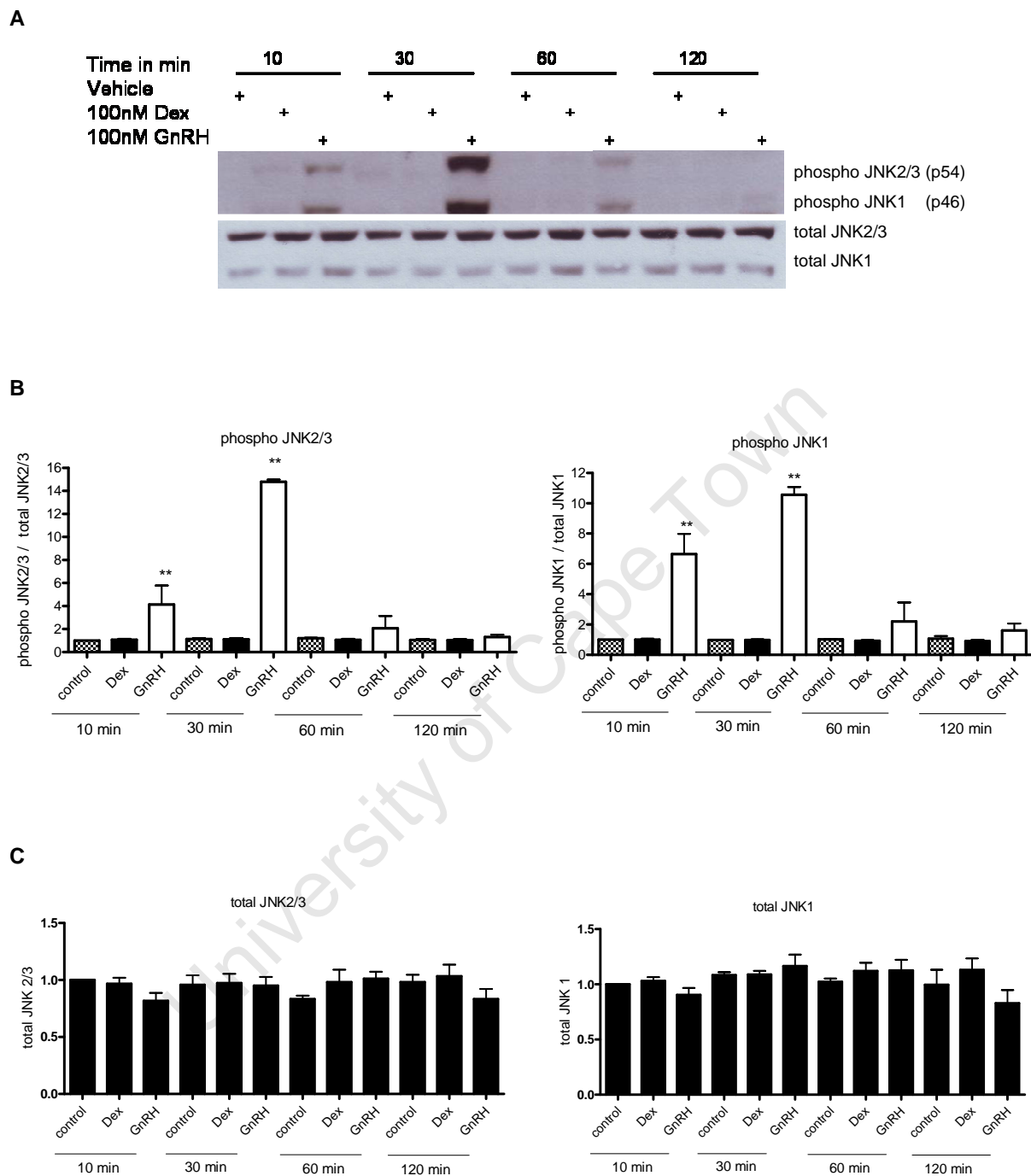


Figure 4.5: Activation of JNK by GnRH in L β T2 cells

A: L β T2 cells were treated with 100 nM dex or 100 nM GnRH in serum-free medium for the indicated times. Proteins were separated on 8% SDS-PAGE gel, electroblotted onto nitrocellulose membrane and probed with anti-phospho JNK antibody. The blots were stripped and reprobed with an anti-total JNK antibody as control. **B:** Pooled and quantified results of 3 independent experiments are presented

as relative amounts of phosphorylated kinase over total kinase for phospho JNK 1 (left) and phospho-JNK 2/3 (right). **C:** Relative kinase expression levels are shown for JNK 1 (right panel) and JNK 2/3 (left panel), with vehicle (control) at 10 min set as 1.

4.3.5.2 p38 is rapidly induced by GnRH and not dex in L β T2 cells

Next the activation of another member of the MAPK family, p38 was investigated. The same samples as in 5.3.1.1 were analyzed by means of Western blotting, using a phospho specific p38 antibody, detecting endogenous p38 proteins phosphorylated at threonine 180 and tyrosine 182. Similarly to the results obtained by others (Bonfil *et al.*, 2004; Liu *et al.*, 2002a), phosphorylated p38 was strongly induced in cells treated with 100 nM GnRH in L β T2 cells (figure 4.6 A and B). The peak of p38 activation was observed after 10 min of stimulation and was found to decrease after 1 h. After 2 h of GnRH treatment p38 phosphorylation was completely abolished in L β T2 cells. Total p38 protein levels remained unaffected in the presence of GnRH compared to unstimulated cells (vehicle control) (figure 4.6 C). As already observed for JNK (figure 4.5) dex failed to induce activation of p38 at any time point investigated (figure 4.6 and Addendum A figure A2).

A

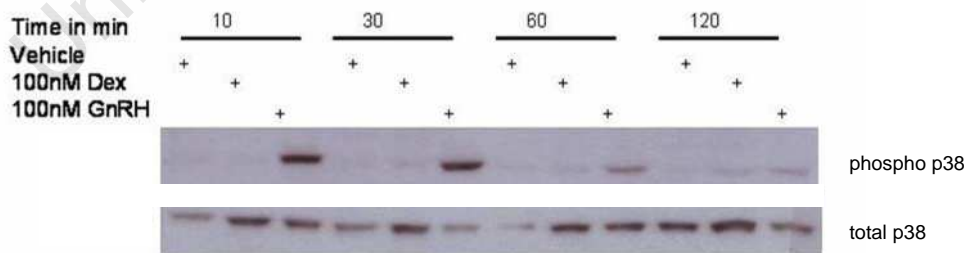


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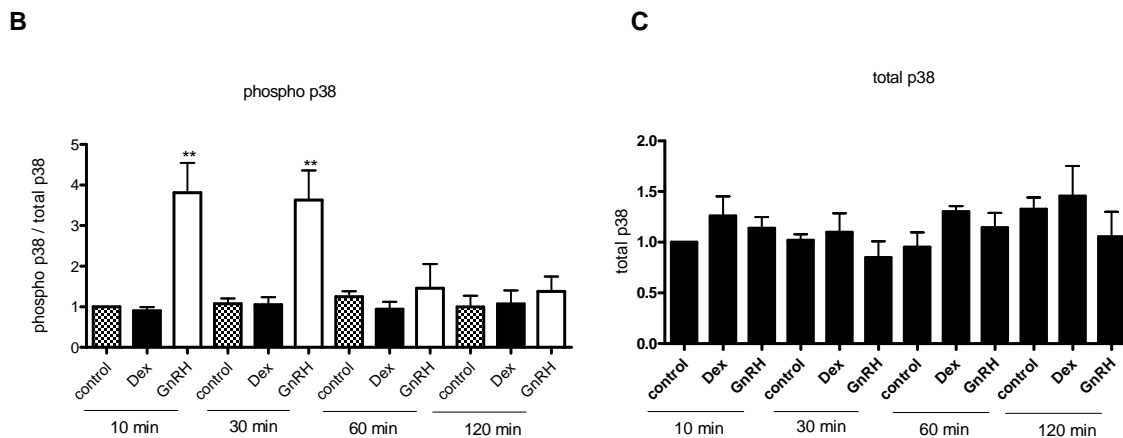


Figure 4.6: GnRH rapidly induces p38 activation in L β T2 cells

A: L β T2 cells lysates as prepared for figure 4.5 were analyzed by Western blotting using an anti-phospho p38 antibody. The blots were stripped and reprobed with anti p38 as control. **B:** Pooled and quantified results of 3 independent experiments are presented as relative amount of phosphorylated p38 over total p38. **C:** Total p38 expression levels are shown, with vehicle (control) at 10 min set as 1.

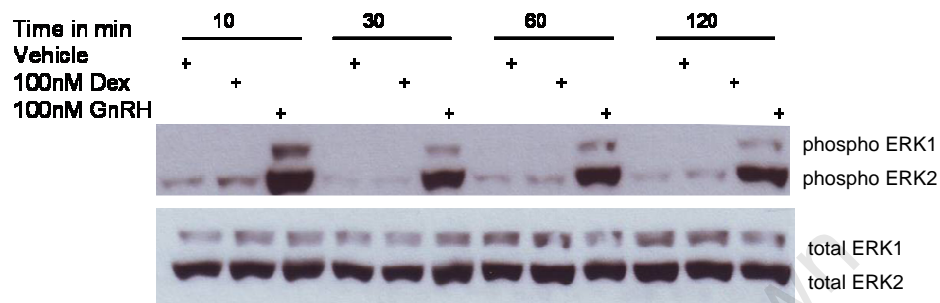
4.3.5.3 ERK is activated by short-term GnRH treatment while only long term dex stimulation results in ERK activation in L β T2 cells

ERK1 (p44) and ERK 2 (p42) were found to be strongly and rapidly activated in GnRH treated L β T2 cells after 10 min (figure 4.7 A and B). In contrast to results obtained with JNK and p38 (figure 4.5 and 4.6), ERK1/2 activation appears to be more sustained since the kinase is still significantly phosphorylated at 8 h of GnRH treatment (Addendum A figure A3). In untreated L β T2 cell lysates weak basal phosphorylation of ERK 2 (p42) was observed which reflects autophosphorylation at the tyrosine residues of the kinase (Sturgill *et al.*, 1988). The total ERK protein expression levels remained unchanged (figure 4.7 C).

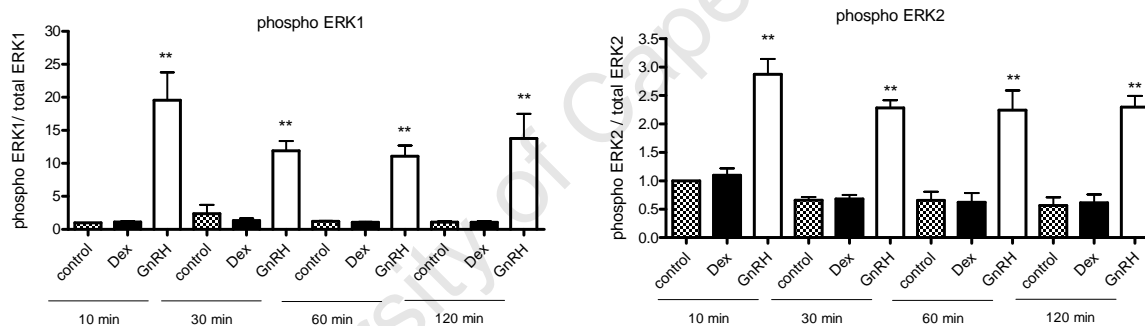
In dex-treated samples no ERK phosphorylation could be detected up until 2 h of continuous treatment (figure 4.7 A and B). However continuous stimulation of L β T2 cells with 100 nM dex for 8 h resulted in increased ERK1/2 phosphorylation while the total kinase levels remained unchanged (Addendum A figure A3). Activation of MAPKs including ERK occurs rapidly (Johnson and Lapadat, 2002) and since 8 h presents a rather long time point it is likely that phosphorylation of ERK1/2 is not a direct result of

dex stimulation. It is feasible that dex increases the levels or activity of upstream kinases such as MKK or other signalling molecules and pathways eventually leading to activation of ERK.

A



B



C

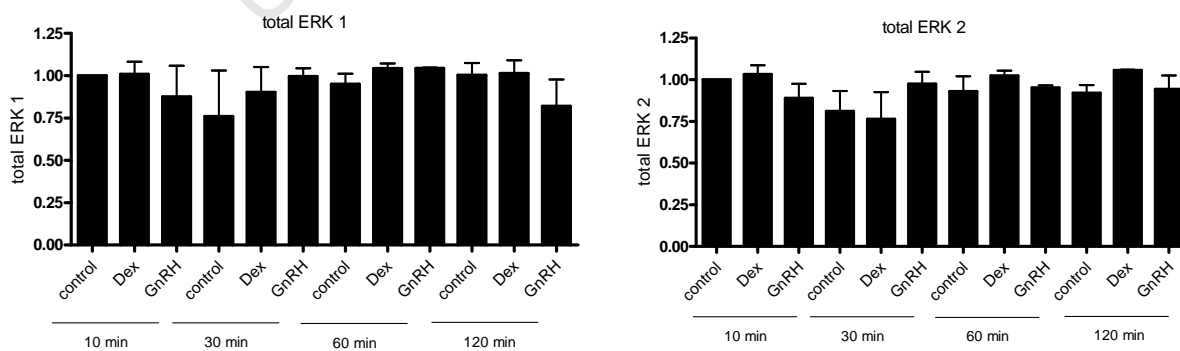


Figure 4.7: Activation of ERK1/2 by GnRH (legend to follow on next page)

A: L β T2 cells were treated as described for figure 4.5 and Western blotting performed using an anti-phospho-ERK1/2 antibody. The blots were stripped and reprobed with anti-ERK1/2 antibody as control. **B:** Pooled and quantified results of 3 independent experiments are presented as relative amount of phosphorylated kinase over total kinase on separate graphs for phospho-ERK 1 (left panel) and phospho-ERK 2 (right panel) **C:** Relative kinase expression levels are shown for ERK 1 (left panel) and ERK 2 (right panel), with vehicle (control) at 10 min set as 1.

4.3.6 Role of kinases in dex- and GnRH-mediated transactivation and GR phosphorylation

4.3.6.1 MAPKs are involved in GnRH- and dex-mediated transactivation of the GR in L β T2 cells

Having established that the MAPK JNK, p38 and ERK are expressed and activated by GnRH in L β T2 cells, their possible involvement in the dex- or GnRH-mediated GR activation was next investigated. L β T2 cells transfected with the GRE-luciferase construct were stimulated with 100 nM GnRH or 100 nM dex, in the absence or presence of selective kinase inhibitors (figure 4.8). Dex-induced transactivation of the reporter gene was significantly increased by approximately 3.5 fold in the presence of the JNK inhibitor while inhibition of p38 appeared to slightly decrease the response, although statistical significance was not established (figure 4.8 A). The results are difficult to interpret since ERK but not p38 or JNK appeared to be activated directly or indirectly at 8 h in response to dex (Addendum A figures A1-3). Thus, it is likely that p38 and JNK are activated by dex at a time point that has not been investigated in the present study. The GnRH-mediated transcriptional activity of the luciferase construct was significantly decreased in the presence of ERK pathway inhibitor (~50%) and p38 inhibitor (~30%) which is in agreement with findings presented in figure 4.6 and 4.7 showing strong induction of these kinases by GnRH. By contrast, GnRH-mediated transactivation was significantly increased by approximately 2 fold when cells were co-treated with JNK inhibitor (figure 4.8 B).

The increase in transcription in the presence of the JNK inhibitor in response to both dex and GnRH was unexpected and suggests that activated JNK displays an inhibitory effect on GR-mediated transactivation. To further investigate this hypothesis, a dose-response experiment was performed with increasing concentration of JNK inhibitor varying from 1 μ M to 10 μ M (figure 4.8 C and D). As shown in figure 4.8 C in the presence of 1 μ M JNK inhibitor dex-mediated transactivation was

significantly increased compared to dex alone. Increasing amounts of JNK inhibitor resulted in a further increase in GRE-reporter activity. Similarly, the presence of 5 μ M JNK inhibitor significantly increased GnRH-mediated transactivation (figure 4.8 C). Importantly, the JNK inhibitor did not significantly increase basal transcriptional activity of the reporter construct (figure 4.8 C and D), indicating that the increased responses are not due to a general activation of the basal transcription machinery. The results strongly support the hypothesis that the active form of JNK exhibits an inhibitory effect on transactivation in this system. By Western blotting it was shown that the JNK inhibitor is efficient and specific since GnRH-induced JNK phosphorylation was completely abolished in the presence of 10 μ M JNK inhibitor, while it had no effect on GnRH-mediated activation of ERK or p38 (Addendum A figure A4D, A5D and A6D).

Take together these data highlight both similarities and differences in the kinase pathways involved in GR-mediated transactivation of a GRE-reporter construct via dex and GnRH. JNK inhibits while p38 seems to stimulate both dex- and GnRH-mediated transactivation. Interestingly ERK appears to be involved in GnRH-mediated transactivation but has no effect on the dex response.

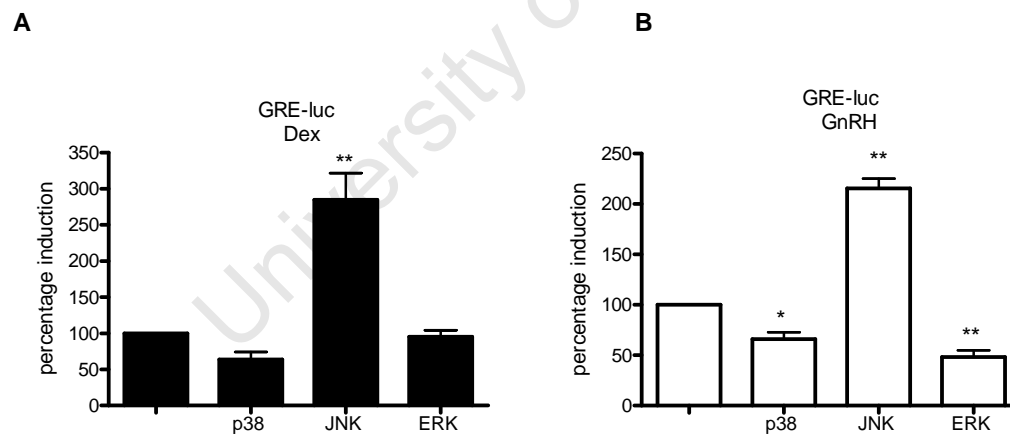


Figure 4.8: (continued on next page)

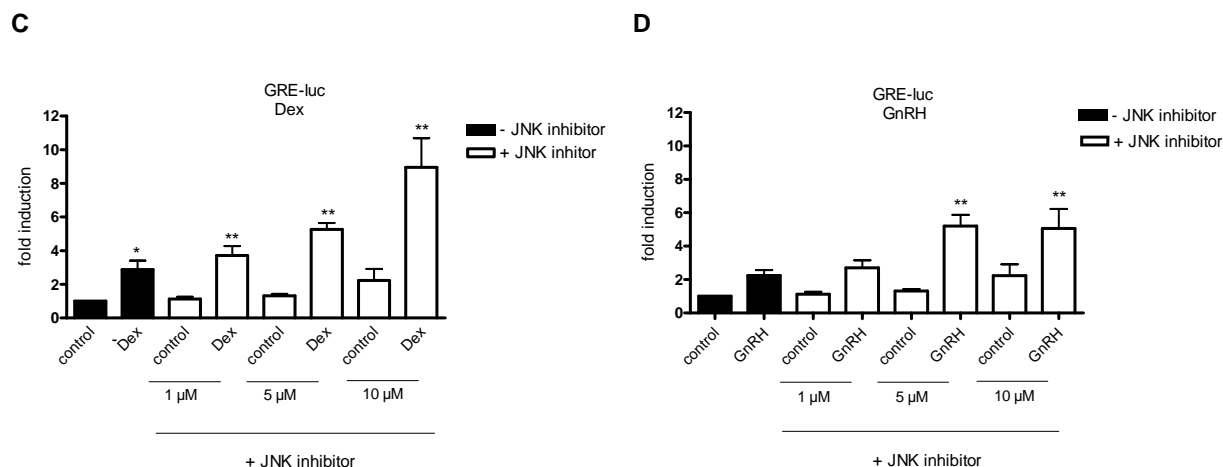


Figure 4.8: MAPKs are involved in GnRH- and dex-mediated transactivation of the GR in LBT2 cells

A,B: LBT2 cells were transfected with 250 ng TAT-GRE-luciferase construct and 24 h later stimulated with 100 nM dex (left panel) or 100 nM GnRH (right panel) for 8 h in serum-free medium in the absence or presence of the following inhibitors (A and B): 10 μM p38 inhibitor (SB203580), 10 μM JNK inhibitor (SP600125) or 50 μM ERK pathway inhibitor (PD98059). The graph shows pooled results of 3 independent experiments carried out in triplicates, where the promoter activity is expressed as percentage of induction relative to dex (A) or GnRH (B) which were set to 100%. **C,D:** LBT2 cells were transfected as described above and stimulated with 100 nM Dex or 100 nM GnRH in the absence or presence of increasing concentrations of JNK inhibitor as indicated below the relevant bars. The hormone-stimulated increase in GRE-promoter activity was calculated relative to vehicle control. The graphs show combined results of at least 3 independent experiments, each performed in triplicate.

4.3.6.2 MAPK are involved in GnRH-mediated AP-1 transactivation

It was next thought to investigate the possible involvement of the MAPKs JNK, p38 and ERK in GnRH-mediated AP-1 transactivation shown in figure 3.2. Therefore, similar experiments as in section 4.3.6.1 were carried out in LBT2 cells to determine the requirement of several MAPKs in activation on an AP-1 promoter construct. The GnRH-mediated transcriptional activity of the luciferase construct was significantly decreased in the presence of ERK pathway inhibitor to approximately 20% and in the presence of the p38 inhibitor to about 50% (figure 4.9). In addition, a significant increase in AP-1 promoter activity by approximately 2 fold was observed when cells were co-treated with JNK inhibitor (figure 4.9). The result is similar to the increased reporter activity seen for the GRE-promoter construct in the presence of GnRH and JNK inhibitor (compare with figure 4.8 B) and suggests an inhibitory role for JNK in GRE and AP-1 transactivation. This was an unexpected finding. Since the AP-1 binding

protein c-Jun is a known substrate for JNK (Smeal *et al.*, 1991) and GnRH has been shown to increase JNK activity in L β T2 cells (figure 4.5) it was anticipated that the JNK inhibitor would have an inhibitory effect on GnRH-mediated AP-1 transactivation. However, the present data suggest that JNK has a negative effect on activation of an AP-1 reporter construct and that a novel mechanism might be involved in AP-1 transactivation in L β T2 cells.

Given that the endogenous GnRHR promoter contains an AP-1 site and that this promoter element has been shown to be important in GnRH-induced gene expression (figure 3.2, Addendum B figure B2 B and figure 3.4) it can be speculated that the MAPKs p38, ERK and JNK are important pathways involved in a positive and a negative fashion in transcriptional regulation of the GnRHR in L β T2 cells.

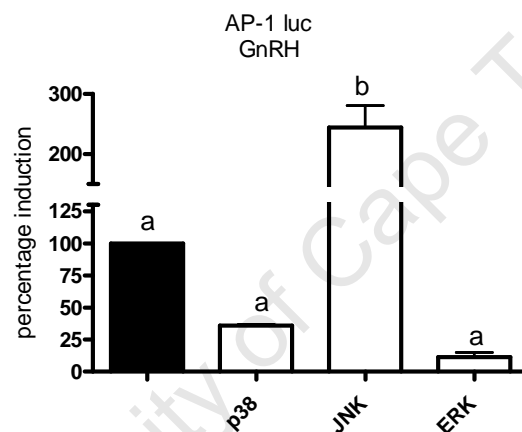


Figure 4.9: MAPKs are involved in GnRH-induced transactivation of a pAP-1 construct in L β T2 cells

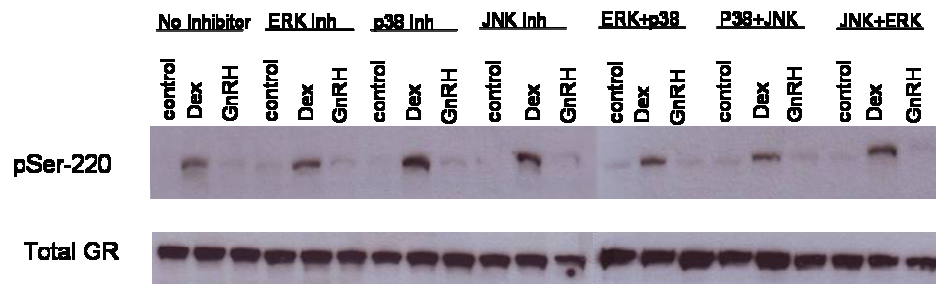
L β T2 cells were transfected with 250 ng pAP-1-luc construct and 24 h later stimulated with 100 nM GnRH in serum-free medium in the presence of MAPK inhibitors as described in figure 4.8. The luciferase activity was normalized to β -galactosidase activity. The graph shows pooled results of 3 independent experiments carried out in triplicates, where the promoter activity is expressed as percentage of induction relative to GnRH which was set as 100%. Please note that the GnRH-mediated AP-1 transactivation is shown in figure 3.2.

4.3.6.3 GnRH-stimulated phosphorylation of the unliganded GR at Ser-234 is mediated by a combination of JNK, p38 and ERK

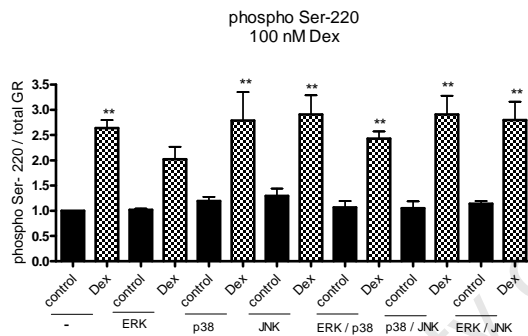
Having shown in figure 4.4 A and B that both dex and GnRH induce site-specific GR phosphorylation it was next investigated which MAPKs are required for GnRH-stimulated phosphorylation of the endogenous GR. Several individual MAPK inhibitors failed to completely abolish dex- or GnRH-

stimulated transactivation of a GRE (figure 4.8 A), indicating that more than one kinase may be required to act in parallel to mediate these effects. In order to further investigate this, the effect of different MAPK kinase inhibitors was determined, individually or in combination, on dex induced Ser-220 as well as on GnRH- and dex-induced Ser-234 phosphorylation of endogenous GR in L β T2 cells. The result showed a significant decrease in Ser-220 phosphorylation in response to dex in the presence of a ERK pathway inhibitor while p38, JNK or combinations of the inhibitors did not effect phosphorylation at that specific site (figure 4.10 A and B). As found previously (figure 4.4 A) GnRH treatment did not result in increased phosphorylation at Ser-220 (figure 4.10 A and C). The total GR protein levels remained unaffected by any MAPK inhibitor (figure 4.10 D and H). Furthermore, the results presented in figures 4.10 E and F show that inhibition of the individual kinases ERK, p38 or JNK had no influence on the dex-stimulated phosphorylation of Ser-234. However, treatment with dex in the presence of both p38 and JNK inhibitors, or with both JNK and ERK pathway inhibitors, lead to a small but statistically significant decrease in GR phosphorylation at that specific site (figure 4.10 E and F). GnRH-stimulated phosphorylation at Ser-234 was similarly not influenced by inhibition of p38 or JNK alone (figure 4.10 E and G). However, inhibition of ERK alone did cause a significant decrease in phosphorylation of the GR, consistent with the results for GnRH-induced transactivation (figure 4.10 E and G). These results strongly support a role for ERK in GnRH-mediated phosphorylation of the unliganded GR. Interestingly; combinations of any two MAPK inhibitors did result in about 30 - 45% inhibition of Ser-234 phosphorylation in response to GnRH. This suggests that in addition to ERK, both JNK and p38 kinases cooperate in a complex interplay to mediate GnRH-induced phosphorylation of the unliganded GR in L β T2 cells. Taken together, these results suggest that dex- and GnRH- induced GR phosphorylation at Ser-234 involves a combination of the MAPKs JNK, p38 and ERK most likely involving a complex interplay of kinase events.

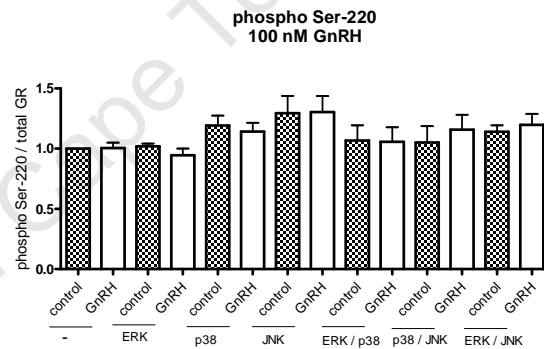
A



B



C



D

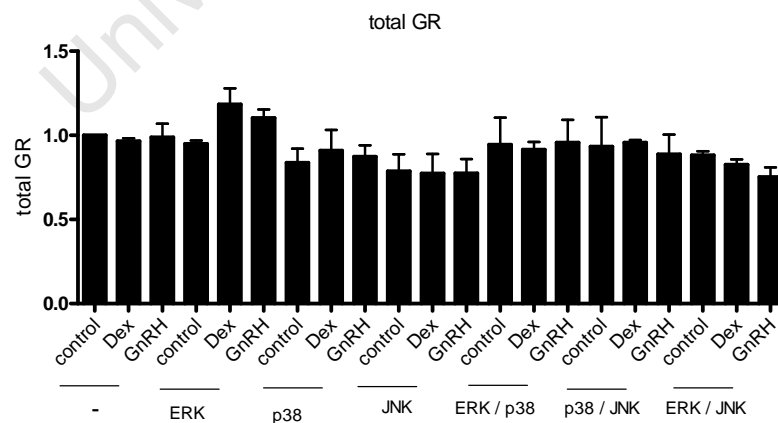
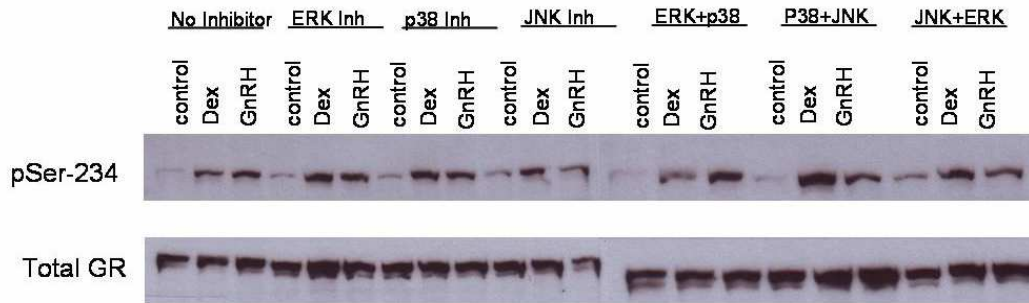
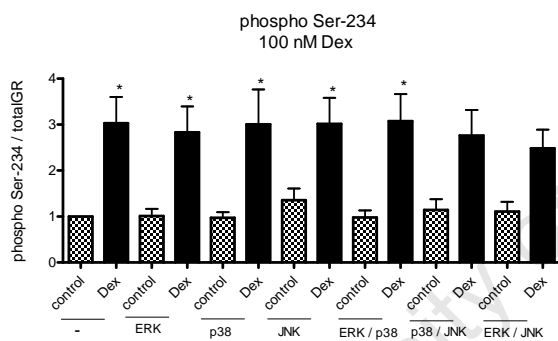


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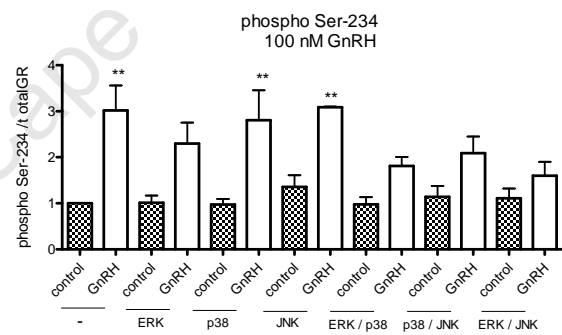
E



F



G



H

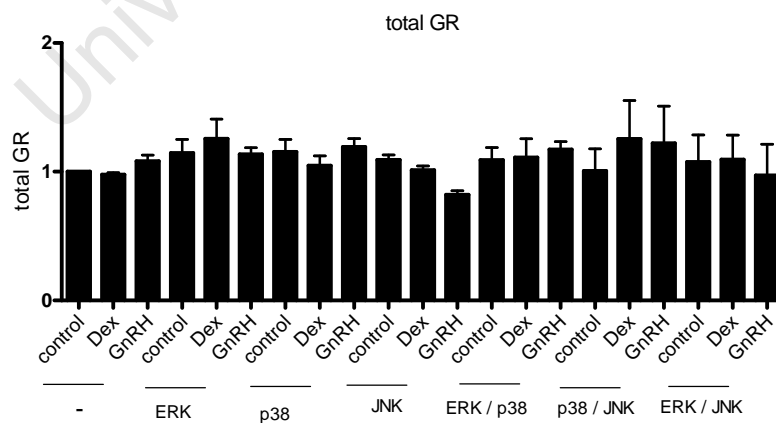


Figure 4.10: Several MAPKs are involved in site specific phosphorylation of the liganded and unliganded GR at Ser-234 in LBT2 cells (legend to follow on next page)

L β T2 were stimulated for 30 min with 100 nM GnRH or 100 nM dex in the absence or presence of the following inhibitors: 10 μ M JNK inhibitor (SP600125), 10 μ M p38 inhibitor (SB203580), 50 μ M ERK pathway inhibitor (PD98059) or a combination thereof as indicated. Western blotting was performed using an anti-GR phospho Ser-220 (A) or an anti-GR phospho Ser-234 antibody (E). The blots were stripped and reprobbed with anti-GR antibody as control (A and E). The combined results from three independent experiments for dex (B and F) and GnRH (C and G) are presented on separate graphs, where phospho GR levels were normalized to total GR, with the value for control (vehicle) set as 1. The total GR protein levels are shown on separate graphs with vehicle (control) set as 1 (D and H).

4.3.6.4 PKC and c-src are involved in GnRH-mediated transactivation

Several groups in the field have studied kinase pathways in gonadotrope cells including L β T2 cells as described in chapter 1 (section 1.14). Since PKC, PKA (Bonfil *et al.*, 2004; Liu *et al.*, 2002a; Liu *et al.*, 2003; Mulvaney *et al.*, 1999; Roberson *et al.*, 1999; Sundaresan *et al.*, 1996) and c-src (Benard *et al.*, 2001; Levi *et al.*, 1998) have been implicated in GnRH-mediated activation of MAPKs it was interesting to examine next whether PKA, PKC or c-src participate in dex- and GnRH-mediated transcriptional activation in L β T2 cells. Please note that the specificity of the PKC-, PKA -, or c-src inhibitor was not determined in the present study.

The stimulatory effect of GnRH on GRE-transactivation was reduced (~40%) by the specific PKC inhibitor BIM (figure 4.11 B) and the tyrosine kinase inhibitor PP2 (figure 4.11 D) while no statistically significant effect was seen for dex-mediated transcriptional activation of the reporter in the presence of these inhibitors (figure 4.11 A and C). Inhibition of PKA did not significantly affect transactivation induced by dex or by GnRH (figure 4.11 A and B). Due to the lack of a positive control for the tyrosine kinase inhibitor PP2, the PKA inhibitor (H89) or the PKC inhibitor (BIM) the efficiency of these inhibitors was not determined. However, together with results in figure 4.8 B and reports in the literature (Benard *et al.*, 2001; Sundaresan *et al.*, 1996) the results would be consistent with a mechanism that possibly involves PKC- and c-src-dependent activation of ERK by GnRH in L β T2 cells.

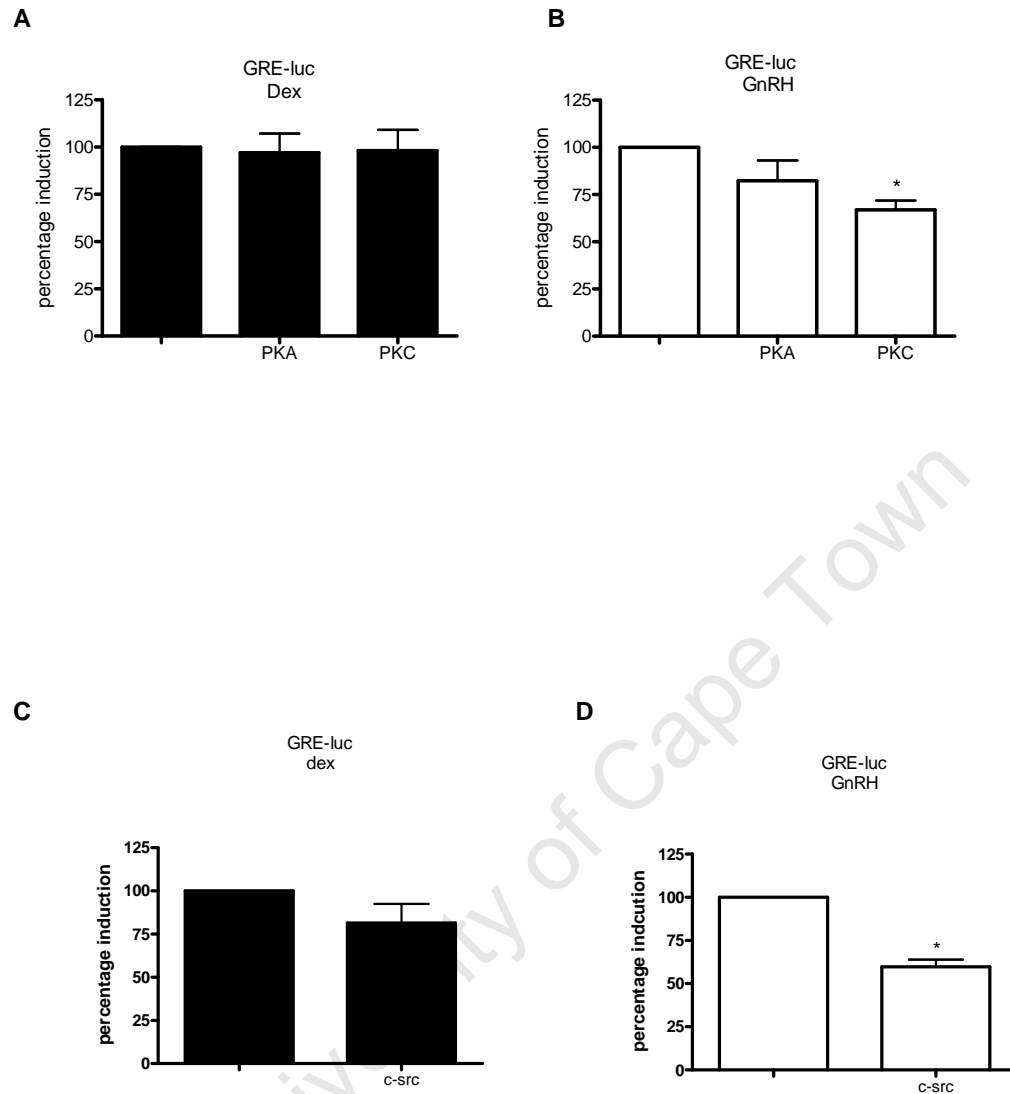


Figure 4.11: PKC and c-src are involved in GnRH-mediated transactivation

A-D: L β T2 cells were transfected with 250 ng TAT-GRE-luciferase construct and 24 h later stimulated with 100 nM dex (left panel) or 100 nM GnRH (right panel) for 8 h in serum-free medium in the absence or presence of the following inhibitors (A and B): 1 μ M PKA inhibitor (H89), 100 nM PKC inhibitor (BIM), or 100 μ M c-src inhibitor (PP2) (C and D). The graphs show combined results of at least 2 independent experiments carried out in triplicate, where the luciferase activity was normalized to β -galactosidase and is expressed as percentage of induction relative to dex (A and C) or GnRH (B and D), set at 100%.

4.3.6.5 PKC and c-src are involved in AP-1 transactivation by GnRH

To examine the effect of PKC, PKA and c-src on AP-1 reporter gene activity, similar experiments as presented in section 4.3.6.4 were carried out in L β T2 cells transfected with an AP-1 reporter construct. As shown in figure 4.12 the GnRH-induced activity of the reporter gene was significantly reduced in the presence of the PKC- or c-src inhibitor while inhibition of PKA appeared to have no effect (figure 4.12). These results further support the hypothesis that PKC and c-src play an important role in GnRH-mediated signalling in L β T2 cells.

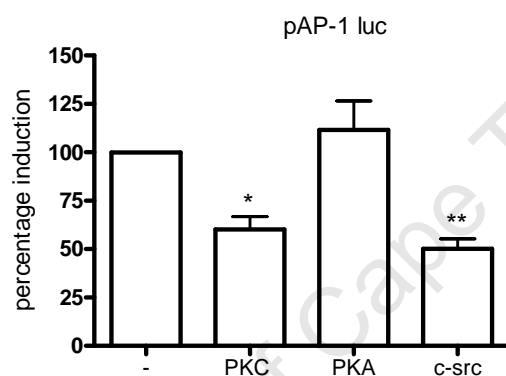


Figure 4.12: PKC and c-src are involved in AP-1 transactivation by GnRH

L β T2 cells transfected with 250 ng pAP-1 luc construct were stimulated with 100 nM GnRH in serum-free medium in the presence of 1 μ M PKA inhibitor (H89), 100 nM PKC inhibitor (BIM), or 100 μ M c-src inhibitor (PP2). The luciferase activity was normalized to β -galactosidase activity. The graph shows pooled results of 3 independent experiments carried out in triplicates, where the promoter activity is expressed as percentage of induction relative to GnRH set at 100%. Please note that the GnRH-mediated AP-1 transactivation is shown in figure 3.2.

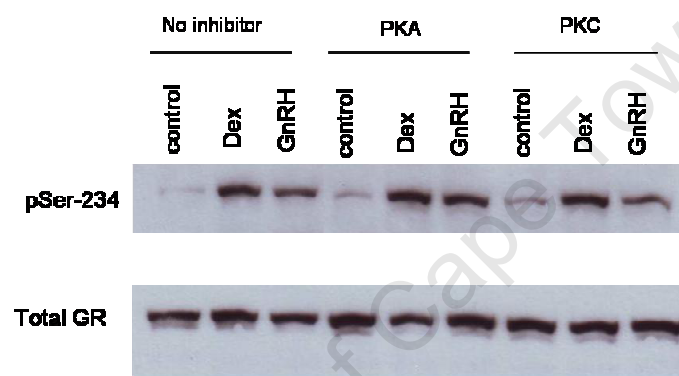
4.3.6.6 PKC is involved in GnRH-mediated GR phosphorylation of Ser-234

In the next set of experiments the role of PKC, PKA and c-src in GnRH-induced GR phosphorylation was investigated in L β T2 cells. Therefore L β T2 cells were stimulated with 100 nM dex or 100 nM GnRH in the presence or absence of PKC, PKA or c-src inhibitors and the phosphorylation status of the GR was analyzed by Western blotting. For these experiments only phosphorylation at Ser-234 of the GR was investigated since GnRH failed to induce phosphorylation at Ser-220 (figure 4.4 A).

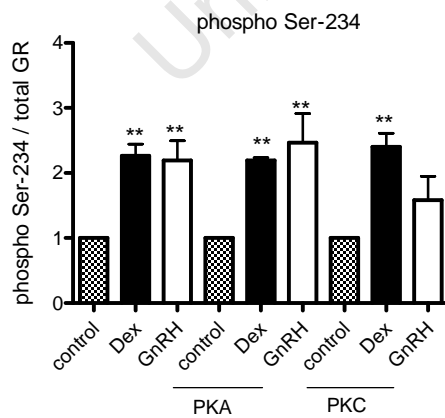
The results presented in figure 4.13 A and B suggest that PKA is not required for dex- or GnRH-induced Ser-234 phosphorylation since a PKA inhibitor did not effect the extent of phosphorylation at

that site. By contrast, inhibition of PKC resulted in a significant decrease for GnRH-, but not dex-induced phosphorylation at Ser-234 (figure 4.13 A and B). These results are consistent with the results obtained for GnRH-induced transactivation of the GRE-reporter construct (figure 4.13 B) where PKC but not PKA was found to inhibit GnRH-mediated transactivation. Inhibition of c-src had no effect on both dex- or GnRH-induced phosphorylation at Ser-234 (figure 4.13 D and E), although it was found that c-src inhibitor decreased GnRH-mediated GRE activation (figure 4.11 D). Total GR protein expression levels were found to be unaffected by PKA, PKC or c-src inhibitors (figure 4.13 C and F).

A



B



C

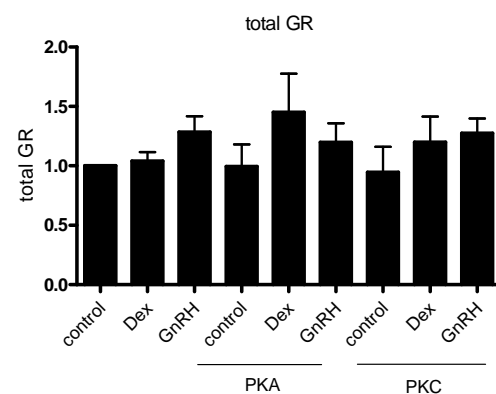
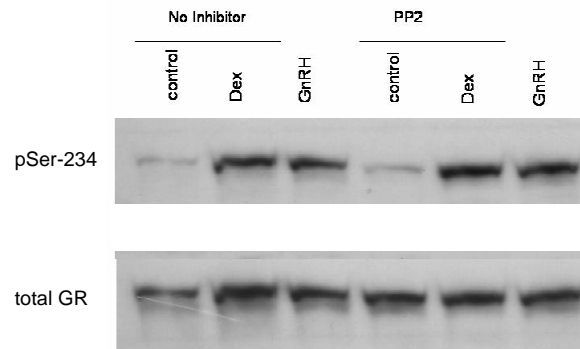
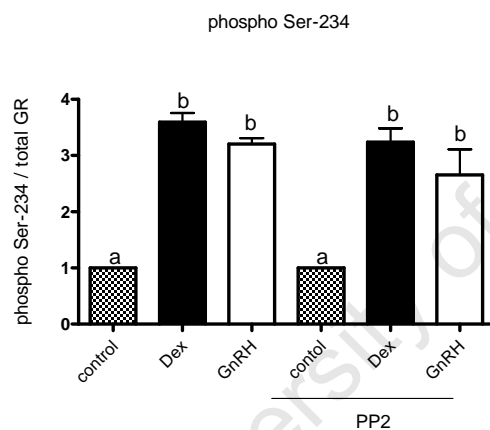


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D



E



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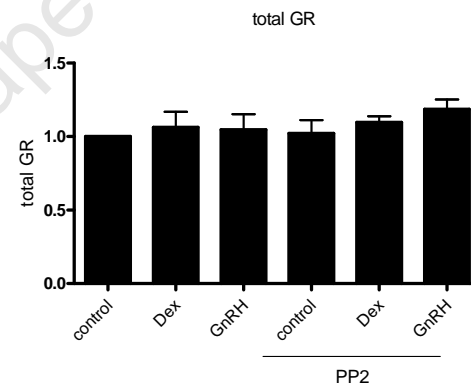


Figure 4.13: PKC is involved in GnRH-mediated GR phosphorylation of Ser-234

A-F: L β T2 were stimulated for 30 minutes with vehicle (control), 100 nM GnRH or 100 nM dex in the absence or presence of 1 μ M PKA inhibitor (H89), 100 nM PKC inhibitor (BIM) (A-C) or 100 μ M c-src inhibitor (PP2) (D-E) as indicated. Proteins were separated on an 8% SDS-PAGE gel, electroblotted onto a nitrocellulose membrane and probed with a phospho GR Ser-234 antibody. The blots were stripped and reprobbed with anti-GR antibody as a control. Representative Western blot are shown in A and D. The graphs in B and E show combined results of 3 independent experiments where levels of GR phosphorylated at Ser-234 were normalized to total GR levels with the value for control (vehicle) set to 1. The total GR expression levels are presented on separate graphs with vehicle (control) set as 1 (C and F).

CHAPTER 5

RESULTS

AN INVESTIGATION INTO THE SYNERGISTIC EFFECT OF DEX AND GnRH IN L β T2 CELLS

5.1 BACKGROUND

In the literature it has been reported that GnRH acts synergistically with activin, estradiol and DHT (Kowase *et al.*, 2007; Norwitz *et al.*, 2002a; Norwitz *et al.*, 2002b; Yasin *et al.*, 1995). Activin A was shown to augment GnRH-mediated transcriptional activation of the mouse GnRHR gene in α T3-1 cells (Norwitz *et al.*, 2002a; Norwitz *et al.*, 2002b) as well as the rat (Gregory *et al.*, 2005) and human (Wang *et al.*, 2008) FSH β gene in L β T2 cells. It was further suggested that the synergism between Activin A and GnRH is mediated via AP-1 (Jun/Fos) and SMAD proteins acting at the conserved AP-1 element of the FSH β promoter in L β T2 cells (Gregory *et al.*, 2005; Wang *et al.*, 2008). Furthermore, GnRH and estradiol were shown to synergistically upregulate LH β and α -subunit promoters and endogenous mRNA levels in L β T2 cells (Kowase *et al.*, 2007). Kowase *et al.* postulated that the mechanism of the synergistic effect of estradiol in part involves transcriptional regulation of both activators and suppressors of transcription (Kowase *et al.*, 2007).

GCs have also been found to result in synergistic effects in combination with other hormones in rat pituitary cells and other cell lines (D'Agostino *et al.*, 1990; Leal *et al.*, 2003; McGillivray *et al.*, 2007; Rosen *et al.*, 1991). GCs were found to synergistically augment the activin-induced increase in FSH β gene expression in both primary rodent pituitary cells (Leal *et al.*, 2003) and in L β T2 cells (McGillivray *et al.*, 2007). In contrast, the presence of both activin and dex reduced the dex-induced transactivation of a transfected human glycoprotein hormone α -subunit (α GSU) luciferase reporter plasmid in L β T2 cells (Sasson *et al.*, 2008). Sasson *et al.* further report that co-treatment with dex and GnRH resulted in a synergistic increase in α GSU promoter activity. Whether these synergistic effects occur on endogenous genes and the precise mechanisms thereof remain to be determined.

5.2 AIMS

In the present study it was found that both dex and GnRH increase GnRHR gene expression in L β T2 cells to a similar extent (figure 3.1). In addition, it could be established that both dex and GnRH require an intact AP-1 promoter element bound by c-Jun and c-Fos (figure 3.2 and figure 3.4 A and B), induce recruitment of the GR to the AP-1 region on the endogenous GnRHR promoter in intact cells (figure 3.6) and that the GR appears to be an essential requirement in both dex- and GnRH-mediated transcriptional regulation of the GnRHR (figure 3.8 C). These results demonstrate that both hormones utilize a similar mechanism to induce transcription of the GnRHR and thus, it was important to determine the effect on gene transcription when cells were co-stimulated both with dex and GnRH.

The detailed aims of this study were the following:

- To determine whether stimulation with dex plus GnRH results in a synergistic increase in expression of the endogenous GnRHR gene and a transfected GRE-promoter construct in L β T2 cells and COS-1 cells;
- To establish a requirement for the GR and the GnRHR for synergism with dex plus GnRH in both cell lines;
- To investigate the dex plus GnRH-mediated phosphorylation status of the endogenous GR and examine the role of MAPKs, PKA, PKC and c-src kinase pathways involved in GR phosphorylation in L β T2 cells in response to dex plus GnRH;
- To determine which proteins are recruited to the AP-1 region of the endogenous GnRHR promoter in intact cells in response to dex plus GnRH as compared to these hormones alone in L β T2 cells

5.3 RESULTS

5.3.1 GnRH and dex synergistically increase gene expression

5.3.1.1 GnRH and dex synergistically increase expression of the endogenous mouse GnRHR gene and a transfected GRE in a GR- and GnRHR-dependent fashion

In order to investigate the effects of co-incubation with both dex and GnRH on gene expression, L β T2 cells were stimulated with 100 nM dex, 100 nM GnRH or both dex plus GnRH for 8 h in serum-free medium. Total RNA was isolated, reverse transcribed and relative levels of GnRHR transcripts were determined by quantitative real-time PCR. As shown in figure 5.1 A, co-incubation with GnRH plus dex resulted in a dramatic synergistic increase in expression of the endogenous GnRHR gene compared to dex or GnRH alone. As already mentioned above, the GR was shown in chapter 3 to play an important role in dex- and GnRH-induced transcription of the GnRHR. Thus, using specific mouse GR siRNA, it was established that the synergistic effect on GnRHR gene expression was also dependent on the presence of the GR since GnRHR expression levels were significantly reduced by approximately 50% in the presence of GR siRNA (figure 5.1 B).

Similarly, co-incubation with GnRH plus dex also resulted in a synergistic increase in transcriptional activity of a transfected GRE-reporter construct in L β T2 cells after 8 h of treatment (figure 5.1 C). To further investigate the role of the GR in the synergistic effect on GRE-transactivation, L β T2 cells were co-transfected with a GR expression construct and stimulated with dex plus GnRH. As shown in figure 5.1 D, overexpression of exogenous GR led to a significant increase in transactivation when cells were treated with both dex and GnRH compared to cells only expressing endogenous GR (figure 5.1 D first bar compared to third bar). As a control, L β T2 cells co-transfected with a GRE-reporter and an expression plasmid for GR were stimulated with only dex to determine that co-treatment of both hormones and not overexpression of GR yields enhanced transcriptional activity (figure 5.1 D second bar). Moreover, the synergistic increase in transcription with dex plus GnRH was found to be decreased in the presence of the GnRHR antagonist Antide (figure 5.1 D fourth bar). Since the response was not completely abolished it can be speculated that Antide specifically antagonized the GnRH- but not the dex-mediated transactivation. Similarly, dex plus GnRH-induced GRE-transactivation was decreased but not abolished in the presence of a GC-antagonist RU486, possibly because the dex- and not the GnRH-mediated response was blocked by the antagonist (figure 5.1 D last bar).

To exclude the possibility that the synergistic effect is a result of increased GR protein levels in response to dex plus GnRH, Western blotting was performed on samples treated for 8 h with dex, GnRH or a combination thereof. Figure 5.1 E and F show that dex or GnRH alone did not have an effect on endogenous GR protein levels, while co-treatment with both hormones resulted in reduced GR protein levels clearly demonstration that synergism is not due to increased GR protein levels (figure 5.1 E and F). Taken together the results suggest that dex plus GnRH induces a synergistic increase in GnRHR mRNA levels as well as in GRE-transactivation which is dependent upon the presence of both the GR and the GnRHR.

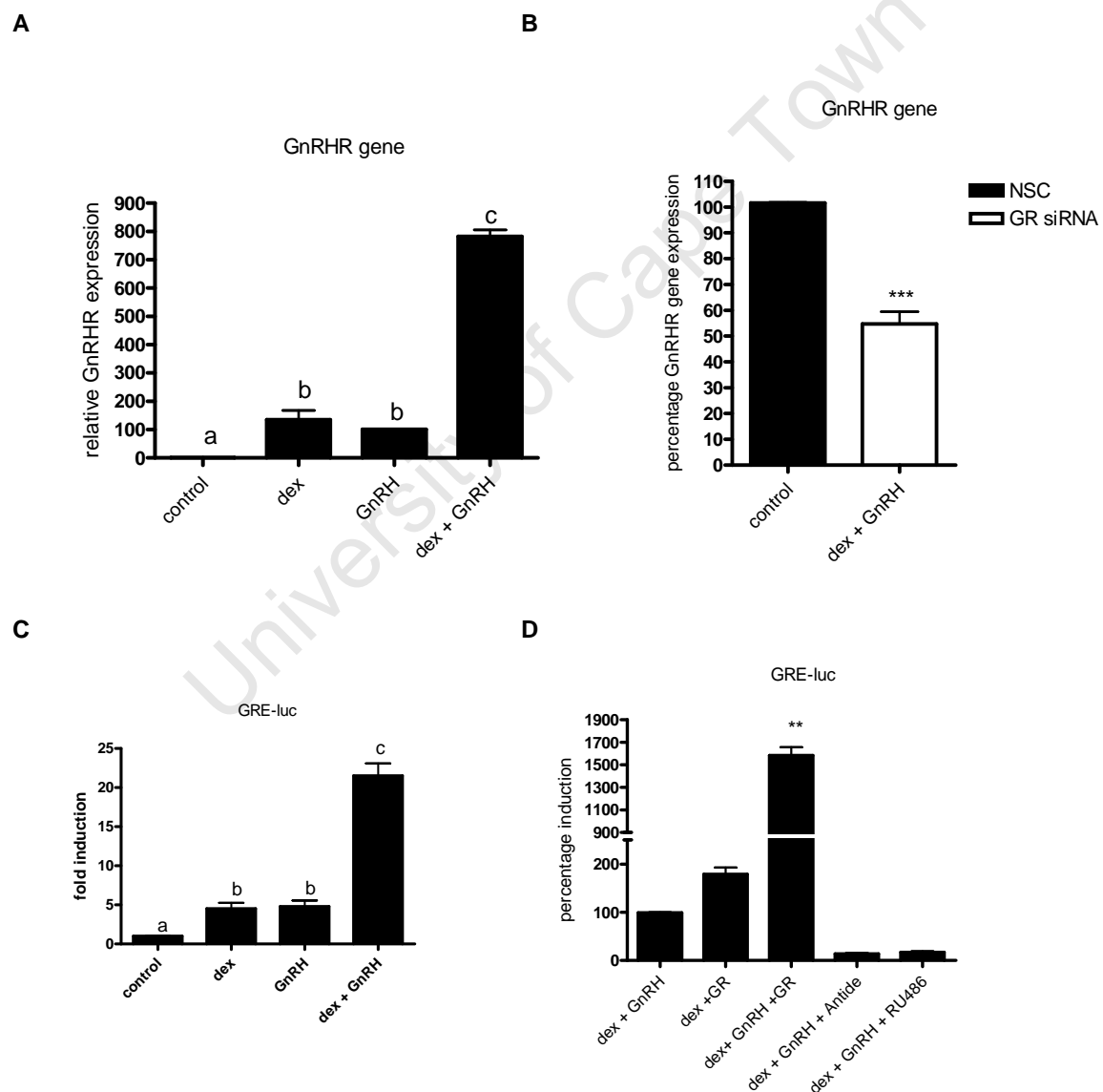


Figure 5.1: (continued on next page)

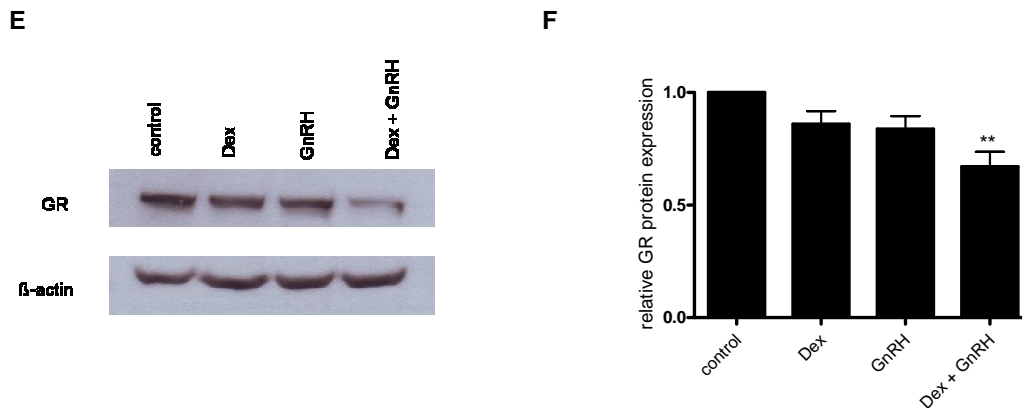


Figure: 5.1: GnRH and dex synergistically increase expression of the endogenous mGnRHR gene and a transfected GRE-reporter in a GR- and GnRH-dependent fashion

A,B: L β T2 cells were treated with vehicle (control) or 100 nM dex, 100 nM GnRH or combinations of both for 8 h in the presence of non silencing control siRNA (B, first four bars) or presence of specific mouse GR siRNA as indicated (B, last bar). Total RNA was isolated, 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) samples. **C,D:** L β T2 cells were transfected with 250 ng TAT-GRE-luciferase construct and 24 h later stimulated with vehicle (control) or 100 nM dex, 100 nM GnRH or combinations of both for 8 h in serum-free medium in the absence (C) or presence of cotransfection with 125 ng HA-GR (+GR), 100 nM Antide or 100 nM Ru486 as indicated (D). **E,F:** L β T2 cells serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex or a combination of both for 8 h. Equal amounts of cell lysates were loaded on a 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific anti-GR or anti- β -actin antibodies (E). The graph in F shows combined results of at least 4 independent experiments. The relative amounts of protein was normalized the β -actin and quantified with the value for control (vehicle) set as 1.

5.3.1.2 The GR and GnRHR are essential for the the GnRH- and dex-induced synergistical increase in GRE-transactivation in COS-1 cells

The results presented in figure 5.1 demonstrate a synergistic effect on GnRHR-, and GRE-transcription mediated by GnRH plus dex in a GR- and GnRHR-dependent fashion in L β T2 cells. In order to explore whether the synergistic effect is cell-specific, COS-1 cells, lacking endogenously expressed GnRHR or GR (van Biljon 2003), were cotransfected with a synthetic GRE-reporter and expression plasmids for the GR and the GnRHR. As shown in figure 5.2 A, promoter activity in cells overexpressing GR and the GnRHR was increased with dex or GnRH stimulation as previously shown in figure 4.3. Importantly, co-incubation with dex plus GnRH significantly increased GRE-

transactivation compared to dex or GnRH alone, similar to results obtained in LBT2 cells (figure 5.1 C), suggesting that synergism is not cell or tissue specific (figure 5.2 A). As expected, GRE-transactivation was only significantly increased in cells overexpressing the GR as shown in figure 5.2 B. Importantly, reporter activity was further increased when cells were cotransfected with both GR and GnRHR expression plasmids suggesting a requirement for both the GR and the GnRHR for the synergistic response (figure 5.2 B).

To ensure that the synergism was not due to increased GR levels in response to dex plus GnRH in COS-1 cells, the expression levels were monitored by Western blotting. As shown in figure 5.2 C untransfected COS-1 cells did not display any detectable GR expression levels while transfected cells were found to strongly express exogenous GR. Furthermore, since an increase in the total GR levels for any incubation condition as compared to vehicle control was not observed upon Western blotting it can be concluded that the synergy was not due to changes in GR levels (figure 5.2 C and D). Interestingly, in dex-treated samples GR levels were found to be significantly decreased compared to vehicle control (figure 5.2 C and D). This is in agreement with findings by others showing dex-dependent protein degradation of overexpressed GR in COS-1 cells (Webster *et al.*, 1997). In contrast GnRH treatment did not decrease GR protein levels at 8 h of continuous stimulation as observed for dex and moreover co-stimulation with both dex and GnRH prevented dex-induced down-regulation of the GR (figure 5.2 C and D).

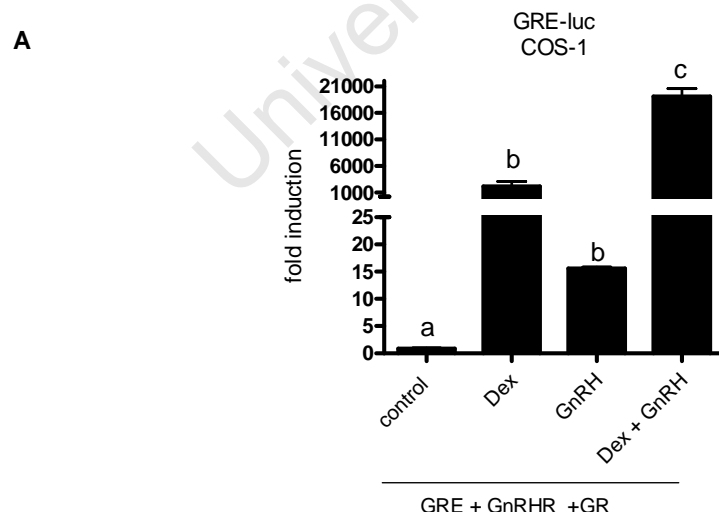
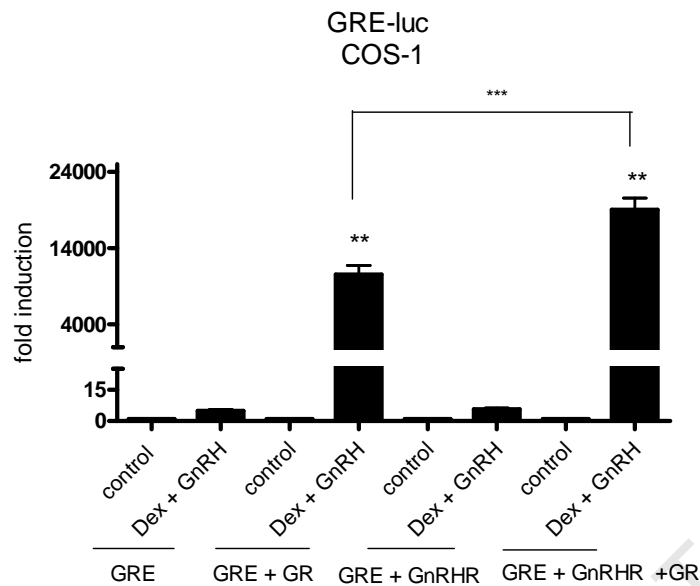
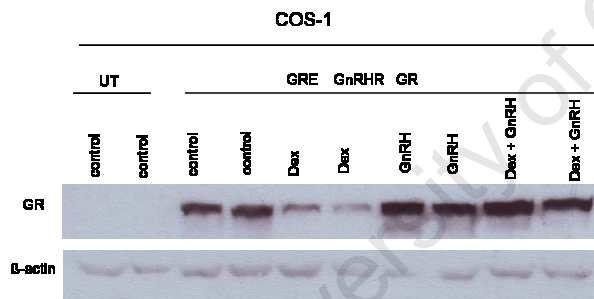


Figure 5.2: (continued on next page)

B



C



D

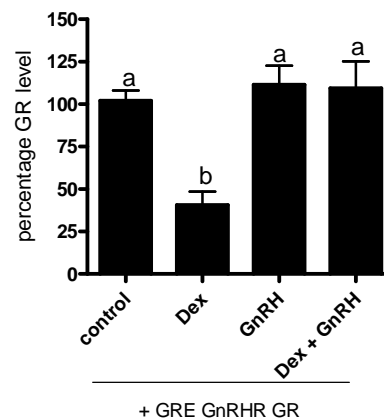


Figure 5.2: The GR and GnRHR are essential for GnRH- and dex-induced synergistically increase in GRE-transactivation in COS-1 cells

A,B: COS-1 cells were transfected with 250 ng TAT-GRE-luciferase construct (GRE) in the absence or presence of cotransfection with 6 ng mouse GnRHR expression vector (GnRHR) and/or 125 ng HA-human GR expression vector (GR), as indicated below relevant bars. Transfected cells were incubated for 8 h in serum-free medium with vehicle (control), 100 nM dex, 100 nM GnRH or co-stimulated with both dex plus GnRH. The luciferase activity was normalized to β -galactosidase and the hormone-stimulated increase in promoter activity was calculated relative to the vehicle control. The graph in A shows promoter activity in the presence of both the GnRHR and the GR. The graph in B

shows dex plus GnRH-induced promoter activity with the individual control transfections as indicated. The results present the promoter activity relative to vehicle (control) of combined results of at least 2 independent experiments, each performed in triplicate. **C,D:** COS-1 cells were transfected with 250 ng TAT-GRE-luciferase construct (GRE) in the absence (UT) or presence of cotransfection with 6 ng mouse GnRHR expression vector (GnRHR) and/or 125 ng HA-human GR expression vector (GR) and serum-starved overnight before treatment with 100 nM GnRH or 100 nM dex for 8 h. Equal amounts of cell lysates were loaded on a 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific anti-GR or anti- β -actin antibodies (C) The graph in D shows combined results of 3 independent experiments. The relative amounts of protein was normalized the β -actin and quantified with the value for control (vehicle) set as 1.

5.3.2 Co-stimulation with dex plus GnRH does not change the GR phosphorylation levels relative to dex alone at Ser-220 or Ser-234 in LBT2 cells

As established previously, dex induces strong GR phosphorylation at both Ser-234 and Ser-220, while GnRH only increased phosphorylation at Ser-234 and not Ser-220 (figure 4.4 A and B). Towards understanding the mechanism of synergism, the phosphorylation status of the endogenous GR was investigated in response to co-treatment with dex plus GnRH. The results presented in figure 5.3 show that co-incubation with dex plus GnRH induced phosphorylation at both Ser-220 and Ser-234 at levels comparable to the phosphorylation levels observed for dex alone (figure 5.3 A and B). The results suggest that differential phosphorylation at these specific GR residues appears not to be responsible for the synergistic transcriptional effect with dex plus GnRH. However, since GR phosphorylation has been reported to modulate maximal transcriptional activity (Avenant, 2009; Chen *et al.*, 2008; Hoeck and Groner, 1990; Kino *et al.*, 2007) it cannot be excluded that other GR phosphorylation sites, not investigated in this study, play a role in synergism.

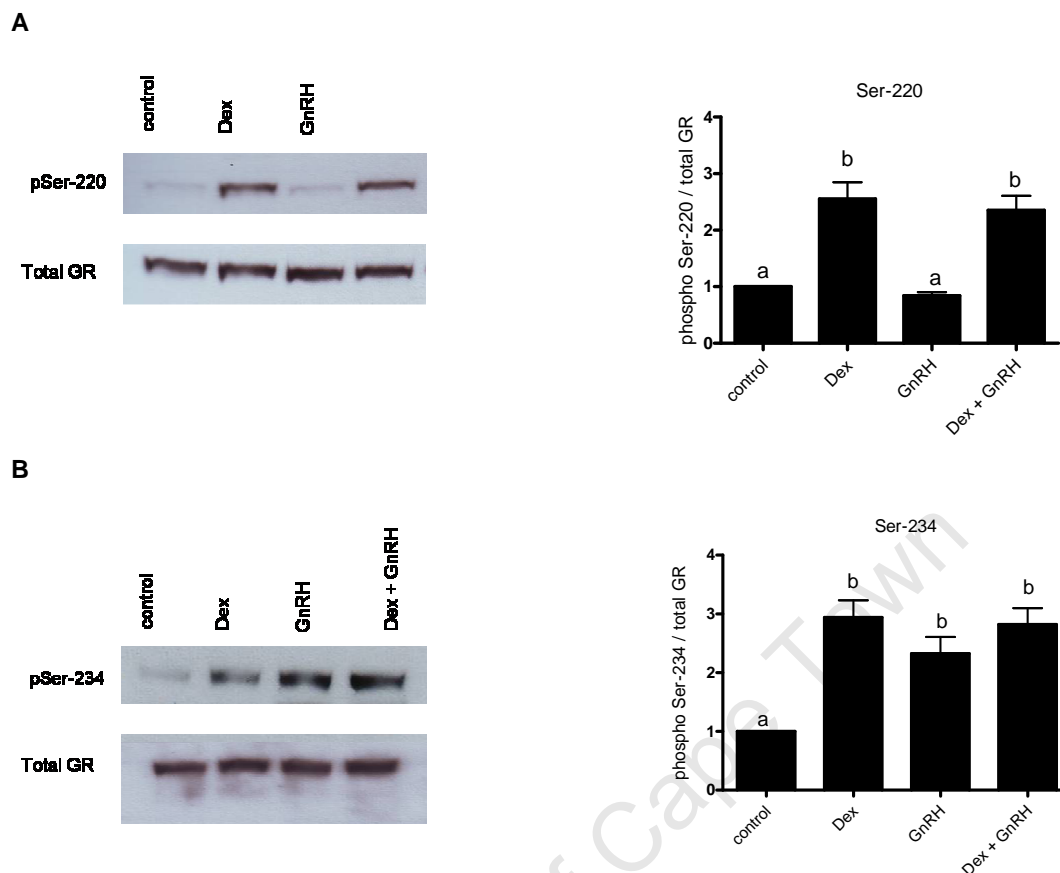


Figure 5.3: Co-stimulation with dex plus GnRH does not change the GR phosphorylation levels at Ser-220 or Ser-234 as compared to dex alone in L β T2 cells

L β T2 cells were grown and serum-starved overnight before treatment with 100 nM GnRH, 100 nM dex or combination of both for 8 h. Equal amounts of cell lysates were loaded on a 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific antibodies for GR phosphorylated at Ser-220 (A) or Ser-234 (B). The blots were stripped and reprobed with an anti-GR antibody as control (Total GR). The graphs show combined results of at least 3 independent experiments. The relative amounts of phosphorylation at the specific serine residues were quantified and expressed as phosphorylated GR content relative to total GR, with the value for control (vehicle) set as 1.

5.3.3 GnRH plus dex-stimulated phosphorylation of the GR is mediated by a combination of JNK, p38 and ERK

Having shown that co-stimulation with dex plus GnRH induces GR phosphorylation levels at Ser-220 and Ser-234 similar to dex alone in L β T2 cells it was next investigated which MAPKs are required for the phosphorylation event in response to co-treatment.

The results presented in figure 5.4 A show that phosphorylation at Ser-220 is significantly decreased only in the presence of both JNK and ERK MAPK inhibitors. This is in contrast to results shown in figure 4.10 A and B where ERK appears to be involved in dex-induced Ser-220 phosphorylation. Inhibition of PKA, PKC or c-src had no effect on the dex plus GnRH-induced Ser-220 phosphorylation (figure 5.4 A and B).

Similarly, phosphorylation at Ser-234 was unaffected by PKA, PKC or c-src inhibitors (figure 5.4 B), while it was previously found that PKC is involved in GnRH- but not dex-mediated phosphorylation at that specific serine residue (figure 4.13 A and B). Inhibition of the individual JNK and p38 kinases appeared to have no effect on Ser-234 phosphorylation whereas inhibition of ERK led to a statistically significant small decrease in GR phosphorylation at Ser-234 (figure 5.4 B). Furthermore, inhibition of any two MAPK led to a significant decrease in dex plus GnRH-mediated phosphorylation consistent with the results for GnRH alone at Ser-234 (figure 4.10 C and D).

A

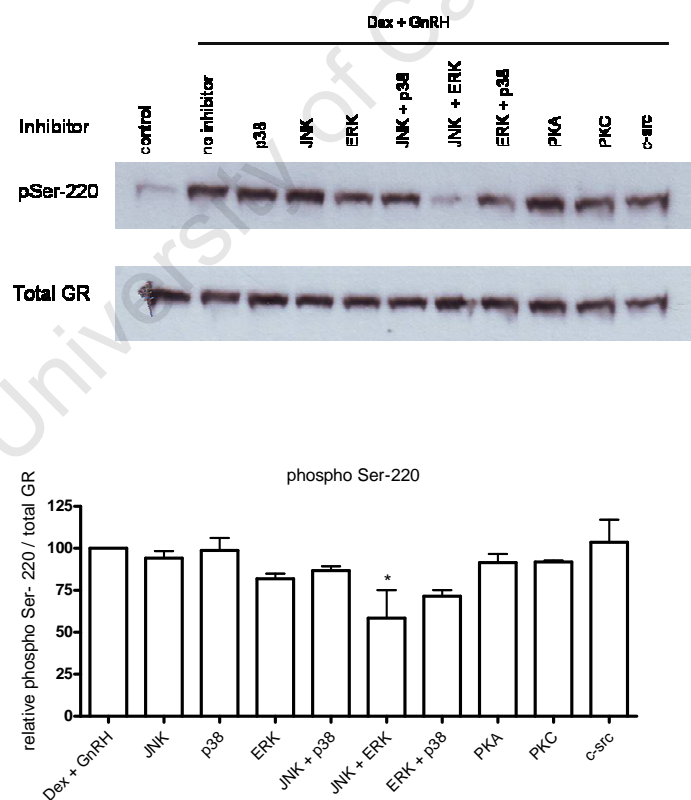


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B

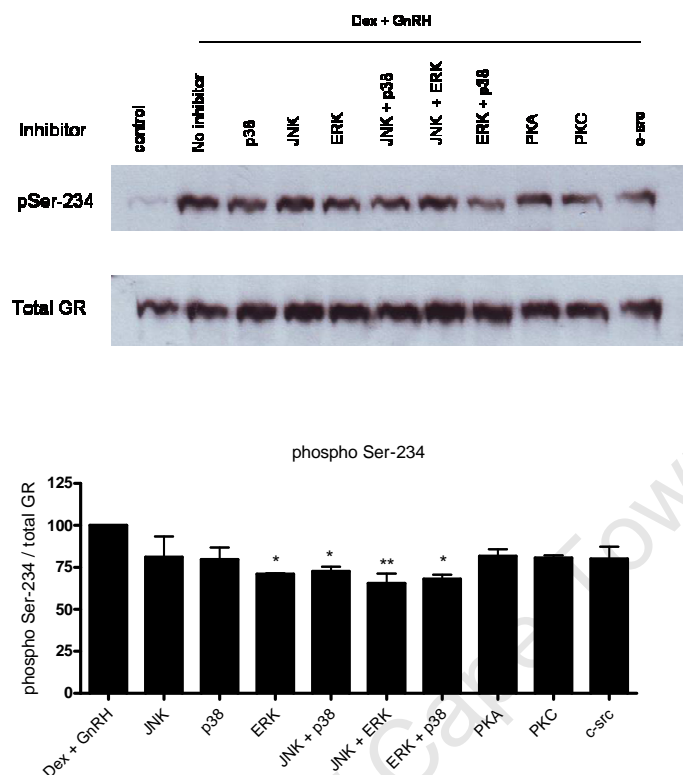


Figure 5.4: GnRH plus dex-stimulated phosphorylation of the GR is mediated by a combination of JNK, p38 and ERK

L β T2 were stimulated for 30 min with either vehicle or 100 nM GnRH plus 100 nM dex in the absence or presence of the following inhibitors: 1 μ M PKA inhibitor (H89) or 100 nM PKC inhibitor (BIM), 100 μ M c-src inhibitor (PP2), 10 μ M JNK inhibitor (SP600125), 10 μ M p38 inhibitor (SB203580), 50 μ M a ERK pathway inhibitor (PD98059) or combinations thereof as indicated. Western blotting was performed using anti-GR phospho Ser-220 antibody (A) or anti-GR phospho Ser-234 antibody (B). The blots were stripped and reprobbed with anti-GR antibody as a control. Results shown in the graphs are combined results from 2 independent experiments, where phospho-GR levels were normalized to total GR, with the value for control (vehicle) set as 1.

5.3.4 Transcription factors involved in synergism

5.3.4.1 AP-1 protein levels bound to the AP-1 region of the GnRHR-promoter remain unchanged in response to dex plus GnRH in intact LBT2 cells

As already shown in figure 3.4 A and B both c-Jun and c-Fos were found to occupy the AP-1 site under basal as well as dex- and GnRH-stimulated conditions. To further study the mechanism underlying the synergistic effect with dex plus GnRH, the promoter occupancy with AP-1 proteins was

examined using ChIP assays. The results presented in figure 5.5 confirm previous results by showing that both c-Jun and c-Fos are bound to the AP-1 region of the endogenous GnRHR promoter under basal as well as dex- and GnRH-stimulated conditions. Co-stimulation with dex plus GnRH did not result in increased or decreased binding of either c-Jun (figure 5.5 A and B) or c-Fos (figure 5.5 A and C) to the promoter suggesting that the composition of the AP-1 heterodimer is not changed when cells are subjected to both hormones.

Interestingly, it was found that co-stimulation with dex plus GnRH had a differential effect on c-Jun but not c-Fos protein expression compared to GnRH on its own (Addendum A figure A7 and A8). As shown in Addendum A in figure A7 GnRH-induced c-Jun expression declines after 2 h while co-treatment with dex plus GnRH resulted in a further increase in c-Jun protein expression after 2 h of incubation. This could possibly indicate a mechanism whereby dex plus GnRH have a differential effect on gene transcription compared to the hormones on their own. However, the results presented in figure 5.5 show that co-treatment did not result in altered recruitment of c-Jun or c-Fos to the GnRHR gene suggesting that different levels of these AP-1 proteins are not involved in the synergistic effect in L β T2 cells.

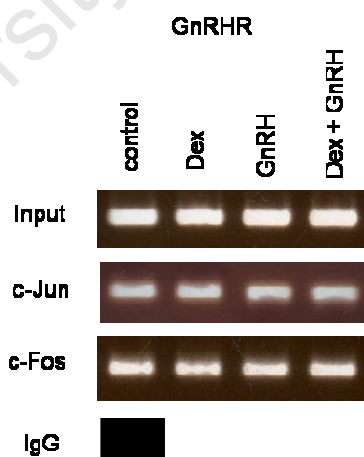
A

Figure 5.5: (continued on following page)

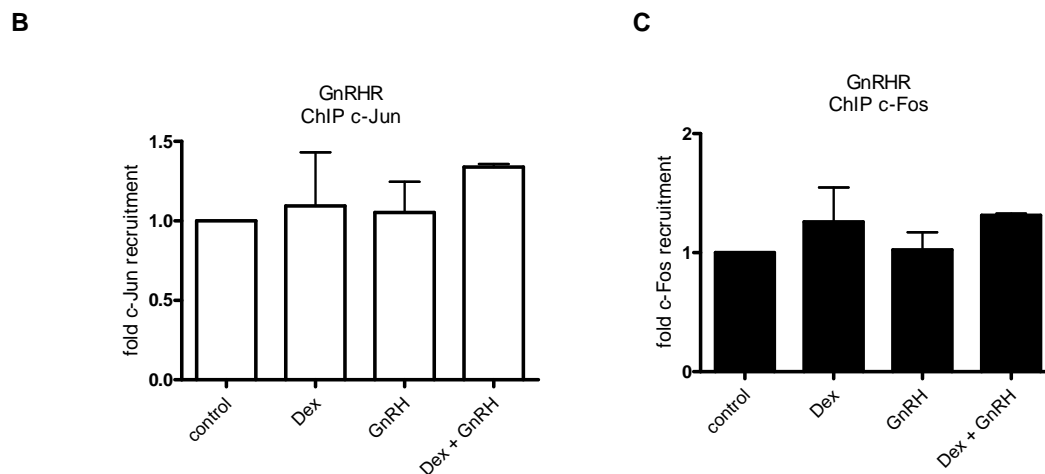


Figure 5.5: Effect of dex plus GnRH treatment on AP-1 protein recruitment to the endogenous GnRHR promoter L β T2 cells

ChIP in L β T2 cells treated with vehicle (control), 100 nM GnRH, 100 nM dex or a combination of both for 1 h. ChIP was performed with anti-c-Jun (A and B) or anti-c-Fos (A and C) specific antibodies. The co-immunoprecipitated DNA fragments and input DNA were analyzed by conventional PCR (A) and by real-time PCR (B and C) using primer spanning the AP-1 region of the mouse GnRHR promoter. Pooled and quantified results of 2 independent experiments are shown normalized to input and expressed as fold-recruitment relative to control values (B and C).

5.3.4.2 GR recruitment to the endogenous GnRHR promoter in response to dex plus GnRH is similar to that observed for dex alone in intact L β T2 cells

Having shown that there is no change in levels of bound AP-1 proteins to endogenous GnRHR promoter it was next investigated whether the GR is differentially recruited when cells are stimulated with both dex and GnRH. It was found by ChIPs that the extent of GR recruitment to the AP-1 region of the endogenous GnRHR promoter was similar to that observed for dex alone suggesting that differential levels of recruitment of the GR is also not responsible for the synergism (figure 5.6).

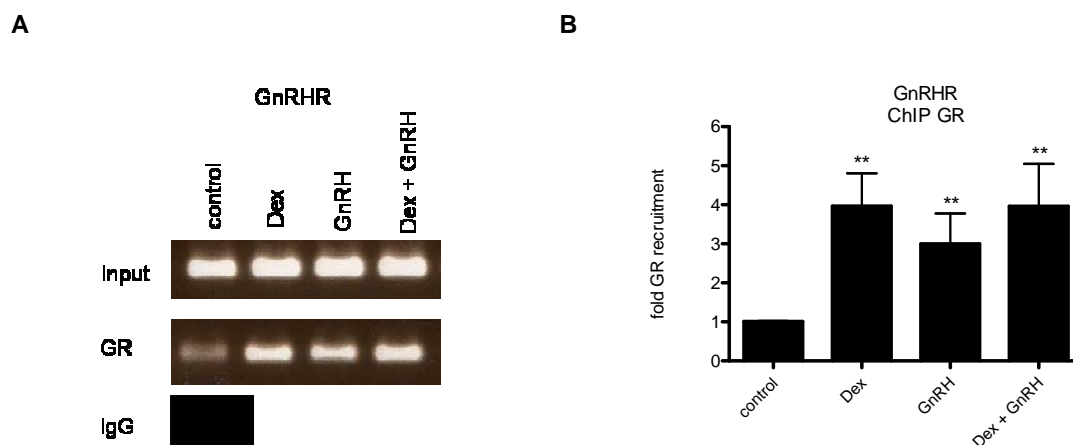


Figure 5.6: GR recruitment on the endogenous GnRHR promoter does not change in response to dex plus GnRH

C,D ChIP was performed in LBT2 cells treated with vehicle (control) 100 nM GnRH, 100 nM dex or combination of both for 1 h using a specific anti-GR antibody. ChIP was analyzed by conventional PCR (A) or real-time PCR (B). Pooled and quantified results of at least 3 independent experiments are shown normalized to input and expressed as fold-response relative to control values.

5.3.4.3 Synergism between dex and GnRH involves recruitment of SRC-1 to the GnRHR promoter in LBT2 cells

The activated GR can interact with many components of the transcriptional machinery, as well as co-activators (Kumar and Thompson, 2005). A recent study demonstrated a direct correlation between the transcriptional response mediated by the GR and GR binding of co-activators, in particular GRIP-1 (Ronacher *et al.*, 2009).

Therefore the possibility of differential GR-mediated assembly of cofactors to the endogenous GnRHR promoter was explored by ChIP using specific antibodies for known GR cofactors. The results presented in figure 5.7 A and B show that dex stimulation strongly induced GRIP-1 recruitment to the promoter while GnRH stimulation did not lead to GRIP-1 binding in intact LBT2 cells. Importantly, co-stimulation with dex plus GnRH resulted in GRIP-1 binding to levels similar to that observed for dex alone (figure 5.7 A and B). In addition, it was found that p300 appears to occupy the AP-1 promoter region in the absence of hormone (control) while stimulation with dex, GnRH or combination thereof resulted in reduced binding of p300 (figure 5.7 A and C). Although the responses varied between experiments and statistical significance could not be established, it was repeatedly found that p300 is

bound under basal conditions while treatment with dex, GnRH or a combination of both decreased p300 occupancy on the GnRHR promoter. Interestingly, it was found that unlike for dex or GnRH alone, GnRH plus dex resulted in SRC-1 recruitment to the AP-1 region of the GnRHR promoter (figure 5.7 A and D). The result suggests that SRC-1 is involved in the synergistic effect of dex plus GnRH on the GnRHR gene, as only co-stimulation induces recruitment of that cofactor.

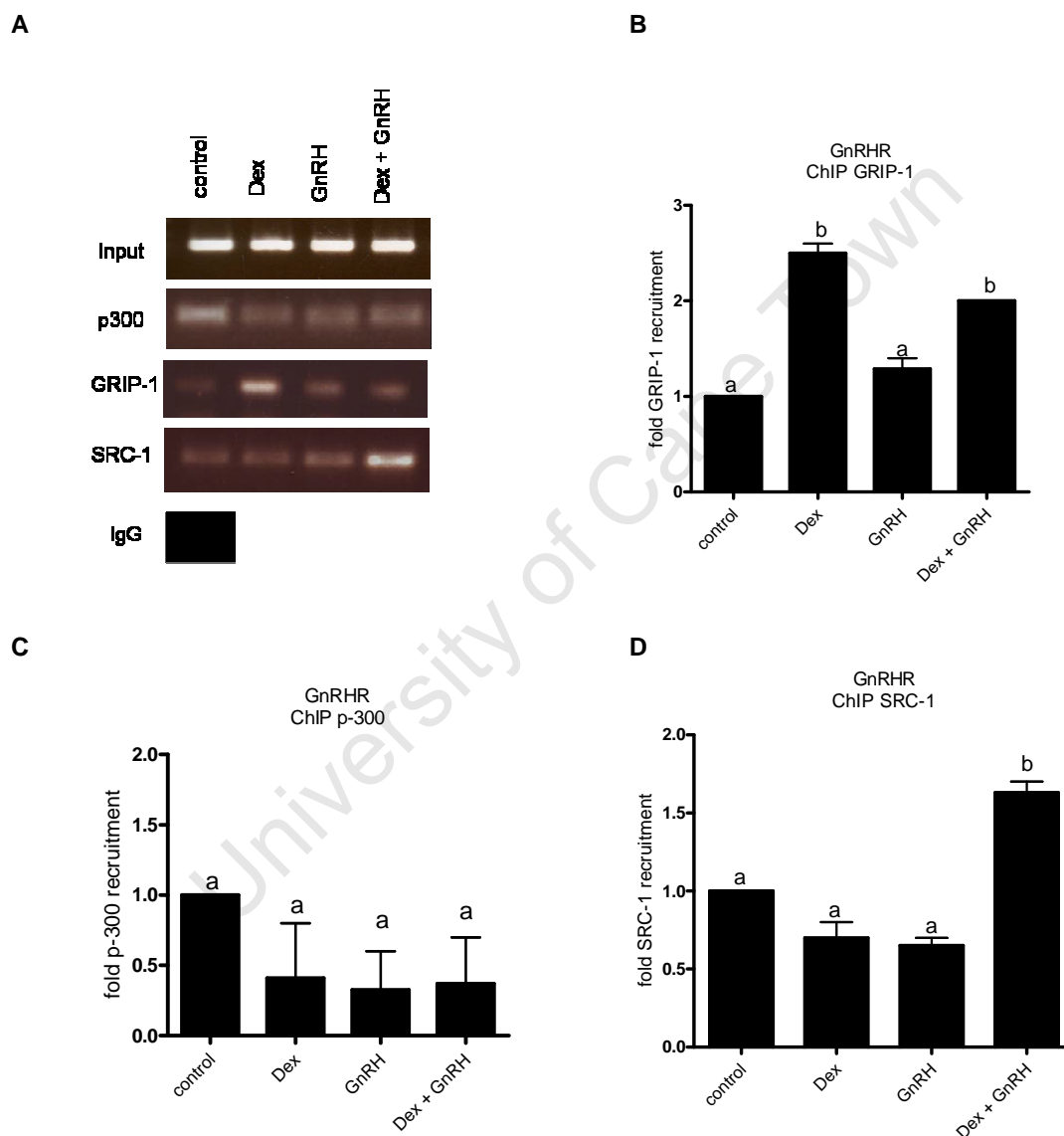


Figure 5.7: Synergism involves recruitment of SRC-1 to the AP-1 region of the GnRHR promoter in LBT2 cells

ChIP was performed in LBT2 cells treated with vehicle (control) 100 nM GnRH, 100 nM dex or combination of both for 1 h using anti-GRIP-1 (B), anti-p300 (C) or anti-SRC-1 (D) specific antibodies.

Conventional PCR was performed, using primers as describes before on input chromatin and precipitated chromatin and products were resolved on agarose gels (A). Pooled and quantified results analyzed by real-time PCR of at least 2 independent experiments normalized to input and expressed as fold-response relative to control values are shown in B-D.

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CHAPTER 6

DISCUSSION AND CONCLUSION

6.1 Both GnRH and dex upregulate the GnRHR gene via the GR by a genomic cross talk mechanism converging on the AP-1 site on the mouse GnRHR promoter in L β T2 cells

The mechanisms underlying transcriptional regulation of the GnRHR gene have been a focus of numerous research groups in the field. Several hormones including GnRH were found to have a significant effect on GnRHR levels *in vivo* (Kaiser *et al.*, 1993; Wu *et al.*, 1994; Yasin *et al.*, 1995). Whether transcriptional regulation of GnRHR expression occurs by GnRH in gonadotrope cells has also been the focus of several groups in recent years. Studies in α T3-1 cells describe homologous regulation of GnRHR transcription (Norwitz *et al.*, 1999a; Sadie *et al.*, 2003; White *et al.*, 1999) while only some reports have been published showing homologous regulation of GnRHR gene expression in L β T2 cells. Bedecarrats *et al.* reported that continuous stimulation for 10 h with 10 nM GnRH has a small effect (approximately 1,5 fold) on the activity of the transfected GnRHR promoter-reporter construct and resulted in decreased GnRHR numbers (Bedecarrats and Kaiser, 2003). Previous data from Prof. Hapgood's laboratory showed that continuous stimulation with 100 nM GnRH increased the expression of a synthetic transfected mouse GnRHR promoter-reporter construct (pLG) by about 2,5 fold (Addendum B figure B1 B) (Sadie, 2006). It was further established that 8 h of continuous stimulation with 100 nM GnRH resulted in maximal transcriptional response while longer incubation times were found to decrease the promoter activity (Fernandes, 2007; Sadie, 2006). In addition, preliminary results from Prof. Hapgood's laboratory showed that endogenous GnRHR mRNA levels are upregulated after 8 h of continuous stimulation with GnRH in L β T2 cells supporting the earlier findings obtained with the transfected promoter-construct (Addendum B figure B1 B) (Sadie, 2006). This observation was confirmed in the present study since it was found that GnRH treatment results in a significant increase in GnRHR transcripts (figure 3.1). In support of these findings White *et al.* reported that continuous stimulation with 100 nM GnRH for 4 h resulted in maximal increase in transcriptional activity of a mouse GnRHR construct and endogenous mRNA levels in α T3-1 cells

(White *et al.*, 1999) and similarly Norwitz *et al.* reported that continuous stimulation with 100 nM GnRH (4 h) increased the transcriptional activity of a transfected mouse GnRHR construct in the same cells (Norwitz *et al.*, 1999a). Bedecarrats *et al.* found that pulsatile GnRH stimulation (1 pulse every 30 min) for 10 h resulted in a 2-fold increase in GnRHR promoter activity as well as a 2-fold increase in GnRHR numbers on the cell surface which is similar to results obtained from our laboratory when cells were treated continuously for 8 h with 100 nM GnRH (Bedecarrats and Kaiser, 2003). Taken together the results above suggest that the GnRHR is transcriptionally activated under both pulsatile and continuous treatment in L β T2 cells.

In addition to GnRH, GCs have also been found to play an important role in modulating GnRHR levels *in vivo* (Breen and Karsch, 2004; Daley *et al.*, 1999; Rosen *et al.*, 1991; Tilbrook *et al.*, 2000). Previously, it was established that GCs exert a direct effect on GnRHR transcription in gonadotrope cells of the pituitary. In the somatolactotrope GGH₃ cell line dex was found to significantly upregulate a transfected mouse GnRHR promoter construct (Maya-Nunez and Conn, 2003). However, GGH₃ cells are rat somatolactotrope cells that artificially express the GnRHR receptor. Evidence for a direct transcriptional effect of GCs on GnRHR gene expression comes from previous reports showing that dex upregulates expression of both a transfected promoter-reporter construct (McGillivray *et al.*, 2007) and the endogenous GnRHR gene in L β T2 cells (McGillivray *et al.*, 2007; von Boetticher, 2008). In the present study the stimulatory effect of dex treatment on endogenous GnRHR gene expression could be confirmed (figure 3.1). Interestingly, when comparing the responses of dex versus GnRH on the synthetic reporter-gene (Addendum B figure B1) and on the endogenous GnRHR gene (figure 3.1) it was found that the magnitude is comparable suggesting that both hormones exhibit a similar transcriptional potential. However, the magnitude of the responses observed for the endogenous gene was much greater than the response of the transfected promoter for both dex and GnRH (Addendum B figure B1 and figure 3.1) (Fernandes, 2007; Sadie, 2006; von Boetticher, 2008). Since the pLG promoter-construct contains only 580 bp of the mouse GnRHR promoter relative to the translational start site it is likely that upstream promoter elements are involved in maximal transcriptional activity. Also, transiently transfected promoters lack natural promoter architecture and chromatin structure which could explain the different extent in fold induction compared to the endogenous gene.

In the literature there are several reports demonstrating that GnRH responsiveness of the mouse GnRHR promoter in α T3-1 cells is mediated via two regions namely SURG-1, containing binding sites for nuclear factor Y (NF-Y) and Oct-1 (Kam *et al.*, 2005), and SURG-2 which contains the AP-1 binding site (Duval *et al.*, 1997b; Norwitz *et al.*, 1999a). In L β T2 cells it was shown that mutation of the AP-1 site decreased the basal activity of the promoter by about 50% (Addendum B figure B2 A) (Fernandes, 2007). In addition the GnRH responsiveness of the promoter-reporter construct containing the AP-1 mutation was significantly decreased compared to the wild type promoter clearly demonstrating that an intact AP-1 element is essential to confer transcriptional activity by GnRH in L β T2 cells (Addendum B figure B2 B) (Sadie, 2006). This result is consistent with findings by other groups in α T3-1 cells where the AP-1 site was shown to play an important role in transcriptional regulation of both basal and GnRH-stimulated mouse GnRHR gene expression (Duval *et al.*, 1997b; Kam *et al.*, 2005; Norwitz *et al.*, 1999a). Interestingly, two AP-1 sites are found in the human GnRHR promoter, one of which has been shown to regulate GnRH responsiveness, showing the importance of this site for homologous regulation in both mouse and human (Hapgood *et al.*, 2005). Furthermore, the loss of the AP-1 site was found to significantly decrease the dex-mediated transactivation of the transfected mouse GnRHR promoter-construct (Addendum B figure B2 C) (Fernandes, 2007). The critical role for the AP-1 element in the activation of the mouse promoter by GCs has been reported by Maya-Nunez *et al.* (Maya-Nunez and Conn, 2003), however that particular study was carried out in GGH₃ cells and therefore the result obtained previously in Prof. Hapgood's laboratory is more significant since it demonstrates the requirement of the AP-1 promoter element for GC-mediated transcription in a gonadotrope cell line. In addition the results obtained in the present study presented in figure 3.2 show that GnRH was able to trigger transactivation of an AP-1 promoter (pAP-1-luc) giving further insight into the mechanism of GnRH-mediated transcriptional activation. Indeed several investigators have shown that GnRH treatment increases the mRNA levels (Kakar *et al.*, 2003; Wurmbach *et al.*, 2001) and protein expression levels (figure 3.3) (Coss *et al.*, 2004; Sadie, 2006) of several Jun and Fos proteins in L β T2 cells providing a mechanism whereby the transcriptional effects of GnRH can be mediated via AP-1. By contrast, dex did not activate the AP-1 promoter-reporter construct (figure 3.2) suggesting that the AP-1 site in the GnRHR promoter is not the sole requirement for dex-induced gene expression.

The present finding that c-Jun proteins are associated with the AP-1 region of the endogenous GnRHR promoter in the absence of both GnRH and dex (figure 3.4 A and B) is consistent with results from another study by Jeong *et al.* also showing the presence of c-Jun proteins on the endogenous promoter under basal conditions in L β T2 cells (Jeong *et al.*, 2004). In addition, the findings are consistent with previous results (Addendum B figure B2 A) (Fernandes, 2007) showing that the AP-1 site is required for maximal basal expression of the GnRHR gene in L β T2 cells. By ChIP analysis it was established that GnRH- or dex-treatment did not change the levels or relative composition of c-Jun or c-Fos proteins associated with the AP-1 region of the promoter (figure 3.4 A and B). This is in contrast to a report in α T3-1 cells where GnRH was found to induce an increase in AP-1 proteins, specifically c-Jun, binding to the promoter in intact cells (Kam *et al.*, 2005), as well as *in vitro* results showing that c-Jun and not c-Fos proteins are able to bind to the AP-1 element in response to dex in GGH₃ cells (Maya-Nunez and Conn, 2003). However, this inconsistency could be due to cell-specific differences or differences in cell culturing conditions. Although c-Jun and c-Fos are only present at low levels in the absence of GnRH (Coss *et al.*, 2004; Liu *et al.*, 2002a), the results of the present study suggest that these levels are sufficient to occupy the GnRHR promoter under basal conditions. It has previously been shown that the transcriptional activity of AP-1 proteins can be induced via post-translational mechanisms, such as the phosphorylation of c-Jun by JNK (Ellsworth *et al.*, 2003b; Yokoi *et al.*, 2000). Several transcription factors including c-Jun and c-Fos are constitutively expressed at low levels and associated with genes in the nucleus without activating these. The transcriptional activity of transcription factors can be increased via post-translational modifications such as the phosphorylation and thereby inducing gene transcription (Brivanlou and Darnell, 2002; Ellsworth *et al.*, 2003b; Treisman, 1995; Yokoi *et al.*, 2000). These proteins may have a structural role in maintaining the chromatin in a certain state to ensure rapid transcriptional activation or they might be involved in conferring basal gene expression. Thus it can be speculated that GnRH-induced signalling pathways could change the phosphorylation status of the AP-1 proteins bound to the DNA and thereby in part enhance GnRHR gene expression.

The above mentioned results suggest that c-Jun and c-Fos are the key components in regulating basal- and hormone-induced expression of the mouse GnRHR gene. Thus, it is likely that the AP-1 proteins form a platform for other transcription factors to bind in response to hormone. Consistent with this hypothesis the GR was found to be recruited to the AP-1 region of the GnRHR promoter (figure

3.6). It is tempting to speculate that the GR does not bind directly to the promoter but rather via a tethering mechanism to AP-1 proteins since no glucocorticoid response element (GRE) has been reported to be present in the mouse GnRHR promoter (Albarracin *et al.*, 1994; Norwitz *et al.*, 1999b). Direct or indirect interaction between the GR and members of the AP-1 protein family members has been well described in the literature (Herrlich, 2001; Kassel and Herrlich, 2007). This interaction does not require direct DNA binding of the GR (Touray *et al.*, 1991) and mostly results in transrepression of AP-1-controlled genes, under conditions where AP-1 consists of c-Jun/c-Fos heterodimers (Yamamoto *et al.*, 1998). By contrast, GR tethering to c-Jun homodimers has been shown to activate AP-1-regulated promoters (Teurich and Angel, 1995; Yamamoto *et al.*, 1998). Results shown in the present study indicate a novel mechanism of GR transactivation via an AP-1 element, since both c-Jun and c-Fos appear to occupy the promoter. In addition, it was shown that the GR is recruited to the AP-1 region of the GnRHR promoter in response to GnRH in the absence of its ligand (figure 3.6). That exciting finding was unexpected as there are no reports in the literature showing that this or any other peptide hormone can activate the GR in the absence of a GR ligand. Results in figure 3.8 established the requirement for the GR in GnRHR gene expression for both dex and GnRH since decreasing of GR protein levels by specific mouse GR siRNA reduced the hormone-mediated GnRHR gene expression. These novel results illustrate that the liganded and the unliganded GR play a critical role in GnRHR gene expression via an intact AP-1 response element bound by c-Jun and c-Fos.

Attempts to investigate whether GR and AP-1 proteins occur in the same complex by co-immunoprecipitation from cell lysates and re-ChIP experiments with endogenous proteins were unsuccessful, possibly due to the low abundance of the endogenous complexes. Although the present results do not provide direct evidence for GR-AP-1 interaction on the GnRHR promoter, it can be speculated that the dex- and GnRH-induced upregulation of the mouse GnRHR gene involves a tethering mechanism via direct or indirect binding of the GR to c-Jun/c-Fos heterodimers. Consistent with results reported in this study, the estrogen receptor (ER) has been shown to increase the transcriptional activity of bound c-Jun/c-Fos heterodimers (Kushner *et al.*, 2000). Kushner *et al.* proposed that the ER does not directly interact with c-Jun/c-Fos proteins but rather binds to a complex of co-activators recruited to AP-1, including CBP/p300 and p160s. Similarly, it has been reported that nTRIP6, a member of the zyxin family of LIM domain-containing proteins acts as a transcriptional co-

activator for AP-1 target genes, such as collagenase I occupied by c-Jun/c-Fos heterodimers, while nTRIP6 is also required for GR-mediated transrepression of AP-1 regulated genes (Kassel *et al.*, 2004). Therefore, it is likely the dex-activated GR requires the presence of another *cis*-element plus associated transcription factor which enables GnRHR promoter activation in response to dex in L β T2 cells.

The result presented in figure 3.2 is striking since dex treatment failed to induce transcription of an AP-1 promoter-construct, suggesting that other *cis*-regulatory elements besides the AP-1 site in the GnRHR promoter are involved in mediating the response. The mouse GnRHR promoter harbours functional elements for Oct-1, NF-Y, SF-1 and CREB adjacent to the AP-1 site (Hapgood *et al.*, 2005). Since the GR is known to cross talk with other transcription factors (Kassel and Herrlich, 2007) it is plausible that the receptor might interact with another transcription factors bound to the promoter and forming a multiprotein complex including AP-1 proteins. A study by Kam *et al.* showed binding of Oct-1 to the GnRHR gene *in vitro* in α T3-1 cells (Kam *et al.*, 2005) making Oct-1 a possible candidate to facilitate GR binding to the promoter. Unpublished data from Prof. Hapgood's laboratory suggest that the AP-1 site and the CRE site of the mouse GnRHR promoter are required for GnRH responsiveness (Sadie, 2006) while the AP-1 site and a novel NRS adjacent to the AP-1 region, binding SF-1 proteins *in vitro*, were found to be essential for dex responsiveness of the GnRHR promoter in L β T2 cells (Fernandes, 2007). Thus it is tempting to speculate that SF-1 and/or CREB are involved in GR binding to the GnRHR promoter in L β T2 cells.

6.2 GnRH activates the unliganded GR by a rapid non-genomic cross talk mechanism involving GnRHR-dependent GR phosphorylation at Ser-234

Several lines of evidence support the finding that the GnRHR signals via rapid non-genomic cross talk to the unliganded GR. Using siRNA technology, it was demonstrated that the GR is required for transcriptional upregulation of the endogenous mouse GnRHR in L β T2 cells in response to GnRH (figure 3.8). Immunofluorescence experiments revealed that GnRH induces nuclear translocation of the unliganded endogenous GR in L β T2 cells (figure 4.1). This is in agreement with findings presented in figure 3.6 showing GnRH-mediated recruitment of the GR to the endogenous GnRHR promoter after 1 h of hormone treatment. It has previously been reported that GnRH causes nuclear translocation of the PR in α T3-1 cells after 1 h of stimulation (An *et al.*, 2006). In primary human lung

fibroblasts β_2 -agonists were shown to induce nuclear translocation of the GR after 4 h of stimulation (Eickelberg *et al.*, 1999). The authors further showed by nuclear fractionation that dex stimulation induced a more rapid depletion of the GR from the cytosol compared to β_2 -agonist. Similarly Tanaka *et al.* demonstrated that stimulation with the bile acid ursodeoxycholic acid (UDCA) results in nuclear import of the GR after 2 h of treatment but to a lesser extent than observed with dex in Chinese hamster ovary cells (Tanaka *et al.*, 1996). However, this is the first study demonstrating that GnRH treatment results in altered cellular distribution of the GR in L β T2 cells compared to unstimulated cells. Furthermore it was shown that GnRH can activate the unliganded GR resulting in transactivation of a synthetic GRE-reporter in both L β T2 and in COS-1 cells, in a GnRHR-dependent fashion (figure 4.2 and 4.3). These results suggest that the ability of the GnRHR to activate the unliganded GR is not gonadotrope-specific, and that the unliganded GR can regulate the GnRHR gene via recruitment to the AP-1 region of the GnRHR promoter. Additionally, it can be speculated that the GR can regulate other genes via direct binding to GREs in the absence of its ligand providing a potential novel mechanism of action for the nuclear receptor. Since the GnRHR is expressed in many extra-pituitary tissues (Hapgood *et al.*, 2005), cross talk between the GnRHR and the GR could occur in many cell types endogenously expressing both receptors to fine tune the biological effects of each other.

Since the GnRHR antagonist Antide abolished the GnRH-induced transcriptional response of a GRE-reporter construct (figure 4.2 E) it was further investigated whether the GnRH-mediated activation of the unliganded GR requires the activity of specific kinase pathways activated by the GnRHR. The study provides direct experimental evidence that GR phosphorylation is required for the GnRHR-mediated GnRH response of the GRE-reporter construct. Figure 4.8 and 4.11 show that MAPKs and PKC are involved in GnRH-mediated transactivation of the GRE by the GR in L β T2 cells. By probing the phosphorylation status of the GR using specific antibodies, it was found that GnRH mediates site-specific phosphorylation of the GR at Ser-234 in the absence of dex in intact L β T2 cells in a GnRHR-dependent fashion, since this phosphorylation can be inhibited with a GnRHR antagonist (figure 4.4). In addition, it was found that this GnRH-stimulated phosphorylation of the unliganded GR is mediated by a combination of the MAPKs JNK, p38 and ERK in L β T2 cells, since combinations of specific inhibitors thereof resulted in decreased GR phosphorylation in intact cells. Furthermore a PKC inhibitor was found to inhibit GnRH-induced GR phosphorylation in these cells. In addition several

reports (Bonfil *et al.*, 2004; Harris *et al.*, 2002; Liu *et al.*, 2002a) have shown by Western blotting and probing for phosphorylated MAPKs that GnRH treatment rapidly (within ten minutes) induces JNK, p38 and ERK activation in intact L β T2 cells (figures 4.5 – 4.7). These data support a direct role for GnRHR in phosphorylation of the unliganded GR via JNK, p38 and ERK. The findings are consistent with previous studies in L β T2 cells showing that GnRH induces phosphorylation of JNK and ERK, mediated predominantly by PKC and c-src, to activate several promoters such as LH- β , FSH- β or COX-1 (Bonfil *et al.*, 2004; Harris *et al.*, 2002; Liu *et al.*, 2002a; Naor *et al.*, 2007). Thus, it can be speculated that GnRH activation of MAPK signal transduction pathways via the GnRHR also leads to the activation and nuclear translocation of the endogenous GR protein. Moreover, inhibition of PKC (figure 4.13 A and B) or ERK alone (figure 4.10 E and G) decreased GnRH-mediated GR Ser-234 phosphorylation, which suggests that PKC may induce ERK, which in turn might be directly upstream of the GR phosphorylation event.

Protein kinases involved in GC-mediated GR phosphorylation have been studied intensively in cells other than L β T2 cells (Ismaili and Garabedian, 2004; Krstic *et al.*, 1997; Rogatsky *et al.*, 1998; Wang *et al.*, 2007). The results suggest that GC-mediated GR phosphorylation is dependent on multiple kinases in a cell-, promoter- and GC-dose-dependent manner (Chen *et al.*, 2008; Ismaili and Garabedian, 2004; Kino *et al.*, 2007; Webster *et al.*, 1997). However, to the present author's knowledge, agonist-mediated GR-phosphorylation at Ser-220 and Ser-234 and transactivation have not to date been directly linked to a particular kinase in intact cells, possibly due to a complex interplay of kinases involving multiple sites. MAPK pathways, in particular JNK and ERK, have been shown to be involved in phosphorylation of the rat GR at Ser-246 or human GR at Ser-211 (equivalent to mouse GR Ser-234) and to be involved in dex-mediated transcription supporting a role for JNK and ERK in GR phosphorylation and transcription (Krstic *et al.*, 1997; Miller *et al.*, 2007; Rogatsky *et al.*, 1998; Tsutsumi *et al.*, 1995). Miller *et al.* provided evidence for a positive role for p38 and a negative role for JNK in phosphorylation of the human GR at Ser-211 (equivalent to mouse GR Ser-234) in CEM-C7-14 cells (Miller *et al.*, 2005). The authors did not investigate the effect of the kinase inhibitor on total GR levels, thus making it difficult to interpret the data (Miller *et al.*, 2005). Chen *et al.* recently showed that turmeric, the yellow colouring agent in curry powder, inhibited human GR phosphorylation at Ser-211 and Ser-226 as well as transactivation via a GRE. However the kinase(s) targeted by the turmeric was

not established and specific inhibition of a wide range of MAPKs had no effect on GR phosphorylation in U2OS-hGR cells (Chen *et al.*, 2008). In the present study it was shown that combinations of JNK, ERK and p38 MAPK inhibitors decreased dex-induced phosphorylation of the endogenous mouse GR at Ser-234 after 30 min of ligand stimulation while the inhibitors on their own had no effect (figure 4.10 E and F). The data are difficult to interpret since short-term dex stimulation (up to 2 h) did not induce ERK, p38 and JNK phosphorylation while long-term (8 h) stimulation was found to only induce ERK activation in L β T2 cells. In the literature it is well established that GCs can cross talk with signalling pathways such as MAPK signalling (Hulley *et al.*, 1998; Kassel and Herrlich, 2007; Lasa *et al.*, 2001; Miller *et al.*, 2005; Pelaia *et al.*, 2001; Sasson *et al.*, 2003; Swantek *et al.*, 1997; Zhang *et al.*, 2000). In contrast to results presented in this study GCs have been shown to rapidly induce ERK and JNK phosphorylation within 30 min in rat ovarian follicular cells and human eosinophils (Sasson *et al.*, 2003; Zhang *et al.*, 2000) while Miller *et al.* reported dex-induced p38 phosphorylation after 20 h of continuous stimulation in human lymphoid cells (Miller *et al.*, 2005). It is possible that the discrepancies between these reports and results from the present study may be due to cell-specific effects and culturing conditions. Furthermore, GCs have been reported to interfere negatively with MAPK signalling (Engelbrecht *et al.*, 2003; Pelaia *et al.*, 2001). The underlying mechanism was shown to involve GC-mediated expression of the MAPK inhibitor, MAPK phosphatase (MKP) which dephosphorylates and inactivates p38 and ERK rapidly within 2 h (Imasato *et al.*, 2002; Kassel *et al.*, 2001; Lasa *et al.*, 2001). However, since ERK phosphorylation was observed at 8 h of dex stimulation it is rather unlikely that MKP-1 activity can explain the failure to detect MAPKs phosphorylation in L β T2 cells after short-term GCs treatment. Inhibition of JNK was reported to occur within less than a minute via direct interaction of JNK and the liganded GR in HeLa and COS-7 cells (Bruna *et al.*, 2003; Caelles *et al.*, 1997). In the above examples dex was proposed to attenuate activation of MAPK pathway induced by proinflammatory stimuli as an anti-inflammatory mechanism mediated by GCs binding to the GR. It is however possible that dex activates the MAPKs p38 and JNK in L β T2 cells at a time point not investigated in this study. Furthermore, the decrease in dex-mediated GR phosphorylation in the presence of combinations of JNK, ERK and p38 MAPK inhibitors could not be directly link with an effect of the kinase inhibitors on GR-mediated transactivation, since the p38 inhibitor slightly decreased transactivation of the GRE construct while the ERK pathway inhibitor alone had no effect on dex-mediated transactivation. Additionally, it was found that JNK inhibition increased

transactivation about 2-fold (figure 4.8 A). This result would suggest that the active form of JNK has an inhibitory effect on GRE-transactivation or that the JNK inhibitor inhibits other signalling molecules in L β T2 cells. Evidence for the latter hypothesis comes from reports in the literature showing that high concentration of a specific p38 inhibitor enhanced NF- κ B-driven promoter activity via activation of the ERK pathway (Birkenkamp *et al.*, 2000). Similarly, another study showed that the p38 inhibitor induced the activation of c-Raf which is an upstream activator of ERK, *in vivo* (Hall-Jackson *et al.*, 1999). Although in the present study the JNK inhibitor was shown to have no effect on ERK or p38 activation it cannot be excluded that other pathways or proteins such as RNA-polymerase II or phosphatases might be affected by the JNK inhibitor resulting in increased transactivation. Interestingly, it was found that inhibition of p38 resulted in a significant increase in JNK phosphorylation (Addendum A figure A4) and decreased GRE-transactivation. This is in agreement with the hypothesis that the active form of JNK exerts an inhibitory effect on transactivation. Taken together the results from the present study suggest that a complex interplay between MAPKs and possibly other kinases and phosphatases occurs in response to dex treatment to modulate mouse GR phosphorylation and transcription in L β T2 cells. However, further experiments are required to validate the results obtained with the pharmacological kinase inhibitors.

Interestingly, GnRH stimulation induced hyperphosphorylation of the GR at Ser-234 while dex stimulation induced hyperphosphorylation of the GR at both Ser-234 and Ser-220 in L β T2 cells (figure 4.4). Strikingly, the GnRH-induced phosphorylation was as rapid (within ten minutes) and similar in magnitude to the phosphorylation induced by dex at Ser-234, suggesting that non-genomic signalling cascades activated by GnRH are responsible for the unliganded GR phosphorylation. The finding that GnRH-induced GR phosphorylation was completely abolished in the presence of the GnRHR antagonist supports the idea that signalling pathways downstream of the GnRHR are mediating the response. To the present author's knowledge, this is the first report showing hyperphosphorylation of the GR with a non-GC ligand, resulting in a differential phosphorylation pattern as compared to dex. The significance of the differential pattern of GR phosphorylation obtained with GnRH versus dex is unclear, but suggests that the GR activated by GnRH may exert differential biological effects as compared to the GR activated by dex. Unpublished data from the laboratory of the present author as well as others has shown that phosphorylation of the human GR at Ser-211, equivalent to Ser-220 in

the mouse GR, has a positive effect on transactivation via a GRE while phosphorylation at Ser-226 of the human GR, equivalent to Ser-234 in the mouse GR, has a negative effect on transactivation via a GRE (Avenant, 2009; Ismaili and Garabedian, 2004). Furthermore, Blind *et al.* suggested that different GR phospho-isoforms selectively occupy different GR target genes (Blind and Garabedian, 2008). Consistent with the idea that GnRH-activated GR exhibits differential biological effects as compared to dex-activated GR, results presented in the present study show by ChIP assays in L β T2 cells that GnRH-induced recruitment of the GR to the endogenous GnRHR promoter is about 50% less than dex-mediated recruitment of the GR (figure 3.6), and that about 50% less GR is translocated to the nucleus upon GnRH versus dex stimulation (figure 4.1). The above results would be consistent with a mechanism involving a less efficient release of cytosolic GR inhibitory proteins and/or nuclear translocation of the GR induced by GnRH as opposed to dex in L β T2 cells. Additionally, the efficacy of dex-induced GR-mediated transactivation of a synthetic GRE in COS-1 cells overexpressing exogenous GR and GnRHR was considerably greater than the GnRH-mediated effect (figure 4.3 A and B). COS-1 cells express very low levels of GR and do not express detectable endogenous GnRHR protein (van Biljon, 2006). Therefore it is possible that some of the components downstream of the GnRHR that are required for maximal GnRHR-mediated activation of the unliganded GR are present in lower amounts in COS-1 cells than in L β T2 cells, resulting in weaker transcriptional activation of the unliganded GR by GnRH compared to activation of the GR by dex in COS-1 cells. These results further suggest that the extent of GnRHR-mediated activation of the unliganded GR may vary in different cell types depending on expression levels of both the GR and GnRHR.

6.3 Synergism occurs between the dex and GnRH response

The finding that dex synergistically enhances the GnRH-induced increase in endogenous GnRHR gene expression in the L β T2 gonadotrope cell line is consistent with other reports of synergistic action of these hormones in the pituitary. GnRH has been found to act synergistically with the steroid hormones estradiol and progesterone, as well as with activin (An *et al.*, 2006; Kowase *et al.*, 2007; Yasin *et al.*, 1995). GnRH and estradiol synergistically upregulate pituitary GnRHR mRNA production in female rats (Yasin *et al.*, 1995). Furthermore, co-stimulation with GnRH and estradiol was found to synergistically increase LH β and α -subunit promoters (Kowase *et al.*, 2007) but not an ERE-reporter construct (Chen *et al.*, 2009) in L β T2 cells. An *et al.* reported that GnRH-induced PRE-promoter

activity was synergistically augmented in the presence of progesterone in α T3-1 cells (An *et al.*, 2006). However, the precise mechanisms thereof remain to be determined. GCs have been shown to synergistically enhance the GnRH-induced increase in endogenous GnRHR pituitary mRNA in rats (Rosen *et al.*, 1991), as well as FSH secretion in rat pituitary fragments (D'Agostino *et al.*, 1990). An important synergistic physiological role for GCs and the GR in stimulating reproductive function in rat pituitary gonadotropes is further supported by the finding that GCs synergistically enhance the activin-induced increase in FSH β gene expression in both primary rodent pituitary cells (Leal *et al.*, 2003) and in L β T2 cells (McGillivray *et al.*, 2007). In contrast, the presence of both activin and dex did not have a synergistic effect on transactivation of a GnRHR promoter-reporter construct (McGillivray *et al.*, 2007) while others found that activin reduced the dex-induced transactivation of a transfected human glycoprotein hormone α -subunit (α GSU) luciferase reporter plasmid in L β T2 cells (Sasson *et al.*, 2008) suggesting that synergism between activin and the GR is promoter-specific. In the same study it was shown that co-treatment with dex and GnRH resulted in a synergistic increase in α GSU promoter activity. Interestingly, results in figure 5.1 show that GnRH and dex act synergistically on both a synthetic GRE-reporter in COS-1 and L β T2 cells, as well as on the endogenous GnRHR gene in L β T2 cells, suggesting that the mechanism of synergism between the GR and the GnRH receptor is not gonadotrope- or promoter-specific.

The finding that dex plus GnRH results in a similar pattern of GR phosphorylation at Ser-220 and Ser-234 compared to that obtained with dex alone (figure 5.3) suggests that differential phosphorylation at these GR residues does not play a role in the synergism. Since in the present study only two specific serine residues were investigated, Ser-220 and Ser-234 respectively, it cannot be excluded that other GR phosphorylation sites may be involved.

In L β T2 cells it was further shown that synergism is not due to increased GR protein levels since co-treatment with dex and GnRH was found to significantly decrease GR protein levels relative to vehicle control (figure 5.1 E and F). In fact it is possible that the dex plus GnRH-mediated synergistic increase in transactivation results in faster GR turnover compared to the hormones on their own in L β T2 cells. Unpublished data from our laboratory demonstrate a correlation between ligand-induced maximal transcriptional activity and GR turnover (Avenant, 2009) with strong GR ligands inducing maximal transactivation and rapid GR turnover. In the present study it was also found that in COS-1 cells dex

treatment decreased GR protein levels relative to control which is in agreement with reports in the literature (Webster *et al.*, 1997), while co-stimulation with both dex and GnRH prevented dex-induced down-regulation of the GR (figure 5.2 C and D). The results indicate that a different mechanism is involved for the liganded versus the unliganded GR in these cells. It is interesting to point out that GR levels were affected differentially in L β T2 cells (figure 5.1 E and F) and COS-1 cells (figure 5.2 C and D) in response to dex, GnRH or a combination of both hormones which could be explained by differential GR activation and turnover occurring in a cell - and/or species-specific fashion.

Furthermore it was shown by ChIPs that synergism does not involve differential recruitment of GR (figure 5.6), AP-1 proteins (figure 5.5), GRIP-1 or p300 (figure 5.7), as compared to dex or GnRH alone (illustrated in figure 6.1 D). By contrast the results suggest a role for SRC-1 since dex plus GnRH resulted in increased recruitment of this co-activator to the AP-1 region of the GnRHR promoter (figure 5.7). This finding might in part explain the synergistic effect of dex plus GnRH on transcriptional expression of the GnRHR gene. It is tempting to speculate that SRC-1 is recruited to the promoter to further enhance the transcriptional effect of other cofactors already bound to the promoter, such as GRIP-1 (figure 6.1 D). A study by Smith *et al.* supports this hypothesis showing that simultaneous expression of exogenous SRC-1 and CBP synergistically enhances ER and PR transcriptional activity of a reporter gene in HeLa cells (Smith *et al.*, 1996). However, the precise mechanisms involved in the synergistic transcriptional effect of dex plus GnRH requires further investigation. Collectively the results suggest that cross talk between steroid receptor and peptide hormone receptor signaling pathways may be a common strategy, involving different mechanisms, for fine-tuning the reproductive response of the pituitary gonadotropes to changes in the hormonal milieu.

6.4 Ligand-independent activation of the GR and other steroid receptors: physiological implications

A few reports in the literature suggest that ligand-independent activation of the GR occurs in cells other than L β T2 cells. Tanaka *et al.* found that in hamster ovary cells UDCA promotes nuclear translocation of the GR mediated by the ligand-binding domain of the GR (Tanaka *et al.*, 1996). In primary human lung fibroblasts and vascular smooth muscle cells the GR was rapidly depleted from the cytosol, translocated into the nucleus, and activated a GR-inducible luciferase reporter gene in response to β_2 -agonists (Eickelberg *et al.*, 1999). The authors suggested a possible role for

cAMP/PKA pathways in the ligand-independent activation of the GR in these cells (Eickelberg *et al.*, 1999). However, whether these examples of ligand-independent activation of the GR occur by rapid non-genomic mechanisms such as GR phosphorylation, or cross talk with others receptors is not known.

Several reports have been published describing ligand-independent activation of steroid receptors other than the GR. The estrogen receptor (ER) has been shown to be activated in a ligand-independent fashion by EGF via mechanisms involving PKA and MAPKs and phosphorylation of the ER at Ser-118 (Bunone *et al.*, 1996; El-Tanani and Green, 1997). Phosphorylation of the human PR at Ser-400 has been shown to be mediated by cyclin-dependent protein kinase 2 (CDK2) in response to EGF in breast epithelial cells (Pierson-Mullany and Lange, 2004). By contrast, An *et al.* reported that GnRH-induced rapid ligand-independent phosphorylation of the mouse PR at Ser-294 and not at Ser-400 in α T3-1 cells (An *et al.*, 2006). They also reported nuclear translocation of the unliganded mouse PR and a requirement for SRC-3 in PR transactivation in response to GnRH in a GnRHR-dependent manner (An *et al.*, 2006). Similarly, a later study by the same authors demonstrated GnRH-induced increase in FSH β mRNA levels in a PR-, and SRC-3-dependent fashion in L β T2 cells (An *et al.*, 2009). The results from the latter study further suggest that GnRH-induced phosphorylation of the PR at Ser-243 is required for increased PRE-promoter activity and FSH β mRNA levels in these cells. Moreover, a recent study by Chen *et al.* demonstrates that GnRH-induced rapid phosphorylation of the ER and transactivation of an ERE-reporter via the GnRHR in L β T2 cells (Chen *et al.*, 2009). The authors further show a requirement for the ER and pCAF for the GnRH-mediated gene expression of fosB in these cells (Chen *et al.*, 2009). Taken together with findings from the present study, these results suggest that the GnRHR can cross talk to both the GR and the PR via a mechanism involving steroid receptor phosphorylation. Thus it appears that ligand-independent activation of steroid receptors by phosphorylation may be a common mechanism of receptor activation in several different cells, via several different pathways.

The present study provides the first evidence that GnRH acting via a GPCR, the GnRHR, can cross talk with the GR. Furthermore the results suggests that this occurs via a novel non-genomic mechanism resulting in rapid site-specific GR phosphorylation at Ser-234 involving PKC and MAPK's, leading to nuclear translocation and transactivation of a GRE in L β T2 cells. Since GnRH regulates

several genes in pituitary gonadotropes, this non-genomic cross talk represents a mechanism whereby the GnRHR and unliganded GR may modulate expression of several GnRH and GR target genes. Thus cross talk may have important physiological implications since GR expression is widespread and the GnRHR is expressed in pituitary gonadotropes as well as many extra-pituitary tissues (Hapgood *et al.*, 2005). This could be a mechanism for fine-tuning interplay between reproductive, stress and immune responses. Several lines of evidence show that the neuroendocrine, immune, inflammatory and stress-response systems are functionally integrated and bidirectionally regulated (An *et al.*, 2006; Chrousos and Gold, 1992; Da Silva *et al.*, 1993; Elenkov and Chrousos, 1999). For example, stress or chronic activation of the HPA-axis suppresses reproduction via elevated GC levels that exert their effects at all levels of the HPG-axis (Rivier and Rivest, 1991). In the present study a direct transcriptional effect of GCs on GnRHR gene expression via the GR in a pituitary gonadotrope cell line was shown, representing another mechanism whereby the HPA axis could modulate the HPG axis.

6.5 Proposed model for genomic and non-genomic cross talk of GnRHR and GR signalling pathways in LBT2 cells

The results from the present study are summarized as proposed models shown in figure 6.1 and 6.2. In the absence of hormone the endogenous GnRHR promoter appears to be occupied by c-Jun and c-Fos heterodimers which was discussed under section 6.1. In addition, by CHIP it was shown that p300 occupies the proximal promoter as indicated in panel A of figure 6.1. Current models suggest that binding of p300 assists in positioning histone acetyltransferases (HATs) in target gene promoter regions and it has been proposed that p300 can interact with components of the general transcriptional machinery such as RNA-polymerase II or transcription factor (TF) IID or TFIIB (Sterner and Berger, 2000; Vo and Goodman, 2001). Conversely, p300 is also known to play a negative role in transcription (Santoso and Kadonaga, 2006). Although the role of p300 in GnRHR gene expression was not established in the present study, the results suggest that p300 has a negative effect on GnRHR gene expression. Unpublished data from our laboratory suggest that SF-1 occupies the nuclear receptor sites (NRSs) of the GnRHR promoter located at positions -244/-236 and -15/-7 relative to the translational start site, under unstimulated conditions *in vitro*, possibly recruiting a corepressor (Sadie, 2006). Furthermore it was found that GnRH stimulation results in decreased SF-1

binding to these NRSs *in vitro* (Sadie, 2006). Since p300 has been proposed to bind to SF-1 (Monte *et al.*, 1998; Vo and Goodman, 2001) it can be speculated that p300 and SF-1 might interact under basal conditions on the endogenous GnRHR promoter in L β T2 cells resulting in transcriptional inhibition. This would be in agreement with the ChIP data presented in figure 5.7 showing that p300 binding is decreased under stimulated conditions although significance could not be established due to variation in the response and more repeats are needed to establish significance. Alternatively, the AP-1 proteins c-Jun and c-Fos that associate with the GnRHR promoter could recruit p300 and thus maintain basal transcriptional activity (indicated by an arrow in figure 6.1 A). The role of p300 in GnRHR gene regulation as well as the specific binding site, however, remain to be determined in future experiments.

Besides the possibility that p300 is removed from the promoter in response to hormone it is also possible that p300 mediates the assembly of transcription factors which reduces the accessibility of the epitope recognized by the p300 antibody. In a recent report by Stossi *et al.* p300 was proposed to act as a bridging factor between the ER α and coregulator complexes (Stossi *et al.*, 2009). It is tempting to speculate that p300 bound to c-Jun/c-Fos enables recruitment of the GR and GRIP-1 in response to dex. Interestingly, Rogatsky *et al.* demonstrated recruitment of GRIP-1 to a complex containing GR directly bound to AP-1 proteins in intact U2OS cells at a collagenase3 response element regardless of the composition of AP-1. However, this interaction resulted in transcriptional repression of the gene dependent on the liganded GR (Rogatsky *et al.*, 2001). To the present author's knowledge this is the first report showing GRIP-1 recruitment to a positively GR regulated gene without a DNA-binding site for the GR. Although a direct interaction between GR and GRIP-1 was not established the data from the present study indicate that both GR and GRIP-1 are recruited to the AP-1 region of the GnRHR promoter without direct binding of the GR to the DNA thus presenting a novel mechanism of GR dependent transcriptional activation mediated via cross talk with AP-1 (figure 6.1 B).

Results from the present study indicate that a complex interplay between MAPKs and possibly other kinases and phosphatases occurs in response to dex treatment to modulate mouse GR phosphorylation and transcription in L β T2 cells. However, within the scope of this study the detailed kinase cascade involved in dex-mediated transcriptional regulation of the GnRHR was not fully

established. Interestingly, progestin binding to PR-B has been shown to mediate non-classical gene transcription through rapid extra-nuclear activation of the epidermal growth factor receptor (EGFR), c-src, and ERK MAPK cascade to stimulate phosphorylation of PR-B at Ser-345. PR phosphorylated at Ser-345 was found to tether to Sp1 to regulate EGFR and p21 gene transcription (Favre *et al.*, 2008). A similar non-classical mechanism could be utilized by the GR, possibly involving cross talk with membrane receptors such as the EGFR or the GnRHR. A study by Asadi *et al.* supports this hypothesis demonstrating that the FMS-like tyrosine kinase 3 directly interacts with the GR thereby affecting GR signalling in rat hepatoma cells (Asadi *et al.*, 2008).

The finding that the GR is recruited to the GnRHR promoter by GnRH strongly suggests another novel mechanism of action of the receptor in the absence of ligand possibly mediated via receptor cross talk (figure 6.1 C). Sex steroid hormone receptor functions have been shown to be affected by 7 transmembrane receptors (TMR) (An *et al.*, 2006; Bunone *et al.*, 1996; Maudsley *et al.*, 2006; Turgeon and Waring, 1994). GnRH was found to induce nuclear translocation of the PR in rat anterior pituitary cells (Turgeon and Waring, 1994) and α T3-1 cells (An *et al.*, 2006) in a steroid-independent fashion. However, the mechanism involved including signalling pathways or target domains of these steroid receptors were not investigated by these authors. In the present study the data suggest that PKC as well as MAPKs might be mediators of GnRH signalling leading to activation of the GR. It is intriguing whether cross talk occurs via direct interaction of the GR and the GnRHR, G-proteins or kinases. Recent reports demonstrate that steroid receptors are associated with the plasma membrane. Jain *et al.* provide the first evidence for a functional contribution of membrane-raft associated GR in Hep3B cells (Jain *et al.*, 2005) while another group found that the ER is associated with the plasma membrane via caveolae in endothelial cells (Razandi *et al.*, 2002; Razandi *et al.*, 1999). In addition the GnRHR has been proposed to co-localize with membrane rafts in association with $G_{\alpha q/11}$ and raf (Bliss *et al.*, 2007). Thus it is tempting to speculate that binding of GnRH to the GnRHR in L β T2 cells triggers the assembly of a multiprotein complex including components of signalling pathways and the GR in association with specialized membrane rafts at the plasma membrane to exhibit the activation of the steroid receptor.

The finding that GnRH fails to induce GRIP-1 recruitment to the GnRHR promoter suggests that GRIP-1 acts as a selective co-activator for the dex-activated GR and not for the unliganded GR. Recent studies demonstrate that differences in the phosphorylation status of the GR influence the interaction of the GR with other proteins (Chen *et al.*, 2008; Galliher-Beckley *et al.*, 2008; Ismaili *et al.*, 2005; Kino *et al.*, 2007). Studies on the ER show that the phosphorylation status of one specific serine residue of the ER determines the recruitment of co-activator versus co-repressor (Endoh *et al.*, 1999; Gburcik *et al.*, 2005; Likhite *et al.*, 2006; Weigel and Moore, 2007). Results from the present study demonstrate differential GR phosphorylation for dex and GnRH at Ser-220 and Ser-234. Thus, in L β T2 cells GRIP-1 recruitment to the GnRHR promoter could be determined by the phosphorylation status of specific serine residues of the GR, i.e. Ser-220 which is rapidly and transiently hyperphosphorylated by dex and not GnRH. This is in agreement with a recent study showing that phosphorylation of the human GR at one or more of residues Ser-226, Ser-211 and Ser-203 (equivalent to mouse Ser-220, Ser-234 and Ser 212) is required for maximal transactivation efficacy and importantly for GRIP-1 recruitment to the MMTV promoter in intact COS-1 cells (Avenant, 2009). Interestingly, An *et al.* found that GnRH promotes PR interaction with SRC-3 and co-recruitment of both PR and SRC-3 to a PRE in the gonadotropin α -subunit gene (An *et al.*, 2006). Although the recruitment of SRC-3 to the GnRHR promoter was not investigated in the present study it is possible that SRC-3 or another cofactor not investigated in this study is recruited to the AP-1 region to regulate the activity of the unliganded GR in response to GnRH (illustrated as “Y” in figure 6.1 C).

The commonly held hypothesis is that GR dimerization is essential for binding of the receptor to a GRE and induction of gene expression but dispensable for repression of genes under the control of AP-1 or NF- κ B (De Bosscher *et al.*, 1997; Heck *et al.*, 1994; Reichardt *et al.*, 1998; Reichardt *et al.*, 2001; Tuckermann *et al.*, 1999). Although the stoichiometric composition of the GR has not been investigated in the present study it can be hypothesized that the liganded-GR binds to the AP-1 region in the GnRHR promoter as a dimer (figure 6.1 B), although this theory needs to be tested with additional experiments. Support for the involvement of a GR dimer rather than a GR monomer in dex-mediated GnRHR transactivation on the GnRHR promoter (figure 6.1 B) comes from results in the present study showing that the AP-1 site is not the sole *cis*-regulatory element in conferring the response, thus making it likely that a GR dimer is required for GR binding to both AP-1 and factor “X”

as illustrated in figure 6.1 B. Furthermore, since GnRH treatment appears to recruit GR to a lesser extent than dex to the GnRHR promoter (figure 3.6) it is tempting to speculate that the unliganded-GR might act as a monomer on the GnRHR promoter to regulate gene expression (figure 6.1 C). It is possible that GnRH induces a differential GR conformation compared to dex in favour of the formation of active GR monomers. This is in agreement with results from the present study showing that dex but not GnRH induces GRIP-1 binding where a different binding surface of the GR, as presented by a monomer versus a dimer, could in part explain the differential co-factor recruitment (figure 5.7). In addition, since co-stimulation with dex plus GnRH induces GR recruitment to the AP-1 region of the GnRHR promoter with levels similar to that observed with dex alone it is tempting to speculate that the presence of both hormones induces formation of a GR dimer possibly consisting of a ligand-dependent and ligand-independent activated GR monomer (figure 6.1 D). Furthermore, the formation of this novel dimer might thereby enable interaction with differential co-factors such as SRC-1 through a novel binding site formed by this GR dimer. However, it is clear that more experiments are needed to establish the role of GR dimerization in GnRHR regulation.

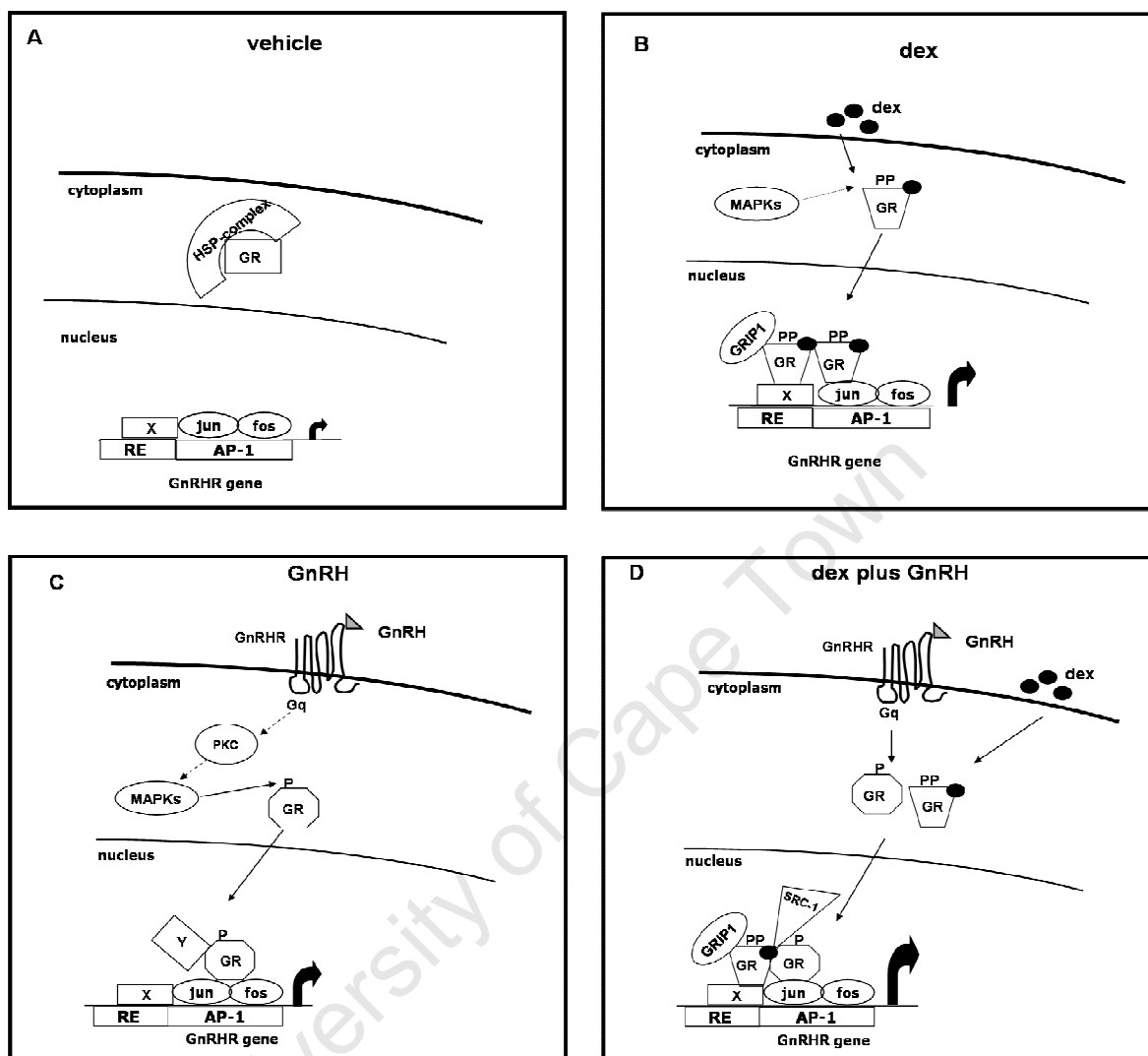


Figure 6.1: Model for genomic and non-genomic cross talk of the gonadotropin-releasing hormone receptor and glucocorticoid receptor signalling pathways converging at the GnRHR gene in L β T2 cells

A proposed model for GR-mediated regulation of the GnRHR promoter in L β T2 cells in the absence (A), or presence of dex (B) or GnRH (C) or both dex and GnRH (D) is shown. The c-Jun and c-Fos proteins are shown bound to the AP-1 site in the GnRHR promoter under basal and hormone-stimulated conditions. The cofactor p300 is shown associated with the AP-1 region only under basal condition (A). The GR is depicted in two different conformations, one activated by dex with the GR phosphorylated at Ser-220 and Ser-234 (indicated by two "Ps"), and another activated by GnRH which binds the GnRHR to activate Gq (Liu *et al.*, 2002b), resulting in activation of PKC and MAPKs, in particular ERK, to mediate GR site-specific phosphorylation at Ser-234 (indicated by one "P") via a rapid non-genomic mechanism. It can be speculated that a transcription factor X bound at an as yet unidentified response element (RE) is required for the dex response in B, since dex did not found

to increase the transcriptional response on a simple AP-1 promoter-reporter. The co-activator GRIP-1 is shown associated with the dex-activated GR in B and D, consistent with the results. It can be speculated that another as yet unidentified co-activator Y is associated with the GnRH-activated GR in C. Consistent with the data, recruitment of SRC-1 co-activator is only shown in D. The model would be consistent with the data, although it should be noted that this study does not provide proof for the monomer versus dimer status of the GR, or for a direct interaction between the GR and AP-1 proteins or factor X, or for a lack of involvement of other cofactors, kinases or phosphatases not investigated.

Result from the present study suggest that non-genomic cross talk between the GR and the GnRHR signalling pathways do not only converge at the GnRHR promoter but also at GRE-containing genes since GnRH induced a minimal GRE in both L β T2 cells and COS-1 cells (figure 4.2 and 4.3). Although it was established that both the GR and the GnRHR are required for this response, the present study did not investigate whether the effect occurs on endogenous genes or the precise mechanism thereof. However, it can be assumed that the dex-activated GR binds to the GRE-promoter as a dimer to induce transactivation (illustrated in figure 6.2 B) as reported in the literature (Chalepakis *et al.*, 1990; Griekspoor *et al.*, 2007; Reichardt *et al.*, 1998). In addition it is tempting to hypothesize that the dex-activated GR recruits a co-activator, most likely GRIP-1 to the minimal reporter thereby inducing transactivation (figure 6.1 B). Evidence for this hypothesis comes from recent reports showing dex-dependent GRIP-1 binding to GRE-bound GR *in vitro* and to the MMTV-promoter in COS-1 and COS-7 cells, respectively (Avenant, 2009; Ronacher *et al.*, 2009). Furthermore, since it has been reported in the literature that GR dimerization is a requirement for GR-mediated transactivation of simple GRE's it can be speculated that GnRH-induced increase in transcriptional activity of a GRE-reporter construct is mediated by GR dimers as illustrated in figure 6.2 C (Reichardt *et al.*, 1998; Reichardt *et al.*, 2001). Interestingly, it has been shown that GnRH-activated PR and ER recruit co-factors to simple hormone-response elements in mouse pituitary cell lines (An *et al.*, 2006; Chen *et al.*, 2009). While An *et al.* showed recruitment of the PR and SRC-3 to the PRE on a reporter plasmid and the endogenous gonadotropin α -subunit gene promoter in α T3-1 cells, a recent study by Chen *et al.* demonstrated that GnRH-activated ER directly interacts with the p300/CBP-associated factor (pCAF) and furthermore the authors showed that the ER and pCAF are recruited to an ERE-reporter construct as well as the endogenous fosB gene containing a known ERE (An *et al.*, 2006; Chen *et al.*, 2009). Thus, it can be speculated that GnRH-mediated GRE-transactivation found in the present study involves co-factor recruitment to mediate the response in L β T2 cells (figure 6.2 C). Interestingly, it was found that co-

stimulation with dex plus GnRH results in a synergistic effect on GRE-transactivation as observed for the endogenous GnRHR gene which lacks a GRE. Thus, it is tempting to speculate that co-treatment with both hormones induces the formation and binding of a novel dimer composed of a ligand-dependent and ligand independent activated GR as described for the GnRHR gene above (figure 6.1 D). This novel dimer might enable the recruitment of similar co-factors such as GRIP-1 and SRC-1 thereby resulting in synergism (illustrated in figure 6.2 D). However, it is clear that further investigations are needed to establish the mechanism involved in transcriptional activation of a GRE by GnRH in these cells.

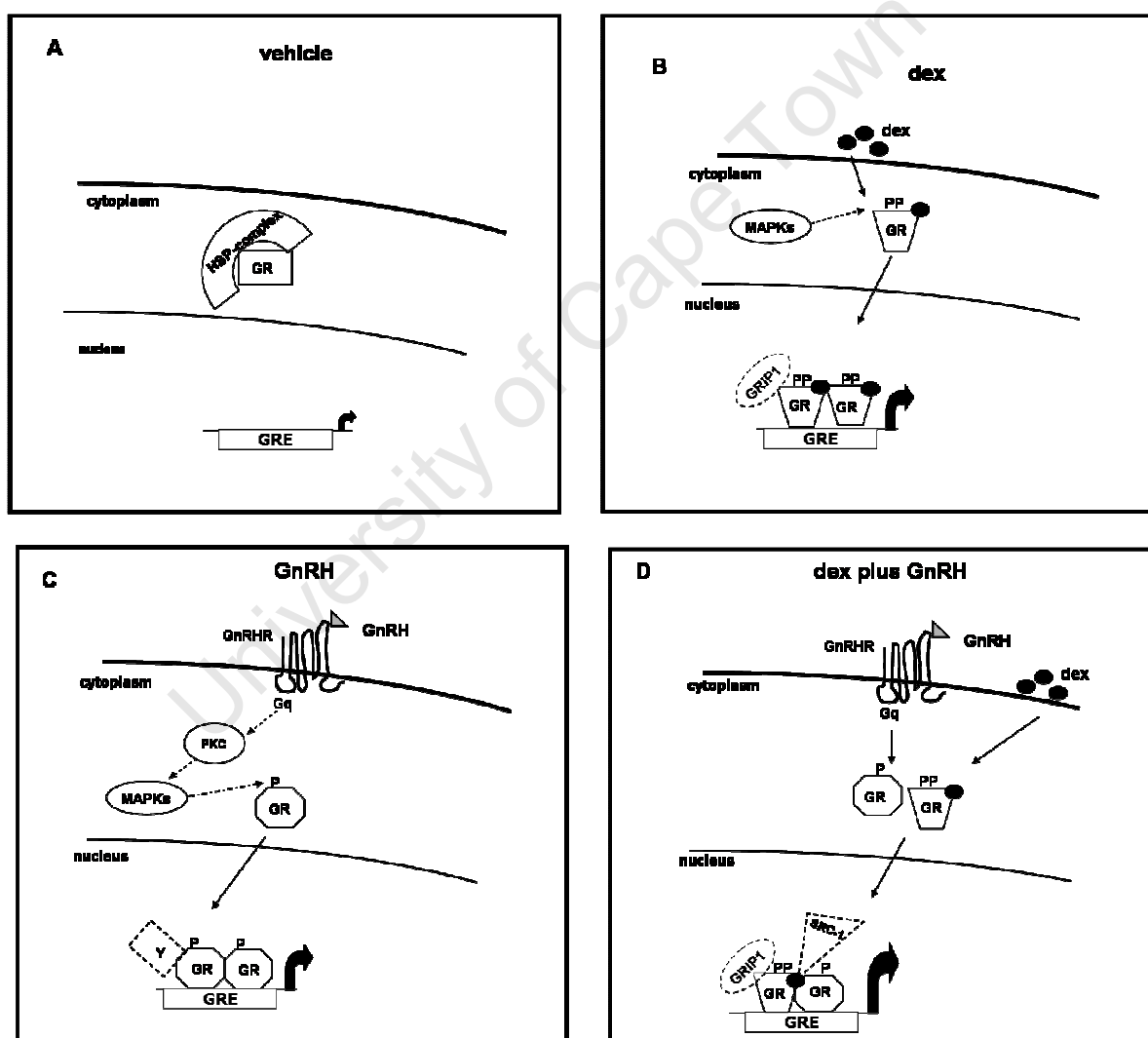


Figure 6.2: Proposed model for transcriptional regulation of a GRE in response to dex, GnRH and a combination thereof in L β T2 cells (legend to follow on following page)

A proposed model for GRE-transactivation in the absence (**A**), or presence of dex (**B**) or GnRH (**C**) or both dex and GnRH (**D**) is shown. The GR is depicted in two different conformations, one activated by dex with the GR phosphorylated at Ser-220 and Ser-234 (indicated by two "Ps"), and another activated by GnRH resulting in GR site-specific phosphorylation at Ser-234 (indicated by one "P") via a rapid non-genomic mechanism. Although the present study does not provide proof for the involvement of a GR dimer or the recruitment of co-activators to the GRE promoter it can be speculated that the dex-activated GR is associated with GRIP-1 (B and D) while an unknown co-activator Y is associated with the GnRH-activated GR in C. It can further be hypothesized that the synergistic effect of co-treatment with dex and GnRH on GRE-transactivation might be due to the recruitment of yet another cofactor such as SRC-1 (as observed for the GnRHR gene).

The data presented in this study strongly suggest that simultaneous activation of the GR by GCs and the GnRHR by GnRH induces simultaneous interaction of several transcription factors including AP-1, the GR, GRIP-1 and SRC-1 which could contribute to transcriptional synergy. It can be speculated that cross talk between the GR and the GnRHR may occur at several levels including modulation of each others signalling pathways whereby GnRHR signalling pathways modify ligand-dependent as well as ligand-independent GR transactivation and in turn the activated GR modulate GnRHR signalling pathways. There is support for this hypothesis in the literature. Kino *et al.* demonstrated that the liganded GR directly interacts with G-protein- β and comigrates to the plasma membrane in human colon carcinoma cells HCT116 (Kino *et al.*, 2005). Thus, it is likely that the GR associated with G-proteins modulates the activity of the GnRHR at the plasma membrane via direct protein - protein interactions with GnRHR-coupled G-proteins (Kino *et al.*, 2005). Further evidence for an effect of the liganded GR on other receptor pathways is supported by findings from another group demonstrating that GCs inhibit insulin signalling through a GR-dependent and transcription-independent mechanism in adipocytes and T lymphocytes (Lowenberg *et al.*, 2006a).

6.6 Conclusions

In summary the present study demonstrates for the first time that rapid non-genomic as well as genomic cross talk occurs between the GnRHR and GR signalling pathways in L β T2 cells. It was established that GnRH and dex increase expression of the endogenous GnRHR in a GR-dependent fashion converging on the same AP-1 site on the mouse GnRHR promoter in L β T2 cells. Additionally this study provides strong evidence for ligand-independent activation of the GR by GnRH. This novel mechanism was found to be mediated by the GnRHR, resulting in GR phosphorylation, nuclear translocation and GR-mediated transcriptional regulation in both L β T2 cells and COS-1 cells. The results suggests that the ability of the GnRHR to activate the unliganded GR is not gonadotrope-specific, and that the unliganded GR can regulate the GnRHR gene via recruitment to the AP-1 region of the GnRHR promoter as well as regulate other genes via direct binding to GREs. Furthermore it was established that GnRH and dex together act synergistically to upregulate the mouse GnRHR promoter in a GR- and GnRHR-dependent fashion, most likely involving increased recruitment of SRC-1.

Taken together the results reported in the present study underscore the importance of cross talk between membrane-associated receptors and intracellular receptors and the functional consequences of cross talk. Furthermore the results suggest a novel mechanism whereby the HPG axis could modulate the HPA axis and immune function where GR-dependent stress, immune or inflammatory response could be modulated by GnRH via GnRHR-dependent activation of the unliganded GR.

6.7 Future perspectives

The results from the present study show that the GR and GnRHR pathways cross talk to each other by rapid non-genomic as well as genomic mechanisms. Yet, many questions remain unanswered. For example, what is the function of c-Jun, c-Fos or p300 on the GnRHR promoter under basal conditions? Whether c-Jun, c-Fos, or p300 have a structural role or a direct positive activation role in transcription of the GnRHR can be investigated by knock down experiments or by utilizing dominant-negative proteins that cannot bind DNA or that have no transcriptional capacity. In addition, it would be of interest to investigate the phosphorylation status of c-Jun and c-Fos under basal as well as dex- and GnRH-mediated conditions and further determine by ChIP whether the phosphorylated forms of c-Jun and c-Fos occupy the AP-1 region of GnRHR promoter under hormone stimulated conditions. Since the AP-1 site in the GnRHR promoter was found not to be the sole regulatory region in

mediating the dex-response, 5'-deletion mutants of the GnRHR promoter or constructs carrying mutations in one or more cis-regulatory element(s) transfected into L β T2 cells could be analysed to identify other regulatory *cis*-elements (indicated as "RE" in figure 6.1 B) in the GnRHR promoter required for the response. The results obtained with such promoter-constructs could be further investigated by ChIPs or knock down experiments establishing a requirement as well as occupancy of this regulatory element (illustrated as "X" in figure 6.1 B). In addition co-immunoprecipitation assays may reveal a direct interaction of the GR with the transcription factors identified in experiments described above. Additionally, it would be important to identify by ChIPs which cofactor is recruited by the unliganded GR to induce GnRHR transcription (indicated as "Y" in figure 6.1 C). By Western blotting the protein expression levels of already identified cofactors such as GRIP-1, SRC-1 and p300 as well as cofactors and transcription factors identified in experiments as described above could be investigated in response to dex, GnRH or a combination thereof.

An intriguing question is how GnRH activates the GR and whether the receptor activated in the absence of GCs has different biological activity compared to the dex-activated GR. As shown in figure 4.2 GnRH stimulation resulted in a differential phosphorylation pattern to that obtained with dex. It would be useful to analyze several other phospho-specific serine residues of the mouse GR since phosphorylation of the receptor has been proposed to play an important role in regulating transcription (Avenant, 2009; Bodwell *et al.*, 1991; Chen *et al.*, 2008; Hoeck and Groner, 1990; Kino *et al.*, 2007; Mason and Housley, 1993; Webster *et al.*, 1997). Moreover, other post translational modifications could be investigated, such as the acetylation status of the GR, to gain further insight into the mechanism whereby GnRH activates the receptor. Additionally, it would be interesting to study the receptor domains of the GR that are essential for GnRH-induced activation, possible structural changes in GR conformation as well as the implications thereof for GR function. In order to do so the effect of several GR constructs containing mutations in the structural domains such as AF-1, AF-2/LBD the DBD and the hinge region as well as the effect of isolated receptor domains on transactivation in response to ligand can be investigated.

When considering the result presented in figure 4.2 and 4.3 demonstrating transactivation of a minimal GRE in both L β T2 cells and COS-1 cells it appears that GnRH regulates GRE-containing genes via cross talk with the GR. To confirm that GnRH induces gene expression in a GRE-dependent fashion, a mutant of a GRE-promoter construct carrying mutations in the GRE sites or GRE deletion mutants

could be transfected into L β T2 cells. The results obtained with such mutant promoter constructs could establish a requirement of a GRE for GnRH-induced transcription. Similarly, the observation that dex plus GnRH induce a synergistic increase in GRE-transactivation suggests that GRE-containing genes are regulated by both dex and GnRH via a non-classical GR mechanism. Thus, it would be particularly interesting to identify endogenous GRE-containing GR target genes which are regulated by dex and GnRH synergistically and unravel the molecular mechanism thereof. In addition it would be important to establish the recruitment of the GR as well as co-factors to the GRE in response to dex, GnRH or a combination thereof in L β T2 cells.

In order to explore whether GR dimerization is a requirement for the dex- and GnRH-mediated transcriptional activation of the GnRHR gene and a GRE-reporter, GR mutants unable to dimerize could be utilized in overexpression studies in both L β T2 and COS-1 cells. Furthermore, it would be of interest to investigate other genes that have been shown to be regulated by GnRH such as LH β (Kowase *et al.*, 2007), FSH β (Gregory *et al.*, 2005; Wang *et al.*, 2008) as well as genes recently identified by microarray analysis involved in transcriptional regulation, cell growth or metabolism (Kakar *et al.*, 2003) and determine whether transcriptional regulation of these genes involves ligand activated or unliganded GR as found for the GnRHR gene in the present study.

Another fascinating question is whether cross talk of the GnRHR and the GR involves direct interaction of the GR with components involved in GnRHR signalling such as G-proteins or kinases and whether these interactions might be associated within microdomains at the plasma membrane. Since it has been shown that the GR is rapidly phosphorylated in response to GnRH (within 10 min, figure 4.4) and that this phosphorylation event could be blocked with the GnRHR antagonist, it appears that signalling pathways downstream of the GnRHR are involved. Thus, it would be sensible to investigate the presence of microdomains, such as caveolae or membrane rafts, in L β T2 cells and to determine whether the GR, the GnRHR, G-proteins, kinases such as PKC or MAPKs are associated in a multiprotein complex in the absence or presence of hormone. Ligand-independent activation of the GR by the GnRHR could occur in these specialized membrane domains by assembling a distinct protein complex at the plasma membrane. This hypothesis would be particularly interesting to follow up since reports in the literature indicate that steroid receptor and GPCRs can interact at the plasma membrane (Asadi *et al.*, 2008; Bliss *et al.*, 2007; Dobkin-Bekman *et al.*, 2009;

Navratil *et al.*, 2003; Razandi *et al.*, 2002; Roberson *et al.*, 2005). Additionally it would be of great interest to establish whether posttranslational modifications such as phosphorylation of the GR occur within these microdomains. Similarly, it would be important to investigate the possible role of proteins associated with the GR such as heat shock proteins (Griekspoor *et al.*, 2007) or phosphatases (Wang *et al.*, 2007) in cross talk with the GnRHR. The results might yield valuable information on the mechanism of non-classical and non-genomic modulation of GR activity via a GPCR.

GPCR and GR signalling pathways could bidirectional modulate each other's activity: Activation of the GR by GCs could potentially modulate a GPCR signalling pathway. Thus, it would also be exciting to investigate whether ligand-activated GR can modulate GnRHR signalling. It could be explored whether the GR associates with the GnRHR in the above mentioned membrane domains in the presence of GCs and thereby directly activating or inhibiting GPCR signalling by modulating coupling to G-proteins, and activating or inhibiting downstream kinase or transcription factors of the GPCR pathway. Furthermore, it would be interesting to examine whether GCs directly interfere with GPCR function by modulating post-translational modifications of the membrane receptor such as phosphorylation, glycosylation or palmitoylation.

Multiple ligands act simultaneously on pituitary gonadotropes. In addition to GnRH, gonadal steroids, peptide hormones, neurotransmitters and pituitary hormones have been demonstrated to act as regulators (Evans, 1999). Thus, stimulation of cells with a single ligand *in vitro* does not reflect the effects of several ligands acting collectively *in vivo* and raises the question of how relevant the results obtained in immortalized cell lines like LBT2 cells are. In addition, the anterior pituitary contains a heterogenic cell population and the effect of GnRH and dex might be more complex and exhibit differential effects in a mixed cell population compared to an isolated cell type due to paracrine effects. Hence, the results obtained in immortalized cell lines should be confirmed in primary pituitary cell cultures. The effect of the endogenous GC corticosterone on GnRHR gene expression should be investigated to further establish that data obtained with the synthetic GC dex are relevant *in vivo*. Furthermore, it would be interesting to study the role of the GR in gonadotrope function *in vivo* by analyzing gene expression in knock-out mice deficient in GR exclusively in the gonadotropes. Gene expression profiles of primary pituitary cells stimulated with dex, GnRH or a combination thereof could be obtained by performing microarray analysis. This would allow the identification of various novel

responsive genes and potentially differential regulation mediated by these hormones. In the future it might also be relevant to investigate the effects of dex, GnRH and dex plus GnRH in primary cells from GR knock out mice to further establish the role of the GR in gene regulation.

University of Cape Town

ADDENDUM A

SUPPLEMENTARY DATA

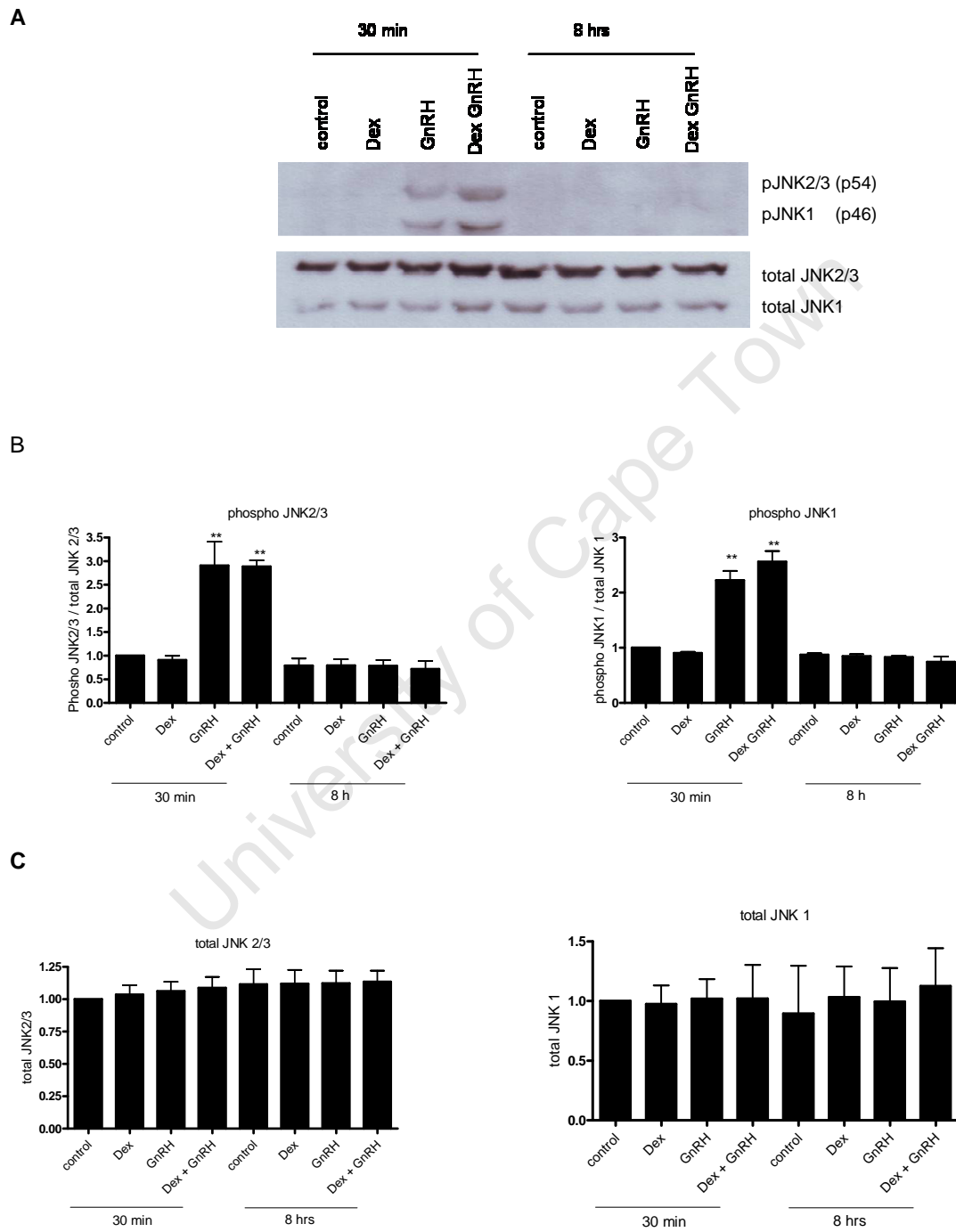
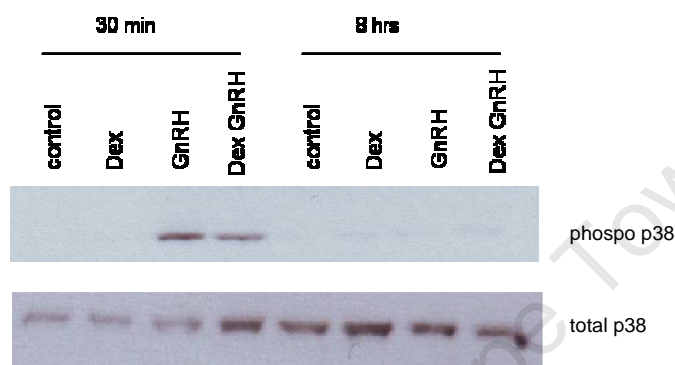


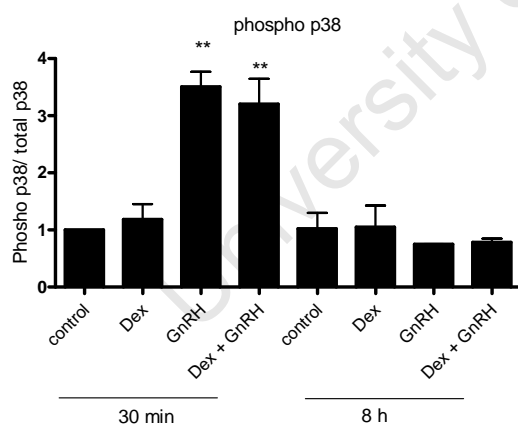
Figure A1: Activation of JNK kinases by GnRH in LβT2 cells (figure legend to follow on next page)

A: L β T2 cells were treated with 100 nM dex, 100 nM GnRH or a combination thereof in serum free medium for the indicated times. Proteins were separated on 8% SDS-PAGE gel, electroblotted onto nitrocellulose membrane and probed with anti-phospho JNK antibody. The blots were stripped and re probed with an anti-total JNK antibody as a control. **B:** Pooled and quantified results of 3 independent experiments are presented as relative amounts of phosphorylated kinase over total kinase for phospho JNK 1 (left) and phospho-JNK 2/3 (right). **C:** Relative kinase expression levels are shown for JNK 1 (right) and JNK 2/3 (left), with vehicle (control) at 30 min set as 1.

A



B



C

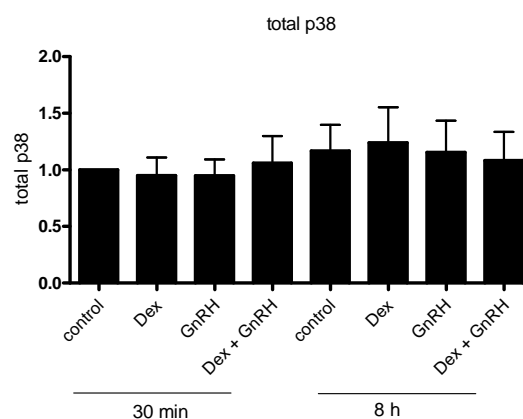
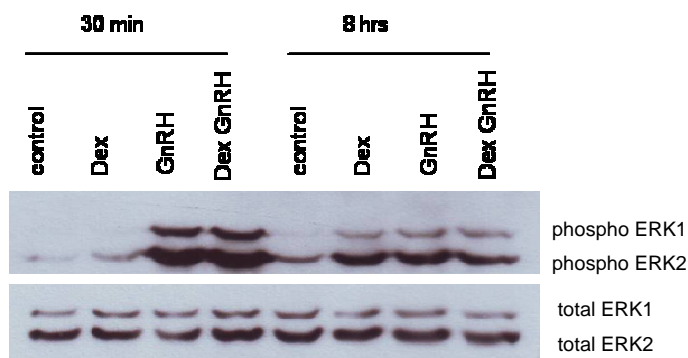


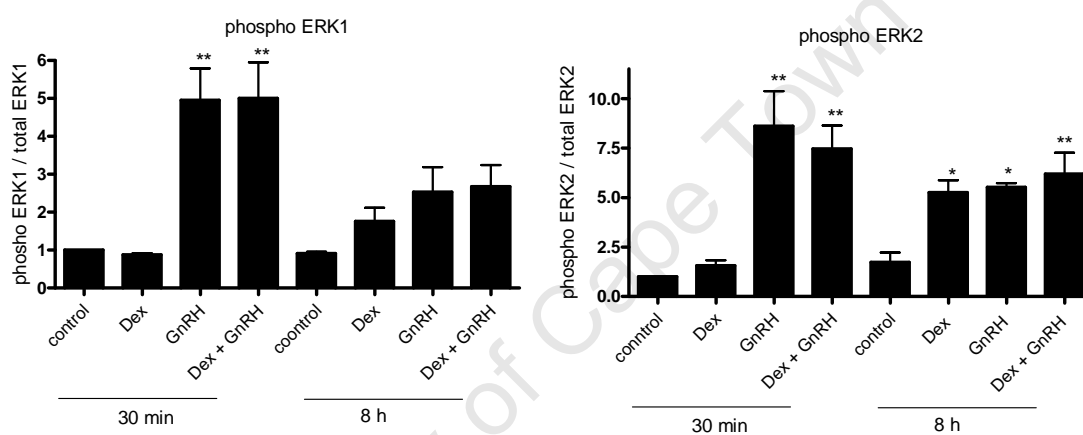
Figure A2: GnRH rapidly induces p38 activation in L β T2 cells

A: L β T2 cells lysates as prepared for figure A1 were analyzed by Western blotting using an anti-phospho-p38 antibody. The blots were stripped and re probed with anti JNK1/2 as control. **B:** Pooled and quantified results of 3 independent experiments are presented as relative amount of phosphorylated p38 over total p38. **C:** Total p38 expression levels are shown, with vehicle (control) at 30 min set as 1.

A



B



C

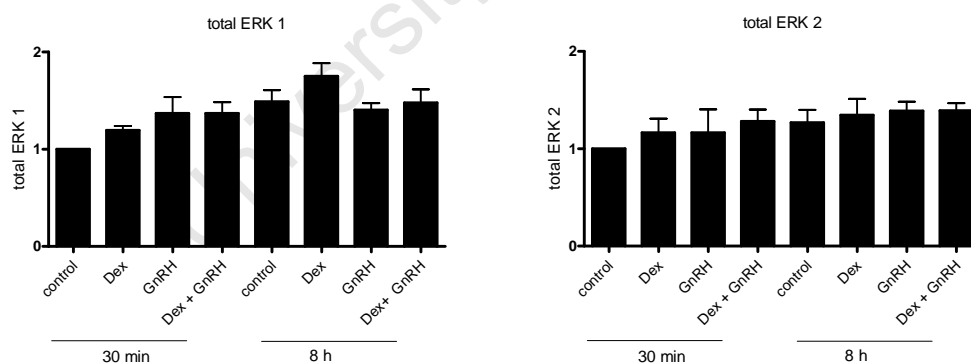


Figure A3: Activation of ERK kinase by GnRH and dex

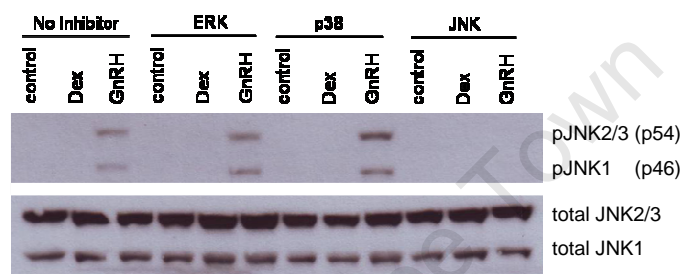
A: LβT2 cells were treated as described for figure A1 and Western blotting performed using an anti-phospho-ERK1/2 antibody. The blots were stripped and re-probed with anti-ERK1/2 antibody as control.

B: Pooled and quantified results of 3 independent experiments are presented as relative amount of phosphorylated kinase over total kinase on separate graphs for phospho-ERK 1 (left panel) and phospho-ERK 2 (right panel)

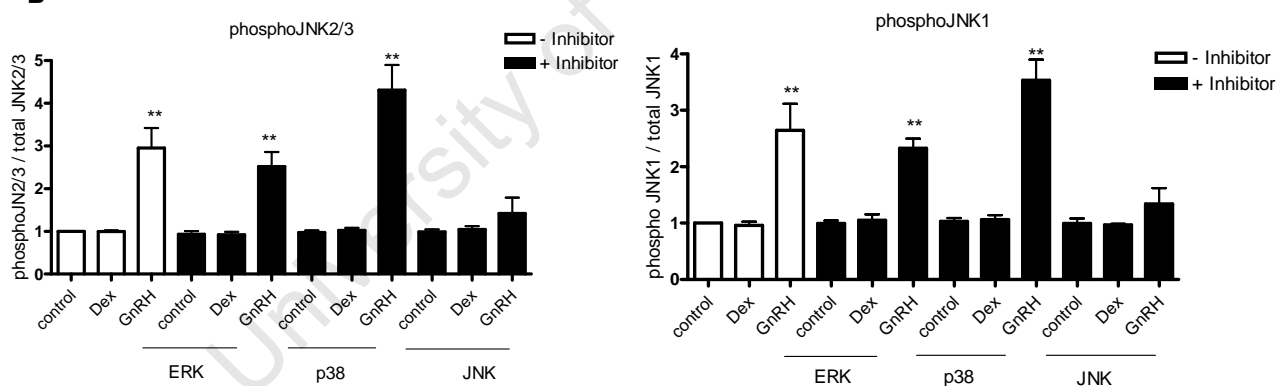
C: Relative kinase expression levels are shown for ERK 1 (left panel) and ERK 2 (right panel), with vehicle (control) at 30 min set as 1.

The JNK inhibitor SP600125 used in the study has been characterized by Bennett *et al.* in 2001 as an anthrapyrazolone inhibitor of JNK (Bennett *et al.*, 2001) These authors found that in the presence of the JNK inhibitor SP600125 stimuli-induced phosphorylation of JNK was reduced to a similar extent as phosphorylation of the downstream JNK substrate c-Jun. They further suggest that this effect may be due to MKK4 inhibitory activity which is upstream of JNK. Therefore the effect of the JNK inhibitor SP600125 on JNK phosphorylation was investigated to determine the efficiency of the inhibitor (figure A4)

A



B



C

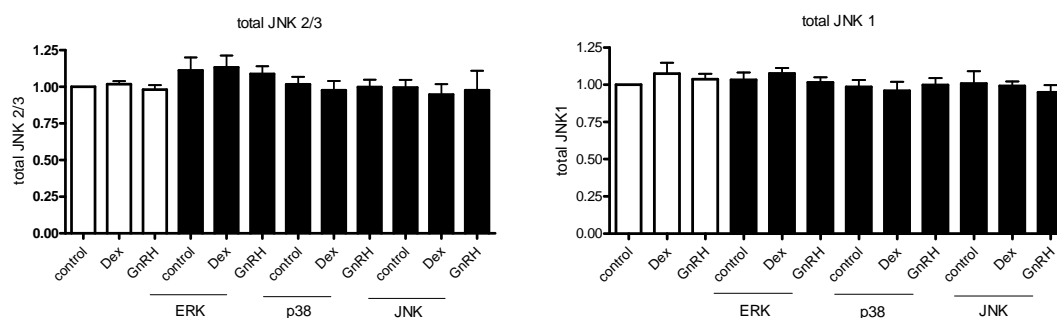


Figure A4: (continued on next page)

D

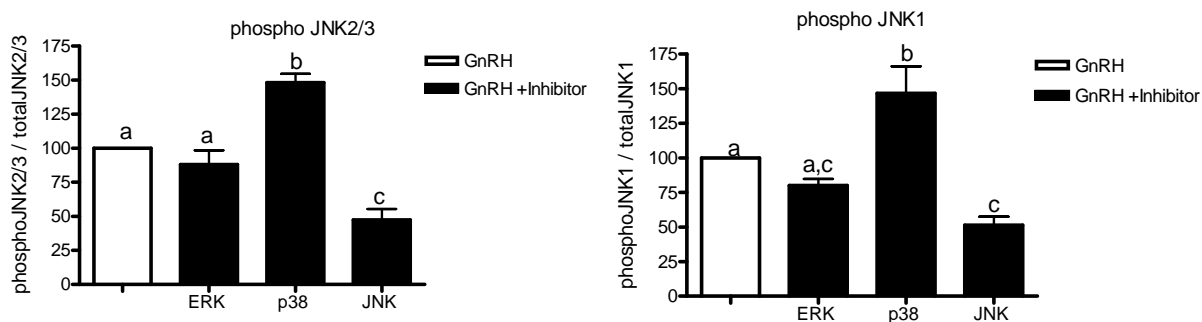
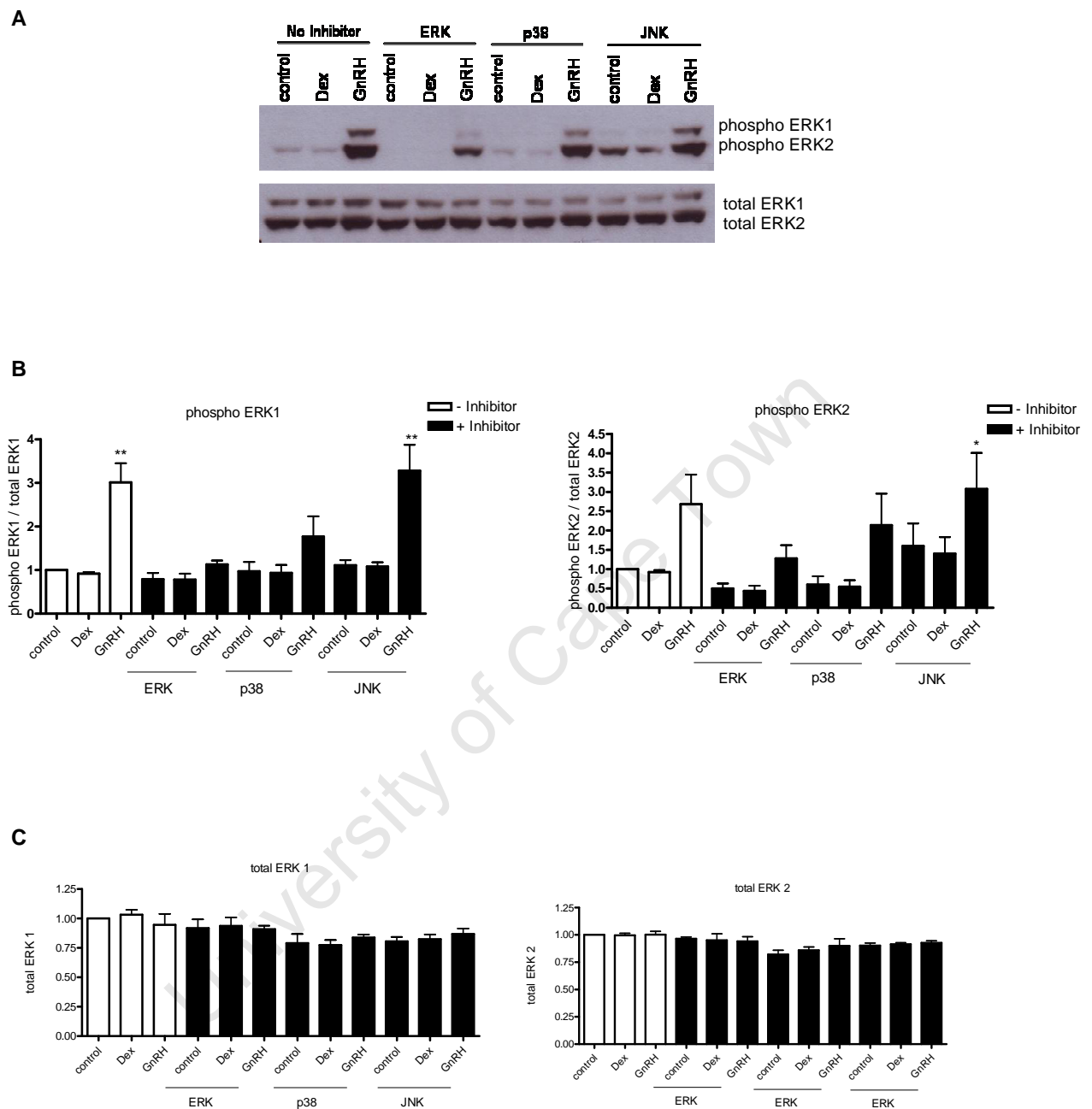


Figure A4: Effects of MAPK inhibitors PD98059, S203580 and SP600125 on GnRH-mediated activation of JNK

L β T2 were stimulated for 30 minutes with 100 nM GnRH in the absence or presence of the following inhibitors: 10 μ M JNK inhibitor (SP600125), 10 μ M p38 inhibitor (SB203580), 50 μ M ERK pathway inhibitor. Proteins were separated on an 8% SDS-PAGE gel, electroblotted onto a nitrocellulose membrane and probed with phospho JNK antibody (A). The blots were stripped and reprobbed with antibodies for total JNK (A). The combined results from three independent are presented in B, where phospho JNK levels were normalized to total JNK with the value for control (vehicle) set to 1. The total JNK protein levels are shown on separate graphs with vehicle (control) at as 1 (C). **D:** Results are presented as phospho JNK over total JNK relative to GnRH induced phosphorylation which was set to 100%.



D

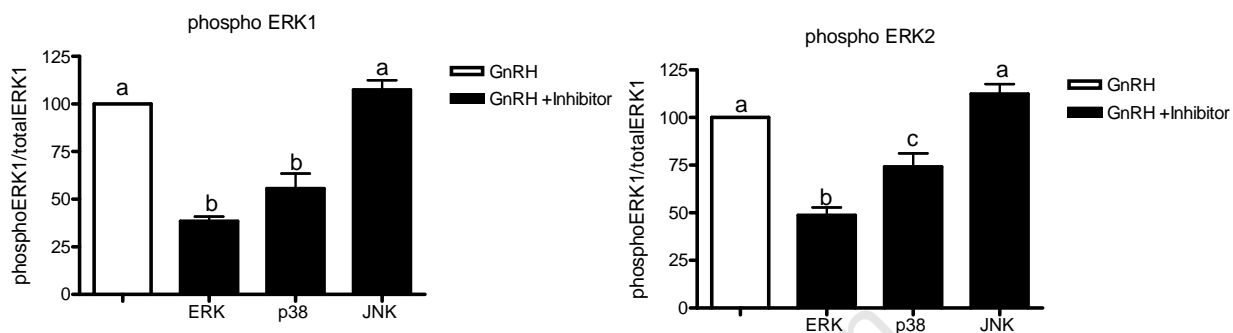
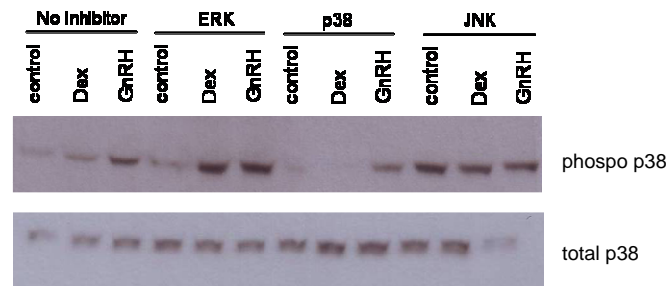


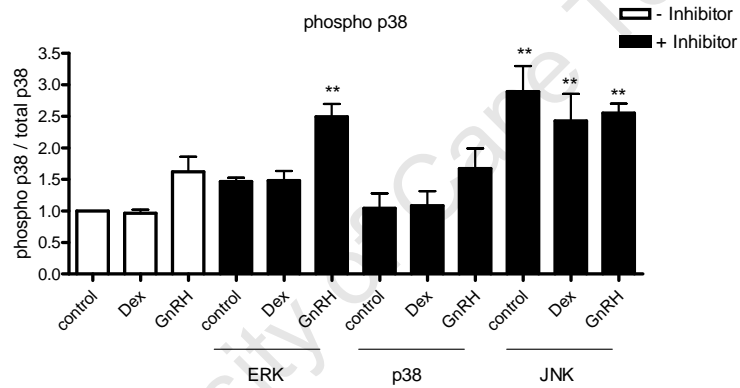
Figure A5: Specificity of MAPK inhibitors on ERK induction

A-C: L β T2 were stimulated as described in figure A4. Proteins were separated on an 8% SDS-PAGE gel, electroblotted onto a nitrocellulose membrane and probed with phospho ERK antibody. The blots were stripped and reprobed with antibodies for total ERK as a control. The combined results from three independent are presented in B, where phospho JNK levels were normalized to total JNK with the value for control (vehicle) set to 1. The total ERK protein levels are shown on separate graphs with vehicle (control) at as 1 (C). **D:** Results are presented as phospho ERK over total ERK relative to GnRH induced phosphorylation which was set to 100%.

A



B



C

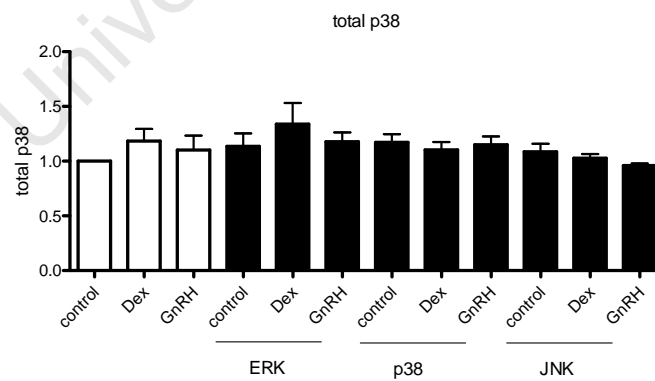


Figure A6: (continued on next page)

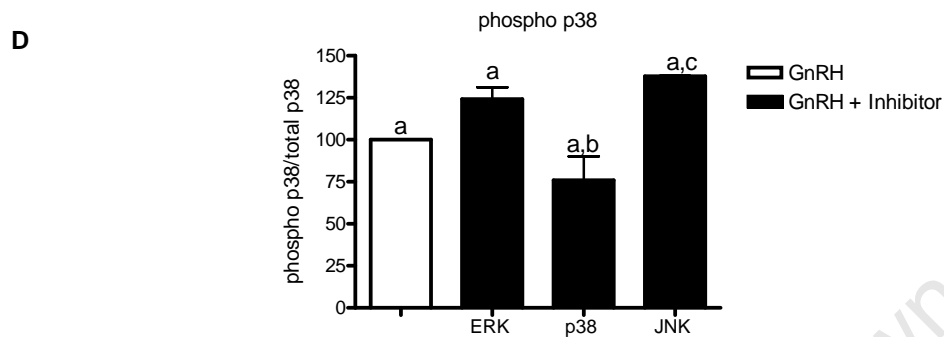
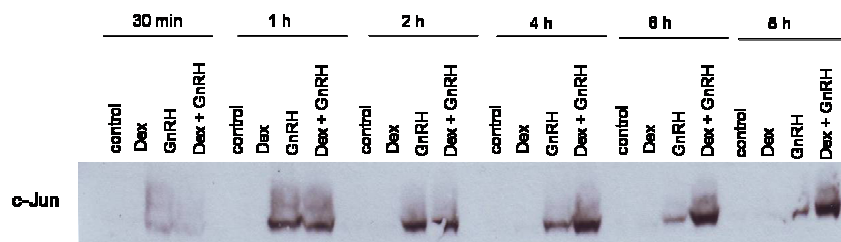


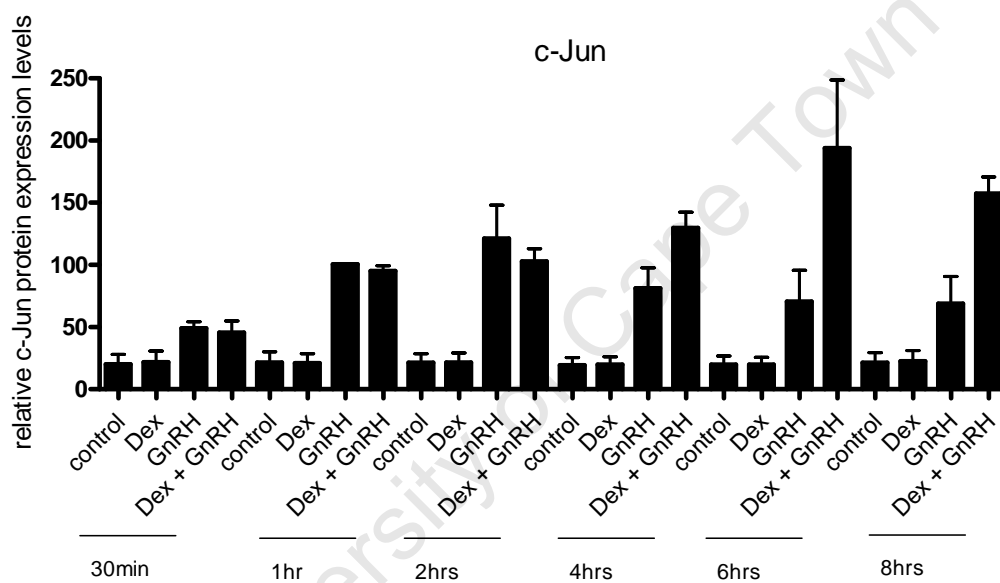
Figure A6: Specificity of MAPK inhibitors PD98059, S203580 and SP600125

A-C: L β T2 were stimulated as described in figure A4. Proteins were separated on an 8% SDS-PAGE gel, electroblotted onto a nitrocellulose membrane and probed with phospho p38 antibody (A). The blots were stripped and reprobed with antibodies for total p38 (A). The combined results from three independent experiments are presented in B, where phospho JNK levels were normalized to total JNK with the value for control (vehicle) set to 1. The total p38 protein levels are shown on separate graphs with vehicle (control) set to 1 (C). **D:** Results are presented as phospho p38 over total p38 relative to GnRH induced phosphorylation which was set to 100%.

A



B



C

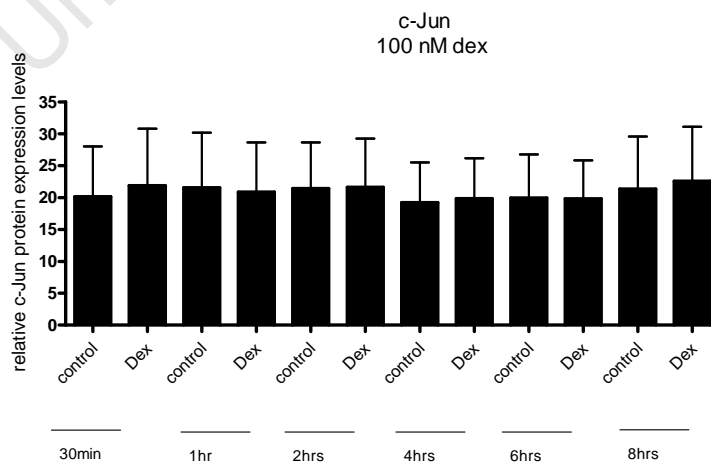
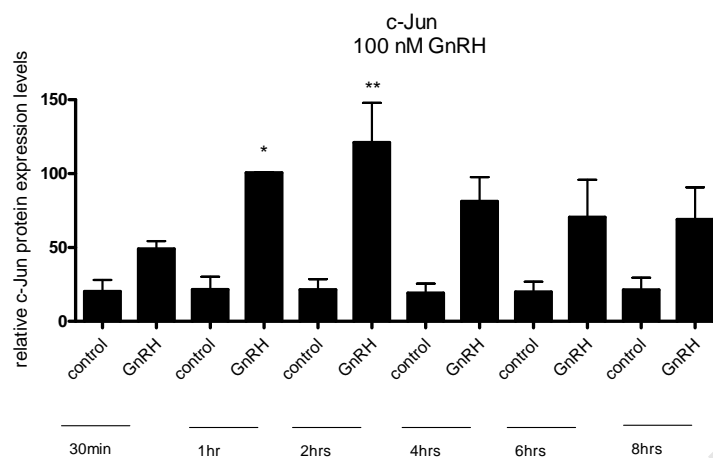


Figure A7: (continued on next page)

D



E

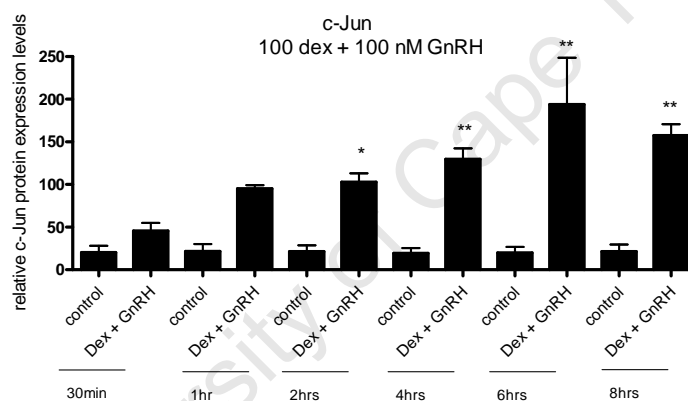
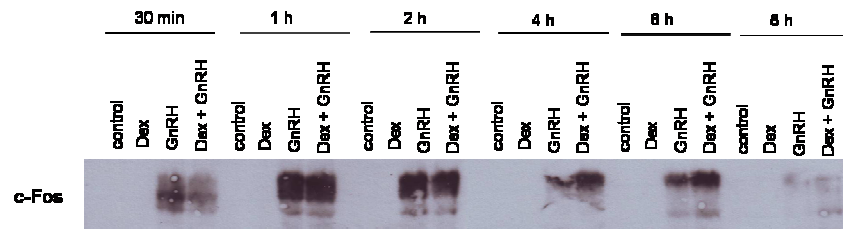


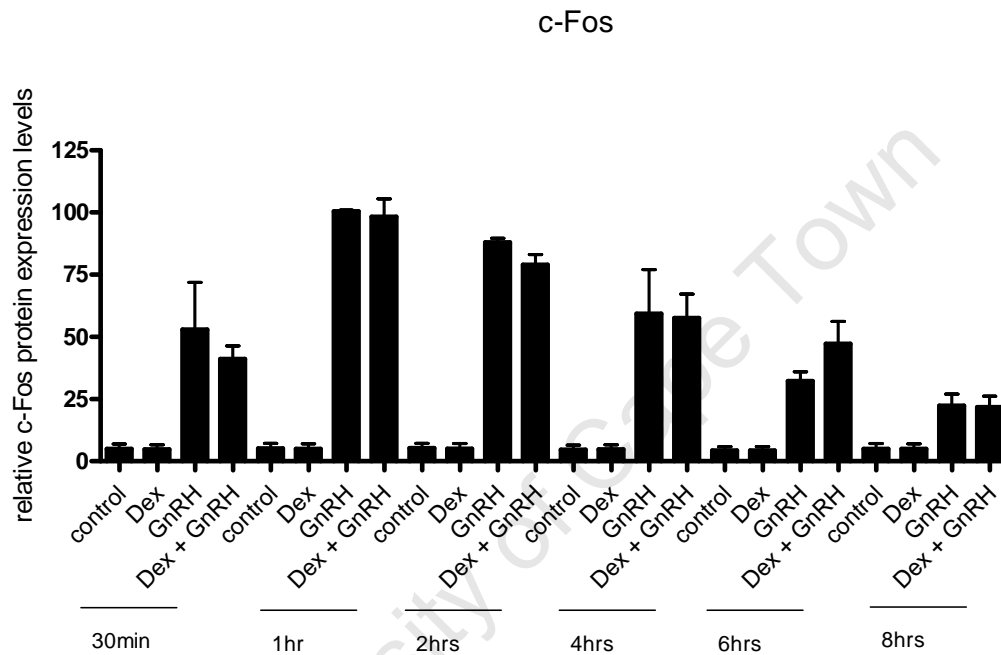
Figure A7: Protein expression of c-Jun is rapidly induced by GnRH and not dex in LBT2 cells

LBT2 cells were grown and serum-starved overnight before treatment with vehicle, 100 nM GnRH, 100 nM dex or a combination thereof for the different times as indicated. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific antibodies for c-Jun (A). The graphs in B shows combined results of at least 2 independent experiments. The graphs in C, D and E show relative c-Jun protein expression levels in response to dex (C), GnRH (D) or dex plus GnRH (E).

A



B



C

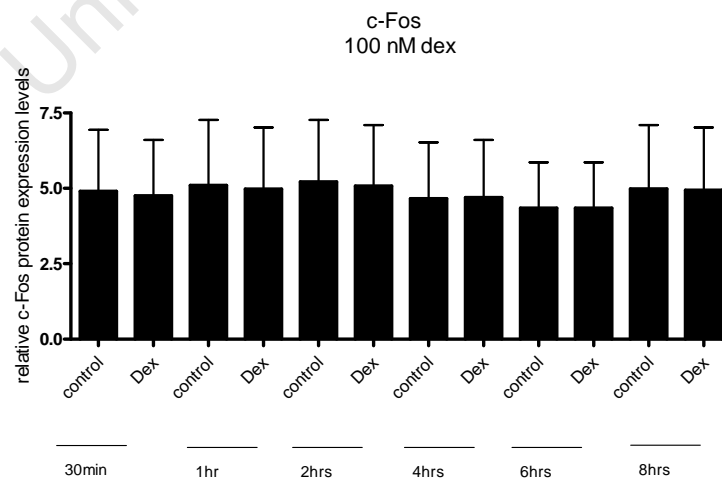
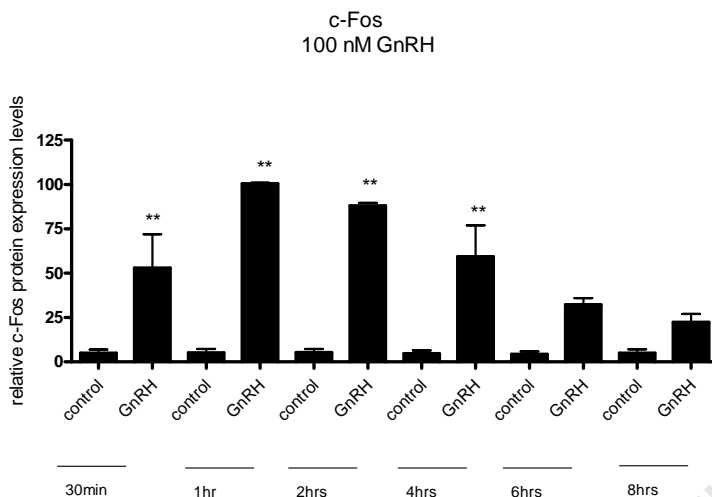


Figure A8: (continued on following page)

D



E

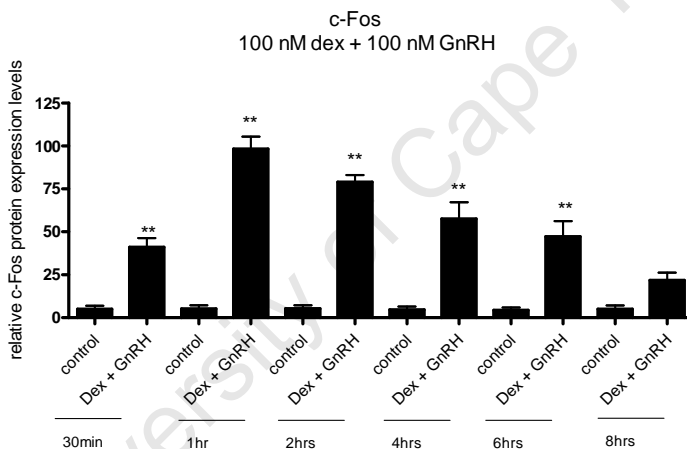


Figure A8: GnRH but not dex increases c-Fos protein expression in LβT2 cells

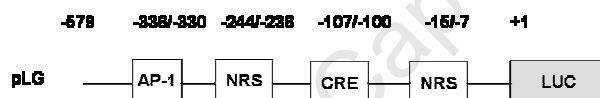
LβT2 cells were grown and serum-starved overnight before treatment with vehicle, 100 nM GnRH, 100 nM dex or a combination thereof for the indicated times as indicated. Equal amounts of cell lysates were loaded on an 8% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed using specific antibodies for c-Fos (A). The graphs in B shows combined results of at least 2 independent experiments and the graphs in C, D and E show relative c-Fos protein expression levels in response to dex (C), GnRH (D) or dex plus GnRH (E).

ADDENDUM B

RESULTS RELEVANT TO THE PRESENT STUDY OBTAINED BY OTHER'S IN PROF. HAPGOOD'S LABORATORY

The results presented in figure B1 (B) and B2 (B) were obtained by Dr. Hanél Sadie-Van Gijzen (Sadie, 2006) and the figures presented in figure B1 (C) and B2 (A) and B2 (B) were obtained from Sandra Fernandes (Fernandes, 2007). (previous students in Prof. Hapgood's research group).

A



B



C

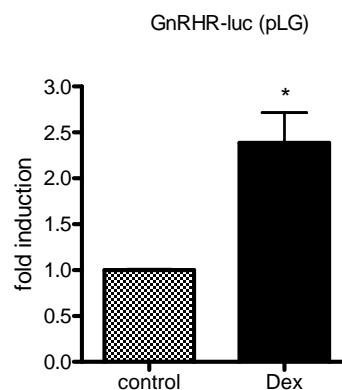
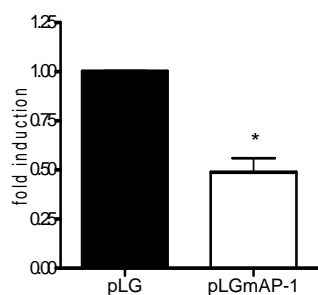


Figure B1: Both GnRH and dex increase expression of the transfected GnRHR promoter-reporter in L β T2 cells

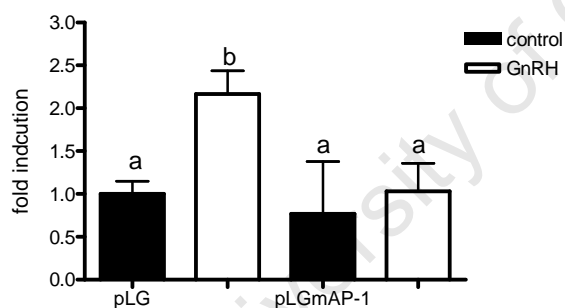
A: Schematic of the mouse GnRHR promoter-luciferase construct (pLG). Transcription factor binding sites are indicated as boxes, with positions relative to translation start site indicated at the top. NRS: nuclear receptor binding site; AP-1 activator protein-1; CRE: cAMP response element. **B,C:** L β T2 cells were transfected with 100ng of pLG and 24 h later stimulated with vehicle (control), 100 nM GnRH (B)

or 100 nM dex (C) for 8 h in the absence of serum. The luciferase activity was normalized to β -galactosidase activity and the hormone-stimulated increase in wild-type GnRHR promoter activity was calculated relative to the control. Graphs show combined results of at least 3 independent experiments, each performed in triplicate.

A



B



C

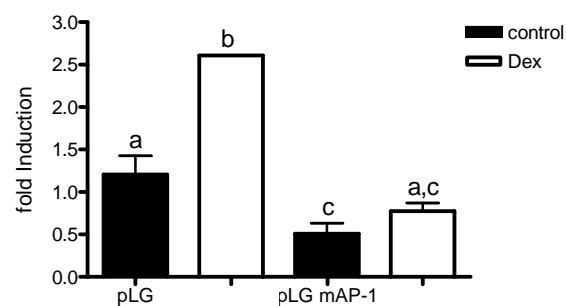


Figure B2: Basal activity plus GnRH- and dex-induced stimulation of the synthetic mouse GnRHR promoter require an intact an AP-1 site

L β T2 cells were transfected with 100 ng of pLG or pLGmAP1 and after approximately 24 h cells were incubated for 8 h in serum-free medium in the absence (A) or presence of 100 nM GnRH (B) or 100 nM dex (B). The luciferase activity was normalized to β -galactosidase activity and the hormone-stimulated increase in promoter activity was calculated relative to the control. The graphs show the combined results of at least 3 independent experiments, each performed in triplicate.

ADDENDUM C

Primer Sequences

C1: Primers used for quantitative real-time PCR

mouse GnRHR gene (product size: 192 bp) (Sadie, 2006)

forward 5'CCACAGTGGTGGCATCAGGCCTTC 3'

reverse 5'TAGCGTTCTCAGCCGAGCTCTTGG 3'

mouse GAPDH gene (product size: 263 bp) (Overbergh *et al.*, 1999)

forward 5' TTCACCACCATGGAGAAGGC 3'

reverse 5' GGCATGGACTGTGGTCATCA 3'

C2: Primers used in ChIP assays

mouse GnRHR promoter* (product size: 168 bp) (Kam *et al.*, 2005)

forward 5' GTATCTGTCTAGTCACAACAG 3'

reverse 5' TCCTGAAGGCCAAGTGTAACC 3'

mouse FSH β promoter* (product size: 280 bp) (Coss *et al.*, 2004)

forward 5'GGTGTGCTGCCATATCAGATTCGG 3'

reverse 5' GCATCAAGTGCTGCTACTCACCTGTG 3'

* spanning the AP-1 site of the mouse promoter

ADDENDUM D

BUFFERS AND SOLUTIONS

D1. Growth medium

SOC medium

2% (w/v) tryptone

0.5% (w/v) yeast extract

0.05% (w/v) NaCl

2.5 mM KCl

10 mM MgCl₂

20 mM glucose

LB medium

1% (w/v) tryptone

0.5% yeast extract

1% NaCl

containing 50 µg/mL ampicillin

for LB-agar plates add 1,5% agar

D2: RNA isolation

DEPC-treated H₂O

1 ml diethyl pyrocarbonate (DEPC) in 1 L dH₂O (1:1000 dilution)

Incubate 2 h at 37°C, autoclave twice to inactivate DEPC

10X Morpholinopropanesulfonic acid (MOPS) buffer

0.2 M MOPS

0,05 M sodium acetate

0,01M EDTA, pH 8.0, adjust pH to 7.0 with 10 M NaOH

Denaturing formaldehyde gel mix (1% 100 ml)

Dissolve 1 g agarose in 70 ml DEPC-treated H₂O and bring to boil. Add 10 ml 10X MOPS buffer and 20 ml formaldehyde in fume hood. Mix well and pour.

RNA sample loading buffer

1,8 ml DEPC H₂O

0,8 ml Bromophenol blue solution (saturated)

1 ml glycerol

1.5 ml 10x MOPS

2,6 ml 12.3 M formaldehyde

7,3 ml formamide

Add 2,5 µl 10 mg/ml ethidium bromide per 1 ml RNA sample loading buffer just before use.

RNA electrophoresis buffer (500 ml)

50 ml 1x MOPS

14 ml formaldehyde

436 ml DEPC-treated water

D3: Western blot analysis**5X SDS sample buffer**

100 mM Tris-HCL, pH 6.8

5% (w/v) SDS

20% (v/v) glycerol

2% (v/v) β-mercaptoethanol

0.1% (w/v) bromophenol blue

Running buffer

25 mM Tris-HCL

250 mM glycine

0,1% SDS (w/v), pH 8,4

Transfer buffer

25 mM Tris
200 mM glycine
10% (v/v) methanol

Tris buffered saline (TBS)

50 mM Tris
150 mM NaCl

TBS-Tween (TBS-T)

50 mM Tris
150 mM NaCl
0.1% (v/v) Tween

Stripping buffer

100 mM β -mercaptoethanol
2% (w/v) SDS
62.5 mM Tris-Cl, pH 6.7

D4: Chromatin immunoprecipitation (ChIP) buffers (Ma *et al.*, 2003)**nuclear lysis buffer**

1% SDS
50 mM Tris-HCL, pH 8.0
10 mM EDTA,
1 x Complete Mini Protease Inhibitor Cocktail per 10 ml buffer

IP dilution buffer

0.01 % (w/v) SDS
20 mM Tris-HCL, pH 8.0

1.1% Triton X-100

167 mM NaCl

1.2 mM EDTA

1 x Complete Mini Protease Inhibitor Cocktail per 10 ml buffer

Wash buffer I

0.1% (w/v) SDS

1% (v/v) Triton X-100

2 mM EDTA

20 mM Tris-HCL pH 8.0

150 mM NaCl

Wash buffer II

0.1% (w/v) SDS

1% (v/v) Triton X-100

2 mM EDTA

20 mM Tris-HCL pH 8.0

500 mM NaCl

Wash buffer III

1% (v/v) NP-40

1% (w/v) sodium deoxycholate

500 mM LiCl

1 mM EDTA

10 mM Tris-HCL pH 8.0

TE

10 mM Tris-HCL pH 8.0

0.1 mM EDTA

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