Modulation of GR transcriptional signalling by HIV-1 Vpr: insights into regulation by progestins

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ACKNOWLEDGMENTS

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ABSTRACT

It has been 30 years since HIV was first discovered, yet the molecular mechanisms whereby the virus mediates its pathogenic effects have not yet been completely elucidated. The glucocorticoid receptor (GR) is a ligand-activated host transcription factor, which mediates anti-inflammatory effects in response to stimulation with glucocorticoids (GC). One of the HIV-1 accessory proteins, Vpr, is highly immunosuppressive and contributes to suppression of the immune system thereby creating an environment favourable for viral proliferation. Vpr has been previously reported to act as a GR co-activator on glucocorticoid response element (GRE) containing promoters. Thus, the GR appears likely to play a role in HIV-1 pathogenesis. Contraceptive usage is also likely to affect HIV-1 pathogenesis as some hormonal contraceptives can bind to and activate the GR. Progesterone (P4) regulates the female reproductive system and the synthetic progestins medroxyprogesterone acetate (MPA) and norethisterone acetate (NET-A) are extensively used as injectable contraceptives. MPA has been shown to act as a partial or full GR agonist and recent evidence indicates that injectable MPA increases HIV-1 acquisition and transmission. The molecular mechanisms of this remain unclear, but may involve decreasing the thickness of the vaginal epithelium as well as actions via the GR that affect gene expression in the cervo-vaginal environment and/or elsewhere. This study aims to investigate the actions of GC's, P4, MPA and NET-A via the GR in the absence and presence of Vpr protein towards gaining some insight into the potential interplay between the host GR, contraceptive use, HIV-1 pathogenesis, and the mechanisms thereof.

Using promoter-reporter assays it was found that Vpr greatly increased dexamethasone, cortisol, and MPA-mediated, but not NET-A or progesterone-mediated transactivation via overexpressed GR in COS1 monkey kidney cells but did not change the biocharacter of any of the tested ligands at this promoter. The extent of the Vpr-mediated increase in GR-dependent transcription of the reporter gene was found to depend on both the concentration and efficacy of the activating GR ligand, with higher concentrations and more efficacious ligands resulting in the highest level of reporter gene activity. Vpr enhanced the efficacy of dexamethasone but did not

affect either potency for transactivation or change the Hill co-efficient and thereby affect the co-operation of ligand binding to the GR. Vpr did not enhance ligand-dependent transactivation of two endogenous GRE-containing genes, MKP-1 and IκBα in COS1 and human endocervical END cells. However, Vpr appeared to increase the partial agonist activities of some ligands in a gene-specific manner, causing them to act like full agonists. These discrepancies regarding the effect of Vpr on transactivation of the reporter gene and two endogenous genes suggest that the ligand-dependent effects of Vpr on the GR may be highly promoter-specific, depend on the absence of native chromatin, and/or be sensitive to the relative concentrations of Vpr, GR, and DNA target sites. Interestingly Vpr induced a marginal but statistically significant increase in basal GR-mediated transactivation of the reporter gene. Investigating various steps in the pathway of GR function showed that Vpr did not significantly increase ligand-dependent GR phosphorylation or nuclear translocation, but did significantly increase GR turnover and may have exerted a moderate effect on the basal level of S226 phosphorylation and GR nuclear translocation. Taken together these data suggest Vpr could potentially be a weak, non-classical GR-activator. However, data from this study could not firmly establish whether Vpr was a classical GR co-activator, acted as a non-classical GR agonist, or reflected the actions of both.

Of great potential significance for disease and choice of contraceptive, high levels of reporter gene transactivation were obtained by MPA, but not NET-A or progesterone, in the presence of Vpr, which are greater than those observed with the full agonist dexamethasone in the absence of Vpr. This suggests that MPA may have very different effects on HIV-1 pathogenesis as compared to NET-A and progesterone, via GR-mediated transcriptional effects, and likely exerts deleterious GC like effects. It remains to be determined if the observed effects of Vpr at the reporter gene are physiologically relevant by investigation of further GRE containing endogenous genes. The results on reporter genes however do suggest that Vpr may lead to enhanced GR signalling on select target promoters which could be a possible mechanism leading to increased susceptibility to HIV-1 acquisition and transmission or even disease progression.

ABBREVIATIONS

aa	Amino Acid
ACTH	Adrenocorticotropic Hormone
AF	Activation Function
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
A_{max}	Maximal Response
AP-1	Activator Protein 1
AR	Androgen Receptor
ATCC	American Type Culture Collection
bp	Base Pair
BTM	Basal Transcriptional Machinery
CBG	Corticosteroid Binding Globulin
CBP	CREB Binding Protein
CDK	Cyclin Dependent Kinase
CMV	Cytomegalovirus
Col-3	Collagenase-3
CORT	Cortisol
CREB	cAMP Response Element Binding Protein
CRH	Corticotropin Releasing Hormone
DBD	DNA Binding Domain
DEAE	Diethylaminoethyl
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagles Medium
E2	Oestradiol
EC_{50}	Effective Concentration required for 50% of maximal response
ER	Oestrogen Receptor
EtOH	Ethanol
END	E6/E7 endocervical cell line
F6	FuGene 6
FCS	Foetal Calf Serum
FRET	Fluorescent Resonance Energy Transfer

FSH	Follicle Stimulating Hormone
FWD	Forward (primer)
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GC	Glucocorticoid
GILZ	Glucocorticoid Induced Leucine Zipper
GLS	GRE like site
GnRH	Gonadotropin Releasing Hormone
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GRU	Glucocorticoid Response Unit
HA	Haemagluttinin
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
hGR	Human Glucocorticoid Receptor
HIV-1	Human Immunodeficiency Virus Subtype 1
HPA/G	Hypothalamic Pituitary Adrenal/Gonadal
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
HSF	Heat Shock Protein Factor
IDV	Integrated Density Value
IgG	Immunoglobulin-G
IκBα	Inhibitor of NFκB type α (NFKBIA)
IL	Interleukin
JNK	c-Jun N-terminal Kinase
kDa	kiloDalton
KSF	Keratinocyte Serum Free
L	Leucine
LBD	Ligand Binding Domain
LH	Luteinising Hormone
LSM	Laser Scanning Microscope
LTNP	Long Term Non Progressor
LTR	Long Terminal Repeat
Luc	Luciferase

M	Molar
MAPK	Mitogen Activated Protein Kinase
MeOH	Methanol
MKP-1	MAP Kinase Phosphatase 1
MPA	Medroxyprogesterone Acetate
MR	Mineralocorticoid Receptor
MW	Molecular Weight
NCoR	Nuclear Receptor Co-Repressor
NET	Norethisterone
NET-A	Norethisterone Acetate
NET-EN	Norethisterone Enanthate
NFκB	Nuclear Factor κB
nGRE	Negative GRE
NLS	Nuclear Localisation Signal
NRID	Nuclear Receptor Interacting Domain
p300	Adenovirus E1A binding protein 300
P4	Progesterone
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pCAF	p300/CBP Activating Factor
POMC	Pro-opiomelanocortin
PP	Protein Phosphatase
PR	Progesterone Receptor
RANKL	Receptor of Activated NFκB Ligand
REV	Reverse (primer)
rGR	Rat Glucocorticoid Receptor
RNA pol II	Ribonucleic Acid Polymerase type II
SEM	Standard Error of the Mean
SDS	Sodium Dodecyl Sulphate
SF	Serum Free
siRNA	Small Interfering RNA
SIV	Simian Immunodeficiency Virus

SMRT	Silencing Mediator for Retinoic Acid and Thyroid Hormone Receptors
SR	Steroid Receptor
SRC	Steroid Receptor Co-activator
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-related Modifier
SWI/SNF	Switch/Sucrose Non Fermentable
TAT	Tyrosine Amino Transferase
TBP	TATA-box Binding Protein
TF	Transcription Factor
TIC	Transcription Initiation Complex
TNF-α	Tumour Necrosis Factor-α
TSS	Transcriptional Start Site
Vpr	Viral Protein R
wt	Wild Type
w/v	Weight per unit volume
x	Any amino acid/nucleotide

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1. LITERATURE REVIEW

Cortisol (CORT) is the natural glucocorticoid (GC) in humans and mediates its effects via binding to the ligand-activated transcription factor (TF), the glucocorticoid receptor (GR). The GR is a member of the nuclear receptor superfamily type III, which includes the Androgen (AR), Oestrogen (ER), Mineralocorticoid (MR) and Progesterone (PR) receptors (Nuclear Receptors Nomenclature, 1999), each of which has a cognate ligand and specialised function. Dexamethasone (DEX) is a synthetic derivative of cortisol, which is more potent but has similar effects via the GR (Meikle and Tyler, 1977). The GR has profound anti-inflammatory and immunosuppressive effects (Ashwell *et al.*, 2000; Galon *et al.*, 2002) which are widely exploited in medicine (Boumpas *et al.*, 1993). It is also involved in a diverse array of physiological processes including homeostasis, growth, reproduction, metabolism, neuroendocrine and cardiovascular functions (Chrousos, 2004; Chrousos *et al.*, 2004; Sapolsky *et al.*, 2000).

1.1. The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal-axis (HPA) is a stress regulated pathway responsible for activation of the GR (Tsigos and Chrousos, 2002). When the body senses stress, corticotropin releasing hormone (CRH) is released from the hypothalamus (Bloom *et al.*, 1982). This stimulates the pituitary gland to produce adrenocorticotropic hormone (ACTH), which in turn activates the synthesis of GC's in the adrenal cortex, leading to the release of cortisol (Chrousos, 1995; Webster *et al.*, 2002). Cortisol is a homeostatic mediator which plays a pivotal role in the maintenance of blood glucose levels, carbohydrate metabolism and most notably suppression of inflammation and the immune response (Ashwell *et al.*, 2000; Herold *et al.*, 2006; Tait *et al.*, 2008). Once released into the blood, 90% of the cortisol will associate with corticosteroid binding globulins (CBG), distributing the steroid around the body (Breuner and Orchinik, 2002).

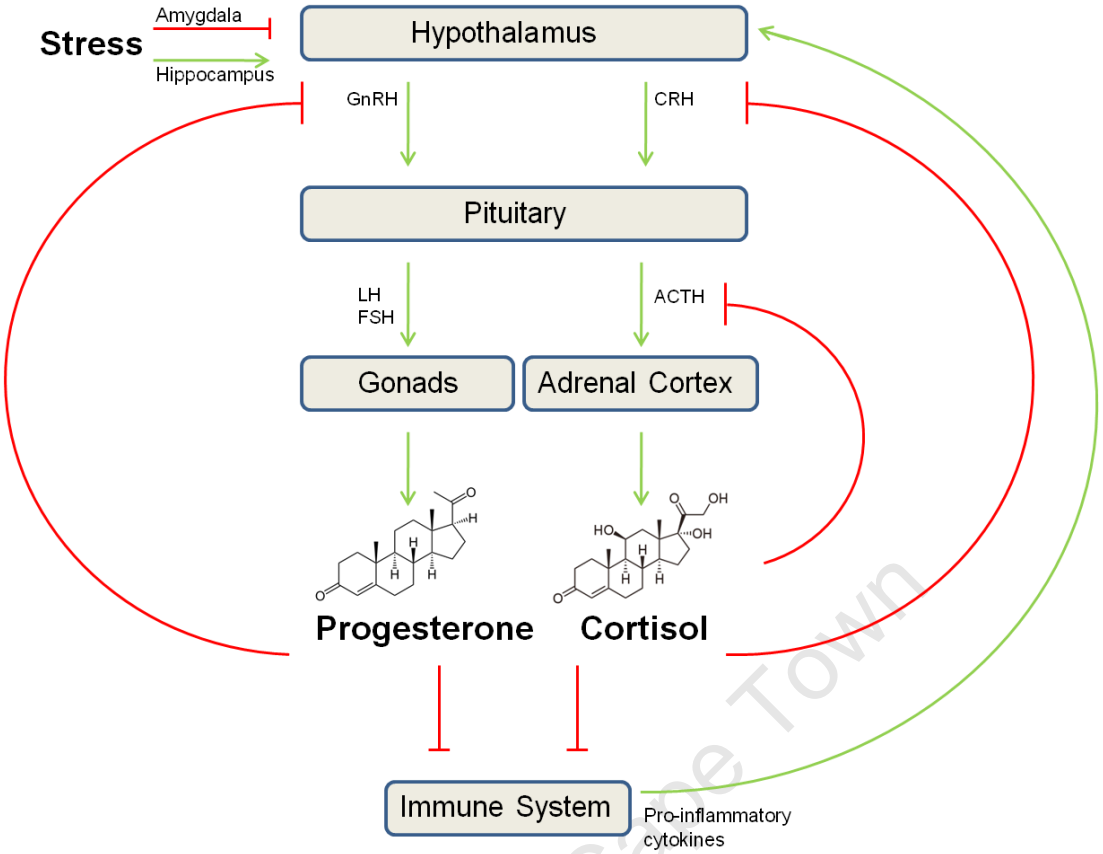


Figure 1.1: Regulation of the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HGA) axis. Stress leads to activation of the HPA axis through hypothalamic release of corticotropin releasing hormone (CRH) and associated pituitary release of adrenocorticotropic hormone (ACTH). This results in adrenal gland synthesis of cortisol. The release of the sex hormone, progesterone (P4), is under control of the hypothalamic-pituitary-gonadal (HPG) axis, where gonadotropin-releasing hormone (GnRH) synthesis in the hypothalamus causes anterior pituitary release of follicle-stimulating hormone (FSH) and luteinising hormone (LH) thereby inducing the gonads to synthesise P4 (Adapted from Backstrom *et al.*, 1982; Reichardt and Schutz, 1998; Tait *et al.*, 2008).

1.2. The Glucocorticoid Receptor

1.2.1. The GR gene and expression

The GR gene is comprised of ten exons spanning 110 kb, encoded on the fifth chromosome at region 5q31p (Encio and Detera-Wadleigh, 1991). GR mRNA is highly expressed in lung, spleen, brain, and liver tissue (Kalinyak *et al.*, 1987). Ligand activation of the GR causes a decrease in mRNA expression in a negative-feedback manner (Burnstein *et al.*, 1994; Okret *et al.*, 1986). Alternative splicing of the 9α or 9β exon in the pre-mRNA yields either the GRα or the GRβ, two highly homologous receptor isoforms with molecular weights of 97 and 94 kDa,

respectively. Both are identical between amino acids (aa) 1-727, with the α-isoform containing an additional 50 residues at the carboxyl terminal, harbouring an activation function (AF), whereas the β-isoform has only 15 non-homologous residues (Hollenberg *et al.*, 1985). Three further GC resistant splice variants have also been discovered (GR-γ, GR-A, and GR-P) which have much lower expression than the α-isoform, but are not yet fully characterised. These may be related to aberrant GR signalling and diseases (Beger *et al.*, 2003; Krett *et al.*, 1995; Moalli *et al.*, 1993).

The physiological role of GRβ is unclear, but is thought to be involved in transcriptional repression by recruiting histone deacetylase (HDAC) (Kelly *et al.*, 2008) and regulating the selective recruitment of co-factors (Charmandari *et al.*, 2005). It is expressed at much lower levels than GRα (Oakley *et al.*, 1997) and resides primarily in the nucleus (Oakley *et al.*, 1996). The GRβ lacks the ligand binding domain of the GRα (de Castro *et al.*, 1996), and has been reported to function as a dominant negative regulator of GRα, by forming transcriptionally inactive heterodimers with the ligand-activated GRα (Bamberger *et al.*, 1995; Oakley *et al.*, 1999). The GRβ has been reported to also repress transcription independently of GRα (Kino *et al.*, 2009).

Alternate translation of GRα mRNA may yield eight further isoforms truncated at the amino terminal. In descending order of size these are termed GRα-A, -B, -C1, -C2, -C3, -D1, -D2, or -D3, and result from translational initiation at alternate start codons (Lu and Cidlowski, 2004; Lu and Cidlowski, 2005). These eight isoforms are transcriptionally active and exhibit differential expression according to tissue type (Lu and Cidlowski, 2005) and exert different magnitudes of transcriptional responses (Lu and Cidlowski, 2006). This is thought to contribute to the cell-specific manner of GRα-mediated transcription (Revollo and Cidlowski, 2009).

1.2.2. Structure of the GR protein

The full length (GRα-A) protein consists of 777 aa comprising three functional domains; the amino terminal domain (NTD), the DNA binding domain (DBD) which encompasses a hinge region (HR), and the ligand binding domain (LBD) (Figure 1.2) (reviewed in Nicolaides *et al.*, 2010; Revollo and Cidlowski, 2009; Zhou and Cidlowski, 2005).

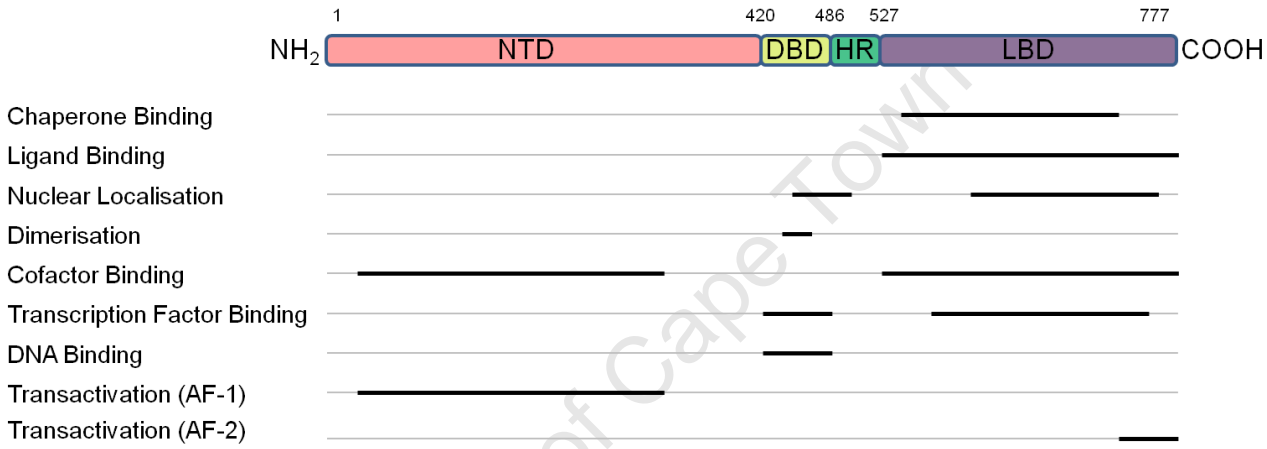


Figure 1.2: Structural organisation of the full length GRα protein. The GR consists of three functional domains, each harbouring regions which mediate discrete functions of GR signalling. NTD- Amino terminal domain, DBD- DNA binding domain, HR- Hinge Region, LBD- Ligand Binding Domain (adapted from Beck *et al.*, 2011; Nicolaides *et al.*, 2010).

The NTD is the least well conserved amongst both the different isoforms of the GRα (Lu and Cidlowski, 2005) and other steroid receptors (SR's) (Griekspoor *et al.*, 2007). It resides between amino acids 1-419 and harbours a constitutive activation function (AF-1) between residues 77-262 (Godowski *et al.*, 1987; Kumar *et al.*, 2001). The AF-1 physically interacts with the basal transcriptional machinery (BTM) (Kumar and Thompson, 2003), and the NTD regulates GR association with chromatin remodelers, co-activators, RNA polymerase-II, TATA binding protein (TBP) and the general transcription factor IID (TFIID) complex (Ford *et al.*, 1997; Heitzer *et al.*, 2007; Henriksson *et al.*, 1997; Hittelman *et al.*, 1999). Many of the residues which undergo post-translational modifications reside in the NTD (reviewed in Duma *et al.*, 2006; Faus and Haendler, 2006).

The receptor makes direct DNA contact through the DBD (residues 420-480). Two highly conserved zinc finger motifs containing four cysteine residues co-ordinate a zinc atom in a tetrahedral fashion to make direct contact with DNA (Freedman *et al.*, 1988) in the promoters of target genes at the major groove of the double helix (La Baer and Yamamoto, 1994). GR homodimerisation has been shown to be dependent on an intact DBD (Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991). The DBD contains a flexible, hinge region (HR) around which the NTD and carboxyl terminal may rotate. The amino part of the HR overlaps with a nuclear localisation signal (NLS) which is required for GR nuclear translocation upon agonist binding (Jewell *et al.*, 1995; Picard and Yamamoto, 1987).

The carboxyl terminus (residues 527-777) is termed the ligand binding domain (LBD) and contains the second, ligand-dependent, activation function (AF-2) (reviewed in Duma *et al.*, 2006; Nicolaides *et al.*, 2010) and other NLS (Cadepond *et al.*, 1992; Picard and Yamamoto, 1987). Two key residues in the LBD have also been demonstrated to be critical for GR homodimerisation (Bledsoe *et al.*, 2002). The LBD is responsible for the recognition and association with steroids, which bind the receptor at a hydrophobic pocket created by twelve α-helices and four β-sheets resulting in a conformational change of the receptor thereby determining the nature of co-factor interaction with the GR and ultimately the transcriptional response elicited by the GR (Bledsoe *et al.*, 2004; Kauppi *et al.*, 2003; Leers *et al.*, 1998).

1.3. GR mechanism of action

In the absence of ligand, the monomeric and inactive GR is held in a heteromeric chaperone complex that is predominantly cytoplasmic (Jewell *et al.*, 1995) but does cycle in and out of the nucleus (Hache *et al.*, 1999). The components of this complex include the Heat Shock Proteins (HSP) 90, 70, 40, and 23, HSP organising Protein (HOP), immunophilins such as FK506 binding protein 51 (FKBP51), and phosphatases such as Protein Phosphatase 5 (PP5), as well as p23 (Figure 1.3) (Dalman *et al.*, 1989; Echeverria *et al.*, 2009; Echeverria and Picard, 2010; Galigniana *et al.*, 2010). HSP90 and the associated chaperones hold the receptor in

a conformation essential for allowing ligand binding (Bohen, 1995; Bohen *et al.*, 1995; Dittmar *et al.*, 1997).

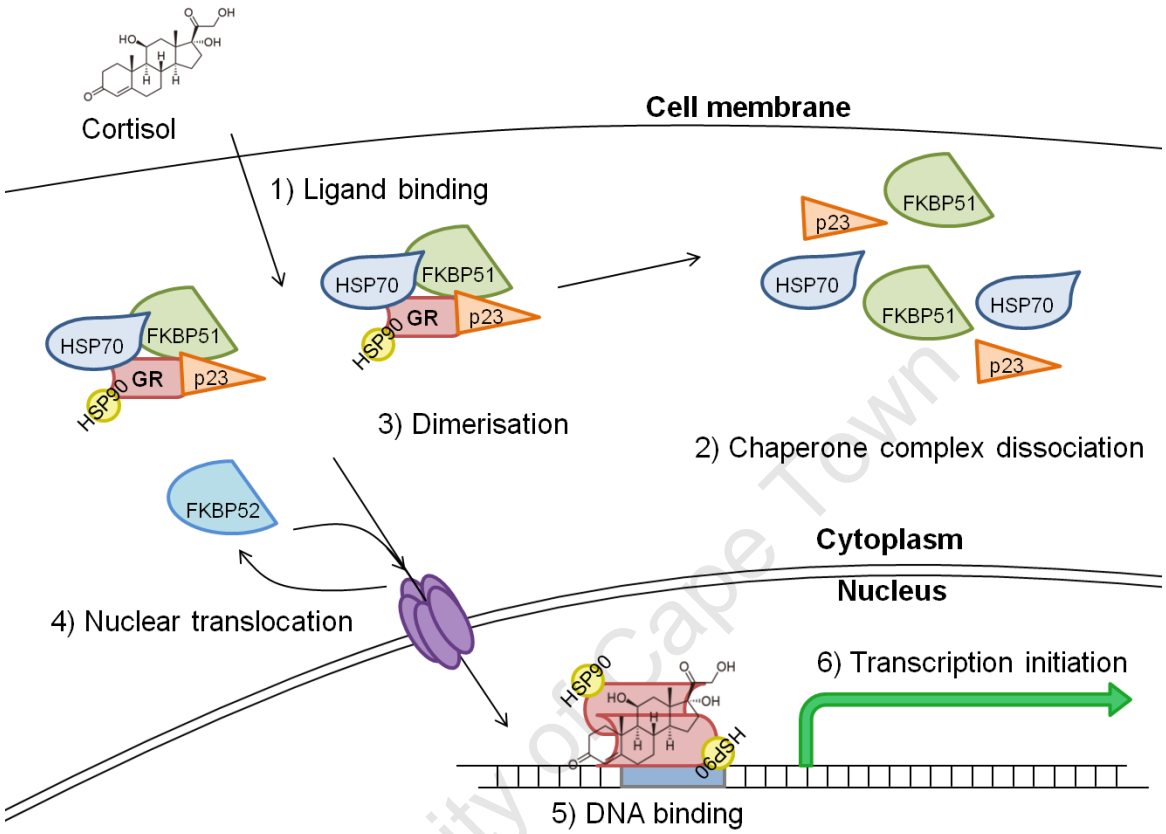


Figure 1.3: Mechanism of GR transcriptional signalling. Ligand binding causes chaperone complex dissociation, nuclear translocation, receptor dimerisation, and binding to DNA response elements resulting in GR-mediated modulation of transcription (Adapted from Revollo and Cidlowski, 2009).

1.3.1. Ligand binding to the GR

Due to their lipophilic nature, cortisol and other GC's passively diffuse from the blood across the plasma membrane after their release from CBG's. Ligands bind the GR directly at a hydrophobic pocket in the LBD (Bledsoe *et al.*, 2004). Several lines of evidence suggest that electrostatic interactions between ligands and the residues in this pocket are specific to the ligand and cause a conformational change to the tertiary structure of the LBD according to the nature of the bound ligand (Gass *et al.*,

1998; Lind *et al.*, 2000; Ray *et al.*, 1999). This is critical for the induction of transcription because it dictates co-factor association and DNA binding of the GR (Figure 1.4) (Kauppi *et al.*, 2003).

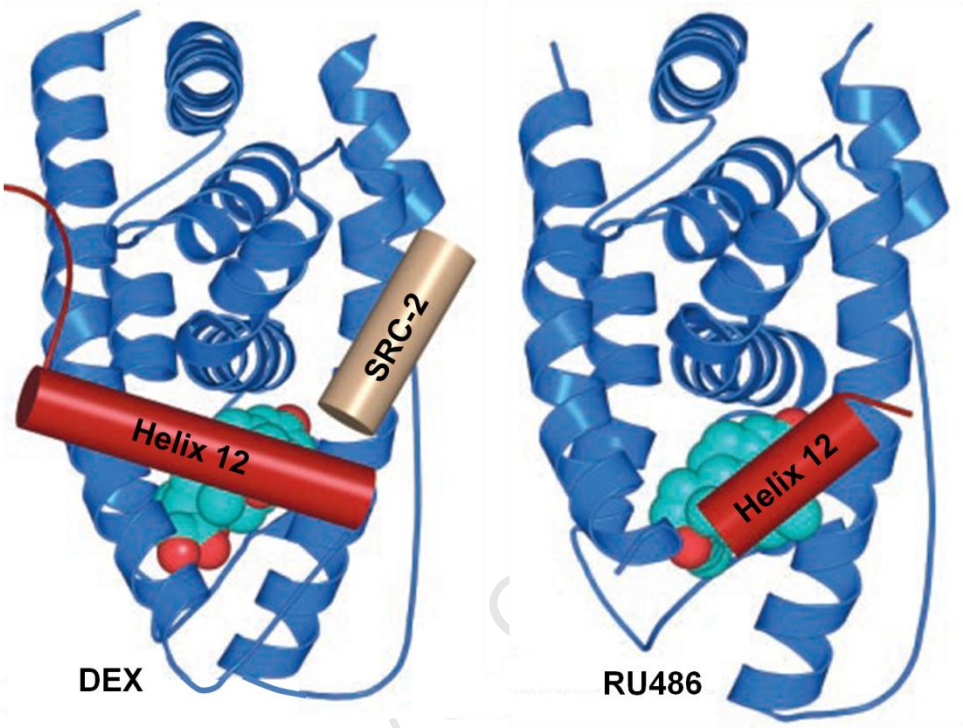


Figure 1.4: Ribbon diagram showing DEX vs. RU486 binding to the LBD hydrophobic pocket in the human GR α isoform (hGRα). Agonist binding to the GR shifts the position of helix-12 (red) allowing a co-factor, in this case, Steroids Receptor Co-activator 2 (SRC-2) to associate with the GR through LXXLL co-activator motifs (Voegel *et al.*, 1998). Antagonist binding causes helix-12 to block this site and prevents co-factor interaction (taken from Kauppi *et al.*, 2003).

Electrostatic and hydrophobic interactions between the receptor and bound ligand compact the helices around this pocket, changing the conformation of the receptor. Agonist binding causes the twelfth α-helix to close over the steroid which allows co-factors to bind (Kauppi *et al.*, 2003) at the AF-2 domain through LXXLL co-activator (Heery *et al.*, 1997), or LXX I/H I XXX I/L co-repressor motifs (Perissi *et al.*, 1999). It is generally accepted that the specific conformation the helices adopt, and thus the extent of co-factor interaction varies according to whether an agonist, partial agonist, or antagonist is bound. Antagonist binding blocks exposure of the AF-2 domain on helix-12 and prevents co-factor association (Figure 1.4) (Wang *et al.*, 2004c). A GR

partial agonist is a steroid which displays less than the maximal transactivation efficacy of an agonist such as cortisol or dexamethasone, and an antagonist is able to inhibit the transactivation induced by a full agonist (Figure 1.5).

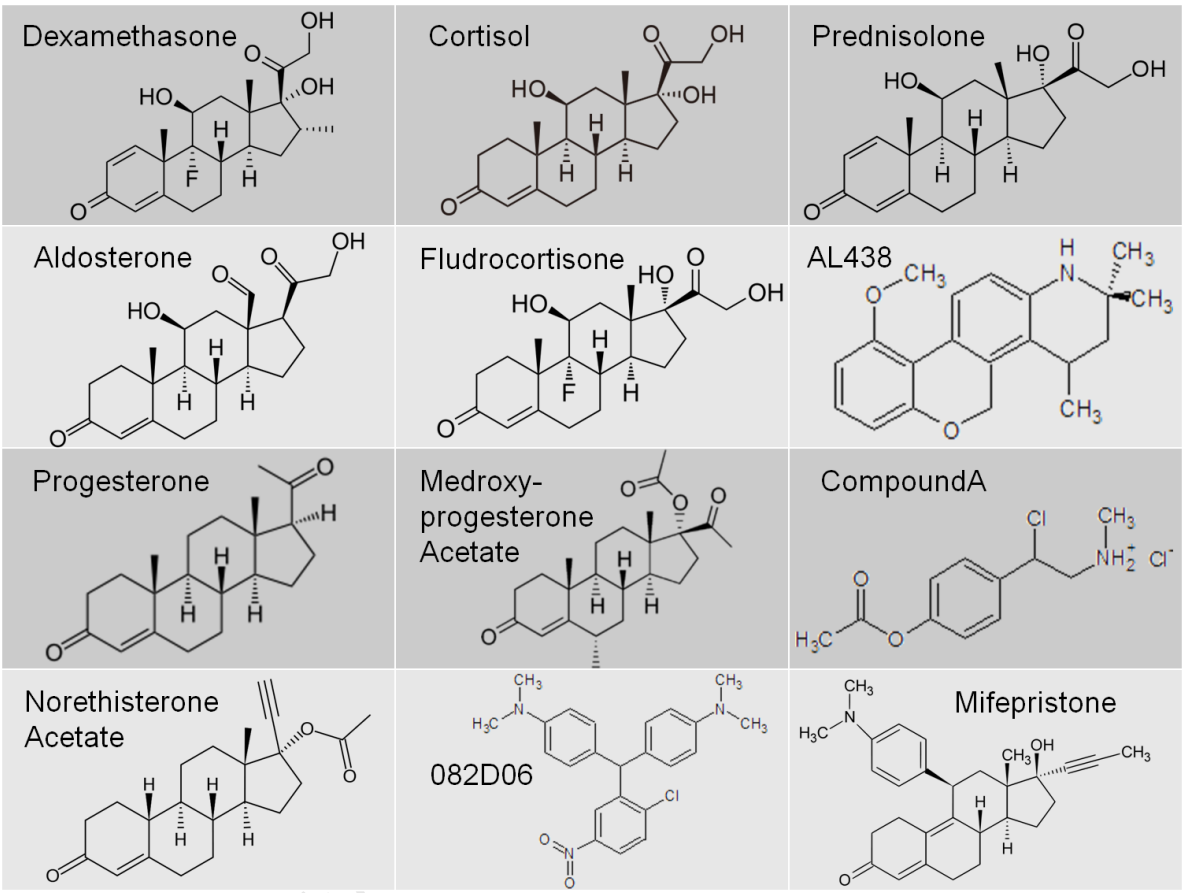


Figure 1.5: Structures of select GR ligands, indicated as full or partial agonists, dissociated, or antagonists by GR-mediated transactivation efficacies. Dexamethasone, Cortisol and Prednisolone are full GR agonists and induce the highest extent of GR-mediated transactivation. Aldosterone, Fludrocortisone, and Medroxyprogesterone Acetate (MPA) may act as either full agonists or partial agonists, and Abbott Ligand AL438 and Progesterone are partial GR agonists as their relative efficacies are much lower than that of dexamethasone. Mifepristone (RU486) and CompoundA are dissociated GC's, as they may selectively act as an agonist or antagonise GR-mediated transcription in a cell type and promoter-specific manner. Norethisterone Acetate (NET-A exerts nearly no effect on GR-mediated transactivation and Abbott Ligand 082D06 is able to antagonise GR-mediated transactivation. It should be noted that the biocharacter of these ligands varies in a cell type and promoter-specific manner (Avenant *et al.*, 2010b; Koubovec *et al.*, 2005; Ronacher *et al.*, 2009).

Apart from conformational changes in the LBD, it is generally accepted that upon association with ligand, the whole GR protein undergoes a conformational change,

causing much of the chaperone complex to dissociate and exposing the NLS's (reviewed in Nicolaides *et al.*, 2010). The unliganded GR associates with FKBP51 through a direct interaction with HSP90, which upon ligand binding is switched for the nucleophilic FKBP52 (Banerjee *et al.*, 2008; Davies *et al.*, 2002; Galigniana *et al.*, 1998; Galigniana *et al.*, 2010; Silverstein *et al.*, 1999). Recent evidence indicates that HSP90 translocates into the nucleus along with the GR and can be found in association with DNA, acting as a regulator of GR function and modulating GR mobility within the nucleus (Echeverria *et al.*, 2009; Echeverria and Picard, 2010; Elbi *et al.*, 2004).

1.3.2. Dimerisation

Chaperone complex dissociation exposes hydrophobic residues in the LBD and DBD which cause GR homodimerisation (Bledsoe *et al.*, 2002; Luisi *et al.*, 1991). This occurs prior to (Savory *et al.*, 1999; Savory *et al.*, 2001), but is not strictly required for nuclear translocation or DNA binding (Adams *et al.*, 2003). Certain mutations in the LBD and DBD prevent homodimerisation and abrogate transactivation, but do not affect nuclear translocation or transrepression (Reichardt and Schutz, 1998; Reichardt *et al.*, 2001). CompoundA is a dissociated GC which causes nuclear localisation and GR-mediated transrepression, but not homodimerisation or transactivation (De Bosscher *et al.*, 2005; Robertson *et al.*, 2010). Thus several lines of evidence suggest that dimerisation is important for GRE-mediated transactivation/DNA binding but not for transrepression or nuclear translocation, although transactivation has been reported to also occur via a monomeric GR (Segard-Maurel *et al.*, 1996).

1.3.3. Nuclear translocation

The two GR NLS's are exposed upon chaperone complex dissociation (Urda *et al.*, 1989). NLS-1 is found at the DBD, with the less well defined NLS-2 at the LBD (Picard and Yamamoto, 1987; Wan *et al.*, 2001). The exposure of these motifs causes a direct interaction between the GR and nucleophilins (importin-α, -7, and -8) responsible for translocation through nuclear pores (Figure 1.3) (Echeverria *et al.*, 2009; Elbi *et al.*, 2004; Freedman and Yamamoto, 2004). The switch between FKBP51 and FKBP52 is also responsible for GR nuclear translocation. The unliganded GR is associated with FKBP51, which is switched for the nucleophilic FKBP52 upon ligand stimulation (Banerjee *et al.*, 2008; Davies *et al.*, 2002). FKBP52 associates with dynein and translocates the HSP90/GR complex into the nucleus along the microtubule network (Czar *et al.*, 1994; Galigniana *et al.*, 1998; Silverstein *et al.*, 1999). Knockout mouse models have shown that the absence of FKBP52 does not cause defects in GR physiology, indicating a degree of redundancy in GR nuclear import (Wolf *et al.*, 2009). DEX stimulation causes GR nuclear translocation in 10 minutes (Picard and Yamamoto, 1987) and the proportion of nuclear GR correlates well with the transactivation efficacy of the stimulating ligand (Hadley *et al.*, 2011).

1.3.4. Post-translational modifications

The GR is expressed in nearly all mammalian cells, but regulates transcription in a cell type- and promoter-specific manner. One mechanism that explains some of these variable effects of the GR is covalent modifications that occur on the receptor after translation (Figure 1.6). These include acetylation (A) (Kino and Chrousos, 2011), nitrosylation (N) (Galigniana *et al.*, 1999), phosphorylation (P) (Orti *et al.*, 1993), sumoylation (S) (Tian *et al.*, 2002), and ubiquitination (U) (Kinyamu *et al.*, 2005). GR post-translational modifications may have a positive or negative effect on GR-mediated transcription (reviewed in Duma *et al.*, 2006; Faus and Haendler, 2006).

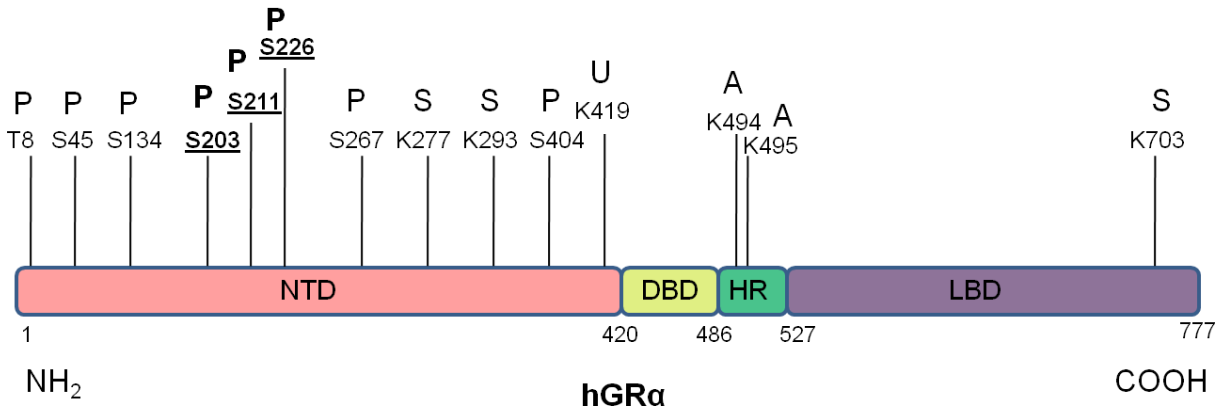


Figure 1.6: Structure of the GR protein showing residues that may undergo covalent post-translational modifications. Serine (S) and threonine (T) residues may be phosphorylated (P) and specific lysine (K) residues may be sumoylated (S), ubiquitinated (U) or acetylated (A) under appropriate stimuli (Adapted from Beck *et al.*, 2011; Faus and Haendler, 2006; Galliher-Beckley and Cidlowski, 2009; Ismaili and Garabedian, 2004).

1.3.4.1. Phosphorylation

GR phosphorylation has been reported to regulate many aspects of GR signalling, including nuclear localisation (Wang *et al.*, 2002), cofactor association (Avenant *et al.*, 2010a; Krstic *et al.*, 1997), promoter association (Blind and Garabedian, 2008), GR protein stability (Webster *et al.*, 1997), and gene expression (Lynch *et al.*, 2010). The unliganded GR is partially phosphorylated, and hyperphosphorylation occurs at specific residues in response to agonist (Avenant *et al.*, 2010a; Avenant *et al.*, 2010b; Chen *et al.*, 2008; Orti *et al.*, 1993; Wang *et al.*, 2002) but not antagonist binding (Hoeck and Groner, 1990; Orti *et al.*, 1989; Wang *et al.*, 2002). Most GR phosphorylation sites reside in the AF-1 domain (Figure 1.6), with three serine (S) residues; S203, S211, and S226 being the best characterised (reviewed in Beck *et al.*, 2011; Galliher-Beckley and Cidlowski, 2009; Ismaili and Garabedian, 2004).

S211 is not phosphorylated in the absence of ligand and becomes hyperphosphorylated upon agonist binding (Wang *et al.*, 2007) in a manner that correlates with ligand efficacy (Avenant *et al.*, 2010b; Wang *et al.*, 2002). S211 phosphorylation has been implicated in GR promoter association (Blind and Garabedian, 2008) and to increase GR-mediated transactivation, as an S211A

mutation causes lower transactivation efficacy of reporter (Avenant *et al.*, 2010b) and endogenous genes (Chen *et al.*, 2008) in a variety of mammalian cells. This phosphorylation mutant also fails to induce apoptosis to the same extent as the wild type (wt) GR does (Almlof *et al.*, 1995; Miller *et al.*, 2005), and taken together this indicates that S211 phosphorylation is likely required for maximal GR-mediated transactivation. The p38 mitogen-activated protein kinase (MAPK) (Miller *et al.*, 2005) and cyclin-dependent kinases (CDK's) 5 and 2 (Kino *et al.*, 2007; Krstic *et al.*, 1997) have both been implicated in S211 phosphorylation indicating some redundancy or cell specificity in the kinases responsible for S211 phosphorylation (Chen *et al.*, 2008; Lynch *et al.*, 2010).

The GR may be phosphorylated simultaneously at S211 and S226 (Wang *et al.*, 2007) with transcriptional activation being greatest when the ratio of S211/S226 phosphorylation is highest (Chen *et al.*, 2008). S226 is partially phosphorylated in the absence of ligand, but is hyperphosphorylated by ligand stimulation (Blind and Garabedian, 2008). S226 phosphorylation is kept at a low level through the actions of PP5 in the chaperone complex (Wang *et al.*, 2007). The pro-inflammatory c-Jun N-terminal kinase (JNK) ligand-dependently increases phosphorylation of S226, which inhibits GR-mediated transcription (Lynch *et al.*, 2010) and enhances nuclear export (Itoh *et al.*, 2002). In support of this, an S226A mutation increases GR-mediated reporter (Avenant *et al.*, 2010b) and endogenous gene transactivation (Chen *et al.*, 2008).

Recent evidence has indicated S226 phosphorylation to be a marker for ligand-independent GR activation, as GnRH (Kotitschke *et al.*, 2009) and TNF-α (Verhoog *et al.*, 2011) have both been shown to increase S226 phosphorylation and ligand-independently activate the GR. GnRH induced phosphorylation of S226 causes enhanced SRC-1 association with the GR and increases transactivation of the GnRH gene (Kotitschke *et al.*, 2009), whereas TNF-α further suppresses GR-mediated repression of IL-6 (Verhoog *et al.*, 2011).

In contrast to S211 and S226, a significant proportion of the GR is phosphorylated at S203 in the absence of ligand, and S203 phosphorylation is only slightly increased upon treatment with DEX (Blind and Garabedian, 2008; Wang *et al.*, 2002). S203

phosphorylation occurs through either CDK2 or 5 (Chen *et al.*, 2008; Kino *et al.*, 2007). The GR phosphorylated at S203 alone is not recruited to the promoters of the TAT, SULT, or GILZ genes, whereas receptors phosphorylated at S211 and/or S226 are (Blind and Garabedian, 2008; Wang *et al.*, 2002). S203 phosphorylation causes peri-nuclear (Chen *et al.*, 2008) cytoplasmic retention of the GR (Blind and Garabedian, 2008), and its dephosphorylation may be related to nuclear import.

1.3.4.2. Acetylation

Acetylation is the covalent attachment of an acetyl group to a protein. Upon ligand stimulation all SR's, except the ER become acetylated in the hinge region (Faus and Haendler, 2006) at a lysine rich motif which appears to be phylogenetically conserved (Fu *et al.*, 2003a; Fu *et al.*, 2003b; Wang *et al.*, 2011a; Wang *et al.*, 2011b). The GR contains an acetylation motif (KXKK/RXKK) between amino acids 492-495 with lysine residues K494 and K495 becoming acetylated in the presence of DEX (Ito *et al.*, 2006). Acetylation of the GR by p300 has been shown to decrease transactivation of the 2XGRE-TATA and SV40-luc reporter promoter constructs (Fonte *et al.*, 2007), but increase transactivation of the MMTV promoter (Li and Verma, 2002), indicating the effects of acetylation on GR transactivation are promoter-specific. It has been reported that histone deacetylase 2 (HDAC2) can also deacetylate the GR, allowing GR association with the p65 subunit of NFκB. Deacetylation therefore allows GR-mediated transrepression to occur through a tethering mechanism (Ito *et al.*, 2006).

1.3.4.3. Nitrosylation

Nitrosylation is thought to occur at several GR cysteine residues (Galigniana *et al.*, 1999). Increased Nitric Oxide (NO) synthase activity leads to increased covalent NO attachment to thiol groups, resulting in a decreased affinity for GC's without affecting the HSP90 interaction (Galigniana *et al.*, 1999).

1.3.4.4. Sumoylation

Sumoylation is the covalent attachment of the small-ubiquitin-related modifier-1 (SUMO-1) peptide to three lysine residues in the GR (K277, K293, and K703), through the actions of the SUMO conjugase Ubc9. Whilst this is not ligand-dependent, it is enhanced in the presence of steroids (Tian *et al.*, 2002) and increases GR-mediated transactivation. Sumoylation also decreases stability of the receptor by targeting it for proteasomal degradation (Le Drean *et al.*, 2002).

1.3.4.5. Ubiquitination

Ubiquitination is the covalent attachment of a 76 aa peptide through the actions of three ubiquitinating enzymes: an E1 activator, E2 conjugase, and E3 ligase (Rogers *et al.*, 1986), which may be reversed by de-ubiquitinating enzymes (Nijman *et al.*, 2005). Ubiquitination occurs at “PEST” (Pro-Glu-Ser-Thr) motifs (Rechsteiner and Rogers, 1996), specifically on the GR at lysine residue K419 (Wallace *et al.*, 2010). Ligand binding to the GR leads to poly-ubiquitination and increases proteasomal degradation (Kinyamu *et al.*, 2005), thereby abrogating the transcriptional response (Deroo *et al.*, 2002; Wallace and Cidlowski, 2001). Ubiquitination acts as a measure of control to decrease excess GR-mediated transcription (Wallace and Cidlowski, 2001).

1.3.5. Transcriptional Regulation

Ligand binding, chaperone complex dissociation, dimerisation, post-translational modifications and nuclear import are all steps which are required for and precede activation of the GR as a transcription factor. Once inside the nucleus, the GR can begin to modulate transcription, in a positive (transactivation) or negative (transrepression) manner, either by binding directly to DNA, or by associating with other transcription factors (TF) (Figure 1.7). The GR recruits the BTM, co-activators or co-repressors, and chromatin remodelling proteins to the promoter which

culminates in a change in the rate of RNA synthesis by RNA polymerase II (RNA pol II) (reviewed in Nicolaides *et al.*, 2010).

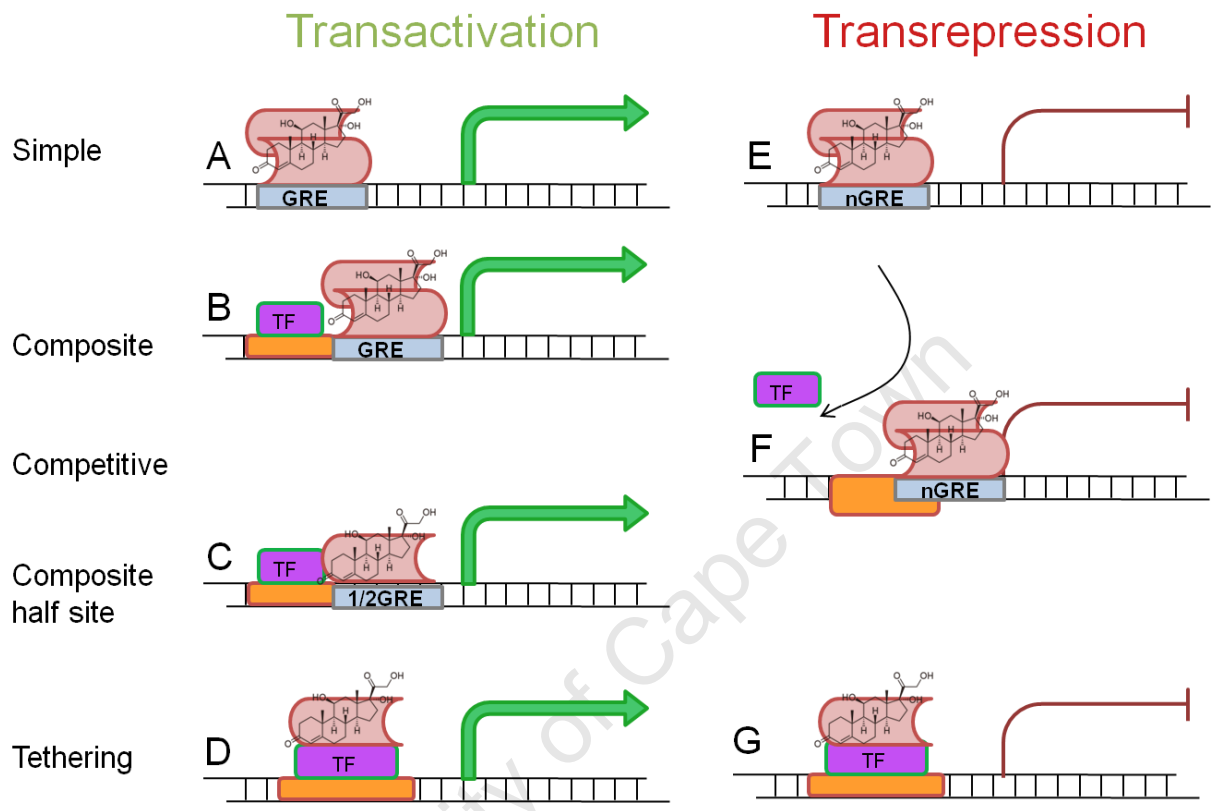


Figure 1.7 (A-G): Mechanisms of GR gene regulation by interaction directly with DNA, or protein regulatory elements. The GR (show in pink) may associate directly with DNA at a glucocorticoid response element (GRE) or negative GRE (nGRE) as a ligand bound dimer to increase (A) or repress (E) gene expression, or a liganded monomer may associate with other DNA bound transcription factors (TF) to increase (D) or repress (G) gene expression. DNA bound and liganded GR monomers (C) or dimers (B) are also capable of increasing gene expression by acting in concert with other DNA bound TF's when bound to GRE half sites (1/2GRE). DNA binding of a liganded GR dimer may also block the DNA binding of other TF's, preventing gene transactivation (F) (adapted from Schoneveld *et al.*, 2004b).

1.3.5.1. Direct DNA binding

Glucocorticoid response elements (GRE's) are palindromic DNA sequences comprised of two hexameric halves separated by three random nucleotides (Beato *et al.*, 1989; Strahle *et al.*, 1987). In humans a consensus GRE has the sequence GGTACAnnnTGTTCT (Nordeen *et al.*, 1990). The exact sequence of each half of

the GRE is not tightly constrained and allows for variability in GR signalling exerted through variances at the GR/DNA binding interface (Kumar *et al.*, 1999; Meijsing *et al.*, 2009; So *et al.*, 2007). GR/DNA binding causes the unstructured AF-1 region to adopt a specific conformation (Kumar and Thompson, 2003) leading to differential recruitment or exclusion of ancillary TF's, thereby regulating transcriptional specificity (Hard *et al.*, 1990; La Baer and Yamamoto, 1994). An interesting feature of GRE's is that their position in DNA is not tightly constrained (Bulger and Groudine, 1999) and may be found in equal frequencies up- or down-stream from the transcription start site (TSS). Up to 63% of GRE's were shown to be greater than 10 kb from the TSS with others having been found in intronic regions of the genes they regulate (So *et al.*, 2007; Wang *et al.*, 2004b).

Many anti-inflammatory genes are upregulated through the liganded GR binding GRE's in their promoters (Figure 1.7A). DEX activation of the GR rapidly induces expression of MAP Kinase Phosphatase-1 (MKP-1) (Lasa *et al.*, 2002; Wu *et al.*, 2005) by acting on at least two consensus GRE's (Shipp *et al.*, 2010; Tchen *et al.*, 2010). MKP-1 is a component of the inflammatory Mitogen Activated Protein Kinase (MAPK) cascade which prevents activation of the ERK and p38 MAPK's (Lang *et al.*, 2006). The GR can also mediate repression of cytokine genes by inhibiting NFκB signalling through enhancing expression of an inhibitor, IκBα (Auphan *et al.*, 1995), which sequesters the RelA subunit of NFκB in the cytoplasm (Li and Verma, 2002). Several GRE half sites exist in the IκBα promoter (Deroo and Archer, 2001; Deroo and Archer, 2002) and two full length GRE's reside within the first intron (Reddy *et al.*, 2009), both of which have been observed to bind the liganded GR and cause transactivation.

A GRE may form part of a larger regulatory complex termed a glucocorticoid response unit (GRU) (Figure 1.7B), where the GR functions in conjunction with other TF's to achieve more dynamic control of transcription. A GRU is variable in its spatial organisation and constituents, and the inclusion of tissue-specific TF's in certain GRU's contributes an additional measure of specificity in GR signalling. The compilation of GRE's into a GRU allows a superior level of transcriptional induction as the GR co-operates with other TF's bound at adjacent sites (Kassel and Herrlich, 2007; Schoneveld *et al.*, 2004a). Genes regulated in this manner include the

carbamoylphosphate synthase gene (Schoneveld *et al.*, 2004a) and the Phosphoenol Pyruvate Carboxy Kinase gene (Scott *et al.*, 1998).

The GR may also function as a monomer when directly binding DNA (Figure 1.7C). A GRE half-site is composed of only one of the two hexameric halves of a GRE (GGTACA or TGTTCT). A liganded monomer may bind directly and induce transactivation, although DNA association of the GR monomer alone is insufficient for induction of transcription (Adams et al., 2003). Half-sites do not function alone as a classical GRE might, but rather are regulated in conjunction with other TF's (Dschietzig *et al.*, 2009a; Dschietzig *et al.*, 2009b; Tseng *et al.*, 2001). Genes regulated by the monomeric receptor include relaxin (Dschietzig *et al.*, 2009a; Dschietzig *et al.*, 2009b) and the β_1 adrenergic receptor (Tseng *et al.*, 2001).

The GR can repress transcription when binding directly to DNA through negative GRE's (nGRE) (Figures 1.7E and F). These have similar sequences to consensus GRE's, albeit with more variation. No consensus nGRE has yet been found, but transrepression is mediated through GR binding to DNA (Dostert and Heinzel, 2004; Surjit *et al.*, 2011). An nGRE may function as full length 15 bp element or as nGRE half-sites, as seen in the CRH (Malkoski *et al.*, 1997; Malkoski and Dorin, 1999), prolactin (Sakai *et al.*, 1988) and osteocalcin promoters (Meyer *et al.*, 1997a; Meyer *et al.*, 1997b) (Figure 1.7 E). At the prolactin and osteocalcin promoters the activated GR binds directly to DNA, which overlaps other TF binding sites, preventing their DNA binding and thus transcriptional induction (Figure 1.7F). In the case of CRH, the GRE overlaps an AP-1 site, preventing TNF-α induced c-Jun/Fos signalling and in the osteocalcin promoter the nGRE incorporates the genes TATA box, which is where RNA-pol II would otherwise bind. An unusual mechanism of GR-mediated transrepression occurs at the pro-opiomelanocortin (POMC) gene. The GR first binds DNA as a homodimer at a consensus GRE. This alone is insufficient to mediate transrepression, but allows the association of a further GR monomer at the opposite side of the DNA, resulting in repression of POMC expression. It is unclear whether the GR monomer associates with the GR homodimer, or whether a conformational change to the DNA induced by GR dimer binding allows association of the monomer with DNA directly (Drouin *et al.*, 1993).

1.3.5.2. Interactions with chromatin

For a long time it has been thought that the GR acts as a “pioneer” factor which, upon ligand activation enters the nucleus and associates with GRE’s at otherwise inaccessible chromatin. Thereafter, the GR recruits chromatin remodelling TF’s, forming stable promoter bound complexes and increasing promoter accessibility for RNA pol II association, which then begins transcription at the nearby TSS and gene expression (Lemon and Freedman, 1999; Urnov and Wolffe, 2001).

This paradigm of stable one-dimensional GR gene regulation is overly simplistic and appears to be changing. Studies have indicated that the “spreading model”, which stipulates that GR signalling is transmitted directly downstream via a *cis* element bound GR may be overly simple and slightly incorrect. GRE’s have been found many kilobases away from the TSS and within intronic regions of GR regulated genes (So *et al.*, 2007; Wang *et al.*, 2004b). A “looping model” appears more favourable; in which distal response elements, which occur on separate chromosomes, may regulate gene expression by looping the DNA bound GR into contact with the TSS (Hakim *et al.*, 2010). DNA is flexible and many regions of a gene may make fleeting contact with many millions of other regions of promoters and other genes, simply by random movement within the nucleus. For the NR family, looping is mediated by a greater than random association frequency (Gothard *et al.*, 1996; Hakim *et al.*, 2009). Indeed many NR regulated genes do not display any familiar response elements near the promoter (John *et al.*, 2008; Wang *et al.*, 2004b). It is not known whether the DNA loop is a cause, or consequence of this distal method of gene regulation (Hakim *et al.*, 2009).

The concept of promoters being inaccessible prior to ligand activation of the GR has also been disputed, as it has been found that many of the GRE’s in classically regulated genes are already in a constitutively DNase hypersensitive state and do not require actions of the Switch/Sucrose Non Fermentable (SWI/SNF) chromatin remodelling complex (John *et al.*, 2008). Chromatin-remodelling is involved with rapid association and disassociation of the GR with a chromatin template, in a far more dynamic mode than postulated with the spreading model (Fletcher *et al.*, 2002;

Nagaich and Hager, 2004). Numerous GR molecules rapidly associate and disassociate with the promoter (McNally *et al.*, 2000; Nagaich *et al.*, 2004). The association is dependent on the nature of the chromatin (Turner *et al.*, 2008), with greater frequency of association when the DNA encompassing the GRE is in a hypersensitive state. It also appears that the structure of the chromatin at the promoter itself is a regulator of GR-mediated gene expression, through selective TF association (John *et al.*, 2011). Chromatin architecture is cell type-specific, and it is becoming increasingly apparent that this pre-determines GR association, and by inference may account for specificity in GR signalling (George *et al.*, 2009). Many genes highly expressed by the GR in 3134 mammary cells are refractive to GR-mediated expression in otherwise identically treated AtT20 cells owing to the absence of a promoter in a hypersensitive site (John *et al.*, 2008). GR/DNA association is a stochastic process owing to Brownian motion; however the frequency of a productive association is greater than that of non-productive associations (George *et al.*, 2009; Hager, 2002; Hager *et al.*, 2004; Hager *et al.*, 2006).

1.3.5.3. Protein-protein interactions

1.3.5.3.1. Tethering

The GR may regulate transcription independently of DNA binding, as part of the transcriptional initiation complex (TIC). In this sense the GR acts as co-regulator of other TF's. Whilst this predominantly occurs in a transrepressive manner (Figure 1.7G) (Reichardt *et al.*, 2001) it may increase the expression of some genes (Figure 1.7D) (Lerner *et al.*, 2003). This process is termed tethering and occurs when a liganded GR monomer modulates transcription by forming heterocomplexes with DNA bound TF's (Doppler *et al.*, 2001; Kassel and Herrlich, 2007). This process is independent of dimerisation and DNA binding (Ray and Prefontaine, 1994; Reichardt *et al.*, 2001) but does require key residues in the DBD and LBD of the GR (De Bosscher *et al.*, 1997; Heck *et al.*, 1994; Liden *et al.*, 1997).

Transactivation of α_2-Macroglobulin occurs through the signal transducer and activator of transcription pathway-3 (STAT-3), a transcription factor activated by IL-6 induction of the Janus Kinase (JAK). However, stimulation of cells with both IL-6 and DEX leads to a much higher level of expression than IL-6 stimulation alone does. The α_2-Macroglobulin promoter lacks GRE's, and ligand activation of the GR alone is insufficient to induce transactivation (Lerner *et al.*, 2003). Furthermore if STAT-3 is absent the GR is not recruited to this promoter (Stoecklin *et al.*, 1997; Takeda *et al.*, 1998). It is likely that the GR increases transactivation by associating with the promoter bound STAT-3 and recruiting further co-factors to the promoter (Figure 1.7F) (Lerner *et al.*, 2003; Takeda *et al.*, 1998).

Tethering is predominantly a transrepressive mechanism of gene regulation (Figure 1.7G). The majority of GR anti-inflammatory effects are mediated in this manner and it is the mechanism of gene regulation targeted by therapeutic use of GC's for the beneficial effects of anti-inflammation (De Bosscher and Haegeman, 2009; Herrlich, 2001; Reichardt *et al.*, 2001). Transrepression occurs at a much lower GC concentration than transactivation through DNA binding does (Adcock and Ito, 2000; Ito *et al.*, 2000; Reddy *et al.*, 2009; Ronacher *et al.*, 2009). The best characterised mechanisms of the GR tethering to other TF's and resulting in transrepression is through interactions with AP-1 (fos-jun) or NFκB (p65/RelA-p50) (Kassel and Herrlich, 2007). Many cytokines, such as IL-1β, IL-2, IL-6, IL-8, and TNF-α contain AP-1 and/or NFκB sites in their respective promoters (De Bosscher *et al.*, 2003). Under agonist, but not antagonist stimulation (Fryer *et al.*, 2000) the GR attenuates transcription of these genes by associating with NFκB and/or AP-1 which are bound at their response elements in the promoters (Brostjan *et al.*, 1997; Kassel and Herrlich, 2007). The effects of GR-mediated transrepression are well documented at the collagenase-3 (Col-3) promoter. Expression of this gene is regulated by AP-1, and GC stimulation causes the GR to associate with the promoter by tethering to the DNA bound AP-1 without altering the composition of the AP-1 subunits (c-Jun and c-Fos). GR association thereby serves to repress transactivation of the Col-3 promoter (Kassel *et al.*, 2004; Rogatsky *et al.*, 2001; Rogatsky *et al.*, 2002). Ligand-independent GR-mediated transrepression through tethering has also been reported for expression of IL-6 and IL-8 (Verhoog *et al.*, 2011).

1.3.5.3.2. Co-factor recruitment

Co-factors are required for the induction or repression of transcription and are accordingly classed as co-activators or co-repressors (van der Laan *et al.*, 2008). Generally these associate with the GR at the AF-2 region at the 12th α-helix (Figure 1.3) (Bledsoe *et al.*, 2002; Bledsoe *et al.*, 2004; Kauppi *et al.*, 2003) but have also been reported to bind the AF-1 region in the NTD (Kumar *et al.*, 2001; Warnmark *et al.*, 2000). The conformation the GR adopts upon DNA binding is dependent on the sequence and nature of the promoter (Deroo and Archer, 2002; Kumar *et al.*, 2001; Meijsing *et al.*, 2009) and allows for the selective recruitment of co-factors (Korzus *et al.*, 1998; Lefstin and Yamamoto, 1998; Rogatsky *et al.*, 2003) thereby contributing to highly specific gene regulation.

The interaction of the GR with co-activators is associated with a change in the nature of chromatin (John *et al.*, 2008). Co-activators associate with the GR through penta-peptide LXXLL motifs (L- leucine, X- any aa), termed nuclear receptor interacting domains (NRID) (Heery *et al.*, 1997; Voegel *et al.*, 1998). The p160 family of steroid receptor co-activators (SRC) is particularly well characterised and includes SRC1 (NcoA-1), SRC-2 (GRIP-1 or TIF2), and SRC3 (pCIP, RAC3, ACTR or AIB1) (Anzick *et al.*, 1997; Carapeti *et al.*, 1998; Ning *et al.*, 1999), all of which exert a low level of histone acetyltransferase (HAT) activity. Histone acetylation diminishes the electrostatic interaction between nucleosomes and DNA, resulting in chromatin becoming transcriptionally permissive (Eberharter and Becker, 2002; Hsiao *et al.*, 2002). This increases accessibility of the promoter and allows association of co-factors including p/CAF, p300, CREB Binding Protein (CBP), the arginine methyltransferase CARM1, and coiled co-activator (Kim *et al.*, 2003; Lee *et al.*, 2005; Vo *et al.*, 2001; Vo and Goodman, 2001) and affords a platform for the BTM to assemble on (Archer *et al.*, 1992; Hebbar and Archer, 2003). A multi-protein complex forms at the promoter, bending DNA so as to bring the GR/GRE associated TFIID and the TATA binding protein (TBP) into proximity with the TATA box (Ford *et al.*, 1997; Horikoshi *et al.*, 1992).

The chromatin remodelling protein, brahma-related gene 1 component of the switch/sucrose non-fermentable (SWI/SNF) complex (Fryer and Archer, 1998) results in ATP-dependent nucleosomal rearrangement at the transcription start site TSS (Logie and Peterson, 1997; Owen-Hughes *et al.*, 1996; Whitehouse *et al.*, 1999) thereby resulting in greater chromatin accessibility (Fletcher *et al.*, 2000; Owen-Hughes *et al.*, 1996; Owen-Hughes and Workman, 1996). Thereafter RNA pol II associates so as to initiate transcription (Beato and Sanchez-Pacheco, 1996).

SRC-2 is a well documented co-activator which associates directly with the liganded GR (Kauppi *et al.*, 2003) and enhances GR-mediated transactivation from GRE driven promoters by associating with the promoter bound GR (Avenant *et al.*, 2010a; Cho *et al.*, 2005). The association of SRC-2 is ligand-dependent and correlates well with the efficacy of the bound ligand (Ronacher *et al.*, 2009). Although SRC-2 has been reported to simultaneously increase ligand potency and efficacy (Cho *et al.*, 2005; Gehin *et al.*, 2002; Hong *et al.*, 1997; Onate *et al.*, 1995), these functions are separable and mediated independently of each other (Awasthi and Simons, 2012; Lee and Simons, 2010; Luo and Simons, 2009).

Transrepression is mediated through chromatin condensation by histone deacetyltransferases (HDAC's) and methyltransferases which include the GR co-repressors: nuclear receptor co-repressor (NCoR) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (Ordentlich *et al.*, 1999; Seol *et al.*, 1996; Zamir *et al.*, 1996). In a manner similar to the LXXLL co-activator motif, these associate with the GR though a co-repressor NRID (LXX I/H I XXX I/L) (Nagy *et al.*, 1999; Perissi *et al.*, 1999; Wang *et al.*, 2004c), but may associate in the absence of ligand (Horlein *et al.*, 1995). NCoR and SMRT can recruit the HDAC's 1, 2 (Jones *et al.*, 2001; Li *et al.*, 2000b; Wen *et al.*, 2000), 4 and 5 (Hu and Lazar, 1999; Huang *et al.*, 2000) which further condense DNA and prevent TF recruitment to promoter elements (Li *et al.*, 2001).

Interestingly, SRC-2 may also further repress expression of genes regulated by GR-mediated transrepression (Verhoog *et al.*, 2011). As indicated, GR represses AP-1 mediated activation of the Col-3 gene. In the presence of DEX, SRC-2 is recruited to

the AP-1 bound GR and serves to enhance transrepression. Similar effects of SRC-2 co-repression have also been observed for NFκB-induced transcription of IL-8 and IL-6, and of the osteocalcin gene, to which the GR mediates transrepression through binding to an nGRE (Rogatsky *et al.*, 2001; Rogatsky *et al.*, 2002; Verhoog *et al.*, 2011). These alternate co-activator/co-repressor modes of SRC-2 are mediated by separate domains (Rogatsky *et al.*, 2002). GR allosteric interactions with other proteins or DNA probably dictates which of the surfaces of SRC-2 are activated and able to modulate transcription (Lonard and O'Malley, 2007).

1.3.6. Ligand-dependent turnover

As a measure to control against excessive GR signalling, the GR is degraded through the ubiquitin-proteasome pathway (Dong *et al.*, 1988; Webster *et al.*, 1997) after periods of activation (Wallace and Cidlowski, 2001). Degradation may occur either in the nucleus or the cytoplasm after export of the receptor (Liu and DeFranco, 2000), with ligand dissociation accelerating the recruitment of the proteasome to the GR complex (Stavreva *et al.*, 2004). Proteasomal inhibition increases the extent of GR-mediated transactivation, indicating the importance of this process in attenuation of GR signalling (Deroo *et al.*, 2002; Garside *et al.*, 2006).

The GR is continually turned over, although the turnover rate of the unliganded receptor is slow if compared to the turnover rate under agonist stimulation (Dong *et al.*, 1988). The half-life of the unliganded GR is roughly 44 hours in COS1 cells and DEX stimulation decreases this to 10 hours (Avenant *et al.*, 2010b). In COS1 cells the rate of GR degradation or turnover also correlates with efficacy of the bound steroid. DEX causes greatest reduction in GR half life, followed respectively by stimulations with prednisolone, CORT, aldosterone, MPA and RU486. Stimulation of cells with P4, CompoundA and NET did not result in a significantly reduced GR half-life. In the same study, after 24 hours DEX, CORT, prednisolone, aldosterone and MPA had significantly reduced GR levels, whereas stimulation with RU486, P4, NET, Ursadiol and CompoundA did not (Avenant *et al.*, 2010b). A previous study has indicated that stimulation with RU486 resulted in a moderate decrease in GR levels

(Hoeck *et al.*, 1989). In HeLa cells saturating concentrations of DEX and CORT lead to the greatest extent of turnover, with a much lower extent induced by P4. Oestradiol and dihydrotestosterone, which do not function as GR agonists, have no effect on GR protein levels (Cidlowski and Cidlowski, 1981).

1.4. Progesterone and synthetic progestins

Cortisol is not the only steroid able to activate the GR. To exploit the therapeutic benefits of anti-inflammatory signalling for use in immune related disorders, many synthetic GC's have been developed (Figure 1.5) to act via the GR (Coghlan *et al.*, 2003). Apart from these artificial hormones, a degree of cross-talk between members of the SR family exists owing to their related structures and function (Thornton, 2001), and conserved ligand binding domains (Griekspoor *et al.*, 2007). Aldosterone and progesterone, two endogenous hormones which exert effects via the MR (Messaoudi and Jaisser, 2011) and PR (Gadkar-Sable *et al.*, 2005), respectively, can also can act via the GR. Progesterone is a weak GR agonist (Avenant *et al.*, 2010b) and has been reported before as an antagonist (Rupprecht *et al.*, 1993b), whilst aldosterone is a relatively potent GR partial agonist (Avenant *et al.*, 2010b). As synthetic analogues to cortisol have been developed for anti-inflammatory therapy, so too have synthetic progestins been developed for use in contraception (Draper *et al.*, 2006; Erkkola and Landgren, 2005). An interesting feature of these hormones is that they have long term stability, circulate at high concentrations and certain progestins have GR agonist or partial agonist properties (Koubovec *et al.,* 2005; Ronacher *et al*., 2009). Progesterone itself (and by inference, its synthetic derivatives) also acts via the PR on the HPA/G axis which regulates GR function and immune responses (Figure 1.1).

1.4.1. Physiological function

The menstrual cycle is divided into two phases; the follicular phase, in which the maturing follicle produces oestrogen (predominantly oestradiol - E2) leading to proliferation of the endometrium, and the luteal phase in which the corpus luteum in the ovaries produces P4 (Figure 1.8) (Graham and Clarke, 1997). Where E2 is an immune activator (Salem *et al.*, 2000), P4 is anti-oestrous (Gellersen *et al.*, 2009; Tait *et al.*, 2008) and antagonises the proliferative effects of E2 (Beagley and Gockel, 2003; Selgrade *et al.*, 2009) by stabilising the lining of the endometrium in preparation for embryo implantation (Graziano *et al.*, 2005; Wood *et al.*, 2007). P4 also suppresses immune activation through negative feedback inhibition of the HPA/G axis (Figure 1.1). This is essential for embryo implantation and the maintenance of pregnancy (Lydon *et al.*, 1995), as an immune response triggered by foetal antigens would otherwise be detrimental to development (Huddleston and Schust, 2004).

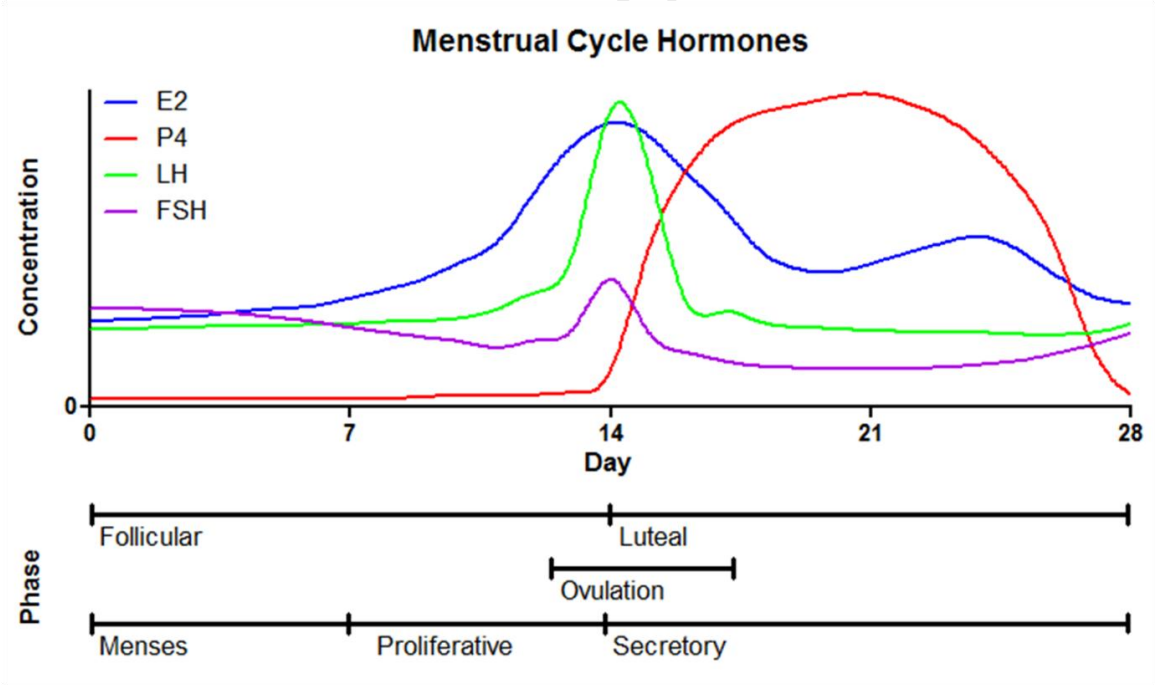


Figure 1.8: Relative concentrations of oestradiol (E2), progesterone (P4), Luteinising Hormone (LH), and Follicle Stimulating Hormone (FSH) during the menstrual cycle. The first two weeks of the menstrual cycle are termed the follicular phase and are characterised by low levels of P4 and higher levels of E2, as produced from the maturing follicle. Around the two week mark, an LH surge is observed and hormonal concentrations begin to change as the corpus luteum secretes P4, having an anti-oestrous and anti-proliferative effect (adapted from Backstrom *et al.*, 1982; Hel *et al.*, 2010).

1.4.2. Contraception

1.4.2.1. Mechanism of action

The short half life, limited bioavailability and rapid metabolism of P4 led to the development of synthetic P4 mimicking analogues which are more stable and also prevent ovulation (Whitehead *et al.*, 1980). MPA and NET are two such synthetic progestin contraceptives (Bray *et al.*, 2005) which function by decreasing the pulsatile frequency of GnRH secretion from the hypothalamus, thereby preventing synthesis of the gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH). The absence of these gonadotropins prevents follicle development and ovulation, which in turn prevents an increase in E2 levels, and thus menstruation (Jeppsson *et al.*, 1982; Kaunitz, 2000a; Mishell, 1996). In addition, progestins cause endometrial thinning and increase cervical mucus thickness. This reduces sperm mobility, increases sperm atrophy and hinders implantation of fertilised eggs (Ildgruben *et al.*, 2003). Due to the lack of pulsatile hormone synthesis, women on progestin-only injectable contraceptives often experience amenorrhea (Kaunitz, 2000b; Mainwaring *et al.*, 1995). MPA and NET elicit similar progestogenic responses via the PR (Bergink *et al.*, 1983; Deckers *et al.*, 2000; Kontula *et al.*, 1975; MacLaughlin and Richardson, 1979) and both have higher relative binding affinities for the PRthan P4 (Africander *et al* 2011b). However, the physiological effects of these steroids differ markedly not only from each other but also from that of P4 when acting through other SR's (Africander *et al.*, 2011a; Africander *et al.*, 2011b; Hadley *et al.*, 2011; Koubovec *et al.*, 2005; Sitruk-Ware, 2004).

1.4.2.2. Non-progestogenic MPA and NET effects

Progestin based contraception may inhibit the immune system and ovary function through activation of the HPA/G axis (Figure 1.1) according to the dose and type of steroid used (Erkkola and Landgren, 2005). Newer generation progestins attempt to mimic as closely as possible the effects (or otherwise) P4 has on other members of the SR family so as to reduce side effects, and undesirable non-progestogenic activity. MPA and NET-EN are first generation progestins and do exert some side

effects (Draper *et al.*, 2006; Haider and Darney, 2007). Both steroids interact with the MR, AR and GR (Africander *et al.*, 2011a; Africander *et al.*, 2011b; Africander, 2010; Bentel *et al.*, 1999; Ghatge *et al.*, 2005; Koubovec *et al.*, 2005; Winneker *et al.*, 2003) leading to cross-reactivity and non-progestogenic activity.

Table 1.1: P4, MPA and NET relative binding affinities and efficacies for the PR, MR and GR (adapted from Africander *et al.*, 2011b). Relative binding affinities are indicated as a percentage of the total specific binding of the endogenous ligand (progesterone, cortisol, or aldosterone respectively) and determined from *in vitro* recombinant human steroid receptor binding studies (Philibert *et al.*, 1999).

		P4	MPA	NET
Relative Binding Affinity	**PR**	100	298	134
	GR	11	58	1.4
	MR	1000	3.1	2.7
Efficacy	**PR**	Agonist	Agonist	Agonist
	GR	Partial Agonist	Partial Agonist	-
	MR	Antagonist	-	-

P4 and some progestin contraceptives have been found to act as potent MR antagonists and inactivate this receptor by competitive inhibition with its ligand, aldosterone. MPA and NET-A have relatively low MR affinities, and do not significantly moderate transcription form the SR (Table 1.1). This lack of MR antagonism may result in increases in weight, cardiovascular risk and blood pressure during progestin-only contraception (Rupprecht *et al.*, 1993a; Sitruk-Ware, 2004). In terms of transactivation efficacy via the GR, P4 is a weak agonist (Avenant *et al.*, 2010b; Fuhrmann *et al.*, 1996; Koubovec *et al.*, 2005; Ronacher *et al.*, 2009). Due to their stability and affinity for the GR, synthetic progestins may exert GC like transcriptional effects. MPA has a much higher relative binding affinity for the GR (79.1%) compared to NET-A (0.88%), NET (0.1%) or P4 (5.57%), and exerts numerous GC like effects (Koubovec *et al.*, 2005; Schoonen *et al.*, 2000). MPA is a GR partial agonist for transactivation (Avenant *et al.*, 2010b; Ronacher *et al.*, 2009), but may become a full agonist at high GR concentrations (Zhao *et al.*, 2003). Indeed MPA binds the GR with a greater affinity than does the endogenous ligand cortisol (Kontula *et al.*, 1983). MPA mediates GR transrepression to a greater extent than it does transactivation, in a manner resembling a dissociated GC (Bamberger *et al.*,

1999) but has a much higher GR transactivation efficacy than either NET or P4 (Avenant *et al.*, 2010b). NET-A is ineffective at GR transactivation, but has been reported to repress transcription from the IL-8 promoter, as do agonistic GC's. As a result of its high GR efficacy, MPA is likely to have more GC like side effects than NET-A and P4 (Africander, 2010; Draper *et al.*, 2006; Ishida and Heersche, 2002). MPA increases GR activity, resulting in repression of some immune related genes by mediating transrepression via the GR. Repressed genes include IL-1; IL-2; IL-6 and IL-8 (Africander *et al.*, 2011a; Bamberger *et al.*, 1999; Brunelli *et al.*, 1996; Hapgood *et al.*, 2004; Koubovec *et al.*, 2004; Koubovec *et al*., 2005; Kurebayashi *et al.*, 2003; Malarkey *et al.*, 1997; Wakatsuki *et al.*, 2002). A loss of bone mineral density or osteoporosis is widely reported in MPA users and most likely arises from its anti-oestrogenic and GR agonist properties (Ishida and Heersche, 2002; Ishida *et al.*, 2008; Kaunitz, 2002; Scholes *et al.*, 2002).

1.4.2.3. Progestin contraception and HIV-1

The concentrations of E2 and P4 during the menstrual cycle have been shown to influence HIV-1 pathogenesis (Asin *et al.*, 2008; Cu-Uvin *et al.*, 2000; Greenblatt *et al.*, 2000), with high P4 levels increasing disease progression and risk of infection or HIV-1 acquisition (Hel *et al.*, 2010). Oestrogens are inflammatory and enhance endothelial cell function, thereby protecting the vagina against viral infection (Smith *et al.*, 2004) whereas P4 is anti-oestrous and anti-proliferative (Parr *et al.*, 1994; Smith *et al.*, 2000). P4 increases the genetic diversity of the HIV-1 populations and is linked to an increase in secondary infections such as *Chlamydia* and *Lactobacillus spp*. (Baeten *et al.*, 2005; Jacobson *et al.*, 2000; Lavreys *et al.*, 2004b; MacLean, 2005).

Trials in Rhesus Macaque monkeys have indicated MPA administration to increase risk of Simian Immunodeficiency Virus (SIV) infection and cause higher viral loads (Abel *et al.*, 2004; Marx *et al.*, 1996; Veazey *et al.*, 2003). This is possibly due to the anti-oestrogenic effects of MPA and P4 (Smith *et al.*, 2004). Subcutaneous injection also caused marked vaginal mucosal epithelium thinning, resulting in increased

susceptibility to SIV vaginal challenge (Genesca *et al.*, 2010; Hild-Petito *et al.*, 1998; Marx *et al.*, 1996) and caused immunosuppression in Macaques (Trunova *et al.*, 2006).

Progesterone-only based contraception may increase the risk of HIV-1 disease transmission in certain groups of people (Hel *et al.*, 2010; Marx *et al.*, 1996; Morrison, 2007; Morrison *et al.*, 2007; Morrison *et al.*, 2012). HIV-1 positive MPA users have been found to have higher viral loads, a greater genetic diversity of viral variants and more cell associated viral DNA in the cervo-vaginal environment (shedding) than infected women not using MPA (Kreiss *et al.*, 1994; Lavreys *et al.*, 2004a; Mostad *et al.*, 2000; Royce *et al.*, 1997). The extent of viral shedding has been shown to correlate with hormonal contraception (Clemetson *et al.*, 1993; Mostad *et al.*, 1997; Mostad *et al.*, 2000; Richardson *et al.*, 2007; Wang *et al.*, 2004a). MPA may increase susceptibility to cervo-vaginal viral and bacterial infections (Hapgood *et al.*, 2004; MacLean, 2005; Trunova *et al.*, 2006; Wang *et al.*, 2004a), through altering immune function and vaginal physiology and suppression of the humoral and cell-mediated immune responses (Gillgrass *et al.*, 2003; Kaushic *et al.*, 2003; Sonnex, 1998). MPA may also increase HIV-1 transmissibility through increasing recruitment of inflammatory HIV-1 target cells to the genital tract (Ghanem *et al.*, 2005; Martin *et al.*, 1998; Prakash *et al.*, 2002).

The vaginal and cervical lining is a formidable barrier to invading pathogens, with epithelial cells expressing high levels of cytokines and chemokines to ensure constant immune surveillance and a thick mucosal layer to hinder pathogen mobility (Fichorova *et al.*, 2001a; Fichorova *et al.*, 2001b; Miller and Shattock, 2003). Progestin contraceptives may decrease the barrier protection of the endometrium (Sorensen *et al.*, 2002; Torgrimson *et al.*, 2011), thereby increasing the risk of HIV-1 infection. MPA may increase the risk of HIV-1 infectivity by antagonising the proliferative and protective effects of oestrogen and reducing the barrier protection of the vaginal epithelium and reducing the protective effect normally exerted by normal vaginal flora, *Lactobacillus spp*. (Bahamondes *et al.*, 2000; Mauck *et al.*, 1999; Miller *et al.*, 2000). Intra-vaginal progestin administration also increases the presence of Langerhans cells in the epithelial layers of the vagina (Huber and Gruber, 2001; Wieser *et al.*, 2001) and MPA has been found to increase the number of

intraepithelial leukocytes, specifically of Langerhans cells, $CD8^+$ T-lymphocytes and dendritic cells, all of which are HIV-1 target cells (Freed, 2004; Ildgruben *et al.*, 2003; Miller, 1998a; Miller, 1998b; Turville *et al.*, 2001). The direct effect of MPA on vaginal endometrium is controversial, as it is unclear whether MPA usage reduces (Miller *et al.*, 2000), has no effect (Bahamondes *et al.*, 2000; Hild-Petito *et al.*, 1998; Mauck *et al.*, 1999), or increases (Ildgruben *et al.*, 2003) the endometrial thickness. Increased HIV-1 pathogenesis in MPA users is likely due to changes to cervical and vaginal physiology and the number and types of HIV-1 target cells at the cervical mucosa (Mauck *et al.*, 1999).

Conflicting reports exist, with some suggesting no correlation between HIV-1 acquisition and hormonal contraception (Allen *et al.*, 1991; Bulterys *et al.*, 1994; Kapiga *et al.*, 1994; Kiddugavu *et al.*, 2003; Kleinschmidt *et al.*, 2007; Mati *et al.*, 1995; Morrison *et al.*, 2007; Morrison *et al.*, 2011; Myer *et al.*, 2007; Siraprapasiri *et al.*, 1991), whilst others have found its use to increase HIV-1 acquisition, albeit for specific sub-populations (Baeten *et al.*, 2005; Blish and Baeten, 2011; Heffron *et al.*, 2012; Jain, 2012; Lavreys *et al.*, 2004a; Leclerc *et al.*, 2008; Martin *et al.*, 1998; Morrison *et al.*, 2005; Morrison *et al.*, 2010; Morrison *et al.*, 2012; Rehle *et al.*, 1992). Similarly, no consensus has been reached as to whether MPA has no effect on (Cejtin *et al.*, 2003; Morrison *et al.*, 2009; Richardson *et al.*, 2007), or increases viral levels and accelerates the loss of $CD4^+$ T-cells in HIV-1 positive women (Stringer *et al.*, 2007).

Much evidence also indicates a role for the GR in HIV-1 pathogenesis (reviewed in Hapgood and Tomasicchio, 2010) and as progestins may be anti-inflammatory themselves or when acting via this receptor (Bamberger *et al.*, 1999; Brunelli *et al.*, 1996; Koubovec *et al.*, 2004; Koubovec *et al.*, 2005; Kurebayashi *et al.*, 2003; Malarkey *et al.*, 1997; Parr *et al.*, 1994; Wakatsuki *et al.*, 2002), it may be that injectable contraceptives render users more susceptible to HIV-1 acquisition through modulating immune responses via the GR.

1.5. The GR and HIV-1 pathogenesis

Many reports in the literature indicate that GR activity and function becomes enhanced with HIV-1 infection, leading to GC hypersensitivity and many HIV-1 positive patients often display chronic over-activation or dysregulation of the HPA axis causing excess production of GC's (Kino and Chrousos, 2001; Kino and Chrousos, 2002; Kino *et al.*, 2003a; Kino *et al.*, 2003b; Norbiato *et al.*, 1997a; Norbiato *et al.*, 1997b; Norbiato *et al.*, 1997c; Norbiato *et al.*, 1998; Wang *et al.*, 1998a). It is entirely possible that enhanced GR function is mediated in part through the actions of Viral Protein R (Vpr), a small virion associated, accessory protein reported before as a GR co-activator (Fakruddin and Laurence, 2005; Kino *et al.*, 1999; Kino *et al.*, 2002a; Kino *et al.*, 2002b; Mirani *et al.*, 2002; Muthumani *et al.*, 2006; Thotala *et al.*, 2004). This may be a viral strategy to utilise the immunosuppressive properties of the receptor to ensure pathogenic success and continued transmission (reviewed in Hapgood and Tomasicchio, 2010).

Altered function of the HPA axis has been reported previously in HIV-1 positive subjects (Dluhy, 1990; Kino and Chrousos, 2007; Sellmeyer and Grunfeld, 1996). HIV-1 disease progression is marked by a reduction in the innate and T-helper cell driven immunities, muscle wasting and myopathy, and a switch from the Th1 (IL-12; IFN-γ and TNF-α) to Th2 (IL-4; IL-10 and IL-13) cytokine profiles (Clerici *et al.*, 1997a; Clerici *et al.*, 1997b; Clerici and Shearer, 1997; Pantaleo *et al.*, 1993; Wang *et al.*, 1998b; Yun *et al.*, 2004) consistent with effects an overactive GR would have (Elenkov and Chrousos, 2006; Mirani *et al.*, 2002; Norbiato *et al.*, 1997b). IL-12 is a T-cell stimulating factor secreted from leukocytes and a regulator of Th1 immunity (Ma and Montaner, 2000; Ma *et al.*, 2000). GC's suppress expression of both the p40 and p70 subunits of this gene (Visser *et al.*, 1998). The decrease in IL-12 levels is also a key marker of HIV-1 induced immunosuppression and the progression to AIDS (Smed-Sorensen *et al.*, 2004; Tcherepanova *et al.*, 2009) and has been reported to be a GR target during HIV-1 infection (Mirani *et al.*, 2002).

1.5.1. Vpr

HIV-1 encodes six accessory proteins: Tat, Rev, Vpu, Vif, Nef, and Vpr (Gramberg *et al.*, 2009). Vpr is a small, 96 aa 14 kDa protein with a diverse array of functions and a high degree of sequence conservation amongst primate lentiviruses, although in SIV and HIV-2 the functions are split between Vpr and a similar accessory gene, Vpx, as a result of a gene duplication (Tristem *et al.*, 1992). In HIV-1 positive individuals Vpr is packaged directly into HIV-1 virions (Bachand *et al.*, 1999), and has been found to occur unassociated with other proteins in serum and has the ability to passively diffuse across cell membranes (Balasubramanyam *et al.*, 2007; Levy *et al.*, 1995; Tungaturthi *et al.*, 2003; Xiao *et al.*, 2008). This allows Vpr the ability to exert pathogenic effects in infected and uninfected cells alike, in a variety of tissues and organs (Coeytaux *et al.*, 2003; Godet *et al.*, 2010; Sherman *et al.*, 2002).

Vpr consists of three α-helices folded around a hydrophobic core (Figure 1.9). The first two helices are arranged in a helix-turn-helix motif (Morellet *et al.*, 2003; Wecker and Roques, 1999) with the third helix offering a stretch of hydrophobic residues resembling a leucine zipper motif (Bourbigot *et al.*, 2005; Schuler *et al.*, 1999; Wang *et al.*, 1996b), which may also contribute to oligomerisation (Schuler *et al.*, 1999; Venkatachari *et al.*, 2010; Zhao *et al.*, 1994b) and interact with host proteins (Zhao *et al.*, 1994a).

Vpr is associated with many pathogenic functions, including cell-cycle arrest at the G2/M interface (Di Marzio *et al.*, 1995; He *et al.*, 1995), the induction of apoptosis (Patel *et al.*, 2000; Snyder *et al.*, 2010), nuclear import of the viral preintegration complex (Haffar *et al.*, 2000), increasing the fidelity of reverse transcription (Fenard *et al.*, 2009), transcriptional regulation of host genes (Fakruddin and Laurence, 2004; Fakruddin and Laurence, 2005; Janket *et al.*, 2004; Tcherepanova *et al.*, 2009) and transactivation of the viral long terminal repeat (LTR) (Ayyavoo *et al.*, 1997a; Cohen *et al.*, 1990). Vpr is also highly immunosuppressive (Muthumani *et al.*, 2005b) and modulates the activity of the pro-inflammatory TF, NFκB (Ayyavoo *et al.*, 1997b) resulting in decreased production of the cytokines IL-12 and TNF-α (Tcherepanova *et al.*, 2009). A single arginine residue (R90) is highly conserved amongst viral

subtypes and has been reported to be important in mediating these immunosuppressive properties. Mutation of this residue alleviates IL-12 suppression and causes rapid Vpr degradation (Tcherepanova *et al.*, 2009).

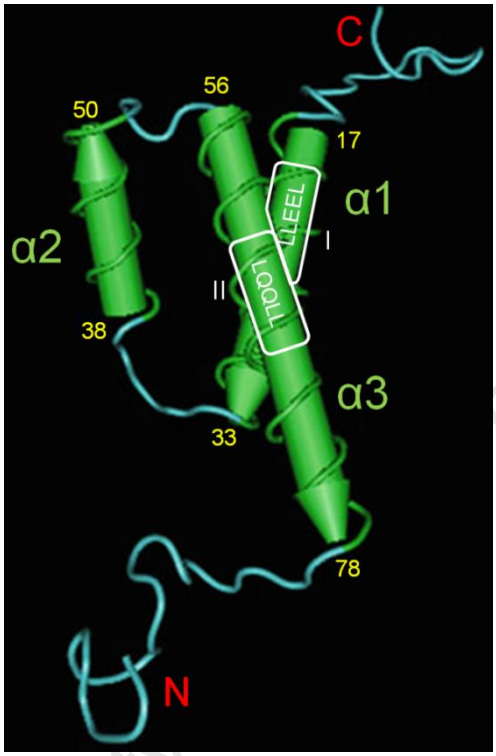


Figure 1.9: Three dimensional structure of Vpr showing the positions of the three α-helices and the two NRID co-activator motifs. Vpr is a small protein consisting of three amphipathic α-helices (α1, α2, and α3). Two of these helices contain on one surface LXXLL NRID motifs which may cause a direct interaction with the GR, these are indicated as I and II and occur between residues 22-26 (I) and 64-68 (II) (Graphic: Zhao *et al.*, 2011, with modifications: Morellet *et al.*, 2003; Sherman *et al.*, 2000).

Evidence from Rhesus Macaque monkey models has illustrated a role for Vpr (in conjunction with Vpx) in the progression towards AIDS and death, by using Vpr and Vpx knockout SIV viral particles. Vpx is an SIV gene homologous to Vpr, arising from a gene duplication event of the common ancestor of Vpr and Vpx (Tristem *et al.*, 1992). In SIV, Vpx serves the function of nuclear targeting viral protein, and replication in non-dividing cells. It is also packaged directly into the virion (Mahalingam *et al.*, 2001). Monkeys infected with an SIV mutant lacking both these genes (together these are homologous to Vpr in HIV-1) do not progress to AIDS and

death (Gibbs *et al.*, 1995; Hirsch *et al.*, 1998), whereas infection with SIV lacking a functional copy of Vpr only showed a delayed disease progression and lower rates of viral replication although this did result in AIDS and death (Hoch *et al.*, 1995; Lang *et al.*, 1993). A Vpx deletion retards the ability of SIV to replicate in primary cells, is associated with lower viral burdens, and delays the onset of AIDS, but does not prevent death altogether as the double mutant does (Gibbs *et al.*, 1994; Gibbs *et al.*, 1995).

A long term non-progressor (LTNP) is an HIV-1 infected individual who does not progress to AIDS, even in the absence of antiretroviral treatment (Lambotte *et al.*, 2005). This phenomenon may be linked to a Vpr point mutation, F72L. Vpr molecules isolated from a LTNP were found to carry this mutation, and were unable to translocate to the nucleus as the wt does (Caly *et al.*, 2008). Studies have indicated that patients who display delayed disease progression and immunosuppression may carry non-functional copies of Vpr (Jacquot *et al.*, 2009; Saksena *et al.*, 1996; Somasundaran *et al.*, 2002; Wang *et al.*, 1996a).

1.5.2. GR and Vpr interactions

Vpr and the GR share common functions, including the induction of apoptosis in dendritic and T-cells, and chronic immunosuppression (Herold *et al.*, 2006; Muthumani *et al.*, 2004). Vpr contains two NRID co-activator motifs, between amino acids 22-26 and 64-68, (Figure 1.9) albeit with the former in reverse orientation (Kino *et al.*, 1999; Sherman *et al.*, 2000). It is believed that Vpr acts as a GR co-activator, in a manner analogous to SRC-2 (Kino *et al.*, 1999; Sherman *et al.*, 2000; Thotala *et al.*, 2004). Transfection of Vpr encoding plasmids has been observed to enhance ligand-dependent GR-mediated transactivation of the MMTV and TAT-GRE synthetic GRE containing reporter genes in 293T, Jurkat, A204, HS729, CEM, HeLa and CV1 cell lines (Kino *et al.*, 1999; Kino *et al.*, 2002b; Sherman *et al.*, 2000) and of the endogenous receptor of activated NFκB ligand (RANKL) gene in PBMC's (Fakruddin and Laurence, 2005). The sodium hydrogen exchanger (NHE1) gene is repressed by both GC's (Muto *et al.*, 2000), and Vpr. Repression by Vpr is reversible by treatment

with RU486, but is it unknown whether Vpr enhances GR-mediated transrepression (Janket *et al.*, 2007). In support of these studies, Vpr has been found to associate directly with the GR *in vitro* (Kino *et al.*, 1999; Kino *et al.*, 2002a; Sherman *et al.*, 2000; Thotala *et al.*, 2004). Further studies have implicated the cellular factors human Vpr interacting protein (Ramanathan *et al.*, 2002) and poly ADP-ribose polymerase-1 (Muthumani *et al.*, 2006) to mediate this interaction *in vivo.*

Vpr is constitutively nucleophilic (Kamata and Aida, 2000; Thotala *et al.*, 2004; Vodicka *et al.*, 1998), through the actions of an atypical two-part NLS (Gallay *et al.*, 1997; Haffar *et al.*, 2000; Karni *et al.*, 1998). Vpr nuclear import is regulated through the importin-α/β pathway (Popov *et al.*, 1998; Vodicka *et al.*, 1998). This is the same mechanism which the GR uses to gain nuclear access (Freedman and Yamamoto, 2004). Vpr mimics importin-β (Vodicka *et al.*, 1998), by associating with importin-α (Nitahara-Kasahara *et al.*, 2007; Suzuki *et al.*, 2009). This association increases the affinity of importins for classical NLS's (Popov *et al.*, 1998). Mutations to the Vpr LXXLL motifs in helices I and III prevent Vpr nuclear localisation (Thotala *et al.*, 2004) and association with importin (Kamata and Aida, 2000).

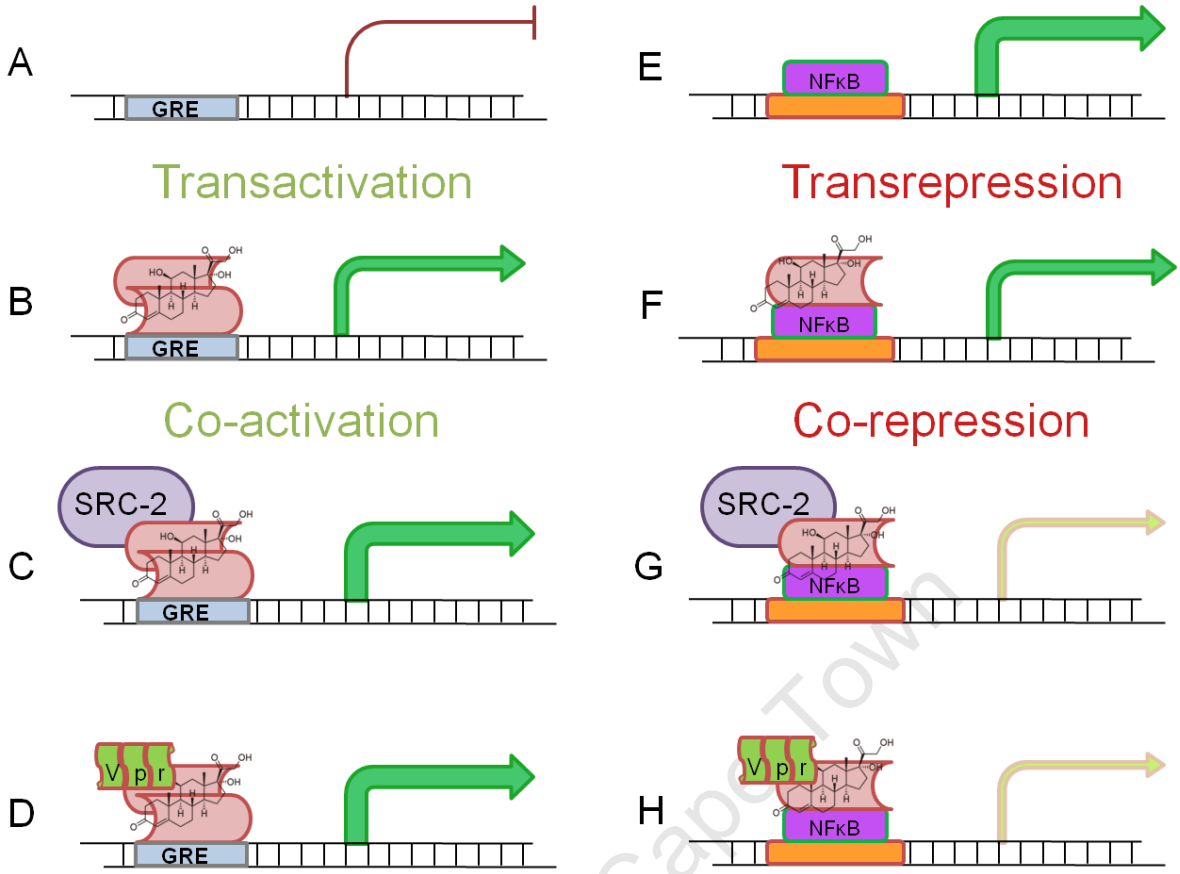


Figure 1.10 (A-H): Select models of co-activation and co-repression of the GR by Vpr. On GRE containing promoters, transcription does not occur in the absence of GC's (A). Ligand activation of the GR causes transactivation (B). This is enhanced by the presence of a co-activator such as SRC-2 (C) or by Vpr (D). In the absence of GC's NFκB activates transcription (E). When GC's are present the GR tethers to and represses transcription from NFκB (F). The presence of SRC-2 (G), and Vpr (H), will enhance the extent of transrepression.

Vpr has a high affinity for DNA (Zhang *et al.*, 1998), but due to its small size Vpr alone is unlikely to be able to remodel chromatin itself. HAT's are generally large MW, multi subunit, enzyme complexes (Roth *et al.*, 2001) and Vpr does not display any conserved enzymatic active site sequences. Rather altered GR transcriptional activities in the presence of Vpr are likely to stem from its propensity to bind multiple proteins *in vitro* (reviewed in Kino and Pavlakis, 2004). Numerous TF's which increase GR-mediated transactivation have been shown to associate with Vpr. These include p300/CBP (Kino *et al.*, 2002a), MED-28, 14-3-3 proteins (Kino *et al.*, 2005), and components of the general TFIIB and TFIIH complexes (Agostini *et al.*, 1996; Agostini *et al.*, 1999; Kino *et al.*, 2002b). Through these interactions it may be

that Vpr enhances GR-mediated transcription and immunomodulatory properties through recruitment of additional transcriptionally active co-regulators to the GR.

Some lines of evidence suggest that Vpr may exert effects through actions of the GR independently of DEX. In the presence of the GR antagonist RU486, Vpr-mediated suppression of IL-12 (which occurs via the GR) is inhibited, likely by disrupting an interaction between Vpr and the GR (Mirani *et al.*, 2002). Additionally in the absence of DEX, the extent of Vpr-mediated transactivation of the LTR is abrogated by RU486 (Soudeyns and Wainberg, 1997), in a manner that correlates with the concentration of antagonist present (Schafer *et al.*, 2006). Vpr-induced apoptosis of T-cells may similarly be prevented by treatment of cell lines with RU486 (Muthumani *et al.*, 2005a). As RU486 is known to disrupt LXXLL GR/co-factor interactions (Kauppi *et al.*, 2003), the above evidence indicates that Vpr may act as a weak GR agonist itself, as has been implied before (Thotala *et al.*, 2004).

The LTR serves as a promoter for viral gene expression and replication and Vpr alone induces a moderate level of LTR transactivation (Forget *et al.*, 1998). The GR can also transactivate the HIV-1 LTR (Bressler *et al.*, 1993; Kinter *et al.*, 2001; Kolesnitchenko and Snart, 1992; Mitra *et al.*, 1995; Vanitharani *et al.*, 2001; Wiegers *et al.*, 2008). The -278 to -168 bp region of the LTR contains three GRE like sites (Ghosh, 1992; Kolesnitchenko and Snart, 1992; Mitra *et al.*, 1995; Soudeyns *et al.*, 1993) which is also the region primarily acted upon by Vpr (Vanitharani *et al.*, 2001). RU486 stimulation can reduce GR-mediated transactivation of the LTR in cell lines and primary macrophages (Schafer *et al.*, 2006; Soudeyns and Wainberg, 1997). Vpr is thought to act as an adaptor molecule, linking the ligand bound GR and p300/CBP to the GRE like site in the LTR closest to the TSS (Felzien *et al.*, 1998; Kino *et al.*, 2002a; Mitra *et al.*, 1995).

1.6. Thesis rationale

HIV-1 has claimed the lives of more than 30 million people since 1981. It has a particularly high prevalence in Sub-Saharan Africa, if compared to the global rate amongst adults: 5.0% compared to 0.8%, equating to 68% of all HIV-1 positive people in this region. South Africa is at the epicentre of the pandemic with near 5.24 million HIV-1 positive adults, representing 10.5% of the population; the greatest number of infected people in any country (UNAIDS, 2011).

One of the HIV-1 accessory genes, Vpr, is highly immunosuppressive and in many ways mimics actions of GC's (Tcherepanova *et al.*, 2009; Tungaturthi *et al.*, 2003), and may enhance GR-mediated transrepression (Mirani *et al.*, 2002; Muthumani *et al.*, 2006) or transactivation (Fakruddin and Laurence, 2005). Indeed in this manner Vpr has widely been reported as a GR co-activator (reviewed in Hapgood and Tomasicchio, 2010). Although much still needs to be resolved, it appears that Vpr actions are reversible by treatment with RU486, an SR antagonist (Schafer *et al.*, 2006), although other reports indicate that Vpr modulation of GR activation requires ligand to be present (Kino *et al.*, 2002a; Sherman *et al.*, 2000). Vpr and the GR have been found to associate *in vivo*, although it is also unclear if this interaction is ligand-dependent (Kino *et al.*, 1999; Kino *et al.*, 2002a), or occurs independently of GR agonists (Sherman *et al.*, 2000; Thotala *et al.*, 2004).

Progesterone regulates female reproduction and the maintenance of pregnancy (Graham and Clarke, 1997; Spencer and Bazer, 2002) and exerts its effects primarily through the PR (Rivera *et al.*, 1999). Due to structural similarities between members of the SR superfamily, progesterone may also act via the AR, MR, and notably the GR (Africander *et al.*, 2011a; Africander *et al.*, 2011b; Africander, 2010; Bentel *et al.*, 1999; Ghatge *et al.*, 2005; Koubovec *et al.*, 2005; Moore *et al.*, 2012; Philibert *et al.*, 1999; Winneker *et al.*, 2003). Endogenous female sex hormones affect replication of HIV-1 in certain cell types (Asin *et al.*, 2008) and the stage of the menstrual cycle has a substantial effect on viral replication rates (Greenblatt *et al.*, 2000). Synthetic P4 mimicking steroids are commonly used as contraceptives and similarly exert their effects via the PR. Depo-Provera® and Noristerate® are two synthetic progestins

widely used as contraceptives in South Africa. Depo-Provera® contains the active compound Medroxyprogesterone Acetate (MPA) and Norestirate® is administered as the inactive enanthate (NET-EN) form before it is hydrolysed to Norethisterone (NET) (Stanczyk and Roy, 1990). As with P4, these hormones may also interact with other members of the SR family. MPA is particularly notable in this regard as it has a high affinity for the GR and acts as a full agonist (Koubovec *et al.*, 2005). These injectable contraceptives are among the most widely used in developing nations (Erkkola and Landgren, 2005), and MPA is administered in some form to more than 20 million women (Affandi, 2002a; Affandi, 2002b). MPA has been available for use in South Africa for over 40 years (Westhoff, 2003). Generally MPA is injected every three months as a 150 mg aqueous suspension reaching serum concentrations of 2.6-3.9 nM (Mathrubutham and Fotherby, 1981; Mishell, 1996) whereas NET-EN is administered every second month as a 200 mg oily suspension (Garza-Flores *et al.*, 1991) with a peak serum concentration of 1.5-59 nM (Fotherby, 1983). These compounds low cost and minimal user participation make them ideal for use in developing countries, particularly in Africa (Erkkola and Landgren, 2005), and nearly half of HIV-1 positive women require access to effective contraception (UNAIDS, 2011).

There is an abundance of literature available supporting the safety of MPA and the many non-contraceptive benefits its use involves (reviewed in Westhoff, 2003), although an accumulating amount of evidence indicates MPA may enhance risk of HIV-1 transmission and shedding (Heffron *et al.*, 2012; Hel *et al.*, 2010; Kleinschmidt *et al.*, 2007; Kreiss *et al.*, 1994; Moss *et al.*, 1991; Mostad *et al.*, 1997; Mostad *et al.*, 2000; Royce *et al.*, 1997; Stringer *et al.*, 2007; Wang *et al.*, 2004a).

Different agonists will differently affect GR function. This study aims to give insight into if Vpr will change how different steroids act via the GR and thereby contribute to an alteration in GR function. As Vpr is a GR co-activator, this may translate into greater changes with strong GR agonists such as MPA than it would for NET-A, due to these steroids differing GR efficacies.

1.7. Hypothesis, aims, and strategies

To investigate a possible link between hormonal contraceptives, GR-mediated transactivation and the HIV-1 accessory gene Vpr the following hypotheses were formulated. The central hypothesis was that Vpr would increase ligand-dependent GR-mediated transactivation and that different progestins will have different GR-mediated responses in the presence of Vpr. To investigate this, a number of specific hypotheses were developed and experimentally investigated.

HYPOTHESIS 1:

Vpr is a GR co-activator and will ligand-dependently increase GR-mediated transactivation. This will occur to a greater extent in the presence of more efficacious ligands than it will for weaker ligands.

To test this hypothesis, the following aims were investigated:

Aim: To determine whether Vpr exerts detectable co-activation of the GR under full agonist stimulation but not with partial or weak agonists, or antagonists, or in the absence of ligand, on GR-mediated transactivation of GRE-containing promoter-reporter constructs..

This will be investigated through use of the TAT-GRE reporter construct in COS1 cells by overexpressing the hGRα in the presence or absence of HA Vpr. Cells will be stimulated with a panel of ligands with different biocharacters and GR transactivation efficacies. The resulting level of reporter gene activity will be compared between corresponding stimulations to observe if HA Vpr enhances ligand-dependent GR-mediated reporter gene transactivation.

Aim: To determine whether Vpr exerts detectable ligand-dependent co-activation of the GR for GR-mediated transactivation of GRE containing endogenous genes in the same cell line.

This will be investigated through quantitative real time PCR in COS1 cells by overexpressing the hGRα in the presence or absence of HA Vpr. Cells will be stimulated with a panel of ligands with different GR efficacies and the resulting mRNA levels compared between corresponding stimulations to observe if HA Vpr enhanced transcription of the endogenous genes.

Aim: To determine whether Vpr exerts detectable ligand-dependent co-activation of the GR for GR-mediated transactivation of the same endogenous genes in a different cell line, which expresses endogenous GR.

This will be investigated through quantitative real time PCR in END1 cells in the presence or absence of expressed HA Vpr. Cells will be stimulated with a panel of ligands with different GR efficacies and the resulting mRNA levels compared between corresponding stimulations to determine if HA Vpr enhanced transcription of the endogenous genes.

HYPOTHESIS 2:

Vpr increases both DEX efficacy and potency for GR-mediated transactivation

Aim: To determine if the concentration of ligand influences the effect of Vpr on GR transcriptional activity.

This will be done by using DEX dose response curves, performed by incubating COS1 cells overexpressing the hGRα, with a range of DEX concentrations. This will be done in the absence and presence of two different amounts of HA Vpr to observe if the level of Vpr protein has an effect on ligand potency and efficacy. The resulting DEX dose response curves will then be compared to determine the effects of Vpr on DEX potency and efficacy.

HYPOTHESIS 3:

Vpr regulates GR levels and turnover.

Aim: To determine whether ligand-dependent GR turnover is decreased in the presence of Vpr, causing higher GR levels and thereby explaining how GR-mediated transactivation is increased by Vpr.

This will be investigated by overexpressing the hGRα in COS1 cells and stimulating cells with the same panel of ligands as used above, in either the presence or absence of HA Vpr followed by Western blot analysis of lysates in order to quantify the relative hGRα and HA Vpr levels.

HYPOTHESIS 4:

Vpr enhances ligand-dependent GR phosphorylation at S203, S211, and S226.

Aim: To determine whether Vpr co-activation of the GR is mediated by modulating post-translational and ligand-dependent phosphorylation of the GR at serine residues 203, 211 and 226.

This will be investigated by overexpressing the hGRα in COS1 cells and stimulating cells with the same panel of ligands as used above, in either the presence or absence of HA Vpr followed by Western blot analysis of lysates in order to quantify the relative extents of S203, S211 or S226 phosphorylation, and if these levels are changed in the presence of HA Vpr.

HYPOTHESIS 5:

Vpr increases nuclear localisation of the rGR in the absence and presence of ligand.

Aim: To determine whether Vpr increases the nuclear localisation of the GR in the presence of a full agonist or without ligand stimulation.

This will be investigated through immunofluorescence in COS1 cells overexpressing the rGR in the presence and absence of HA Vpr and stimulated with a saturating concentration of DEX or of vehicle alone. The nuclear localisation of the rGR will be determined by quantifying the extent of nuclear GR signal as a proportion of total cell GR signal (nuclear/whole cell GR signal intensity). This will then indicate how the nuclear fraction of GR changes in response to ligand stimulation, the presence of Vpr, or both.

HYPOTHESIS 6:

Progestins will differently affect GR function when in the presence of Vpr, leading to changes in biocharacter and partial agonist activity.

Aim: To determine whether any of the progestins (or other ligands) have different GR-mediated responses (biocharacter or partial agonist activity) when in the presence of Vpr.

This will be analysed by plotting data as a relative percentage of the maximal response, obtained with DEX stimulation thereby indicating the partial agonist activity or biocharacter. A post-hoc Fishers least significant differences statistical test will determine if any GC or progestin causes a significantly different response via the GR if compared to the response it exerted in the absence of HA Vpr. This will indicate if the biocharacter of any of the tested ligands changes in the presence of HA Vpr.

2. MATERIALS & METHODS

2.1. Cell lines

COS1 African Green Monkey Kidney Fibroblast cells were purchased from the American Type Culture Collection. These were cultured in high glucose (1 g/ml) Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich, South Africa) supplemented with 10% (v/v) Foetal Calf Serum (FCS, Sigma-Aldrich, South Africa) and 1% v/v Penicillin/Streptomycin (100 IU/ml Penicillin and 100 µg/ml Streptomycin, Gibco BRL Life Technologies, UK) (DMEM-10). Cells were passaged twice weekly at a 1/15 dilution using 0.25% Trypsin/0.1% EDTA in calcium- and magnesium-free PBS (Highveld Biologicals, South Africa) for 3 minutes at 37°C and pelleted by centrifugation.

E6E7 human endocervical cells (END1) expressing high amounts of endogenous GR, were also purchased from ATCC and were cultured in keratinocyte serum free medium (KSF, Sigma Aldrich, South Africa) supplemented with 50 µg/ml bovine pituitary extract (BPE, Sigma Aldrich, South Africa) and 1% v/v Penicillin/ Streptomycin. END1 cells were passaged once weekly to a 1/4 dilution using 5 ml 0.25% Trypsin/0.1% EDTA in calcium- and magnesium-free PBS for 10 minutes at 37°C before addition of 5 ml DMEM-10 to prevent further trypsinisation and pelleted by centrifugation. Medium was aspirated and cells rinsed with at 37°C with PBS before addition of fresh medium once weekly.

Both cell lines were maintained at 37°C in a water jacket incubator (5% CO_2, 90% humidity) in 75 cm^2 tissue culture flaks (Greiner Bio-one, South Africa) and regularly tested for Mycoplasma contamination through Hoechst staining (Battaglia *et al.,* 1980) with only Mycoplasma-negative cell lines being used in experimentation.

2.2. Test compounds and antibodies

Dexamethasone (DEX), Medroxyprogesterone Acetate (MPA), Norethisterone Acetate (NET-A), Progesterone (P4), Cortisol (CORT) and Mifepristone (RU486) were purchased from Sigma-Aldrich, South Africa and dissolved in absolute ethanol. Ethanol (EtOH) was used as a vehicle and compounds were always delivered in such a way that the same volume (1.0 µl/ml) was always delivered to cells. Cells were stimulated with 100 nM DEX, 100 nM MPA, 10 µM NET-A, 1 µM P4, 100 nM Cort, 100 nM DEX/1 µM RU486 or a 0.1% v/v dilution of vehicle alone (EtOH) for 24 hours. These ligand concentrations were calculated to result in GR saturation, based on the K_D's and were used for all experiments, unless otherwise indicated (Kontula *et al.*, 1983; Ronacher *et al.*, 2009).

The α-phospho serine 203, 211 and 226 (P-S203, P-S211 and P-S226 respectively) hGRα-specific antibodies were a generous gift from Dr M.J. Garabedian (New York University, School of Medicine, USA). The polyclonal mammalian GR (H-300; sc-8992) antibody was purchased from Santa Cruz Biotechnology (USA). The GAPDH antibody (SC) was used as a loading control and purchased from Santa Cruz Biotechnology (USA). The α-HA tag (12CA5) antibody was purchased from Roche (South Africa). The secondary α-mouse (sc-2005) and α-rabbit (sc-2313) HRP conjugated antibodies were both purchased from Santa Cruz Biotechnology (USA). The α-HA antibody used for immunofluorescence (Y11, sc-805) was purchased from Santo Cruz Biotechnology (USA). The α-mouse GR antibody (BuGR22) was purchased from Cell Signalling (USA). The fluorescent anti-rabbit immunoglobulin-G (IgG) AlexaFluor488 (A21206) antibody was purchased from Invitrogen and α-mouse IgG Cy3 antibody (715-166-150) was purchased from Jackson Immuno Research (USA).

2.3. Plasmids

The pCMV4-3HA-Vpr plasmid (HA Vpr) expresses an HA tagged variant of Vpr protein obtained from the HIV-1 type B NL4-3 clone and is under control of a constitutive CMV promoter in the CMV4 vector plasmid. It was obtained from Dr Warner Greene (University of California, San Francisco) and has been described previously (Sherman *et al.,* 2000). The p-hGRα (DR) (hGRα) plasmid was a kind gift from Prof. David Ray (Manchester University), which constitutively expresses the full length and untagged human GRα isoform under control of the CMV promoter cloned into the pcDNA3 vector, and has been described before (Ray *et al.*, 1999). The pcDNA3.1+ (empty vector) plasmid contains a CMV promoter in the pcDNA vector with no inserted downstream DNA sequence. This was used to control for equal amounts of transfected DNA and was purchased from Invitrogen (UK). The pTAT-GRE-E1b-Luc (TAT-GRE) luciferase reporter gene plasmid is controlled by the inducible E1b promoter and contains two GRE's from the rat Tyrosine Amino Transferase gene, and was a gift from Dr G. Jestner (Erasmus University of Rotterdam, Netherlands) and has been described previously (Sui *et al.,* 1999). The rat GR encoding plasmid, pSVL-rGR (rGR), expresses the full length wt rGR under control of a constitutive late SV40 promoter cloned into the pSVL vector plasmid and was a kind gift from Dr Stoney Simons Jr. (National Institute of Health, USA) and has been described previously (Cho *et al.,* 2005).

Plasmids were transformed into *Escherichia coli* DH5α strain by standard heat shock method (Sambrook *et al.,* 1989). Briefly, 10 ng of the desired plasmid and 100 µl of competent cells in Luria broth (1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl) were incubated on ice for 30 minutes before heat-shock at 42°C for 2 minutes and placed back on ice for a further 2 minutes. Cells were recovered by the addition of 900 µl of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM Glucose) and incubated at 37°C for 1 hour. Cells were plated onto Luria agar plates (Luria broth with 1.5% (w/v) agar) containing 100 µg/ml ampicillin (Sigma-Aldrich, South Africa) and incubated overnight at 37°C. For plasmid purification a single colony was picked and inoculated into 100 ml LB containing 100 µg/ml ampicillin and incubated while shaking overnight

at 37°C. Plasmid DNA was isolated and purified using a Promega Pureyield Plasmid Midi-prep kit (Promega Corp., USA) according to the manufacturer's instructions. Plasmid integrity and purity were analysed by restriction enzyme digestion followed by 0.8% agarose gel electrophoresis at 65 V containing 10.0 µg/ml Ethidium Bromide.

2.4. Reporter assays

COS1 cells were plated into 10 cm dishes at $2.0x10^5$ cells per dish 24 hours prior to bulk transfection using the DEAE dextran method as previously described (Luthman and Magnusson, 1983) with a few changes. A transfection mix was prepared in serum free (SF) DMEM containing 2.5% FCS with 100 µM chloroquinediphosphate equilibrated to 37°C. Cells were transfected with 3 600 ng TAT-GRE, 1 600 ng hGRα, and 3 600 µg HA Vpr or empty vector with 100 µg/ml DEAE-Dextran. Media was aspirated and 2.5 ml of transfection mix was added to cells for 1 hour at 37°C. Medium was aspirated and replaced with 2.5 ml 10% (v/v) DMSO in PBS for 3 minutes at room temperature. Cells were then washed with PBS and grown in DMEM-10 for a day prior to re-plating at a density of $1.4x10^5$ cells/ml in 24 well experimental plates (Greiner Bio-one, South Africa). This equated to roughly 112 ng hGRα and 250 ng HA Vpr per ml DMEM-10. After 24 hours of incubation, cells were stimulated with the test compounds in SF-DMEM at the indicated concentrations.

This method of bulk transfection was found to cause a high measure of error and subsequent reporter assays (as indicated in figure legends) were plated and transfected directly using FuGENE6 transfection reagent (F6, Roche Applied Science, South Africa) according to the manufacturer's instructions. Briefly cells were plated directly into 24 well plates at a density of $1.0x10^5$ cells/ml and grown for 24 hours in DMEM-10 prior to transfection. F6 was added to 100 µl of SF-DMEM in a ratio of 2 µl F6 to 1 µg DNA and left at room temperature for 5 minutes before the addition of hGRα and HA Vpr to final concentrations of 175 ng hGRα per well and 50 or 175 ng HA Vpr per well as indicated (this equates to 350 ng hGRα and 100 or 350 ng HA Vpr per ml). This was left at room temperature for 30 minutes before addition directly to cells in culture medium. One day later cells were washed in PBS and

stimulated with a range of DEX concentrations or EtOH as indicated for 24 hours according to experimental requirements.

After stimulation cells were washed twice with ice cold PBS and harvested with 50 µl reporter lysis buffer (Promega Corp., USA). The luciferase assay (Luciferase Assay System, Promega, USA), was carried out with 10 µl of lysate and 50 µl luciferase substrate in white 96 well plates in a Modulus Microplate reader (Turner Biosystems, USA). Luciferase units were normalised to total protein concentration, as measured by Bradford's Assay (Bio-Rad, South Africa) and expressed relative to control values. The Bradford assay consisted of adding 250 µl of Bradford reagent to 2 µl of protein samples of unknown concentration (measured in duplicate) from each of the 24 wells on the experimental plate. The absorbance of the mixture at 595 nm was measured by spectrophotometer, and using a set of bovine serum albumin protein standards (2 mg/ml BSA, Pierce, South Africa) diluted in reporter lysis buffer and thus of known concentration, the concentration of unknown samples was calculated (Bradford, 1976).

2.5. SDS-PAGE and Western blot

For sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), COS1 cells were seeded in DMEM-10 directly into 12 well plates at a density of $1.0x10^5$ cells/ml. Twenty four hours later cell were transfected with 350 ng hGRα, and 350 ng HA Vpr or empty vector per ml (per well) unless otherwise indicated in figure legends using F6 according to the manufacturer's instructions. One day later cells were washed in PBS and stimulated in SF-DMEM with test compounds or vehicle (EtOH) as indicated in figure legends for 1, 4 or 24 hours according to experimental requirements. Where END1 cells were used in Western blotting, cells were seeded at $4x10^4$ cells per well in a 12 well plate and did not require hGRα transfection but were similarly transfected with 1 000 ng of either HA Vpr or empty vector 24 hours before stimulation in KSF-SF medium for four hours with saturating concentrations of ligand.

Samples were harvested in 50 µl 2x SDS sample buffer (diluted from 5x SDS sample buffer: 100 mM TRIS-Cl pH 6.8; 5% (w/v) SDS; 20% (v/v) glycerol; 2% (v/v) β-mercaptoethanol; 0.1% (w/v) Bromophenol-blue). Equal amounts (9 µl) of sample were boiled (100°C) for 5 minutes and resolved by 10% SDS-polyacrylamide gel in running buffer (25 mM TRIS-Cl pH 8.4; 250 mM glycine and 0.1% (w/v) SDS) using a Bio-Rad Mini Protean-II electrophoresis cell chamber at 75 V for 30 minutes followed by 120 V for 1½ hours (Maniatis *et al.,* 1975).

Proteins were transferred from the gel onto a HyBond-ECL nitrocellulose membrane (AEC-Amersham, South Africa) at 180 mA in transfer buffer (25 mM TRIS, 250 mM glycine and 20% (v/v) MeOH) for 1 hour using the Mini Protean-II blotting apparatus (Bio-Rad, South Africa). Membranes were blocked in 4% (w/v) ECL advance blocking powder (AEC-Amersham South Africa) prepared in TRIS buffered saline tween (TBST; 50mM TRIS-Cl pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween-20) for 1 hour at room temperature and incubated with primary antibodies (Table 2.1) prepared in blocking solution at 4°C overnight. The following day membranes were washed once for 15 minutes and three times for 5 minutes, in TBST at room temperature before incubation with Horseradish Peroxidase (HRP) conjugated secondary antibodies in 5% (w/v) non-fat milk powder in TBST for an hour at room temperature. Blots were washed as above followed by rinsing with TBS and developed using Pierce Western blotting detection re-agents (Pierce, South Africa) and Hyperfilm MO high performance autoradiography film (AEC Amersham, South Africa), according to the manufacturer's instructions. Exposed bands on the film were scanned and quantified with the AlphaEase FluorChem5500 software (Alpha Innotech). After probing, blots were stripped of antibodies at 60°C for 30 minutes in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) SDS and 62.5 mM TRIS-Cl pH 6.8) before being re-blocked and re-probed, if necessary.

Table 2.1: Dilutions of primary and secondary antibodies used in Western blot analysis

Antibody	Dilution	Secondary	Dilution
GAPDH	1/20 000	α Mouse	1/15 000
GR	1/4 000	α Rabbit	1/5 000
HA	1/1 000	α Mouse	1/2 000
P-S203	1/10 000	α Rabbit	1/5 000
P-S211	1/10 000	α Rabbit	1/5 000
P-S226	1/10 000	α Rabbit	1/5 000

2.6. Quantitative real time PCR

COS1 and END1 cells were seeded at densities of $1.0x10^4$ or $4.0x10^4$ cells/well respectively in 12 well plates, twenty four hours prior to transfection with F6 according to the manufacturer's instructions. COS1 cells were transfected with 350 ng hGRα and 350 ng HA Vpr or empty vector, and END1 cells were transfected with 1 000 ng HA Vpr or empty vector per ml (per well). After 24 hours of incubation at 37°C, cells were stimulated in SF-DMEM or KSF (without BPE) respectively, for four hours with 100 nM DEX, 100 nM MPA, 10 μM NET-A, 1 μM P4, 100 nM CORT or vehicle (EtOH). RNA was isolated using TRIZOL reagent (Sigma-Aldrich, South Africa) according to the manufacturer's instructions and RNA integrity was verified on a denaturing 1% agarose gel, containing 10% morpholinopropanesulphonic acid (0.2 M morpholinopropanesulphonic acid; 0.05 M Sodium Acetate; 0.01 M EDTA, pH 7.0) 20% (v/v) formaldehyde and electrophoresed at 65 V for 30 minutes.

RNA was stored at -80°C before 500 ng RNA was reverse transcribed with oligo-dT priming using the Transcriptor First Strand cDNA Kit (Roche Applied Science, South Africa) according to the manufacturer's instructions.

Table 2.2: Primers used in real time PCR

Primer	Sequence (5'-3')	Product (bp)	Reference
GAPDH FWD	TGA ACG GGA AGC TCA CTG G	307	Ishibashi *et al* ., 2003
GAPDH REV	TCC ACC ACC CTG TTG CTG TA		
IκBα FWD	ACT CGT TCC TGC ACT TGG CC	238	Emmerich *et al* ., 1999
IκBα REV	TGC TCA CAG GCA AGG TGT AG		
MKP-1 FWD	AGT ACC CCA CTC TAC GAT CAG G	250	Rauhala *et al* ., 2005
MKP-1 REV	TGA TGG AGT CTA TGA AGT CAA TGG		

Equal volumes of each cDNA synthesis reaction were used as a template for quantitative real-time PCR, using primers for IκBα, MKP-1 and GAPDH as control (Table 2.2) as well as the SensiMix dT kit (Quantace, UK) and a Corbett real time PCR machine, according to the manufacturer's instruction. The cycling parameters consisted of an initial 10 minute denaturation at 95°C, followed by 30-35 cycles of 10 sec at 95°C, 15 sec annealing and 10 sec elongation at 72°C and a final 10 minute extension at 72°C.

Melt curve analysis and 1% agarose resolving gel electrophoresis were performed to confirm the correct product had been amplified. Relative transcript levels were calculated and normalised to GAPDH abundance using the Pfaffl equation (Pfaffl, 2001).

$$relative\ abundance = \frac{[E_S]^{\Delta C_t\ (control-sample)}}{[E_R]^{\Delta C_t\ (control-sample)}}$$

Where E_S is the primer efficiency of the target/sample gene, E_R is the efficiency of the reference gene and C_t is the cycle number at the threshold crossing.

2.7. Immunofluorescent microscopy

COS1 cells were seeded at a density of 4.0×10^5 cells per well in DMEM-10 in 6 well tissue culture plates (containing sterile glass coverslips which had been washed

twice in 3.2% HCl for 30 minutes) twenty four hours before changing medium to charcoal stripped DMEM-10 and transfection. Cells were transfected with 1.0 µg of the rGR plasmid and 1.5 µg HA Vpr per well where appropriate (750 ng rGR and 500 ng HA Vpr per ml) using F6, according to manufacturer's instructions (Roche). Twenty four hours later cells were stimulated for an hour in SF-DMEM with 100 nM DEX or vehicle (EtOH).

After stimulation the cells were washed twice with ice-cold PBS, permeablised with methanol (MeOH) for 10 minutes at -20°C, before three washes with PBS at room temperature and blocked with 5% (w/v) BSA (Roche Diagnostics, South Africa) prepared in PBS, for 1 hour at room temperature. After blocking, cells were incubated with primary antibodies (Table 2.3) (diluted in 5% BSA) for 1 hour in a humidified environment at room temperature, and subsequently washed three times with 1% BSA/PBS containing 0.05% triton X-100 (Sigma Aldrich, South Africa). Cells were then stained with fluorophore-conjugated secondary antibodies (prepared in 5% BSA) in a humidified environment in the dark for an hour at room temperature and washed as indicated above.

Table 2.3 Dilutions of primary and conjugate fluorophores used in immunofluorescence

Primary	Antibody	Dilution	Fluorophore	Dilution
mouse GR	BuGR22	1/250	Cy3	1/1 000
HA	Y11	1/200	Alexa488	1/250

Cells were stained in 100 µg/ml Hoechst (Sigma Aldrich, South Africa) for 5 minutes at room temperature and mounted using Mowiol (475904, Calbiochem, USA). Slides were allowed to set overnight and stored at 4°C in the dark until visualisation. Imaging of the slides was performed with a Zeiss Axiovert 200M LSM 510 Meta NLO Confocal Microscope using the 40X water immersion objective lens. A multi-track scanning configuration using the 488 nm (HeNe gas laser), 561 nm (solid state laser) and 633 nm Argon laser to photo-excite and visualise the fluorophores was used. At

least three different fields of view from three independent experiments were collected and analysed using LSM image analysis software.

The pSVL-rGR plasmid (rGR) was used here because the 12CA5 (Roche) primary HA antibody bound non-specifically to the mitochondria. This was rectified through using a different HA primary antibody (Y11 Santa Cruz) which required a rabbit secondary antibody. As the human GRα antibody (p300, Santa Cruz) is also generated in rabbits, a different GR expressing plasmid had to be used. The rGR was most favourable as it was generated in mice, and could be used in conjunction with the only other available fluorophore, Cy3, which is conjugated to a mouse secondary antibody. The BuGR22 rGR antibody resulted in non-specific nuclear red spots in COS1 cells (Figure 5.2). Although this may have led to interference with the quantification process and cast doubt over the certainty of the receptor subcellular distribution, it was assumed it would be of equal intensity across all conditions.

2.8. Statistical and graphical analysis

All results were plotted using Graph Pad Prism version 5 (Graph Pad Software Inc.) and statistical analysis performed with STATISTICA version 10 (Statsoft Inc.). A two-way ANOVA test was used to determine whether in general the whole -Vpr group was significantly different to the whole +Vpr group (A). A Fishers Least Significant Differences post-hoc test was then used to compare all columns to each another. Selected columns were paired to note the effect of ligand stimulation vs. vehicle in cells lacking Vpr and similarly the effect of ligand stimulation vs. vehicle in cells expressing Vpr, or similarly each ligand's partial agonist activity, expressed as a percentage of DEX (B). In cells expressing Vpr, stimulations were matched and compared to the corresponding stimulation in the absence of Vpr (C) (e.g. -Vpr EtOH vs. +Vpr EtOH and -Vpr DEX vs. +Vpr DEX etc.). The level of significance was indicated as *, **, or *** to indicate $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

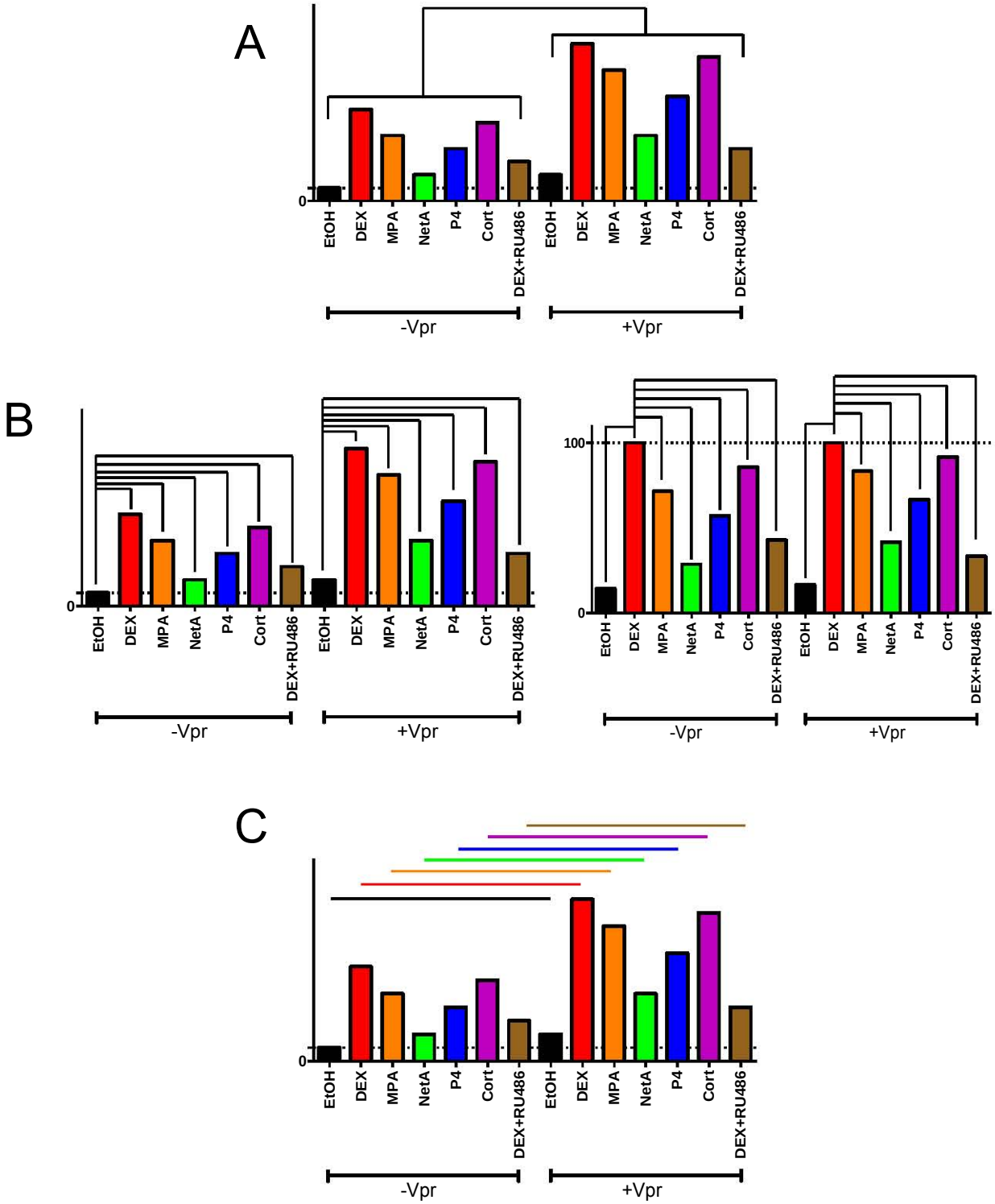


Figure 2.1 (A-C): Indications of statistical analysis. (A) A two way Analysis of Variance (ANOVA) test, comparing groups of ligand stimulations to test if Vpr caused a significant change in the group as a whole. (B) Fishers least significance differences post-hoc test, bars were compared to their corresponding condition's vehicle only control (left) or to maximal DEX activity, indicating the partial agonist activity (right). (C) Fishers least significance differences post-hoc test, ligand stimulations were compared between the group expressing Vpr and group lacking Vpr.

3. RESULTS

3.1. Vpr increases GR-mediated transactivation of the TAT-GRE reporter gene

Previous reports in the literature have indicated that Vpr acts as a GR co-activator, increasing GR-mediated transactivation of both endogenous genes and synthetic promoter-reporter constructs in the presence of the GR agonist DEX (Fakruddin and Laurence, 2005; Kino *et al.*, 1999; Kino *et al.*, 2002a; Sherman *et al.*, 2000). In order to determine whether Vpr can act as a GR co-activator and increase transactivation in the presence of different GC and progestin ligands, COS1 cells were transiently transfected with a synthetic reporter (TAT-GRE) construct, as well as the hGRα in the presence and absence of HA Vpr, before stimulation with GC's and progestins.

Results show that in the absence of Vpr, DEX induced a statistically significant level of reporter gene activity, roughly 26 fold higher than basal. Significant induction was also observed with CORT, MPA, and P4, approximately 20, 17, and 5 fold respectively as compared to unstimulated cells. Stimulation with NET-A did not significantly increase reporter gene activity. Co-stimulation with DEX and RU486 did not significantly increase reporter gene activity above control, indicating that RU486 antagonised the significant induction by DEX. In the presence of Vpr, a significantly higher level of reporter gene activity (A_{max}) was observed in general when comparing the +Vpr group to the -Vpr group (Figure 3.1A). Similar to the results obtained in the absence of Vpr; DEX, CORT, MPA, and P4 were able to significantly induce transcription above basal in the presence of Vpr (Figure 3.1A). Furthermore, when comparing the level of transactivation observed with MPA, P4, and NET-A stimulation in the absence of Vpr with the corresponding stimulations in the presence of Vpr, a significant increase was observed (coloured bars), indicating that Vpr acted like a co-activator under these ligand stimulations. Although Vpr dramatically increased reporter gene activity induced by DEX and CORT (26 to 57 and 20 to 45 fold above basal respectively), both conditions had a high degree of error and these differences were not statistically significant (Figure 3.1A).

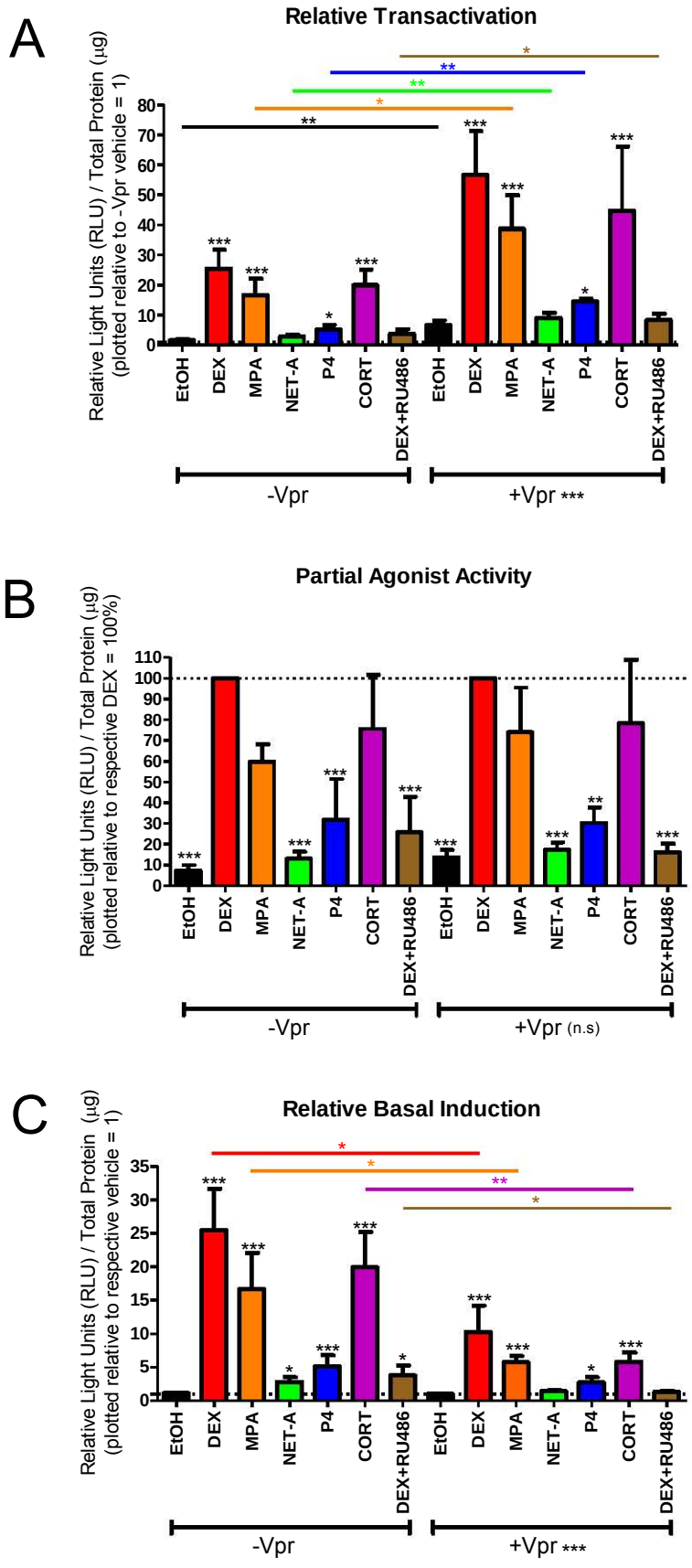


Figure 3.1 (A-E): Vpr increases GR-mediated transactivation of a TAT-GRE promoter-reporter plasmid (Continued).

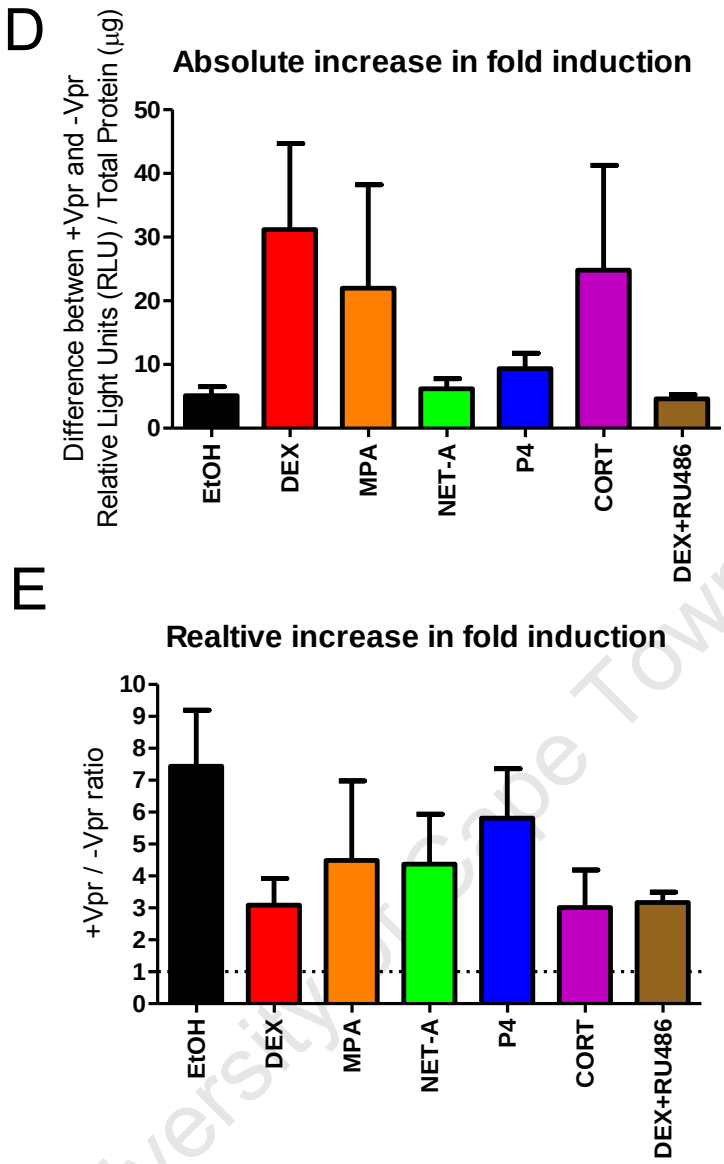


Figure 3.1 (A-E): Vpr increases GR-mediated transactivation of a TAT-GRE promoter-reporter plasmid. COS1 cells were transfected in 10 cm dishes with hGRα, TAT-GRE and either HA Vpr or empty vector 24 hours prior to re-plating in 24 well plates. After re-plating the relative concentrations of GR and Vpr were 112 and 250 ng/ml respectively. After 24 hours incubation, cells were stimulated with ligands at the concentrations indicated in materials and methods for a further 24 hours. Samples were harvested in reporter lysis buffer before luciferase quantification in a Luminex luminometer with the Promega luciferase assay and normalisation to total protein as determined by Bradford protein assay. Data were either plotted as (A) a fold increase above vehicle in the absence of Vpr, which was set as equal to 1, (B) relative to each DEX response set as 100%, showing the partial agonist activity, or (C) as the fold increase of each stimulation above its respective basal, set as equal to 1. Figures D and E respectively show the difference (absolute increase) and fold induction (relative increase) in reporter gene activity mediated by Vpr from Figure 3.1A. Graphs show pooled data from 4 biological repeats each performed in triplicate. A Fishers least significant differences post-hoc test was used to determine if ligand stimulation caused a significant increase by comparing each stimulation to its respective vehicle (A and C), or if any ligand caused a significantly different efficacy to DEX (B). Additionally this test was used to compare ligand stimulations in the absence and presence of Vpr (coloured bars above histograms). A two way ANOVA was used to compare the groups containing or lacking Vpr as a whole. Statistical analysis was marked *, ** or *** to indicate significance levels of $P<0.05$, $P<0.01$ or $P<0.001$ respectively.

Interestingly Vpr significantly increased reporter gene transactivation under co-stimulation with DEX and RU486, as well as the basal level of reporter gene activity, in the absence of ligand (Figure 3.1A). This suggests that Vpr may transactivate the TAT-GRE reporter gene in the presence of antagonist or absence of GC's, an observation inconsistent with previous reports (Kino *et al.*, 1999; Kino *et al.*, 2002a).

Setting maximal reporter gene activity (DEX) as 100% more clearly represents the partial agonist activity of other ligands and allows a clear representation of their relative biocharacters. The rank-order of transactivation efficacies in the absence of Vpr (Figure 3.1B) was similar to an earlier report (Ronacher *et al.*, 2009) which had showed that DEX≥CORT>MPA>P4>NET-A, although in the published report NET-A did not show any transactivation agonist activity, whereas in Figuer 3.1B NET-A did increase reporter gene transactivation via the GR. NET-A, P4, and RU486/DEX all had significantly lower efficacies than DEX, with partial agonist activities of 13, 31, and 26% respectively. CORT and MPA had partial agonist activities of 76% and 60% respectively, which were not significantly different to DEX. This was an expected trend as CORT is a full agonist and MPA may act as a partial or full agonist. Vpr did not significantly change the biocharacter of any ligand or significantly increase percentage partial agonist activity of a ligand relative to its DEX response (Figure 3.1B). Therefore, Vpr did not affect any ligands biocharacter, and the rank-order of efficacy for GR-mediated transactivation was unchanged in the presence of Vpr. This indicates that the relative fold increase in transactivation mediated by Vpr for each of the tested ligands and vehicle alone is roughly equal. Vpr slightly increased the basal level of reporter gene activity independently of ligand (Figure 3.1A), but does not cause the basal level of reporter gene activity to increase when compared to the maximal response (Figure 3.1B). It is possible that the increased GR-mediated transactivation in the presence of ligands and Vpr may be simply be due to the effects Vpr alone mediates on TAT-GRE transactivation in addition to ligand-dependent GR-mediated transactivation, as opposed to Vpr acting as a GR co-activator.

In order to determine if the effect Vpr exerts on ligand-dependent GR-mediated transactivation is simply additive between the effects Vpr ligand-independently exerts and ligand-dependent GR-mediated transactivation, or is greater than the increase

that each of these alone mediate, data were plotted with both vehicle conditions set to 1 and reporter gene activity shown as a fold increase (Figure 3.1C). If Vpr was acting independently of, or in an additive manner with ligand stimulation, this would be indicated in Figure 3.1C as roughly equal fold inductions in the presence or absence of Vpr with each ligand stimulation, and if Vpr were acting as a ligand-dependent co-activator, the fold induction of each ligand relative to its own basal control would be higher in the presence of Vpr. Neither of these is true as in the presence of Vpr the fold increase above basal for all ligand stimulations excepting NET-A, were significantly lower than the fold increase above basal in the absence of Vpr. It appears that Vpr did increase the maximal level of ligand-dependent, GR-mediated transactivation, but in a manner that was less than additive with ligand stimulation, and that Vpr exerted more of an effect on the unliganded GR than it did on the ligand bound GR (Figure 3.1C).

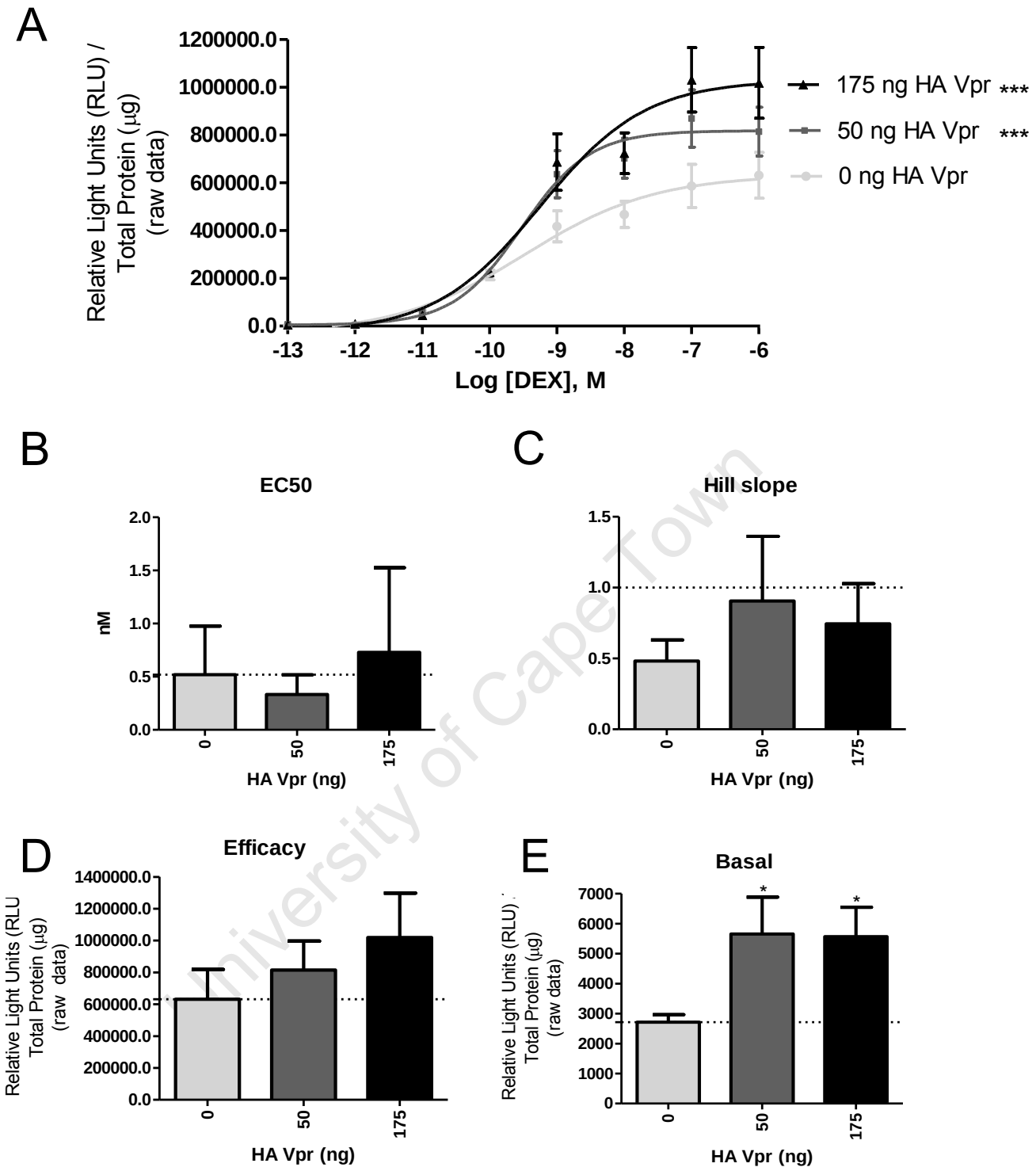
Data from Figure 3.1A was plotted alternatively to better give an indication of the extent by which Vpr increases reporter gene activity. Plotting the absolute differences in reporter gene activity, calculated by subtracting the luciferase/total protein values obtained in the absence of Vpr from the values obtained in the presence of Vpr reveals the absolute level by which Vpr increases reporter gene activity for each ligand. Vpr increased the absolute level of reporter gene activity to a greater extent when in the presence of strong agonists than it did in the presence of partial agonists (Figure 3.1D). Plotting the ratio by which reporter gene activity is enhanced by Vpr (+Vpr / -Vpr luciferase/total protein values) revealed that Vpr-mediated a similar increase in reporter gene activity for each of the tested ligand stimulations, which ranged between a 3 and 6 fold increase (Figure 3.1E). Taken together, Vpr increases the absolute level of transactivation with all the GR ligands and progestins, but does not change the relative order of efficacies for GR-mediated transactivation of any of the ligands.

3.2. Vpr-mediated increases in GR driven TAT-GRE transactivation are dependent on DEX concentration

The relationship between Vpr and ligand-dependent GR-mediated transactivation is not clear. Vpr may have increased reporter gene transactivation independently of GC stimulation (Figure 3.1A) or, as reported in literature (Kino *et al.*, 1999; Sherman *et al.*, 2000) requires ligand to be present for transcriptional effects to be observed. To determine if the concentration of ligand influences the effect of Vpr on GR transcriptional activity, a dose response curve using a range of DEX concentrations; from 10^{-13} to 10^{-6} M (Figure 3.2A) and two different transfected amounts (50 or 175 ng per well) of HA Vpr was performed. Two different amounts of Vpr were transfected to additionally observe if the amount of Vpr influenced the relationship between Vpr, DEX concentration, and reporter gene transactivation.

A standard sigmoidal dose response curve was observed with DEX stimulation of the GR (Figure 3.2A). This curve was then compared to dose response curves in which Vpr was present so that it could be elucidated which concentrations of DEX, if any, were required for Vpr to show an observable effect on GRE transactivation. All data were plotted as raw, relative light units / total protein instead of being shown as relative to any particular measure, as five different aspects of transactivation were investigated and this allowed all the data to be interpreted together.

In the absence of Vpr, stimulation with DEX resulted in transactivation of the reporter gene in a sigmoidal dose-response manner, consistent with previous reports in literature (Ronacher *et al.*, 2009). Very low levels of reporter gene transactivation were observed without ligand. Concentrations of DEX in excess of 10^{-11} M were required to induce an observable level of reporter gene activity (Figure 3.2A). The response increases exponentially with ligand concentration to 10^{-9} M before reaching a plateau or A_{max}, at $6.3x10^5$ relative light units per unit protein at 10^{-7} M DEX (Figures 3.2A and D). Higher DEX concentrations did not further transactivation of the reporter gene with the same GR quantity, as the receptor is saturated.



Variable slopes

Figure 3.2 (A-G): Vpr increases the relative efficacy of DEX via the GR without affecting the potency on a TAT-GRE reporter (Continued).

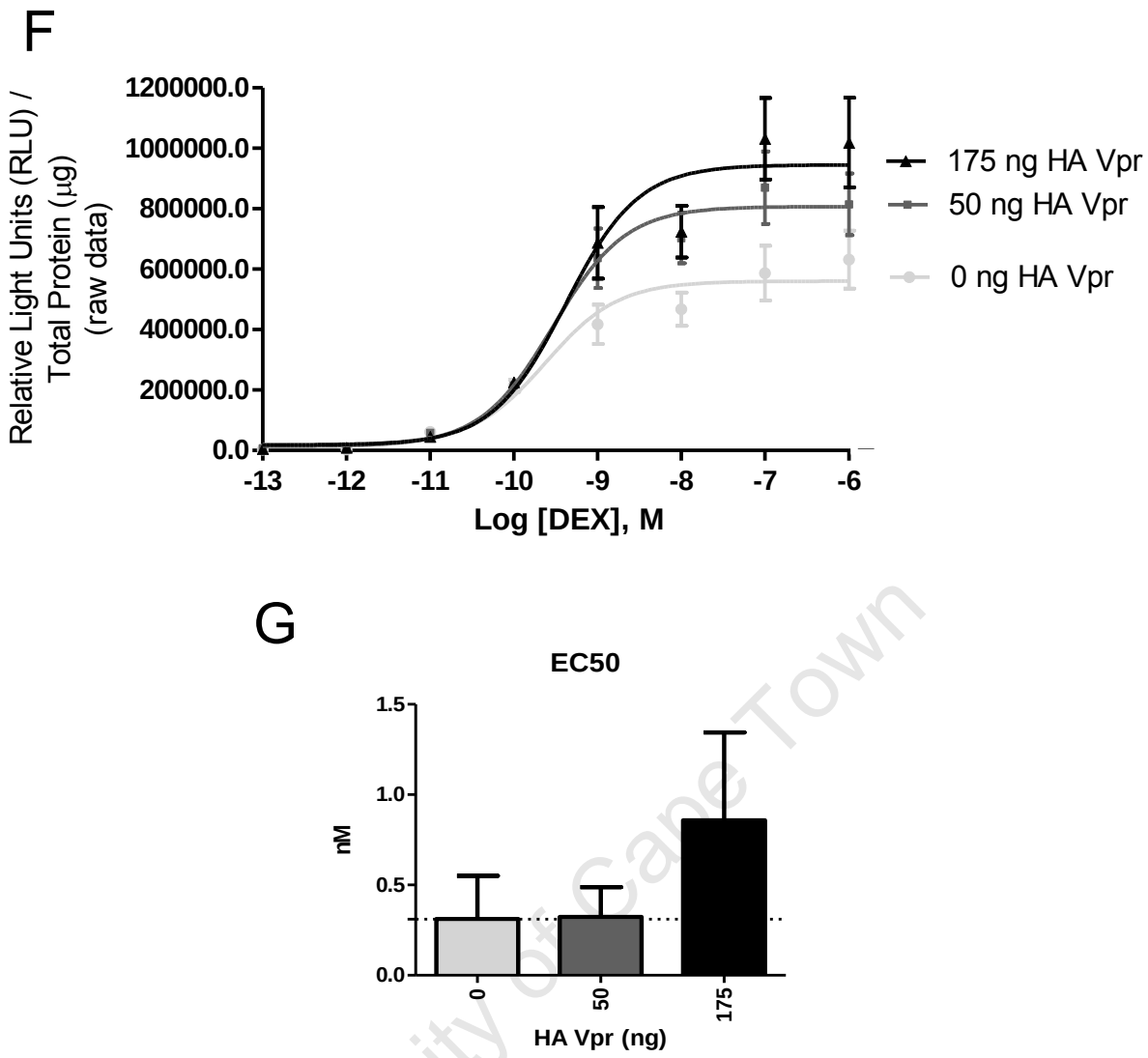


Figure 3.2 (A-G): Vpr increases the relative efficacy of DEX via the GR without affecting the potency on a TAT-GRE reporter. COS1 cells were seeded directly into 24 well plates and grown for 24 hours prior to FuGene transfection with hGRα, TAT-GRE and 0, 50, or 175 ng HA Vpr or corresponding amounts of empty vector DNA as indicated. This equated to 350 ng/ml hGRα, and 100 or 350 ng/ml HA Vpr. 24 hours later cells were stimulated with DEX at concentrations indicated for 24 hours prior to harvesting, luciferase quantification, and total protein determination by Bradford's assay. Graphs show three pooled results each performed in triplicate plotted as a variable log [agonist] vs. response curve. A two-way ANOVA (continuous variables) and Fishers least significant differences post-hoc test was used for statistical analysis and marked *, ** or *** to indicate significance of $P<0.05$, $P<0.01$ or $P<0.001$, respectively. Significant differences are all shown compared to 0 ng HA Vpr; indicating the effects of Vpr on GR-mediated reporter gene transactivation across a range of DEX concentrations (A), how Vpr affects DEX potency (B), The effect of Vpr on the Hill slope (C), how Vpr effects maximal DEX efficacy (D), and how Vpr effects the basal level of reporter gene activity (E). Additionally data were analysed with fixed log [agonist] vs. response slopes (F) and the EC_{50}'s also shown (G).

All three dose response curves at DEX concentrations lower than 10^{-10} M are indistinguishable from each other. At higher DEX concentrations Vpr was observed to greatly increase reporter gene activity above the level that DEX alone exerts. Vpr increases GR-mediated transactivation dependently on DEX concentration, with greater levels of reporter gene activity induced by Vpr being observed at higher DEX concentrations (Figure 3.2A). A greater amount of Vpr also caused a greater increase in reporter gene activity, indicating a relationship between the amount of Vpr present and the level of ligand-dependent GR-mediated reporter gene transactivation. Vpr increases the efficacy of DEX, increasing the A_{max} above the level which DEX alone induces for transactivation of the TAT-GRE reporter gene.

Comparing the EC_{50} values obtained in the absence and presence of Vpr showed that Vpr did not significantly change DEX potency. In the absence of Vpr the EC_{50} was 0.5 nM. In the presence of 50 and 175 ng transfected Vpr the EC_{50}'s were 0.3 and 0.7 nM respectively. Both were similar to the EC_{50} of DEX in the absence of Vpr and neither of these values is a statistically significant difference, indicating Vpr does not change DEX potency (Figure 3.2B). A co-activator that increases ligand efficacy might have been expected to decrease, rather than increase the EC_{50} values.

The Hill slope is a measure of the co-operative binding of ligand to a macro-molecule. This was not significantly changed in the presence of Vpr. If Vpr were acting as a GR ligand and increasing receptor dimerisation and steroid binding affinity this would have increased the Hill slope. The Hill co-efficient for the GR alone was 0.5. As this value is less than 1 it indicates that DEX binding was negatively co-operative and that DEX binding reduces GR affinity for further ligand binding. In the presence of both amounts of Vpr the Hill slopes were slightly increased (0.9 for 50 ng and 0.7 for 175 ng), but still less than 1, indicating DEX/GR binding is negatively co-operative in the presence of Vpr (Figure 3.2C). These are insignificant increases, and it was assumed that Vpr did not affect the co-operation of DEX/GR binding.

If Vpr does increase the Hill co-efficient or affect the EC_{50} value of DEX, the changes it mediates are lower than the level of error and thus not observable in an assay of this type.

Showing reporter gene activity obtained at saturating (10^{-6} M) ligand concentrations indicates the effects of Vpr on DEX efficacy. Neither amount of Vpr significantly increased the A_{max}, although both had mean values higher than the absence of Vpr, and the greater amount of Vpr resulted in a greater extent of transactivation of the reporter than the lower amount did (Figure 3.2D). This indicated that the transactivation of the reporter gene by Vpr in conjunction with DEX is dependent on the amount of Vpr present. It is difficult to determine if Vpr increases the A_{max} significantly without further experimental repeats being done as these observed increases are also below the level of experimental error.

The effect of Vpr in the absence of ligand is too small to be directly observed on the dose response curve. However, plotting reporter gene activity obtained with vehicle alone, shows there is a slight but significant increase from the basal level of reporter gene activity resulting from transfection of Vpr (Figure 3.2E). Both amounts of Vpr induced similar increases, which were less than 0.01% of the magnitude mediated by 1 μM DEX stimulation.

Although Vpr did mediate a significant increase in reporter gene activity in the absence of ligand (Figure 3.2E), a larger Vpr-mediated increase in reporter gene activity was observed at greater ligand concentrations, arguing against Vpr acting independently of DEX stimulation of the GR. This indicates that Vpr acts in a manner that is related to DEX concentration and DEX mediated increases in reporter gene activity, and thus also acts in combination with the GR.

Plotting the same data with fixed, as opposed to variable slopes (Figure 3.2F) did not alter the relative positions of the A_{max} relative to each other, nor did it show an observable increase in the basal level of reporter gene activity. Using fixed slope curves (Hill co-efficient = 1) to calculate the EC_{50} values does not show Vpr to have an effect of changing the EC_{50} above the level of error or statistical power of the experiment (Figure 3.2G).

3.3. Vpr increases GR turnover

Having investigated the effects that Vpr exerts on GR-induced reporter gene transactivation, non-transcriptional markers of GR activation were studied. Several parameters that indicate GR activation, including GR turnover, phosphorylation, and nuclear localisation were investigated. This was done to try and understand the mechanism by which Vpr increased GR-mediated reporter gene transactivation. Firstly, to determine how turnover was affected by Vpr, GR levels were quantified after 24 hours of ligand stimulation, either in the absence or presence of Vpr.

In the absence of Vpr, 24 hours of stimulation with DEX caused a significant reduction in GR levels (Figures 3.3A lanes 2 vs. 1 and 3.3B). Turnover under DEX stimulation was expected owing to the full agonist nature of this steroid and the short GR half-life it causes (44 hours). MPA, NET-A, P4 and CORT did not increase turnover (Figure 3.3A, lanes 3, 4, 5, and 6 vs. 1) and showed GR levels not statistically different to that of the vehicle (Figure 3.3B). This was unexpected in the case of CORT, and to a lesser degree MPA, as these are full agonists and as such would be expected to cause ubiquitination and the subsequent degradation of the GR. Additionally both of these steroids decrease the GR half life to 12 and 17 hours respectively, both of which are less than the time point investigated in this study (24 hours) (Avenant *et al.*, 2010b). GR levels in the presence of MPA, NET-A, P4 and CORT were slightly but insignificantly higher than the level of vehicle alone (Figure 3.3B).

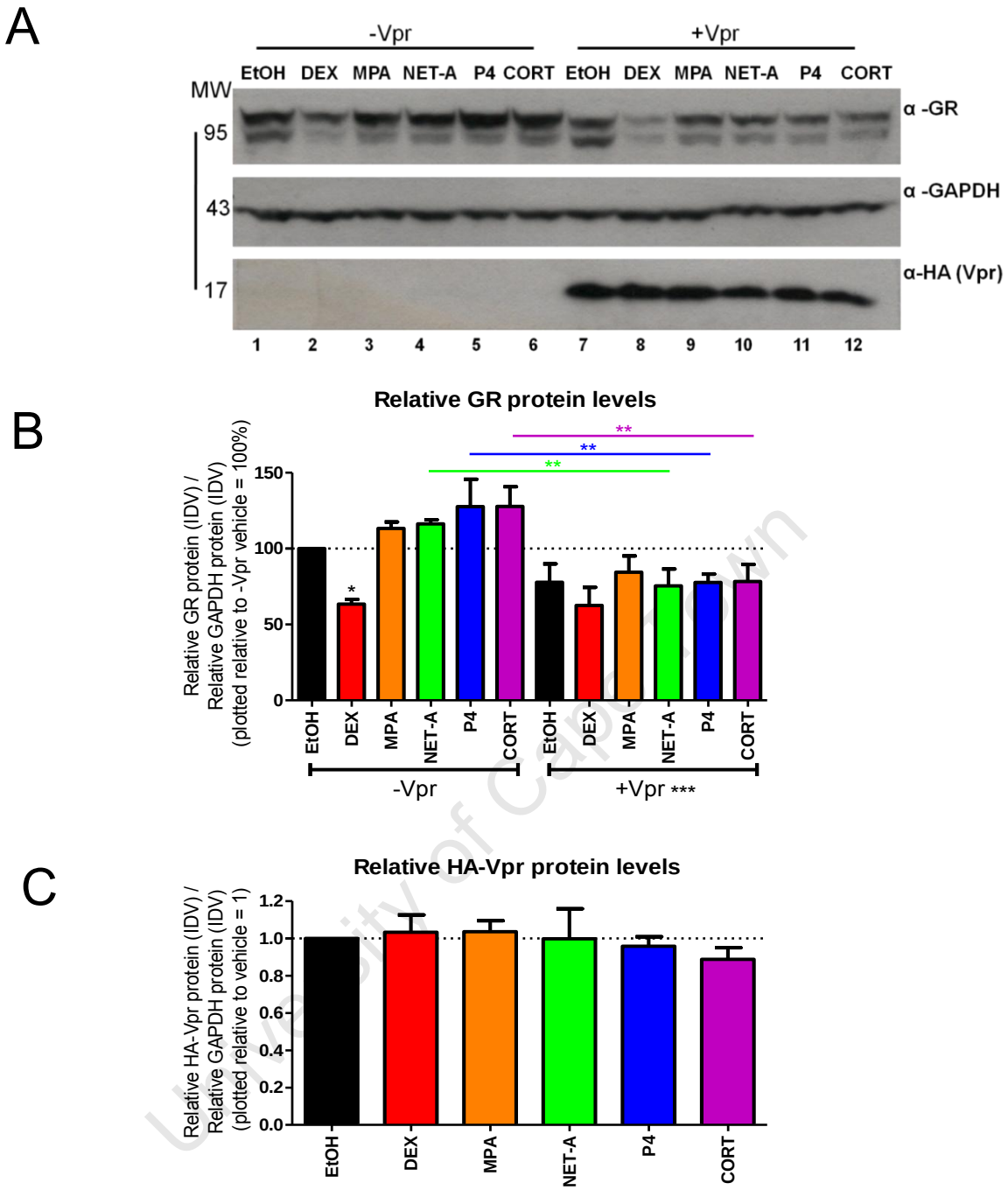


Figure 3.3 (A-C): Vpr decreases GR levels. COS1 cells were seeded directly into a 12 well TC plate and incubated at 37°C for 24 hours prior to transfection with HA Vpr or empty vector (350 ng/ml) and hGRα (350 ng/ml) using FuGene. Cells were stimulated with ligand at the concentrations indicated in materials and methods for 24 hours before harvesting in a 2x SDS sample buffer and resolved on 10% PAGE before transfer to nitrocellulose membrane probed for the presence of GR, GAPDH, and the HA tag. Protein bands were scanned and quantified with Alpha-ease FC software, and total GR integrated density value (IDV) was normalised to the intensity of the GAPDH IDV. A representative Western blot is shown (A) and histograms (B and C) show pooled data from three biological repeats. A Fishers least significant differences post-hoc test was used to determine if ligand stimulation caused a significant increase by comparing each stimulation to its respective vehicle (B), or if ligand stimulations in the absence and presence of Vpr were different (coloured bars above histograms). A two way ANOVA was used to compare the groups containing or lacking Vpr as a whole. Statistical analysis was marked *, ** or *** to indicate significance levels of $P<0.05$, $P<0.01$ or $P<0.001$, respectively.

In the presence of Vpr, a significantly greater extent of turnover was observed with NET-A, P4, and CORT stimulations when compared to the corresponding stimulations in the absence of Vpr (Figure 3.3B, coloured bars). A slight degree of DEX-dependent turnover was observed, but not statistically significant (Figure 3.3A, lanes 8 vs. 7 and Figure 3.3B), and was found to be similar to the level of GR stimulated with DEX in the absence Vpr (Figure 3.3B). Vpr caused a slight reduction in unliganded GR levels, but this was not significant (Figure 3.3B). Considering the transfection conditions as a whole, Vpr caused an overall significant reduction in GR levels (Figures 3.3B, right vs. left). This indicates that the overall increased reporter gene activity in the presence of Vpr (Figure 3.1A) cannot be explained simply by increased GR levels or stability.

Vpr levels were unchanged by ligand stimulation (Figure 3.3C), indicating that Vpr is not degraded in the proteasome in conjunction with the GR and that ligand stimulation of the GR does not affect expression of Vpr.

3.4. Vpr does not affect ligand-dependent serine 203, 211, or 226 GR phosphorylation

Phosphorylation of serine residues 203, 211 and 226 has previously been shown to increase in response to ligand stimulation (Avenant *et al.*, 2010b; Weigel and Moore, 2007). The effect Vpr has on phosphorylation of these residues is not yet known. It was hypothesised that Vpr may increase ligand-dependent GR phosphorylation at S203, S211 and S226. Additionally phosphorylation of one or more of these residues is required for co-activator recruitment (Avenant *et al.*, 2010a) which could explain how GR-mediated transactivation is increased in the presence of Vpr (Figures 3.1A and 3.2A). In order to determine how Vpr affects GR phosphorylation, COS1 cells transiently transfected with hGRα and HA Vpr or empty vector were stimulated with different ligands for an hour and a Western blot was performed with GR phospho-specific antibodies for phosphorylated serine residues 203, 211, or 226. Blots were stripped and re-probed for normalisation of phosphorylation to total GR levels.

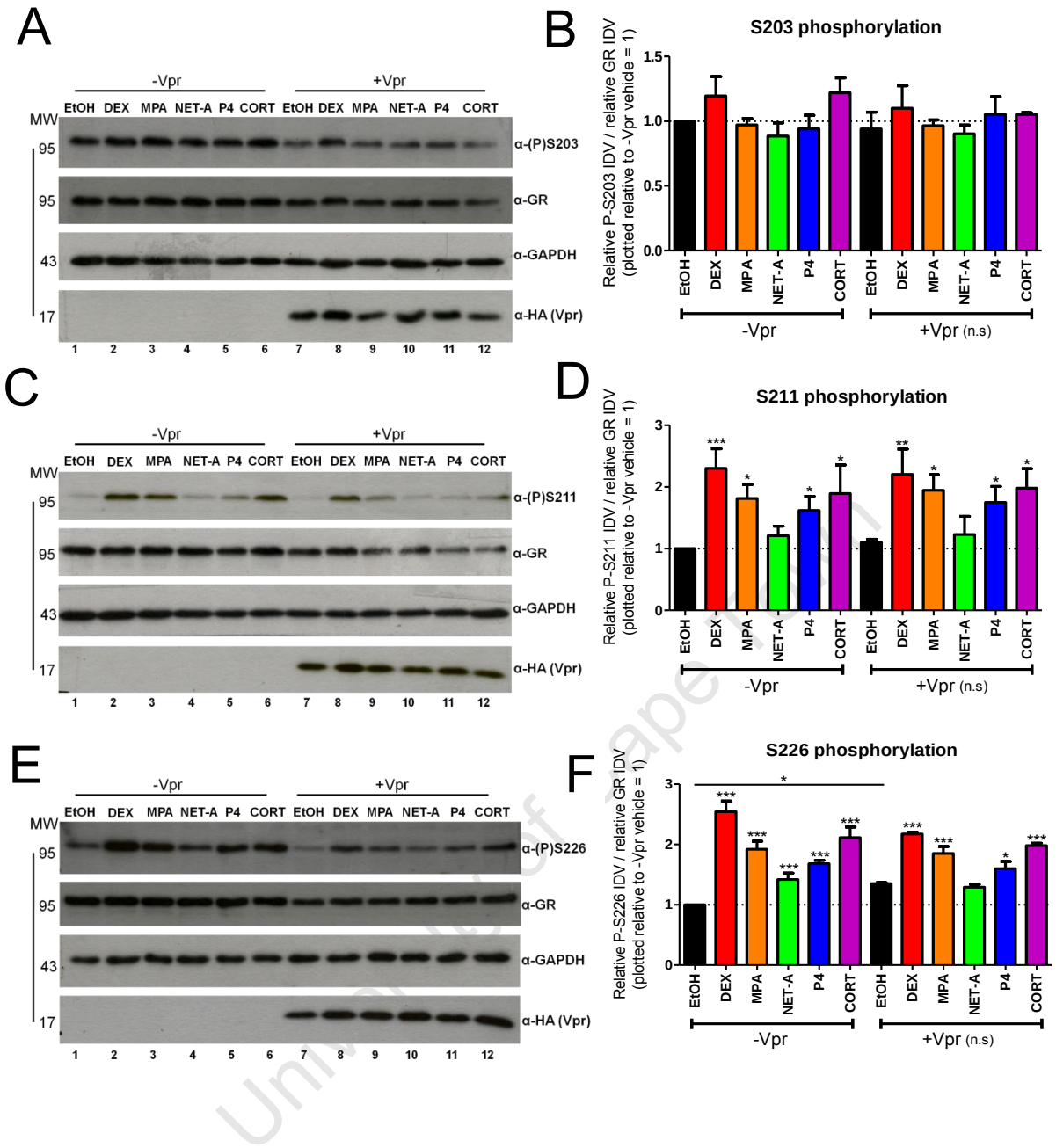


Figure 3.4 (A-F): Vpr does not affect the phosphorylation of the GR at S203, S211, or S226 in the absence or presence of ligands. COS1 cells were plated directly into 12 well plates and grown for 24 hours prior to transfection with 350 ng hGRα and either 350 ng HA Vpr or empty vector using FuGene. Cells were stimulated for an hour using concentrations of ligand as indicated in materials and methods before harvesting in 2x SDS sample buffer and resolved on 10% PAGE and transferred to a nitrocellulose membrane. Membranes were probed for the presence of GR, GAPDH, HA tag, and phosphorylated GR serine residue 203 (A and B), anti phosphorylated GR serine residue 211 (C and D), or phosphorylated GR serine residue 226 (E and F). Protein bands were scanned and quantified with Alpha-ease FC software, and phospho-GR IDV was normalised to the intensity of the total GR IDV. Representative Western blots are shown (A, C, E) and histograms (B, D, F) show pooled data from at least two biological repeats. A Fishers least significant differences post-hoc test was used to determine if ligand stimulation caused a significant increase by comparing each stimulation to its respective vehicle, or if ligand stimulations in the absence and presence of Vpr were different (bars above histograms). A two way ANOVA was used to compare the groups containing or lacking Vpr as a whole. Statistical analysis was marked *, ** or *** to indicate significance levels of $P<0.05$, $P<0.01$ or $P<0.001$, respectively.

In the absence of Vpr, ligand stimulation did not significantly change the extent of GR S203 phosphorylation in COS1 cells after an hour of stimulation (Figures 3.4A, lanes 1-6 and 3.4B) although DEX and CORT did show a small but insignificant increase above basal. Vpr had no effect on the phosphorylation status of GR S203, either in the absence or presence of any ligand (Figure 3.4B).

All tested ligands excepting NET-A, the weakest ligand in terms of GR transactivation efficacy tested here, significantly increased the extent of S211 phosphorylation in the absence of Vpr when compared to basal. DEX induced phosphorylation above basal to the greatest extent, followed by CORT, MPA, and P4 respectively (Figure 3.4D). The rank-order of ligand induced phosphorylation of this residue was not inconsistent with the rank-order of ligand induced reporter gene transactivation efficacy from a previous report (Ronacher *et al.*, 2009). In the presence of Vpr, S211 phosphorylation was also ligand-dependent, with the tested ligands displaying a very similar biocharacter in the presence of Vpr as they did in its absence (Figure 3.4D). Vpr did not change the basal level of S211 phosphorylation, or significantly increase the extent of ligand-induced S211 phosphorylation (Figure 3.4D).

GR S226 phosphorylation was increased significantly in response to ligand stimulation (Figure 3.4E lanes 2, 3, 4, 5, and 6 vs. 1). DEX induced a 2.5 fold increase, while CORT, MPA, P4, and NET-A all significantly increased phosphorylation, by 2.1, 1.9, 1.7, and 1.5 fold, respectively (Figure 3.4F). Ligand-dependent phosphorylation of S226 was found to be highly reproducible, and as with phosphorylation of S211, the rank-order of ligand induced phosphorylation of this residue was also not inconsistent with the established rank-order of ligand induced reporter gene transactivation efficacy (Ronacher *et al.*, 2009). Transfection of Vpr exerted no overall significant effect on S226 phosphorylation (Figure 3.4F, right vs. left), although Vpr did induce a small and statistically significant increase in S226 phosphorylation in the absence of ligand (Figure 3.4E lanes 7 vs. 1 and Figure 3.4F). Ligand-dependent S226 phosphorylation was not affected by transfection of Vpr, as all ligand stimulations showed similar levels of S226 phosphorylation when compared to their corresponding stimulations in the absence of Vpr (Figure 3.4F).

3.5. Vpr does not result in ligand-independent GR phosphorylation at S226

The previous result showed that Vpr increased S226 phosphorylation in the absence of ligand (Figure 3.4F). To further investigate this, five different concentrations of the HA Vpr construct were transfected in the absence of ligand and the relative extent of phosphorylated S226 was compared to a control not transfected with Vpr (0 ng). It was hypothesised that the extent of S226 phosphorylation would correlate with amount of Vpr present.

The same amount of DNA transfected per well in Figure 3.4 (350 ng per well), had no effect on S226 phosphorylation. Indeed, in the absence of ligand, no amount of transfected Vpr significantly increased S226 phosphorylation above the basal level, and no trend correlating the amount of transfected Vpr with S226 phosphorylation was observed (Figure 3.5B). No transfected amount of Vpr affected expression of the GR (Figure 3.5C) and as expected, expression levels of Vpr protein significantly increased with increasing amounts of transfected Vpr expression vector (Figure 3.5D).

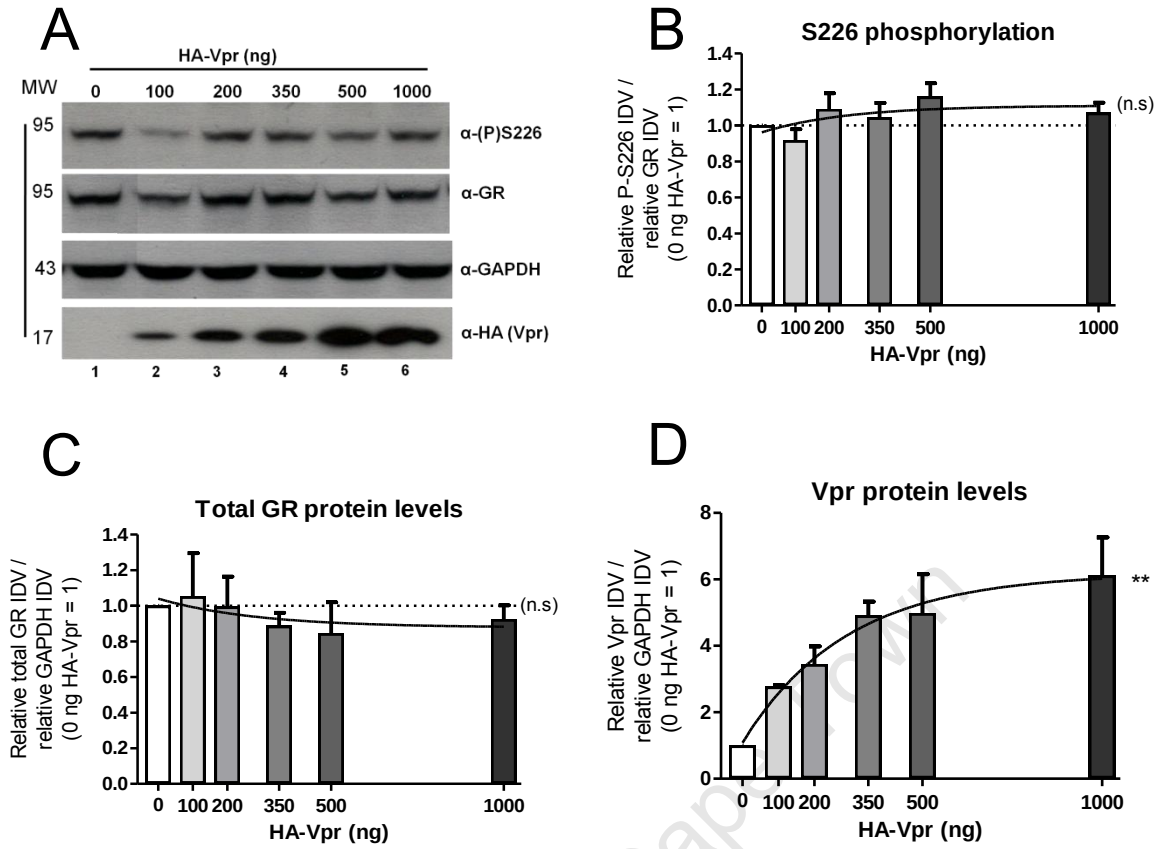


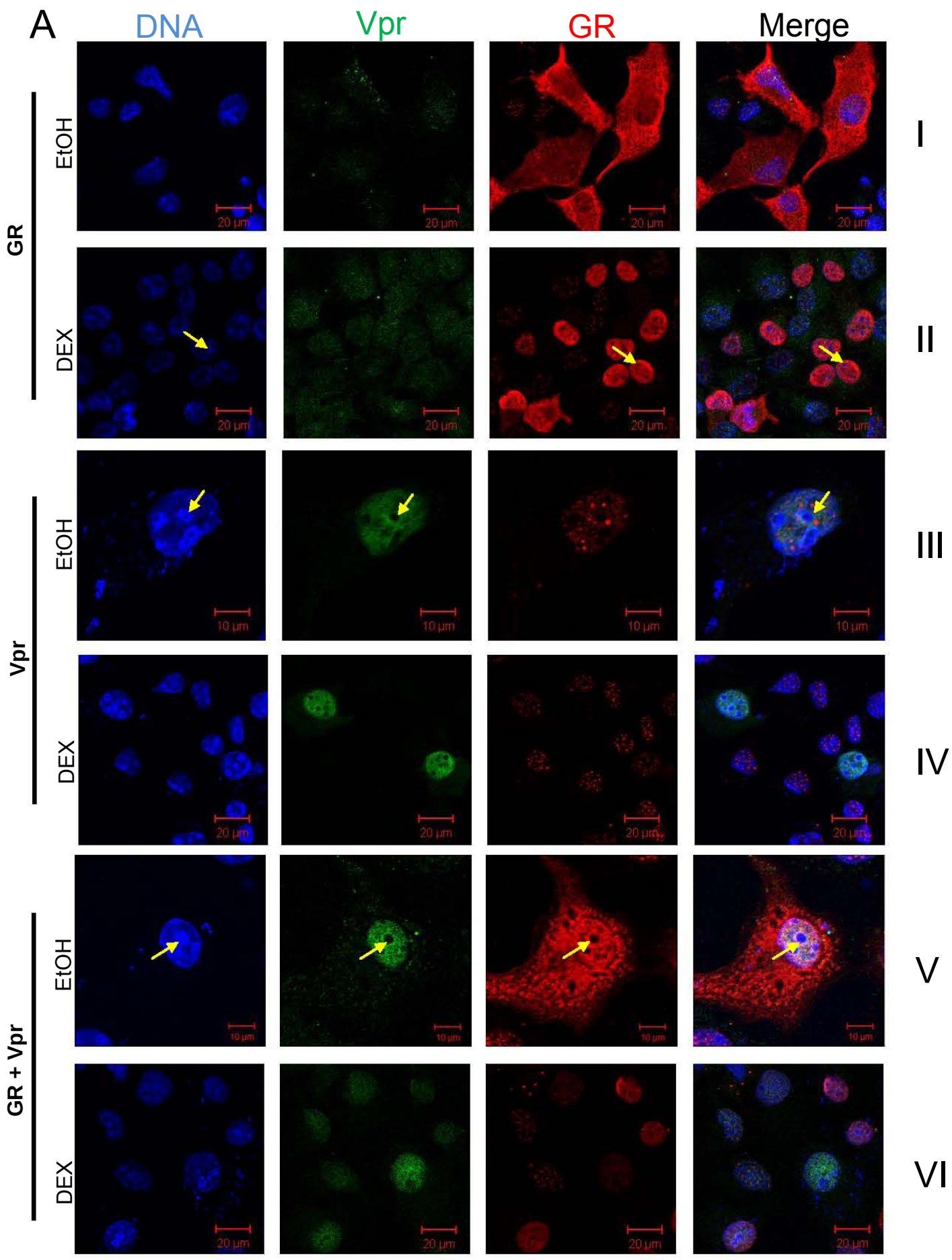
Figure 3.5 (A-D): Vpr does not increase GR phosphorylation at S226 in the absence of ligand. COS1 cells seeded directly into 12 well plates were grown for 24 hours prior to transfection with 350 ng hGRα and the indicated amount of either the HA Vpr or empty vector per well using FuGene. Twenty four hours after transfection cells were serum starved without ligand for an hour. Cells were harvested in a 2x SDS buffer and resolved on 10% PAGE before transfer to nitrocellulose membrane. Protein bands were scanned and quantified with Alpha-ease FC software. A Western blot, representative of two biological repeats is shown (A). Histograms show the average of two biological repeats. A two-way ANOVA using continuous variables test was used to indicate the significance of the correlation between the amount of transfected HA Vpr and phosphorylation of S226 (B), total GR levels (C), or Vpr protein levels (D). Histograms were marked *, ** or *** to indicate significance of $P<0.05$, $P<0.01$ or $P<0.001$, respectively.

3.6. Vpr does not increase rGR nuclear translocation

Prior to the induction of transcription, the GR needs to access the nucleus and associate with responsive promoters. The effects Vpr may have on GR nuclear translocation were investigated through fluorescent microscopy. It was hypothesised that Vpr would cause an increase in the nuclear proportion of the GR in both the presence and absence of DEX stimulation thereby increasing transactivation of the TAT-GRE reporter gene as observed in Figures 3.1A and 3.2A.

The presence of extra-nuclear DNA in all conditions (Figure 3.6A I-VI) was thought to be contamination from *Mycoplasma spp.* an intracellular pathogen which reveals a similar staining pattern if cells are contaminated. The cytoplasmic DNA spots were found not to be due to contamination but rather due to excess plasmid DNA. This was determined by a transfection negative control showing no cytoplasmic DNA, whereas cells that had been transfected showed a Hoechst stain in this manner (Figure 5.2). High amounts of DNA needed to be transfected in order for sufficient protein expression to allow visualisation under immunofluorescence, and accordingly COS1 cells were transfected with 750 ng rGR and 500 ng HA Vpr per ml culture medium which was in excess of the concentrations used in the previous assays.

In the absence of ligand the GR resides predominantly in the cytoplasm. The nucleus is not entirely vacant of Cy3 signal, indicating a small quantity of unliganded GR is present in the nucleus (Figure 3.6A I), in agreement with previous literature (Hache *et al.*, 1999; Savory *et al.*, 1999). GR nuclear localisation was calculated by expressing Cy3 fluorescent signal in the nucleus as a fraction of the total cell Cy3 fluorescent signal (nuclear/whole cell GR signal). This was then plotted as a relative fold induction above the unstimulated basal not expressing Vpr, as a measure to indicate the increase in nuclear translocation that DEX and/or Vpr mediate (Figure 3.6B). Stimulation of COS1 cells with 100 nM DEX for an hour causes the Cy3 fluorescent signal to be located nearly entirely in the nucleus (Figure 3.6A II). Areas of intense Hoechst stain represent densely packaged chromatin. The liganded GR is vacant from such areas (Figure 3.6A II, yellow arrows). DEX stimulation causes a significant 3.2 fold increase in rGR nuclear translocation (Figure 3.6B).


A
DNA
Vpr
GR
Merge
GR
EtOH
DEX
Vpr
EtOH
DEX
GR + Vpr
EtOH
DEX
I
II
III
IV
V
VI
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Hoechst
Alexa488
Cy3

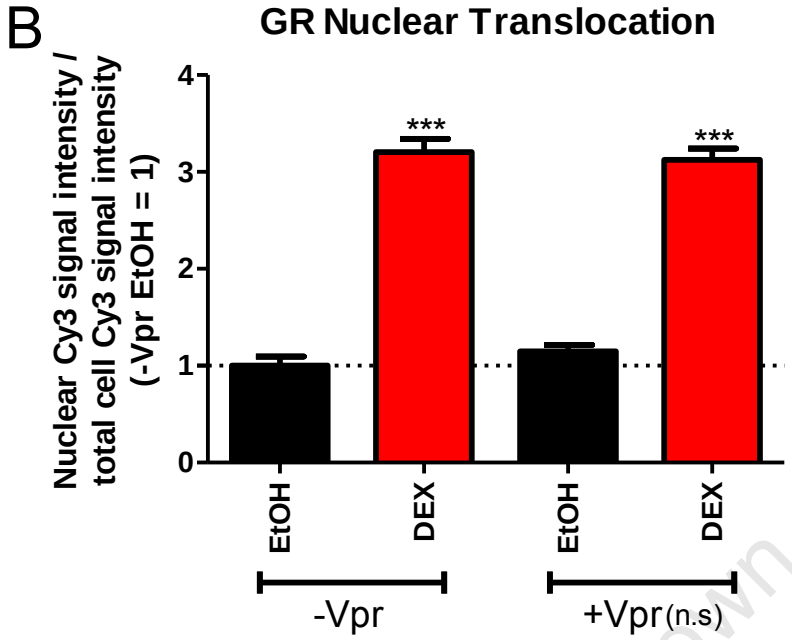

Figure 3.6 (A-B): HA Vpr does not affect the extent of rGR nuclear translocation. COS1 cells were seeded at a density of $4x10^4$ cells/ml directly into a 6 well plate on sterile glass cover slips. After 24 hours the medium was changed to charcoal stripped DMEM-10 and cells were either transfected with 1 µg of the rat-GR (rGR) only, 1.5 µg HA Vpr only, or both 1 µg rGR and 1.5 µg HA Vpr as indicated per well using FuGene. After an additional 24 hours, cells were stimulated in SF DMEM for an hour with 100 nM DEX or vehicle (EtOH). Cells were fixed and permeablised with methanol before addition of the primary α-mouse GR and α-HA (Vpr) antibodies. After an hour cells were washed and stained (according the manner described in the materials and methods). The Cy3 secondary antibody emits a red signal, indicating the location of the GR, and the Alexa488 conjugated fluorophore emits a green signal, indicating the subcellular location of the HA tag (Vpr), and the Hoechst dye stains DNA light blue. Yellow arrows indicate areas of densely stained chromatin which lack GR and/or Vpr fluorescent signal. Three biological repeats were done, each with at least three different fields of view being studied on each slide with a representative image shown (A). Slides were viewed with a Zeiss LSM510 Meta confocal microscope using the 40x objective lens, and quantified for fluorescent intensity using the LSM image analyser software. The histogram shows the pooled average of GR nuclear localisation. This was measured by quantifying the Cy3 fluorescent intensities, and plotted as a ratio of nuclear/whole cell intensity (B). A two-way ANOVA and Fishers least significant differences post-hoc test was used for statistical analysis and marked *, ** or *** to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively when comparing DEX stimulation to the basal for each condition.

Vpr has been extensively reported to be a constitutively nucleophilic protein (Agostini *et al.*, 2002; Thotala *et al.*, 2004). In agreement with this, when Vpr was transfected into COS1 cells without the rGR, it was found to be completely nuclear and the presence or absence of DEX had no effect on the subcellular localisation of Vpr (Figures 3.6A III and IV). This indicates that Vpr nuclear translocation is not dependent on DEX stimulation or the presence of the receptor. In a manner similar to the liganded GR being vacant from areas of heterochromatin, Vpr was not found

at areas of intense Hoechst stain, indicating that Vpr likely resides at euchromatic DNA regions (Figure 3.6A III, yellow arrows).

In the absence of DEX, it appears that Vpr may have a small effect in the subcellular distribution of the rGR. Unstimulated cells expressing both Vpr and the rGR were frequently shown to have a higher GR nuclear signal (Figure 3.6A V) than unstimulated cells expressing the rGR only (Figure 3.6A I). Inside the nucleus, the unliganded GR and Vpr are both vacant from the same sub-nuclear regions (Figure 3.6A V, yellow arrows). However, quantification of nuclear and total cell GR signal intensity using LSM image analyser software revealed that Vpr had no significant effect on the nuclear/cytoplasmic distribution of the unliganded rGR (Figure 3.6B). The discrepancy between the representative images and the fluorescent quantification data may be explained by one of two phenomena. Firstly, the data in Figure 3.6B is an average of all quantified slides, and whilst it did appear that Vpr frequently caused increased nuclear translocation of the GR (Figure 3.6A V), this may not accurately represent the situation in every single quantified slide, as the image in Figure 3.6A V represented a frequent occurrence that was apparent to an observer. However, it is possible that the trend could not be accurately measured using this type of fluorescent quantification. Secondly, the presence of non-specific nuclear red spots (Appendix 5.2) may have interfered with the quantification process, rendering the quantification of nuclear GR fluorescence inaccurate, and consequentially the data in Figure 3.6B not to be a true reflection of the GR subcellular distribution.

Vpr also did not affect the extent of nuclear localisation of the DEX-activated rGR (Figure 3.6A VI). As DEX stimulation causes almost total GR nuclear translocation, any increase in liganded rGR nuclear import through Vpr would be difficult to separate from the nuclear import caused by ligand activation alone.

Taken together, Vpr did not increase the nuclear proportion of the rGR in the absence of ligand or in the presence of DEX (Figure 3.6B). Reciprocally, the subcellular localisation of Vpr is unaffected by the presence of the receptor whether inactive (Figure 3.6A V) or ligand bound (Figure 3.6A VI).

3.7. Vpr does not enhance GR-mediated regulation of endogenous MKP-1 mRNA

MKP-1 was chosen as a suitable endogenous target gene to study the effects of ligand stimulation and Vpr as it has previously been shown to be regulated by the GR and is maximally expressed after short term GC stimulations (1-8 hours) (Shipp *et al.*, 2010; Wu *et al.*, 2005). The expression of MKP-1 mRNA was studied in both COS1, and human END cells which express endogenous GRα. As END cells express endogenous GR they are a more physiologically relevant system to study the effects of Vpr on GR signalling. END cells also express the MR, AR, and ER but not the PR-B (Verhoog, 2010).

In COS1 cells MKP-1 mRNA expression was significantly increased approximately 2.7 fold in the presence of DEX and nearly 2.0 fold in response to CORT stimulation in the absence of Vpr. MPA, NET-A and P4 did not significantly increase MKP-1 mRNA abundance (Figure 3.7A). However in the presence of Vpr, only DEX and P4 resulted in a significant increase in MKP-1 mRNA expression above vehicle. When comparing corresponding stimulations in the presence and absence of Vpr, no ligand showed any significant difference in MKP-1 mRNA levels. Furthermore, as a whole, the +Vpr group is not significantly different to the -Vpr group (Figure 3.7A).

In the absence of Vpr the tested ligands all display similar partial agonist activities which are significantly lower than the DEX response. In the presence of Vpr, P4 has a partial agonist activity that is not significantly different to that of DEX, indicating Vpr has mediated a change to its biocharacter (Figure 3.7B).

Variances in expression of MKP-1 mRNA with ligand stimulation cannot be attributed to variances in GR expression levels. Although the Western blot is only indicative of one biological repeat, the intensity of the GR signal with DEX stimulation appears to be only slightly reduced below basal indicating turnover after four hours (Figure 3.7C). Other than DEX, no form of stimulation increased turnover and the expression of Vpr had no ligand-dependent or -independent effect on GR levels in COS1 cells at this time point.

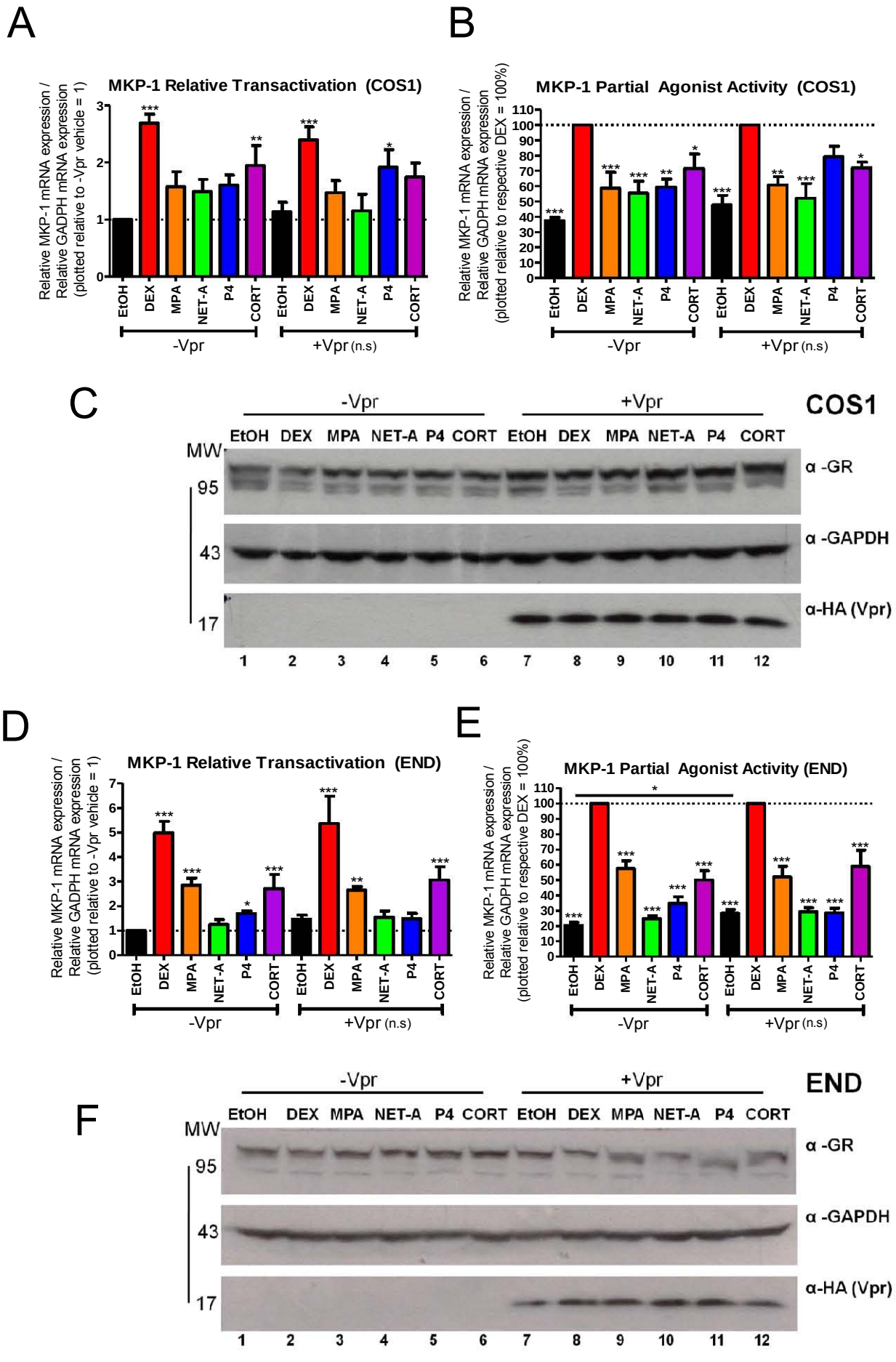
A
MKP-1 Relative Transactivation (COS1)
Relative MKP-1 mRNA expression / Relative GADPH mRNA expression (plotted relative to -Vpr vehicle = 1)
EtOH DEX MPA NET-A P4 CORT EtOH DEX MPA NET-A P4 CORT
-Vpr
+Vpr (n.s)
B
MKP-1 Partial Agonist Activity (COS1)
Relative MKP-1 mRNA expression / Relative GADPH mRNA expression (plotted relative to respective DEX = 100%)
EtOH DEX MPA NET-A P4 CORT EtOH DEX MPA NET-A P4 CORT
-Vpr
+Vpr (n.s)
C
-Vpr
+Vpr
COS1
EtOH DEX MPA NET-A P4 CORT EtOH DEX MPA NET-A P4 CORT
MW
95
43
17
α -GR
α -GAPDH
α-HA (Vpr)
1 2 3 4 5 6 7 8 9 10 11 12
D
MKP-1 Relative Transactivation (END)
Relative MKP-1 mRNA expression / Relative GADPH mRNA expression (plotted relative to -Vpr vehicle = 1)
EtOH DEX MPA NET-A P4 CORT EtOH DEX MPA NET-A P4 CORT
-Vpr
+Vpr (n.s)
E
MKP-1 Partial Agonist Activity (END)
Relative MKP-1 mRNA expression / Relative GADPH mRNA expression (plotted relative to respective DEX = 100%)
EtOH DEX MPA NET-A P4 CORT EtOH DEX MPA NET-A P4 CORT
-Vpr
+Vpr (n.s)
F
-Vpr
+Vpr
END
EtOH DEX MPA NET-A P4 CORT EtOH DEX MPA NET-A P4 CORT
MW
95
43
17
α -GR
α -GAPDH
α-HA (Vpr)
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.7 (A-F): Vpr has no effect on MKP-1 mRNA abundance in the presence of different GC's and progestins. COS1 (A-C) and END (D-F) cells seeded in 12 well plates were grown for 24 hours before transfection with HA Vpr or empty vector, and hGRα (COS1 only). Cells were stimulated for four hours at the concentrations shown in materials and methods. Total RNA was isolated, reverse transcribed into cDNA, and MKP-1 mRNA abundance was measured by real-time PCR and quantified relative to GAPDH (A, B, D, E). Graphs show pooled data of at least two biological repeats. For Western blotting (C, F) cells were harvested in 2x SDS sample buffer before 10% PAGE. A two-way ANOVA and Fishers Least Significant Differences post-hoc test was used for statistical analysis and marked *, ** or *** to indicate P<0.05, P<0.01 or P<0.001, respectively. Statistics show significance as compared to each EtOH (A and D) or DEX (B and E) and between corresponding stimulations in the presence and absence of Vpr.

In END cells, stimulation with DEX, MPA, CORT and P4 significantly increased MKP-1 mRNA abundance 5.0, 2.9, 2.7, and 1.7 fold respectively, whereas NET-A did not (Figure 3.7D). In the presence of Vpr DEX, CORT and MPA stimulations significantly increased MKP-1 mRNA expression above vehicle, but none of the ligands exhibited a significantly different effect compared to their corresponding stimulation in the absence of Vpr and the +Vpr group was not significantly different from the -Vpr group. Vpr did appear to increase the level of MKP-1 mRNA in the absence of ligand by 1.5 fold although this was insignificant (Figure 3.7D).

In the absence of Vpr, the relative trend of transactivation efficacies with DEX>MPA≥CORT>P4>NET-A was found to be in accordance with an earlier report (Ronacher *et al.*, 2009), and what was observed in Figure 3.1. Vpr did not significantly change the partial agonist activity of any ligands, but the absolute basal level of MKP-1 expression, relative to DEX was observed to be significantly increased by the presence of Vpr (Figure 3.7E).

Although the Western blot is only representative of one biological repeat, it appears that ligand stimulation did not cause an observable level of GR turnover or regulate Vpr expression, and Vpr had no effect on GR levels in END cells under these conditions (Figure 3.7F).

3.8. Vpr does not enhance GR-mediated regulation of endogenous IκBα mRNA

Due to inconsistent results obtained with the reporter constructs, where Vpr increased GR-mediated transactivation, and for MKP-1 mRNA expression, where Vpr did not increase GR-mediated transactivation, the ability of the Vpr to increase GR-mediated transactivation on another endogenous GRE-containing gene was also investigated. IκBα represents a suitable candidate gene for study as it has a role in inhibition of pro-inflammatory signalling, which may be favourable for HIV-1 replication and the promoter architecture, as with MKP-1, consists of at least two consensus GRE's which are responsible for upregulation in response to ligand activation of the GR (Reddy *et al.*, 2009).

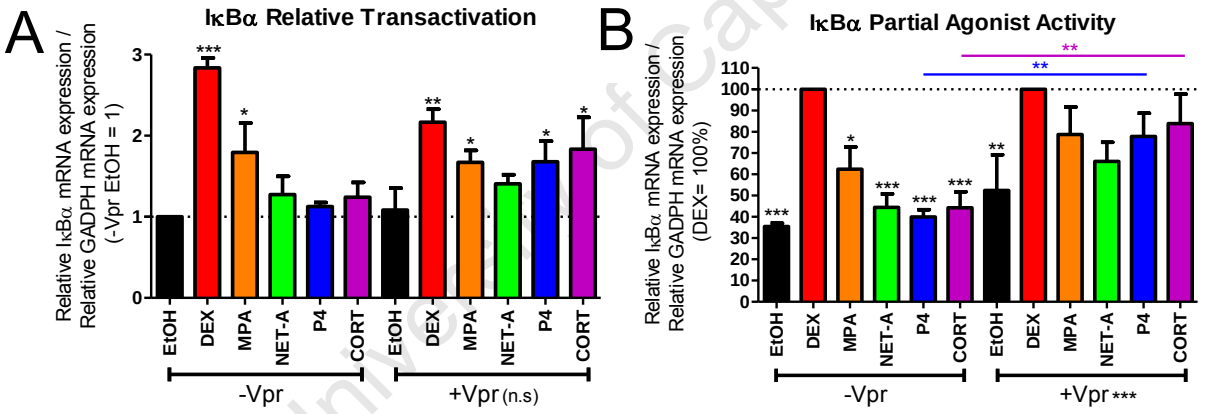

Figure 3.8 (A-B): Vpr has no effect on IκBα mRNA abundance in the presence of different GC's and progestins but does increase the partial agonist activity for some ligands relative to DEX. The same END cDNA samples used for MKP-1 real time analysis was also analysed for expression of the IκBα gene. END cells in 12 well plates were transfected with the HA Vpr empty vector 24 hours before ligand stimulation at the concentrations shown in materials and methods for four hours. Total RNA was isolated, reverse transcribed into cDNA, and IκBα abundance was measured by real-time PCR, before quantification relative to GAPDH. This is indicative of three biological repeats. A Western blot indicative of one biological repeat is shown in Figure 3.7F. A two-way ANOVA and Fishers Least Significant Differences post-hoc test was used for statistical analysis and marked *, ** or *** to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively. Statistics show significance compared to each EtOH (A) or DEX (B) and between corresponding stimulations in the presence and absence of Vpr.

In the absence of Vpr, IκBα mRNA expression in END cells was significantly upregulated by DEX and MPA, 2.8 and 1.8 fold, respectively (Figure 3.8A). Stimulations with CORT, NET-A and P4 did not significantly increase IκBα expression. The presence of Vpr did not significantly increase IκBα mRNA expression for any tested ligand stimulation.

Interestingly, under DEX stimulation, the abundance of IκBα mRNA appeared to be lower in the presence of Vpr than it was in the absence of Vpr (Figure 3.8B). However, this difference is not statistically significant. When plotted relative to their corresponding DEX stimulation, CORT and P4 were found to have a significantly higher partial agonist activity when in the presence of Vpr. Moreover, in the presence of Vpr, the biocharacter of all ligands was found not to differ significantly from DEX, whereas these same ligands partial agonist activities were significantly lower than DEX in the absence of Vpr. It was found that in general, the presence of Vpr caused the whole group of ligands to attain a higher relative biocharacter. The relative trend of transactivation efficacies was found to differ from what was observed previously, as in the absence of Vpr CORT did not act as a full GR agonist (Figure 3.8B).

4. DISCUSSION AND CONCLUSIONS

Six different hypotheses were presented at the beginning of this study, each of which had an aim and strategies for investigation. For each of the hypotheses, the Figures showing results relevant to that hypothesis, and the relevant section in the discussion is included in Table 4.1 below.

Table 4.1: Hypotheses, results and relevant sections in discussion

SECTION	PAGE	HYPOTHESIS		RESULTS
4.1	82	**Vpr increases ligand efficacy for transactivation of the TAT-GRE reporter gene, but not of endogenous MKP-1 and IκBα mRNA**		
		1	Vpr is a GR co-activator and will ligand-dependently increase GR-mediated transactivation. This will occur to a greater extent in the presence of more efficacious ligands than it will for weaker ligands	3.1A, 3.1D, 3.2A, 3.7A, 3.7D, 3.8A
4.2	90	**Vpr increases GR-mediated reporter gene transactivation dependently on DEX concentration but does not affect the DEX EC50**		
		2	Vpr increases both DEX efficacy and potency for GR-mediated transactivation	Figure 3.2
4.3	95	**Vpr increases GR turnover but does not change serine 203, 211, or 226 phosphorylation or nuclear translocation of the rGR**		
4.3.1	96	**Turnover**		
		3	Vpr regulates GR levels and turnover	Figure 3.3
4.3.2	99	**Phosphorylation**		
		4	Vpr enhances ligand-dependant GR phosphorylation at S203, S211, and S226	Figures 3.4, 3.5
4.3.3	101	**Nuclear translocation**		
		5	Vpr increases nuclear localisation of the rGR in the absence and presence of ligand	Figure 3.6
4.4	103	**Vpr mediates a much larger increase in MPA efficacy than it does for NET-A, and changes biocharacter in a ligand- and gene-specific manner**		
		6	Progestins will differently affect GR function when in the presence of Vpr, leading to changes in biocharacter and partial agonist activity	Figures 3.1B, 3.3, 3.4, 3.7B, 3.7E, 3.8B
4.5	107	**Vpr effects resemble those of a weak, non-classical GR activator**		
		n/a	not hypothesised	Figures 3.1A, 3.2D, 3.3B, 3.4F, 3.7A, 3.7D

4.1. Vpr increases ligand efficacy for transactivation of the TAT-GRE reporter gene, but not of endogenous MKP-1 and IκBα mRNA

Previous reports in the literature have described Vpr as a GR co-activator (Agostini *et al.*, 1999), with the ability to enhance DEX-mediated GRE driven reporter gene activity from the MMTV promoter and other synthetic GRE containing promoter-reporter constructs in CEM, Jurkat, A204, HS27, (Kino *et al.*, 1999; Kino *et al.*, 2002a; Kino *et al.*, 2002b), HEK293, and CV-1 cells (Sherman *et al.*, 2000). In this present study it was hypothesised that Vpr would act ligand-dependently (Table 4.1), and increase reporter gene transactivation to a greater extent in the presence of agonists than partial agonists.

To investigate the hypothesis, reporter gene analysis with transiently expressed hGRα and HA Vpr was performed in COS1 cells. Vpr was observed to significantly increase the efficacy or maximal reporter gene activity (A_{max}) after a 24 hour stimulation with the ligands MPA, NET-A, P4 and DEX/RU486. A statistically significant increase to the efficacy of DEX or CORT was not observed in the presence of Vpr, most likely owing to the high degree of error observed with these ligand stimulations (Figure 3.1A). Interestingly, Vpr also mediated a small but statistically significant increase in reporter gene activity in the absence of ligand. RU486 has been observed by others to abrogate Vpr co-activation of the DEX activated GR in 293T and A204 cells (Kino *et al.*, 1999; Sherman *et al.*, 2000). In this present study, RU486 was found to abrogate DEX induction of the TAT-GRE reporter gene in both the absence and presence of Vpr, although a higher level of reporter gene activity was observed when in the presence of Vpr (Figure 3.1A). If the absolute increases in reporter gene activity mediated by Vpr are compared across ligand stimulations, it becomes clear that Vpr did not increase GR-mediated reporter gene transactivation equally for each ligand simulation. Rather, Vpr appears to mediate greater increases in the absolute level of reporter gene activity when the GR is stimulated with efficacious agonists than when it is stimulated with partial agonists. However these were not significant differences compared to each other (Figure 3.1D). The relative fold induction by which Vpr increases GR-mediated reporter gene

transactivation, however appears similar in the absence and presence of all ligand stimulations (Figure 3.1E). In support of the original hypothesis, Vpr did significantly increase GR-mediated transactivation of the TAT-GRE reporter gene in the presence of ligands, and this appeared to be to a greater extent when in the presence of full agonists. It thus appears that Vpr enhanced ligand efficacy for transactivation of the reporter gene. Hypersensitivity to GC's has been reported before in AIDS patients (Kino and Chrousos, 2001), and hence the observation of Vpr increasing GR ligand efficacies in this present study suggests a mechanism whereby this could occur.

COS1 cells generally have very low GR expression (de Lange *et al.*, 1997; Verhoog, 2010) making the use of transiently transfected hGRα and the TAT-GRE reporter gene very practical in terms of measuring the specific effect of transfected Vpr on GR-mediated transactivation without significant interference from other SR's. The TAT-GRE promoter contains two full length consensus GRE's (Table 4.2), which ensures a high level of transcription driven from this promoter. For these reasons promoter-reporter assays in COS1 cells are a useful tool for investigating GR-specific transcriptional affects, but are conversely not a physiologically relevant model. In addition to COS1 cells, END cells were also used for endogenous gene PCR as they express endogenous GR, negating the need for transfection of a GR expression vector. END cells also express the AR, MR, and ER, and are highly responsive to steroids (Verhoog, 2010). These cells are human in origin and derived from the endocervical epithelial cells of the female genital tract, a region which provides a barrier to sexually transmitted diseases making them a physiologically relevant system in which to study the effects of HIV-1 Vpr (Fichorova *et al.,* 2002; Kaushic *et al.,* 2010; Wira *et al.,* 2005).

In the present study, two candidate endogenous genes were selected to study the effect of Vpr on GR-mediated transactivation by real time PCR, primarily based on their responsiveness to GC stimulation through GRE's in the promoter regions (Table 4.2), and secondarily on their likely relevance to HIV-1 infection through reported effects on immune function. These were MKP-1, a nuclear phosphatase which prevents MAPK signal transduction (Furst *et al.*, 2007), and IκBα, a gene which inhibits NFκB by sequestration of the RelA subunit in the cytoplasm (Auphan *et al.*, 1995). Based on the above promoter-reporter results, Vpr was also expected

to increase GR-mediated transactivation of both the MKP-1 and IκBα genes owing to their reported responsiveness to GC stimulation and the presence of putative GRE's in their promoters (Table 4.2).

Table 4.2: GRE-sequences from selected gene promoters relevant to this study

GR transactivated genes containing GRE's			
Gene name	**Number of GRE's**	**Sequence**	**Reference**
Consensus	n/a	AGA ACA nnn TGT TCT	Strähle *et al.,* 1987
TAT	2	TGT ACA gga TGT TCT GGA CTT gtt TGT TCT	Grange *et al.,* 1991
MMTV-LTR	4	GTT ACA aac TGT TCT GGT ATC aaa TGT TCT ACG TCT tag TGT TCT ATT TTC tag TGT TCT	Ham *et al.,* 1988
RANKL	1	TTT CCC gac TGT TCT	Kitazawa *et al.,* 1999
MKP-1	5	TGG CCA ccc TGC GTC GGA ACA ttc TGC CGT TGG ACA gcg GAG CCT GGG CCA ttg TGG CGC CGC TCA ctg TGT ATA	Shipp *et al.,* 2010
IκBα	2	GGA CGA agc CAT TCT unknown	Heck *et al.,* 1997 Reddy *et al.,* 2009
GILZ	3	GGA ACC caa TGT TCT CAA ACA ccg TGT TCA CTG CAG ctt TGT TCT	Wang *et al.,* 2004

GC induction of MKP-1 expression has been shown by others to be greatest between one and two hours of ligand stimulation in a variety of cell types, through five GRE like sites (Table 4.2) (Furst *et al.*, 2007; Lasa *et al.*, 2002; Shipp *et al.*, 2010; Tchen *et al.*, 2010; Wu *et al.*, 2005). In this present study, in both COS1 and END cells the abundance of MKP-1 mRNA was up regulated after four hours of ligand stimulation in a ligand-dependent manner, but expression was not significantly increased by Vpr (Figures 3.7A and 3.7D). IκBα expression has been previously shown to be upregulated by GC stimulation in a tissue specific manner (Han *et al.*, 1999; May and Ghosh, 1997; Scheinman *et al.*, 1995) through several putative GRE half sites (Deroo and Archer, 2001) and two full length GREs (Table 4.2) (Reddy *et al.*, 2009) and is greatest after four hours of ligand stimulation in A1-2 cells (Deroo and Archer, 2001). In the present study, after four hours of ligand stimulation in END cells it was found that although GC's increased expression, Vpr did not significantly

affect the absolute level of IκBα mRNA transcripts for any of the tested ligands (Figure 3.8A). IκBα expression in COS1 cells could not be analysed as the available human primers did not anneal to cDNA from COS1 cells (data not shown). Published primers specific for IκBα from monkey cell lines could not be found online and the monkey genomic sequences are not available on the databases. Based on the effects Vpr had on IκBα expression in END cells, it was thought that designing new primers for use in COS1 cells would be a futile effort.

Vpr has been observed to enhance GR-mediated mRNA and protein expression of the Receptor of Activated NFκB Ligand (RANKL) gene, which contains one GRE in the promoter (Table 4.2), in both PBMC and Jurkat cells (Fakruddin and Laurence, 2005).Expression of the GRE containing Glucocorticoid Induced Leucine Zipper (GILZ) gene (Table 4.2) has previously been shown in both A549 and U2OS cells to respond to ligand stimulation of the GR with the same relative rank-order of GR transactivation efficacies that the tested ligands induced for TAT-GRE transactivation in published reports (Hadley *et al.*, 2011; Ronacher *et al.*, 2009). However, Vpr was not found to enhance GR-mediated transactivation of the GILZ gene in COS1 cells (Avenant *et al.,* 2012, unpublished data). Besides the effect Vpr exerts on the GR at some GRE-containing promoters, Vpr also reduces induction of the synthetic NFκB driven promoter-reporter gene without DEX being present (Muthumani *et al.*, 2006). Previous work in our laboratory has found Vpr to abrogate NFκB and AP-1 induction by TNF-α but not to enhance DEX-mediated suppression of these reporter genes (Avenant et al., 2012, unpublished data). Vpr has however been reported by others to enhance ligand-dependent, GR-mediated transrepression of the NFκB and AP-1 regulated endogenous IL-12 gene (Mirani *et al.*, 2002). The effects of Vpr on GR-mediated transrepression were not investigated here, as it was beyond the scope of this project to study transrepression, and this present study was confined to transactivation only. These effects of Vpr on GR-mediated expression of the endogenous RANKL and IL-12 genes are consistent with Vpr acting as a GR co-activator. However, unpublished data from our laboratory, as well as the limited published data for endogenous GR regulated genes, suggests that whether Vpr acts as a GR co-activator on all endogenous genes is controversial and requires further investigation. The effects of Vpr on global gene expression have been analysed through a microarray, where numerous genes regulated by Vpr were identified. This

study did not indicate whether the GR is also involved with expression of these genes (Janket *et al.*, 2004), but did indicate that transactivation of Heat Shock Protein Factor 1 gene (HSF1) is increased 14-fold in the presence of Vpr (Janket *et al.*, 2004). This is notable as HSF1 is a component of the GR chaperone complex and a major contributor to stress-induced activation of the GR (Li *et al.*, 2000a), suggesting another indirect mechanism whereby Vpr could affect GR function.

The differences in the results obtained on the reporter gene compared to the endogenous genes in the present study are interesting and intriguing and require some explanation. Discrepancies in GR-mediated transactivation between synthetic reporter and endogenous genes with similar promoter elements have been reported before. For example, the MMTV-Luc reporter gene and endogenous IκBα promoters share common GRE response elements, yet the endogenous IκBα promoter responded to both R5020 (a synthetic PR agonist) and GC stimulation, whereas a stably integrated MMTV promoter was only responsive to GC stimulation in T47D cells (Deroo and Archer, 2002). This was found to be a result of the "closed" chromatin structure of the stably integrated reporter gene, and the open/permissive nature of the chromatin around the endogenous IκBα promoter (Deroo and Archer, 2001; Deroo and Archer, 2002). However, this example is not analogous to the comparison in the present study, which involves a transiently transfected reporter plasmid versus an endogenous gene, the promoters of which will differ markedly in their chromatin structure.

The structure of chromatin appears to dictate the nature of the GR interaction with a promoter and the resulting transcriptional outcome (John *et al.*, 2008). The reporter gene plasmid used in this study was transiently expressed in COS1 cells and as a result is not packaged into a native chromatin state. Thus the most likely explanation for the inconsistency in the observed reporter and endogenous gene results is that Vpr cannot access the GR and endogenous GRE-regulated gene promoters in the context of native chromatin, whereas it may do so for transiently transfected synthetic genes. Chromatin structure determines how the GR associates with DNA (John *et al.*, 2011), and a GRE sequence confers specific conformational changes to the GR AF-2 domain (Meijsing *et al.*, 2009) which is responsible for the GR association with transcriptional co-regulators (Kauppi *et al.*, 2003). It is possible that

at the endogenous promoter, the GR occurs in a transcription initiation complex (TIC) with other transcription factors that is different to the TIC found at the promoter-reporter gene. It is plausible that the TIC found at the promoter-reporter gene favours a Vpr interaction with the GR, whereas the GR-TIC complex at the endogenous gene did not, owing to either an alternate GR conformation or the blocking of co-factor interacting sites by other co-regulatory proteins. These results do not exclude the possibility that the effects of Vpr are highly promoter specific and that Vpr can regulate some, but not all GRE-containing endogenous genes. The GRE sequences of GR-regulated genes which have been reported in the literature to be affected by the presence of Vpr (RANKL, MMTV-LTR, and TAT-GRE) contain the TGT TCT half site whereas the two endogenous genes investigated in this study do not (Table 4.2). Further investigation will need to determine if this specific DNA sequence is a marker for Vpr enhancement of GR-mediated transactivation.

It is important to note that in the reporter gene assay in COS1 cells (Figure 3.1) different amounts of the GR (112 ng/ml) and Vpr (250 ng/ml) expression vectors were transfected when compared to the real time PCR assay (Figure 3.7), and all subsequent experiments in COS1 cells in which 350 ng/ml of either Vpr and/or the GR expression vectors were transfected unless otherwise indicated. COS1 cells were plated at the same densities so that the amount of medium was proportional to the amount of cells. END cells did not require GR transfection, but did require much higher levels of Vpr transfection (1 000 ng/ml) in order to detect similar levels Vpr expression as in the COS1 cell line (Figure 3.7). This may have been due to poorer transfection efficiency of this cell line. The discrepancy in the amounts of transfected DNA between the reporter assay and real time experiments was a consequence of the bulk DEAE-Dextran transfection, and re-plating method used in Figure 3.1 to correct for transfection efficiency. This bulk transfection and re-plating method was found to cause a much higher degree of experimental variability and error, and less reproducibility than a direct FuGene6 transfection. As a result, subsequent assays after Figure 3.1 were transfected directly with FuGene6. The commonly used β-Galactosidase assay for transfection efficiency could not be used however as the two available β-Galactosidase encoding plasmids were found to have expression regulated by either Vpr or steroids respectively, making it impossible to correct for variances in transfection efficiency. Without correcting for transfection efficiency it is

impossible to know if co-transfection of the Vpr expression vector affected the transfection efficiency of other plasmids. The relative concentrations of GR, Vpr, other components of the transcriptional machinery and GRE promoter sequences may determine the outcome of the effect of Vpr on GR-mediated transactivation. As different amounts and ratios of the GR and Vpr expression vectors were transfected in Figures 3.1, 3.7, and 3.8, these factors vary substantially when comparing results of reporter gene experiments with overexpressed GR in COS1 cells to those of endogenous genes regulated by either overexpressed GR in COS1 or the endogenous GR in END cells. This confounds a direct comparison of these obtained results by making a direct comparison between the above reporter gene assay and real time PCR results difficult. Thus, comparison of the results obtained via overexpressed GR in COS1 cells for the reporter gene versus the endogenous MKP-1 gene suggest that these differing results may not be due to the gene promoter alone, but also could have been an artefactual observation owing to the differing methods of transfection and relative GR/Vpr ratios, both of which are confounding factors when drawing a conclusion based on a comparison of these results.

It was thus difficult to conclude whether the original hypothesis that Vpr is a classical GR co-activator is true (Table 4.1). Taken together, Vpr significantly increased the efficacy of some ligands at the TAT-GRE promoter-reporter plasmid, which is consistent with a role of Vpr as a GR co-activator, but had no effect on the mRNA abundance of the two tested endogenous genes. The discrepancy between these results could be due to a whole host of confounding factors. Furthermore, showing Vpr to have no effect on two endogenous genes does not indicate that Vpr has no effect on all GRE regulated endogenous genes nor disprove the co-activator model for Vpr. Insights gained from this present study indicate that before conclusive statements can be made regarding Vpr-mediated co-activation of the GR, many further studies and strategies would need to be investigated, especially regarding a direct interaction between Vpr and the GR in whole cells and the identification of at least one endogenous gene which Vpr enhances GR mediated transcription of.

A co-activator should associate directly with the GR on the promoter through, for example LXXLL motifs and serve to increase recruitment of additional proteins to enhance agonist-mediated effects on transcription. The presence of LXXLL motifs is

common to both Vpr and the p160 steroid receptor co-activators (SRC), and Vpr has been reported to immunoprecipitate *in vitro* with the DEX bound GR (Kino *et al.*, 1999; Kino *et al.*, 2002a), although other reports indicate that this may not be ligand-dependent (Sherman *et al.*, 2000; Thotala *et al.*, 2004). Mutation of either of the two Vpr LXXLL motifs reportedly abolishes Vpr immunoprecipitation with the GR (Sherman *et al.*, 2000). Alternatively, Vpr may interact with the GR through an intermediate protein *in vivo* (Muthumani *et al.*, 2006; Ramanathan *et al.*, 2002). However, no evidence for a direct, *in vivo* association between Vpr and the GR has yet been shown. Similar to an SRC, Vpr has been reported to immunoprecipitate with numerous transcription factors (Kino *et al.*, 2002a; Kino and Pavlakis, 2004). The association of SRC-2 (alternatively GRIP-1 or TIF2) with the GR is blocked by stimulation with RU486 (Kauppi *et al.*, 2003). However, in the present study RU486 treatment did not prevent Vpr from mediating an increase in DEX-mediated reporter gene transactivation (Figure 3.1A and 3.1D), suggesting Vpr not to act exactly as the co-activator SRC-2 does via the GR. RU486 stimulation in the absence of DEX has been found to reduce both Vpr/GR-mediated transactivation of the HIV-1 LTR (Schafer *et al.*, 2006), and to prevent Vpr/GR-mediated transrepression of the NHE1 gene (Janket *et al.*, 2007) suggesting Vpr itself to act more like a GR agonist than a ligand-dependent co-activator. This is supported by the observation in this current study where Vpr induced a significant increase in reporter gene transactivation in the absence of ligand (Figure 3.1A).

4.2. Vpr increases GR-mediated reporter gene transactivation dependently on DEX concentration but does not affect the DEX EC_{50}

Classical GR co-activators, such as members of the p160 SRC family have been reported to ligand-dependently increase efficacy (A_{max}), decrease EC_{50} (a measure of potency), and change the partial agonist activity of certain ligands for both transiently expressed reporter and endogenous gene expression (He and Simons, 2007; Hong *et al.*, 1997; Lee and Simons, 2010; Luo and Simons, 2009; Onate *et al.*, 1995; Ronacher *et al.*, 2009; Sun *et al.*, 2008; Szapary *et al.*, 1999). SRC's increase the A_{max} by associating with numerous TF's and histone acetyltransferases (HAT) which cause nucleosomal re-arrangement in the region of the promoter and result in increased transcriptional rates (Li *et al.*, 2003).

In order to gain insight into the possible co-activator role of Vpr via the GR, the relationship between ligand concentration and Vpr mediated increases in GR-driven reporter gene transactivation was investigated through DEX dose response curves. Two different amounts of Vpr expression vector were transfected as it was expected that higher concentrations of Vpr protein would result in greater reporter gene transactivation, as was observed previously in the presence of 100 nM DEX at the TAT-GRE reporter gene (Sherman *et al.*, 2000). Non-saturating concentrations of ligand indicate how Vpr affects EC_{50}, and the saturating concentration indicates the effects on A_{max}. Owing to the well-documented effects of SRC-2 on GR-mediated transactivation, and the resemblance of some Vpr mechanisms to SRC-2, it was thought that in addition to the already observed effect Vpr has on increasing ligand efficacy, Vpr would also increase the potency of DEX (Table 4.1).

In the absence of ligand, both amounts of Vpr expression vector induced a similar, very low but statistically significant induction of the reporter gene. This was less than 0.01% of the effect induced by 1 μM DEX, making the physiological relevance unclear. At DEX concentrations less than 10^{-10} M, dose response curves in the presence of Vpr were indistinguishable from that in the absence of Vpr. At higher DEX concentrations reporter gene activity is increased by Vpr in a manner that is

greater than additive between ligand stimulation and the level of reporter gene activity that Vpr caused in the absence of ligand. Vpr did not increase reporter gene activity equally across all concentrations of DEX, but rather increased reporter gene transactivation to greater extents in the presence of higher DEX concentrations. Additionally increased reporter gene activity was observed with the greater amount of transfected Vpr expression vector if compared to the lower amount (Figure 3.2A). As DEX is a highly specific GR agonist, this illustrates a relationship between Vpr and DEX stimulation of the GR and excludes the possibility that Vpr acts entirely independently of DEX-mediated GR transactivation (Figure 4.1A). Vpr did not significantly decrease the EC_{50} as it had been expected to. The EC_{50} of DEX in the absence of Vpr was 0.5 nM. Transfection of 50 ng of Vpr expression vector insignificantly decreased this, in the direction expected, to 0.3 nM. However, transfection of the higher amount (175 ng) caused an insignificant increase, rather than decrease in the DEX EC_{50}, to 0.7 nM, thereby suggesting that Vpr did not change the affinity of DEX for the GR. Transfection of Vpr also did not significantly change the Hill slope, which is a measure of the degree of co-operative ligand binding. Previous work by collaborators has shown that at high GR concentrations, positive co-operation occurs, suggesting that ligand binding to one GR monomer increases ligand binding to the other GR monomer within a GR dimer, whilst at low GR concentrations there is no such co-operativity, with a Hill slope of 1 (Robertson, 2011). As Vpr resulted in no change in the Hill slope in the present study, the results suggest that Vpr did not change the degree of co-operation of ligand binding to the GR. Interestingly, in the absence of Vpr, the Hill slope was less than 1, suggesting negative co-operativity, a finding not observed in the previous study (Robertson, 2011). This should however be investigated further, as it indicates that under these conditions that ligand binding to one GR monomer hampers ligand binding to the other GR monomer within a GR dimer. When drawing conclusions from these data, it should be noted that it is possible that Vpr mediated a small change to the EC_{50} and Hill slope that was undetectable above the level of error and biological variability in this experiment. More accurate values for the EC_{50} and Hill slopes may be obtained using more concentrations of DEX in the range of the steep slope on the dose response curve, in addition to performing more repeat experiments (increasing the n-value), preferably in a cell line expressing endogenous GR.

The finding that Vpr increased the A_{max} for the DEX dose response curves is consistent with the result in Figure 3.1 as well as several reports in the literature. In 293T cells Vpr enhanced DEX-mediated TAT-GRE reporter gene transactivation above the level of the GR alone, dependently on ligand concentration, similar to the classical co-activator SRC-2, albeit to a lower extent (Sherman *et al.*, 2000). Similar results were observed in A204 cells where Vpr enhanced a GR-mediated DEX dose response of the MMTV promoter-reporter gene (Kino *et al.*, 1999). The EC_{50}'s were not however determined in either published study.

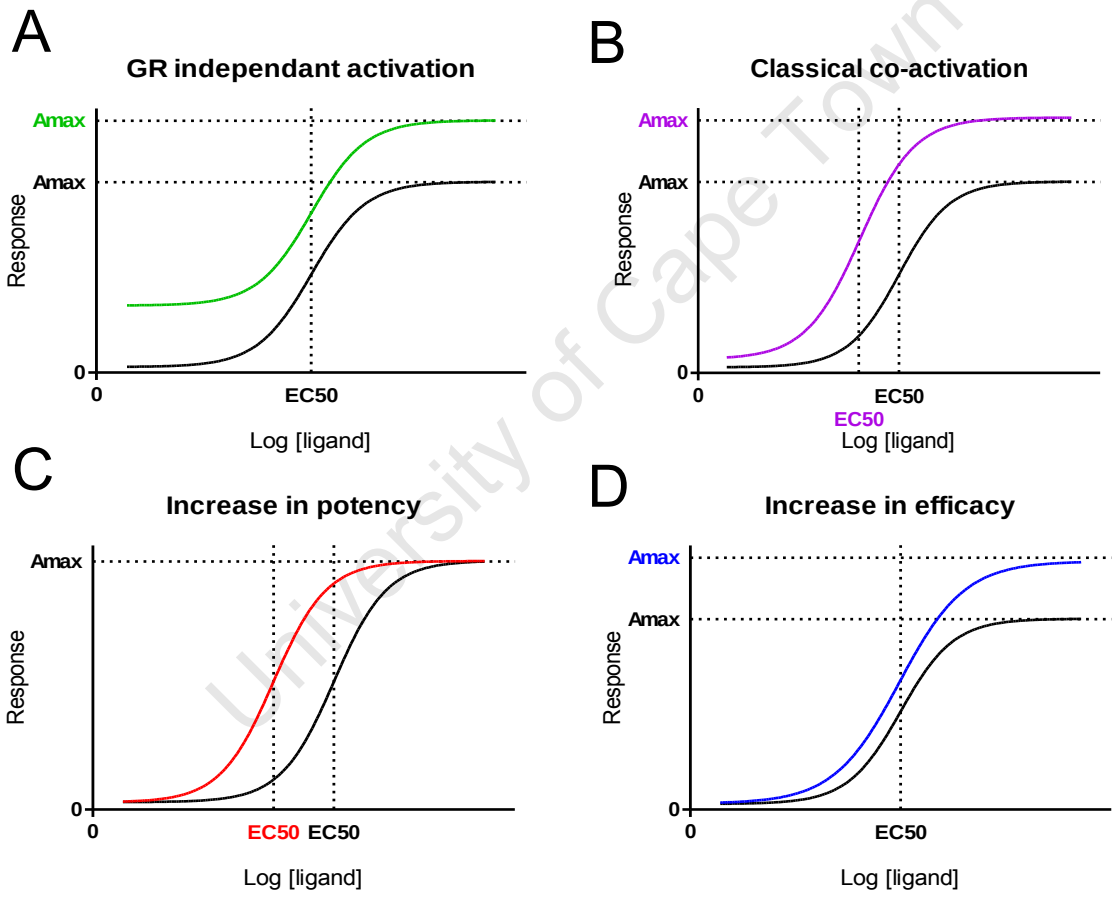

Figure 4.1 (A-D): Models representing different possible outcomes for increasing ligand-dependent transactivation. A steroidal dose response curve is shown in black, with different possible outcomes for increased transactivation shown in colour. (A) GR independent activation would increase efficacy by an equal magnitude independently of steroid concentration without affecting EC_{50}, (B) classical co-activation (frequently observed with SRC-2) simultaneously increases efficacy and decreases EC_{50}. (C) Some co-activators may increase ligand potency but have no effect on efficacy, (D) whilst others may increase the A_{max} without affecting the EC_{50}.

When assessing what insights can be obtained from the dose response curves regarding Vpr mechanism of action and its possible function as a GR co-activator, a comparison with data obtained by others with established GR co-activators is helpful. SRC-2 co-activation of the GR was found to be dependent on ligand concentration (Hong *et al.*, 1997; Onate *et al.*, 1995; Voegel *et al.*, 1996) and the extent of SRC-2 recruitment to the GR was found to be dependent on ligand efficacy (Ronacher *et al.*, 2009). Vpr does mediate more of an increase in GR-mediated reporter gene activity in the presence of agonists than it does for partial agonists (Figure 3.1D), and induces more of an increase in reporter gene activity at higher DEX concentrations (Figure 3.2A). This suggests that Vpr, like SRC-2, serves to enhance ligand induced GR-mediated transactivation rather than mediate a ligand independent general increase in GR-mediated transactivation. The effects of Vpr are thus dependent on the degree of GR activation, and will be greater in the presence of efficacious steroids and high concentrations of ligand.

The established co-activator SRC-2 simultaneously increases ligand potency and efficacy (Figure 4.1B), in what is termed classical co-activation (Hong *et al.*, 1997; Onate *et al.*, 1995; Szapary *et al.*, 1999). However, mutational analysis has revealed that modulation of the EC_{50} and A_{max} are in fact separable effects, mediated through different regions of the GR (Awasthi and Simons, 2012). Select mutations in the GR LBD which affect the interaction with SRC's can selectively modulate one of these parameters without affecting the other, indicating that the EC_{50} and A_{max} may be modulated by co-factors independently (Lee and Simons, 2010; Luo and Simons, 2009; Russcher *et al.*, 2005; Tao *et al.*, 2008; Vottero *et al.*, 2002). The exact effects of a GR co-activator appear to be highly variable according to context, and no firm rule about the relationship between A_{max} and EC_{50} exists. Rather, there are some reports in which a co-activator only affects EC_{50} and not A_{max} (Figure 4.1C), while others report that a co-activator may affect only the A_{max} and not EC_{50} (Figure 4.1D) (as reviewed in Ong *et al.*, 2010). Overexpression of SRC-1 in Astrocytes and SRC-2 in MSC80 cells has been reported to not affect the A_{max} of a GRE containing reporter gene (Grenier *et al.*, 2006) and SRC-1 has been reported to only increase ligand potency via the GR, but not the A_{max} (Szapary *et al.*, 1999). In contrast, SRC-2 knockdown has been shown to have no effect on DEX EC_{50} for expression of the endogenous GILZ and CD163 genes, but did reduce the A_{max} (Luo and Simons,

2009). TSA, VPA and Ubc9 have also been reported to ligand-dependently increase the A_{max} of a synthetic reporter gene, without affecting the EC_{50} (Kaul *et al.*, 2002; Kim *et al.*, 2006; Lee and Simons, 2010), similar to the effects obtained with Vpr in this present study.

Although it had been expected that Vpr would decrease the DEX EC_{50}, an analysis of the literature revealed that under certain conditions co-activators may increase the A_{max} without rendering a ligand more potent. Data from Figure 3.2 is thus not inconsistent with a role of Vpr as a GR co-activator, but alone is insufficient as proof thereof. Published results show there is a high degree of variability regarding the effects a co-activator may exert on GR-mediated transactivation. SRC-2 is a constituent member of the p160 SRC family, and around 350 further co-regulators have been identified (York and O'Malley, 2010). As Vpr is not a human gene, it may not perfectly mirror all functions of SRC-2, but does increase GR-mediated transactivation of a reporter gene, similar to the effects of a co-activator. This may be through a similar mechanism or in a manner distinct from classical co-activation. Data obtained from Figures 3.1 and 3.2 could not determine the mechanism by which Vpr enhanced reporter-gene activity.

The SRC-1 and SRC-3 members of the p160 family have intrinsic HAT activity, whilst it is controversial as to whether SRC-2 harbours a HAT function (Leo and Chen, 2000; Marmorstein, 2001). Due to its small size it is unlikely that Vpr itself may have HAT activity, as HAT's are generally large multi-subunit enzyme complexes (Roth *et al.,* 2001), and Vpr does not display any recognised chromatin remodelling motifs (Neuwald and Landsman, 1997; Yuan and Giordano, 2002). Although sequence alignment between SRC-2 and Vpr proteins does not yield any conserved domains apart from the Leucine rich domains, it is possible that the mechanism by which Vpr increases reporter gene transactivation is similar to the mechanism by which SRC-2 does. SRC's increase GR driven transcription by recruiting further co-regulators and HAT's to the promoter (Horwitz *et al.,* 1996; Onate *et al.,* 1995; Voegel *et al.,* 1996). SRC-2 recruits further co-regulators to the TIC to increase transcription. The homo- and hetero- oligomerisation properties of Vpr (Kino and Pavlakis, 2004) may also result in increased recruitment of HAT's or other chromatin remodelling proteins to the TIC. One such TF this has already been reported for is

p300, which is responsible in part for enhanced GR activity in the presence of Vpr (Felzien *et al.*, 1998; Kino *et al.*, 2002a).

Several factors and some data indicate that Vpr differs in its mechanism of action compared to SRC-2. Vpr was able to increase GR-mediated transactivation in the presence of RU486 (Figure 3.1A), which is thought to block SRC-2 association with the GR, and thereby prevent SRC-2 induced transcriptional effects (Bledsoe *et al.*, 2002; Kauppi *et al.*, 2003), however, the presence of DEX in this experiment may have contributed to the greater level of reporter gene transactivation observed, and the concentration of RU486 may not have been sufficient to prevent co-activator association. Vpr did not mediate a change in DEX potency (Figure 3.3B), as SRC-2 does, nor did it increase the relative extent of partial agonist activity of any ligand.

In conclusion, many lines of evidence indicate that the effects of Vpr closely resemble those of SRC-2. In contrast, other evidence both from this study and reported by others indicates that Vpr may act in a manner that is very similar to, but actually discrete from classical co-activation by p160 SRC's. The results from this present study indicate the need for alternate strategies before the role of Vpr as a GR co-activator can be proven. The increase mediated by Vpr to the reporter gene A_{max} is insufficient to classify Vpr as a GR co-activator. Indeed, any reporter gene analysis cannot be relied upon as the sole indicator of Vpr co-activation of the GR. Rather an endogenous gene regulated in a similar mechanism would need to be identified and co-occupancy of the GR and Vpr at a promoter would be required before such a statement could be made.

4.3. Vpr increases GR turnover but does not change serine 203, 211, or 226 phosphorylation or nuclear translocation of the rGR

To determine if Vpr changes GR-mediated reporter gene transactivation by a mechanism that is discrete from classical co-activation, which predominantly increases protein-protein interactions at the promoter, three steps involved with the

activation of the GR were investigated. Since ligand efficacy was enhanced in the presence of Vpr, it was hypothesised that Vpr could have: changed the abundance of the GR itself, caused selective hyperphosphorylation of key GR serine residues, or increased the extent of GR nuclear translocation.

4.3.1. Turnover

Ligand binding destabilises the GR by causing poly-ubiquitination, which enhances GR affinity for the proteasome (Kinyamu *et al.*, 2005). The greatest extent of ligand-dependent GR degradation occurs with the most efficacious ligands (Avenant *et al.*, 2010b). Decreasing GR levels after ligand activation serves as a mechanism to prevent excessive GR signalling (Wallace and Cidlowski, 2001). Thus decreased GR levels reflect increased transactivation efficacy. In an apparent paradox, proteasomal inhibition prevents GR degradation, thereby increasing GR levels. This also has the effect of increasing GR mediated transcription (Deroo *et al.*, 2002; Wallace and Cidlowski, 2001). Vpr could have stabilised the GR against degradation thereby increasing GR levels prior to stimulation, or could have either acted like a ligand itself or increased ligand efficacy, which would have had the effect of reducing GR levels. Both mechanisms can plausibly explain how Vpr resulted in an increase in reporter gene transactivation.

The current study showed that stimulation of the GR with DEX for 24 hours significantly decreased GR levels, while stimulation with CORT, MPA, P4, or NET-A did not (Figure 3.3B). This may have been expected with the weaker agonists NET-A and P4, as these have low GR efficacies, but was not expected with CORT or MPA stimulations, two ligands which significantly induced reporter gene transactivation. It has been previously shown that the half-life of the unliganded GR in this cell line is 44 hours. This is reduced to 10 hours upon stimulation with DEX, 12 hours with CORT, 17 hours with MPA, 28 hours with P4, and 37 hours with NET-A. Stimulation of the GR with each of these ligands has been found to significantly decrease GR levels after 24 hours (Avenant *et al.*, 2010b). Both the unliganded and liganded GR are continually turned over, although the liganded GR is degraded much faster than

the unliganded GR (Avenant *et al.*, 2010b). The experimental design in the present study and those of the published report differ as *de novo* protein synthesis had been prevented in the published report, allowing determination of the GR half-life, whereas the present study effectively measured GR turnover, which includes both *de novo* GR synthesis and degradation. Thus the observation of no change in GR levels for stimulations with all ligands except DEX could plausibly be explained by a rate of GR expression which is as fast or faster than GR degradation and that a change in ligand-dependent GR turnover may have been masked by new GR protein synthesis.

Use of a two-way ANOVA test allowed a comparison of the two whole groups (-Vpr and +Vpr) by excluding ligand stimulation as a variable. COS1 cells transfected with the GR and Vpr were found to have significantly lower GR levels than cells transfected with the GR and an empty vector plasmid. DEX dependent GR turnover was equal in the absence and presence of Vpr, and the levels of GR stimulated with MPA were not significantly different in the absence and presence of Vpr. However, significantly lower GR levels in the presence of Vpr were observed with CORT, P4, and NET-A if compared to the corresponding stimulations in the absence of Vpr. Vpr also decreased levels of the GR stimulated with vehicle alone, suggesting that it may exert some measure of activation to the unliganded GR. However, this difference was not found to be statistically significant (Figure 3.3B).

Increased turnover of the GR mediated through the actions of Vpr has not been reported before, but does suggest that the increased reporter gene activity induced by Vpr in the present study cannot be explained simply by higher levels of GR protein. Vpr enhanced the efficacy of the tested ligands and as previously reported, ligands with greater efficacies cause greater levels of GR degradation (Avenant *et al.*, 2010b). It is thus possible that the lower levels of GR observed in the presence of Vpr reflect enhanced efficacy, although it should be noted that the extent of turnover did not correlate with the ligands transactivation efficacies. Vpr itself may have acted as a weak, non-classical GR activator thereby causing enhanced GR degradation in a manner similar to how a ligand would. It is also possible that Vpr increases GR ubiquitination and affinity for the proteasome (Kinyamu *et al.*, 2005), thereby lowering GR levels. The cell cycle arresting abilities of Vpr are dependent on an

interaction with DCAF/VprBP. These proteins form a direct linkage with DDB1, a core subunit of the Cul4 ubiquitin ligase (Le Rouzic *et al.*, 2007). The recruitment of an ubiquitin ligase to the GR/Vpr complex may have resulted in increased GR ubiquitination and subsequently more rapid degradation.

Vpr has been found to immunoprecipitate with the GR in the presence of DEX (Kino *et al.*, 1999; Kino *et al.*, 2002a), although such an interaction has not been observed from studies in our lab (Avenant et al., 2012, unpublished data). In this current study, in the presence of Vpr, all ligand stimulations were found not be significantly different to stimulation with vehicle alone, although DEX stimulation did cause a slight reduction in GR levels in the presence of Vpr (Figure 3.3B) and no ligand stimulation, inclusive of DEX had any effect on Vpr levels (Figure 3.3C). This therefore suggests that Vpr is not degraded alongside the DEX activated GR. Thus if a complex containing both these proteins exists *in vivo*, it is likely to be dissociated prior to GR degradation or Vpr may be resistant to proteasomal degradation.

To ensure that each condition within an experiment was transfected with an equal quantity of DNA and thus treated with an equal volume of transfection reagent, a quantity of empty vector DNA equal to the quantity of Vpr expression vector was transfected into cells that were required not to express Vpr. As discussed in section 4.1 above, transfection efficiency was unable to be determined, and accordingly it was impossible to determine if transfection of Vpr decreased transfection of the GR expression vector, which would have also resulted in decreased GR levels. Alternatively, the cytotoxic (Gummuluru and Emerman, 1999) and apoptotic (Snyder and Ross, 2009; Snyder *et al.*, 2010) effects of Vpr may have also contributed to lower GR plasmid expression, increased proteolytic activity, or caused cell lysis and a consequential loss of the expressed proteins into the culture medium. Apoptosis and cell death alone are unlikely causes however, as the GAPDH loading control was not found to be lower in the presence of Vpr (Figure 3.3A).

4.3.2. Phosphorylation

The relative phosphorylation status of three key GR serine (S) residues; 203, 211, and 226 has been reported to change with GR nuclear import and export (Dean *et al.*, 2001; Itoh *et al.*, 2002), affect co-factor association (Avenant *et al.*, 2010a; Chen *et al.*, 2008) and reflect ligand efficacy for transactivation of reporter genes (Avenant *et al.*, 2010b). All three residues are ligand-dependently hyperphosphorylated after an hour of stimulation (Blind and Garabedian, 2008; Chen *et al.*, 2008; Ismaili and Garabedian, 2004; Wang *et al.*, 2007). Ligand transactivation efficacy also reflects the extent of SRC-2 association with the GR (Ronacher *et al.*, 2009), which is dependent on the GR being phosphorylated at one or more of these residues (Avenant *et al.*, 2010a). Changes in phosphorylation status were thus thought of as a likely target mechanism for Vpr mediated modulation of GR signalling. If Vpr is causing an increase in GR ligand efficacy, it could be expected to also enhance phosphorylation of these serine residues in the same manner that ligands do (Table 4.1). If, however Vpr resulted in GR activation via a non-classical mechanism, i.e. other than through the ligand-binding domain as for classical GR ligands, Vpr could increase basal S226 phosphorylation, as has been reported for GnRH at the corresponding residue on the mouse GR (Kotitschke *et al.*, 2009) or TNF-α on the human GR (Verhoog *et al.*, 2011).

Ligand-dependent S203 hyperphosphorylation was not observed in this study (Figure 3.4B), in agreement with previous reports indicating S203 phosphorylation is only weakly increased upon DEX stimulation in U2OS and A549 cells (Wang *et al.*, 2002). The basal level of S203 phosphorylation is reportedly much higher than that of S211 (Ismaili and Garabedian, 2004; Wang *et al.*, 2002), causing the ligand-induced fold increases above basal to appear lower. Consistent with this, in the present study in the absence of Vpr, DEX stimulation insignificantly increased S203 phosphorylation only 1.2 fold above basal, and a high level of basal S203 phosphorylation was present (Figure 3.4A). This causes the relative fold increases induced by ligand to appear lower when compared to basal. No ligand significantly increased S203 phosphorylation above stimulation with vehicle alone, and Vpr had no effect on S203 phosphorylation, either in the absence or presence of any of the

tested ligands (Figure 3.4B). Others have shown that phosphorylation of S203 alone slightly impairs S211 phosphorylation (Wang *et al.*, 2002), and causes the GR to attain a peri-nuclear distribution, but not gain nuclear access (Wang *et al.,* 2002). The GR phosphorylated at S203 has not been found associated with the TAT-GRE promoter (Blind and Garabedian, 2008), and thus it was not entirely surprising that Vpr exerted minimal observable effect on S203 phosphorylation.

Hyperphosphorylation of S211 reflects higher levels of GR-mediated transactivation (Avenant *et al.*, 2010b), and the P-S211/P-S226 ratio is linked to increased GR-mediated transactivation (Chen *et al.*, 2008). It was therefore hypothesised that Vpr would enhance ligand-dependent S211 phosphorylation. Significant phosphorylation of both residues S211 (Figure 3.4D) and S226 (Figure 3.4F) was found to occur after an hour of ligand stimulation, in agreement with previous findings indicating that these residues may be simultaneously phosphorylated (Wang *et al.*, 2007). The established rank-order of GR reporter gene transactivation efficacies from an earlier report was DEX≥CORT>MPA>P4>NET-A (Ronacher *et al.*, 2009). Ligands result in different levels of S211 and S226 hyperphosphorylation correlating with their transactivation efficacies in this same rank-order (Avenant *et al.*, 2010b). The relative extents of S211 and S226 phosphorylation obtained in the current study were not inconsistent with this rank-order (Figure 3.4). However, Vpr had no significant effect on ligand-dependent phosphorylation at either of the tested residues (Figures 3.4D and 3.4F), which was not expected.

Phosphorylation of S226 alone decreases GR efficacy for transactivation of the TAT-GRE reporter gene in COS1 cells (Avenant *et al.*, 2010b). Phosphorylated S226 leads to a decrease in phosphorylation of S211, consistent with ligand-dependent S226 phosphorylation attenuating GR function (Chen *et al.*, 2008; Krstic *et al.*, 1997; Wang *et al.*, 2007). S226 has been reported by others to be partially phosphorylated in the absence of ligand, and to become hyperphosphorylated upon ligand stimulation. However, ligand-dependent S226 hyperphosphorylation does not occur to the same extent as S211 hyperphosphorylation does in rat hepatoma cells (Blind and Garabedian, 2008). Although Vpr had no effect on ligand-dependent S226 phosphorylation in this present study, it did cause a statistically significant increase in S226 phosphorylation in the absence of ligand (Figure 3.4F), consistent with

previous reports regarding the effects of the non-classical GR activators, GnRH and TNF-α (Kotitschke *et al.*, 2009; Verhoog *et al.*, 2011).

The effect of Vpr on ligand-independent S226 phosphorylation was tested further by transfecting differing amounts of the Vpr expression vector with the expectation that higher amounts of Vpr would reflect as increased extents of ligand-independent S226 phosphorylation. However, no tested concentration of Vpr expression vector, including the concentration previously shown to increase S226 phosphorylation (350 ng/ml), had any effect (Figure 3.5). These two conflicting results therefore make a conclusion regarding the effect of Vpr on ligand-independent S226 phosphorylation difficult to reach based on the available data from this present study. Further experimentation would be required before a conclusive statement may be made regarding the effects of Vpr on ligand-independent S226 phosphorylation.

This report is the first investigating the effect of ligand selective phosphorylation of GR residue S203, and the effects of Vpr on S203, S211, and S226 GR phosphorylation.

4.3.3. Nuclear Translocation

It was hypothesised that Vpr would increase nuclear translocation of the GR in the presence and absence of DEX owing to its nucleophilic nature and the shared nuclear import pathway (Freedman and Yamamoto, 2004; Refaeli *et al.*, 1995). This may be the mechanism whereby Vpr increases GR-mediated transactivation of the reporter gene in both the absence and presence of ligand (Table 4.1).

Using immunofluorescence analysis and confocal microscopy, the subcellular distribution of the rGR and Vpr in the absence and presence of DEX was investigated in COS1 cells (Figure 3.6A and B). The rGR was predominantly cytoplasmic, but not entirely vacant from the nucleus in the absence of ligand, in agreement with previous literature reports indicating that the unliganded GR is constantly shuttled between the nucleus and cytoplasm (Hache *et al.*, 1999; Madan

and DeFranco, 1993; Savory *et al.*, 1999). Stimulation of the receptor with DEX for an hour caused the rGR to translocate almost entirely to the nucleus, which equates to a roughly three-fold increase in the nuclear/whole cell proportion of the rGR. This is also consistent with literature which indicates that almost entire rGR nuclear translocation occurs upon ligand stimulation (Baumann *et al.*, 1999; Htun *et al.*, 1996). In both A549 and U2OS cells, the extent of GR nuclear translocation correlates with ligand efficacy (Hadley *et al.*, 2011). Vpr was found to be both constitutively and entirely nuclear, independent of DEX stimulation or the presence of the rGR. This is in agreement with earlier studies showing Vpr to be nucleophilic (Mahalingam *et al.*, 1997; Sherman *et al.*, 2001; Suzuki *et al.*, 2009) and to have a general affinity for nucleic acids (Kichler *et al.,* 2000; Zhang *et al.,* 1998). Co-expression of the rGR with Vpr does not cause a statistically significant change in the subcellular distribution of the unliganded or DEX-activated rGR if compared to results obtained in the absence of Vpr (Figure 3.6B). Because DEX caused near total nuclear translocation of the GR, it was difficult to observe how Vpr might have increased this.

Areas of intense Hoechst stain indicate highly condensed chromatin, which is likely to be transcriptionally inactive (yellow arrows). This staining pattern indicates that the liganded GR is vacant from such areas (Figure 3.6A II) as is Vpr (Figure 3.6A III) and that inside the nucleus Vpr and the GR display a similar distribution (Figure 3.6A V). Visual inspection of these images prior to quantification indicated that in the presence of Vpr, the unliganded GR had higher nuclear signal intensity than it did in the cytoplasm (Figure 3.6A V). In addition, there was a greater proportion of the unliganded rGR in the nucleus in the presence of Vpr (Figure 3.6A V) if compared to the absence of Vpr (Figure 3.6A II). Quantification of the fluorescent intensities with LSM image analyser software determined that the nuclear proportion of the unliganded rGR was not significantly increased by Vpr (Figure 3.6B). However, increased nuclear localisation may have been masked by the high level of non-specific nuclear rGR staining by the primary GR antibody (Figure 5.2).

Vpr may have mediated a small increase in rGR nuclear translocation in the absence of ligand that was too small below the background level to be established as statistically significant. Such an observation of Vpr increasing nuclear import of the

unliganded GR would have supported previous observations from this present study which indicate Vpr to increase transactivation via the unliganded GR, as a weak, non-classical GR activator may (Figures 3.1A and 3.2D). The experimental design was unable to conclude if Vpr did increase DEX-mediated GR nuclear translocation, as the saturating DEX concentration used caused nearly entire GR nuclear translocation (Figures 3.6A II and VI). Fluorescent quantification of the slides determined that the nuclear proportion of the rGR was not significantly affected by Vpr in the presence of 100 nM DEX (Figure 3.6B).

4.4. Vpr mediates a much larger increase in MPA efficacy than it does for NET-A, and changes biocharacter in a ligand- and gene-specific manner

Agonists and partial agonists have different relative binding affinities for the GR which correlates well with potency, and to a lesser extent efficacy (Ronacher et al., 2009). These differences cause transcriptional responses that vary according to the nature of the bound steroid, in a cell type and promoter specific manner (Africander *et al.*, 2011b; Hapgood *et al.*, 2004; Ishida *et al.*, 2008; Ronacher *et al.*, 2009). It was hypothesised that Vpr would alter the biocharacter of the GR partial agonists, in addition to generally mediating an increase for the reporter gene transactivation efficacies of the tested steroids (Table 4.1). The five ligands tested (DEX, MPA, NET-A, P4 and CORT) and the antagonist (RU486) have different GR transactivation efficacies, and accordingly exhibit different levels of reporter gene transactivation when acting via the GR. Reports in the literature have established the rank-order of these ligands as DEX≥CORT>MPA>P4>NET-A for reporter gene transactivation efficacy in COS1 cells (Ronacher *et al.*, 2009) and HEK293 cells (Koubovec *et al.*, 2005). DEX and CORT are full GR agonists, and result in greatest induction of the TAT-GRE reporter gene via the GR. MPA and P4 are partial agonists, although under high enough GR concentrations MPA may act as a full agonist (Zhao *et al.*, 2003), and NET-A has a very low GR binding affinity and efficacy and is not reported as a GR agonist for transactivation (Koubovec *et al.*, 2005; Schindler *et al.*, 2003; Schoonen *et al.*, 2000; Sitruk-Ware, 2004).

The partial agonist activity is a measure of the transactivation that a ligand induces relative to that of a full agonist, which in this present study is DEX. Co-activators which enhance the A_{max} have been reported before to also affect the partial agonist activity of non-agonist steroids (Lee and Simons, 2010; Luo and Simons, 2009; Sun *et al.*, 2008). The effects of Vpr on ligand biocharacter were also investigated as many confounding factors, other than Vpr alone may have mediated a change to ligand efficacy and the absolute increases in GR-mediated transcription.

Plotting the reporter gene activity induced by each of the tested ligands relative to DEX revealed that Vpr did not cause any ligand to attain a significantly higher different biocharacter relative to DEX. No ligand was found to change biocharacter from a partial agonist, with a significantly lower partial agonist activity than DEX, to a full agonist which is not significantly different to DEX (Figure 3.1B). Although statistically significant differences were not observed between the tested ligands, a rank-order for reporter gene transactivation efficacy that was consistent with previous literature (DEX>CORT>MPA>P4>NET-A) was observed in both the absence and presence of Vpr (Figure 3.1B) (Koubovec *et al.*, 2005; Ronacher *et al.*, 2009). The relative fold increase by which Vpr increased reporter gene activity for each ligand was similar (Figure 3.1E), which therefore indicated that whilst Vpr did increase ligand transactivation efficacy, it did not alter the relative biocharacter of these ligands for induction of the TAT reporter gene.

The effect of Vpr on ligand biocharacter for the induction of the two endogenous genes was also investigated. In COS1 cells in the absence of Vpr, the partial agonist activity of P4 is significantly lower than that of DEX, as it is acting as a partial agonist, whereas in the presence of Vpr the partial agonist activity of P4 was at a level of significance comparable with DEX (Figure 3.7B). This suggests that Vpr changed the biocharacter of P4 from a partial to a full agonist for expression of MKP-1 (Figure 3.7B). Additionally, although significance was not established, the relative trend of transactivation efficacies in the absence of Vpr was not consistent with that observed in the presence of Vpr.

END cells not expressing Vpr appeared to induce expression of MKP-1 mRNA in accordance with the observed and published rank-order for relative GR

transactivation efficacies of these ligands, although significant differences between ligands could not be established given the small differences and large number of variables. All ligands were found to act as partial agonists in both the absence and presence of Vpr (Figure 3.7E), indicating Vpr to not alter any ligands biocharacter for expression of MKP-1 in END cells. Interestingly, a significantly higher basal level of MKP-1 expression was observed if the absolute partial agonist activities are compared between the corresponding conditions, although the relevance of such an observation is disputable.

Transfection of Vpr into END cells significantly increased the partial agonist activities of P4 and CORT when expressed as a percentage of the DEX response and corresponding stimulations compared in the absence and presence of Vpr (Figure 3.8B). In the presence of Vpr all ligands were found to have a partial agonist activity that was at a level insignificantly different to DEX, whereas in the absence of Vpr all ligands were found to have a partial agonist activity significantly lower than DEX (Figure 3.8B). It was concluded that Vpr increased the partial agonist activity for all ligands for expression of the IκBα gene in END cells, especially the relative biocharacter of P4 and CORT relative to DEX, such that the partial agonists behaved like agonists. This is an interesting observation as P4 and CORT were the only two endogenous steroids used in this present study, and supports the observation regarding the effect of Vpr in changing the biocharacter of P4 from a partial agonist to a full agonist shown in Figure 3.7B. It is possible that Vpr preferably increases the partial agonist activity if the GR is bound to endogenous hormones as opposed to synthetic exogenously administered steroids such as MPA and NET-A. This result should be interpreted with caution since the relatively high error of the data in the presence of Vpr may have masked differences in the maximal responses of the ligands in the presence of Vpr as compared to in the absence of Vpr. A clearer result should be obtained through more biological repeats, with fewer variables so as to give a greater power of statistical analysis, to confirm whether Vpr significantly changes the biocharacter of partial agonist to agonists on some endogenous genes.

Steps in the GR pathway other than transcription were also investigated to observe any progestin-specific and Vpr-dependent effects. None of the progestins or GC's caused differing levels of S203, S211, or S226 phosphorylation when in the

presence of Vpr. Interestingly, stimulations with CORT, P4, and NET-A were all found to have significantly lower GR levels in the presence of Vpr whereas DEX and MPA did not (Figure 3.3B).

The partial agonist activities of MPA and NET-A relative to DEX were not found to differ for transactivation of the reporter gene or either of the endogenous genes in the absence and presence of Vpr. It was hypothesised that the progestins MPA and NET-A could act differently via the GR when in the presence of Vpr (Table 4.1), but this was found not to have occurred as Vpr enhanced reporter gene transactivation with both these ligands roughly 4.5 fold (Figure 3.1E). Vpr increased all the tested ligands A_{max} at the TAT-GRE reporter gene in a manner that reflected the ligand efficacies. However, the Vpr-mediated increase in reporter gene A_{max} with MPA (a full to strong partial agonist), was much greater than it was for NET-A (a weak partial agonist) (Figure 3.1D). This Vpr mediated increase on reporter gene A_{max} raises concerns regarding the use of exogenously administered steroids with high GR transactivation efficacies. If endogenous genes are found which respond to Vpr in the same manner as the reporter gene does, then use of MPA should be treated with caution where exposure to Vpr is concerned. If Vpr is present in conjunction with efficacious GC's, such as MPA, it could lead to an enhanced level of GR-mediated responses, than if compared to the presence of a weak agonist such as NET-A. This would affect endocrine and immune functions and the contraceptive side effect profile. Such an effect is noteworthy regarding bone related disorders, as long-term use of MPA has been reported to enhance osteoporosis, likely through its GR agonist properties (Greydanus *et al.*, 2001; Ishida and Heersche, 2002; Ishida *et al.*, 2008; Kaunitz, 2000c; Ott *et al.*, 2001; Scholes *et al.*, 2002). Vpr potentiates GR-mediated induction of the RANKL gene (Fakruddin and Laurence, 2005) which is implicated to play a role in HIV-1 related bone density loss through enhancing bone resorption (Fakruddin and Laurence, 2004). It is therefore possible that bone density loss during HIV-1 infection, and after long-term use of MPA are mediated by similar mechanisms, namely through the GR. Vpr is thus highly relevant to the choice of contraception, and NET-A could prove favourable to be used in place of MPA where Vpr exposure is concerned. NET-A would exert less GC like effects than MPA, and thus Vpr would most likely not dramatically enhance GC like side effects of this contraceptive.

4.5. Vpr effects resemble those of a weak, non-classical GR activator

It was not hypothesised that Vpr would mediate an effect on the GR in the absence of ligands (Table 4.1). Rather it was thought that Vpr would only enhance ligand-dependent stimulation of the GR. However, statistically significant increases in the basal level of GR-mediated reporter gene activity in the presence of Vpr were observed twice in this present study (Figures 3.1A and 3.2E). This is a novel finding and was not reported in previous publications which investigated the effects of Vpr on GR-mediated reporter gene transactivation (Kino *et al.*, 1999; Kino *et al.*, 2002a; Sherman *et al.*, 2000). However it is possible that these authors may not have noticed these effects which may have been masked by their method of normalisation.

It was understood that Vpr increased TAT-GRE transactivation through the GR. If Vpr were acting independently of the GR altogether, then the additional measure of reporter gene activity induced by Vpr would be equal under all conditions and concentrations, including the absence of ligand (Figure 4.1A). This was not observed as it was found that the Vpr-mediated increases in reporter gene activity were larger at higher DEX concentrations (Figure 3.2A) or with more efficacious GR agonists (Figure 3.1D). As DEX is a specific GR agonist, this refuted the possibility that Vpr increased reporter gene activity independently of the GR. A negative control is present in Appendix 5.1 indicating that COS1 cells not transfected with the GR expression vector show a negligible level of reporter gene activity, and are not responsive to DEX stimulation. Ideally, this negative control should have also been included for all of the tested ligands from this study.

In addition to increased reporter gene transactivation, results presented in this study suggest that Vpr increased GR turnover in a manner that was both independent of which ligand was used for GR stimulation, and occurred in the absence of ligand (Figure 3.3B). Under stimulation with vehicle alone, S226 phosphorylation (Figure 3.4F) and rGR nuclear localisation (Figure 3.6A IV), both appeared to also be enhanced by the presence of Vpr. These observations are consistent with Vpr itself

acting as a GR ligand, although statistical significance of these changes in the individual experiments was not established. This does not however, discount the possibility that Vpr does have a significant effect on the unliganded GR, but rather only indicates that the experimental design used in this current study was unable to detect any possible effects conferred by Vpr as they may have been within the range of experimental error. Reducing the number of variables from 12, and increasing the number of biological repeats should effectively resolve this issue. It would be highly co-incidental that several different assays, each of which was independently performed, would all suggest ligand-independent GR activation mediated by Vpr. It is possible that Vpr activates the GR in a manner analogous to GnRH (Kotitschke *et al.,* 2009) or TNF-α (Verhoog *et al.*, 2011). The mechanism by which GnRH and TNF-α activate the GR has not been fully established, but is not thought to involve direct binding to the GR. Results thus far suggest that activation is via indirect effects of GnRH or TNF-α on kinases via their respective receptors, which then modulate GR phosphorylation and nuclear translocation. Another possibility is that Vpr may create an intracellular environment more conducive to GR activation and GRE transactivation, as has been reported for the LTR, where the cell-cycle arresting capabilities of Vpr cause increased GR-mediated transactivation (Gummuluru and Emerman, 1999), although such an effect may have been expected to also be evident for transactivation of the endogenous genes.

Taken together a putative model may be proposed to explain increases in unliganded GR activity in the presence of Vpr. Given that results in the literature suggest that Vpr associates with the GR, this suggests that a mechanism of GC ligand-independent activation by Vpr is different to that of GnRH and TNF-α. In the absence of ligand, nuclear Vpr may associate with the GR (Sherman *et al.*, 2000; Thotala *et al.*, 2004) as it shuttles between the cytoplasm and nucleus (Hache *et al.*, 1999; Madan and DeFranco, 1993; Savory *et al.*, 1999), leading to increased GR nuclear retention. Owing to the capability of Vpr to interact with multiple proteins (Kino and Pavlakis, 2004) and homo-oligomerise (Schuler *et al.*, 1999; Zhao *et al.*, 1994b), the GR may associate with multiple Vpr molecules, which in turn associate with GR co-activators, including p300 (Felzien *et al.*, 1998; Kino *et al.*, 2002a; Wang *et al.*, 1995). As Vpr displays an affinity for nucleic acids (Kichler *et al.*, 2000; Zhang *et al.*, 1998), this may cause increased association of the GR/Vpr/co-activator

complex with any accessible promoters and marginally increase transcription rates (Figure 4.2). It is possible that the GR/DNA association is weak owing to the absence of ligand, and that GR binding to DNA would have only occurred at highly accessible promoters, such as those not in a condensed chromatin formation. The absence of native chromatin on the reporter gene promoter fits well with this hypothesis, as it could explain why Vpr mediated an effect for unliganded GR-mediated transactivation of the promoter-reporter, but not for either of the tested endogenous genes.

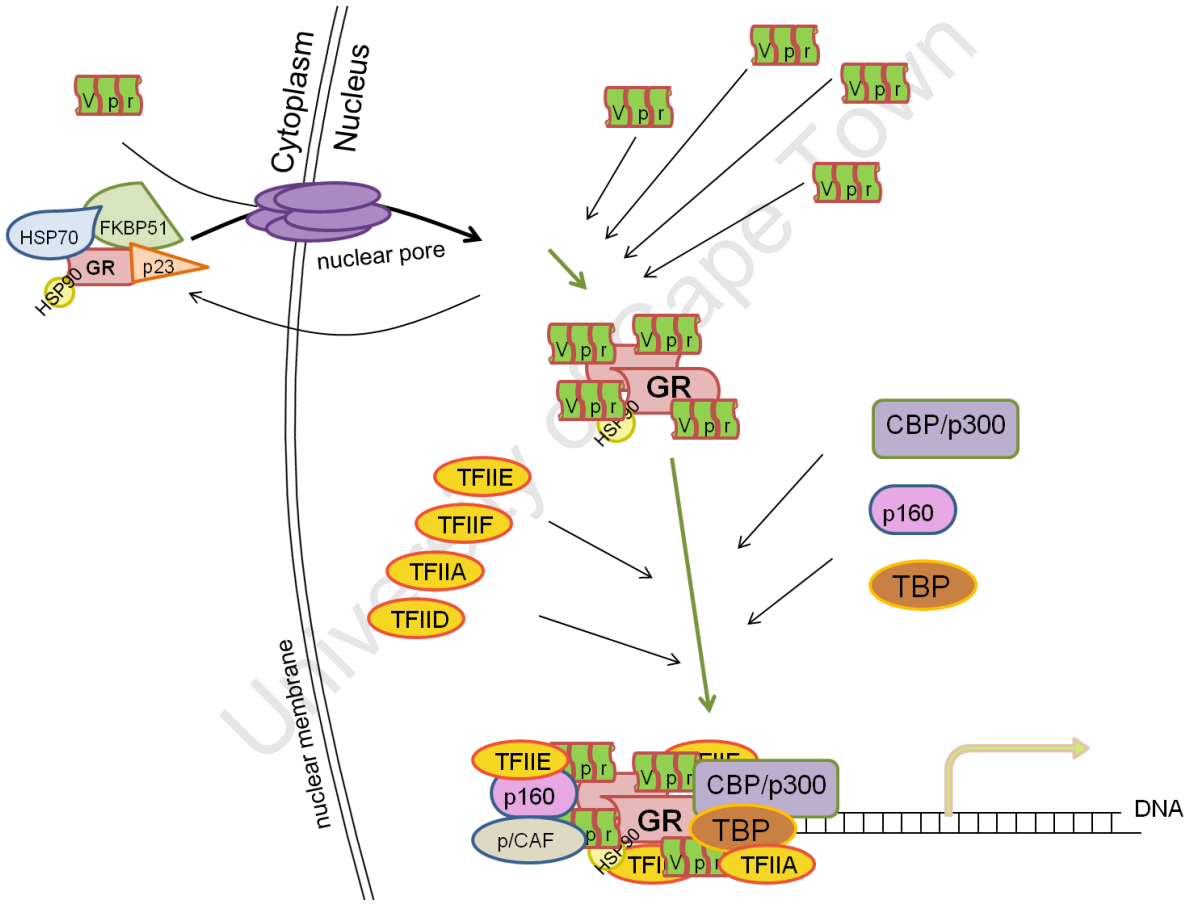

Figure 4.2: A putative model indicating how Vpr may increase GR-mediated transcription in the absence of ligand. The unliganded GR (shown here in pink) shuttling between the nucleus and cytoplasm associates with newly translated Vpr in the cytoplasm or already in the nucleus, increasing GR nuclear retention. Owing to its propensity to interact with numerous proteins and DNA, Vpr non-specifically enhances transcription co-factor binding to the GR, and GR association with DNA, leading to a small level of transactivation from some accessible GRE containing genes.

4.6. Conclusions

Vpr increases the efficacy of all tested ligands for GR-mediated transactivation of the TAT-GRE reporter gene, without affecting the potency of DEX, whereas transcription of the endogenous genes MKP-1 and IκBα was not affected by the presence of Vpr. The extent by which Vpr increases reporter gene activity is dependent on the concentration of DEX and is greater in the presence of a higher amount of Vpr. In the absence of ligand Vpr also marginally increases reporter gene activity. The ligand-dependent effects of Vpr on GR-mediated transcription are likely either to be promoter specific or dependent on the presence and/or architecture of chromatin at the promoter. Additionally, the relative concentrations of promoter-promoter genes, GR molecules, Vpr molecules, and other endogenous co-regulators may determine ligand-dependent Vpr effects. The mechanism by which Vpr increases ligand-dependent GR-mediated transactivation was not fully determined, although a mechanism similar to that of the classical co-activator SRC-2 is a likely possibility, and several other possible mechanisms were excluded. Vpr does not stabilise the GR against ligand-dependent degradation, but rather increases GR turnover in the presence of several ligands, possibly indicative of a more transcriptionally "capable" GR that is responsible for enhanced reporter gene activity, as opposed to simply higher GR levels. Vpr does not detectably increase GR phosphorylation or nuclear localisation in the presence of ligand, although experimental design may have been unable to reveal such differences. Results did show that Vpr is constitutively nuclear, and like the rGR, was not found at areas of heterochromatin. In the absence of ligand, Vpr significantly increased reporter gene transactivation in some experiments, and appeared to enhance GR turnover, S226 phosphorylation, and rGR nuclear translocation, indicating Vpr may act as a weak non-classical GR partial agonist.

The enhanced ligand-dependent reporter gene efficacy mediated by Vpr raises the possibility that Vpr may increase the absolute level of GR signalling with therapeutic administration of steroids which have high GR efficacies. Vpr was observed to change the biocharacter of P4 relative to DEX for transcription of both tested endogenous genes, but in general did not mediate large changes to the partial

agonist activity of the tested steroids. Vpr did however; increase GR-mediated reporter gene activity in a manner that was dependent on both ligand efficacy and concentration. MPA is a steroid, where if exposure to HIV-1 and thus Vpr is likely, use should be treated with caution. MPA may lead to excessive GR activity if Vpr is present, more so than would NET-A, which has very little GR transactivation efficacy. Excessive GR signalling will have numerous endocrine effects, not least of which will be immunosuppression, a condition already associated with AIDS.

4.7. Future perspectives

Much of the data from this study supports the novel finding that Vpr acts as a non-steroidal GR activator. However, due to the large number of variables as well as the relatively small effects compared to the technical and biological error in the experiments, statistical significance was not always obtained. Thus future experiments should include some repeat experiments from this present study, but with fewer variables and a greater n-value, and using a cell line which expresses endogenous GR, such as END cells to confirm this finding. Thereafter, future studies building on the work presented in this thesis, should focus on investigating in more depth the mechanisms by which Vpr increases ligand-dependent and ligand-independent GR-mediated transactivation. These are presented below as questions and the proposed approach.

1) Does phosphorylation of GR serine residues 203, 211, and/or 226 determine whether Vpr can enhance ligand-dependent GR-mediated transactivation? The current study showed that Vpr did not enhance ligand-dependent GR phosphorylation at these three serine residues, but did not determine if phosphorylation of one or more of these residues is required for Vpr to associate with, and thus enhance transactivation via the GR, as has been reported for SRC-2 (Avenant *et al.*, 2010a; Wang *et al.*, 2007). Using GR expression constructs harbouring the point mutations S203A, S211A, and S226A will prevent phosphorylation of that particular residue. If Vpr is unable to mediate an increase in reporter gene activity with a mutant GR this would support the hypothesis that

phosphorylation of the particular residue is required for Vpr to enhance GR-mediated transactivation. Such an effect could be further confirmed by failure of Vpr to co-immunoprecipitate with such a GR mutant. GR mutants should be used as single, double or triple mutants to allow the specific location and number of mutations which prevent the effects of Vpr on the GR to be established. These studies would be required to be performed in COS1 cells owing to the very low level of endogenous GR, which would otherwise confound results, but should use Vpr peptides or purified protein to allow exact control over the amount of Vpr added. The represents a physiologically relevant setting as Vpr protein has the ability to occur unassociated in serum and passively transverse the cell membrane (Tungaturthi *et al.*, 2003).

2) Does Vpr increase GR S134 and/or S404 phosphorylation in the absence of GR ligand? Phosphorylation of GR residue S404 regulates the CBP/p300 interaction with the GR (Galliher-Beckley *et al.*, 2008), a co-factor which has been previously reported to directly associate with Vpr (Kino *et al.*, 2002a), and S134 is phosphorylated ligand-independently (Galliher-Beckley *et al.*, 2011). The phospho-status of these residues should be investigated by Western blotting with phosphorylation specific antibodies to determine if S134 and/or S404 phosphorylation is enhanced by Vpr. In order to gain better control over the levels of the GR and Vpr, this experiment should be performed in the physiologically relevant END cell line which express endogenous GR, and with the addition of Vpr peptide or purified protein as opposed to transient transfection of Vpr expression vector.

3) Does the sequence of the GRE dictate whether Vpr will enhance GR-mediated transactivation of an endogenous gene, in the absence and presence of GR ligands? It appears that the TGT TCT GRE half-site (Table 4.2) is common to genes on which Vpr mediates an effect. Using END cells with endogenous GR, and adding Vpr peptide or purified protein in the presence and absence of DEX followed by microarray analysis, will allow identification of any endogenous genes co-regulated by both the GR and Vpr. Microarray analysis has the capacity to analyse thousands of different genes for changes in expression at one time. After GR-regulated genes on which Vpr has an effect have been identified, the promoters of these genes can be analysed and the GRE sequence elucidated. This assay will have the advantage of identifying novel GR-regulated genes which Vpr may regulate

in a manner similar to a co-activator. To confirm the effects of Vpr occur through the endogenous GR, siRNA knockdown can also be used to inhibit GR expression, and thus observe if changes in gene expression induced by Vpr is concomitantly lost when GR levels are decreased, thereby indicating Vpr to act via the GR.

4) Are the effects of Vpr on GR-mediated transactivation cell-type specific in the absence and presence of GR ligands? To determine if the effects of Vpr are variable across cell type, the effects of Vpr on GR-mediated transactivation should be compared in different cells for transactivation of the same gene. This will require a gene, at which Vpr enhances GR-mediated transactivation, such as endogenous RANKL or the TAT-GRE reporter gene. Using cell types with endogenous GR will present variances in GR levels that cannot be controlled for, but will present a more physiological setting in which to study the effects of Vpr on gene transcription. END and Jurkat cells should also be used owing to their human origin, physiological significance for HIV-1 actions, and expression of endogenous GR. Both represent cell types in which Vpr may be expected to be found after HIV-1 infection. To control for possible variances in Vpr levels that may be caused by transfection into different cell types, the cells should be treated with equal amounts of Vpr peptide or purified protein. The relative extents of RANKL and TAT-GRE expression could then be analysed (by qPCR or luciferase quantification respectively) in the absence and presence of DEX, and absence and presence of Vpr to determine if the effects of Vpr on GR-mediated transactivation are cell-type specific.

5) Does Vpr associate directly with the GR *in vivo*? GR and Vpr co-immunoprecipitation experiments have been done before, but do not indicate whether the interaction occurs *in vivo*, or if it is a direct interaction. A Fluorescence Resonance Energy Transfer (FRET) assay could be performed to determine if these two proteins interact directly. For matters of consistency, it would be best to use END cells with endogenous GR and exogenously added and tagged Vpr protein to allow the greatest measure of control in the relative levels of these proteins. Alternatively tagged Vpr could be delivered using HIV-1 pseudovirus or engineered HIV-1 infectious molecular clones. FRET analysis uses photo-excitation to excite an electron on a fluorophore tag, which, after falling back to a ground state releases a photon that may excite a second, adjacent fluorophore tag which is then detected.

As this energy transfer can only occur over very small distances, detection of the second fluorophore indicates that the two tagged proteins must be in direct contact with each other. This assay also has the advantage of being able to show both an *in vivo* association and the sub-cellular location where such an interaction occurs. If the GR and Vpr are found to associate directly, follow up experiments should determine if the interaction is ligand-independent or ligand-dependent. If the former, this would support the finding that Vpr acts like a GR agonist. If ligands with greater efficacy cause a greater extent of Vpr recruitment to the GR and if antagonists reverse Vpr association with the GR, this would support the hypothesis of Vpr being a GR-co-activator.

6) Does Vpr change components of the complexes at the promoter or alter chromatin structure in the absence and presence of GR ligands? To further investigate such an association, the co-localisation of Vpr and the GR at an endogenous promoter should also be investigated. For consistency with the FRET analysis, END cells with endogenous GR and a similar method of delivery of Vpr should be used. Vpr is only known to enhance GR mediated transactivation of one endogenous gene, RANKL (Fakruddin and Laurence, 2005). Using chromatin-immunoprecipitation analysis (ChIP), analysis of this gene to isolate promoter bound GR molecules will reveal if Vpr is present in the promoter bound GR complex. ChIP-on-ChIP analysis will also confirm that these two proteins are present in the same promoter bound complex. As with FRET analysis above, treatment of cells with DEX or RU486 may reveal if the association of Vpr with the GR is ligand-dependent and similar to how SRC-2 associates with the GR. The components of GR-promoter bound complexes may be compared in the absence and presence of Vpr to determine what additional proteins Vpr recruits to the promoter. Vpr may increase the relative abundances of the GR, GR associated TF's, recruit novel co-factors and chromatin-modifying proteins to the promoter so as to enhance gene expression. By performing ChIP analysis with antibodies specific to modified (specifically acetylated or methylated) histones, some insight will be obtained as to whether Vpr is responsible for altered chromatin structure at the promoter. DNase I hypersensitive site mapping could also prove another strategy to investigate whether Vpr alters chromatin structure, making promoters more or less accessible.

7) Does Vpr only enhance GR-mediated transactivation in the absence of chromatin? This may be determined by comparing the effect of Vpr on the extent of GR-induced transcription of the endogenous TAT gene with that of the same promoter-reporter plasmid which is not organised into native chromatin, in the same cells. If Vpr only increases GR-mediated reporter gene activity, but not transactivation of the endogenous TAT gene, this would be consistent with the hypothesis that chromatin inhibits the effects of Vpr on the GR, although other explanations would be possible. This hypothesis may be tested further through DNase I hypersensitive site mapping on endogenous genes where Vpr is known to enhance GR-mediated transactivation, such as RANKL, and comparing this to a similar GRE containing gene, where Vpr is known not to affect GR-mediated transactivation, such as GILZ. Results should determine if hypersensitive chromatin is a prerequisite for Vpr to enhance GR-mediated gene expression. Ideally such experiments should be done in a cell line which expresses endogenous GR, such as END cells, using expressed or exogenously added Vpr protein.

8) Does Vpr increase the potency of MPA and NET-A via the GR? The effects of Vpr on a GR dose-response curve under MPA and NET-A stimulations should be investigated and compared. Whilst it is established that Vpr increases these ligands' efficacies under certain conditions, it is not known how Vpr may affect their potencies, and GR-mediated transactivation at sub-saturating concentrations. If Vpr were to increase these ligands' potencies, MPA would exert more GC like side effects at lower concentrations than expected when in the presence of Vpr. Accordingly, a GR dose response curve in a cell line which expressed endogenous GR, such as END cells should be done. For simplicity this experiment should be investigated on a reporter gene, and the GR stimulated with a range of MPA or NET-A concentrations to give an accurate value of the EC_{50}'s of MPA and NET-A in the absence and presence of Vpr.

9) Does Vpr increase MPA-mediated induction of any GR-regulated endogenous genes to a greater extent than it does for NET-A? One of the main findings of this study was that Vpr enhanced GR-mediated reporter gene activity to a greater extent with MPA stimulation than it did with NET-A and P4 stimulation. The identification of an endogenous gene regulated in a similar manner would have

profound clinical significance for HIV-1 infection and the choice of contraception. Using cell lines with endogenous GR which resemble HIV-1 target cells, and cells likely to encounter Vpr during HIV-1 infection such as Jurkat and END cells, a GR-regulated target gene on which Vpr enhances transcription should be identified. This should be done through a microarray experiment as discussed above. Upon identification of a suitable endogenous gene, GR siRNA knockdown analysis should be used to prove the effects of MPA or NET-A on gene expression are mediated by the actions of the GR, by investigating transcription rates (as determined by qPCR) in the presence of lower amounts of GR. Thereafter, the relative extents of MPA and NET-A stimulation of GR-mediated transcription of the endogenous gene should be compared in the presence of Vpr to determine if Vpr potentiates MPA driven transcription of the gene to a greater extent than it does for NET-A. As such a finding would have profound clinical significance, the experiment should be repeated in primary cells and tissue extracts with endogenous GR with different methods of Vpr delivery, including using virus, as well as physiological relevant concentrations of MPA and NET-A, so as to resemble as closely as possible a physiological setting.

5. APPENDICES

5.1. Reporter gene control

Transactivation of the TAT-GRE reporter gene requires the presence of the hGRα. A representative Western blot indicates that COS1 cells do not express detectable levels of the GR (Figure 5.1A). Cells not transfected with the hGRα do not show an increase in reporter gene activity with DEX stimulation and transfection of the hGRα causes a roughly 100-fold increase in reporter gene activity when DEX is not present and near 2 500 fold induction in the presence of DEX (Figure 5.1B).

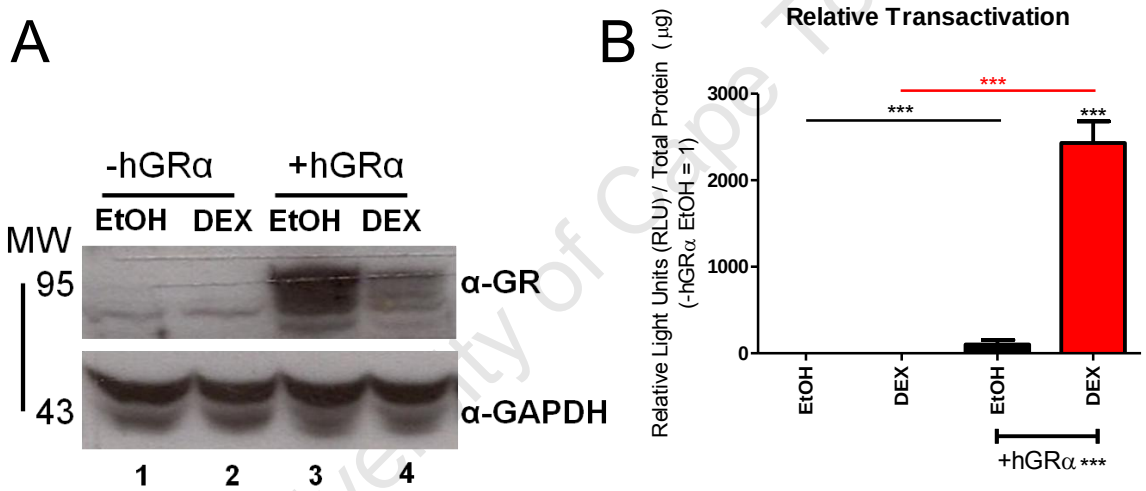

Figure 5.1: COS1 cells do not express a detectable level of hGRα (A) and transfection of the hGRα construct is required for observable reporter gene transactivation (B). COS1 cells were transfected in 10 cm dishes with TAT-GRE, and either hGRα or empty vector 24 hours prior to re-plating in 24 well plates. After a further 24 hour incubation, cells were stimulated with 100 nM DEX for 24 hours. Cells were harvested in a 2x SDS sample buffer and resolved on 10% PAGE before transfer to nitrocellulose membrane (A) or harvested in reporter lysis buffer before luciferase quantification in a Luminex luminometer with the Promega luciferase assay and normalised to total protein as determined by Bradford protein assay. Data was plotted as a fold induction above the unstimulated condition in the absence of hGRα (B). The histogram shows pooled data from 3 individual biological repeats each performed in triplicate. A two-way ANOVA and Fishers least significant differences post-hoc test was used for statistical analysis and marked *, ** or *** to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively with significance shown relative to the corresponding vehicle control.

5.2. Immunofluorescence controls

5.2.1. Transfection negative control

Cells were not transfected, but were incubated with primary antibodies, fluorophores, and Hoechst stain (Figure 5.2A). The cytoplasm exhibits undetectable staining with Hoechst (DNA) stain, revealing that cells are not contaminated with *Mycoplasma spp.*, and the cytoplasmic DNA observed previously is likely transfected plasmid DNA. The Alexa488 stain exhibits low intensity background without HA Vpr transfection and does not preferentially occur in the cytoplasm or nucleus. Without rGR transfection, red nuclear spots are visible indicating this not to be an effect of the presence of rGR protein, but rather due to non-specific binding of the BuGR22 antibody to an endogenous nuclear COS1 protein. Cells were not stimulated with DEX making it highly unlikely that this is endogenous GR, as the signal is nuclear and endogenous GR would be cytoplasmic if not stimulated.

5.2.2. Primary antibody negative control

Cells were transfected with both the rGR and HA Vpr expression vectors but were not incubated with the α-mouse GR or α-HA antibodies. Thereafter slides were stained with the secondary fluorophores (Figure 5.2B). This control was performed as it determined the extent of background signal owing to the fluorophores, thereby allowing a threshold to be set above which any fluorescent intensity was deemed to be specific. This control indicates that the fluorescence observed in Figure 3.6 is a result of the fluorophores specifically adhering to the primary antibodies. The absence of red and green stain indicates that the primary antibodies are required in order for fluorescence to occur. The red spots appear to have been caused by the α-mouse GR BuGR22 antibody adhering to non-specific nuclear proteins in the COS1 cells, as the red nuclear spots observed in Figure 5.2A are absent in this control which lack the primary antibody. Hoechst staining does detect cytoplasmic DNA in these cells, as they had been transfected with both the rGR and HA Vpr expression vectors.

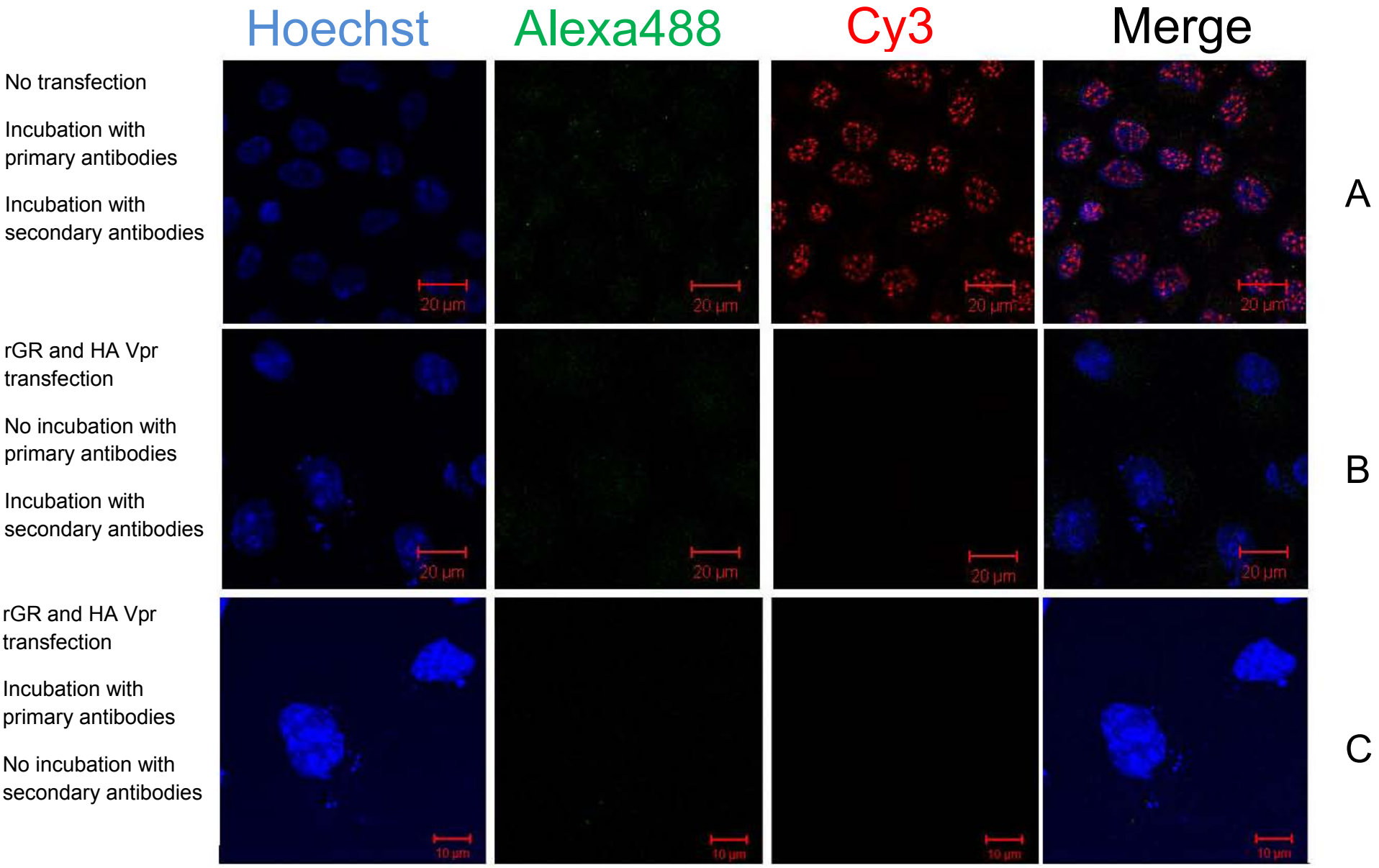

Figure 5.2 (A-C): Immunofluorescence controls. (A) Transfection negative control (B) Primary antibody negative control (C) Fluorophore negative control.

5.2.3. Fluorophore negative control

Cells were transfected with both the rGR and HA Vpr, followed by incubation with primary antibodies for an hour (Figure 5.2C). The fluorophore conjugated secondary antibodies Cy3 and Alexa488 were not used to stain these slides and accordingly no fluorescence is observed in these cells. This control indicates that the fluorescence observed in Figure 3.6 is a specific result of the fluorophore conjugated secondary antibodies and not an alternate source of fluorescence.

5.3. Representative qPCR C_t values, melt curves, and agarose gel analysis

In order to optimise primer concentrations for qPCR, a standard PCR was first performed on cDNA from COS1 and END cells using the GoTaq-buffer and -Flexi DNA polymerase kit according to the manufacturer's instructions (Promega Corp., USA). Once effective primer and $MgCl_2$ concentrations had been optimised, qPCR was performed. It was however found that IκBα primers could not anneal at any tested concentration of $MgCl_2$ to COS1 cDNA, as assessed by the lack of a detectable conventional PCR product. Vpr was not observed to increase the abundance of either MKP-1 or IκBα mRNA transcripts relative to basal and no primer standard curve was performed. Without a standard curve, a primer efficiency of 2 is assumed. The correct primer efficiency allows an accurate determination of absolute RNA levels. If the primer efficiency for either of the tested genes were different to 2, this would not have affected the relative expression levels, and as such, it was deemed unnecessary to perform a PCR standard curve.

As all PCR products were found to be of the expected size (Figures 5.3A and 5.4A and Table 5.3), and displayed uniform melt curve analyses (Figure 5.3B and 5.4B) it was determined that the assay was valid and that the Ct values represented values corresponding to the original amount of cDNA present.

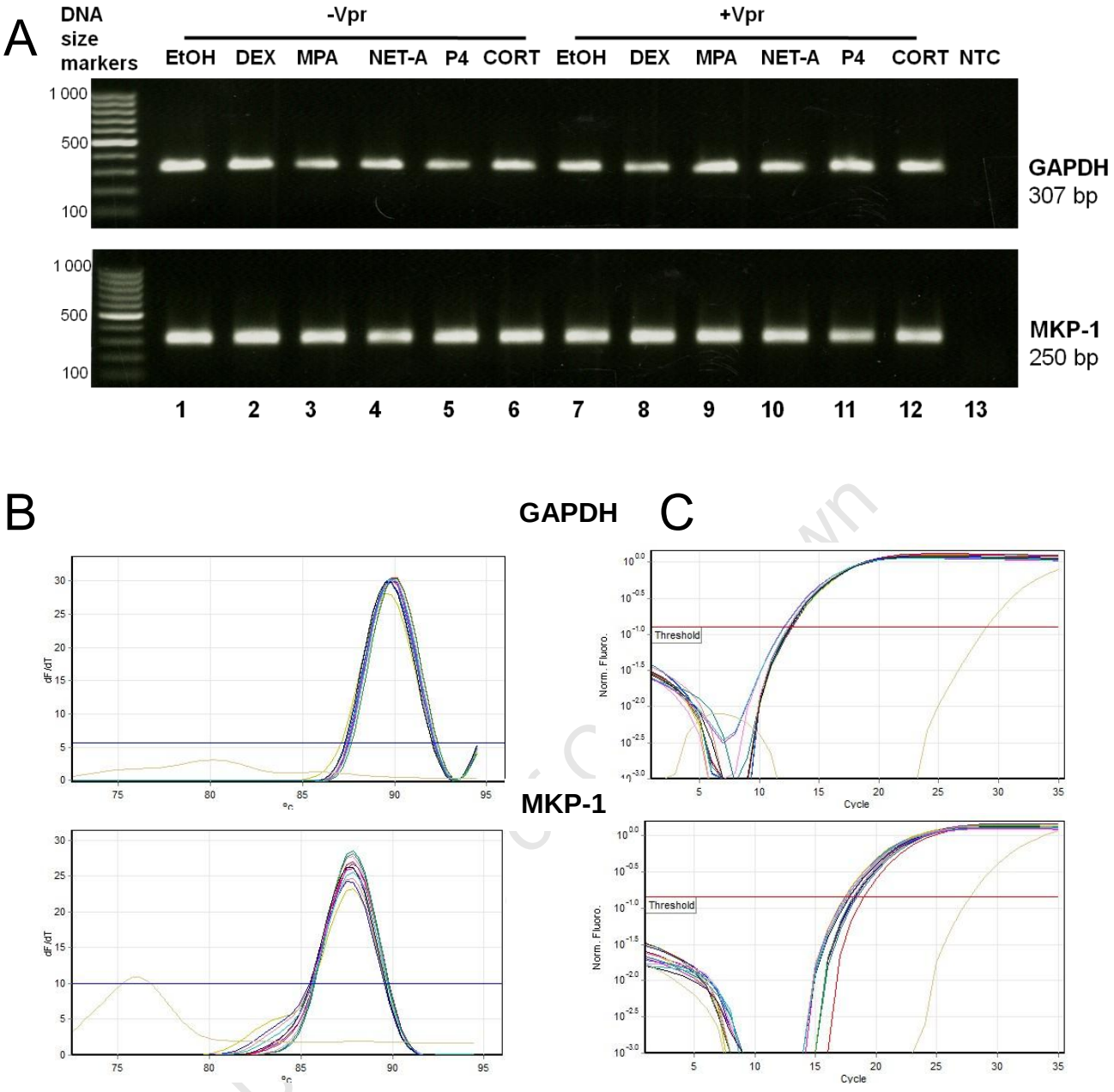

Figure 5.3: (A) Representative agarose gel analysis, (B) melt curve analysis, and (C) Ct threshold value of qPCR amplified GAPDH and MKP-1 cDNA in COS1 cells.

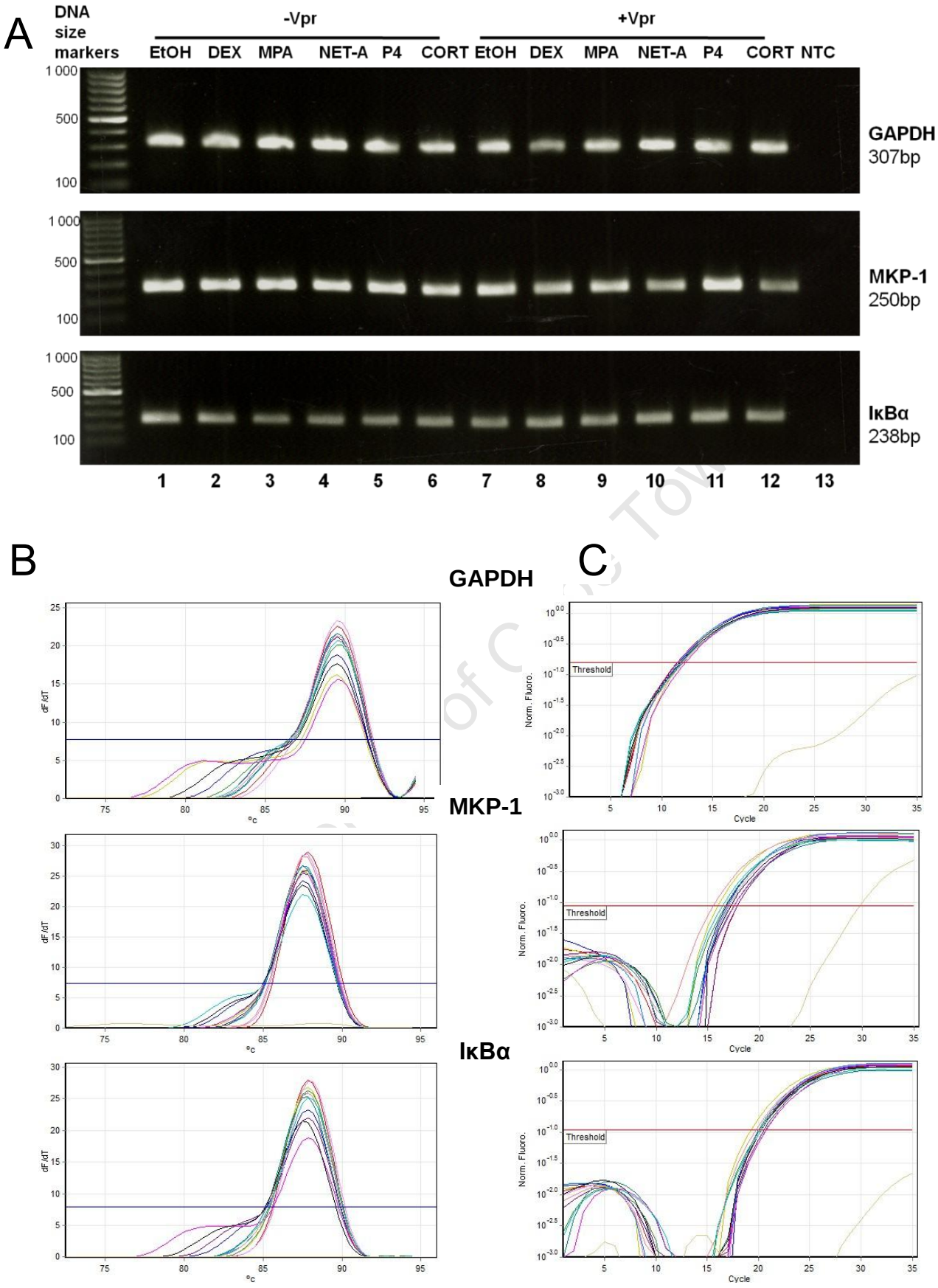

Figure 5.3: (A) Representative agarose gel analysis, (B) melt curve analysis, and (C) Ct threshold value of qPCR amplified GAPDH, MKP-1, and IκBα cDNA in END cells.

Table 5.1: Result showing qPCR Ct values of GAPDH and MKP-1 in COS1 cells from a single representative experiment.

COS1	Name	GAPDH Ct	MKP-1 Ct
	EtOH	12.75	18.98
	DEX	12.71	17.36
	MPA	12.53	18.20
	NET-A	12.67	18.30
	P4	12.09	17.54
	CORT	12.49	17.42
	Vpr/EtOH	12.69	18.43
	Vpr/DEX	12.41	17.44
	Vpr/MPA	12.56	18.10
	Vpr/NET-A	12.07	17.82
	Vpr/P4	12.57	17.77
	Vpr/CORT	12.11	17.61
	NTC	29.00	27.70

Table 5.2: Result showing qPCR Ct values of GAPDH, MKP-1, and IκBα in END cells from a single representative experiment.

END	Name	GAPDH Ct	MKP-1 Ct	IκBα Ct
	EtOH	11.49	17.87	20.28
	DEX	11.90	15.98	19.28
	MPA	11.67	16.80	20.13
	NET-A	11.52	17.87	20.55
	P4	11.61	17.07	20.13
	CORT	11.66	16.94	19.99
	Vpr/EtOH	12.03	17.54	20.73
	Vpr/DEX	12.12	15.53	19.72
	Vpr/MPA	11.87	16.69	20.09
	Vpr/NET-A	12.28	17.57	20.76
	Vpr/P4	11.81	17.24	20.27
	Vpr/CORT	11.93	16.41	20.28
	NTC	n/a	29.79	n/a

Table 5.3: Quantitative PCR primers

Primer	Sequence (5'-3')	Theoretical Anneling Temp (°C)	Final Conc. (nM)	Length (bp)	GC %	Product (bp)	Reference
GAPDH FWD	TGA ACG GGA AGC TCA CTG G	56	100	19	58	307	Ishibashi *et al*., 2003
GAPDH REV	TCC ACC ACC CTG TTG CTG TA		100	20	55		
IκBα FWD	ACT CGT TCC TGC ACT TGG CC	60	250	20	60	238	Emmerich *et al*., 1999
IκBα REV	TGC TCA CAG GCA AGG TGT AG		250	20	55		
MKP-1 FWD	AGT ACC CCA CTC TAC GAT CAG G	56	250	22	55	250	Rauhala *et al*., 2005
MKP-1 REV	TGA TGG AGT CTA TGA AGT CAA TGG		250	24	42		

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