Author:
Paul, Susana N

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Mineralocorticoid and Glucocorticoid Receptor Interactive Complexes and their Cellular Localisation

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Mineralocorticoid and Glucocorticoid Receptor Interactive Complexes and their Cellular Localisation

By

Susana N Paul-Stols

Bristol Medical School

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Supervisors: Dr. Caroline Rivers & Prof. Stafford Lightman

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Abstract

Adrenal glucocorticoids (GCs) released into circulation exhibit circadian and ultradian profiles, which are important in regulating homeostasis and physiological pathways. GCs induce gene expression changes in targeted cells via ligand activated transcription factors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which produce both physiological and adaptive responses to stress. MR and GR are highly abundant in the hippocampus, an area vital for learning and memory, and effective responses to stressful events involve modification of cognitive processes in the brain to improve coping mechanisms to future stressors. MR and GR regulate transcription of GC targets, often as homodimers, but the existence and significance of MR/GR heteromers in gene regulation remains unclear.

Using a proximity ligation assay (PLA) for the first time, MR and GR have been shown to be in very close proximity and are consequently likely to be interacting together. MR:GR complexes were detected both in cultured cells (3617 and Neuro-2A) transfected with MR/GR expression vectors and in rat hippocampal tissue where endogenous MR and GR are co-expressed. Nuclear MR:GR complexes were localised to heterochromatic regions, in proximity to the nuclear lamina. The interaction appears to require neither the DNA binding domain, nor the dimerization interface, indicating another mode of MR:GR interaction. In the absence of ligand, MR:GR complexes were unexpectedly detected in the cytoplasm, predominantly at the nuclear envelope. Using RT-qPCR, the transcriptional consequences of MR/GR alone or together with 'physiological' levels of CORT suggested cooperative regulation of some GC target genes.

An optimised protocol for *in situ* PLA and the modular image analysis (MIA) pipeline are presented, for the detection and analysis of MR:GR complexes in Neuro-2A cells and hippocampal tissue, which are applicable to other receptor-receptor interaction studies in order to reveal dynamic spatio-temporal protein-protein interactions.

Dedication and Acknowledgements

I offer my gratitude and appreciation to my supervisors, Caroline Rivers and Stafford Lightman, for the deft ways in which you lovingly challenged and supported me throughout the lifetime of this work. The last four years have been an incredible and challenging experience – the opportunity has been invaluable, and I cannot imagine this journey without you both. Thank you to Gavin Welsh and Stephen Lolait, for your advice and support during my progress reviews and anytime that I have needed it. I could always knock on your door for a chat.

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I am grateful to my sisters Victoria and Connie; for our hilarious silliness together and the bond that we share, you are my best friends. Thank you to my mother Maria, for the love and support, encouragement and provision of various delicious foods – essential in PhD survival. I am also grateful to my other family members and friends who have supported me along the way.

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To my beautiful boys Ricardo and Rubén who are my most treasured projects to date. This thesis is for the both of you, but I realize that you are probably too young to understand (or want to read) pages filled with neuroscience stuff and probably by the time you are older, you will comment on how ancient the printing and binding is, and most likely the content. But some day you might read it and

appreciate that mummy pursued and persevered in her passions, and that despite inevitable challenges, self-belief and hard work can make dreams come true.

I would like to also dedicate this thesis to my dad, who cultured my science interests from the time I was able to sit still long enough to listen. I would much prefer it if you were still alive to tell me – but I do know that this would have made you very proud. Thank you and I miss you every day.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Abbreviations

A640T	Dimerisation interface mutants
Ab	Antibody
ACTH	Adrenocorticotropic Hormone
ACTR	Activator of retinoid and thyroid receptors
AF	Regulatory Region
AF1	N-terminal Transactivation Region
AF2	C-terminal Transactivation Region
AIB1	Amplified in breast cancer 1
AKT	Activation Function-1
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AP1	Activator Protein-1
AR	Androgen Receptor
ATP	Adenosine Triphosphate
AVP	Arginine Vaospressin
BBB	Blood Brain Barrier
BDNF	Brain-derived neurotrophic factor
BMAL	Brain and Muscle ARNT-like 1
BNST	Bed Nucleus of the Stria Terminalis
CBP	CREB Binding Protein
CBG	Corticosterone Binding Protein
CDK1	Cyclin Dependant kinase 1
CDK8	Cyclin Dependant kinase 8
CDK19	Cyclin Dependant kinase 19

ChIP	Chromatin Immunoprecipitation
CIP	CDK interacting protein
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	Central Nervous System
CORT	Corticosterone or cortisol
C2B	C-terminal C2B domain
CRH	Corticotropin Releasing Hormone
CRF	Corticotropin Releasing Factor
CSS	Charcoal Stripped Serum
DBD	DNA Binding Domain
DBS	DNA Binding Sequence
Ddc	Dopa Decarboxylase
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EM	Electron Microscopy
EGR1	Early growth response protein 1
ER	Estrogen Receptor
EtOH	Ethanol
FBS	Fetal Bovine Serum
FKBP5	FK506 binding protein 5
FKBP	FK506 binding protein (number)
FOX	Forkhead Transcription Factor
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer

GBS	GR Binding Sequence
GC	Glucocorticoids
GRE	Glucocorticoid Response Elements
GRE	Glucocorticoid Response Element
GR	Glucocorticoid Receptor
GRIP1	Glutamate Receptor Interacting Protein 1
HAT	Histone Acetyltransferase
HDAC2	Histone Deacetylase 2
hGR	human Glucocorticoid Receptor
HSP -	Heat Shock Protein (number)
HPA axis	Hypothalamic Pituitary Adrenal Axis
HR	Hinge Region
HRE	Hormone Response Elements
IF	Immunofluorescence
IGF1	Insulin like Growth Factor 1
IHC	Immunohistochemistry
INM	Inner Nuclear Membrane
I.P.	Intraperitoneal
IR-GBS	Inverted Repeat- GR Binding Sequence
JNK	c-Jun N terminal kinase
LAD	Lamina Associated Domain
LBD	Ligand Binding Domain
MED1	Mediator Complex Subunit 1
MED14	Mediator Complex Subunit 14
MG132	Proteasome Inhibitor

MIA	Modular Image Analysis
Min	Minute
MR	Mineralocorticoid Receptor
MR:GR	Interacting complex with MR and GR
mRNA	messenger ribonucleic acid
MTC	Multi-protein transcriptional complex
NAD	Nucleoli Associated Domain
NCOA1	Nuclear Receptor Coactivator 1
NCOA2	Nuclear Receptor Coactivator 2
NCOA3	Nuclear Receptor Coactivator 3
NE	Nuclear Envelope
NF1	Nuclear Factor 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
nGRE	Negative Glucocorticoid Response Element
NL	Nuclear Lamina
NLS	Nuclear Localisation Signal
NMDA	N-Methyl-d-aspartate
NPC	Nuclear Pore Complex
NTD	N-terminal Domain
ONM	Outer Nuclear Membrane
PAX5	Paired box protein Pax-5
PCAF/KAT2B	P300/CBP-associated factor/ K(lysine) acetyltransferase 2B
Per1	Period Circadian Regulator 1
PFA	Paraformaldehyde
PFC	Pre-frontal Cortex

PLA	Proximity Ligation Assay
PPI	Protein-protein Interaction
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone Receptor
PRMT1	Protein Arginine Methyltransferase 1
PVN	Paraventricular Nucleus
RIME	Rapid Immunoprecipitation of Endogenous Proteins
RNAPII	Ribonucleic Acid Polymerase II
RT-qPCR	quantitative Polymerase Chain Reaction
RU486	Mifepristone
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SGK1	Serum/Glucocorticoid Regulated Kinase 1
SGRMs	Selective Glucocorticoid Receptor Modulators
SP3	Sp3 transcription factor
SRC-2	steroid receptor coactivator- 2
STAT	Signal Transducer and Activator of Transcription
STAT3	Signal transducer and activator of transcription 3
SUMO	Small Ubiquitin-related modifier
SWI/SNF	Switch/sucrose non-fermentable
Syt2	Synaptotagmin 2
TAD	Topologically Associated Domains
TATA/TBP	Tata binding Protein
TET	Tetracycline
TF	Transcription factors

Thr	Threonine
TIF2	Transcriptional mediators/intermediary factor 2
TNFalpha	Tumour Necrosis factor alpha
TRAM1	Translocation Associated Membrane Protein 1
TSS	Transcription Start Site
VEH	Vehicle
VTA	Ventral Tegmental Area
Wt	Wild type
XDBD	DNA Binding Domain Mutant

1 Introduction

Glucocorticoids (GCs), are steroid hormones which regulate numerous biological processes and are critical in the adaptive response to stress. A relevant target organ is the brain, where GC effects, corticosterone (CORT) in the rat and cortisol in humans, are mediated by the high-affinity type I corticosteroid receptor (MR), encoded by the NR3C2 gene, and the lower affinity type II corticosteroid receptor (GR), encoded by the NR3C1 gene (Oitzl *et al.*, 2010).

Due to its high affinity, MR is maximally occupied at basal hormone levels during the circadian nadir and peak and greatly activated throughout the inter-pulse interval (Reul and de Kloet, 1985; Lightman *et al.*, 2008). Conversely, GR requires higher hormone levels and is only ligand-activated during the circadian peak or stress responses (Reul, van den Bosch and de Kloet, 1987). This results in underlying ultradian CORT pulses producing fluctuations in GR activity and DNA binding (Stavreva *et al.*, 2009; Lightman and Conway-Campbell, 2010) reflecting the rise and fall of CORT levels. Cortisol and corticosterone are the main ligands of brain MRs, and in addition distinct brain areas bind aldosterone to control physiology and behaviour related to salt balance. MR expressing neurons in the nucleus of the solitary tract, convert GCs into cortisone, its inactive state, via enzyme 11β-hydroxysteroid dehydrogenase type 2, permitting the access of aldosterone to MR (Geerling and Loewy, 2009; Gasparini *et al.*, 2019).

MR and GR form a dual receptor system with balancing actions regulating the effects of circulating GCs in context of individual receptor affinities and differential expression profiles that will determine the physiological outcomes. GR is located in most of the brain and cell types, whilst the localisation of MR is more restricted to forebrain and limbic brain regions, such as in the hippocampus, amygdala and the prefrontal cortex (Reul and de Kloet, 1985). MR and GR regulate various physiological pathways involved in acute and chronic responses to stress, such as neuronal differentiation (Fitzsimons *et al.*, 2013) and excitability (Joels, 2006), cognitive processes, memory formation and retrieval, and behavioural reactivity (E R de Kloet, Joëls and Holsboer, 2005). During the initial phase of an acute stress, MR activation is required for appraisal processes and memory retrieval and this is supported by activated GR promoted memory consolidation and learnt adaptive behaviours (E Ron de Kloet, Joëls and Holsboer, 2005). As well as their complementary actions, MR and GR produce converse effects, identified at the cell level, namely MR stimulated hippocampal CA1 neurone excitation that is suppressed by GR activation (Joels, 2006). These converse effects of the dual receptor system also extend to the immune system, whereby MR mediated pro-inflammatory pathways differ to GR regulated immune suppression (Chantong *et al.*, 2012), and in the

cardiovascular system (Oakley *et al.*, 2019). In these ways, the balance of MR and GR activation determines physiological outcomes.

In the brain, GC effects exerted via MR and GR are implicated in various psychopathologies. Commonly, GR overactivation is suggested to participate in the development of mood conditions. For example, patients with Cushing's disease, which causes excessive release of ACTH and thus excessive production of cortisol, often suffer with mood disorders such as anxiety, irritability and depression, as well as affecting concentration and memory. Treatment involves management of symptoms such as with GR antagonists (Nieman *et al.*, 1985; van der Lely *et al.*, 1991; Pereira, Tiemensma and Romijn, 2010). Opposingly, decreased MR activation is implicated in mood disorder development, whereby low MR expression was observed in psychiatric patients, such as depression and schizophrenia (ter Heegde, De Rijk and Vinkers, 2015). MR activity has been shown to be enhanced via MR gene variant haplotype 2 and linked to a decrease risk of depression in females. This further supports interactions with other steroid receptors, such as GR (M D Klok *et al.*, 2011) and even with other nuclear receptors. An MR agonist has been trialled as additional therapy for major depressive disorder (MDD) treatment and an enhanced anti-depressive effect was observed (Otte *et al.*, 2010). It is believed that increased MR activation in the brain may modulate excitability in the limbic regions and decreased MR activation may disrupt its tonic inhibitory effects of the hypothalamic pituitary adrenal axis (HPA axis), and as such leads to increased chronic GC levels (ter Heegde, De Rijk and Vinkers, 2015; de Kloet *et al.*, 2016).

The use of synthetic GCs has been implicated in mood disorder development (Fardet, Petersen and Nazareth, 2012; Judd *et al.*, 2014). For example, Dexamethasone (DEX), a highly selective synthetic GR agonist (Reul *et al.*, 2000), causes HPA axis suppression and a loss of endogenous cortisol production, therefore removing MR of its activating ligand. In addition, disturbed psychological symptoms and disrupted sleep patterns as a consequence of DEX treatment, were reversed with an add on cortisol treatment, re-establishing MR activation (Warris *et al.*, 2016). These observations further allude to the importance of balanced MR and GR activity (Meijer and de Kloet, 2017), and the potential effects of dysregulated MR/GR activation, and how this dual receptor system is important in mood disorders.

The differential intrinsic effects of MR and GR at the cell level requires further investigation, especially since they inherently share basic mechanistic function and have been identified to regulate the same target genes. For the purpose of this chapter, the structural and transcriptional mechanisms are discussed with respect to GR, though applicable to both receptors (MR and GR evolved from a common ancestral corticosteroid receptor gene and share basic nuclear receptor structure elements) and so the differences in ligand binding affinities between MR and GR are highlighted.

1.1 The Glucocorticoid Receptor

GR is a ligand induced transcription factor encoded by the nuclear receptor subfamily 3 group C member 1 (NR3C1) gene, which is located on chromosome 5 (5q31) and is closely related to its paralogue NR3C2, which encodes the mineralocorticoid receptor. GR is activated by its endogenous corticosteroid or by synthetic GCs such as dexamethasone, to modulate the up- or down-regulation of thousands of cell specific genes (Reddy *et al.*, 2009). Unliganded GR remains mainly cytoplasmic as part of a chaperone complex containing heat shock proteins (HSPs) 70, HSP90, binding protein p23 (Picard *et al.*, 1990), immunophilins, such as FKBP51, FKBP52, Cyp44, and PP5, and factors that avert receptor degradation and development. Such GR-HSP associations prioritises high affinity hormone binding whilst curbing other receptor activities, such as nucleoplasmic location and associations at the DNA. Liganded GRs undergo conformational changes, altering its chaperone complex to reveal the nuclear localisation sequences (NLS) at the hinge and ligand binding domain (LBD) regions. Following nuclear translocation, the transcription regulatory complex containing GR will interact at specific genomic GRE sites, forming interactions with other Transcription Factors (TRFs) and co-regulatory proteins, promoting the assembly of a multiprotein complex for the activation and repression of GR responsive genes. Co-regulatory proteins can be either co-activators or co-repressors and their stoichiometry is believed to define the efficacy of steroid receptor signalling. Steroid receptor co-activators (SRCs) are important in GR signalling pathways, such as transactivation, whereby SRCs interact with activated GR to enhance transcription via chromatin re-organisation and the recruitment of other co-activators such as CBP/p300. Conversely, co-repressors NCoR and SMRT bind to unliganded GR and actively repress transcription (McKenna, Lanz and O'Malley, 1999). Co-activators involved in transcription activation complexes are discussed in further detail in section 1.2.3.1. *In vitro*, GR promotes formation of stable complexes at the DNA. Although not fully understood, DNA contact by the GR complex *in vivo*, occurs via a rapid ON-OFF exchange (McNally *et al.*, 2000; Becker *et al.*, 2002) whereby the specific GR complex interacts at the DNA where it regulates transcription followed by disassembly of the complex (Stavreva *et al.*, 2004; Meijsing *et al.*, 2007).

1.2 Glucocorticoid Receptor Structure

GR and MR are part of a set of nuclear receptor family transcription factors that modulate a specific set of genes in a context-specific manner, and yet can adjust their regulatory effects relative to the their brain region, cell type and physiological state (Sacta, Chinenov and Rogatsky, 2016), providing several end-results. GR (figure 1) and MR both share a typical modular protein structure consisting of an amino-acid or N-terminal domain (NTD), a carboxy-terminal ligand binding domain (LBD), and hinge region and a zinc-finger DNA binding domain (DBD) (Oakley and Cidlowski, 2013). In fact, the LBD and

DBD are highly conserved between GR and MR, in particular the DBD, which is 94% identical (Arriza *et al.*, 1987) and permits the heterodimerisation at the same glucocorticoid response elements (GREs) (Trapp and Holsboer, 1996; Mifsud and Reul, 2016; Weikum *et al.*, 2017). These domains bear regions that control GR activity; the activation function domain 1 (AF1 - also referred to as transactivating domain 1, tau1 and τ1) is contained in the NTD, and the tau2 (can be referred to as transactivating domain 2 and τ2) and AF2 are contained in the LBD. The regions surrounding the NTD, in particular the NTD-AF1 domain between GR and MR (and in fact across all nuclear receptor family transcription factors) differ most in amino acid structure, sequence length and size, as a result of variation in translation induction (Lu and Cidlowski, 2005; Viengchareun *et al.*, 2007; Wu *et al.*, 2013; Oakley *et al.*, 2018). Importantly, the AF-1 region does not require ligand activation for transcriptional induction and is 'intrinsically' disordered', and thus its conformation is influenced by receptor-protein interactions (Kumar and Thompson, 2012). However, the specific effects of the NTD domain variations between GR and MR has not been shown. The LBD has a ligand binding pocket that controls the specificity and affinity of ligand binding. It also houses amino acid residues that initiate conformational changes in the event of ligand binding, initiating receptor nuclear translocation, where the receptor can bind at specific genomic sites and/or to proteins involved in transcriptional regulation. The LBD contains the AF-2 transcriptional output domain that under ligand dependency interact with downstream proteins that modulate receptor driven transcription (Vandevyver, Dejager and Libert, 2012; Starick *et al.*, 2015). The DBD contains a proximal box (P-box) domain that house three amino acid residues, which make contact with the major groove of the DNA binding sequence to define the specificity of the hormone response element.

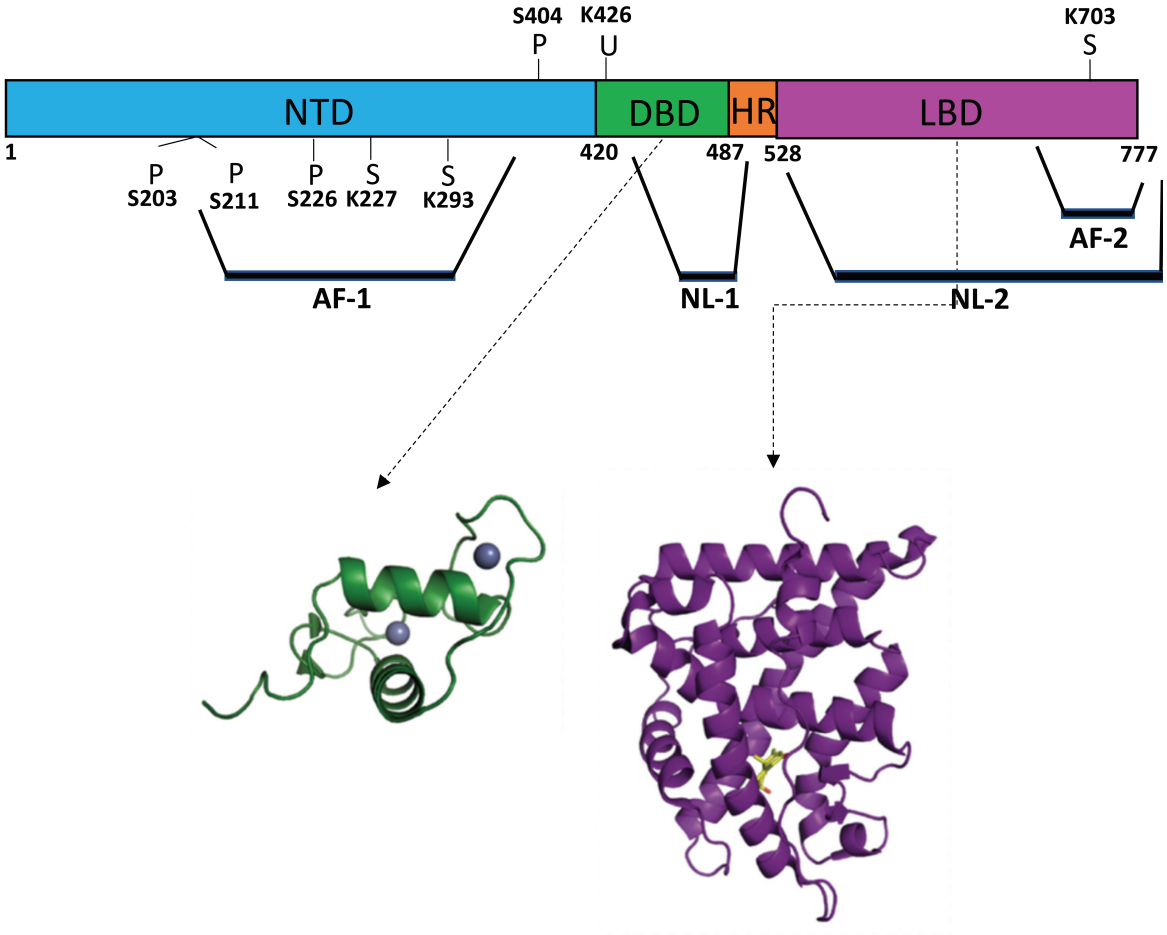


Figure 1 Linear domain structure of glucocorticoid receptor (GR).

GR consists of the amino terminal domain (NTD), DNA-binding domain (DBD), hinge region (HR) and ligand binding domain (LBD). Within these domains are regions that are context specifically involved in transcription regulation, including, activation function domain 1 (AF1) and AF2. The crystal structures are provided for a single DBD (in green), as well as identifying the coordinated zinc ions (in grey). The LBD can be seen (in purple) liganded with cortisol (in yellow) and complexed with steroid receptor co-regulator 2 (SRC-2) peptide that is not included here (Eick et al. 2012). Crystal structures have been modified from RCSB Protein Data Bank identifier (PDB ID): 1R4R (Luisi et al. 1991; Eick et al. 2012). NL-1/2 = Nuclear Factor 1 and 2.

1.3 GR splice variants and translational isoforms

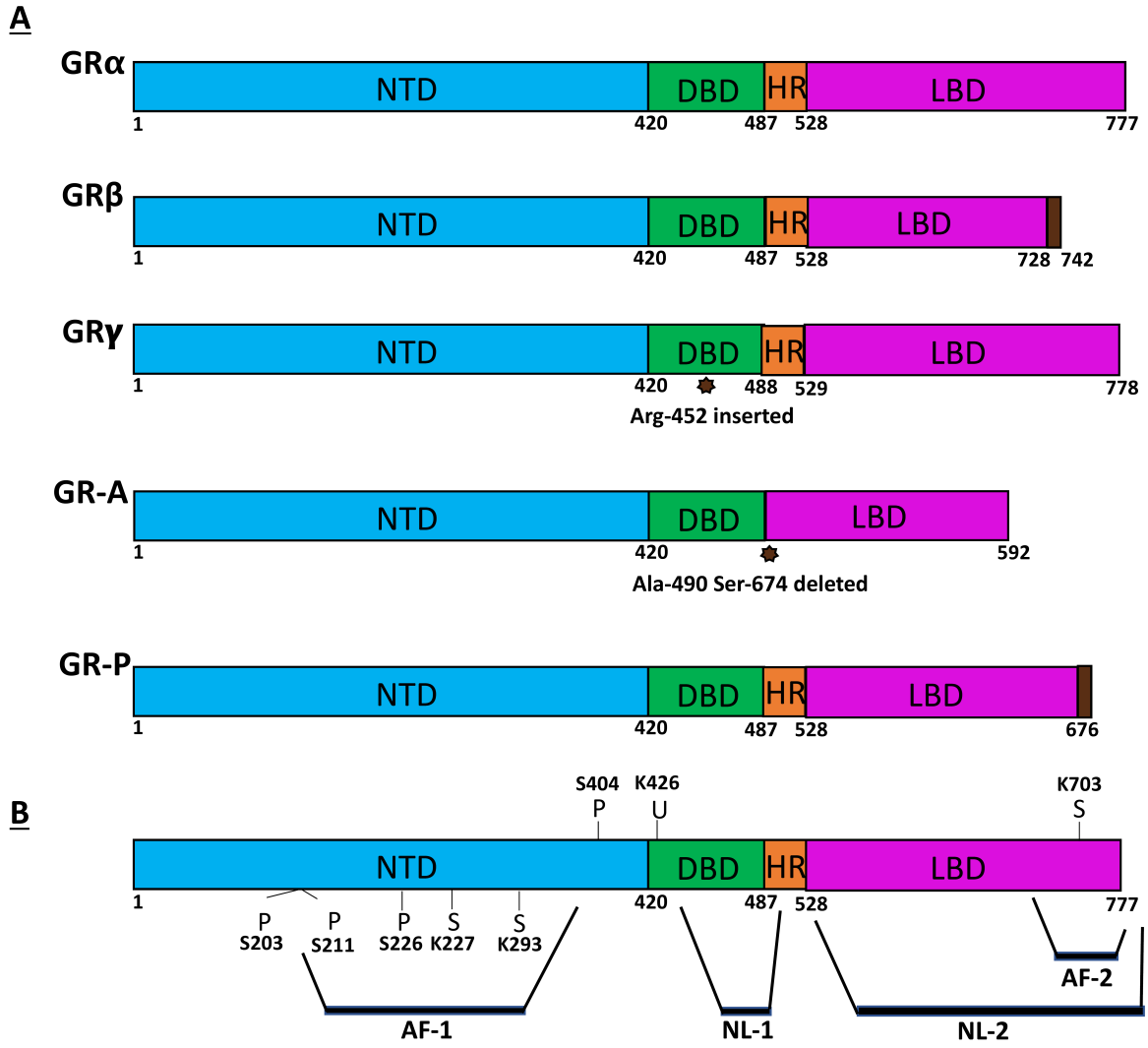


Figure 2 Splice variants and translational isoforms of the human glucocorticoid receptor (GR).

(A) GRα is the most highly expressed isoform. The C-terminal LBD of the GRβ does not bind GCs and GRβ functions as a dominant negative regulator of GRα. GRγ's DBD has an additional arginine residue inserted, which alters its ability to regulate specific GC-responsive genes. GR-A and GR-P have truncated LBDs and cannot bind GCs (Oakley and Cidlowski, 2013). (B) Linear domain structure of glucocorticoid receptor (GR) displays the several residues that are post-translationally modified. AF, activation function; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain; NLS, nuclear localization signal; NTD, N-terminal domain; P, phosphorylation; S, sumoylation; U, ubiquitination.

The predominantly expressed form of GR is GRα but numerous variant forms exist from its encoding gene NR3C1. The varied isoforms have differing expression profiles and transcriptional regulatory protein complexes, and numerous post translational modifications (PMTs) can generate further varied

GR isoforms (figure 2). Splicing near the 3'UTR of the primary hGR transcript produces two alternatively spliced C-terminal isoforms, hGRα and a minor variant hGRβ. The most abundant form, hGRα are spliced at exons 8-9 whereas in minor variant hGRβ exon 8 is spliced at an alternative downstream acceptor site, exon 9β (Lewis-Tuffin and Cidlowski, 2006; Kino *et al.*, 2009; Kino, Su and Chrousos, 2009). hGRα and β share the same amino acid structure up to amino acid position 727. The C-termini ends diverge for each isoform, such that hGRα has an additional 50 amino acids to complete a 777 amino acid structure and hGRβ contains an extra 15 amino acids to complete its protein structure of 742 amino acids. Thus, hGRβ is unable to bind GCs due to a short LBD, however mifepristone, a GR antagonist, is able to bind to the exclusively nuclear receptor (Lewis-Tuffin *et al.*, 2007). Recently, a mouse-GRβ (mGRβ) was confirmed to exist and in rat (Hinds *et al.*, 2010), possessing a similar structure and function to hGRβ, but instead of splicing at exon 9 as in the human isoform, it arises from differential splicing in intron 8. A study in mice reported GRβ inhibition of GC action via GRα and a pro-inflammatory role for GRβ (Marino *et al.*, 2016). What is more, with its nuclear localisation hGRβ remains transcriptionally active and regulates expression of specific genes that are unregulated by GRα (Lewis-Tuffin *et al.*, 2007; Kino *et al.*, 2009; Oakley and Cidlowski, 2011). hGRβ mediates transcriptional repression via recruitment of co-repressors like histone deacetylase (Kelly *et al.*, 2008). hGRβ regulated gene transcription can be determined by an antagonistic interaction with hGRα, such that when both variants are co-expressed together, a dominant negative function is produced by hGRβ onto hGRα, thus blocking the regulatory action of the latter variant (Oakley, Jewell, *et al.*, 1999; Oakley, Webster, *et al.*, 1999; Yudt *et al.*, 2003). Because of this action, hGRβ is involved in GC resistance and this has been shown at some degree to be involved in certain pathologies with aberrant GC signalling and in resistance to GC treatment (Webster *et al.*, 2001). A widely expressed variant, GRγ contains an insertion of a single arginine residue between the two zinc fingers of the DBD (Ray *et al.*, 1996; Rivers *et al.*, 1999; Meijsing *et al.*, 2009). GRγ has similar GC and DNA binding affinities as GRα however is unable to induce GRE reporters as efficiently thus producing differential transcriptional outputs. Furthermore, GRγ has been linked to GC resistance. There are additional hGR splice variants that have been identified, however extensive understanding on these is limited: hGR-A, hGR-P (Gaitan *et al.*, 1995; de Lange *et al.*, 2001), hGRδ, hGR-S1 (Baker *et al.*, 2012), hGR-NS1 and hGR-DL1 (Tung *et al.*, 2011), but these will not be discussed in further detail.

1.4 Mechanism of Action

Up to 95% of GR binding occurs in pre-accessible chromatin regions which is kept open by other DNA-binding proteins, whereas a small number of GR binding sites are inaccessible, closed chromatin regions where activated GR induces chromatin remodelling. In eukaryotic cells, GR and chromatin

remodelling proteins have been shown to control chromatin organisation by the ligand dependant alteration of histone interactions at the DNA and as such secondary factors are recruited (Zaret and Yamamoto, 1984; Richard-Foy and Hager, 1987). What is more, GR can increase accessibility of closed chromatin regions via interactions with chromatin remodelling proteins (John *et al.*, 2011). Transcription factors, such as AP1 motif, can interact with GR to assist in receptor binding at targeted genomic sequences by maintaining an accessible chromatin landscape and recruiting secondary regulatory proteins in a cooperative context (Biddie *et al.*, 2011).

1.5 Types of Glucocorticoid Receptor-Genomic Associations

GR can transactivate and repress gene transcription through various mechanisms including (figure 3): 1. Direct GR-DNA binding at glucocorticoid response elements (GRE) stimulating recruitment of and cooperation with coregulator proteins to initiate transcription. 2. GR tethers to a DNA bound transcription factor and regulates transcription of a specific gene, as in trans-repression by binding to NF-κB (Weikum *et al.*, 2017). 3. GR associates at the GRE and interacts with a proximal or co-bound TFs at the DNA to control targeted genes in a receptor dependant manner (Weikum *et al.*, 2017).

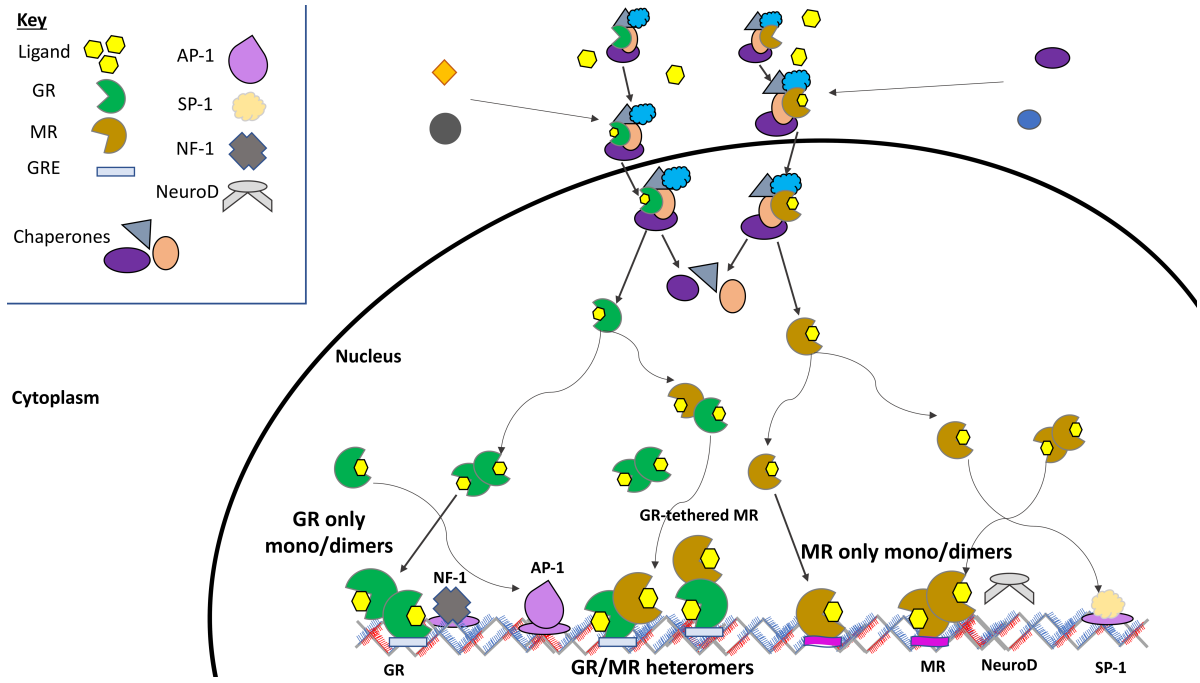


Figure 3 Graphical representation of MR and GR specific, and general interactions.

MR and GR are held in the cytoplasm bound within a chaperone complex (e.g. Hsp90, p23, immunophilins, Fkbp5). Following ligand binding, MR/GR-chaperone complex translocates to the

nucleus and dissociates from the chaperone proteins. At this point, MR and GR are able to form and function as monomers, dimer or heteromers. MR and GR homo- or heteromers bind to glucocorticoid response elements (GREs) at specific GC inducible genes. MR and GR targeted binding mostly occur alongside binding motifs of other transcription factors (TFs) (e.g. NF-1 for GR, and SP-1 for MR) demonstrating GR/MR-TF interactions.

The zinc finger within the GR DBD is highly conserved and has four tetrahedrally organised Cysteine (Cys) residues that coordinate one of two zinc ions, each followed by an α-helix and a peptide loop. The P-box is located within the first subdomain and contains three residues that make targeted contact at the base of the major groove of the genomic sequence. Here, nucleotide specific contacts can be formed, such that this sequence outlines the response element (Luisi *et al.*, 1991). Within the second subdomain helix, nonspecific contacts occur at the DNA helix backbone and minor groove and instead the peptide loop contains distal box (D-box) residues involved in GR dimer formation (Luisi *et al.*, 1991; Kumar and Thompson, 1999). The DBD guides GR localisation at targeted genomic loci through either direct GR-DNA interactions or indirect GR-protein interaction.

Liganded GRs bind directly via their DBD to canonical positive GREs DNA-Binding Sequences (DBS) within the promotor region of target genes to regulate the transcription of genes signalled by GCs. The GRE sequence, GGAACAnnnTGTTCT, is an imperfect palindrome that contains two pseudo-palindromic hexameric AGAACA repeats divided by a three-base spacer (Luisi *et al.*, 1991; Meijsing *et al.*, 2009). The three-base pair spacer between the two half sites is essential for the dimerization of GR at this element. Often referred to as a positive GRE, at this site GC dependant GR-coactivator interactive complexes mediate transactivation of genes. Amino acid side chains Arg-447, Lys-442 and Val-443 of the targeted DNA attaches to the three base-specific sites within the major groove of the GBS. This head to head binding of GR supports the dimerization between two GR DBDs, facilitating interactions between two GR proteins and GR at the DNA. GR can also bind at GREs and GRE half sites that can be proximal enough to one another for functional cooperativity (Liu *et al.*, 1996; Adams *et al.*, 2003), further supporting GR homodimer and GR-DNA interactions (Thomas-Chollier *et al.*, 2013; Watson *et al.*, 2013). Within the D-box are 5 amino acids that create important protein associations that enforce the GR DBD-DNA interaction. Mutations in the dimerization disrupt GR transcriptional regulation (Heck *et al.*, 1994; Reichardt *et al.*, 1998), as reported in the presence of GR-GR binding at the DNA but with lessened cooperativity (Watson *et al.*, 2013). In addition, GR occupancy at composite GREs containing GR-DBSs were identified from genome wide analyses to cooperatively regulate transactivation or repression (Luisi *et al.*, 1991; Meijsing *et al.*, 2009; Uhlenhaut *et al.*, 2013),

suggesting roles for epigenetic regulators and chromatin dependant factors outside of the GRE sequence.

GR is also able to interact at the DNA via the selective binding at canonical half site sequences (AGAACA), although this is not structurally defined (Schiller *et al.*, 2014; Lim *et al.*, 2015). This interaction is stabilised by indirect mechanisms between GR and other co-regulatory or transcriptional proteins in contact with the DNA, close to the point of binding of the GBS. However, what constituents forming such transcriptional complexes and their enrichment at GBS half sites and how they regulate gene expression, requires further understanding. Another mode by which GR indirectly binds at specific genomic loci is by tethering, whereby the DBD interacts with other TFs precisely bound to their known functional sequence motifs, as observed with activator protein 1 (AP-1) or nuclear factor κ-B (NFκB) (De Bosscher, Vanden Berghe and Haegeman, 2001, 2003; Luecke and Yamamoto, 2005).

GRs are also able to bind at DNA through the inverted repeat negative glucocorticoid response elements (IR nGREs) defined by an alternative GR-binding motif. The nGRE sequence, CTCC(n)0-2GGAGA, contains a variable spacer (between 0 to 2 nucleotides) and is occupied by two GR monomers that do not dimerise as they are bound on opposing sides of the DNA at inverted repeat GR Binding Sequence (IR-GBS) (Surjit *et al.*, 2011; Hudson, Youn and Ortlund, 2013). Specifically, a monomer binds at three high-affinity sites facilitated by side chains Lys-442 and Val-443, which are also involved distinguishing DNA in the GR DBD-GBS structure. Hydrogen bonds occur between the Arg-447 sidechain and guanine in canonical GBS structures, however in the IR-GBS structure Arg-447 does not bind to a guanine due to steric conflicts with a thymine (Hudson, Youn and Ortlund, 2013). The other monomer establishes its base specific interaction between sidechain Arg-447 and guanine. At nGREs, the monomeric GR DBD associates at an everted repeat with negative cooperativity (Hudson, Youn and Ortlund, 2013). Here they can undergo SUMOylation at the NTD and recruit co-repressors (Hudson, Youn and Ortlund, 2013; Hua, Paulen and Chambon, 2016), such as ligand activated GR-corepressor (SMRT/NCoR and HDACs) interactive complexes to induce the trans-repression of genes (Surjit *et al.*, 2011). IR-nGREs possess silencing properties, whereby ligand activated formation of a repressive complex prevents enhancer-proximal promotor interactions (Surjit *et al.*, 2011). nGREs contribute to HPA-axis regulation by GR mediated transrepression of CRH in the hypothalamus and POMC in pituitary corticotrophs (Surjit *et al.*, 2011; Oakley and Cidlowski, 2013; Ramamoorthy and Cidlowski, 2013). Of note, MR is unable to associate at nGREs, where the particular mutation required for binding at such sites is specific to GR only (Hudson, Youn and Ortlund, 2013; Hudson *et al.*, 2016).

GR has been shown to bind IR-GBS elements, however when a monomer is bound the tendency of another monomer binding is reduced. On the other hand, cooperative binding is seen at canonical GBS sites (Hudson, Youn and Ortlund, 2013). What is more, nuclear magnetic resonance (NMR) data show considerable alterations in the chemical nature of D-loop residues similar to dimerization when bound at canonical GBS, however no changes are observed if bound to an IR-GBS (Hudson *et al.*, 2016). As such, these *in vitro* studies demonstrate that GR monomers can be presumed to bind the same elements *in vivo*. Furthermore, MR has been shown to bind to a canonical GBS, although binding to IR-GBS is restricted to GR (Hudson *et al.*, 2016).

Chromatin accessibility partially explains why most putative GR binding sequences are not occupied by receptors and do not constitute actual GREs. The functionality of GRE sequences is further defined by GR interaction with transcriptionally active proteins (So *et al.*, 2008), and determines the evolutionary conservation of functional GRE sequences (Datson *et al.*, 2011). Further, mutations in the DBD dimer interface interfere with receptor binding (Liu *et al.*, 1995; Reichardt *et al.*, 1998), but GRE sites exist where receptors can bind (Lim *et al.*, 2015).

1.6 Models of Chromatin Interaction and Glucocorticoid Regulation Targeted Transcription

The oligomeric configuration of GR, and MR, during the stages of signal transduction, continues to be debated (Gebhardt *et al.*, 2013; Presman *et al.*, 2014, 2016; Starick *et al.*, 2015). Full-length ligand activated GR has been described as a monomer in solution *in vitro*, including if exposed to increased ligand concentrations (Bain *et al.*, 2012; Robblee, Miura and Bain, 2012). GRs were originally believed to bind to DNA as monomers or dimers, however following DNA contact, tetramers have been reported to cause interdomain allosteric regulatory change within the receptor looping of distal chromatin regions bound with GR dimers (Presman *et al.*, 2016). In classic GR mediated pathways, activated GR translocates through the perinuclear space and into the nucleus and binds at targeted GREs within specific genomic regions. Studies have demonstrated the association of GR monomers at GRE palindromic and half sites producing varying transcriptional outputs (Eriksson and Wrange, 1990; Perlmann, Eriksson and Wrange, 1990). MR and GR can bind directly at GREs or indirectly (through tethering mechanisms via transcription factors) (figure 3). Direct interactions between GRs and specific DNA sites were usually associated with transactivation and indirect tethering interactions transrepressive effects. However, evidence has shown indirect tethering mechanisms to also stimulate transcriptional effects. Langlais *et al* (2012) investigated the genome wide prevalence of tethering between GR and STAT3, revealing extensive transcriptional interactions between GR and STAT3.

MR and GR interactions at specific genomic sites have been investigated in genome wide studies using chromatin immunoprecipitation (ChIP)-sequencing analysis. These DNA binding sequences were subjected to motif analysis to identify GRE-like sequences which are likely to represent direct interaction sites for MR and/or GR and to identify alternative motifs including other transcription factor binding sites associated with the GR/MR binding sites.

A study in a human kidney cell line found a GRE sequence as the predominant motif for MR binding sites, however it was revealed that the most prevalent mechanism of interaction was through indirect-tethering to other transcription factors, such as FOX, EGR1, AP1, and PAX5 (Le Billan *et al.*, 2015). This has also been observed in several tissue types for GR contact at the DNA. Conversely, data from ChIP-sequencing of hippocampal samples in the rat show a majority of MR and GR regulated DNA sites to occur via direct binding at GREs (Polman, de Kloet and Datson, 2013; Pooley *et al.*, 2017; van Weert *et al.*, 2017) as well as identifying DNA motifs proximal to some of these sites. Furthermore, when mapped GRE sequences were overlapped with CORT regulated genes in the rat hippocampus, several GREs were revealed surrounding particular CORT regulated genes; and these were enriched with transcription factor binding motifs (Datson *et al.*, 2013). Recent genome wide experiments by Pooley *et al* (2017) discovered binding motifs for NF-1 in half of the GR binding sites in the hippocampus. Furthermore, this study identified motif for NeuroD transcription factors in ~15% of GR binding loci (Pooley *et al.*, 2017). Genes where MR and GR bind at the same GRE sequences exerting their transcriptional regulatory effects include Per1, Fkbp5, Gilz, and Sgk1 (Webster *et al.*, 1993; D'Adamio *et al.*, 1997; S. Y. Chen *et al.*, 1999; Robert-Nicoud *et al.*, 2001; Mifsud and Reul, 2016).

It is known that MR and GR exert distinct receptor effects, and their inherently differential receptor mediated effects in the same cell type are reported to occur via genomic pathways (Joels, Hesen and de Kloet, 1991; Karst *et al.*, 2000). It is possible that these receptors exert their effects by independent interactions by alternately binding DNA interaction sites. However, MR and GR may be interacting together, and the overall transcriptional responses may be due, in part, to MR-GR interactions. This study addresses whether it is likely that MR and GR are interacting to direct transcriptional effects.

1.7 Glucocorticoid Receptor Regulation

GR regulates transcription by recruiting various other co-regulators and transcriptional proteins to form large transcriptional regulatory complexes. These complexes determine functional or conformational alterations within the chromatin landscape and/or transcriptional machinery, consequently controlling the production of mRNA via activation or repression of target genes. Around 300 co-regulators have been identified and many form into specific multi-factor complexes that confer multiple functions, although it is not known if they all interact with GR (Jenkins, Pullen and Darimont,

2001; Bannister and Kouzarides, 2011). The highly context specific interactions between GR and co-regulatory complexes depends on access to regulatory proteins and types of GR regulating signals (hormone, DBSs, PMTs and interactions with other TFs). In addition, the Mediator complex, also known as Mediator, interacts with numerous TFs and other well-defined complexes/factors to regulate transcription of target genes (Weber and Garabedian, 2018). Co-regulatory proteins read GR facilitated signalling by converting the signal induced allosteric regulation of specific interfaces of the GR defining transcriptional output. Co-regulators are usually defined by their activation or repression of targeted genes, however some regulatory proteins both activate and repress transcriptional output (Millard *et al.*, 2013).

1.7.1 Co-regulators

The *p*160 SRC family fall within the structural and enzyme functional classes of GR co-regulators. Within this functional class are SRC-1 (also named NCoA-1), SRC-2 (also named NCoA-2, TIF2 and GRIP1) and SRC-3 (also named NCoA-3, CIP, AIB1, ACTR and TRAM1) (Jenkins, Pullen and Darimont, 2001). These co-regulators form supporting frameworks and can interact with up to ten other regulatory proteins (Dasgupta, Lonard and O'Malley, 2014), for example coiled-coil coactivator (COCOA) cell division cycle and apoptosis regulator 1 (CCAR1), histone acetyltransferases (CREB-binding protein (CBP)/p300 and CBP/p300-associated factor (PCAF or KAT2B) and histone methyltransferases (coactivator associated Arg methyltransferase 1 (CARM1 or PRMT4) and protein Arg *N*-methyltransferase 1 (PRMT1)) (Kim, Li and Stallcup, 2003; Stallcup *et al.*, 2003; Fonte *et al.*, 2005; Kim *et al.*, 2008; Xu, Wu and O'Malley, 2009). Studies have shown opposing effects of SRC proteins on GR regulated gene expression (Szapary, Huang and Simons, 1999; Trousson *et al.*, 2007).

The three functional domains of each SRC include an N-terminal domain that binds to GRs AF1 domain to stabilise the receptor and improve the α-helical content (Khan *et al.*, 2012), the central receptor interaction domain (RID) containing three LXLL motifs and two transcription activation domains AD1/AD2, which interact with GRs AF2 site contained within the LBD (Darimont *et al.*, 1998; Voegel *et al.*, 1998), and C-terminal activation domains that associate with HATs and histone methyltransferases (Xu, Wu and O'Malley, 2009).

GR favours interactions with co-regulatory protein SRC-2, which can form distinctive foci within the nucleoplasm together with other co-regulators such as p300 and its associated factor PCAF, as well as the steroid receptors such as GR and MR (Kurihara *et al.*, 2002; Li *et al.*, 2003). Nuclear localised DEX-activated GR was observed to co-localise to these co-regulators, however this was not observed in the presence of RU486 (Ogawa *et al.*, 2004). Further, GC induced GR interaction-dependant phosphorylation of SRC-2 imparts modifications at these sites determining co-regulatory function and

targeted genes are repressed (Dobrovolna *et al.*, 2012). Moreover, SRC-2 was primarily demonstrated to organise individual receptor domains involved in GR-specific transcriptional regulation (Kamei *et al.*, 1996; Sheppard *et al.*, 1998; De Bosscher, Vanden Berghe and Haegeman, 2001; Rogatsky *et al.*, 2002).

Mediator is a large complex made up of ~30 proteins (1.2 MDa) that establish contacts between GR and machinery involved in transcriptional regulation. Mediator regulates RNA polymerase II (RNAPII), essential in transcribing protein coding and almost all non-coding RNA genes and transcription factor II Human (TFIIH) activity that is involved in transcriptional of various protein coding genes and DNA nucleotide excision repair pathways, to determine transcriptional output (Allen and Taatjes, 2015). Several studies investigating protein structure of Mediator complexes have alluded to allosteric mediation of its activity (Taatjes *et al.*, 2002; Knuesel *et al.*, 2009; Meyer *et al.*, 2010; Knuesel and Taatjes, 2011). Interactions between the LBD within steroid receptors and other TRFs induce modification in receptor conformation establishing a Mediator pocket interface and permits the interaction between Mediator and RNAPII (Meyer *et al.*, 2010; Bernecky *et al.*, 2011). Primary constituents of the Mediator complex form interactions with kinase molecules, such as CDK8 or CDK19, and these themselves employ enzymes, including HAT GCN5L (also named KAT2A) (Meyer *et al.*, 2008). The Mediator complex can be bound by GR at two particular Mediator subunits, Mediator of RNAPII transcription subunit 1 (MED1) and MED14. MED1 associates at the GR LBD if ligand activated at the LXXLL motifs while MED14 forms associations at the AF1 domain without ligand activation (Hittelman *et al.*, 1999). GR modulated gene transcription is supposed to rely on effects of MED1 or MED14 interactions (Chen, Rogatsky and Garabedian, 2006). Co-activators HIC-5, COCOA and CCAR1 can form direct or indirect interactions with GR, that together establish such multi-constituent complexes involved in context dependent transcriptional regulation (Yang *et al.*, 2000; Kim, Li and Stallcup, 2003; Stallcup *et al.*, 2003; Kim *et al.*, 2008; Chodankar *et al.*, 2014).

Other TFs that GR interacts with include AP-1 and Nuclear factor (NF)-κB. GR has been shown to mediate immune and anti-inflammatory effects of GCs by interacting with pro-inflammatory transcription factors, such as AP-1 and NF-κB. Both are inducible TFs with opposing roles, with NF-κB regulating transactivation of cytokine regulated genes and GR evoking suppressive effects in inflammatory pathways by inhibiting the transcription of several cytokine induced genes. Their interactive interplay is important in the regulation of genes involved in inflammation and immunity (Zhong, Voll and Ghosh, 1998; McKay and Cidlowski, 1999). AP-1 transcriptionally regulates genes involved in proliferation and differentiation, as well as cytokine upregulation. Similarly, to NF-κB, AP-

1 is repressed by GR via direct interactions, ensuing opposing transcriptional effects. AP-1 is DNA binding protein that is made up of Jun or Fos protein dimers (Petta *et al.*, 2016).

1.7.2 DNA Binding Domain

Thousands of proteins that make contact with DNA have been discovered using several high-throughput techniques (Hudson and Ortlund, 2014), of which the most common are TRFs with sequence-specific DNA-binding activity. These sequence-specific DNA-binding platforms appear to have allosteric effects on transcriptional regulators (Lefstin and Yamamoto, 1998; Meijsing *et al.*, 2009; Watson *et al.*, 2013).

Protein-protein interactions (PPIs) at the DBD is enhanced by DNA binding at canonical GBSs (Luisi *et al.*, 1991). In addition to GR interacting at sequence-specific DNA platforms with each hexameric half site, the three base pair spacer separating these half sites within particular GBSs facilitates effective protein-DNA contacts and dimerization (Meijsing *et al.*, 2009). Further, allosteric regulation from one hexameric half site sequence can transmit its effects across the dimer interface, structurally changing complex conformations by changing binding of and replacement of proteins (Watson *et al.*, 2013). The sequences of each three base pair spacer will influence the DNA shape produced at the DNA-sequencing helix. This allosteric effect is carried via the lever arm altering GRs D-box and consequently influences the regulation of GR activity (Watson *et al.*, 2013). These distinct conformational structures can be observed in different GR DBD-GBS structures. Studies using NMR and reporter methods *in vitro*, have identified sequences at the +8 and -8 positions proximal to the GBS, which change DNA conformation and also disrupt GR DBD structure, and shown with endogenously expressed GR *in vivo* (Schone *et al.*, 2016). Taken together it can be assumed that types of GR conformations depend on contacts with certain sequence-specific DNA platforms that influence the structure of GR interfaces where interactions take place and ultimately regulate transcriptional output.

In addition, NMR studies reported allosteric signalling between GR monomer-dimers at canonical GBSs, and dynamic stimulation modelling to assess movements of atoms within a macromolecule over specific time intervals, allude to allosteric regulation between GR monomers at IR-GBSs *in vitro* (Watson *et al.*, 2013; Hudson *et al.*, 2016). Here, DNA induces allosteric signalling that might influence GRs affinity for transcriptional proteins (i.e. co-regulators) and their output of transcriptional regulation, which suggest that allosteric induced signalling reaches the NTD and LBD. Zhang *et al*. (2001) demonstrated that DNA binding alters coactivator interaction surfaces of the intact vitamin D receptors (VDR)-retinoid X receptor (RXR) complex, corroborating allosteric signalling across heterodimer complexes when bound at the DNA (Zhang *et al.*, 2011). Although, this has only been demonstrated at isolated domains within the GR-protein complex and not in full-length GR. They went

on to demonstrate conformational alterations of the intact VDR-RXR-DNA complex, upon interaction with ligand regulatory proteins and at the DNA. The allosteric effects of change in the VDR DNA-binding sequence (VBS) were transmitted through the VDR DBD, altering the access LBD interfaces of the RXR molecule (Zhang *et al.*, 2011).

1.7.3 Ligand Binding Domain

Steroid receptors are ligand activated nuclear transcription factors with the ability to distinguish their specific endogenous steroid hormones and exogenous substances, making them examples for the evolution of specificity (Eick *et al.*, 2012). Specific ligands for GR and MR, for example, bind at an internal pocket within the evolutionary conserved ligand binding domain (LBD), where they share significantly similar sequences but with differences in structure, of the interface, determining selective ligand affinity (Thornton, 2001). Ligand binding to the LBD induces the recruitment of co-activator proteins and conformational changes, modulating gene transcription (Gronemeyer, Gustafsson and Laudet, 2004). Steroid receptors have evolved through a series of gene duplications, producing in humans two phylogenetic categories of steroid receptors each able to distinguish their specific ligands compatible to each nuclear receptor LBD. These include the estrogen receptors (ERs), whose ligands contain an aromatised A-ring and a OH-C3 attachment on the steroid skeleton and the nonaromatised steroid receptors, such as with gluco- or mineralocorticoids, which possess a nonaromatised A-ring, an extra methyl group at C19 and mostly a ketone at C3. Each of the nonaromatised nuclear receptors due to size and the interaction of the C17 and C21 functional groups with different polar residues of the LBD, has high sensitivity. Although functional groups at other locations might effect sensitivity, they are able to discriminate its endogenous NR3C family member 3-keto ligands from related NR3A family estrogen receptor (ER) 3-hydroxy-specific ligand recognition (Orchinik, Murray and Moore, 1991; Katzenellenbogen, 1995; Orchinik M, 2006). Bledsoe *et al.* (2002) demonstrated the crystal structure of GR LBD activated with dexamethasone (DEX) revealing a network of polar and nonpolar residues at the interface, such that every DEX polar atom binds with the GR LBD, demonstrating highly significant binding specificity of DEX to GR (Bledsoe *et al.*, 2002). Although DEX demonstrates high affinity, almost half of the internal ligand binding pocket is left available for binding by other ligands (He *et al.*, 2014), and this is similarly the case with cortisol. What is more, following binding at GRs LBD by cortisol and mifepristone, the LBD structure was deformed enabling further binding with different ligands (Kauppi *et al.*, 2003) and may suggest regulatory and transcriptional outcomes as a result of structural alterations to the LBD. The challenges from GR ligands leaving portions of the LBD structure vacant for binding by other ligands and deformation of the binding pocket, has also assisted in ligand design. Arylpyrazole compounds (non-steroidal ligand) were designed according to observed

DEX binding at the LBD pocket and demonstrated different pharmacological actions in comparison to DEX effects (Wang *et al.*, 2006). GR modulators with anti-inflammatory and immunosuppressive properties but without adverse effects continue to be researched, however this is difficult and will likely involve consideration of context specific allosteric, conformational and enzymatic changes in regulatory complexes to further understand properties and mechanism, as well as the molecular features of therapeutic candidates under varying phenotypic and pathological outcomes.

The importance of context-specific GR ligands is essential in regulatory outcomes and communicating allosteric signalling and control (Ricketson *et al.*, 2007). These will influence GR recruitment of chaperone and transcriptional proteins or actions and stimulate GR NLSs and interaction at the DNA. Slight changes in GR ligand conformation or dosing will impact on gene specific structure of GR occupied regions (Wang *et al.*, 2006; Chen *et al.*, 2013), regulatory protein interactions and action, such as modification of histone acetyltransferase (HAT) while avoiding disrupting GR occupied regions (Wang *et al.*, 2006).

1.7.4 Post Translational Modifications for Glucocorticoid Receptors

Covalent post translational modifications (PTMs) of GR act as endpoints to signal transduction of context specific information different to non-covalent ligand induced cell communication. Such modifications can determine allosteric signalling, alter protein binding interfaces and influence subcellular localisation, stability, interaction with the DNA, ligand response and regulatory activity that is context specific.

1.7.4.1 Phosphorylation

Protein phosphorylation (the addition of phosphate groups) leads to regulation and alteration of protein function. GR phosphorylation is involved in ligand binding, enzymatic activity, nucleoplasmic localisation in the nucleus, DNA interaction, and associations with co-regulatory proteins (Itoh *et al.*, 2002; Ismaili and Garabedian, 2004; Ward and Weigel, 2009). GR is under a basal level of phosphorylation, with further sites being phosphorylated when bound by its ligand (Housley and Pratt, 1983; Bodwell *et al.*, 1998; Wang *et al.*, 2007). More than 20 residues, mainly serine (Ser) and threonine (Thr) have been identified in the human GR (hGR) structure as phosphorylated sites. The identified phosphorylated sites within GRs NTD include at least the following phosphorylation sites in humans: Ser113, Ser134, Ser141, Ser203, Ser211, Ser226 and Ser404 (Ismaili and Garabedian, 2004). The biological activity of phosphorylated GR *in vitro* using ATP as the source of phosphate are thought to be regulated by kinase enzymes MAPK, CDK, JNK, GSK-3 which can induce conformational changes in GR, although not every phosphorylation will activate GR (Krstic *et al.*, 1997), which is suggestive of various signalling pathways which communicate with GR (Mason and Housley, 1993; Webster *et al.*,

1997). Phosphorylation increases GRs half-life, and mutations at Ala within certain phosphorylated sites initiates fast GR degradation (Webster *et al.*, 1997; Wallace and Cidlowski, 2001). For example, cyclin dependant kinase 5 (Cdk5) phosphorylation of Ser2013, Ser211 and Ser226 located within the AF1 region are believed to modulate the exposure of GR interfaces crucial for the recruitment of co-factors, such as the histone acetyl-transferases (HAT), CBP and p300 and adenosine triphosphate (ATP) dependant chromatin remodelling factors such as the SWI/SNF complex (Wallace and Cidlowski, 2001; Itoh *et al.*, 2002; Wang, Frederick and Garabedian, 2002; Kino *et al.*, 2007; Blind and Garabedian, 2008; Chen *et al.*, 2008; Garza, Khan and Kumar, 2010).

1.7.4.2 Ubiquitylation

Ubiquitination is an enzymatic post translational modification whereby multiple ubiquitin residues are covalently attached to GR, and is involved in proteasomal degradation of GR. Prior to ubiquitination, is the phosphorylation of PEST sequences (namely: Proline, Glutamic acid, Serine and Threonine residues) by E1 activating, E2 conjugating and E3 ligase enzymes (Jadhav and Wooten, 2009). The ubiquitin molecule is covalently bound to Lys residues on the GR protein and initiates the protein turnover by directing GR to the proteasome for degradation. When Lys426 residue was mutated to alanine the PEST sequence could not be ubiquitinylated and disrupted GR nuclear export and degradation (Wallace and Cidlowski, 2001; Wallace *et al.*, 2010). Further, when the carboxyl-terminus of hsp70 interacting protein, a co-chaperone of GR regulating E3 ligase, was overexpressed, the level of GR transcripts decreased, pointing to the involvement of chaperone complexes in protein degradation (Ballinger *et al.*, 1999; Vandevyver, Dejager and Libert, 2014). GR is degraded by the proteasome pathway and studies suggest ubiquitin is also involved. GR has further been shown to be degraded by the proteasomal pathway since inhibitor MG132 treatment resulted in increased GR protein levels (Wallace and Cidlowski, 2001; Deroo *et al.*, 2002; Han *et al.*, 2009; Wilkinson, Verhoog and Louw, 2018).

1.7.4.3 Sumoylation

Sumoylation (SUMO) is the addition of a small ubiquitin-related modifier-1 (SUMO-1) (Le Drean *et al.*, 2002) that covalently bind (under ATP-dependant processes) to lysine residues on GR, effecting GR's stability, PPIs, subcellular localisation and transcriptional activity (Muller *et al.*, 2001; Wallace and Cidlowski, 2001; Verger, Perdomo and Crossley, 2003; Gill, 2005). Ubiquitin and SUMO molecules share a degree of homology (20%) and are modulated by SUMO-1 (50%), and SUMO-2 and SUMO-3 proteins whose homology is higher (95%) (Mahajan *et al.*, 1997; Kamitani *et al.*, 1998; Tatham *et al.*, 2001). Sumoylation of human GR occurs predominantly at residues Lys-277 and -293 within the N-Terminus Domain (NTD) and Lys-703 in the LBD of the CTD and effecting GR in a context dependant

manner (Le Drean *et al.*, 2002; Tian *et al.*, 2002; Holmstrom *et al.*, 2008; Oakley and Cidlowski, 2013). SUMO-1 modification of GR can determine receptor function in promotor driven transcriptional regulation, whereby the disruption of SUMO polypeptides for GR increased the recruitment of GR to DNA modifying the regulation of genes involved in cell growth, proliferation and survival (Paakinaho *et al.*, 2014).

SUMO of TFs, including GR, is usually associated with reduced or repressed transcription (Treuter and Venteclef, 2011; Flotho and Melchior, 2013), whereby SUMO promotes the recruitment of co-repressor complexes or by disrupting PTMs required in transcriptional regulation, such as phosphorylation. Furthermore, alteration in levels of TFs interacting at their targeted DNA regions can occur by SUMO, either by reducing effective binding or causing removal of TFs at the chromatin or effecting regulation of TF populations at the chromatin. Studies observed repression of inflammatory genes where GR binds at regions within IR-GBS (Hua, Ganti and Chambon, 2016; Hua, Paulen and Chambon, 2016). Specifically, this resulted from SUMO at GRs Lys-293 residue within the NTD to promote IR-GBS mediated repression and which repressive effects did not occur following mutations at any other residues. In addition, SUMO at GRs Lys-293 residue stimulates formation of SUMO repressing complexes containing coregulators, SUMO-SMRT-NCoR-HDAC3, that are required for GC activated IR-nGRE mediated transrepression without effecting activation by GR regulated genes related to canonical GBS containing GR occupied regions (Hua, Paulen and Chambon, 2016). In addition SUMO of GR supports the receptors association at weak regions within IR-GBS GR occupied regions and when SUMO is prevented, a reduction in GR driven repression at sites capable of being tethered is observed, such as AP-1 and NF-κB (Hua, Ganti and Chambon, 2016), possibly due to inability to recruit co-regulators into mediating complexes.

1.7.4.4 Acetylation

Acetylation of hGR involves the addition of an acetyl group from acetyl coenzyme A (Ac-CoA). hGR has four acetylation sites within its hinge region, namely residues 480, 492, 494 and 495, which are acetylated by numerous Histone acetyltransferases (HAT) proteins CBP, p300, circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like1 (BMAL1) (Nader, Chrousos and Kino, 2009) in a daily oscillation pattern following the rise and fall of blood serum concentration of cortisol and are involved in the regulation of gene expression (Ito *et al.*, 2006; Nader, Chrousos and Kino, 2009). The acetylation of GRs NTD tail of chromatin attached histones by HATs are required for transcription initiation by exposing the promotor sites to TFs, chromatin remodellers and the RNAPII complex (An, 2007). As well, HAT coactivators can acetylate other co-activator proteins, other TFs including steroid receptors and chaperone molecules such as HSP90 (Kovacs *et al.*, 2005; Minucci and

Pelicci, 2006). Deacetylation of GR can occur by HDAC classes I, II and III, including HDAC1, 2, 3 and Sirt1. Clock associates with GRs LBD within the C-terminal region, where GR is acetylated at its lysine residues clustered within the hinge region (Nader, Chrousos and Kino, 2009) and is important in repression of NF-B controlled genes (Ito *et al.*, 2006). GRs lysine residues are found within the KXKK motif (K is a lysine and X is any amino acid) and is conserved across MR, AR and PR, but not ER α and β (Faus and Haendler, 2006). Acetylation of the KXKK motif diminished GRs affinity to canonical GBSs sites and decreased GR regulated transcription. CLOCK mediated post translational modifications are essential in circadian regulation (Nader, Chrousos and Kino, 2009).

1.7.4.5 Nitrosylation

Nitrosylation is another post translational modification whereby a nitric oxide is covalently attached to a thiol group on a Cys residue. GRs house two zinc finger motifs (as discussed in chapter 1.1.1 and 1.3.1), each with four Cys sites. A nitric oxide can attach at these sites to release the bound zinc ions. Studies have demonstrated inhibition of ligand binding as a result of this modification (Galigniana, Piwien-Pilipuk and Assreuy, 1999).

1.8 Chromatin Structure

1.8.1 Chromatin Structure and Histone Exchange

The genome in eukaryotic cells is a highly dynamic and complex structure that is organised by and packaged into chromatin and located in the confines of the nucleus. Chromatin assists in regulating the accessibility of DNA for transcription, recombination, DNA repair and replication, and is made up of strings of nucleosomes. Each nucleosome is surrounded by tightly coiled DNA (coiled ~1.65 times around an octamer core containing two replicates of each of the four canonical histone proteins (H2A, H2B, H3 and H4) (Kornberg, 1974; Hayes, Clark and Wolffe, 1991). These histone proteins share a common structural domain containing three α-helices (α1, α2 and α3) separated by two loops, L1 and L2, known as the histone fold, facilitating the interactions of H2A-H2B and H3-H4, during nucleosome organisation and forms the dimeric structural unit of the nucleosome (Luger *et al.*, 1997). Each interacting histone pair is arranged head-tail in a motif from the two histone fold interfaces. As a result, the L1 and L2 loops are at opposite dimer ends, which are pivotal in nucleosome-DNA interaction (Luger *et al.*, 1997). These interacting histone pairs associate and go on to form a four-helix bundle. The α-helices of H3 interact, pulling the two H3-H4 pairs in to construct the tetrameric backbone of the nucleosome (Luger *et al.*, 1997). The four-helix bundle consisting of H2B and H4 interacting dimers positions on either side of tetramer forming an organised octamer (Smith and Stillman, 1991). This organised structural configuration of the H3-H4 tetramer core is more stable, compared to the H2A-H2B dimers that can be removed more easily (Kulaeva, Hsieh and Studitsky,

2010). *In vivo*, this increased exchange of H2A-H2B dimer pairs was shown, in comparison to the H3-H4 tetramer (Jamai, Imoberdorf and Strubin, 2007). In addition, removal of the H3-H4 tetramer to be partially or completely exchanged for newer or altered components cannot occur before the removal of the H2A-H2B dimers and replaced (English *et al.*, 2006; Henikoff, 2008; Luger, Dechassa and Tremethick, 2012). This identifies the necessary steps required for H3 and H4 exchange. The ordered disassembly of nucleosomes allows regulatory factors and RNAPII to access the DNA template. On the other hand, the ordered reassembly of nucleosomes restricts access, consequently inhibiting transcription and mRNA synthesis.

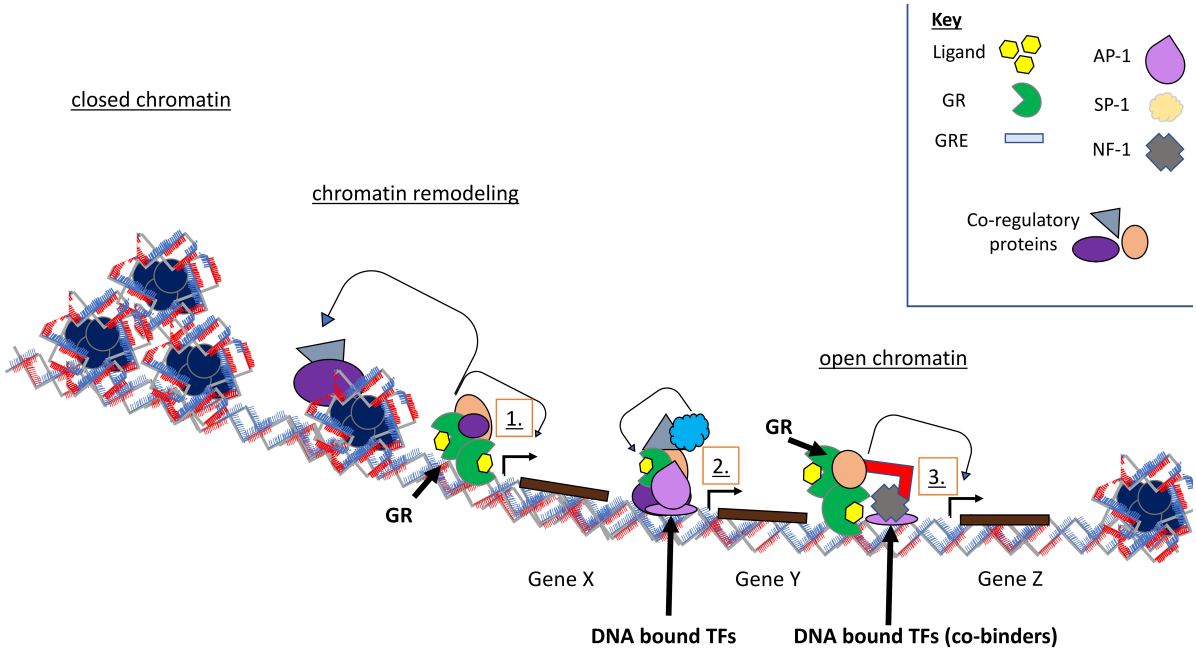


Figure 4 MR/GR-DNA interactions.

GRE targeting by MR/GR requires access to the specific DNA sequence in open chromatin. In addition, GR actively binds to chromatin remodelling proteins, increasing accessibility of closed chromatin. MR/GR activate or repress gene expression by 1. Initiation of transcription by direct DNA binding to the GRE by multi protein complexes containing co-regulatory proteins and other TFs, 2. Tethering mechanism whereby MR/GR binds to DNA-bound TFs and control transcriptional regulation of the targeted gene, 3. MR/GR bind at the DNA alongside DNA bound TFs resulting in MR/GR regulation of specific genes.

Nucleosome stability and chromatin configuration depends on histone variants and PTMs, the recruitment of chromatin re-modellers and modifiers, and the degree of DNA methylation (John *et al.*, 2011) (figure 4). Disruption of histone-DNA interactions initialises histone exchange whereby histones are removed in a sequential manner. Histone exchange is regulated by factors that weaken the histone octamer interaction at the DNA and the inter histone interactions following PTMs, or by

histone chaperone mediated alteration of nucleosome structure and/or via ATP-dependant chromatin remodelling proteins. Within histones are amino acids that can be either acetylated, phosphorylated, methylated, ubiquitylated, and sumoylated, and each will influence the regulation of chromatin structure and cellular output, such as in differentiation of tissue types (Smolle and Workman, 2013). PTMs impact on nucleosome stability through changes in the chemical binding within nucleosomes or with proximal nucleosomes, causing the chromatin to be either open or closed (Williams, Truong and Tyler, 2008; Tropberger *et al.*, 2013). Chromatin regions that are less dense are described as 'open' (also referred to as heterochromatin) and more transcriptionally active, whereas regions containing denser chromatin are described as 'closed' (also referred to as euchromatin) and transcriptionally inactive (Archer *et al.*, 1991; John *et al.*, 2008). Alterations in chromatin structure can be caused by direct effects of PTMs on histone-DNA interactions (Shogren-Knaak *et al.*, 2006), or via the regulation of local transcriptional processes. PTMs regulate local transcription by functioning as a scaffold at which 'reader' proteins are able to associate to (Lee *et al.*, 2007). Post translationally modified histones also restrict certain proteins from binding at particular genomic regions (Venkatesh *et al.*, 2012). Histones are either activators or repressors depending on their transcriptional effects (Smolle and Workman, 2013).

Post translationally modified histones can be reversed to their prior state via separate 'writer' or 'eraser' enzymes that add or remove such marks, and as such modulates their localisation within the chromatin landscape and modulates the duration of PMT at a specific locality on the genome. Papamichos-Chronakis, *et al*. (2011), identified that localisation of histone variant H2A.Z via the INO80 chromatin re-modelling factor is vital in genomic regulation and acetylation of H2A.Z by acetyltransferases at the promotor prevented its removal from promotor regions (Papamichos-Chronakis *et al.*, 2011). In addition, PTMs on histones are thought to be effects of histone altering enzymes at specific targets and not as a result of their position within genomic regions (McKittrick *et al.*, 2004). For example, the H3.3 variant contains active chromatin marks including acetylated H4 acetylation, and methylated H3 lysine 4 (H3K4) and H3K36, caused by active transcription at the particular site where they are localised to (McKittrick *et al.*, 2004). Research has predominantly looked at PTMs of histone's amino terminal tail extension, however recently, PMTs in the histone-fold domains involved predominantly in nucleosome lateral surface formation, have been discovered (Tropberger and Schneider, 2013). Post translational modifications at these sites interfere with effective histone-DNA interactions, weakening the nucleosome and assisting histone exchange, such as in yeast where H3K56 is acetylated and as seen in the acetylation of H3K122 residues that are essential in the unravelling of nucleosomes (Tropberger *et al.*, 2013).

1.8.2 Chromatin Remodelling

In human cells, SWI/SNF-related ATPases activate DNA translocase via ATP hydrolysis, to slide or evict nucleosomes from certain genomic regions and thus cause several alterations to the usual organisation of chromatin within the nucleosome (Pazin and Kadonaga, 1997; Narlikar, Sundaramoorthy and Owen-Hughes, 2013). Consequently, the DNA will 'open' and allow access for targeted histone chaperones to bind (Becker and Workman, 2013). These multi-protein ATP dependent chromatin re-modellers include the homologous Brahma (BRM) and BRG1 (also referred to as SMARCA4) complexes, which associate with GR under specific conditions (Fryer and Archer, 1998; Engel and Yamamoto, 2011) and change nucleosome arrangement to permit DNA access by TFs and other transcriptional regulators (Ostlund Farrants *et al.*, 1997; Collingwood, Urnov and Wolffe, 1999; King, Trotter and Archer, 2012). Direct interactions between GR and numerous subunits of the BRM/BRG1 complexes has been shown to occur at the DBD, LBD and AF1 interfaces of the receptor under context dependent conditions (Wallberg *et al.*, 2000; Muratcioglu *et al.*, 2015).

Histones and other proteins can be methylated by protein methyltransferase CARM1, and G9a/EHMT2 (a lysine methyltransferase). Methyltransferase proteins are able to directly interact with GR or indirectly via co-regulatory proteins already bound at the receptor, such as p160 or p300, where under context specific conditions, GR regulated genes can be activated or repressed (D. Chen *et al.*, 1999; Bittencourt *et al.*, 2012). GRs and histones can also be modified in a context specific manner (Fonte *et al.*, 2005; Shahbazian and Grunstein, 2007), by histone acetyltransferase complexes (HATs), whereby a ε-N-acetyl-lysine is formed at certain Lys residues. GR can be bound directly by CBP, p300, and PCAF at the AF1 domain, or by indirectly interacting with a AF2 bound p160 co-regulator, for transcriptional regulation (Yao *et al.*, 1996; Almlof *et al.*, 1998; Voegel *et al.*, 1998; Wallberg *et al.*, 1999; Jenkins, Pullen and Darimont, 2001), usually activation (Verdin and Ott, 2015). Further, HDACs are part of NCoR-SMRT multi-protein complexes (Stewart and Wong, 2009) that house CoRNR boxes that can bind with GRs LBD. Although not physiologically relevant, this interaction is observed more when GR is activated by RU486 rather than the usual GCs (Ronacher *et al.*, 2009), because RU486-GR LBD binding preferentially positions helix 12 of the GR for CoRNR association (Schoch *et al.*, 2010). However, NCoR-SMRT complexes directly or indirectly associate at IR-GBS or NF-κB- or AP-1 bound to GR occupied regions where for example DEX activated GRs are sumoylated within the NTD (Hua, Ganti and Chambon, 2016; Hua, Paulen and Chambon, 2016).

1.9 Receptor Localisation

Receptor driven transcriptional effects on cellular processing depends on differential expression of MR and GR, and their localisation across brain regions. Reul and De Kloet, identified that MR and GR

differ in their expression patterns in the brain (Reul and de Kloet, 1985). GR is expressed throughout the mouse brain at varying degrees, with the exception of the suprachiasmatic nucleus (Sheppard *et al.*, 1998; Balsalobre *et al.*, 2000). Conversely, MR is more restricted in its expression, but highly expressed in the human and rodent hippocampus. In fact, MR expression equals or exceeds that of GR in the CA3 pyramidal cells (Mahfouz *et al.*, 2016). Further, MR is expressed in the prefrontal cortex and amygdala (Venkova, Foreman and Greenwood-Van Meerveld, 2009; McEown and Treit, 2011; Qi *et al.*, 2013).

MR and GR are expressed in neuronal cells and in a neurone specific context GC effects are modulated via these receptors (Joels, 1997; Ambroggi *et al.*, 2009; Hartmann *et al.*, 2017). Furthermore, oligodendrocytes (van Gemert *et al.*, 2006), astroglia (Koyanagi *et al.*, 2016; Piechota *et al.*, 2017), and microglia (Tentillier *et al.*, 2016) have all been shown to express GR. The role of MR and GR co-expression within the brain vasculature might be important regarding neuronal processes (Gomez-Sanchez, 2014), such as where MR in endothelial and vascular smooth muscle cells may selectively respond to aldosterone rather than cortisol. This is as a result of inactivation of endogenous GCs by 11β-hydroxysteroid dehydrogenase type 2 enzyme expression into their 11-dehydro metabolites, such as with cortisone from cortisol, due to the expression of cortisol's inactivating 11β-hydroxysteroid dehydrogenase type 2 enzyme (Bender *et al.*, 2013).

1.10 Ligand-Activated Glucocorticoid Receptor Transport to the Nuclear Membrane: Cytoplasmic-Nuclear Translocation

In the cytoplasm and in the absence of activating hormone levels, MR and GR are sequestered in a multi-protein complex which retain the receptors in a ligand-binding conformation and are protected from proteolysis (Pratt and Toft, 1997). The multi-protein complex includes a hsp90 dimer, p23 and a tetratricopeptide repeat (TPR)-containing co-chaperone, such as the immunophilin fk506-binding proteins (FKBP) 51 or 52, cyclophilin 40 (CyP40), or protein phosphatase 5 (PP5). The multi protein complex is assembled via ATP-driven pathways and necessitates the contribution of hsp70, hop and Aha1 (Ratajczak, 2015). FKBP51 acts to decrease GR ligand affinity by GR-induced upregulation of FKBP51 expression producing a negative feedback mechanism to decrease ligand sensitivity and reduces the ability of the receptor to translocate into the nucleus (Davies, Ning and Sanchez, 2005; Wochnik *et al.*, 2005; Banerjee *et al.*, 2008, 2015; Cluning *et al.*, 2013). Further, FKBP51 has been suggested to be regulated by MR, where both MR and GR were shown to bind at similar GRE sites (Mifsud and Reul, 2016). Of note, this cellular pathway has been implicated in the development of psychiatric diseases, where epigenetic changes have occurred in the GRE that modulate FKBP51 induction (Klengel *et al.*, 2013). Several studies using both cell lines or animals, although

experimentally different (i.e. endogenous vs exogenous corticosteroids), have demonstrated the fast and effective cytoplasmic-nuclear translocation of the GR for rapid GC signalling to targeted GREs (Picard and Yamamoto, 1987; Hache *et al.*, 1999; Savory *et al.*, 1999; Conway-Campbell *et al.*, 2007).

In the presence of ligand, the complex disassociates or re-structures itself, and FKBP51 is replaced with FKBP52 initiating GR activation. Following these modifications, there are two mechanisms by which GR is translocated into the cell nucleus as multi-protein complex containing FKBP52 and Hsp90, where once within the nucleus the receptors will then dissociate from (Galigniana *et al.*, 2010).

The first, consists of GR binding to Importin molecules (90-120 kDa) (karyopherins) to expose nuclear receptor localisation sequences (NLSs) following dissociation of the complex. GR possess two NLSs (Picard and Yamamoto, 1987) and MR three NLSs (Walther *et al.*, 2005). The N-end of the importin molecule contains an interface able to bind to Ras-related nuclear proteins or GTPase Ran and at the same time the C-end binds to the GRs NLS sequences in preparation for transport through the nuclear pore complex (NPC) and into the nucleus (Nakielny and Dreyfuss, 1999). The predominant GR NLS, NL1, which is located in GRs hinge region, makes contact with heterodimeric importins α- and β-, as well as importins 7, 8 and 13, whereas GRs NL2 sequence housed it the LBD is only identified by importins 7/8 (Freedman and Yamamoto, 2004; Vandevyver, Dejager and Libert, 2012). In both cases this is dependent on ligand binding. Importin molecules regulate the interaction with nuclear pore proteins and upon GR translocation into the nucleus, the importins dissociate from the receptor by binding with a Ran GTPase (Marfori *et al.*, 2011).

Secondly, the GR-chaperone protein complex is re-organised in its conformation, rather than dissociated from the chaperone proteins. When ligand free, FKBP51 is part of the complex, whereas following ligand activation, the former immunophilin is replaced with FKBP52 in order to initiate nuclear translocation of the receptor (Davies, Ning and Sanchez, 2002). From here, FKBP52 will bind the motor molecule Dynein, which attaches to the microtubule cytoskeleton where, with operational support of the complex via an ATP, and nuclear import of MR and/or GR multi-protein complex is passed through the NPC (Harrell *et al.*, 2004).

Nuclear-cytoplasmic exportation of the nuclear receptors occurs by binding of exportin Calreticulin, in a Ca2+ dependant mechanism, to a nuclear export signal (NES) housed within the DBD between the two zinc fingers. The exportin-NES complex makes contact with a Ran GTP and is thus moves out of the nucleus via the NPC.

In both nuclear translocation mechanisms, an equilibrium between the import and export of GRs will determine their subcellular distribution. This seems to occur in a context specific manner, whereby IF

analysis of GR distribution in the ADX rat hippocampus observed abundant detection of MR and GR in the DG, however not in CA1 pyramidal cells (Sarabdjitsingh *et al.*, 2009). Following nuclear translocation, re-association of GR with particular components of the multi-protein complex such as p23 and Hsp90 (although weakly) disrupted receptor driven transcription *in vitro* and *in vivo* (Freeman and Yamamoto, 2002). The microtubule-associated protein doublecortin-like (DCL) was shown to support GR transport in neuronal progenitor cells (Fitzsimons *et al.*, 2008). Here, knockdown of DCL hampered GR translocation, therefore implicating DCL expression in GR transport and signalling regulation. Effective movement of GR from the cytoplasm to the nucleus relies on cooperative interactions with a network of accessory, transport and mechanical proteins, and which will determine GR positioning for cellular processes.

1.11 Localisation of Glucocorticoid Receptors within the Nucleus

As previously described, MRs and GRs bind at specific GRE sites to regulate transcription. The spatial distribution of GR within chromatin regions is advancing with methodological development in microscopic capabilities using proteins with fluorescently labelled tags, thus permitting studies on protein movement patterns (van Royen *et al.*, 2011; Mueller *et al.*, 2013). Fluorescent recovery after photobleaching (FRAP) and single-molecule microscopy experiments performed together observed similar movement of MR and GR within the nucleoplasm (Groeneweg *et al.*, 2014). Schaaf, M.J.M. *et al* (unpublished) discovered differing receptor motility coefficient rates of diffusion throughout the nucleoplasm, revealing four types of GR proteins within the nucleus. Two of the GR molecular complexes are immobile where the duration is determined in a ligand dependent manner (0.5 or 3 seconds). The other two GR molecular complexes readily move through the nucleus at a diffusion coefficient rate of 3.1 $\mu m^2/s$, or with an approximately six-fold lower diffusion coefficient. These GR complexes were able to readily diffuse was assumed as freely moving through the nucleus, whilst the slower diffusing GR receptors involves a brief (< 1 ms) contact at the DNA. They suggested the longer immobilisations as direct or indirect binding events between the receptor and the DNA. Further analysis revealed GRs to be in a longer relatively free diffusion state for >7.5 seconds, usually followed by transition into a slow diffusion state. Proceeding this is a final transition into an immobile state before beginning the diffusion cycle once more from free diffusion to slow diffusion to an immobile state. The transitioning between slow diffusion to immobilised states is referred to as a repetitive switching mode (Meijer, Buurstede and Schaaf, 2019).

1.12 Glucocorticoid Receptors in the Nucleoplasm

Once within the nucleus, the ligand-receptor complex will bind at GREs located at specific DNA sites where it will recruit a number of cofactors and chromatin remodelling proteins, such as the SWI/SNF

complex, and can re-organise the chromatin landscape allowing the recruitment of basal transcription factors RNAPII initiating transcription (Kino, Nordeen and Chrousos, 1999; Wallberg *et al.*, 2000). Once GR is ligand free, the receptor will export from the nucleus and into the cytoplasm and completes the nuclear-cytoplasmic protein cycling (Vandevyver, Dejager and Libert, 2012). Vandevyver *et al* (2012) observed complete ligand activated GR nuclear translocation to occur in as little as 3-5 minutes. On the other hand, nuclear export of GR requires several hours to complete and thus it can be said that the duration of GR retention is over a long period (Vandevyver, Dejager and Libert, 2012). Cycling on and off the chromatin is known to occur, where the ligand-receptor complex binding-dissociation at GREs is repeated under tens of seconds. For example, in a cell line containing a tandem array of MMTV LTRs with various GREs (800–1200 GREs in a single cluster) that were transfected with a combination of GR, SWI/SNF complex and ATP proteins, GR was found to cyclically interact with the LTR fragment for up to one minute and followed by dissociation for 4-5 minutes. This was similarly observed with the SWI/SNF complex (McNally *et al.*, 2000; Nagaich and Hager, 2004). Dissociation of the ligand-receptor complex enables the available GR to interact once more with the same nuclear chaperones and regulatory proteins enabling ligand binding. The ligand-receptor complex then dissociates from the chaperone proteins within the nucleus and interacts at the specific DNA sites. This GR turnover within the nucleus is modulated by chaperone binding and referred to as the chaperone cycle, and is thought to explain the long term retention of GR within the nucleus (Stavreva, Varticovski and Hager, 2012; Miranda, Morris and Hager, 2013).

1.13 The Nuclear Lamina

Recent research has suggested the involvement of disrupted Lamin Associated Domains (LADs) in molecular pathways such as nuclear mechanotransduction and disease development. The nuclear lamina (NL) supports the nuclear structure and rigidity as well as maintaining heterochromatin and transcriptional proteins organisation. The NL is a sub nuclear compartment composed of a thin network of V-type filaments lining the inner nuclear membrane, thereby establishing a platform for chromatin binding and facilitating chromatin arrangement. The region of chromatin networks which associate with the NL are named lamin associated domains (LADs) and house an abundance of genes weakly transcribed and are enriched for repressive histone modifications. LADs adapt and change their spatial arrangement in a cell type specific manner and in response to gene expression during differentiation and development. In eukaryotic cells, lamin A/C is required for efficient organisation of the heterochromatin at the nuclear periphery. In Drosphilia S2 cells, disruption of the NL was responsible for overall chromatin compaction and compromised the three-dimensional organisation of active and inactive chromatin regions. NL disruption also led to decompaction of some NL-attached

TADs related to fractional de-repression of their chromatin (Ulianov *et al.*, 2019). The NL are known to be involved in transcriptional regulation of GR specific chromatin regions. For example, silencing lamin A/C was found to significantly increase expression of shear stress induction and alterations in histone protein function following DEX treatment. PCR data revealed increased expression for NF-κB inhibitor alpha and dual specificity phosphatase 1 (DUSP1) genes when lamin A/C was silenced. This suggests that in the absence of lamin A/C, functional transcriptional regulation of specific GREs is hindered, but GR nuclear translocation is not affected (Nayebosadri and Ji, 2013).

1.14 Steroid Receptors at the Membrane: Non-Genomic Effects.

Corticosteroid hormones are also able to elicit fast membrane elicited or non-genomic effects, independent of the classical DNA binding. These rapid signalling events modulated by MRs and GRs may be localised at or near the membrane, although how remains to be elucidated. These nongenomic effects are thought to proceed the receptors dissociation from chaperone complexes within the cytoplasm (Gutierrez-Mecinas *et al.*, 2011). In addition, it is important to consider the increased corticosteroid concentrations required for initiation of membrane bound GR effects in comparison to those required in classical genomic modulation (Karst, 2005; Nahar *et al.*, 2015). Studies have identified a facilitative role for nongenomic GR mechanisms in initial responses to a stressor, whereby rapid mediators such as noradrenalin and MR-dependant effects will influence the adaptive response in the brain (Joels, Sarabdjitsingh and Karst, 2012; Jiang, Liu and Tasker, 2014). Of note, these nongenomic GR mechanisms not only occur prior to classical genomic pathways within the same pulse of phase of corticosteroid activation, but can also determine genomic mechanisms during metaplasticity (Karst *et al.*, 2010), as demonstrated with ion channels in hippocampal cells (Groeneweg *et al.*, 2011, 2012; Chatterjee and Sikdar, 2014).

A rodent study using patch clamping observed the corticosterone inhibition of NMDA receptor currents in cultured hippocampal primary neurons (Liu *et al.*, 2007). Furthermore, nerve impulse transduction in peripheral neurons was blocked when a topical corticosteroid was applied to the cells (Johansson, Hao and Sjölund, 1990). Recently, the role of peripheral MR in neuronal and glial cells in pain modulation has increased attention. Shaqura *et al* (2016) identified a crucial role for MR, under increasing doses of MR agonist aldosterone, on peripheral nociceptive neurons and their behaviour, suggested to be via non-genomic pathways under healthy and significantly in pathological conditions (Shaqura *et al.*, 2016).

Although these rapid nongenomic effects can occur independent of receptor binding (Gasser and Lowry, 2018), studies using MR/GR knockout mice observed many cellular processes mediated via these receptors were abolished (Karst, 2005; Nahar *et al.*, 2015).

Nongenomic modulation has been reported in the vasculature via aldosterone activated MR pathways (Dooley, Harvey and Thomas, 2012). In endothelial cells and murine heart tissue, aldosterone was able to disrupt striatin and MR interactomes, which could not be recovered following spironolactone treatment (Pojoga *et al.*, 2012; Ashton *et al.*, 2015). In vascular cells and tissue of mice, striatin levels were increased under high aldosterone induced MR activation (Ricchiuti *et al.*, 2011; Pojoga *et al.*, 2012). Upon reduction of striatin levels in endothelial cells, aldosterone-regulated non-genomic MR-induced ERK phosphorylation was decreased, without interfering with EGF mediated ERK phosphorylation or genomic MR transduction (Coutinho *et al.*, 2014). A study using heterozygous striatin KO mice, a nominated non-genomic MR signalling pathway, the pAKT:AKT ratio, is diminished and MR expression and genomic transduction is enhanced (Garza *et al.*, 2015). Petta *et al* (2017) identified novel GR-STRN3 interactions, a protein of the striatum family, and reported STRN3 overexpression to decrease GR transactivation via decreased phosphorylation of GR at S211, while silencing enhanced GR transactivation following enhancement of GR phosphorylation at the same residue. In addition, absence of STRN3 expression enhanced endogenous mRNA levels of GR activated specific genes DUSP1, encoding MKP1, a potent anti-inflammatory mediator, PER1 and ZFP (Petta *et al.*, 2017). STRN family is believed to be a prominent regulator of steroid and peroxisome proliferator induced nuclear receptors, and has been reported to modulate GR, MR, ER and PR function. In these ways, experimental data using knockout models and comparisons with other nuclear receptors suggest signalling by membrane-bound GRs is important for key cellular processes.

1.15 Differential Mineralocorticoid and Glucocorticoid Receptor Effects

Primarily, the MR and GR expression and/or activation across different cell types, throughout the brain for example, will determine which receptor will modulate the effects of circulating corticosteroids. In the middle temporal gyrus of human cortex, single-nucleus RNA-sequencing analysis was used to perform a comprehensive study of cell types, whereby 69 types of excitatory and inhibitory neurons were identified (Hodge *et al.*, 2019) and which variable expression levels of GR and MR in all individual 45 inhibitory and 24 excitatory neuronal cell types of the human temporal cortex have been reported. Currently, MR appears to have increased expression in C3 pyramidal cells in the hippocampus and in a subset of GABA-ergic cortical neurons, compared to GR. Although cells expressing MR only have not been identified, it can be suggested that GR would be minimally active if at all, in neurons only expressing aldosterone selective MR (Sarabdjitsingh, Meijer and de Kloet, 2010).

Co-expression of MR and GR produces differential regulation of cellular processes. MR mediated corticosteroid, follow a U-shaped effect, stimulating the excitability of hippocampal CA1 neurons by supressing the responsiveness to 5-HT1A receptor and calcium currents. Concurrent GR activation

produces the reverse effects seen with MR. As such, MR and GR regulate differential cellular effects on neuronal excitability via varying corticosteroid affinities (Joels, 2006). Differential receptor effects are also further complicated by MRs and GRs varying N-terminus lengths as a result of alternative mRNA translation (Lu and Cidlowski, 2005; Faresse, 2014) and relative activity between the receptors as a result of PTMs (Vandevyver, Dejager and Libert, 2014).

MR and GR can differentially determine transcriptional activity dependant on their presence in the same cell and whether they bind at targeted GREs (via both direct and indirect mechanisms), the ability for differential binding to nGREs, differential protein-protein interactions between transcription factors, and differential associations with downstream coregulators following receptor-DNA binding. GR binds to nGREs (Hudson, Youn and Ortlund, 2013) and exerts GR dominant transpressional effects of transcription factor AP-1. Whereas, GR and MR differentially regulate NF-κB activation and neuroinflammatory parameters in BV-2 cells. Conversion of inactive 11-dehydrocorticosterone to active corticosterone, 11β-HSD1 was shown to mediate the coordinated action of GR and MR. Specifically, an MR-dependent potentiation of IL-6 and tumour necrosis factor-α (TNF-α) expression and NF-κB activation at low/moderate concentrations and a GR-dependent suppression at high concentrations was observed (Joels, 2006; Chantong *et al.*, 2012).

1.16 MR:GR Balance Hypothesis

The role of MR:GR balance in modulating various cellular and allosteric pathways, and stress adaptation, has gained momentum over recent years. The MR:GR balance changes according to context specific changes during a lifespan (Reul and de Kloet, 1985; De Kloet and Reul, 1987; E Ron de Kloet, Joëls and Holsboer, 2005; Joels, Sarabdjitsingh and Karst, 2012; de Kloet, 2014). MR and GR are expressed throughout the limbic system and together function in a complementary manner to regulate HPA-axis activity (Joels, Hesen and de Kloet, 1991; McEwen and Stellar, 1993; McEwen and Gianaros, 2010). The appropriate exposure to GCs following stress initiate the selection of a suitable behavioural response and memory development (Karst, 2005; Joels *et al.*, 2008; Melanie D Klok *et al.*, 2011). However, prolonged activation by glucocorticoids, as occurs in long term exposure to stress, or high levels of GCs, are thought to dysregulate the HPA axis thus hindering an efficient stress response (de Kloet, Oitzl and Joëls, 1999). Corticosterone was shown to supress stress induced HPA axis activity more potently than cortisol and highlights varying affinities of cortisol and corticosterone to MR and GR. GR is activated by cortisol and cortisone, but not 11-deoxycorticosterone, whereas MR is activated by 11-deoxycorticosterone and cortisol, but not cortisone (Baker and Katsu, 2017). In the mammalian brain, cortisol is an MR agonist in tissues that possess sufficient 11β-hydroxysteroid

dehydrogenase type 2, which converts cortisol to cortisone (Funder *et al.*, 1988; Geerling and Loewy, 2009).

GR expression in the brain is directly associated with autonomous functional HPA axis regulation, irrespective of pituitary GR activity and are thought to contribute to symptoms of anxiety, depression and executive functioning (Kolber, Wieczorek and Muglia, 2008). GR activity in heterozygotic (Ridder *et al.*, 2005) or forebrain restricted (Boyle *et al.*, 2005) GR knockout mouse models have also been shown to elicit depression-related behaviours. Conversely, anxiolytic symptoms decrease along with an absence of any behavioural effects in mouse models where GR has been depleted in neuronal or glial cells (Tronche *et al.*, 1999), and with antisense GR inhibition (Montkowski *et al.*, 1995). Although, in transgenic mice, overexpressed forebrain GR produces heightened anxiety and depressive symptoms (Wei *et al.*, 2004). However, it must be noted that such contrasting observation may be due to experimental designs with restricted tissue regions where the receptor may produce opposing or complicated effects (Whirledge and DeFranco, 2017).

In neuroendocrine functions of stress, anxiety and cognition, MR plays a significant role. In mouse models of disrupted MR expression or altered using MR antagonists (de Kloet *et al.*, 2000; Kolber, Wieczorek and Muglia, 2008; Baker and Katsu, 2017), inhibition or knockout of brain MR negatively effects cognitive function such as memory retention and dysregulates the HPA axis (Oitzl and de Kloet, 1992; Conrad *et al.*, 1997; Berger *et al.*, 1998; Douma *et al.*, 1998; Yau, Noble and Seckl, 1999; Schwabe *et al.*, 2013; de Kloet and Joëls, 2017). On the other hand, overexpressed forebrain MR in transgenic mouse models improves anxiety related behaviours.

1.17 Gene Regulation

It is suggested that MR and GR can bind at receptor specific and shared DNA binding sites, either as homodimers or heterodimers, as well as at specific target genes. Whole genome MR and GR binding comparison studies have been performed, as well as subsequent motif analysis of genes bound separately by either receptor or both together, and whether these contain GRE type sequences or available tethering proteins. In human kidney cells, observations of MR-DNA bound fragments revealed the most prevalent motif contained a GRE sequence, however most MR bound sites involved indirect DNA binding via other transcription factors (Le Billan *et al.*, 2015; van Weert *et al.*, 2017) and this has been similarly observed for GR in rat tissue (John *et al.*, 2011; Uhlenhaut *et al.*, 2013). Conversely, various rat hippocampal ChIP experiments propose MR/GR to exert their effects mostly by direct binding at GRE sequences (Polman et al. 2013; Pooley *et al.*, 2017; van Weert *et al.*, 2017). Intriguingly, DNA motifs were observed proximal to GREs (Polman et al. 2013; Pooley *et al.*, 2017; van Weert *et al.*, 2017). Further comparative analysis of these GREs at known GC inducible target genes

in rat hippocampal tissue showed several highly conserved GRE sequences at these genes (Datson *et al*. 2013). Although, GR binding was reported at only half of these GREs and in which several TF binding motifs (Datson *et al*. 2011) such as NF-1 were identified. In addition, binding motifs for TFs of NeuroD were observed for ~15% of GR binding sites (Pooley *et al.*, 2017). As previously discussed, MR/GR can regulate transcription by binding to the same GRE sequences of known genes where they can exert receptor specific effects within the same cell type via particular genomic pathways (Joëls *et al*. 1991; Karst *et al*. 2000). A recent study revealed common genomic sites for binding of both receptors, as well as specific loci at which only MR or GR were bound to. For example, proximal to the identified MR targeted loci, the NeuroD sequence was detected, but not at GR specific sites (van Weert *et al.*, 2019). Specifically, chromatin immunoprecipitation experiments (CoIP) on rat hippocampal DNA revealed NeuroD2 protein proximal to the MR targeted loci (~300 nucleotides distance) and in forebrain MR depleted mice, NeuroD2 remained at such loci, indicating that NeuroD2 binds before MR (van Weert *et al.*, 2019). From this it can be suggested that NeuroD may specifically regulate the binding of MR to targeted GREs in hippocampal neurones. Furthermore, lack of MR expression was not replaced by GR binding at the shared receptor genes, and neither did GR associate at MR targeted genomic sites. However, in the MR-knockdown mouse model, GR binding was enhanced at the Per1 gene (van Weert *et al.*, 2019), although this may be due to the high affinity of GR to the locus (Reddy *et al.*, 2012). This further supports early indications of specific genomic sites where MR and GR can bind as homo- or hetero- dimers (Liu *et al.*, 1995).

A study applied rat hippocampal MR chromatin binding profile to identify unique MR targeted genes. Site selection required MR to be localised in intronic regions or within 5 kilobases of transcription start sites (TSSs). Where MR was found to bind the same sites in the mouse hippocampus (Berger *et al.*, 2006), these were applied to mouse models with MR knocked down in the forebrain. Expression of Jdp2. Nos1ap, and Supv3l1 mRNA decreased (~50%) in MR deficient mice indicating such genes as specific to MR (van Weert *et al.*, 2019), and there may be many more MR-unique targets to discover. Identifying MR controlled genes could provide functional insight into the unique role played by MR in stress and steroid pathways.

Identifying unique effects for each receptor, separately or together, *in vitro* and then their clinical function is essential. GREs can be targeted by both MR and GR and thus several targeted mRNAs for assessing GC effects, such as Perl (Reddy *et al.*, 2012; Le Billan *et al.*, 2018; Fletcher *et al.*, 2019), Sgk1 (Webster *et al.*, 1993; S. Y. Chen *et al.*, 1999), GILZ (D'Adamio *et al.*, 1997; Soundararajan *et al.*, 2005), and FKBP5 (Mifsud and Reul, 2016), have been shown to be stimulated by both receptors. Furthermore, proximal to several MR specific genes, basal levels of FKBP5 mRNA were considerably

downregulated in the mouse hippocampus (van Weert *et al.*, 2019). The ability of both receptors to bind at and regulate the same transcripts independently and/or together, indicates a cooperative regulatory effect on cellular responses to GCs. This is further corroborated whereby MR and GR are both involved in the negative feedback on the HPA axis. GR regulates negative feedback in the hypothalamic-pituitary brain regions, whilst MR regulates feedback in the hippocampus (De Kloet and Reul, 1987; Bradbury *et al.*, 1991; van Haarst, Oitzl and de Kloet, 1997). This cooperative regulation of the HPA axis is likely to occur progressively from trough to peak GC levels. Of note, it is important to consider that the regulation of certain genes and pathogenic epigenetic changes in the gene, such as with FKBP5, are usually attributed to GR function only (Klengel *et al.*, 2013; Yehuda *et al.*, 2013).

Genetic variants for both receptors and epigenetic alterations can dysregulate the MR:GR balance (M D Klok *et al.*, 2011; Melanie D Klok *et al.*, 2011). Genetic polymorphisms of MR include the common MR -hap1, -hap2 and -hap3, and MR-hap4, although the latter rarely occurs and possesses low frequency and transactivation strength. Sex dependant differences have been observed, whereby MR haplotypes moderated via progesterone activation influenced depression susceptibility following childhood maltreatment (Vinkers *et al.*, 2015; Hamstra *et al.*, 2016). MR variants influence electrolyte and volume regulation (van Leeuwen *et al.*, 2010), and the stress response (M D Klok *et al.*, 2011). For example, the MR functional c.-2G > C variant controls blood pressure, renin and aldosterone levels (van Leeuwen *et al.*, 2010) and in transfected cell models an MR haplotype 2 produces the most increased expression and transactivation compared to other genetic polymorphisms (M D Klok *et al.*, 2011). This variant has also been identified in patients to produce decreased chronic stress levels (van Leeuwen *et al.*, 2011). Furthermore, mouse studies using MR knockout models were not able to survive past postnatal day 10, and elevations in plasma renin, angiotensin II, aldosterone, hyperkalemia and hyponatremia were observed (Berger *et al.*, 1998). Sex differences bias the function of MR haplotype 2 in childhood maltreatment and depressive symptoms. Supporting this, females with this genetic polymorphism observe a higher predisposition to optimistic traits and some resilience against depression and which is further corroborated in MR haplotype-2 homozygotes demonstrating positive affect. MR haplotypes regulate the effects of estradiol, progesterone, menstrual cycle and emotional information processing, and are indicated in contraceptive use (Hamstra *et al.*, 2015, 2016, 2017).

1.18 Glucocorticoids and the Hypothalamic Pituitary Adrenal Axis

In the 1950s, Hans Selye, was the first to classify stress as a non-specific biological response and the significant role played by the HPA axis in modulating the adrenal axis stress response. He termed the stress response as *"a nonspecific response of the body to any demand made upon it"* (SELYE, 1950).

This led to future discoveries for critical roles of the adrenal cortex stress response in functional regulation of the immune system, cognitive processes and metabolism (SELYE, 1950; McEwen, 2007; Lightman and Conway-Campbell, 2010). The stress response is mediated through anatomical structures found both in the central nervous system (CNS) and peripheral tissues, namely the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland situated caudally and above the optical chiasm, and the adrenal gland, which together form the HPA axis. Also involved in mediation of the adaptive stress response are the brain stem noradrenergic neurons, sympathetic adrenomedullary circuits and parasympathetic systems (Chrousos, 1992; Whitnall, 1993; Habib, Gold and Chrousos, 2001).

1.19 Steroid Hormones

In 1929, the first extracts of steroid hormone preparation were taken from the adrenal cortex and the development of cortisone as a therapeutic agent began. Upon realisation of symptom management of adrenal insufficiency both in adrenalectomized animals and in Addison's disease patients, in 1935 cortisone was separated as a new compound. Following this, in 1949 synthetic steroid compounds were developed and trialled for their potent anti-inflammatory effects. Out of twenty-eight compounds that were originally separated from the adrenal cortex, four possessed physiological activity, and were originally termed A, B, E, and F. Compounds B and F, cortisone and cortisol, respectively, successfully treated inflammatory conditions such as rheumatoid -arthritis and -fever, and today remains the first line treatment (Hench and Kendell, 1949; Kendall, 1949; Hench *et al.*, 1950).

Peaks of corticosterone release during ultradian and circadian cycles are involved in control of basal metabolic demand and the adaptive stress response (Lightman and Conway-Campbell, 2010). GCs are lipophilic by nature and accordingly diffuse into various tissue during circulation, including the brain (Jones, Hillhouse and Burden, 1977). The endogenous GCs secreted form the adrenals are 'cortisol' in humans and 'corticosterone' in the rat. In humans, the circulating blood concentration ratio of cortisol:corticosterone fold increase is 20 and in the brain this reduces by 6 fold to 3 times higher (Karssen *et al.*, 2005). Although circulating blood plasma levels of corticosteroids are high, under 10% of total levels are freely circulating, with the rest bound to corticosteroid binding globulin (CBG) which are major transport proteins. The half-life of free corticosterone is dependent on its systemic localisation, but in healthy female rats the half-life of total corticosterone was reported to be 8.6 ± 1.4 min from a 10-minute blood sampling window (Windle *et al.*, 1998, 2006). On the other hand, the half-life of the main corticosteroid in humans, cortisol, was calculated to be between 62-97 minutes (Weitzman *et al.*, 1971; Weitzman, 1976; Veldhuis *et al.*, 1989).

1.19.1 Synthetic Glucocorticoids

Exogenous ligands can dysregulate the MR:GR balance. DEX is a potent synthetic GC and causes HPA axis suppression which is observed by significant declines in endogenous circulating concentrations of corticosterone and cortisol. In the brain, low levels of administered DEX struggle to cross the blood brain barrier (BBB) but with increasing doses of DEX will bind GR, however interestingly MR is rendered absent of corticosterone and cortisol (De Kloet, Wallach and McEwen, 1975; Meijer *et al.*, 1998; Karssen *et al.*, 2005). Studies have shown in rodents with DEX dysregulated HPA axis, observed impairments in cognitive function that with corticosterone intervention were restored (Liston and Gan, 2011; Liston *et al.*, 2013).

Adverse effects from DEX treatment regimens are often reported in human studies. Observed interruption of effective and restorative sleep during DEX treatment was restored by administering at the same time fludrocortisone, an MR agent, and this restoration success is thought to be attributed to the first sleep phase where slow wave sleep is initiated by central MR activation (Born *et al.*, 1991; Groch *et al.*, 2013). In addition, in humans acute DEX treatment was accompanied with reports of increased energy and chronic administrated patients were depressed, anxiolytic, aggressive, poor self-esteem, physiological stress and with disturbed sleep patterns. These symptoms from chronic DEX treatment were all ameliorated with co-treatment of fludrocortisone (Plihal *et al.*, 1996). Another clinical application for co-administered fludrocortisone with high chronic DEX is in types of childhood leukaemia which recovered previously reported serious psychiatric disorders and disruption to sleep patterns in a third of patients. This further highlights the importance of imitating as best, exogenously the endogenous cortisol release rhythms, with synthetic GCs (de Kloet, 2014; Nixon *et al.*, 2016; Meijer and de Kloet, 2017).

1.20 Anatomy

Stress mediated activation from aversive physiological of psychological inputs, stimulate the HPA axis (Pariante and Lightman, 2008; Koch *et al.*, 2017). The suprachiasmatic nucleus (SCN) located in the anterior hypothalamus of the brain is considered the main ultradian and circadian regulator ('the master clock') of pulsatile GC release and has efferent projections to neuroendocrine cells in the PVN which activate the HPA axis (Engeland and Arnhold, 2005). The SCN regulates the HPA axis by innervating to the PVN, a medial forebrain bundle which houses parvocellular neurosecretory cells, as well as the hypothalamic preoptic area and dorsomedial nucleus. Parvocellular neurosecretory cells synthesise corticotropin-releasing hormone (CRH) or -factor (CRF) and arginine vasopressin (AVP) which is released via the median eminence into the hypophyseal portal vessels to the anterior pituitary gland (Vale *et al.*, 1981; Rivier and Vale, 1983; Lightman *et al.*, 2008) (figure 5). CRF binds to

corresponding receptors on pituitary corticotrophs to regulate adrenocorticotropic hormone (ACTH) synthesis and secretion from the anterior pituitary gland into the pulmonary network where it is circulated diffusing into various tissues and in the zona fasicullata of the adrenal cortex stimulates GC production. GCs are released into circulation and regulate physiological processes upon binding to their GRs, as the downstream HPA axis effectors (Munck, Guyre and Holbrook, 1984; Bamberger, Schulte and Chrousos, 1996).

Corticotropin-releasing factor (CRF) is the primary regulator of the highly synchronised and oscillating release of ACTH from the anterior pituitary corticotropes (Rivier and Vale, 1983) with a 10 minute delay between release from the pituitary to the synthesis and release of GCs from the adrenal cortex (Carnes *et al.*, 1989; Iranmanesh *et al.*, 1990; Jasper and Engeland, 1991; Henley *et al.*, 2009; Rankin *et al.*, 2012; Walker *et al.*, 2012). The action of CRF are mediated by two CRF receptors classified within the B family of G-protein coupled receptors (Perrin and Vale, 1999). The gene encoding CRF type 1 receptor (CRFR1) produces one functional variant (α) in humans and rodents (as well as non-functional splice variants) (Chang *et al.*, 1993; Chen *et al.*, 1993; Vita *et al.*, 1993) and the CRF type 2 receptor (CRFR2) gene encodes functional genetic variants α, β, and γ in humans and α and β in rodents (Perrin *et al.*, 1995; Stenzel *et al.*, 1995). CRF is further believed to be involved in the regulation of the autonomous nervous system (ANS), learning and memory and reproductive behaviours (Valentino, Foote and Aston-Jones, 1983; Valentino and Foote, 1988; Chatterton, 1990; Petraglia *et al.*, 1994; Contarino *et al.*, 2000; Croiset, Nijsen and Kamphuis, 2000; Richard, Lin and Timofeeva, 2002). CRF's are ubiquitously distributed within the CNS and in select peripheral tissues. CRF is predominantly present in the parvocellular neurosecretory cells of the PVN but is also present at lower levels in the olfactory bulb, bed nucleus of the stria terminalis (BNST), medial pe-optic area, lateral hypothalamus, central nucleus of the amygdala, Barington's nucleus, dorsal motor complex, and inferior olive (Sawchenko *et al.*, 1993). In peripheral tissues, CRF has been identified in the adrenal medulla, testis, placental cells, gastrointestinal tract, thymus, and skin (Bruhn *et al.*, 1987; Audhya *et al.*, 1989; Bale and Vale, 2004).

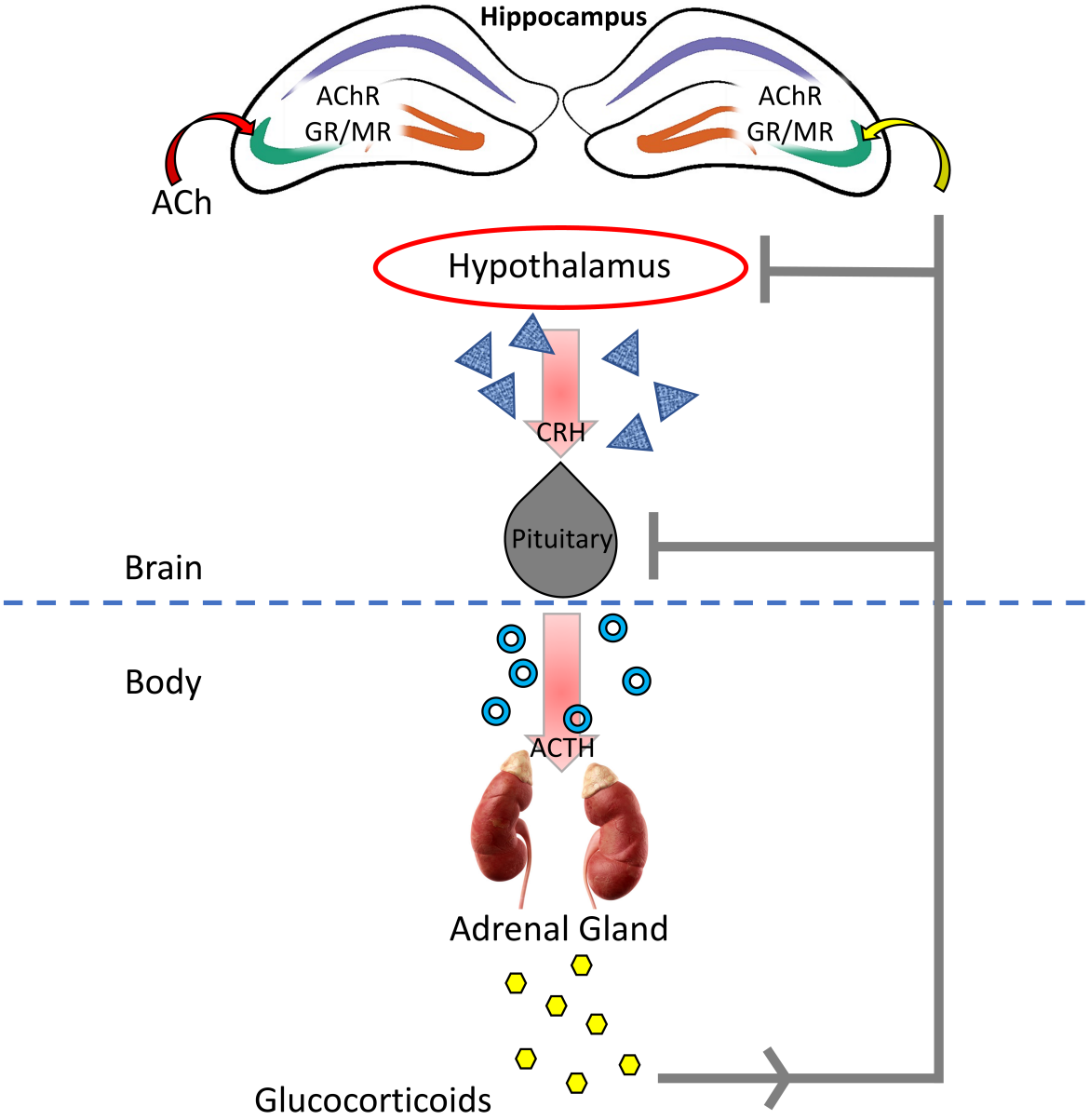


Figure 5 The role of the hippocampus in modulating the HPA (hypothalamic pituitary adrenocortical) axis.

Following a stressor, glucocorticoids are secreted, which exerts feedback to the hippocampus, hypothalamus, and pituitary. CRH, released from the hypothalamus activates the secretion of ACTH from the anterior pituitary, which in turn stimulates the secretion of glucocorticoids (cortisol in humans) from the adrenal glands and are released into circulation. When a threshold of glucocorticoids blood concentration is achieved, further secretion of glucocorticoids is inhibited via a negative feedback mechanism. Acetylcholine (ACh) is also involved in mediating neuroendocrine, emotional, and physiological responses in tandem with the HPA axis. AChR, acetylcholine receptors; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone.

1.21 Fast and Delayed Stress Responses in the Brain

The HPA axis follows a ultradian profile, whilst corticosteroid concentrations are under a circadian rhythm that build to peak secretion levels in humans during the early waking hours of the morning, but in rodents these peaks are observed in the beginning of the evening (Lightman and Conway-Campbell, 2010). As GC concentrations increase, a negative feedback loop is triggered whereby CRH neurons within the PVN of the hypothalamus and ACTH synthesising corticotropes of the anterior pituitary gland directly and rapidly cease CRH production and ACTH secretion (Jones, Hillhouse and Burden, 1977; Plotsky and Sawchenko, 1987; Harbuz and Lightman, 1989). Not only is the adaptive stress response reliant on physiological structures and cellular pathways, but the timing and length of stress exposure and the consequential events are pivotal to the response (de Kloet, 2013). Disruption of the negative feedback look in HPA axis activation can cause prolonged chronic exposure to GCs (Russell, Kalafatakis and Lightman, 2015) which can dysregulate homeostasis in the brain and are suggested to underpin the development of various pathologies (Munck, Guyre and Holbrook, 1984; McEwen and Stellar, 1993). Mediating neural and endocrine systems produce adaptive responses within seconds following identification of a stressor and requires regulation of limbic cortical circuits (Bains, Wamsteeker Cusulin and Inoue, 2015). During an acute response, the PVN modulates the 'fight or flight' response through neural and endocrine pathways (Dallman, 2005). Following GC release, the rate of PVN excitatory postsynaptic currents (mEPSCs) are subdued driving glutamatergic excitability reduction and enhancement in GABA inhibition (Di *et al.*, 2005), predominantly via non genomic GR and endocannabinoid signalling pathways (Di *et al.*, 2003; Verkuyl, Karst and Joels, 2005). On the other hand, MR modulated GC effects increase mEPSC excitatory activity in CA1 pyramidal cells (Karst and Joëls, 2005; Olijslagers, 2008; Groeneweg *et al.*, 2012) and in the BLA neurons even following GC washout, of which the latter occurs via GR and cannabinoid receptor 1 (Karst *et al.*, 2010). Furthermore, GR driven glutamate release is increased following acute stress stimulus in the PFC.

The immediate responses to acute stress stimuli are proceeded by a delayed effects in limbic-cortical structures (Joels, Sarabdjitsingh and Karst, 2012), such as in CA1 neurons where via GR control mEPSC amplitude is increased (Karst and Joëls, 2005; Martin, 2009). Furthermore, delayed GC effects via GR, modulate synaptic plasticity of CA1 neurons, stimulating long term depression (LTD) and disrupting long term potentiation (LTP), whereas MR increases LTP activation (Pavlides *et al.*, 1996). Excitability of neurones in the BLA is increased following delayed responses to GC releases (Duvarci and Pare, 2007), and in the PFC glutamatergic transmission is increased and inhibitory post-synaptic currents is decreased (Hill *et al.*, 2011). As such, the interplay between these limbi-cortical structures maintains

a homeostatic environment enhancing the retention of appropriate stress response behaviours for the future (Joels, Pasricha and Karst, 2013).

When there is prolonged exposure to GCs, alterations in limbic cortical structures and in reward systems occur (Joels, Pasricha and Karst, 2013; Russo and Nestler, 2013). Reductions in dendritic complexity of hippocampal and pre-frontal cortex (PFC) areas have been observed (McEwen and Magarinos, 1997; Holmes and Wellman, 2009), as well as the converse increase in dendritic density and excitatory tone and decrease in inhibitory tone by neurones of the basolateral amygdala and the nucleus accumbens (Christoffel *et al.*, 2011; Muhammad, Carroll and Kolb, 2012). LTP in CA1 cells of the hippocampus is dysregulated under chronic stress, as well as decreasing AMPA and NMDA-mediated synaptic transmission (Joels, Sarabdjitsingh and Karst, 2012; Yuen *et al.*, 2012). As such, alterations in brain structures, such as hypertrophy in the amygdala were reported to cause anxiolytic behaviours (Mitra and Sapolsky, 2008) and changes in hippocampal and PFC structures associated cognitive decline (Joels, Sarabdjitsingh and Karst, 2012). Furthermore, decreases in brain-derived neurotrophic factor (BDNF) expression, a canonical nerve growth factor involved in growth and maturation of neurons, in the ventral tegmental area (VTA) were observed (Krishnan *et al.*, 2007).

1.22 Protein-protein interactions (PPIs)

Evaluation of intracellular PPIs can provide significant developments in hormone signalling in various hormone dependant cellular pathways. What is more, the detection and quantification of protein complexes can enhance diagnostic and prognostic assessment in comparison to the measurement of individual proteins. Various techniques have been developed for the detection of PPIs, including xenograft or transgenic mice for *in vivo* imaging (Sun *et al.*, 2016; Komatsu *et al.*, 2018). PPIs are predominantly studied using *in vitro* cell culture models, despite advances in gene recombination and Ab manufacturing for this purpose. The dynamic aspects to PPIs can be assessed in live cells *in vitro* using advancement in technologies and methods (Michnick *et al.*, 2010; Yurlova *et al.*, 2014; Malleshaiah, Tchekanda and Michnick, 2016). What is more, novel technologies of *in vitro* PPI analysis are emerging, such as the advancements in molecular probe design (Choi *et al.*, 2018; Lin *et al.*, 2018; Wang *et al.*, 2018). However, identifying subcellular PPIs by histological and pathological detection in tissue samples *in vivo* is proving difficult in comparison to in vitro assessment *in vitro*.

Dimeric proteins of nuclear receptors have mainly been identified by bioluminescence resonance energy transfer (BRET)/förster resonance energy transfer (FRET) analyses, double label indirect immune fluorescence (DIIF) microscopy and recently structured illumination microscopy (SIM) has been developed, however in the latter the resolution of conventional fluorescence microscopy is restricted to no more than 200 nm due to the diffraction limit of light. The intracellular dynamics of

GRs have followed subcellular diffusion of MR and GR in living cells using a GFP tagged receptor in combination with its other Fluor variants (Htun *et al.*, 1996; Fejes-Tóth, Pearce and Náray-Fejes-Tóth, 1998; Nishi *et al.*, 2001; DeFranco, 2002). McNally et al (2000), identified the rapid exchange of activated GR between chromatin and subnuclear compartments using fluorescent recovery after photobleaching technique (FRAP) (McNally *et al.*, 2000; Reits and Neefjes, 2001). The earliest study investigating receptor complex formation reported binding as homodimers to hormone response elements (HREs). Following this, successive physiological studies in numerous models proposed MR and GR to interact (Gomez-Sanchez *et al.*, 1990; Oitzl and de Kloet, 1992; Joels and de Kloet, 1994), which was later suggested to form as a MR-GR heterodimer with functional interactions using a luciferase reporter system (Trapp, Rupprecht, Castrén, *et al.*, 1994; Liu *et al.*, 1995; Savory *et al.*, 2001). Though this was not assessed in relation to the sub cellular compartmentalisation of the receptor complex in intact cell structures, but in extracted cell lysates only. The visualisation of MR:GR interactions is limited; however, a study reported MR and GR heterodimers following ligand activation using green fluorescent protein-based fluorescence resonance energy transfer (FRET) microscopy in cultured hippocampal neurons and non-neural cells, and identified dynamic interactions between MR and GR in a spatiotemporal-specific manner (Nishi *et al.*, 2004). Forster resonance energy transfer (FRET) by lifetime measurement, and fluctuation analysis by cross-correlation number and brightness assay (ccN&B) was previously used to assess MR-GR interactions in living 3617ChMR cells (Pooley, J., Presman, D., Lightman, S., & Hager, 2015). The accumulated fluorescent GFP-tagged GRs and mCherry-MRs at the tandem array of the MMTV long terminal repeat (800–1200 GREs) driving viral Harvey-Ras expression was used to study MR and GR interactions at chromatinised DNA, together with standard co-immunoprecipitation (CoIP) and computational predictions for MR-GR interactions.

Proximity based labelling methods are another way to discover catalogues of protein associations, over varying time frames in living cells. Proximity-labelling based methods are able to identify candidate interacting proteins or the components involved in subcellular compartments such as with proximity dependant biotin identification (BioID). BioID is a proximity dependent biotinylation method capable of identifying PPIs by labelling proteins in proximity to the protein of interest in living cells (Roux *et al.*, 2012; Roux, Kim and Burke, 2013). The biotin ligase BioID is fused to the targeted protein of interest and exploits a mutant BirA found in Escherichia coli, whereby the conserved biotin- and bioAMP binding domain is mutated (Choi-Rhee, Schulman and Cronan, 2004). The mutated site lowers its affinity to bioAMP and this is believed to release biotin that covalently binds to primary lysine side chains on any proteins within 10 nm of the target (Kim *et al.*, 2014). Biotinylation of proteins by BioID fusion protein permits specific isolation and identification with standards biotin affinity capture. The captured proteins become candidate interacting proteins for the targeted

protein. The advantages of BioID include decreased background and increased sensitivity, as well as improved spatial resolution in cells. A limitation of BioID is the potential for the ligated 35 kDa biotin to the targeted protein to interfere with its sub cellular positioning and cellular function, as well as producing false positives. This has been addressed by production of a smaller ligase variant to enhance the selectivity to the protein. In addition, there are a number of inherent caveats in this proximity dependent labelling system including the accessibility of the Biotin in secretory pathways and decreases efficient labelling in particular cellular compartments. BioID techniques have long labelling protocols, which prevents isolated analysis of events occurring within a shorter time frame. In reference to the longer labelling time frame for BioID, the biotynilation of proteins can affect normal protein function. As seen in dual labelling systems, BioID may only identify proteins within a proximate distance to the protein of interest, which may not necessary directly interact. Importantly, identification of protein candidates associated to the biotinylated target protein will not explain their biological role (Kim and Roux, 2016; Li *et al.*, 2017)

In situ technologies have not been described further restricting the identification of homo- or heterodimers in hormone responsive cells and tissues. At the protein level, identifying the relative abundance of MR:GR protein complexes is restricted by its dependence on Abs, but also made complicated by technical issues including sample processing. The analysis of proteins can be improved using a dual recognition by Abs in a sandwich immunoassays in place of single Ab binding (Wide, Bennich and Johansson, 1967). The immunochemistry technique was developed over 75 years ago (Albert H. Coons2, Hugh J. Creech, 1942) and since, the detection of protein-protein interactions by imaging techniques has predominantly used overexpressed fluorescently tagged proteins and individual Ab preparations, which can interfere with the biological property of the protein or the unspecific detection of proteins and lead to misinterpretation of results (Raykova *et al.*, 2016; Andersson *et al.*, 2017). The proximity ligation assay (PLA) uses a dual detection system to detect target proteins by binding pairs of a specific primary and species specific secondary conjugated-oligonucleotide Abs, where short DNA strands will ligate forming a circular template, which can be amplified via rolling circle amplification (RCA) and visualised with an added fluorescent probe (Soderberg *et al.*, 2006), revealing a distinct puncta representative of a single PPI complex. *In situ* PLA has improved sensitivity, specificity, and enhanced the methodological scope for localised protein detection in tandem with other techniques such as western blotting (Liu *et al.*, 2011), flow cytometry (Leuchowius *et al.*, 2009; Burns *et al.*, 2017) and sandwich enzyme-linked immunosorbent assay (ELISA) (Tong *et al.*, 2016; Ebai *et al.*, 2017). PLA was developed to visualise PPIs with a high degree of sensitivity, with improved spatial resolution, result independence, exposure time and the digital amplification of the microscope channels.

PLA has been used to identify PPIs involved in various cellular processes such as organelle crosstalk in the exchange of proteins, lipids and ions, or proteins involved in cell proliferation. Recently, studies using PLA have identified PPIs involved in ER–mitochondrial crosstalk involved in oxidative stress in neuronal cells (Honrath *et al.*, 2017) and ERα homodimers and ERα/β heterodimers were detected in breast cancer cells and tissues (Iwabuchi *et al.*, 2017), as well as interactions with ERα-associated candidate proteins (Papachristou *et al.*, 2018). To date, PLA has not been used to detect PPIs between GRs. PLA has several advantages in the detection of PPIs in cells or tissues provided the specific Abs are available. PLA relies on the recognition of proteins of interest that are within 40 nm of each other by species specific affinity probes, that produce amplifiable circular DNA strands via rolling circle amplification (RCA) as a detection signal (Soderberg *et al.*, 2006). Where PLA identifies the amplified product as dots, several interacting molecules within a complex, for example, will produce a robust and ambient visible signal. An optimised PLA is a highly sensitive and relatively fast way to assay protein interactions without too much manipulation of cells. This dual recognition system produces enhanced signal strength from the amplified product at the specific location thus improving specificity, sensitivity and target range.

1.23 Project Outline

In the presented work, the dynamic nuclear organisations of MR and GR, and their presence within a protein-protein interacting complex, has been investigated.

The biological significance of the interaction of GR complexes (such as MR:GR) with targeted nuclear foci and cellular structures still remains to be fully understood. Transcription factors (TFs) such as GRs regulate transcriptional events by interacting dynamically at specific chromatin sites and recently it has been shown that MR-tethered GR regulates gene transcription differently to GR or MR dimers alone. The sub nuclear localisation and spatial organisation of MR:GR complexes within the nucleoplasm and at nuclear structures brings into question the influence of nuclear localisation on the mechanistic function of these nuclear receptors and their dynamic movement through structural compartments over time or under varying ligand conditions, for example. The cell nucleus is a major organelle that together with the nuclear envelope is involved in mechanotransduction and disease development. Elements of the nuclear envelope such as the nuclear lamina (NL) provide structural support and connect the cytoskeleton to chromatin, where the organisation and regulation of heterochromatin and transcriptional proteins are maintained (Kirby and Lammerding, 2018; Mathieu and Manneville, 2019). The role and organisation of dynamic endogenous protein-protein interactions, such as MR:GR, is integral to the accurate molecular characterisation of biological systems.

Here, I use a combination of transcriptomics and advanced fluorescence microscopy and proximity ligation assay, a highly specific protein detection method, to investigate the dynamics and interactions of MR:GR protein complexes throughout the nucleoplasm under differing ligand treatment designs, indicating their localisation throughout the nuclear compartments. Further advancing our understanding of MR:GR mediated cellular effects requires the integration of transcriptional events following receptor recruitment to the DNA and combined with spatial and temporal distribution of the MR:GR complex, modulated by nuclear compartmentalisation. I further describe the use of confocal microscopy to detect nuclear distribution of MR and GR, and PLA to detect MR:GR interactions using both 3617 and Neuro-2A cells, and rat hippocampal tissue.

1.24 Hypothesis

We hypothesised that the spatial localisation of nuclear MR:GR may be influenced by concentration and duration of corticosteroid activation, and that MR:GR regulation could provide a mechanism of transcriptional regulation.

1.25 Aims and Objectives

1. Optimise and develop the proximity ligation assay (PLA) method for the analysis of protein-protein interactions (PPIs) in neuronal cells and develop analysis pipeline to process the PLA data effectively.
2. Explore the existence of MR:GR interactive complexes expressed in cultured cells and endogenously expressed in brain tissue: identify and analyse their subcellular spatial localisation.
3. Identify gene targets that may be differentially regulated by GR, MR and MR:GR transcriptional complexes.

2 General Methods

2.1 Cell lines and cell culture

2.1.1 3617 cells

3617 cells (a gift from Gordon Hager, NCI, USA) derive from the 3134 mouse mammary adenocarcinoma cell line which contain a tandem head to tail array of 200 copies of the MMTV long terminal repeat (LTR) with 800 to 1200 binding sites for GR which produces a viral Harvey Ras reporter RNA. The 3617 cell line (McNally *et al.*, 2000) also contains a GFP-tagged GR with a C656G mutation under a tetracycline-off CMV promoter, which has a high sensitivity to the synthetic GC dexamethasone under the CMV promoter (Walker, Htun and Hager, 1999). Without antibiotics, expression of the integrated GFP-GR (and mCherry-MR for 3617ChMR) was prevented during routine culture by addition of 5 µg/ml tetracycline (Sigma). Cells were maintained in DMEM high glucose (Life Technologies, CA; Thermo Fisher, Paisley, UK) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA; Thermo Fisher, UK) and 2 mM L-glutamine (Life Technologies, CA; Thermo Fisher, UK) in a 5% CO2 humidified incubator at 37^{o}C. 3617 cells were cultured up to passage 20, to minimise clonal variation.

2.1.2 Neuro-2A male mouse neuroblast cell line (N2a)

Neuro-2A mouse neuroblast cells (purchased from the European Collection of Authenticated Cell Cultures (*RRID:CVCL_0470*) (Sigma-Aldrich (St. Louis, MO) were cultured in DMEM media (Thermo Fisher Scientific, Waltham, MA) supplemented with 7.5% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific), cysteine, HCl 0.3 mM, L-alanine 0.4 mM, asparagine 0.45 mM, L-aspartic acid 0.4 mM, L-proline 0.4 mM and L-glutamate 0.4 mM (Sigma-Aldrich) (Oortgiesen 1989) in a 5% CO2 humidified incubator at 37^{o}C. Neuro-2A cells were cultured up to passage 20, to minimise clonal variation, and the cells demonstrated consistent neuronal-type morphology with the ability to produce neurite projections upon differentiation (Oortgiesen and Vijverberg, 1989).

Experimental cells for 3617 and Neuro-2A cells were seeded in growth media wherein the FBS was replaced with charcoal stripped serum (CSS) and supplemented as above following 2 washes with Dulbecco's PBS (Thermo Fisher Scientific) at 200,000 cells per well in Nunc imaging dishes (for PLA experiments) and 250 000 per well in 6-well plates (for RNA experiments). Cells were transfected ~18 hours after seeding, using a ratio of 1:2 (w/v) DNA to jetPRIME® (Polyplus) following the manufacturer's instructions. 26-27 hrs after transfection, cells were treated with: (i) corticosterone

15 nM, (ii) corticosterone 100 nM, (iii) vehicle (ethanol, final concentration 0.01%), followed by fixation with PFA (4%) in CSS media at the defined time points for PLA or RNA extraction.

2.2 Plasmid constructs

pC1-EGFP-rGR (gift from Gordon Hager, National Cancer Institute) (Presman *et al.*, 2016) encodes EGFP fused to the N-terminal of rat GR cDNA under the control of the CMV promoter in the mammalian expression vector C1 (Clontech/Takara Bio, Mountain View, CA). pC1-mCherry-rMR (subcloned from pC1-EGFP-rMR, gift from David Pearce, University of California San Francisco). Plasmids encoded the EGFP fused to the N-terminal of rat GR alpha cDNA and the mCherry protein fused to the N-terminal of rat MR cDNA inserted into pC1 vectors (Clontech/Takara Bio, Mountain View, CA). pC3 (gift from Gordon Hager, National Cancer Institute) and untagged mouse MR [gift of pcDNA4/TO-mMR from Diego Alvarez de la Rosa (Aguilar-Sanchez *et al.*, 2012) in pSF-PGK-EMCV-Puro (Oxford Genetics, Oxford, UK)] were expressed. Construction of XDBD mutants involved a combination of PCR and cloning methods to change three amino acids in the P-box region of GR (amino acids 446, 447 and 450) and MR (amino acids 621, 621, and 625) to tryptophans:

- GR-XDBD mutants DNA sequence change: from GGAAGCTGTAAAGTC to

TGGTGGTGTAAATGG.

- MR-XDBD mutants DNA sequence change: from GGCAGCTGCAAAGTC to

TGGTGGTGCAAATGG.

For both GR and MR, amino acids changed from Gly-Ser-Cys-Lys-Val to Trp-Trp-Cys-Lys-Trp.

A640T MR mutants gifted by (Rivers *et al.*, 2019) contain a single base of mouse MR (amino acid 640) to substitute alanine (GCT) with threonine (ACT). The encoded DNA binding domain D-loop amino acid sequence of these mutants is altered from

EGQHNYLCAGRNDCIIDK to EGQHNYLCTGRNDCIIDK.

2.3 Transfection

Unless specified otherwise cells in 35 mm Nunc™ glass bottom dishes and 6-well plates were transfected with 4 µL of jetPRIME® (Polyplus Transfection, Illkirch-Graffenstaden, France) [ratio of 1:2 (w/v)] per well and a total of 2 µg of DNA. For western blot analysis pC1-rGRwt and pC1-rMR expressing untagged GR and MR were used. For PLA and RNA expression, expression vectors pC3-mGR and pcDNA4/TO-mMR expressing untagged GR and MR were used. DNA binding domain mutants (XDBD) and A640T mutants (Rivers *et al.*, 2019) PLA experiments used untagged MR-

XDBD/GR-XDBD/wtMR/GR and A640T-MR/GR-XDBD/wild-type MR/GR, respectively. For IF, pC1-EGFP-rGR and pC1-mCherry-rMR plasmids expressing tagged GR and MR were used. In order to maintain the same total amount of DNA in the transfection reactions, the pcDNA3.1 empty vector was used, when either a GR or MR expression vector was not required for the conditions.

2.4 Animal Husbandry

Adult male Sprague-Dawley rates were group housed in rooms under standard conditions with 14:10 light:dark cycles (lights on at 05:00 hours) with standard chow and water available *ad libitum*. All procedures were carried out in accordance with the UK Home Office animal welfare regulations.

2.5 Perfusion and harvest of hippocampal tissue

Rats were given an intraperitoneal (IP) injection of 3 mg/kg corticosterone followed by 30 minutes exposure prior to an overdose of pentobarbital. For unfixed samples, rats were decapitated, the brains were removed and placed into cold PBS on ice. For perfusion fixed tissue, animals were perfused intra-cardiacally with 200 mLs ice cold 0.1 M PBS and then 300 mLs ice cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Following decapitation, whole brains were harvested and transferred into 4% PFA on ice. Brains were fixed overnight and then incubated overnight in 30% sucrose solution in 0.1 M PBS at 4^{o}C. For both perfused and fresh brains, samples were placed in a weighing boat and gently passed over the surface of liquid nitrogen for gentle freezing. They were then stored at -80 C. Coronal dorsal hippocampus (Bregma -3.14 to -4.30) sections (30 µm) were cut using a cryostat (Leica) and then mounted on slides. Slides were kept at -20^{o}C until processing.

2.6 Antibodies (Abs)

For IF and PLA, anti-rMR1-18-1D5 polyclonal Ab was used to detect MR (rMR1-18 1D5 was deposited to the DSHB by Gomez-Sanchez,C. Purchased from DSHB, Hybridoma Product rMR1-18-1D5). Epitope location/sequence is the N-terminus A/B domain (aa 1-18) and recognises all MR isoforms. The sequence of the rMR 1–18 peptide (METKGYHSLPEGLDMERR-C) is identical in the rat, human, and mouse (Gomez-Sanchez; Gomez-Sanchez *et al.*, 2006). Anti-GR M20 polyclonal Ab was used to detect GR (purchased from Santa Cruz Biotechnology, Cat. No. sc-1004, CA, USA).

2.7 Primary antibodies and PLA kit

Ab combinations tested during PLA optimisation can be seen in chapter 3 and the validated Abs are further described here. Duolink® PLA Probes specific to rabbit mouse and rabbit (anti-mouse PLUS and anti-rabbit MINUS) and Duolink® PLA Detection Reagents (red) were purchased from Duolink/Sigma Aldrich (MO, USA). The combined PLA kits contain secondary probes, blocking solution,

wash buffers A and B, amplification, ligase and detection reagents. Duolink™ PLA protocols require two validated primary Ab and raised in two different species. It is recommended that Abs be IgG class and specific to the targeted proteins. Both monoclonal and polyclonal are compatible with the PLA.

2.8 Preparation of cells and tissue for immunofluorescence, immunohistochemistry and proximity ligation assay.

3617- and Neuro-2a cells were cultured in 35mm Mat-Tak dishes after two washes with Dulbecco's PBS (Thermo Fisher Scientific) and seeded in the appropriately supplemented CSS media [as previously listed] at 200,000 cells per dish. Transient transfection was performed ~18 hours after plating, and cells were treated with vehicle (ethanol, final concentration 0.01%), 15 mM or 100 nM CORT (Sigma-Aldrich) ~24 hours later. All cells were fixed with 4% paraformaldehyde for 15 minutes with gentle rotation (~40 rpm). Three PBS washes were performed to rehydrate the cells before the Duolink Blocking Solution was applied for 1 hr at 37°C. The cells were then washed two more times for 5-minutes with PBS. Primary Abs anti-MR 1D5 (Gomez-Sanchez) and/or anti-GR M-20 (Santa Cruz Biotechnology) in their incubation solution (permeabilisation listed in chapter 2) were added to the cells and incubated for 72 hours at 4°C. Cells were washed three times in 1X PBS for 10 minutes under gentle shaking following incubation. For PLA, all incubations were performed in a humidity chamber at 37°C according to the Duolink® PLA Probes and Duolink® In Situ Detection Reagents Red kit instructions (DUO92008, Sigma-Aldrich, Gillingham, UK). All wash buffers were prepared according to the Duolink In Situ PLA Kit instruction.

2.9 Immunofluorescence (IF) in cultured cells

3617 and Neuro-2A cells were transfected with pC1-EGFP-rGR and pC1-mCherry-rMR plasmids and following CORT treatment were fixed with 4% paraformaldehyde at room temperature for 15 mins. Rat brain tissue was fixed under the same conditions. After rehydration in 1X PBS (one 5-minute and two 1-minute washes) on an orbital shaker, cells were blocked for at least 2 h in 2% BSA/PBS at 4°C. All cells were incubated for 72 hours with anti-GR M20 with/without anti MR 1D5 overnight at 4°C in 0.3% Triton-X100 for permeabilisation, followed by three 1X PBS 10-minute washes at RT at ~40 rpm on an orbital shaker. Cells were then incubated with Alexa Fluor-labelled secondary antibodies donkey anti-rabbit (Alexa Fluor-488 cat #ab150061, Abcam) and/or goat anti-mouse (Alexa Fluor-594 cat #ab150120, Abcam) both at a 1:1000 in 1X PBS and 0.3% Triton-X in sterile MQ water at RT. Finally, x3 washes in 1X PBS for 10 mins were performed, followed by replacement with fresh 1X PBS for storage at 4°C until imaging. Images were acquired using confocal microscopy (Leica LASX imaging software) at 488 and 594 nM with a 40x magnification oil lens (Wolfson Bioimaging Facility, University of Bristol).

2.10 Immunohistochemistry (IHC) in endogenous tissue

Fixed-perfused hippocampal slices were rinsed in 1X PBS and three slices were mounted onto a Superfrost™ Plus Microsoft slide (Thermo Fisher Scientific). Slides with hippocampal slices were air dried in a chemical fume hood for 10-15 minutes onto the slide to prevent detachment during the rinsing steps. In a chemical fume hood, each dry tissue slice was encircled using an ImmEdge™ Hydrophobic Barrier Pen (Vector Laboratories) to create a hydrophobic barrier to contain the reagents applied on the slice within the selected imaging area. The hydrophobic pen solution required 20-30 minutes to dry. Once dried, the microscope slides were immersed into 1%PBS with 0.01% triton X-100 in a coplin jar and agitated at 20-30 rpm for 15 minutes. Slides were then rinsed in 1X PBS three times for 5 minutes per wash in a coplin jar. Slides were then transferred onto flat and moist tissue paper in a sterilised Tupperware and 70 µl of PLA blocking solution (yellow cap) was pipetted onto each section and covered with a lid and incubated for 1hr at 37^{o}C. The following blocking solution can also be used if the PLA blocking solution is not an option [0.3% Triton X-100 and 2% BSA Fraction V in 0.1M (1X) PBS for 2hrs at 37 ^{o}C]. Ensure complete coverage. Following blocking, slides were overturned carefully onto blotting paper to discard blocking solution followed by two 5-minute 1X PBS washes. Samples were not allowed to completely dry from this point. Slides were returned to their moist tupperware container and 70 µl of permeabilisation solution containing the primary abs was applied to each slice, ensuring complete coverage. Samples were incubated for 72 hours at 4^{o}C. Slides were washed three times in 1X PBS for 10 minutes to remove any unbound primary ab. Excess liquid was removed by careful blotting away. The wash steps were repeated as described following 'blocking step' but primary ab was replaced with the secondary ab in solution [0.3% Triton X-100, 2% BSA in 1X PBS, abs 1:1000 for both donkey anti-rabbit AlexaFluor-488 #ab150061, goat anti-mouse AlexaFluor-594 #ab150120] incubated for 2 hrs at RT with a foil cover to avoid bleaching of the sample. A final set of washes was performed (three 10-minute washes in 1X PBS to remove unbound secondary ab and slides were airdried in the dark (e.g. slide storage box). Once dried, 4 – 6 µl of Duolink *In Situ* Mounting Medium with DAPI was applied to the tissue slice using a 20 µl pipette tip with the end cut off to widen the bore size (at the end of the tip). Images were acquired using confocal microscopy (Leica LASX imaging software) at 488 and 594 nM with a 10x magnification dry lens and a 40x magnification oil lens (Wolfson Bioimaging Facility, University of Bristol).

2.11 Proximity Ligation Assay (PLA)

The PLA method has been described extensively in Chapter 3: optimisation and development of PLA using confocal microscopy. In brief, following cell culture and CORT treatment, all cells were fixed in 4% PFA, blocked and incubated with primary antibodies anti-GR M20/anti-MR 1D5 (dilution at 1:500)

in a 2 mL 0.3% triton-X100 permeabilization solution, at 4°C for 72 hours. Time and temperature dependant incubations were performed, namely: hybridisation using Duolinks (DUO92002) PLA probes, ligation of the probes with a ligase enzyme, rolling circle amplification (RCA) with a polymerase enzyme, and confocal microscopy images were acquired for image analysis.

2.12 IF/PLA quantification and visualisation

2.12.1 Microscopy and fluorescence Imaging

Images were acquired with a Leica TCS SP5II confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an oil immersion Objective Plan-Apochromat 40x/1.4 Oil DIC M27 (FWD=0.13mm), (UV)VIS-IR, with three standard PMTs plus two high enhanced photon sensitivity 'hybrid' GaAsP detectors, and using Immersion oil 518 F (Wolfson Bioimaging Facility, University of Bristol). Wavelengths were used at 405 nm (DAPI: 4',6-diamidino-2-phenylindole), 488 nm (FITC (Cyanine 2), Zeiss Filter set 38) and 594 nm (Texas Red®, Zeiss Filter set 31). When required, 3D stacks were obtained on a Märzhäuser scanning stage that enabled multi-position acquisition and tiled imaging. High resolution images were obtained in the form of a z-stack with a 0.02 µm z-interval. To avoid cross talk between the different fluorophores, sequential scanning was used.

2.12.2 Image Analysis

2.12.2.1 Software

Fiji, based on ImageJ 1.51h (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). PLA images were analysed using the 'Fiji' software. 3D rendering and orthogonal processing were presented across each available axis (XY, XZ, YZ) and presented in transparent frames which can be manually rotated for optimal viewing.

2.12.2.2 Modular Image Analysis

Spatial distribution of puncta in nuclei was measured using the Modular Image Analysis plugin for Fiji (Schindelin *et al.*, 2012; Rueden *et al.*, 2017; Cross, 2019). In order to identify nuclei, the blue fluorescence channel was processed with 3D Gaussian and median filters (both sigma of 2px) to reduce noise whilst preserving a sharp nuclear edge profile. The filtered image was then binarised using the Huang thresholding method (Huang and Wang, 1995). Individual nuclei were identified as regions of contiguous foreground-labelled pixels, as such, prior to this step a 3D distance-based watershed filter (Legland, Arganda-Carreras and Andrey, 2016) was used to split adjacent nuclei which had become merged during the binarisation process. Nuclei clipped across the image edge and those with volume less than 200 μm^3 were removed from further analysis. Puncta were identified using the 3D spot detection functionality of TrackMate (Tinevez *et al.*, 2017). The shortest distance between the puncta

and the nuclear surface was then calculated, with negative values corresponding to puncta inside the nucleus and positive values for those outside. Fluorescence intensity in the blue and green channels was measured as a function of distance from the nuclear surface. Average intensity measurements were divided into 21 distance bins from -5 μm from the nuclear surface to +5 μm from the nuclear surface.

2.13 Western Blot Analyses

Whole cell samples from proteasome inhibition experiments were separated by SDS–PAGE and transferred to PVDF membranes. Blots were probed with primary antibodies anti-GR M-20 (1:5000, Santa Cruz Biotechnology), anti- MR 1D5 (1:4000, gift from Celso Gomez- Sanchez, University of Mississippi Medical Centre, USA) or anti-beta-tubulin (1:5000, Sigma, cat# T4026) in Tris-buffered saline (TBS) containing 5% nonfat dry milk, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibody (Santa Cruz Biotechnology). Beta tubulin was used as a loading control to normalize detected levels of protein by verifying that protein loading is equal in each well of the gel. All blots were visualized with the ECL kit (Supersignal by Thermo Scientific) and protein bands were imaged with the GeneSys software.

2.14 RNA extraction and reverse transcription

Total RNA was extracted from Neuro-2A cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and treated with TURBO^{TM} DNase (Ambion/Thermo Fisher Scientific) in order to remove residual DNA contamination from the RNA samples. Reverse transcription was performed with 1 μg of total RNA using an iScript™ cDNA (Bio-Rad Laboratories Ltd) synthesis kit, according to the manufacturer's instructions. RNA transcript levels were assessed by real-time PCR using the SYBR Green Fast PCR master mix system (Applied Biosystems, Thermo Fisher Scientific). As an endogenous control, Mcm3ap was used which demonstrated constant expression in the presence and absence of CORT treatment. Primers were designed using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA). PCR specificity and efficiency were validated by melt curve analysis and amplification over a serial dilution of template.

2.15 Statistical analysis

Reverse transcription quantitative PCR (RT-qPCR) analyses were performed with a minimum of five independent biological replicates ($n \geq 5$), and each qPCR measurement was calculated from the mean of two separate Ct values which were technical repeats. RT-qPCR analyses were compared by one-way ANOVA with a Bonferroni multiple comparisons test, and data represented as a mean $\pm$ SEM. Statistical significance is labelled by the following: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

For PLA analysis, data was analysed by one-way ANOVA with a Bonferroni multiple comparisons test, and data represented as a mean $\pm$ SEM.

3 Development and Optimisation of Proximity Ligation Assay using Confocal Microscopy.

3.1 Introduction

The detection of proteins can be enhanced when the detection system consists of a dual recognition using Abs in a sandwich immunoassay instead of single bound Abs (Wide, Bennich and Johansson, 1967). Such detection systems are commonly applied to the high-performance solution-phase protein detection techniques used in scientific research. Conversely, since the development of immunohistochemistry over 75 years ago (Albert H. Coons2, Hugh J. Creech, 1942), the majority of *in situ* protein measurements depended on Ab-protein specificity, which often times lead to cross reactivity and non-specific binding (Raykova *et al.*, 2016; Andersson *et al.*, 2017). Soderberg *et al.* (Soderberg *et al.*, 2006) first described the *in situ* proximity ligation assay (PLA) ten years ago, a system where in situ target measurement relies on ligation of pairs of oligonucleotide-conjugated Abs that produce circular DNA strands, which with a polymerase addition, are amplified by rolling circle amplification (RCA). As a result, the dual recognition enhances specificity and localised amplification via RCA improves signal strength (Lizardi *et al.*, 1998). Protein-protein interactions (PPIs) in cells and endogenous samples and post translational modifications (Koos *et al.*, 2014; Smith *et al.*, 2015), such as methylation and phosphorylation (Jarvius *et al.*, 2007), have been measured using *in situ* PLA. Detection of proteins in interactive complexes or individual proteins and their subcellular localisation, help to understand the proteomic profile and modulation of cellular processes such as gene transcription, between cell types, such as in malignant cells.

In situ PLA, is an extension to conventional immunoassays for detection of PPIs that occur in the same cellular environment and between different cells via cell surface proteins (Fredriksson *et al.*, 2002), and for endogenous *in situ* PPI in cell lines (Soderberg *et al.*, 2008). *In situ* PLA has been combined with other techniques for measurement of localised protein, such as western blotting (Liu *et al.*, 2011), flow cytometry (Leuchowius *et al.*, 2009; Burns *et al.*, 2017), and sandwich enzyme linked immunosorbent assay (ELISA) (Tong *et al.*, 2016; Ebai *et al.*, 2017). Typically, co-immunoprecipitation with western blotting technique is used to detect PPIs. Using a fluorescent probe, PLA can detect, visualise and quantify endogenous proteins, protein-protein interactions (PPI) and their corresponding co-factors, and post translational modifications in cells and tissues processed for microscopy. Whereas, traditional immunocytochemistry techniques only allow for the co-localisation of proteins (Fredriksson *et al.*, 2002; Soderberg *et al.*, 2006; Trifilieff *et al.*, 2011; Fichter *et al.*, 2014; Roussis *et*

al., 2016). PPI's can be interrupted or inhibited by antibodies, small molecules or peptides, providing an opportunity for therapeutic intervention.

In situ PLA uses a pair of 'oligonucleotide-conjugated' Abs or 'proximity probes', species specific to two primary Abs targeting the proteins of interest. Once applied and if within ≤40 nm of each other, the short oligonucleotide strands attached to the PLA probes will ligate forming a circle DNA template. The circular DNA template amplifies via rolling circle amplification (RCA) primed by one of the proximity probes, thus creating a concatemeric amplification product that remains covalently attached to the proximity probe, generating signals at Ab bound sites. The RCA products each constitute a singular DNA strand, with hundreds of complements of the DNA circle that are compressed and following hybridization with a fluorophore-labelled oligonucleotide are visualised by microscopy and enumerated data can be extracted (Clausson *et al.*, 2015). PLA can be applied to various samples including protein suspensions, such as cell lysates, and fixed tissues, such as cell culture dishes, cytospin preparations or frozen tissues sections (Soderberg *et al.*, 2008; Zieba *et al.*, 2010).

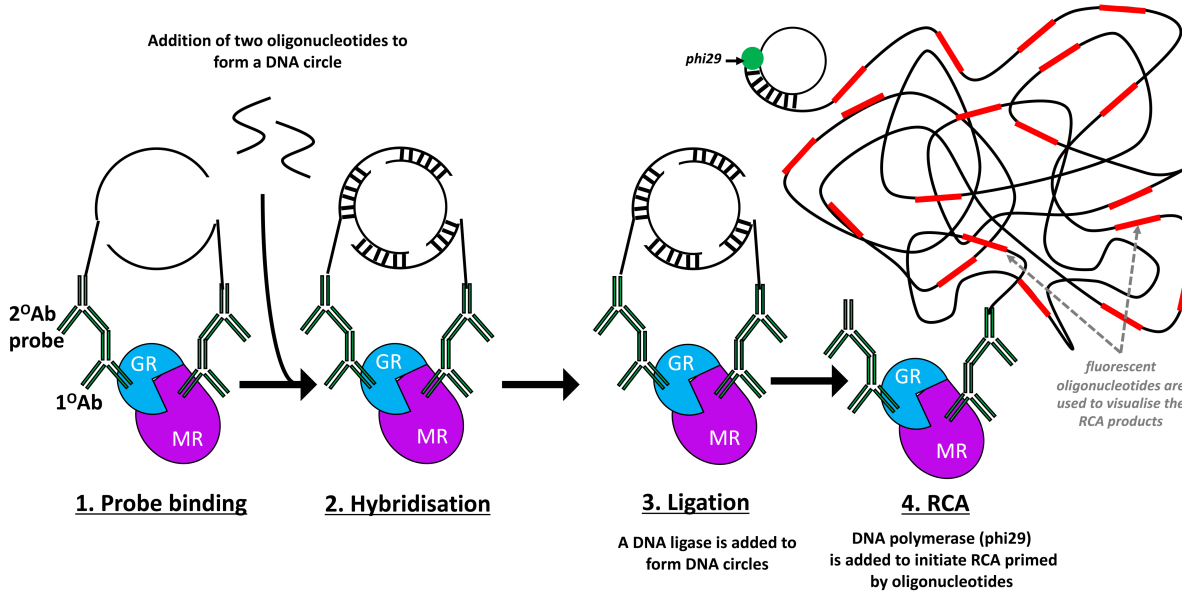


Figure 6 Schematic drawing of different antibody binding to two different proteins and visualisation steps of PLA adapted from Klaesson et al. (2018).

1. Following incubation with primary Abs that selectively bind to each receptor (blue and purple), secondary PLA probes are added and incubated, according to the protocol. 2. Two additional oligonucleotides hybridise to the two PLA probes. 3. A ligase enzyme is applied to the oligonucleotides to form a closed circle. 4. Using the ligated circle as a template, a polymerase enzyme is added to

induce rolling circle amplification (RCA). Fluorescence labelled oligonucleotides (red) hybridise to and amplify the RCA product, which can be visualised via fluorescent microscopy.

It is vital to acknowledge that *in situ* PLA relies on Abs that recognise a given protomer (a structural unit of an oligomeric protein) and possesses a high level of selectivity to restrict the risks for signal artefacts. A reliable antibody is essential for successful IHC; therefore, it is sensible to source the Ab from recommended providers, together with product information detailing IHC compatibility and applicability. Furthermore, commercial companies can withhold the antigen sequences used to raise the Ab (Saper, 2009), further supporting the need for Ab validation prior to IHC experiments.

Epitope specificity is further determined by the selection and suitability of polyclonal or monoclonal primary Ab's for use in IHC (Bordeaux *et al.*, 2010). Polyclonal Ab's have higher overall affinity to the target due to recognition ability of several epitopes, increased sensitivity for detecting low-quality proteins, less sensitive to protein changes, such as denaturation, and a higher ability of capturing the target protein. Monoclonal Abs are homogenous and stable, possessing a higher purity and concentration compared to polyclonal (Kohler and Milstein, 1975). The performance of an IHC assay relies on the use of a sensitive protein detection system where the antigen-antibody reaction can be visualised. Enzyme and fluorophore-mediated detection systems are commonly used in the visualisation of such reactions.

3.1.1 Antibodies for validation in cells and tissue

Using cell models: the 3617-cell line containing the mouse mammary tumor virus [MMTV] array, derived from the 3134-mouse mammary adenocarcinoma line; and Neuro 2A mouse neuroblastoma cells, PLA was optimised as a method for the detection of interactions between the glucocorticoid and mineralocorticoid receptors. This was later tested in tissue expressing endogenous GR and MR. The optimisation involved extensive validation of Abs (Table 1), incubation times and confirmation of interactions.

Table 1 Primary antibodies for GR and MR, and their combination for IF/IHC and PLA.

anti-GR	dilution	source	identifier	anti-MR	dilution	source	identifier
anti-GR (clone M20)	*1:5000*	*Santa Cruz*	*Cat# sc-1004*	*anti-MR (clone rMR1-18 1D5)*	*1:4000*	*DSHB – (depositor) Gomez Sanchez*	*Cat# AB_1157909*
anti-GR (clone H300)	*1:500*	*Santa Cruz*	*Cat# sc-8992*	*anti-MR (clone rMR1-18 1D5)*	*1:4000*		
anti-GR (clone G5)	*1:500*	*Santa Cruz*	*Cat# sc-393232*	*anti-MCR (clone H300)*	*1:200*	*Santa Cruz*	*Cat# sc-11412*
anti-GR (clone FiGR)	*1:200*	*Santa Cruz*	*Cat# sc-12763*	*anti-MR (clone ab62532)*	*1:500*	*Abcam*	*Cat# ab62532*
anti-GR (clone PA1510A)	*1:250*	*ThermoFischer Scientific*	*Cat# PA1-510A*	*anti-MR (clone rMR1-18 1D5)*	*1:4000*		
anti-GR (clone D6H2L)	*1:200*	*Cell signaling*	*Cat# 12041*	*anti-MR (clone rMR1-18 1D5)*	*1:4000*		
anti-GR (clone BuGR2)	*1:500*	*Novus Bio*	*Cat# NB300-731*	*anti-MCR (clone H300)*	*1:200*		

The following protocol for indirect PLA is provided and used with cells and brain tissue endogenously expressing receptors, and information on data analysis. A schematic diagram of the developed PLA is shown in Figure 6. Using this approach, I was able to confirm an interaction between the glucocorticoid and mineralocorticoid receptor overexpressed in the 3617 and Neuro-2a cells, and in hippocampal tissue slices. The optimised PLA method can be applied to other cell types and tissues to assess protein interactions under different experimental conditions.

3.2 Material & methods

3.2.1 Cell lines

See chapter 2.

3.2.2 Plasmids

See Chapter 2.

3.2.3 Perfusion and harvest of hippocampal tissue

Rats were given an intraperitoneal injection (IP) of 0.75 mg corticosterone per ~250g for 30 minutes and then given an overdose of pentobarbital. For fresh sample, rats were decapitated, and the brains removed and placed into cold PBS on ice. For perfusion fixed samples, animals were perfused intra-

cardiacally with 200 mLs ice cold 0.1 M PBS and then 300 mLs ice cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Following decapitation, the whole brains were harvested and transferred into 4% PFA on ice. Brains were fixed overnight and followed by an overnight incubation in 30% sucrose solution in 0.1 M PBS at 4^{o}C. For both perfused and fresh brains, samples were placed in a weighing boat and gently passed over the surface of liquid nitrogen for gentle freezing. They were then stored at -80 C. Coronal dorsal hippocampus (Bregma -3.14 to -4.30) sections (30 µm) were cut produced using a cryostat (Leica) and then mounted on slides. Slides were kept at -20^{o}C until processing.

3.2.4 Antibodies (Abs)

All cells were permeabilised with a 2 mL 0.3% triton-X100 solution containing the validated primary Abs anti-GR M20 and anti-MR 1D5 (dilution at 1:500), at 4^{o}C for 72 hours. Abs were prepared according to the manufacturer's instructions. According to the PLA instructions, primary Abs can either be poly- or mono-clonal, but should be from IgG grade, target specific and ideally affinity purified. Primary Abs for GR and MR were validated using IF/IHC and PLA probes were selected to be raised against rabbit and mouse, respectively.

3.2.5 Primary antibodies and PLA kit

Duolink^{TM} PLA protocols require two validated primary Ab and raised in two different species. It is recommended that Abs be IgG class and specific to the targeted proteins. Both monoclonal and polyclonal are compatible with the PLA. Anti-rMR1-18-1D5 polyclonal Ab was used to detect MR (rMR1-18 1D5 was deposited to the DSHB by Gomez-Sanchez, C. Purchased from DSHB, Hybridoma Product rMR1-18-1D5). Epitope location/sequence is the N-terminus A/B domain (aa 1-18) and recognises all MR isoforms. The sequence of the rMR 1–18 peptide (METKGYHSLPEGLDMERR-C) is identical in the rat, human, and mouse (Gomez-Sanchez, no date; Gomez-Sanchez *et al.*, 2006). Anti-GR M20 polyclonal Ab was used to detect GR (purchased from Santa Cruz Biotechnology, Cat. No. sc-1004, CA, USA). Duolink® PLA Probes specific to rabbit mouse and rabbit (anti-mouse PLUS and anti-rabbit MINUS) and Duolink® PLA Detection Reagents (red) were purchased from Duolink/Sigma Aldrich (MO, USA). The combined PLA kits contain secondary probes, blocking solution, wash buffers A and B, amplification, ligase and detection reagents.

3.2.6 Preparation of cells and tissue for immunofluorescence, immunohistochemistry and proximity ligation assay.

3617- and Neuro-2a cells were cultured in 35mm Mat-Tak dishes after two washes with Dulbecco's PBS (Thermo Fisher Scientific) and seeded in the appropriately supplemented CSS media [as previously listed] at 200,000 cells per dish. Transient transfection was performed ∼18 hours after plating, and

cells were treated with vehicle (ethanol, final concentration 0.01%) or 100 nM CORT (Sigma-Aldrich) ~24 hours later. Cells were fixed with 4% paraformaldehyde for 15 minutes with gentle rotation (~40 rpm). Three PBS washes were performed to rehydrate the cells before the Duolink Blocking Solution was applied for 1 hr at 37°C. The cells were then washed two more times for 5-minutes with PBS. Primary Abs anti-MR 1D5 (Gomez-Sanchez) and/or anti-GR M-20 (Santa Cruz Biotechnology) in their incubation solution (permeabilisation listed in chapter 2) were added to the cells and incubated for 72 hours at 4°C. Cells were washed three times in 1X PBS for 10 minutes under gentle shaking following incubation. For PLA, all incubations were performed in a humidity chamber at 37°C according to the Duolink® PLA Probes and Duolink® In Situ Detection Reagents Red kit instructions (DUO92008, Sigma-Aldrich, Gillingham, UK). All wash buffers were prepared according to the Duolink In Situ PLA Kit instruction.

3.2.7 Immunofluorescence (IF) in cultured cells

3617 and Neuro-2A cells were treated for 90 and 45 minutes, respectively, with 100nM corticosterone to ensure nuclear translocation and fixed using 4% paraformaldehyde at room temperature for 15 minutes. After rehydration in 1X PBS (one 5-minute and two 1-minute washes) on an orbital shaker, cells were blocked for at least 2 h in 2% BSA/PBS at 4^{o}C. Cells were incubated for 72 hours anti-GR M20 with/without anti MR 1D5 overnight at 4^{o}C in 0.3% Triton-X100 for permeabilisation, followed by three 1X PBS 10-minute washes at RT at ~40 rpm on an orbital shaker. Subsequently, the cells were incubated with Alexa Fluor-labelled secondary antibodies donkey anti-rabbit (Alexa Fluor-488 cat #ab150061, Abcam) and/or goat anti-mouse (Alexa Fluor-594 cat #ab150120, Abcam) both at a 1:1000 in 1X PBS and 0.3% Triton-X in sterile MQ water at RT. Finally, three 10-minute 1X PBS washes were performed, followed by replacement with fresh 1X PBS for storage at 4^{o}C until imaging. Images were acquired using confocal microscopy (Leica LASX imaging software) at 488 and 594 nM with a 40x magnification oil lens (Wolfson Bioimaging Facility, University of Bristol).

3.2.8 Immunohistochemistry (IHC) in endogenous tissue

Fixed-perfused hippocampal slices were rinsed in 1X PBS and three slices were mounted onto a Superfrost™ Plus Microsoft slide (Thermo Fisher Scientific). Slides with hippocampal slices were air dried in a chemical fume hood for 10-15 minutes. Slices were completely dried onto the slide to prevent detachment during the rinsing steps. In a chemical fume hood, each dry tissue slice was encircled using an ImmEdge™ Hydrophobic Barrier Pen (Vector Laboratories) to create a hydrophobic barrier to contain the reagents applied on the slice within the selected imaging area. The hydrophobic pen solution required 20-30 minutes to dry. Once dried, the microscope slides were immersed into 1%PBS with 0.01% triton X-100 in a coplin jar and agitated at 20-30 rpm for 15 minutes. Slides were

then rinsed in 1X PBS three times for 5 minutes per wash in a coplin jar. Slides were then transferred onto flat and moist tissue paper in a sterilised Tupperware and 70 µl of PLA blocking solution (yellow cap) was pipetted onto each section and covered with a lid and incubated for 1hr at 37°C. The following blocking solution can also be used if the PLA blocking solution is not an option [0.3% Triton X-100 and 2% BSA Fraction V in 0.1M (1X) PBS for 2hrs at 37 °C]. Ensure complete coverage. Following blocking, slides were overturned carefully onto blotting paper to discard blocking solution followed by two 5-minute 1X PBS washes. Samples were not allowed to completely dry from this point. Slides were returned to their moist tupperware container and 70 µl of permeabilisation solution containing the primary abs was applied to each slice, ensuring complete coverage. Samples were incubated for 72 hours at 4°C. Slides were washed three times in 1X PBS for 10 minutes to remove any unbound primary ab. Excess liquid was removed by careful blotting away. The steps were repeated (primary ab) but was replaced with the secondary ab in solution [0.3% Triton X-100, 2% BSA in 1X PBS, abs 1:1000 for both donkey anti-rabbit AlexaFluor-488 #ab150061, goat anti-mouse AlexaFluor-594 #ab150120] incubated for 2 hrs at RT with a foil cover to avoid bleaching of the sample. A final set of washes was performed (three 10-minute washes in 1X PBS to remove unbound secondary ab and slides were airdried in the dark (e.g. slide storage box). Once dried, 4 – 6 µl of Duolink In Situ Mounting Medium with DAPI was applied to the tissue slice using a 20 µl pipette tip with the end cut off to open up the tip. Images were acquired using confocal microscopy (Leica LASX imaging software) at 488 and 594 nM with a 10x magnification dry lens and a 40x magnification oil lens (Wolfson Bioimaging Facility, University of Bristol).

3.2.9 Microscopy and fluorescence Imaging

Fluorescence was visualised using a Confocal 8 –SP5-II tandem scanner confocal with three standard PMTs plus two 'hybrid' GaAsP detectors, which offer much greater sensitivity, boosting of low signal and photon counting modes (Wolfson Bioimaging Facility, University of Bristol). A 40x PL APO CS lens with oil was used for the higher magnification images (with zoom when necessary) at wavelengths at 405 (nur DAPI), 488 (FITC (Cyanine 2), Zeiss Filter set 38) and 594 (Texas Red®, Zeiss Filter set 31) nM. When required, high resolution images were obtained in the form of a z-stack with a 0.2 µm z-interval. To avoid cross talk between the different fluorophores, sequential scanning was used.

3.2.10 Image Analysis

PLA images were analysed using the 'Fiji' software. Hyper-stacks have been used and can store up to 5 dimensions with clarity and minimal dimension related errors that can occur during command updates. The PLA images have been presented in several ways to reveal observations seen in the original 2D images generated using Image-J (or FIJI). 3D data (puncta) is presented in densely packed

transparent boxes that can be viewed from different angles, usually producing attractive images or short video clips, for example. However, it can prove difficult to translate as their interpretation relies on perspective and if pixels overlap from a previous viewing angle.

3.3 Results

3.3.1 Expression of MR and GR in cells using microscopy

Indirect PLA was employed with the aim of visualising the co-localisation between GR and MR in 3617 cells, followed by testing in Neuro-2A cells and then in hippocampal tissue. MR and GR were both transiently transfected into the cells. Cells and tissues were processed to observe the localisation of overexpressed and endogenous GR/MR, respectively, using conventional immunofluorescence. In addition to characterising the expression patterns of GR/MR, extensive ab validation was performed and in addition to testing the specificity of the assay by the absence of non-specific staining in the negative controls. This was demonstrated by a combination of extensive negative controls where MR and GR plasmids and antigen specific primary antibodies were omitted (-) or with their respective IgG controls.

3.3.1.1 Immunofluorescence: Ab validation

PLA success depended on two abs that selectively and specifically recognised MR and GR and that restrict the risks for signal artefacts. Various antibodies against MR and GR are available. Six different pairs of antibodies against overexpressed MR and GR protomers were tested in 3617 cells to identify the most appropriate Ab pairing for PLA in Neuro-2a cells and for eventual testing in hippocampal tissue. Antibody pairing anti-MR (1D5) + anti-GR (M20) was revealed to be the most effective and was subsequently used in the eventual transition to Neuro-2A cells and hippocampal tissue.

3.3.1.1.1 3617 mouse mammary adenocarcinoma cells

3617 cells were cultured, and the expression and localisation of overexpressed GR and MR were observed using IF. In addition, the binding specificity and selectivity of abs against GR and MR was assessed under a corticosterone treatment condition. 3617 cells were treated with 100 nM corticosterone for 90 minutes and transiently transfected with expression constructs for untagged GR/MR alone (balanced with empty vector pcDNA3.1), GR and MR together, or an pcDNA3.1 control. Upon ligand activation, GR/MR translocated into the nucleus and remained cytoplasmic under vehicle control, when their relative species specific primary and secondary abs were incubated in succession together (figure 7 H). Where both protomers were overexpressed and incubated with only one primary Ab, either anti-GR (Figure 7, A) or anti-MR (Figure 7, B), green or red nuclear staining were observed, respectively. When incubated with their opposing primary Abs (i.e. Cl-rGRwt+anti-MR 1D5;

pX-rMRwt+anti-GR) no staining was revealed, Figure 7(C) and Figure 7(D), respectively. In the absence of both protomers (pcDNA3.1 only), no staining was observed (Figure 7, E), confirming the non-existence of non-specific binding to any endogenous antigens or aldehyde groups. Further, antigen controls in place of anti- GR or MR as shown in Figure 7 (F) and (G) reveal no staining. Overall the specificity and selectivity of the primary abs to their targeted proteins was confirmed.

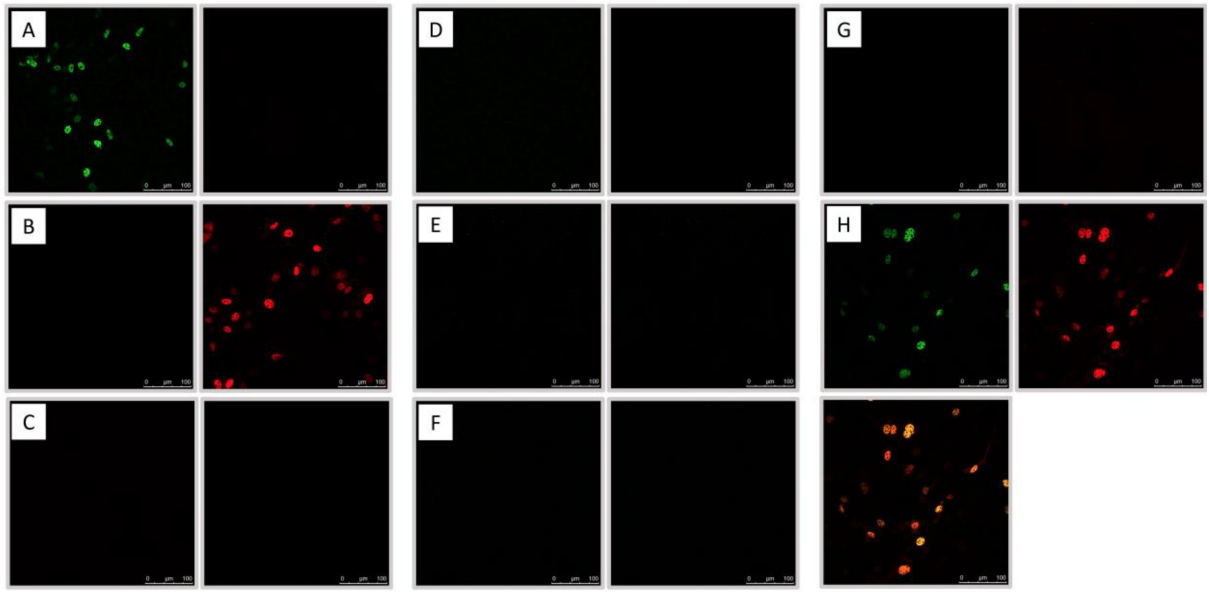


Figure 7 Validation of antibodies for PLA in 3617 cells.

Detection of GR and MR proteins overexpressed in PFA fixed 3617 cells. 3617 cells treated with 100nM corticosterone for 90 minutes and fixed in 4% PFA. IHC of anti-GR M20 and anti-MR 1D5 were stained by their respective secondary antibodies 488 (green) and 594 (red), respectively. As a background control MR and GR proteins and primary antibodies were omitted/incubated individually, and in addition to extensive antigen controls: (-) (A) GR only (anti-GR M20), (B) MR only (anti-MR 1D5), (C) GR only (anti-MR 1D5), (D) MR only (anti-GR M20), (E) pcDNA3 (anti-GR M20+anti-MR 1D5), (F) GR only (rIgG), (G) MR only (mIgG) and together (+) (H) GR+MR (anti-GR M20+anti-MR 1D5). Studies were performed as two independent experiments.

Transfected Protein	Primary Antibody applied	Results	Comments
None	anti-GR M20, anti-MR 1D5	No staining (-ve)	Expected –ve signal showing Abs do not bind to other antigens.
MR+GR	anti-GR M20, anti-MR 1D5	Staining for both receptors	Expected +ve signal for both Abs anti-GR and anti-MR for their respective antigens.
MR	anti-MR 1D5	Positive control (+ve)	Expected +ve signal for MR only with anti-MR 1D5 only.
GR	anti-GR M20	Positive control (+ve)	Expected +ve signal for GR only with anti-GR M20 only.
MR	anti-Mouse IgG	Antigenic negative control (-ve)	Expected –ve signal where primary Ab for MR is absent.
GR	anti-Rabbit IgG	Antigenic negative control (-ve)	Expected –ve signal where primary Ab for GR is absent.
MR	anti-GR M20	Cross reactivity control (-ve)	Expected –ve signal where anti-GR M20 does not recognise MR.
GR	anti-MR 1D5	Cross reactivity control (-ve)	Expected –ve signal where anti-MR 1D5 does not recognise GR.

Table 2 Table showing extensive genetic and primary controls in 3617 cells.

No staining was observed without MR or GR. Staining was observed for both MR/GR when both receptors were overexpressed. Positive staining was observed by the correct Alexa fluor to the correct antigen and cross reactivity controls were negative supporting the specificity of the secondary Ab to its primary Ab. Antigenic negative controls were negative.

The GR Ab did not recognize its closest relative MR, and vice versa, which was important to confirm as this could produce a PLA signal if both antibodies bound to the same protein (detailed in Table 2). The 3617-cell line does not express MR endogenously and therefore there should not be anything to stain the 1D5 unless transfected in. Preliminary luciferase experiments showed near undetectable levels of endogenous GR, which was controlled for using the tetracycline ON/OFF system.

3.3.1.2 Proximity ligation assay: testing of combinations of Abs

Ab combinations specific to GR and MR were tested in 3617 cells using indirect PLA. Cells were treated with 100 nM corticosterone for 90 minutes. Three primary Ab pairings that recognise GR/MR revealed GR:MR interactions, however Ab pair 'anti-GR (M20) + anti-MR (1D5)' produced the most reliable and detectable signal across repeats (Figure 8, C, D and E). A PLA signal, although weak and inconsistent,

was detected with anti-GR (511) + anti-MR (1D5) and anti-GR (510) + anti-MR (H300) (Figure 8 A and B, respectively). To visualise the nucleus, two options were explored: a GFP tagged MR as seen in Figure 8 (A-D), and a DAPI stain shown in Figure 8 (E). Experiments with GFP-tagged MR produced weak signals suggesting that the large GFP tag was interfering with the PLA process, such as diffusion of or contact of the two PLA probes, as the number of interactions was reduced in the nucleus (Figure 8, D). Therefore, GFP tagged MR was replaced with an untagged construct (pX-rMRwt) and the nucleus was stained with DAPI when required, as shown in Figure 8 (E). Following GFP removal PLA signal was improved and consistent.

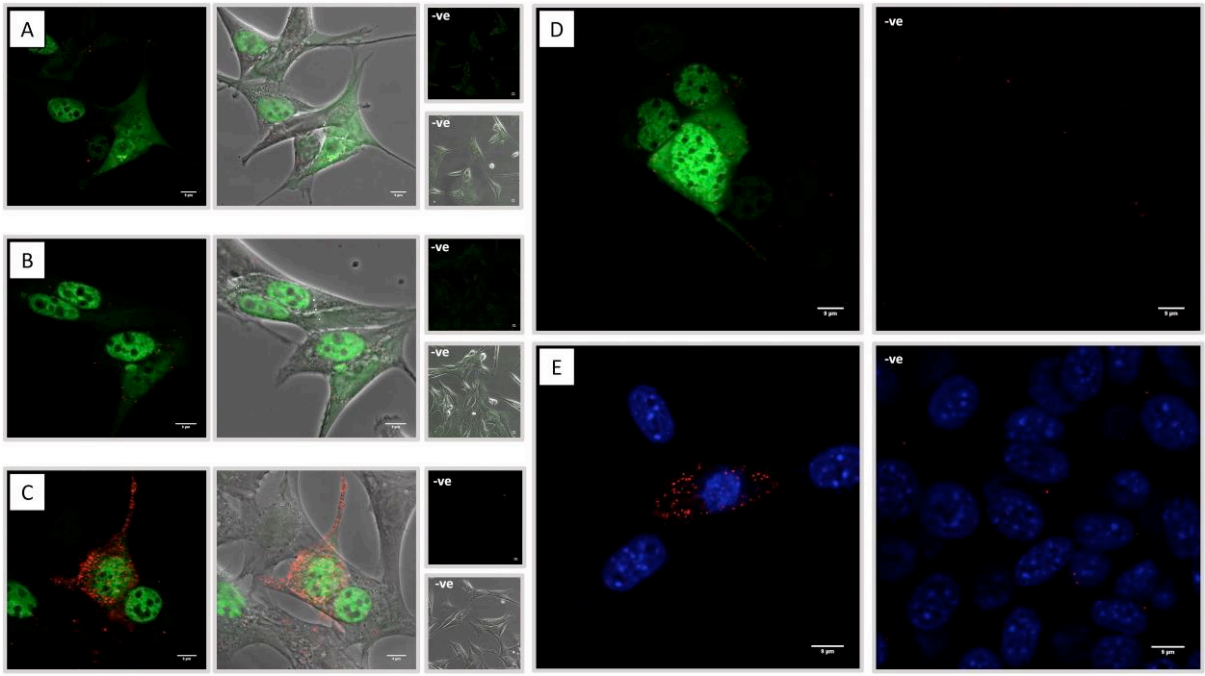


Figure 8 Validation of anti-GR and anti-MR pairs for PLA and detection of GR and MR proteins in 4% PFA fixed 3617 cells.

3617 cells treated with 100nM corticosterone for 90 minutes and fixed for 15 minutes. Expression of MR+GR protein complex under corticosterone treatment for 90 minutes was detected using antibody pairs: anti-GR 511+anti-MR 1D5 (A), anti-GR 510+anti-MR H300 (B) and anti-GR M20+anti MR 1D5 (C). As a background control (-ve) MR was omitted and GR translocated only (+pcDNA3) with both respective primary antibodies. Detection of 100nM corticosterone treatment induced the MR+GR complex and cytoplasmic-nuclear translocation for 90 minutes using PLA in (D) and (E). MR+GR complex represented in red dots (Texas red) and nucleus identification in green (GFP-GR) and blue (DAPI) for (D) and (E), respectively. Studies were performed as six independent experiments.

3.3.2 GR:MR interactions in 3617 cells

3617 cells are well a characterised cell line that contains a mouse mammary tumour virus (MMTV) promoter driving a *ras* reporter (Htun *et al.*, 1996; Collingwood, Urnov and Wolffe, 1999) and were available for use during these experiments. A tetracycline regulatory system permits an inducible ON/OFF switch of a (GR)/GFP chimera. Thus, intracellular concentrations of GFP-GR can be modulated, allowing for experimental assessment of the subcellular movement of GR and co-localisation to other targets (Walker, Htun and Hager, 1999). Cells were transiently transfected with GR/MR and treated with 100 nM corticosterone for 90 minutes to further explore GR:MR interactions using indirect PLA. For these analyses, the tested Ab pair anti-GR (M20) + anti-MR (1D5) was used. Each PLA puncta represents the product of the proximal GR and MR proteins. Since ligand activated GR is nuclear, abundant GR:MR interactive complexes (red puncta) were predominantly dispersed throughout the nucleoplasm (Figure 9), indicating a close proximity between GR and MR. As a negative control to detect any potential unspecific signal (Figure 10), GR/MR was omitted, or a primary antibody removed or replaced by their respective IgG antigen. Thus, there was no protein or primary Ab for the primary or secondary Ab to bind to, respectively, and no PLA signal was generated.

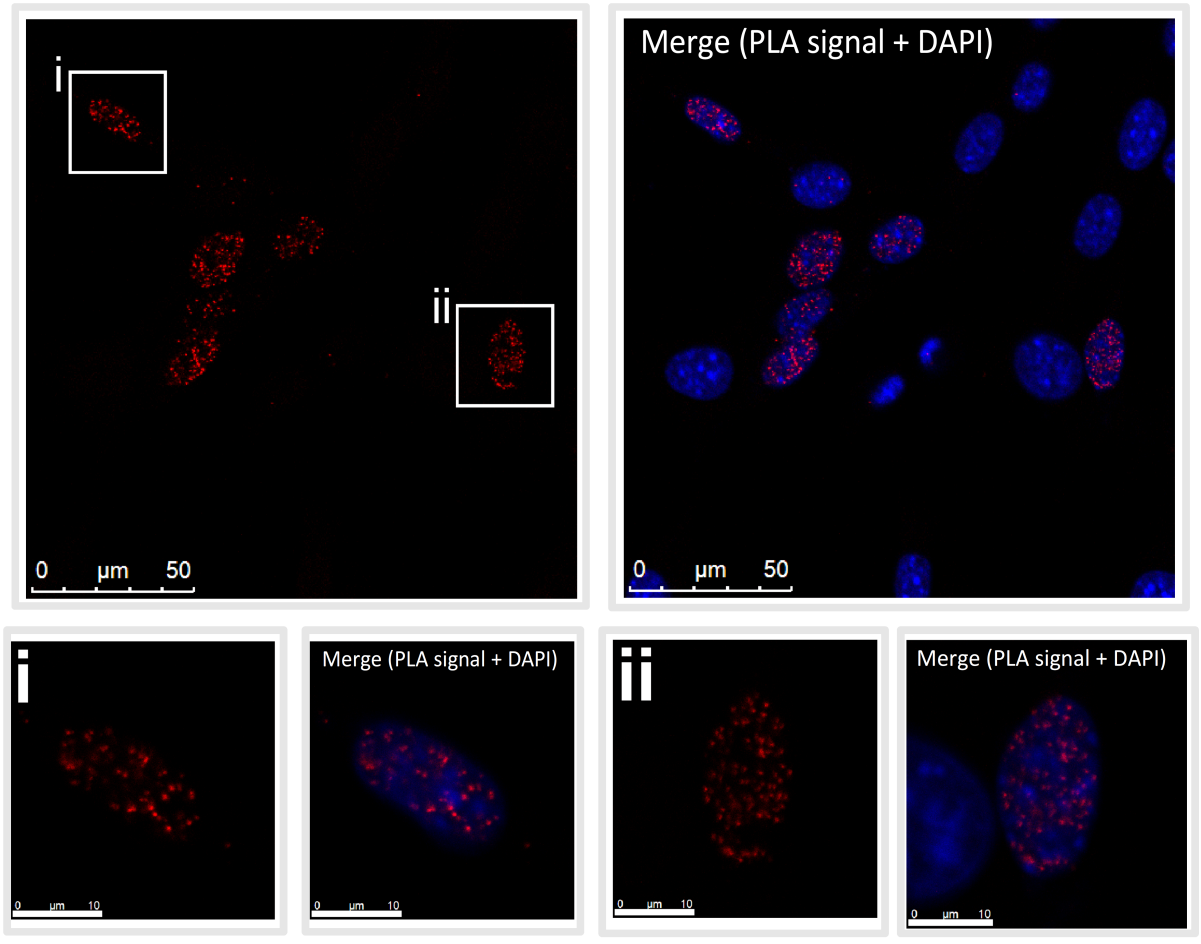


Figure 9 Expression of MR+GR protein complex under corticosterone treatment for 90 minutes in 4% PFA fixed 3617 cells.

Detection of 100nM corticosterone treatment induced the MR+GR complex and cytoplasmic-nuclear translocation for 90 minutes using PLA. MR+GR complex represented in red dots (Texas red) and nuclei blue (DAPI). Top two large images represent the same widefield view: left, PLA stain only; right, merged nuclei and PLA stain. Selected cells (i) and (ii) from widefield view are further assessed in bottom magnified images, each represented as PLA only and merged PLA+nuclei windows. Each picture is representative of a typical cell staining observed in 5 fields randomly chosen. Studies were performed as four independent experiments.

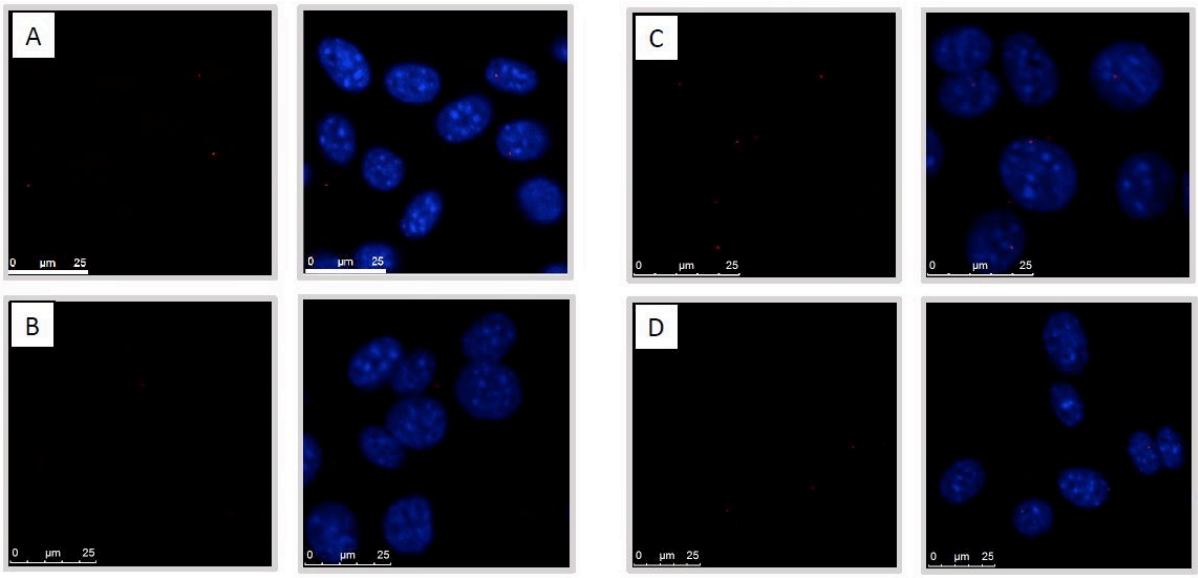


Figure 10 Technical control demonstrates the specificity of PLA signals in 3617 cells and the proximity between two proteins (MR+GR).

No PLA signal was detected. Nuclei were stained with DAPI. As a background control MR and GR proteins were omitted (A) or primary antibodies replaced with their respective antigenic IgG controls: anti-rabbit IgG + anti-MR 1D5 (B), anti-mouse IgG+anti-GR M20 (C), anti-rabbit IgG + anti-mouse IgG (D). Studies were performed as four independent experiments.

3.3.3 GR:MR interactions in Neuro-2A cells

3.3.3.1 Immunofluorescence

It was necessary to test PLA in a more appropriate (neuronal-like) cell line in order to more accurately understand MR/GR receptor behaviour. Eventually, PLA experiments were to be performed in

endogenous brain tissue. The neuronal-like Neuro-2a cell line has been widely used to test cellular and molecular responses to novel compounds for therapeutic and neurotoxic properties, as well as their associated mechanisms (LePage *et al.*, 2005). Furthermore, the neuro-2a cell line was available and set up during the time of these experiments. Although IF staining for MR/GR was previously observed in 3617 cells, specificity of the Abs was necessary in Neuro-2As, which was to be the modelled cell line for future experiments. IF was performed in Neuro-2A cells to assess the specificity of primary anti-GR (M20) and anti-MR (1D5) to their targeted proteins. Following treatment with 100 nM corticosterone for 90 minutes, Neuro-2As expressed nuclear GR and MR, confirming specificity of the targeted primary and secondary Ab combinations, shown in Figure 9 (D). Overexpression of both GR and MR together, and incubation with only anti-GR (Figure 11, A) or anti-MR (Figure 11, B), and with both secondary abs, produced green or red nuclear staining, respectively. As a negative control, pcDNA3.1 replaced GR/MR and no staining was observed (Figure 11, C). Western blotting confirmed protein expression of GR and MR in Neuro-2A cells at the correct molecular weight across repeats as shown in Figure 11 (E) and (F). All western blot analysis was performed in triplicate and are seen as representative images from the whole cell lysates. Further, extensive antigen controls in place of anti-GR or MR as performed in 3617 cells reveal no staining (not shown).

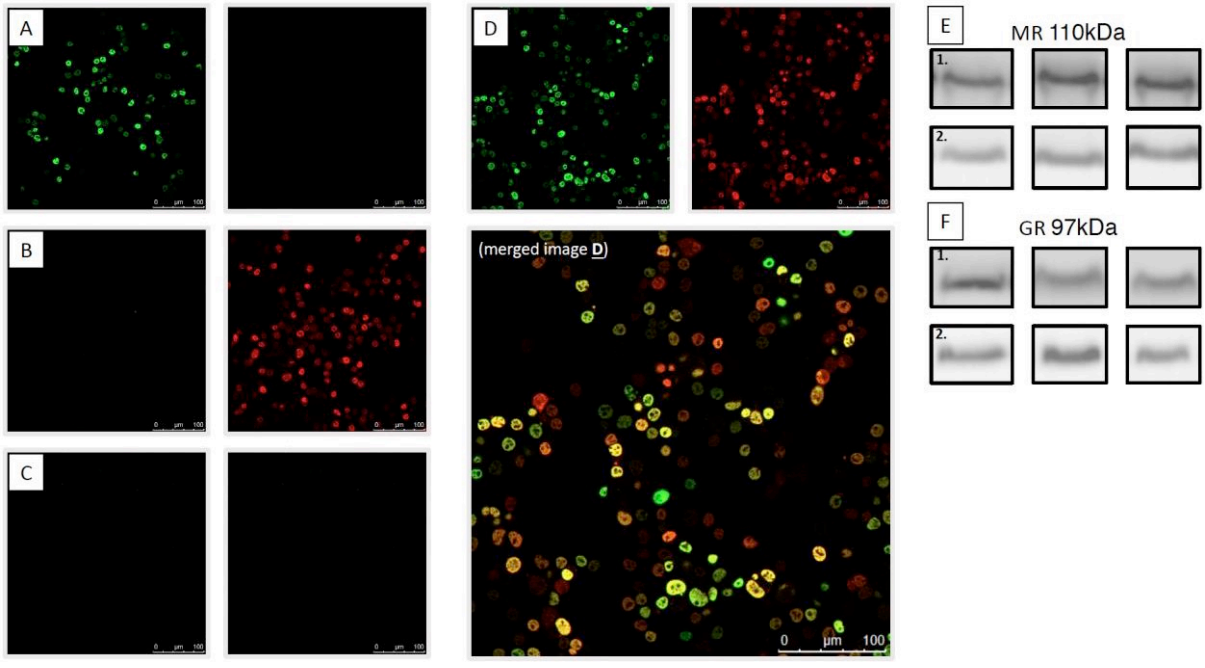


Figure 11 Validation of antibodies for PLA in Neuro-2a cells.

Detection of GR and MR proteins overexpressed in PFA fixed Neuro-2a cells. Neuro-2a cells treated with 100nM corticosterone for 90 minutes and fixed in 4% PFA. IHC of anti-GR M20 and anti-MR 1D5 were stained by their respective secondary antibodies 488 (green) and 594 (red), respectively. As a

background control MR and GR primary antibodies were omitted/incubated individually (-) (A, B, and C), and together (+) (D, merged image). Studies were performed as two independent experiments. Western blot analysis showed protein expression of GR and MR in Neuro-2a cells. All western blot analysis was performed in triplicate and are representative images from the whole cell lysates.

3.3.3.2 Detection of overexpressed Glucocorticoid receptors by proximity ligation assay in neuroblastomas (Neuro-2A)

Neuro 2A cells have been extensively used to study neuronal differentiation, axonal growth and signaling pathways. Cells were treated with 100nM corticosterone for 90 minutes and PLA was performed. Incubation with anti-GR (M20) and anti-MR (1D5), which are directed to GR and MR (Figure 11), respectively, produced an abundance of PLA puncta thus confirming the expression of GR/MR, validating the use of selected Abs for PLA and the detection of a GR:MR interaction in Neuro 2A cells (Figure 12). In the presence of 100nM corticosterone, the GR:MR complex was localised in the nucleus and predominantly peripheral to the perinuclear membrane (Figure 12). By contrast, treatment with a vehicle control (Figure 13) revealed GR:MR interactions in the cytoplasm. Specificity of the assay in these cells was shown by a lack of or minimal fluorescent signal, observed by the omission of one or both proteins and primary antibodies in the negative controls (Figure 14). A DAPI stain was applied to visualize the cell nucleus for localisation purposes (visible in merged photographs of Figure 12 and Figure 13). These results show that the optimized indirect PLA protocol is highly sensitive, selective and sufficient to detect protein-protein interactions in different cell types. Here I have validated the efficiency of this technique to show an GR:MR interaction in Neuro 2A cells using PLA. This is evidence of a dual receptor system, that further supports recent reports of a cooperative receptor system shown to modulate transcriptional responses (Mifsud and Reul, 2016; Rivers *et al.*, 2019).

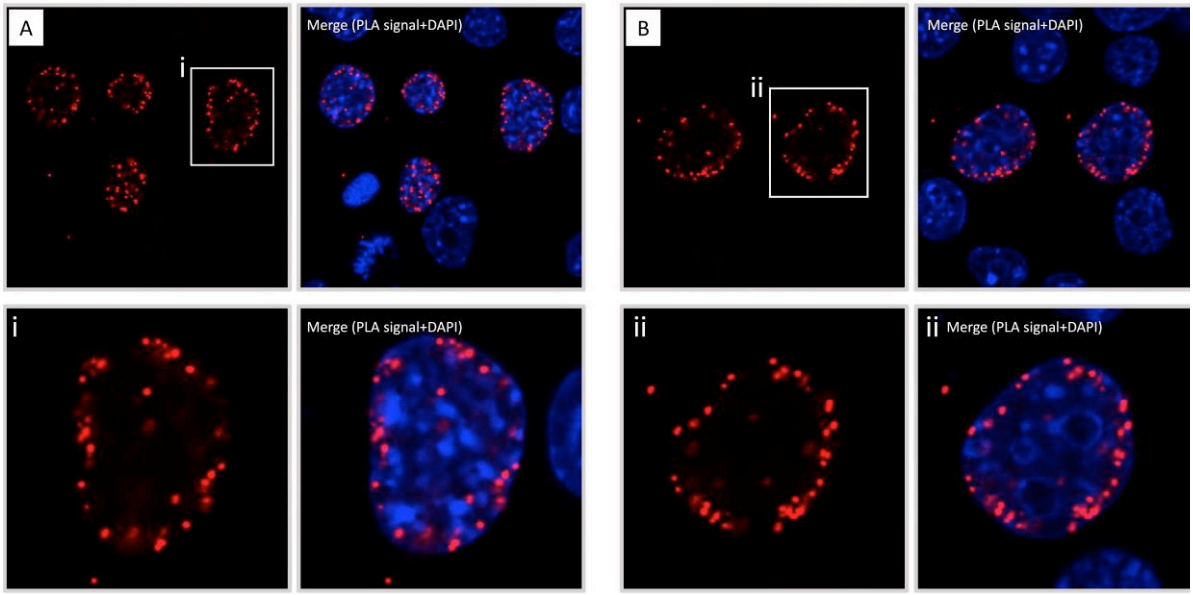


Figure 12 PLA: Ligand activated MR:GR interactions.

Expression of MR+GR protein complex under corticosterone treatment for 90 minutes in 4% PFA fixed neuro-2a cells. Detection of 100nM corticosterone treatment induced the MR+GR complex and cytoplasmic-nuclear translocation for 90 minutes using PLA. A and B show positive PLA signal (red). DAPI enables visualization of the cell nucleus (blue) combined with PLA signal (red) seen in 'merge' window. Individual cells A (i) and B (ii) are magnified for closer visualization. PLA positive signals are present and appear to be localized within the nucleus and close to the nuclear membrane edge. Studies were performed as four independent experiments.

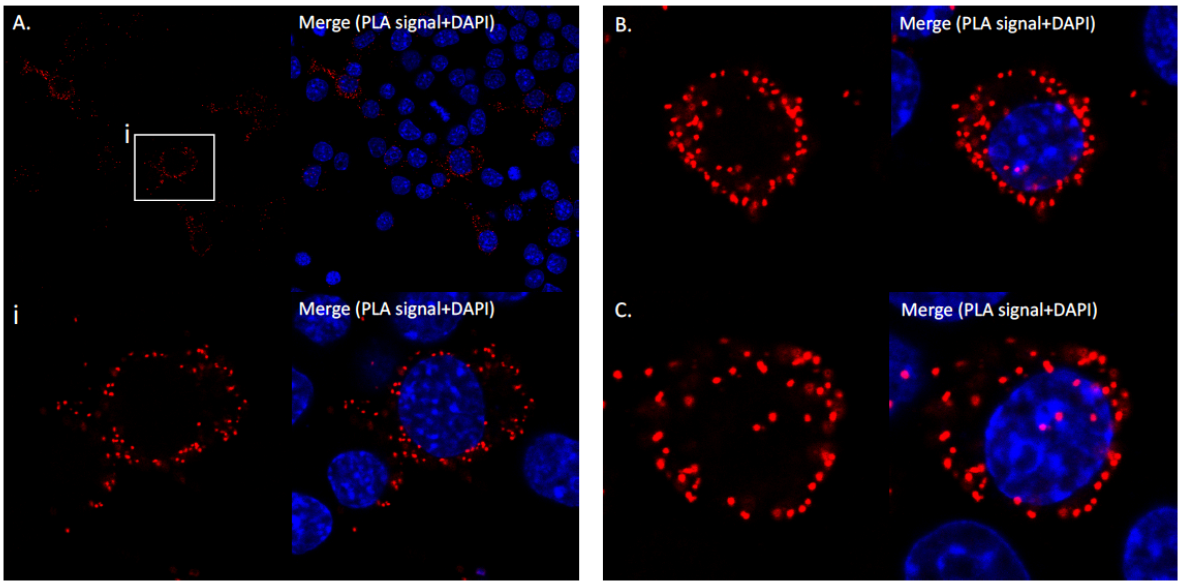


Figure 13 MR:GR interactions under vehicle treatment.

Expression of MR+GR protein complex under vehicle (0.01% EtOH) treatment for 90 minutes in 4% PFA fixed neuro-2a cells. Detection of vehicle treatment induced MR+GR complex within the cytoplasm for 90 minutes using PLA. Images: A (widefield view) and A(i); B and C, show positive PLA signal (red). DAPI enables visualization of the cell nucleus (blue) combined with PLA signal (red) seen in 'merge' window. Individual cell A(i) is magnified from window (A) for closer visualization. Additional magnified cells can be viewed in (B and C). PLA positive signals are present and appear to be localized around the outside of the nuclear membrane edge and within the cytoplasmic body. MR+GR complex represented in red dots (Texas red) and nuclei blue (DAPI). Studies were performed as four independent experiments.

Technical controls

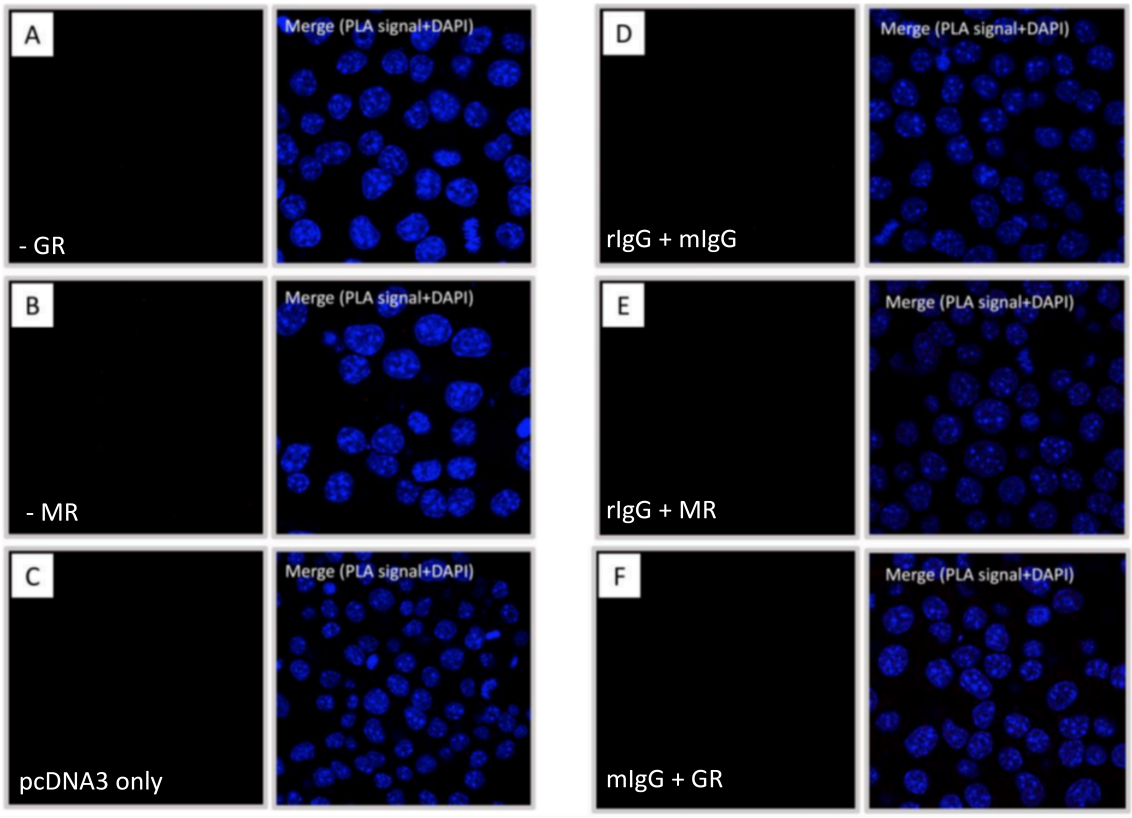


Figure 14 Validation of PLA in Neuro-2a cells: background controls.

Technical control demonstrates the specificity of PLA signals in Neuro-2a cells and the proximity between two proteins (MR+GR) following corticosterone treatment for 90 minutes and 4% PFA fixation. Cells were negative for PLA signal. Nuclei were stained with DAPI. As a background control MR and GR primary antibodies were omitted (-) or with their respective IgG controls. Each image is representative of a typical cell staining observed in 5 fields randomly chosen, and in a minimum of four

repeats. The quantification of the number of PLA dots per nucleus is presented with the mean values ± SD.

3.3.4 Detection of endogenous mineralo- and glucocorticoid receptor expression

IF was performed in tissue to assess physiological endogenous protein expression of GR/MR in primary tissue to test the specificity and adequate sensitivity of Abs to detect dynamic levels of the protomers when conducting IHC and PLA. Initial PLA in tissues gave rise to non-specific background staining due to endogenous peroxidase that was tested using a DAB substrate solution after rehydration to water. Therefore, pre-treatment of tissue with a 0.3% hydrogen peroxide solution prior to incubation with the primary Abs was performed as well as a change of anti-GR Ab (replacing anti-GR- M20 with 24050-1-AP), resulting in a substantial reduction in non-specific background and improved PLA signal. This suggests that anti-GR (24050-1-AP) is more specific and sensitive in detecting changeable endogenous GR protein.

The expression and localisation of GR/MR was assessed via IF, initially with primary Abs anti-GR (M20) + anti-MR (1D5) (Figure 15.1). Nuclear hippocampal IF staining was observed for GR/MR in hippocampal tissue after a 30 minutes *ip* corticosterone treatment (3mg/kg) (Figure 15.1B-E). Non-specific background staining can be observed, predominantly with anti-GR (M20) compared to anti-MR (1D5). In the absence of primary Abs, no IF staining was observed (Figure 15.1A). Abs anti-GR (24050-1-AP) + anti-MR (1D5) were tested in hippocampal tissue that had previously received a 30-minute restraint stress condition. Nuclear staining was observed for GR and MR, when incubated with their appropriate primary and secondary Ab combinations, shown in Figure 15 (photographs 2 and 3). No staining was observed when both primary abs were excluded as a negative control (not shown). Negative controls were captured at the same laser powers as the antibody + condition. Thus, it can be said that the staining is real and is not bleed-through from the DAPI channel.

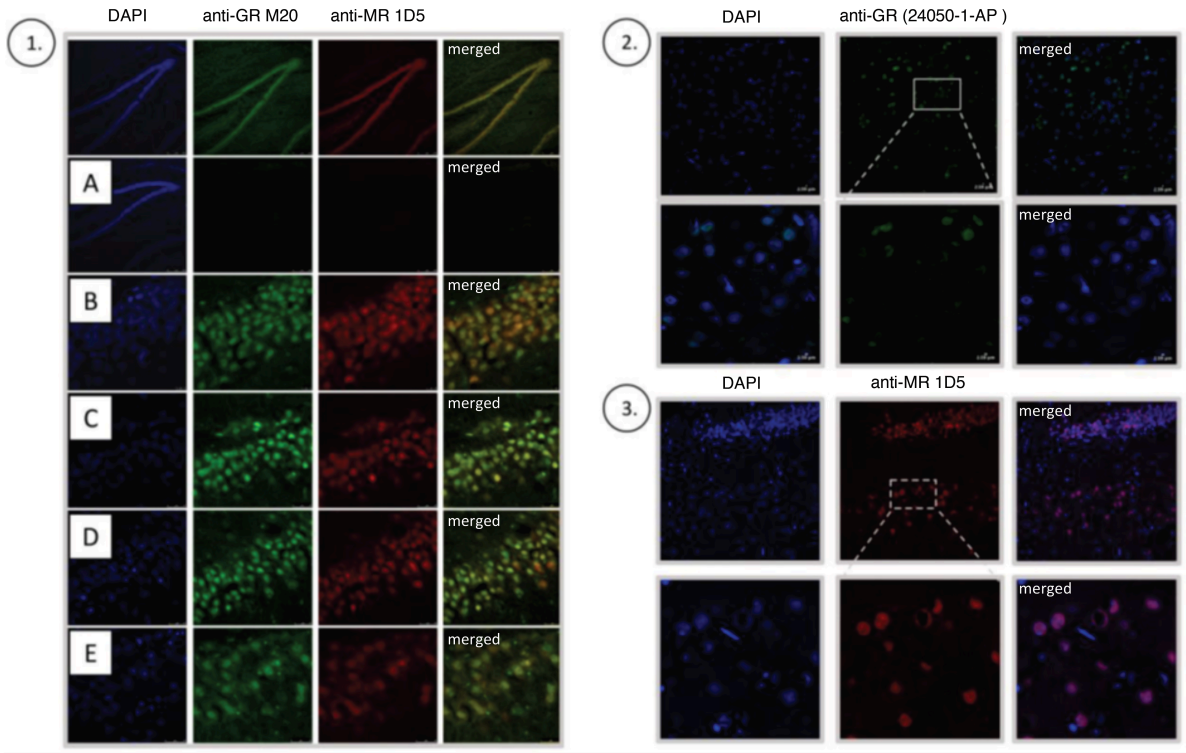


Figure 15 Detection of GR and MR proteins and validation of antibodies for PLA in hippocampal rat tissue.

IHC of anti-GR M20 and anti-MR 1D5 were stained by their respective secondary antibodies 488 (green) and 594 (red), respectively. All images show the positive localisation of GR/MR in the cell nuclei. DAPI was used for visualization of the nucleus. Overlay of channels can be seen in farthest (1) column (yellow). (1) animals were treated for 30 minutes with 3mgs/kg corticosterone (A: -ve primary Ab, B: DG, C: CA3, D: CA2, E: CA1. When primary Ab was omitted no staining was observed (A) and when both primary Abs were included staining was present in (B-E). (2,3): 30-minute restraint stress in (2) amygdala and (3) DG and CA3. As a background control MR and GR primary antibodies were omitted (-) (1.A). Top photographs for (1), (2) and (3) are of a lower magnification and the photographs below are at a higher magnification. Studies were performed as three independent experiments.

3.3.5 Detection of endogenous mineralo- and glucocorticoid receptor interactions

GR:MR interactions in endogenous tissue were investigating using an optimised indirect PLA protocol for use in tissue. To that aim, using indirect PLA, GR:MR interactions were detected in hippocampal tissue (Figure 16). Following ligand activation, PLA puncta or GR:MR complex were observed at and around the edge of the DAPI staining. Red and green detection kits were used in administered high corticosterone and restraint test in endogenous corticosterone activation, respectively. The negative control replaced anti-GR (24050-1-AP) with a species-specific antigen IgG control and displayed very

low background and no detectable fluorescent puncta (Figure 17). It can be concluded that the optimised indirect PLA can be applied to different cell types overexpressing the protein/s of interest and endogenous expressing tissue, assisting the analysis of PPIs and their subcellular localisation.

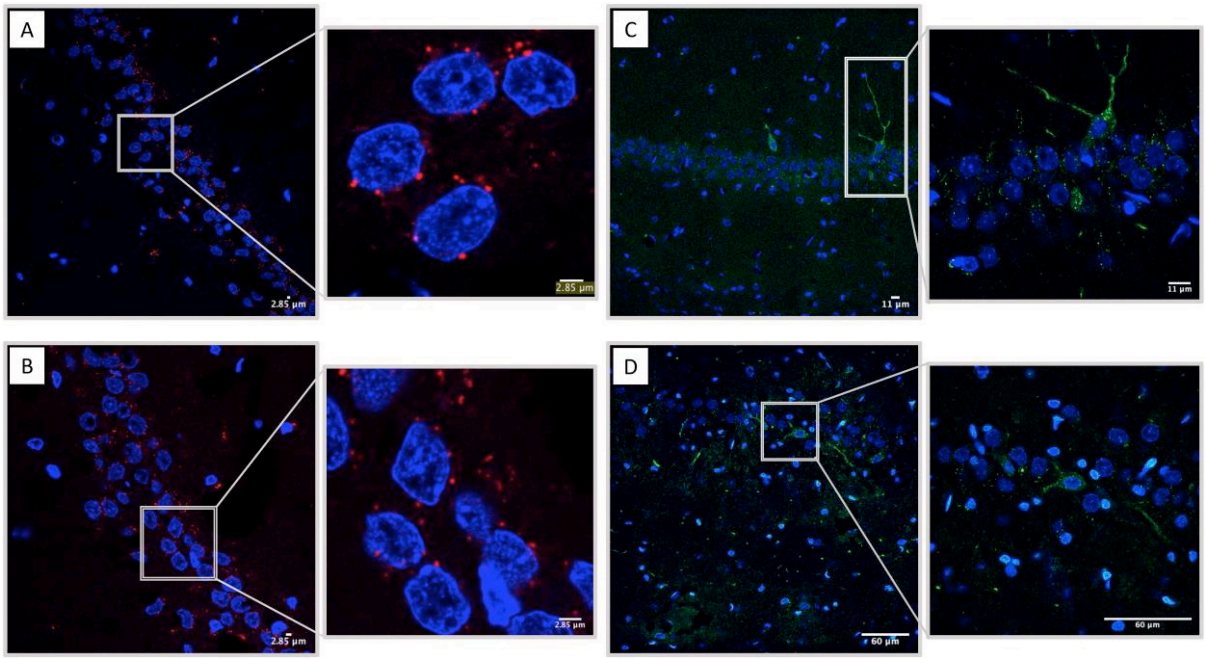


Figure 16 Detection of MR+GR proteins and interaction in hippocampal tissue by PLA.

Tissue was perfused PFA fixed. MR and GR antibodies were incubated together according to the protocol outlined at the end of this chapter. PLA signals [red (A and B) or green (C and D) dots] were detected peripheral to the inner nuclear membrane of cells in the CA1 (A), DG (B and C), and CA3 (D). Nuclei were stained with DAPI (blue) and PLA signals observed using the red and green detection kits across two separate experiments. Magnified cells are observed adjacent to the right of their respective wide field magnification photographs. Studies were performed as three independent experiments.

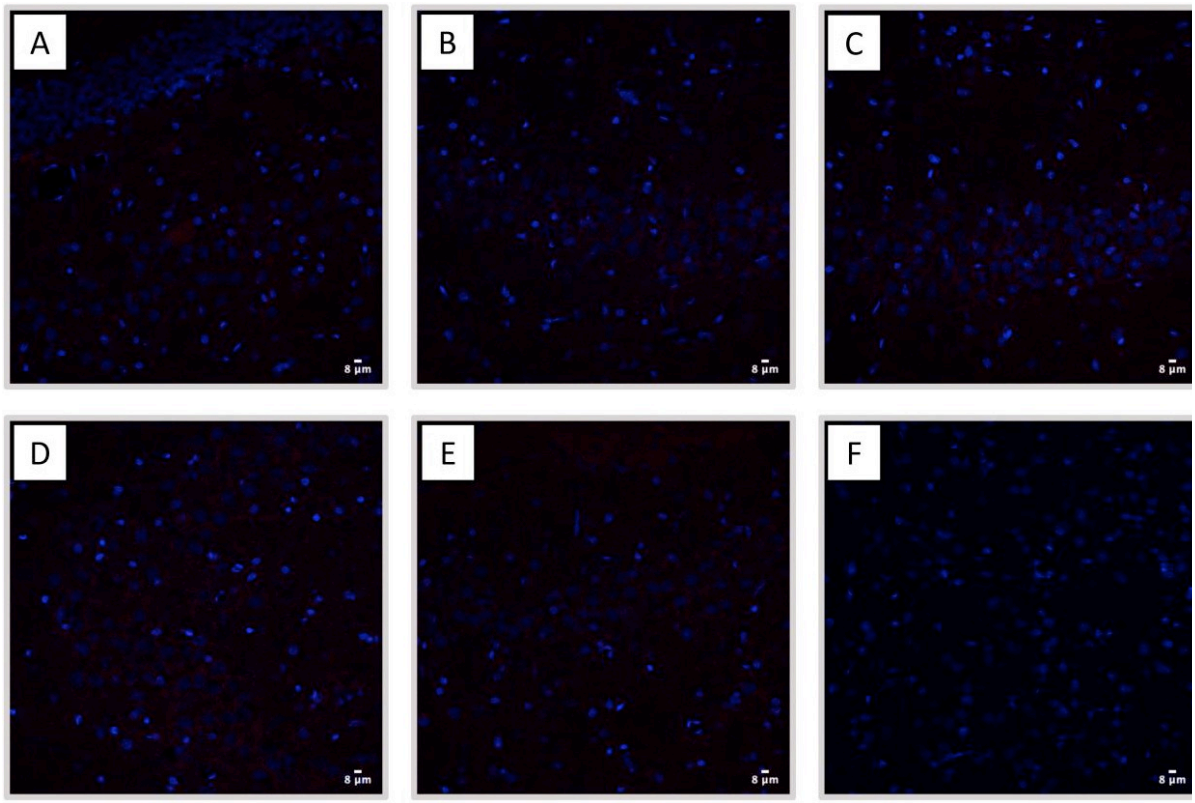


Figure 17 Validation of PLA in hippocampal tissue: background controls.

Technical control demonstrates the specificity of PLA signals in hippocampal tissue and the proximity between two proteins (MR+GR). As a background control GR primary antibody was omitted (-) and replaced with a rIgG antigen, together with anti-MR 1D5. Each photograph is representative of a typical cell staining observed in 5 fields randomly chosen. Studies were performed as three independent experiments.

3.4 Discussion

The aim was to develop an optimised protocol for indirect PLA to explore the interaction between GR and MR in overexpressing cells and endogenous expressing tissue with a commercial PLA set and standard laboratory materials. For the first time colocalization between GR and MR can be observed. The optimised indirect PLA protocol is suitable for detection of a GR:MR complex across different sample types, one that can be applied to assess other protein-protein interactions and its potential role in medical research. PLA is powerful tool in molecular biology to analyse specific PPIs and their co-localisation within the cell and encompasses other advantageous methods including microscopy, necessitating low cell numbers and in situ visualisation. What sets PLA aside from other methods of detecting PPIs is its molecular precision where PPIs are detectable between 0-40 nm. Here, robust

negative and positive controls were provided, demonstrating specificity of the optimised indirect PLA for total protein detection.

A strong and abundant positive PLA signal was observed in both cells [using GR (M20) and anti-MR (1D5)] and in tissues [using anti- and anti-GR (24050-1-AP) and anti-MR (1D5)], representing a co-localised MR:GR complex, validating both the optimized indirect PLA protocol, GR/MR expression and interaction in cellular compartments (relative to the treatment), and the specific and selective performance of primary Abs. Early observations in the testing of the PLA revealed a specific localisation of the GR:MR complex in the cytoplasm under a vehicle (EtOH) treatment control and peripheral to the perinuclear membrane in Neuro-2A cells. PLA in 3617 cells revealed GR:MR dispersed throughout the nucleoplasm following ligand activation with 100nM corticosterone.

It must be noted that the nature of the interaction, whether direct or indirect, cannot be demonstrated using PLA, the short DNA strand conjugated secondary Ab probes technically permit proteins within a proximity of <40 nm to produce a PLA signal (Soderberg *et al.*, 2006). However, Rivers *et al*. chromatin-immunoprecipitates of characterized MR and GR DNA binding profiles in Neuro-2A cells demonstrated recruitment to highly similar DNA binding sites, with differential transcriptional regulation of genes depending on the type of cooperative interaction between MR and GR (Rivers *et al.*, 2019). This observation is in agreement with the GR:MR interaction shown in the preliminary PLA experiments.

Of interest is the specific localisation of GR:MR complex in Neuro-2A cells where the PLA signal (red puncta) is consistently observed at the nuclear periphery proximal to the inner nuclear membrane, as denoted by the edge of the nuclear DAPI stain and possibly within heterochromatic regions. This is discussed further in chapter 4 and presumably highlights the co-localisation of GR:MR complexes to particular subnuclear proteins and/or structures, i.e. those involved in the regulation of cell structure and genomic regulation. Inactive parts of the genome, also referred to as the silenced genome, are organised into topologically associated domains (TADs) that contain transcribed genes positioned into unfolded, less dense and lose chromatin. The genome can be separated into spatially inaccessible blocks that permit these TADs to develop into self-regulating genomic blocks with semi-autonomous mechanistic regulation (Razin *et al.*, 2016; Razin and Ulianov, 2017). Throughout, these functional domains are modulated, and it is believed that their disruption is involved in the dysregulation of genes involved in disease.

There are differences in the number of PLA puncta observed when comparing Neuro-2A cells and hippocampal tissue. Considerably more puncta are observed in Neuro-2a cells than in hippocampal tissue. This could be explained by the overexpression of GR/MR in cells and the physiologically lower

endogenous protein levels observed at the perinuclear membrane *in vivo*. Nevertheless, the optimised PLA protocol is highly specific and suited for detection of molecular proximity between MR and GR *in vitro* and *in vivo*. Efficiency of the PLA method was successfully tested in cells and tissues for the detection of glucocorticoid receptors MR and GR.

Of note, growing and adherence of semi-adherent neuro-2a cells to the chamber surface at times was problematic, despite chemical fixation, however this was managed during the incubation and washing steps using optimised PBS and PLA 'wash' buffer solutions. Solutions and reagents were applied to and removed at the inner chamber wall by careful tilting of the chamber containing cells and not directly onto the cells. Furthermore, incubation on a shaking platform was performed at a low rpm. The number of washing steps was decreased from four to three to avoid agitating the attached cells more than necessary, and the duration of each wash step increased to ten minutes. A variety of modifications to the manufacturers protocol were introduced. Buffers and reagents were prepared and stored according to the manufacturer's instructions and for optimal Ab performance so as to avoid any background signal (non-fluorescent). The antibody buffer used across the different samples was made according to titration optimisation of the permeabilization factor in the laboratory and not from the reagent provided in the commercial PLA kit, and this was always prepared before addition of the antibody and stored at 4^{o}C. The blocking step from endogenous tissue was increased to two hours despite the one-hour detail in the manufacturers protocol. In addition, a hydrogen peroxidase antigen retrieval step was applied for the purpose of minimising non-specific background signal. The secondary-probe and ligation incubation steps were increased by 30 minutes, as detailed in the commercial protocol, in each case.

Antibody testing is essential to confirm target specificity and sensitivity supporting IF/IHC performance across a dynamic range of PPIs across different experimental conditions and if to be used in pathological samples where protein expression or interactions may be weaker, as an example. Early validation is essential in cases where the selected Ab will be applied for use in developing further cellular understanding and drug development processes where the target may be used as the method by which the therapy is applied. Further supporting the need for Ab validation, more often than not, Ab information regarding epitope specificity or optimal titrated concentrations is scarce if at all provided in commercial data sheets and academic publications.

The specificity of the indirect PLA is reliant on the chosen pair of primary abs. The abs work in conjunction with and specificity to each other:

- antigen/protein target ← primary ab ← secondary ab

Specificity and efficiency of the detection system will be compromised with a sub-optimal ab pair and without validation testing. It is recommended that abs be validated via IF/IHC and followed by PLA testing. Control material for ab validation include positively and negatively cultured cells, and where transfection can be manipulated, such as introducing different dose levels of the targeted protein in what may be weakly expressing or negative cell lines. Cell lines can reveal are useful in expression and ab validation, which compared to non-IHC techniques, are very useful, however the use of positive and negative endogenous tissue will complete a full validation profile for the ab and assessment of any non-specific binding to other endogenous molecules. For the purpose of validating expression levels in disease it would be ideal to test expression in a selection of disease in addition to matched control (healthy) tissue. In advance of validation in tissue, the composition of the tissue will require assessment and against a screen of ab candidates. The PLA validation, either by single or dual recognition of a protein/s, will demonstrate which ab combinations contain the limiting ab by observing a reduction in fluorescent puncta.

Due to the sensitivity and fragility of the PLA methodology, it is important to adhere strictly to the recommended or optimised protocol for the sample type being used. Incubation conditions should be followed. The correct storage of enzymes and abs is crucial as their performance will degrade over time and should only be prepared as close to their sample application where possible. Wash buffers should be prepared in high purity water and where possible sterile conditions, and stored at 4^{o}C in the dark, as this will extend its duration. I recommend replacing wash buffer solutions every 6-8 weeks if they have been prepared and stored appropriately.

The duration of the protocol from plating of 3617 or Neuro-2A cells using the validated ab pair is 7 days. Cells should be calculated at a seeding density to produce a maximum ~70% confluency as this will support the performance of the PLA through ab/probe/enzyme accessibility via diffusion into the sample and for visualisation clarity using microscopy. This is due to the ab pair requiring 72 hours incubation, but other abs will vary in their incubation durations and conditions for optimal performance. It is worth considering that when overexpressing the proteins of interest, 100% transfection efficiency is rarely achieved, and a subset of cells will remain negative or weakly stained with fluorescent signal. However, this can be useful in distinguishing IF/IHC/PLA signal to background noise. Following application of the polymerase enzyme, the samples should not be exposed to light as this will decrease the fluorescence. Samples should be stored appropriately in the dark at 4^{o}C and quality of image acquisition will reduce after 1 week.

The indirect PLA has been optimised for use on 3617 and Neuro-2A cells, and brain tissue. Cellular mechanisms and biochemical pathways are disrupted in disease as a result of altered subcellular

location of proteins and PPIs. The optimised protocol for indirect PLA provides a means to observe PPIs and localisation.

3.5 Conclusion

IF/IHC based techniques are pivotal in histopathology and plays a significant role in diagnostics, such as in biomarker mediated tumour identification, and in targeted drug therapies. IF/IHC, and more uniquely as seen in PLA, are distinct from tinctorial staining methods where the methods employed are unique to PLA, highly specific and sensitive, and reproducible if strictly followed. The data shows that the optimised protocol for indirect PLA provides opportunities for detection of PPIs, such as GR+MR interactions, and their subcellular localisation and is a valuable tool in pathogenesis research. The optimised protocol for indirect PLA is reliable and accurate and has a variety of applications.

3.6 Method for the optimized proximity ligation assay (PLA) for detecting glucocorticoid receptor (GR) and mineralocorticoid receptors (MR)

3.6.1 Indirect Proximity Ligation Assay (PLA) for cells

Seeding of cells in 35mm Mat-Tak dishes.

1. Approximately 200,000 3617 and Neuro-2a cells were added to each dish and grown to 70% confluency and incubated for 24 hours at 37°C with 5% CO_2.
2. 1 microgram of pC1-rGRwt and 1 µg of pX-rMRwt were transiently transfected into 3617 and Neuro-2a cells using 4 µL of jetPRIME® reagent (PolyPlus, New York, NY) according to the manufacturer's instructions. at a ratio of 1:2 (w/v).
3. 3617 and Neuro-2a cells were treated with 100nM corticosterone for 90 and 45 minutes, respectively, at 37°C.

Fixing and blocking.

4. The dishes were gently held at an angle and the media + corticosterone carefully aspirated from the corners.
5. Cells were covered in 4% paraformaldehyde and fixed at RT for 15 minutes. Cells were washed twice for 5 minutes at RT using 1X PBS for re-hydration and in preparation for blocking.
6. Prior to aspirating final PBS wash, blocking solution was gently vortexed.
7. Two drops (~80 µl) of blocking solution (yellow capped bottle from the Duolink PLA probe kit) were added to the entire imaging area of the Mak-Tak chamber dish and incubated for 60 minutes at 37°C.
8. Blocking solution was discarded by gentle tilting of the dish allowing the solution to run off independently and followed by two 10-minute 1X PBS washes at RT.

NOTE: Cells mustn't become totally dry from this point as this will create background signal. All incubations at any temperature must be performed in a moist environment.

Primary antibody incubation

9. 2 mLs of antibody diluent with permeabilisation were applied to each dish and primary antibodies (anti-GR M20 and anti-MR 1D5) were added at the desired dilution (Table 1).

10. Cells were incubated at the optimal time and temperature for the selected Ab, which in this case was for 72 hours at 4°C with gentle rotation.

NOTE: The antibodies that recognise the targeted protomer and PLA probes must be raised in the same species. Depending on the primary antibody and sample type (cells or tissue), titration of dilution concentrations may be required.

11. Primary antibody was carefully aspirated, and cells washed 3 times for 10 minutes in wash buffer A at RT.

NOTE: During the final wash in step 11, PLA probes were prepared and always left on ice.

PLA probe incubation

12. PLA probes (PLUS and MINUS) and Ab diluent were gently vortexed.
13. PLA probes were diluted at the recommended 1:5 concentration to allow for a 60 µL reaction per dish.
14. Final wash was carefully aspirated and 60 µL of the PLUS/MINUS probe solution was pipetted directly to the imaging area and incubated for 1 hour at 37°C.
15. The dish was carefully tilted to allow the Ab probe solution to run off for aspiration.
16. Cells were washed in buffer A twice for 10 minutes at RT.

NOTE: During the washes in step 16, the ligation buffer was completely thawed and vortexed. The ligase buffer solution was prepared and left on ice, and the ligase enzyme only added immediately before being pipetted onto sample dish. The ligase enzyme was retrieved form -20 storage using a freezer block.

Ligase incubation and formation of the rolling circle template.

17. For a 60 µL reaction, the 5x ligation buffer was prepared with sterile high purity water at the recommended 1:5 dilution. The ligase enzyme was calculated at a 1:40 dilution and this same quantity was removed from the ligation buffer solution prior to ligase addition to ensure accuracy.
18. Wash buffer A is carefully aspirated. 60 µL of the ligation + ligase solution was directly pipetted onto the imaging area and incubated for 30 minutes at 37°C.
19. Repeat washes in step 16.

NOTE: During the washes in step 19, the amplification buffer was completely thawed and vortexed. The amplification buffer solution was prepared and left on ice, and the polymerase enzyme only added immediately before being pipetted onto sample dish. The polymerase enzyme was retrieved form -20 storage using a freezer block. As the polymerase buffer is very light sensitive, anything containing this buffer was always protected from light or prepared in minimal light.

Amplification and addition of fluorescent oligonucleotides.

20. For a 60 µL reaction, the 5x amplification buffer was prepared with sterile high purity water at the recommended 1:5 dilution. The polymerase enzyme was calculated at a 1:40 dilution and this same quantity was removed from the amplification buffer solution prior to polymerase addition to ensure accurate concentration.

21. Wash buffer A is carefully aspirated. 60 µL of the amplification buffer + polymerase solution was directly pipetted onto the imaging area and incubated for 100 minutes at 37^{o}C.

Final wash phases

22. Cells were washed in buffer B thrice for 10 minutes at RT. A nuclear DAPI stain was added during the second wash. A final wash with 0.01x wash buffer B (made up in high purity water) was performed.

23. Cell were stored in 2 mLs 1x PBS at 4^{o}C in complete darkness until imaging.

3.6.2 Indirect Proximity Ligation Assay (PLA) in rat tissue slices

Primary Abs for endogenous GR and MR are unconjugated and were raised in rabbit and mouse, respectively. These were coupled with species specific PLA probes (anti-rabbit PLUS and anti-mouse MINUS) as secondary abs, i.e. GR was detected with anti-rabbit. All washes were performed in a coplin jar at RT with gentle agitation on an orbital shaker (20-30 rpm).

1. 30-µm thick fixed rat hippocampal tissue slices were cut for the indirect PLA.

2. Cut sections were transferred into 0.1 M PBS in a 6 well plate according to treatment category. These were stored in the fridge at 4^{o}C (expiry 1 month) or at -20^{o}C in an anti-freeze cryoprotectant solution [20% glycerol; 30% ethylene glycol in 50 nM phosphate buffer, pH 7.4].

3. When required for processing, sections were transferred from their storage solutions to fresh 0.1M PBS and washed two times for 5 minutes. A final wash was performed in 0.1M PBST to begin permeabilization.

4. Mounting rat hippocampus onto slides

5. Three to four hippocampal sections were mounted per 'Superfrost™ Plus Microsoft slide' using 1X PBS. 0.2% gelatin in 0.05 M Tris Cl, pH 7.6 was tested for section mounting and this did not interfere with the results. However, it was an additional preparation step that was not essential to indirect PLA performance.

6. Slides were airdried at RT until completely dry to prevent detachment from the slide surface during wash steps.

7. Each coronal slice was encircled with an ImmEdge™ Hydrophobic Barrier Pen to maintain the sequential reagents applied on the sample and within the imaging area (Figure 18). Allow ~30 minutes to dry.

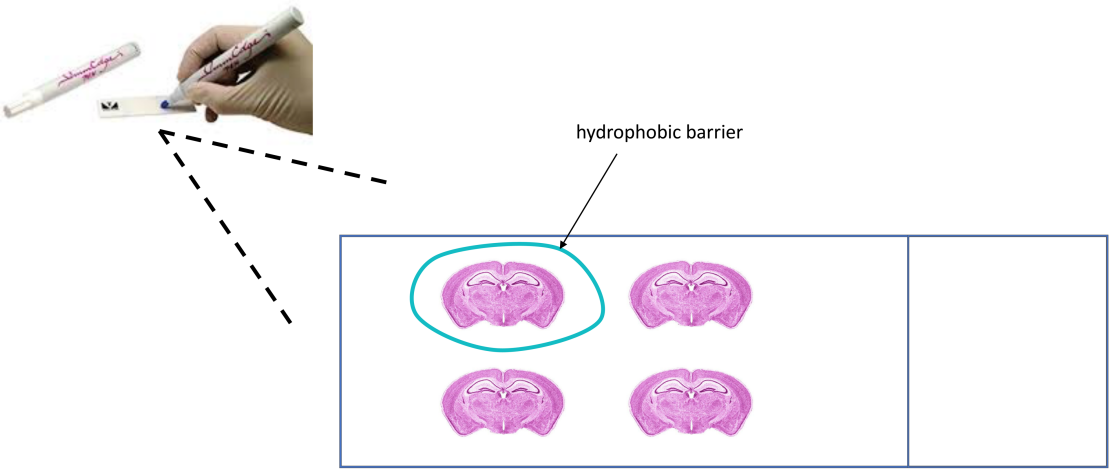


Figure 18 Schematic representation of tissue section arrangement and application of a hydrophobic barrier.

8. Once dry, the slides were permeabilised with 0.01% Triton X-100 in 1X PBS and agitated on an orbital shaker at 20-30 rpm for 15 minutes.

9. Keeping the slides in the coplin jar, the permeabilization solution was discarded and replaced with 1X PBS for two 10-minute washes.

10. The slides were transferred into a suitable container (in this case a large petri dish with a lid) and laid flat on top of wet tissue and into the incubating chamber ready for blocking.

Blocking

11. Two drops (~80 µl) of blocking solution (yellow capped bottle from the Duolink PLA probe kit) were added to the entire imaging area within the hydrophobic barrier per slide and incubated with the lid on for 60 minutes at 37°C.

12. Blocking solution was discarded by gentle tilting of the slide onto tissue allowing the solution to run off independently and followed by two 10-minute 1X PBS washes.

NOTE: It is important that the cells do not become totally dry from this point as this can cause background signal. Slides were transferred from PBS solution back into the petri dish once the secondary ab probes were ready to use. All incubations at any temperature must be performed in a moist environment.

PLA probe incubation

13. PLA probes (PLUS and MINUS) and Ab diluent were gently vortexed.

14. PLA probes were diluted at the recommended 1:5 concentration to allow for a 70 µL reaction per hippocampal section.

15. The slides were returned to their container and carefully tilted to allow excess PBS to run off. Slides were laid flat on top of moist tissue and 70 µL of the PLUS/MINUS secondary probe solution was pipetted directly to the imaging area and incubated for 1 hour at 37°C.

16. The slides were carefully tilted to allow the Ab probe solution to run off for aspiration.

17. Slides were washed in buffer A twice for 10 minutes.

NOTE: During the washes in step 16, the ligation buffer was completely thawed and vortexed. The ligase buffer solution was prepared and left on ice, and the ligase enzyme only added immediately before being applied. The ligase enzyme was retrieved form -20 storage using a freezer block.

Ligase incubation and formation of the rolling circle template.

18. For a 70 µL reaction, the 5x ligation buffer was prepared with sterile high purity water at the recommended 1:5 dilution. The ligase enzyme was calculated at a 1:40 dilution and this same quantity was removed from the ligation buffer solution prior to ligase addition to ensure accuracy.

19. Slides were returned to their incubation container and 70 µL of the ligation + ligase solution was directly pipetted onto each imaging area and incubated for 30 minutes at 37°C.

20. Repeat washes in step 16.

NOTE: During the washes in step 19, the amplification buffer was completely thawed and vortexed. The amplification buffer solution was prepared and left on ice, and the polymerase enzyme only added immediately before being pipetted onto sample dish. The polymerase enzyme was retrieved form -20 storage using a freezer block. As the polymerase buffer is very light sensitive, from this point on samples were always protected from light or kept in the dark.

Amplification and addition of fluorescent oligonucleotides.

21. For a 70 µL reaction, the 5x amplification buffer was prepared with sterile high purity water at the recommended 1:5 dilution. The polymerase enzyme was calculated at a 1:40 dilution and this same quantity was removed from the amplification buffer solution prior to polymerase addition to ensure accurate concentration.

22. Slides were returned to their incubation container and 70 µL of the amplification buffer+polymerase solution was directly pipetted onto the imaging area and incubated for 100 minutes at 37^{o}C.

Final wash phases and application of DAPI mounting media

23. Slides were washed in buffer B thrice for 10 minutes (ensure covering of coplin jar with foil). A nuclear DAPI stain was added during the second wash. A final wash with 0.01x wash buffer B (made up in high purity water) was performed.

24. Slides were air dried and 8 µl of Duolink mounting medium with DAPI was applied with a widened cavity pipette tip.

25. Glass coverslips were immediately placed on top with care to avoid air bubbles and the edges were adhered to the slide with a clear acrylic solution.

26. Hippocampal sections were visualised under a confocal microscope using a 10x lens dry and a 40x lens oil.

4 Modular Image Analysis

4.1 Introduction

Advances in imaging techniques over the last three decades, the introduction of imaging hardware platforms and fluorescent probes has allowed visualisation of protein pathways in tissue and in cells. Immunofluorescence microscopy (IF) is a popular mode to investigating the effects of proteins and their interactions in protein localisation and interaction studies. Changes in puncta properties such as number, density and intensity provide information regarding the subcellular distribution and any changes in the localised numbers of the proteins of interest within particular cells.

Image analysis has been applied to study nuclear receptors including in situ PLA analysis of oestrogen receptor dimers (ER-ER) by measuring the area of dots localised in the nuclei to predict response to endocrine therapy for breast cancer (Iwabuchi *et al.*, 2017). Image analysis has been extensively applied to neuronal studies, for example, PSD-95, a post synaptic scaffold protein was identified as a marker of excitatory synapses (El-Husseini *et al.*, 2000). Unfortunately, the quantification of proteins in various neuronal cell types using fluorescent images is tediously rigorous and time consuming. Analysing puncta properties manually involves counting and tracing individual proteins and their clusters throughout the cellular structures, and also introduces user-bias. Publicly available image analyses packages such as Fiji or MetaMorph (Collins, 2007) are commonly used. These packages have less user bias and are more rapid, and thus are have effective multipurpose use in image analysis, however when applied for specified tasks such as puncta analysis, numerous measurements must be recorded, re-organised and then re-combined to obtain the output data. A common caveat is the image analysis packages' automated particle detection algorithm frequently will identify numerous clusters proximal to each other as a single object, requiring intervention by manual correction or resulting in a false result. Publicly available analysis packages have difficulty in processing large image data sets and some require various detection settings to be manually adapted to each individual image, which both ultimately extends the time required for analysing fluorescent images.

Therefore, the need for image analyses packages in quantitative protein analysis, such as puncta identification and its spatial relation within the cell, exists. The analysis of dynamic GR protein interactions, specifically MR:GR, and their regulation by GCs, has not been previously performed. In conjunction with the PLA technique for analysis of PPIs, it was essential to develop a method that would objectively and accurately identify and measure PPIs, as well as determine their subcellular localisation.

This chapter provides a detailed workflow for protein interactions and localisation experiments using the developed MIA (modular image analysis), a modular framework for Fiji in which image and object analysis modules can be formulated. There is a need for such modular frameworks to analyse protein interaction and localisation experiments, particularly nuclear localised proteins, puncta detection and its distance relation to the inner nuclear edge. The principles of the modular framework within the MIA are introduced, as well as introduced control features, and then discussed. Basic microscopy recommendations in image acquisition that may need to be performed for later analysis are briefly discussed. In summary but discussed in further detail in this chapter, a defined nuclear channel is pre-processed with a 3D Gaussian filter to reduce noise and with an applied threshold to identify the nucleus. Nuclei are subsequently detected as regions of connected pixels (Legland, Arganda-Carreras and Andrey, 2016) above the threshold and any mis-detected nuclei are removed based on a size threshold (they must be larger than 200 μm^3). The red-channel objects (puncta) are detected using the TrackMate plugin (Tinevez *et al.*, 2017) and the distance of each object to the surface of the closest nucleus is measured. TrackMate is a Fiji plugin available in the public domain, that can be applied at varying degrees of manual and/or the automated tracking of single particles. The intensity of pixels within a specific range (-5um to +5um in my version) are then measured for the red and blue channels. Furthermore, the analysis is set for batch processing as a default mode enabling large datasets to be processed. The development of this workflow has enabled the counting and relating of puncta to subnuclear structures (i.e. NE) in multiple 3D images, for the analysis of cellular processes such as protein interactions.

4.2 Method: Modular Image Analysis

Modular Image Analysis (MIA) is a Fiji plugin that allows for modular framework assembly of image and object analysis pipelines. The analysis involves modules for object (nuclei or puncta) transformation and filtering that are required prior to measurement and relating of puncta (MR:GR puncta) to the inner nuclear edge (Figure 19). These are usually batch enabled which permit analysis of large datasets. The module structures and processes shall be discussed here in further detail. A summarised description is available in the previous methods section of this chapter.

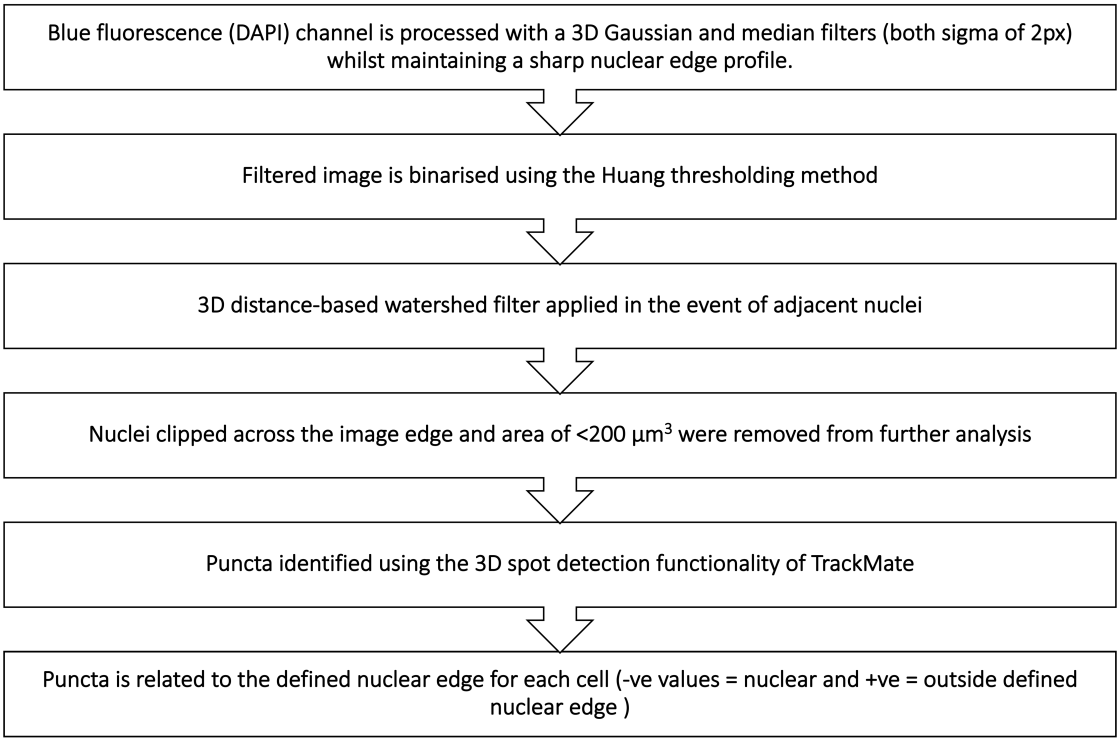


Figure 19 Simplified workflow of the Modular Image Analysis (MIA) for measuring the spatial distribution of puncta in nuclei.

The plugin identifies the nucleus as a whole (i.e. all DAPI-stained pixels). When calculating the edge, it's referring to the outermost shell of nuclear pixels. As such, the edge will technically be "inside" the nucleus and measured in microns. A 3D Gaussian and median filter are applied to reduce noise and a defined edge is maintained, prior to binerisation by the Huang thresholding method. A 3D watershed filter is applied to split any adjacent nuclei. TrackMate identifies puncta according to a pre-defined threshold and the puncta are then related to the defined nuclear edge.

4.3 Method Description

4.3.1 Modular Image Analysis plugin

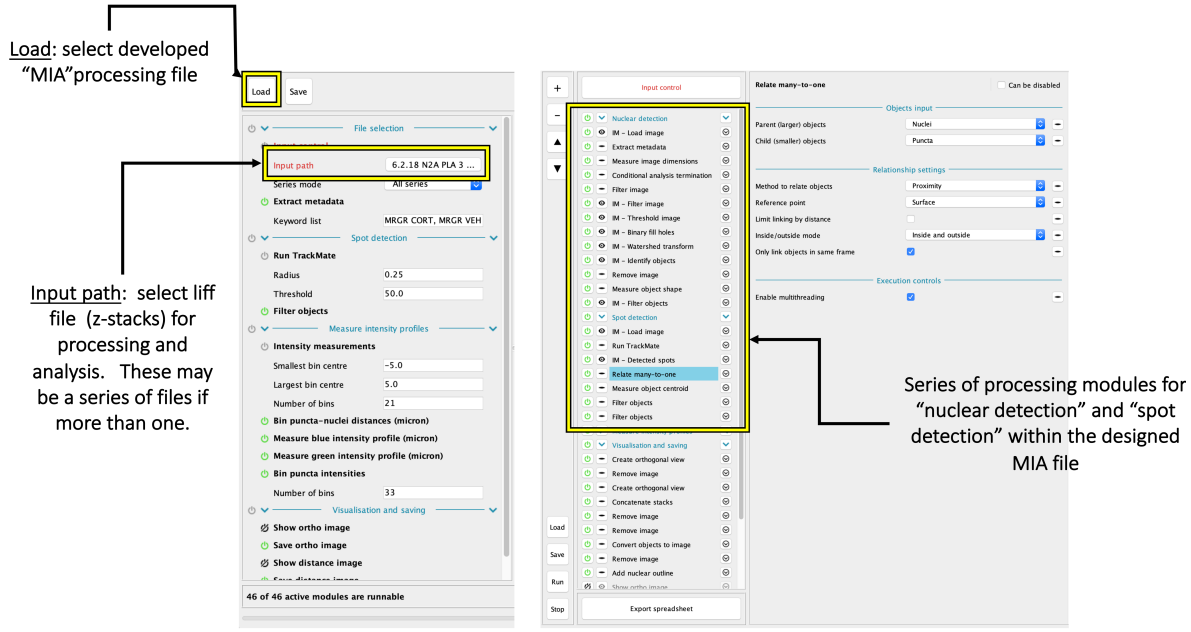


Figure 20 Modular Image Analysis Fiji plugin.

Objects can be detected for transformation, filtered, measured and related. Analysis pipelines can be batch enabled or in single file mode. Load: the. mia workflow file is uploaded into the pipeline. Input path: the liff file is loaded into the input control framework. The summary of the ongoing task is viewable and once finished will display as complete.

The MIA plugin is run from a .jar file which requires installation into the Fiji installation plugins directory. In Fiji, the plugin is found in Plugins > Bristol WBIF > Modular Image Analysis. Next (figure 20), the analysis workflow is loaded by selecting the .mia workflow file. This workflow configuration will present various controls that will be visible in the MIA pipeline window. In the right panel, modules available can be added or removed from the workflow using the '+' and '-' buttons, and their processing order can be reorganised using the arrow buttons. Selecting a module will display parameter details in the right panel. Input files are specified using the input control and the analysis is started by clicking 'Run'. In batch mode processing the status panel displays the number of processed files and in single file mode, the summary of the task being processed. Once the task is finished, 'complete' will be displayed in the status panel and an XLS file is exported and saved in the input directory. The modules within the MIA workflow will now be discussed in further detail.

4.3.2 Nuclear detection

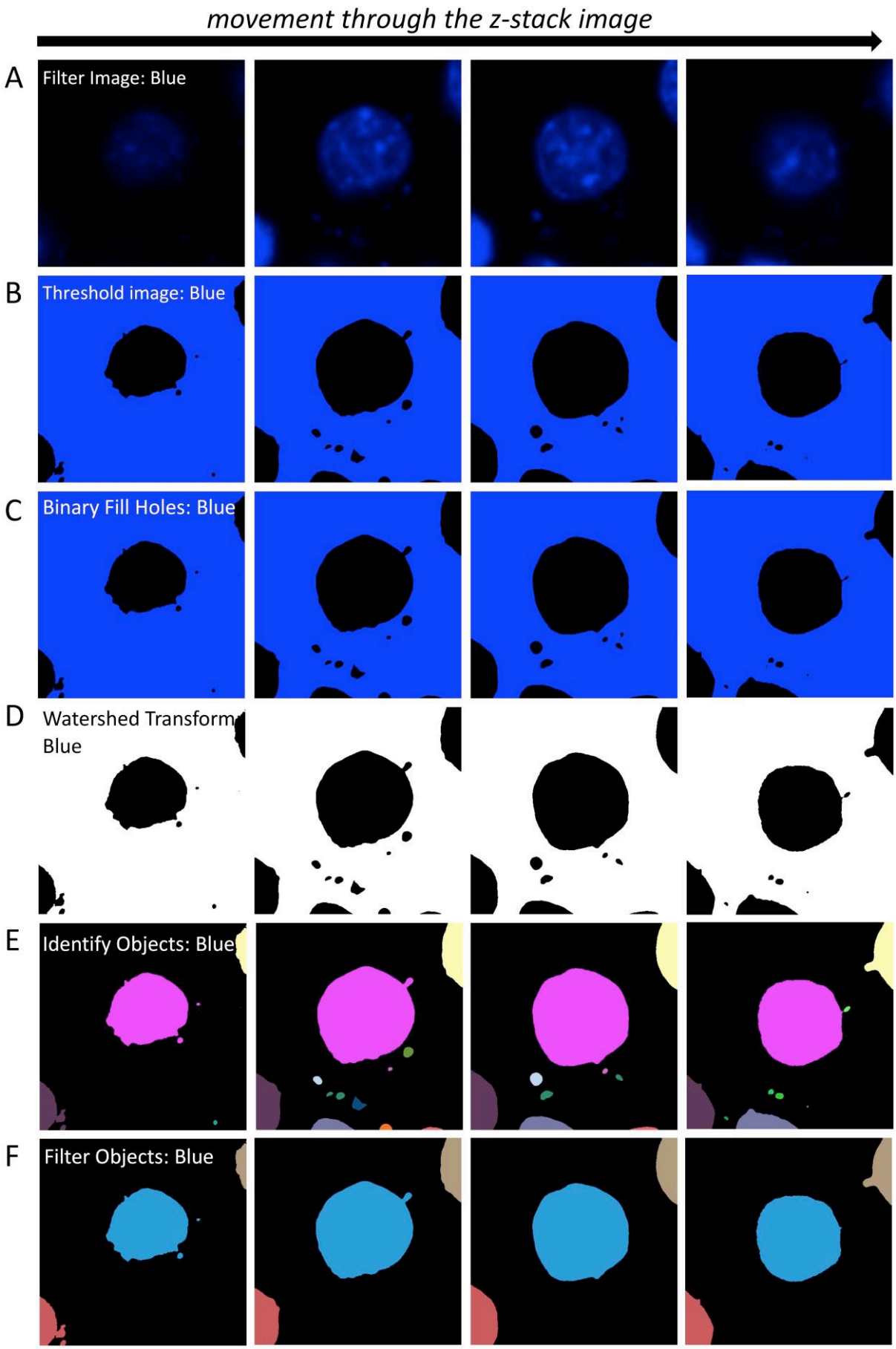

movement through the z-stack image
A
Filter Image: Blue
B
Threshold image: Blue
C
Binary Fill Holes: Blue
D
Watershed Transform
Blue
E
Identify Objects: Blue
F
Filter Objects: Blue

Figure 21 Modular Image Analysis for nuclear detection.

A-F represent the processing modules and are shown across 4 representative z planes for each module. (A) Filter image: input image is processed using 3D Gaussian to smooth and reduce noise levels in preparation for the edge-detection output. (B) Threshold image: the threshold value is set based on the voxel value as the inclusion function as determined by the Huang algorithm (Huang and Wang, 1995). (C) Binarising the nuclear image: The input image is binarised in case of background voxels or 'holes' that may be identified and split at this point and result in the computation of the distance transform. (D) Watershed transform: input image is segmented where objects are touching via detection of different catchment basins. (E) Identify objects: objects are identified as contiguous regions of foreground labelled voxels and their coordinates stored. (F) Filter objects: components or 'nuclei objects' that fall outside of the defined threshold value (26-way connectivity), such as noise or foreign particles are removed.

4.3.2.1 Filter Image

A 3D Gaussian (or Gaussian smoothing) is a representation of the "point spread function" of each point of light, hence, the shape of the light profile and how the light blurs as it passes through the microscope (Figure 21 A). 3D Gaussian smooths out the blur to produce an image approximating what would be observed through a translucent screen. The gaussian filter is a well-characterised process applied to image processing to reduce image noise and detail (Huang and Wang, 1995).

The 3D Gaussian filter is common in edge detection, where edge-detection algorithms such as the Laplacian filter, are prone to non-specific noise. As such, Gaussian smoothing is applied prior to detection of the edge to minimise image noise levels and improve the edge-detection output. Next, a "edge preserving" image filter is applied to remove further noise and without causing too much blurring of edges, where the edges between objects and the background must be maintained as sharp as possible for good segmentation (Fisher, Perkins, 2003).

4.3.2.2 Threshold Image

The level at which the threshold is set is determined by the Huang algorithm (Huang and Wang, 1995) (Figure 21 B). A threshold value is set to determine the inclusion function of a voxel value using the absolute difference between the gray level and the average gray level of its source location, such as the object or the background. If the total value discrepancy increases, the inclusion value decreases. The pixel/voxel inclusion function of an input image is presumed to be maximal. Furthermore, it uses two fuzziness measures, Shannon's function and Yager's measure, of an input image to define the appropriate threshold value:

- *maximum fuzziness is measured at* $\mu_{\mathrm{X}}(\chi_{mn}) = 0.5$

- *minimum fuzziness is measured at* $\mu_{\mathrm{X}}(\chi_{mn}) = 1$

Using this, the most appropriate threshold can be set by reducing the fuzziness value of an input image, where the pixel/voxel inclusion value are between 0.5 and 1, and accordingly the actual inclusion value per pixel/voxel can be presumed to be nearer to 1, enabling the most decreased fuzzy value per pixel.

4.3.2.3 Binarising the nuclear image -> Binary Fill Holes

In a later step, a 'watershed' segmentation is applied to split any adjacent nuclei and define them as separate structures. Prior to this, the nuclear image must be binarized in the event of 'holes' (background pixels) being present within the binarised nuclear object, which places the watershed algorithm at risk of splitting the object at the 'holes' and thus produce inaccurate results (Figure 21 C). This is because the watershed segmentation calculates distances of all foreground pixels to the closest background pixel.

4.3.2.4 Watershed transform: Separates any adjacent nuclei.

The watershed transform algorithm is used to segment touching objects (nuclei). Its function involves incorporating the grey level image to a digital elevation model for identification of different catchment basins. The catchment basins correspond to dark regions surrounded by bright structures (Figure 21 D and Figure 22).

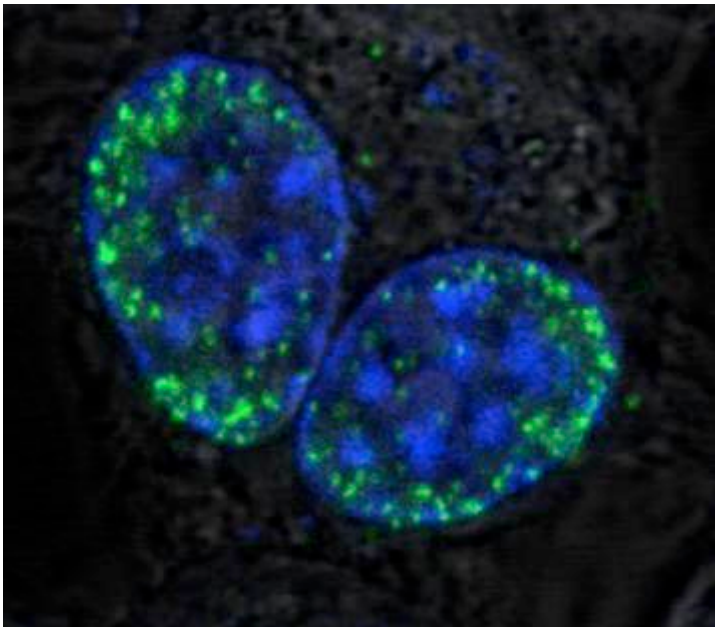


Figure 22 Watershed transform: example pictograph of touching nuclei that require splitting to identify them as separate structures.

4.3.2.5 Identify Objects

The identify objects module utilises 'connected components operators' (CCOs) that pool information within an image according to the connectivity between pixels or voxels. Individual objects are identified as contiguous regions of foreground labelled voxels. A morphological area or volume using a 26-way (pixels/voxels) connectivity threshold value will remove any 'nuclei objects' containing less than 26 voxels (Figure 21 E). This includes diagonals of the defined object so that elongated objects can be identified as well as rounded, where nuclei can take on various forms. Within CCOs, is a morphological reconstruction sub-operator that permits reformation of a marker image by confining it to a mask. This sub-operator can be further developed to allow detection of minima and maxima in grey scale images and is applied as a marker detection for segmentation (Breen and Jones, 1996).

Upon completion of the 'identify objects' module, objects are stored in the plugin as sets of coordinates, which will allow for the objects to be related to each other. In the output image (Figure 21 E) the different objects are displayed in different and randomly applied colours. This enables the viewer to distinguish those objects which were identified as the same or different.

The nuclei object shape is measured by counting the number of voxels for each object and then the known spatial calibration is applied to get a measurement in um^3. Spatial calibrations are real-world distances per pixel and come from the setup of the microscope.

4.3.2.6 Filter Objects

The filter object module aims to remove components or 'nuclei objects' of an input image based on the defined threshold value (26-way connectivity). This will remove any images with noise, or which have been contaminated by foreign particles, and are falsely identified as nuclei (Figure 21 F). In addition, detected objects are filtered to identify and remove any nuclei objects that are touching the image border as it cannot be assumed how much of the nucleus is missing and might create a false nuclear surface where the image edge is assumed to be part of the nuclear edge.

4.3.3 Spot detection

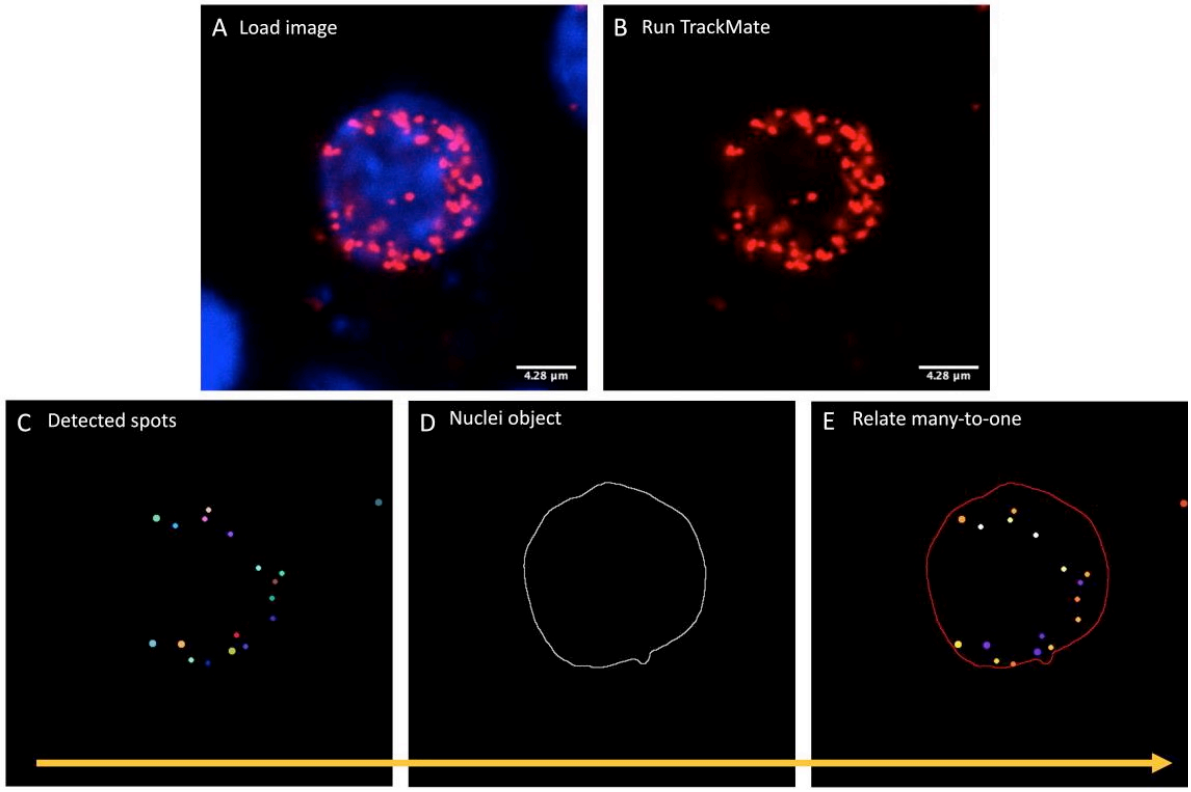


Figure 23 Modular Image Analysis for spot detection.

A-E represent modular stages for spot detection and TrackMate processing. (A) Load image: the composite image [nuclei (blue) and MR:GR puncta (red spots)] is accessed in the input control. (B) Run TrackMate: the input image is prepared for sport detection using a Laplacian of Gaussian (LoG) spot detector. (C) Detected spots: identified single particle features and position coordinates are recorded. (D) Nuclei object: previously detected nuclei object is loaded. (E) Relate many-to-one: the stored nuclei and spots detected are now related to each other.

4.3.3.1 Run TrackMate module

TrackMate's Laplacian of Gaussian spot detector module is used for spot detection within an image (Figure 23 B). This module can identify single particles or "spot like" features, which are bright objects within a dark background and the extracted information is stored according to their specific X/Y/Z coordinates within the image (the centroid of that spot). TrackMate computes and expands the numerical features for each 3D spot based on mean, max, min and median intensity and the input radius and orientation for each spot (Tinevez *et al.*, 2017).

The spot radius is approximated (based on pixel/voxel units = 0.25), and a Laplacian of Gaussian filter is applied to the image with this specific radius (Figure 23 C). This enhances spots of this size, which then allows TrackMate to detect points within the image which are brighter than the specified threshold. The output image exhibits coloured overlap spots that were detected according to its position.

4.3.3.2 Relate many-to-one module

At this point, the detected 'spot' objects can be related to the previously detected 'nuclei' object, using the stored coordinates (Figure 23 D). Using the extracted coordinates for both detected objects, the shortest distance is measured from each spot centroid to the closest nuclei surface (Figure 23 E). For each puncta, a "bin" value is assigned and stored and can be exported to an XLS spreadsheet for further analysis. The bin value is based on the distance to the nuclear surface:

- The distance measured is increasingly positive the further away from the nuclei surface the spots are.
- The distance measured is increasingly negative the further inside the nuclear edge the spots are.

**The MIA workflow for puncta analysis was developed in discussion with Dr. Stephen Cross, Research Associate in Bioimage Analysis at Wolfson Bioimaging Facility (University of Bristol). The code for data analysis was formulated by Dr. Cross and is stored in a ZIP file in Online supplemental material. The plugin (v0.11.18) and the source code (http://doi.org/10.5281/zenodo.3530751) are also publicly accessible on a new GitHub repository*

(https://github.com/SJCross/MIA/tree/v0.11.23). This is connected to Zenodo (https://zenodo.org/record/3530751#.XcMA8JBxc6Q) where the DOI series for this version is stored. The Modular Image Analysis (MIA) requires the plugin Modular_ Image_Analysis-v 0.11.23.jar and the MIA workflow file used for the final analysis (Puncta-in-nuclei.mia). Installation and usage texts are available (GitHub) on how to install the plugin and run the analysis, and a list of other associated functions required for the workflow.

4.4 Discussion

The MIA (modular image analysis) was developed as a modular framework for Fiji in which image and object analysis modules can be formulated, such as with puncta detection in relation to a subcellular structure. The described specific analysis module has been designed to analyse puncta properties in relation to the periphery of the inner nucleus using a DAPI stain as the nuclear edge. The MIA plugin can identify the nucleus as a whole using all DAPI stained voxels and calculation of the edge is referred to as the outermost shell of nuclear pixels. As such, the edge is technically inside of the nuclear membrane. The nuclear channel is pre-processed with a 3D Gaussian filter to reduce noise and then is thresholded to identify the nucleus. Nuclei are subsequently identified as regions of connected voxels that exceed the threshold. Any mis-identified nuclei are removed based on the pre-defined size threshold; in this case they must be larger than 200 μm^3. This calculates the average (and standard deviation) distance of intensity to the edge of an object. The intensity can either be inside, outside or both for the object. The intensity of pixels/voxels is measured within a specific range, in this case between (-5 μm to +5 μm), for both the red and blue channels. This distance range was set where initial 3D volume slicing, and orthogonal viewing revealed no puncta beyond this distance within the nucleus. Red channel objects are also detected using the TrackMate plugin, and the positioning information is used to calculate the distance of each object to the surface of the closest nucleus. In general, the bins with negative distance, e.g. -3 μm corresponds to pixels -3 μm from the inner nuclear surface, as opposed to those with positive values, which are 3 μm outside of the defined nuclear edge, suggested to be predominantly at the outer nuclear membrane or within the perinuclear space. Bins without values occur when there or no detected pixels/voxels at this distance from the 'nuclear' edge, inside or outside.

TrackMate was initially designed to study *C. elegans* (Tinevez *et al.*, 2012) and was mostly developed to handle trafficking across cellular networks and since, it has been used in protein studies from tracking the movement of molecules (Mueller *et al.*, 2013; Mossuto *et al.*, 2014; Tanenbaum *et al.*, 2014) to identifying their presence and localisation in subcellular compartments. Previously, assessment of 'spot' like objects or particles was detected by manual identification and quantification of individual fluorescent spots. This is a time-consuming process and invites the potential for user bias due to the challenging and subjective determination of where the spots are and what parameters define a spot. TrackMate avoids such issues where its ability in 'spot' detection surpasses that of the user, by the integrated quality threshold parameter (Tinevez *et al.*, 2017). It must be considered that the ability of some modules within the MIA framework, such as TrackMate to objectively identify 'spot' objects relies on pre-defined parameters by the user, and as such a certain degree of user bias is introduced. Nonetheless, the pre-defined parameters are applied across every sample, which

removes unintended errors and user bias in spot detection. TrackMate avoids the labour-intensive process of manually identifying and analysing dynamic events in microscopy data.

The development of the MIA workflow file has seen several versions during its evolution for the detection of puncta (MR:GR) and calculation of distance to the defined nuclear edge. At the start of its development, the spot detection module was of concern as background noises (false detections) were being identified as real objects, producing dramatic increases in puncta numbers. Therefore, a 'threshold enable' control was integrated into the MIA workflow, whereby the spot detection could be made less selective or more sensitive. In addition, a control for 'spot radius' on the main workflow panel (in μm) permitted where necessary, changes dependant on the magnification of the image, such as 0.25 μm for zoomed in samples and 0.4 μm for low magnification. Although this was rarely altered as images were taken at the same magnifications across samples. Non-trivial issues consisted of incompatibility of the Fiji plugin with the MIA workflow pipeline following any updates and was easily resolved by simply uploading the newest version of Fiji from Fiji.sc or the MIA file. As such a fundamental requirement for efficient image processing is optimising memory handling to avoid such system crashes, particularly if processing several z-stack files in 'batch mode'. The most memory demanding part of the analysis is the "Relate objects" step. This is where the detected spots are related to the nuclei. At times, processing of large 'single mode' files that had increased z-stacks number taken from a cell or group of cells within one frame, would cause a system crash during processing only after a few stacks. Fortunately, this issue was resolved by optimising the memory handling of the plugin, however shorter z-stack distances (thus more z-stack slices take within the image file) will require more powerful computers with larger RAMs capable of coping with large data files, particularly when analysing more dense and complex samples such as in tissue, and it is advised not to exceed more than 80% of the total working RAM of the device. Lastly, although the thresholding for detected nuclei (which is based on the Huang threshold, so will adapt to slight variations in image intensity arising the fluctuations in laser power or different sample staining), the puncta detection currently uses a fixed threshold. This doesn't appear to be problematic but will require further optimisation. Similarly, all parameters for the analysis require manual setting in the first place, so the element of user bias isn't completely removed – although issues of sample-to-sample variation by using fixed parameters are avoided.

MIA is a Fiji enabled plugin that acts as a scaffold in which you can input modular pipelines for image and object analysis. MIA is freely available and open source, which now allows download of the same workflow file to run the same analysis. In this case, the nuclei and puncta are transformed and filtered, before being measured and related to each other to ascertain the distance of the MR:GR complex to

the inner nuclear edge. This work has enabled the identification and analysis of MR:GR complexes, with the intention of developing our understanding of proteins in cellular processes.

5 Detection and analysis of proximity between the mineralocorticoid and glucocorticoid receptor using proximity ligation assay

5.1 Introduction

The spatio-temporal localisation of MR and GR requires further investigation, particularly where dynamic interactions of MR and GR have been reported to produce differential transcriptional effects. Investigating their subcellular localisation is important in understanding their interactive relationship within the cell and in regulating transcription of CORT inducible genes. Previous research has identified partition and trafficking of ligand induced GR and MR in living cells using fluorescently tagged receptor expression vectors, and observed their distribution throughout the nucleus (Htun *et al.*, 1996; Fejes-Tóth, Pearce and Náray-Fejes-Tóth, 1998; Nishi *et al.*, 2001; DeFranco, 2002). Furthermore, using GFP-fusion proteins, the formation of numerous nuclear GR foci throughout subcellular compartments were identified (Griekspoor *et al.*, 2007). Moreover, following DEX treatment GFP-GR appeared partitioned around the nucleus, between the inner and outer layers of the nuclear membrane, where highly condensed chromatin is found, and is suggested to regulate the amount of available receptor for cellular interactions (Stortz *et al.*, 2017). Despite the interest in GR and its PPIs, the dynamic interaction and movement of MR and GR within the subcellular space is not fully understood. The androgen receptor (AR), for example, is a steroid receptor which relies on PPIs for effective transcriptional regulation, whereby AR interactions with an activity modulating chaperone, Bag-1L, enhances AR action and regulates transactivation of ligand inducible genes (Shatkina *et al.*, 2003; Dehm and Tindall, 2007), which upon disruption of ARs NTD, prevented AR-Bag-1L interactions and produced dysregulated transcriptional effects (Dehm and Tindall, 2007).

A range of cellular and tissue-based methods exist for identifying the co-localisation of proteins and techniques for identifying PPI's (discussed in chapter 1), such as double label indirect immune fluorescence (DIIF) microscopy and Förster resonance energy transfer (FRET) microscopy (Pietraszewska-Bogiel and Gadella, 2011) or fluorescent recovery after photobleaching technique (FRAP) (Reits and Neefjes, 2001). Co-immunoprecipitation (Co-IP) does not confirm if the proteins exist in the same cells and fails to account for cell to cell variability or spatial information (Gerace and Moazed, 2014). ELISAs or intracellular immunofluorescence are typically used in assessing single protein, such as GR activation and subcellular location (Sarabdjitsingh, Meijer and de Kloet, 2010), by measuring fluorescence intensity throughout the subcellular compartments, however although used at high resolution this would bot demonstrate functional proximity. Dynamic interactions between GR and MR were reported using FRET in living cells (Nishi *et al.*, 2004). However, common problems include spectral bleed-through (SBT) or contributions of donor and acceptor fluorescence emission

into the FRET channel. In specific brain regions such as the hippocampus where MR/GR are co-expressed in the same cells (van Steensel *et al.*, 1996), their proximity may produce differential transcriptional output of specific GC regulated genes.

PLA is a recent addition that involves the ligation of short strand DNA probes attached to proximal protein targets to visualise proteins and their interactions with a high degree of sensitivity. PLA utilises two primary antibodies (Abs) raised in different species to recognise two different protein targets or complexes of interest (Soderberg *et al.*, 2006). This is described in further detail in chapter 3: Development and optimisation of PLA using confocal microscopy. PLA has been used to identify PPIs of other steroid receptors, such as ER-ER dimers (Hu *et al.*, 2016; Iwabuchi *et al.*, 2017) or the interactions of ER within a co-regulatory complex, such as GREB-1, SMARCC2 (BAF170) and HDAC1 (Papachristou *et al.*, 2018), due to the receptors role in hormone related growth in hormone derived cancers, such as in the breast or endometrial.

Using proximity ligation assay (PLA) and advanced fluorescence microscopy techniques, the existence of dynamic MR:GR interactions are reported here, both in vitro in Neuro-2As (N2a) cells and in vivo in rat hippocampus. The PLA data reveals the subcellular localisation of MR:GR complexes, after different CORT treatment times. Following CORT treatment, MR:GR complexes were visible close to the inner nuclear membrane. The spatial and temporal localisation to this nuclear compartment informs our understanding of the functional role of MR:GR complexes, how they may be exerting their effects together, which other proteins and structures they may interact with and suggests a role for MR:GR complexes in heterochromatin and the control of gene transcription.

5.2 Method

5.2.1 Plasmids and Constructs

See chapter 2.

5.2.2 Cell culture

Neuro-2A cells were washed twice with Dulbecco's PBS (Thermo Fisher Scientific) and seeded in CSS media (DMEM with 7.5% (v/v) charcoal-stripped fetal bovine serum and the other supplements described in chapter 2) at 0.2 million per well on Thermo Scientific™ Nunc Glass Bottom Dish with a 12 mm viewing area. Cells were transfected ~18 hours following seeding, treated with vehicle control (ethanol, final concentration 0.01%) or 15 nM CORT (Sigma-Aldrich) 24 hours later for 15, 30, 45, 60 and 90 minutes.

5.2.3 Proximity Ligation Assay

The PLA method has been described in Chapter 2: methods and materials and extensively in Chapter 3: optimisation and development of PLA using confocal microscopy. In brief, following cell culture, and CORT treatment, cells were fixed in 4% PFA, blocked and incubated with primary antibodies anti-GR M20/anti-MR 1D5 (dilution at 1:500) in a 2 mL 0.3% triton-X100 permeabilization solution, at 4^{o}C for 72 hours. Time and temperature dependant incubations were performed, namely: hybridisation using Duolinks (DUO92002) PLA probes; ligation of the probes with a ligase enzyme; rolling circle amplification (RCA) with a polymerase enzyme; image acquisition by microscopy and image analysis.

5.2.4 Microscopy and Fluorescence Imaging

See chapter 2.

5.2.5 Image Analysis

See chapter 2 and 4.

5.3 Results

5.3.1 Localisation of MR:GR interactive complexes with and without ligand induction

Interactions between MR and GR were detected and are shown in (figure 24). MR:GR complexes are represented as a red puncta. Neuro-2A cells were cultured according to the protocol described in chapter 2 and transiently transfected with different combinations of plasmids, untagged wild-type rat MR and GR (MR/GR alone, MR+GR together, or an empty vector control (pcDNA3.1) and treated with vehicle control (ethanol, final concentration 0.01%) or 15 nM CORT, for 60 minutes. The 4% PFA fixed cells on glass coverslip chambers were incubated with a blocking solution to reduce the possibility of unspecific binding of proximity probes and then incubated in their appropriate conditions with their primary Abs prior to proximity probe incubation, as described in chapter 3. The observation of a very low non-specific background signal demonstrates the sensitivity of PLA. PLA specificity depends on antibody-recognition which was confirmed earlier with IF using a combination of Abs and receptors where one or both receptors were omitted or replaced with an IgG control. The Ab specificity was further demonstrated using western blot and this is shown in chapter 3.

Puncta were measured in relation to the nuclear membrane denoted by the edge of the DAPI staining, with -ve numbers being within the nucleus and +ve numbers outside the nucleus. Under treatment with a vehicle control (figure 1 A) the MR:GR complex is predominantly cytoplasmic in its localisation. Puncta were detected at distances between (+ve) 5 and 0 microns (figure 2), with an increased presence of MR:GR complexes at 0-microns, where 0 is denoted by the edge of the DAPI staining.

With 15 nM CORT treatment for 60 minutes (figure 24 B) MR:GR complexes appear in the nucleus localised at the inner nuclear periphery. The CORT activated MR:GR interactive complexes are detected at distances between (-ve) 5 and 0 microns, with a small number remaining within the cytoplasm (figure 24 A). An abundance of MR:GR complexes are detected between -0.5 and -1.5 microns (figure 25). The captured z-stack files were displayed as texture-based 3D volume renderings to enable visualisation of ortho slices and the 3D surface. Figure 24 (A and B) bottom right pictographs show 3D renderings of a blown-up selected cell treated with either vehicle control or 15 nM CORT, and which demonstrate the respective cytoplasmic and nuclear localisation.

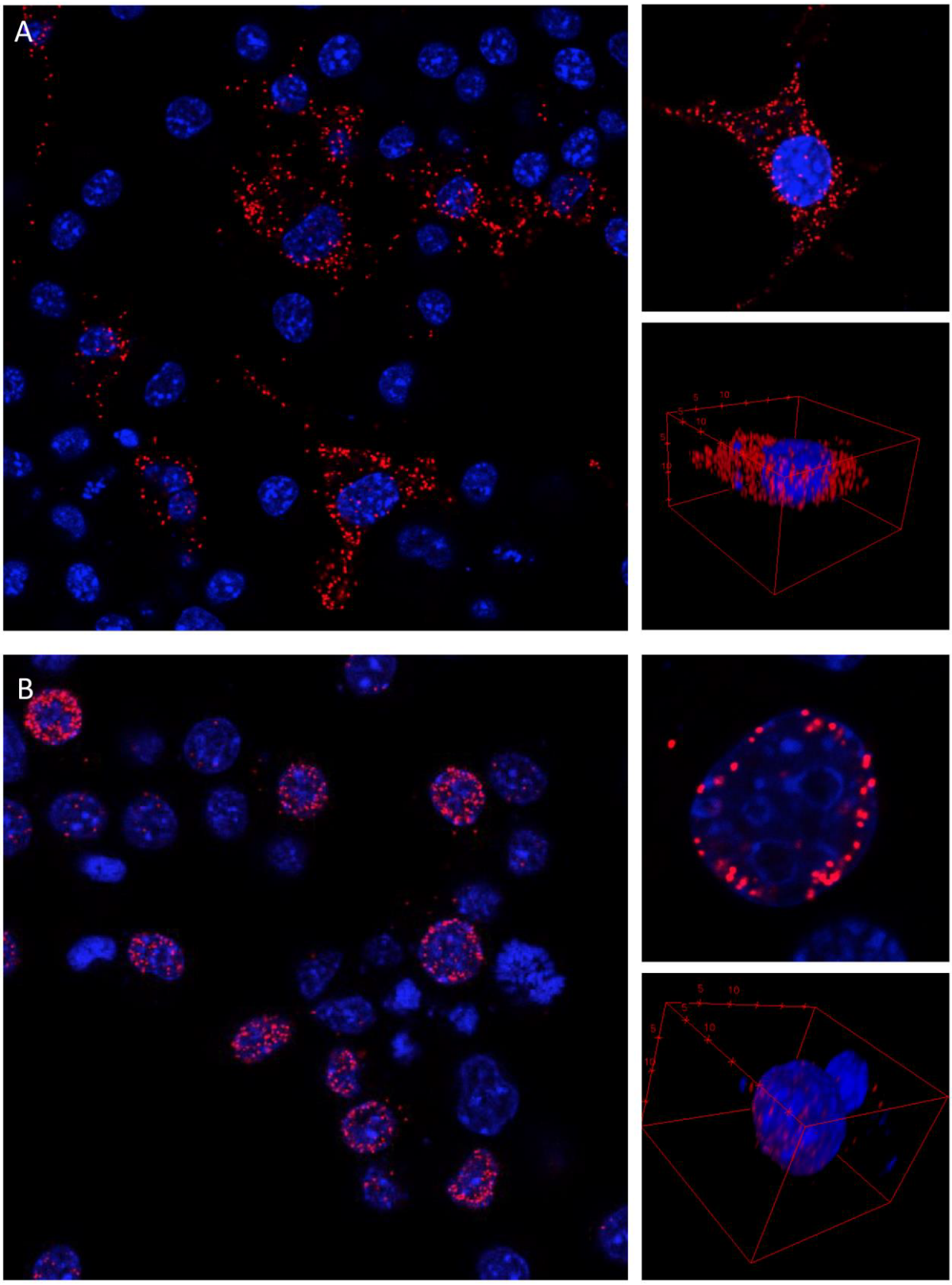


Figure 24 Proximity ligation assay in Neuro-2A cells: vehicle control vs 15 nM CORT, 60 minutes. *(A) detection of MR:GR complexes with vehicle (ethanol, final concentration 0.01%) showing cytoplasmic localisation vs (B) detection of MR:GR complexes with 15 nM CORT showing nuclear localisation, in 4% PFA fixed Neuro-2a cells. 3D volume rendering can be observed for (A) and (B) in*

respective bottom right pictographs displaying cytoplasmic vs nuclear MR:GR interactive complexes. MR:GR complexes are represented as red dots (Texas red) and nuclei blue (DAPI). Studies were performed as four independent experiments.

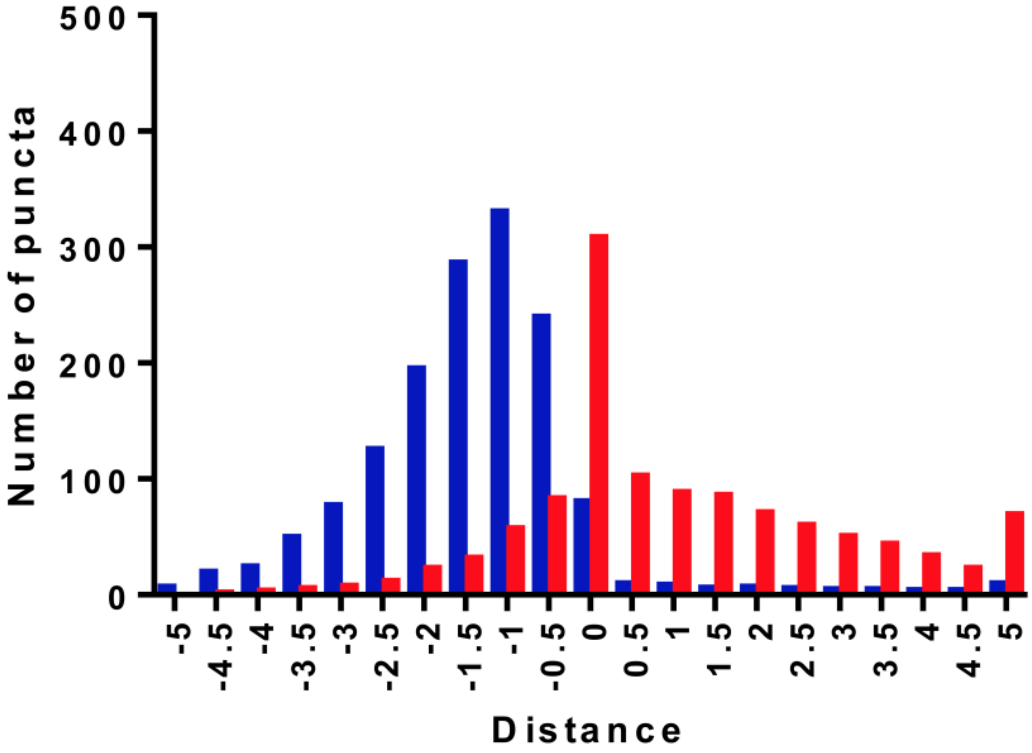


Figure 25 Proximity ligation assay in Neuro-2A cells.

MR:GR complex localisation for vehicle control (ethanol, final concentration 0.01%) vs 15 nM CORT, for 60 minutes. Graph represents subcellular position and distance of MR:GR protein complex from the inner nuclear membrane (represented by the DAPI edge) in 4% PFA fixed Neuro-2a cells under vehicle control (red bars) and 15 nM CORT (blue bars). Nuclear membrane position on the 'x' axis is denoted at '0' (distance in microns). Studies were performed as four independent experiments.

5.3.2 Ligand activated protein expression and distribution of the mineralocorticoid and glucocorticoid receptor

Ligand activated steroid receptors and their co-activators are heterogeneously dispersed throughout the nucleoplasm (van Steensel *et al.*, 1995; Kumar *et al.*, 2006). However, this has only been observed for GR and MR separately, and not for both receptors within an interacting complex. Where preliminary PLA data demonstrated a specific localisation of MR:GR puncta at the nuclear periphery, it was important to assess the distribution of each receptor under increasing exposure time to CORT treatment. IF was used to investigate the subcellular distribution of total MR and GR in Neuro-2A cells transfected with fluorescently tagged MR and GR and treated with 15 nM CORT for 15, 45 and 90 minutes (Figure 26).

Discrete changes in MR and GR distribution expression can be observed at each time point for both receptors. At 15 minutes, intensity of GR and MR fluorescence is low throughout the nucleoplasm with a slight increase at the nuclear membrane, as the receptors are translocating into the nucleus. At 45 and 90 minutes, GR fluorescence appears to increase over time and is more dispersed towards the center of the nucleus and surrounding the nucleoli structures, and discrete clusters of intense green fluorescence can be observed. On the other hand, MR fluorescence intensity appears to remain more peripheral within the nucleus only at 45 and 90 minutes. The overlay shows similar peripheral localisation is observed for GR, suggesting that these could be regions of MR:GR complexes. This indication prompted an investigation of the dynamic localisation of MR:GR complexes.

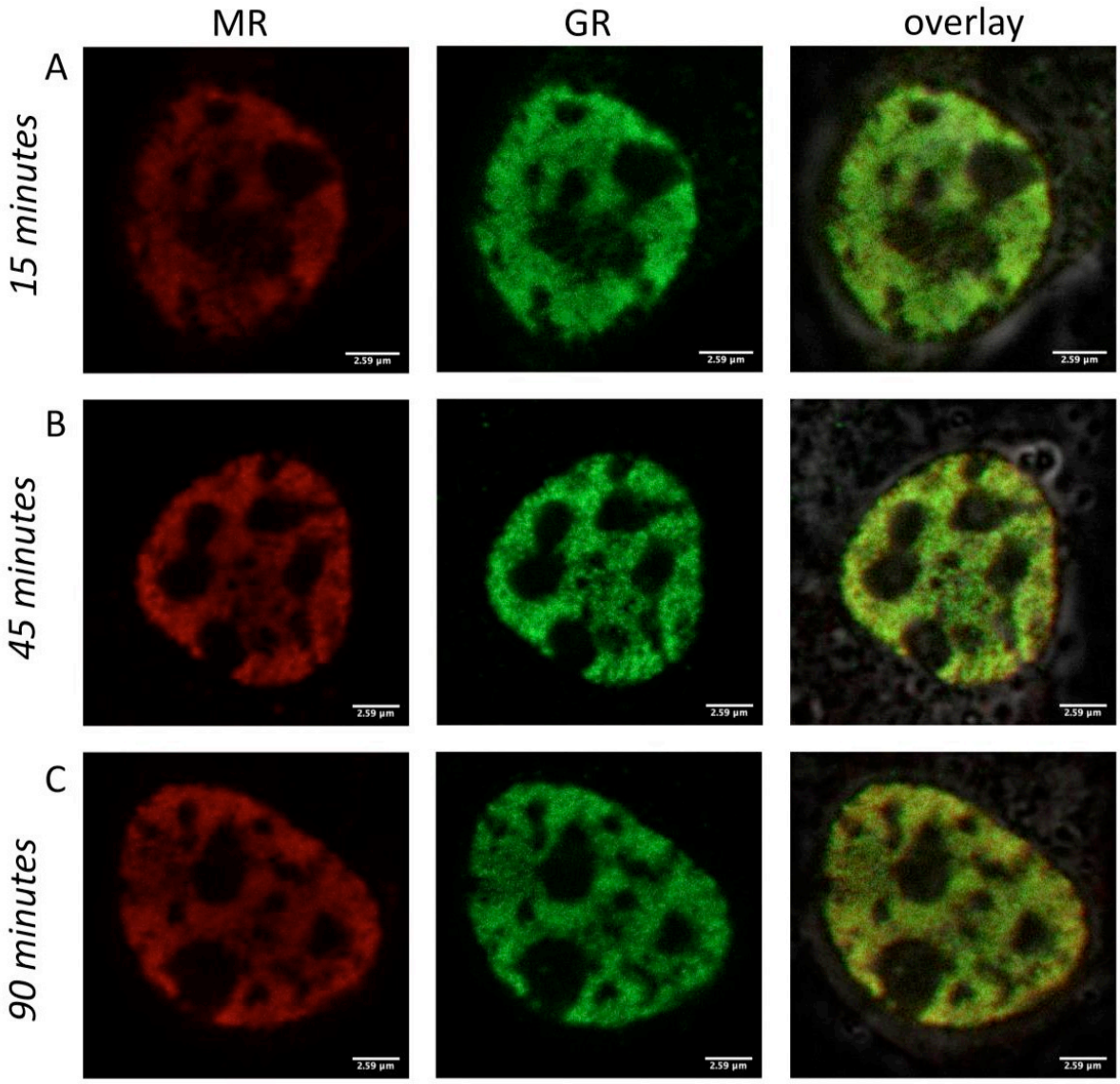
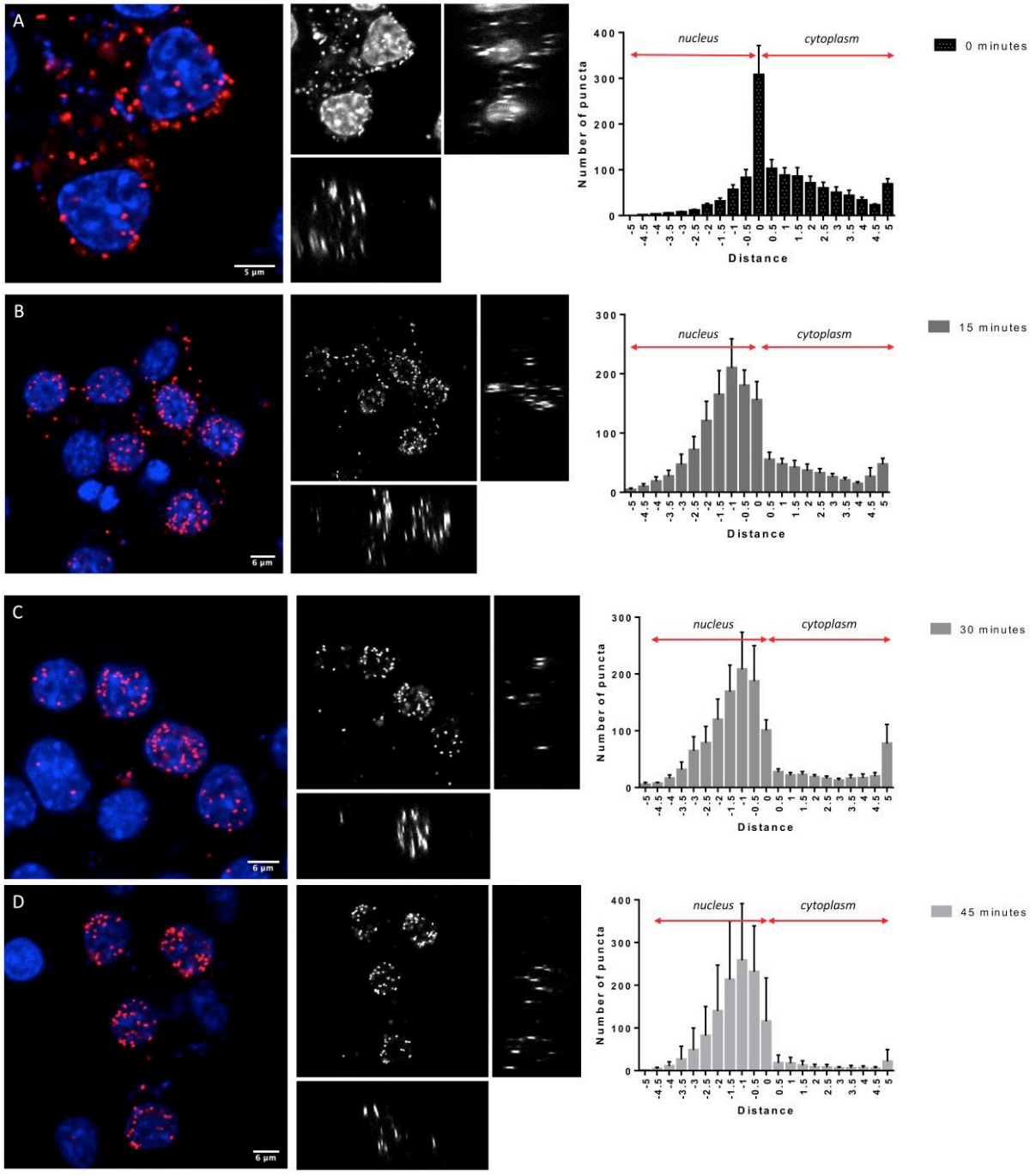


Figure 26 Immunofluorescence detection and distribution of nuclear MR and GR proteins.

Pictographs show protein expression distribution in 4% PFA fixed Neuro-2A cells transfected with fluorescently tagged MR (red images) and GR (green images) expression vectors and treated with 15 nM CORT for (A) 15, (B) 45 and (C) 90 minutes. An overlay of nuclear MR and GR protein expression are observed in the far-right images (yellow). Studies were performed as two independent experiments.

5.3.3 MR:GR dynamically and preferentially partition at the nuclear periphery



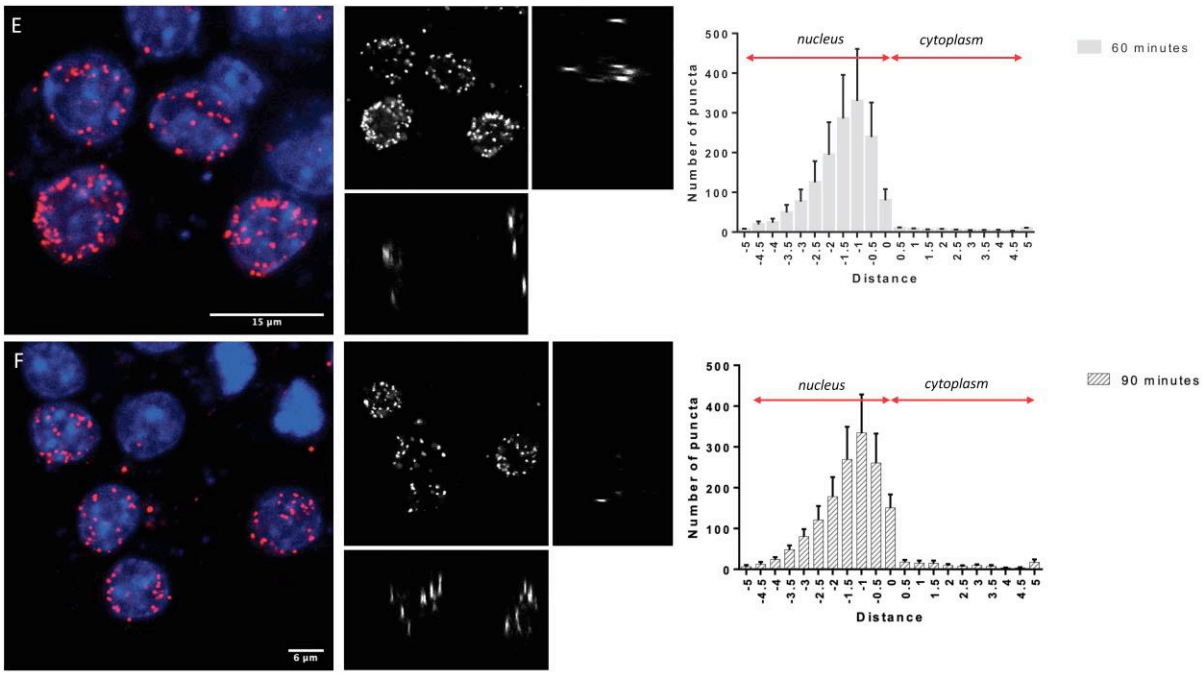


Figure 27 Visualisation of MR:GR interactions by PLA over time.

Neuro-2A cells were transfected with MR or GR only, MR+GR expression plasmids and/or empty vector control (pcDNA3.1). Cells were treated with vehicle control (ethanol, final concentration 0.01%) for 45 minutes (A), or 15 nM CORT for 15 minutes (B), 30 minutes (C), 45 minutes (D), 60 minutes (E) and 90 minutes (F). MR:GR complexes are represented in red dots (Texas red) and nuclei blue (DAPI). Over time the MR:GR complex can be observed to translocate into the nucleus where it is predominately localised at the inner periphery of the nucleus. Orthogonal processing of (A-F) PLA data is shown alongside (displayed as black image/white puncta) to visualize MR:GR puncta at each available axis (XY, ZY, and XZ). Graphs represent mean subcellular position and distance of MR:GR complexes from the inner nuclear membrane (defined by the DAPI edge). Inner nuclear membrane position on the 'x' axis is denoted at '0' (distance = microns: where positive numbers indicate cytoplasmic position and negative numbers nuclear position). Studies were performed as four independent experiments.

In order to measure the position of MR:GR complexes within the cell over time, Neuro-2A cells were transfected with untagged MR and GR, and following vehicle/CORT stimulation, cells were fixed and a DAPI stain applied prior to PLA experiments. The DAPI stain was used to determine the inner nuclear edge and processed using the 3D Gaussian and median filters, followed by the Huang thresholding method (Huang and Wang, 1995), and by which the spatial distribution of puncta in nuclei was measured as described in the developed Modular Image Analysis for Fiji (Cross, 2019), chapter 4. MR:GR interactive complexes are detected as red puncta using the 3D spot detection functionality of TrackMate (Tinevez *et al.*, 2017). The shortest distance between the puncta and the nuclear surface

was then calculated, with negative values corresponding to puncta inside the nucleus and positive values for those outside. Fluorescence intensity in the blue and red channels was measured as a function of distance from the nuclear surface. Average intensity measurements were divided into 21 distance bins from -5 μm from the nuclear surface to +5 μm from the nuclear surface.

In the absence of CORT (vehicle control: Figure 27 A), MR:GR interactive complexes are cytoplasmic and predominantly localised between 0 and 5 microns from the outer DAPI edge. An abundance of puncta can be observed at 0 microns. Following 15 minutes of CORT exposure, many MR:GR complexes were present in the nucleus and positioned at the nuclear periphery (between 0 and -1.5 microns), and a number of interactive complexes remain cytoplasmic (15 minutes: Figure 27 B). After 30 minutes of CORT exposure, most MR:GR complexes are present within the nucleus between -0.5 and -1.5 microns, and localised at the periphery, with few cytoplasmic complexes observed (30 minutes: Figure 27 C). Distinct changes occur between 15 and 30 minutes, with the number of cytoplasmic MR:GR complexes decreasing and a high number of MR:GR complexes (>200 puncta) present around -1 microns inside the nucleus. After 45 (Figure 27 D), 60 (Figure 27 E) and 90 (Figure 27 F) minutes of CORT exposure, the number of cytoplasmic MR:GR complexes is notably decreased, indicating that the majority of interactive complexes are now nuclear. Puncta continue to be localised just within the nucleus (between -0.5 and -1.5 microns) across time points and interestingly at 45 minutes MR:GR complexes are reduced (≤250 puncta at -1 microns) in comparison to levels observed at 60 and 90 minutes (>300 puncta at -1 microns), perhaps indicative of MR:GR complexes dissociating. The MR:GR complexes appear to be localised to nuclear regions where DNA is mostly heterochromatic, following translocation up to 90 minutes. Unlike in regions containing euchromatin, heterochromatic regions consist of chromatin that is more compact, and while numerous sequences are transcribed in heterochromatin, these usually regulate gene silencing.

5.3.4 Disrupting the DNA-Binding Domain does not affect the presence of GR:MR interactive complexes

The stoichiometry of MR:GR complexes is unclear but are regularly described as GR or MR monomers, homodimers or heterodimers binding at DNA. Recently, live cell studies reported oligomerisation of GR to a tetrameric state at chromatinised DNA arrays following GR-DNA binding and that these higher order oligomerisation states are implicated in specific GC mediated GR regulated gene transcription (Paakinaho *et al.*, 2019).

In an attempt to gain insight into the molecular mechanisms involved in MR:GR interactions, expression vectors for both receptors were used with mutated DNA binding domains (XDBD). XDBD mutants were constructed by replacing three amino acids within the DBD P-box, necessary for

recognising the DNA binding site (Zilliacus *et al.*, 1993; Rivers *et al.*, 2019) and were recently used to determine the nature of MR/GR interactions at the DNA, which were producing augmented transcriptional effects (Rivers *et al.*, 2019). In addition, this particular DBD mutation was shown to prevent interactions at the DNA by an ER variant.

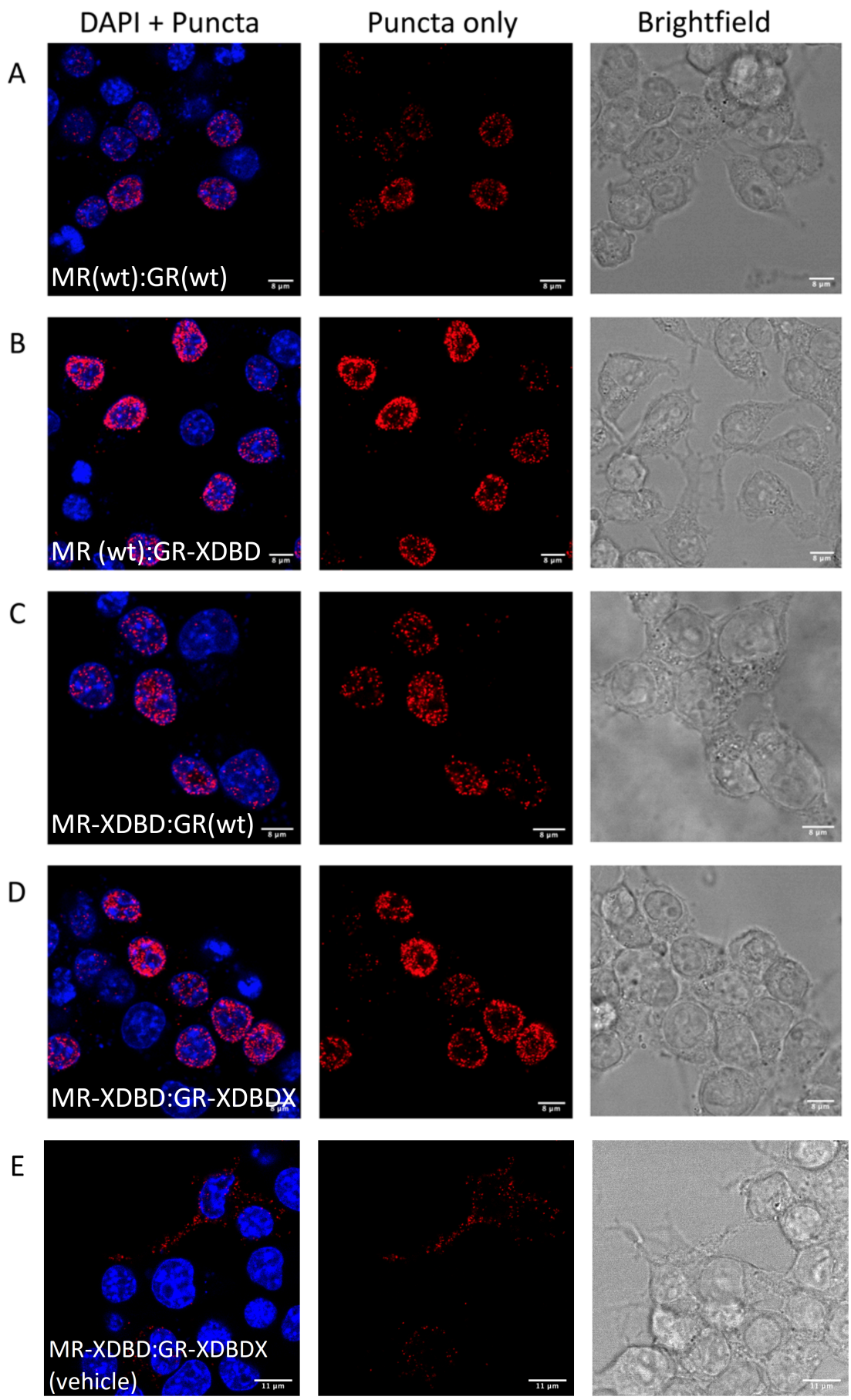

DAPI + Puncta
Puncta only
Brightfield
A
MR(wt):GR(wt)
B
MR (wt):GR-XDBD
C
MR-XDBD:GR(wt)
D
MR-XDBD:GR-XDBDX
E
MR-XDBD:GR-XDBDX
(vehicle)

Figure 28 The effects of wild-type MR/GR and MR-XDBD/GR-XDBD on MR:GR interactions.

Proximity ligation assay in 4% PFA fixed Neuro-2A cells with over expressed with a combination of wild-type (wt) MR/GR and MR-XDBD/GR-XDBD mutants and treated with 15 nM CORT for 60 minutes: (A) MR(wt):GR(wt) (B) MR(wt):GR-XDBD, (C) MR-XDBD:GR(wt), (D) MR-XDBD:GR-XDBD. (E) Cells transfected with MR-XDBD:GR-XDBD and treated with vehicle (0.0% sterile EtOH) for 60 minutes. The interactive complex is represented in red dots (Texas red), nuclei blue (DAPI), cellular structure (brightfield – grey). Studies were performed as two independent experiments.

Neuro-2a cells were transfected with over expressed wild-type MR/GR together or combined with MR-XDBD/GR-XDBD mutants, as well as both receptor XDBD mutants together and treated with 15 nM CORT for 60 minutes (shown in Figure 28 A-D). Cells were fixed as described and PLA was performed. MR:GR interactive complexes were present in all conditions where the XDBD in one or both receptors were mutated and can be observed in pictographs (A) MR(wt):GR(wt), (B) MR(wt):GR-XDBD, (C) MR-XDBD:GR(wt) and (D) MR-XDBD:GR-XDBD. MR:GR interactive complexes for all conditions A-D are located at the nuclear periphery, however when the MR-XDBD is co-expressed with a wild type GR the puncta appeared more dispersed at the periphery and located further into the nucleoplasm (C). When GR-XDBD mutants are co-transfected with MR-XDBD or wild type MR, the number of puncta at the periphery appears increased (B and D). Cells transfected with GR/MR DBD mutants (E) and treated with vehicle observed cytosolic MR:GR complexes proximal to the membrane and along neurite outgrowths. Puncta analysis using MIA was not performed for these experiments where they were only performed as two independent experiments.

In addition, MR-A640T mutants constructed by Rivers *et al* (2019) with a disrupted dimerization interface (an Ala to Thr mutation within the D-loop of MR with the same DBD structure), were used to asses any interference with the interacting MR:GR complex (figures not shown). MR:GR interactive complexes were detected despite when combinations of MR-A640T or MR-A640T-XDBD mutants were co-expressed with either wild type GR or GR-XDBD mutants. These results suggest another mode for MR:GR interactions other than at the dimerization interface and that when MR/GR are unable to bind to DNA, MR:GR complexes still form.

5.4 Discussion

IHC and IF are commonly used techniques in scientific research and more recently in imaging of single or multi-protein complexes in various samples using binding of single labelled Abs to assess spatial organisation and dynamic function. PLA is a technology that relies on the binding of Abs pairs to produce a signal by localised rolling circle amplification reactions. *In situ* PLA is a dual binding assay able to interrogate interactions or proximally localised proteins that are each bound by a primary Ab and then each bound by a species-specific secondary probe. Furthermore, the likelihood of cross-reactivity between non-specific proteins is decreased due to the specificity of the targeted Abs that are required to produce a signal, and dramatically reduces background signal. The antibodies were robustly tested for the purpose of these experiments. In addition, PLA negative control testing did not produce signal with only one MR/GR plasmid or with IgG control antibody (further details are available in chapter 3).

In these experiments, PLA was used to identify dynamic MR:GR interactions in Neuro-2A cells. Ligand activated MR and GR are likely to be within the same interactive complex as observed here using PLA proximity (<40 nm). Because a PLA signal is only obtained if the Abs are in close proximity (<40 nm), this is indicative of close molecular interactions. Following prolonged exposure to CORT, the MR:GR complex is observed to clearly localise within peripheral regions of the nucleus, as detected between -0.5 and -1.5 microns. There was previously no evidence of where MR:GR complexes were localised within the subcellular space.

Immunofluorescent (IF) experiments were conducted to assess the distribution of MR/GR throughout the nucleus following CORT induction. From these experiments it can be said that MR/GR are dispersed throughout the nucleus and with regions of overlay within parts of the nucleus. Notably in contrast, PLA data shows MR:GR complexes predominantly at the nuclear periphery and the presence of these complexes declines as you move away from the nuclear membrane towards the center of the nucleus. This further suggests that these are genuine interactive MR:GR complexes and not stochastic events.

The specific localisation of MR:GR complexes restricted to the nuclear periphery and not throughout the centre of the nucleus, raises further questions regarding their role in CORT induced transcriptional regulation. Within these regions are specific genomic compartments specific to the nuclear periphery. The inner nuclear membrane of a metazoan cell is lined by the nuclear lamina (NL) that is involved in structural function and transcriptional regulation in the cell nucleus. Closely localised to the NL, and less frequently around nucleoli, are lamina-associated domains (LADs) that correspond to B-type compartments (A-type compartments correspond to inter-LADs) (Kind *et al.*, 2015). These are

described as AT-rich genomic regions of tightly condensed heterochromatin (Guelen *et al.*, 2008; Harr *et al.*, 2015), and contain genes which are mostly transcriptionally silent or expressed at low levels (Guelen *et al.*, 2008; Wen *et al.*, 2009). When MR:GR complexes were observed more centrally within the nucleus, it was observed that the MR:GR complexes were detected in nucleoli-associated domains (NADs). The regions intense DAPI staining, appeared inhabited by increased clustering of MR:GR complexes. However, relating the puncta to nucleoli for quantitative measurement was not possible with the MIA pipeline (chapter 4) where the nuclei were being detected as separating regions and were thus segmented as separate nuclei. In addition, the Neuro-2A cell line has an extensive network of nucleoli which would be difficult to quantify manually. The capability to relate MR:GR puncta to nucleoli regions is currently being developed.

In the absence of ligand, MR:GR complexes were detected in the cytoplasm and in close proximity to the nucleus. Further analysis through the MIA pipeline detected the highest number of MR:GR complexes following a vehicle control (0.01% EtOH) localised predominantly at 0 and +0.5 microns, and where the edge of the DAPI stain denotes the very inner nuclear membrane (0 microns), this may represent the perinuclear and the cytoplasmic space. The presence of MR:GR complexes in the cytoplasm and in proximity to the nuclear membrane was an unexpected observation. There is no evidence to suggest that MR:GR interactive complexes form in the absence of ligand and in the cytoplasm and perinuclear space. Whether they are directly interacting or not remains unclear. In the absence of ligand, the NLS is protected within accessory protein complexes (e.g. hsp90, hsp70, hsp56, p23, and immunophilins). Depending on the nature of their interactive proximity, an interaction may occur via low affinity interaction at the NTD. This has been previously observed with GR dimers using medium and high concentrations of GRwt (wild type), which were shown to dimerise in the cytoplasm and at the nuclear membrane independent of ligand (vehicle control) and thought to prime the system via ligand independent DNA loading and transactivation (Robertson *et al.*, 2013). A decrease in dimerization was reported with dimerization mutants, although interactions still were detectable (Robertson *et al.*, 2013). Interestingly, using GRwt maximal dimerization was achieved and not further increased by addition of GCs, and transactivation of GRE sites was enhanced significantly (Robertson *et al.*, 2013). Unliganded GR has previously been reported to induce genomic effects through direct or indirect transactivation or transrepression (Sapolsky, Romero and Munck, 2000). A small number of MR:GR complexes were detected in the nucleus (figure 2). Unliganded GR has been reported to translocate into the nucleus in a cytoskeleton independent manner, via induction by reactive oxygen and nitrogen species, shear stress conditions and particular cytokines (Sundahl *et al.*, 2015). However, this is not fully understood.

Interestingly and although not fully evaluated, in a subset of cells that developed neurite projections, MR:GR complexes were observed at the cytoplasmic membrane and along the neurite outgrowth. Although this was repeatedly observed, it was not pursued at the time due to low number. This observation may refer to GCs interacting with membrane bound receptors inducing non-genomic effects (Groeneweg *et al.*, 2011, 2012; Meir Drexler and Wolf, 2017) and at increased GC concentrations can occur via GR independent pathways (Baschant and Tuckermann, 2010).

The PLA experiments were conducted with untagged MR and GR receptors. During optimization, GFP-tagged receptors were used, which caused a dramatic reduction in PLA signal (chapter 3) and suggests compromised GR epitope availability and proximity when the tags are present. The use of fluorescent protein tags, such as GFP, although useful in providing dynamic information on subcellular localisation may however alter protein function or localisation. By performing PLA with untagged MRs and GRs it was possible to avoid any potential impact of protein tags on nuclear receptor localisation.

In these experiments, MR and GR were over-expressed from mammalian gene expression plasmids. There are many things to consider when expressing genes of interest in a mammalian platform, such as expression constructs that result in the protein of interest being expressed at the correct expression level. Overexpressing proteins can be detrimental to the cell in numerous ways, such as overloading the resources involved in protein production and transport (Stoebel, Dean and Dykhuizen, 2008), overload and activation of cellular pathways (Makanae *et al.*, 2013; Tang and Amon, 2013), and disrupting regulation. Also, they can also interfere with liquid phase separation within the cell (Birchler and Veitia, 2012; Bolognesi *et al.*, 2016). Hence, PLA was optimised for use in endogenous MR and GR in *in vivo* rat tissue where endogenous levels of MR and GR are expressed due to more physiologically representative protein behaviours in cells (chapter 3).

The MR:GR complexes that localised to the nuclear periphery did not appear to be interacting via the DBD or the dimerization interface. Since mutation of the DBD and dimerisation interface did not hinder puncta formation, the data suggest another mode of MR:GR interaction, in place of MR:GR dimers. One possible type of interaction is within a liquid-liquid phase separated droplet region that form following transcription factor binding at the DNA and encompass other TFs and proteins within the vicinity following DNA binding (Nair *et al.*, 2019).

Using PLA to investigate MR and GR interactions has demonstrated close proximity and provides evidence that MR and GR are present in the same interactive complexes, *in vitro* and *in vivo*. In addition, the dynamic localisation to the nuclear periphery suggests MR:GR complexes modulate transcriptional regulation of GC responsive genes by interacting with heterochromatin, provides evidence for a specific and novel function for MR:GR complexes.

6 Differential effects of the mineralocorticoid receptor and glucocorticoid receptor on gene regulation

6.1 Introduction

The effects of MR and GR are dependent on their activation by CORT. The effects of endogenous CORT follow hourly ultradian pulses with levels fluctuating in non-stressed basal conditions. The ultradian rhythm occurs within a characteristic circadian cycle (a 24-hour period), where low levels of CORT are present during the inactive phase with levels increasing in amplitude toward the start of the active period in preparation for the start of the day (Droste *et al.*, 2008; Lightman and Conway-Campbell, 2010; Russell *et al.*, 2010). It is the hourly pulses that regulate GC responsivity.

The highly homologous receptors, type I high-affinity MR and the type II low-affinity GR, are abundantly expressed throughout cortico-limbic regions (De Kloet and Reul, 1987), such as the hippocampus, amygdala and some cortical areas (Ahima and Harlan, 1990; Ahima, Krozowski and Harlan, 1991). GR is however, more widely distributed throughout the brain compared to MR, which is more restricted. MR is activated at very low CORT concentrations of the circadian nadir and remains activated during the inter-pulse interval (Lightman *et al.*, 2008), whereas GR requires higher concentrations for maximal activation (Reul and de Kloet, 1985). GR-DNA binding therefore occurs according to the ultradian pulsatility of CORT release (Stavreva *et al.*, 2009; Lightman and Conway-Campbell, 2010). MR and GR are both activated during the circadian peak or at CORT levels associated with stress. The combination of unique MR/GR CORT affinities and their cerebral distribution contribute to a fine-tuned cycle of transcriptional expression patterns.

Ligand activated MR and GR undergo dynamic conformational changes and remodelling of stabilising chaperone complexes, allowing accessibility to the nuclear localisation signal (NLS). The receptor-ligand complex then translocates into the nucleus where it associates with glucocorticoid response elements (GREs) in the promotor regions of glucocorticoid target genes where they can modulate transcription (Madalena and Lerch, 2017). MR and GR simultaneously regulate transcriptional activity of ligand inducible genes via several proposed manners:

(i) In parallel as separate MR or GR homodimers, monomers or other heteromers.

(ii) In combination as DNA-binding heterodimer of MR and GR.

(iii) In alternative arrangements e.g. such as the mode of interaction that does not require MR DNA-binding (Rivers *et al.*, 2019).

The appropriate exposure to GCs following stress supports the dynamic balance between MR and GR regulated transcription and the selection of a suitable behavioural response and memory development (Karst, 2005; Joels *et al.*, 2008; Melanie D Klok *et al.*, 2011). The functional balance between MR and GR regulated transcription is important and produce differing effects in the brain, and these may be due to MR or GR effects or by MR:GR interactive effects. However, overexposure are thought to dysregulate the HPA axis thus hindering an efficient stress response (de Kloet, Oitzl and Joëls, 1999). Prolonged activation of GR cause deleterious effects of GCs on neuronal cells and forms of memory formation, whereas MR activation mediates beneficial effects such as neuroprotection (Lai *et al.*, 2007; Mitra, Ferguson and Sapolsky, 2009). To be able to produce an appropriate response to stressful experiences and disease susceptibility, the dynamic balance between MR and GR exposure to GCs and activation must be achieved. It is believed that dysregulation of receptor activation and consequently transcriptional regulation of specific genomic targets determines an ineffective stress response, and susceptibility to disease (de Kloet, Oitzl and Joëls, 1999).

The effects of MR/GR individually and in combination on RNA expression levels of several well-known and more recently shown targets, such as *Per1* and *Sgk1*, to which GR and MR have recently been shown to be bound in Neuro-2A ChIP-nexus (Rivers *et al.*, 2019), was measured and compared using quantitative polymerase chain reaction (RT-qPCR). Measurements were taken with much lower levels of CORT than are commonly reported, in order to represent a more physiologically relevant response.

6.2 Materials and Methods

6.2.1 Cell Culture

See chapter 2.

Cells were washed twice with Dulbecco's PBS (Thermo Fisher Scientific) and seeded in CSS media (DMEM with 7.5% (v/v) charcoal-stripped fetal bovine serum and the other supplements described in chapter 2) at 0.25 million per well on 6-well plates for RNA expression analysis. After 18 to 24 hours, cells were transfected with MR, GR, and empty vector expression plasmids (see chapter 2) and 24 hours later were treated with vehicle (ethanol 0.01% final concentration) or 15 nM CORT for 15, 30, 45, 60 and 90 minutes prior to RNA extraction.

6.2.2 Plasmid constructs and transient transfections

See chapter 2.

6.2.3 RNA extraction and reverse transcription

Total RNA was extracted from Neuro-2A cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with treated with TURBO™ DNase in order to remove residual DNA contamination from the RNA samples. Reverse transcription was performed with 1 μg of total RNA using an iScript™ cDNA synthesis kit as described in the provided protocol. Levels of transcripts were assessed by real-time PCR using the SYBR Green Fast PCR master mix system (Applied Biosystems, Thermo Fisher Scientific). As an endogenous control, Mcm3ap was selected and demonstrated constant expression in the presence and absence of CORT treatment. Primers were designed and tested using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA). PCR specificity and efficiency were validated by melt curve analysis and amplification over a serial dilution of template.

6.2.4 Statistical analysis

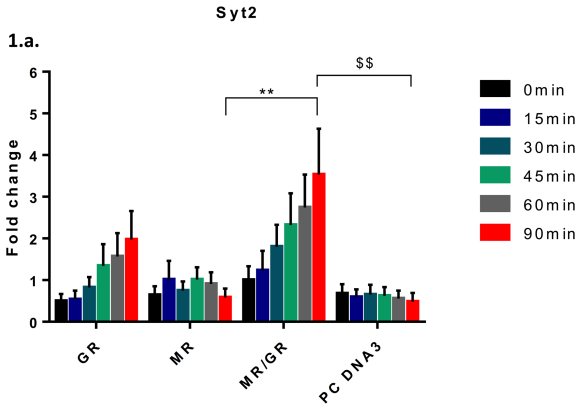
Reverse transcription quantitative PCR (RT-qPCR) analyses were performed with a minimum of five independent biological replicates ($n \geq 5$), and each qPCR measurement was calculated from the mean of two separate Ct values. RT-qPCR analyses were compared by one-way ANOVA with a Bonferroni multiple comparisons test, and data represented as a mean $\pm$ SEM. Statistical significance is labelled by the following: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

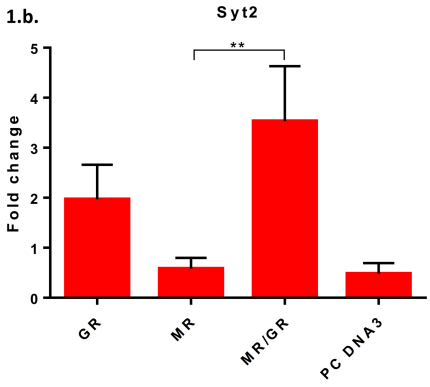
6.3 Results

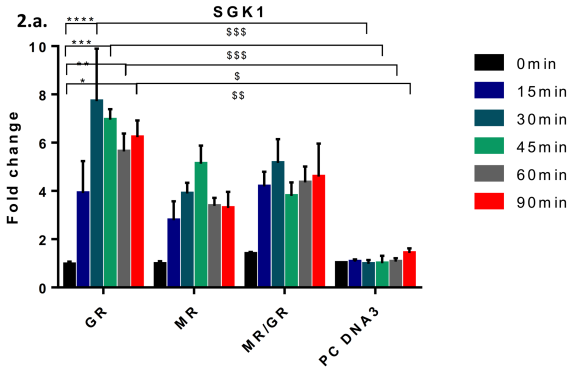
6.3.1 Differential effects of MR and GR on transcriptional regulation of target genes

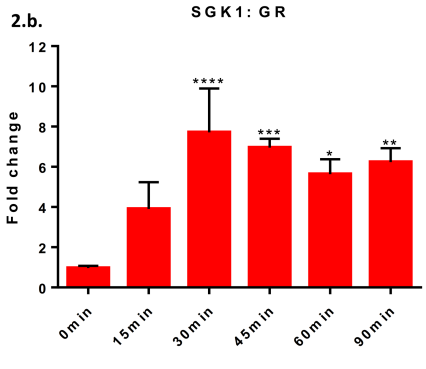
The expression levels of selected genes were measured by RT-qPCR. The targeted genes were selected where MR and GR has been previously shown to bind to in Neuro-2A cells using ChIP-nexus. RNA was extracted from Neuro-2A cells transiently transfected with expression constructs for untagged MR/GR alone, MR and GR together, and empty vector control (pcDNA3.1) and treated with 15 nM CORT for 0, 15, 30, 45, 60 and 90 minutes. Preliminary experiments using a MMTV luciferase reporter showed gene activation and transcriptional effects of endogenous GR in Neuro-2A cells to be minimal (Rivers *et al.*, 2019). Gene expression included upregulation of *Per1, Syt2* and *Sgk1* (figure 29) relative to the pcDNA3.1 control. When transfected alone, GR elicited significant changes in *Sgk1* expression at 30, 45, 60 and 90 minutes of constant CORT treatment, when compared to the '0' time point (no CORT) control. Although not significant (p = 0.0657), *Sgk1* mRNA expression in the presence of MR was increased at 45 minutes compared to vehicle control (p = 0.66) and empty vector control (p = 0.072). Interestingly, when both MR and GR were co-transfected and at 90 minutes of CORT exposure, MR and GR elicited significantly increased upregulation of *Syt2* mRNA when compared with MR alone and the pcDNA3 control. In the presence of GR only, *Per1* expression significantly increased at 30 minutes

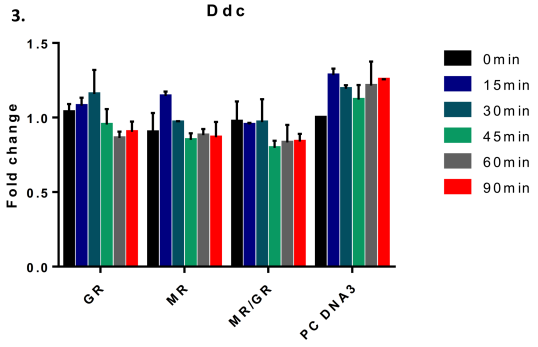
compared to the pcDNA3.1 control. *Ddc* mRNA expression did not change significantly, although there appears to be a decreasing trend observed with co-expression of MR and GR in comparison to '0' (no CORT) time point and the pcDNA3 control.











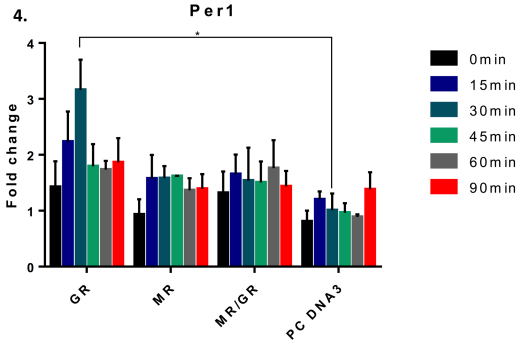


Figure 29 Effects of CORT-activated MR and GR on mRNA expression.

*Graphs show (1.a) Syt2 mRNA and (1.b) is a focus on significant results; (2.a) Sgk1 mRNA and (2.b) is on GR only significant results, (3) Ddc mRNA and (4) Per1 mRNA changes in Neuro-2A cells with expressed wild type MR and GR individually or combined (MR+GR). RT-qPCR data from Neuro-2A cells transfected with GR/MR/ MR+GR/ pcDNA3.1 and treated with 15 nM CORT for 0 (no CORT), 15, 30, 45, 60 and 90 minutes. Data are represented as mean fold changes relative to experimental conditions or pcDNA3 transfected controls ± SEM (n ≥ 5). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; $p < 0.05; $$p < 0.01; $$$p < 0.001; $$$$p < 0.0001.*

The gene expression data suggests enhanced expression when MR and GR are co-expressed, such as shown with upregulation of *Syt2* mRNA at 90 minutes of constant CORT treatment compared to expression of MR but not GR alone. GR elicited changes in *Sgk1* mRNA expression was observed following 30 minutes of CORT treatment.

6.4 Discussion

This data shows differential transcriptional regulation of *Per1*, *Syt2* and *Sgk1* genes by GR and MR alone, and co-expressed MR and GR, within physiologically representative levels of CORT activation (15 nM). Although most responses are small, this data represents physiologically relevant levels of ligand activation. At 15 nM both GR and MR are expected to be activated. Published data using higher 100 nM CORT levels shows more robust mRNA expression (Rivers *et al.*, 2019) and changes in gene expression levels included upregulation of *Syt2* and *Sgk1* and downregulation of *Ddc* genes, relative to the control. Notably, further upregulation of *Syt2* was observed when MR and GR were co-expressed together compared to the expression of MR or GR alone, suggesting potential interactive effects of MR/GR giving rise to altered activated/repressive effects (Rivers *et al.*, 2019). A somewhat similar trend is apparent at the more physiological concentration of 15 nM whereby interacting MR and GR show an enhanced transcriptional regulation of *Syt2* in response to GC activation, however although the effect of co-expressed MR:GR on fold change is significantly different from the effect of expressed MR alone, this isn't the case from the effect of expressed GR alone and suggests additive effects of the individual receptors when co-expressed together. For this to be a 'more than additive' effect, the effects of the co-expressed receptors should be significantly higher than both MR and GR when expressed alone. Further, it is possible that increasing experimental repeats may have revealed significant upregulation of co-expressed MR:GR compared to expressed GR alone. *Syt2*, or synaptotagmins, are found in vertebrates and are integral membrane proteins of synaptic vesicles and function as a calcium sensor for neurotransmitter release, where Ca^{2+} binds to the C2B domain to drive

fast synchronous vesicle fusion at synapses (Littleton *et al.*, 1993; Mackler *et al.*, 2002; Pang *et al.*, 2006). *Syt2* is preferentially expressed in the hindbrain and spinal cord, and specifically acts as the predominant sensor for vesicle fusion at excitatory synapses formed within hindbrain and spinal cord neurones. In addition, *Syt2* has been identified in GABA-ergic nerve terminals of PV interneurons in the hippocampus and cortex, particularly in aged animals, and is necessary for effective feed forward inhibition at these junctions observed in the cerebellum (Chen *et al.*, 2017). *Syt2* has been shown to be bound by MR and GR and produce augmented mRNA expression (Rivers *et al.*, 2019).

Serum and glucocorticoid inducible kinase (*Sgk1*) is an intracellular signalling molecule (Miyata *et al.*, 2011) believed to be involved in oligodendrocyte functional plasticity required for myelination in the brain (Demerens *et al.*, 1996; Gibson *et al.*, 2014) and is suggested to be modulated in response to exploratory events (Fields, 2015). *Sgk1* mRNA expression was shown to be quickly upregulated following an acute stress in the rodent brain (Miyata *et al.*, 2011), and since GC induced increases in *Sgk1* mRNA expression have been reported (Webster *et al.*, 1993; Itani *et al.*, 2002; Leong *et al.*, 2003; Anacker *et al.*, 2013; Xydous, Prombona and Sourlingas, 2014), in addition to increased *Sgk1* protein synthesis (Webster *et al.*, 1993; Webster, Goya and Firestone, 1993), following GR binding at specific GREs associated at the *Sgk1* gene (Webster *et al.*, 1993; Itani *et al.*, 2002), have been demonstrated. In addition, within 30 minutes rapid increases in *Sgk1* mRNA expression were observed in the corpus callosum following an acute stress (Hinds *et al.*, 2017). As such, this data demonstrates rapid increases in GC induced *Sgk1* mRNA expression by GR alone and observed at 30 minutes, which continued up to 90 minutes.

Period Circadian Clock 1 (*Per1*) is a CLOCK gene involved in the regulation of various cellular networks and oscillates following a circadian rhythm in the SCN, the master circadian clock, and plays a key role in SCN signalling networks with peripheral clocks. GCs are reported to drive circadian and ultradian transcription bursting of clock genes such as *Per1,* and this rhythm is disrupted in diseases such as major depressive and inflammatory disorders (Wiley, Higgins and Athey, 2016). *Per1* has been reported to be transcriptionally involved in long term memory formation in the hippocampus (Deibel *et al.*, 2015), which together with the circadian rhythm, becomes impaired with the aging brain (Kwapis *et al.*, 2018). GC induced *Per1* mRNA expression by GR is observed to increase between 15 and 30 minutes, with significance at 30 minutes. Although, *Per1* is a known GC target with GREs within clock gene promotors, this data shows GR mediated induction at low 'physiological levels' of GCs.

Augmented transcriptional regulation by MR:GR interactions (Trapp, Rupprecht, Castren, *et al.*, 1994), as well as inhibition of GR activation (Liu *et al.*, 1995), were initially observed using reporter constructs. More recently, RNA expression of GC inducible genes following a timed forced swim test in rats

observed increases in *Sgk1* mRNA expression which were significant at 30 minutes and peaking at 60 minutes in the dorsal dentate gyrus and CA hippocampal regions. A similar observation is reported here where GR elicits a significantly increased GC induced response for *Sgk1* at 30 minutes and significance is maintained up to 90 minutes. In addition, MR and GR were identified at the same DNA fragments *in vivo* (Mifsud and Reul, 2016). Furthermore, the differential up- and down-regulation of genes within a native chromatin context by tethered MR observed an increase in GR elicited GC responses for specific genes (Rivers *et al.*, 2019) and demonstrated MR and GR to bind to some of the genes investigated here (*Syt2* and *Ddc)* in addition to a mode of interaction other than heterodimerisation as determined by augmented mRNA expression of genes despite co-transfection with MR-A640T (disrupted D-loop of the dimerization interface). Other residues where MR and GR interact include MRs NTD with GR (Planey *et al.*, 2002), and a sequence in the hinge region of GR was able to mediate GR-MR interaction, necessary for the MR mediated nuclear translocation of a GR variant unable to translocate (Savory *et al.*, 2001) and might explain the observed cytoplasmic MR:GR complexes in chapter 5. Although the described expression changes do not strongly demonstrate evidence of a real interactive effect as previously reported (Mifsud and Reul, 2016; Rivers *et al.*, 2019) it is worth noting that lower CORT levels were used here compared to high CORT concentrations or stress levels. However, expression changes were observed in *Sgk1* and *Syt2*, that have been previously shown to bind both proteins.

Using Neuro-2A cells these results suggest differential expression of *Syt2* by CORT activated co-expressed MR:GR in comparison to MR expressed alone. However, it cannot be known if these are interactive effects as significant changes were not similarly observed in comparison to GR expressed alone, augmented upregulation of this gene has previously been reported to occur via a MR-tethered GR mode of action at higher CORT levels (Rivers *et al.*, 2019). The augmented changes in mRNA expression might result from additive effects of MR and GR individually, or they could be due to MR:GR interactions producing differential activation compared to MR alone. *Per1* is shown to be significantly regulated in the presence of GR at 30 minutes, with mRNA expression peaking at 30 minutes before decreasing. *Sgk1* is shown to be significantly regulated by GR, with mRNA expression increasing from 15 minutes and peaking at 30 minutes following CORT exposure, with significance continuing up to 90 minutes. These results might reflect results by Mifsud *et al* (2016) that showed increased binding of MR and GR homodimers following a forced swim test at the *Sgk1* gene and alluded to predominantly homodimer regulated gene expression at this site.

In vitro GR and MR investigations have used high doses of glucocorticoids (100 nM dexamethasone, 100 nM CORT) to reveal gene expression changes. This study used physiologically relevant levels of glucocorticoids applied to neuroblastoma cells, which may not have activated GR sufficiently. The

findings here differ from published data using higher levels of CORT which showed more-than-additive effects of MR and GR in combination at *Syt2* and *Ddc* loci (Rivers *et al.*, 2019), where this data shows mRNA changes at more physiologically relevant CORT levels. Of note, further experimental repeats may have produced significant changes. While it is not possible to say whether these results are due to direct GR:MR interactions, rather than individual additive effects, earlier results (PLA data chapter 5) show GR:MR proximity occurs in the nucleus at these time points and at the 15 nM CORT concentration.

7 Discussion chapter

7.1 Summary of findings

In this thesis, using a proximity ligation assay (PLA), MR and GR have been shown to be in very close proximity and are consequently likely to be interacting together. MR:GR complexes have been detected both in cultured cells (3617 and Neuro-2A) transfected with MR/GR expression vectors and in rat hippocampal tissue expressing endogenous MR/GR.

PLA in cultured cells has revealed the precise localisation of MR-GR complexes which suggests and informs a functional role for these complexes. Prolonged activation (>45 mins) with 15 nM CORT showed MR:GR complexes are clearly localized at the nuclear periphery, while number of cytoplasmic MR-GR complexes decreased over time. The nuclear detection of MR:GR complexes suggests they localise to regions that are heterochromatic. The mode of interaction appears to be neither by the DBD or the dimerization interface, since MR:GR interactive signals were produced when using DBDX and A640T-XDBD mutants indicating another mode of MR:GR interaction. In the absence of ligand, MR:GR complexes were unexpectedly detected in the cytoplasm being most abundant at the outer edge of the DAPI stain in the perinuclear space.

Using PLA, the first evidence of endogenous MR-GR complexes has been shown in rat hippocampal cells, following an "acute" IP injection of CORT. As observed in cultured cells, MR:GR complexes were found to be peripherally located within the nucleus following ligand induction and in the saline group.

The consequences of MR and GR, individually or in combination, on transcription were explored using physiological levels of CORT activation (15 nM), rather than high saturating level, in Neuro-2A cells. The well-known GC-target *Sgk1* showed increased expression with 15 nM CORT in the presence of GR alone. Expression of GR+MR together augmented *Syt2* mRNA induction, a recently described GC target, compared to the expression of MR alone suggesting possible additive effects of GR and MR individually, or they could represent interactions between GRs and MRs producing altered transcription of GC inducible genes.

An optimised protocol for *in situ* PLA and the modular image analysis (MIA) pipeline, were developed for the detection and analysis of MR:GR complexes in Neuro-2A cells and hippocampal tissue, which are applicable to future studies involving receptor-receptor interactions.

7.2 Discussion of findings

The main focus of project has been to investigate the dynamic behaviour of MR and GR, and analyse if their proximity to one another was close enough to suggest interactive effects. MR:GR complexes

have not been observed before, but spatio-temporal studies have co-localized GR and MR separately, with an emphasis on GR (Nishi *et al.*, 2001, 2004; Han *et al.*, 2005; Sarabdjitsingh *et al.*, 2009; Stortz *et al.*, 2017; Stavreva *et al.*, 2019). MR and GR interactions have mostly only been previously studied by methods using whole cell extracts, which does not address any dynamic spatiotemporal detail of the interacting complex within living cells (Trapp, Rupprecht, Castrén, *et al.*, 1994; Mifsud and Reul, 2016). FRET has been used to assess MR:GR interactions (Nishi *et al.*, 2004), however positive and false FRET signals are challenging to separate, which can lead to inaccurate conclusions when using FRET efficiency as it is not consistently translatable to distance, particularly in the case of heterogeneous multi-protein systems (Shrestha *et al.*, 2015). Recently, an acute stressful challenge was shown to increase the interaction of both MRs and GRs on the same DNA fragments (Mifsud and Reul, 2016). These experiments used tandem ChIP to test for *in vivo* interactions between GR and MR, and demonstrated MR and GR were associated with the same DNA fragments. However, since the DNA fragments were around 400-500 bp long, the receptors could be interacting at separate binding sites within the same DNA fragment rather than as a MR-GR heterodimer. Therefore, it was timely to use a new approach to investigate the question of whether or not MR and GR are proximal enough to be interacting each other.

Having demonstrated the existence of MR:GR complexes, their localisation within the nucleus and temporal distribution following CORT application was further investigated. PLA is a recent technique capable of visualizing PPIs in cell lines and tissue. Robust Ab validation experiments supported the development of an optimized PLA protocol and analysis pipeline, which is able to accurately detect proximity between MR and GR in cells and tissue. It must be noted that the nature of the interaction, whether direct or indirect, cannot be specified using PLA, as the short DNA strand conjugated secondary Ab probes technically permit proteins within a proximity of <40 nm to produce a PLA signal (Soderberg *et al.*, 2006). However, at this distance (<40 nm) the proteins are so close that they are most likely to be within the same complex and consequently interact with each other. In this thesis, MR and GR were detected to be within <40 nm of each other using PLA, which is the first time MR and GR have been reported to be within interacting distances in vivo in rat hippocampus and in vitro in cultured cells.

7.2.1 Development and optimization of proximity ligation assay using confocal microscopy

In this work, a combination of IF, PLA and confocal microscopy were used to explore how GR and MR are organised in the nuclei of 3617 cells, Neuro-2a cells and hippocampal tissue. Cell microscopy studies investigating MR and GR distribution have usually relied on overexpressed fluorescently tagged receptors. During PLA optimisation in chapter 3, GFP-tagged GR plasmids were used to

visualise the distribution of the GR and to define the nucleus, however in comparison to conditions using untagged receptors combined with a nuclear stain to highlight the nucleus, PLA signals were dramatically reduced. This may be due to the attached fluorophore sterically hindering ligation of the short strand DNA probes or preventing epitope recognition by the antibody. Whether the fluorescently tagged receptors exhibit exact endogenous receptor behaviour cannot be completely known, however studies have confirmed normal biological functions of GFP tags , such as in transcription, oligomerisation (Presman *et al.*, 2014) and subcellular movement and localisation (van Steensel *et al.*, 1995). To remove the possibility of tags interfering with protein localisation and function, untagged MR and GR proteins were used for PLA experiments together with a DAPI stain for nuclei definition.

In chapter 3, a selection of primary Abs pairs for MR/GR were screened for PLA optimization. Selected Abs, anti-MR 1D5 (mouse) and anti-GR M20 (rabbit), were titrated to an optimal working concentration. As the MR:GR complex appeared localized at the nuclear periphery, it was desirable to use fluorescently tagged Abs to nuclear membrane components, with the intention of precisely defining the nuclear structure to which MR-GR complexes co-localise. However, the commercially available Abs for lamins, nuclear pore complexes (NPCs) and emerins, for example, are raised in either mouse or rabbit and therefore not compatible with the optimised PLA system which requires two antibodies derived from separate species (rabbit and mouse). Double species staining was attempted several times (with concentration titration), however the background staining interfered with the MIA analysis and reduced confidence in the result.

An alternative approach is to detect nuclei from the brightfield channel images, distinguishing nuclear and non-nuclear regions, and in principle to segment based on this. This would identify the nuclear membrane structure from the outer nuclear membrane (ONM), however this approach is incompatible with 3D analysis as a single slice would need to be selected. Alternatively, provided there is a DAPI stain, a "convex hull" could be applied around the regions that have been segmented. This is a smooth surface, which could approximate the whole nuclear shape in 2D as well as in 3D, however this analysis has not been developed yet. Other approaches that can be applied to enable the visualisation of associated nuclear components may include immunolabelling techniques with light microscopy (Murphy, 2013), electron microscopy (EM) with transmission electron microscopy (TEM) or scanning electron microscopy (SEM) (Russell, 1999), and gold (immunogold labelling) (Cafiero *et al.*, 2011).

7.2.2 MIA analysis

In chapter 4, a framework containing image and object analysis modules, the MIA pipeline, was developed to determine the number and distance of MR:GR complexes from the inner nuclear membrane. MIA avoids the laborious and user-bias error drawbacks of manually counting each puncta or filtering of each individual stack. Although the threshold of puncta definition is pre-determined by the user for processing of an input image, this is applied across all samples. Through a series of modular steps (described in chapter 4), the identified objects are processed with a 3D Gaussian and median filter and then binarized (Huang and Wang, 1995), followed by a watershed filter to split any adjacent nuclei. Nuclei are subsequently identified as regions of connected voxels that exceed the threshold. Any mis-identified nuclei are removed based on the pre-defined size threshold, in this case they must be larger than 200 μm^3. 3D spot detection function TrackMate identifies the MR:GR complexes to be related to the previously identified nuclei edges by calculating average (and standard deviation) distance of intensity to the edge of an object. The intensity can either be inside, outside or both for the object. The intensity of pixels/voxels is measured within a specific range, in this case between (-5 μm to +5 μm), for both the red and blue channels. This distance range was set where initial 3D volume slicing, and orthogonal viewing revealed no puncta beyond this distance within the nucleus. Increasing the number of distance bin intervals (i.e. 0.05, 0.1, 0.15 microns etc) in the future could provide a more specific location of MR:GR complexes within these areas, although this will inevitably increase the size of the data file which may cause processing issues. The application of this MIA pipeline produced results demonstrating clear localisation patterns within the nucleus which have informed MR:GR function.

The MIA pipeline could be applied to imaging techniques combined with fluorescent probes for the visualisation and analysis of protein networks. Understanding the role and spatial organisation of dynamically regulated proteins is vital in accurately developing paradigms of biological systems. MIA can be applied to the analysis of proteins under varying experimental or physiological conditions, such as GR/MR agonist/antagonist combinations or selective glucocorticoid receptor modulators (SGRMs), to investigate changes in MR:GR complex concentration, density (are they arranged in protein clusters) and spatial movement or positioning within a cell, as examples. MR/GRs are involved in several different biological functions in various organs and the ability to evaluate the status of GR complexes and their interactions with other transcription factors or co-regulatory proteins involved in transcriptional regulation might clarify the clinical significance of MR:GR complexes and GC effects in disease. Image analysis has been applied to study nuclear receptors including *in situ* PLA analysis of oestrogen receptor dimers (ER-ER) (Iwabuchi *et al.*, 2017) and ER interactions with co-regulatory proteins within a multi protein transcriptional complex (Papachristou *et al.*, 2018). ER dimers were

detected in cells lines and for the first time in breast cancer tissue (Iwabuchi *et al.*, 2017), and analysed using the Lumina Vision analysis pipeline (Mitani Corp, Japan). This study compared different cell lines and visualised variants of ER dimer subtypes at varying concentrations with increasing exposure to oestrogen. The software system used in this study provides several bio-imaging analysis operations for use with fluorescent images and includes image capture, observation and measurement of fluorescent intensities and particles. However, as with several publicly available analysis packages, numerous measurements for detection and thresholding must be pre-determined, re-organised and then re-combined at various steps throughout the analysis to obtain the output data. In addition, files cannot be processed in batch mode, and must be processed singularly. This ultimately renders the technique as laborious and time consuming, as well as including a degree of user bias. The MIA pipeline that has been established in this project avoids the hurdles of pre-determining thresholds, intermediate steps and single image processing. This MIA pipeline allows multiple files to be completely processed in batches and enables un-biased localisation data to be collected.

7.2.3 Detection and analysis of proximity between the mineralocorticoid and glucocorticoid receptors using proximity ligation assay

Following CORT induction (15 nM CORT), MR:GR complexes were clearly located within the nucleus and as the duration of CORT exposure increased a more peripheral location with close proximity to the NE was observed. Unliganded GR is complexed with hsp90, hsp70, hsp56, p23, and immunophilins within the cytoplasm, concealing its NLS and is available for ligand activation (Merkulov, Klimova and Merkulova, 2016). Once activated by GCs, other synthetic ligands or SGRMs, the GR-protein complex translocates via dynein along microtubules, to NPCs where it interacts with importins and nucleoporins and is imported into the nucleus. GR disassociates from the chaperone complex and together with specific co-regulatory protein interactions, associates at the DNA to induce gene transcription (van Weert *et al.*, 2019). The discovery of MR:GR complexes proximal to the nuclear lamina (NL) suggests a functional interaction with the NL and/or chromatin re-modellers and indicates a role for MR:GR complexes in transcriptional regulation within these regions. The NL is composed of fibrous (lamin) proteins in type V intermediate filaments, which control the structural function and chromatin organisation in the cell nucleus (de Leeuw, Gruenbaum and Medalia, 2018). The NL contains and mediates spatial organisation of the genome in the nucleus, and facilitates the bi-directional transport of molecules involved in transcriptional processes across the nuclear membrane (Stancheva and Schirmer, 2014). MR:GR complexes have not been detected before and therefore their role within these nuclear regions is unknown, although lamina-associated proteins are often involved in chromatin remodelling and transcriptional regulation (de Leeuw, Gruenbaum and Medalia, 2018). As well as being localised to the NL, MR-GR complexes were found around the nucleoli. These

regions are lamina-associated domains (LADs) that correspond to B-type compartments of the genome (A-type compartments correspond to inter-LADs and include mostly active genes) (Kind *et al.*, 2015). LADs are AT-rich genomic regions of tightly condensed chromatin with overlapping heterochromatic features (histone modifications H3K9me2, H3K9me3 and in some cells H3K27me3 (Guelen *et al.*, 2008; Harr *et al.*, 2015)), and contain genes which are mostly transcriptionally silent or expressed at low levels (Guelen *et al.*, 2008; Wen *et al.*, 2009). Finding MR-GR complexes in two regions, the NL and around the nucleoli, associated with heterochromatic DNA suggests MR-GR complexes are regulating gene transcription by gene silencing. Furthermore, DAPI binds strongly to AT rich DNA and in the PLA images, puncta appear in proximity to compact regions of intense 'blue' fluorescence can be observed within regions usually associated with LADs and NADs (Guelen *et al.*, 2008; Peric-Hupkes *et al.*, 2010), and this is further evidence that MR:GR complexes are associated with heterochromatin. To determine whether MR-GR complexes are really present in densely stained DNA regions will require automated tracking of fluorescence intensities through the blue and red channels concomitantly, which currently using the MIA pipeline is not possible due to segmentation at the nucleoli but is in development.

There are differences in the number of PLA puncta observed when comparing Neuro-2A cells and hippocampal tissue. Considerably more puncta are observed in Neuro-2a cells than in hippocampal tissue. This could be explained by overexpression of the receptors in cells compared to actual endogenous protein levels observed in rat hippocampal tissue. Nevertheless, the MR:GR puncta were localised to the nuclear periphery in both, demonstrating MR:GR proximity of <40 nm in cultured cells and hippocampal tissue.

Specific NE proteins, such as nuclear lamins interact with DNA and regulatory proteins within the nuclear periphery and are reported to bring chromatin into contact with the NE via tethering mechanisms (Mattout-Drubezki and Gruenbaum, 2003; Stancheva and Schirmer, 2014). Several studies have shown NL mediated RNAPII dependent gene regulation (Kumaran, Muralikrishna and Parnaik, 2002; Spann *et al.*, 2002; Ansari *et al.*, 2016) and have shown TFs to bind lamina proteins (Leatherman, Kaestner and Jongens, 2000). Insulin promoter factor-1 (IPF-1) regulates transactivation of the insulin promotor in β-cells of pancreatic islets, which at low transcellular glucose levels induces inactivation of IPF-1 located peripherally to the NE and at high concentrations moves towards the nuclear centre, where it can bind to and activate its target gene (Rafiq, Kennedy and Rutter, 1998). Another example includes Sp3, a GC box-binding TF, that can both activate and repress gene dependant expression. Sp3 is sumoylated by SUMO-1 and located within LADs as nuclear spots which evokes a repressive transcriptional role. When the SUMO-1 site was removed, Sp3 localised throughout the nucleoplasm and activated targeted genes (Ross *et al.*, 2002). Furthermore, studies

have demonstrated circadian genes, such as Per1, to be rhythmically recruited to the NE and interact with specific LAD targets such as transcriptional modulator MAN1, which via PMTs attenuates gene expression (Bu *et al.*, 2019) and upon disassociation from specific lamina proteins at the NE, believed to define the duration of the circadian cycle, sees cyclic transcriptional activities restored (Lin *et al.*, 2014; Zhao *et al.*, 2015; Bu *et al.*, 2019). Since MR-GR complexes were found at the NL, it is possible they are directly interacting with proteins, such as lamins, and have a chromatin tethering role, whereby they regulate transcriptional activities, or they may be involved in chromatin organisation.

Several studies describe how mechano-transduction can impact on nuclear functions such as transcriptional regulation. This has been looked at for GR, where the NL was not necessary for DEX or shear stress induced nuclear translocation of GR, however using a luciferase reporter assay, GRE transcription was dysregulated (Nayebosadri and Ji, 2013). In this study, the NL is suggested to mediate shear stress-induced gene regulation, rather than HDAC-mediated transcriptional regulation under DEX initiation (Nayebosadri and Ji, 2013). This supports recent suggestions that nearby and distal co-bursting transcription sites are stimulated by mechanical forces by the NE and cytoskeleton (Stavreva *et al.*, 2019). LAD-NL attachments have been assessed using beads (observed as GFP spots) attached to cellular membranes and following force exposure movement of GFP spots led to rapid nuclear chromatin elongation and transcription (Tajik *et al.*, 2016). What is more, lamin and emerin proteins have been shown to interact (Dutta, Bhattacharyya and Sengupta, 2016) and knockdown of both NE proteins dysregulated the expression of specific gene subsets implicating an interactive role in mechanical and structural organisation of chromatin dynamics and function in the interphase nucleus (Ranade *et al.*, 2019). In addition, NPCs that control molecule movement across the NE have been observed to associate with super-enhancer regions peripherally located at the nuclear interior and their depletion effected the transcriptional output of enhancer regulated genes (Ibarra and Hetzer, 2015). This might suggest MR:GR complexes are involved in regulation of super enhancer regions, since they have been detected peripherally at the nuclear interior and in proximity to the NE. Similarly, acute ligand induced ERα-dependent MegaTrans enhancer condensates produced cooperative spatial alterations in chromosomal architecture (Nair *et al.*, 2019) which might explain the similar arrangement of the MR:GR complex at the nuclear periphery. In consideration of this evidence, it is possible that following prolonged CORT exposure, MR:GR complexes localise within the above described nuclear periphery that house usually 'repressive' heterochromatic domains such as LADs and are spatially and transcriptionally regulated by their interactions with each other and with NE associated proteins. This could be interrogated using combinations of immunolabelling with a brightfield multi-complex proximity ligation assay that utilises electron microscopy to visualise interacting protein as DAB precipitates.

In chapter 5, Neuro-2a cells treated with vehicle control (ethanol, final concentration 0.01%) for 45 minutes, MR:GR complexes were detected in the cytoplasm between 0 and +5 microns (Figure 2 and 3A). Given that the mammalian perinuclear space is usually between 30-50 nm wide, their detection in bin '0' indicates localisation of MR:GR complexes within the cytoplasmic space and perhaps even the perinuclear space. The MIA can analyse puncta properties in relation to the calculated outer most shell of DAPI stained nuclear voxels and as such, the edge is technically inside of the nuclear membrane. Since an abundance of MR:GR complexes are detected between 0 and 0.5 microns following a vehicle control treatment, a number of complexes appear localised within the perinuclear space (30-50 nm) between the inner and outer nuclear membranes of the NE, a bi-layer lipid membrane structure (Franke *et al.*, 1981; Alberts, 2002). Increased protein concentrations are predominantly found at the outer nuclear membrane (Hetzer, 2010) which is contiguous with the endoplasmic reticulum (Tabares, L., Mazzanti, M. & Clapham, 1991; Alberts, 2002) and further into the cytoplasm, which is consistent with the abundance of MR:GR complexes detected from bin '0' following a vehicle control. Unliganded MR:GR interactions within the cytoplasm and at the NE, was an unexpected result which has not been previously reported. Since unliganded GRs are held in accessory protein complexes in the cytoplasm that allow GC binding and additionally inactivate GRs NLS, it is surprising to learn that MR:GR exist in pre-formed complexes in the cytoplasm prior to ligand activation. A study suggested a sequence in GR's hinge region to mediate MR:GR dimerization in the cytoplasm (Savory *et al.*, 2001). However, beyond this, MR:GR complexes have not been identified before. Unliganded MR:GR pre-formed conformations in proximity to the nuclear membrane structure may exert genomic and nongenomic effects, and which has been previously described for ERα mediated cellular effects (Norman, Mizwicki and Norman, 2004). GR dimers were reported using varying concentrations of overexpressed GRwt (wild type) in COS-1 cells. Pre-formed GR homodimers were reported to form independent of ligand and which were shown to enhance transactivation by cooperative DNA loading at GRE promotors (Robertson *et al.*, 2013). Interestingly, maximal dimerization was achieved in the absence of ligand, which did not increase following GC exposure, and transactivation of GRE sites remained significantly increased (Robertson *et al.*, 2013). What is more, unliganded GR has previously been reported to induce genomic effects through direct or indirect activation or repression of specific genes (Sapolsky, Romero and Munck, 2000). Unliganded GR has been reported to interact with co-regulatory proteins via the LBD (Pfaff and Fletterick, 2010; Monczor *et al.*, 2019). Although, a small number, unliganded MR:GR complexes were observed in the nucleus and their translocation may be explained by other mechanisms as observed with unliganded GR, which has been reported to translocate into the nucleus via cytoskeleton independent mechanisms such as free radicals and reactive molecules from oxygen and nitrogen species, as well as

various cytokines (Sundahl *et al.*, 2015). Although some studies describe GR as resistant to ligand independent activity (Weigel and Zhang, 1998), GR activation in the absence of ligand has also been reported (Cenni and Picard, 1999; Eickelberg *et al.*, 1999) and PTMs or mutations have produced a constantly active GR without ligand induction (Godowski *et al.*, 1987). Although these studies were predominantly looking at GR only, they might offer some explanation for the detected unliganded MR:GR complexes observed predominantly in and around the NE space, as well as in the nucleus, in this project.

As described in chapter 1, MR-tethered GR binding at the DNA was shown to produce differential gene expression. This was shown using a combination of wild type and XDBD mutant MR/GR expression vectors. Significant changes in the up- and down-regulation of specific genes were observed in Neuro-2a cells that were transfected with XDBD-MR with an intact GR, compared to the receptor alone. This data concluded that MR could exert transcriptional effects, in combination with GR, without MR binding at the DNA (Rivers *et al.*, 2019). With this in mind, combinations of MR and GR DBD mutants, MR-A640T and MR-A640T-XDBD mutants were used in combination with each other and with wild type receptors, to assess if this would prevent the formation of MR:GR PLA complexes. Despite the two types of interface disruptions, MR:GR complexes were still detected at a similar number and within the same peripheral locations. This supports the novel study by Rivers *et al* (2019), which questioned the mode of receptor-XDBD mutants tethered by wild type intact receptors to be either at the dimerization domain, in the classically described MR:GR dimer conformation or by another mode. However, similarly despite disruption of the dimerization interface, MR-A640T and MR-A640T-XDBD mutants, when co-expressed with wild type GR demonstrated similar augmented up- and down-regulation of genes, compared to the receptor alone. This observation is similar to one made when GR dimerization was reported even when the dimerization interface was disrupted, although the number of dimers was less (Robertson *et al.*, 2013). Together, this suggests another mode of interaction between MR and GR, whether their proximity is due to indirect binding to other co-regulatory or TF proteins, or via direct interaction between alternative domains. A possible mode of interaction is co-localisation within a liquid-liquid phase separated droplet, which can be initiated by TF-DNA binding (Nair *et al.*, 2019).

7.2.4 Differential effects of the mineralocorticoid and glucocorticoid receptor on gene regulation

MR:GR interactions have been previously reported to produce differential functional effects on gene transcription *in vitro* (Trapp, Rupprecht, Castrén, *et al.*, 1994; Rivers *et al.*, 2019) and as well in physiological studies (Gomez-Sanchez *et al.*, 1990; Joels and De Kloet, 1992). *In vitro* GR and MR investigations have used high doses of glucocorticoids (100 nM dexamethasone, 100 nM CORT) to

reveal gene expression changes (Savory *et al.*, 2001; Rivers *et al.*, 2019). Chronic exposure to high levels of CORT, as observed during stress (Russell, Kalafatakis and Lightman, 2015), causes consistent MR activation, and prolonged activation of GR, which can cause uninterrupted RNA production (Stavreva *et al.*, 2009, 2015). This study used physiologically relevant levels of CORT (15 nM) and consequently only small changes in mRNA expression were detected, however these changes are physiologically relevant and co-occur with the dynamic changes that were observed in MR:GR complex formation, since the experiments use the same CORT treatment and time-points. The affinity of MR to CORT is 10-fold higher than for GR, and therefore MR is activated at lower levels of circulating CORT (Rupprecht *et al.*, 1993), however at 15 nM CORT both MR and GR are expected to be activated. Published data using higher levels (100 nM CORT) show more robust responses, producing potentially additive or interactive effects of MR and GR at GC inducible genes (Rivers *et al.*, 2019). Transcriptional data in this thesis did not support the previously aforementioned published data as such, however this might be as a result of using more physiological concentration of 15 nM CORT, which may influence activation of GR and therefore necessitating higher concentrations of CORT. In addition, increased experimental repeats may be required in order to achieve significance. Overall, this explores the possibility of GR:MR interactions underlying greater than additive gene expression effects. Although it cannot be said if MR and GR are both present at these sites to produce interacting effects instead of individual additive effects, the PLA results show GR:MR proximity occurs in the nucleus at these time points and at the 15 nM CORT concentration.

Liganded GR regulates transcriptional regulation of targeted genes in a context specific manner requiring specific interactions within multi-protein transcriptional complexes consisting of co-regulatory proteins and genomic HREs. Transcriptional regulation by MR and GR are in part governed by factors that are organised and interact within multi-protein transcriptional complexes (Britten and Davidson, 1969; Yamamoto, 1985; Bain *et al.*, 2012, 2014; van Weert *et al.*, 2019). The recent study by Rivers *et al.* (2019) describes dynamic transcriptional responses that are enhanced when MR+GR are co-expressed together compared to MR/GR alone, and therefore provide increased sensitivity and responses by CORT. In addition, the concentration of available receptor that is ligand activated will have transcriptional effects (Chow *et al.*, 2011) where glucocorticoid receptor function is affected by ligand concentration and chemical composition, ligand exposure and kinetics, interactions with co-regulators, DBSs and chromatin accessibility (Darimont *et al.*, 1998; Collingwood, Urnov and Wolffe, 1999; Rogatsky *et al.*, 2003; Reddy *et al.*, 2009; Stavreva *et al.*, 2009; van Weert *et al.*, 2019). Several of these regulatory factors, particularly those within the multi-protein transcriptional complex, can allosterically alter receptor conformation and effect transcriptional regulation (Meijsing *et al.*, 2009; Millard *et al.*, 2013; Watson *et al.*, 2013). These factors are important in understanding liganded GR

mediated gene expression, and are context specific across cell and tissue type (Rogatsky *et al.*, 2003; Oakley and Cidlowski, 2011, 2013), and discrepancies in receptor binding have been observed across sample-specific chromatin landscapes (Grontved *et al.*, 2013; Pooley *et al.*, 2017). Following ligand activation, associations with transcription factors, coactivators and co-repressors that determine gene expression and DNA binding, take place prior to the induction of the targeted gene (Meijer, 2002). What is more, some co-repressors can stabilise chromatin via HDACs (Collingwood, Urnov and Wolffe, 1999) and coactivators allosterically modulate GR and effect ligand binding (Pfaff and Fletterick, 2010), further exemplifying the importance of protein-protein interactions in transcriptional regulation.

GCs are involved in the homeostatic regulation of all organs and tissues under basal and stress conditions. A large proportion of which exert their effects via the GR to induce transcription of target genes. Upon ligand activation, nuclear translocation of GR occurs, where the formation of multi-protein transcriptional complexes occurs at GREs and regulates transcription (Darimont *et al.*, 1998; Rogatsky *et al.*, 2003; Luecke and Yamamoto, 2005). The dysregulation of GC exposure and their GR mediated effects is believed to be involved in several disease pathways and as such is of pharmacological importance (Gessi, Merighi and Borea, 2010; Kadmiel and Cidlowski, 2013; Oakley and Cidlowski, 2013).

Endogenous GCs modulate neuronal processes via the MR and GR and regulate transcriptional processes of target genes. This transcriptional control requires specific interactions within multi-protein transcriptional complexes consisting of co-regulatory proteins and genomic HREs. Transcriptional regulation by MR and GR are in part governed by factors that are organised and interact within multi-protein transcriptional complexes (Britten and Davidson, 1969; Yamamoto, 1985; E R de Kloet, Joëls and Holsboer, 2005; Bain *et al.*, 2012, 2014; Vogel *et al.*, 2016; Meijer and de Kloet, 2017; van Weert *et al.*, 2019). In addition, the concentration of available receptor that is ligand activated will have transcriptional effects (Chow *et al.*, 2011). The amount of ligand will determine MR or GR activated responses due to the high and low affinity's innate to each receptor, respectively (Reul and de Kloet, 1985; E R de Kloet, Joëls and Holsboer, 2005; Joels *et al.*, 2008) and their expression alone or together in the same cells will determine the transcriptional output (E R de Kloet, Joëls and Holsboer, 2005). *In vivo*, CORT induced GR occurs during the ultradian peak of CORT secretion (Lightman *et al.*, 2008), or at stress induced levels. Thus, we would expect the GR:MR complexes detected following 15 nM CORT to be binding at DNA, possibly in heterochromatin, where DNA binding would mostly occur during ultradian peaks in CORT secretion or with prolonged CORT exposure. The role of MRs is predominantly associated with rapid non genomic effects via membrane bound MRs (Karst, 2005; Joels, Sarabdjitsingh and Karst, 2012), and their detection at the nuclear membrane in these studies may suggest another role for MR mediated regulation of cellular

processes. In addition GR:MR may display pulsatile profiles as observed with dynamic GR-DNA binding (McNally *et al.*, 2000; George *et al.*, 2017; Stavreva *et al.*, 2019). This is important where hippocampal control of memory and cognitive processes, emotional processing and adaptive behaviours, are regulated by differential GC-activated MR and GR effects. These differing effects have been observed in pyramidal neuron excitation in cells of the CA1 region (Joels and de Kloet, 1990; Karst *et al.*, 2000) and MR modulated behavioural adaptation responses by MR compared to memory consolidation by GR (Oitzl and de Kloet, 1992; Oitzl *et al.*, 2001). Furthermore, differing MR mediated effects to those of GR are further supported by the discovery of MR-regulated genes (Datson *et al.*, 2001; van Weert *et al.*, 2017), as well as the identification of co-regulatory proteins specific to MR (Pascual-Le Tallec and Lombes, 2005; Yang *et al.*, 2014). MR:GR interactions within the nuclear periphery suggests a specific role in transcriptional regulation, and potentially spatial organisation, of heterochromatic regions.

7.3 Future work

In addition to MR:GR heteromers, it is important to further evaluate dimerization of MR/GR, MR-MR and GR-GR under different types of ligand. The endogenous GR ligands are proposed to augment the formation of GR-GR dimers in comparison to other synthetic GCs, such as DEX, and as such produce molecularly more effective anti-inflammatory properties and with less adverse effects (Souffriau *et al.*, 2018). Investigating changes in spatial organization is important to identify transcriptional roles unique to varying MG/GR dimer conformations. In addition, assessing dimerization patterns of MR/GR splice variants and their subtypes may provide insight into expression profiles of the receptor which could impact transcriptional control. This has been looked at for ER splice variant interactions in breast cancer tissue (Omoto *et al.*, 2003; Iwabuchi *et al.*, 2017). However, the possibility of this work will depend on the availability and ability of Abs to detect MR/GR subtypes.

Exposing MR:GR complexes to different ligands, such as CORT vs SGRMs, will provide information regarding changes in activated receptor concentrations and dynamic spatial behaviour. In addition, proteomic analysis using Rapid Immunoprecipitation of Endogenous Proteins (RIME) could be used to further understand existing or reveal novel interacting partners of the MR:GR complex, where previously different ligands were reported to alter GRs interaction with co-regulatory proteins (Zalachoras *et al.*, 2013). As MR:GR complexes were detected at the nuclear periphery, candidate interacting partners of the receptor complex include structural proteins lamin, emerin, and nucleoporins, which could be tagged together with MR and GR using a multi complex PLA. Further, the dynamic lamin-MR:GR complex, may change in response to the type of and duration of ligand (CORT vs SGRMs) exposure, possibly affecting interactions between the NL and transcriptional protein

complex and impacting on the transcriptional regulation of specific genes. Tagging particular foci together with the MR:GR complex, may further determine if MR:GR are involved in heterochromatin organisation, as interactions between ligand bound GR and co-regulatory proteins further determines the regulation of specific genes (Lachize *et al.*, 2009) by modifying chromatin structure. In addition, conducting EM-PLA has the potential to deviate away from limitations of fluorescent studies where the number of targets that can be fluorescently tagged are limited either by species compatibility and fluor colour limits (usually 4) that can be used in a single experiment due to issues with bleed through. Finally, identifying how they interact under specific contexts: interactions via proteins domains or via liquid-liquid phase separation droplets. Clarification of these factors would lead to better understanding of the functional role of MR-GR complexes.

7.4 Conclusion

Taken together, MR and GR were found to be in very close proximity and are consequently likely to be interacting together. Since the complexes were localized to heterochromatic regions and MR and GR are transcription factors, they are likely to be involved in 3D chromatin organization and are implicated in regulating global gene transcription profiles. Since MR:GR complexes are localised at the periphery of the NE following prolonged GC exposure, this might allude to interactions with structural proteins and transcriptional regulatory roles specific to heterochromatic regions (Stancheva and Schirmer, 2014; de Leeuw, Gruenbaum and Medalia, 2018), which might regulate gene shut-down. Despite the nature of the MR:GR conformation remaining elusive, identifying its dynamic nuclear localisation within the nuclear periphery suggests a role in transcriptional regulation, and potentially spatial organisation, of heterochromatic regions. This reveals a mode of global transcriptional regulation by MR:GR complexes and further contributes to the understanding GC regulated cellular processes.

Chapter 8 References

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