3D RECONSTRUCTION OF SYNAPTIC AND NUCLEAR CORTICOSTEROID RECEPTORS DISTRIBUTION DENSITY IN THE AMYGDALA: A FEASIBILITY STUDY

Stephanie Koo

BA Social Science (Psychology) (Honours)

Submitted in fulfilment of the requirements for the degree of

Masters of Applied Science (Research) HL84

Translational Research Institute (TRI) and
Institute of Health and Biomedical Innovation (IHBI)

School of Psychology and Counselling
Queensland University of Technology (QUT)

2017
Keywords

Adrenal Glands, Amygdala, Brain, Cytosol, Dendrite, Fear, Glucocorticoids, Membrane, Mineralocorticoids, Neuron, Nucleus, Post Synaptic Density, Spine, Stress, Synapse.
Abstract

Disruptions to neuronal populations of corticosteroid receptors (glucocorticoid receptors; GR and mineralocorticoid receptors; MR) have been implicated in a range of stress-related pathologies; referred to as the Receptor Balance Hypothesis. Traditionally, however, the receptor balance hypothesis only focuses on genomic populations of corticosteroid receptors, and does not account for membrane-associated corticosteroid receptors. In this thesis, we tested the feasibility of using novel methods of reconstructing subcellular structures in order to characterise the distribution densities of GR and MR within the nucleus, and at excitatory post-synaptic terminals in the rat amygdala. We used triple-label immunofluorescence in conjunction with confocal imaging to characterise the labelling of corticosteroid receptors. Using Imaris™ software, we found that we could three-dimensionally reconstruct corticosteroid receptors, and perform object-based colocalisation analysis, in order to quantify the populations of corticosteroid receptors located at excitatory post-synaptic sites. This provides a novel method of quantifying corticosteroid receptors in amygdala tissue. The adaptability of the method suggests that it could be applicable to a range of applications in stress research.
# Table of Contents

Keywords ........................................................................................................... i

Abstract ........................................................................................................... ii

Table of Contents ............................................................................................ iii

List of Figures ................................................................................................. vii

List of Tables .................................................................................................. xiii

List of Abbreviations ....................................................................................... xiv

Statement of Original Authorship ................................................................... xv

Acknowledgements ........................................................................................ xvi

Chapter 1: Introduction .................................................................................... 1

  Functional Role of Corticosteroids ................................................................. 2

  The Amygdala and Corticosteroids ................................................................. 5

  Corticosteroid Receptors ............................................................................... 10

  A Rationale for Quantifying Corticosteroid Receptor Subpopulations ........ 13

  Fluorescent Imaging and Reconstruction of Corticosteroid Receptor Subtypes 14

  Thesis Objectives and Outline ....................................................................... 18

Chapter 2: Corticosteroid Receptors ................................................................. 21

  Dosage Effects of Corticosteroids on Corticosteroid Receptors .................. 21

  Corticosteroid Receptors in the Amygdala .................................................. 22

  Temporal Effects of Corticosteroid Receptors ............................................ 24

  Receptor Balance Hypothesis ...................................................................... 30

  Summary and Implications ............................................................................ 33

Chapter 3: General Method ............................................................................. 37
Chapter 5: Corticosteroid Receptor Densities in the Amygdala

Method

Subjects and Procedure

Design

Results

Controls

Mosaic Images

Deconvolution of Images

Nuclear Surfaces

Creation of Genomic GR and MR

Creation of Extra-nuclear GR and MR and Post-synaptic Terminals

Colocalisation of Corticosteroid Receptors at Post-synaptic Terminals

Corticosterone levels

Analysis

Descriptive Statistics

Genomic Corticosteroid Receptors vs. Corticosteroid Receptors at Post-Synaptic Terminals

Proportion of Synapses that contain Corticosteroid Receptors

Chapter 6: Discussion

Applicability of 3D Reconstruction for Characterising Corticosteroid Receptors

GR and MR labelling can be Reconstructed as Spots

Nuclei can be reconstructed as Surfaces to identify gGR and gMR populations

3D Reconstruction of Synaptic and Nuclear Corticosteroid Receptors Distribution Density in the Amygdala: A Feasibility Study
3D Reconstruction of Synaptic and Nuclear Corticosteroid Receptors Distribution Density in the Amygdala: A Feasibility Study
List of Figures

Figure 1. The release of corticosteroids (Cort) via the Hypothalamic Pituitary Adrenal (HPA) Axis ................................................................. 4

Figure 2. Depiction of the organisation of subnuclei (labelled for GR) in a coronal section of the rat amygdala, under wide-field epifluorescence. Overlay adapted from Figure 31 of Stereotaxic Coordinates (Paxinos & Watson, 1997). 4-point axis refers to the orientation of the section: D, dorsal; V, ventral; M, medial; L, lateral. LA, lateral amygdala; BA, basal amygdala; CeA, central amygdala. ............................................ 6

Figure 3. The amygdala receives excitatory inputs from the hippocampus, thalamus and mPFC during stress – these circuits underlie Pavlovian conditioning and drive activation of the HPA axis from the CeA. Excitatory intra-amygdaloid circuits are also activated during stress by corticosteroids. ................................................................. 9

Figure 4. Factors that interact with MR and GR to affect Cognition and Behaviour........ 12

Figure 5. Distribution of Genomic and Synaptic GR and MR within a neuron. Genomic GR and MR are located within the cytoplasm, and translocate to the nucleus when bound. Synaptic GR and MR are located near or within the membrane at synapses; when activated, these receptors can affect neurotransmission. ............................................... 25

Figure 6. Corticosteroid receptors in the BLA-complex mediate neuronal excitability differently to the hippocampus. Corticosteroids increase neuronal excitation in the BLA-complex, through mMR. This excitability is maintained through gGR. Further application of corticosterone depressed neuronal excitability through mGR.

3D Reconstruction of Synaptic and Nuclear Corticosteroid Receptors Distribution Density in the Amygdala: A Feasibility Study
Adapted from research by Karst et al. (2010), Groeneweg et al. (2011), and Sarabdjitsingh and Jöels (2014).

**Figure 7.** Excitation and Emission Spectrum for DAPI, Alexa Fluor 488 and Alexa Fluor 594. Adapted from Life Technologies (2015a).

**Figure 8.** The LA, BA and CeA in a coronal section, regions sampled in grey. Sections were taken -2.04mm to -3.36mm from the bregma according to the rat brain atlas (Paxinos & Watson, 2007). Adapted from “The Rat Brain in Stereotaxic Coordinates 6th edition,” by G. Paxinos and C. Watson, 2007, p.56.

**Figure 9.** Groups involved in MR titration of antibodies.

**Figure 10.** Representative image of fluorescent labelling of PSD-95-like immunoreactivity at concentrations of 1:500, 1:750 and 1:1000 (epifluorescence), in rat brain tissue. A non-linear contrast was applied in Photoshop using the curves function. Transformation was applied uniformly to all three images to improve the contrast. Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm.

**Figure 11.** a), b), c), and d) show tissue sections incubated with rMR-1D5 at a dilution of 1:200; where the top two images are sections incubated for 24 hours, and the middle two images are sections incubated for 48 hours. Images e) and f) were diluted at 1:500 incubated for 48 hours. Images in the left column have been incubated for 2 hours with the secondary antibody and images in the right column have been incubated for 4 hours. Epifluorescent images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm.
Figure 12. Confocal imaging of rMR-1D5 labelled slices at a concentration of 1:200 with an incubation time of 48 hours. Tissue sections were incubated with IgG Alexa Fluor 488 for 2 hours (a) or 4 hours (b). Images were taken with a 60x (1.35 NA) oil objective with 2.5x sensor zoom. Scale bar: 10µm. ................................. 63

Figure 13. Control sections, sample: rat brain tissue. Single-label controls for GR (a) and MR (b) in the GFP channel, green. Figures (c) and (d) show secondary-only controls of GR and MR respectively. Images (e) and (f) show the cross-reactivity controls for GR and MR respectively, in the cy3 channel. Epifluorescent images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm. ................................. 65

Figure 14. Single-label control in rat brain tissue, for anti-PSD-95 shows puncta labelling (a) with cy3 filter. Alexa Fluor 594, secondary-only control (b) with cy3 filter shows minimal labelling. Cross-reactivity control for anti-PSD-95 primary antibody (c) shows minimal immunoreactivity in GFP channel. Epifluorescent images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm. ................................. 66

Figure 15. Epifluorescent images of brain tissue sections labelled with one primary antibody and both secondary antibodies: GR single-double control in the (a) GFP channel (green), and (b) cy3 channel (red); MR single-double control in the (c) GFP channel (green) and the (d) cy3 channel (red). Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm. ................................. 67

Figure 16. Triple labelling for GR sections in rat brain tissue, under the epifluorescent microscope. For the same region, in the GFP channel (green) GR-like immunoreactivity was observed (a). In the cy3
channel (red) PSD-95-like labelling was seen (b). In the DAPI channel (blue) nuclei-labelling was seen (c). All three channels were artificially merged (d). Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm.

**Figure 17.** Confocal imaging of GR-section in rat brain tissue. GR-like labelling can be seen in green, PSD-95-like labelling in red, and DAPI labelling in blue. Colocalisation of GR-like labelling with PSD-95 like labelling is shown in yellow (indicated by white arrows). Images were taken with a 60x (1.35 NA) oil objective with 2.5x sensor zoom. Scale bar: 10µm.

**Figure 18.** Triple labelling for MR sections in rat brain tissue, under the epifluorescent microscope. a) In the GFP channel (green) MR-like immunoreactivity was seen. b) In the cy3 channel (red) PSD-95-like labelling was seen. c) In the DAPI channel (blue) nuclei-labelling was seen. d) All three channels were artificially merged. Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm.

**Figure 19.** Confocal imaging of MR-section in rat brain tissue. MR-like labelling can be seen in green, PSD-95-like labelling in red, and DAPI labelling in blue. The nuclei labelling was selected for optimal MR labelling – the DAPI labelling is not representative of the DAPI nuclei staining obtained throughout. Colocalisation of MR-like labelling with PSD-95 like labelling is indicated with white arrows. Images were taken with a 60x (1.35 NA) oil objective with 2.5x sensor zoom. Scale bar: 10µm.

**Figure 20.** Mosaic image of rat brain tissue, taken at 20x (0.86 NA) oil objective. White dots depict the coordinates of images sampled for analysis.
Overlay in white displays the boundaries of the different subregions of the amygdala.

Figure 21. Maximum intensity projections for DAPI and PSD-95 are depicted, before and after deconvolution. The images depicted contained 52 stacks, with a thickness of 15.6µm. Deconvolved images b) and d) show reduced light scattering.

Figure 22. Maximal z-projections are displayed for GR and MR labelling before and after deconvolution. Z-thickness of deconvolved images for GR and MR show the correction for noise, and the change in the size of puncta: changes in GR puncta can be seen in c) and d); changes in MR puncta can be seen in g) and h).

Figure 23. Creation of nuclear surfaces for GR and MR sections. First row of images a) and d) display unfiltered fluorescence. Second row of images b) and e) display filtered DAPI labelling. Third row c) and f) displays Imaris-generated 3D surfaces. Scale bar: 10µm.

Figure 24. Intra-nuclear GR- and MR-like labelling was filtered based on nuclei surfaces. The first row a) and d) displays unfiltered labelling. The second row b) and e) displays the filtered labelling. The third row c) and f) displays detected labelling that was reconstructed as spots. Scale bar: 10µm.

Figure 25. Creation of extra-nuclear spots for GR-like labelling and MR-like labelling and PSD-95. Fluorescence labelling before and after filtering is also shown. Scale bar: 10µm.

Figure 26. Creation of colocalised spot objects from overlaps between corticosteroid receptors and PSD-95 (indicated by white arrows).
Asterisk indicates seemingly colocalised fluorescence that was not labelled in 3D reconstruction. Scale bar: 3µm.

*Figure 27.* Mean densities of genomic and colocalised GR and MR spots. Symbols represent section averages. Error bars display ±1 Standard Error of the Mean (SEM).

*Figure 28.* Mean density of receptor spots located within DAPI surfaces, adjusted by the surface volume. Light grey bars represent density of GR spots, per amygdala subregion. Dark grey bars represent density of MR spots, per amygdala subregion. Error bars represent ±1 SEM.

*Figure 29.* Amount of PSD-95 spots that contain colocalised GR or MR spots. The mean volumetric density of PSD-95 spots for GR or MR sections is indicated by the black bars. The mean volumetric density of colocalised GR or MR spots is indicated by light bars.

*Figure 30.* Bar graph depicting the proportion of extra-nuclear GR spots that were colocalised with PSD-95 spots, within each brain region. Error bars represent ±1 SEM.

*Figure 31.* Bar graph depicting the proportion of extra-nuclear GR spots that were colocalised with PSD-95 spots, within each brain region. Error bars represent ±1 SEM.

*Figure 32.* Image of genomic and colocalised GR and MR spots after 3D reconstruction in Imaris. Scale bar: 5µm.
List of Tables

Table 1 39
Table 2 48
Table 3 98
Table 4 101
Table 5 102
Table 6 103
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic Releasing Hormone</td>
</tr>
<tr>
<td>BLA</td>
<td>Basolateral Amygdala</td>
</tr>
<tr>
<td>BA</td>
<td>Basal Nuclei of the Amygdala</td>
</tr>
<tr>
<td>CeA</td>
<td>Central Nuclei of the Amygdala</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin Releasing Hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>gGR</td>
<td>Genomic Glucocorticoid Receptor</td>
</tr>
<tr>
<td>mGR</td>
<td>Membrane-associated Glucocorticoid Receptor</td>
</tr>
<tr>
<td>HPA-axis</td>
<td>Hypothalamic-Pituitary-Adrenal Axis</td>
</tr>
<tr>
<td>LA</td>
<td>Lateral Amygdala</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term Potentiation</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid Receptor</td>
</tr>
<tr>
<td>gMR</td>
<td>Genomic Mineralocorticoid Receptor</td>
</tr>
<tr>
<td>mMR</td>
<td>Membrane-associated Mineralocorticoid Receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
</tbody>
</table>
Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature

Date: 07/02/2017
Acknowledgements

I would like to begin by thanking my supervisors, Luke, Andrew, and Arno. Thank you Luke and Andrew for this opportunity, and for your patience and understanding while I transitioned from psychology to neuroscience; at the start, even basic concepts were difficult for me to grasp. My complete and utter thanks goes to Arno, for your willingness to teach me and guidance you gave me along the way. Not only did I learn about neuroscience, I also learnt from you the importance of collaboration– your attitude towards science is truly inspiring, and your patience even more so.

I would also like to thank all the genuine, and genuinely intelligent people in both the Bartlett and Johnson Lab that I was fortunate enough to meet. I am so grateful to everyone for all the support I received, but even more so, for the friendships made. The lab was made a much warmer place with all of you there. I would like to thank my housemate Eric, for his dinners, his patience, and his understanding. I was not a very fun person to be around, especially during the stressful periods, but your unbelievable patience and understanding kept me motivated. I would also like to thank Vinnie, Lyndon, Maddy, Cody, Dana and Sarah, for offering me a bed in Brisbane when I needed to work long hours in the lab. Knowing that I would not have to travel too far, to be in good company, after those long, stressful days, really kept me going –I really cannot thank you all enough.

Finally, I would like to thank my friends and family back home in Melbourne. To my wonderful parents, Cheong and Meng, who have given me so much: thank you for guiding me and teaching me; but also for all the unconditional love and support you’ve shown me – I could not have done any of this without you. To my partner Alex; despite the distance, knowing that I could come home every day to see your lovely face gave me the stability I needed in the tumultuous world of the lab. Thank you.

From a Bachelor’s degree in Psychology to a Master’s degree in Neuroscience, the learning curve has not only been steep, but it has wound its way through different labs, across cities, and through a mile of paperwork. There are more people who have had an impact than I can fit in one page, and I don’t even feel that I have effectively expressed how grateful I am to the people mentioned here. But I am so thankful for all the support I’ve received, both academically and emotionally, which has pushed me, taught me and encouraged me to get to where I am today.

_The ironic thing about writing a thesis on stress, is the sheer amount of stress it causes you. So without further ado..._
Chapter 1: Introduction

Acute stress helps the body respond to threats, potentially interacting with memory systems to support long-term memory of dangerous events and places (Sapolsky, Romero, & Munck, 2000). In contrast to the beneficial effects of acute stress, stress can also alter pathologies such as addiction, anxiety disorders, and mood disorders (Daskalakis, Lehrner, & Yehuda, 2013; Millan et al., 2012). Moreover, long-term stress has negative impacts on human health (Millan et al., 2012). In the brain, these negative impacts can include the re-shaping of neurons and their synaptic connections. These important and varied actions of stress are mediated by corticosteroids (cortisol in humans; corticosterone in rodents) a hormone released from the adrenal glands in response to stress.

In the brain, corticosteroids act on both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). Collectively known as corticosteroid receptors, disruptions to these receptors have been implicated in a range of disorders (Millan et al., 2012; Wingenfeld & Wolf, 2015). GR and MR are both transcription factors – they interact with the genome to influence protein synthesis (Polman, de Kloet, & Datson, 2013). In addition, both GR and MR have been identified at the membrane, where they are reported to have rapid membrane and sub membrane-signalling effects (Groeneweg, Karst, de Kloet, & Joels, 2011). Nonetheless, the subcellular organization and distribution of membrane-associated MR and GR among brain subnuclei is still being characterised.

The aim of this thesis was to provide new data about the distribution of membrane-associated MR and GR in the amygdala, including their relationship to synapses. In order to understand the current research around these corticosteroid
receptors, it is important to firstly understand the role of cortisol— the main ligand for these receptors. Thus, in this chapter I provide an introduction to the functional role of corticosteroids in the brain, and how corticosteroids in the amygdala have been proposed to modulate the brain’s emotional response to stress (Schwabe, Joels, Roozendaal, Wolf, & Oitzl, 2012).

**Functional Role of Corticosteroids**

To understand the importance of corticosteroid receptors, it is important to understand the functional role of the corticosteroids in the brain and the body. In this thesis, the term *corticosteroids* is used to refer specifically to cortisol in humans or corticosterone in rodents; which are the body’s naturally occurring glucocorticoids (Groeneweg, 2014). Corticosteroids have an active role within the body, both, under basal conditions (Horrocks et al., 1990) and under stress. Corticosteroids can differentially affect cognition and behaviour, and these effects are dependent upon the distribution of corticosteroid receptors in the brain (Santos, Cespedes, & Viana, 2014; Wingenfeld & Wolf, 2015). In the brain, corticosteroids have been found to bind to two types of corticosteroid receptors: glucocorticoid receptors (GR, a Type II adrenocorticosteroid receptor); and mineralocorticoid receptors (MR; a Type I adrenocorticosteroid receptor) (Herman & Spencer, 1998; Reul & de Kloet, 1985; Teng, Zhang, Zhao, & Zhang, 2013). I review these receptors in more detail in Chapter 2.

Corticosteroids play a major role in the stress response. When the stress response is triggered, usually during a threatening event (Iwasaki-Sekino, Mano-Otagiri, Ohata, Yamauchi, & Shibasaki, 2008), the hypothalamic-pituitary-adrenal (HPA) axis is stimulated. Corticotropin releasing hormone (CRH) is secreted from the hypothalamus (Herman et al., 2003), which activates the pituitary gland, resulting
in the release of adrenocorticotropic hormone (ACTH) into the blood stream (Zalachoras, 2014). ACTH subsequently stimulates the adrenal glands, causing the release of corticosteroids (Bouchez et al., 2012). Corticosteroids travel in the bloodstream, and the lipophilic nature of corticosteroids allows them to cross the blood-brain barrier, resulting in elevated levels of corticosteroids in the brain (Dedovic, Duchesne, Andrews, Engert, & Pruessner, 2009). Once in the brain, they can have both acute and long-term effects on cognition and behaviour (Joels & Baram, 2009). Finally, corticosteroids act on receptors in the HPA-axis via negative feedback, subsequently inhibiting further release of corticosteroids (Groeneweg, Karst, de Kloet, & Joels, 2012), which is illustrated in Figure 1.

Corticosteroids are also secreted during homeostasis (de Kloet, 2014). At basal conditions, corticosteroid levels oscillate in time with the body’s circadian rhythms (Lightman et al., 2008). The HPA-axis (Figure 1) secretes corticosteroids in pulses, resulting in peaks and troughs of corticosteroid levels (Lightman et al., 2008). In rodents, corticosterone trough concentration in the morning and peaks in the evening (Chaudhury & Colwell, 2002; Reul & de Kloet, 1985). In humans, cortisol levels peak in the morning, and taper off during the day (Horrocks et al., 1990). Cortisol pulses are significantly reduced between 6pm to midnight (Horrocks et al., 1990), with the trough occurring around midnight (Buckley & Schatzberg, 2005).
Figure 1. The release of corticosteroids (Cort) via the Hypothalamic Pituitary Adrenal (HPA) Axis

Circadian oscillations of corticosteroids are important for normal functioning (de Jong et al., 2000). For example, corticosterone peaks have been demonstrated to promote dendritic spine formation after learning, and corticosterone troughs are important for stabilising learning-related dendritic spines (Liston et al., 2013). These circadian pulses may also be important in maintaining the body’s responsiveness to stress (de Jong et al., 2000), mediating the duration of the stress response and facilitating stress recovery (Jacobson, Akana, Cascio, Shinsako, & Dallman, 1988). Stress increased corticosteroid levels can disrupt these natural corticosteroid oscillations, affecting HPA axis responsiveness (Millan et al., 2012). This can have negative effects such as sleep disorders (Buckley & Schatzberg, 2005), depression (Keller et al., 2006; Solberg, Olson, Turek, & Redei, 2001), PTSD (Chaudhury & Colwell, 2002), epilepsy (Kumar et al., 2007), drug addiction (Simms, Haass-Koффler, Bito-Onon, Li, & Bartlett, 2012) and pain (A. C. Johnson & Meerveld, 2015).
The Amygdala and Corticosteroids

Stress is an emotionally arousing experience, affecting cognition and behaviour (Schwabe et al., 2012). In the brain, the amygdala is central to the stress response (Roozendaal, 2003). Not only does the amygdala directly affect stress-related learning and behaviour (Roozendaal & McGaugh, 1996; Roozendaal, Portillo-Marquez, & McGaugh, 1996; Roozendaal, Quirarte, & McGaugh, 2002), it also modulates memory processing in various brain regions (Harris, Holmes, de Kloet, Chapman, & Seckl, 2013; L. R. Johnson, McGuire, Lazarus, & Palmer, 2012; Kolber et al., 2008; Quaedflieg et al., 2015), such as the hippocampus (McReynolds et al., 2010), and medial prefrontal cortex (mPFC) (Schwabe et al., 2012). On a cellular level, corticosteroid action in the amygdala affects gene transcription and neuronal excitability (Groeneweg et al., 2012). On a systems level, corticosteroids in the amygdala affect its neuronal connectivity with other brain regions, differentially influencing learning and memory (Roozendaal, 2003; Schwabe et al., 2012).

The structural organisation of the amygdala appears to affect the functional outcomes, where different subregions have different roles in the processing and behavioural outcomes of stressful situations (Sah, Faber, Lopez de Armentia, & Power, 2003). The amygdala can be broadly divided into three subregions; the basolateral amygdala complex (BLA), the central amygdala (CeA) and the intercalated cells (ITC) (Giustino & Maren, 2015; Pape & Pare, 2010). Moreover, the BLA nuclei can be further divided into two regions: the lateral amygdala (LA) and the basal nuclei (BA; referring to both the basolateral nuclei and the accessory basal nucleus); which differ in structure and function (Giustino & Maren, 2015; Pape & Pare, 2010). During stress, the LA, BA and CeA subregions receive a range of sensory information from various pathways including: olfactory projections,
somatosensory inputs, gustatory and visual areas, and auditory and visual projections (Sah et al., 2003). As these three subregions have been shown to be differentially influenced by corticosteroids (Roozendaal, 2003; Roozendaal & McGaugh, 1996), they were the primary subnuclei targeted in this thesis. The organisation of the LA, BA and CeA nuclei in the rain brain can be seen in Figure 2.

Figure 2. Depiction of the organisation of subnuclei (labelled for GR) in a coronal section of the rat amygdala, under wide-field epifluorescence. Overlay adapted from Figure 31 of Stereotaxic Coordinates (Paxinos & Watson, 1997). 4-point axis refers to the orientation of the section: D, dorsal; V, ventral; M, medial; L, lateral. LA, lateral amygdala; BA, basal amygdala; CeA, central amygdala.

The amygdala subnuclei have different functional roles in stress-related emotion and behaviour. The LA is considered the input nuclei, and the CeA and BA as output nuclei for fight and flight associated responses (Amorapanth, LeDoux, & Nader, 2000). The LA is proposed to be the region where the acquisition, but not the
expression, of fear learning. LA lesion studies have demonstrated that lesions to the LA inhibits acquisition of fear memories (Amorapanth et al., 2000). Single unit recording studies have further differentiated the LA as the primary input site, where neuronal excitability was shown to increase during threat acquisition (Bauer, Schafe, & LeDoux, 2002; Rosenkranz & Grace, 2002) but prior to any behavioural response (Repa et al., 2001). Alternatively, the CeA has been demonstrated to be responsible for behavioural responses in stressful situations. As the primary output of stress expression (Penzo et al., 2015), the CeA has been shown to project to numerous extra-amygdaloid brain regions (Cassell, Freedman, & Shi, 1999; Jolkkonen & Pitkänen, 1998), resulting in behavioural and physical changes, such as: increased heart rate, freezing or increased corticosteroid release (Parsons & Ressler, 2013). Lesion studies support this, showing suppression of the CeA only affected threat expression, but not threat learning (Amorapanth et al., 2000; Roozendaal & McGaugh, 1996). Corticosteroids in the CeA, but not the BLA, influence drug-seeking behaviours (Simms et al., 2012), and the knockdown of corticosteroid receptors in the CeA increased the expression of pain (A. C. Johnson & Meerveld, 2015). Finally, the BA is suggested to act as the interface between the LA and the CeA. Selective inactivation of the LA or the CeA prior to fear conditioning has been demonstrated to inhibit fear expression, however, prior lesions of the BA had no effect on fear acquisition (Anglada-Figueroa & Quirk, 2005). Alternatively, lesions to BA nuclei after learning blocked fear expression (Amano, Duvarci, Popa, & Pare, 2011; Anglada-Figueroa & Quirk, 2005) suggesting that fear expression cannot occur without the BA acting as an intermediary between the LA and the CeA (Anglada-Figueroa & Quirk, 2005). In summary, during fight and flight associated responses: the LA is the ‘input centre’ where acquisition occurs, the CeA is the ‘output centre’
responsible for fight and flight behaviour, and the BA acts as the interface between the two nuclei (Anglada-Figueroa & Quirk, 2005; Giustino & Maren, 2015; Pape & Pare, 2010).

These functional differences in the subnuclei of the amygdala are affected by differences in morphology and connectivity (Sah et al., 2003). The BA, LA and the CeA receive excitatory inputs from various brain regions (Sah & Lopez de Armentia, 2003; Stefanacci & Amaral, 2002), which are activated during stress and fear (Parsons & Ressler, 2013). The BLA-complex receives excitatory inputs from various brain regions including the hippocampus (Jin & Maren, 2015), thalamus (Bauer et al., 2002; Y. Zhou, Won, Karlsson, Zhou, & Rogerson, 2009) and mPFC (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). The CeA also receives inputs from other brain regions (Stefanacci & Amaral, 2002); however a lot of the glutamatergic inputs into the CeA appear to be intra-amygdaloid connections (Sah et al., 2003) from the LA (Sah & Lopez de Armentia, 2003) and the BA (Paré, Smith, & Paré, 1995; Vidal-Gonzalez et al., 2006). Alternatively, the LA receives both excitatory and inhibitory inputs from the BA (Savander, Miettinen, LeDoux, & Pitkänen, 1997), and very limited input from the CeA (Jolkkonen & Pitkänen, 1998). During fear and stress, these excitatory pathways become disinhibited (Z. Liu et al., 2014; Pape & Pare, 2010) resulting in increased neuronal activity in the LA, BA (Bauer et al., 2002; Vidal-Gonzalez et al., 2006; Y. Zhou et al., 2009), and CeA (Maras, 2014). These excitatory inputs are summarised in Figure 3.
Figure 3. The amygdala receives excitatory inputs from the hippocampus, thalamus and mPFC during stress – these circuits underlie Pavlovian conditioning and drive activation of the HPA axis from the CeA. Excitatory intra-amygdaloid circuits are also activated during stress by corticosteroids.

Corticosteroids interact with the amygdala and its connections, affecting learning and behaviour in both rodents and humans. In rodents, stress has been shown to increase corticosterone levels in the amygdala and has been associated with changes in cognition and behaviour (Bouchez et al., 2012; Iwasaki-Sekino et al., 2008; Kolber et al., 2008). The effect of corticosteroids on the amygdala are further supported in studies that administer corticosteroids to rodents, demonstrating that corticosteroids in the LA (Monsey et al., 2014) the BA (Yang, Chao, Ro, Wo, & Lu, 2007) and the CeA (A. C. Johnson & Meerveld, 2015; Shepard, Barron, & Myers, 2000; Simms et al., 2012) can directly affect learning and behaviour. The effects of
corticosteroids in the amygdala are also evident in human studies. One fMRI study revealed the functional connectivity of the amygdala-mPFC pathway was mediated by cortisol. During acute stress, participants were separated into two groups based on their salivary cortisol levels. The researchers found that the group with an increased cortisol response had significantly stronger functional connectivity in the amygdala-mPFC pathway and the amygdala-hippocampus pathway (Quaedflieg et al., 2015). This effect was further corroborated by Henckens, van Wingen, Joels, and Fernandez (2012), who directly administered hydrocortisone (a corticosteroid agonist) to participants, before conducting an emotional Stroop test. They found that participants administered hydrocortisone had significantly less correct responses on the emotional Stroop task compared to the placebo group. Moreover, fMRI scans displayed reduced amygdala inhibition in response to aversive words, and an increased coupling between the amygdala-mPFC pathway and amygdala-insula pathway (Henckens et al., 2012). In summary, corticosteroids appear to interact with the amygdala and its pathways in both rodents and humans, affecting cognition and behaviour.

**Corticosteroid Receptors**

The effects of corticosteroids are limited to the cellular expression of GR and MR (Joels & Baram, 2009; Joels, Pasricha, & Karst, 2013). The downstream effects of these receptors are dependent upon a variety of factors (Figure 4), such as the individual’s genetic history, the context and presentation of stress, the dosage of corticosteroids, the time domain of corticosteroids, and the brain region where the receptors are located (Groeneweg et al., 2011; Joels & Baram, 2009). While the interaction between these factors is quite complex, the general view is that MR is involved in stress appraisal (Hamstra, de Kloet, van Hemert, de Rijk, & Van der
Does, 2015) and GR is involved in the body’s return to homeostasis (de Kloet, 2014). In accordance with these effects, de Kloet (1991) developed the *Receptor Balance Hypothesis* (explored in more detail in Chapter 2), which postulates that a balance between GR and MR populations regulate neuronal excitability (de Kloet, 2014) and is necessary for maintaining homeostasis and adaptive functioning (de Kloet, 2013). Imbalances in receptor density between GR and MR can have profound effects on learning and memory (Harris et al., 2013) and is a potential factor in the development of stress-related mental illnesses (de Kloet, 2014; Han, Ding, & Shi, 2014). In Chapter 2, I review the literature surrounding corticosteroid receptors in more detail; however, due to the scope of this thesis, I focus primarily on the dosage and temporal factors of GR and MR located in the amygdala.
Factors that affect (and interact with) MR & GR

1. Context
   - Type of Stressor
     - Acute
     - Chronic
     - Single Prolonged Stress
   - Presentation of stressor
     - Order
     - Strength/Saliency

2. Dosage
   - MR - high affinity for cortisol
     Occupied at low levels of circulating cortisol, basal levels of stress
   - GR - low affinity for cortisol
     Occupied at high levels of circulating cortisol, higher levels of stress

3. Temporal
   - Genomic - located within the cytoplasm and nucleus
     Slow acting, transcription factors
   - Synaptic - membrane-associated, located at the synapse
     Fast acting effects of stress

4. Region Specific
   - Prefrontal Cortex (PFC)
   - mPFC
   - Hippocampus
     - CA1, CA2, CA3
     - Dentate Gyrus
   - Amygdala
     - Basal Lateral Amygdala (BLA)
     - Central Amygdala (CeA)

MR and GR

1. Neurotransmitters
   - Glutamate
   - GABA
   - Norepinephrine (NE)
   - Dopamine (DA)
   - Serotonin (5HT)

   Function
   - Stimulate release
   - Increase release probability
   - Inhibit release
   - Decrease release probability

2. Hormones
   - Corticotrophic Releasing Hormone (CRH)
   - Adrenocorticotropic Hormone (ACTH)

Affect Neuronal Excitability

3. Brain Connectivity
   - Factors affected (mediated) by MR & GR

4. Cognition and Behaviour
   - Memory
     - Emotional
     - Spatial

Figure 4. Factors that interact with MR and GR to affect Cognition and Behaviour
A Rationale for Quantifying Corticosteroid Receptor Subpopulations

GR and MR in the brain are important for maintaining homeostasis (Dedovic et al., 2009), and disruptions to the populations of GR and MR have been implicated in a range of disorders (described earlier). The receptor balance hypothesis suggests that these disorders are influenced by an imbalance between the populations of GR and MR (de Kloet, 2014). Traditionally the receptor balance hypothesis of GR and MR refers to an imbalance between genomic populations of GR and MR (de Kloet, 2013), and the role of membrane-associated populations is unclear. Membrane-associated corticosteroid receptors (described in more detail in Chapter 2) have been located at synapses in the LA (L. R. Johnson, Farb, Morrison, McEwen, & LeDoux, 2005; Prager, Brielmaier, Bergstrom, McGuire, & Johnson, 2010) and functional effects of fast-acting, effects have been demonstrated within the BA (Sarabdjitsingh & Joels, 2014; Sarabdjitsingh, Kofink, Karst, De Kloet, & Joels, 2012). However, the relative distributions of these membrane-associated receptors in relation to genomic receptors, are yet to be explored – especially in the different subnuclei of the amygdala. Understanding the relative importance of these receptors could add a new dimension to understanding the balance hypothesis; especially within the amygdala, which has not been demonstrated to be sensitive to the balance hypothesis (Caudal, Jay, & Godsil, 2014; Han et al., 2014).

The literature to date has not directly compared the populations of both genomic and synaptic receptors of GR and MR within the brain. We aimed to address this by comparing the genomic and synaptic populations of GR and MR within the amygdala. In this thesis, we validated a protocol for immunofluorescence and piloted a novel approach of visualising and quantifying corticosteroid receptors...
at synapses. Using this approach, we were able to provide some preliminary data about these distributions for use in future experiments.

Another gap in the literature is the proportion of corticosteroid receptors located at synapses. Previous electron microscopy studies have identified both GR and MR within some excitatory synapses in the LA. However, there was limited data on the number of excitatory synapses that actually contained GR and MR (Johnson 2005; Prager et al, 2010). In this thesis, we were interested in testing the feasibility of a new method, in order to provide some preliminary data that characterised the distribution of excitatory post-synaptic terminals containing GR or MR, in the amygdala. Finally, due to the different effects of fast-acting corticosteroid receptors, between the LA, CeA and BA (Karst et al., 2010); we were interested in seeing if there were any subregional differences in the populations of GR and MR located at excitatory post-synaptic sites.

**Fluorescent Imaging and Reconstruction of Corticosteroid Receptor Subtypes**

The distribution densities of corticosteroid receptors have been characterised using a range of methods that included: autoradiography (Reul & de Kloet, 1985; Reul, van den Bosch, & De Kloet, 1987), cell fractionation (Aronsson et al., 1988; Caudal et al., 2014; Han et al., 2014) and microscopy (Ahima & Harlan, 1990; Moutsatsou, Psarra, Paraskevakou, Davaris, & Sekeris, 2001; van Steensel et al., 1996). However, most of these studies either focus on genomic corticosteroid receptors, or do not distinguish between different receptor subpopulations at all. As such, the distribution of membrane-associated receptors in the brain is still unclear. In this section I will discuss the novel method we used to investigate the localisation of membrane-associated corticosteroid receptors at synapses. The first part describes the use of multiple labelling immunofluorescence in colocalisation analysis, and the
second part describes the novel object-based analysis used in this study for colocalisation analysis.

Multiple labelling immunofluorescence can be used to visualise the distribution of receptor proteins (Daly & McGrath, 2003), and to further characterise the colocalisation of subcellular organelles (Bolte & Cordelières, 2006). Colocalisation studies in neuroscience tend to use antibodies coupled to fluorescent markers which label the desired structures (e.g. receptor protein) on a sample (Bolte & Cordelières, 2006; Zinchuk & Grossenbacher-Zinchuk, 2009). These markers can be subsequently imaged using a confocal microscope, and the association between two fluorescent markers would suggest an association between two subcellular structures (Dunn, Kamocka, & McDonald, 2011). Previous studies have used confocal immunofluorescence to characterise corticosteroid receptors. For example, van Steensel et al. (1996) used confocal immunofluorescence to determine the spatial distribution of GR and MR within neuronal nuclei in the hippocampus. van Steensel and colleagues (1996) were able to simulate the relative distributions of GR and MR in CA1 nuclei, and provide qualitative information about the clustering of GR and MR. Immunofluorescence has also been used to measure membrane-associated corticosteroid receptors. Oppong et al. (2014) measured the movement of GR to the membrane by fluorescently labelling the plasma membrane of mast cells in red, and GR in green. When the GR was colocalised with the membrane, yellow fluorescence was emitted.

For colocalisation analysis, confocal imaging provides high resolution images (Bolte & Cordelières, 2006). Confocal imaging eliminates out of focus light, making it appropriate for use with thick brain sections (Bolte & Cordelières, 2006). The low temporal resolution, however, restricts imaging to fixed sections (Bolte &
Cordelières, 2006). Compared to other imaging techniques such as electron microscopy or light microscopy, confocal imaging has an added z-resolution, making it easier to three-dimensionally reconstruct neuronal structures (Bacallao, Kiai, & Jesaitis, 1995). Moreover, the added z-resolution allows for object-based colocalisation analysis and volumetric quantification (Fogarty, Hammond, Kanjhan, Bellingham, & Noakes, 2013). Finally, the use of immunofluorescence in conjunction with confocal imaging also had the added advantage of providing descriptive information about the distribution densities of GR and MR; as opposed to cell fractionation and radiography methods (Daly & McGrath, 2003; Zinchuk & Grossenbacher-Zinchuk, 2009).

Advances in technology (Zinchuk & Grossenbacher-Zinchuk, 2009) has led to an increase in the use of object-based methods in colocalisation analysis (Bolte & Cordelières, 2006; Lagache, Sauvonnet, Danglot, & Olivo-Marin, 2015). Object-based methods allow for spatial exploration of the colocalised signals along the z-axis (Bolte & Cordelières, 2006), and is suited for puncta labelling (Fogarty et al., 2013; Lagache et al., 2015), such as that of GR and MR (van Steensel et al., 1996). Fogarty et al. (2013) developed a semi-automated object-based method, which utilised the commercial software Imaris (Bitplane) to map the distribution of synaptic inputs. By performing a three-dimensional reconstruction of fluorescently labelled structures, Fogarty et al. (2013) subsequently used object-based analysis for colocalisation. Fogarty et al. (2013) have stated that their method, which I refer to in this thesis as Fogarty’s approach, can be used to visualising various subcellular structures. In this thesis, we tested the feasibility of adapting this approach to quantify the 3D distribution of GR and MR; both within the nucleus and at excitatory-post synaptic terminals.
Object-based methods of colocalisation have multiple advantages compared with traditional methods of colocalisation (Lagache et al., 2015). Object-based methods are less sensitive to noise and pixel-shift, and are more accurate and robust than traditional colocalisation methods (Lachmanovich et al., 2003; Lagache et al., 2015). Fogarty’s approach applied colocalisation parameters to images in batch (Fogarty et al., 2013); as such it provided a more objective and consistent method of quantifying synapses than traditional counting (Lachmanovich et al., 2003). Furthermore, the accuracy of colocalised synaptic sites calculated using their approach was further corroborated by electrophysiological recordings and electron microscopy data (Fogarty et al., 2013). Another strength of this approach is the sensitivity of the software used. Signals in the middle of a section are not always visible to the human eye, these objects could be missed during manual counting. As the software is more sensitive to the signal, it is more accurate at quantifying objects (Fogarty et al., 2013). Finally, the semi-automated nature of this approach allows for increased throughput.
Thesis Objectives and Outline

Disruptions to the corticosteroid receptors have implications in a range of stress-related disorders (Millan et al., 2012). Understanding the distribution of corticosteroid receptors in the amygdala could further unravel the stress system. The overarching objective was to adapt Fogarty’s approach to visualise the distribution of GR and MR in the amygdala. More specifically I aimed to:

1. Validate the antibodies and reagents from the electron-microscope studies conducted by L. R. Johnson et al. (2005) and Prager et al. (2010), for use in multiple labelling immunofluorescence.

2. Investigate the distribution of GR and MR in nuclei and at excitatory post-synaptic terminals.

3. Quantify the balance between these receptors in stress-naïve animals in order to provide a foundation for future studies into stress.

To investigate these aims, I begin by reviewing the literature surrounding corticosteroid receptors in Chapter 2. More specifically, I review the dual system of GR and MR, and subsequently describe their distribution within the subnuclei of the amygdala. I further describe the temporal effects of corticosteroid receptors, and review the literature surrounding the two subpopulations of GR and MR within the different amygdala subnuclei. Finally, I review the role of GR and MR in the Receptor Balance Hypothesis, and how the subpopulations of GR and MR could add a new dimension to understanding the ratio of GR:MR in the amygdala.

In Chapter 3, I describe the general method used in this thesis. In this chapter I provide an overview of the variables and the procedure; I also discuss the biometric properties of the antibodies chosen and their respective labels. Chapter 3 also details the controls used, which were further tested in Chapter 4 (protocol validation).
Finally, in Chapter 3, I describe the ethical implications of animal research, and briefly discuss the applicability of using animals to investigate corticosteroid receptors.

In Chapter 4 (protocol validation), we test the feasibility of using multiple-label immunofluorescence as a method of visualising the distribution of GR and MR. In this chapter, we optimised the reagents used in this thesis, and standardised a protocol for future research. We also tested the feasibility of using triple-label immunofluorescence for visualising the distributions of GR and MR.

In chapter 5 (corticosteroid receptor densities in the amygdala), we tested the feasibility of adapting Fogarty’s approach, in order to three-dimensionally reconstruct GR and MR in the amygdala. Using the protocol developed in Chapter 4, we used confocal microscopy to image amygdala tissue sections, and subsequently reconstructed these images in 3D in Imaris (Bitplane). Following this, we used object-based methods to create colocalised objects for quantification and using the data from this technique, we performed some preliminary analyses to see if there were any differences in volumetric density.

In Chapter 6, I discuss the findings from Chapter 5. I discuss the strengths and weakness of using Fogarty’s approach, and provide suggestions for future research. Moreover, I also discuss some potential implications of our findings in relation to stress. Finally, all results are summarised in Chapter 7, and discussed in a broader context.
Chapter 2: Corticosteroid Receptors

The distribution of corticosteroid receptors are the limiting factor for the downstream signalling effects of corticosteroids (Joels & Baram, 2009), and disruptions to the populations of these receptors have been implicated in a range of stress-related pathologies (Millan et al., 2012). In this chapter, I review the current literature surrounding the corticosteroid receptors, GR and MR, in the brain. In the first section I provide a brief overview of the dual system of GR and MR. In the second section, I subsequently review the previous research surrounding the distribution of GR and MR in the amygdala. In the third section, I introduce the two subtypes of GR and MR; slow genomic receptors and fast membrane-associated receptors. In this section I also review amygdala-specific effects of these receptor subpopulations. In the fourth section of this literature review I discuss the Receptor Balance Hypothesis, where I aim to consolidate the temporal effects of corticosteroid receptors into the receptor balance hypothesis. Additionally, I provide a rationale for the current research. In the final section of this chapter, I summarise the literature and discusses the current gaps in our understanding of these receptors.

Dosage Effects of Corticosteroids on Corticosteroid Receptors

One manner in which MR and GR affect learning and memory, appears to be dependent on the dosage of corticosteroid levels in the brain (Prager & Johnson, 2009; Sandi, 2011). Corticosteroids have been demonstrated to have different binding affinities for MR and GR. Corticosteroids have a higher binding affinity for MR, and at low levels of circulating corticosterone, more MR will be bound than GR (Conway-Campbell et al., 2007; Reul et al., 1987). The opposite effect is seen in the
case of GR, which has a ten-times lower binding affinity than MR, and is only activated during higher levels of circulating corticosteroids in the brain (Conway-Campbell et al., 2007; Reul et al., 1987). Consequently, it has been suggested that MR are bound during basal levels, at homeostasis (Joels, Karst, DeRijk, & de Kloet, 2008) and GR are occupied during the circadian peaks and higher levels of stress (de Kloet, Karst, & Joels, 2008; Joels et al., 2008). However, the mechanisms of these receptors are not simply dosage dependent, they are also affected by their location, both within the neuron, and the region of the brain (Quaedfliege et al., 2015; Sarabdjitsingh & Joels, 2014; Q. Wang et al., 2014).

**Corticosteroid Receptors in the Amygdala**

Corticosteroid receptors are distributed throughout the body and the brain (Reul & de Kloet, 1985), however the density of GR and MR populations differ across locations. GR has been found to be widely expressed in the central nervous system with a higher density in cortical regions such as the hippocampus and amygdala (Reul & de Kloet, 1985; Teng et al., 2013). MR is less ubiquitous and found to be densest in limbic areas, in particular the hippocampus and the amygdala, as well as the pre-frontal cortex (Ahima, Krozowski, & Harlan, 1991; Joels et al., 2008). Both populations of corticosteroid receptors have both been found to be expressed in the amygdala (Reul & de Kloet, 1985), and are believed to be involved in mediating the cognitive and behavioural effects that arise from stress and fear (Roozendaal, 2000). It should be noted that corticosteroid receptors are not limited to neurons, but also located in glial cells (Matsusue, Horii-Hayashi, Kirita, & Nishi, 2014). Moreover, the role of GR and MR in glial cells in the amygdala may also be implicated in stress-related pathologies, such as depression (Q. Wang et al., 2014).
The distribution densities of GR and MR also vary between the subnuclei of the amygdala (introduced in Chapter 1). Autoradiography studies have shown that in naïve animals, with higher levels of GR in the amygdala compared to MR (Marlier et al., 1997). It has been found that the CeA has higher levels of GR compared to MR (Reul & de Kloet, 1985; Reul et al., 1987), however there is limited information about these receptor populations in the other subnuclei of the amygdala. Studies measuring receptor mRNA, provided more detail about receptor densities and in naïve rodents, significantly higher levels of GR mRNA in the amygdala, compared to MR mRNA (Han et al., 2014; Patel et al., 2000). The highest densities of GR mRNA were found within the CeA, followed by the LA and BA (Aronsson et al., 1988; Morimoto, Morita, Ozawa, Yokoyama, & Kawata, 1996). For MR, a similar trend followed, with highest mRNA densities in the CeA and then the BA (Herman, 1993). It should be noted that mRNA quantification is a limited indicator of final receptor protein densities (Herman, 1993). For proteins like GR and MR that are affected by post-transcriptional factors (Groeneweg, 2014; Ratman et al., 2013), mRNA expression may not be the most accurate indicator of receptor density (Helmstetter, Parsons, & Gafford, 2008; Rizzo et al., 2014).

Studies examining GR and MR immunoreactivity tend to provide a higher resolution distribution of corticosteroid receptors compared to mRNA in situ hybridisation and autoradiography (Morimoto et al., 1996). While some studies have shown that these methods have similar trends in receptor density, the density of immunolabelled receptors are not completely concordant with mRNA results (Marlier et al., 1997; Morimoto et al., 1996). Between GR and MR populations, Han et al. (2014) found that GR mRNA levels in the amygdala were significantly higher than MR mRNA, and although GR immunoreactive neurons were higher than MR,
the difference was not significant. Morimoto et al. (1996) compared GR populations in naïve rodents and found a similar trend between GR mRNA levels and GR immunoreactive neurons. The highest densities of GR were in the CeA and moderate densities were found in the LA and BA (Morimoto et al., 1996). Studies of MR density have found more variable results (Ahima et al., 1991). Autoradiography studies found MR was largely restricted to limbic areas (Reul & de Kloet, 1985), however, mRNA and immunoreactivity studies found MR distributed throughout the brain (Ahima et al., 1991). Within the amygdala subnuclei, MR immunoreactivity was had the highest density in the CeA, followed by the LA, with the lowest densities in the BA (Ahima et al., 1991). Although this followed a similar trend to MR mRNA levels, mRNA numbers appeared to overestimate MR populations compared to immunoreactive studies (Herman, 1993). Overall, MR and GR densities in the subregions of the amygdala are not characterised as well as the subregions of the hippocampus (Cohen, Kozlovsky, Matar, Zohar, & Kaplan, 2014; Reul et al., 1987; Sampedro-Piquero, Begega, & Arias, 2014; Zhe, Fang, & Yuxiu, 2008). Moreover, studies that employ immunolabelling, to provide higher cellular resolution of receptor density, are even more scarce (Ahima et al., 1991).

**Temporal Effects of Corticosteroid Receptors**

The location of GR and MR within a neuron can affect the temporal effects of these corticosteroid receptors. Classically, GR and MR were found to exist within the cytoplasm as genomic GR (gGR) and genomic MR (gMR) (Reul & de Kloet, 1985); operating in a “slow” manner (hours to days), affecting gene transcription (Datson, van der Perk, de Kloet, & Vreugdenhil, 2001; Groeneweg et al., 2014; van Steensel et al., 1996). However, genomic receptors could not account for the “fast” effects (seconds to minutes) of corticosteroids; thus it was proposed that GR and MR
must operate through non-genomic mechanisms (de Kloet et al., 2008). Fast-acting, non-genomic corticosteroid receptors were further differentiated from genomic receptors via their intracellular localisation, and were proposed to reside at the plasma-membrane of neurons as membrane-associated GR (mGR) and MR (mMR) (Joels et al., 2013). Thus, GR and MR have two further subtypes: slow-acting, genomic receptors; and fast-acting, membrane-associated receptors (Groeneweg et al., 2012; Prager & Johnson, 2009); the differential distributions are depicted in Figure 5.

Figure 5. Distribution of Genomic and Synaptic GR and MR within a neuron.

Genomic GR and MR are located within the cytoplasm, and translocate to the nucleus when bound. Synaptic GR and MR are located near or within the membrane at synapses; when activated, these receptors can affect neurotransmission.

When inactive, unbound genomic corticosteroid receptors typically reside within the cytoplasm of a neuron (Ahima & Harlan, 1990; Nishi, Ogawa, Ito, Matsuda, & Kawata, 2001). When corticosteroids enter a neuron, they bind with gGR or gMR in the cytoplasm, forming cortisol-receptor complexes (Groeneweg et al., 2014; Nishi et al., 2001). Now in their active state, these ligand-receptor complexes homodimerise as: GR-GR or MR-MR complexes (Herman & Spencer,
1998; van Steensel et al., 1996); or heterodimerise as: GR-MR complexes (W. Liu, Wang, Sauter, & Pearce, 1995). These ligand-receptor complexes subsequently translocate to the nucleus (Groeneweg et al., 2014) where they bind to glucocorticoid binding sites (GBS). Within the nucleus gGR or gMR can directly or indirectly interact with DNA to, respectively, activate or suppress gene transcription (Nishi et al., 2001; Polman et al., 2013). While these two receptors have some overlap in the DNA binding domain, they can differentially regulate these genes and affect function (Le Menuet & Lombès, 2014).

These genomic receptors can have a range of biological and behavioural effects (Hamstra et al., 2015; Ratman et al., 2013). gGR has been shown to affect Arc transcription in the hippocampus, affecting dendritic plasticity and long-term fear memories (Chen, Bambah-Mukku, Pollonini, & Alberini, 2012). Chronic administration of corticosterone has been demonstrated to mediate the enhancement of anxious behaviour (Santos et al., 2014), and administration of GR antagonists have been shown to later suppress fear conditioning (M. Zhou et al., 2010). The slow effects of MR have been shown to be involved in acquisition of fear memories (M. Zhou et al., 2010). However, it has also been shown that increased expression of gMR has been implicated in improved emotional resilience (Hamstra et al., 2015; Klok et al., 2011).

The effects of genomic receptors are usually visible hours to days after the initial onset of a stressor (Groeneweg et al., 2011), and do not account for immediate (second to minutes) biological changes to stress (Popoli, Yan, McEwen, & Sanacora, 2011; Sandi, 2011). For example, rats that underwent a 15 minute forced-swim stress test showed increases in corticosterone levels in the BLA-complex (Bouchez et al., 2012), and administration of spironolactone (MR antagonist) rapidly reduced the
expression of conditioned fear (de Oliveira & Reimer, 2014). In humans, Henckens et al. (2012) aimed to determine the temporal effects of cortisol on the amygdala in the presence of emotional stimuli. In a randomised, placebo-controlled trial, experimental participants were administered hydrocortisone (a cortisol agonist) and given an emotional interference task. The rapid effects (60 minutes) of hydrocortisone, increased participants’ sensitivity to emotional interference compared to the slower effects (270 minutes), which did not differ from the control group. An fMRI was used to measure neuronal activity and found that for the rapid effects of corticosteroids, the amygdala was less inhibited towards emotional interference. van Ast, Cornelisse, Meeter, Joels, and Kindt (2013) also found differences in the temporal effects of cortisol. Administration of hydrocortisone found that the rapid effects (30 minutes) of cortisol impaired the contextualisation of emotional memory; alternatively, the slow effects (270 minutes) enhanced it.

Similarly, Quaedflieg et al. (2015) found that, during acute stress, participants with increased cortisol levels had increased functional connectivity between the amygdala and the mPFC, and between the amygdala and the hippocampus. Interestingly, these biological differences did not translate behaviourally as Quaedflieg et al. (2015) found that hydrocortisone did not appear to have an effect on perceived stress.

These “fast” cognitive and behavioural effects were suggested to be initiated by corticosteroid receptors located at the membrane, as opposed to receptors within the nucleus (Joels et al., 2013). These membrane-associated receptors were demonstrated to exist in the neuron through electrophysiological recordings (Karst et al., 2010; Karst et al., 2005; Sarabdjitsingh & Joels, 2014), synaptosome fractions (Moutsatsou et al., 2001; C. Wang & Wang, 2009), and electron microscopy (L. R. Johnson et al., 2005; Komatsuzaki et al., 2012; Prager et al., 2010). Moreover these
membrane-associated receptors have been found at synapses (Prager & Johnson, 2009), at both pre- and post-synaptic sites on dendrites and dendritic spines (Komatsuzaki et al., 2012). These membrane-associated receptors are believed to be coupled with signalling proteins which can affect neurotransmission (Groeneweg et al., 2012; Popoli et al., 2011), possibly influencing glutamate release at pre-synaptic sites, and affecting glutamate receptor mobility at post-synaptic sites (Joels et al., 2013; Sandi, 2011) (Figure 5). In addition, membrane-associated receptors may even have effects further downstream, affecting protein synthesis (Roozendaal et al., 2002).

While the literature is still emerging, the membrane-associated effects of corticosteroid receptors have been better characterised in the hippocampus than in the amygdala (Groeneweg et al., 2012; Popoli et al., 2011; Prager & Johnson, 2009). The literature available, however, has suggested that corticosteroid receptors in the amygdala function in a very different manner to the hippocampus (Groeneweg et al., 2011). Within the hippocampus, MR and GR neuronal excitability follows an inverted-U trend. Moderate doses of corticosteroids were shown to act on MR, inducing long-term potentiation (LTP) and increasing neuronal activity (Chatterjee & Sikdar, 2014; Karst et al., 2005). Alternatively, high doses of corticosteroids acted on both MR and GR, subsequently depressing post-synaptic excitability in neurons (Chatterjee & Sikdar, 2014; Karst et al., 2005). The amygdala has displayed an opposite trend. In the BLA-complex, higher levels of corticosteroids increased excitability at the post-synaptic terminal (Karst et al., 2010; Sarabdjitsingh & Joels, 2014). Moreover, GR-MR-mediated excitability was slower in onset, and less transient than in the hippocampus (Karst et al., 2010; Sarabdjitsingh & Joels, 2014). It was suggested that mMR has a lower affinity towards corticosteroids than gMR,
and is only activated during stress at higher levels of corticosteroids (Karst et al., 2005). When activated, mMR mediates the initial increase of LTP, and this increase is maintained by gGR (Karst et al., 2010). When corticosteroid levels were increased further, through a second pulse of corticosterone, mGRs were activated, which depressed neuronal excitability (Karst et al., 2010). It should also be noted, that corticosteroid receptor-mediated excitability was also dependent on the history of stress. Animals that were stressed prior to corticosterone administration had elevated corticosteroid levels similar to exogenous application of corticosterone. Thus, further administration of corticosterone resulted in depressed neuronal activity (Karst et al., 2010; Sarabdjitsingh & Joels, 2014), suggesting that repeated exposure to stressors can depress neuronal excitability via interactions with GR and MR (Figure 6).

![Figure 6](image)

**Figure 6.** Corticosteroid receptors in the BLA-complex mediate neuronal excitability differently to the hippocampus. Corticosteroids increase neuronal excitation in the BLA-complex, through mMR. This excitability is maintained through gGR. Further application of corticosterone depressed neuronal excitability through mGR. Adapted from research by Karst et al. (2010), Groeneweg et al. (2011), and Sarabdjitsingh and Jöels (2014).
Interestingly, out of the LA, BA and CeA, the highest densities of both GR and MR have been found in the CeA (Ahima & Harlan, 1990; Ahima et al., 1991). However, this does not appear to be reflected functionally, as corticosterone application to CeA neurons did not seem to affect excitation at post-synaptic terminals (Karst et al., 2010). This may be due to higher density of inhibitory connections in the CeA, which mediate the excitability in CeA neurons (Cassell et al., 1999; Jolkkonen & Pitkänen, 1998). This is further reflected in the morphology of neurons in rats exposed to single prolonged stress. Cui, Sakamoto, Higashi, and Kawata (2008) found that, after stress, there was a significant increase in dendritic spines in the BLA but not in the CeA, suggesting the CeA may not be as susceptible to stress-related changes as the BLA. Moreover, there was an increase in GR and MR labelling in excitatory neurons in the BLA, but no difference in the CeA between the stress and control groups (Cui et al., 2008). The distribution of membrane associated corticosteroid receptors at CeA synapses is currently unclear. Previous research on distributions of GR and MR have been limited to mRNA or genomic receptors; as such further investigation is warranted.

**Receptor Balance Hypothesis**

The *Receptor Balance Hypothesis* (introduced in Chapter 1) states that a balance between the dual receptor system of GR and MR is necessary for an adaptive stress response (de Kloet, 1991). GR and MR are believed to operate in a complementary fashion where MR maintains homeostasis during basal levels of stress, and GR returns the body to homeostasis after stress (de Kloet, 2014). Furthermore, the receptor balance hypothesis posits that an imbalance between the populations of GR and MR results in a dysregulation of the HPA-axis and a vulnerability to mental illness (de Kloet, 2014; Dedovic et al., 2009).
Support for the receptor balance hypothesis has been found within the regions of the mPFC and hippocampus (Caudal et al., 2014; Zhe et al., 2008), where stress-induced increases in corticosterone can result in decreases in MR or increases in GR (de Kloet, 2014), creating imbalances in between GR and MR. It should be noted that the balance between GR and MR populations are not zero-sum. That is, an increase in GR does not necessarily result in a decrease in MR, or vice versa (Harris et al., 2013). Although the signalling pathways between GR and MR appear to interact, the expression of GR and MR populations appear to be quite independent of each other (Harris et al., 2013). Thus the receptor balance hypothesis is not about the sole expression of GR or MR, rather it is the ratio of GR:MR expression (Cotella, Durando, & Suarez, 2014; de Kloet, 2014). Within the hippocampus, the ratio of gGR:gMR binding can be altered by varying the concentration of corticosteroids (Polman et al., 2013). As such, de Kloet (2014) suggested that imbalances between the genomic populations of corticosteroid receptors in the hippocampus are responsible for the maladaptive effects of stress. Within the amygdala, however, evidence supporting the receptor balance hypothesis is mixed.

Studies that support the receptor balance hypothesis in the amygdala suggest that over- or under-expression in GR and/or MR can negatively affect individuals. In rodents, increased populations of MR, but not increased populations of GR, appear to be protective against stress. Mitra, Ferguson, and Sapolsky (2009) found that, at basal levels of stress, over-expression of MR in the BLA-complex resulted in increased exploration behaviours in rats. In stressed rats, an over-expression of MR in the BLA resulted in reduced plasma corticosterone levels and reduced anxious behaviours (Mitra et al., 2009). Deletion of GR in the CeA in mice has resulted in significantly lower neuronal activity in the CeA at basal levels, and in both the CeA
and the BLA-complex during stress (Kolber et al., 2008). Moreover, GR-knockout mice were observed to have reduced freezing, suggesting that lower levels of activated GRs may weaken fear memory consolidation, and in turn, anxious behaviours (Kolber et al., 2008). In humans, Q. Wang et al. (2014) found that humans with depression had a higher density of GR in both neurons and astrocytes in the amygdala compared to bipolar patients and healthy controls. These studies, however, did not compare the ratio of GR:MR directly. Thus, while the population of these stress receptors may differ from healthy controls, it is arguable whether the ratio between GR:MR is significantly different.

Studies which have directly compared ratios of GR:MR have found that the amygdala does not seem to be sensitive to the receptor balance hypothesis. After single prolonged stress, Han et al. (2014), found that, while expressions of GR and MR were significantly reduced after stress, the GR:MR ratio did not change. Caudal et al. (2014) found that stress increased gMR expression in the nucleus, albeit not significantly. In accordance with previous literature, they also found that the GR:MR ratio of genomic receptors was significantly different after stress in the hippocampus and mPFC, but not within the amygdala (Caudal et al., 2014). In support of this, other research has also demonstrated that GR expression in both the lateral amygdala and the central amygdala do not vary, even after administration of corticosterone, although this may be due to ceiling effects as control animals also had high levels of GR (Santos et al., 2014).

These studies suggest, that while the amygdala may be sensitive to differences in GR expression or MR expression it is possible that the ratio of GR:MR does not change. Alternatively, differentiating between genomic and membrane-associated receptors may provide a new dimension to understanding the receptor
balance hypothesis in the amygdala. As discussed earlier, genomic receptors interact with membrane-associated receptors with different functional effects in the amygdala compared to the hippocampus (Joels et al., 2013; Karst et al., 2010; Pasricha, 2013; Sarabdjitsingh et al., 2012). While the hippocampus may be sensitive to genomic GR:MR imbalances, the amygdala may be sensitive to membrane-associated GR:MR balances. Moreover, de Kloet (2014) has stated that the emotional processing of stressful stimuli occurs within seconds to minutes, potentially acting on mMR and mGR. Consequently, the expression of mGR and mMR in the amygdala may add a new dimension to the receptor balance hypothesis.

**Summary and Implications**

In summary, stress can have a range of cognitive and behavioural effects (Santos et al., 2014; Vogel et al., 2014; Wingenfeld & Wolf, 2015; Zalachoras, 2014, which is believed to be mediated by two populations of corticosteroid receptors in the brain: GR and MR (Joels & Baram, 2009). These corticosteroid receptors can have fast, membrane-associated effects or slow genomic effects within the nucleus (Groeneweg et al., 2012) but the relative distribution between these receptor subpopulations is still yet to be understood; especially within the subnuclei of the amygdala. The amygdala has not been demonstrated to be sensitive to the receptor balance hypothesis, which typically focuses on genomic receptors (de Kloet, 2014). However, understanding the relative importance of these receptor subpopulations within the amygdala may add a new dimension to the receptor balance hypothesis, and help us to better understand the effects of stress.

As of yet, there is no known differences in gene encoding or receptor structure between genomic and membrane-associated corticosteroid receptors (Prager & Johnson, 2009). As such, the most optimal way of investigating these receptor
subpopulations was to visualise them. Fogarty and colleagues (2013) developed a robust method of visualising the distribution of synaptic markers through 3D reconstruction. The aim of this thesis was to test the feasibility and applicability of this approach to corticosteroid receptor, in order to characterise the distribution densities of GR and MR within the nucleus, and at post-synaptic sites.

Firstly, we aimed to test the feasibility of using multiple label immunofluorescence to identify receptors located at excitatory post-synaptic sites. Previous studies have used immunofluorescence to characterise the distribution of steroid receptors at the membrane. Schindelin et al. (2012) found, using electron microscopy, that not all inhibitory boutons contained estrogen receptors. In order to investigate the amount of boutons that actually contained estrogen receptors, Schindelin and colleagues (2012) used immunofluorescence to differentially label GABA-boutons and estrogen receptors in rat hippocampal tissue. Colocalisation of these markers allowed them to quantify the amount of inhibitory boutons that contained estrogen receptors. In guinea pig corti tissue, Kil and Kalinec (2013) used confocal immunofluorescence to characterise the populations of genomic and membrane-associated GR and MR. The researchers labelled corticosteroid receptors with a green fluorescent marker, and differentiated between the membrane and nuclei by labelling the actin in the plasma membrane with a red fluorescent marker, and the nuclei with a blue fluorescent marker. Using this method, the researchers were able to visualise colocalisation between these markers, and subsequently quantify the different populations of GR and MR. Based on these previous, studies, we used triple-labelling immunofluorescence as a method of differentiating between genomic and membrane-associated populations of GR and MR.
Secondly, we aimed to test the feasibility of adapting Fogarty’s approach to visualise GR and MR at different neuronal locations. Electron microscopy studies have shown that membrane-associated receptors are located in the post synaptic density (PSD) at asymmetrical (excitatory) synaptic sites (L. R. Johnson et al., 2005; Prager et al., 2010) in the LA. Interestingly, GR and MR were not found in all synapses (L. R. Johnson et al., 2005; Prager et al., 2010). Using fluorescent markers, we labelled corticosteroid receptors and excitatory post-synaptic sites to determine what population of excitatory post-synaptic sites contained GR or MR. Karst and colleagues (2010) have suggested that genomic and membrane-associated receptors may interact in a “yin-yang” fashion in the amygdala. In order to better characterise genomic and membrane-associated populations, we also labelled nuclei within tissue sections to characterise the populations of gGR and gMR.

Finally, we aimed to not only visualise the distribution, but to see if Fogarty’s approach could be used to potentially quantify these subpopulations of GR and MR. Most of the literature described uses traditional pixel-based methods of colocalisation, which is more prone to error than object-based methods of colocalisation (Lagache et al., 2015); and GR and MR data obtained from object-based colocalisation is still lacking. Moreover, there appears to be functional differences of membrane-associated GR and MR in the amygdala (Karst et al., 2010; Sarabdjitsingh & Joels, 2014; Sarabdjitsingh et al., 2012); however, relatively little is known about the distribution density in the different amygdala subnuclei. As such, we were interested in testing the feasibility of using Fogarty’s approach to perform some preliminary analysis, on the volumetric densities of GR and MR in the amygdala, to further characterise these different subpopulations in stress-naive animals.
Chapter 3: General Method

In this thesis, I describe two studies we performed to characterise the distribution of GR and MR: the first study demonstrated the feasibility of using multiple labelling immunofluorescence to visualise the distribution of GR and MR; the second study adapted Fogarty’s approach to reconstruct the GR and MR for further analysis. Both studies involved similar methods; thus in order to minimise repetition, this chapter describes the general materials and procedures shared by both studies. Details specific to each study are provided in their respective chapters.

In this chapter I begin by describing the animals used in the study. I subsequently provide an overview of the antibodies and reagents used throughout the thesis, and review the specificity and sensitivity of the antibodies and their corresponding fluorescent labels. In this chapter, I also describe the procedures involved: tissue preparation; corticosterone assay; immunohistochemistry; confocal imaging; and finally, image processing and analysis. This chapter also includes the controls employed to ensure specificity of labelling, and provides operationalised definitions of the variables involved. In the last section of this chapter, I discuss the ethical implications and application of animal research.

Subjects

The research was conducted on eight stress-naïve, male Sprague-Dawley rats sourced from University of Queensland Biological Resources (Queensland, Australia). Rats were used as they provide a basic model for mammalian studies in neuroscience (Fogarty et al., 2013; Lupien & McEwen, 1997) and have displayed similarities to humans with respect to stress and memory (Lupien & McEwen, 1997;
Rats were 20 weeks old, and weighed between 240g to 350g. Due to sample attrition, three rats were used for protocol validation, and three rats were used to test the feasibility of three-dimensionally reconstructing corticosteroid receptors in the amygdala. The sample size was in accordance with previous literature testing the feasibility of imaging techniques (Fogarty et al., 2013; Sarabdjitsingh, Meijer, & de Kloet, 2010; van Steensel et al., 1996)

Rats were housed in a temperature-controlled room (24°C) maintained on a 12hr/12hr light/dark cycle (6:30am-6:30pm). There were two rats per cage and food and water were available ad libitum. The animals were housed for seven days after arrival and handled for around five minutes every two to three days in order to habituate them to human handling (Deutsch-Feldman, Picetti, Seip-Cammack, Zhou, & Kreek, 2015). All procedures were conducted in compliance with National Health and Medical Research Council (2013) as mandatory practice under the Queensland Animal Care and Protection Act (2016); all efforts were made to minimise animal suffering.

**Antibodies**

The density distributions of corticosteroid receptors have been shown to differ based on the antibody used (Sarabdjitsingh et al., 2010). Moreover, some antibodies are better suited to detecting GR or MR in certain types of samples, e.g. tissue culture vs. fixed tissue compared to other (Sarabdjitsingh et al., 2010). The reporting of antibodies used in research have been criticised for the lack of clarity (Bordeaux et al., 2010; Helsby, Fenn, & Chalmers, 2013). As such, we described the biometric properties of the antibodies used, and reviewed their applicability to the current research in order to provide a standardised protocol for future studies.
Primary antibodies were raised in two different host species (Staines, Meister, Melander, Nagy, & Hökfelt, 1988); BuGR2 and rMR-1D5 antibodies were raised in mouse, and the anti-PSD-95 antibody was raised in rabbit. Both secondary antibodies were raised in a different species to either primary antibody (Staines et al., 1988). The host species of the primary and secondary antibodies, as well as their antigens are summarised in Table 1. Further details about the antibodies used (e.g. lot number) are available in Appendix A.

Table 1

Summary of the primary antibodies used, as well as their corresponding secondary antibodies

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host Species</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>BuGR2</td>
<td>Mouse</td>
<td>Rat GR</td>
</tr>
<tr>
<td>rMR-1D5</td>
<td>Mouse</td>
<td>Rat MR</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Rabbit</td>
<td>Rat PSD-95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Corresponding secondary antibody</th>
<th>Host Species</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 488</td>
<td>Goat</td>
<td>Mouse</td>
</tr>
<tr>
<td>Alex Fluor 594</td>
<td>Goat</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

Primary Antibodies

Mouse, anti-GR, BuGR2 (Thermofisher Scientific, Australia; catalogue number #MA1-510). The BuGR2 is a primary antibody, against glucocorticoid receptor (Ahima & Harlan, 1990). The BuGR2 antibody has been used to identify GR within the central nervous system of rodents(Azic, Djordjevic, Demonacos, Krstic-Demonacos, & Radojcic, 2009; Virgonlini, Chen, Weston, Bauter, & A.,
2005) and has shown consistent labelling in cell cultures and brain tissue (Sarabdjitsingh et al., 2010). BuGR2 is a monoclonal antibody that has demonstrated high specificity towards GR; the epitope targets amino acids 407-423, close to the DNA binding domain (Ahima & Harlan, 1990; Howard, Holley, Yamamoto, & Distelhorst, 1990), but still discrete from the MR binding domain (Ahima & Harlan, 1990). Moreover, the BuGR2 antibody identifies both active and non-active sites (L. R. Johnson et al., 2005) and has identified GR in a number of locations including the nucleus and cytoplasm, at the synapse and within the neuropil (Ahima & Harlan, 1990; L. R. Johnson et al., 2005). The BuGR2 antibody has been used in fluorescence imaging (L. R. Johnson et al., 2005; Sarabdjitsingh et al., 2010), where increased immunoreactivity suggests increased receptor density (Ahima & Harlan, 1990; L. R. Johnson et al., 2005). In our current research, the BuGR2 antibody was used to identify GR populations within neurons.

Mouse, anti-MR, rMR-1D5 (Developmental Studies Hybridoma Bank, IA, USA; catalogue #rMR1-18 1D5). rMR-1D5 is a monoclonal, primary antibody, against mineralocorticoid receptor protein in rat, human and mouse (Gomez-Sanchez et al., 2006). The rMR-1D5 has been demonstrated to be an accurate identifier of MR within rat brain tissue (Gomez-Sanchez et al., 2006) and has been used to label MR within the rat hippocampus (Gomez-Sanchez et al., 2006; Minuth, Denk, Hu, Castrop, & Gomez-Sanchez, 2007) and amygdala (Prager et al., 2010). The epitope of rMR-1D5 targets amino acids 1-18 of the antibody domain (Gomez-Sanchez et al., 2006; Prager et al., 2010), identifying both active and inactive MR (Prager et al., 2010). Aldosterone is the endogenous ligand for MR (Funder, 2005), however rMR-1D5 has been demonstrated to label corticosterone-specific MR, as opposed to aldosterone-MR in rats (Prager et al., 2010). rMR-1D5 identifies gMR, mMR, and
MR in the neuropil (Prager et al., 2010). Increased immunoreactive labelling indicates increased concentration of MR protein (Minuth et al., 2007). There has been limited research showing the use of rMR-1D5 in fluorescence microscopy. Thus, we standardised a protocol for the use of rMR-1D5 in immunofluorescence in Chapter 4 (protocol validation).

**Rabbit, anti-PSD-95 (Life Technologies, VIC, Australia; catalogue #51-6900).** Most excitatory synapses have a pronounced post-synaptic density (Allison, Chervin, Gelfand, & Craig, 2000), and the PSD-95 protein is a prominent scaffolding protein specific to the post-synaptic density membrane (Hunt, Schenker, & Kennedy, 1999; Niethammer, Kim, & Sheng, 1996). Consequently, an anti-PSD-95 antibody was used as a membrane marker of excitatory post-synaptic terminals. The anti-PSD-95 antibody used in this study was a polyclonal antibody derived from the C-terminal region of PSD-95 in humans, and has confirmed reactivity in rat tissue (Invitrogen, 2015). This antibody was selected based on its host species, immunoreactivity and its application for fluorescence imaging (Invitrogen, 2015). The anti-PSD-95 primary antibody was raised in rabbit, thus limiting cross-reactivity between primary antibodies (Staines et al., 1988); moreover it has been used in the fluorescence imaging of neurons in rat tissue (Soria Van Hoeve & Borst, 2010). However, a Western blot has shown that this antibody has some cross-reactivity for an unknown protein at ~50kDa within rat brain homogenates (Invitrogen, 2015).

**Fluorescent Labels**

In order to visualise receptor localisation, fluorescent labels were used to mark the subcellular structures of interest (Daly & McGrath, 2003). Primary antibodies were labelled with species-specific IgG (secondary antibody) coupled to a fluorochrome and nuclei were labelled with a fluorescent stain. The sensitivity and
specificity of the reagents used described below. The excitation and emission spectra for the fluorescent labels are shown in Figure 7.

Figure 7. Excitation and Emission Spectrum for DAPI, Alexa Fluor 488 and Alexa Fluor 594. Adapted from Life Technologies (2015a).

4',6-diamidino-2-phenylindole (DAPI; Life Technologies, VIC, Australia). DAPI is a blue fluorescent stain that binds to sites in the DNA (Kapuściński & Skoczylas, 1978; Life Technologies, 2015b) and has been used to identify the nuclei in the neurons within the amygdala (Y. Zhou et al., 2009). DAPI had an excitation maximum of 358nm, and an emission maximum of 461nm (Invitrogen, 2006), displayed in Figure 7. For the colocalisation of genomic receptors, DAPI was used to label nuclei within neurons.

Goat Anti-mouse IgG, Alexa Fluor 488 (Abcam, VIC, Australia). Both BuGR2 and rMR-1D5 have demonstrated high specificity to goat anti-mouse IgG coupled to an Alexa Fluor 488 fluorochrome (L. R. Johnson et al., 2005; Minuth et al., 2007). Alexa Fluor 488 had an excitation maximum of 495nm and an emission maximum of 519nm (Abcam, 2015a)(displayed in Figure 7) labelling receptors in green. The laser line of Alexa Fluor 488 was narrow, and relatively distinct from the other two fluorochromes used. The bleed-through between the emission spectra of
Alexa Fluor 488 and DAPI (Figure 7) was controlled for by using sequential scanning (North, 2006).

**Goat, Anti-rabbit IgG, Alexa Fluor 594 (Abcam, VIC, Australia).** Goat, anti-rabbit IgG coupled to Alexa Fluor 594, was used to label the anti-PSD-95 antibody. The excitation maximum of Alexa Fluor 594 was 590nm and its emission maximum was 617nm (Abcam, 2015b), displayed in Figure 7. The emission spectrum of Alexa Fluor 594 fluorochromes was quite separate from Alexa Fluor 488, which reduced the chance of cross-talk between the two fluorochromes (North, 2006).

**Procedure**

**Tissue Preparation**

Animals were anaesthetised with ketamine/xylazine (1mg/kg/0.1mg/kg ip). Animals were subsequently transcardially perfused (Peristaltic Pump Model 3200, Welch-IImvac) with 200mL of physiological saline for 1 minute, and then perfused with 250mL of 4% paraformaldehyde (PFA; w/v) and 0.1M phosphate buffer (PB; v/v) in order to fix the brain tissue. Brain tissue was removed from the animal and post fixed in 4% PFA in 0.1M PB for 4 hours and then washed in 0.1M phosphate buffer solution (PBS) three times at 10 minutes each. Brains were placed in a 1mm rat brain matrix and sectioned on the coronal plane using the anterior and posterior of the mammillary bodies as a guide. The tissue was attached to the specimen plate with cyanoacrylate adhesive and divided into two hemispheres. Tissue was sectioned at 40µm using a vibratome and collected in PBS to be processed via immunochemistry.
**Corticosterone Assay.** In order to ensure that animals were not stressed, the plasma concentration of corticosterone was determined for each rat using a competitive ELISA (Liston et al., 2013). After rats were anaesthetised, blood samples were collected via cardiac puncture and incubated on ice. Blood samples were collected at around ~12pm, at neither the peak or trough of the circadian cycle (Liston et al., 2013). Blood samples were centrifuged for 20 minutes at 3500rpm, 4°C, to separate the plasma fraction, which was aliquoted and stored at -80°C until assayed.

The plasma concentration of corticosterone was measured using a commercial ELISA kit (Enzo Life Sciences, Catalogue #ADI-900-097). Before assay analysis, all plasma samples and kit reagents were brought to room temperature. Samples were then diluted: first with steroid displacement reagent at a dilution of 1:2 (incubated for 10 minutes); and subsequently with assay buffer (final dilution 1:40). Samples were loaded, in duplicate, into donkey, anti-sheep coated wells containing equal volumes of alkaline-phosphate conjugated corticosterone and sheep anti-corticosterone polyclonal antibody. Samples were incubated in wells for 2 hours, with agitation, at room temperature and were subsequently washed three times with wash buffer. Wells were incubated with P-nitrophenyl phosphate (developer buffer) for 1 hour at room temperature and the reaction was stopped with trisodium phosphate. The well-plate was measured using PHERAstar FS spectrophotometer (BMG Labtech, Germany) at an absorbance wavelength of 405nm.

**Immunohistochemistry**

Free floating, hemispheric, coronal sections were blocked for 60 minutes with the blocking solution: 0.1M PBS, 1% Bovine Serum Albumin (BSA; w/v), 4%
Normal Goat Serum (NGS; v/v), 0.3% Triton-X 100 (v/v), 0.05% Tween-20 (v/v) to prevent non-specific binding (Burry, 2011). A simultaneous double-staining protocol was used: the primary antibody for receptor type (BuGR2 or rMR-1D5) was applied to tissue slices at concentrations of 1:200, in conjunction with primary antibody PSD-95 (1:500) and incubated for 48 hours at 4°C. Tissue was washed two to three times with the diluent buffer (0.1M PBS/1% BSA/4% NGS/0.3% Triton-X 100/0.05% Tween-20), prior to the application of secondary antibodies. Both secondary antibodies were applied simultaneously at a concentration of 1:400, and incubated for 3 hours before being washed two to three times in diluent buffer. DAPI was applied to the tissue at a concentration of 1:1000 for 10 to 15 minutes. The tissue was washed two times in diluent buffer, and then three times with PBS prior to mounting. Tissue was mounted onto slides with Mowiol mounting medium and covered with No. 1.5 coverslips (Starfrost, Germany). After the mounting medium had dried, slides were sealed using clear, non-fluorescent nail polish and stored at 4°C in the dark. The optimisation of the protocol is described in more detail in Chapter 4 (protocol validation).

**Confocal Imaging**

Slides were imaged using an Olympus FV1200 Confocal Microscope equipped with 405nm, 473nm and 559nm solid state lasers; acquisition parameters are detailed in Appendix B. Mosaic images were taken at 20× (0.86 NA) oil objective to accurately determine the presentation of the LA, BA and CeA regions for each section; two images were selected per region for quantification. Images for quantification were taken at a resolution of 1024×1024, with a magnification of 60× (1.35 NA) oil objective and 2.5× sensor zoom, and a z-step of 0.3µm. To avoid cross-talk (North, 2006), scanning was performed sequentially (473 and 405+559)
with a dwell time of 2µs/px. Nyquist sampling theorem was used to ensure adequate sampling (North, 2006), and the sampling parameters (Appendix C) were within 1.7× the Nyquist calculations (Bolte & Cordelières, 2006).

Image Processing

Deconvolution techniques (here we used deconvolution to refer to both deconvolution and background correction algorithms) have been used to improve the Signal to Noise Ratio (SNR; i.e. the ratio between signal intensity to background intensity, where a higher ratio is preferred; Bolte & Cordelières, 2006), and the reliability of colocalisation analysis (Bolte & Cordelières, 2006; Zinchuk & Grossenbacher-Zinchuk, 2009). Huygens Professional software (Scientific Volume Imaging) was used to reduce the amount of light scattering, and increase the SNR in confocal images (Bolte & Cordelières, 2006; Zinchuk & Grossenbacher-Zinchuk, 2009). The deconvolution parameters used, and the resulting images are described in more detail in Chapter 5. Subsequent to deconvolution, the images were saved in the “Imaris Classic” file format for further processing in Imaris (Version 8.2, Bitplane).

In order to perform the colocalisation analysis, subcellular structures were mapped and reconstructed in Imaris (Version 8.2, Bitplane) based on the methods by Fogarty et al. (2013). As stated in Chapter 1, Fogarty et al. (2013) developed a robust method of reconstructing labelled synapses, and this approach can be used for to accurately perform colocalisation analysis. We adapted this approach to measure colocalisation of corticosteroid receptors in different sub-cellular structures. That is, GR or MR within nuclei or GR or MR colocalised with PSD-95. The precise parameters are described in more detail within the results of Chapter 5 (corticosteroid receptor densities in the amygdala).
Design

Controls

**Labelling Controls.** We firstly controlled for *autofluorescence*. Brain tissue has endogenous fluorescence which can impact the quality of the fluorescent signal, and particulate autofluorescence can be incorrectly mistaken for labelling (Burry, 2011). Fixation techniques also present a potential threat to autofluorescence, increasing the background signal and lowering the SNR (Burry, 2011). In order to control for autofluorescence, sections were blocked in blocking serum for one hour before being mounted on slides and observed with the same acquisition settings as experimental sections. A lower signal indicated less autofluorescence.

*Single-label* controls were used to characterise the labelling and to ensure that the labelling in triple-labelled sections was similar to that in single-label controls (Staines et al., 1988). Single-label controls also served as a microscopy control to ensure that the fluorescent-label corresponded to the correct filter (North, 2006).

*Secondary-only* controls were sections that omitted the primary antibody in order to characterise any non-specific labelling (Staines et al., 1988). Sections were blocked and incubated in the secondary antibody (primary antisera were omitted). There were three groups: sections that were incubated in goat, anti-mouse IgG Alexa Fluor 488; sections that were incubated in goat, anti-rabbit IgG Alexa Fluor 594; and sections that were incubated in both secondary antisera.

*Secondary antibody controls.* Multiple labels were used, as such, it was necessary to confirm the specificity of the secondary antibody to its primary antibody (Burry, 2011; Staines et al., 1988). The first secondary antibody control used a *single-double* labelling method (Burry, 2011) to test the specificity of the secondary antibody to the primary antibody. Tissue sections were incubated with one primary
antibody, but stained with both secondary antibodies – groups summarised in Table 2. Specificity of the secondary antibody was determined by labelling in the channel congruent with the primary antibody and no labelling in the incongruent channel.

Table 2

A Summary of the Single-double Secondary Antibody Control

<table>
<thead>
<tr>
<th>Control Type</th>
<th>Conditions</th>
<th>First Primary</th>
<th>Second Primary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>Primary</td>
<td>BuGR2 or rMR-1D5</td>
<td>Anti-PSD-95</td>
</tr>
<tr>
<td></td>
<td>Secondary</td>
<td>Alexa Fluor 488</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td>Label Expected</td>
<td>Normal (Green)</td>
<td>Normal (Red)</td>
</tr>
<tr>
<td>No First Primary</td>
<td>Primary</td>
<td>None</td>
<td>Anti-PSD-95</td>
</tr>
<tr>
<td></td>
<td>Secondary</td>
<td>Alexa Fluor 488</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td>Label Expected</td>
<td>None</td>
<td>Normal (Red)</td>
</tr>
<tr>
<td>No Second Primary</td>
<td>Primary</td>
<td>BuGR2 or rMR-1D5</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Secondary</td>
<td>Alexa Fluor 488</td>
<td>Alexa Fluor 594</td>
</tr>
<tr>
<td></td>
<td>Label Expected</td>
<td>Normal (Green)</td>
<td>None</td>
</tr>
</tbody>
</table>


The second secondary antibody control was to ensure there was no cross-reactivity between antigen species (Burry, 2011). This was done by staining the same tissue section with a primary antibody and its incongruent secondary antibody.

In more detail, tissue stained with the primary antibody for receptor-protein (BuGR2 or rMR-1D5) was labelled with Alexa Fluor 594, and tissue stained with the anti-PSD-95 antibody was labelled with Alexa Fluor 488. Labelling would indicate that cross-reactivity occurred between antibodies, and present as potential threats to the validity of the results.
Operationalisation of Variables

**Plasma Corticosterone Levels.** Plasma corticosterone levels were measured using an ELISA kit (procedure described above) to determine animal stress levels. Using Microsoft Excel, the absorbance values were averaged for each pair of wells. To calculate the net optical density, the average optical density from the “blank” and non-specific binding values were subtracted from the average optical density of bound wells. Maximum binding wells and standard wells were used to generate a standard curve – creating a precision profile, and the absorbance values of the samples were plotted against this precision profile. Corticosterone concentrations, were determined from the line of best fit, multiplied by the dilution factor (1:40) and expressed as ng/mL.

**Operationalisation of Amygdala Subnuclei.** The regions of interest (ROI) were the three subnuclei of the amygdala, LA, BA and CeA. These subnuclei were identified using the topological layout of the amygdala in the rat brain atlas (Paxinos & Watson, 2007), displayed in Figure 8. Sections were required to have all three structures on the same slice, limiting the sections to (anterior to posterior) Bregma – 2.04mm to -3.36mm (Paxinos & Watson, 2007). Anatomical landmarks were used to determine the boundaries of the LA; the external capsule was the lateral boundary of the LA; the striatum (Olucha-Bordonau, Fortes-Marco, Otero-Garcia, Lanuaz, & Martinez-Garcia, 2015) and the ITC as the medial boundaries of the LA. This involved all subregions (Olucha-Bordonau et al., 2015), presented in the rat brain atlas as dorsal lateral (LaDL), ventral medial (LaVM), dorsal ventral (LaVL) (Paxinos & Watson, 2007). The BA was operationalised as the basolateral amygdala anterior part (BLA) and posterior part (BLP) (Paxinos & Watson, 2007). The BA was directly ventral to the LA, and bordered laterally by the external capsule. As the
boundary between the LA and the BA was not always distinct, we sampled from the most ventral parts of the BA, using the most ventral part of the external capsule as a guide. The CeA presented as a ring-like structure adjacent to the BLA-complex (Olucha-Bordonau et al., 2015). The CeA has been further subdivided into central amygdala central, central amygdala lateral, and central amygdala medial (Sah et al., 2003), which are abbreviated in the rat brain atlas as CeC, CeL, CeM, respectively (Paxinos & Watson, 2007). For the current study, we did not differentiate between the CeC, CeL and CeM, and operationalised all three sub-regions as the CeA.

Figure 8. The LA, BA and CeA in a coronal section, regions sampled in grey. Sections were taken -2.04mm to -3.36mm from the bregma according to the rat brain atlas (Paxinos & Watson, 2007). Adapted from “The Rat Brain in Stereotaxic Coordinates 6th edition,” by G. Paxinos and C. Watson, 2007, p.56.
**Corticosteroid receptors.** The labelling of corticosteroid receptors (GR or MR) was varied between groups. That is, tissue was either incubated with the BuGR2 or rMR-1D5 primary antibody. As both BuGR2 and rMR-1D5 were raised in mouse, they were labelled with the same secondary antibody: goat, anti-mouse IgG Alexa Fluor 488. Both groups had tissue from the same rats and tissue sections were assigned as GR sections or MR sections by alternating serial slices. Both GR and MR sections were processed in parallel, and all other labels were consistent across groups. The acquisition settings were slightly different for GR and MR. That is, the laser power, offset, and gain for the 473nm laser, was varied between GR sections and MR sections.

**Parameters of nuclei and excitatory post-synapses.** Corticosteroid receptors (GR or MR) were labelled using green fluorescent markers (IgG Alexa Fluor 488). Excitatory post-synaptic terminals (PSD-95) were labelled using a red fluorescent marker (IgG Alexa Fluor 594). Nuclei were labelled using DAPI, a blue fluorescent marker. Filters were set to detect the emission wavelength of corticosteroid receptors between 527nm to 583nm, PSD-95 labelling was read at 668nm, and DAPI-labelled nuclei were read at 461nm. Channels were pseudo-coloured as green, red and blue respectively.

Volumetric quantification of colocalised corticosteroid receptors, was performed in Imaris (Bitplane, Version 8), using Fogarty’s approach and were operationalised based on the 3D objects rendered in Imaris (Bitplane). Nuclei were reconstructed as “surfaces”. GR, MR and PSD-95 puncta were reconstructed as “spots”. The parameters are described in more detail in Chapter 5. In order for puncta-labelling to be reconstructed as spots, Fogarty’s approach had two criteria that needed to be met: a) spot-like labelling needed to be well defined, with a
minimum of diameter of 0.25µm; and b) the signal intensity of the puncta needed to be strong enough, relative to the background, for the software to accurately demarcate between labelling and noise (Fogarty et al., 2013).

Corticosteroid receptor-like spots, inside of nuclei surfaces, were classified as *genomic GR spots* or *genomic MR spots*. Receptor-like spots outside of nuclei surfaces were operationalised as *extra-nuclear GR spots* or *extra-nuclear MR spots*. Corticosteroid receptor-like spots, colocalised with PSD-95 spots were referred to as *colocalised spots*.

**Parameters for colocalisation.** Colocalised objects were calculated using the *nearest-neighbour distance approach* (Bolte & Cordelières, 2006). That is, objects are deemed to be colocalised if they are within a predetermined distance to each other. Distances are calculated between the centres of two objects (Lachmanovich et al., 2003). Thus, a maximum distance of 0µm assumes a complete overlap between the centre points of the receptor spot and the PSD-95 spot. While corticosteroid receptors have been found to be located within the PSD (L. R. Johnson et al., 2005; Komatsuzaki et al., 2012; Prager et al., 2010), it was not necessarily the case that the centres of both spot objects would completely overlap. As such, a distance of 0µm was too stringent and would lead to underestimating the degree of colocalisation. Instead, the maximum distance between two objects was set at 0.1µm. This was because the smallest structures were receptors spots, which had a diameter of 0.2 µm and a threshold of 0.1µm ensured that at least half of the smallest object would be covered (Lachmanovich et al., 2003).

**Statistical Analysis.** Data was extracted from Imaris (Bitplane, Version 8) into Microsoft Excel. Statistical analysis was performed in SPSS, and statistical significance was accepted as $p < 0.05$. 


Ethics and Limitations

Ethics and Handling

All procedures were approved by the University Animal Ethics Committee at The University of Queensland, under the Approval Number: TRI/QUT/262/15/IHBI; and by the University Animal Ethics Committee from Queensland University of Technology under Approval Number: 1500000739, see Appendix D. As stated in the Subjects section, all research was conducted in compliance with Australian code for the care and use of animals for scientific purposes 8th Edition (2013) as embodied in the Queensland Animal Care and Protection Act (2016).

Handling-induced stress has been demonstrated to significantly increase corticosterone levels in rats (Balcombe, Barnard, & Sandusky, 2004). As our study aimed to understand corticosteroid receptors in stress-naive animals, handling-induced stress threatened the validity of the study. Playful handling is a method that has been shown to decrease the emotional stress displayed by rats, as well reduced fear towards experimenters (Cloutier, Panksepp, & Newberry, 2012). In order to control for handling-associated stress, playful handling was used to habituate the rats to the experimenter and decrease variability in corticosterone levels (Deutsch-Feldman et al., 2015). Additionally, a corticosterone assay was performed to confirm animals were not stressed.

The applicability of Animal Research to Humans in Stress

At first glance, rodents and humans appear quite different, thus the use of animals presented as a potential threat to the validity of the study. The primary ligand for GR and MR differs between rodents and humans (Prager & Johnson, 2009). In rodents, corticosterone is the primary stress hormone released in rats,
whereas in humans, cortisol is the primary stress hormone (de Kloet, 2014). However, cortisol and corticosterone are chemically similar, and corticosterone has also been found in human brain tissue (Karssen et al., 2000). Furthermore, rodent models of GR and MR have been shown to have structural and functional similarities compared with humans. Humans and rodents have displayed high expressions of GR and MR in the brain (Reul & de Kloet, 1985; Seckl, Dickson, Yates, & Fink, 1991; Q. Wang et al., 2014), although the subregional distributions have been shown to differ between species (Seckl et al., 1991). The behavioural and cognitive data of both humans and rats, in response to corticosteroids, suggest that animal models have at least some applicability to humans (Lupien & McEwen, 1997; Schwabe, 2013). In accordance with behavioural changes, corresponding biological changes to GR and MR expression have been demonstrated in rats and humans (López, Chalmers, Little, & Watson, 1998; Schwabe, 2013). Although there are physiological differences, the evidence would suggest that rodent models can be used to provide an understanding of GR and MR populations.
Chapter 4: Protocol Validation

Immunofluorescence has been used in previous research to characterise the densities of GR and MR in neurons (Han et al., 2014; van Steensel et al., 1996). However, in order to view mGR and mMR labelling at excitatory post-synaptic terminals, we needed to perform fluorescence imaging at a higher resolution than previous studies investigating density. Although GR- and MR-labelling have been previously observed at the post-synaptic density under the electron microscope (L. R. Johnson et al., 2005; Prager et al., 2010), it was unknown if the corticosteroid receptor labels would be colocalised with the PSD-95 labels. Moreover, in order to perform the 3D reconstruction of “spots” for colocalisation analysis, the labelling of corticosteroid receptor proteins needed to meet certain criterion (Fogarty et al., 2013). Thus, the aim of the study was two-fold: first, we wanted to test the specificity of the labels and optimise the reagents for triple-labelling immunofluorescence; second, we wanted to determine if the characterisation of the labelling was suitable for Fogarty’s approach. The first part of this chapter describes the methods used to validate the protocol and the reagents. Subsequently, the results are displayed in the form of representative images, and their implications discussed.

Method

Subjects and Procedure

Four Sprague-Dawley rats, weighing between 250g to 350g, were housed for one week (more details are provided in Chapter 3). Animals were handled during the week in order to familiarise them to the researchers, and reduce stress (Cloutier et al., 2012). All procedures were approved by the animal ethics committee for
Queensland University of Technology, and The University of Queensland (Appendix D).

Some tissue was lost in processing; as such, samples were obtained from three Sprague-Dawley rats. Rats were transcardially perfused with 0.9% saline solution, followed by 250ml of 4% PFA (w/v) in 0.1M PB. Tissue was post-fixed for 4 hours and cut using a vibratome (Pelco Easislicer) at 40μm to 60μm thick sections. Immunohistochemistry was performed as described in Chapter 3. Sections were incubated in a blocking buffer for 1 hour at room temperature, and primary antisera was incubated between 24 to 48 hours at 4°C depending on the condition. After the washing step, tissue was incubated in secondary antisera (1:400) between 2 to 4 hours at room temperature, depending on the condition. Some sections were incubated in DAPI (1:1000) for ~15 minutes and washed three times in PBS before being mounted onto slides. Slides were mounted with Mowiol and covered with No. 1.5 coverslips (Starfrost, Germany).

Sections were imaged using an Olympus IX73 epifluorescent microscope equipped with a metal halide lamp, in three channels (Emission$_{max}$/Absorbance$_{max}$): DAPI (350/455), GFP (470/510), and cy3 (560/656). In order to observe puncta labelling, images were taken using a 60× (1.25 NA) oil objective. Multi-channel images were manually captured in sequence: first in the GFP channel, then the cy3 channel and finally the DAPI channel. Some sections were further imaged on the confocal microscope to determine if the resolution would be suitable for further analysis.

Epifluorescent images were taken using a XM10 monochrome camera. As such, all images were pseudo-coloured and adjusted for brightness and contrast in Fiji (Schindelin et al., 2012). GR- or MR-like labelling was pseudo-coloured green,
PSD-95-like labelling was pseudo-coloured red and DAPI-labelling was pseudo-coloured blue. Epifluorescent images were merged in Fiji (Schindelin et al., 2012) using the merge channels function. Images were arranged for display using Adobe Photoshop CS6.

**Design**

**Anti-PSD-95 Titration.** Protocol optimisation for the anti-PSD-95 primary antibody was based on the supplier’s recommended dilutions for immunofluorescence (Invitrogen, 2015). Sections were incubated for 24 hours at 4°C, with anti-PSD-95 antibody and diluted with 0.1M PBS/1% BSA/4% NGS/0.3% Triton-X 100/0.05% Tween-20. Dilutions were performed at 1:500, 1:750 and 1:1000.

**rMR-1D5 Titration.** The immunofluorescent protocol for the MR primary antibody, rMR-1D5, was not well established in the literature compared to the primary GR antibody, BuGR2 (Gomez-Sanchez et al., 2006). In order to optimise the labelling and provide a standardised protocol, titrations were conducted for the MR antibody, rMR-1D5, and diluted in 0.1M PBS/1% BSA/4% NGS/0.3% Triton-X 100/0.05% Tween-20. An epifluorescent microscope was used to image all groups, and the conditions that displayed the best labelling were further examined under the confocal microscope.
Titrations were done at dilutions of 1:200, 1:500 and 1:1000 and 60µm thick sections were incubated for 24 hours or 48 hours at 4°C. The secondary antibody incubation time was also varied, at 2 hours and 4 hours. A summary of these groups can be seen in Figure 9.

**Buffers.** Two different protocols were used. The first protocol was based on the protocol described in Prager et al. (2010); tissue was blocked with 1% BSA (w/v) in 0.1M PBS, antibodies were diluted with 0.1M PBS, 0.3% Triton-X 100 (v/v) and the washed with 0.1M PBS. The second protocol used a blocking serum that not only blocked for the primary IgG, but also the secondary IgG to improve specificity (Xing, 2007). The blocking serum was 0.1M PBS, 1% BSA (w/v), 4% NGS; (v/v), 0.3% Triton-X 100 (v/v), 0.05% Tween-20 (v/v). This solution was also used as the diluent buffer and the wash buffer.

**Controls.** Controls were performed as described in Chapter 3. Firstly, autofluorescence controls were performed in order to determine the amount of endogenous background fluorescence from the tissue. Secondly, single label controls were imaged. For single-label controls, sections were incubated with one primary antibody and its corresponding secondary antibody. Labelling of single-
label controls was compared to secondary-only controls and cross-reactivity controls to determine the specificity of the fluorescent label to the primary antibody (Burry, 2011).

**Triple-labelling.** All sections were stained for PSD-95 immunoreactivity and DAPI. However, corticosteroid receptor labelling was varied between groups. Tissue was either incubated with the BuGR2 or rMR-1D5 primary antibody, which we refer to as *GR sections* or *MR sections* respectively. Triple labelling was initially characterised using the epifluorescent microscope. Upon confirmation of labelling, sections were further imaged at a higher resolution, under the confocal microscope to ensure that labelling met the criterion for 3D spot reconstruction according to Fogarty et al. (2013).

**Results and Discussion**

**Reagent optimisation**

**PSD-95 Titration.** Titrations were conducted for the anti-PSD-95 primary antibody at dilutions of 1:500, 1:750 and 1:1000. Under the epifluorescent microscope, a dilution of 1:500 provided the strongest labelling, but also had a relatively higher background signal compared to the other dilutions (Figure 10). The best SNR under the epifluorescent microscope was seen at a dilution of 1:750. Under the confocal microscope, there was limited background at a concentration of 1:500. Sections incubated at a concentration of 1:500 showed good penetration of the antibody and required less laser power and exposure time. As a result, the anti-PSD-95 primary antibody was diluted at a concentration of 1:500 in Chapter 5.
Figure 10. Representative image of fluorescent labelling of PSD-95-like immunoreactivity at concentrations of 1:500, 1:750 and 1:1000 (epifluorescence), in rat brain tissue. A non-linear contrast was applied in Photoshop using the curves function. Transformation was applied uniformly to all three images to improve the contrast. Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm

MR Titration. In order to optimise the fluorescence labelling for rMR-1D5, the concentration and incubation time of the primary antibody, rMR-1D5 was varied, as was as the incubation time of the secondary antibody, IgG Alexa Fluor 488. Observation under the epifluorescent microscope showed that an incubation time of 4 hours for the secondary antibody provided the strongest labelling, regardless of the primary antibody conditions, albeit with higher amounts of background noise (Figure 11b, 11c and 11d). At an incubation time of 24 hours, the dilution that provided the strongest labelling was 1:200, while still having a relatively little background (Figure 11a and 11b). An incubation time of 48 hours had clearer labelling at dilutions of 1:200 and 1:500, compared with an incubation time of 24 hours (Figure 11). The background labelling at 1:500 was relatively reduced compared to 1:200 (Figure 11f and 11d respectively), however, the puncta labelling at 1:200 was a lot stronger. As the strongest labelling of rMR-1D5 was at 1:200 for 48 hours, these sections were further examined using the confocal microscope to confirm if they met the criterion for spot creation (Fogarty et al., 2013).
Figure 11. a), b), c), and d) show tissue sections incubated with rMR-1D5 at a
dilution of 1:200; where the top two images are sections incubated for 24 hours,
and the middle two images are sections incubated for 48 hours. Images e) and f)
were diluted at 1:500 incubated for 48 hours. Images in the left column have been
incubated for 2 hours with the secondary antibody and images in the right column
have been incubated for 4 hours. Epifluorescent images were taken with a 60x
(1.25 NA) oil objective. Scale bar: 10µm
Sections incubated in rMR-1D5 at 1:200 for 48 hours were examined under the confocal microscope. Contrary to the epifluorescent microscope, the best SNR was seen when the secondary antibody was incubated at 2 hours instead of 4 hours, displayed in Figure 12. Although the puncta staining was stronger in the 4-hour condition, the background labelling was also a lot stronger. Based on this, we decided to use an incubation time of 3 hours for the secondary antibody in Chapter 5. In addition, the use of 60μm thick sections could have contributed to the high background noise. This was also corrected in Chapter 5 by reducing the thickness of the tissue sections.

Punctate labelling was observed in clusters within the nuclei, but also sparsely scattered throughout the section (Figure 12). MR-like puncta were measured to have a diameter between 0.3μm to 0.5μm; thus meeting the minimum criterion for Fogarty’s approach (Fogarty et al., 2013). It was interesting to note that MR-like puncta in the nucleus, but in some nuclei, MR-like labelling appeared to be homogenously distributed within the nucleus. The homogenous labelling provided a potential threat to later spot creation. As extra-nuclear MR-like labelling was distinctly punctate, Fogarty’s approach could still be used for colocalisation analysis. No labelling was seen within the nucleolus (Figure 12) which was consistent with previous literature (van Steensel et al., 1996).
Figure 12. Confocal imaging of rMR-1D5 labelled slices at a concentration of 1:200 with an incubation time of 48 hours. Tissue sections were incubated with IgG Alexa Fluor 488 for 2 hours (a) or 4 hours (b). Images were taken with a 60x (1.35 NA) oil objective with 2.5x sensor zoom. Scale bar: 10µm

Buffer comparison. The more sophisticated diluent buffer appeared to have less non-specific labelling at the surface of sections. Antibody penetration also appeared to be better when using the more sophisticated diluent buffer. As a result, the buffer solution comprising of 0.1M PBS, 1% BSA (w/v), 4% NGS (v/v), 0.3% Triton-X 100 (v/v) and 0.05% Tween-20 (v/v), was used in Chapter 5.

Labelling Specificity

Autofluorescence. Autofluorescence was uniformly diffused across the tissue. At higher magnifications, some particulate autofluorescence could be seen, but it was quite dim. As the autofluorescence of the tissue was relatively dim, the same fixation parameters were used in the study conducted in Chapter 5.

Specificity of Fluorescent Labels. Single-label controls consisted of sections incubated with one primary antibody and its congruent secondary antibody.
The labelling of these sections was compared to secondary-only controls and cross-reactivity controls. Both GR-like and MR-like labelling was observed in single-label controls (Figure 13a and 13b). Comparatively, secondary-only controls displayed minimal immunoreactivity outside of background labelling (Figure 13). It should be noted that background labelling in the GFP channel of secondary-only controls was still brighter than autofluorescence. Finally, cross-reactivity controls revealed limited cross reactivity. Random blebs could be seen but they were not characteristic of GR-like or MR-like labelling (Figure 13e and 13f). Moreover, the blebs appeared to be present only at the surface of the sections and were not seen in the middle of the slice.
**Figure 13.** Control sections, sample: rat brain tissue. Single-label controls for GR (a) and MR (b) in the GFP channel, green. Figures (c) and (d) show secondary-only controls of GR and MR respectively. Images (e) and (f) show the cross-reactivity controls for GR and MR respectively, in the cy3 channel. Epifluorescent images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm

Accordingly, single-labelled sections for excitatory post-synapses revealed PSD-95-like immunoreactivity. PSD-95-like labelling was consistent with previous
literature, and presented as puncta-shaped labelling (Fogarty et al., 2013) distributed throughout the amygdala (Hunt et al., 1999), see Figure 14a. Secondary-only controls (sections incubated in goat, anti-rabbit IgG, Alexa Fluor 594) displayed minimal immunoreactivity and no punctate staining was observed (Figure 14b). This suggests the labelling of the secondary antibody is specific to PSD-95. Cross-reactivity controls also did not show any puncta staining in the GFP channel (Figure 14c) further suggesting the specificity of the secondary label.

Figure 14. Single-label control in rat brain tissue, for anti-PSD-95 shows puncta labelling (a) with cy3 filter. Alexa Fluor 594, secondary-only control (b) with cy3 filter shows minimal labelling. Cross-reactivity control for anti-PSD-95 primary antibody (c) shows minimal immunoreactivity in GFP channel. Epifluorescent images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm

We also found that single-label controls only displayed labelling in their respective channels. This suggested that there was no bleed-through between channels (North, 2006). Tissue incubated with both secondary antisera (primary antisera omitted) also showed minimal immunoreactivity in the GFP and cy3 channels, further supporting the specificity of the fluorescent labels.

**Single-double labelling.** In order to test the specificity of the secondary antibodies to each primary antibody, tissue was incubated in one primary antibody and both secondary antibodies (Burry, 2011), displayed in Figure 15. Tissue incubated in primary antiserum, BuGR2, showed GR-like labelling under the GFP
channel and minimal labelling in the cy3 channel, similar to the cross-reactivity controls. Similarly, tissue incubated in the primary antiserum rMR-1D5 also showed MR-like labelling under the GFP channel and limited labelling the cy3 channel. It was observed that the GR- and MR-like labelling was brighter in the GFP channel compared to single label controls. No labelling was observed for the PSD-95 single-double control for either channel.

![Epifluorescent images of brain tissue sections labelled with one primary antibody and both secondary antibodies: GR single-double control in the (a) GFP channel (green), and (b) cy3 channel (red); MR single-double control in the (c) GFP channel (green) and the (d) cy3 channel (red). Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm](image)

**Figure 15.** Epifluorescent images of brain tissue sections labelled with one primary antibody and both secondary antibodies: GR single-double control in the (a) GFP channel (green), and (b) cy3 channel (red); MR single-double control in the (c) GFP channel (green) and the (d) cy3 channel (red). Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm
Characterisation of Triple Labelling

**Fluorescent Labelling in GR sections.** We were interested in characterising the appearance of triple-labelling in GR sections. Tissue sections were initially imaged using the epifluorescent microscope (Figure 16). GR, PSD-95 and DAPI immunoreactivity was seen in the GFP, cy3 and DAPI channel respectively. GR-like labelling was seen within the nucleus and distributed throughout the amygdala (Figure 16a). GR-like labelling was distinctly punctate shaped and clustered within the nucleus (Figure 16d).
Figure 16. Triple labelling for GR sections in rat brain tissue, under the epifluorescent microscope. For the same region, in the GFP channel (green) GR-like immunoreactivity was observed (a). In the cy3 channel (red) PSD-95-like labelling was seen (b). In the DAPI channel (blue) nuclei-labelling was seen (c). All three channels were artificially merged (d). Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm
In order to see if GR was colocalised with PSD-95, higher resolution images were captured under the confocal microscope. Under the confocal microscope, GR-like labelling presented as well defined puncta, both within the nucleus and distributed throughout the section, which was consistent with previous research (Sarabdjitsingh et al., 2010). The diameter of GR-like puncta were around 0.2µm to 0.7µm, similar to that in previous literature (van Steensel et al., 1996). Moreover, puncta labelling was relatively distinct from the background, meaning that GR-like labelling was suitable for 3D reconstruction (Fogarty et al., 2013). The diameter of PSD-95-like punctate were measured to be between 0.3µm to 0.7µm, which was in accordance with previous research (Hunt et al., 1999). Additionally, PSD-95-like puncta were well defined, and also met the criterion for spot quantification (Fogarty et al., 2013). GR-like labelling appeared to overlap with PSD-95-like labelling, which was indicated by yellow fluorescence (white arrows in Figure 17). Some PSD-95-like labelling was found inside the nuclei. As stated in Chapter 3, this particular anti-PSD-95 antibody has displayed some cross-reactivity within rat homogenates (Invitrogen, 2015). This was controlled for during analysis by excluding any labelling within nuclei.
Figure 17. Confocal imaging of GR-section in rat brain tissue. GR-like labelling can be seen in green, PSD-95-like labelling in red, and DAPI labelling in blue.

Colocalisation of GR-like labelling with PSD-95 like labelling is shown in yellow (indicated by white arrows). Images were taken with a 60x (1.35 NA) oil objective with 2.5x sensor zoom. Scale bar: 10µm
**Fluorescent labelling in MR sections.** We were also interested in testing whether triple-labelling fluorescence on MR sections was a suitable for use with Fogarty’s approach. Labelling in MR sections were initially characterised using the epifluorescent microscope (Figure 18). MR-like immunoreactivity was not as intense as GR-like immunoreactivity. Moreover, the labelling of MR-like structures was not as well defined as GR-like structures. Some neurons displayed homogenous distribution of MR-like labelling within the nucleus. The DAPI and PSD-95-like labelling was similar to labelling seen in GR sections, suggesting that the staining conditions of DAPI and PSD-95 were constant between GR and MR sections.
**Figure 18.** Triple labelling for MR sections in rat brain tissue, under the epifluorescent microscope. a) In the GFP channel (green) MR-like immunoreactivity was seen. b) In the cy3 channel (red) PSD-95-like labelling was seen. c) In the DAPI channel (blue) nuclei-labelling was seen. d) All three channels were artificially merged. Images were taken with a 60x (1.25 NA) oil objective. Scale bar: 10µm
Further examination under the confocal microscope showed MR-like structures were not as distinct as GR-like structures (Figure 19). MR-like puncta labelling could be seen, distributed throughout the section. Moreover, MR-like puncta showed some overlap with PSD-95-labelling (indicated by white arrows in Figure 19). Within the nucleus, MR-like puncta could be seen, however, they were not well defined. Moreover, MR-like labelling in the nucleus was more homogenously distributed which made it difficult to threshold MR-like puncta in the nucleus. In the literature, MR-like labelling in the nucleus appeared to be more variable. Some research displayed MR-like labelling to be distinctly puncta shaped (Kil & Kalinec, 2013; van Steensel et al., 1996), while others suggested that MR labelling had a relatively homogenous distribution within nuclei (Groeneweg, 2014). MR-labelling has also been reported to have a higher distribution within the cytoplasm compared to GR-labelling (Han et al., 2014).
Figure 19. Confocal imaging of MR-section in rat brain tissue. MR-like labelling can be seen in green, PSD-95-like labelling in red, and DAPI labelling in blue. The nuclei labelling was selected for optimal MR labelling – the DAPI labelling is not representative of the DAPI nuclei staining obtained throughout. Colocalisation of MR-like labelling with PSD-95 like labelling is indicated with white arrows. Images were taken with a 60x (1.35 NA) oil objective with 2.5x sensor zoom. Scale bar: 10µm
Summary

Previous studies have used triple-labelling immunofluorescence as a method of distinguishing between different subcellular populations of GR and MR in other cell types (Kil & Kalinec, 2013). In this study, we aimed to test the feasibility of using triple-labelling to characterise the populations of GR and MR in brain cells and whether the labelling would be suitable for 3D reconstruction in Imaris. As the validity of the primary antibodies used had been well characterised (Ahima & Harlan, 1990; Gomez-Sanchez et al., 2006), we aimed to optimise the protocol for multiple labelling immunofluorescence. A titration of the anti-PSD-95 antibody revealed good penetration and labelling at a dilution of 1:500. Titration of the primary MR antibody, rMR-1D5, showed the best puncta labelling at a dilution of 1:200 and an incubation time of 48 hours.

Next we tested the specificity of the fluorescent labels. Single-label controls revealed labelling of GR- and MR-like structures that was similar to previous research (Groeneweg, 2014; L. R. Johnson et al., 2005; Prager et al., 2010; van Steensel et al., 1996). Autofluorescence controls and secondary-only controls had limited immunoreactivity, suggesting that the labelling seen was not merely an artefact produced by the secondary antibodies (Burry, 2011). Cross-reactivity controls revealed limited labelling, suggesting that the fluorescent labels were specific to the primary antibody. Single-double controls further supported the specificity of the secondary label to its corresponding secondary antibody.

Finally, we described the triple-labelling for GR and MR sections. GR- and PSD-95-like structures presented as well defined puncta, with a minimum diameter above 0.2μm; meeting the criteria for spot reconstruction (Fogarty et al., 2013). Extra-nuclear MR-like structures also had well defined puncta and met the criteria.
for reconstruction and colocalisation analysis (Fogarty et al., 2013). However, MR-like puncta within nuclei was not as well defined and presented a potential threat to the reconstruction and quantification analysis. Confocal imaging also showed that both GR and MR sections showed some overlap in the fluorescence signals of receptor-like labelling and PSD-95-like labelling. This suggested that GR- and MR-like structures may be colocalised with PSD-95-like structures.

Based on these results we were able to confirm the use of the reagents for visualising GR-like and MR-like structures in fixed brain tissue. Moreover, puncta labelling for GR, PSD-95 and extra-nuclear MR clearly met the criterion for Fogarty’s approach (Fogarty et al., 2013), although nuclear MR structures were less well defined. Thus, we were able to establish a standardised protocol for triple-labelling immunofluorescence of corticosteroid receptors to be used in colocalisation analysis in Chapter 5.
Chapter 5: Corticosteroid Receptor Densities in the Amygdala

The neuronal distribution of corticosteroid receptors in the amygdala have been described in previous studies (Ahima & Harlan, 1990; Ahima et al., 1991; Morimoto et al., 1996), however the distributions of mGR and mMR are still being characterised. In the LA, mGR and mMR have been found in the PSD at some synapses (L. R. Johnson et al., 2005; Prager et al., 2010), but the overall distribution of mGR and mMR located at post-synaptic sites is unclear. In this chapter, we used the method developed in Chapter 4 for confocal imaging, and we adapted Fogarty’s approach to reconstruct the labelled structures for colocalisation analysis (Fogarty et al., 2013). Using this approach, we were interested in obtaining preliminary data for the characterisation of GR and MR at excitatory post-synaptic sites within the different subnuclei of the amygdala.

This chapter begins with an overview of the methods used. This involves the parameters for deconvolution using Huygens Professional Software (Scientific Volume Imaging), and the 3D reconstruction of fluorescent structures in Imaris (Version 8.2, Bitplane). Subsequently we present the results of adapting Fogarty’s approach to GR and MR fluorescent labelling, and provide detailed instructions of the process used. Finally, we used the data obtained from 3D reconstruction to perform some preliminary calculations on the distribution densities of GR and MR in the amygdala.
Method

Subjects and Procedure

Tissue was collected from four Sprague-Dawley rats (255-260g), although some samples were lost during processing. As such, the final analysis was performed on three animals. This was still a reasonable sample size for a feasibility study using stress naïve animals, and was in accordance with similar research (Fogarty et al., 2013; Sarabdjitsingh et al., 2010; van Steensel et al., 1996). Animals were acclimatised to the environment for one week prior to any procedures. Housing conditions and handling procedures are described in more detail in Chapter 3. Animals were anesthetised with ketamine/xylazine (1mg/kg/0.1mg/kg ip). Blood, for immunoassay, was collected via cardiac puncture and stored on ice. Animals were perfused with 0.9% saline solution and subsequently with ~250ml of 4% PFA (w/v) in 0.1M PB. Brains were post-fixed for three hours and washed three times in PBS. Brains were sectioned using a 0.1mm brain matrix and further cut using a vibratome (Leica, Australia) at 40μm thick sections. Tissue sections were serially assigned to conditions in the following order: Controls; GR sections; MR sections. The controls were processed as described in Chapter 3. Immunohistochemistry for GR and MR sections were processed in parallel.

Imaging. Sections were imaged using an Olympus FV1200 Confocal Microscope equipped with 405nm, 473nm and 559nm solid state lasers. First, a mosaic image of the amygdala was created using the “Multi Area Time Lapse” function in the Olympus Fluoview software (Olympus), to ensure accurate sampling. The mosaic was imaged on one z-plane, using 473nm and 559nm lasers, with a 20× (0.86 NA) oil objective. From the mosaic image, six ROI were selected to be imaged using the 60× (1.35 NA) oil objective with a 2.5× sensor zoom. Two ROIs were selected from the
LA, two from the CeA, and two from the BA (operationalised in Chapter 3). The start and end points were individually set for each ROI, and added to the “Registered Point List” and subsequently scanned. Each image was acquired using a 1024×1024 scan format, and contained 65 z-stacks, with a step size of 0.3µm. The maximum optical resolution of each image was 83.97µm × 83.97µm with a z-depth of 19.5µm.

**Deconvolution.** Images were deconvolved using Hyugen’s Professional software (Scientific Volume Imaging), in order to improve the SNR, and restore the resolution along the z-axis by removing signals that were not present in at least two stacks (Bolte & Cordelières, 2006). The deconvolution parameters were set at a maximum of 20 iterations and a quality threshold of 0.01. For DAPI-labelling, the SNR was set at 10, and for puncta labelling (i.e. receptor- and PSD-95-labelling), the SNR was set at 5.

**3D reconstruction and colocalisation in Imaris.** Images were further processed in Imaris (Version 8.2, Bitplane). Firstly, images were imported into Arena View and then individually cropped along the z-axis to remove any artefacts and blebs at the top and/or bottom of sections. Subsequently, a random image was selected to establish the parameters for batch processing. First the parameters for nuclei surfaces were established. DAPI labelling was reconstructed as a “surface object” layer. Parameters for the surface object layer were as follows: smoothing and background subtraction were enabled with a threshold of 1.5µm; the maximum diameter of the largest sphere was set at 15µm; and the interactive histogram threshold minima was set at 2.42 arbitrary units for GR sections, and 1.00 arbitrary units for MR sections. These parameters were saved and applied to all images.

The surface object layer was subsequently used to create three new mask channels: GR or MR labelling within nuclei; GR or MR labelling outside of nuclei;
and PSD-95 labelling outside of nuclei. The effect of mask channels and the resultant labelling was described in more detail in the Results section. For each of the masked channels, we digitally reconstructed the immunoreactive labelling into “spot objects”. Using a random image, receptor labelling inside of surfaces was reconstructed into 3D spot objects with the following parameters: the minimum diameter for both genomic GR and MR spots was set at 0.2μm; the interactive histogram was filtered on quality where GR spots had a threshold minima of 7.996 arbitrary units and MR spots had a threshold minima of 4.638 arbitrary units. Extra-nuclear GR and MR spots were created from the mask channel containing receptor-like labelling outside of surfaces. The minimum diameter for extra-nuclear receptor spots was also set at 0.2μm. The interactive histogram was used to filter spots based on quality; for GR, the minima was set at 2.789 arbitrary units, while MR was set at 3.456 arbitrary units. PSD-95 spots were reconstructed from the mask channel containing PSD-95 labelling outside of nuclei surfaces. PSD-95 spots had a minimum estimated diameter of 0.35μm, with background subtraction enabled. The interactive histogram threshold for PSD-95 spots was filtered by quality; in GR sections the minima was set at 2.797 arbitrary units and in MR sections the minima was set at 3.769 arbitrary units. Once these parameters were finalised, they were applied to the batch to maintain consistency between images.

Colocalisation analysis was performed using the nearest-neighbour distance approach (Bolte & Cordelières, 2006; Lagache et al., 2015), described in detail in Chapter 3. In Imaris, the “spot colocalisation” ImarisXT plugin was used to create new spot objects. Extra-nuclear GR or MR spots that were within 0.1μm of PSD-95 spots were classified as colocalised. Two layers of colocalised spot objects were created: the number receptor spots within 0.1μm of PSD-95 spots; and the number of
PSD-95 spots within 0.1µm of receptor spots. An online supplementary video is provided in Appendix E, which demonstrates the process of 3D reconstruction and the three-dimensional nature of the surface and spot objects.

**Design**

In line with object-based analysis (Lagache et al., 2015), we were able to quantify the number of spot objects for analysis. As stated above, colocalised spots referred to the number of PSD-95 spots within 0.1µm of GR or MR spots outside of DAPI nuclei surfaces. We referred to the spots located inside of DAPI surfaces as: *genomic GR spots* and *genomic MR spots*. The density of genomic spots was calculated: per nuclei volume, or per total volume; most comparisons were made based on the spot density, per total volume. The number of genomic GR and MR spots, however, was compared between amygdala subregions, based on the density per nuclei volume. This was calculated by diving the number of spots by the volume within DAPI surfaces; which accounted for any differences in nuclei volume between amygdala subregions. All statistical analysis was performed in SPSS, where statistically significant results were accepted as $p < .05$.

**Results**

**Controls**

Immunoreactive labelling in control sections were similar in presentation to the fluorescent and reagent controls used in protocol validation (Chapter 4). This suggested that the labelling seen was specific.
Mosaic Images

Mosaic images were taken for each section in order to ensure accurate sampling of amygdala subnuclei. Mosaic images were captured at 20× (0.86 NA) magnification using 473nm (green) and 559nm (red) lasers. Coordinates, for the images used for 3D reconstruction and colocalisation analysis, were plotted on the mosaic image (white dots in Figure 20). Two images from each subnuclei (three subnuclei: LA, CeA and BA) were sampled from each section.
Figure 20. Mosaic image of rat brain tissue, taken at 20× (0.86 NA) oil objective. White dots depict the coordinates of images sampled for analysis. Overlay in white displays the boundaries of the different subregions of the amygdala.
Deconvolution of Images

Images were deconvolved using Huygens Professional (Scientific Volume Imaging). Deconvolution parameters were differentially set for each channel. For DAPI-labelling, the SNR was set at 10, to prevent light scattering. The quality threshold was set at 0.01 with a maximum of 20 iterations. For PSD-95-labelling, SNR was set at 5, with a quality threshold of 0.01 and a maximum of 20 iterations. These parameters were held constant for all sections. After deconvolution, the appearance of light scattering in both blue and red channels was greatly reduced, (Figure 21) and the labelling relative to the background was more distinct. For PSD-95 labelling, the size of PSD-95 puncta was reduced (Figure 21c and Figure 21d).
Figure 21. Maximum intensity projections for DAPI and PSD-95 are depicted, before and after deconvolution. The images depicted contained 52 stacks, with a thickness of 15.6µm. Deconvolved images b) and d) show reduced light scattering.
GR- and MR-like labelling was also deconvolved using Huygens Professional. We found that using conservative parameters removed an excessive amount of pixel values. Due to the small size of GR- and MR-puncta, the conservative parameters treated some faint labelling as background, and subsequently removed smaller puncta; resulting in an underestimation of receptor density. To avoid over correction, less conservative parameters were used. This resulted in a higher SNR without excessive removal of pixel values, but it did not completely resolve out-of-focus light. Labelling that was not present for two or more stacks were removed, suggesting that only real puncta labelling was preserved (Bolte & Cordelières, 2006). Deconvolution improved the SNR (Figure 22) resulting in more well defined puncta. In addition, deconvolution improved shape of the puncta along the x, y and z-axes (Figure 22). Puncta were more distinct and there was less smearing of objects along the z-plane (Figure 22c and 22d depict GR puncta, and Figure 22g and 22f depict MR puncta.

MR-like labelling in raw images displayed less homogenous labelling compared to the labelling seen during protocol validation (Chapter 4). Moreover, deconvolution also improved the SNR and distinctiveness for both nuclear (and extra-nuclear) puncta (Figure 22f and 22h). This rendered MR sections suitable for 3D reconstruction and object-based colocalisation (Fogarty et al., 2013).
Figure 22. Maximal z-projections are displayed for GR and MR labelling before and after deconvolution. Z-thickness of deconvolved images for GR and MR show the correction for noise, and the change in the size of puncta: changes in GR puncta can be seen in c) and d); changes in MR puncta can be seen in g) and h).
**Nuclear Surfaces**

DAPI-labelling was used to create *nuclei surfaces*. To create an unbroken surface, smoothing was enabled at a threshold of 1.5μm and background subtraction thresholding was enabled. The diameter of largest sphere was set at 15μm, based on the maximum diameter of neuronal nuclei in previous literature (Chareyron, Lavenex, Amaral, & Lavenex, 2011). Using the interactive histogram, a minimum threshold was used to correct for any artefacts and blebs (Fogarty et al., 2013), with arbitrary units of 2.42 for GR-sections, and 1.00 for MR-sections (Figure 23).
Figure 23. Creation of nuclear surfaces for GR and MR sections. First row of images a) and d) display unfiltered fluorescence. Second row of images b) and e) display filtered DAPI labelling. Third row c) and f) displays Imaris-generated 3D surfaces.

Scale bar: 10µm
Creation of Genomic GR and MR

The presentation of puncta labelling was characterised during protocol validation (Chapter 4), and found to meet the criterion for spot creation (Fogarty et al., 2013). After the reconstruction of nuclei surfaces, we demarcated between intra- and extra-nuclear labelling. Using the DAPI-surfaces, we filtered the intra-nuclear fluorescent labelling of genomic GR- or MR-like structures. We selected the DAPI surface objects layer and used the “mask all” function under the edit tab. As we wanted to mask for receptor-like labelling inside surfaces, we selected the GR (or MR) channel and set “voxels outside of surface” to 0.0. We also enabled “duplicate channel before applying mask”, in order to preserve the original channel.

Consequently, a new source channel was created for GR-like labelling or MR-like labelling inside of DAPI-surfaces (Figure 24b and figure 24e).

Next, 3D genomic GR or MR spot objects were reconstructed from the filtered fluorescence. We selected the new source channel, which contained the filtered receptor-labelling, used it to add a spot objects layer. Background subtraction was enabled, and GR- and MR-labelling was determined to have an estimated diameter of 0.2 µm, based on previous literature (van Steensel et al., 1996). We used the histogram threshold in order to detect as many spots as possible without creating artificial objects; around the point of inflection on the histogram (Fogarty et al., 2013). For GR-like labelling, spots were filtered on quality, with a threshold minima of 7.996 arbitrary units (Figure 24c). For MR-like labelling, spots were also filtered on quality, with a threshold minima of 4.638 arbitrary units (Figure 24f). Genomic GR- and genomic MR-spots generated from GR- and MR-like labelling could subsequently be used for analysis (link to online supplementary video in Appendix E).
Figure 24. Intra-nuclear GR- and MR-like labelling was filtered based on nuclei surfaces. The first row a) and d) displays unfiltered labelling. The second row b) and e) displays the filtered labelling. The third row c) and f) displays detected labelling that was reconstructed as spots. Scale bar: 10µm
**Creation of Extra-nuclear GR and MR and Post-synaptic Terminals**

Spot objects were also created for GR- and MR-like structures outside of DAPI-surfaces. We selected the surface objects layer and used it to filter receptor-like labelling outside of nuclei surfaces. Again, we used the “mask all” option under the edit tab, and selected the original channel containing the unfiltered GR or MR fluorescence. However, to filter the fluorescence outside of nuclei surfaces, we set “voxels inside of surfaces” (as opposed to voxels outside of surfaces) to 0.0. This mask channel was also created as a duplicate channel in order to preserve the original data (Figure 25).

Extra-nuclear GR and MR spots were constructed from the filtered fluorescence. Firstly, we selected the new channel created, which contained the filtered extra-nuclear labelling, to create a new spots objects layer. For both GR- and MR-like labelling, background subtraction was enabled, and extra-nuclear spots had an estimated diameter of 0.2μm, maintaining consistency with the diameter of genomic spots. For GR-like labelling, the histogram threshold was used to filter spots with quality above 2.789 arbitrary units. For MR-like labelling the histogram threshold was set to filter spots with quality above 3.456 arbitrary units (Figure 25c and Figure 25f).

PSD-95-like labelling was also filtered using the DAPI-surfaces. Using the DAPI-surfaces “mask all” option, the PSD-95 channel was selected, and the “voxels inside of surfaces” were set to 0.0. This mask was also created as a duplicate channel, in order to preserve the original PSD-95 labelling (Figure 25). Spot objects were created from the filtered PSD-95 layer, with an estimated diameter of 0.35μm (background subtraction enabled) based on the PSD-95 labelled spines measured in previous studies (Cooke & Woolley, 2005; Fogarty et al., 2013). The histogram
threshold for PSD-95 spots in GR sections were filtered by quality with a minima of 2.797 arbitrary units. PSD-95 spots in MR sections had a minima of 3.769 arbitrary units (Figure 25).

Figure 25. Creation of extra-nuclear spots for GR-like labelling and MR-like labelling and PSD-95. Fluorescence labelling before and after filtering is also shown. Scale bar: 10µm

Colocalisation of Corticosteroid Receptors at Post-synaptic Terminals

Object-based colocalisation analysis was performed using Imaris. Colocalised spots were calculated using the nearest-neighbour distance approach
(Bolte & Cordelières, 2006), described in Chapter 3. We used the ImarisXT plugin, “spot colocalisation”, to create a new layer of *colocated spots* (Figure 26, and online supplementary video 2, linked in Appendix E). As we were interested in an overlap between receptor spots with PSD-95 spots, colocated spots were created by selecting the extra-nuclear receptor spot layer and the PSD-95 spot layer, from the menu. We chose a distance threshold for analysis that ensured that at least half of the smallest object would be covered (Lachmanovich et al., 2003). As the smallest object was the receptor spot, which had an estimated diameter of 0.2µm, the maximum distance threshold was set at 0.1µm. Using Imaris, two new sets of collocated objects were created: the first set contained the number of extra-nuclear receptor spots that were within 0.1 µm from PSD-95 spots; and the second set contained the number of PSD-95 spots within 0.1µm from extra-nuclear receptor spots. There were no differences in the number of colocalised objects between the two sets.

We also observed some yellow fluorescence (suggesting a colocalised signal; indicated by the asterisk in Figure 26c), that was not reconstructed as a colocalised object (Figure 26c and Figure 26f). This further supports the importance of the added z-resolution used in object-based analysis (Lagache et al., 2015). Moreover, in some images, we also observed overlapping spot objects that were not recreated as colocated spots as they exceeded the maximum distance threshold. This demonstrates the consistency of the criterion used, as these overlaps could have been misattributed as colocalised objects during manual counting (Bolte & Cordelières, 2006).
Figure 26. Creation of colocalised spot objects from overlaps between corticosteroid receptors and PSD-95 (indicated by white arrows). Asterisk indicates seemingly colocalised fluorescence that was not labelled in 3D reconstruction.

Scale bar: 3µm
Corticosterone levels

Corticosterone levels were measured using a competitive binding ELISA (Table 3). Rat 1 weighed 255g and had a corticosterone level of 18.61ng/ml. Rat 2 weighed 250g and had corticosterone level of 29.55ng/ml. Rat 3 weighed 260g and had corticosterone level of 68.62ng/ml. The corticosterone levels of Rats 1 and 2 were similar to previous literature of rats familiar to handling (Deutsch-Feldman et al., 2015). Rat 3 had higher levels of corticosterone, similar to that of rats that were not familiar with handling procedures (Deutsch-Feldman et al., 2015). Corticosterone levels were below that of rats previously reported as “stressed”, which typically have plasma corticosterone levels around 250ng/ml (Dhabhar, McEwen, & Spencer, 1993).

Table 3

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Plasma Corticosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stressed Rat&lt;sup&gt;a&lt;/sup&gt; 200 to 300</td>
<td>200</td>
</tr>
<tr>
<td>Rat 1 255</td>
<td>18.61</td>
</tr>
<tr>
<td>Rat 2 250</td>
<td>29.55</td>
</tr>
<tr>
<td>Rat 3 260</td>
<td>68.62</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average corticosterone level of “stressed rat” were adapted from “Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels - a comparison between Sprague-Dawley, Fischer 344 and Lewis rats,” by F. S. Dhabhar, B. S. McEwen and R. L. Spencer, 1993, Brain Research, 616, p. 91.

Analysis

Descriptive Statistics

A total of 155 images were analysed. There was a total of 81 images for GR sections (n<sub>rats</sub> = 3), and 74 images for MR sections (n<sub>rats</sub> = 2). To compare between images, spots were adjusted based on their volumetric density. The number of spots
for each image was divided by its total volume and multiplied by 1000 and the volumetric density was displayed as spots per 1000\(\mu m^3\) (or \(10^3\mu m^3\)).

The variability between rats, of the volumetric densities of genomic and colocalised GR and MR, are reported in Tables 4 to 6. Rat 1 had the lowest amount of plasma corticosterone (18.63 ng/ml). The highest mean density of genomic GR, spots per 1000\(\mu m^3\), was in the CeA (\(M = 52.71, SD = 25.34\)), and the highest density of colocalised GR spots was in the LA (\(M = 3.22, SD = 1.88\)). Skewness and kurtosis suggested that the volumetric density was more normally distributed for colocalised GR spots compared to genomic GR spots (Table 4).

Rat 2 showed the highest levels of genomic GR (\(M = 62.82, SD = 19.77\)) and genomic MR (\(M = 42.09, SD = 21.97\)) in the CeA. In rat 2, genomic GR spots had the lowest mean density in the BA (\(M = 19.39, SD = 8.82\)), whereas genomic MR spots had the lowest mean density in the LA (\(M = 14.68, SD = 8.46\)). For both colocalised GR and MR spots, the lowest mean density was within the LA, where the mean densities per 1000\(\mu m^3\) (standard deviation in parentheses) were = 3.10 (0.87) and = 1.70 (0.64) respectively. Distribution densities of colocalised GR receptors were skewed slightly towards the right (more images with a volumetric density above the mean), whereas colocalised MR receptors were skewed slightly towards the left (more images with a volumetric density below the mean; displayed in Table 5).

Rat 3 had the highest amount of blood corticosterone (68.82ng/ml), more than two times as high as Rat 2 (29.55 ng/ml). Unlike rat 2, rat 3 had a higher mean density of genomic MR spots (\(M = 39.19, SD = 32.26\)) compared to genomic GR spots (\(M = 14.40, SD = 9.62\)) per 1000\(\mu m^3\). Similar to rat 1 and rat 2, rat 3 had the highest density of genomic GR spots in the CeA (\(M = 24.48, SD = 9.89\)). Skewness
and kurtosis for the densities of genomic GR and MR spots were also higher compared to rat 2, indicating that the volumetric density of images, sampled from rat 3 were less normally distributed. The highest density of colocalised GR spots in rat 3, was found in the CeA ($M = 3.68$, $SD = 0.79$), however, the highest density of colocalised MR spots were found in the BA ($M = 2.57$, $SD = 1.01$), which can be seen in Table 6.
Table 4

*Descriptive statistics for Rat 1: The distribution density of GR-like spots per 1000µm³*

<p>|          | Genomic |                                 |                                 |                                 |                                 |                                 |                                 |                                 |                                 |
|----------|---------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|                                 |</p>
<table>
<thead>
<tr>
<th></th>
<th>NImages</th>
<th>M (SD)</th>
<th>Range</th>
<th>Skewness</th>
<th>Kurtosis</th>
<th>M (SD)</th>
<th>Range</th>
<th>Skewness</th>
<th>Kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid Receptor(^b)</td>
<td>12</td>
<td>38.06 (24.67)</td>
<td>76.99</td>
<td>1.22</td>
<td>0.63</td>
<td>2.84 (1.14)</td>
<td>3.74</td>
<td>0.89</td>
<td>0.16</td>
</tr>
<tr>
<td>LA</td>
<td>4</td>
<td>42.96 (27.11)</td>
<td>59.91</td>
<td>1.08</td>
<td>0.11</td>
<td>3.22 (1.88)</td>
<td>3.74</td>
<td>0.15</td>
<td>-5.07</td>
</tr>
<tr>
<td>CeA</td>
<td>4</td>
<td>52.71 (25.34)</td>
<td>57.96</td>
<td>1.62</td>
<td>3.04</td>
<td>2.62 (0.76)</td>
<td>1.60</td>
<td>0.76</td>
<td>-1.77</td>
</tr>
<tr>
<td>BA</td>
<td>4</td>
<td>18.50 (5.11)</td>
<td>11.27</td>
<td>-0.24</td>
<td>-2.86</td>
<td>2.67 (0.60)</td>
<td>1.46</td>
<td>-0.68</td>
<td>1.70</td>
</tr>
</tbody>
</table>

\(^a\) corticosterone level = 18.63 ng/ml, \(^b\) n(sections) = 2
Table 5

Descriptive statistics for Rat 2: The distribution density of GR-like spots and MR-like spots per 1000µm³

<table>
<thead>
<tr>
<th></th>
<th>Genomic</th>
<th>Colocalised</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N_images</td>
<td>M (SD)</td>
</tr>
<tr>
<td><strong>Glucocorticoid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor⁹</td>
<td>40</td>
<td>38.04 (24.21)</td>
</tr>
<tr>
<td>LA</td>
<td>13</td>
<td>33.36 (18.29)</td>
</tr>
<tr>
<td>CeA</td>
<td>13</td>
<td>62.82 (19.77)</td>
</tr>
<tr>
<td>BA</td>
<td>14</td>
<td>19.39 (8.82)</td>
</tr>
<tr>
<td><strong>Mineralocorticoid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor⁸</td>
<td>34</td>
<td>23.84 (18.98)</td>
</tr>
<tr>
<td>LA</td>
<td>11</td>
<td>14.68 (8.46)</td>
</tr>
<tr>
<td>CeA</td>
<td>11</td>
<td>42.09 (21.97)</td>
</tr>
<tr>
<td>BA</td>
<td>12</td>
<td>15.51 (9.17)</td>
</tr>
</tbody>
</table>

⁹corticosterone level = 29.55 ng/ml, ⁸n(sections) = 7, ⁷n(sections) = 7
Table 6

*Descriptive statistics for Rat 3: The distribution density of GR-like spots and MR-like spots per 1000µm<sup>3</sup>*

<table>
<thead>
<tr>
<th></th>
<th>Genomic</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N&lt;sub&gt;images&lt;/sub&gt;</td>
<td>M (SD)</td>
<td>Range</td>
<td>Skewness</td>
<td>Kurtosis</td>
<td>M (SD)</td>
<td>Range</td>
</tr>
<tr>
<td>Glucocorticoid Receptor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29</td>
<td>14.40 (9.62)</td>
<td>40.55</td>
<td>1.35</td>
<td>1.99</td>
<td>3.08 (0.91)</td>
<td>3.81</td>
</tr>
<tr>
<td>LA</td>
<td>10</td>
<td>10.65 (5.35)</td>
<td>14.91</td>
<td>0.57</td>
<td>-1.18</td>
<td>3.03 (0.89)</td>
<td>2.40</td>
</tr>
<tr>
<td>CeA</td>
<td>9</td>
<td>24.48 (9.89)</td>
<td>31.56</td>
<td>0.89</td>
<td>0.48</td>
<td>3.68 (0.79)</td>
<td>2.56</td>
</tr>
<tr>
<td>BA</td>
<td>10</td>
<td>9.07 (4.85)</td>
<td>14.35</td>
<td>0.79</td>
<td>-0.13</td>
<td>2.60 (0.77)</td>
<td>2.61</td>
</tr>
<tr>
<td>Mineralocorticoid Receptor&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40</td>
<td>39.19 (32.26)</td>
<td>135.77</td>
<td>1.63</td>
<td>2.55</td>
<td>2.23 (0.78)</td>
<td>3.04</td>
</tr>
<tr>
<td>LA</td>
<td>13</td>
<td>24.10 (19.22)</td>
<td>58.95</td>
<td>0.98</td>
<td>-0.05</td>
<td>2.08 (0.46)</td>
<td>1.42</td>
</tr>
<tr>
<td>CeA</td>
<td>14</td>
<td>58.47 (39.03)</td>
<td>115.74</td>
<td>1.28</td>
<td>0.22</td>
<td>2.07 (0.71)</td>
<td>2.67</td>
</tr>
<tr>
<td>BA</td>
<td>13</td>
<td>33.52 (25.44)</td>
<td>91.18</td>
<td>1.43</td>
<td>2.33</td>
<td>2.57 (1.01)</td>
<td>2.78</td>
</tr>
</tbody>
</table>

<sup>a</sup>corticosterone level = 68.82ng/ml, <sup>b</sup>n(sections) = 6, <sup>c</sup>n(sections) = 7
Genomic Corticosteroid Receptors vs. Corticosteroid Receptors at Post-Synaptic Terminals

The densities of genomic and colocalised GR and MR from rats 2 and 3 were pooled into section means (n_{sections} = 13). Two paired samples $t$ tests were performed as part of preliminary analysis (Figure 27). The first paired samples $t$ test was performed on the densities of colocalised GR spots and colocalised MR spots. A statistical difference was found $t(12) = 4.74, p < .001, 95\% \ CI [0.62, 1.97]$ between the densities of colocalised GR and MR, where colocalised GR ($M = 3.19, SD = 0.85$) had a significantly higher density than colocalised MR ($M = 2.04, SD = 0.52$). The second paired samples $t$ test was performed between the densities of genomic GR spots and genomic MR spots. No significant difference was found between the densities of genomic GR spots ($M = 26.77, SD = 16.27$), and genomic MR spots ($M = 27.89, SD = 14.10$), $t(12) = -0.18, p = .858$. 

![Section Average](image)
Genomic GR spot density was compared between amygdala subregions. The number of genomic GR spots were adjusted by the volume of DAPI-surfaces and multiplied by 1000 (genomic spots per 1000\(\mu\)m\(^3\) of DAPI). For each section, the mean density for each amygdala subregion was calculated (\(n_{\text{rats}} = 3\)). A repeated measures, one-way analysis of variance (ANOVA) was performed on the amygdala subregions (LA vs. CeA vs. BA) from 12 sections. The CeA had the highest density of genomic GR (\(M = 256.21, SD = 89.61\)), followed by the LA (\(M = 159.45, SD = 87.19\)), and finally the BA (\(M = 127.15, SD = 59.09\)). Mauchley’s test of sphericity was not violated (\(p = .721\)) and sphericity was assumed. A significant difference was found between amygdala subregions, \(F(2, 22) = 21.88, p < .001\), partial \(\eta^2 = .665\). Pairwise comparisons (Bonferroni adjustment) revealed that the CeA had a significantly higher density of genomic GR spots compared to the LA (\(p = .004\)), and the BA (\(p < .001\)).

We conducted some preliminary analyses to see if there was a difference in genomic MR spot density between amygdala subregions (\(n_{\text{rats}} = 2\)). Density of MR spots was also represented as spots per 1000\(\mu\)m\(^3\) of DAPI, and the densities for each subregion were averaged for each section (\(n_{\text{section}} = 13\)). Missing values resulted in the attrition of one section. A one-way repeated measures ANOVA was performed on amygdala subregions (LA vs. CeA vs. BA), where the CeA had the highest density of genomic MR spots (\(M = 272.61, SD = 139.61\)) followed by the BA (\(M = 195.25, SD = 119.70\)), and the LA (\(M = 129.49, SD = 68.35\)). Assumption of sphericity was met (\(p = .206\)), and a significant difference was found \(F(2, 24) = 8.55,\)
Pairwise comparisons (Bonferonni adjustment) showed a significant difference between the densities in the CeA and LA subregions ($p = .001$).

The mean densities for genomic GR and MR spots are depicted in Figure 28.

![Figure 28](image)

**Figure 28.** Mean density of receptor spots located within DAPI surfaces, adjusted by the surface volume. Light grey bars represent density of GR spots, per amygdala subregion. Dark grey bars represent density of MR spots, per amygdala subregion. Error bars represent ±1 SEM.

**Proportion of Synapses that contain Corticosteroid Receptors**

Next, we calculated the volumetric density of PSD-95 spots in GR and MR sections (dark bars in Figure 29). In GR sections ($n_{rats} = 3$), PSD-95 had a mean density (standard deviation in parentheses) of 34.45 (10.39) spots per $1000\mu m^3$. The mean density of PSD-95 spots was higher in MR sections ($n_{rats} = 2$), with a mean density of 40.97 (18.35) spots per $1000\mu m^3$. 

$p = .002$, partial $\eta^2 = .416$.
The proportion of post-synaptic sites containing corticosteroid receptors, per 1000µm\(^3\), was calculated by dividing the number of colocalised spots by the number of PSD-95 spots (Bolte & Cordelières, 2006). When multiplied by 100, this provided an indication of the percentage of PSD-95 spots that contained GR or MR spots. GR sections had a higher percentage of PSD-95 spots that contained GR spots was 9.28% (2.86), compared with MR sections where the number of PSD-95 spots that contained MR was 5.27% (2.17).

![Bar chart showing the mean volumetric density of PSD-95 spots and colocalised spots for GR and MR sections.](image)

**Figure 29.** Amount of PSD-95 spots that contain colocalised GR or MR spots. The mean volumetric density of PSD-95 spots for GR or MR sections is indicated by the black bars. The mean volumetric density of colocalised GR or MR spots is indicated by light bars.

A preliminary analysis was performed in order to see if there was any difference between amygdala subregions. Two, one-way repeated measures ANOVAs were performed: one for GR sections; and one for MR sections. For each
section, the average proportion was calculated for each amygdala subregion, and subregions were compared within sections.

For GR sections, analysis was performed on 12 sections (n_{rats} = 3), with an attrition of three sections due to missing values. A one-way repeated measures ANOVA conducted on amygdala subregions (LA vs. CeA vs. BA). The CeA had the highest degree of colocalisation (M = 11.75, SD = 3.33), followed by the LA (M = 8.88, SD = 1.87), and then the BA (M = 8.46, SD = 1.91); displayed in Figure 30. Mauchly’s test of sphericity was not violated, p = .106, and sphericity was assumed. The ANOVA revealed a significant difference between brain regions, F(2, 24) = 11.12, p < .001, partial η² = .503. Pairwise comparisons (Bonferroni adjustment) showed that the mean proportion of PSD-95 spots that contain GR spots was significantly higher in the CeA compared to the LA (p = .006), and in the CeA compared to the BA (p = .016).

Figure 30. Bar graph depicting the proportion of extra-nuclear GR spots that were colocalised with PSD-95 spots, within each brain region. Error bars represent ±1 SEM.

For MR sections, a one-way repeated measures ANOVA was also conducted on amygdala subregions (LA vs. CeA vs. BA) to determine the proportion of PSD-95
spots that were colocalised with MR-spots. Analysis was performed on 13 sections (n<sub>rats</sub> = 2), after missing values lead to the attrition of one section. Descriptive statistics showed that the CeA had the highest degree of colocalisation (M = 5.62, SD = 2.33), followed by the BA (M = 5.17, SD = 1.50), and finally the LA (M = 4.70, SD = 2.16); displayed in Figure 31. One-way repeated measures ANOVA was performed on amygdala subregions (LA vs. CeA vs. BA) to see if there was a difference in the number of PSD-95 spots that were colocalised with MR spots. Assumption of sphericity was not violated (p = .717). There were no significant differences found in proportion of PSD-95 spots containing MR spots, between amygdala subnuclei, F(2, 26) = 1.72, p = .199.

![Bar graph depicting the proportion of extra-nuclear GR spots that were colocalised with PSD-95 spots, within each brain region. Error bars represent ±1 SEM.](image)

*Figure 31.* Bar graph depicting the proportion of extra-nuclear GR spots that were colocalised with PSD-95 spots, within each brain region. Error bars represent ±1 SEM.
Chapter 5: Corticosteroid Receptor Densities in the Amygdala
Chapter 6: Discussion

The dual system of GR and MR have also been shown to have two further subpopulations that have different temporal domains; fast and slow (Joels et al., 2013). Fast corticosteroid receptors have been located at the membrane of synapses, whereas slow corticosteroid receptors are located within the cytoplasm and nuclei of neurons (Prager & Johnson, 2009). It is currently unknown whether there is a structural difference between the two subpopulation of corticosteroid receptors (Prager & Johnson, 2009). As such, there are fewer methods available to delineate the subpopulations of these receptors; limiting the available data (Groeneweg, 2014).

Previous studies have used electron microscopy (L. R. Johnson et al., 2005; Komatsuzaki et al., 2012; Prager et al., 2010) or cell fractionation (Moutsatsou et al., 2001) to quantify these subpopulations, however the data around the distribution of membrane-associated corticosteroid receptors is still emerging. In this study, we used confocal imaging and colocalisation analysis to characterise the subpopulations of GR and MR, as confocal imaging has a larger field of view than electron microscopy (Bacallao et al., 1995), and better spatial resolution than fractionation assays (Ahima & Harlan, 1990).

Recent advances in technology have introduced more accurate and reliable colocalisation techniques (Zinchuk & Grossenbacher-Zinchuk, 2009). One such technique, developed by Fogarty et al. (2013), used 3D reconstruction in Imaris (Bitplane) for more accurate object-based colocalisation analysis. In this study we aimed to test the feasibility of adapting Fogarty’s approach of 3D reconstruction to quantifying corticosteroid receptors at the excitatory post-synaptic sites. Following this, we aimed to use the data obtained from 3D reconstruction to perform some
initial analyses on the distribution densities of GR and MR. The first part of this chapter discusses the feasibility of using Fogarty’s approach for characterising the distribution of GR and MR. The second part of this chapter describes the findings, and discusses how Fogarty’s approach can be further applied to future stress research.

**Applicability of 3D Reconstruction for Characterising Corticosteroid Receptors**

**GR and MR labelling can be Reconstructed as Spots**

In Chapter 5, we aimed to test the feasibility of adapting Fogarty’s approach, in order to delineate between populations of genomic corticosteroid receptors and membrane-associated corticosteroid receptors at synapses. Fogarty et al. (2013) utilised the commercially available software Imaris (Bitplane), in order to three-dimensionally reconstruct neuronal surfaces and pre- and post-synaptic terminals. In order to adapt Fogarty’s approach of spot reconstruction to corticosteroid receptors, the labelling of GR, MR, and PSD-95, needed to present as: a) well-defined spots; and b) easily distinguishable from background fluorescence (Fogarty et al., 2013). In our study, we found that PSD-95 labelling was similar to that of previous research (Fogarty et al., 2013; Soria Van Hoeve & Borst, 2010), and presented as distinct puncta scattered throughout the sections. We also found that GR- and MR-like labelling was spot-like; which was consistent with the fluorescent labelling seen in previous confocal imaging studies (Sarabdjitsingh et al., 2010; van Steensel et al., 1996), but not as distinct as PSD-95 labelling.

Using Huygen’s deconvolution software (Scientific Volume Imaging), we found that the amount of background fluorescence was reduced and the SNR was improved. Moreover, we found that after deconvolution, the distinctiveness of GR-
and MR-like structures was increased, and were more suitable for 3D reconstruction. Deconvolution was not only used to increase the resolution of the images; previous research has shown that deconvolution has been used to restore images to more closely resemble the original object (Dodt et al., 2007). Consequently, deconvolved images are less likely to result in false positives, thus improving the overall accuracy of colocalisation analysis (Zinchuk & Grossenbacher-Zinchuk, 2009).

Subsequent to performing deconvolution, we were able to reconstruct GR, MR and PSD-95 structures as spot objects, and perform colocalisation using the nearest neighbour approach for object-based colocalisation (Lagache et al., 2015). Object-based methods of colocalisation have been shown to be more accurate and statistically robust compared to traditional pixel based methods (Lagache et al., 2015). While pixel based methods are widely used (Dunn et al., 2011), they have reduced resolution compared to object-based methods, and the reduced resolution increases the likelihood of false positives (Bolte & Cordelières, 2006; Lagache et al., 2015). Alternatively, object-based methods plot the spatial coordinates of labelled structures within an image. As such, object-based methods have added resolution along the z-axis improving the overall accuracy of quantification (Lagache et al., 2015). Much of the literature reviewed used pixel-based methods of colocalisation (Han et al., 2014; L. R. Johnson et al., 2005; Kil & Kalinec, 2013; van Steensel et al., 1996), and this is the first study that uses object-based colocalisation analysis to investigate the distribution of GR and MR at post-synaptic sites in the amygdala.

While this study used object-based analysis to characterise the colocalisation of GR and MR at excitatory post-synaptic terminals, this approach could be adapted to characterising GR and MR within other types of synapses. For example, GR and MR have also been located within the membrane at pre-synaptic sites (Komatsuzaki
et al., 2012; Prager et al., 2010). By using a marker for pre-synaptic component (e.g. anti-VGLUT2; Fogarty et al., 2013), this method could be used to characterise GR and MR that are located at pre-synaptic sites. Moreover, the semi-automated nature of this approach allows for increased throughput, and the use of computer algorithms standardises image treatment (Zinchuk & Grossenbacher-Zinchuk, 2009), and provides more consistent quantification (Lagache et al., 2015).

The reconstruction of spots for colocalisation, however, is dependent on consistent spot-like labelling of GR and MR. In previous literature, GR and MR labelling does not always present as puncta. For example, GR and MR labelling in amygdala neurons have been shown to have increased cytoplasmic distribution after stress (Han et al., 2014), and labelling appeared more homogenously diffused in the cytoplasm and nucleus. Moreover, hippocampal cells have also been shown to have a higher density of GR and MR compared the amygdala (Ahima et al., 1991; Caudal et al., 2014; Marlier et al., 1997; Morimoto et al., 1996). As such, GR and MR labelling in hippocampal neurons appear more homogenous and are less likely to present as well defined spots (Sarabdjitsingh et al., 2010). In such situations, it would be difficult to use Imaris 3D reconstruction to quantify genomic GR and MR, limiting 3D reconstruction to membrane-associated populations. However, homogenous labelling may also have been due to the choice of antibodies (Sarabdjitsingh et al., 2010) or the absence of light-scattering correction (Bolte & Cordelières, 2006; van Steensel et al., 1996). As a result, we strongly suggest that future studies adapting this method firstly characterise the labelling of the antibodies used, and subsequently employ reliable deconvolution techniques prior to colocalisation analysis.
Nuclei can be reconstructed as Surfaces to identify gGR and gMR populations

In their original method, Fogarty and colleagues (2013) originally created neuronal surfaces, which we adapted, to create nuclei surfaces. We used DAPI to stain the nuclei within the amygdala, and subsequently reconstructed the DAPI-labelling as surface objects. This allowed us to demarcate between the populations of intra-nuclear corticosteroid receptors from extra-nuclear corticosteroid receptor populations. Additionally, the nuclei surfaces allowed us to quantify the amount of genomic corticosteroid receptor spots, and subsequently compare its distribution density with corticosteroid receptors spots located at excitatory-post-synaptic terminals (Figure 32).

![Figure 32. Image of genomic and colocalised GR and MR spots after 3D reconstruction in Imaris. Scale bar: 5µm](image)

In this study, however, we did not distinguish between the types of nuclei we were labelling. As glial cells have also been found to contain GR and MR (Matsusue et al., 2014), it was possible that we were also quantifying genomic GR and MR within glia. Future studies can control for this by using fluorescent markers specific
to neurons, and additionally recreate neuronal membrane surfaces. Alternatively, researchers could also adapt this approach to study the populations of GR and MR within various glial cells as GR and MR in glia have been implicated in various pathologies. In mice tissue, the BLA-complex has been demonstrated to express GR, but not MR in oligodendrocytes, which may have implications for neurological pathologies such as multiple sclerosis (Matsusue et al., 2014). Additionally (as stated in Chapter 2), patients with depression have shown increased GR populations in astrocytes in the LA, BA and CeA, compared to healthy controls (Q. Wang et al., 2014). Our method of immunofluorescence and 3D reconstruction for colocalisation could be utilised in future research, to further investigate the role of GR and MR in glial cells, which is still poorly understood.

**Distribution Densities of GR and MR**

**Distribution of gGR and gMR in Amygdala Subnuclei**

The distribution of receptors have been shown to follow the same pattern in corticosteroid binding (Reul et al., 1987), and understanding the distribution density within the amygdala could further our understanding of the stress response. As such, volumetric quantification was performed using Fogarty’s approach, which allowed us to compare the relative distribution densities between GR and MR. Genomic GR and MR are responsible for the slow effects of stress (Prager & Johnson, 2009), and are expressed in high density in the amygdala (Reul & de Kloet, 1985). In our study, we found that genomic GR was found to have the highest density within the CeA with minimal differences in density between the LA and BA. This was consistent with previous light microscopy studies (Ahima & Harlan, 1990; Ahima et al., 1991) where the highest density of GR-immunoreactivity was within CeA neurons, while
LA and BA neurons had similar levels of immunoreactivity. With respect to genomic MR spots, the CeA showed the highest density, followed by the BA and then the LA. This was somewhat consistent with previous research where Ahima et al. (1991) found that the CeA had the highest amount of MR immunoreactive neurons, followed by a moderate density in the LA, and the lowest density in the BA. It should be noted that the distribution density of MR has shown mixed results, although the CeA is consistently shown to have the highest density (Morimoto et al., 1996), which is consistent with our findings. Additionally, mosaic images increased the confidence that the images used in analysis were sampled from the correct subnuclei. However, due to low power of the current feasibility study, generalisability of the results is limited. On the other hand, preliminary analysis appears to be mostly consistent with previous research, which further supports the feasibility of using 3D reconstruction in Imaris for quantification.

We found that genomic receptors had a much higher density compared with the receptors colocalised with PSD-95; with nearly ten times as many spots per cubic micron. The high density of genomic receptors was also consistent with previous research. Light microscopy studies have shown that GR and MR immunoreactivity was strongest in the nuclei of neurons, with weak labelling in the cytoplasm (Ahima & Harlan, 1990; Ahima et al., 1991). Confocal imaging has shown similar results, where the strongest labelling was observed within the nucleus, and weak labelling was seen within the cytoplasm of neurons (Han et al., 2014; van Steensel et al., 1996). Thus, the density of reconstructed spots was also consistent with the labelling seen in previous research, providing further support for the feasibility of the approach.
**Distribution of Genomic and Colocalised Corticosteroid Receptors**

Preliminary analyses did not show a statistically significant difference between genomic GR and MR within the amygdala. This was consistent with previous research that tested the receptor balance hypothesis. For example, Caudal et al. (2014) investigated the ratio of GR:MR in the amygdala and found that the nuclear levels of GR and MR were quite similar in rat brain homogenates. This was further corroborated by Han et al. (2014), who showed that the neurons in the amygdala of stress-naïve rats displayed a similar level of expression of GR and MR. Our preliminary analyses did suggest, however, that there could be a statistically significant difference between the densities of GR and MR located at the PSD. In the amygdala, we found a higher density of GR spots located at excitatory postsynaptic terminals compared to MR. Studies testing the receptor balance hypothesis have suggested that the amygdala is not sensitive to changes in the GR:MR ratio (Caudal et al., 2014; Han et al., 2014; Harris et al., 2013). These studies, however, either did not demarcate between genomic or membrane-associated receptors, or alternatively focused on the GR:MR ratio between genomic receptors (de Kloet, 2014). The high density of genomic receptors could potentially mask the effects of membrane-associated populations, which have a central role in our acute response to stress (Quaedflieg et al., 2015; Vogel et al., 2014). Moreover, mGR and mMR have less transient effects in the amygdala compared to mGR and mMR in the hippocampus (Sarabdjitsingh & Joels, 2014; Sarabdjitsingh et al., 2012), which is an area that is sensitive to the receptor balance hypothesis (de Kloet et al., 2008). This, in conjunction with our findings, could provide a new direction for research investigating the receptor balance hypothesis in the amygdala. The ratio between
mGR:mMR could add a new dimension to the receptor balance hypothesis and further our understanding of the stress system.

**Proportion of Excitatory Post-synaptic Terminals Containing Corticosteroid Receptors**

In the amygdala, excitatory inter- and intra-cellular pathways are activated during stress (Z. Liu et al., 2014; Maras, 2014). As a result, we were interested in examining the proportion of excitatory post-synaptic terminals containing corticosteroid receptors in the amygdala. Moreover, previous electron microscopy studies have shown that while some excitatory synapses contain mGR and mMR, the overall distribution is unclear (L. R. Johnson et al., 2005; Prager et al., 2010). In this study, we used Fogarty’s approach to perform some preliminary analyses on the amount of excitatory post-synaptic terminal that contained GR or MR. In the amygdala, we found that the proportion of excitatory post-synaptic terminals containing GR was around 9.28%, nearly twice as high the amount of excitatory post-synaptic terminals containing MR (~5.27%). This was interesting as activation of mMR in the amygdala has been shown to increase neuronal excitability at post-synaptic sites, whereas mGR has been shown to attenuate neuronal excitability (Karst et al., 2010; Sarabdjitsingh et al., 2012). Additionally, mGR appears to have a larger functional role upon repeated exposures to stress (Karst et al., 2010), as opposed to the animals used in this study, which were stress-naïve. As yet, it is unknown how the densities of mGR and mMR at synapses translate to functional differences; however, the method detailed in this thesis could be applied to future studies to better understand this.

Despite being part of the amygdala, the LA, CeA and BA differ both structurally and functionally (Sah et al., 2003; Sah & Lopez de Armentia, 2003). As
such, we were also interested to see whether the proportion of GR and MR at excitatory post-synaptic sites differed within the different amygdala subnuclei. Our results, while tentative, suggested that the CeA contained a higher proportion of excitatory post-synaptic terminals containing GR compared with the LA and the BA. But no statistically significant difference between amygdala subregions was found for post-synaptic terminals containing MR. These results were interesting, as previous electrophysiological studies have shown that the application of corticosterone affects excitatory post-synaptic currents in the BA, but not the CeA (Karst et al., 2010). However, the CeA, while containing a high density of inhibitory interneurons, also receives many excitatory inputs from the LA and BA (Sah et al., 2003; Sah & Lopez de Armentia, 2003). In addition, CeA knockout studies have shown that GRs have a large role in modulating neuronal excitability compared with MR (A. C. Johnson & Meerveld, 2015; Kolber et al., 2008). Corticosteroids in the CeA affect the behavioural output in stressful situations (Kolber et al., 2008; Roozendaal, 2000; Simms et al., 2012) and further delineating region-specific differences could have further functional implications.

Due to the small sample size of this study, however, further research is needed to corroborate these results. Nonetheless, we were able to use Fogarty’s approach to demonstrate the distribution densities of genomic GR and MR in fixed amygdala sections – which was in line with the available literature (Ahima & Harlan, 1990; Ahima et al., 1991; Morimoto et al., 1996; Reul & de Kloet, 1985; Reul et al., 1987). Using 3D reconstruction for object-based colocalisation analysis, we were also able to see the distribution densities of GR and MR located at excitatory post-synaptic sites. In future studies, PSD-95 markers could be substituted with other subcellular labels. Moreover, this approach can be used to visualise the effects of
behavioural or drug interventions. The wide applicability of this approach can be used to provide a better resolution of the functional role of membrane-associated GR and MR in the stress response.
Chapter 7: Conclusions

While there have been previous studies characterising the neuronal distribution of corticosteroid receptors, this was the first study to test feasibility of using 3D immunofluorescent reconstruction to characterise and quantify GR and MR at synaptic sites. In this thesis I aimed to test the feasibility of adapting Fogarty’s approach to visualising mGR and mMR at excitatory post-synaptic terminals in fixed amygdala sections. As stated in Chapter 1, the thesis objectives were as follows:

1. Validate the antibodies and reagents from the electron-microscope studies conducted by L. R. Johnson et al. (2005) and Prager et al. (2010), for use in multiple labelling immunofluorescence.

2. Adapt Fogarty’s approach to investigate the distribution of GR and MR in nuclei and at excitatory post-synaptic terminals.

3. Quantify the balance between these receptors in stress-naïve animals in order to provide a foundation for future studies into stress.

Reagent and Protocol Validation

In Chapter 4, we established a standardised protocol for confocal imaging. We used primary antibodies that had demonstrated sensitivity and specificity to their respective proteins (detailed in Chapter 3), and optimised their concentrations for fluorescent imaging. We also established the specificity of the fluorescent labels for each primary antibody and developed a protocol which was used in for 3D reconstruction and colocalisation analysis in Chapter 5. We also found that PSD-95- and GR-like labelling met the criterion for 3D spot reconstruction. MR-like puncta were not as well defined; however, we corrected for in Chapter 5. Using triple-
labelling immunofluorescence, we found that GR and MR were located within the DAPI-stained nuclei, and also appeared to be colocalised with PSD-95 which was consistent with previous electron microscopy studies (L. R. Johnson et al., 2005; Prager et al., 2010). In our design we varied GR- and MR-labelling between sections, but as GR and MR are largely colocalised within neurons (Han et al., 2014; van Steensel et al., 1996), characterising the distribution of GR and MR puncta within the same sample could be another application for this technique; although different primary antibodies would need to be used. As antibody labelling can vary (Sarabdjitsingh et al., 2010), future studies using different reagents should be optimised accordingly, to ensure that labelling meets the criteria for 3D spot reconstruction (Fogarty et al., 2013).

3D reconstruction of Corticosteroid Receptors

In Chapter 5, we applied the protocol established in Chapter 4, to further characterise the distribution of GR and MR labelling in the amygdala. We observed puncta labelling of receptors and PSD-95 in the raw image, although the quality of the puncta labelling improved after deconvolution. The distinctiveness of the puncta labelling subsequent to deconvolution techniques was in line with previous research; improving the reliability of colocalisation analysis (Dodt et al., 2007; Zinchuk & Grossenbacher-Zinchuk, 2009). As such, it is strongly recommended that deconvolution techniques are used in future studies employing this technique.

From the fluorescent labels that were imaged, DAPI-labelling was three-dimensionally recreated as neuronal surfaces, and puncta-labelling was recreated as spots. We found that re-creation of DAPI-surfaces allowed us to view and quantify the spatial distribution of genomic GR and MR. The presentation of labelling, coupled with preliminary analysis, suggested that the distribution density of genomic
spots was in accordance with previous literature (Ahima & Harlan, 1990; Ahima et al., 1991; van Steensel et al., 1996).

We also found that GR and MR spots were colocalised with PSD-95 spots. Subsequent to 3D reconstruction we were able to perform object-based colocalisation analysis. This allowed us to perform preliminary analyses on the subpopulations of GR and MR located at excitatory post-synaptic sites. Our findings indicated that there could potential be a higher amount of GR colocalised with PSD-95 compared to MR. As synaptic GR and MR in the amygdala have been implicated in the maintenance of maladaptive cognitions and behaviours (Karst et al., 2010), it is possible that the amygdala may be sensitive to differences between ratios of mGR and mMR, and the high density of genomic receptors mask this effect. However, due to the limited power of this study, these findings are still tentative. Future studies employing 3D reconstruction for object-based colocalisation are needed to confirm this.

Finally, we found that the approach developed here was feasible for investigating the distribution densities of GR and MR. We conclude, based on our initial quantifications, that this approach can be used in future studies to directly test for differences within the different amygdala subnuclei. Our results suggested that there could be a higher amount of GR at excitatory post-synaptic sites in the CeA. As the CeA is responsible for behavioural responses to stressful stimuli (Kolber et al., 2008; Roozendaal, 2000; Simms et al., 2012), further delineating these differences could have implications for various stress related pathologies (Hermans et al., 2014).

Stress can cause acute changes to cognition and behaviour (Groeneweg et al., 2012), and chronic stress can even lead to the development of various pathologies.
At a cellular level, a balance between the populations of GR and MR appear important for maintaining homeostasis (de Kloet, 1991), and a disruption to these corticosteroid receptors have been implicated in a variety of maladaptive behaviours (Harris et al., 2013). Further understanding the role of GR and MR at the synapse, may help us better understand stress. In this thesis, we tested a relatively novel method for characterising these receptors; with the hopes of providing a robust technique that can be adapted to various forms of stress research to better understand the role of these receptors in stress.
References


doi:10.1016/j.psyneuen.2012.08.007

synthesis, distributed synaptic plasticity and fear conditioning. *Neurobiology  

Helsby, M. A., Fenn, J. R., & Chalmers, A. D. (2013). Reporting research antibody  
use: How to increase experimental reproducibility. *F1000Research, 2*(153),  
1-11. doi:10.12688/f1000research.2-153.v2

dependent effects of cortisol on selective attention and emotional  
interference: A functional MRI study. *Frontier in Integrative Neuroscience,  

Herman, J. P. (1993). Regulation of adrenocorticosteroid receptor mRNA expression  
in the central nervous system. *Cellular and Molecular Neurobiology, 13*(4),  
349-372. doi:10.1007/BF00711577

Herman, J. P., Figueiredo, H., Mueller, N. K., Ulrich-Lai, Y., Ostrander, M. M.,  
integration: Hierarchical circuitry controlling hypothalamo–pituitary–  
doi:10.1016/j.yfrne.2003.07.001

receptor gene transcription and protein expression in vivo. *The Journal of  
Neuroscience, 18*, 7462-7473.

Roozendaal, B. (2014). How the amygdala affects emotional memory by


doi:10.1016/j.psyneuen.2008.09.003


doi:10.1210/endo-122-4-1343#sthash.6pZnO2mx.dpuf
Jin, J., & Maren, S. (2015). Fear renewal preferentially activates ventral hippocampal neurons projecting to both amygdala and prefrontal cortex in rats. *Scientific Reports*, 5(8388), 1-5. doi:10.1038/srep08388


Comparative Neurology, 395, 53-72. doi:10.1002/(SICI)1096-9861(19980525)395:1<53::AID-CNE5>3.0.CO;2-G


Klok, M. D., Van der Does, A. J. W., Geleijnse, J. M., Antypa, N., Penninx, B. W. J.
mineralocorticoid receptor haplotype enhances optimism and protects against
depression in females. *Translational Psychiatry, 1*, e62.
doi:10.1038/tp.2011.59

Muglia, L. J. (2008). Central amygdala glucorticoid receptor action promotes
doi:10.1073/pnas.0803216105

Kawato, S. (2012). Corticosterone induces rapid spinogenesis via synaptic
doi:10.1371/journal.pone.0034124

Kumar, G., Couper, A., O’Brien, T. J., Salzberg, M. R., Jones, N. C., Rees, S. M., &
Morris, M. J. (2007). The acceleration of amygdala kindling epileptogenesis
by chronic low-dose corticosterone involves both mineralocorticoid and
glucocorticoid receptors. *Psychoneuroendocrinology, 32*, 834-842.
doi:10.1016/j.psyneuen.2007.05.011

Lachmanovich, E., Shwartsman, D. E., Malka, Y., Botvin, C., Henis, Y. I., & Weiss,
membrane proteins by computerized fluorescence microscopy: Application to
immunofluorescence co-patching studies. *Journal of Microscopy, 212*(2),
122-131. doi:10.1046/j.1365-2818.2003.01239.x


Australian code for the care and use of animals for scientific purposes, (2013).


. PHERAsystem FS spectrophotometer. [Software]. Offenburg, Germany: BMG Labtech.


Simms, J. A., Haass-Koffler, C. L., Bito-Onon, J., Li, R., & Bartlett, S. E. (2012). Mifepristone in the central nucleus of the amygdala reduces yohimbine...


Yang, Y. L., Chao, P. K., Ro, L. S., Wo, Y. P., & Lu, K. T. (2007). Glutamate NMDA receptors within the amygdala participate in the modulatory effects of glucocorticoids on extinction of conditioned fear in rats. *Neuropsychopharmacology, 32*, 1042-1051. doi:10.1038/sj.npp.1301215


# Appendix A

## Antibody Details

Table 1

*Details of Antibodies Used*

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Manufacturer</th>
<th>Country</th>
<th>Catalogue Number</th>
<th>Lot Number</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>BuGR2</td>
<td>Thermofisher Scientific</td>
<td>Australia</td>
<td>PIEMA1-510</td>
<td>P1200288</td>
<td>1mg/ml</td>
</tr>
<tr>
<td>rMR-1D5</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>IA, USA</td>
<td>rMR1-18 1D5</td>
<td>6/5/14-516ug/M1g</td>
<td>0.1ml</td>
</tr>
<tr>
<td>PSD-95 Antibody</td>
<td>Life Technologies</td>
<td>VIC, Australia</td>
<td>51-6900</td>
<td>QA213131</td>
<td>0.25mg/ml</td>
</tr>
<tr>
<td>Goat Anti-mouse IgG H&amp;L (Alexa Fluor 488)</td>
<td>Abcam</td>
<td>VIC, Australia</td>
<td>A8150113</td>
<td>-</td>
<td>2.00mg/ml</td>
</tr>
<tr>
<td>Goat Anti-rabbit IgG H&amp;L (Alexa Fluor 594)</td>
<td>Abcam</td>
<td>VIC, Australia</td>
<td>A8150084</td>
<td>-</td>
<td>1.96mg/ml</td>
</tr>
<tr>
<td>4',6- diamidino-2-phenylindole, dihydrochloride (DAPI)</td>
<td>Life Technologies</td>
<td>VIC, Australia</td>
<td>D1306</td>
<td>1711782</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Acquisition Parameters

Table 2

*Acquisition Parameters of Images with Olympus FV1200*

<table>
<thead>
<tr>
<th></th>
<th>Solid State Laser</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD 405nm (50nw)</td>
</tr>
<tr>
<td>Laser Power</td>
<td>0.9%</td>
</tr>
<tr>
<td>HV</td>
<td>855</td>
</tr>
<tr>
<td>Offset</td>
<td>1</td>
</tr>
<tr>
<td>Gain</td>
<td>37</td>
</tr>
</tbody>
</table>
Appendix C

Nyquist Sampling Parameters

Table 3

*Nyquist Sampling Parameters from Scientific Volume Imaging (n.d.)*

<table>
<thead>
<tr>
<th>Microscopy Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope Type</td>
<td>Confocal</td>
</tr>
<tr>
<td>Numerical Aperture</td>
<td>1.35</td>
</tr>
<tr>
<td>Excitation Wavelength</td>
<td>488</td>
</tr>
<tr>
<td>Emission Wavelength</td>
<td>520</td>
</tr>
<tr>
<td>Number of Excited Photons</td>
<td>1.0</td>
</tr>
<tr>
<td>Lens Immersion Refractive Index</td>
<td>Oil, 1.515</td>
</tr>
</tbody>
</table>


Retrieved from: https://svi.nl/NyquistCalculator
Appendix D

Ethics

Animal Ethics Approval Certificate

Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details:
Chief Investigator: Dr Luke Johnson
Title: Sympathetic properties of glucocorticoid and mineralocorticoid receptors
AEC Approval Number: QUT TRJ/262/15/1HBI
Previous AEC Number: QUT TRJ/115/15/
Approval Duration: 29-Jun-2015 to 29-Jun-2018
Funding Body: Health Sciences
Other Staff/Student: Stephanie Koo, Lisa Craig, Rebecca Davies, Andrew Battle, Kim Bitman
Location(s): PA Hospital Translational Research Institute (TRI)

Summary

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>Strain</th>
<th>Class</th>
<th>Gender</th>
<th>Source</th>
<th>Approved</th>
<th>Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats - non genetically modified</td>
<td>Sprague Dawley</td>
<td>Adults</td>
<td>Male</td>
<td>Commercial breeding colony</td>
<td>$</td>
<td>$</td>
</tr>
</tbody>
</table>

Permit:

Proviso:

Approval Details:

<table>
<thead>
<tr>
<th>Description</th>
<th>Amount</th>
<th>Balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats - non genetically modified (Sprague Dawley, Male, Adults, Commercial breeding colony)</td>
<td>$</td>
<td>$</td>
</tr>
</tbody>
</table>

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration.

Please use this Approval Number:
1. When ordering animals from Animal Breeding Houses
2. For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all project animal details be made available to Animal House OIC. (UAEC Rating 14/12/2003)

The Chief Investigator takes responsibility for ensuring all legislative, regulatory and compliance objectives are satisfied for this project.

This certificate supersedes all preceding certificates for this project (i.e. those certificates dated before 12-Jan-2016)
Appendix E

Supplementary Videos

Table 4

*Links to Supplementary Videos of 3D Objects in Imaris (Bitplane)*

<table>
<thead>
<tr>
<th>Video</th>
<th>URL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Video 1, Process of 3D Reconstruction</td>
<td><a href="https://youtu.be/9kgq69dt1ls">https://youtu.be/9kgq69dt1ls</a></td>
</tr>
<tr>
<td>Video 2, High Magnification view of 3D objects in Imaris</td>
<td><a href="https://youtu.be/cs3e4UxgAdY">https://youtu.be/cs3e4UxgAdY</a></td>
</tr>
</tbody>
</table>