The Effects of Short Term Stress on the Central and Peripheral Nitrergic System

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BSc (Hon)

A thesis submitted for the degree of Doctor of Philosophy at The University of Queensland in 2016
School of Biomedical Sciences
Abstract

The mammalian stress response is generated by several integrated neural systems that allow rapid adaptation to homeostatic disturbances. This is principally mediated through the hypothalamic-pituitary-adrenal axis which mobilises energy reserves by adrenal glucocorticoids. This in turn accelerates cellular metabolism which consequently increases free radical formation through the mitochondrial electron transport chain. Excess generation of free radicals such as reactive oxygen and nitrogen species (RNS) may potentially cause damage to fatty acids, proteins, and DNA by oxidative and nitrosative stress. Nitric oxide is a ubiquitous signalling molecule belonging to the family of RNS and is endogenously synthesised from l-arginine by nitric oxide synthase (NOS). Three distinct subtypes of NOS have been identified, with the neuronal and endothelial isoforms being constitutively expressed enzymes responsible for synaptic signalling and smooth muscle relaxation. The inducible isoform is primarily present in cells of the inflammatory immune system where its activity is transcriptionally induced by cytokines and other inflammatory agents and thus, is thought to play an important role in immunomodulation. Recently, a number of studies have demonstrated that the nitrergic system have significant implications in neuropsychiatric disorders including anxiety and depression. Specifically, chronic stress-induced hippocampal neuronal NOS upregulation is responsible for glucocorticoid receptor downregulation, a factor linked to the development of depressive disorders. Although stress and nitrergic signalling are intrinsically linked in pathological states, little is known about the acute physiology between these two systems. Therefore, the primary aim of this thesis was to establish the profile of nitrosative changes in peripheral blood and brain regions known to be impacted by stress following an acute psychological challenge. The observed changes were subsequently investigated by employing a pharmacological intervention using a novel stress-alleviating and anti-inflammatory endocannabinoid enhancer.

To achieve the aims for this thesis, Male Wistar rats aged 5-7 weeks postnatal were maintained under control conditions or subjected to acute stress. Blood samples were collected at time-points immediately before, during, and following treatment in order to estimate the levels of stress hormones and redox parameters including oxidative/nitrosative status, glutathione and glutathione disulphide ratio, and lipid peroxidation. Post mortem neural tissue was collected and specific brain regions were isolated for the determination of nitrosative status, NOS enzymatic activity, and relative gene expression of stress and nitrergic-related genes. Following the establishment of these measurements, a separate group of animals was used to examine the effects of endocannabinoid modulation on the nitrergic and inflammatory-related indicators following acute stress.
We found that exposure to a single episode of psychological stress causes early and marked changes to both oxidative and nitrosative status sufficient to induce oxidative damage in peripheral blood. Within the hippocampus, there was a rapid increase in levels of nitric oxide due to an initial and transient increase in the activity of calcium-dependent constitutive NOS following acute stress. Transcriptional activation of inducible NOS following stress resulted in sustained elevations in nitric oxide within the hippocampus. In comparison, there was a delayed stress-induced increase in striatal nitrosative status which was mainly driven by the inducible isoform. Furthermore, preventing endocannabinoid hydrolytic degradation with a fatty acid amide hydrolase inhibitor, PF-3845, successfully suppressed hippocampal inducible NOS upregulation and the associated neuroinflammation following acute stress exposure.

In conclusion, this thesis has established an acute profile of stress-induced central and peripheral nitrergic activation. By demonstrating changes in the oxidative and nitrosative systems, and linking these to indices of cellular oxidative damage, it has confirmed the involvement of the nitrergic system in the rapid redox response to stress. Short term restraint stress also rapidly increases hippocampal nitrergic activity involving both the constitutive and inducible isoforms of NOS while striatal nitrosative status following stress is predominately inflammatory-mediated. The amelioration of these stress-induced modifications in the hippocampus via central augmentation of endogenous cannabinoid signalling could serve as an alternative treatment for stress-related disorders.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

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Publications included in this thesis


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Contributions by others to the thesis

The majority of the work presented in this thesis was completed by Hsiao-Jou Cortina Chen. The project was conceptualised by Hsiao-Jou Cortina Chen, Jereme G. Spiers, Conrad Sernia, and Nickolas A. Lavidis. Jereme G. Spiers assisted with animal treatment, redox, nitrosative, hormone, and real-time PCR assays and was involved in data interpretation, reviewing and editing of manuscripts. Stephen T. Anderson helped with the prolactin and insulin assays in Chapter III. Conrad Sernia and Nickolas A. Lavidis assisted in data interpretation and manuscript revisions.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.
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<tr>
<td>2-AG</td>
<td>2-Arachidonoyl glycerol</td>
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<tr>
<td>3-NT</td>
<td>3-Nitrotyrosine</td>
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<td>7-NI</td>
<td>7-Nitroindazole</td>
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<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-n-propylamino)tetralin</td>
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<td>ACTH</td>
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<td>Anadamide</td>
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<td>AIBN</td>
<td>Australian Institute of Bioengineering and Nanotechnology</td>
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<tr>
<td>AM</td>
<td>Adrenal medulla</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>aPT</td>
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<td>ATP</td>
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<td>Arginine vasopressin</td>
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<td>Cyclooxygenase-2</td>
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<td>2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>Cu^{2+}</td>
<td>Copper</td>
</tr>
<tr>
<td>Cu, Zn-SOD</td>
<td>Copper, zinc-superoxide dismutase</td>
</tr>
<tr>
<td>DAF-FM DA</td>
<td>4-Amino-5-methylamino-2’, 7’-difluorofluorescein diacetate</td>
</tr>
<tr>
<td>DAM-FM T</td>
<td>Trizole form of 4-amino-5-methylamino-2’, 7’-difluorofluorescein</td>
</tr>
<tr>
<td>DCF</td>
<td>Dichlorofluorescein</td>
</tr>
<tr>
<td>DCFH</td>
<td>Dichlorofluorescin</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2’, 7’-Dichlorofluorescin diacetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FAM</td>
<td>6-Carboxyfluorescein</td>
</tr>
<tr>
<td>Fe^{3+}</td>
<td>Iron</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEA</td>
<td>N-pamitoyl ethanolamine</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAM</td>
<td>Sympatho-adrenomedullary</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylyl cyclase</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-α convertase</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRIM</td>
<td>1-(2-Trifluoromethylphenyl)-imidazole</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>VIC</td>
<td>2’-Chloro-7’-phenyl-1, 4-dichloro-6-carboxyfluorescein</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
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</table>
Chapter I

Introduction
1.0 Thesis Overview

Depression is characterised by persistent dejected mood, loss of interest and concentration, decreased liveliness and low self-confidence, disturbed sleep or appetite, helplessness, and suicidal ideation. It is a chronic disorder often accompanied by symptoms of anxiety, and is a significant contributor to the global burden of disease estimated to affect 350 million people with adverse societal costs. Due to the complexity and aetiological heterogeneity of depressive disorder, it has been difficult to fully elucidate its pathophysiology and identify proper diagnostic tools and efficacious treatment. Stress is one of the major risk factors in the aetiology of psychological illnesses and is often used as a model system in basic research and clinical studies to identify neurochemical and cellular alterations underlying depression and other stress-related disorders. Nitric oxide, a free radical with physiological, as well as pathological functions, has recently been shown to mediate stress-induced depressive-like behaviour in mice. Moreover, pharmacological inhibition of nitric oxide production results in anxiolytic- and antidepressant-like effects in animal models. Genetic polymorphisms in genes encoding nitric oxide synthesizing enzymes have been suggested to contribute to the risk of developing recurrent depressive disorder. Therefore, a dysfunction of the nitrergic system has been implicated in the neuropathogenesis of both anxiety and depression. However, the delineation between physiological function and pathological insult of nitrergic system activation remains unclear. Therefore, the principle aim of this thesis was to examine the effects of acute psychological stress on the central and peripheral nitrergic system.
1.1 Neuroendocrine Responses to Stress

1.1.1 The stress systems

Living organisms survive by maintaining a complex and dynamic homeostasis that is consistently challenged by intrinsic and extrinsic factors. When faced with excessive stress, whether physical or psychological, a subject’s adaptive responses react in a relatively stereotypic and non-specific nature, referred to by Hans Selye as the ‘general adaption syndrome’ (Selye, 1936, 1950). It consists of an initial alarm reaction, a second stage of resistance, and a final exhaustion stage with the second stage being favourable as it allows the body to compensate and restore homeostasis (Selye, 1936, 1976). Selye also proposed that not all states of stress or threatened homeostasis are deleterious, terming healthy stress ‘eustress’ and distress as ‘pathogenic’ stress. The more severe and uncontrollable situations of physical or psychological distress trigger disease and pathology as a consequence, while brief and controllable challenges may be perceived as pleasant or exciting and could act as positive stimuli for emotional and intellectual growth (Selye, 1976). This model was later revisited by McEwen (1998) with a focus on the long term effects of physiological response to stress and proposed the concept of adaptation to potentially stressful challenges involving the activation of neural, neuroendocrine, and neuroendocrine-immune mechanisms. For this reason, Sterling and Eyer (1988) introduced the term ‘allostasis’ or ‘stability through change’. McEwen further elaborate Selye’s eustress concept as an allostatic response whereas distress represented allostatic load or overload resulting from either too much stress or inefficient management of allostasis (McEwen, 1998). Two types of allostatic overload have been described, acute (type I) and chronic (type II) (McEwen and Wingfield, 2003). Type I overload is the stress response to different threats that increases the demand for energy whereas many chronic stress-related diseases such as hypertension, stroke, obesity, metabolic syndrome, memory impairment, and autoimmune diseases have been related to type II allostatic overload (McEwen and Wingfield, 2003; McEwen, 2005, 2007).

There are three discrete and dedicated systems required to maintain and reinstate homeostasis during a response to stress (Fig. 1.1). The autonomic nervous system quickly responds within seconds to stress exposure through the sympathetic and parasympathetic divisions, which provide rapid alterations in physiological states through neural innervation of end organs. Activation of the sympa-tho-adrenomedullary (SAM) system leads to release of catecholamines, adrenaline and noradrenaline, from the adrenal medulla into blood circulation generating a ‘fight-or-flight’ response (Jansen et al., 1995). This rapidly increases heart rate and blood pressure while enhancing arousal and vigilance. Within the same timeframe, an increased secretion of pituitary prolactin and growth hormone, and pancreatic glucagon is often observed following different types of stressors.
(Lennartsson and Jonsdottir, 2011; Ranabir and Reetu, 2011; Jones et al., 2012). However, these secondary stress systems are activated as required to meet a stress-induced demand and those involving prolactin are still incompletely understood, with controversial roles in the dynamics of a response to stress. A less well characterised multisynaptic autonomic hypothalamic-spinal-adrenocortical (HSA) axis is activated almost simultaneously to the SAM system. This axis sends projection from the hypothalamus, primarily the paraventricular nucleus, to the intermediolateral cell column of the spinal cord and via the splanchnic nerve to innervate the adrenal cortex (Lowry, 2002). The HSA axis, through direct innervation of the glucocorticoid synthesizing adrenal cortex, has demonstrated important roles in modulation of ultradian and circadian rhythms and stress-induced neuroendocrine function (Jasper and Engeland, 1994; Buijs et al., 1999). Ulrich-Lai and colleagues (2006) demonstrated that adrenal splanchnic innervation modulates the stress hormone, corticosterone (in rodents), by increasing adrenal sensitivity to adrenocorticotropic hormone (ACTH). Following activation of the SAM system and HSA axis, the stress system is primed and subsequent activation of the hypothalamic-pituitary-adrenocortical (HPA) axis results in amplified elevations in circulating corticosteroids with peak plasma levels occurring approximately ten to fifteen minutes after the initiation of stress (Chrousos and Gold, 1992; Droste et al., 2008). It should be noted that each of these systems have important roles in normal homeostatic physiology. For example, under basal unstressed conditions, the HPA axis exhibits a prominent daily circadian rhythm characterised by rapid ultradian oscillations with peak levels of corticosteroids occur during the active phase over a period of approximately 24 hours (Windle et al., 1998). The circadian rhythmicity is generated and synchronised by photic entrainment of the hypothalamic suprachiasmatic nuclei via the retino-hypothalamic tract (Moore and Lenn, 1972; Mohawk and Takahashi, 2011). This rhythmic activity of the suprachiasmatic nuclei gives rise to a transcriptional-translational autoregulatory network involving ‘clock genes’ generating oscillating biochemical signals which in turn regulate downstream behavioural and physiological processes (Stratmann and Schibler, 2006; Maywood et al., 2007). Numerous peripheral clocks have also been identified including studies suggesting that steroidogenesis can be regulated independently from the suprachiasmatic nuclei by a peripheral intra-adrenal circadian pacemaker (Oster et al., 2006; Son et al., 2008). Although the HPA axis has a well characterised circadian rhythm, during acute exposure to stress, this rhythm is superseded by the HPA axis to facilitate the secretion of glucocorticoids (Windle et al., 1998).

The HPA axis involves the activation of parvocellular neuroendocrine cells within the paraventricular nucleus of the hypothalamus which secrete corticotropin-releasing hormone (CRH) and arginine vasopressin into the hypophyseal portal system (Tsigos and Chrousos, 1994). Arginine vasopressin is a potent synergistic factor acting concurrently with CRH on the anterior pituitary,
and via activating their respective transmembrane receptors, vasopressin V1b receptor and corticotropin-releasing hormone receptor 1, stimulates ACTH release to the systemic circulation (Smith and Vale, 2006). Circulating ACTH subsequently binds to the melanocortin type 2 receptor on the inner adrenal cortex (zona fasciculata) to initiate de novo synthesis through transcriptional activation of a number of steroidogenic genes to transport cholesterol across the mitochondrial membrane and ultimately increase the release of corticosteroids (Tsigos and Chrousos, 2002). The activity of parvocellular neuroendocrine cells is tightly regulated by the hippocampus via gamma-aminobutyric acid (GABA)ergic synaptic input from its interneuron populations that are in close proximity to the paraventricular nucleus including the bed nucleus of the stria terminalis or other hypothalamic nuclei within the dorsomedial hypothalamus and medial preoptic area (Roland and Sawchenko, 1993). This topographically organised output from the hippocampus involving subcortical GABAergic systems serves as a ‘gateway’ for inhibitory signals to affect the HPA and HSA pathways (Lowry, 2002). Injection of GABA-A antagonist into the paraventricular nucleus profoundly increases neural activity and circulating corticosterone levels indicating that GABA exerts an inhibitory effect which constrains parvocellular neuroendocrine cell activity under basal conditions (Borycz et al., 1992). It is believed that removal of this GABAergic inhibition is necessary for the initiation of the neuroendocrine response to stress (Borycz et al., 1992; Cole and Sawchenko, 2002). Hewitt and colleagues (2009) demonstrated that following acute restraint stress, the parvocellular neuroendocrine cells undergo a depolarizing shift in the reversal potential for chloride ions by activation of α1-adrenoceptors thereby inhibiting the activity of transmembrane potassium-chloride-cotransporter 2. This impaired chloride ion extrusion within the parvocellular neuroendocrine cells leads to impaired GABA-A receptor-mediated inhibitory control of the HPA axis following the onset of stress (Wamsteeker and Bains, 2010).
Figure 1.1. The three stress systems.

Schematic representation of the three systems required to maintain and reinstate homeostasis during stress. Activation of the sympatho-adrenomedullary system (green) through the locus coeruleus (LC) within the brain stem leads to release of catecholamines, adrenaline and noradrenaline, from the adrenal medulla (AM) through the intermediolateral cell column (IML) of the spinal cord. The multisynaptic autonomic hypothalamic-spinal-adrenocortical axis (orange) is concurrently activated and sends projections from the paraventricular nucleus (PVN) of the hypothalamus to the IML and via the splanchnic nerve to innervate the adrenal cortex (AC). Activation of the hypothalamic-pituitary-adrenal axis (red) stimulates release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the PVN into the pituitary portal circulation. These act concurrently on the anterior pituitary (aPT) causing release of adrenocorticotropic hormone (ACTH) that travels through the systemic circulation to induce glucocorticoid release from the AC.
1.1.2 Negative Feedback of the Hypothalamic-Pituitary-Adrenocortical Axis

Under stressful conditions, the HPA axis is regulated by glucocorticoid-mediated negative feedback to extra-hypothalamic centres, hypothalamus, and anterior pituitary to terminate the stress response and restore homeostasis (Pardridge and Mietus, 1979; De Kloet et al., 1998). This feedback is mediated by biologically active glucocorticoids that are able to cross the blood brain barrier (Reul et al., 2000). In circulation, corticosterone can be found in free form, but approximately 95% of corticosterone is protein bound, mainly to the high-affinity corticosteroid-binding globulin (CBG; formally transcortin) and low-affinity albumin (Siiteri et al., 1982). Thus, plasma corticosterone can be partitioned into free corticosterone, CBG-bound and albumin-bound reservoirs. It has been shown that following acute stress, the rapid release of CBG from the liver restrains free corticosterone for approximately 20 minutes when compared with the total corticosterone response in the blood (Qian et al., 2011). Both the free and albumin-bound corticosterone pools (via dissociation) are capable of crossing the blood brain barrier and act as the final effectors of the HPA axis mediating negative feedback (Pardridge and Mietus, 1979). Slow transcription-dependent actions of biologically active glucocorticoids occur at each of the glucocorticoid feedback target sites and represent a longer form of negative feedback following stress exposure. Glucocorticoids regulate the transcription and translation of pro-opiomelanocorticotropic hormones thus lowering ACTH levels, as well as reducing CRH and arginine vasopressin expression within the pituitary and hypothalamus (Birnberg et al., 1983; Sawchenko, 1987; Makino et al., 1995; Aguilera and Rabadán-Diehl, 2000). The combined effects of decreased ACTH levels, CRH, and vasopressin expression contribute to a suppression of the HPA hormone response to stress.

Two types of corticosteroid receptors have been identified in the rat brain; type I mineralocorticoid receptor (MR) with a higher binding affinity and type II glucocorticoid receptor (GR) with a ten-fold lower binding affinity for endogenous corticosterone (Reul and de Kloet, 1985). Moreover, the distribution of these two receptors within the rodent brain differs with MR found predominantly in limbic areas, particularly the hippocampus, while GR are more widely distributed across all brain regions (Reul and de Kloet, 1985; Herman et al., 1989). Based on these differentiating properties, it is thought that corticosterone acts via MR to regulate circadian rhythmicity and maintain homeostasis, while GR become occupied concurrently with increasing plasma corticosterone following exposure to a stressor or circadian and ultradian peak and are therefore involved in regulation of negative feedback through repression of Crh and Avp gene expression (Reul et al., 1987; Watts, 2005). This genomic effect of glucocorticoids is characterised by a lag-period ranging from at least 30 minutes to several hours or days from entry into the cell to the manifest action of its hormonal response.
Recently, corticosteroids have been shown to influence cognition, adaptive behaviour and neuroendocrine output within minutes, a time frame that is too rapid to be explained by genomic effects (Haller et al., 2008). These acute, non-genomic effects of glucocorticoids on the main feedback target sites tend predominantly toward the rapid suppression of HPA axis. For example, stress-induced corticosterone causes direct feedback on the hypothalamic PVN secretory neurons resulting in decreased excitability via reduced miniature excitatory postsynaptic currents in response to glutamate (Di et al., 2003). This effect was not able to be reversed with washout of corticosterone and was suggested to be mediated via a post-synaptic G-protein couple receptor mechanism triggering retrograde endocannabinoid signalling (discussed below). Conversely, the rapid effects of glucocorticoids in the hippocampus cause an increase in the probability of glutamate release onto CA1 pyramidal neurons resulting in an increase in the miniature excitatory postsynaptic currents mediated through transcription-independent actions at the MR (Karst et al., 2005). Furthermore, this increased miniature excitatory postsynaptic current frequency in the hippocampus can be reversed following removal of available corticosterone. The increase in corticosterone-induced excitability in this region reflects the inhibitory nature of the hippocampus on activity of the HPA axis, an effect mediated via intermediary projections to GABAergic regions such as the bed nucleus of the stria terminalis.

As mentioned above, it has also being shown that corticosteroids rapidly reduce HPA axis activity in a non-genomic, membrane-associated manner via endocannabinoid signalling (Di et al., 2003; Evanson et al., 2010). The endocannabinoid system is a lipid signalling network throughout the brain and periphery composed of two G-protein coupled receptors. The cannabinoid receptor type 1 (CB1R) is expressed ubiquitously throughout most regions of the brain with some expression in peripheral tissue whereas the cannabinoid receptor type 2 (CB2R) is expressed predominately in cells of the immune system such as microglia (Cabral and Griffin-Thomas, 2009). The two major endogenous ligands for both cannabinoid receptors, N-arachidonylethanolamine (anadamide; AEA) and 2-arachidonoylglycerol (2-AG), are synthesised on demand in the postsynaptic membrane from phospholipid precursors. Furthermore, endocannabinoids are rapidly eliminated, with anandamide hydrolysis being catalysed by the enzyme fatty acid amide hydrolase (FAAH), while 2-AG is primarily catabolised by monoacylglycerol lipase. The endocannabinoids are released in a retrograde fashion to activate presynaptic cannabinoid receptors and act as inhibitory signalling messengers at glutamatergic and GABAergic synapses (Freund et al., 2003; Mackie, 2008). They are capable of inhibiting voltage-gated calcium channels, and hence, neurotransmitter release including glutamate, GABA, acetylcholine, serotonin, and norepinephrine (Schlicker and Kathmann, 2001; Freund et al., 2003). Therefore, the aforementioned non-genomic feedback is achieved via rapid induction of glucocorticoid-induced endocannabinoid synthesis and release,
resulting in the suppression of excitatory glutamatergic neurotransmission to CRH neurosecretory cells thereby dampening the HPA response. To further clarify the profile of endocannabinoids following stress, an in vivo study demonstrated that 30 minutes of acute restraint stress in rats produced an elevation in 2-AG but not AEA content in the hypothalamus (Hill and McEwen, 2010). Interestingly, Hill and colleagues (2009) has also demonstrated a rapid loss of AEA levels in the basolateral amygdala may be involved in the natural activation of the HPA axis in response to stress. The mechanism behind this reduction is partly through a rapid 3-fold induction of FAAH activity. Nevertheless, the link between glucocorticoid and endocannabinoid signalling represents an important mechanism of rapid steroid hormone action in the brain and dysregulation of this system may be responsible for stress-related disorders (Hill et al., 2010; Hill and Tasker, 2012). Furthermore, due to the neuromodulating role, the endocannabinoid system could be involved in several other physiological functions such as memory processing, pain perception, and inflammation (Pacher et al., 2006). Recently, specific FAAH inhibitors have demonstrated anxiolytic, anti-depressant, and anti-inflammatory efficacies, implicating the endocannabinoid system as a potential therapeutic target (Ahn et al., 2009a).

1.1.3 The physiological and pathophysiological effects of glucocorticoids
Circulating corticosteroids regulate a broad spectrum of physiological changes through at least 16 different isoforms of ubiquitously distributed intracellular GR which belong to the nuclear receptor superfamily of transcription factors. During the inactive state, GR is part of a multiprotein complex that undergoes repeated cycles of dissociation and adenosine-5’-triphosphate (ATP)-dependent re-association with regulatory proteins such as heat shock protein (HSP)-90 kDa, HSP-70, p59 immunophilin, FK506-binding protein 4, and p23 phosphoprotein (Nicolaides et al., 2010). Upon ligand binding, GR dissociates from the complex and translocates into the nucleus and interacts with specific DNA motifs termed glucocorticoid response elements (GREs) in the promoter region of target genes as homo- or heterodimers, thus influencing the transcription rate in a positive or negative manner. However, GR has been shown to modulate gene expression independent of GRE-binding through direct protein-protein interaction with other transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (Yang-Yen et al., 1990; Ray and Prefontaine, 1994). During times of stress, the biological effects of glucocorticoids are usually adaptive and include enhanced cognition, mobilisation of necessary energy substrates, immunosuppressive functions, and alteration of stress-related behaviours. These are beneficial as they permit the ‘fight-or-flight’ response and attempt to regain and maintain homeostasis.

One of the foremost metabolic effects of glucocorticoids is their ability to increase circulating glucose through immediate stimulation of glycogenolysis, hepatic gluconeogenesis, and inhibition
of peripheral glucose transport and utilisation (Andrews and Walker, 1999; Marik and Raghavan, 2004). Additionally, glucocorticoids decrease hepatic insulin sensitivity and enhance lipolysis in fat cells and proteolysis in various muscle types to mobilise lipids and amino acids to meet the increased energy demand following stress exposure (Simmons et al., 1984; Divertie et al., 1991; Ling et al., 1998). Glucocorticoids are also well known for their role in maintaining immune homeostasis following activation of the stress response. It is generally accepted that glucocorticoids are anti-inflammatory as they have been widely documented to (1) counteract the production of pro-inflammatory cytokines; (2) stimulate the production of anti-inflammatory cytokines; and (3) directly suppress innate inflammatory mediators such as the pro-inflammatory transcription factor NF-κB via protein–protein interactions between activated GR and the p50/p65 subunits (De Bosscher et al., 2003; Silverman and Sternberg, 2012; Frank et al., 2013). Interestingly, suppression of pro-inflammatory cytokines by glucocorticoids exhibits a hierarchy of sensitivity with tumor necrosis factor-alpha (TNF-α) being the most sensitive, while IL-1 inhibition occurs only at supraphysiological levels, and IL-6 is relatively resistant when compared to other cytokines (DeRijk et al., 1997). However, it seems maladaptive to decrease central and peripheral immune activity during a ‘fight/flight’ emergency, as these are periods of increased risk for infection and injury, especially if one considers immunological challenge itself a stressor. To justify this paradox, several studies have demonstrated that there is a rapid immune activation prior to adrenocortical release of corticosteroids following exposure to endo- or exotoxins, infectious microorganisms or antigen, as well as non-infectious causes such as psychological stressors (Harbuz and Lightman, 1992; Stenzel-Poore et al., 1993; Zhou et al., 1993). Therefore, Munck and colleagues (1984) proposed that the anti-inflammatory actions of glucocorticoids function to mediate recovery from this initial immune activation to prevent an organism suffering from hypersensitivity and autoimmunity. This is further supported by a study which demonstrates that following stressful events, there is enhanced free corticosterone due to a drop in CBG levels via an IL-6 dependent hepatic posttranscriptional blockade that suppresses further inflammatory responses (Pugeat et al., 1989). In terms of the initial increases in immune activity immediately following stress, it has being reported that it may be predominantly mediated by catecholamine release where α1- and β-adrenoceptor antagonists inhibit tail shock-induced increases in cytokine production in stress-related brain regions (Johnson et al., 2005). However, it is important to note that basal corticosteroid levels are required for this immune activity as proper catecholamine signalling is modulated by glucocorticoids (Joels and de Kloet, 1989). Interestingly, the rapid rise in prolactin has also being shown to mediate stress-induced immune deregulation and promote pro-inflammatory immune responses via NF-κB (Brand et al., 2004a; Wu et al., 2014). Despite the aforementioned anti-inflammatory role of glucocorticoids, it is believed that it has the ability to enhance both the availability and activity of specific immune cells.
It has been shown that following a single episode of psychological stress, the resultant corticosterone decreases circulating leukocyte numbers and directs these cells to leave circulation and redistribute to other compartments of the body that might be in need (McEwen et al., 1997). It was proposed that this glucocorticoid-induced change in leukocytes is a passive process compared to the active recruitment of leukocytes by chemotactic factors following an active immune challenge. Based on this observation, McEwen and colleagues (1997) hypothesised that the stress-induced relocation of immune cells by glucocorticoids may have significant contribution to the ability of the immune system to perform its surveillance function and ensure appropriate defence is present at the right place and the right time to potential or ongoing immune threats. In agreement with this view, there is increasing evidence that acute stress exposure promotes morphological transformation of microglia from a ramified-resting state to an ‘activated state’ (Walker et al., 2013). Typically, ‘activated’ microglia retract and thicken the fine processes that extend from the soma and release a significant amount of pro-inflammatory molecules including TNF-α and, in some instances, become antigen presenting cells with many macrophage-like properties such as phagocytosis. Therefore, it has been proposed that during a ‘fight/flight’ emergency, in addition to the well-known anti-inflammatory actions, stress-induced corticosteroids function as a warning signal to the peripheral and central innate immune systems and perhaps primes the immune response for potential noxious insults (Sorrells et al., 2009; Frank et al., 2013). Following the emergency, levels of glucocorticoids dissipate, the peripheral and central (microglia) immune defences are now primed which enable a further potentiated inflammatory response to any persisting injury or infection (Fig. 1.2).
Figure 1.2. Priming of the neuroinflammatory response following peak glucocorticoid levels within the central nervous system.

During the initial phase of a stress response, glucocorticoid levels rise and exert anti-inflammatory effects while simultaneously initiating a process which primes the pro-inflammatory response within the brain via microglial sensitization. Glucocorticoids return to baseline values following cessation of a stressor and the body enters a recuperative phase where a pro-inflammatory response is favoured. (Figure from Frank et al., 2013)
While acute stress promotes adaptation and survival, prolonged impairments in HPA activity can have detrimental effects on metabolic, immune, and neural functions especially the limbic system. It is well known that the HPA axis plays an important role in the pathophysiology of stress-related psychiatric disorders such as anxiety and depressive disorders (de Kloet et al., 2005; McEwen, 2008). Patients with psychiatric illnesses have being identified with symptoms such as (1) high concentrations of CRH in the central nervous system; (2) hyperactive CRH neurons; and (3) abnormal CRH receptor expression patterns (Kasckow et al., 2001; Claes, 2004). Moreover, positron emission tomography and functional magnetic resonance imaging studies in patients suffering from major recurrent depression demonstrated altered patterns and volume in stress-related brain regions such as the hippocampus, amygdala and prefrontal cortex (Drevets et al., 1997; Sheline et al., 1999; Sheline et al., 2003). There is a plethora of animal models linking disturbed HPA response to depression. For example, Healy and colleagues (1999) reported that rodents administered with the corticosteroid synthesis inhibitor, metyrapone, exhibited anti-depressant-like behaviour. In an attempt to model human anxiety and depressive disorders in rodents, a wide range of behavioural testing has been developed. Elevated plus-maze is a well-established paradigm in assessing anxiety-like behaviours in laboratory animals. One of the earliest studies on depression in rodents began in 1967 with Overmier and Seligman’s work characterizing how prior exposure to an uncontrollable trauma termed ‘learned helplessness’ affects subsequent traumatic events (Overmier and Seligman, 1967). These initial observations were adapted by Porsolt (1977) utilising the same principles in rats using a forced swimming test until resigned despair was learned. Porsolt’s forced swimming model of behavioural despair was particularly powerful due to the sensitivity displayed by several antidepressant treatments (Porsolt et al., 1977; Porsolt et al., 1978). However this sensitivity was only displayed if animals were subjected to a pre-test (Borsini et al., 1989). Subsequently, a set of clearly defined active behaviours was characterised by Detke and colleagues (1995) which allowed discrimination between compounds specific or selective for particular neurotransmitters based on their distinct modulation of active behaviour patterns. Particularly, selective norepinephrine reuptake inhibitors specifically increased climbing behaviour, and selective serotonin uptake inhibitors specifically increased swimming behaviour while both decreased behavioural immobility (Detke et al., 1995; Detke and Lucki, 1996). The forced swim test was quickly adopted as a behavioural screen for predicting the efficacy of antidepressant compounds, and allowed investigation into the physiological and neurochemical changes associated with depression (Swiergiel et al., 2007; Dunn and Swiergiel, 2008). Recently, a series of molecules from the reduction/oxidation (redox) system have been implicated in various aspects of stress response generation at both the mechanistic and modulatory levels with further implications in depressive behaviour.
1.2 Oxidative and Antioxidative Systems

1.2.1 Free radical production

Normal cellular metabolism depends on oxygen to generate ATP, with a theoretical reduction of oxygen to water by the electron transport chain involving a coordinated four-electron transfer (Halliwell and Gutteridge, 1989). During this process, 1-3% of all electrons ‘leak’ from the electron transport chain to react with molecular oxygen, generating superoxide radicals instead of being reduced to water (Liu et al., 2002; Muller et al., 2004; Cash et al., 2007). Although this occurs at both complex I and complex III of the electron transfer chain, the majority occurs at complex I where it is facilitated by succinate (Liu et al., 2002). Most of the cellular superoxide is produced inside the inner mitochondrial membrane where the mitochondrial concentration of superoxide can be between 5-10 times that of the cytosol or nucleus (Cadenas and Davies, 2000). The remainder of mitochondrial superoxide is primarily formed by complex III on both sides of the mitochondrial membrane and by extra-mitochondrial flavoenzymes (Zimmerman and Granger, 1994; Cadenas and Sies, 1998; Brand et al., 2004b). Superoxide then undergoes spontaneous or enzymatic dismutation via superoxide dismutase (SOD) to generate hydrogen peroxide. Although hydrogen peroxide is relatively stable, subsequent interactions with superoxide radicals and/or transition metals such as Fe$^{2+}$ or Cu$^{2+}$ induce production of the highly toxic hydroxyl radical by Haber-Weiss and Fenton chemistry. This radical has been suggested to cause more damage to biological systems than any other reactive oxygen species (ROS) due to the extreme reactivity and very short in vivo half-life of $\approx$9-10 milliseconds (Pastor et al., 2000).

Outside the mitochondrion, there are three major processes responsible for the production of free radicals, principally in the form of reactive oxygen and nitrogen species. The first process involves the production of hydrogen peroxide as a by-product of fatty acid catabolism by peroxisomes (Ames et al., 1993; Wanders and Waterham, 2006). Although technically not a free radical, hydrogen peroxide is still classed as a ROS for its role in Fenton and Haber-Weiss chemistry (Cimen, 2008). Within the peroxisome, the majority of hydrogen peroxide is neutralised via canonical catalase activity or peroxidation to another catalase substrate (Wanders and Waterham, 2006; Valko et al., 2007). However, under some conditions hydrogen peroxide can avoid degradation and escape the peroxisome, ultimately leading to cellular and nucleic acid damage (Kasai et al., 1989). The second process involves the reliance of the innate immune system on the ability of phagocytic cells such as neutrophils to engulf and digest foreign pathogens. Following the encapsulation of the foreign body into a phagosome, neutrophils increase their oxygen consumption specifically to supply the dormant nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase with molecular oxygen (Dahlgren and Karlsson, 1999). This enzyme catalyses the oxidation of
NADPH to form two superoxide radicals which, together with reactive metabolites of superoxide including hydrogen peroxide and hypochlorite, constitutes the respiratory burst responsible for killing the pathogen (Ames et al., 1993; Dahlgren and Karlsson, 1999; Stadtman et al., 2007; Valko et al., 2007). Hayashi and colleagues (2008) have also demonstrated that NADPH-oxidase derived ROS can also be produced via a non-genomic mechanism following aldosterone administration in rat cardiac myocytes. The third process involves redox metals such as Fe^{2+/3+}, Cu^{2+}, and Mn^{2+} which are essential for electron transfer in many enzymatic reactions, including the antioxidant enzymes of the oxidative cascade. However, these transitional metal ions can also undergo reactions resulting in the production of hydroxyl radicals (Rovira et al., 2007).

Although there is a general negative connotation associated with ROS production, they have important cellular functions under normal physiological conditions. Even low levels of the extremely reactive hydroxyl radical have been shown to activate guanylate cyclase, stimulating the production of a cyclic guanosine monophosphate (cGMP) second messenger cascade (Mittal and Murad, 1977). In fact, the physiological roles of ROS vary significantly, ranging from specific oxidations of cysteine groups affecting enzyme activity and function, to cellular redox sensing in the determination of cell differentiation fate (Nicotera et al., 1985; Dalton et al., 1999; Wang et al., 2011). Progression of the cell cycle itself has demonstrated dependence on radicals produced by NADPH-oxidase modulating mitogenic pathways (Burhans and Heintz, 2009). Several transcription factors are also regulated directly by ROS-induced modifications, thereby modulating the downstream expression of several gene families (Dalton et al., 1999). Notably, the dimerised protein products of immediate-early response genes FOS and JUN, AP-1, is activated by ROS through redox reactions and post-translational modification of the individual FOS and JUN proteins (Buscher et al., 1988; Abate et al., 1991; Devary et al., 1991). Under normal conditions, any excessive ROS not participating in these physiological functions are reduced by the antioxidant system. However, an imbalance between the production of ROS and the ability of the antioxidant defence system to readily detoxify the reactive intermediates, termed oxidative stress, leads to damage of biological macromolecules and dysregulation of normal metabolism (Sies, 1997; Nordberg and Arner, 2001).

1.2.2 The endogenous antioxidant system

In order to neutralise ROS, cells use a suite of enzymatic and non-enzymatic antioxidants, ultimately attempting to neutralise the radical by reduction to water. In the typical ROS reduction cascade, SOD is the top-tier antioxidant, catalyzing the dismutation of this radical to hydrogen peroxide. This is achieved through the transfer of electrons across the catalytic metal core of the enzymes to reduce the superoxide radicals. In mammals, the two major isoforms of SOD are the
copper, zinc-SOD which are found throughout most cell compartments, and the manganese-SOD that is specific for mitochondria. Catalase is centred around an iron-containing ferriheme group that acts as the transition metal during the reduction of hydrogen peroxide. Access to this active site is fairly specific as the channel opening is narrow and does not allow the passage of large molecules. High concentrations of superoxide anions are able to inactivate catalase by oxidizing the heme group in the active site. To prevent this, catalase binds NADPH to maintain this group in the reduced state (Nordberg and Arner, 2001; Fridovich et al., 2007). Hydrogen peroxide can also be reduced directly by both peroxiredoxins, which allow the oxidation of an active cysteine thiol group to degrade one molecule of hydrogen peroxide into two molecules of water, and the glutathione-glutathione peroxidase system. Reduced glutathione (GSH) is the most abundant intracellular thiol-based antioxidant which protects cells against oxidative stress by acting as a substrate for the selenium-containing glutathione peroxidase, subsequently forming oxidised glutathione disulphide (GSSG). In turn, GSSG is regenerated to GSH by glutathione reductase in a NADPH-dependent mechanism (Barycki et al., 2007). The cellular concentrations of this soluble tripeptide range from 1 to 11 mM in the cytosol, 3 to 15 mM in the nucleus, and 5 to 11 mM in the mitochondria, although mitochondrial GSH requires membrane transport even against a concentration gradient (Shen et al., 2005; Valko et al., 2007). Glutathione also acts as a substrate for the glutaredoxins, which reduce proteins that have been glutathionylated by reducing GSSG to a mixed disulphide protein and GSH. As the occurrence of mixed disulphides increases with increasing concentrations of GSSG, the ratio of the reduced to oxidised fractions (GSH/GSSG) of GSH within cells is often used as a reliable indicator of redox imbalance and has been shown to strongly influence cell cycle progression in proliferating cells (Menon et al., 2003; Öztürk and Gümüslü, 2004; Rose et al., 2012). The transcription factor nuclear factor-erythroid-2-related factor 2 (Nrf2) is essential for the coordinated induction of cytoprotective enzymes and related proteins in response to oxidative and electrophilic stresses (Itoh et al., 1999; Urano and Motohashi, 2011). This transcription factor regulates a battery of redox genes such as the glutathione synthesis enzyme gamma-glutamylcysteine synthetase, glutathione peroxidase, glutathione disulphide reductase, glutathione S-transferase, thioredoxin-1, and heme oxygenase-1 through their antioxidant response element (Inamdar et al., 1996; Moinova and Mulcahy, 1999; Kim et al., 2003; Kwak et al., 2003; Gorrini et al., 2013). Under basal conditions, Nrf2 activity is sequestered in part by the actin-associated Keap1 protein within the cytoplasm. Activation of Nrf2 in response to oxidative and electrophilic agents is thought to be initiated by disruption of this Nrf2-Keap1 complex, releasing Nrf2, which translocates into the nucleus to regulate the expression of downstream targets.
1.2.3 Adrenal glucocorticoids and oxidative stress

One of the underlying initiators of oxidative stress is the increased rate of aerobic metabolism generated by exposure to physical and/or psychological stress. Mammalian erythrocytes are particularly susceptible to oxidative damage due to their high polyunsaturated fatty acid content within the membranes and high cellular concentrations of oxygen (Pandey and Rizvi, 2010). An accumulating number of rodent studies evidence the enhanced oxidative damage and altered antioxidant activity in erythrocytes following exposure to both acute and chronic stressors (Gumuslu et al., 2002; Öztürk and Gümüslü, 2004; Sahin et al., 2004). Given that erythrocytes are continuously exposed to ROS from extracellular sources, through anion channels and surrounding tissues, the accumulated oxidative damage within these cells can reflect the general oxidative status of many tissues and organs (Nwose et al., 2007). Although erythrocytes lack mitochondria, two mechanisms of intracellular ROS formation occur via haemoglobin auto-oxidation and plasma membrane deoxygenation (Kinoshita et al., 2007; Cimen, 2008). Additionally, erythrocytes exposed to an oxidative challenging environment initiate other ROS-generating mechanisms such as the Fenton reaction and peroxyl radical production that further exacerbate ROS formation (Minetti and Malorni, 2006). In our laboratory, we have examined the temporal relationship between stress and the onset of oxidative challenge (Spiers et al., 2013). Particularly, while known physiological stress markers like blood glucose and plasma corticosterone rapidly return to baseline values, changes in redox state suggest a slightly delayed onset after which persistent increases remain following the cessation of the stressor (Fig. 1.3).
Figure 1.3. Temporal relationship between stress and the onset of oxidative challenge.
Serial blood samples collected at 0 (baseline), 30, 60, 120, and 240 minutes from male Wistar rats with indwelling jugular catheters. Rats were randomly divided into Control (●) or 120 minute Stress (■) groups (n=6-9/group). Serial samples were used to determine (A) blood glucose levels; (B) plasma corticosterone concentrations; (C) general oxidative status measured by dichlorofluorescein (DCF) formation in erythrocytes; and (D) glutathione to glutathione disulfide ratio (GSH/GSSG) stress in erythrocytes of control or stressed rats. The black filled bar indicates time of exposure to acute restraint stress. Data are expressed as mean ± standard error of the mean (SEM) where *p<0.05, **p<0.01 and ***p<0.001. (Figures from Spiers et al., 2013)
Neuronal redox status has recently undergone intense scrutiny due to the association of oxidative stress with the pathology of neurodegenerative diseases (Bond and Greenfield, 2007; Cotella et al., 2012). It has been shown that both chronic oxidative stress and glucocorticoid exposure promote gliogenesis over neurogenesis in hippocampal neural stem cell progenitors and may be the direct result of accumulated mitochondrial oxidative stress (Wang et al., 2011; Chetty et al., 2014). Glial cells play an important but poorly understood role in the modulation of neuronal redox state. It has recently been shown that astrocyte-derived L-lactate potentiates N-methyl-D-aspartate (NMDA) receptor activity by modulating neuronal redox status (Yang et al., 2014). This neuron-glia interaction can also increase noradrenaline release from the locus coeruleus and hypothalamic ATP production (Cortes-Campos et al., 2011; Tang et al., 2014). Furthermore, Reyes and colleagues (2012) have demonstrated that neuronal NADPH oxidase-derived superoxide can traverse the extracellular space to modulate the redox state in neighbouring neurons and astrocytes. However, in comparison to astrocytes, neurons are known to have relatively poor expression of endogenous antioxidants, making them highly susceptible to oxidative stress.

Glucocorticoids induce neuronal oxidative stress directly through enhanced mitochondrial respiration and oxidative phosphorylation. This was demonstrated clearly in a study by Du and colleagues (2009), showing that acute incubation of cortical neurons with corticosterone increased mitochondrial oxidation, membrane potential, and calcium-holding capacity in a dose and time-dependent manner. This was further clarified by You and colleagues (2009) using the oxidation product dichlorofluorescien as a ROS indicator in organotypic hippocampal slice cultures exposed to the synthetic glucocorticoid, dexamethasone, and the glucocorticoid receptor antagonist, RU486. The single and combination use of these compounds demonstrated that hippocampal neuronal death, marked by propidium iodide, was selectively induced by glucocorticoid exposure, while other steroid hormones had no effect. Acute incubation with dexamethasone increased the hippocampal oxidative status by approximately 200% in a dose dependent manner, an effect that was ameliorated with pre-treatment of RU486 or the ROS-scavenger, N-acetyl-L-cysteine. Furthermore, four hours of dexamethasone incubation, induced the highest increase in oxidative status, with concurrent gene expression up-regulation of the ROS-producing enzyme NADPH-oxidase, while the antioxidant enzyme glutathione peroxidase was significantly down-regulated (You et al., 2009). This indicates that the increase in oxidative status is produced by a glucocorticoid-dependent and transcriptional increase in pro-oxidative drive, with concurrent inhibition of the antioxidant defence system, ultimately leading to increased neuronal cell death. Cortical and hippocampal neural cultures have also established that 24 hours of glucocorticoid exposure increases basal ROS production and exacerbates the concomitant ROS produced by adriamycin redox cycling, which negatively affects survival in hippocampal neurons (McIntosh and
Sapolsky, 1996). This is supported by in vivo evidence that administration of exogenous corticosterone over a 14 day period increases hippocampal oxidative indicators including lipid peroxidation and protein carbonyls, while the enzymatic antioxidants SOD, catalase, and glutathione peroxidase activities are all decreased (Sato et al., 2010). This study, performed by Sato and colleagues (2010), also demonstrated increased apoptosis and decreased glucocorticoid receptor expression, a hallmark of chronically high glucocorticoids, in the hippocampus. Furthermore, this study also utilised a serum measurement of iron-induced superoxide formation in blood serum, establishing that this peripheral marker increased with chronic corticosterone administration. Further in vivo studies have shown that both corticosterone administration and restraint stress for 21 days induce an overall decrease in GSH in addition to SOD, catalase, glutathione transferase, and glutathione reductase activities in whole brain, liver, and heart tissues (Zafir and Banu, 2009). This study, by Zafir and Banu (2009), also demonstrated that these treatments increased lipid peroxidation and oxidised protein carbonyl groups in the same tissues. However, the overall increase in neural tissue is driven by specific increases in particular subregions that are susceptible to stress-induced oxidative stress. However, this overall increase in oxidative stress is not generalised in all areas of the brain but is likely due to increases in specific subregions. For example, Mendez-Cuesta and colleagues (2011) used an acute immobilisation stress to induce increased lipid peroxidation and decreased SOD activity in a highly oxidative-vulnerable region, the striatum. Interestingly, they found that the decrease in SOD activity was exclusively due to the mitochondrial isoform, manganese-SOD, while the copper, zinc-SOD showed little change. Furthermore, Lucca and colleagues (2009) used a chronic mild stress regime to show that SOD decreased and oxidised protein carbonyl groups increased in several cerebral regions including the striatum and hippocampus, while the cerebellum remained largely unaffected. We have also observed the induction of regional specific oxidative stress in the hippocampus but not the amygdala following an acute restraint stress, demonstrating that this process is not exclusive to chronic stress exposure (Spiers et al., 2013). Some of the aforementioned aspects of the redox system have been summarised in Figure 1.4.

With the majority of studies focusing on the canonical stress-induced modulation of the redox system, several groups have also demonstrated regulatory role of ROS on the limbic stress axis. Asaba and colleagues (2004) demonstrated that ROS attenuate the glucocorticoid-induced down-regulation of pro-opiomelanocortin in pituitary corticotrophs, thereby promoting an increase in HPA axis activity via dampened negative feedback. The expression and nuclear internalisation of GR have also demonstrated susceptibility to highly pro-oxidative environments (Zhou et al., 2011). Using a cultured fluorescently labelled chimeric GR, Okamoto and colleagues (1999) demonstrated that nuclear translocation of GR following acute dexamethasone treatment is impaired in the
presence of hydrogen peroxide. This effect was reduced in the presence of exogenous antioxidants or following substitution of serine for a redox-sensitive cysteine residue. The dissociation of heat shock proteins from the cytosolic GR was also impaired in a pro-oxidative environment, indicating that there may be multiple redox regulatory roles involved in the cellular response to glucocorticoids (Okamoto et al., 1999; Tanaka et al., 1999). These observations highlight that maintenance of a balanced redox state is critical for normal cellular homeostatic function within the neuroendocrine system.
Figure 1.4. The oxidation/reduction system.

Stress causes an increase in corticosterone which activates cytosolic glucocorticoid receptors (GR). These translocate into the nucleus to modulate gene transcription through glucocorticoid responsive elements (GRE), or co-localise with the anti-apoptotic Bcl2 protein and translocate into the mitochondria. This increases mitochondrial membrane potential, calcium holding capacity, and mitochondrial oxidation. The increase in cellular metabolic rate promotes ATP synthesis in addition to spontaneous superoxide (O$_2^-$) production via complex I and III of the electron transport chain. This is dismutated to hydrogen peroxide (H$_2$O$_2$) by manganese superoxide dismutase (Mn-SOD) and can be further converted to hydroxyl radical (OH$^-$) or reduced to water by the mitochondrial antioxidant pathway. In the cytosol, a major source of superoxide production is via the oxidation of NADPH via NAPDH oxidase. Cytosolic superoxide is dismutated to hydrogen peroxide by copper, zinc-superoxide dismutase (Cu, Zn-SOD). Hydrogen peroxide is neutralised by catalase (CAT) or glutathione peroxidase (GPx) which oxidises the reduced form of glutathione (GSH) to oxidised glutathione (GSSG). GSH is then regenerated from GSSG via the glutathione reductase (GSR) enzymatic system. Hydrogen peroxide can also interact with superoxide radicals and/or transition metals such as Fe$^{2+}$ or Cu$^{2+}$ to produce the highly toxic hydroxyl radical by Haber-Weiss and Fenton chemistry. An increase in the production of superoxide, hydrogen peroxide, and hydroxyl radicals leads to a state of cellular oxidative stress which causes oxidative damage to DNA, protein carbonyl formation, and membrane lipid peroxidation (LPO). Hydrogen peroxide is membrane permeable and moves freely from mitochondrial to cytosolic compartments, in addition to traversing the extracellular space to affect neighbouring neurons and glial cells. Superoxide radicals can also induce oxidative stress in neighbouring cells by diffusing through membrane-bound anion channels. The majority of neuronal and astrocytic reactive oxygen species are produced by mitochondrial oxidation, while other cell types such as microglia rely heavily on the cytosolic NADPH-oxidase system to produce a respiratory burst in response to invading pathogens. However, in comparison to glial cells, neurons display a relatively poor expression of endogenous antioxidants, making them more vulnerable to oxidative stress. (Figure from Spiers et al., 2014a)
The Nitrergic System

1.3.1 Nitric oxide biosynthesis and functions

Nitric oxide, a gaseous diatomic free radical belonging to the family of reactive nitrogen species (RNS), is synthesised through the conversion of amino acid L-arginine to L-citrulline by nitric oxide synthase (NOS) in the presence of oxygen, NADPH, and cofactors including flavin adenine dinucleotide, flavin mononucleotide, and (6R)-5, 6, 7, 8-tetrahydro-L-biopterin (Andrew and Mayer, 1999). There are three main isoforms, each with a specific distribution profile; neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (Stuehr, 1999). Though nNOS is predominantly active in the cytosol of central and peripheral neurons for signalling and regulation, it has also been found in the sarcolemma and cytoplasm of all muscle fibres (Frandsen et al., 1996). Interestingly, nNOS is present in the hippocampus, hypothalamus, pituitary, and adrenal gland, suggesting co-localisation with the HPA axis (Lai et al., 2005; Gadek-Michalska et al., 2012). Furthermore, several studies have demonstrated transcriptional regulation of nNOS by glucocorticoids in the hippocampus, implicating its importance in the stress response, although the upstream promoter of NOS1 does not carry a glucocorticoid responsive element (López-Figueroa et al., 1998; Reagan et al., 1999; Zhou et al., 2011). There are four nNOS splice variants, α, β, γ, and μ, with nNOSα being the most dominant and therefore being physically and functionally coupled to the glutamate receptors of the NMDA subtype through their mutual postsynaptic density-95/discs-large/zona occludens-1 (PDZ) binding motif (Eliasson et al., 1997). Within the hippocampus, local calcium influx through NMDA receptors can trigger the production of NO, which subsequently activates its receptor, soluble guanylyl cyclase, leading to release of second messenger cGMP (Fig. 1.5). This NO-cGMP signalling has been implicated in the induction of hippocampal long-term potentiation which is known to be one of the principal mechanisms in learning and memory (Schuman and Madison, 1991; Arancio et al., 1996; Kelley et al., 2010). The nNOSμ mainly localises in the skeletal muscles interacting with the dystrophin-associated glycoprotein complex and muscles deficient in this isoform become myopathic (Percival et al., 2008). The β-variant lacks the PDZ domain while nNOSγ has very little to no enzymatic activity (Eliasson et al., 1997). Endothelial NOS contains a putative shear stress responsive element in the promoter region of the NOS3 gene while the protein is membrane-bound to the golgi apparatus and caveolae, producing NO mainly in the endothelium of blood vessels responsible for vasodilation and smooth muscle relaxation (Smith et al., 2006). The inducible form of NOS was first identified in mouse macrophages, it is almost undetectable in resting cells but is induced at the transcriptional level by immunostimulatory cytokines, viral infection, or bacterial products such as lipopolysaccharides (Zamora et al., 2000; Aktan, 2004). Furthermore, lipopolysaccharides or
inflammatory cytokine-induced iNOS are thought to contribute to inflammation by increasing vascular permeability and leukocyte infiltration to inflamed tissues (Nagy et al., 2007). Within the central nervous system, iNOS expression is observed in astrocytes, microglia, endothelial cells, immature neurons, and invading macrophages where the iNOS-mediated release of NO by astrocytes and microglia has a major role in antimicrobial and tumoricidal activity (Heneka and Feinstein, 2001; Hua et al., 2002; Aktan, 2004; Brantley et al., 2010). Moreover, upon transcriptional activation, this soluble subtype can produce micromolar levels of NO and is known to be associated with diseases such as atherosclerosis, rheumatoid arthritis, diabetes, septic shock, and multiple sclerosis (Kuhlencordt et al., 2001; Hill et al., 2004; Maki-Petaja et al., 2008; Heemskerk et al., 2009; Soskic et al., 2011). Interestingly, despite most of its deleterious role, iNOS has recently being demonstrated to acts as an important signalling intermediate in plasma cell survival pathways (Saini et al., 2014). Both nNOS and eNOS are constitutively active isoforms producing low concentrations of NO (in the nanomolar range) over long periods and are activated by calcium ions though transient binding to the calcium-binding protein, calmodulin (Knott and Bossy-Wetzel, 2009). Comparatively, the inducible form of NOS can produce high concentrations of NO in relatively short periods and is calcium independent due to a high binding affinity to calmodulin (Aktan, 2004). A fourth NOS isoform, mitochondrial NOS (mtNOS) has been proposed, localising at the inner mitochondrial membrane controlling mitochondrial energetics (Ghafourifar et al., 2001; Ghafourifar and Cadenas, 2005). However, the existence of mtNOS as a unique enzyme is still a matter of debate (Tay et al., 2004; Venkatakrishnan et al., 2009).

1.3.2 Nitric oxide metabolism and clearance

In the vasculature, erythrocytes are the major scavenger of nitric oxide due to the high haemoglobin concentration and thus, nitric oxide is sequestered via the dioxygenation reaction in which nitric oxide reacts with oxygenated haemoglobin to form methaemoglobin and the more stable metabolite nitrite (Han et al., 2003). Excessive production of nitric oxide can also be counteracted by conjugation with reduced glutathione, forming the stable adduct S-nitrosoglutathione which can in turn be cleaved directly by mammalian thioredoxin (Nikitovic and Holmgren, 1996). The inorganic ions, nitrate and nitrite (NOx), were previously thought to be the end products of NO metabolism. However, recent studies have demonstrated a NOS-independent pathway in which NO can be produced by reducing NOx, a reaction catalysed by xanthine reductase under low oxygen tension and low pH environment. The NO produced by this nitrate-nitrite-NO pathway may have similar roles to NO generated from the L-arginine-NOS pathway representing an important secondary pool (Lundberg et al., 2008).
1.3.3 Reactive nitrogen species and nitrosative stress

High levels of NO and its derivatives are destructive to cellular components such as proteins, lipids and DNA. Nitric oxide can react directly with molecular oxygen to produce two relatively strong oxidants, nitrogen dioxide and dinitrogen trioxide. However, at physiological levels of NO these reactions are relatively slow. A primary reaction in the production of RNS is the combination of NO and superoxide anions to form the highly reactive metabolite, peroxynitrite, a potent neurotoxin (Lipton et al., 1993). It has been suggested that NO and peroxynitrite can disrupt ATP synthase and almost all components of the mitochondrial respiratory chain (Almeida and Bolanos, 2001; Sarti et al., 2012). These RNS reversibly or irreversibly inhibit mitochondrial oxygen consumption, particularly at complex IV (also known as cytochrome c oxidase), and may lead to cellular energy deficiency and ultimately cell death in pathological conditions (Sarti et al., 2012). Inhibition of cytochrome c oxidase by NO and peroxynitrite causes neuronal dysfunction and, in addition to high iNOS expression, has been observed in the cortex of Alzheimer’s patients (Mutisya et al., 1994; Haas et al., 2002).

S-nitrosylation is the covalent attachment of NO to the thiol side chain of the amino acid cysteine, forming other NO derivatives termed S-nitroso-proteins. Under physiological conditions, it has been demonstrated that NO is converted to the nitrosonium ion which subsequently S-nitrosylates the NMDA receptor, thereby preventing glutamate excitotoxicity by blocking calcium influx, promoting cell survival (Lipton and Stamler, 1994). Excessive production of NO can be counteracted by conjugation with reduced glutathione, forming the stable adduct S-nitrosoglutathione which has important role in signal transduction and regulation of a variety of protein functions (Klatt and Lamas, 2000; Anand and Stamler, 2012). Abnormal S-nitrosylation to proteins such as apolipoprotein E, cyclin-dependent kinase 5, dynamin-related protein 1, parkin, peroxiredoxin 2, protein disulfide isomerase, heat-shock protein 90, and X-linked inhibitor of apoptosis have all being linked to neurodegenerative conditions such as Alzheimer’s and Parkinson’s diseases (Anand and Stamler, 2012). Lastly, peroxynitrite provokes protein nitrotyrosination, an irreversible chemical addition of a nitro group to the tyrosine residue in target proteins generating 3-nitrotyrosine. This posttranslational modification usually impairs the normal physiological function of the proteins and therefore nitrotyrosination has been used as a marker in several neurodegenerative conditions such as amyotrophic lateral sclerosis (Peluffo et al., 2004). These aspects of the nitrergic system have been summarised in Figure 1.5.
Figure 1.5. A schematic representation of the nitrergic system and its downstream effects in hippocampal neurons following stress exposure.

In hippocampal neurons, the majority of nitric oxide (NO) production occurs via the conversion of L-arginine to L-citrulline by the neuronal isoform of nitric oxide synthase (nNOS) ①. High concentrations of NO can then covalently bond with protein thiol groups (protein-SH) to form S-nitroso-proteins (protein-SNO) ② or interact with the reduced form of glutathione (GSH) forming S-nitroso glutathione (GSNO) ③. This can be regenerated back to GSH via an initial conversion to oxidised glutathione (GSSG) by S-nitroso glutathione reductase (GSNOR), and subsequent reduction of GSSG by glutathione reductase (GSR) ④. Interaction of NO with the superoxide radical (O₂⁻) results in the formation of the neurotoxic radical, peroxynitrite (ONOO⁻) which irreversibly reacts with protein tyrosine (Tyr) residues to form 3-nitrotyrosine (3-NT) ⑤. Increased NO and ONOO⁻ are capable of causing cellular energy deficiency by inhibition of all components of the electron transport chain (Complex I - IV), including ATP synthase, resulting in decreased ATP production ⑥. Both post-synaptically produced NO, and NO produced by the inducible isoform of nitric oxide synthase (iNOS), can act as a neurotransmitter on pre-synaptic neurons ⑦. This pre-synaptic NO causes glutamate release, which activates post-synaptic NMDA receptors (NMDAR) to increase calcium (Ca²⁺) concentration and, in the presence of calmodulin, further potentiate nNOS-derived NO ⑧. Stress exposure increases NO by activating inflammatory cytokines to potentiate glial/astrocyte iNOS activity ⑨, and by increasing circulating corticosterone (CORT) which induces nNOS activity via a mineralocorticoid receptor (MR)-mediated pathway ⑩. This increase in NO results in downregulation of hippocampal glucocorticoid receptor (GR) thereby modulating stress-related behaviour in animal models ⑪. (Figure from Chen et al., 2015)
1.3.4 Stress-evoked modulation of the nitrergic system

It has been generally accepted that psychophysiological stress is associated with upregulation of NOS mRNA expression and enzymatic activity. For example, a single six hour acute immobilisation stress induces upregulation of iNOS expression and activity in the cerebral cortex which is mediated by the NMDA receptor and subsequent activation of the transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Madrigal et al., 2001). The acute stress-induced activation of the NMDA receptor also increases TNF-α via upregulation of TNFα-convertase. Antagonism of TNFα-convertase prevents the stress-induced translocation of NF-κB and subsequent iNOS expression, thus confirming the involvement of TNF-α (Madrigal et al., 2002). This is also supported by Shirakawa and colleagues (2004) who demonstrated glutamatergic activation and not catecholaminergic drive of the hypothalamic paraventricular nucleus to be responsible for the acute stress-induced increase in NO metabolites. Interestingly, biting activity is capable of suppressing the stress-induced increase in hypothalamic nNOS mRNA expression in rats (Hori et al., 2005). A single two hour acute restraint stress significantly increases the density of neurons expressing nNOS visualised by nicotinamide adenine dinucleotide phosphate-diaphorase histochemistry in the amygdaloid nucleus, an effect delayed by 5 days in the hippocampus and entorhinal cortex (Echeverry et al., 2004). Predator-induced posttraumatic stress significantly increases nNOS positive neurons and total NOx in the medial prefrontal cortex seven days after the 10 minute predator stress treatment (Campos et al., 2013). Conversely, Chakaborti and colleagues (2014) demonstrated that acute restraint stress causes a reduction in total NOx and an increase in the major endogenous NOS inhibitor, asymmetric dimethylarginine, in whole brain homogenates. This suggests that the stress-induced NOx increases in regions such as the hippocampus and hypothalamus may hold a high degree of functional significance. These biochemical changes in NOx and asymmetric dimethylarginine were observed alongside anxiety-like behaviour and were more pronounced in male compared to female rats. The pharmacological blockade of oestrogen biosynthesis exacerbated these biochemical and behavioural changes in females, suggesting that the observed sex differences are due to a protective role of oestrogen. Interestingly, bilateral injection of an NMDA receptor antagonist, NOS inhibitor, or NO scavenger into the dorsal hippocampus attenuated autonomic responses such as hypertension and tachycardia following a 60 minute acute restraint stress, suggesting that NMDA/NOS activation within the hippocampus plays a role in autonomic modulation during stress (Moraes-Neto et al., 2014).

Another study from the same group proposed a glutamatergic NMDA receptor-NO-cGMP signalling pathway in modulating contextual fear conditioning within the dorsal hippocampus, where intra-hippocampal injection of NMDA receptor antagonist DL-AP7, NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), and cGMP inhibitor 1H-
[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, attenuated the fear-conditioned response (Fabri et al., 2014).

Chronic immobilisation stress has been shown to increase NOx, iNOS activity, and peroxynitrite-induced 3-nitrotyrosine accumulation in cortical neurons (Olivenza et al., 2000). Notably, de Pablos and colleagues (2014) recently found a degree of regional specificity associated with this chronic stress-induced iNOS expression, with little to no constitutive expression in the substantia nigra following 9 days of unpredictable stress exposure. However, this same unpredictable stress model potentiates iNOS expression following exposure to exogenous immunostimulatory stressors such as lipopolysaccharides. Recent studies in several animal paradigms have demonstrated that inhibitors of NOS significantly modulate stress-related behaviours. In support of these findings, the commercially available antidepressant paroxetine, a selective serotonin reuptake inhibitor, also possesses NOS inhibition capability (Finkel et al., 1996). Wegener and Volke (2010) have reviewed and summarised these studies including data on each of the NOS inhibitor’s specificity and potency, and their anxiolytic- and antidepressant-like properties. Chronic unpredictable mild stress increases plasma nitrite levels and iNOS mRNA expression in the cortex, in addition to damaging cortical neurons and inducing depressive-like behaviour (Wang et al., 2008; Peng et al., 2012). These effects can be attenuated or prevented using NOS inhibitors, which was demonstrated by intra-hippocampal injection of the selective iNOS inhibitor, aminoguanidine, resulting in suppression of the chronic unpredictable mild stress-induced depressive-like behaviour in rats (Wang et al., 2008). Regional infusion of a selective nNOS inhibitor 7-nitroindazole (7-NI) into the hippocampus showed antidepressant-like effects similar to those with the iNOS inhibitor, aminoguanidine (Joca and Guimaraes, 2006). Likewise, the anxiogenic-like behaviours observed in rats during ethanol withdrawal is inhibited by administration of the selective iNOS inhibitor, 1400W, into the dorsolateral periaqueductal gray (Bonassoli et al., 2013). The data with intra-cerebral NOS inhibition is further supported by studies using systemic treatment. Intraperitoneal injection of 1400W increases survival of cortical neurons and decreases the depressive-like behaviour in mice (Peng et al., 2012). The nNOS inhibitor 1-([2-trifluoromethylphenyl]-imidazole (TRIM) given systemically 30 minutes prior to testing induces anxiolytic-like behaviours shown by increased time spent in the light compartment of a light-dark compartment test (Volke et al., 2003). Furthermore, TRIM administration decreased the immobility time in the forced swimming test, demonstrating an antidepressant-like effect comparable to the tricyclic antidepressant imipramine. In agreement with these observations, Ulak and colleagues (2008) injected TRIM intraperitoneally 50 minutes before a forced swim test and showed the involvement of the serotonergic system in the antidepressant-like actions of TRIM. This was further clarified in a later study in which the serotonin type II receptors were found to be responsible for this effect (Ulak et al., 2010). Furthermore, Joung and colleagues...
demonstrated that following a two hour immobilisation stress, the selective inhibitor 7-NI produced its anxiolytic-like effects shown by an increase in the time spent on the open arms of the elevated plus-maze through the direct reduction of NO metabolites in the PVN and locus coeruleus. A less specific NOS inhibitor, l-N\(^\text{G}\)-Nitroarginine methyl ester (l-NAME), injected systemically 30 minutes prior to testing shows protective effects against chronic swim stress-induced impairment of passive avoidance learning and hyperalgesia in rats (Nazeri et al., 2014). In a similar vein, Ferreira and colleagues (2012) performed behavioural, genomic, and proteomic analyses in rats and suggested that the antidepressant-like effects of NOS inhibition may involve the expression of additional factors including members of the glutathione redox system.

Genetic animal models have also contributed to the current understanding of nitrergic changes in stress. Thus, inhibition of NO production by nNOS gene deletion in mice suppressed hippocampal neurogenesis and exhibited antidepressant-like properties while nNOS over-expression in the hippocampus was essential for chronic stress-induced depression (Zhou et al., 2007). Recently, a number of studies have proposed a regulatory role of NO on the limbic HPA stress axis. Zhang and colleagues (2010) used mice lacking the nNOS gene to demonstrate an anxiolytic-like phenotype when tested using an elevated plus-maze, similar to normal mice treated with intra-hippocampal microinjection of the selective nNOS inhibitor 7-NI. The authors proposed a signalling pathway involving the activation of serotonin type IA receptors which mediate, via an unknown mechanism, the downregulation of hippocampal nNOS, leading to a decrease in NO and subsequent inhibition of cAMP response element-binding (CREB) protein phosphorylation. A follow up study elucidated further the link between NO and the HPA axis by showing that chronic mild stress and glucocorticoid exposure lead to hippocampal nNOS overexpression via activating hippocampal mineralocorticoid receptor (MR) (Zhou et al., 2011). The excessive nNOS-derived NO significantly downregulated local glucocorticoid receptor (GR) expression through either the soluble guanylyl cyclase/cGMP or peroxynitrite/extracellular signal-regulated kinase (ERK) signalling pathways. The significant downregulation of GR in the hippocampus leads to an elevation in hypothalamic corticotropin-releasing hormone and the depressive-like behaviours in mice as illustrated in Figure 1.5. It is important to note that nNOS deletion, infusion of intrahippocampal nNOS inhibitor, and NO-cGMP signalling blockade prevented the chronic mild stress-evoked behavioural modification. Conversely, Gao and colleagues (2014) have demonstrated a significant decrease in constitutive NOS activity and neuronal NOS immunoreactivity in the hypothalamic paraventricular nucleus of rats exposed to chronic unpredictable stress. However, the chronic glucocorticoid-induced MR-nNOS-NO pathway may not be present in the hypothalamus and is exclusive to the MR-rich hippocampus which drives HPA axis hyperactivity through impaired negative feedback (Zhu et al., 2014).
The considerable body of evidence from animal models is progressively expanding and supported by modest but significant clinical studies. Several reports have shown that increased levels of NO metabolites are present in depressed and autistic patients (Suzuki et al., 2001; Sogut et al., 2003; Lee et al., 2006). Patients with recurrent depressive behaviour displayed higher plasma NOx concentrations which were associated with cognitive impairment (Talarowska et al., 2012). Galecki and colleagues (2010, 2011) discovered single nucleotide polymorphisms in exon 22 of the NOS2A gene (iNOS) and exon 29 of the NOSI gene (nNOS) in depressed Caucasian individuals. Furthermore, three single nucleotide polymorphisms located at the regulatory region of NOSI gene are responsible for the susceptibility of an individual to depressive disorders (Sarginson et al., 2014). The intrinsic cross talk between neuroendocrine stress and the nitrergic system activation is now an important physiological consideration. Further understanding the role of this system is important in identifying early players in stress-induced pathological conditions.
1.4 Thesis Rationale

A growing body of evidence suggests that the aetiology of anxiety and depressive-related conditions can be derived from the sensitization of particular stress circuits that are ‘primed’ following exposure to a short-term stressor. The duration for stress-related circuitry priming far exceeds responses to adrenergic and glucocorticoid-mediated signalling. Understanding the mechanisms underlying the induction of this long latency will provide a significant link between stress and the pathogenesis of anxiety and depressive disorders. The nitrergic system has been implicated in the regulation of the neuroendocrine stress response with chronic mild stress increasing hippocampal nNOS expression via a MR-dependent mechanism causing chronic production of NO, ultimately leading to down-regulation of GR. This augmentation in hippocampal GR is associated with abnormal negative feedback of glucocorticoids upon the HPA axis and has been linked to the pathogenesis of depression. However, this represents changes occurring in chronic stress conditions, while the acute physiology of altered nitrergic status in both peripheral and central tissues is poorly understood. Therefore, the present thesis used an acute stress model to establish the time course and extent of nitrosative changes in peripheral blood and stress-related neural regions such as the hippocampus. Another signalling pathway with an important role in mediating the stress response is the endogenous cannabinoid (endocannabinoid) system. Interestingly, it has been suggested that inhibition of endocannabinoid degradation with an enzyme inhibitor suppresses the inflammatory-related nitrergic activation following traumatic brain injury in mice. However, little is known on the effects of endocannabinoid degradation on the nitrergic system following short term stress. Therefore, the involvement of endocannabinoid-mediated regulation of the nitrergic system following acute stress was also examined.
1.5 Overall Aim

This thesis aims to establish the temporal profile and functional output of nitrergic indicators responsive to an acute psychological stress in peripheral and central tissues. In addition, this thesis examined the relationship between endocannabinoid signalling and the nitrergic-related inflammatory response in the hippocampus following short term stress.

To achieve these overall aims, three separate but closely related studies were undertaken:

1. To establish the temporal profile of nitrosative changes in peripheral plasma and erythrocytes following short term stress. (Chapter III)

2. To establish the physiological and functional output of nitrergic indicators in brain regions displaying differential stress sensitivity following short term stress. (Chapter IV)

3. To determine if an increase in endocannabinoid signalling will prevent the acute stress-mediated activation of inducible nitric oxide synthase. (Chapter V)
Chapter II

General Materials and Methods
2.0 General Methods
The following describes in length the general methods used in the generation of the present thesis as this is often truncated in manuscript preparation.

2.1 Ethics Clearance
Experiments carried out in Chapter III received ethical clearance from The University of Queensland Animal Ethics Committee under approval number SBS/363/11/URG. Experiments carried out in Chapter IV and V were performed under approval number SBMS/018/11/URG and SBS/456/14/URG respectively. All experiments strictly adhered to the Animal Care and Protection Act Qld (2002), Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition 2004), and the University Animal Ethics Committee policies and standard operating procedures.

2.2 Animal Housing
Outbred male Wistar rats (Rattus norvegicus) aged 5-7 weeks postnatal were sourced from The University of Queensland Biological Resources breeding colony. Rats were housed in standard animal cages within a colony room with temperature controlled between 21-23°C and humidity kept between 50-60% in the Australian Institute of Bioengineering and Nanotechnology (AIBN) animal house (The University of Queensland, St Lucia, Queensland, Australia). The colony room was fitted with an automatic timer maintaining a 12:12h light-dark cycle with lights off at 12:30 h. Rats were given ad libitum access to standard rat chow and water at all times outside of experimental procedures.

2.3 Habituation and Transportation
Upon arrival to the AIBN animal facility, rats were given a period of habituation (7 days) to this new environment prior to any experimental habituation. Rats were then habituated to human handling for 10 minutes per day over 6 days. During this habituation period, only the two experimenters were in contact with the animals. On each experimental day, rats were transported in their home cages immediately prior to the changeover from light to dark cycle to an experimental room that was under low light and noise within the same animal facility and given one hour acclimatization to this new environment. Upon arrival to the experimental room, rat cages were housed within a larger containment chamber (68 cm (L) x 45 cm (W) x 38.5 cm (H)) to minimise sound, light and noise. Over the duration of the treatment, only the two experimenters entered this room to minimise any additional stress.

2.4 Acute Stress
In Chapter III, rats were immobilised according to a well-established protocol by Hori and colleagues (2004) and Tsukinoki and colleagues (2006). Rats were immobilised on a Perspex™
board (18×25 cm) in the supine position using a polyester strapping with their legs fixed at an angle of 45 degrees to the midline with adhesive tape. Acute immobilisation stress was applied within the individual home cages enclosed with the larger containment chamber. In Chapter IV and V, acute restraint stress was applied using adjustable wire mesh restrainers in order to minimise overheating and avoid unnecessary body compression (Dayas et al., 1999). The restrainers were constructed from a polyvinyl chloride (PVC) pipe skeleton (20 cm (L) x 7.5 cm (D)) externally coated with welded wire mesh (6 mm x 6 mm grid), an adjustable internal nasal insert allowing ventilation, and a rear tail aperture. In order to isolate the effects of acute stress for both models, control animals were deprived of food and water for an equal time during experiments using these two stressors.

2.5 PF-3845 Administration

In Chapter V, the fatty acid amide hydrolase (FAAH) inhibitor, PF-3845 (5 mg/kg), was dissolved in 2% dimethyl sulfoxide (DMSO) in normal saline (0.9% sodium chloride) and injected intraperitoneally (i.p.) 60 minutes before stress treatment with an injection volume of 10 mL/kg. The PF-3845 hydrate was purchased from Sigma under catalogue number PZ0158 (St. Louis, MO). The dose and injection route was adapted according to studies by Ahn and colleagues (2009b) and Tchantchou and colleagues (2014). Figure 2.1 demonstrates injection of PF-3845 significantly elevated AEA levels by 10-fold in mouse brain as well as other N-acylethanolamines including N-pamitoyl ethanolamine and N-oleylethanolamine which peaked at 3 hours post injection and maintained at relatively maximal levels for up to 7 to 12 hours.

![Figure 2.1](image)

**Figure 2.1.** Levels of (A) endocannabinoid N-arachidonoyl ethanolamine (anandamide; AEA), and two other N-acylethanolamines with affinity to cannabinoid-like G-coupled receptors (B) N-pamitoyl ethanolamine (PEA) and (C) N-oleylethanolamine (OEA) in brain tissue following intraperitoneal injection of two different fatty acid amide hydrolase inhibitors, PF-3845 and URB597 (10 mg/kg final dose). n=4 C57Bl/6J mice/group; *p<0.05, **p<0.01. *(Figure from Ahn et al., 2009b)*
2.6 Experimental Groups

Table 2.1. An overview of the treatment groups used in three experimental chapters of this thesis.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong> <em>(Chapter III)</em></td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td><strong>Experiment 2</strong> <em>(Chapter IV)</em></td>
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<tr>
<td>Control</td>
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<tr>
<td><strong>Experiment 3</strong> <em>(Chapter V)</em></td>
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<tr>
<td>Control with Vehicle injection</td>
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<tr>
<td>60 min restraint stress with Vehicle injection</td>
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<tr>
<td>240 min restraint stress with Vehicle injection</td>
</tr>
<tr>
<td>360 min restraint stress with Vehicle injection</td>
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</table>

2.7 Sample Collection

In Chapter III, baseline blood samples were collected via tail-tip sampling into both heparinised (20 IU/mL blood) and ethylenediaminetetraacetic acid (EDTA)-coated (1.5 mg/mL blood) Eppendorf tubes prior to treatment. Subsequent sampling at 60, 90, and 120 minutes during stress was achieved by gently removing clotted blood. Blood samples were each around 150 µL to guarantee that the total amount of blood collected remains under 10% of the calculated blood volume. The EDTA-treated samples were used for total nitrite and nitrate determination, as heparin in plasma may produce precipitation upon addition of the Griess reagent. Heparinised whole blood (2.5 µL aliquot) was used for the determination of glucose using a standard glucometer (Accu-Chek® Performa®, Roche Diagnostics Aust. Pty. Ltd., Castle Hill NSW 2154, Australia). The remaining blood samples were immediately centrifuged at 200 x g for 5 minutes to avoid degradation. Phosphate buffered saline (PBS; 50 mM, pH=7.4)-washed erythrocytes were immediately used to determine the general oxidative status, while aliquots of erythrocytes and supernatant plasma were stored at -80 °C for later analysis of nitric oxide production, plasma oxidative status, reduced and oxidised glutathione, lipid peroxidation, haemoglobin, and plasma hormones. At the end of the
treatment, rats were weighed and sacrificed with sodium pentobarbital (100 mg/kg i.p. injection, Lethabarb, Virbac).

In Chapter IV, following each allocated treatment, terminal blood samples were collected following sodium pentobarbital injection via cardiac puncture into ice-chilled heparinised tubes (20 IU/mL blood). Whole blood was centrifuged at 200 × g for 5 minutes and supernatant plasma was collected and stored at -80°C for later determination of corticosterone. Brains were rapidly removed following cervical dislocation and frozen immediately on a sheet of aluminium foil embedded in powdered dry ice. The brain regions of interest were then cryo-dissected prior to analysis. This was achieved by mounting the cerebellar face of the brain with Optimum Cutting Temperature (OCT) compound and sectioning in a cryostat. The striatum (Bregma 2.52 to 0.36 mm) and hippocampus (Bregma -2.64 to -4.44 mm) were identified using a rat brain atlas (Paxinos and Watson, 2007) and then removed from individual serial sections on a metallic dry ice-embedded platform using a scalpel and dissection microscope. Regionalised tissue was stored in Eppendorf tubes at -80°C until later determination of nitric oxide production and metabolism, enzymatic activity of nitric oxide synthases, and relative gene expression.

In Chapter V, post-treatment samples were collected via tail-tip sampling into ice-chilled heparinised tubes (20 IU/mL blood). Blood samples were each around 150 µL to guarantee that the total amount of blood collected remains under 10% of the calculated blood volume. Whole blood was centrifuged at 200 × g for 5 minutes and supernatant plasma was collected and stored at -80°C for later determination of corticosterone. Following each allocated treatment, rats were sacrificed with sodium pentobarbital. Brains were rapidly removed following cervical dislocation and frozen immediately. The brain regions of interest were then cryo-dissected as described previously and regionalised hippocampus was stored in Eppendorf tubes at -80°C until later determination of relative gene expression.

2.8 Plasma Prolactin

A commercially available ELISA (Demeditec Diagnostics, GmbH, Kiel, Germany) was used to determine prolactin concentrations in Chapter III with a lower detection limit of 0.6 ng/mL. A 10 µL plasma aliquot was diluted with 15 µL of rat prolactin calibrator/sample diluent, standards were serial diluted to range of 2.5-80 ng/mL, both diluted samples and standards were loaded into a monoclonal anti-rat prolactin antibody-coated microplate. To these 25 µL aliquots, 50 µL of sample buffer was added and shaken for 2 hours at room temperature. The contents of the microplate were discarded and the wells washed 4 times with 300 µL washing buffer prior to the addition of 200 µL of enzyme-labelled rat prolactin antibody conjugated to horseradish peroxidase and shaken again for 1 hour. The plate was washed 4 times with wash buffer followed by the addition of 200 µL of 3,
3', 5, 5'-tetra-methyl-benzidine in buffered peroxide solution and incubated for 30 minutes in the dark. A 50 µL aliquot of 2 M hydrochloric acid stop solution was subsequently loaded to each well and the absorbance was measured at 450 nm using a Sunrise™ microplate absorbance reader (TECAN, Groedig, Austria). Prolactin concentrations were determined using the log-linear standard curve and corrected for dilution.

2.9 Plasma Corticosterone

Plasma corticosterone concentrations were determined in duplicate using an in-house radioimmunoassay with a minimum detection of 10 pg/mL. Unlabelled steroids were reconstituted in phosphate buffered saline (0.1 M, pH=7.0) containing 0.1% (w/v) porcine gelatin (PBS-G) to generate a standard curve with a range of 0-10,000 pg/mL. Anti-rat corticosterone polyclonal antibody raised in sheep using corticosterone-3-monoxime coupled to keyhole limpet hemocyanin was purchased from Sapphire Bioscience Pty. Ltd., Waterloo, Australia. The antibody was diluted with PBS-G to yield an optimum working titer that binds 50% of the tritium radiolabelled corticosterone tracer in the absence of competing unlabelled steroid. A 5 µl aliquot of vortex homogenised plasma was extracted by adding 2 mL of glass-distilled dichloromethane followed by a 5 minute vortex allowing the separation of aqueous phase and steroid-containing organic phase. The aqueous phase was frozen along the wall of a large test tube (16 × 100 mm) using liquid nitrogen while the organic phase will be decanted to a smaller glass tube (12 × 75 mm) for radioimmunoassay. Extracts was dried at 55°C under a stream of nitrogen gas and reconstituted in 2 mL of PBS-G. A 100 µL aliquot of unlabelled corticosterone standard or reconstituted sample was incubated with 100 µL of the highly specific anti-rat corticosterone polyclonal antibody and 100 µL of [1, 2, 6, 7-3H]-corticosterone tracer (100 Ci.mmol⁻¹, Perkin Elmer) overnight at 4°C after a gentle vortex and centrifuge at 1000 x g for 1 minute. The following day, samples were loaded with 500 µL of dextran-coated charcoal (50 mM, pH=7.4) to strip any unbound steroids and centrifuged at 1000 x g for 10 minutes at 4 °C. Radioactivity was counted in a 100 µL aliquot of the resulting supernatant with 2 mL of liquid scintillation cocktail (Ultima Gold™, Perkin Elmer) using a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer). The counts per minute were then converted to equivalent corticosterone concentrations from the standard curve and corrected for dilution and dichloromethane extraction recovery.

2.10 Plasma Insulin

Plasma insulin concentrations were measured in Chapter IV using a commercial radioimmunoassay kit (Coat-a-Count ® Insulin, Siemens Healthcare, Frimley, UK) and the assay sensitivity was 0.25 mIU/L. A 40 µL plasma aliquot was diluted to 200 µL of assay buffer and standards were serial diluted to a range of 2.5-80 mIU/L. Diluted samples and standards were pipetted directly to the
bottom of the insulin antibody-coated polypropylene tubes followed by a 1 mL addition of \[^{125}\text{I}]\)-insulin tracer. Samples were incubated at room temperature overnight after which the contents within each tube were decanted thoroughly. Radioactivity was then counted using a 2470 Automatic Gamma Counter (WALLAC Wizard\(^2\), Perkin Elmer). The counts per minute were corrected for non-specific binding and then converted to equivalent insulin concentrations from a standard curve and corrected for dilution.

2.11 DAF-FM DA Assay

2.11.1 Plasma assay

Intracellular nitric oxide were assayed with 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA) developed by Kojima and colleagues (1998) to measure low concentrations of nitric oxide. This pH-insensitive fluorescent compound is cell-permeable and passivelydiffuses across cell membrane. It is essentially non-fluorescent, however, once inside the cell, it is de-acetylated by intracellular esterases to become a weakly fluorescent DAF-FM. After reacting with nitric oxide specifically, the produced trizole form (DAF-FM T) shows stable and intense fluorescence which can be detected with excitation/emission maxima of 495/515 nm. Itoh and colleagues (2000) have demonstrated that DAF-FM does not interact with nitrite, nitrate, or other ROS including peroxynitrite, superoxide, and hydrogen peroxide. Briefly, a 4 µM DAF-FM DA working solution was prepared fresh in PBS (50 mM, pH=7.4). Frozen plasma was rapidly defrosted and a 1% (v/v) plasma suspension was prepared in PBS. Four replicates were plated on a 96-well plate containing final concentrations of 0.5% (v/v) plasma suspension and 2 µM of DAF-FM DA under low light conditions. Plates were incubated for 60 minutes at room temperature and sample fluorescence was measured by a POLARstar OPTIMA microplate reader (BMG Labtechnologies). Data were corrected with corresponding protein concentrations and expressed as the percentage change in arbitrary fluorescence units relative to baseline/control values.

2.11.2 Tissue assay

A 4 µM DAF-FM DA working solution was prepared fresh in PBS (50 mM, pH=7.4). Four replicates of PBS-diluted tissue supernatant (0.1% w/v) were plated on a 96-well plate containing a final concentration of 2 µM of DAF-FM DA. Plates were incubated for 60 minutes and sample fluorescence was measured by a POLARstar OPTIMA microplate reader (BMG Labtechnologies). Data were corrected with corresponding protein concentrations and expressed as the percentage change in arbitrary fluorescence units relative to control values.
2.12 Total Nitrite and Nitrate Levels

2.12.1 Plasma assay
The total nitric oxide metabolites, NOx, were measured with the Griess reaction using a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A) with a minimum sensitivity of 2.5 µM/L. Plasma samples using EDTA as an anticoagulant were ultra-filtered through a 10-kDa molecular weight cut-off centrifugal device with omega membrane (Pall Nanosep®) to reduce background absorbance due to the presence of haemoglobin. These filters were pre-rinsed twice with 500 µL of MiliQ water (centrifuged at 10,000 x g for 5 minutes each). For assaying NOx, 20 µL of ultra-filtered plasma was diluted with 60 µL of assay buffer followed by a 10 µL addition of enzyme cofactor mixture. The plate was incubated at room temperature for 3 hours on an orbital shaker after the addition of nitrate reductase (10 µL). Following incubation, 50 µL of Griess reagent R1 was added to each wells and immediately followed by 50 µL of Griess reagent R2. Colour was developed for 10 minutes at room temperature and read at 540 nm using a Sunrise™ microplate absorbance reader. Within the assay, nitrate standards were serial diluted to a range of 5-40 µM. Samples were corrected with corresponding protein concentrations and expressed as a percentage change relative to baseline values.

2.12.2 Tissue assay
The commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to measure NOx in the hippocampus and striatum. Tissue homogenate (20X in PBS) was ultra-filtered through the centrifugal device (Pall Nanosep®) at 10,000 x g for 20 minutes as this will improve colour development with the Griess reagent. Assay protocol is as described above in the plasma assay. Samples were corrected with corresponding protein concentrations and expressed as µM per mg of protein.

2.13 Plasma Oxidative Status
A fluorometric assay modified from the erythrocyte assay in Spiers and colleagues (2013) involving the direct oxidation of dichlorofluorescin (DCFH) by non-specific ROS to a highly fluorescent product, dichlorofluorescein (DCF) was used to determine the overall cellular oxidative status in plasma. This fluorometric assay relies on the nonionic and nonpolar 2’, 7’-dichlorofluorescin diacetate (DCFH-DA) crossing the cell membrane when applied to intact cells. It is then hydrolysed by non-specific intracellular esterases to non-fluorescent DCFH. In the presence of ROS, non-fluorescent DCFH is rapidly oxidised to a highly fluorescent DCF. A 1 mM DCFH-DA stock solution was prepared in dimethyl sulfoxide and ethanol in a ratio of 1:4 to a final volume of 1 mL. The stock solution was wrapped in aluminium foil to prevent photo-oxidation and stored at -20°C. The DCFH-DA working solution was prepared fresh each day by diluting 100 µL of the stock
solution with 4.9 mL of PBS to achieve a final concentration of 200 µM. A 1% plasma suspension (v/v) was prepared in PBS. Eight replicates each containing 50 µL of 1% plasma suspension and 50 µL of 200 µM DCFH-DA were plated on a 96-well plate. To ensure sample reliability and reproducibility, the loaded plate was manually shaken prior to double-orbital shaking in a POLARstar OPTIMA multidetection microplate reader for 30 seconds. Sample fluorescence was analysed with absorbance and emission filters set at 485 nm and 520 nm respectively. After the initial reading, the microplate was wrapped with aluminium foil and kept in the dark at room temperature for 10 minutes until the final reading. Samples fluorescence was corrected with corresponding protein concentrations and data expressed as a percentage change relative to baseline values.

2.14 Reduced and Oxidised Glutathione Assay in Erythrocytes

A fluorometric assay modified from Senft and colleagues (2000) for the measurement of both reduced glutathione (GSH) and glutathione disulphide (GSSG) was used employing the fluorophore O-phthalaldehyde (OPA). Briefly, 50 µL of erythrocytes were suspended with 450 µL of 5% metaphosphoric acid and vortexed thoroughly to deproteinate the sample. The acidic suspension was left on ice for 10 minutes and centrifuged at 2300 x g for 5 minutes. Since OPA reacts with GSH only (not GSSG), 150 µL of deproteinated sample was redox quenched with 250 µL of potassium phosphate buffer (1 M; pH=7.0) and incubated with 20 µL of OPA (4 mg/mL of methanol) at room temperature for 30 minutes to specifically quantify GSH. The concentration of GSH was corrected with the non-glutathione dependent fluorescence by a corresponding volume matched 7.5 mM N-ethylmaleimide-reacted sample. The protocol for quantifying GSSG specifically requires a GSH quencher to remove all GSH thus preventing the reaction with OPA while GSSG is unaffected. A reducing agent was then added to remove any excess GSH quencher and to convert GSSG to GSH. This converted GSH was then measured fluorometrically using OPA. Duplicates of the deproteinated supernatant (150 µL) were incubated with 20 µL of 7.5 mM N-ethylmaleimide for 5 minutes at room temperature to quench GSH and this was subsequently redox quenched in 1 M potassium phosphate buffer. A 30 µL aliquot of the reducing agent sodium dithionite was then added to one duplicate to remove any excess N-ethylmaleimide and convert GSSG to GSH. This converted GSH was measured fluorometrically following a 30 minute incubation with 20 µL of OPA. The concentration of GSSG was calculated by the difference between sodium dithionite-reacted sample and its corresponding blank using 0.1 M potassium buffer with EDTA (pH=7.5). The OPA-derived fluorescence was measured at 365 nm (excitation) and 430 nm (emission) with a POLARstar OPTIMA multidetection microplate reader. Sample values were determined directly from linear GSH and GSSG standard curves ranging between
5.085-81.353 µM. Data was corrected with corresponding hemoglobin concentrations and expressed as a percentage of total glutathione equivalents [GSH+2GSSG] and GSH/GSSG ratio relative to baseline values.

### 2.15 Plasma Lipid Peroxidation Assay

The peroxidation of lipids was assessed using the thiobarbituric acid reactive substances (TBARS) assay through quantification of malondialdehyde (MDA) production using a modified protocol from Zavodnik and colleagues (2002). To prevent further sample peroxidation occurring during the assay process, 10 µL of butylated hydroxytoluene in methanol was added to 50 µL of diluted samples or 1, 1, 3, 3-tetraethoxypropane standards ranging from 3.125 to 100 nmol/mL. A 125 µL aliquot of 1% (w/v) thiobarbituric acid in 0.1 M sodium hydroxide pH adjusted with concentrated hydrochloric acid to pH=3.5 was loaded and vortexed vigorously. Samples were then acidified with 315 µL of a 20% (w/v) trichloroacetic acid solution and incubated in an 85°C water bath for 30 minutes using glass marbles as condensers. Glass test tubes were subsequently cooled for 10 minutes on ice and loaded with 500 µL of n-butanol and centrifuged at 1000 x g for 5 minutes at 4°C. The extracted pink adduct in the supernatant was loaded to a 96-well plate and measured fluorometrically at 544 nm excitation and 590 nm emission with a POLARstar OPTIMA microplate reader (BMG Labtechnologies). Sample values were quantified directly from the linear standard curve, adjusted for protein content, and data were expressed as a percentage change relative to baseline values.

### 2.16 Nitric Oxide Synthase Activity in Tissue

The activity of nitric oxide synthase in brain regions of interest were determined by a standard citrulline formation assay as previously described (Weissman and Gross, 2001). The assay is based on the biochemical conversion of L-arginine to nitric oxide along with the stoichiometric production of L-citrulline by nitric oxide synthase. This assay includes the use of radioactive substrates, L-[14C(U)]-arginine (274 mCi/mmol, Perkin Elmer) enabling sensitivity at the picomole level. Briefly, tissue was homogenised in 15X (w/v) of PBS (50 mM, pH=7.4) using a homogeniser (Ultra Turrax® T10) for 15 seconds. The homogenate was centrifuged at 10,000 x g for 10 minute at 4°C. Tissue supernatant (10 µL) was then added with 12.5 µL of assay buffer (50 mM Tris-HCl; pH=7.4 with 1.25 mM CaCl₂, 1 mM of MgCl₂, 0.6 mM of L-valine, and 1 mM of dithiothreitol). A 12.5 µL aliquot of a mixture of cofactors containing 50 mM Tris-HCl with 100 nM calmodulin, 40 µM of (6R)-5, 6, 7, 8-tetrahydrobiopterin dihydrochloride, 8 µM of flavin adenine dinucleotide, and 8 µM of riboflavin 5’-monophosphate was then added. A 2.5 µL aliquot of either MiliQ or 20 mM ethylene glycol tetraacetic acid (EGTA) was added to determine total or calcium-independent iNOS activity respectively. An enzyme blank was included for every sample which requires a 12.5 µL of
8 mM Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) instead of the cofactors followed by the addition of a 2.5 µL EGTA (20 mM). Reaction was initiated by the addition of radiotracer mix (12.5 µL) containing 50 mM of Tris-HCl, 2 µCi/mL of L-[14C(U)]-citrulline, and 4 mM of NADPH at 25°C and this was incubated for 30 minutes. The reaction was stopped by 700 µL of 1 part Dowex AG 50W-X8 (100-200 mesh, Na+ form) to 2 parts EDTA-HEPES buffer (pH=5.5; v/v) with 80 µM of NOG-nitro-L-arginine methyl ester (L-NAME) followed by 2 mL of MiliQ. L-[14C(U)]-citrulline was specifically eluted by centrifugation at 1000 x g for 5 minutes and the radioactivity was quantified with a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer). Enzyme activities were calculated as pmol/min/mg protein accounting for individual enzyme blanks and the presence or absence of EGTA and data were expressed relative to control values.

2.17 Ribonucleic Acid Extraction and Preparation of cDNA

Total RNA was extracted from each brain region of interest using lysis buffer RLT with β-mercaptoethanol (10 µL/mL) provided in a RNasy mini kit (QIAGEN, Doncaster, Australia). Tissues were homogenised with an Ultra-Turrax® T8 homogeniser (IKA®, Labtek, Brendale, Australia) and cellular debris was pelleted by centrifugation for 3 minutes at top speed. The resulting supernatant was mixed with an equal volume of 70% ethanol in RNAase-free water and transferred to the provided spin columns which binds nucleic acid and allows the eluent to pass through and discard. The RNA isolation was completed with the presence of deoxyribonuclease I (approximately 30 Kunitz units; QIAGEN, Doncaster, Australia) for 15 minutes to digest any genomic DNA contaminating the RNA preparation. The total RNA was eluted in 30 µL of RNase/DNase-free water prior to determining sample concentration and quality by the A260 reading with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Scoresby Vic, Australia). The quantity of RNA was determined using the Beer-Lambert law and the optical density at 260 nm, while the quality of the RNA yield was taken as the A260/A280 ratio (acceptable 260/280 ratio ≥1.80). The RNA preparations were stored frozen at -80°C until use.

The synthesis of cDNA was performed using 1 µg of total RNA from each sample and reverse transcribed with the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) using an optimised blend of oligo(dT) and random primers according to the manufacturer's instructions. Negative samples were generated using extracted RNA under the same conditions with the omission of reverse transcriptase present in the reaction mixture.
2.18 mRNA Expression

Taqman gene expression ‘assay-on-demand™’ kits (Life Technologies, Mulgrave, Australia) with optimised primers and reporter dye labelled-probes (FAM; 6-carboxyfluorescein) were used to detect gene expression for the following targets in Table 2.2.

Table 2.2. Applied Biosystems™ by Life Technologies inventoried ‘assay-on-demand™’ probes used in this thesis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Gene Aliases</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adam17</td>
<td>ADAM metallopeptidase domain 17</td>
<td>Tumor necrosis factor-α converting enzyme (TACE)</td>
<td>Rn00571880_m1</td>
</tr>
<tr>
<td>Ciita</td>
<td>Class II, major histocompatibility complex,</td>
<td>Major histocompatibility complex II transactivator (Mhc2ta)</td>
<td>Rn01424725_m1</td>
</tr>
<tr>
<td></td>
<td>transactivator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II1b</td>
<td>Interleukin 1 beta</td>
<td>IL-1β</td>
<td>Rn00580432_m1</td>
</tr>
<tr>
<td>II6</td>
<td>Interleukin 6</td>
<td>IL-6</td>
<td>Rn01410330_m1</td>
</tr>
<tr>
<td>MiF</td>
<td>Macrophage migration inhibitory factor</td>
<td>MIF or glycosylation-inhibiting factor (GIF)</td>
<td>Rn00821234_g1</td>
</tr>
<tr>
<td>Nfkbia</td>
<td>Nuclear factor of kappa light polypeptide</td>
<td>Inhibitor of nuclear factor kappa B (NF-κB) alpha (IκB-α)</td>
<td>Rn01473657_g1</td>
</tr>
<tr>
<td></td>
<td>gene enhancer in B-cells inhibitor, alpha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nos1</td>
<td>Nitric oxide synthase 1, neuronal</td>
<td>Neuronal nitric oxide synthase (nNOS)</td>
<td>Rn00583793_m1</td>
</tr>
<tr>
<td>Nos2</td>
<td>Nitric oxide synthase 2, inducible</td>
<td>Inducible nitric oxide synthase (iNOS)</td>
<td>Rn00561646_m1</td>
</tr>
<tr>
<td>Nr3c1</td>
<td>Nuclear receptor subfamily 3, group C, member 1</td>
<td>Glucocorticoid receptor (GR)</td>
<td>Rn00561369_m1</td>
</tr>
<tr>
<td>Nr3c2</td>
<td>Nuclear receptor subfamily 3, group C, member 2</td>
<td>Mineralocorticoid receptor (MR)</td>
<td>Rn00565562_m1</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>Cyclooxygenase-2 (COX-2)</td>
<td>Rn01483828_m1</td>
</tr>
<tr>
<td>Tnf</td>
<td>Tumor necrosis factor</td>
<td>TNF</td>
<td>Rn01525859_g1</td>
</tr>
</tbody>
</table>

All expression assays were multiplexed with 2’-cholo-7’-phenyl-1, 4-dichloro-6-carboxyfluorescein (VIC)-labelled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). The fold change in expression of each target gene was calculated using the ΔΔCT method relative to the control group.
2.19 Haemoglobin Determination

Haemoglobin concentrations were used to standardise reduced and oxidised glutathione assay in erythrocytes. This was determined using a modification to the cyanmethemoglobin method of Fairbanks and Klee (1999). Drabkin’s solution was prepared by dissolving 0.2 g potassium ferricyanide (K₃Fe[CN]₆), 0.05 g potassium cyanide (KCN), and 1.0 g sodium bicarbonate (NaHCO₃) successively in 1000 mL of MiliQ water. To this solution, 0.5 mL of 30% Brij 35 (w/v) was added as a surfactant to minimise the turbidity created by erythrocytes. This assay was adapted for microplates allowing the measurement of 10 µL of diluted samples in 125 µL of Drabkin’s solution read at 540 nm on a spectrophotometer with a 700 nm reference wavelength. Sample values were determined directly from a linear standard curve constructed from bovine haemoglobin ranging 0.07-9 mg/mL.

2.20 Protein Estimation

Protein was routinely measured to standardise assays performed with plasma and tissue samples utilising the RED 660™ Protein Assay (G-235 Biosciences, St Louis, MO, USA) using bovine serum albumin as a standard ranging between 37.5-2000 µg/mL. This kit was scaled for microplates allowing the determination of protein content in 6 µL diluted samples directly incubated in 120 µL of assay reagent. This was developed at room temperature for 15 minutes and the absorbance read at 660 nm with a 700 nm reference on a Sunrise™ microplate absorbance reader.

2.21 Statistical Analysis

Data were analysed using GraphPad Prism (Versions 5.0.3 and 6.0, GraphPad Software Inc., San Diego, CA, USA). Two-way repeated measure ANOVAs with Bonferroni post-test were used to compare control and stress data for the intra-individual time course analysis in Chapter III. One-way ANOVAs followed by Dunnett’s multiple comparisons test were used to compare control and stress groups sacrificed at different time intervals in Chapter IV. Where data were not normally distributed, appropriate non-parametric Kruskal-Wallis ANOVA with Dunn’s multiple comparisons test was utilised. Lastly, two-way repeated measure ANOVAs with Fisher’s Least Significant Difference (LSD) test were used to compare between vehicle and PF-3845-injected groups in Chapter V. Results were expressed as mean ± standard error of the mean (± SEM) and p-values less than 0.05 were considered statistically significant.
Chapter III

Reactive nitrogen species contribute to the rapid onset of redox changes induced by acute immobilisation stress in rats

This chapter has been published in: Stress 17(6), 520-527.
3.1 Abstract

Acute stress leads to the rapid secretion of glucocorticoids, which accelerates cellular metabolism, resulting in increased reactive oxygen and nitrogen species generation. Although the nitrergic system has been implicated in numerous stress-related diseases, the time course and extent of nitrosative changes during acute stress have not been characterised. Outbred male Wistar rats were randomly allocated into control (n=9) or 120 minute acute immobilisation stress (n=9) groups. Serial blood samples were collected at 0 (baseline), 60, 90, and 120 minutes. Plasma corticosterone concentrations increased by approximately 350% at 60, 90, and 120 (p<0.001) minutes of stress. The production of nitric oxide, measured as the benzotriazole form of 4-amino-5-methylamino-2′,7′-difluorofluorescein, increased during stress exposure by approximately 5%, 10%, and 15% at 60 (p<0.05), 90 (p<0.01) and 120 (p<0.001) minutes respectively compared to controls. Nitric oxide metabolism, measured as the stable metabolites nitrite and nitrate, showed a 40% to 60% increase at 60, 90, and 120 (p<0.001) minutes of stress. The oxidative status of 2′, 7′-dichlorofluorescein in plasma was significantly elevated at 60 (p<0.01), 90, and 120 (p<0.001) minutes. A delayed decrease of approximately 25% in the glutathione redox ratio at 120 minutes (p<0.001) also indicates stress-induced cellular oxidative stress. The peroxidation of plasma lipids increased by approximately 10% at 90 (p<0.05) and 15% at 120 (p<0.001) minutes, indicative of oxidative damage. It was concluded that a single episode of stress causes early and marked changes of both oxidative and nitrosative status sufficient to induce oxidative damage in peripheral tissues.
3.2 Introduction

Living organisms maintain a complex and dynamic homeostasis that is continually challenged by intrinsic and extrinsic stressors. Three discrete and dedicated systems maintain and reinstate homeostasis during stress. The autonomic nervous system (ANS) quickly responds to stress exposure through the sympathetic and parasympathetic divisions. The rapidly activated sympathetic catecholamines are responsible for generation of the ‘fight or flight’ response in addition to potentiating the pituitary gland to subsequent hypothalamic activation (Wamsteeker and Bains, 2010). The hypothalamic-spinal-adrenocortical (HSA) axis modulates adrenal sensitivity to adrenocorticotropic hormone (ACTH) through a nitric oxide-mediated pathway (Jansen et al., 1995; Mohn et al., 2005; Ulrich-Lai et al., 2006). In addition, prolactin facilitates adrenal sensitivity to ACTH by a neural pathway that is independent of glucocorticoid-mediated negative feedback (Jaroenporn et al., 2009). Together, stress-induced activation of both the ANS and HSA axis prime the stress system, and subsequent activation of the hypothalamic-pituitary-adrenocortical (HPA) axis produces maximal ACTH-induced release of adrenal glucocorticoids. This results in an increase in gluconeogenesis and cellular metabolism leading to a spontaneous increase in different forms of free radicals including both reactive oxygen species (ROS) and nitrogen species (RNS) which ultimately lead to both oxidative and nitrosative stress (Zhou et al., 2011; Liu and Zhou, 2012).

The gaseous RNS, nitric oxide, is endogenously synthesised through conversion of L-arginine to L-citrulline by nitric oxide synthases or the nitrate-nitrite-nitric oxide pathway (Lundberg et al., 2008). Nitric oxide is involved in the regulation of many physiological processes, such as vascular tone, neurotransmission, and the immune system (Ignarro et al., 1987; Hibbs et al., 1988; Knowles et al., 1989). However, the pro-oxidant actions of nitric oxide cause nitrosative stress, which is defined as an increase in RNS occurring in response to the by-products formed from an oxidative challenge (Ghafourifar et al., 2001). A primary reaction in the production of RNS is the combination of nitric oxide and superoxide anions to form the highly reactive metabolite, peroxynitrite, a potent neurotoxin. It is now considered that nitric oxide and peroxynitrite can inhibit components of the mitochondrial respiratory chain such as cytochrome c oxidase, thereby decreasing the affinity to oxygen, potentially leading to cellular energy deficiency and ultimately cell death (Bolanos et al., 1994; Guidarelli et al., 2000). In the circulation, erythrocytes are the major scavenger of nitric oxide in which the reaction of nitric oxide with oxyhemoglobin produces the more stable metabolite nitrate and non-oxygen binding methemoglobin (Lundberg et al., 2008).

Assessment of total nitrite and nitrate (NOx) content in biological fluids is routinely used as an indirect estimate of nitric oxide production and metabolism in vivo. There is considerable evidence
for a link between altered NOx content and neurodegenerative conditions including Alzheimer’s, Huntington’s, and Parkinson’s diseases (Boje, 2004). Clinical studies have also demonstrated an increase in total nitrate and nitrite levels in major depression and autistic patients using peripheral measures (Suzuki et al., 2001; Sogut et al., 2003). However, these represent changes occurring in chronic conditions, while the acute physiology of altered nitrosative status is poorly understood. We hypothesised that the production of reactive nitrogen species contribute to the acute increase in oxidative status observed following stress exposure. Therefore, in the present study, an acute immobilisation stress model was used to establish the temporal profile of nitrosative changes and its involvement in the rapid onset of redox imbalance.

3.3 Materials and Methods

3.3.1 Experimental animals
Outbred male Wistar rats (Rattus norvegicus) aged 6-7 weeks postnatal, weighing 255.6 ± 2.82 g, were sourced from the University of Queensland Biological Resources breeding colony. Animals were housed individually under standard laboratory conditions (22 ± 2°C; 55 ± 5% humidity) with a 12 hour light-dark cycle (lights off at 12.30). Standard rat chow and water were available ad libitum. All experimental procedures were in accordance with regulations and policies outlined by The University of Queensland Animal Ethics Committee with AEC approval number SBS/363/11/URG.

3.3.2 Acute immobilisation stress
Acute immobilisation stress was applied according to a well-established protocol by Hori and colleagues (2004). Rats were immobilised on a Perspex® board (18×25 cm) in the supine position using a polyester strapping and adhesive tape with their legs set at 45 degrees to the midline. Acute immobilisation stress was applied for 2 hours (stress treatment starts at 13:30) within individual home cages enclosed with a larger containment chamber (68 cm (L) × 45 cm (W) × 38.5 cm (H)) to reduce external disturbances. To isolate the effects of immobilisation stress, control animals were deprived of food and water during the treatment period.

3.3.3 Experimental protocol
Animals were habituated to human handling for 10 minutes per day one week prior to experimentation. On each experimental day, animals were transported in their home cages from the colony room to an experimental room that was under low light and noise within the same animal facility. They were acclimatised to the novel experimental room for one hour and then randomly allocated to either control (n=9) or 2 hour immobilisation stress (n=9) treatment groups. The distal 1 to 2 mm of the tail was clipped once only and a baseline (0 minute) blood sample was collected into both ethylenediaminetetraacetic acid (EDTA)-coated Eppendorf tubes (1.5 mg mL⁻¹ blood) and ice-
chilled heparinised tubes (20 IU mL⁻¹ blood). Subsequent collection at 60, 90, and 120 minutes was achieved by gentle removal of clotted blood. Blood samples were around 150 µL to guarantee that the total amount of blood collected remains under 10% of the calculated blood volume. A 2.5 µL aliquot of heparinised whole blood was used for the determination of glucose using a standard glucometer and test strips (Accu-Chek® Performa®, Roche Diagnostics Aust. Pty. Ltd., Castle Hill, Australia) and the remaining sample was centrifuged at 200 × g for 5 minutes. Supernatant plasma was collected and stored at -80°C for later determination of nitric oxide production, general oxidative status, lipid peroxidation, prolactin, and corticosterone concentrations. Supernatant plasma with EDTA as anticoagulant was stored at -80°C for measuring nitric oxide metabolism, NOx. Aliquots of heparinised erythrocytes were stored at -80°C for later analysis of reduced and oxidised glutathione and hemoglobin. Following the final sample, animals were weighed and overdosed with the anesthetic sodium pentobarbital (100 mg kg⁻¹ i.p. injection, Lethabarb, Virbac, Peakhurst, Australia).

3.3.4 Hormone assays
Plasma prolactin concentrations were assayed using commercially available ELISA kits (Demeditec Diagnostics, GmbH; Germany) with a lower detection limit of 0.6 ng mL⁻¹ according to the manufacturer’s protocols. The intra- and inter-assay coefficients of variation were 4.2% and 4.0% respectively. Plasma corticosterone concentrations were determined in duplicate using radioimmunoassay with a minimum sensitivity of 10 pg mL⁻¹ as described in Spiers and colleagues (2013). A 5 µL plasma aliquot was extracted in 2 mL of glass-distilled dichloromethane. Extracts were dried under a stream of nitrogen gas and reconstituted in phosphate buffered saline (PBS; 100 mmol L⁻¹, pH=7.0) containing 0.1% (w/v) porcine gelatin. A 100 µL aliquot of unlabeled corticosterone standard or reconstituted sample was incubated with 100 µL of anti-rat corticosterone polyclonal antibody (Sapphire Bioscience Pty. Ltd., Waterloo, Australia) and 100 µL of [1, 2, 6, 7-³H]-corticosterone tracer and incubated overnight at 4°C. The following day, samples were loaded with 500 µL of dextran-coated charcoal (50 mmol L⁻¹, pH=7.4) and centrifuged at 1000 × g for 10 minutes at 4 °C. Radioactivity was counted in a 100 µL aliquot of the resulting supernatant using a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer, Waltham, MA). Unknown sample concentrations were determined from a standard curve and corrected for dilution and extraction efficiency. Dichloromethane extraction efficiency was 89.6% and intra- and inter-assay coefficients of variation were 4.5% and 3.1% respectively. Plasma insulin concentrations were measured using a commercial radioimmunoassay kit (Coat-a-Count® Insulin, Siemens Healthcare, Frimley, UK) according to manufacturer's protocol. Assay sensitivity was 0.25 mIU L⁻¹ with intra- and inter-assay coefficients of variation of 3.1 % and 5.0% respectively.
3.3.5 Nitrosative status

The production of nitric oxide was assayed in plasma with 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA) synthesised by Kojima and colleagues (1999). This pH-independent fluorescent dye undergoes de-acetylation by esterases to become a weakly fluorescent DAF-FM with a fluorescence quantum yield of around 0.005 (Kojima et al., 1999). After reacting directly with nitric oxide in the presence of molecular oxygen, the resultant benzotriazole form (DAF-FM T) shows stable and an approximately 160-fold increase in fluorescence intensity which can be detected with excitation and emission maxima of 485 nm and 520 nm respectively (Kojima et al., 1999). Briefly, a 4 µmol L\(^{-1}\) DAF-FM DA working solution was prepared fresh in PBS (50 mmol L\(^{-1}\), pH=7.4). Frozen plasma was defrosted and a 1% (v/v) plasma suspension was prepared in PBS. Four replicates were plated on a 96-well plate containing final concentrations of 0.5% (v/v) plasma suspension and 2 µmol L\(^{-1}\) of DAF-FM DA under low light conditions. Plates were incubated for 60 minutes and sample fluorescence was measured by a POLARstar OPTIMA microplate reader (BMG Labtechnologies). Data were corrected with corresponding protein concentrations and expressed as the percentage change in arbitrary fluorescence units relative to baseline values. The intra-assay coefficient of variation was 2.4%.

The total nitric oxide metabolites, NO\(_x\), were measured with the Griess reaction using a commercially available colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI) with a minimum sensitivity of 2.5 µmol L\(^{-1}\) according to the manufacturer’s instructions. Plasma samples using EDTA as an anticoagulant were ultra-filtered through a 10-kDa molecular weight cut-off filter using commercially available centrifugal devices (Pall Nanosep\(^{\circledR}\), Cheltenham, Australia). Samples were corrected with corresponding protein concentrations and expressed as a percentage change from baseline values. All samples were run on the same day with an intra-assay coefficient of variation of 3.3%.

3.3.6 General oxidative status assay

General oxidative status in plasma was measured fluorometrically using methods modified from Spiers and colleagues (2013). Frozen plasma was vortex-defrosted and a 1% (v/v) plasma suspension was prepared in PBS. Four replicates were plated on a 96-well plate containing final concentrations of 0.5% (v/v) plasma suspension and 100 µmol L\(^{-1}\) of 2’, 7’-dichlorofluorescin diacetate (DCFH-DA). Sample fluorescence was measured by a POLARstar OPTIMA microplate reader (BMG Labtechnologies) with excitation and emission filters set at 485 nm and 520 nm respectively. Data were corrected with corresponding protein concentrations in plasma and expressed as the percentage change in arbitrary fluorescence units relative to baseline values. The intra-assay coefficient of variation was 2.5%.
3.3.7 Reduced and oxidised glutathione assay

Reduced (GSH) and oxidised (GSSG) glutathione disulfide were measured with the fluorophore O-phthalaldehyde (OPA) using a procedure modified from Senft and colleagues (2000). Briefly, a 50 µL aliquot of erythrocytes was suspended in 450 µL of 5% (w/v) metaphosphoric acid. The denatured protein precipitate was removed by centrifugation (2300 x g for 5 minutes). Aliquots of the supernatant were redox quenched with potassium phosphate buffer (1.0 M, pH=7.0) and incubated with 20 µL of OPA (4 mg mL⁻¹ in methanol) to specifically quantify GSH. The concentration of GSH was corrected with the non-glutathione dependent fluorescence by a corresponding volume matched 7.5 mmol L⁻¹ N-ethylmaleimide (NEM)-reacted sample. For the determination of GSSG, deproteinated supernatant was incubated with 20 µL of 7.5 mmol L⁻¹ NEM for 5 minutes to remove GSH and redox quenched in potassium phosphate buffer. A 30 µL aliquot of 100 mmol L⁻¹ sodium dithionite was added to one duplicate to convert GSSG to GSH. This converted GSH was measured fluorometrically following incubation with OPA. The concentration of GSSG was calculated by the difference between sodium dithionite-reacted sample and its corresponding blank using 0.1 mol L⁻¹ potassium buffer with EDTA (KPE buffer; pH=7.5). The OPA-derived fluorescence was measured using a POLARstar OPTIMA microplate reader (BMG Labtechnologies) with excitation and emission filters set at 365 nm and 430 nm respectively and sample values were determined directly from linear GSH and GSSG standard curves with a minimum sensitivity of 2.5 µmol L⁻¹. The intra- and inter-assay coefficients of variation were 1.5% and 8.1% respectively for the GSH assays and 4.0% and 8.3% respectively for GSSG assays. Data were corrected with corresponding hemoglobin concentrations and expressed as the GSH/GSSG ratio relative to baseline values.

3.3.8 Lipid peroxidation in plasma

The peroxidation of lipid in plasma was determined according to method previously described by Zavodnik and colleagues (2002) by quantifying the production of malondialdehyde using a thiobarbituric acid reactive substances (TBARS) assay. 1, 1, 3, 3-tetraethoxypropane was used as an external standard for malondialdehyde (MDA) with a minimum detection sensitivity of 3.1 nmol L⁻¹. To prevent further peroxidation during the assay, 10 µl of butylated hydroxytoluene in methanol was added into 50 µl of standards or diluted plasma. A 125 µl aliquot of 1% (w/v) thiobarbituric acid (TBA) in 0.05 mol mol⁻¹ sodium hydroxide solution (pH=3.5) was added and vortexed vigorously. Following the addition of 315 µl of 20% trichloroacetic acid (w/v), the mixture was incubated in an 85 °C water bath for 30 minutes using glass marbles as condensers. After cooling on ice for 10 minutes, the coloured product was extracted with n-butanol (500 µl) and the mixture was centrifuged at 1000 x g for 5 minutes. The extracted adduct was plated on a 96-well plate and measured fluorometrically at 544 nm (excitation) and 590 nm (emission) using a POLARstar...
OPTIMA microplate reader (BMG Labtechnologies). Samples were corrected for corresponding protein concentrations and expressed as the percentage change in MDA-TBA adduct formation relative to baseline values. The intra- and inter-assay coefficients of variation were 4.3% and 7.5% respectively.

3.3.9 Haemoglobin and protein determination

Haemoglobin was determined by a modification of the cyanmethemoglobin method of Fairbanks and Klee (1999) using bovine haemoglobin as a standard. Protein content was determined with a commercially available RED 660™ Protein Assay (G-Biosciences, St Louis, MO, USA) using bovine serum albumin as a standard.

3.3.10 Statistics

Data were analysed using statistical software GraphPad Prism (Version 5.0.3, GraphPad Software Inc., San Diego, CA, U.S.A). Two-way repeated measure ANOVAs with Bonferroni post-test were used to compare control and stress data for all time course analyses. Results were expressed as mean ± standard error of the mean (± SEM) and p-values less than 0.05 were considered statistically significant.
3.4 Results

3.4.1 Endocrine and metabolic

This study associated specific indicators of nitrosative activation with profiles of known endocrine and oxidative parameters following an acute immobilisation stress. Plasma prolactin significantly increased during immobilisation stress \( [F(1,16)=36.60, \ p<0.0001] \) in a time-dependent manner \( [F(3,48)=14.59, \ p<0.0001] \). A comparison of group means showed highly significant increases in prolactin at 60, 90, and 120 minutes of immobilisation when compared with control concentrations (Fig. 3.1A). The changes in plasma corticosterone following immobilisation stress are shown in Fig. 3.1B. Baseline concentrations of corticosterone prior to immobilisation were not statistically different between control and stress groups. Immobilisation significantly increased corticosterone concentration above controls \( [F(1,16)=70.11, \ p<0.0001] \) over the time-course of the experiment \( [F(3,48)=16.35, \ p<0.0001] \). The effect of treatment over time was also significant \( [F(3,48)=11.26, \ p<0.0001] \). Corticosterone concentrations increased approximately 150-300% above controls at 60, 90, and 120 minutes of immobilisation while no significant differences were observed in controls. The accompanying changes in blood glucose following the onset of immobilisation are illustrated in Fig. 3.1C. A Two-way repeated measures ANOVA demonstrated a time-dependent effect \( [F(3,48)=4.77, \ p=0.005] \), and a significant interaction between time and stress treatment \( [F(3,48)=6.74, \ p=0.0007] \), while the main effect of stress was not significant \( [F(1,16)=3.12, \ p=0.097] \). Post-test analysis revealed a significant increase in blood glucose after 60 minutes following the onset of stress. Plasma insulin decreased in a stress \( [F(1,14)=7.36, \ p=0.017] \) and time \( [F(3,42)=2.89, \ p=0.047] \) dependent manner with no significant interaction between stress and time \( [F(3,42)=1.18, \ p=0.33] \). Post-test analysis showed a significant decrease in plasma insulin following 90 minutes of immobilisation stress exposure (Fig. 3.1D).
Figure 3.1. The effect of acute immobilisation stress on (A) plasma prolactin, (B) plasma corticosterone, (C) blood glucose, and (D) plasma insulin concentrations from control (○) and stressed (■) rats (n=9 per group for plasma prolactin, corticosterone, and blood glucose; n=8 per group for plasma insulin). All measures were determined from serial blood samples collected at 0 (baseline), 60, 90, and 120 minutes. Data are expressed as mean ± SEM (Two-way ANOVA with Bonferroni post-test, *p<0.05, **p<0.01, ***p<0.001).
3.4.2 Nitrosative status

The nitricergic system forms a subgroup of reactive species, including nitric oxide, responsible for numerous important physiological functions. Following the onset of immobilisation stress, there was a linear increase in nitric oxide production with a correlation coefficient of $r^2=0.99$ [p<0.001] while there was no significant change in controls (Fig. 3.2). A Two-way repeated measures ANOVA demonstrated stress treatment significantly increased nitric oxide production [$F_{(1,16)}=15.57$, p=0.0012] over the duration of the experiment [$F_{(3,48)}=3.32$, p=0.0274], and the interaction between stress treatment and time was also significant [$F_{(3,48)}=8.77$, p<0.0001]. Post-test analysis showed this increase became significantly different from controls at 60, 90, and 120 minutes.

**Figure 3.2.** The effect of acute immobilisation stress on nitric oxide production measured by the benzotriazole derivative of 4-amino-5-methylamino-2’, 7’-difluorofluorescein (DAF-FM T) formation in plasma from control (○) and stressed (■) rats (n=9 per group). Relative DAF-FM T formation was determined from serial blood samples collected at 0 (baseline), 60, 90, and 120 minutes. Data are expressed as mean ± SEM (Two-way ANOVA with Bonferroni post-test, *p<0.05, **p<0.01, ***p<0.001).
Nitric oxide metabolism, measured as the stable metabolites NOx, also demonstrated linear increases [$r^2=0.94$, $p<0.05$] following application of immobilisation stress. NOx production increased [$F(1,16)=41.68$, $p<0.0001$] in a time dependent manner [$F(3,48)=5.47$, $p=0.0026$] and the interaction of stress treatment and time was also highly significant [$F(3,48)=10.25$, $p<0.0001$]. NOx increased above controls at 60, 90, and 120 minutes (Fig. 3.3).

**Figure 3.3.** The effect of acute immobilisation stress on total nitrite and nitrate (NOx) levels from control (○) and stressed (■) rats ($n=9$ per group). Relative total NOx was determined from serial blood samples collected at 0 (baseline), 60, 90, and 120 minutes. Data are expressed as mean ± SEM (Two-way ANOVA with Bonferroni post-test, ***$p<0.001$).
3.4.3 Redox status

Figure 3.4 shows the changes in plasma general oxidative status following immobilisation stress. Plasma oxidative status demonstrated highly significant linear increases \([r^2=0.98, p<0.05]\) following exposure to immobilisation \([F_{(1,16)}=30.57, p<0.0001]\) in a time-dependent manner \([F_{(3,48)}=15.64, p<0.0001]\). The interaction between treatment and time also demonstrated high significance \([F_{(3,48)}=17.36, p<0.0001]\). Post-test analysis showed immobilisation stress increased plasma oxidative status above controls at 60, 90, and 120 minutes.

**Figure 3.4.** The effect of acute immobilisation stress on the general oxidative status measured by dichlorofluorescein (DCF) formation in plasma from control (○) and stressed (■) rats (n=9 per group). Relative DCF formation was determined from serial blood samples collected at 0 (baseline), 60, 90, and 120 minutes. Data are expressed as mean ± SEM (Two-way ANOVA with Bonferroni post-test, **p<0.01, ***p<0.001).
Over the duration of the experiment, concentrations of total erythrocytic GSH did not change significantly. However, application of immobilisation stress increased the oxidised fraction while decreasing the reduced fraction of glutathione equivalents relative to baseline values. This resulted in delayed decreases in the ratio of GSH/GSSG from baseline, indicative of oxidative stress onset following immobilisation stress [$F_{(1,16)}=15.78$, $p=0.011$]. The interaction of stress with time was also significant overall [$F_{(3,48)}=5.38$, $p=0.0028$]. Bonferroni post-test analysis demonstrated that this increase due to immobilisation did not occur until 120 minutes of stress exposure (Fig. 3.5).

**Figure 3.5.** The effect of acute immobilisation stress on the erythrocytic glutathione to glutathione disulfide (GSH/GSSG) ratio indicative of oxidative stress from control (○) and stressed (■) rats (n=9 per group). The relative ratio was determined from serial blood samples collected at 0 (baseline), 60, 90, and 120 minutes. Data are expressed as mean ± SEM (Two-way ANOVA with Bonferroni post-test, ***$p<0.001$).
3.4.4 Lipid peroxidation

Immobilisation stress effectively elevated MDA production [$F_{(1,16)}=7.52$, $p=0.0145$]. The interaction of stress with time was also statistically significant [$F_{(3,48)}=5.49$, $p=0.0025$]. Post-test analysis showed that this increase became significant at 90, and 120 minutes of stress exposure (Fig. 3.6).

**Figure 3.6.** The effect of acute immobilisation stress on plasma lipid peroxidation measured by malondialdehyde-thiobarbituric acid (MDA-TBA) adduct formation from control (○) and stressed (■) rats ($n=9$ per group). Relative plasma MDA-TBA was determined from serial blood samples collected at 0 (baseline), 60, 90, and 120 minutes. Data are expressed as mean ± SEM (Two-way ANOVA with Bonferroni post-test, $^*p<0.05$, $^{***}p<0.001$).
3.5 Discussion

This study has demonstrated that alongside known hormonal parameters, acute psychological stress increases the production and metabolism of nitric oxide, likely contributing to the observed increase in the general oxidative status assay. The marked 250% increase in plasma prolactin from baseline observed at 60 minutes of immobilisation stress is in agreement with Ren and colleagues (2010). Moreover, the 350% increase in plasma corticosterone at 60 minutes is comparable with previous findings by Lee and colleagues (2008) using the same stressor. Acute immobilisation stress increased circulating blood glucose by approximately 25% at 60 minutes, which then progressively declined towards baseline. These results are supported by the findings of Sanchez and colleagues (2002) who reported a similar initial increase in plasma glucose of tape-immobilised mice at 20 minutes, which significantly declined between 20- and 180-minutes. The decline in circulating glucose during stress noted in the present study does not seem to be insulin-dependent, as we found that stress decreased insulin concentrations. Such a reduction in plasma insulin is suggested to be adrenergic-mediated and has been demonstrated in previous reports accompanied by a rise in the hyperglycemic hormone glucagon (Mioduszewski and Critchlow, 1982; Perez-Llamas et al., 1992; Yamada et al., 1993; Amrani et al., 1994; Machado et al., 1995). The fall in blood glucose in rats exposed to immobilisation stress is likely a result of increased glucose utilisation due to enhanced skeletal muscle activity that has been observed in this particular stress model (Sanchez et al., 2002).

The present study demonstrated a 7% increase in nitric oxide measured by DAF-FM T formation at 60 minutes in response to immobilisation stress, with a further linear increase to 15% at 120 minutes. Several experiments have utilised DAF-FM DA to assess nitric oxide dynamics, such as bioimaging intracellular nitric oxide in smooth muscle cells pretreated with various cytokines showing a fluorescence increase following DAF-FM DA loading (Itoh et al., 2000). The increased fluorescence was reversed using a nitric oxide synthase inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME). Sicard and colleagues (2008) measured nitric oxide with DAF-FM DA using flow cytometry as an oxidative/nitrosative stress index in blood monocytes of normal and hypertensive rats. Under these conditions, DAF-FM T fluorescence increased significantly by approximately 25% in genetically hypertensive rats compare to the normotensive strain. It has been reported that these diaminofluorescein probes react specifically with nitric oxide without interacting with the metabolites, nitrite and nitrate, or other ROS and RNS including superoxide, hydrogen peroxide, and peroxynitrite (Kojima et al., 1998). It was later confirmed that both DAF-FM and the diacetate form is pH-insensitive above pH 5.5 and more photostable and sensitive for nitric oxide than DAF-2 and DAF-2 DA (Kojima et al., 1999; Itoh et al., 2000; Freitas et al., 2009). Balcerczyk and colleagues (2005) reported an induction of DAF-FM fluorescence using various peroxynitrite
concentrations. However, this was limited to less than one fluorescent unit in the diacetate form even in the presence of supra-physiological concentrations of peroxynitrite. Moreover, DAF-FM DA fluorescence induced by peroxynitrite is 300 times less than DAF-FM at similar peroxynitrite concentrations. In a recent study, the sensitivity of DAF-FM DA was examined by incubating plant tissue with the nitric oxide donor or scavenger, sodium nitroprusside (SNP) and 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) respectively (Kolbert et al., 2012). Incubation with SNP significantly increased fluorescence intensity of DAF-FM T over all concentrations tested. However, this SNP-induced increase was abolished with co-incubation of cPTIO. No change was observed in DAF-FM T fluorescence when tissues were incubated with increasing concentrations of hydrogen peroxide. Based on these studies, it can be concluded that DAF-FM DA is specific for nitric oxide as long as precaution is taken to limit photobleaching. The present study and previous reported observations demonstrate that DAF-FM DA is a reliable indicator of nitric oxide in biological samples. However, to validate this stress-induced increase in nitric oxide, this study utilised the conventional method of assessing the stable metabolites, NOx.

Immobilisation stress induced an increase in nitric oxide metabolites by approximately 40-60% from baseline over the duration of stress exposure. The magnitude of changes in NOx is in the range reported in blood from patients suffering major depression, suicidal ideation, and autism. (Suzuki et al., 2001; Sogut et al., 2003; Lee et al., 2006). The significance of this comparison lies in the link between increased nitric oxide and release of the excitatory amino acid glutamate in the central nervous system (Segieth et al., 1995). It is a common observation in stress models that glutamate release is enhanced while glial-mediated glutamate cycling is reduced, leading to altered synaptic transmission in limbic and cortical areas (Yuen et al., 2009). Although the underlying mechanisms between stress and the nitrergic system remain unclear, a number of studies have demonstrated that various inhibitors of nitric oxide synthase possess anxiolytic- and antidepressant-like properties (Volke et al., 2003; Joca and Guimaraes, 2006; Zhang et al., 2010). Interestingly, Zhang and colleagues (2010) demonstrated that selective serotonin type 1A receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and selective serotonin reuptake inhibitor fluoxetine reduce neuronal nitric oxide synthase protein expression in the hippocampus and display a reduction in anxiety-like behaviours. Furthermore, knock out mice lacking neuronal nitric oxide synthase also demonstrated a reduction in anxiety-like behaviour and were no longer sensitive to serotonin agonists or uptake inhibitors. A follow up study revealed that chronic mild stress and glucocorticoid exposure lead to hippocampal neuronal nitric oxide synthase overexpression (Zhou et al., 2011). This increase in neuronal nitric oxide synthase-derived nitric oxide caused the downregulation of hippocampal glucocorticoid receptor expression, ultimately leading to depressive-like behaviour in mice.
In addition to changes in central nervous system activity, persistent increases in nitric oxide lead to cellular damage directly, or by interaction with superoxide, leading to the formation of peroxynitrite radicals. Damage via protein carbonyl formation and lipid peroxidation has been shown in erythrocytes and neural tissues following exposure to different stressors (Sahin et al., 2004; Sahin and Gumuslu, 2007). In the present study, oxidative status increased by approximately 30% at 120 minutes of immobilisation stress. Increases in GSH oxidation to GSSG should follow increases in cellular oxidative status, as is evident in our data after 120 minutes of stress exposure. This coincided with increased plasma malondialdehyde production, a widely accepted general indicator of oxidative damage. Liu and colleagues (1994) demonstrated a significant increase in plasma lipid peroxidation using similar methods following 6 hours of immobilisation stress which could be reversed by reduced glutathione treatment, implicating directly the causative role of oxidative damage.

By showing changes in the oxidative and nitrosative systems, and linking these to indices of cellular oxidative damage, the present study has confirmed the involvement of RNS in the rapid redox response to stress. Together with ROS, these likely contribute to the depletion of the cellular glutathione ratio and increase in markers of lipid peroxidation. Interestingly, it has been suggested that NOx, particularly nitrite, act as a nitric oxide reservoir. The rapid oxidation of nitric oxide to nitrite allows this stable metabolite to increase bioavailability by requiring only a single electron for regeneration to nitric oxide, a step catalysed enzymatically by xanthine oxidoreductase and non-enzymatically by deoxygenated haem proteins under low oxygen tension. This regeneration of nitric oxide can partially offset local hypoxic conditions where production by the nitric oxide synthase system would not be possible. Under stress, particularly in tissues with increased cellular metabolism, localised hypoxia caused by increased oxygen consumption may be offset by nitric oxide production in this manner. Moreover, preferential metabolism of nitric oxide to nitrite and nitrate would limit the production of other reactive metabolites such as peroxynitrite, thereby playing a potential protective role in the acute response to psychological stress. The time domain and recovery of these physiological changes following stress exposure in the current study is an important consideration for future studies utilising oxidative measures in peripheral blood. Furthermore, the mechanistic link between stress exposure, glucocorticoids, and increased nitric oxide production will need to be elucidated in both blood and tissues.
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3.7 Conflict of Interest
The authors declare no competing financial interests.

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Chapter IV

Acute restraint stress induces specific changes in nitric oxide production and inflammatory markers in the rat hippocampus and striatum

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4.1 Abstract

Chronic mild stress has been shown to cause hippocampal neuronal nitric oxide synthase (NOS) overexpression and the resultant nitric oxide (NO) production has been implicated in the aetiology of depression. However, the extent of nitrosative changes including NOS enzymatic activity and the overall output of NO production in regions of the brain like the hippocampus and striatum following acute stress has not been characterised. In this study, outbred male Wistar rats aged 6-7 weeks were randomly allocated into 0 (control), 60, 120, or 240 minute stress groups and neural regions were cryodissected for measurement of constitutive and inducible NOS enzymatic activity, nitrosative status, and relative gene expression of neuronal and inducible NOS. Hippocampal constitutive NOS activity increased initially but was superseded by the inducible isoform as stress duration was prolonged. Interestingly, hippocampal neuronal NOS and interleukin 1β mRNA expression was downregulated, while the inducible NOS isoform was upregulated in conjunction with other inflammatory markers. This pro-inflammatory phenotype within the hippocampus was further confirmed with an increase in the glucocorticoid-antagonizing macrophage migration inhibitory factor, Mif, and the glial surveillance marker, Ciita. This indicates that despite high levels of glucocorticoids, acute stress sensitises a neuroinflammatory response within the hippocampus involving both pro-inflammatory cytokines and inducible NOS while concurrently modulating the immunophenotype of glia. Furthermore, there was a delayed increase in striatal inducible NOS expression while no change was found in other pro-inflammatory mediators. This suggests that short term stress induces a generalised increase in inducible NOS signalling that coincides with regionally specific increased markers of adaptive immunity and inflammation within the brain.
4.2 Introduction

In response to a stressor, sympathetic activation results in the release of epinephrine and norepinephrine from the adrenal medulla, causing a catecholamine surge in the blood circulation, generating a ‘fight-or-flight’ response (Jansen et al., 1995). Subsequent activation of the hypothalamic-pituitary-adrenal (HPA) axis leads to an increase in the amplitude and synchronization of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) secretions from the parvocellular neuroendocrine cells (Tsigos and Chrousos, 2002). Synergistically, AVP acts with CRH on the anterior pituitary corticotrophs, potentiating the release of adrenocorticotropic hormone (ACTH), which in turn stimulates adrenal glucocorticoid production. The stress-induced hyperactivity also activates the renin-angiotensin-aldosterone system which, via the action of angiotensin II on the paraventricular nucleus of the hypothalamus, facilitates stimulation of CRH, ACTH, and adrenal glucocorticoids (Ganong and Murakami, 1987; Veltmar et al., 1992; Saavedra and Benicky, 2007; Bali and Jaggi, 2013). The biologically available fractions of corticosterone not bound to high affinity carrier proteins are capable of crossing the blood-brain barrier and are responsible for mediating negative feedback (Pardridge and Mietus, 1979). This feedback mechanism depends on the binding of corticosteroids to cytosolic glucocorticoid receptors (GR) which translocate into the nucleus and interact as homodimers with specific glucocorticoid responsive elements, thus repressing Crh and Avp gene expression to terminate the stress response and restore homeostasis (Watts, 2005).

Following the release of the stress hormone corticosterone, blood glucose concentrations rapidly elevate through processes of hepatic gluconeogenesis and insulin resistance necessary for the adaptive responses to stress, including increased mitochondrial ATP production via oxidative phosphorylation (Teague et al., 2007; Du et al., 2009; Li et al., 2013). This glucocorticoid-driven increase in cellular metabolism is subject to a small percentage of electron ‘leakage’ from the electron transport chain, ultimately generating free radicals such as superoxide in place of water molecules, in addition to other members of the reduction/oxidation (redox) system such as nitric oxide (NO) (Spiers et al., 2014a). We have previously demonstrated that peripheral measures of oxidative and nitrosative status are affected significantly by an acute psychological stress and can persist beyond the cessation of the stressor (Spiers et al., 2013; Chen et al., 2014). Recently, there has been considerable interest in elucidating the role of the redox system in the downstream cellular events initiated by stress exposure. Zhou and colleagues (2007) found that the expression and activity of the constitutive enzyme, neuronal nitric oxide synthase (nNOS), which produces NO in central and peripheral neurons, increased following chronic mild stress in the hippocampus of adult mice. No change was observed in the constitutive endothelial isoform (endothelial nitric oxide...
synthase; eNOS) and the transcription-dependent inducible nitric oxide synthase (iNOS). However, Olivenza and colleagues (2000), using a chronic immobilisation paradigm, and Peng and colleagues (2012), using unpredictable chronic mild stress, demonstrated that iNOS expression was increased in the cerebral cortex. The latter group suggested that this iNOS-derived NO following chronic stress may contribute to depressive-like behaviours in mice. A single episode of 6 hours acute restraint stress in rats also significantly increased iNOS activity in the cerebral cortex, an effect mediated by nuclear factor Kappa-light-chain-enhancer of activated B cells (NF-κB) signalling (Madrigal et al., 2001; Madrigal et al., 2002).

A number of studies have now implicated NO at both the modulatory and mechanistic levels of the stress system. Mohn and colleagues (2005) discovered that corticosterone is released via a NO and prostaglandin-dependent pathway stimulated by either ACTH or the NO donor, sodium nitroprusside, highlighting the importance of redox molecules in the neuroendocrine stress system under physiological conditions. Using a chronic mild stress paradigm, Zhou and colleagues (2011) showed that stress increases hippocampal nNOS expression via a MR-dependent mechanism causing chronic production of NO, ultimately leading to down-regulation of GR, a factor linked to the development of depression. Together, these studies demonstrate that the neuronal isoform of NOS in limbic regions is predominately associated with chronic stress exposure, while iNOS in cortical regions is responsive to both acute and chronic stress. However, the cellular, physiological, and functional effects of the nitrergic system following short term stress are poorly understood. In this study, we used the hippocampus and striatum, regions that display differential stress sensitivity and redox responses, to characterise the temporal changes in the neuronal and inducible NOS isoforms and pro-inflammatory markers following acute psychological stress.

### 4.3 Materials and Methods

#### 4.3.1 Experimental animals

Outbred male Wistar rats (Rattus norvegicus) aged 6-7 weeks postnatal, weighing 297.9 ± 3.8 g were sourced from The University of Queensland Biological Resources breeding colony. Rats were housed individually under standard laboratory conditions (22 ± 2°C; 55 ± 5% humidity) with a 12:12h light-dark cycle (lights off at 12.30h). Standard rat chow and water were available ad libitum. All experimental procedures were in accordance with regulations and policies outlined by The University of Queensland Animal Ethics Committee with AEC approval numbers 018/11.

#### 4.3.2 Habituation and transportation

Rats were habituated to human handling for 10 minutes per day one week prior to experimentation. On each experimental day, rats were transported in individual home cages from the colony room to an experimental room within the same animal facility. Rats were acclimatised to the novel
experimental room under low light and noise for one hour prior to treatment. To isolate the effects of restraint stress, all rats were deprived of food and water during the one hour habituation and treatment period.

4.3.3 Acute restraint stress
Rats were subjected to acute restraint stress using adjustable wire mesh restrainers to avoid overheating and body compression. The wire mesh restrainer was made of a PVC skeleton coated externally with welded wire mesh (6 mm × 6 mm grid). Acute restraint stress was applied for 60, 120, and 240 minutes (stress treatment starting at 13:30h) within individual home cages enclosed with a larger ventilated containment chamber (68 cm (L) × 45 cm (W) × 38.5 cm (H)) to reduce any external disturbances.

4.3.4 Treatment protocol and tissue collection
Animals were randomly allocated to 0 (control), 60, 120, or 240 minutes stress treatment groups (n=5-8 per group). At the end of each treatment period, rats were weighed and sacrificed with sodium pentobarbital (100 mg/kg i.p. injection, Lethabarb, Virbac) and a single blood sample was collected via cardiac puncture into ice-chilled heparinised tubes (20 IU/mL blood). The brain was rapidly removed and frozen on powdered dry ice for storage at -80°C. Whole blood was centrifuged at 200 × g for 5 minutes and supernatant plasma was collected and stored at -80°C for later determination of corticosterone. Frozen brains were sectioned on a cryostat and the hippocampus and striatum were dissected out on a powdered dry ice-embedded metal platform according to the rat brain atlas (Paxinos and Watson, 2007). Regionalised neural tissues were stored at -80°C for later determination of the enzymatic activity of NOS, NO metabolism, 4-amino-5-methylamino-2', 7'-difluorofluorescein (DAF-FM) activation, and relative gene expression.

4.3.5 Plasma corticosterone assay
Corticosterone concentrations were determined using an in-house radioimmunoassay employing a highly specific ovine anti-rat corticosterone polyclonal antibody (Sapphire Bioscience Pty. Ltd.) and tritiated [1, 2, 6, 7 - ³H]-corticosterone tracer as previously described in Spiers and colleagues (2013). Radioactivity was counted in liquid scintillation cocktail (Ultima Gold™, Perkin Elmer) using a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer). Concentrations of unknown sample were determined from a standard curve and corrected for dilution and extraction efficiency. Dichloromethane extraction recovery was 89.65% and intra- and inter-assay coefficients of variation were 4.70% and 4.59% respectively.

4.3.6 Neural tissue preparation
The hippocampus and striatum were homogenised (IKA® Ultra-Turrax® T10) in 20 volumes (w/v) of phosphate buffered saline (PBS; 50 mM, pH=7.4) and the crude homogenates were centrifuged
at 10,000 x g for 10 minutes at 4°C. The resulting supernatant was used for the determination of NOS enzymatic activity, DAF-FM activation, and protein concentrations. For the quantification of total nitrite and nitrate (NO\textsubscript{x}), 60 µL of the 20 volume supernatant was ultra-filtered through a 10-kDa molecular weight cut-off filter using commercially available centrifugal devices (Pall Nanosep\textsuperscript{®}, Cheltenham, Australia).

4.3.7 Assay of calcium-independent and dependent nitric oxide synthase activity
The enzymatic activity of calcium-independent and dependent NOS isoform was determined by monitoring the conversion of L-[\textsuperscript{14}C(U)]-arginine to L-[\textsuperscript{14}C(U)]-citrulline as described by Weissman and Gross (2001) with modifications. In brief, 10 µL of tissue supernatant was added with 12.5 µL of assay buffer (50 mM Tris-HCl; pH=7.4 with 1.25 mM CaCl\textsubscript{2}, 1 mM of MgCl\textsubscript{2}, 0.6 mM of L-valine, and 1 mM of dithiothreitol). A 12.5 µL aliquot of a mixture of cofactors containing 50 mM Tris-HCl with 100 nM calmodulin, 40 µM of (6R)-5, 6, 7, 8-tetrahydrobiopterin dihydrochloride, 8 µM of flavin adenine dinucleotide, and 8 µM of riboflavin 5′-monophosphate was then added. A 2.5 µL aliquot of either MiliQ or 20 mM EGTA was added to determine total or calcium-independent iNOS activity respectively. An enzyme blank control was included for every sample using a non-specific NOS inhibitor which required 12.5 µL of 8 mM \(N_\omega\)-nitro-L-arginine methyl ester hydrochloride (L-NAME) instead of the cofactors followed by the addition of 2.5 µL EGTA (20 mM). Reactions were initiated by the addition of radiotracer mix (12.5 µL) containing 50 mM of Tris-HCl, 2 µCi/mL of L-[\textsuperscript{14}C(U)]-citrulline, and 4 mM of NADPH at 25°C and incubated for 30 minutes. The reaction was stopped by addition of 700 µL of 1 part Dowex AG 50W-X8 (100-200 mesh, Na\textsuperscript{+} form) to 2 parts EDTA-HEPES buffer (pH=5.5; v/v) with 80 µM of L-NAME followed by 2 mL of MiliQ. L-[\textsuperscript{14}C(U)]-citrulline was specifically eluted by centrifugation at 1000 x g for 5 minutes and the radioactivity was quantified with a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer). Enzyme activities were calculated as pmol/min/mg protein after accounting for individual enzyme blank controls and the presence or absence of EGTA and data were expressed relative to control values.

4.3.8 Nitric oxide metabolism in neural tissue
The total NO metabolites, NO\textsubscript{x}, were measured in ultra-filtered supernatant using a commercially available colorimetric assay kit according to the manufacturer’s instructions (Cayman Chemical Company, Ann Arbor, MI, USA). Samples were corrected with corresponding protein concentrations and expressed as µM per mg of protein.

4.3.9 Nitric oxide production in neural tissue
Production of NO was assayed with 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate (DAF-FM DA) according to method previously described in Chen and colleagues (2014). Briefly, a
4 µM DAF-FM DA working solution was prepared fresh in PBS (50 mM, pH=7.4). Four replicates of diluted tissue supernatant were plated on a 96-well plate containing a final concentration of 2 µM of DAF-FM DA under low light conditions. Plates were incubated for 60 minutes at room temperature and sample fluorescence was measured by a POLARstar OPTIMA microplate reader (BMG Labtechnologies). Data were corrected with corresponding protein concentrations and expressed as the percentage change in arbitrary fluorescence units relative to control values.

4.3.10 Real-time PCR
Total RNA from each representative region was extracted using the RNeasy mini kit (Qiagen, Doncaster, Australia) treated with deoxyribonuclease I and reversed transcribed into cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Gladesville, Australia) according to the manufacturer’s instructions. Taqman gene expression ‘assay-on-demand™’ kits (Life Technologies, Mulgrave, Australia) with optimised primers and reporter dye labelled-probes (FAM; 6-carboxyfluorescein) were used to detect gene expression of Nr3c1 (glucocorticoid receptor; Rn00561369_m1), Nr3c2 (mineralocorticoid receptor; Rn00565562_m1), Nos1 (neuronal nitric oxide synthase; Rn00583793_m1), Nos2 (inducible nitric oxide synthase; Rn00561646_m1), Il1b (interleukin 1 beta; Rn00580432_m1), Il6 (interleukin 6; Rn01410330_m1), Nfkbia (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; Rn01473657_g1), Cita (class II, major histocompatibility complex, transactivator; Rn01424725_m1), and Mif (macrophage migration inhibitory factor; Rn00821234_g1). All expression assays were multiplexed with 2’-chloro-7’-phenyl-1, 4-dichloro-6-carboxyfluorescein (VIC)-labelled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). The fold change in expression of each target gene was calculated using the ∆∆C_T method relative to the control group.

4.3.11 Protein estimation
Protein content was determined with a commercially available RED 660™ Protein Assay (G-Biosciences, St Louis, USA) using bovine serum albumin as a standard.

4.3.12 Statistical analysis
Data were analysed using statistical software GraphPad Prism (Version 6.0, GraphPad Software Inc., San Diego, CA, USA). Initially, data were tested for equal group variance and normality using Brown-Forsythe and Bartlett’s tests. Any data with significant group variability were compared using a non-parametric Kruskal-Wallis ANOVA with Dunn’s multiple comparisons post-test. All other normally distributed data were compared using one-way ANOVA with Dunnet’s multiple comparisons post-test to compare control and stress data. Results were expressed as mean ± standard error of the mean (± SEM) and p-values less than 0.05 were considered statistically significant.
4.4 Results

4.4.1 Indicators of HPA activity

To indicate that restraint in the dark-cycle is a highly effective stressor, we determined concentrations of the plasma stress hormone, corticosterone, which demonstrated significant increases above values of unrestrained controls (288.6 ± 21.01 ng/mL) when compared using a non-parametric Kruskal-Wallis test (p<0.001). Dunn’s post-test analysis demonstrated a greater than 2-fold increase above controls at 60 [711.0 ± 95.54 ng/mL; p<0.01], 120 [609.7 ± 72.82 ng/mL; p<0.01], and 240 [591.6 ± 39.08 ng/mL; p<0.01] minutes (Fig. 4.1A). High levels of circulating corticosterone are known to cause down-regulation of GR mRNA in HPA-regulatory regions such as the hippocampus. One-way ANOVA of hippocampal GR mRNA [F(3,20)=4.023, p=0.0216] revealed significant decreases relative to controls at 60 [0.88 ± 0.03; p<0.05], 120 [0.85 ± 0.03; p<0.05], and 240 [0.87 ± 0.04; p<0.05] minutes of stress exposure (Fig. 4.1B). No significant changes were observed in mRNA expression of the hippocampal MR (Fig. 4.1C).
Figure 4.1. The effect of acute restraint stress on (A) plasma corticosterone, (B) hippocampal glucocorticoid receptor (Nr3c1), and (C) hippocampal mineralocorticoid receptor (Nr3c2) mRNA expression in control and stressed rats (n=6-8 per group). Plasma corticosterone concentration was determined in blood samples collected via cardiac puncture and the relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM. *p<0.05, **p<0.01.
4.4.2 Hippocampal nitricergic activity

The synthesis of NO was determined using an established radiometric assay of citrulline formation by the NOS enzyme. This assay measures the total, calcium-dependent activity of both neuronal and endothelial isoforms, and the calcium-independent activity of the inducible isoform. One-way ANOVA \(F_{(3,16)}=6.375, \ p=0.0048\) demonstrated stress was effective at increasing total NOS activity. A Dunnet’s post-test analysis revealed significant increases at 60 [140.7 ± 5.83%; \(p<0.01\)], 120 [126.7 ± 5.02%; \(p<0.05\)], and 240 [131.8 ± 10.29%; \(p<0.05\)] minutes (Fig. 4.2A). This increase was due in part to calcium-dependent NOS activity which was shown to increase after stress \(F_{(3,16)}=5.595, p=0.0081\). This increase was only significantly elevated above unstressed controls at 60 minutes [142.6 ± 5.32%; \(p<0.01\)] of stress exposure (Fig. 4.2B). A one-way ANOVA also demonstrated a significant effect of stress on calcium-independent NOS activity \(F_{(3,16)}=3.73, \ p=0.0331\). However, in contrast to the calcium-dependent isoforms, post-test analysis demonstrated that the activity of the iNOS isoform was only significantly elevated following 240 [219.2 ± 41.22%; \(p<0.05\)] minutes of stress exposure (Fig. 4.2C).
Figure 4.2. The effect of acute restraint stress on (A) total nitric oxide synthase (NOS), (B) constitutive NOS, and (C) inducible NOS enzymatic activity in the hippocampus of control and stressed rats (n=5 per group). Enzymatic activities were determined in isolated hippocampal tissue collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, *p<0.05, **p<0.01.
Figure 4.3A shows the effects of stress on the stable metabolites of NO, NOx. A one-way ANOVA [F(3,28)=4.952, p<0.007] showed that the concentration of NOx increased significantly relative to controls (5.27 ± 0.37 µM/mg protein). Dunnet’s post-test analysis demonstrated increases at 60 [8.67 ± 1.03 µM/mg protein; p<0.01] and 120 [7.98 ± 0.60 µM/mg protein; p<0.05] minutes of stress. Figure 4.3B shows the overall hippocampal DAF-FM activation assessed using a novel semi-quantitative assay monitoring the formation of the highly fluorescent benzotriazole derivative of DAF-FM. One-way ANOVA of DAF-FM T formation [F(3,28)=4.105, p=0.0156] demonstrated increases relative to unstressed controls (26.3 ± 0.92 AU/mg protein). Post-test analysis revealed significantly increased DAF FM T at 60 [32.76 ± 1.96 AU/mg protein; p<0.05], 120 [33.56 ± 1.78 AU/mg protein; p<0.05], and 240 [33.55 ± 2.05 AU/mg protein; p<0.05] minutes of stress exposure.

![Figure 4.3](image.jpg)

**Figure 4.3.** The effect of acute restraint stress on hippocampal (A) total nitrite and nitrate (NOx) and (B) nitrosative status measured by the benzotriazole derivative of 4-amino-5-methylamino-2', 7'-difluorofluorescein (DAF-FM T) formation in control and stressed rats (n=8 per group). The metabolism and general production of nitric oxide were determined in isolated hippocampal tissue collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, *p<0.05, **p<0.01.
4.4.3 Hippocampal mRNA expression of NOS and inflammatory markers

To further evaluate the changes observed in the hippocampal nitrergic system, mRNA levels for the neuronal and inducible NOS isoforms were measured by quantitative real-time PCR. One-Way ANOVA \( [F(3,20)=3.141, p=0.0481] \) of hippocampal nNOS showed that stress decreased the mRNA expression (Fig. 4.4A). Post-test analysis showed that this was significantly lower relative to unstressed controls at 60 \([0.82 ± 0.03; p<0.05]\) and 120 \([0.83 ± 0.05; p<0.05]\) minutes. Figure 4.4B shows the effects of stress on iNOS expression. A non-parametric Kruskal-Wallis test \((p<0.01)\) with Dunn’s post-test analysis revealed a substantial increase in iNOS mRNA expression of approximately 10.5-fold at 240 \([13.84 ± 5.76; p<0.01]\) minutes. Expression of the inhibitory subunit, Nfkbia (or IκB-α), were also monitored following stress exposure (Fig. 4.4C). A one-way ANOVA \([F(3,20)=29.71, p<0.0001]\) of IκB-α mRNA demonstrated stress was highly effective at increasing the expression of this subunit. Post-test analysis revealed significant increases at 60 \([1.83 ± 0.12; p<0.001]\), 120 \([2.13 ± 0.04; p<0.001]\), and 240 \([1.81 ± 0.09; p<0.001]\) minutes.

To determine if the changes in iNOS expression were accompanied by other inflammatory indicators, mRNA levels of the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6) were determined. One-way ANOVA \([F(3,18)=3.836, p=0.0276]\) of IL-1β showed stress significantly decreased expression of this cytokine in the hippocampus. Post-test analysis indicated that 60 \([0.63 ± 0.05; p<0.05]\) minutes of stress exposure significantly decreased IL-1β expression, with subsequent stress durations returning towards control values (Fig. 4.4D). Figure 4.4E shows hippocampal IL-6 expression following stress exposure. A one-way ANOVA \([F(3,18)=3.896, p=0.0262]\) of this data showed a significant increase in expression of this cytokine following stress exposure. Dunnett’s post-test analysis revealed this increase was significant at 60 \([1.71 ± 0.21; p<0.05]\), 120 \([1.66 ± 0.09; p<0.05]\), and 240 \([1.70 ± 0.18; p<0.05]\) minutes of stress exposure relative to controls. Expression of the novel pro-inflammatory cytokine, Mif, was also shown to increase in a one-way ANOVA \([F(3,18)=3.461, p=0.0382]\), becoming significantly upregulated at 240 \([1.24 ± 0.04; p<0.05]\) minutes of stress exposure (Fig. 4.4F). Figure 4.4G demonstrates the effects of stress exposure on the glial-specific transcriptional coactivator of class II major histocompatibility complex, Ciita, in the hippocampus. A non-parametric Kruskal-Wallis test \((p<0.001)\) with Dunn’s post-test analysis revealed a significant increase in Ciita expression following 240 \([1.52 ± 0.16; p<0.05]\) minutes of stress relative to controls.
Figure 4.4. The effect of acute restraint stress on hippocampal (A) neuronal nitric oxide synthase (Nos1), (B) inducible nitric oxide synthase (Nos2), (C) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Nfkbia), (D) interleukin-1β (Il1b), (E) interleukin-6 (Il6), (F) macrophage migration inhibitory factor (Mif), and (G) class II, major histocompatibility complex, transactivator (Ciita) mRNA expression in control and stressed rats (n=6 per group). The relative expression was determined in isolated hippocampal tissue collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.
4.4.4 Striatal NO production, metabolism, and synthesis

Figure 4.5A shows the changes observed in total NOS activity in the striatum. A one-way ANOVA \(F(3,16)=3.53, \ p=0.039\) demonstrated significant increases in total activity at 120 [131.2 ± 9.25%; \(p<0.05\)], and 240 [129.6 ± 6.93%; \(p<0.05\)] minutes. Although no changes were observed in constitutive NOS activity (Fig. 4.5B), calcium-independent activity was significantly increased following stress exposure (Fig. 4.5C). Post-test analysis of these results indicated that this inducible activity was increased above non-stressed controls at 120 [212.5 ± 23.97%; \(p<0.05\)] and 240 [233.9 ± 22.44%; \(p<0.01\)] minutes.

**Figure 4.5.** The effect of acute restraint stress on (A) total nitric oxide synthase (NOS), (B) constitutive NOS, and (C) inducible NOS enzymatic activity in the striatum of control and stressed rats (n=5 per group). The enzymatic activities were determined in isolated striatal tissue collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, *\(p<0.05\), **\(p<0.01\).
There were no significant changes observed in striatal NOx concentrations (Fig. 4.6A). However, Figure 4.6B highlights the changes in striatal DAF-FM T formation following stress exposure. A one-way ANOVA \([p=0.08]\) and post-test of these results suggested that there may be a non-significant trend towards an increase at 240 \([p<0.05]\) minutes of stress exposure.

**Figure 4.6.** The effect of acute restraint stress on striatal (A) total nitrite and nitrate (NOx) and (B) nitrosative status measured by the benzotriazole derivative of 4-amino-5-methylamino-2’, 7’-difluorofluorescein (DAF-FM T) formation in control and stressed rats \((n=8\text{ per group})\). The metabolism and general production of nitric oxide were determined in isolated striatal tissue collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, *\(p<0.05\).
4.4.5 Striatal mRNA expression of NOS and inflammatory markers

Similar to the observations in NO synthesis, no changes were observed in nNOS mRNA expression in the striatum (Fig. 4.7A). Furthermore, iNOS mRNA demonstrated significant increases relative to controls in a non-parametric Kruskal-Wallis test (p≤0.021) at 120 [5.06 ± 1.10; p<0.05] and 240 [7.70 ± 2.77; p<0.05] minutes (Fig. 4.7B). These results were also observed alongside increases in IκB-α mRNA [F(3,20)=25.91, p<0.0001] which demonstrated a similar expression profile to the hippocampus, with significant increases at 60 [1.92 ± 0.04; p<0.001], 120 [2.09 ± 0.10; p<0.001], and 240 [1.66 ± 0.14; p<0.001] minutes of stress exposure (Fig. 4.7C). Figure 4.7D shows the changes in striatal IL-1β following stress exposure. A one-Way ANOVA [F(3,20)=3.48, p=0.0351] indicated stress significantly decreased expression of IL-1β relative to controls. A Dunnett’s post-test analysis showed, similar to the hippocampus, this decrease occurred at 60 [0.53 ± 0.09; p<0.05] minutes of stress exposure, with a recovery at subsequent time points. No differences were observed in striatal expression of IL-6 (Fig. 4.7E) or Mif (Fig. 4.7F), while Ciita (data not shown) was not reliably detectable following stress exposure.
Figure 4.7. The effect of acute restraint stress on striatal (A) neuronal nitric oxide synthase (Nos1), (B) inducible nitric oxide synthase (Nos2), (C) nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Nfkbia), (D) interleukin-1β (Il1b), (E) interleukin-6 (Il6), and (F) macrophage migration inhibitory factor (Mif) mRNA expression in control and stressed rats (n=6 per group). The relative expression was determined in isolated striatal tissue collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, *p<0.05, ***p<0.001.
4.5 Discussion

This study examined the physiological changes occurring in the nitrergic system following 60, 120, and 240 minutes of restraint stress in the hippocampus and striatum, regions known to display differential redox responses following stress (Liu et al., 1996). There was a rapid increase in hippocampal nitrosative status due to an initial increase in the activity of calcium-dependent constitutive NOS at 60 minutes of restraint stress. Interestingly, nNOS mRNA levels decreased following 60 and 120 minutes of stress exposure. Transcriptional activation of iNOS at 240 minutes of stress was observed with increased iNOS activity which sustains the elevation in overall hippocampal DAF-FM activation. Specific changes in inflammatory markers were also observed alongside an increase in Ciita, a transactivation marker of antigen presenting cells predominately expressed in glia. In comparison, there was a delayed stress-induced increase in striatal nitrosative status at 240 minutes of stress which was driven by the inducible isoform, while changes in inflammatory markers remained consistent with the anti-inflammatory properties of increased glucocorticoid concentrations. Furthermore, increases in mRNA expression of the inhibitory subunit, IκB-α, indicate activation of NF-κB in both the hippocampus and striatum.

It is well known that corticosterone mediates adaptation to stress through intrinsic negative feedback mechanisms. In the present study, acute restraint stress elicited increases in plasma corticosterone secretion of approximately 150% at 60 minutes, and 100% at both 120 and 240 minutes following stress induction. Within the hippocampus, ligand-activated cytosolic GR translocate into the nucleus to regulate target gene expression, including down-regulation of GR itself. As a positive control for neural gene expression, we assessed hippocampal GR mRNA levels which demonstrate an approximately 15% reduction at all stress periods relative to controls. This is comparable to similar time-course experiments suggesting intact negative feedback mechanisms (Noguchi et al., 2010). No change was observed in hippocampal MR mRNA level, a result similar to the study by Paskitti and colleagues (2000) who demonstrated unchanged full-length MR mRNA. However, they also demonstrated a 50% reduction in MR heterogeneous nuclear RNA in rat hippocampus 60-120 minutes following a 30 minute restraint stress, suggesting an increase in the stability of full-length MR mRNA following acute stress.

Under basal conditions, NO serves many important functions in the central nervous system including synaptic plasticity, neurotransmitter release, immune-like activity, and hormonal secretion via activating soluble guanylyl cyclase (sGC), leading to the release of second messenger cyclic guanosine monophosphate (cGMP) (Cao et al., 1996; Bogdan, 2001; Calka, 2006; Garthwaite, 2008). The present study demonstrated a 25-30% stress-induced increase in hippocampal DAM-FM activation at all restraint periods. However, this method requires an initial oxidative reaction of NO
to NO₂ which reacts with DAF-FM to form a non-fluorescent intermediate before a second reaction with NO forms the highly fluorescent DAF-FM triazole product (Namin et al., 2013). Alternatively, NO production can be estimated by determining the concentrations of total NO metabolites, NOₓ. Restraint stress significantly induced an increase in hippocampal NOₓ by approximately 65% at 60 minutes and 50% at 120 minutes of stress exposure, with a non-significant 20% increase at 240 minutes. In comparison, Moraes-Neto and colleagues (2014) observed an increase in rat dorsal hippocampal NOₓ following 10 and 30 minutes of acute restraint stress, while no significant changes were detected following 60 minutes of stress applied in the early light phase of the circadian cycle. Basal NOS activity within the rat brain, including the hippocampus, displays prominent circadian oscillations, with the highest NOS activity found toward the end of the behaviourally active dark phase (Ayers et al., 1996). This flux in NOS activity may account for the differential results observed in these two studies and highlights an important experimental consideration.

To determine which class of NOS isoforms produced the overall increase in the functional output of NO within the hippocampus, the enzymatic activity of constitutive NOS and iNOS were measured by monitoring the conversion of L-[¹⁴C(U)]-arginine to L-[¹⁴C(U)]-citrulline. Total NOS activity was elevated by approximately 30-40% at all stress periods when compared to unstressed controls. Furthermore, there was an initial 40% increase in constitutive NOS activity at 60 minutes of stress, with a non-significant 25% and 20% increase at 120 and 240 minutes respectively. In the brain, nNOS is tethered to the glutamatergic NMDA receptor, with calcium influx through this receptor triggering NO production. Using one hour restraint stress in rats, Lowy and colleagues (1993) reported an initial increase in glutamate release in the hippocampus at 10 minutes of stress which peaked at 20 minutes, and returned to baseline values by 40 minutes. Furthermore, nNOS-derived NO produced through the NMDA-mediated Ca²⁺ influx acts as a retrograde transmitter on presynaptic neurons to enhance neurotransmitter release and long term potentiation in the hippocampus, with the majority of these effects being mediated via NO-stimulated sGC binding and cGMP synthesis (Prast and Philippu, 2001; Chen et al., 2015). Additionally, Kotsonis and colleagues (1999) demonstrated that under defined conditions, the enzyme-derived reactive nitrogen species such as NO, and oxygen species such as hydrogen peroxide, have the potential to autoinhibit nNOS enzyme activity. Together, phasic activation of the glutamatergic NMDA receptor and NO/oxygen species-induced autoinhibition may begin to explain the observed decrease in nNOS activity following 60 minutes of stress. Conversely, iNOS activity in the hippocampus increased non-significantly by 15% and 35% at 60 and 120 minutes of stress, followed by a substantial and significant increase of approximately 120% at 240 minutes compared to controls. This production of NO can be sustained over a period of days as iNOS remains very stable at both
the mRNA and protein level (Vodovotz et al., 1993). In comparison, Gadek-Michalska and colleagues (2012) demonstrated that 10 minutes of restraint stress increased hippocampal nNOS protein content two hours post-stress, while no increase in iNOS was observed. Although this is likely due to the shorter duration of stress exposure employed, the measurement of protein content during the early light cycle where NOS protein expression is lowest may also be a contributing factor. Mechanistically, the observed increase in iNOS activity may be the result of glutamate receptor activation following stress, which induces up-regulation of TNF-α convertase, hence TNF-α, and the subsequent activation of NF-κB (Madrigal et al., 2002).

Interestingly, there was a 20% reduction in hippocampal nNOS mRNA levels at 60 and 120 minutes of restraint stress. Schwarz and colleagues (1998) have demonstrated down-regulation of nNOS expression by glucocorticoids in a concentration-dependent manner without changing the activity of the enzyme in a murine neuroblastoma cell line. Notably, NO and IL-1β have been reported to exert tonic inhibitory action on the sympathetic nervous system. However, within the brain, increased angiotensin II can inhibit mRNA expression of both nNOS and IL-1β to induce greater sympathetic nervous activity (Campese et al., 2002). In the present study, the observed down-regulation of both nNOS and IL-1β mRNA following stress likely results from the combination of increased corticosterone and angiotensin II release. Typically, iNOS is not expressed in resting cells, and yet there is a significant 10.5-fold increase in hippocampal iNOS mRNA levels following a 240 minute restraint stress which corresponds to the increased iNOS activity. Madrigal and colleagues (2002) demonstrated that the induction of iNOS protein expression and subsequent activity is dependent on NF-κB signalling. Under basal conditions, NF-κB is sequestered in the cytoplasm complexed to its inhibitor, IκB-α. In response to extracellular signalling, mainly by inflammatory cytokines, IκB kinases are rapidly activated and phosphorylate two critical serines in the N-terminal regulatory domain of the IκB molecule (Hayden and Ghosh, 2012). Phosphorylated IκB molecules undergo polyubiquitination and subsequent degradation, leading to NF-κB translocation to the nucleus and activation of target gene expression such as iNOS. Newly synthesised IκB-α can enter the nucleus to terminate NF-κB-dependent signals by removing the DNA binding and exporting the complex back to the cytoplasm to restore the original latent state. However, Madrigal and colleagues (2002) also demonstrated that the increase in TNF-α convertase activity, and subsequent TNF-α production, required for NF-κB activation are completely dependent on NMDA receptor signalling during stress exposure. The time domain of each of these events likely favour NF-κB activation as increased NMDA receptor signalling occurs rapidly in comparison to the inhibitory protein-protein interactions and increased IκB-α which require sufficient GR activation and protein synthesis respectively. Bottero and colleagues (2003) have proposed a different method for monitoring NF-κB transactivation potential by quantifying IκB-α mRNA levels using real-time PCR. In the present
study, stress-induced increases in IκB-α mRNA levels, indicative of NF-κB activation, supports the extensive expression of iNOS. Furthermore, the observation of increased Cita expression in the hippocampus indicates that glial cells likely play a role in these stress-induced mechanisms. Expression of Cita is most prominent in glia and functions as a critical coactivator required for transcription of the antigen-presenting major histocompatibility complex II (Otten et al., 1998). It has also been suggested that iNOS expression in the CNS is primarily localised to astrocytes and microglia, becoming induced under pro-inflammatory conditions (Calabrese et al., 2007). Interestingly, while the inflammatory profile of IL-1β expression is consistent with the anti-inflammatory effects of increased corticosterone, IL-6 expression is capable of overcoming this to be significantly elevated in hippocampal tissue. However, IL-6 is known to be resistant to glucocorticoid-mediated repression and acts as a potentiator of HPA activity (DeRijk et al., 1997; Lenczowski et al., 1999). Furthermore, the increase in Mif expression may begin to explain the rapid recovery of IL-1β expression in the presence of increased glucocorticoids. In the brain, this pro-inflammatory cytokine is released during stress and functions as a counter-regulator of glucocorticoid anti-inflammatory activity, allowing the expression of other pro-inflammatory cytokines such as IL-1β (Bucala, 1996). It has also been suggested that Mif can induce iNOS expression, though this may be an indirect effect via antagonism of glucocorticoid-induced NF-κB inhibition (Daun and Cannon, 2000; Bogdan, 2001).

It is known that NO is one of the key regulators of neuronal activity in the striatum where production by nNOS interneurons is stimulated following concurrent activation of the glutamatergic corticostriatal and dopaminergic nigrostriatal pathways (West and Grace, 2000; Fino et al., 2009). Many studies have focused on the augmented striatal NO-sGC-cGMP signalling following dopamine depletion, the hallmark feature in the pathogenesis of Parkinson’s disease (West and Tseng, 2011; Friend et al., 2013; Schwenkgrub et al., 2013). However, the effect of stress on striatal nitrergic activity is not yet well characterised. Total NOS activity in the striatum was elevated at both 120 and 240 minutes of stress by approximately 40% when compared to unstressed controls. No change in activity was detected in the constitutive forms and nNOS mRNA levels across all stress periods examined, despite nNOS being relatively abundant in the dorsal striatum and nucleus accumbens (Bredt et al., 1990; Vincent, 1994). Acute restraint stress caused a delayed increase in the formation of DAF-FM T of approximately 25% compared to controls at 240 minutes in the striatum, while no change was observed in striatal total NOx. The output of NO in the striatum was entirely driven by iNOS activity, with an apparent 110-130% increase at 120 and 240 minutes of restraint stress exposure alongside a 4 to 6-fold elevation in iNOS mRNA levels at 120 and 240 minutes of stress respectively. Expression of iNOS was also observed alongside increased IκB-α mRNA, though striatal IL-1β expression showed significant stress-induced repression, and both IL-
6 and Mif expression were not significantly elevated due to stress exposure. This increase in NF-κB signalling could be the result of other rapidly accumulating inflammatory cytokines and oxidative/nitrosative stress caused by increased peroxynitrite and hydrogen peroxide production and subsequent glutathione depletion (Christman et al., 2000; Oliveira-Marques et al., 2009). Moreover, expression of iNOS and IκB-α mRNA is also observed in the medial prefrontal cortex, indicating that this may be a generalised response to stress exposure (Appendix Fig. A2 and A3).

In summary, this study has demonstrated that short term restraint stress rapidly increases hippocampal nitrergic activity through a switch from constitutive to inducible NOS signaling accompanied by increased expression of inflammatory cytokines. This was consistent with a primed pro-inflammatory phenotype and shift towards adaptive immunity involving major histocompatibility complex-expressing glial elements. Conversely, striatal nitrosative status following stress is predominately mediated by iNOS with no change in other pro-inflammatory markers. Therefore, it is thought that despite the anti-inflammatory action of glucocorticoids, they may function as a warning signal to the central immune system and sensitise an array of specific immune adaptations including iNOS activation following acute stress. It is interesting to note that this increased iNOS activity appears to be a generalised response, while nNOS expression specifically decreased in the hippocampus, remained neutral in the striatum, and increased significantly within the medial prefrontal cortex by four hours of restraint (Appendix Fig. A1). This regional specificity in the expression pattern of nNOS may be due to the crucial role played by the limbic system in HPA axis activation. Taken together, the response to acute psychological stress involves regional and isoform specific changes in the nitrergic system observed concurrently alongside priming of central adaptive immunity. The link between nitrergic activity and inflammatory processes should be further explored to determine the contribution of immunologic priming to the pathogenesis of stress-related disorders such as depression.

4.6 Acknowledgements
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4.7 Conflict of Interest
The authors declare no competing financial interests.

4.8 Role of Funding Source
This work was supported by a University of Queensland Research Grant.
Chapter V

Inhibition of fatty acid amide hydrolase by PF-3845 alleviates nitrergic-related neuroinflammatory response following acute restraint stress
## 5.0 Contributions by others

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5.1 Abstract

Psychological stressors are undoubtedly amongst the most examined risk factors in studying depressive disorders. An increasing body of evidence suggests that immune dyshomeostasis following stress plays a major role in the pathophysiology of stress-related illnesses. Long term exposure to stress has been demonstrated to cause neuroinflammation through a sustained overproduction of free radicals, including nitric oxide, via an increased inducible nitric oxide synthase (iNOS) activity. This enzyme is regulated by cytokines and inflammatory complexes following noxious stimuli and consequently mediates cytotoxicity through oxidative/nitrosative stress under chronic stress conditions. We have previously demonstrated that iNOS activity and mRNA expression are upregulated in the hippocampus following just 4 hours of acute restraint stress. Interestingly, the nitrergic system has recently demonstrated interactions with the endocannabinoid system following stressful or aversive situations. Similar to nitric oxide, the endogenous cannabinoids are synthesised on demand and have been classified as atypical neurotransmitters, with preclinical findings suggesting that cannabinoid receptor agonists and endocannabinoid enhancers inhibit nitrergic activity and display antidepressant-like properties. Specifically, pharmacological enhancement of endocannabinoid content by fatty acid amide hydrolase inhibitor (FAAH), PF-3845, has been demonstrated to alleviate neuroinflammation by decreasing the number of iNOS-expressing microglia following traumatic brain injury. However, this describes cannabinoid modulation during pathological conditions and therefore the present study aim to examine the effects of systemically injected PF-3845 in the modulation of nitrergic and inflammatory-related genes within the hippocampus after acute stress exposure. Following vehicle or PF-3845 injections (5 mg/kg in normal saline; i.p.), outbred male Wistar rats aged 5-6 weeks postnatal were exposed to a single episode of 0 (control), 60, 240, or 360 minute of restraint stress (n=5-6 per group). The hippocampus was cryodissected and assayed for relative expression of nitrergic and inflammatory-related genes. The results demonstrate that pretreatment with the FAAH inhibitor rapidly reduces restraint stress-induced plasma corticosterone release. This was accompanied by a reduction in the stress-induced pro-inflammatory response including iNOS, interleukin-1β, interleukin-6, and cyclooxygenase-2 mRNA in the hippocampus. Furthermore, transcriptional potential of NF-κB was inhibited by PF-3845 suggesting that enhanced endocannabinoid levels in the hippocampus have an overall anti-inflammatory effect following acute stress exposure.
5.2 Introduction

During stress exposure the hypothalamic-pituitary-adrenal (HPA) axis functions synergistically with the autonomic nervous system to orchestrate appropriate adaptive responses in central and peripheral tissues. Glucocorticoids are the final hormonal effector of an activated HPA-axis which exerts a plethora of physiological functions through the ubiquitously expressed glucocorticoid receptors. One of the essential roles of glucocorticoids along with protecting the host from noxious insults is the regulation of immune homeostasis (Chrousos and Gold, 1992; Cancedda et al., 2002). Stress and glucocorticoids are generally regarded to be anti-inflammatory as numerous studies have demonstrated their ability to suppress gene transcription of several pro-inflammatory cytokines (Barnes, 1998; Coutinho and Chapman, 2011). However, this anti-inflammatory action exhibits a hierarchy, with tumour necrosis factor (TNF) having the greatest sensitivity to glucocorticoid suppression at physiological levels, while interleukin-1β (IL-1β) displays intermediate sensitivity and interleukin-6 (IL-6) is comparatively resistant (DeRijk et al., 1997). Nevertheless, in addition to the anti-inflammatory actions, it has become increasingly apparent that the initial rise in glucocorticoids following stress exposure primes the immune effector cells through innate immune signalling pathways such as toll-like receptor upregulation (de Pablos et al., 2006; Frank et al., 2007; Wohleb et al., 2011; Frank et al., 2012). This is considered to be an important adaptive action by glucocorticoids, with dissipation of the ‘fight/flight’ response heightening immune defences and vigilance beyond basal levels and promoting more vigorous reactions to persisting or impending threats (Sapolsky et al., 2000; Johnson et al., 2002; Sorrells et al., 2009). Specifically within the central nervous system (CNS), it has been demonstrated that short term stress can modulate microglial immunophenotype to sensitise a neuroinflammatory response which may be exaggerated in subsequent inflammatory challenges (Blandino et al., 2006; Frank et al., 2007; Sugama et al., 2007; Blandino et al., 2009; Sugama et al., 2009; Sugama et al., 2011). Activated microglia upregulate antigen presentation molecules such as major histocompatibility complex, increase phagocytic activity, generate pro-inflammatory cytokines and reactive oxygen/nitrogen species, overall inducing a neuroinflammatory response (de Pablos et al., 2006; Frank et al., 2007; Colton, 2009).

Pro-inflammatory cytokines including TNF and IL-1β are among the principle messengers responsible for initiating and coordinating the acute phase inflammatory response, with IL-1β acting through autocrine/paracrine signalling to further potentiate IL-6 production (Dinarello, 1998). It has been reported that following acute immobilisation stress, there was an increase in soluble TNF which was released by membrane-anchored zinc metalloproteinase, commonly known as TNF-α convertase (TACE), in the rat brain cortex (Madrigal et al., 2002). This cytokine has been
implicated in the pathogenesis of various neurodegenerative conditions including Alzheimer’s and Parkinson’s disease (Fischer and Maier, 2015). Central administration of the archetypal pro-inflammatory cytokine, IL-1, has been shown to produce endocrine, neurochemical, and behavioural modifications similar to those elicited by psychological stressors (Song et al., 2006). Several studies have demonstrated that IL-1 activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways which subsequently initiate downstream inflammatory, oxidative, and nitrosative signalling (Mercurio and Manning, 1999; Albrecht et al., 2007; Solt et al., 2007). Specifically, cytokine-induced NF-κB transactivation is capable of increasing the activity of inducible nitric oxide synthase (iNOS), a widely accepted pro-inflammatory mediator which may contribute to cellular toxicity by extensive and prolonged production of nitric oxide (NO) and the downstream oxidant, peroxynitrite (Chen et al., 2015). Madrigal and colleagues (2001) have demonstrated that inhibition of NF-κB translocation in the rat brain cortex decreased iNOS activity and expression following acute stress. Natural compounds such as curcumin, α-tocopherol, and resveratrol have also been shown to inhibit NF-κB activation and the associated neuroinflammatory response including both cytokines and iNOS induction (Suzuki and Packer, 1993; Lu et al., 2010; Olivera et al., 2012). Another important inflammatory signalling cascade involves cyclooxygenase-2 (COX-2), a constitutively expressed enzyme in the brain that converts arachidonic acid into prostaglandin. Upon stimulation, it can be rapidly upregulated in cells that are involved in inflammation, thereby mediating fever and sensitivity to pain (Dubois et al., 1998). A number of recent studies have demonstrated its involvement in HPA axis activation, with inhibition of COX-2 demonstrating anti-depressant like properties following lipopolysaccharide-injection without affecting major cytokine responses (de Paiva et al., 2010; Teeling et al., 2010; Ma et al., 2013).

Recently, cannabinoids have received extensive research interest due to their roles in increasing sleep and fat storage while attenuating inflammatory response and stress-induced behaviours (Pacher et al., 2006). The two major endogenous cannabinoids, N-arachidonoylethanolamine (AEA; commonly known as anandamide) and 2-arachidonoylglycerol (2-AG), are produced on demand post-synaptically as a neuromodulator in response to stress and generally function in opposition to the HPA response. For example, selective cannabinoid receptor type 1 (CB1R) antagonist has been reported to modulate corticosterone release and possess anxiogenic effects (Wade et al., 2006). Hill and colleagues (2011) demonstrated that endocannabinoid signalling played an important role in suppressing corticosteroid secretion following stress through inhibition of GABAergic neurotransmission. Pharmacological inhibition of fatty acid amide hydrolase (FAAH), an enzyme responsible for rapid physiological degradation of anandamide, robustly blocked stress-induced corticosterone release (Patel et al., 2004). In addition to its critical involvement in stress modulation, endocannabinoids have demonstrated anti-inflammatory properties as FAAH knock out
mice have a reduced lipopolysaccharide-induced hyperalgesia and edema, an effect mediated mainly through the cannabinoid receptor type 2 (CB2R) (Naidu et al., 2010). Studies have reported AEA and other cannabinoid-like fatty acid amides, including N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA), exert their anti-inflammatory effects by acting on the peroxisome proliferator-activated receptors to induce expression of anti-inflammatory proteins such as nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkB-α) while repressing expression of TNF (D'Agostino et al., 2007; Rettori et al., 2012; Paterniti et al., 2013; Alhouayek and Muccioli, 2014). Interestingly, Tchantchou and colleagues (2014) have recently demonstrated effective suppression of traumatic brain injury-induced neuroinflammation, including iNOS and COX-2 expression, in microglia/macrophages by PF-3845, a novel FAAH inhibitor with high sensitivity and long duration. However, this represents modulation occurring in a condition of chronic injury, while the effects of endocannabinoid modulation by FAAH inhibitor on the nitrergic and inflammatory indicators following short term stress remains unclear. Therefore, the present study will examined the effects of systemic injection of PF-3845 on the nitrergic and inflammatory-related genes following acute stress.

5.3 Materials and Methods

5.3.1 Ethical approval and experimental animals

All experimental procedures were in accordance with regulations and policies outlined by The University of Queensland Animal Ethics Committee with AEC approval numbers SBS/456/14/URG. Outbred male Wistar rats (Rattus norvegicus) aged 5-6 weeks postnatal, weighing 206.6 ± 2.04 g were sourced from The University of Queensland Biological Resources breeding colony. Rats were housed individually under controlled laboratory conditions (22 ± 2°C; 55 ± 5% humidity) with a 12:12h light-dark cycle (lights off at 12.30h). Standard rat chow and water were available ad libitum.

5.3.2 Experimental protocol

Rats were habituated to human handling for 10 minutes per day 6 days prior to experimentation. On each experimental day, rats were transported in individual home cages from the colony room to an experimental room within the same animal facility. Rats were randomly allocated to 8 treatment groups with control, or restraint stress for 60, 240, and 360 minutes with either vehicle or PF-3845 injection. Rats were subjected to acute restraint stress using adjustable wire mesh restrainers within individual home cages (stress treatment starting at 13:45h). The FAAH inhibitor, PF-3845 (5 mg/kg; Sigma, St. Louis, MO), dissolved in 2% DMSO in normal saline (0.9% sodium chloride) was injected 60 minutes before stress treatment with an injection volume of 10 mL/kg (i.p.). During this period, rats were acclimatised to the novel experimental room under low light and noise. To
isolate the effects of restraint stress, all rats were deprived of food and water during the one hour habituation and stress treatment period. Following each allocated treatment, a blood sample was collected in sodium heparin (20 IU/mL blood) via tail-tipping. Heparinised blood samples were centrifuged at 200 x g for 5 minutes immediately after collection with the resulting plasma supernatant stored at -80 for later determination of corticosterone concentrations. Rats were then overdosed with 100 mg/kg of sodium pentobarbital (i.p. injection; Lethabarb, Virbac, Peakhurst, Australia). Whole brain were rapidly removed and frozen on powdered dry ice for storage at -80°C. Frozen brains were sectioned on a cryostat and the hippocampus was cryo-dissected from sections according to a rat brain atlas (Paxinos and Watson, 2007) on a dry ice-embedded metal platform. Regionalised hippocampus was stored at -80°C for later relative gene expression analysis.

5.3.3 Plasma corticosterone assay
Corticosterone concentrations were measured by an in-house radioimmunoassay using anti-rat corticosterone polyclonal antibody (Sapphire Bioscience Pty. Ltd.) and tritiated [1, 2, 6, 7- ³H]-corticosterone tracer as previously described in Spiers and colleagues (2013). Radioactivity was counted in liquid scintillation cocktail (Ultima Gold™, Perkin Elmer) using a Liquid Scintillation Spectrometer (Tri-Carb 3100 TR, Perkin Elmer). Dichloromethane extraction recovery was 79.14% and intra- and inter-assay coefficients of variation were 5.79% and 2.12% respectively.

5.3.4 Real-time PCR
Total RNA was extracted from the hippocampus using a RNeasy mini kit (Qiagen, Doncaster, Australia) treated with deoxyribonuclease I and reversed transcribed into cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Gladesville, Australia) according to the manufacturer’s instructions. Taqman gene expression ‘assay-on-demand™’ kits (Life Technologies, Mulgrave, Australia) with optimised primers and FAM-labelled probes were used to detect gene expression of Nos2 (inducible nitric oxide synthase; Rn00561646_m1), Nos1 (neuronal nitric oxide synthase; Rn00583793_m1), Tnf (tumor necrosis factor; Rn01525859_g1), Il1b (interleukin 1 beta; Rn00580432_m1), Il6 (interleukin 6; Rn01410330_m1), Nfkbia (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; Rn01473657_g1), and Ptgs2 (prostaglandin-endoperoxide synthase 2; Rn01483828_m1). The ΔΔCT method was used for all expression assays which were run in a multiplex reaction with VIC-labelled GAPDH as endogenous control using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA).

5.3.5 Statistical analysis
Data were analysed using statistical software GraphPad Prism (Version 6.07, GraphPad Software Inc., San Diego, CA, USA). Two-way repeated measure ANOVAs with Fisher’s Least Significant Difference (LSD) test were used to compare vehicle and PF-3845 data for all time course analyses.
Results were expressed as mean ± standard error of the mean (± SEM) and p-values less than 0.05 were considered statistically significant.

5.4 Results

5.4.1 Inhibition of FAAH dampens acute stress-induced corticosterone release

This study examined the effects of pharmacological enhancement in endocannabinoid contents by administration of selective FAAH inhibitor on inducible nitric oxide synthase and several other inflammatory-related genes in the hippocampus following acute stress. To determine whether increased endocannabinoid signalling modulates HPA output, we determined concentrations of the plasma stress hormone, corticosterone. Pre-treatment with the FAAH inhibitor, PF-3845, significantly inhibited restraint-induced corticosterone release \([F(1, 40) = 5.863, p=0.0201]\) in a time-dependent manner \([F(3, 40) = 24.42, p<0.0001]\). A comparison of group means demonstrated significantly lower corticosterone concentrations in the PF-3845 treated group \([472.27 ± 27.2\ ng/mL, p<0.01]\) at 60 minutes of stress compared to the vehicle-treated group \([638.22 ± 66.71\ ng/mL]\) (Fig. 5.1).

![Graph showing corticosterone levels](image)

**Figure 5.1.** The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on plasma corticosterone levels from control and stressed rats (n=5-7 per group). Plasma corticosterone concentration was determined in blood samples collected via tail-tipping from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, **p<0.01.
5.4.2 Acute stress-induced upregulation of inducible nitric oxide synthase was attenuated by PF-3845

There was a progressive increase in inducible NOS (Nos2) in the hippocampus of vehicle-treated animals as the duration of stress exposure prolonged which was highly significant at 360 minutes of stress [5.48 ± 1.25, p<0.001] compared to controls [1.14 ± 0.2]. Figure 5.2A demonstrates a time-dependent [F(3, 40) = 10.17, p<0.0001] inhibition of hippocampal inducible NOS mRNA levels in the PF-384-treated group [F(1, 40) = 4.191, p=0.047]. Post-test analysis revealed a significantly lower inducible NOS mRNA expression at 360 minutes of stress exposure following PF-3845 treatment [3.25 ± 0.91, p<0.05] compared to vehicle [5.48 ± 1.25]. No significant changes were observed in hippocampal neuronal NOS (Nos1) mRNA levels between vehicle- and PF-3845-treated groups (Fig. 5.2B).

**Figure 5.2.** The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on hippocampal (A) inducible nitric oxide synthase (Nos2), and (B) neuronal nitric oxide synthase (Nos1) mRNA expression from control and stressed rats (n=5-6 per group). The relative expression was determined in isolated hippocampus collected from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, *p<0.05.
5.4.3 Dampening of HPA activity by PF-3845 prevents TNF downregulation and TACE upregulation

Glucocorticoid is known to suppress TNF synthesis contributing to an anti-inflammatory action. Treatment with FAAH inhibitor significantly prevented hippocampal TNF downregulation at the mRNA level [$F_{(1, 36)} = 6.963$, p=0.0122] at 60 and 240 [p<0.05] minutes of stress exposure when compared to the vehicle group (Fig. 5.3).

**Figure 5.3.** The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on hippocampal tumor necrosis factor (Tnf) mRNA expression from control and stressed rats (n=5-6 per group). The relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, *p<0.05.
The pro-inflammatory cytokine, TNF, is synthesised as a 32 kDa transmembrane anchored precursor that is cleaved by TACE, encoded by the gene a disintegrin and metalloprotease 17 (Adam17), yielding a non-glycosylated soluble 17 kDa protein. Stress significantly increases Adam17 mRNA levels at 360 minutes \([1.42 \pm 0.25, \ p<0.05]\) above values of unrestrained controls \([1.03 \pm 0.12]\) in the vehicle group. Administration of PF-3845 significantly reduced Adam17 mRNA levels \(F_{(1, \ 36)} = 15.21, \ p=0.0004\) in the hippocampus. The Fisher’s LSD test revealed significant reduction in Adam17 mRNA levels by PF-3845 at 60 [p<0.05], 240, and 360 [p<0.01] minutes of acute stress when compared to the vehicle-treated animals.

**Figure 5.4.** The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on hippocampal disintegrin and metalloprotease 17 (Adam17) mRNA expression from control and stressed rats (n=5-6 per group). The relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, *p<0.05, **p<0.01.
5.4.4 Inhibition of FAAH constrains inflammatory cytokines including IL-1β and IL-6 upregulation following acute stress

Inflammatory cytokines are rapidly upregulated following activation of immune cells that reside in the CNS including microglia, CNS macrophages, and astrocytes. We found that following acute psychological stress there was an upregulation of IL-1β expression at 240 [p<0.05] and 360 [p<0.001] minutes, while IL-6 expression was upregulated at 60 [p<0.05], 240, and 360 [p<0.001] minutes of stress exposure in the hippocampus. Previous studies have revealed the anti-inflammatory properties exerted by endocannabinoids are partly through attenuation of microglial activation. In the present study, there was a significant time-dependent \[F(3, 36) = 17.21, p<0.0001\] and PF-3845 treatment \[F(1, 36) = 4.358, p=0.044\] effects on IL-1β gene expression following stress exposure. Post-test analysis showed a transient reduction in IL-1β mRNA levels at 60 minutes of stress exposure following PF-3845 treatment \[0.67 ± 0.03, p<0.05\] compared to the vehicle group \[1.18 ± 0.15\]. Furthermore, the FAAH inhibitor \[F(1, 36) = 18.95, p=0.0001\] also inhibited the stress-induced upregulation of IL-6 mRNA in a time-dependent manner \[F(3, 36) = 6.049, p=0.002\] at 60 [p<0.05], 240, and 360 [p<0.01] minutes of restraint stress (Fig. 5.5B).
Figure 5.5. The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on hippocampal (A) interleukin-1β (Il1b) and (B) interleukin-6 (Il6) mRNA expression from control and stressed rats (n=5-6 per group). The relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, *p<0.05, **p<0.01.
5.4.5 PF-3845 acutely suppressed inflammatory COX-2 mRNA expression in the hippocampus

It has been demonstrated that COX-2 is constitutively expressed in the brain and is primarily responsible for the production of prostanoids including prostaglandin E2 which is involved in pathological inflammatory conditions. Notably, PF-3845 treatment \( [F_{(1, 36)} = 4.476, p=0.041] \) resulted in decreased prostaglandin-endoperoxide synthase 2 (or COX-2) mRNA levels in animals exposed to 60 \( [p<0.05] \) minutes of restraint stress compared to the vehicle-injected animals.

**Figure 5.7.** The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on hippocampal prostaglandin-endoperoxide synthase 2 (Ptgs2; also known as cyclooxygenase-2 or COX-2) mRNA expression from control and stressed rats \( (n=5-6 \text{ per group}) \). The relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, \(*p<0.05\).
5.4.6 The FAAH inhibitor reduces the potential of NF-κB transactivation monitored by Nfkbia mRNA expression

The transcription factor, NF-KB, plays a central role in the pro-inflammatory signalling pathway by regulating the expressions of proteins involved in oxidative stress/damage and inflammation including iNOS, pro-inflammatory cytokines, and COX-2. To examine the NF-κB transactivation potential, expression of the inhibitory subunit, Nfkbia (or IκB-α), were monitored following stress exposure in vehicle and PF-3845-treated animals (Fig. 5.5). In the vehicle-treated group, restraint stress significantly increases the expression of IκB-α across all stress time points examined [p<0.001] when compared to unstressed controls. Two-way ANOVA demonstrated PF-3845 was highly effective [F(1, 36) = 19.16, p<0.001] in blunting IκB-α mRNA expression over the time course of the experiment [F(3, 36) = 15.05, p<0001]. Post-test analysis revealed significant reduction at 60 [p<0.001], 120, and 240 [p<0.05] minutes of stress exposure when compared to the vehicle group (Fig. 5.6).

Figure 5.6. The effect of the fatty acid amide hydrolase inhibitor, PF-3845 (5 mg/kg, i.p. injection), on hippocampal nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Nfkbia) mRNA expression from control and stressed rats (n=5-6 per group). The relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to 0 (control), 60, 240, and 360 minutes of acute restraint stress. Data are presented as mean ± SEM, *p<0.05, ***p<0.001.
5.5 Discussion

This study demonstrated that administration of the FAAH inhibitor, PF-3845, robustly suppresses acute stress-induced upregulation of nitrergic and neuroinflammatory responses due to an increased endocannabinoid tone within the hippocampus. The results show that pretreatment with PF-3845 rapidly dampens restraint stress-induced plasma corticosterone release. This was accompanied by a reduction in stress-induced iNOS, TACE, IL-1β, IL-6, and COX-2 mRNA upregulation in the hippocampus. Furthermore, transcriptional potential of NF-κB was inhibited by PF-3845 suggesting that an enhanced endocannabinoid levels in the hippocampus have an overall immunosuppressive effect following acute stress exposure.

In the present study, treatment with PF-3845 negatively modulates HPA axis activation which is in agreement with previous literature using an alternative selective FAAH inhibitor, URB597. Patel and colleagues (2004) demonstrated that URB597 dose-dependently inhibited corticosterone release following a 30 minute restraint stress in mice. Several studies have also demonstrated the importance of endocannabinoid in stress system regulation, for example, modulation of CB₁R signalling by genetic modification or pharmacological blockade of the receptor results in an increased HPA activity under basal or stressful conditions (Wade et al., 2006; Cota et al., 2007; Steiner et al., 2008). Furthermore, Steiner and colleagues (2008) reported that CB₁R receptors are localised on the glutamatergic neurons and via dendritic release and the retrograde actions of endocannabinoids, the excitatory synaptic drive to corticotropin-releasing hormone neurons are suppressed within the paraventricular nucleus of the hypothalamus thereby restraining activation of the HPA axis. Interestingly, following acute stress, it has been demonstrated that FAAH-mediated AEA hydrolysis was enhanced in response to acute stress and therefore the rapid loss of AEA levels in the basolateral amygdala may play an important role in the disinhibition of HPA axis following stress (Hill et al., 2009).

The expression of the calcium-independent NOS isoform is generally restricted but can become rapidly and significantly elevated through stimulation of a variety of factors including pro-inflammatory cytokines (Aktan, 2004). We have previously demonstrated a significant 10.5-fold increase in hippocampal iNOS mRNA levels following 240 minutes of restraint stress which corresponds well with the 2-fold increase in iNOS activity (Chapter IV). Based on an interesting study by Tchantchou and colleagues (2014) where an enhanced endocannabinoid by PF-3845 successfully suppressed the expression of iNOS following traumatic brain injury, the present study aim to test the effect of PF-3845 on acute stress-induced iNOS mRNA upregulation. The FAAH inhibitor significantly alleviated stress-induced hippocampal iNOS mRNA upregulation at 360 minutes of restraint when compared to the vehicle group. This may be an indirect consequence of
the early reduction in corticosterone secretion, indicating the initial response to restraint stress was buffered following PF-3845 administration. In contrast, we observed no significant changes in expression of the constitutive calcium-dependent isoform, nNOS, following PF-3845 administration. You and colleagues (2009) demonstrated the overall oxidative status in the hippocampus, measured using the non-selective and NO-reactive fluorescent probe, 2', 7'-dichlorofluorescin diacetate, is both time-dependent and dose-responsive to glucocorticoid receptor agonists. Although the relative contribution of NO to the fluorescence of dichlorofluorescein is unknown, data from our laboratory indicate that NO-induced activation of the NO-selective fluorescent probe, 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM DA), and the metabolites of NO, nitrate/nitrite, are increased rapidly following restraint stress exposure over a similar timeframe used by You and colleagues (2009). However, it has also been suggested that microglia are the principle cell type responsible for expressing iNOS in the CNS following stress exposure and these cells are known to express low levels of functional FAAH (Heneka et al., 2000; Tham et al., 2007). Tham and colleagues (2007) demonstrated that the FAAH inhibitor, URB597, reduced expression of both COX-2 and iNOS in addition to their corresponding products prostaglandin E₂ and NO following lipopolysaccharide stimulation in cultured cortical microglia. Recent data from our laboratory suggests that the acute stress used in the present study does induce alterations in the essential glial co-activator of class II major histocompatibility complex, Cita, associated with antigen presentation over a similar timeframe to the changes observed in iNOS expression (Chapter IV). This indicates that acute stress induces glial cells to change from a state of quiescence to surveillance, with increases in major histocompatibility complex monitoring the hippocampus for foreign antigens.

The transcriptional activation of iNOS has previously been shown to rely heavily on TNF-induced NF-κB transactivation. Madrigal and colleagues (2002) demonstrated that one hour of immobilisation stress caused an increase in cortical TACE activity and TNF, leading to translocation of NF-κB into the nucleus by 4 hours and a subsequent increase in iNOS activity by 6 hours of stress exposure. Pharmacological inhibition of TACE activity or NMDA receptor antagonism strongly attenuated these changes, indicating that glutamatergic transmission and TNF production are involved in NF-κB activation prior to iNOS transcription. In the present study, one hour of stress exposure increased expression of Adam17 mRNA, the gene responsible for TACE translation. This was mirrored by a corresponding decrease in TNF mRNA. Although this may seem paradoxical in comparison to the results obtained by Madrigal and colleagues (2002) in the cortex, it is likely that the hippocampus does undergo a constrained but significant increase in TNF. The potent immunosuppressive effects of glucocorticoids on TNF mRNA have been documented previously (Steer et al., 2000). However, pro-TNF protein present in the cytosol would still be
subject to cleavage by TACE, and this initial increase in TNF would subsequently potentiate Adam17 mRNA transcription (Gooz, 2010). Interestingly, PF-3845 prevented the stress-induced decrease in hippocampal TNF mRNA and effectively decreased Adam17 mRNA, resulting in significant differences between vehicle and drug treated animals. This is likely due in part to the reduction in corticosterone observed early during stress exposure. However, there is also a likelihood that enhanced endocannabinoids are acting as anti-inflammatory agents as there is also a subtle but significant reduction in hippocampal COX-2 expression. In addition to the TACE/TNF system, stress exposure also altered hippocampal IL1-β and IL-6 mRNA in a time-dependent manner. Both IL1-β and IL-6 mRNA were increased in the hippocampus following stress exposure in vehicle-treated animals. Administration of PF-3845 caused an early inhibition of this increase in IL-1β that was only significantly different to vehicle treatment following 60 minutes of stress. Microglia were the most likely cellular source of this increase in pro-inflammatory IL-1β based on the study by Blandino and colleagues (2006) which evaluated the effects of minocycline on acute foot shock-induced increase in central IL-1β. The results from the study demonstrated that minocycline, a tetracycline derivative which selectively restrict pro-inflammatory cytokine release from microglia by inhibiting its activation and proliferation, abolished the foot shock-induced increase in hypothalamic IL-1β mRNA. PF-3845 markedly prevented stress-induced increases in hippocampal IL-6 mRNA at all stress durations. The magnitude of these changes may indicate that the endocannabinoid system preferentially interacts with IL-6 during a normal response to stress. However, IL-6 also exhibits a positive feed-forward expression, meaning reductions in corticosterone by PF-3845 may prevent transcription and further potentiation of IL-6 mRNA in the hippocampus (Bethin et al., 2000; Spooren et al., 2011).

Together, the hippocampal stress response exhibits a significant expression profile of pro-inflammatory cytokines. Increases in the protein products of these genes, particularly within the TACE/TNF system, would cause the transactivation of NF-κB and result in activation of several downstream targets including transcription of iNOS. In the present study, the status of NF-κB following stress exposure was determined by monitoring the inhibitory subunit IκB-α, responsible for binding and inactivating NF-κB in the cytoplasm. Bottero and colleagues (2003) validated the use of IκB-α mRNA quantification as a simple method of monitoring NF-κB in place of more difficult techniques such as electrophoretic mobility shift assays. This inhibitory subunit is rapidly transcriptionally activated during increased NF-κB signalling to remove the pro-inflammatory transcription factor from the nucleus and restore cellular homeostasis. Although NF-κB can be activated by a number of different upstream pathways, Madrigal and colleagues (2002) demonstrated that nuclear translocation of this transcription factor was required for stress-induced upregulation of iNOS. In the present study, IκB-α levels indicative of NF-κB activity show early
and persistent increases following exposure to stress. This increase is strongly attenuated by inhibition of FAAH at all time points used in this experiment and likely reflects the combined action of decreased corticosterone output, which would rapidly and non-genomically reduce NDMA receptor-dependent calcium currents via a GR-independent mechanism, and reductions in pro-inflammatory cytokines observed in the hippocampus.

In conclusion, psychological stress produces a distinct pro-inflammatory profile in a number of cytokines at different durations of stress exposure in the hippocampus. These exhibit a hierarchy of production, with IL-6 appearing to be the primary cytokine in response to this type of stressor in this region. Inhibition of the enzyme responsible for endocannabinoid degradation, and hence increased endocannabinoid signalling, greatly changes this stress profile both at the peripheral hormone and hippocampal transcriptional level. Overall, this results in decreased indicators of neuroinflammation following psychological stress exposure for up to 6 hours and, with reductions in iNOS transcription, likely prevents the development of stress-induced nitrosative stress on a biochemical level. Neuroinflammation is a key pathogenic mechanism in many disease states including chronic stress-induced depression. The present study highlights the importance of the endogenous endocannabinoid system in the neuroinflammatory response to stress and may offer a useful therapeutic target for conditions exacerbated or caused by prolonged stress exposure.

5.6 Acknowledgements

We would like to thank Ms Mary White for her assistance with general laboratory maintenance and the animal technicians from the University of Queensland Biological Resources animal house.

5.7 Conflict of Interest

The authors declare no competing financial interests.

5.8 Role of Funding Source

This work was supported by a University of Queensland Research Grant. This funding body had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.
Chapter VI

General Discussion
6.0 Thesis Summary

This thesis has focused on establishing an acute profile of stress-induced peripheral and central nitrergic activation (Fig. 6.1). In Chapter III, peripheral tissue was demonstrated to be particularly susceptible to early oxidative and nitrosative changes following a single episode of psychological stress, sufficient to induce oxidative damage. The follow up study, Chapter IV, found that short term stress are capable of initiating rapid and time-dependent changes in hippocampal nitrergic activity. Specifically, there was a rapid increase in hippocampal NO levels due to an initial increase in the constitutive NOS activity. Following transcriptional activation of the inflammatory related-iNOS and the resultant increases in iNOS enzymatic activity withstand the elevation in NO within the hippocampus. In comparison, no change was observed in striatal constitutive NOS, however, the delayed stress-induced increase in striatal NO was utmost driven by the inducible isoform. Furthermore, an increased NF-κB transactivation potential is accountable for iNOS activation in both neural regions examined. Chapter V employed an enzyme inhibitor, PF-3845, to pharmacologically enhanced endocannabinoid content which demonstrated anti-inflammatory properties by decreasing the expression of iNOS and other inflammatory genes following acute psychological stress.
Fig. 6.1. Schematic thesis summary.

Experiment one demonstrated that a single episode of stress causes early and marked changes of both oxidative (reactive oxygen species, ROS; glutathione/glutathione disulfide, GSH/GSSG) and nitrosative status (nitric oxide, NO; nitrite and nitrate, NOx) likely contributing to the observed increase in oxidative damage (lipid peroxidation, LPO) in peripheral blood. The second experiment showed that short term stress rapidly increases hippocampal nitrergic activity through a switch from calcium dependent nitric oxide synthase (NOS) to NF-κB-induced calcium independent NOS signalling. This was observed concurrently with a primed pro-inflammatory response involving major histocompatibility complex (CIITA), macrophage migration inhibitory factor (MIF), interleukins (IL-6 and IL-1β), tumor necrosis factor-α (TNF-α) and the converting enzyme (TACE).

The results from the third experiment demonstrate that pretreatment with the fatty acid amide hydrolase inhibitor, PF-3845, dampens the release of corticosterone (CORT) and the hippocampal pro-inflammatory response including a decrease in inducible NOS following stress. This experiment highlights the importance of endocannabinoid signalling in regulation of the hypothalamic-pituitary-adrenal (HPA) stress axis and adaptive immunity.
6.1 Acute Psychological Stress and the Endocrine System

The experiments performed throughout this thesis have relied on the generation of a relatively strong stress response in order to induce sufficient cellular changes in the nitrergic system. To achieve this, we have utilised two well established models of psychological stress, immobilisation and restraint. Several indicators of endocrine activity were initially assessed in Chapter III to verify the stress models. Plasma prolactin has been used previously in our laboratory as a highly sensitive pituitary-derived hormone that exhibits exquisite stress responsiveness while remaining unresponsive to glucocorticoid-mediated negative feedback (Weiser et al., 2011). The immobilisation stress used in Chapter III was highly effective in producing increased plasma prolactin levels to similar, if not higher than those observed in our previous model of restraint stress (Spiers et al., 2013). Jaroenporn and colleagues (2009) have shown that prolactin acts as an adrenal sensitiser and facilitates the release of corticosterone. In this thesis, stress exposure reliably produced 2-3 fold increases in plasma corticosterone concentration across the time points used in these experiments. The intra-individual blood sample collection using the tail-tipping method in Chapter III produced consistently low corticosterone values for pre-stress and control samples that were well within the expected range for rats sampled in the first three hours of the dark cycle (Ulrich-Lai et al., 2006). Furthermore, peak corticosterone values were in good agreement with previous levels observed in our laboratory using jugular catheter sample collection (Spiers et al., 2013). Tail tipping was also used in Chapter V for collection of endpoint samples. Although basal levels were slightly higher than those observed in Chapter III and likely reflect the addition of the intraperitoneal PF-3845 injection, they were still well within the expected range for rats at the beginning of the dark cycle (Tab. 6.1).

Table 6.1. The effects of blood sampling method on the baseline/pre-stress corticosterone concentrations.

<table>
<thead>
<tr>
<th>Baseline/Control corticosterone concentrations</th>
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<tbody>
<tr>
<td><strong>Chapter III: Tail-tip sampling</strong></td>
<td>140.80 ± 41.57 ng/mL</td>
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<tr>
<td><strong>Chapter IV: Cardiac puncture</strong></td>
<td>288.6 ± 21.01 ng/mL</td>
</tr>
<tr>
<td><strong>Chapter V: Intraperitoneal injection prior to tail-tip sampling</strong></td>
<td>227.84 ± 36.60 ng/mL</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.
Expectedly, animals sampled in Chapter IV demonstrated the highest basal corticosterone levels. These samples were collected using the cardiac puncture sampling method following an intraperitoneal injection of sodium pentobarbital. Vahl and colleagues (2005) have shown that this method of anaesthesia/euthanasia produces the highest concentrations of corticosterone in blood collected from the trunk following decapitation. This is likely due to the close proximity of the adrenal venous vessels to the inferior vena cava and heart in comparison to the relative distance of the tail. However, as the cardiac puncture sampling method also requires loss of reflexive activity, the increase in time and physical stress associated with this sampling method undoubtedly plays a role in the increased levels of corticosterone observed in Chapter IV. In spite of this, concentrations of control samples collected were still in the acceptable range for this part of the light cycle (Ulrich-Lai et al., 2006; Spiers et al., 2013; Spiers et al., 2014b). This experimental chapter also included an assessment of hippocampal GR and MR which are known to be stress responsive. Interestingly, hippocampal GR demonstrated expected downregulation as a result of negative feedback in response to stress exposure while MR was not changed. A similar phenomenon was observed by Paskitti and colleagues (2000) who demonstrated that only the intronic mRNA of MR is responsive to acute stress while full length MR is unaffected. Interestingly, using the same model of restraint used in Chapter IV and V, we have recently demonstrated that both GR and MR are robustly downregulated following 6 hours of acute stress applied in the opposite part of the light cycle (Fig. 6.2A & 6.2B). This may be due to the relative low levels of corticosterone present prior to restraint allowing the MR to remain in the cytosol. In comparison, the high levels of corticosterone observed in the dark cycle would occupy the MR and cause nuclear translocation prior to application of the stressor, rendering the relative change due to stress negligible (Conway-Campbell et al., 2007).
Figure 6.2. The effect of acute restraint stress during the light cycle on (A) hippocampal glucocorticoid receptor (Nr3c1) and (B) hippocampal mineralocorticoid receptor (Nr3c2) mRNA expression in control and stressed rats (n=8 per group). The relative mRNA expression was determined in isolated hippocampal tissue from rats exposed to no stress (control), 1 day, 2 day, or 3 day of restraint stress (6 hours per day). Data are expressed as mean ± SEM, * p<0.05, ** p<0.01, *** p<0.001.
In Chapter V, the administration of PF-3845 produced a significant decrease in stress-induced corticosterone secretion at 60 minutes of stress exposure when tissue concentrations of hormone are expected to peak (Qian et al., 2011). This was anticipated due to the regulatory influence of increased cannabinoids on the activity of the HPA axis (Crowe et al., 2014). Furthermore, similar inhibitors of FAAH have demonstrated an inhibitory influence on HPA output (Patel et al., 2004). Although this effect was only present in the initial stress period measured, it was hypothesised that the decrease in peak HPA activity would effectively blunt the upregulation of inducible NOS in the hippocampus observed in Chapter IV.

Finally, stress was also shown to decrease plasma insulin levels in Chapter III. This decrease facilitates the catecholamine and glucocorticoid-mediated hyperglycaemia observed following stress exposure (Yamada et al., 1993; Amrani et al., 1994). This hyperglycaemic response was observed in Chapter III with an approximately 20% increase in circulating glucose concentrations occurring at 60 minutes of immobilisation stress exposure. Interestingly, this peak response diminished quickly in comparison to our previous observations using restraint stress (Spiers et al., 2013). Although the models are quite similar, there is a significant degree more struggling behaviour observed in immobilised animals in comparison to those restrained in wire mesh tubes. This behaviour likely facilitates the utilisation of glucose and contributes to the greater physical demand of immobilisation over restraint. These transient elevations in circulating glucose have been observed in previously reported immobilisation experiments on rodents (Sanchez et al., 2002).

6.2 Acute Psychological Stress and the Redox Response

One of the major functions of glucocorticoids is increasing cellular energy via the generation of ATP in the mitochondrial oxidative phosphorylation pathway (Teague et al., 2007; Du et al., 2009). The by-product ROS produced in this process can either: 1) be neutralised by the endogenous antioxidant system; 2) induce cellular compartmental oxidative stress; or 3) cross the cell membrane via passive diffusion (uncharged ROS such as hydrogen peroxide) or through specialised anion channels (charged molecules such as superoxide) (Reyes et al., 2012). The ROS traversing the cell membrane to the extracellular space are sequestered by cells and plasma of the circulation which have an enormous reducing capacity in comparison to most tissues (Richards et al., 1998; Valko et al., 2007). The circulation is so well equipped at neutralising ROS, it has been proposed that this is the major secondary function following gas/nutrient exchange (Richards et al., 1998). Markers of oxidative stress in peripheral blood have been observed in several neurodegenerative conditions such as Alzheimer’s, Amyotrophic Lateral Sclerosis, Huntington’s, and Parkinson’s diseases (McGrath et al., 2001; Sohmiya et al., 2004; Klepac et al., 2007; Babu et al., 2008). Chronic administration of corticosterone has also been shown to increase circulating oxidative stress
indicators (Sato et al., 2010). However, we have recently demonstrated that these indicators are responsive to acute stress, with 10-20% increases in oxidative state occurring within 2 hours of stress onset and continual increases in the immediate period following stress cessation (Spiers et al., 2013). Interestingly, the plasma oxidative state reported in Chapter III correlated very highly with the oxidative state reported in red blood cells. There are several distinct advantages to assaying plasma in place of erythrocytes: 1) plasma can be stored and assayed together to reduce inter-assay variability; 2) plasma has very little impact on fluorescence quenching in comparison to the intact membranes of erythrocytes; 3) plasma is easier to pipette and does not require normalisation to haematocrit or haemoglobin. However, care must be taken in sampling as lysed blood can give false positive high results due to the interaction between the DCFH dye and free iron released from damaged haemoglobin (Kalyanaraman et al., 2012). The 25% reduction in GSH/GSSG ratio observed using immobilisation stress in Chapter III is also in good agreement with our previous restraint model (Spiers et al., 2013). This was observed alongside a 10-15% increase in plasma lipid peroxidation, indicative of oxidative damage. As the time-course of this experiment was only two hours, this level of increase was quite surprising given that the increase in plasma lipid peroxidation in rats chronically stressed for 40 days only reaches 30% (Tagliari et al., 2010). However, this increase of approximately 15% within two hours of stress exposure is in good agreement with previous experiments using restraint stress within our laboratory (Spiers et al., 2014b).

6.3 Stress-Induced Activation of the Nitrergic System

6.3.1 Peripheral tissue

One of the major aims of this thesis was determining if acute psychological stress is capable of inducing a response in peripheral nitrergic markers. This was assessed in Chapter III using an established method measuring the NO metabolites, NOx, and an experimental method using a fluorescent probe quite specific for nitric oxide detection. Assay of plasma NOx from intra-individual samples indicated that stress is highly effective at inducing an increase in nitrergic system activity with nearly a 50% increase observed by two hours of stress exposure. Although the increase was more modest, the results seen in the experimental fluorescent method agreed very well with the increase seen in NO metabolites. Kojima and colleagues (1998) have demonstrated that this fluorescent probe exhibits a stable non-fluorescent profile against other RNS including peroxynitrite. The diacetate form is membrane permeable where, once inside the cell, it is subsequently cleaved by intracellular esterases to non-fluorescent DAF-FM which reacts specifically with nitric oxide to produce the highly fluorescent triazole, DAF-FM T. This fluorescent method offers a number of advantages over the measurement of NOx metabolites: 1) the direct interaction of the probe with sample NO simplifies the measurement procedure and removes
the requirement of utilising the enzyme nitrate reductase; 2) the time required for the fluorometric measurement is less than two hours compared to 6-7 hours for NOx determination; and 3) the cost is reduced as samples do not require ultrafiltration. However, the disadvantage of this system is the current lack of a suitable internal standard, rendering the output semi-quantitative relative to controls or baseline samples.

The results from these two measures clearly indicate that stress is capable of rapidly mobilising the nitrergic system. In the case of NOx metabolites, circulating levels can be influenced by dietary intake and metabolism of NO from the l-arginine-NO synthase pathway (Lundberg et al., 2008). However, as food and water are removed prior to experimentation, it is not likely that dietary NOx is heavily contributing to the increases observed. The next likeliest candidate would seem to be metabolism of NO produced from the endothelial isoform. However, using human and rat endothelial cells, Liu and colleagues (2009) demonstrated that the eNOS promotor region contains a suppressive GRE sensitive to 2-3 fold increases in glucocorticoid similar to those observed during a stress response. This decrease in eNOS expression is suggested to contribute to the hypertensive effect of increased glucocorticoids. Moreover, the increase in struggling behaviour observed in Chapter III may indicate increased muscular activity and hence, requirement for nitric oxide-induced vasodilation. Even with decreasing eNOS, this could be achieved directly by skeletal muscular tissue which creates a temporary ‘hypoxic’ state due to the increased oxygen demand. The skeletal muscle can derive NO independent of NOS activity by utilising deoxygenated myoglobin to reduce nitrate to NO, a process that can also occur in blood via haemoglobin, and plasma via xanthine oxidoreductase (Lundberg et al., 2008). However, this would deplete the local nitrite pool in favour of NO production. While this may contribute to the observed DAF-FM T formation, this would be in opposition to the increases observed in NOx. This indicates that the increases in plasma markers of DAF-FM T and NOx ultimately reflect an increased flux of NO metabolism through the l-arginine-NO synthase pathway, signalling activation of the nitrergic system. Furthermore, the observation of a generalised increase in peripheral nitrergic system activity following stress exposure was good evidence that this system is capable of responding to acute challenges and may utilise the circulation as a clearance mechanism. However, the large number of redox active enzymes present in circulation and the enormous NO quenching activity of haemoglobin would make it very difficult to ascertain the originating source of NO production. For example, caeruloplasmin, a major copper-carrying ferroxidase enzyme present in circulation under normoxic conditions acts as a NO oxidase/nitrite synthase by oxidizing NO. Conversely, a number of mechanisms, including reduction by xanthine oxidoreductase are capable of regenerating NO from nitrite. This complex and ubiquitous redox cycle limits the conclusions in terms of originating tissues that can be drawn from peripheral measures of NO.
6.3.2 Central tissue

Utilising the standard Griess NOx determination and a modified protocol of the fluorescent DAF-FM T assay, Chapter IV investigated if psychological stress of up to four hours could induce changes in these nitrosative markers in both hippocampal and striatal tissue. These two regions have previously been shown to exhibit a differential redox response in markers of oxidative damage (Liu et al., 1996). Furthermore, recent studies in our laboratory have shown that the hippocampus exhibits an initial induction of oxidative protection through upregulation of antioxidative enzyme, glutathione peroxidase 1 and 4, an effect not seen in the striatum (Fig. 6.3). This may be the result of the high expression of GR in the hippocampus in comparison to striatal tissue. Interestingly, the hippocampus demonstrated early changes in both DAF-FM T and NOx formation. However, NOx formation exhibited a progressive decline with extended stress exposure. This may indicate that increased NO formation in the hippocampus may initially require NOS activation for NO production and metabolism after which increased levels of NO may be maintained by the alternative NOS-independent pathway utilising nitrite as a substrate for NO production. However, examination of enzymatic activity of the Ca$^{2+}$-dependent and independent isoforms indicated that following a transient increase in Ca$^{2+}$-dependent activity, there was a steady increase in Ca$^{2+}$-independent activity indicative of iNOS activation. This was further supported at the mRNA level with sustained declines in hippocampal nNOS mRNA indicative of transcriptional negative feedback while hippocampal iNOS mRNA increased exponentially over the short time-course of the experiment. This rapid induction has been reported previously in cortical tissue and may indicate the recruitment and activation of microglia (Madrigal et al., 2002). Interestingly, in comparison to the hippocampus, striatal nitrergic activity demonstrated delayed activation with no change in NOx and slight elevations in DAF-FM T formation only appearing after four hours of stress exposure. Enzymatic activity in this region indicated that there was a slight insignificant increase in Ca$^{2+}$-dependent activity after stress, but the majority of the stress-induced nitrergic activation appeared to be driven by the inducible NOS. This was supported by increases in iNOS mRNA. Interestingly, both regions displayed robust increases in Nfkbia, one of the inhibitory subunits of NFKB. Bottero and colleagues (2003) demonstrated that monitoring the mRNA levels of this subunit directly correlated to NF-κB transactivation as this tightly regulated molecule is quickly recruited to inhibit activated NF-κB. This very useful observation allows the employment of relatively simple and clean PCR techniques in comparison to the more technical electrophoretic mobility shift assays that require use of antibodies and radioligands. However, the electrophoretic mobility shift assays was utilised by Madrigal and colleagues (2002) to demonstrate that the increase in cortical iNOS was highly dependent on transactivation of NF-κB signalling, and this also involved neuroinflammatory mediators such as TNF.
Figure 6.3. The effect of acute restraint stress on neural glutathione peroxidase 1 (Gpx1) and glutathione peroxidase 4 (Gpx4) mRNA levels in the hippocampus (A & B) and striatum (C & D) of control and stressed rats (n=6 per group). Hippocampal and striatal mRNA were determined in isolated neural tissue collected from animals exposed to 0 (control), 60, 120, and 240 minutes of restraint stress during the dark cycle. Data are expressed as mean ± SEM. Data were analysed using one-way ANOVA with Fisher’s least significant difference test comparing to unrestrained controls, *p<0.05.
6.4 Acute Psychological Stress and the Inflammatory Response

The observation of increased hippocampal iNOS expression in Chapter IV indicated that neuroinflammation may be playing an important role in stress responsiveness and recovery in this region. Therefore, the final chapter of this thesis examined if this indicator of nitrergic-mediated inflammation could be pharmacologically attenuated using novel modulators of the endocannabinoid system that have anti-inflammatory activity towards the iNOS system. This was chosen over modulators of HPA activity as these often exert differing effects on glucocorticoids that may result in off target effects. For example, as psychological stress does not induce a physical trauma and is routinely used to induce anxiety, the common anxiolytic compound diazepam was initially considered. However, Kalman and colleagues (1997) demonstrated that diazepam increases basal corticosterone markedly to peak stress-induced concentrations following intraperitoneal injection of 6 mg/kg. Furthermore, only this dose was effective at alleviating stress responsiveness to restraint. While still acting as a behavioural anxiolytic, this basal increase and inability to inhibit adrenal output would undoubtedly be sufficient to potentiate iNOS expression, perhaps even in the absence of restraint. The next consideration was the corticosterone synthesis inhibitor, metyrapone, a compound used in the diagnosis of adrenal insufficiency. However, the nature of this inhibition includes decreasing mineralocorticoid production while increasing upstream stress hormones ACTH and CRH via the reduction in negative feedback. This imbalance in HPA dynamics, particularly in the hippocampus, hypothalamus, and pituitary made this an undesirable option as the effect of increased central stress hormones does not wholly represent a reduction in HPA responsiveness. As the stress and inflammatory systems are intrinsically linked, with each playing a modulatory role on the other, a novel enzyme inhibitor, PF-3845, capable of increasing endogenous cannabinoids was selected. Endogenous endocannabinoid signalling has been shown to transiently reduce HPA output via direct feedback onto hypothalamic neurons (Steiner and Wotjak, 2008). In the present study, administration of this compound effectively blocked stress-induced hippocampal inflammatory indicators at the transcriptional including expression of IL-1β, IL-6, COX-2, and iNOS. There was also a decreased induction of IκB-α, indicating reduced NF-κB signalling. It is interesting to note that the anti-inflammatory effects of PF-3845 were most evident on IL-6 and TACE when compared to IL-1β, TNF, and COX-2 expression. In combination with the direct effects of increased endocannabinoids on hypothalamic neurons, the reduction in IL-6 may also contribute to the decrease in corticosterone observed following PF-3845 administration.
6.5 Future Directions

The results presented in this thesis have identified acute activation of the nitrergic system in response to psychological stress. While the aims of the experiments have been fairly rudimentary in nature, mechanistically, the mediators initiating this response have not been identified. Chapter III and IV presented some preliminary findings suggesting that glucocorticoids were involved in nitrergic activation. However, the next principle test in this line of experiments needs to pharmacologically validate the involvement of glucocorticoids using receptor blockade with RU-486/spironolactone, and corticosterone synthesis inhibition with metyrapone. This can allow a more confidant claim towards involvement of glucocorticoids in nitrergic activation. This will need to be further validated to ascertain that nitrergic activation is directly associated rather than acting through secondary/tertiary mechanisms that are not directly related to glucocorticoid signalling.

Conversely, as noted in Chapters IV and V above, a single acute psychological stress induces a rapid hippocampal pro-inflammatory response even in the presence of high concentrations of anti-inflammatory glucocorticoids. Moreover, the anti-inflammatory effects of PF-3845 were preferentially observed on IL-6 and IκB-α which are known to be resistant to glucocorticoid-induced suppression in comparison to other cytokines. However, given the relative energy-intensive requirements of cytokine production in general, the physiological rationale for negating glucocorticoid anti-inflammatory effects during an acute stress response where energy demand in other tissues is also high seems counter-intuitive. Furthermore, the oxidative and nitrosative stress placed on tissues immediately following acute stress exposure also cause generalised damage to lipid membranes, proteins, and DNA, which each require energy in the form of endogenous antioxidants and repair mechanisms to reinstate normal cellular function. Frank and colleagues (2013) suggested that an additional function of stress-induced glucocorticoids is to prime the immune system for subsequent activation as glucocorticoids decrease following peak pulse concentrations. While this may be based on specific increases in cytokine mRNA (not necessarily translated to protein) and alterations in the activation state of microglia, observed as hyper-ramified in post-stress surveillance, little is known of the functional purpose of increasing short-lived radicals. While these have traditionally been labelled as by-products of increased metabolism, Lundberg and colleagues (2008) proposed that preferential oxidation of NO, to nitrite in particular, creates a reservoir of NO accessible in the absence of oxygen required for synthesis. Interestingly, recent experiments in our laboratory have demonstrated that the initial hippocampal oxidative response to stress causes a sharp decrease in the reduced form of glutathione that coincides with increased oxidative status and lipid peroxidation. However, this effect rebounds quickly to restore reduced glutathione. This is an important observation as activation of nitric oxide from nitrite
requires an abundant cellular reducing agent in order to be an effective reservoir. In spite of this, although activation of the nitrergic system and creation of a NO reserve appears to be tethered to the pro-inflammatory immune mediators, the functional role of this response in an acute stress response has not been established. To address this, experiments using psychological stress coupled with a lipopolysaccharide-induced immune challenge should be performed to identify the protective or deleterious nature of acute stress-induced nitrergic activation. This should include an immunohistochemical component to identify preferential protection/destuction in neurons/gliai cells expressing the different NOS isoforms.

6.6 Conclusions
Psychological stress is a powerful causative factor in a myriad of chronic disease states. Detailed knowledge about the regulation of HPA axis activity during physiological conditions is important for the development of novel therapeutic strategies for the treatment of prolonged psychopathologies. While there is a considerable link between altered nitrergic activity and many prevalent neurodegenerative diseases, this thesis has established an acute profile of stress-induced peripheral nitrergic activation. Furthermore, the rapid involvement of nitrergic activity within the CNS following acute stress displayed regional specificity. This observed nitrergic activation within the brain following acute stress is predominately inflammatory-related while an enhanced endocannabinoid signalling has the capability of modulating these responses. Further understanding the role of the nitrergic system is important in identifying early players in the stress-induced aetiology of pathological conditions.
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Appendix
Appendix A

Figure A1. The effect of acute restraint stress on neuronal nitric oxide synthase (Nos1) mRNA expression in the medial prefrontal cortex (mPFC) of control and stressed rats (n=5-6 per group). The relative expression was determined in isolated medial prefrontal cortex collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, **p<0.01.
**Figure A2.** The effect of acute restraint stress on inducible nitric oxide synthase (Nos2) mRNA expression in the medial prefrontal cortex (mPFC) of control and stressed rats (n=5-6 per group). The relative expression was determined in isolated medial prefrontal cortex collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, **p<0.01.**
**Figure A3.** The effect of acute restraint stress on nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Nfkbia) mRNA expression in the medial prefrontal cortex (mPFC) of control and stressed rats (n=5-6 per group). The relative expression was determined in isolated medial prefrontal cortex collected from rats exposed to 0 (control), 60, 120, and 240 minutes of restraint stress. Data are expressed as mean ± SEM, **p<0.01.