Neurobiological alterations in the rat medial prefrontal cortex following exposure to chronic psychological stress

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Submitted January 2013 for the degree of Doctor of Philosophy (Anatomy)
DECLARATION

This thesis contains no material which has been accepted for the award of any other Degree or Diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Deputy Head of Faculty (Research and Research Training), attesting to my contribution to the joint publications.

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MADELEINE HINWOOD January 2013
ACKNOWLEDGEMENTS

Over the course of this PhD I have received support and assistance from a number of people, and it is a pleasure to have an opportunity to thank some of them now. Without the support of these people there would be no thesis.

Firstly, my principal supervisor Dr. Rohan Walker. You have furthered my interest in the study of neuroscience, and fostered a passion for learning new techniques. Whilst you have both inspired and challenged me to be the best scientist I could, you have also been a fantastic friend when needed. Thank you for your personal and professional guidance over the past six years. Looking forward to sharing more fun times (and wine, hopefully) in the future.

Secondly, my associate supervisor Professor Trevor Day. Whilst you have been a very busy man throughout the course of my candidature, you have been consistently guiding and assisting in the direction of my research. I will be satisfied if I am eventually but a fraction of the scientist and mentor to others that you have been to many young scientists.

Thirdly, the people who have worked in the lab over the past few years making this PhD interesting and enjoyable, many of whom have collaborated on projects with me. Firstly, Britt Saxby. We started in the lab within a few weeks of one another and your help and support with my work is greatly appreciated. Ross Tynan. Sometimes the only person in the world who can understand how it feels to be in your situation is a PhD student in the same lab. Thank you Ross for listening, and best wishes for your own work into the future. Janine Charnley and Sarah Beynon. Whilst joining the lab towards the end of my time
there, you have both provided excellent assistance, kindness and understanding when I was at my most fraught towards the end of the experimental work outlined in this thesis.

Fourthly, so many people in the School of Biomedical Sciences and Pharmacy. Among these, Chris Dayas and Doug Smith, for your guidance, kindness and understanding. Thank you. And Amanda Brown, thank you for all the help, wine and social events.

Finally, my wonderful family. I would have never made it to this stage of my career without your support. Thank you for bearing with me through the stressful times and supporting me throughout the course of this degree. Thank you Katy for helping with childcare so many times throughout my final year, when I struggled to find time for writing in between working and parenting. Thank you Nic for your help with the more technical aspects of formatting this finished work. Thank you to mum and dad for all your assistance and unwavering support. Thank you to my 2.5 wonderful children, Isabella, Oliver and Thing 3, who have all arrived (or are due to arrive shortly!) during the course of this degree. Whilst you haven’t made this experience any easier, you have certainly made it a hundred times better. Finally, my dear husband Rocky. Both your support and your removal from my career have kept me grounded and provided me with a serene plane of existence untouched by lab-based stresses! Thank you, my love.
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ABSTRACT

Persistent exposure to stressful events can produce serious disorders of cognitive function and mood state. Globally, depression affects between 5 and 20% of the world’s population, and represents a large burden of disease. Current treatments are not effective for all people with depression. Research efforts directed towards understanding the neurobiology of stress and depression are increasing in an attempt to better understand, treat, and possibly even prevent mood-related psychopathology. One of the first steps is understanding the response of the prefrontal cortex, part of the brain responsible for the control of stress and emotional responses, to chronic stress. With this in mind, the experiments in this thesis were aimed at elucidating cellular changes in the rat medial prefrontal cortex after exposure to chronic psychological stress.

The experiments described in Chapter Two aimed to examine the phenotype of chronically activated neurons in the forebrain following exposure to chronic stress. The medial prefrontal cortex was the only area examined to display a difference in levels of ∆FosB, a marker used for functional imaging of chronic neuronal activation. It was found that these cells were glutamatergic pyramidal projection neurons.

The results of Chapter Two, along with emerging evidence that stress activates the neuroimmune system (microglial cells), and that these cells appear to be able to alter neuronal connectivity, led us to investigate how exposure to chronic stress affects microglial activity in the medial prefrontal cortex in Chapter Three. It was found that chronic psychological stress increased microglial cell immunoreactivity, as well as local neuronal activity. In this study, stress also impaired performance on a working memory task, a cognitive function that is prefrontal cortex dependent. Administration of minocycline hydrochloride, a tetracycline antibiotic which is known to inhibit microglial activation,
reversed the effects of stress on microglial cells. Additionally, minocycline administration reduced the impact of stress on neuronal activation and working memory performance. This suggests that microglia mediate the effects of stress on prefrontal cortex neuronal function and prefrontal cortex dependent behaviour.

In Chapter Four, following on from the results of the previous two studies, we investigated how exposure to chronic stress alters microglial cell morphology. This is an important issue, as experience-dependent changes to microglial cells are only just starting to be elucidated, and form is closely related to function for these cells. Cells from animals exposed to chronic psychological stress were digitally reconstructed and analysed for morphological characteristics. It was found that microglial cells in the medial prefrontal cortex of animals exposed to chronic psychological stress had increased ramification (branching) without an increase in the overall size of the cell. This was associated with an increase in the structural protein β1-integrin, which has been implicated in microglial ramification. These effects were reversed in animals who were administered minocycline. Increased ramification of microglia may be the morphological representation through which microglia exert their effects following exposure to chronic psychological stress.

Overall, the experiments presented in this thesis have contributed to our knowledge of how neurons and microglial cells in the medial prefrontal cortex respond to chronic stress, and how some of the effects of stress may be mediated by a neuroinflammatory response evoked by microglia. These results form an important contribution to further understanding the neurobiology of the stress response, and may have implications for the future development of efficacious pharmacotherapies for stress-induced psychopathologies.
## LIST OF ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
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<td>IEG</td>
<td>Immediate-early gene</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>mPFC</td>
<td>Medial prefrontal cortex</td>
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<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
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<tr>
<td>IL</td>
<td>Infralimbic cortex</td>
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<td>PrL</td>
<td>Prelimbic cortex</td>
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<tr>
<td>ACd</td>
<td>Dorsal anterior cingulate cortex</td>
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<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
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<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
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<tr>
<td>Iba-1</td>
<td>Ionized calcium-binding adaptor protein-1</td>
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<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
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<td>SNRI</td>
<td>Serotonin-noradrenaline reuptake inhibitor</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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CHAPTER 1

Introduction
CHAPTER 1: LITERATURE REVIEW AND INTRODUCTION

Stress

The human body is equipped to deal with challenges on a moment by moment basis, from mild (such as hunger) to extreme (such as a life threatening injury). Stress is a term that is increasing in commonality of use, but is a difficult concept to define. However, there is a characteristic stress response comprising a set of physiological responses that has been identified and extensively researched in humans and animals. The physiologist Walter Cannon (1929) was the first to describe the physiological underpinnings of the stereotypical flight or fight response: where the heart pounds, pupils dilate, blood pressure increases, and digestion is slowed in response to a challenge. He showed that these responses involved the activation of the sympathetic nervous system, and used the term ‘homeostasis’ to refer to equilibrium, or a steady state maintained by the body. Cannon suggested that challenges to homeostasis were capable of eliciting the flight or fight response, and conversely defined a stressor as anything that challenged homeostasis. However, homeostasis is continually being challenged by internal and external stimuli, not all of which are stressful. In the 1930s, Hans Selye, often referred to as the father of stress research, refined this definition to include any threat to an organism that overwhelms usual homeostatic response mechanisms, requiring a non-selective response that engages the organisms cognitive and physiological resources to deal with the challenge (Selye 1980). Our overall response to stress results from the co-ordination of physiological, cognitive and behavioural systems in order to adapt to or overcome whatever threat is presented by the stressor. The response to an acute stressor is fast, reversible and often essential for survival, providing the organism with the necessary resources to overcome the challenge presented by the
stressor. However, it may cause adverse effects when the response is exaggerated, or the application of stress is sustained or repeated over long periods of time. It is well established that stress, whilst a ubiquitous part of the human experience, can lead to disturbances of mood and cognition and is a leading precursor to the development of psychiatric disorders (Staufenbiel et al. 2012).

What causes stress?

It appears that the brain distinguishes between different classes of stressors (stimuli which elicit the stress response), with the most widely used separation being two main categories: physical and psychological (Sawchenko et al. 2000; Dayas, Buller, Crane, et al. 2001). Physical stressors are those stimuli which involve a disturbance of an organism’s physical parameters, such as acute blood loss. Psychological stressors are those which the animal interprets as being potentially disruptive to the organism, such as social conflict. There are many studies showing that the brain is capable of distinguishing between these two classes of stressor, with brain regions activated by physical stress differing from those activated by psychological stress (Sawchenko et al. 2000; Dayas, Buller, Crane, et al. 2001; Dayas, Buller & Day 2001). In the context of human lives, psychological stress, such as unemployment, strain in personal or work relationships, and financial concerns, are all frequently experienced and those most likely to lead to the development of mood disorders. An analysis of trends in workers’ compensation claims showed that overall, claims in Australia decreased 13% between 1997/8 and 2003-04, with decreases occurring across all categories other than the category entitled mental stress (Australian Safety and Compensation Council 2007). Claims under mental stress increased by 83% in the same period. Mental stress under workers’ compensation includes stress caused by work
pressure, harassment, workplace violence, trauma and others. In 2003-04, claims due to mental stress had substantially greater median times lost from work than other claims (9.7 weeks for mental stress compared to 4.0 weeks for other new claims), and a median direct cost of $12 800, compared to a median cost of $5800 for all new claims.

The physiological response to stress

All mammals, from mice to men, exhibit a biologically similar response to stress. The central nervous system works in concert with the endocrine system to regulate the body’s stress related mechanisms, activating the flight or fight response (Kvetnansky et al. 2009). This response begins with the detection of a threat by the brain, and the subsequent rapid activation of the sympathetic nervous system which releases the catecholamines epinephrine and norepinephrine. The sympathetic nervous system redistributes oxygen and nutrients, redirecting blood flow to organs deemed essential for the stress response (such as the brain and major muscles). The activation of the sympathetic nervous system establishes a physiological state most capable of dealing with the demands placed upon the body by the stressor. This is followed by the somewhat slower activation of the hypothalamic-pituitary-adrenal (HPA) axis, where the hypothalamus secretes corticotrophin releasing hormone, which triggers release of adrenocorticotrophic hormone from the anterior pituitary into the bloodstream, which finally stimulates the release of glucocorticoids from the adrenal cortex (Turnbull & Rivier 1999). Glucocorticoids modulate biological activities in the minutes and hours following exposure to stress, and their actions are numerous and diverse (Yehuda et al. 1998). For example, one of the actions of glucocorticoids is to suppress the body’s immune system, which can lead to health problems when the stress response is exaggerated or sustained (McEwen 1998).
Stress neurocircuitry

The stress response begins with the generation of neuronal activity in response to the perception of threat by the brain. Attempts to elucidate the circuitry involved in the response to a stressor have largely focused on those brain regions directly involved in the control of the HPA axis and sympathetic nervous system, for, as previously described, these are responsible for producing the stereotypical fight or flight response to stress (Cullinan et al. 2008; Jankord & Herman 2008; Steiner & Wotjak 2008). In animal studies, the primary method used to image functional activity in the brain has been through the quantification of changes in the expression of immediate-early genes (IEGs) such as c-Fos (Deutch et al. 1991; Kollack-Walker et al. 1997; Chung et al. 2000; Dayas & Day 2002; Gerrits et al. 2003; Choi et al. 2008). Transcription of c-Fos is upregulated in response to many extracellular signals, and detection of this activity serves as an indirect marker of neuronal activity because c-Fos is often expressed when neurons fire action potentials (Dragunow & Faull 1989). Generally, two main approaches are used to quantify changes in IEGs. The first is detection of the protein product using immunohistochemistry, and the other is the detection of mRNA using in-situ hybridization. Immunohistochemical detection of Fos has proven to be a powerful tool for identifying activated neurons and extended circuits that affect neuroendocrine functions. Analysis of the pattern of c-Fos expression in the brain provides cellular resolution of neural activity that can be quantified by counting numbers of labelled cells in a particular brain region of interest (Day et al. 2008). The technique allows mapping of neuronal populations activated by a stimulus, as virtually the entire brain of a single animal can be visualised for analysis of distributed networks. Studies utilising this technique have shown that besides the hypothalamus and the medullary/brainstem nuclei (locus...
coeruleus, nucleus of the solitary tract, and the median and dorsal raphe nucleus), which are responsible for generating the autonomic and neuroendocrine responses to stress, several 'higher' cognitive regions of the brain involved in memory, emotion and decision making (executive function) are also activated following stress (Cullinan et al. 1995; Kollack-Walker et al. 1997; Gerrits et al. 2006).
Figure 1. Fos-like immunoreactivity in the rat prefrontal cortex. A, B, C, and D, show cingulate area 3; E and F, show the lateral orbital area; and G and H, show agranular retrosplenial cortex. Images shown in A, E, and G are from animals exposed to restraint stress, animal in B was treated with bacterial endotoxin lipopolysaccharide (LPS), animal in C was treated with saline and animals in D, F, and H were untreated controls. Scale bars = 200µm. Images from Yokoyama and Sasaki (1999).
There is a large database of c-Fos literature pertaining to regions of the brain that respond to psychological stress (Watanabe et al. 1994; Martinez et al. 1998; Kollack-Walker et al. 1999; Martinez et al. 2002; Nordquist et al. 2003; Spencer et al. 2004). Immunohistochemical studies utilising the expression of c-fos in order to measure neuronal activity after exposure to chronic psychological stress have consistently found the following limbic/forebrain regions responsive to stress: hippocampus, medial prefrontal cortex, anterior bed nucleus of the stria terminalis, medial, central and basolateral amygdala, the paraventricular nucleus of the hypothalamus, the ventral lateral septum, the periaqueductal grey and the ventral tegmental area (Martinez et al. 2002). Whilst these regions appear to be activated by stress, and many have been implicated in the control of the physiological responses to stress, it is now known that certain areas of the brain are modified anatomically following stress. That is, exposure to chronic stress can reshape the physical structure of the brain.

**Stress-induced structural changes within the brain**

Structural changes in the brain- referred to as neural plasticity- are a feature of the brain’s capability to adapt and respond to its surroundings. Elucidating the plastic changes that occur in response to stress is a current subject of much research, particularly because these changes may underlie a susceptibility to stress induced pathology. A substantial body of research has been devoted to understanding the neuroplasticity of the hippocampus, which plays an important role in inhibition of the HPA axis response to stress, and displays structural and functional impairments after exposure to chronic stress (or glucocorticoid administration). For example, the replacement of neurons in the dentate gyrus of the hippocampus is suppressed after exposure to chronic stress, and dendritic retraction occurs
in the CA3 region (McLaughlin et al. 2005; McLaughlin et al. 2007; Bessa et al. 2009). These effects are reversible, with structural alterations returning to baseline within weeks after the cessation of the stressor.

More recently, the medial prefrontal cortex (mPFC) has also become the subject of a similar line of investigation. Like the hippocampus, the mPFC possesses an abundance of glucocorticoid receptors, and plays a role in the negative feedback control of the HPA axis (Furay et al. 2008). The mPFC has been repeatedly shown to inhibit stress-induced HPA axis activity (Diorio et al. 1993; Sullivan & Dufresne 2006). More recently, a growing number of studies have demonstrated that exposure to chronic stress leads to a reduction in dendritic length, and a reduction in branching and spine density in the dendritic arbors of the pyramidal neurons of layers II/III (Radley et al. 2008). These changes are reversible after a recovery period. The functional significance of these changes is currently unknown; however there does appear to be a correlation between these structural modifications and dysfunction in mPFC-dependent cognitive tasks (Liston et al. 2006; Miracle et al. 2006; Dias-Ferreira et al. 2009). Furthermore, neuroimaging studies of depressed patients have shown significant reductions in grey matter density of the mPFC when compared to controls (Drevets et al. 2008). Collectively, these studies demonstrate that the mPFC is highly sensitive to the effects of chronic stress.
Figure 2. Chronic restraint stress causes dendritic remodelling in mPFC dendrites and spines.  (a) a layer II/III pyramidal mPFC neuron filled with Lucifer Yellow to allow for dendrite visualization. (b) and (c) dendrite segments from control and stress groups, demonstrating spine loss. (d) NeuronStudio software reconstruction of a dendritic segment, demonstrating spine size measurements. (e) and (f) 21 days restraint stress causes significant decrease in total apical dendrite length and in spine density. (g) Chronic restraint stress also causes a shift in spine size, with dendrites exhibiting a smaller percentage of large spines. * p < .05. Images from Shansky & Morrison (2009). Images adapted from Radley et al. (2004; 2006; 2008).
Stress-induced changes in cognition

There is accumulating evidence from preclinical studies that stress impacts negatively upon cognitive flexibility and working memory, both of which are executive functions and regulated by the prefrontal cortex (Holmes & Wellman 2009; McEwen & Gianaros 2011). Early studies in rats clearly demonstrated that uncontrollable stress induced significant working memory impairments (Minor et al. 1984; Diorio et al. 1993; Murphy et al. 1996). More recently, it has been shown that these impairments are due to structural alterations within the PFC (Radley et al. 2008; Holmes & Wellman 2009). These observations have now also been confirmed to occur in monkeys and humans exposed to persistently stressful situations (Arnsten & Goldman-Rakic 1998; Arnsten 2009). Specifically, neuroimaging studies of people experiencing severe mood disorders have revealed correlations between the size of reductions in grey matter density in the mPFC and the severity of cognitive and affective changes symptomatic of mental illness (Newport & Nemeroff 2000; Shin et al. 2005; Drevets, Savitz, et al. 2008). Given the association of the prefrontal cortex with executive function, and the impairment of these functions with exposure to stress, an important question is to identify how chronic stress modifies the prefrontal cortex.

The Medial Prefrontal Cortex (mPFC)

Anatomy

The prefrontal cortex in the rodent can be grossly divided into two areas: the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (Uylings et al. 2003).
prefrontal cortex is located over the medial surface of the cerebral hemispheres. It is generally subdivided into three cytoarchitecturally distinct subregions, namely the infralimbic cortex (IL), the prelimbic cortex (PrL), and the dorsal anterior cingulate cortex (ACd) (Ongur & Price 2000). Of these subregions, it is the IL and PL which have been implicated in the response to stress, with the ACd involved in motor control (Morgan & LeDoux 1995). As such, the IL and PrL have been the main focus of the work presented in this thesis. The IL and PL are agranular, that is, lacking a distinct layer IV. The mPFC contains two neuronal subtypes (Homayoun & Moghaddam 2007). Whilst layer I is largely devoid of neurons, layer II/III predominantly possess glutamatergic pyramidal projection neurons which largely project to other regions of the PFC, and those in layer V/VI possess reciprocal connections with subcortical regions. Layers II-VI possess chiefly γ-aminobutyric acid (GABA)-ergic local circuit inhibitory neurons.
Figure 3. Low magnification light micrographs showing the location of the (A) infralimbic cortex; (B) prelimbic cortex; (C) anterior agranular insular cortex; and (D) posterior agranular insular cortex injected with anterograde tracer biotinylated dextran amine into neuron-containing layers 2-5. The line diagram insets in these pictures are taken from the stereotaxic atlas of Paxinos and Watson and show the position of the cortices, with the distance from bregma for each section. From Gabbott et al. (2003).
Connectivity

The mPFC is anatomically well linked with the limbic system, and other regions of the prefrontal cortex, underscoring its role in visceral and cognitive emotional functions (Gabbott et al. 2005). Whilst they share some similarities in connectivity and function, it is largely the case that the IL and PrL project differentially throughout the brain (Vertes 2004). The main projection sites of the IL are the lateral septum, the bed nucleus of the stria terminalis (BNST), the central and medial amygdala, several subregions of the hypothalamus, and the parabrachial and solitary nuclei of the brainstem. The main projection sites of the PrL includes the insular cortex, nucleus accumbens, olfactory tubercle, various subregions of the thalamus, the basolateral amygdala and the dorsal and median raphe nuclei of the brainstem. Note that many of these areas are those shown to increase neuronal activity after exposure to stress, which, when taken into account with the ability of the mPFC to inhibit the glucocorticoid stress response, leaves the mPFC well-placed to exert control over the physiological response to stress (Cook & Wellman 2004). Functionally, the mPFC provides a line of communication between the limbic system and higher cortical structures (Gabbott et al. 2007). It plays a role in higher order functions including learning, memory, task sequencing, decision making and goal-directed behaviour. The mPFC also coordinates behaviour requiring working memory, and inhibits inappropriate responses via the integration of higher cognitive and emotionally relevant information relayed via the limbic system.
Investigating the response of the mPFC to chronic stress.

The results of the aforementioned studies suggest that exposure to stress appears to compromise the structure and function of the mPFC. Studies utilising the expression of the immediate early gene c-fos to assess neuronal activation in response to an acute episode of stress have consistently implicated several brain regions in responding to stress (Chan et al. 1993; Cullinan et al. 1995; Morinobu et al. 1995). However, the human experience of psychological stress that is more likely to lead to the development of psychopathology is chronic or ongoing, such as persistent workplace conflict or financial insecurity. Accordingly, there has been some interest in elucidating those brain regions activated in response to chronic stress. However this has, from an experimental perspective, not been straightforward, as chronic stressors are known to elicit regionally specific desensitization of c-fos production (Watanabe et al. 1994). This phenomenon has resulted in the development of alternate methods to identify those neuronal populations involved in responding to and regulating chronic stress.

Over the past decade many studies investigating the neuronal response to repeated exposure to similar events have utilised the expression of ΔFosB, a protein product of the fosB gene which appears to accumulate within repeatedly activated neurons (Chen et al. 1997; McClung et al. 2004; Zachariou et al. 2006; Berton et al. 2007; Winstanley et al. 2007). Induction of ΔFosB (35-37 kDa isoform) occurs in a stimulus dependent manner, and studies of transgenic mice lacking ΔFosB have shown that the accumulation ΔFosB in certain brain regions after stress promotes active coping responses and increases resilience to the behavioural effects of stress (Berton et al. 2007). Its great degree of stability and high levels of transcriptional activity have led researchers to postulate that the accumulation of

Whilst \(\Delta FosB\) has been examined for its functional roles, it is also commonly used as a marker of repeated neuronal activation. The protein product of the \textit{fosB} gene, \(\Delta FosB\), is a transcription factor which accumulates and persists in the brain in response to chronic stimulation. Many studies have shown that chronic administration of several types of drugs of abuse, such as cocaine, amphetamine, morphine, nicotine and methamphetamine, induce \(\Delta FosB\) particularly within the nucleus accumbens and dorsal striatum, with lower levels variously reported in prefrontal cortex, amygdala, ventral tegmental area and hippocampus (Liu \textit{et al} 2007, McDaid \textit{et al} 2006a2006b, Nye \textit{et al} 1996, Perrotti \textit{et al.} 2005, 2008). Induction of \(\Delta FosB\) has also been observed in the striatum after chronic exposure to natural rewards, such as wheel running and food intake (Werme 2003, Olausson 2006) (Werme, Lindholm, \textit{et al.} 2002; Werme, Messer, \textit{et al.} 2002; Olausson \textit{et al.} 2006). Accordingly, in these studies we have used \(\Delta FosB\) as a marker of neurons repeatedly activated by exposure to chronic stress.
Figure 4. Illustration showing the gradual accumulation of ΔFosB versus the rapid and transient induction of other Fos family proteins. Top graph shows several waves of Fos family proteins (including c-Fos, FosB, ΔFosB (33-kDa isoform), Fra-1 and Fra-2) are induced in a region specific manner within the brain by acute stimulation (for example, stress or drug administration). Also induced are isoforms of ΔFosB (35-37kDa) at low levels.
after an acute stimulus, but are extremely stable and appear to accumulate and persist in region of the brain with continued stimulation. Bottom graph shows that with repeated stimulation, each administration of the stimulus induces a low level of the stable ΔFosB isoforms, indicated by a set of overlapping lines. The result is a gradual increase in certain neuronal populations of the total levels of ΔFosB with repeated stimulation. The gel inset shows a sample of this phenomenon, with the accumulation of various Fos family proteins within brain after chronic or acute exposure to cocaine. Images from McClung et al. (2004).
**Stress and the inflammatory response**

Inflammation is a complex multicellular response of vascular tissue, which usually occurs with disruption of tissue, or exposure to pathogens or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and restore function (Ferrero-Miliani et al. 2007). The inflammatory response in the periphery involves the generation and release of cell derived mediators from leukocytes, including pro-inflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-α (TNF-α). It has been well reported that high stress levels elevate peripheral levels of pro-inflammatory cytokines in humans and in animal studies (O’Connor et al. 2000; Brydon et al. 2005). In animal studies, this so called ‘stress priming’ effect on the immune system has shown that exposure to acute psychological stress prior to an inflammatory challenge can enhance immune function (Maier 2003). For example, mice exposed to acute stress prior to cutaneous application of 2,4-dinitro-1-flurobenzene display a significantly enhanced delayed type hypersensitivity response (Dhabhar et al. 2000). This effect appears to be mediated by glucocorticoid (specifically, corticosterone) induced changes in leukocyte trafficking. Similarly, acute psychological stress appears to enhance immunoglobulin production by B-lymphocytes (Padgett & Glaser 2003).

**Microglial cell responses to stress**

In the periphery, the inflammatory response is handled by a variety of innate immune cells. However, inflammation within the central nervous system is tightly controlled and chiefly handled by neural tissue support cells known as microglia. Microglia are differentiated from
monocytes, and share some of the characteristics of these cells. Whilst considerable effort has been directed into understanding the neuronal alterations that occur in response to acute and chronic stress, much less is known about glial cell responses. Once considered to merely be a support cell for neurons, the role of glial cells in neuroplastic changes associated with exposure to stress, pain or depression is now emerging (Blandino et al. 2006; Trang et al. 2006; Krishnan & Nestler 2008; Eyre & Baune 2012). The known functions of microglia are expanding beyond a purely immunogenic role, with a recent body of work has shown that resting microglia are involved in non-pathological monitoring of dendritic spines, at which over 90% of excitatory synapses occur (Wake et al. 2009; Tremblay 2011). The role that microglia play in potentially modifying both neuronal connectivity and neuroinflammation may be important in stress induced physiological changes in neuronal circuitry. Along with many neuronal subtypes, microglia also possess glucocorticoid receptors. The ability of microglia to modulate neuronal activity and respond to glucocorticoids places them in an ideal position to respond to stress in a way that also modulates neuronal responses.

**Quiescent microglia**

Microglia are of monocyte/macrophage lineage and are the resident cells of the innate immune system in the central nervous system (Tremblay et al. 2011). They play a key role in mediating the response to tissue injury and are activated in a range of pathological conditions, including Alzheimer’s and Parkinson’s diseases (Kreutzberg 1996; Imamura et al. 2003; Streit 2004). Microglial cells in the healthy adult CNS display small nuclei and a branched or ramified morphology. This has been termed their ‘resting’ phenotype, although several recent studies utilising in vivo imaging techniques have shown that in this state,
microglial processes are highly mobile (Nimmerjahn et al. 2005; Wake et al. 2009). This action allows continual sampling of their microenvironment for changes, with some estimates suggesting that the entire parenchyma could be sampled within a three hour period. This dynamic surveillance suggests a sentinel-like role for microglia under normal or resting conditions, as it involves constant movement of the cellular processes and presumably biochemical sensing for any disturbance. Part of the movement of the processes appears to be directed towards monitoring of synapses, exemplifying the potential of microglia to influence information processing in the CNS by modulating neuronal activity.

**The stereotyped microglial response to injury or infection.**

Unlike other cell types in the CNS, microglia are derived from mononuclear phagocytes and are capable of rapid activation in response to pathological changes within the CNS. Microglial activation has traditionally been conceptualised as a stepwise sequence of stereotyped transformations, with a tight coupling existing between microglial morphology and function (Ladeby et al. 2005; Schwartz et al. 2006). In response to an immune challenge or tissue damage, microglia transition from the quiescent or surveillance mode described above and acquire a more reactive profile in order to provide an appropriate response. Following exposure to an ‘activating stimulus’, such as a danger signal posed by a neuron or an astrocyte (pro-inflammatory cytokines or free radicals typically associated with tissue injury), microglia change both morphological and immunophenotypic state. Ramified microglia as described above transition initially into a reactive phenotype, characterised by a shortening and thickening of microglial processes. Activated microglia develop a large cell body with filopodia, enabling movement through the neural tissue. They
are capable of proliferating and migrating to the site of brain injury where, depending upon the extent of the injury, they further transition into phagocytic or amoeboid microglia and become highly phagocytic towards dying neurons or foreign particles. This form is typically defined by the absence of any cellular processes, however recent evidence suggests that in vivo, amoeboid microglia may possess a modified single cellular process to facilitate cellular mobility (Graeber 2010). The effects of microglial activation appear to be a dual phenomenon, with both neuroprotective and neurodegenerative effects described. Whilst the cells are capable of a diverse range of response phenotypes, their response to injury and infection is the best characterised to date, where microglia assume the phagocytic functional phenotype described here.
Figure 5. Morphological remodelling of microglia in response to pathological (classic) and non-pathological signals. Blue Box: Typically ramified quiescent microglial cell within the grey matter of the adult rat brain. Green Box: It is thought that morphological alterations in response to non-pathological stimuli (experience-dependent modifications) can be quite diverse. Fontainhas et al. (2011) recently described hyper-ramification of microglia after ionotropic glutamatergic neurotransmission led to increased microglial process length and branching. Tremblay et al. (2010) have further demonstrated that marked changes in the level of light can induce microglial processes to make more frequent contacts with synapses within the visual cortex, a finding that suggests that hyper-ramified microglia possess the ability to reorientate processes in response to changes in neuronal activity. Red Box: Morphological alterations in response to injury or inflammation have been extensively described and appear to be highly stereotyped (Davis et al. 1994; Streit et al. 1999; Graeber & Streit 2010; Kettenmann et al. 2011). It appears that the initial response of microglia to injury appears to be rapid process extension and reorientation towards the site
of injury (Davalos et al. 2005; Nimmerjahn et al. 2005). Following this, evidence suggests that reactive microglia then transition into phagocytic, amoeboid cells. Stence et al. (2001) suggested that this cell type can be further differentiated into transitional (T-stage), motile (M-stage) and locomotor (L-stage) microglia. One open question with respect to changes in microglial morphology in response to injury is the degree to which these signals promote hyper-ramification. Several groups have previously described hyper-ramification occurring in injury-based models (Wilson & Molliver 1994; Streit et al. 1999) but in one of the only quantitative studies to date, Stence et al. (2001) found no evidence of microglial hyper-ramification occurring in response to slice preparation as an injury model. Image from Beynon and Walker (2012).
**Experience-dependent modifications to microglia.**

The strictly limited concept of microglial cell activation being restricted to the stereotyped sequence of events described above is changing. The original concept was founded upon experimental challenges of microglia using facial nerve axotomy models in animals, historically the experimental paradigm used to investigate microglial function (Graeber, Streit, et al. 1988; Graeber, Tetzlaff, et al. 1988; Kreutzberg 1996). According to this view, microglia would respond to varying stimuli with a uniform profile of singular functions, the classic microglial cell response described previously. This conceptualisation leaves little room for adjustments according to the situational context or feedback regulation within the tissue, particularly where experience-dependent modifications in the absence of any apparent injury are concerned. It also cannot provide a satisfying explanation of the dual phenomenon of protective versus destructive functions of microglia, if the end point of activation is always the same. Activation of microglia is likely to be an adaptive process, with the cells transforming into different phenotypes depending upon the activating stimulus (van Rossum & Hanisch 2004). Much of the evidence for such diversity comes from studies of peripheral macrophages, which acquire distinct reactive phenotypes depending upon which signal they have been exposed to. It is known that microglial morphology is intimately linked with their function, so investigating morphological alterations occurring in response to stress will possibly lead to new understandings about their role in the stress response and associated pathology.

Whilst it is well documented that exposure to chronic stress produces various morphological and physiological changes in dendrites, dendritic spines and synaptic density in the mPFC, the concept of functional plasticity is less well studied in microglia. However, it is possible
that activated microglia may contribute to neuroplastic changes through several of their known functions, including release of proinflammatory cytokines, synaptic remodelling, interactions with excitatory neurotransmission and phagocytosis of neurons and cellular debris.

**Microglia are capable of modifying neuronal activity**

Microglia are responsive to several stress related neurotransmitters, including adrenaline, noradrenaline, dopamine and glutamate (Noda et al. 2000; Farber et al. 2005). They have the ability to sense synaptic release, which allows for communications between neurons and microglia. The finding that resting or quiescent microglia are extremely dynamic, continually extending and retracting their motile processes and interacting with axon terminals and dendritic spines, has led to a further set of studies suggesting a role for microglia in neural circuit function and synaptic plasticity. As an amoeboid phagocyte in the developing CNS, microglial cells participate in the shaping of neuronal circuitry by removal of aberrant cells and synaptic connections, as the developing brain creates far more synapses than are eventually maintained in the adult brain (Paolicelli et al. 2011). The microglial phagocytosis of extra synapses later in life appears to be activity-dependent, however currently the cellular mechanisms by which this occurs have not yet been elucidated (Schafer et al. 2012). Recent research has suggested that the complement system may be involved in tagging synapses for elimination during development (Stevens et al. 2007). The complement protein C3 is enriched at some developing synapses, for which the phagocytic complement receptor is expressed on the surface of microglia (Tremblay et al. 2011). The complement cascade has been shown to be essential for normal developmental elimination of synapses (Schwartz et al. 2006). These findings suggest that, in a similar way to which
the peripheral immune system utilises C3 as a tag for cells or foreign bodies which are to be eliminated by phagocytic macrophages, C3 may tag extraneous synapses for elimination by microglia via C3-mediated phagocytosis.

**Microglia are emerging as a promising target of stress**

Preclinical studies have shown that stress elevates the levels of pro-inflammatory cytokines within the periphery and the brain (O’Connor et al. 2003). Exposure to pro-inflammatory cytokines, such as interleukin-1β (IL-1β) has been shown to induce an inflammatory response in microglia, promoting the activation and proliferation of these cells (Frank et al. 2007; Sugama et al. 2007). This appears to cause a further increase in the release of pro-inflammatory cytokines such TNFα and IL-1β in both in vitro and in vivo models. The link between stress and inflammation was described in a study demonstrating that exposure to a single episode of inescapable shock induced production of IL-1β (O’Connor et al. 2003). Since this initial finding, it has been shown that pre-treatment with minocycline attenuates the increase in IL-1β (Blandino et al. 2006). As minocycline is an inhibitor of microglial cell activity (Tikka et al. 2001), this suggests that microglia play a role in mediating the neuroinflammatory effects of stress.

Following from investigations of central elevations of pro-inflammatory cytokines resulting from exposure to stress, there have been several investigations of the microglial response to stress using immunohistochemistry (Nair & Bonneau 2006; Sugama et al. 2007; Tynan et al. 2010). The primary antibodies most commonly used to detect microglia are those raised against the proteins CD11b, and ionized calcium-binding adaptor protein-1 (Iba-1). CD11b is a β-integrin surface marker of microglia which increases during neurodegenerative
inflammation (Roy et al. 2006). The increase in CD11b expression appears to correspond with the severity of microglial activation (He et al. 2001; Roy et al. 2006). Iba-1 appears to be a specific marker of microglia, which has never been observed in astrocytes, oligodendrocytes or neurons (Imai et al. 2007). Iba-1 co-localises with actin filaments, modulating membrane ruffling. Using these markers, a growing number of studies have used immunohistochemical methods to reveal that stress exerts an effect on microglial activation state, since changes in microglial proliferation, gene expression profile and morphology have all been observed following exposure to stress (Nair & Bonneau 2006; Frank et al. 2007; Sugama et al. 2007; Blandino et al. 2009). A series of recent studies has shown that exposure to various forms of acute stress promotes the transition of microglia from the quiescent to an activated state (Sugama et al. 2007; Sugama 2009; Sugama et al. 2009; Sugama et al. 2012). The first study to examine microglial changes after chronic stress showed that in rats, repeated restraint stress increases the density of Iba-1 immunoreactivity in several stress-responsive brain regions including mPFC, nucleus accumbens, BNST, medial amygdala, hippocampus and the periaqueductal grey (Tynan et al. 2010). Collectively, these findings suggest that the activation of microglia may play an important role in controlling the stress response and/or adapting to stress.

Whilst it is apparent that microglia respond to stress, the exact nature of this response is currently not defined. It has recently been shown that stress-induced microglial activation is not accompanied by the neurotoxic factors (IL-6 and nitric oxide synthase) produced as part of their response to injury (Sugama et al. 2007). These findings indicate that microglial activation during stress differs qualitatively from activation resulting from exposure to injury or infection. Further, a critical question for researchers in the field has been whether the pro-inflammatory cytokines seen within the brain after exposure to stress are generated
there or trafficked in from the periphery. It was initially assumed that pro-inflammatory cytokines generated in the periphery were too large to cross the blood-brain barrier, a vascular-endothelial cell barrier that restricts the diffusion of large molecules and bacteria from the circulation into the cerebrospinal fluid, whilst allowing small molecules such as oxygen and hormones through. Thus, it was thought that pro-inflammatory cytokines could only gain entry to the CNS via the circumventricular organs, sections of the blood-brain barrier where the endothelial cell lining is significantly more permeable (Maier et al. 1998; Roth et al. 2004). Due to the limitations of diffusion, this would suggest that pro-inflammatory cytokines generated within the periphery would have to be located close to the circumventricular organs. However, research evidence at the time suggested that peripherally generated cytokines appeared to influence sites deep within the brain, distant from the circumventricular organs (Munoz-Fernandez & Fresno 1998; Szelenyi 2001). Since then, a series of studies has shown that peripheral cytokines (including TNFα and IL-1β) can gain entry to the CNS via active transport mechanisms (Banks et al. 1989). This suggests that manipulating pro-inflammatory cytokines in the periphery can have central effects.

**Neuroinflammation modulated by microglia**

When microglia detect ‘danger’ signals, such as pro-inflammatory cytokines they become activated and produce a number of pro-inflammatory and cytotoxic molecules, which drive a neuroinflammatory response. This neuroinflammation is the brain’s innate immune response and if usually beneficial in the short term (Walter & Neumann 2009; Napoli & Neumann 2010), but if chronically engaged may be maladaptive, disturbing neuronal cell function and possibly cell damage due to the release of cytotoxic free radicals (Block et al.
It is important to note here that activated microglia release a number of substances that can modulate neuronal signalling (Hansson & Ronnback 2003).

In addition to the microglial response to peripheral pro-inflammatory cytokines, stress is capable of triggering neuroinflammation through a variety of other mechanisms. Stress can trigger the release of pro-inflammatory cytokines directly from microglia (Blandino et al. 2006). It is also recognised that corticosterone, a glucocorticoid hormone that is readily diffused across the blood-brain barrier and elevated during the stress response, can act directly to cause microglial activation and proliferation (Sorrells & Sapolsky 2007; Sorrells et al. 2009). Circulating pro-inflammatory cytokines can also bind to receptors within the blood-brain barrier, triggering the central release of pro-inflammatory molecules (Schiltz & Sawchenko 2002; Konsman et al. 2004).

Neuroinflammation is increasingly being implicated in some cases of major depression. Patients with major depressive disorder frequently display markedly increased levels of peripheral and central inflammatory markers. It has also been shown that antidepressants possess anti-neuroinflammatory properties. It has recently become apparent that the most common pharmacological treatments for depression, the selective serotonin and serotonin norepinehrine reuptake inhibitors (SSRI; SNRI) exert anti-inflammatory effects on microglia. A recent study (Tynan et al. 2012) found that SSRIs were able to suppress microglial responses (production of TNF-α and NO) to an inflammatory stimulus (LPS) in vitro. This finding suggests that some of the therapeutic action of antidepressants may be due to their anti-inflammatory properties within the CNS.
Figure 6. The effects of increasing concentrations of antidepressants on 10ng/mL of LPS-induced BV-2 production of TNF-α (○) and NO (●) after 24h of co-incubation (FLX - fluoxetine; SERT – sertraline; PARO - paroxetine; FLV – fluvoxamine; CIT – citalopram; VEN – venlafaxine). TNF-α production was determined using ELISA, and levels of NO were evaluated using the Griess reagent assay. The dashed line represents where viability was compromised according to the PrestoBlue test. Data are represented as mean (±SEM) percentage of TNF-α and NO levels relative to LPS + vehicle treated cells (assigned a value of 100%). From Tynan et al. (2012).
Determining the effects of stress on microglia function may assist in understanding stress-induced neuroinflammation. Emerging studies using animal models of chronic stress and neurological disorders have shown that antidepressants inhibit the expression of inflammatory mediators (such as pro-inflammatory cytokines) and microgliosis, and conversely that anti-inflammatories can have antidepressant effects (Roumestan et al. 2007; Hashioka et al. 2009; Miller et al. 2009). Neuroinflammation represents a potential underlying cause of psychopathology and a possible new target mechanism for development of antidepressants.

*A dual role for microglial cells*

Microglial activation is observed in many neurological conditions, as well as in response to stress, injury or inflammation. Thus these cells have been implicated in contributing to tissue injury or disease processes, particularly as under certain experimental conditions activated microglia can cause neuronal damage and cell death (Chao et al. 1992; Kreutzberg 1996). However it is unlikely that microglia evolved to be a risk factor in the brain, capable only of causing damage. It is more likely that most microglial activation is transient and successfully resolved without our ever being aware of it. It has been shown that under many conditions microglia actually exert neuroprotective effects, particularly if they are resolving an injury or infection (Streit 2002). However, detrimental consequences of activated microglia do arise, if their activation is exaggerated or sustained for long periods. Further research needs to be directed into characterising the various activation states of microglia, and how these can be either protective or detrimental to neural tissue.
Issues associated with the measurement of microglial activation

The most commonly used method of measuring microglial activation has been the thresholding approach (Sugama et al. 2007; Romero-Sandoval et al. 2008; Shapiro et al. 2008; Sugama et al. 2009). This involves imaging microglial cells which have typically been immunolabelled with either Iba-1 or CD11b, as discussed previously. These images are then converted into a binary image, with pixel intensities above a defined set-point changed to black. Whilst this procedure offers many advantages, such as being sensitive to global changes in labelling, being an objective technique, and can be undertaken relatively quickly, it does present several issues. For the approach to be valid, all tissue sections must be processed simultaneously, and imaged under identical conditions. Variability in the immunolabelling or imaging can confound the results of the experiment. Additionally, in most studies where a difference in the overall level of thresholded material is observed using this technique, it is described as reflecting microglial activation (Burguillos et al. 2011). However, this technique does not provide evidence as to what type of microglial activation is occurring. It is impossible to know whether the increase in the intensity of thresholded material is due to a difference in the length, thickness, or hyper-ramification of microglial processes. Given the multi-faceted response patterns of which microglia are now known to be capable of, this distinction is an important one to make. Differing morphological transitions have been described, from the well-defined response to injury to the seemingly non-pathological adaptation to stimulation.
Figure 7. This panel of images demonstrates common problems associated with standard thresholding-based assessment of microglia within the rat brain. (A) Photomicrograph showing a typical field of microglial cells from the rat mPFC (20x objective) that have been labelled using Iba-1 and imaged at a resolution of 2040 x 2040 pixels. (D) The same image subjected to a standard thresholding routine, with thresholded (presumably representing immunoreactivity) material shown in white overlay. (B) 7x digital magnification of (A) detail, (E) and (F) show 7x digital magnifications of the thresholding shown in (D). Note that the more distal processes of microglia shown in (E) were not detected using the standard thresholding procedure. This occurs as lowering the threshold value further results in a large increase in the amount of nonspecific background labelling included in the analysis (F). These limitations can be overcome by collecting a z-stack of images through the thickness of a section. (C) Minimum intensity projection image representing a stack of images taken through the z-axis of the image shown in (A). (G) The same thresholding routine shown in panels (B) and (E), upon the photomicrograph shown in (C). In comparison to thresholding performed at a single plane on the z-axis, thresholding on a minimum intensity projection more accurately captures the entire length of cell processes by
RATIONALE

The overall purpose of these studies is to ascertain chronic stress induced modifications to neurons and microglial cells of the medial prefrontal cortex. Further investigation into these changes may help increase our understanding of the neurobiological changes that occur as a result of exposure to chronic psychological stress and consequently how these may contribute to stress-induced psychopathology such as depression.

The mPFC has frequently been observed to be altered by, and respond to, stress, and is also a key brain region frequently implicated in our limited understanding of the pathophysiology of depression. Whilst a number of approaches are used to identify changes in the brain that occur as a result of exposure to stress, immunohistochemistry provides a method for localising specific proteins within CNS tissue, whilst retaining morphological features.

The emerging inflammatory hypothesis of depression, based on initial observations by Smith (1991), refers to a complex neuroimmune response coordinated primarily by microglia. As previously described, there is extensive evidence in human populations showing that chronic stress induces a pro-inflammatory state, and recent animal studies have also shown that chronic psychological stress promotes neuroinflammation characterised by microglial activation. Human studies have also linked immune activation to symptoms of mood disorders. Concurrent investigation of neuronal and microglial cell responses to chronic psychological stress, as well as pharmacological blockade of inflammatory microglial cell responses, may provide some insight into the mechanisms by which exposure to chronic stress leads to the development of psychopathology.
EXPERIMENTAL AIMS AND HYPOTHESES

The overall aim of the studies undertaken within this thesis is to characterise neuronal and microglial cell responses in the rat mPFC after exposure to chronic psychological stress. This work may provide a neurobiological basis for some of the behavioural changes that result from exposure to chronic stress, with particular emphasis on stress-related psychopathology.

Experiment 1. Repeated social defeat selectively increases ∆FosB expression and histone H3 acetylation in the infralimbic medial prefrontal cortex.

This study identified regions of the brain where neurons were repeatedly activated with exposure to chronic social defeat stress, a psychological stressor. A social defeat model was used, whereby naïve intruder rats are introduced into the home cage of older resident males, trained to attack and defeat all such intruders. A sham stress control group was used for comparison, where intruder animals were treated identically to the experimental group except that the resident was absent. This allowed control animals to be exposed to the same olfactory and visual environment as animals exposed to stress, as presumably this experience would also create neural activity. We used ∆FosB to assess levels of neuronal activation after 12 episodes of social defeat stress. ∆FosB-positive cells were characterised according to their neurotransmitter phenotype, and we examined stress-responsive region for evidence of chromatin remodelling, an experience-dependent modification that occurs within activated neurons.

It was hypothesised that:
a) Exposure to repeated social defeat would increase levels of ∆FosB immunoreactivity in several stress-responsive brain regions when compared to naïve non-handled controls, however less of these regions would display increased levels of ∆FosB when compared to the sham stress controls;

b) In line with previous studies evaluating the neurotransmitter phenotype of the stress responsive ∆FosB-positive cells in the mPFC, that these consistently activated neurons would be glutamatergic pyramidal projection neurons, located mainly in cortical layers II/III; and

c) That exposure to repeated social defeat stress will increase levels of histone acetylation in the cells of the mPFC.
Experiment 2. Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex.

In this study, changes in microglial immunoreactivity in the mPFC were assessed after exposure to chronic psychological stress (restraint stress). These observed changes were then linked to changes in neuronal activation and functionally linked to working memory, a behaviour which is mPFC-dependent. The observed increase in microglial immunoreactivity was assessed in conjunction with markers for antigen presentation and apoptosis, to evaluate whether the observed changes were due to traditional injury/infection activation of microglia. Finally, a separate experimental group was run where pharmacological blockade of microglia was utilised in order to assess the extent to which stress-induced alterations in behaviour are dependent on microglial activation.

It was hypothesised that:

a) Exposure to repeated psychological stress will increase microglial immunoreactivity and ΔFosB immunoreactivity in the mPFC;

b) The observed changes in microglial immunoreactivity do not correspond with an antigen presenting response;

c) Exposure to repeated psychological stress will reduce working memory capacity, as assessed by performance in a T-maze task; and

d) Inhibition of microglial activity using minocycline will improve working memory performance after exposure to chronic stress.
Experiment 3. *Chronic stress induced remodelling of the prefrontal cortex: Structural reorganization of microglia and the inhibitory effect of minocycline.*

Whilst it has been shown in a range of studies that stress increases microglial immunoreactivity, the corresponding change in microglial phenotype has not yet been elucidated. This is an important research question as microglial phenotype is intimately linked with function, and discoveries about phenotypic alterations have typically preceded and motivated corresponding studies regarding the associated function. In this study, microglial cells from layers II/III of the mPFC from animals exposed to chronic psychological restraint stress were reconstructed using Neurolucida software, and various cellular characteristics assessed in order to determine morphological alterations. The morphological changes associated with the administration of minocycline, a microglial inhibitor, were also assessed. The observed changes in morphology may represent an important mechanism through which microglia mediate the effects of chronic stress in the mPFC. Qualitative observations from previous studies led to the hypotheses that:

a) Exposure to chronic psychological stress will increase the ramification of microglia, leading to greater branching and number of cellular processes; and

b) Treatment with minocycline will reverse this effect, restoring microglial phenotype to that of control animals.
This work in this thesis describes changes to neurons and microglial cell populations in the rat medial prefrontal cortex after exposure to chronic psychological stress. This thesis is subdivided into three published manuscripts:


- **Hinwood M, Morandini JM, Day TA & Walker FR (2012).** Evidence that microglia mediate the neurobiological effects of chronic psychosocial stress on the prefrontal cortex. *Cerebral Cortex*, 22 (6), 1442-1454.

CHAPTER 2

Repeated social defeat selectively increases △FosB expression and histone H3 acetylation in the infralimbic medial prefrontal cortex
CHAPTER 2: Repeated social defeat selectively increases ∆FosB expression and histone H3 acetylation in the infralimbic medial prefrontal cortex


Cerebral Cortex (2011) Vol. 21(2), pp 262-271

Statement of author contributions to manuscript.

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<td>Madeleine Hinwood</td>
<td>Designed and executed the study. Analysed and interpreted the data. Wrote the manuscript.</td>
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<td>Ross J. Tynan</td>
<td>Provided some technical assistance.</td>
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<td>Trevor A. Day</td>
<td>Aided in data interpretation and corrected the manuscript.</td>
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<td>F. Rohan Walker</td>
<td>Designed the study, interpreted the data and corrected the manuscript.</td>
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Date: 17/11/13

PROFESSOR JOHN ROSTAS

Deputy Head of Faculty (Research and Research Training)
Repeated Social Defeat Selectively Increases ΔFosB Expression and Histone H3 Acetylation in the Infralimbic Medial Prefrontal Cortex

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Exposure to social stress has been linked to the development and maintenance of mood-related psychopathology; however, the underlying neurobiological changes remain uncertain. In this study, we examined numbers of ΔFosB-immunoreactive cells in the forebrains of rats subjected to 12 episodes of social defeat. This was achieved using the social conflict model whereby animals are introduced into the home cage of older males (‘residents’) trained to attack and defeat all such ‘intruders’; importantly, controls were treated identically except that the resident was absent. Our results indicated that the only region in which ΔFosB-positive cells were found in significantly higher numbers in intruders than in controls was the infralimbic medial prefrontal cortex (mPFC). This same effect was not apparent using another psychological stressor, noise stress. Cells of the infralimbic mPFC also displayed evidence of chromatin remodeling. We found that exposure to repeated episodes of social defeat increased numbers of cells immunoreactive for histone H3 acetylation, but not for histone H3 phosphoacetylation, in the infralimbic mPFC. Collectively, these findings highlight the importance of the infralimbic mPFC in responding to social stress—a finding that provides insight into the possible neurobiological alterations associated with stress-induced psychiatric illness.

Keywords: chronic stress, infralimbic cortex, layer IV, (medial) prefrontal cortex, social conflict

Introduction

Social defeat has been consistently implicated in both the induction and the exacerbation of mood-related psychopathology (Bjoqvist 2001; Huhan 2006). Because of this, there have been a number of preclinical studies aimed at elucidating the underlying neuronal circuitry. Most such studies have utilized the expression of immediate early genes, particularly c-fos, to assess the defeat-induced pattern of neuronal activation (Kollack-Walker et al. 1997; Martinez et al. 1998; Chung et al. 1999; Kollack-Walker et al. 1999; Martinez et al. 2002). While this approach has been very useful for identifying circuits activated by a single social defeat, it is not well suited to mapping neuronal activation resulting from repeated or chronic defeat. This is because of the declining ability of neurons to continue to express c-fos in the face of repeated activation (Martinez et al. 1998; McClung et al. 2004). This limitation is unfortunate as repeated or chronic stress is a more frequent antecedent to the development of psychopathology than a single episode (Iyngula et al. 2005; Huhan 2006). There has been recent interest in ΔFosB as an alternative marker of neuronal activation in response to chronic events. In contrast with other known fos family members, ΔFosB, a truncated splice variant of fosB, progressively accumulates in repeatedly activated neurons and persists there for several weeks (Chen et al. 1997; McClung et al. 2004; Perrotti et al. 2004). Accordingly, in the present study, we assessed the effect of repeated episodes of social defeat on the expression of fosB-like immunoreactivity in limbic forebrain regions associated with the control of mood (Di Chiara et al. 1999; Scheggi et al. 2002; Boye and Finlay 2005). It is important to recognize that the results from these social defeat studies cannot be generalized to females as defeat can only be reliably elicited in males. Nevertheless, as several other studies have indicated that different forms of stress elicit distinct patterns of neuronal activation in the forebrain (Cullinan et al. 1995; Abraham and Kovacs 2000; Sawchenko et al. 2000; Dayas et al. 2001), we considered it prudent to examine the ΔFosB response to another form of psychological stress, noise stress. These studies failed to produce any effect of repeated noise stress on ΔFosB expression in the forebrain. Intriguingly, this work revealed only one region in which there was a significant increase in numbers of fosB-positive neurons after exposure to repeated social defeat, the infralimbic medial prefrontal cortex (mPFC). In subsequent work, fosB-positive cells in this region were further characterized as to their neurotransmitter phenotype. Additionally, because of recent evidence suggesting that neuronal activation can also induce chromatin remodeling (Miller et al. 2006; Chung and Lien 2009; Sweating 2007), we examined cells in this region for evidence of acetylation, or phosphoacetylation, of histone H3.

Materials and Methods

Animals

Adult Sprague-Dawley male rats (350-450 g; ~70 days old, n = 8-11 per group) were obtained from the University of Newcastle Central Animal House for use in this study. Animals were maintained in temperature-controlled holding rooms (21 ± 1 °C) for the duration of the study, with food and water provided ad libitum. The rooms were held under a 12-h light/dark cycle (lights on at 1400 h), and all experimental procedures were conducted between 0800 and 1200 h. This study was approved by the University of Newcastle Animal Care and Ethics Committee and was performed in accordance with the New South Wales Animals Research Act and the Australian Code of Practice for the use of animals for scientific purposes. All animals were housed in groups of 4 prior to the commencement of the experiment but individually housed thereafter (with the exception of nonhandled controls).

Experimental Design

Subjects were exposed to either noise (NS+ ) or social defeat (SC+) stress, a total of 12 times over a 16-day period. Two types of controls

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were used: sham stress controls (NS- and SC-) that were exposed to exactly the same procedures as the stressed animals except that the stressor was not applied and home cage controls (HCCs) that were simply left in their home cage for the duration of the experiment. An additional group was run where animals were exposed to a single social defeat and sacrificed 24 h later in order to confirm that AFOB was not expressed at this time.

Social Defeat

The social defeat procedure used is adapted with modifications from Miczek (1979). Briefly, subjects (‘intruders’) were exposed to the attacks of a dominant male (the ‘resident’) in the resident’s home cage. Residents (Sprague–Dawley males, 500–800 g) were at least 4 months old and had been co-housed in a large multi-compartment acrylic cage, with a ligated female for at least 6 weeks prior to the commencement of the experiment. Prior to the study commencing, all residents were screened to ensure that they would reliably attack intruders with a latency of less than 1 min. Fifteen minutes prior to each defeat taking place, females were removed from the resident home cage.

Intruders were removed from their home cage and placed directly into a resident home cage. After the first attack by the resident, and subsequent expressions of submissive postures (lying on the back or side with no sign of resistance while being inspected by the resident) by the intruder, a wire mesh barrier was inserted into the cage to separate the 2 animals. This ensured that the animals had auditory, visual, and olfactory contact with one another but prevented further physical contact. The intruder remained in the resident’s home cage for a total of 90 min, including both interaction and separation time.

To allow a proper distinction between the effects of exposure to the resident cage (novelty, handling, olfactory) and the effects of social stress, a group of sham stress control animals (SC-) were exposed to an empty resident home cage for 90 min. Exposures were time-locked to the corresponding social defeat group. As such, these animals differ from the intruders in just one respect: Only intruders were subjected to social defeat.

Noise Stress

Subjects were exposed to white noise at 105 dB for half an hour at a time. Exposure occurred within a soundproof box that the animals’ home cage was placed into. Sham stress control animals (NS-) were removed from their holding room and their home cage placed into the sound chamber but not exposed to any sound.

Immunohistochemistry

Twenty-four hours after the last treatment, animals were deeply anesthetized with sodium pentobarbital and then perfused transcardially with 2% sodium nitrate solution, followed by 1% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Critically, this time of sacrifice was chosen expressly to ensure that AFOB would be the only Fos isoform present as it has been previously shown that all other Fos family proteins have degraded prior to this time (McClellan et al. 2004; Perrotti et al. 2004; Bertron et al. 2007; Nikulina et al. 2008). Additionally, evidence suggests that this same fixation time will reveal maximum expression of acetylated and phosphoacetylated histone H3 proteins (Renthal and Nestler 2009). Stable changes in histone acetylation have been found to persist for at least 2 weeks after cessation of a repeated stimulus (Renthal et al. 2007, Tsankova et al. 2007, Corvington et al. 2009, Renthal and Nestler 2009). After the brains were removed, they were postfixed for 17 h in the same fixative solution. After fixation, brains were transferred to a 12.5% sucrose solution in 0.1 M phosphate-buffered saline for cryoprotection. Serial coronal sections (40 μm) were cut using a freezing microtome (Leica 2000R) and processed for Fos, acetylated histone H3, or phosphoacetylated histone H3 immunoreactivity.

For AFOB, we used a rabbit polyclonal antibody that had been raised against an N-terminal region of FosB and recognizes both FosB and AFOB (SC-48, dilution 1:500; Santa Cruz Biotechnology). Labeling for acetylated histone H3 (Ac(lys9)-Ac(lys14)-H3) and phosphoacetylated histone H3 (P(Ser 10)-Ac(lys14)-H3) was performed with rabbit polyclonal antibodies (06-599, 1:500, and 07-081, 1:250, respectively, both from Upstate). To our knowledge, the antibody for acetylated histone H3 has not been used previously for immunohistochemistry. However, the antibody has been validated for use in western blot procedures both by the manufacturer and in previous studies (Steffan et al. 2001; Kumar et al. 2005; Renthal et al. 2007; Corvington et al. 2009; Persis et al. 2010) and in all instances produces a single band at ~17 kDa. We additionally performed a preabsorption test using the peptide for acetylated H3 to confirm that the antibody is specific for this protein. We observed no labeling in tissue treated with preabsorbed antibody (see Fig. 1 below). The histone H3 phosphoacetylation antibody used in this study has been previously validated for use in immunohistochemistry in a recent series of studies (Chandramohan et al. 2007; Reul and Chandramohan 2007, Chandramohan et al. 2008). A 1-in-4 series of forebrain was processed batch wise for AFOB, or a 1-in-8 series of mpPC was processed for acetylated and phosphoacetylated H3, using the following immunoperoxidase technique. Free-floating sections were first treated with 1% H2O2 in 0.1 M phosphate buffer and then incubated for 30 min in 3% normal horse serum in 0.1 M phosphate buffer. Sections were then incubated overnight at 4°C in 1% normal horse serum, 0.1% bovine serum albumin, 0.3% Triton X-100, and primary antibody. Sections were washed and incubated in the secondary antibody biotinylated goat anti-rabbit (BA1000, 1:500, Vector Laboratories) for 1 h, washed, and incubated in a 1:1000 dilution of Extravidin (Sigma-Aldrich) for 2 h. Peroxidase activity was visualized using a reaction with 3,3′-diaminobenzidine. The reaction was terminated when an optimal contrast between specific cellular labeling and nonspecific background was reached. Sections were then mounted onto chrome-alum-subbed slides, dehydrated in a series of alcohols (70%, 95%, 100%, absolute), cleared in xylene, and coverslipped using Permount (Fisher Scientific).

Analysis

Slides were coded, and an experimentiner blind to the code counted immunoreactive cells for each region of interest. Only those forebrain regions associated with the control of mood and regulation of the stress

Figure 1. Photomicrographs showing results of preabsorption immunohistochemistry test for acetylated histone H3 (lys9-lys14). Left: Infralimbic cortex labeled with untreated primary antibody. Right: Infralimbic cortex labeled with antibody preabsorbed with acetylated histone H3 (lys9-lys14) protein. Scale bars = 10 μm.
response were examined for the presence of FosB-positive cells (Di Chiara et al. 1999; Weiss et al. 2001; Scheggi et al. 2002). No significant labeling was found in the paraventricular nucleus of the hypothalamus; the bed nucleus of the stria terminalis; or the medial, central, or basolateral amygdala. However, significant numbers of FosB-positive cells were present in prefrontal cortex, nucleus accumbens (Nac), and lateral septum. Therefore, more precise quantification was carried out in these regions, specifically the prelimbic and infralimbic cortices of the mPFC (5.0–2.50 and 5.72–2.44 mm from bregma, respectively), the core and shell of the Nac (2.82–0.90 mm from bregma), and the ventral lateral septum (VLS) (2.04 to 0.56 mm from bregma). Regions were defined according to Paxinos and Watson (2005). For each region of interest, immunoreactive cells were counted bilaterally at 520-μm intervals over a number of rostrocaudal levels, their boundaries being determined by cytoarchitectural features mapped in adjacent double-labeled FosB- and Nissl-stained sections (Gerfen 2003). Acetylcholinesterase- and phosphoacetyl-H3-positive cells were counted in mPFC only. Positive cells were counted bilaterally at 520-μm intervals. Analysis was performed using Metanorph Image System software (Universal Imaging Corp., Velpicelli-Duley and Levey 2005).

Double Label Immunofluorescence

Immunohistochemistry was used to double label FosB (SC-48, 1:100; Santa Cruz Biotechnology) and several other proteins. These included neuron-specific nuclear protein NeuN (MAB377, 1:100; Chemicon), parvalbumin (MAB572, 1:500; Chemicon), calbindin (AB1778, 1:100Chemicon), and calretinin (AB5054, 1:1000; Chemicon). Immunohistochemistry was also used to double label acetylated histone H3 (H3-599, 1:100Chemicon) and neuron-specific nuclear protein NeuN (MAB377, 1:100; Chemicon). The proteins were visualized using AlexaFluor-labeled secondary antibodies (488 or 594, 1:400; Molecular Probes). Localization of protein expression was performed using an Olympus BX51 microscope fitted with an Olympus DP71 camera and U-RFL-T burner connected to a personal computer running DP-BSW software.

Data Analysis

Raw counts of positive immunoreactive cells were analyzed using analysis of variance (ANOVA), followed by post hoc testing. In all figures and tables, data are expressed as the mean ± standard error (SEM). The data were considered to reach statistical significance where P < 0.05.

Results

Stress Induction of ΔFosB Protein in Brain

As several previous studies have demonstrated that ΔFosB is only apparent after repeated stimulation, we were interested in confirming that ΔFosB expression was low after a single social defeat episode. Consistent with previous reports, we found no evidence of ΔFosB in tissue collected 24 h after a single social defeat (see Fig. 2 below).

Each of the regions examined are represented in Fig. 3, below. While immunoreactive cells were counted bilaterally, there were no significant differences between the left and the right sides of any brain region counted. A one-way ANOVA was used to test differences between the stress groups in each brain region counted. In the infralimbic (IL) mPFC, there was a significant effect of group, F(2, 38) = 21.54, P < 0.001. Tukey’s post hoc comparisons revealed that the repeated social defeat stress group has significantly higher ΔFosB counts than the sham social defeat group (P < 0.001), the noise stress group (P < 0.001), the sham noise stress group (P < 0.001), and the HCCs (P < 0.001). The sham social stress group was also significantly different to the HCCs (P = 0.045).

In the prelimbic (PrL) mPFC, a one-way ANOVA suggested that there was a significant effect of group, F(2, 38) = 4.21, P = 0.026. Tukey’s post hoc comparisons revealed significant differences

Figure 2. Left: Photomicrograph of HCC infralimbic cortex labeled with AFosB. Center: Photomicrograph of infralimbic cortex from an animal exposed to a single social defeat and sacrificed 24 h later and labeled with AFosB. Right: Photomicrograph of infralimbic cortex from an animal exposed to 12 social defeats and sacrificed 24 h after the final episode and labeled with AFosB. Scale bars = 50 μm.

Figure 3. Images adopted from Paxinos and Watson (2002) depicting the location of each region examined including (A) prelimbic cortex, (B) infralimbic cortex, (C) NAc core, (D) NAc shell, and (E) VLS. Numbers indicate distance from bregma in millimeters.
between the repeated social defeat group and both the HCCs \((P = 0.01)\) and the sham noise stress condition \((P = 0.047)\).

In the shell of the NAc, there was a significant effect of group, \(F_{3,90} = 4.64, P = 0.004\). Tukey’s post hoc comparisons showed significant differences between the repeated social defeat condition and both the HCCs \((P = 0.009)\) and the sham noise stress group \((P = 0.014)\).

In the core of the NAc, a one-way ANOVA revealed a significant main effect of group, \(F_{3,90} = 5.26, P = 0.002\). Tukey’s post hoc testing revealed significant differences between the repeated social defeat group and the noise stress \((P = 0.013)\), sham noise stress \((P = 0.019)\), and HCC \((P = 0.008)\) groups.

In the VLS, a one-way ANOVA was not significant overall \((P = 0.16)\). Tukey’s post hoc analysis revealed a significant difference between the repeated noise stress condition and the HCCs only \((P = 0.017\) \(\text{see Table 1 below}\).

Overall, relative to HCCs, numbers of \(\Delta FosB\)-positive cells were significantly elevated in just one of the regions counted in the forebrains of animals subjected to sham social defeat (SC-), the II mPFC, and 4 of the regions counted in the forebrains of animals subjected to repeated social defeat (SC+), the PFL mPFC, the II mPFC, the NAc core, and the NAc shell. However, relative to the SC-group, only the increase observed in the II mPFC of SC+ animals was statistically significant (see Fig. 4 below).

Relative to HCC animals, numbers of \(\Delta FosB\)-positive cells were not significantly elevated in any of the forebrain regions counted in animals subjected to sham noise stress (NS-), but they were significantly elevated in one of the regions counted in the forebrains of animals subjected to repeated noise stress (NS+), that being the VLS. Importantly, however, this increase in the VLS of NS+ animals was not statistically significant relative to NS- animals.

### Cellular Specificity of \(\Delta FosB\) Induction by Chronic Stress in the Infrahilar Cortex

\(\Delta FosB\)-positive cells observed in the infrahilar cortex of animals subjected to repeated social defeat were further characterized. Tissue immunolabelled for \(\Delta FosB\) was also subjected to a Nissl stain. This showed that \(\Delta FosB\)-positive cells were densest in cortical layers II and III (Fig. 5). Double-label immunofluorescence showed that almost all of these cells were also immunopositive for Neun, a marker of mature neurons (Fig. 6, Magavi and Macklis 2008). However, no \(\Delta FosB\)-positive cells were immunoreactive for calbindin-D28K, parvalbumin, or calcitriol, all of which are established markers of γ-Aminobutyric acid (GABAergic) neurons (Kawaguchi and Kubota 1997). As the cortex contains primarily GABAergic interneurons and glutamatergic pyramidal projection neurons, this suggests that the \(\Delta FosB\)-positive cells were of the latter type (Fig. 7).

### Stress Induction of Changes in Histone Acetylation Status in mPFC

We used immunohistochemistry to quantify changes to histone H3 acetylation status in the II and PFL of animals exposed to repeated social defeat. While there was a significant difference in levels of acetylated histone H3 \([\text{Ac-Lys9, Ac-Lys14}]\) immunoreactivity between repeated social defeat and sham social defeat controls in the II \((P = 0.02\) \(\text{Fig. 8}\), we found no difference between the 2 groups for phosphoacetylated histone H3 \([\text{P(Ser10)-Ac-Lys14}]\) immunoreactivity. We found no differences between repeated social defeat and sham social defeat controls in levels of acetylated \([\text{Ac-Lys9, Ac-Lys14}]\) histone H3 immunoreactivity in the PFL (see Table 2). Further analysis of the acetylated histone H3 \([\text{Lys9-Lys14}]\) response in the II using dual-label immunofluorescence showed partial colocalization with Neun, suggesting that this change occurred in both neurons and glial cells (Fig. 6).

### Discussion

In this study, it was found that, although repeated noise stress was without effect, repeated social defeat led to a significant increase in numbers of \(\Delta FosB\)-immunoreactive neurons in just one forebrain area, the infrahilar mPFC. Because a single social defeat does not elicit detectable \(\Delta FosB\)-immunolabelling in the forebrain, this result is assumed to reflect the cumulative impact of multiple defeat episodes over the preceding 16 days, an interpretation consistent with reports that this protein progressively accumulates in the nuclei of repeatedly activated neurons (McCaug et al. 2004; Nikulina et al. 2008; Renthal et al. 2008).

The current findings are somewhat surprising as there have been few previous studies suggesting that repeated stress (both social defeat and restraint stress) increases \(\Delta FosB\) expression in multiple forebrain regions (Perrotti et al. 2004; Nikulina et al. 2008). However, it is critical to note that, in both these previous studies, the reported increases were relative to levels observed in control animals that were, at most, subjected to brief handling by the experimenters. In contrast, in the current study, the critical controls were animals that were treated identically to the stressed animals, except for application of the stressor itself. For example, sham social defeat controls were, in addition to being handled by the experimenters, also placed in the temporarily vacated home cage of an aggressive conspecific for 30 min. Such a cage would obviously constitute a highly novel environment, being rich in spatial and olfactory stimuli. Previous work suggests that this should elicit neuronal activation well beyond that produced by handling alone (Hosokawa and Chiba 2008; Cavalcante et al. 2006; Nugent et al. 2009, and indeed, we found that \(\Delta FosB\)-positive cell numbers in the II and mPFC of the sham social defeat controls were significantly higher than that in HCCs (see Table 1). It is our view then that, particularly when assessing the impact of complex stressors, the most appropriate control...
Figure 4. Quantification of ΔFosB induction in the infralimbic mPFC of animals exposed to 12 days of repeated social defeat. Levels of ΔFosB immunoreactivity were assessed by immunohistochemistry in (A) animals subjected to repeated social defeat and (B) animals exposed to sham social defeat. (C) Line graph showing the distribution of ΔFosB-positive cells across the rostrocaudal axis of the infralimbic cortex of animals exposed to chronic social stress (○), sham stress controls (■), and nonhandled HCCs (▲). Inset: Bar graph displaying mean totals of ΔFosB-positive cells in the infralimbic mPFC of animals exposed to chronic social stress (SC+), chronic sham social stress (SC−), and nonhandled HCCs. Data are expressed as mean ± standard error (SEM). *P < 0.05 vs midline (layer 1). Scale bars = 50 μm.

Figure 5. Layer-specific ΔFosB labeling in the infralimbic cortex. (A) Light photomicrograph showing laminar distribution of cresyl violet-stained cells in the infralimbic cortex using cortical layer demarcation adapted from Gabbott et al. (2009). Photomicrographs demonstrating layer-specific labeling of ΔFosB with cresyl violet. (B) Animal exposed to chronic social defeat. Inset: High-power photomicrograph with arrows indicating cells double-labeled with both cresyl violet and FosB in layer I/II. (C) Animal exposed to chronic sham social defeat. Inset: Examples of cells from layer I/II. Scale bars = 100 μm (A), 50 μm (B and C), and 10 μm (insets).

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Figure 6. The upper series of 3 panels shows a representative fluorescent photomicrograph displaying colocalization of FosS with NeuN, a marker of mature neurons, in the infralimbic cortex. Left: FosS only. Center: NeuN only. Right: Merged. Arrows indicate double-labeled cells. The lower series of 3 panels shows a representative fluorescent photomicrograph demonstrating incomplete colocalization of acetyl-H3-positive cells with NeuN, a marker of mature neurons. Left: Acetyl-H3 only. Center: NeuN only. Right: Merged. Arrows indicate acetyl-H3-positive cells that are not neurons. Scale bars = 50 μm.

Figure 7. Cellular specificity of ΔFosB induction in the infralimbic mPFC following repeated social defeat. Representative fluorescent photomicrographs show no colocalization of FosB with parvalbumin (A-C), calcitonin (D-F), or calbindin (G-I), markers of GABAergic interneurons. Scale bars = 50 μm.

group is one differing from their experimental counterparts only in the absence of the critical stress element, which, in the case of the social defeat paradigm, is a aggressive social interaction culminating in defeat.

It is particularly interesting that in the present study: 1) Although repeated social defeat elicited a significant increase in ΔFosB expression, repeated noise stress did not and 2) this change occurred only in the infralimbic mPFC. One possibility is that social defeat is simply a more intense stressor than noise stress. This is consistent with the considerable body of evidence showing that the infralimbic mPFC plays a key role in regulating the activity of the subcortical stress response system (Diorio et al. 1993; Figueiredo et al. 2003; Spencer et al. 2004; Radley et al. 2006). However, one problematic aspect of this explanation is that we would expect levels of ΔFosB to be higher in the noise stress group than in the sham social defeat group, a group only exposed to an empty social conflict arena, when in fact they are statistically equivalent. Alternatively, the results could be taken to suggest that the infralimbic mPFC is uniquely responsive to social forms of stress. Certainly, this...
hypothysis is consistent with evidence indicating that the mPFC is involved in modulating social interactions (Ortscharenoff and Braun 2001; Shah and Treit 2003; Kiyokawa et al. 2007; Leussi et al. 2008). Experimentally, teasing out whether the infralimbic mPFC is uniquely responsive to social stressors will certainly be a challenge. One promising avenue may be to examine a wider range of social (social isolation, housing) and nonsocial stressors (predation, shock) that vary in their inherent intensity.

It is generally agreed that neurons located within the mPFC are either glutamatergic pyramidal neurons or GABAergic interneurons with regard to phenotype (Gabbott et al. 1997). Moreover, it is generally accepted that neurons containing the calcium-binding proteins calbindin, calbindin-D28k, or parvalbumin also contain GABA (Kawaguchi and Kubota 1997; Grilli et al. 2003; Molyneaux et al. 2007). Consequently, our finding that none of the FoṣB-positive cells were immunoreactive for any of these 3 calcium-binding proteins suggests that these cells are likely to be glutamatergic pyramidal projection neurons. Notably, most of these presumptive glutamatergic pyramidal neurons were located in cortical layer II/III. Previous studies have shown that the pyramidal neurons of layer II/III are stress sensitive, with prolonged exposure to stress leading to extensive and reversible dendritic remodeling (Radley et al. 2006; Radley, Rother et al. 2008; Radley, Williams et al. 2008; Watakabe 2009). Furthermore, while some of these neurons project to subcortical regions including the basolateral amygdala, lateral hypothalamus, and NAc, it is thought that the majority of pyramidal neurons in layer II/III project to other cortical areas, including orbital, insular, and entorhinal cortices (Vertes 2004; Gabbott et al. 2005; Hoover and Vertes 2007).

As FoṣB is a transcription factor, increased numbers of FoṣB-positive cells in the infralimbic mPFC has been interpreted as a change in the functional capacity of the neurons in this region (McClelland et al. 2004; Perrotti et al. 2008; Wallace et al. 2008). Recent studies indicate that another way in which transcriptional modification of neurons can be achieved is via chromatin remodeling (Bilang-Bieuel et al. 2005; Tsuchiya et al. 2006; Tsuchiya et al. 2007; Jiang et al. 2008). One way in which this can occur is through the posttranslational modification of core histone proteins at their N-terminus, increasing the accessibility of DNA to binding proteins (Kouzarides 2007; Rendahl et al. 2007). Thus, it has been demonstrated in several studies that the acetylation [Ac(Lys9)-Ac(Lys14)] and phosphoacetylation [P(Ser10)-Ac(Lys14)] of histone H3, events associated with the activation and maintenance of transcription, can be modified in several brain regions by exposure to stress (Bilang-Bieuel et al. 2005; Chandramohan et al. 2007; Bical and Chandramohan 2007; Chandramohan et al. 2008; Fuchikami et al. 2009; Sweet 2009).

It was against this background that we examined cells in the infralimbic mPFC for immunoreactivity against acetylated or phosphoacetylated histone H3 after exposure to repeated social defeat. This revealed that, although there was no change in numbers of immunoreactive cells for phosphoacetylated histone H3, there was a significant increase in acetylated histone H3 immunoreactivity. Ideally, we would have wished to demonstrate increases in acetylated histone H3 localized to cells that were FoṣB positive. However, this was not possible as all commercially available antibodies for these proteins are currently raised in the same species, making it impossible to
achieve reliable double labeling. Nevertheless, to our knowledge, it still stands that this is the first provision of evidence that stress-induced chromatin remodeling occurs in the prefrontal cortex. We additionally examined cells in the adjacent prelimbic mPFC for immunoreactive cells for acetylated and phosphorylated histone H3. Interestingly, in line with the null result found in this region for ΔFosB, we also found no significant differences in chromatin remodeling between groups.

One of the crucial functions of the mesocorticolimbic system, which includes the mPFC, is to prevent excessive behavioral and physiological responses to stress (Sullivan 2004). Both electrolytic lesions and injections of dopamine receptor antagonists into the mPFC have previously been shown to exaggerate chronic stress-induced neuroendocrine responses (Gerrits et al. 2003; Sullivan and Dufresne 2006). Our results extend this body of work by elucidating specific neurobiological alterations that occur following exposure to chronic stress within the mPFC. From a clinical standpoint, these results are in keeping with functional neuroimaging studies in humans, which report increased metabolic activity in the subgenual cingulate cortex (the closest analogous region to the rat infralimbic cortex) in patients with depression (Gottlib et al. 2005; Beuregard et al. 2006; Drevets et al. 2008), a condition that may be precipitated by exposure to chronic psychosocial stress (Bjorkqvist 2001).

In conclusion, this study confirms that repeated social defeat leads to an increase in ΔFosB expression in the forebrain of the rat. In contrast to the sole previous report, we demonstrated that this is restricted to a single area in the forebrain, the infralimbic mPFC. In particular, this increase in FosB-like immunoreactivity was localized to glutamatergic pyramidal neurons, largely in layers II/III in that region. Moreover, evidence was provided of histone modifications occurring in this same region after repeated exposure to social defeat, this being the first time any evidence of this functionally important change is occurring in prefrontal cortex. While these results are interesting, it is important to note that these experiments were carried out using male rats only, and at this point, the extent to which they generalize the female brain is unknown. Importantly, however, it has been shown that exposure to a nonsocial form of chronic stress increases the number of ΔFosB-positive nuclei in the mPFC of female rats, suggesting that the mPFC is similarly responsive to chronic stress in females (Gerrits et al. 2006).

Funding

National Health and Medical Research Council of Australia (#G0187604), Hunter Medical Research Institute (#G018876). Notes

We wish to thank Ms. Britt Sadby and Dr. Douglas Smith for their assistance with the laboratory work associated with this study. Conflict of Interest: None declared.

References


Nisgt BM, Wright CL, Zip Zi, McCarthy MM. 2009. Muscular pain induced by neonatal exposure to PGE (2) or estradiol alters c-fos expression by induced estrous odors in adult rats. Physiol Behav. 95:59-63.


CHAPTER 3

Evidence that microglia mediate the neurobiological effects of chronic psychosocial stress on the prefrontal cortex.
CHAPTER 3: Evidence that microglia mediate the neurobiological effects of chronic psychosocial stress on the prefrontal cortex.

Madeleine Hinwood, James Morandini, Trevor A. Day and F. Rohan Walker.


Statement of author contributions to manuscript.

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<td>Madeleine Hinwood</td>
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<td>James Morandini</td>
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Evidence that Microglia Mediate the Neurobiological Effects of Chronic Psychological Stress on the Medial Prefrontal Cortex

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Psychological stress contributes to the development of clinical depression. This has prompted many preclinical studies to investigate the neurobiology of this relationship; however, the effects of stress on glia remain unclear. In this study, we wished to determine, first, how exposure to chronic psychological stress affects microglial activity within the prefrontal cortex (PFC) and, second, whether the observed changes were meaningfully related to corresponding changes in local neuronal activity and PFC-regulated behavior. Therefore, we examined markers of microglial activation, antigen presentation, apoptosis, and persistent neuronal activation within the PFC after exposure to repeated restraint stress. We also examined the effect of stress on spatial working memory, a PFC-dependent function. Finally, we tested the ability of a microglial activation inhibitor (minocycline) to alter the impact of chronic stress on all of these endpoints. Stress exposure produced positively correlated increases in microglial and long-term neuronal activation in the PFC but not antigen presentation or apoptosis. As expected, it also impaired spatial working memory. Importantly, minocycline reduced the impact of stress on neuronal activation and working memory, as well as microglial activation. These results suggest a role for microglia in mediating the effects of stress on PFC neuronal function and PFC-regulated behavior.

Keywords: chronic stress, ΔFosB, iba-1, minocycline, working memory

Introduction

Chronic psychological stress is a critical risk factor in the emergence of major depressive disorder (Kendler et al. 1999; Hammen 2005). In considering the biological basis of the link between stress and depression, considerable attention has been given to the role of the prefrontal cortex (PFC; Di Chiara et al. 1999; Drevets 2000; Shansky et al. 2000). It is well-established that patients with major depressive disorder display structural and functional changes in the PFC (Drevets 2000; McSween 2005) and that stress, independent of frank mood disturbance, can disrupt PFC activity (Figueroa et al. 2003; Cerqueira et al. 2007; Linton et al. 2009; Richter-Levin and Maroun 2010) and PFC-dependent behavioral tasks (Mizoguchi et al. 2000; Linton et al. 2009). Unfortunately, factors such as the limited spatial resolution of clinical neuroimaging methods restrict our ability to understand, at the cellular level, exactly how stress impacts the PFC in humans. Because of this, it has been necessary to resort to the use of animal models. In so doing, it has been important to recognize that significant interspecies differences can exist (Ongur and Price 2000). Nevertheless, it has been possible, using these models, for researchers to generate insights into the stress-induced changes in cellular PFC function that are generally considered to be indicative of what occurs in humans. Recent notable outcomes in this regard include the observation that chronic stress significantly alters neuronal morphology (Radley et al. 2004; Radley, Rocher, et al. 2006), and local release of dopamine (Di Chiara et al. 1999; Mizoguchi et al. 2000; Pani et al. 2000) and glutamate (Gilad et al. 1990; Moghadam 2002), in the rat medial PFC (mPFC). Likewise, our own studies have presented evidence that stress increases levels of ΔFosB in glutamatergic cells of the rat mPFC (Hinwood et al. 2010), a finding consistent with stress producing long-lasting functional changes in mPFC neurons (Perrotti et al. 2004). Intriguingly, however, we have also recently shown that chronic stress triggers regionally selective activation of microglia in rat mPFC (Tyson et al. 2010). This is particularly interesting because there is rapidly accumulating evidence that, in addition to their long-established role in defensively responding to neural tissue insults, microglia also play a critical role in regulating ongoing neuronal activity and connectivity (Walke et al. 2009; Graeber 2010). Accordingly, the present study was conducted in an attempt to determine whether at least some of the changes observed in mPFC neuronal function and mPFC-dependent behavior after exposure to chronic psychological stress might depend upon changes in local microglial activity. To that end, we have now conducted studies in which we have examined the relationship between stress-induced changes in mPFC microglial and neuronal activity and mPFC-dependent behavior. Moreover, to determine whether there might be a causal relationship between microglial activation and corresponding changes in neuronal activity and behavior, we have also examined the consequences of an inhibitor of microglial activation and proliferation, minocycline, on neuronal and behavioral responses to chronic stress.

Materials and Methods

Ethics

Experiments were approved by the University of Newcastle Animal Care and Ethics Committee and was performed in accordance with the NSW Animal Research Act and the Australian Code of Practice for the use of animals for scientific purposes.

Animals

Adult male Sprague-Dawley rats (350-450 g) used in experiments 1 and 2 were 70 days old at the commencement of the experiments and were obtained from the Animal Resource Centre (Perth, West Australia). The animals used in both experiments were held, individually, in temperature-controlled holding rooms (21±1 °C) on a 12-h reversed light-dark cycle (lights on at 1900 h). All experimental procedures were conducted during the dark phase of the light cycle. Animals were adapted to single housing for 7 days prior to any manipulation. Animals in experiment 1 were maintained on standard rat chow and water provided ad libitum. In experiment 2, animals were provided with ad libitum access to water (except during restraint/control procedures)
and were food deprived at 85% of free-feeding weight, consistent with the approach used by Mizoguchi et al. (2000) to motivate performance in the delayed alternating T maze test.

**Experiment 1**

**Experimental Design**

A 2 (drug treatment: minocycline vs. no minocycline) × 2 (stress condition: chronic stress vs. handled controls) between groups design was employed. Animals were exposed to either daily restraint (ST) or twice daily handling (CON) over a 21-day period. Minocycline hydrochloride (M) was administered to half of the stress group (STR × M), and half of the control group (CON × M) via the drinking water for the duration of the study, starting 21 h prior to the initial episode of stress or handling. Remaining animals received normal drinking water. Animals were sacrificed 24 h after the final episode of stress or handling. This protocol gives 4 experimental groups: 1) 21 days of restraint stress; 2) 21 days of brief handling; 3) 21 days of restraint stress and administration of minocycline; and 4) 21 days of brief handling and administration of minocycline.

**Restraint Stress**

Animals assigned to stress groups were exposed to a single 6-h session of restraint per day for 21 days. Control animals were handled twice daily in a separate room at corresponding times to the initiation and cessation of restraint. Handling controls were also food and water deprived for the same duration as the stress protocol. The method of restraint used in the current study is as previously described (Tyman et al. 2010). Restraints were constructed of a fine gage wire mesh (6.0 mm diameter, 6.5 × 6.5 mm grid) secured with butterfly clips. All procedures were conducted within the animals home cage.

**Oral Administration of Minocycline Hydrochloride**

We administered minocycline for the duration of the stress exposure period. This administration procedure was chosen as a variety of studies examining the ability of minocycline to quench microglial activity also use chronic administration paradigms (Tremblay et al. 1998; Raghaven et al. 2003; Hassenzahl et al. 2011). Minocycline hydrochloride (PCCA, Australia) was administered orally via animals drinking water at a dosage of 40 mg/kg/day. Previous studies have demonstrated that doses ranging from 20 to 50 mg/kg/day are effective in attenuating microglial activation in the brain (Kad theta et al. 2003; Raghaven et al. 2003; Liu et al. 2007). Plasma levels of minocycline were assessed on day 21 of oral administration, in a subgroup of animals using a tetracycline enzyme-linked immunosorbent assay (BIBO Scientific Co). Using a minocycline standard, plasma levels were found to be approximately 4.55 µg/ml, which are in the same range as observed following intravenous administration (Colovic and Caccia 2003). Minocycline solution was made up each day at a concentration of 1 mg/ml in animal's usual drinking water. The solution was available ad libitum except during restraint or control handling procedures. Administration began 24 h prior to the initial episode of restraint stress and continued throughout the remainder of the experiment. Consumption was measured daily.

**Perfusion and Tissue Processing**

Twenty-four hours after the final episode of restraint stress or control procedures, animals were deeply anesthetized using sodium pentobarbitone and transcardially perfused via the ascending aorta with 2% sodium nitrite solution (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed for 17 h in the same fixative, then transferred to 12.5% sucrose solution in phosphate-buffered saline (pH 7.4) for cryoprotection. Serial coronal sections of 50 µm were collected using a freezing microtome (Leica 2009) and stored in an ethylene glycol cryoprotectant solution at 4 °C until processed.

**Antisera Used for Immunohistochemical and Immunofluorescent Detection of Microglia and Activated Neurons**

For semi-quantitative assessment of changes in microglial activity and iNOS immunoreactivity, a 1-in-6 series of PFC sections were collected 180 µm apart. For immunoperoxidase labeling of microglia, we used a rabbit (polyclonal) antigen directed against the ionized calcium-binding adapter molecule 1 (Iba-1; 1:100; Zymed, catalog no. 019-1974). According to the manufacturer, this antibody recognizes a single band of approximately 17 kDa using Western blot and in typical neural tissue, is immunoreactive with microglial cells. Five consecutive sections were processed for Iba-1 immunoreactivity, those selected being approximately 5.5, 5.0, 5.18, 5.00, and 2.82 mm rostral to bregma. For immunofluorescent labeling of microglia, we used a goat (polyclonal) antisera directed against Iba-1 (1:100; Santa Cruz Biotechnology, catalog no. sc-48). This antibody has been verified for specificity by both the manufacturer and in several previous studies (McClung et al. 2004; Perrotti et al. 2004; Berton et al. 2007; Nikulina et al. 2008). Producing a band of ~45 kDa using Western blotting 24 h after the final stress or control treatment, we would expect Iba-1 to be the only Iba-1 isoform present, as it has been shown previously that other Fos family proteins undergo proteolytic processing prior to this time (McClung et al. 2004). In total, 5 consecutive sections were processed for Iba-1 immunoreactivity; those selected being approximately 5.51, 5.53, 5.15, 2.97, and 2.79 mm rostral to bregma. Immunolabelling for major histocompatibility complex II (MHC II), CD68, and cleaved caspase-3, as well as an assay for terminal deoxynucleotidyl transferase (TdT) nick end labelling (TUNEL), were each performed on a single mPFC section per animal. All sections were processed simultaneously.

**Immunofluorescent Labeling of Microglia and Activated Neurons**

The following immunoperoxidase technique was used for each of the primary antibodies and has been previously described (Himwood et al. 2010; Tyman et al. 2010). Sections were incubated overnight at 4 °C with the primary antisera at the described dilutions, then washed, and incubated in the secondary antibody (biotinylated donkey anti-rabbit IgG; 1:500, Jackson; or biotinylated donkey anti-mouse IgG; 1:500, Jackson; catalog #711-005-152 and #715-005-150, respectively) at room temperature for 1 h. Immunoreactivity was localized using a nickel-enhanced glucose oxidase (3,3’-diaminobenzidine) reaction. The reaction was terminated when an optimal contrast between specific cellular labeling and nonspecific background was reached. All sections from all treatment groups were processed simultaneously. Control experiments using tissue sections from stressed and control animals without primary, or without primary and secondary antisera, were performed routinely for each antisem and with no nonspecific labeling observed in any case (data not shown). Imaging was performed using an Olympus IX51 microscope fitted with an Olympus DP71 camera.

**Immunofluorescent Labeling of Microglia and Activated Neurons**

A dual immunofluorescence protocol was used to detect levels of Iba-1 and FoSy immunoreactivity in the same section. Sections were incubated with primary antisera for Iba-1 and FoSy overnight at 4 °C. Sections were subsequently incubated for 2 h at room temperature with Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:400, Invitrogen) and Alexa Fluor 488-conjugated donkey anti-goat IgG (1:400, Invitrogen) to localize iNOS and Iba-1, respectively. Control experiments using tissue sections from control and stressed animals were conducted with each primary antisera singly and with both secondary antisera to ensure no cross-reactivity occurred. Imaging was performed using a Confocal Leica TCS SP2 laser scanning microscope equipped with an Olympus DP71 camera and an Olympus U-FL1 10X objective.
available within the Metamorph software package (Version 7.1.3.0; Molecular Devices). Procedures differed somewhat for immunoperoxidase and immunofluorescent images. For assessment of immunoperoxidase labeling, raw colored images were converted to grayscale and then into a binary image. A software routine was then used to quantify the proportion of black material. This form of threshold procedure is commonly used to quantify changes in immunoperoxidase labeled tissue (Abbade et al. 1996; Romero-Sandowal et al. 2008). For the assessment of fluorescent material, single color images (green or red) were thresholded using RGB channels. As changes in Ilol-A expression are known to occur across the entire surface of microglia, we adjusted settings so that when the threshold procedure was performed in the entire cell body, and its associated processes were captured. As such the resulting measure reflects the relative percentage of all thresholded material within the given region, which we have referred to as a percentage (density). As △ FosB is a nuclear antigen, we imposed a size restriction limit whereby only clusters of pixels 20–150 in number were captured. This effectively resulted in only △ FosB labeled nuclei being captured. This approach was highly correlated with manual counts of △ FosB positive cell bodies.

Cortical Layer Determination
Cortical layer depths for the infralimbic and prelimbic prefrontal cortex were determined according to the estimates established by Gabbott for the adult Sprague-Dawley rat (Gabbott et al. 1997) and used in a recent publication (Morshead and Meredith 2007). These layer coordinates were then used to create ROIs, and immunofluorescence was quantitated as set out in the previous section.

Experiment 2
Experimental Design
The same experimental groups and treatments were used in experiment 2 as were used in experiment 1. However, the sequence of events preceding and following the stress and minocycline treatment differed somewhat (see Fig. 8A).

Delayed Alternation T-maze

The T-maze apparatus consisted of a 60 cm (L) × 16 cm (W) × 30 cm (H) stem and two 45 cm (L) × 12 cm (W) × 30 cm (H) branch arms made of 9 mm marine ply. The bottom 20 cm of the stem was partitioned by a guillotine door and covered by a clear Perspex lid, forming a start box. Sliding partitions at the entry to each goal arm allowed blocking of the unused arm on forced trials. Haptic food wells were centered 5 cm from the end of each goal arm (see Fig. 8B).

Pre-DAT Manipulations
Animals were handled daily for 2 weeks to reduce neophobia; in this period, animals were individually housed and placed on a restricted diet to motivate performance. Animals during this time were daily had food rewards (mini M&Ms), in order to familiarize them with this process. Weight gain was maintained at approximately 85% of that of a group of free-feeding weight control animals.

DAT Familiarization
After handling, animals were familiarized to the T-maze apparatus over a period of 5 consecutive days. This involved 2 paired familiarization sessions of 10 min each, and 3 individual familiarization sessions of 5 min. During these sessions, food rewards were initially dispensed throughout the maze and progressively restricted to food wells, in order to shape the animals performance. The apparatus was cleaned between sessions with a 10% ethanol solution.

DAT Training Phase
After familiarization, T-maze alternation training was initiated, with animals learning to alternate arm choice on the T-maze apparatus each time a food reward was successfully retrieved. Training sessions consisted of an initial forced choice trial (in which a partition blocked one of the two goal arms), followed by two choice trials. The blocked arm was alternated between animals across days of training to ensure animals did not develop a place preference. Animals then underwent 9 rewarded alternation trials (where the forced choice partition was removed). Animals were placed in the start box facing the goal arms, and the guillotine door raised to allow access to the goal arms. When an animal reached a baited food well it was allowed to consume the reward and was then placed back at the start box. If the animal entered an incorrect arm, it was allowed to briefly investigate the empty food well and was then returned to the start box. The position of the food reward was alternated if it was successfully retrieved on the previous trial. T-maze training was conducted daily, until average group performance stabilized at 85% accuracy.

DAT Testing
Baseline delayed alternation T-maze (DAT) test performance was measured immediately before the commencement of the stress/ minocycline treatments. Animals were brought into the testing room individually and allowed to habituate (as described above). Each animal performed an initial 0-s delay baseline trial session, followed by a 30-s delay trial session (each trial session consisting of 1 forced and 9 test trials in succession). Animals were briefly returned to their home cage between sessions to allow cleansing and baiting of the apparatus. During the 30-s delay trial sessions, animals were restricted to the start box of the maze for the appropriate delay period and then released (by raising the partition). Poststress DAT Testing
Seventy-two hours after cessation of the stress protocol, poststress DAT performance was measured. Delayed alternation was conducted as described at baseline (involving 1 forced trial and 9 alternating trials at 0- and a 30-s delay).

Administration of Minocycline Hydrochloride
Minocycline hydrochloride was prepared and administered as described in experiment 1.

RestRAINT Stress
Restraint stress was conducted as described in experiment 1.

Data Analysis
Statistical analysis was conducted using PASW, Version 17 (SPSS Inc.) Differences in immunobinding and in T-maze performance were analyzed using either analyses of variance (ANOVAs) or analyses of covariance (ANCOVAs). Specifically, changes in immunoreactivity were analyzed using a repeated measures ANOVA, with treatment group (TG) as the between subjects variable and rostrocaudal level (RL) as the within-subjects variable. Analysis of Ilol-A and △ FosB immunoreactivity across cortical layers was performed using univariate ANOVAs. DAT performance was analyzed using ANCOVA, with baseline performance used as a covariate. A priori comparisons between the treatment groups were performed using independent samples t-test, and post hoc comparisons were performed using Fisher’s Protected Least Significant Difference test. Correlations between the densities of different immunoreactivities were analyzed using independent samples t-test, and post hoc comparisons employed Bonferroni’s corrections. ANCOVA and ANOVA assumptions, including homogeneity of variance, homogeneity of regression and linearity between the covariate and the dependent variable, were satisfied, except where otherwise noted. All statistical analysis was conducted with an α criterion of 0.05.

Results
Stress Induced Changes in Ilol-A Immunoreactivity within the Prefrontal Cortex and the Impact of Minocycline Administration

Infralimbic
We examined differences in immunoreactivity between the 4 treatment groups using a repeated measures ANOVA. This analysis indicated that there was a significant difference
between the means of the 4 groups ($F_{3,21} = 9.2, P < 0.0001$). Further analysis using a priori comparisons indicated that chronic stress group had significantly higher levels of Iba-1 immunoreactivity compared with handled controls ($P = 0.004$), and treatment with minocycline significantly reduced this effect ($P = 0.001$). No statistically significant differences were observed between the other treatment groups. See Figure 1A.

**Prelimbic**

Repeated measures ANOVA indicated that there was a significant difference between the means of the 4 groups ($F_{3,21} = 7.45, P < 0.0001$). Further analysis using a priori comparisons indicated that chronic stress group had significantly higher levels of Iba-1 immunoreactivity compared with handled controls ($P = 0.0008$), and treatment with minocycline significantly reduced this effect ($P = 0.001$). No statistically significant differences were observed between the other treatment groups. See Figures 1F, 2, and 3.

**Infrahmbic**

A repeated measures ANOVA indicated that there was a significant difference between the means of the 4 groups ($F_{3,28} = 5.12, P < 0.01$). Further analysis using a priori comparisons indicated that chronic stress group had significantly higher levels of Iba-1 immunoreactivity compared with handled controls ($P = 0.001$), and treatment with minocycline significantly reduced this effect ($P = 0.001$). No statistically significant differences were observed between the other treatment groups. See Figure 1A.

**Stress Induced Changes in ΔFoxD2 Immunoreactivity within the Prefrontal Cortex and the Impact of Minocycline Administration**

A repeated measures ANOVA indicated that there was a significant difference between the means of the 4 groups ($F_{3,28} = 8.4, P < 0.001$). Further analysis using a priori

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**Figure 1.** Quantification of Iba-1 induction in the infralimbic and prelimbic mPFC (A-E and F-J, respectively). (A) Bar graph showing average density of Iba-1-positive cells (± standard error of the mean) within the infralimbic mPFC of animals exposed to chronic restraint stress (STR), handled controls (CON), chronic restraint stress with minocycline administration (STR + M), or handled controls with minocycline administration (CON + M). * indicates that the level of Iba-1 immunoreactivity in the STR group was significantly greater than all other groups, $P < 0.05$. (B-F) Representative bright-field photomicrographs of Iba-1 immunoreactivity from the infralimbic mPFC of animals exposed to (B) STR, (C) CON, (D) STR + M, and (E) CON + M. (F) Bar graph displaying the average density of Iba-1-positive immunoreactivity (± standard error of the mean) within the prelimbic mPFC of STR, CON, STR + M, and CON + M groups. * indicates that level of Iba-1 in the STR group was significantly greater than all other groups, $P < 0.05$. (G-J) Representative bright-field photomicrographs of Iba-1 immunoreactivity from the prelimbic mPFC of animals exposed to (G) STR, (H) CON, (I) STR + M, and (J) CON + M. Scale bars = 50 μm.
comparisons indicated that chronic stress group had significantly higher levels of Iba-1 immunoreactivity compared with handled controls ($P < 0.0001$), and treatment with minocycline significantly reduced this effect ($P = 0.0390$). In this region, minocycline treatment increased neuronal activation significantly above that of handled controls for both the STR + M group ($P < 0.025$) and the CON + M group ($P < 0.035$). See Figure 4F.

**Impact of Chronic Stress and Minocycline Treatment on MHC-II and Caspase-3 Immunoreactivity in the Prefrontal Cortex**

MHC-II and CD68 labeling was undertaken to determine the extent to which chronic stress increased antigen presentation. Expression of these antigens on microglia are low under normal conditions but can be dramatically increased in response to tissue damage. Using our immunoperoxidase labeling approach we could find no evidence of MHC-II or CD68 within the parenchyma of the PFC (no images shown). In addition to MHC-II labeling, we also determined the levels of activated Caspase-3 and a TUNEL assay, both markers of apoptosis. We could find no evidence of either activated Caspase-3 or TUNEL labeling within the PFC (no images shown). This suggests that the changes we observed in microglial activity are not due to either inflammation or tissue damage.

**Relationship between Iba-1 and ΔFosB Immunoreactivity in mPFC of Stressed and Minocycline-Treated Animals**

Fluorescent double labeling of Iba-1 and FosB was performed on sections corresponding to 3.26-mm rostral to bregma and thus containing both the infralimbic and prelimbic mPFC. There was a strong positive correlation between Iba-1 and ΔFosB immunoreactivity in both the infralimbic ($r = 0.65, P < 0.01$) and prelimbic ($r = 0.66, P < 0.01$) cortices (see Fig. 5 for representative fluorescent images). Scatter plots that illustrate the quantitative relationship between Iba-1 and ΔFosB labeling are presented in Figure 6.

**Assessment of Density of Immunolabeling for Iba-1 and ΔFosB across Prefrontal Cortical Layers**

Separate univariate ANOVAs were used to analyze differences in the density (immunoreactivity/layer size) of Iba-1 and ΔFosB immunolabeling within each of the 5 layers (I, II, III, IV, and VI) of the infralimbic and prelimbic mPFC. The $F$ statistics for these ANOVAs are detailed in Table 1. The mean density levels for each of the layers (± standard error of the mean) are shown in Figure 7. Iba-1 immunoreactivity was increased across all cortical layers in animals exposed to chronic restraint stress, particularly layer V. This increase was significant when compared with all other groups in the IL. In the PL, levels were significantly increased compared with CON and STR + M groups only. ΔFosB immunoreactivity was significantly increased in animals exposed to chronic restraint stress in layers II-VI, particularly in layer II/III, when compared with other groups Figure 8.

**Minocycline Consumption**

Minocycline consumption across the 3 week administration window was compared in stressed and control animals. This comparison indicated that the average consumption of minocycline in the chronically stressed animals was 39.52 mg/day ± 2.5 and 40.31 mg/day ± 2.1 in control animals. An
period but minocycline administration did not.

![Graph showing average number of FoxP3-positive cells for STR, CON, STR + M, and CON + M.](image)

**Figure 4.** Quantification of FoxP3 induction in the infralimbic and prelimbic mPFC (A-E and F-J, respectively). (A) Bar graph showing average number of FoxP3-positive cells (+ standard error of the mean) within the infralimbic mPFC of animals exposed to chronic restraint stress (STR), handled controls (CON), chronic restraint stress with minocycline administration (STR + M), or handled controls with minocycline administration (CON + M), a indicates that FoxP3-positive cell number in the STR group was greater than all other groups, P < 0.05. (B-J) Representative bright field photomicrographs of FoxP3 immunoreactivity from the infralimbic mPFC of animals exposed to (B) STR, (C) CON, (D) STR + M, and (E) CON + M. (F) Bar graph displaying the average number of FoxP3-positive cells (+ standard error of the mean) within the prelimbic mPFC for the 4 experimental groups, STR, CON, STR + M, or CON + M. a indicates that level of FoxP3 in the STR group was greater than all other groups, P < 0.05. b indicates that the level of FoxP3 was lower in the CON group when compared with the STR + M and CON + M groups, P < 0.05. (G-J) Representative bright field photomicrographs of FoxP3 immunoreactivity from the prelimbic mPFC of animals exposed to (G) STR, (H) CON, (I) STR + M, and (J) CON + M. Scale bars = 50 μm.

Independent samples t-test indicated that the difference between groups was not statistically significant (P > 0.05).

**Weight**

The weights of animals in the 4 experimental groups were monitored for the duration of the experiment. Analysis of weight involved calculating the percentage weight gain of the final day of the stress exposure procedure relative to baseline weight. The percentage change figures for each group were compared using ANOVA. The analysis revealed significant group differences in weight gain across the experiment (F(3, 38) = 15.5, P < 0.001). Post hoc comparisons revealed that the STR and the STR + M groups weighed significantly less (28.8% ± 2.1 and 31.3% ± 3.4, respectively) than the CON and CON + M groups (32.3% ± 2.2 and 32.1% ± 2.0, respectively). No differences were found between the STR and STR + M groups or the CON and CON + M groups. These results indicate that the exposure to stress significantly reduced weight gain over the stress exposure period but minocycline administration did not.

**Delayed Alternation T-maze Training Data**

To ensure groups were broadly equivalent before treatment, delayed alternation trial scores for each animal were averaged across the final 5 days of training and compared between groups. ANOVAs indicated that there was no significant main effect of stress condition (P > 0.05), minocycline condition (P > 0.05), or interaction between these conditions (P > 0.05), indicating no preexisting group differences in alternation accuracy following 14 days of training. Averages for accuracy at the end of the 14 day training period for the 4 groups were: STR = 89.2% (±5.6); CON = 88.2% (±7.7); STR + M = 88.2% (±2.2); CON + M = 88.5 (±2.0).

**Poststress Delayed Alternating T-maze Performance**

To assess whether chronic restraint reduced accuracy on the DAT and whether minocycline administration attenuated this reduction, a 2 drug treatment (no drug, minocycline) × 2 stress condition (control, stress) ANCOVA was performed on trial accuracy in each delay condition: 0 and 30 s. The covariate in
end of the decreased accuracy was found to significantly contribute to the observed variance (P < 0.05). Planned comparisons indicated significantly lower accuracy in the chronically stressed animals (STR) compared to control animals (CON) (P < 0.02), the control animals that received minocycline (CON + M) (P < 0.01) and the chronically stressed animals that received minocycline (STR + M) (P < 0.02). No differences were observed between the other groups (P > 0.05) Figure 9.

Discussion
The present results provide evidence that microglia play a pivotal role in modulating the impact of chronic psychological stress on PFC neuronal activity and PFC-regulated behavior. First, it was shown that chronic stress induced an increase in activated microglia in the mPFC that was strongly and positively correlated with changes in local neuronal activity and also corresponded to a decline in working memory performance. Notably, these changes appeared unrelated to any form of tissue insult or neurodegeneration as there was no evidence of increased antigen presentation or apoptosis in the PFC. Secondly, consumption of a drug that dampened stress-induced activation of microglia also reduced PFC neuronal activation and reversed the stress-induced decline in working memory performance.

There is abundant evidence both that chronic psychological stress contributes to the development and progression of psychopathology, including unipolar depression and that the mPFC is likely to be one of the key brain regions involved in this process. Many studies have also shown that the mPFC plays a central role in regulating the stress response (Radley, Arias et al. 2006; Baratta et al. 2009) and displays major structural and functional alterations in response to chronic stress (McEwen 2005; Cerqueira et al. 2007; Czeh et al. 2008; Dias-Ferreira et al. 2009). The current study examined both the prelimbic and infralimbic regions of the mPFC. Although the 2 regions overlap in some functions (regulation of working memory and the stress response), there is also considerable evidence demonstrating that they also regulate discrete activities (Vertes 2004). The prelimbic cortex, for instance, has been found to be involved in “cognitive” processing such as the preparation of organized responses (Yang et al. 1996). In contrast, the infralimbic cortex appears to be involved in conditioned learning, as well as visceral/autonomic control (Gabott et al. 2005; Holmes and Wellman 2009). Our own work, and that of others, has consistently shown that the mPFC responds vigorously to the application of stressors (Figueiredo et al. 2003; Cerqueira et al. 2007; Hinwood et al. 2010; Tynan et al. 2010).

Most previous studies examining the effects of chronic stress on the mPFC have focused solely on neural changes, despite the recent growth in evidence that glia can play a significant role in modulating PFC activity (Cotter et al. 2001, 2002; Rajkowska et al. 2002; Rajkowska and Miguel-Hidalgo 2007; Banzar and Duman 2008; Schipke et al. 2011). Indeed, we are aware of only 3 studies that have examined PFC microglial responses to chronic stress, one of these being from our own group (Nair and Bonneca 2006; Tynan et al. 2010; Wohbeh et al. 2011). In the current study, we assessed microglial activity by quantifying the density of Iba-1 (also known as allograft inflammatory factor-1) immunolabelling. Iba-1 is a protein that acts to modulate membrane ruffling changes during microglial activation (Imai and Kohsaka 2002) and has been extensively used to identify microglia within the central nervous system.
Although it is constitutively expressed by microglia within the parenchyma, it is only moderately expressed by quiescent ramified microglia and not at all by astrocytes, oligodendrocytes, or neurons (Imai et al. 1996), the latter point being consistent with the fact that we saw no double labeling for Iba-1 and FosB in this study. Although equally specific labels for microglia within the parenchyma exist (such as the CD11b antigen), Iba-1 is preferable in at least 2 major ways. First, the quality of labeling obtained with Iba-1 is superior, which in our experience translates directly into significantly reduced variance in quantification. Secondly, and perhaps more importantly, the expression of Iba-1 has been shown to be directly related to microglial activation state. That is, Iba-1 has been shown to be only moderately expressed in quiescent ramified microglia but strongly expressed in the response to activating stimuli (Imai and Kohsaka 2002). Despite chronic stress significantly increasing the density of Iba-1 positive cells, there was no apparent increase in the numbers of cells immunoreactive for MHC-II, which is involved in presentation of processed antigen, or caspase-3, a putative marker of apoptosis. This is significant because it suggests that microglial activation was not the result of tissue insult or stress-induced neurodegeneration. Although it is the case that increased MHC-II expression has often been reported in association with microglial activation, such reports concern studies involving relatively direct insults to neural tissue, such as the intrathecal administration of lipopolysaccharide (Block et al. 2007; Moss et al. 2007).

One interesting aspect of our results was the relative magnitude of the change observed in the density of Iba-1 labeling across the treatment groups. The density of labeling across the entire mPFC was approximately 25% greater in chronically stressed animals. This result while significant is quite moderate when compared with >100% change shown to occur after the CNS has been challenged with, for example, an intracerebral injection of lipopolysaccharide (Fan et al. 2005; Wang et al. 2006). When Iba-1 positive cells from chronically stressed animals were examined at higher magnification, it was clear that they had transitioned from a ramified to a hyper-ramified state, characterized by an increase in cellular processes. Interestingly, however, we could find no obvious anatomical evidence of remodeling progressing past the hyper-ramified state. This phenomenon has elsewhere been referred to as arrested hyper-ramification and is a state not frequently reported in studies examining microglial responses to pathological events. Arrested hyper-ramification, however, has been shown to occur as a result of the normal aging process and after exposure to certain neurotoxins (Wilson and Molliver 1994; Strotet et al. 1999).

Increases in mPFC microglial activation were paralleled by changes in neuronal activation, the latter being assessed on the basis of immunodetection of FosB. FosB positive cells were observed in all cortical layers except layer I, with the highest density observed in layers II/III. FosB is a truncated splice variant of Fos and, in contrast to c-Fos, progressively accumulates in repeatedly activated neurons and persists there for several weeks (Chen et al. 1997; McClung et al. 2004; Perrotti et al. 2001). Because of this, an increasing number of studies use FosB as a marker of neuronal activation in Figure 5. Scatter plots illustrating the relationship between lba-1 and ΔFosB immunolabelling in the infralimbic (A) and the prelimbic (B) mPFC. Dotted line = line of best fit; r = Pearson correlation coefficient.

Table 1

<table>
<thead>
<tr>
<th>Layer</th>
<th>Area of Neurons</th>
<th>ΔFosB</th>
<th>Iba-1</th>
</tr>
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<td>Area</td>
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<td>14.0, P &lt; 0.002*</td>
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<td>Area</td>
<td>11.6, P &lt; 0.002*</td>
<td>11.5, P &lt; 0.002*</td>
</tr>
</tbody>
</table>

Note: *, a statistically significant difference among the 4 treatment groups.
Figure 7. Illustrates the density of lba-1 and ΔFosB immunolabelling (signal intensity/cortical layer size) within each of the 6 layers of the infra and prelimbic mPFC. Panel (A and B) illustrates the intralaminar layer densities for A) FosB and (B) lba-1. Panel (C and D) illustrates the prelimbic layer densities for (C) FosB and (D) lba-1. * indicates that the density of immunolabelling in the STR group was greater than all other groups, *P < 0.05. † indicates that the density of immunolabelling in the STR group was greater than the CON and the STR + M groups, †P < 0.05.

Figure 8. Panel A illustrates the experimental protocol and timeline for the assessment of working memory performance using the delayed alternating T-maze (DAT) test. T-maze Fam: Familiarization to the T-maze apparatus (BDAT). Baseline T-maze performance data collected prior to exposure to stress or minocycline. FD: Food deprivation to 85% of controls. PS(DAT) assessment of poststress working memory performance using the DAT test. The lower panel B depicts the delayed alternation T-maze (DAT) apparatus (see Materials and Methods for dimensions) and the forced trial alternation process with an animal in the "start box" immediately prior to trial. The food reward is located in the right goal arm, with left arm blocked by a sliding partition (forced choice [FC] partition). On the subsequent trial (panel C), the food position is alternated to the left goal arm with the rat successfully retrieving the reward. Panel D shows an incorrect arm choice.
response to repeated challenges, such as the chronic stress paradigm used in the present study. Although the antibody used in this study is a pan-Fos antibody directed against FosB and ΔFosB, it has now been shown in several reports that with a time of sacrifice greater than ~17 h after the final episode of stimulation, the only protein isoforms to be detected are ΔFosB (McClung et al. 2004; Perrotti et al. 2004; Nikulina et al. 2008). Therefore, as the CNS tissue processed in the present study was collected 24 h after the animals last exposure to stress, we will henceforth refer to FosB-like labeling as ΔFosB.

Exposure to chronic restraint stress increased ΔFosB immunolabeling in the mPFC, and this activation was significantly inhibited by the administration of minocycline. As ΔFosB is a marker of repeatedly activated neurons, the reduction of its expression in the mPFC by minocycline administration suggests that minocycline attenuates the neural response to chronic stress. This may be due to the inhibitory actions of minocycline on the local microglia. Previous studies have shown a neuroprotective effect of minocycline using in vivo models of stroke/ischemia (Yenari et al. 2006; Yang et al. 2007), however, to our knowledge, this is the first report of minocycline administration reducing neuronal activation after exposure to chronic stress.

Although our results for the prelimbic and infralimbic regions were generally similar, a difference was observed in the number of ΔFosB-positive cells in the prelimbic cortex between the minocycline-treated groups when compared with the untreated controls. Specifically, minocycline-treated animals had higher levels of ΔFosB than the untreated control group. The significance of this result, however, is unclear as the stress and control groups treated with minocycline were not significantly different from one another and both were still significantly less than the untreated stress group.

We also assessed changes in the Iba-1 and ΔFosB labeling across the layers of the cortex, as it is widely known that each possesses distinct afferent and efferent connections (Goodfellow et al. 2009). We observed a significant increase in Iba-1 density across all layers of the infralimbic and prelimbic cortices and increase in ΔFosB in all layers except layer I. With respect to ΔFosB-positive cells, we observed a peak in number in cortical layers II/III. This finding complements previous work, which has demonstrated that layers II/III of the mPFC undergo significant neuronal remodeling following chronic stress (Wellman 2001; Liston et al. 2006; Radley, Rocher, et al. 2006). Interestingly, Iba-1 density in the untreated stress group was significantly elevated in II/III but peak levels of labeling were found to occur in layer V. Previous work describing the effect of stress on deeper cortical layers has been patchy, although it is known that layers V/VI project to the striatum and play a role in working memory (Yang et al. 1996).

The ability of psychological stress to reduce the capacity of working memory in the rat has been well described (Aronsten and Goldman-Rakic 1998; Cerqueira et al. 2007; Bessa et al. 2009; Qin et al. 2009). Working memory deficits are also a characteristic feature of stress-related psychopathologies such as depression (Elliott et al. 1996; Weiland-Fiedler et al. 2004). In primates, including humans, working memory is primarily underpinned by the dorsolateral PFC (Sawaguchi and Goldman-Rakic 1994; Uylings et al. 2003). In the rat, however, it is the mPFC that subserves this function, among others (Moghaddam 2002). We used the delayed alternating T-maze (DAT) test to assess working memory because the DAT has been validated in numerous studies as a test of spatial working memory (Mizoguchi et al. 2009; Jablonski et al. 2010) and because mPFC damage has been shown to reliably degrade DAT performance in the rat (Brito-GN and Brito LS 1996; Qian et al. 2009; Radley, Rocher, et al. 2006). Spatial working memory in this task can only be assessed once the animals have learnt, over consecutive trials, to retrieve a food reward from the alternate side of the T maze. Once all animals have achieved a certain level of success (typically 85% correct), the load placed on working memory can be increased by holding the animal in the start chamber for a certain period before allowing them to run the maze. With regard to the choice of delay used to investigate working memory in both this and other studies, it is important to recognize that estimates vary both within and across species. In terms of the upper limit of working memory, it has been suggested to be in the vicinity of 30 s (Cowan 1999). As such, delay conditions of up to 30 s are generally considered to assess working memory performance, whereas, beyond this, it is thought to assess other forms of memory, such as long-term memory. In the current study, we observed that animals exposed to chronic stress made significantly more errors in the 30-s delay condition but not in the 0-s delay. These results indicate that exposure to chronic stress reduced the capacity of working memory but did not eliminate it. These findings are completely in line with an earlier study that also used chronic restraint stress (Mizoguchi et al. 2000).

To better determine whether the observed stress-induced changes in mPFC neuronal activity and mPFC-related behavior actually depend on microglial activation, we tested the effects of administration of minocycline. Originally recognized for its
antibiotic properties, this drug displays high bioavailability after oral administration, readily crosses the blood brain barrier and has been repeatedly shown to inhibit microglial activation (Tikka et al., 2001; Colovic and Caccia, 2003). Interestingly, microglial is currently undergoing clinical trials for use in neurodegenerative conditions where microglia are thought to play a significant role (Yong et al., 2001). We found that in addition to the anticipated decrease in stress-induced microglial activation, microglial also significantly reduced numbers of ΔFosB-positive cells in the pFC of stress-exposed animals and reversed the working memory deficit normally apparent in DAT tests of stressed animals. The latter finding constitutes, to the best of our knowledge, the first demonstration that a drug thought to act by modifying microglial activity can alter working memory performance.

Conclusions

Until recently, microglia were thought of primarily in terms of their relevance to neural defense and neuropathology. Their potential contribution to the modulation of signaling within the CNS has yet not been fully realized, and thus, their potential roles as contributors to the dysregulation of such signaling in conditions such as a psychopathology has received much less attention. The results of the experiments described in this report suggest that microglia play a significant role in determining the neuronal and the behavioral responses to chronic psychological stress and, as such, may potentially contribute to the development of stress-related psychopathologies such as depression.

Funding

Australian National Health and Medical Research Council; the Hunter Medical Research Institute; and the University of Newcastle's Centre for Brain and Mental Health Research.

Notes

We thank Ms. Briti Sadby for her technical assistance and Prof. Karin/Lage Maggiori for advice regarding the optimal conditions for using the delayed alternating T-maze. Conflict of Interest: None declared.

References


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Uylings HB, Groenewegen HJ, Kolb B. 2003. Do rats have a prefrontal cortex? Behav Brain Res. 146:3-17.


CHAPTER 4

Chronic stress induced remodelling of the prefrontal cortex:

Structural re-organization of microglia and the inhibitory effect

of minocycline.
CHAPTER 4: Chronic stress induced remodelling of the prefrontal cortex: Structural re-organization of microglia and the inhibitory effect of minocycline.


Submitted to Cerebral Cortex, December 2011.

Statement of author contributions to manuscript.

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<tr>
<td>Madeleine Hinwood</td>
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<td>Ross J. Tynan</td>
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Date: 17/11/13

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Deputy Head of Faculty (Research and Research Training)
Recently, it has been discovered that the working memory deficits induced by exposure to chronic stress can be prevented by treating stressed animals with minocycline, a putative inhibitor of microglial activity. One of the pressing issues that now requires clarification is exactly how exposure to chronic stress modifies microglial morphology, this being a significant issue as microglial morphology is tightly coupled with their function. To examine how chronic stress alters microglial morphology, we have investigated what was previously shown to be the first demonstration that a drug treatment that modifies microglial activity can improve a stress-induced cognitive impairment. 

While previous research has clearly implicated microglial alterations in mediating stress-induced cognitive disturbance, one of the fundamental questions that remain unanswer is how stress alters microglial morphology. This is a highly significant issue as it well recognized that a tight coupling exists between microglial morphology and function. Rio-Hortega (1932) was the first to recognize this relationship, and over the ensuing years, the phases of microglial transformation, and their coupled functional states, have been significantly elaborated (Streit et al. 1999; Stence et al. 2001). It is now recognized that under non-pathological conditions, microglia are frequently found to possess numerous thin processes emerging from the soma, with each primary process exhibiting some secondary and tertiary branching. This form of microglia (often referred to as ramified microglia) do not typically engage in injury-related activities but rather appear to be involved in monitoring synaptic integrity (Tremblay et al. 2010). In contrast, when the central nervous system (CNS) has sustained an injury, microglial processes are frequently observed to thicken and shorten, or retract altogether transitioning into what are referred to as amoeboid microglia. Microglia possessing this type of morphology are commonly found to engage in phagocytosis, and release of free radicals and proinflammatory factors (Graber and Streit 2010). 

To date, all published studies that have examined the effects of stress on microglia have used densitometry. This technique quantifies the changes in the intensity of microglial-specific immunohistochemical or immunofluorescent labeling and reveals population-wide changes within a given region of interest (Sugama et al. 2009; Tynan et al. 2010; Hinwood, Morandini et al. 2011; Wohleb et al. 2011, 2012; Jurgens and Johnson 2012). The limitation of this procedure, however, is that it does not provide specific information on how individual cells are altered. Therefore, at present, it is not known whether the morphological alterations induced by chronic stress are more consistent with an injury or a non-pathological phenotype.

As we have shown that chronic stress drives substantial changes in microglia within the medial prefrontal cortex (mPFC) and that inactivating microglia using minocycline reduces these changes and improves stress-induced working memory deficits, we undertook a study whereby we reconstructed and analyzed microglia of the mPFC to determine exactly how chronic stress alters their phenotypes. Specifically, we focused our attention on those layers of the mPFC that have previously been shown to be most influenced by...
exposure to chronic stress (Raley et al. 2006; Shansky et al. 2009). Our data indicates that chronic stress significantly increased the internal complexity of microglia, enhancing ramification without altering the area occupied by the cell. We found that these effects were restricted to the secondary microglial processes and were more pronounced in larger cells and that minocycline abolished the pro-ramifying effects of stress. In terms of identifying the potential molecular mechanisms involved in driving these alterations, we first examined changes in several proteins linked to CNS injury and inflammation [MHC-II, CD68, activated caspase-3, terminal deoxynucleotidyl transferase (TdT) nick end labeling (TUNEL), and interleukin-1β (IL-1β)]. We found no evidence that exposure to stress (or stress plus minocycline) elevated the levels of any of these markers. This result suggested that the microglial alterations occurring in response to stress were unlikely to be due to injury. Additionally, we examined the expression of β1-integrin (CD29), as recent evidence has demonstrated that this protein is involved in promoting ramification and that its levels are significantly reduced with minocycline treatment (Nutile-McMenemy et al. 2007). Our investigation revealed that chronic stress significantly increased microglial levels of β1-integrin, with greater levels being found in larger cells. We further observed that minocycline dramatically reduced β1-integrin expression in both stress and control groups. Collectively, these results clearly indicate that exposure to chronic stress induces a form of non-injury-related hyper-ramification that appears to be mediated by an increase in β1-integrin.

**Materials and Methods**

**Ethics**

Experiments were approved by the University of Newcastle Animal Care and Ethics Committee and were conducted in accordance with the NSW Animal Research Act and the Australian Code of Practice for the use of animals for scientific purposes.

**Experimental Design**

We used a 2 x 2 (stress condition: chronic restraint stress vs. handled controls) x 2 (drug treatment: minocycline vs. no minocycline) between-group experimental design. This protocol yields 4 experimental groups (n = 10 per group): 1) 21 days of restraint stress (STR); 2) 21 days of brief handling (CON1); 3) 21 days of restraint stress and administration of minocycline (STR + M); and 4) 21 days of brief handling and administration of minocycline (CON + M). All animals were sacrificed 24 h after the final episode of stress or handling.

**Experimental Animals and Treatments**

Adult male Sprague-Dawley rats (350-450 g; 70 days old at the commencement of the experiment) were obtained from the Animal Resource Centre (Perth, Western Australia). Animals were held in individual cages in temperature-controlled animal holding rooms (21 ± 1°C) on a 12 h reversed light-dark cycle (lights on at 19:00 h). All experimental procedures were conducted during the dark phase of the light cycle. Animals were adapted to individual housing for 7 days prior to any experimental manipulation and were maintained on standard rat chow and water provided ad libitum.

Restraint stress was conducted as described previously (Tynan et al. 2010). Rats were placed in wire mesh restrainers secured with butterfly clips for 6 h during the dark period of the light cycle. The restraint stress procedure was repeated once daily for 21 days. Control groups were handled twice daily for 2 min.

**Administration of Minocycline**

As previously described (Hinwood, Moorhead et al. 2011), minocycline hydrochloride (PCGA, Australia) was administered orally via drinking water at a dosage of 40 mg/kg/day. Minocycline was administered for the duration of the stress exposure period. We have previously found this to be an effective form of drug administration in rats, with the administered dose reaching a clinically effective level (Hinwood, Moorhead et al. 2011).

**Tissue Processing and Immunohistochemistry**

Twenty-four hours after the final day of the repeated stress protocol, animals were deeply anaesthetized with sodium pentobarbital and transcardially perfused via the ascending aorta with 2% sodium nitrite followed by 4% ice-cold paraformaldehyde. Brains were removed and post-fixed overnight in the same fixative and then placed into 12.5% sucrose in phosphate-buffered saline (PBS) for storage and cryoprotection. Brains were sectioned into 50 μm slices using a freezing microtome (Leica).

As previously described (Hinwood, Tynan et al. 2011), coronal sections (1 in 6 series) were incubated with a rabbit polyclonal antibody directed against the ionized calcium-binding adapter molecule 1 (Iba-1; 1:10000; Wakó Bioproducts; catalogue #019-1974) with 2% normal horse serum overnight. Iba-1 is a constitutively expressed calcium-binding protein which is specific to microglia (Ahmed et al. 2007; Inui and Koisaka 2002). The specificity of this antibody has been verified by the manufacturer and in a recent study using western blot analysis (Horvath and DeLeo 2009). Sections were washed and incubated with biotinylated donkey anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch; catalogue #711-005-582). Iba-1 immunolabeling was developed using a nickel-enhanced 3,3’-diaminobenzidine reaction. Brain regions were identified anatomically in accordance with the stereotactic rat brain atlas and Watson (2005). Negative control sections, in which one primary antibody was omitted at a time, were performed for all experiments.

Immunolabelling to exclude the possibility of microglial activation as a result of injury or apoptosis was performed for several markers of injury or inflammation. Specifically, we measured levels of MHC-II, a protein involved in the presentation of a processed antigen, CD68, a marker of microglial phagocytosis, and the apoptotic antibodies activated caspase-3 and TUNEL. The pro-inflammatory cytokine IL-1β was also examined, as it has previously been reported that other neuroinflammatory conditions (e.g., peripheral injection of lipopolysaccharide) increase amounts of IL-1β in the brain (Corona et al. 2010) and that microglia are a primary producer of IL-1β in the CNS (Ostermann et al. 2009). These were each performed on a single mPPG section per animal. Mouse monoclonal antibodies were used for the identification of MHC-II and CD68 (Serotec; catalogue: #MCA48G and #MCA41H1A, respectively), which are surface antigens expressed by microglia during the response to tissue damage. For the identification of cells undergoing apoptosis, we used a rabbit polyclonal antiserum directed against the cleaved p17 fragment of activated caspase-3 (Bethyl-Gene, catalogue #AB36253 and a TUNEL assay (Invitrogen). In addition to the experimental tissue, positive control tissue for apoptosis from rat mammary gland post-lactation was labeled to verify antibody effectiveness. For IL-1β, we used a rabbit polyclonal antibody raised against the C-terminus of IL-1β (Santa Cruz Biotechnology, catalogue #SC-7880).

**Reconstruction of Microglia and Associated Processes**

Slides were coded and data analysis was performed by an experimenter blind to the treatment condition, with the code not broken until all analyses were complete. Microglia in cortical layers III/IV of the infralimbic mPPG were reconstructed using a computer-assisted morphometry system consisting of a Zeiss Axiolab photomicroscope equipped with an MRC 6000 XTZ computer-controlled motorized stage and joystick with focus control (Hudl Electronic Products), a Q Imaging video camera (MBF Bioscience), a PC running Windows Vista (Dell Australia), and NeuroLucida morphometry software (MBF Biosciences). Microglia were visualized and reconstructed under a Zeiss Axio Plan NEOFUAR ×100 objective with a numerical aperture

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of 1.3 under oil immersion, using Neurolucida software (MBF Biosciences). Inclusion for analysis required that microglia were located in layer II/III of the infralimbic mPFC and exhibited intact microglial processes unobserved by either background labeling or other cells. A separate sample of cells in layer II/III of the secondary motor cortex, a region not known to display a microglial response to stress, was reconstructed for comparison to those in the mPFC. Microglia were traced throughout the entire thickness of the section, and trace information was then rendered into a 3D diagram of each cell (Fig. 1). Four cells per region were randomly selected, giving a total of 148 reconstructed microglia included for analysis from the mPFC: 40 from each of the STR and CON groups, and 54 from each of the STR+M and CON+M groups.

**Double Label Immunofluorescence and Confocal Laser Scanning Microscopy**

Double labeling for Isl-1 (goat polyclonal antibody to Isl-1; 1:500; Wako Biosciences; catalogue number 4949) was used in order to quantify the amount of Isl-1 within microglial cells of layer II/III of the IL mPFC. Both Isl-1 and β1-integrin were visualized using AlexaFluor-labeled secondary antibodies (488 or 594, respectively; 1:600; Molecular Probes). Briefly, free-floating sections were rinsed using PBS, incubated with a 3% bovine serum albumin (BSA)/0.3% Triton X-100 (TX-100) blocking solution and then incubated overnight at 4°C with a primary antibody cocktail. Antibodies were diluted in a 1% BSA/0.1% TX-100 blocking solution. Sections were rinsed and then incubated with appropriate secondary antibodies for 2 h. Sections were rinsed and mounted using anti-fade mountant (Citifluor). Localization of protein expression was performed using a Nikon CI confocal laser scanning microscope, with a ×100 objective. The double labeling was visualized by acquiring a Z-stack of confocal images (step size 0.5 μm), each with a resolution of 1024×1024 pixels. A minimum of 5 randomly selected cells per animal were imaged, from layer II/III of the IL mPFC.

**Analysis of Reconstructed Cells**

All analyses were conducted using NeuroExplorer software (MBF Biosciences). Initially, we examined several soma and process characteristics including cell body perimeter, number of processes, number of nodes (branch points), and total length and volume of cellular processes. A convex hull analysis was performed, which measures the area of the field defined by the processes, calculated as the area enclosed by a polygon that joins the most distal aspects of the cellular processes. A fractal analysis involved undertaking a box counting procedure (Fernandez and Jelinek 2001), which determines the fractal dimension (D). This value provides a measure of how completely the cell fills the space defined by its boundaries. Due to the considerable range in cell size, we also examined morphological characteristics for large (>2000 μm²) and small cells (<1000 μm²) consistent with the size-based approach to the morphometric analysis approach used by Badley et al. (2008). As the complexity of microglial cells varies as a function of the distance from the soma, we also undertook the Sholl analyses, for both the entire group, and separately for large and small cells. Specifically, this involved creating a series of increasing 5 μm concentric circles (radii) around the soma (Sholl 1956). Nodes (branch points), the number of processes that intersect the concentric radii, and process length, surface area, volume and average diameter were quantified as a function of the distance from the cell soma for each radius (see Fig. 1 for details).

For the quantification of β1-integrin (CD29) colocalized within microglial cells, Z-stacks of individual cells were imported into Neurolucida and condensed to a single tiff file of the maximal intensity profile of the entire image stack. Each level of the Z-stack was checked visually to ensure that β1-integrin labeling was located within the boundaries of the microglial cell. Metamorph imaging software was used to create a region around the cell of interest, and a thresholding procedure was used to quantify the amount of β1-integrin within each cell. In this way, we ensured that only double-labeled Isl-1/β1-integrin was counted.

**Statistical Analysis**

Morphometric parameters were averaged for each experimental group, and between-group differences were analyzed using one-way ANOVAs with post hoc tests. For the Sholl analyses, group means were compared using mixed-design between-subject (treatment groups: CON, STR, STR+M, STR+M) and within-subject (radial distance from the cell soma) ANOVAs. This was followed by planned comparisons of simple effects and post hoc testing to determine specific points of statistical significance. Additionally, we determined whether changes in process morphology occurred equally across the entire spectrum of cells, or whether they varied according to the cell size (similar to the technique used by Badley et al., 2008, in order to determine whether spine density varied according to the spine size). We examined all process characteristics for the lower 25th and upper 75th percentiles of cell area as calculated using a convex hull analysis for each experimental group. Thus, differences in morphology of cells below the 25th percentile, and above the 75th percentile, were examined between groups, using the same analysis as described above.

An independent samples Kruskal–Wallis non-parametric ANOVA was used to determine whether stress has caused a change in the overall numbers of small and large cells within the PFC. The number of small and large cells was compared in each of the 4 experimental groups.

For the analysis of β1-integrin labeling, raw counts of positive immunoreactive material were analyzed using one-way ANOVA to determine differences between the 4 treatment groups. Pearson’s correlations were run to determine the size of the association between the cell size and the amount of β1-integrin. The amount of β1-integrin in the lower 25th and upper 75th percentiles of cells according to the size was also examined.

In all cases, ANOVA assumptions, including homogeneity of variance, homogeneity of regression, and sphericity, were satisfied. Data are expressed as the mean ± SEM, and all statistical analysis was conducted using an α criterion of 0.05.
Results

The precise location of the reconstructed microglia was evaluated using cortical layer depths that have been established for the adult Sprague-Dawley rat (Gabrielli et al. 2005) and used in several recent publications (Monshedi and Meredith 2007; Hirwood, Morandini et al. 2011; Hirwood, Tyman et al. 2013). Layer II/III of the infralimbic mPFC ranges from 17.8% to 46.6% of the distance from the pial surface to the underlying white matter (see Fig. 1 for details).

Stress Increases the Number of Branch Points of Microglial Cells, and Minocycline Causes a Reduction in Cellular Processes and Area Occupied by the Cell

We assessed the average size of the cell body; number, volume and length of processes; branch points; and the total area of the cell as assessed by convex hull analysis for microglial cells from each experimental group using one-way ANOVAs. These analyses revealed that there was a significant effect of the group on branch points (F(3,141) = 3.99, P = 0.02). Post hoc tests revealed that stress increased the degree of branching by approximately 21% relative to handled controls and by approximately 39% relative to both minocycline-treated groups (all P < 0.05). There was a significant effect of the group on the number of processes (F(3,141) = 4.26, P = 0.013), process length (F(3,141) = 4.15, P = 0.01), and convex hull area (F(3,141) = 4.14, P < 0.01). Post hoc tests revealed that these metrics were all reduced in minocycline-treated groups compared with both STR and CON (all P < 0.05). Note that across groups, the k-dim value, cell body size, and total volume of processes remained unchanged by any treatment (see also Table 1).

The range of cell sizes as assessed by convex hull area across all groups varied between 601.56 and 4558.73 μm². We have therefore included descriptive statistics for the 25th (≤1800 μm²) and 75th (>2000 μm²) percentiles of cells according to their convex hull area (μm²), henceforth referred to as the cell’s “footprint” in order to describe the heterogeneity of the sample. In both stress and control groups, 50% of the surveyed cells were over 2000 μm². Note that stress predominantly affects the top 25% of cells. An independent samples non-parametric Kruskal-Wallis ANOVA was performed on all 4 groups to determine whether the distribution of cell sizes was significantly affected by either stress or minocycline administration. This was not significant (P = 0.33), which suggests that neither stress nor minocycline affects the ratio of small versus large microglia (Fig. 2).

The Effect of Stress on Microglial Cells is Magnified in the Upper 75th Percentile of Cells, an Effect Blocked by Minocycline

Analysis revealed that there was a significant effect of group on k-dim values (F(3,39) = 4.54, P = 0.008) branch points (F(3,39) = 6.6, P = 0.001), and process length (F(3,39) = 7.82, P < 0.001). Post hoc tests showed that stress increased the k-dim value (all P < 0.01), branch points (all P < 0.01), and total process length (all P < 0.005) in comparison to all other treatment groups. This suggests that stress increases the internal complexity (degree of branching) of the cells. There was a significant effect of group on convex hull area (F(3,39) = 5.89, P = 0.002). This was due to minocycline administration resulting in reduced cell area when compared with both STR and CON groups (all P < 0.01). Note that there are no differences between groups for soma size, number of processes, and process volume (see also Table 2).

There is no Effect of Stress on the Microglial Cells in the Lower 25th Percentile of Cells

There was a significant effect of group on the number of processes (F(3,39) = 3.4, P = 0.03), branch points (F(3,39) = 3.27, P = 0.05), process length (F(3,39) = 9.82, P = 0.001), and convex hull area (F(3,39) = 13.3, P < 0.001). While there was no effect of stress on the bottom 25% of cells, there was an effect of minocycline, with branch points, process length, and convex hull area reduced in minocycline groups compared with both STR and CON groups. Again, there is no difference between groups in the k-dim values, cell body size, process number, or process volume (see also Table 3).

Sholl Analysis for the Entire Sample Reveals That Stress Increases Branch Points in the Proximal Aspect of the Cell, and Minocycline Causes a Reduction in Primary Cellular Processes

The following parameters were quantified by the Sholl analysis: length, volume, and surface area of processes, average diameter of processes, intersections, and nodes (branch points; Fig. 3). Most effects of stress and drug administration occur in the proximal aspect of the cell. The most striking finding arising from the group-based Sholl analysis was the increase in branch points in cells from stressed animals between 18 and 28 μm from the cell soma. Other cellular properties overall appeared to be unaffected by stress. Minocycline had some effects on intersections, process length, surface area, and volume, reducing all of these measures typically between 8 and 28 μm from the cell soma, when compared with untreated groups. The average diameter of the processes was largely unaffected by either stress or minocycline (Fig. 3).

Branch Points

The proximal aspect of the cell contained the most branch points, with 92% of branch points occurring between 8 and 28 μm from the soma, regardless of treatment. Analysis of differences between the groups across each of the radii revealed a significant interaction between the radius and the

Table 1: Morphological characteristics of total microglial cell sample

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<th>CON</th>
<th>STR</th>
<th>CON + M</th>
<th>STR + M</th>
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| Cell mean
  projection (μm²) | 22.02 (0.08) | 22.02 (0.08) | 22.02 (0.08) | 22.02 (0.08) |
| k-dim | 1.020 (0.002) | 1.020 (0.002) | 1.020 (0.002) | 1.020 (0.002) |
| Number of
  processes originating from soma | 5.23 (0.22) | 5.23 (0.22) | 5.23 (0.22) | 5.23 (0.22) |
| Branch points | 12.00 (0.75) | 15.39 (0.90) | 15.39 (0.90) | 15.39 (0.90) |
| Total process length (μm) | 209.62 (14.9) | 209.62 (14.9) | 209.62 (14.9) | 209.62 (14.9) |
| Total process volume (μm³) | 210.14 (189.10) | 210.14 (189.10) | 210.14 (189.10) | 210.14 (189.10) |

*P < 0.05 compared with STR + M and CON + M groups.
**P < 0.05 compared with CON, STR + M, and CON + M groups.

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The analysis revealed that independent of treatment, 89% of the intersections between cellular processes and the radii superimposed upon the cell for the Sholl analysis occur 8 and 28 μm from the soma. Analysis of differences between the groups across each of the Sholl radii revealed a significant interaction between the radius and the group (F_{24,1104} = 2.44, P < 0.001). Planned comparisons revealed that this interaction was driven primarily by differences between treatment groups at radii 12, 18, and 23 μm from the soma (all P < 0.05). Post hoc comparisons revealed that the stress group was not different from control at any radius. There was, however, a clear effect for minocycline, with both drug-treated groups (STR + M and CON + M) possessing shorter process surface area at each of these levels than either the STR or the CON group (all P < 0.05).

### Surface Area

Analysis showed that independent of any treatment, 87.5% of the surface area of the cellular processes was between 8 and 28 μm from the cell soma. Analysis of differences between the groups across each of the radii revealed a significant interaction between the radius and the group (F_{24,1104} = 2.64, P < 0.001). Planned comparisons revealed that this interaction was driven primarily by group differences at 17, 18, 23, and 28 μm from the cell soma. Post hoc comparisons revealed that the stress group was not significantly different from control at any radius. However, there was a clear effect of minocycline administration, with both drug-treated groups (STR + M and CON + M) possessing a smaller process surface area at each of these levels than either the STR or the CON group (all P < 0.05).
**Volume**

Eighty-one percent of the volume of the cellular processes was between 8 and 23 μm from the cell soma. Analysis of differences between the groups across each of the radii revealed a significant interaction between the radius and the group \( (F_{24,4,094} = 2.43, P < 0.001) \). Planned comparisons revealed that this interaction was driven primarily by group differences at 8, 13, 18, 23, and 28 μm from the soma. Post hoc comparisons revealed that there was an effect of stress at 18 μm (STR significantly greater than all other treatment groups, all \( P < 0.05 \)) and an effect of minocycline, with STR possessing a significantly greater volume than both minocycline-treated groups at 23 and 28 μm from the soma (all \( P < 0.05 \)).

**Average Diameter**

The average diameter of the cellular processes decreases distally from the soma, with processes 42% thicker between 0 and 23 μm from the soma than the remainder. Analysis of differences between groups across each of the radii revealed a significant main effect of radius \( (F_{8,1101} = 161.66, P < 0.001) \).

**Sholl Analysis for Large Cells Reveals That Exposure to Stress Increases Branch Points, Intersections and Total Process Length in the Proximal Aspect of the Cell, an Effect Reversed by Minocycline**

In large cells (top 25%), we found no difference in cell size or soma size, or average diameter of processes. The most
prominent differences were contained to branching indices only; stress increased branch points (nodes), intersections, and process length 18–28 μm from the soma (Fig. 4).

Branch Points
Analysis of differences between groups across radii revealed a significant interaction between the radius and the group (F24,288 = 2.27, P < 0.001). Planned comparisons revealed that group differences were significant at 18, 23, and 28 μm from the soma (all P < 0.05). Post hoc tests indicated that this was due to STR possessing a greater number of branch points at these levels than CON, CON + M, or STR + M (P < 0.05).

Intersections
Analysis of differences between the groups across each radii revealed a significant main effect of both the radius (F24,288 = 145.88, P < 0.001) and the group (F24,288 = 6.85, P < 0.001). Planned comparisons revealed that these effects were driven primarily by significant differences between groups at 18, 23, and 28 μm from the cell soma (all P < 0.05). Post hoc comparisons revealed that there was a clear effect of stress, with STR possessing a greater number of intersections than any other group at 18, 23, and 28 μm (all P < 0.05).

Total Process Length
Analysis of differences between the 4 groups across each of the radii revealed a significant interaction between the group and the radius (F24,288 = 1.95, P = 0.006). Planned comparisons indicated that group differences were significant at levels 8, 18, 23, 28, and 35 μm from the cell soma (all P < 0.05). Post hoc comparisons revealed that there was a clear effect of stress, with STR possessing a greater process length at levels 18, 23, 28, and 35 μm than the other 3 groups (all P < 0.05).

Surface Area
Analysis of differences between groups across each radii revealed that there was a significant interaction between the group and the radius (F24,288 = 1.85, P = 0.011). Planned comparisons revealed that group differences were significant at 18, 23, and 28 μm from the cell soma (all P < 0.05). Post hoc tests indicated that this was due to a clear effect of stress, with STR possessing a significantly larger surface area than other groups at 18, 23, and 28 μm (all P < 0.05).

Volume
Analysis of differences between groups across each radii revealed a significant interaction between the group and the radius (F24,288 = 1.95, P = 0.007). Planned comparisons revealed that group differences were significant at 18 μm from the soma (P < 0.05). Post hoc tests indicated that this was due to an increased process volume in the STR group compared with CON and CON + M groups at this level (all P < 0.05).

Average Diameter
Analysis of differences between groups across each radii revealed that there was a significant main effect of the radius on average diameter (F24,288 = 23.52, P < 0.001). While planned comparisons revealed a group effect, this was at 43 μm from the cell soma only and was due to CON + M group possessing a greater diameter than the other 3 groups (all P < 0.05).

Sholl Analysis for Small Cells Reveals That There Is No Effect of Stress on any Index

Branch Points
Analysis of differences between the 4 treatment groups across radii revealed a significant main effect of the radius (F8,288 = 61.71, P < 0.001) and the group (F24,288 = 3.27, P = 0.032). Planned comparisons revealed that there were significantly greater numbers of branch points at 23 and 28 μm from the cell soma across all groups (P < 0.05). Post hoc tests indicated that there were no significant differences between groups at this level (Fig. 5).

Intersections
Analysis of differences between the 4 treatment groups across radii revealed a significant interaction between the group and the radius (F24,288 = 1.97, P = 0.005). Planned comparisons revealed that this interaction was due to a significant effect of the group at 18 μm from the cell soma (all P < 0.05). Post hoc tests indicated that this was due to CON possessing a greater number of intersections than STR + M, and STR possessing a greater number of intersections than CON + M.

Length
Analysis of differences between the 4 treatment groups across radii revealed a significant interaction between the group and the radius (F24,288 = 2.35, P = 0.001). Planned comparisons revealed that this interaction was due to a significant effect of the group at 18, 23, and 28 μm from the cell soma (all P < 0.05). Post hoc testing indicated that this was due mainly to a small minocycline effect, with STR + M being shorter than STR and CON at 18 and 23 μm.

Surface Area
Analysis of the 4 treatment groups across radii revealed a significant interaction between the group and the radius (F24,288 = 2.29, P < 0.001). Planned comparisons revealed that this interaction was due to a significant effect of the group at 23 and 28 μm from the cell soma (all P < 0.05). Post hoc tests revealed that this was due to an effect of minocycline administration, with STR possessing a greater surface area than STR + M at 18, 23, and 28 μm, and STR possessing a greater surface area than CON + M at 28 μm (all P < 0.05).

Volume
Analysis of differences between the 4 treatment groups across radii revealed a significant main effect of the radius (F8,288 = 97.27, P < 0.001). Planned comparisons revealed that this was due to a significant effect of the group at 23 and 28 μm from the cell soma (all P < 0.05). Post hoc tests indicated that this was due to an effect of minocycline administration, with STR possessing a greater process volume than CON + M and STR + M at both 23 and 28 μm (all P < 0.05).

Average Diameter
Analysis of differences between the 4 treatment groups across radii revealed a significant main effect of the radius (F8,288 = 91.55, P < 0.001). Planned comparisons however revealed no significant effect of group at any level.
Figure 4. Bar graphs depicting results from the Sholl analysis of large cells (top 75th percentile of sample) as a function of radius. Animals exposed to chronic stress exhibited greater numbers than other experimental groups of branch points, intersections, and process length, all indicators of secondary branching. Error bars represent SEM. *STR > CON, CON + M, STR + M. STR > CON and CON + M (P < 0.05).

Sholl Analysis of Secondary Motor Cortex Reveals no Effect of Stress on Microglia
The following parameters were quantified by the Sholl analysis for the secondary motor cortex: length, volume, and surface area of processes, average diameter of processes, intersections, and nodes. There was no effect of stress on any of these characteristics.

Exposure to Stress Causes Significant Reduction in Animal Weight
At the completion of the protocol, stressed rats weighed significantly less (ca. 15%) than handled controls (P < 0.001). This difference was primarily due to weight loss in the first week followed by a slower rate of gain over the remainder of the restraint period and is comparable to weight changes reported in other studies using the same protocol (Radley et al. 2006).

Immunolabeling Indicates That There Was Neither Evidence of Programmed Cell Death Nor Microglial Markers of Tissue Insult/Injury
We found no MHC-II, CD68, or activated caspase-3 immunoreactivity nor any cells positive for the TUNEL labeling in the mPF of animals from any experimental group (images not shown). We also examined sections for pro-inflammatory
cytokine IL-1β, and while there was inter- and extracellular IL-1β present in the tissue, there was no significant effect of stress ($P > 0.05$). This suggests that the changes we observe in microglial activity are not due to inflammation, tissue damage, or programmed cell death.

**Stress Increases β1-Integrin (CD29) Immunoreactivity**
Overall, stress significantly increases the level of β1-integrin immunoreactive material. A one-way ANOVA showed that there was a significant effect of the group on the levels of β1-integrin ($F_{3,122} = 30.74$, $P < 0.01$). Post hoc tests revealed that this was due to an effect of both stress and minocycline administration, with STR possessing a greater amount of β1-integrin than either the CON, STR + M, or CON + M group (all $P < 0.01$). The CON group also possesses a significantly greater amount of β1-integrin immunoreactivity than either the STR + M or CON + M group (all $P < 0.05$). For the lower 25th percentile of cells according to the size, there was a significant effect of the group on CD29 immunoreactivity ($F_{3,59} = 5.03$, $P < 0.01$). Post hoc testing revealed that this was due to the STR group possessing greater levels of CD29 immunoreactivity than either the CON, STR + M, or CON + M group (all $P < 0.05$). There were no significant differences between other groups (Fig. 6).
For the upper 25th percentile according to the cell size, there was a significant effect of group on CD29 immunoreactivity ($F_{3,29} = 16.79, P < 0.01$). Post hoc testing revealed that this was due to the STR group possessing greater levels of immunoreactivity than either the CON, STR + M or CON + M group (all $P < 0.01$). The CON group also had significantly greater levels of immunoreactivity than the CON + M group ($P = 0.012$; Fig. 6).

**Discussion**

To determine how exposure to chronic stress altered microglia, we undertook a study, the first of its type, to reconstruct microglia within the PFC, and quantitatively analyze changes in their morphology. These analyses revealed 6 major results: 1) chronic stress increased the level of microglial process branching, and this enhanced ramification was restricted to the secondary branches and above of the cell; 2) these effects were magnified in a subpopulation consisting of the largest but not the smallest cells in our sample, with stress increasing secondary ramification without altering the overall footprint of the cell; 3) administration of minocycline, an inhibitor of microglial activation, attenuated the effects of stress and appeared to induce mild de-ramification of microglial cells, an effect that was independent of experimental condition; 4) the stress-induced morphological alterations were relatively specific to the mPFC, as no observable changes occurred within the secondary motor cortex; 5) the stress-induced enhancement of microglial ramification is not driven by CNS inflammation or injury as we could find no evidence of increased expression of IL-1β, MHC-II, CD80, TUNEL or activated caspase-3; 6) we did, however, find that exposure to stress significantly increased the microglial-specific expression of β1-integrin and that this was blocked by minocycline treatment. Together, these results provide the first quantitative evidence that chronic stress promotes a specific form of microglial hyper-ramification and that this effect is associated with a stress-induced alteration in β1-integrin expression. Finally, the microglial phenotype induced by exposure to chronic stress is markedly different from those that have traditionally been observed in response to injury and most likely represents an adaptive response to this form of environmental challenge.

In the current study, we focused specifically on changes in the mPFC, as we had previously observed significant microglial changes in this region (Tyman et al. 2010; Hinwood, Mordonini et al. 2011). In these previous studies, we detected microglial alterations by quantifying region-wide changes in immunoreactive (Iba-1) material. While this approach is the most extensively used to examine changes in glial populations, it is based on a thresholding procedure (Sugama et al. 2007; Tyman et al. 2010), which is insensitive to specific

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**Figure 6.** Stress increases levels of β1-integrin immunoreactivity specific to microglia. (A) Levels of β1-integrin immunoreactivity in small microglia. (B) Levels of β1-integrin immunoreactivity in large microglia. In both groups of cells, stress significantly increases levels of β1-integrin relative to control, and minocycline reverses this effect. The effect of minocycline is more significant in large cells. The panel of images below depicts representative photomicrographs of double-labeled cells (Iba-1, green; and β1-integrin, red) from small and large cells of each experimental group. *$P < 0.05$. Scale bar = 10 μm.
changes in the phenotype. Accordingly, when differences are observed using this technique, microglia are simply referred to as ‘activating’. Certainly, differences can be made about the specific state of activation (reactive, amoeboid, etc.) when combined with qualitative observations of labeled cells; however, it is not clear how reliable these inferences are. The reconstruction approach undertaken in the current study circumvents the difficulties associated with an empirically establishing microglial phenotype.

While microglia respond to and interact with other cells within the CNS, they are unique, in that under normal conditions, they are uncoupled from one another (Graber et al. 2010; Graber and Streit 2010). Indeed, stereological evidence concerning the spatial distribution of microglia within the hippocampus has demonstrated that microglia appear to be repulsed from one another, with each cell maintaining its own independent footprint (Jinno et al. 2007). Our observations support this and have also revealed other characteristics of microglia not elsewhere reported. Microglia within the mPFC, independent of treatment (and excluding those treated with minocycline), cover approximately 2000 µm² based on their convex hull area, exhibit 5 processes extending from their soma (primary processes), and on average possess 12 branch points. The Sholl analyses further revealed that almost all features of microglia (length, thickness, and surface area of processes, as well as process branching) reached their maximum levels between 8 and 18 µm from the cell soma.

Our initial analysis of the morphology data gathered in the current study involved examining the cells in each of the 4 groups. This investigation revealed that stress significantly increased the number of primary branch points by approximately 20% in relation to all other groups. The magnitude of this difference is consistent with what we have previously observed when quantifying region-wide changes in immuno-reactive material (Yyan et al. 2010) and stands in marked contrast to the very substantial changes (upwards of 400%) that are frequently reported in response to tissue insults such as facial nerve axotomy (Kreutzberg 1996; Graber et al. 1998). Additional investigation of the branching data revealed one further intriguing difference. Specifically, we observed that there was no effect of stress on the number of primary processes (i.e. those emerging from the soma). This finding indicates that the overall difference in branching is driven primarily by alterations in secondary branching (or above), which suggests that alterations in microglial ramification can be restricted to particular components of the microglial process. Also of note was the fact that these stress-induced changes were not evident in the secondary motor cortex, an area not widely recognized to be altered by exposure to stress, a result indicating that the stress-induced alterations are somewhat circuit-specific.

Minocycline clearly blocked the effect of stress on microglial branching. Indeed, we observed that minocycline, independent of treatment, reduced the number of primary processes (20%), the degree of microglial branching (21%), the combined process length (19%), and the overall area that the cell occupied within the CNS (23%), relative to handled controls. As minocycline decreased several morphological parameters, including primary branching, and did so in both the stress and control groups, it would seem unlikely that minocycline achieves its effect by simply inhibiting the pro-ramification effect of stress. While this is the first in vivo study to quantitatively examine the morphological alterations produced by minocycline, our findings appear to align well with an extensive literature, indicating the ability of minocycline to enhance microglial quiescence (Yordanov et al. 1999; Tikka et al. 2001). One final point concerning the effectiveness of minocycline in blocking the effects of stress is that it remains to be determined how effective the drug, or at attenuating stress-induced changes when the commencement of treatment is delayed with respect to the onset of stress. From a clinical perspective, this is a highly significant issue and one that merits further investigation.

In our second analysis, we investigated whether the effects of stress were distinct for large and small microglia. This analysis was motivated by a previous study which demonstrated that stress-induced changes in neuronal architecture are size-dependent (Bradley et al. 2008). The results from this additional analysis revealed that the morphology of microglia was similar for small cells from the stress and control groups. For large cells, however, we observed pronounced changes, with stress substantially increasing branching and total process length relative to all other groups. Given that the overall area of large microglia from the stress and control groups was different, the stress-induced increase in branching and process length indicates that the “internal complexity” of the cell had increased. Indeed, this suggestion is supported by the fact that stress resulted in a significantly higher rlim, a measure that reflects how completely a fractal object (i.e. the microglial cell) fills the area defined by its boundaries. These alterations are intriguing as it suggests that stress produces a form of morphological alteration that would allow microglia to make more contacts, or scan more efficiently, within its microenvironment. Again we observed that minocycline reversed the effects of stress, significantly reducing the complexity of the cell branching.

One additional issue that emerges with regard to the ability of stress to increase the complexity of large cells is whether stress has caused a change in the overall numbers of small and large cells within the PFC. To address this issue, we examined the ratio of small to large cells in each of the 4 experimental groups. This analysis revealed that the ratio of small to large did not differ significantly across treatment groups. Accordingly, it appears that the ability of stress to enhance ramification is quite specific to the large microglial cells within the PFC.

The transition of ramified microglia to a hyper-ramified state has frequently been described in the literature (Streit et al. 1999). In the earliest study we could identify, Wilson and Molliver (1994) demonstrated that p-chloroamphetamine, which causes the degeneration of fine serotoninergic axon terminals, appeared to increase the number of microglial processes. It is worthwhile noting, however, that this study did not directly quantify whether processes had in fact increased. Indeed, none of the studies described within the literature that we could identify, which referred to hyper-ramification directly quantified whether microglial processes (primary or secondary) had increased (Hurley and Coleman 2003; Roberts et al. 2004; Herber et al. 2006). The question of what drives hyper-ramification is a significant one, as it has previously been proposed that hyper-ramification represents one of the earliest microglial transformations in response to injury (Streit et al. 1999). This proposition, however, appears to have been...
studies to observe microglial responses to injury in real time, Stence et al. (2001) found no evidence that microglia transitioned into a hyper-ramified state, either initially or at any other time. Given this situation, it will be of great interest to establish whether hyper-ramification should be considered as part of the injury schema or whether in fact it is a response to intense neuronal activity.

In investigating the signals responsible for driving the stress-induced hyper-ramification, we first examined a variety of markers associated with inflammation and neurodegeneration. Specifically, we examined changes in M1/M2 expression, a marker of antigen presentation; CD68 (ED-1), a marker frequently associated with microglial phagocytosis; IL-1β, a pro-inflammatory cytokine; and activated caspase-3 and TUNEL, both commonly deployed markers of apoptosis. This analysis revealed that none of these markers were elevated in animals exposed to chronic stress (or stress plus minocycline). This result, in combination with our previous densitometry data indicating that the magnitude of changes induced by stress is quite moderate, suggests that stress-induced changes in microglial morphology are unlikely to be due to CNS injury.

We next examined changes in the expression of β1-integrin (CD29), a heterodimeric cell adhesion transmembrane protein that has been shown to play a role in microglial ramification (Kloss et al. 2001). Specifically, it has been shown that microglial-specific β1-integrin expression is dramatically up-regulated in a variety of pathological conditions. Further, Obasawa et al. (2010) have provided compelling in vitro evidence, demonstrating that β1-integrin mediates adenosine triphosphate-induced process extension. In the current study, we observed that chronic stress significantly increased microglial β1-integrin expression. While this was significant at the group level, we wished to determine whether this effect was size-dependent, in line with our other results. This analysis revealed that β1-integrin expression was increased in both small and large microglia, a result that indicates if β1-integrin does mediate the increased ramification of large cells it does so in concert with yet unidentified factors that are not present in smaller cells. We further observed that minocycline treatment significantly reduced the expression of β1-integrin following stress in both small and large cells. These results suggest that increased ramification observed following chronic stress may be mediated by stress-induced disruption of interactions between the cell and the extracellular matrix.

Summary and Future Directions

The major finding to emerge from our current study is that exposure to chronic stress induces several readily quantifiable alterations in microglial morphology. Specifically, we have observed that stress induces a unique form of hyper-ramification, whereby only branching in secondary processes (and above) is increased and this occurs without any significant change in process diameter. Strikingly, chronic stress does not alter the overall area occupied by the cell within the brain (i.e. its footprint), a result, which when taken together with the observed change in branching, indicates that exposure to stress significantly increases the internal complexity of PFC microglia. This result significantly extends existing knowledge concerning the effects of chronic stress on microglia. Previously, densitometric analysis of microglial changes in microglial-specific cytoskeletal-linked proteins (β1-integrin) has only been able to indicate that the microglia had changed, and now, it is clear at the cellular level exactly how they have been altered.

With respect to signals driving the stress-induced secondary hyper-ramification, we have again confirmed that chronic stress, using the repeated restraint paradigm, does not induce any measurable alterations in proteins associated with inflammation or neurodegeneration (Mattsson et al. 2006; Graber et al. 2011). This finding, rather than being surprising, is consistent with observations that microglial de-ramification is associated with an increased level of inflammatory signaling within the brain (Wynne et al. 2009; Kettemann et al. 2011).

In contrast to the rich literature concerning microglial de-ramification, research on hyper-ramification is sparse. Certainly, several in vitro investigations have investigated this issue, but they have primarily addressed the question of what conditions are necessary for amoeboid microglia (the forms that are typically observed in standard culture conditions) to develop processes equivalent to those seen in vivo. This research has identified a variety of factors that promote ramification such as astrocyte conditioned medium (Wilms et al. 1997), gangliosides (Park et al. 2008), and antioxidants (Heppner et al. 1998). Among the most consistently identified molecules associated with ramification has been β1-integrin (Kloss et al. 2001; Obasawa et al. 2010). In the current study, the effect of stress on β1-integrin was unequivocal. This finding is extremely interesting as it suggests that stress may be involved in disrupting either the extracellular matrix, and/or the preparations that microglia employ to adhere and interact with the matrix. This is a completely novel area of stress-related microglial research, and accordingly, many issues are yet to be investigated. Perhaps, most pressing is the temporal profile of alterations induced in microglial specific β1-integrin expression, and changes in the expression of other integrins that are also known to contribute to extracellular matrix adhesion.

Finally, in terms of placing the current set of results within a broader neurobehavioral context, we have previously reported that the chronic stress paradigm used in the current study produces a significant working memory impairment and that this impairment occurs in conjunction with an increase in the density of PFC microglial labeling (Hinwood, Morandini et al. 2011). We also reported that the microglial alterations and working memory deficit could be reversed by minocycline treatment (Hinwood, Morandini et al. 2011). Merging our current results with our prior findings, we now have reasonable evidence to suggest that stress-induced microglial hyper-ramification is associated with the emergence of working memory deficits and that reversal of this hyper-ramification is associated with the restoration of working memory performance. Accordingly, we propose that the increased ramification of microglia represents an important neurobiological mechanism mediating the neurobiological effects of chronic psychological stress on the PFC.

Funding

These studies were supported by funding from the Australian National Health and Medical Research Council, the Hunter Medical Research Institute, and the University of Newcastle's
References


CHAPTER 5

General discussion and future directions
CHAPTER 5: GENERAL DISCUSSION

Depression is a leading cause of disability worldwide (Mathers et al. 2006), and has the highest amount of non-fatal burden of disease measured in terms of disability-adjusted life years (Ustun et al. 2004). Current recommended pharmacotherapies for moderate to severe depression are not efficacious for all users. This unmet need represents our inadequate understanding of the neurobiology underpinning depression. The focus of the body of work presented in this thesis was to develop a greater understanding of changes to neurons and microglia following exposure to chronic psychological stress, a frequent antecedent to the development of depression in humans.

The link between stress and depression

It is thought that chronic stress disrupts or alters the activity of vulnerable brain regions, and thus leads to psychopathology. The mPFC, the focus of the work presented in this thesis, has been implicated as one such region in both human and animal studies. Imaging studies in humans have shown that mPFC dysfunction is correlated with the cognitive and affective alterations symptomatic of stress-induced psychopathology (Shin et al. 2005; Drevets, Price, et al. 2008). Further human studies have shown that mPFC dysfunction is correlated with abnormalities in neuroendocrine control (Drevets, Price, et al. 2008; Heim & Nemeroff 2009). Animal models have implicated the mPFC in the regulation of HPA axis activity in response to psychological stress and control of the emotional stress response (Cerqueira et al. 2008). Whilst these studies have shown that the mPFC can modulate the HPA axis response to stress, recent evidence suggests that this region is also a target of the effects of
repeated stress. Chronic exposure to psychological stress has been shown in a number of report to reversibly reduce dendritic length and spine density within the rat mPFC (Cook & Wellman 2004; Radley et al. 2005; Liston et al. 2006; Radley, Rocher, et al. 2006; Radley et al. 2008; Shansky et al. 2009). The mPFC controls a number of cognitive processes including working memory and cognitive flexibility, and performance on mPFC-dependent tasks is susceptible to decline with exposure to stress, particularly chronic stress (Arnsten & Goldman-Rakic 1998; Cerqueira et al. 2007). The neurobiological correlates of this stress-induced decline in the mPFC structure and function remain essentially unknown, particularly in regards to specific cellular changes. Glial cell responses to chronic stress in particular have remained largely unexplored. Investigation of how repeated psychological stress affects neuronal responses and microglial cell morphology may assist in providing a cellular basis for the behavioural changes associated with exposure to chronic stress, particularly those relevant to stress-related psychopathology. As such, the experiments outlined in this thesis were aimed at increasing our knowledge of the changes to neurons and microglia in the rat mPFC after exposure to chronic psychological stress. The results of these experiments have allowed us to add to a growing body of literature regarding the role of microglia in brain and behavioural responses to psychological stress. It is hoped that improved understanding of the cellular responses to chronic stress in the healthy adult rat brain will lead to a better understanding of how stress-related psychopathology may develop, and subsequently to the development of better treatments for these disorders.

This studies presented herein have utilised primarily immunohistochemical techniques to characterise neuronal and glial cell changes in the mPFC following exposure to chronic psychological stress. Additionally, we examined how changes to microglial cell activation influence stress-induced deficits in working memory.
Study 1:

As demonstrated in Chapter 2, exposure to repeated social defeat stress increased numbers of neurons immunoreactive for ΔFosB in the infralimbic medial prefrontal cortex. As this protein accumulates within neurons with repeated stimulation, this represents the cumulative, or longer term, impact of psychological stress on the mPFC. Significant findings from this study include highlighting the importance of the use of appropriate controls when assessing specific patterns of neuronal activation resulting from exposure to an experience, in order to partial out those neuronal responses specifically associated with the exposure of interest. The FosB positive neurons in experimental animals were shown to be glutamatergic pyramidal projection neurons, mainly located in layers II/III of the infralimbic cortex. The specificity of this unique result may be due to differences in afferent and efferent connections of the infralimbic vs. the prelimbic cortex, which is discussed in this chapter. This result concurs with findings from other experiments which show that this population of neurons is particularly sensitive to the effects of stress. This includes studies demonstrating an accumulation of ΔFosB in the same cells of the mPFC with exposure to chronic stress or drugs of abuse (Perrotti et al. 2004; Olausson et al. 2006; Berton et al. 2007; Nikulina et al. 2008), and further a developing body of evidence showing that these neurons display evidence of dendritic retraction and spine loss after exposure to chronic stress (Cook & Wellman 2004; Liston et al. 2006; Radley, Rocher, et al. 2006; Radley et al. 2008). Whilst it has been known for some time that the mPFC inhibits the HPA axis response to stress, particularly once it is perceived that the immediate threat presented by the stressor has passed, the specific neurobiological alterations in this region after chronic stress are incompletely described. This study provides some further evidence towards this
goal, showing that exposure to chronic stress selectively activates certain neurons repeatedly in layers II/III of the mPFC.

Study 2:

In this study, we showed that microglia become activated in the mPFC following exposure to chronic psychological stress. Administration of minocycline, a pharmacological inhibitor of microglial activity, reversed the neurobiological and behavioural effects of stress. This suggests that microglia may play a pivotal role in modulating the effects of stress on FosB-positive neurons and mPFC-dependent behaviour. The changes to the microglial cells were not due to injury, infection or neurodegeneration, and so are thought to represent experience-dependent modifications to these cells, as opposed to a traditional injury or infection phenotype. Qualitative observations suggested that the microglia in the stressed animals were presenting a hyper-ramified phenotype, which isn’t commonly described in the stereotypical microglial response to injury. These observations add to the literature regarding stress induced modifications to microglia. Interestingly, administration of minocycline attenuated both the microglial and the neuronal response to stress, suggesting that the activity of microglia (and its suppression by minocycline) impacts upon the neuronal activation in response to stress. Additionally, we showed that minocycline administration improved stress induced deficits in working memory, an aspect of cognition that is mPFC-dependent. This may provide a cellular basis for the effects of repeated psychological stress on working memory, a cognitive change symptomatic of a variety of stress-induced psychopathologies.
Study 3:

In the final study presented in this thesis, we found that exposure to chronic restraint stress increased the level of branching, or ramification, in the secondary processes of microglia. This effect was magnified in a subpopulation consisting of the largest microglial cells. Administration of minocycline reversed the effects of stress, and caused some deramification of cells independent of experimental condition. The hyper ramification promoted by stress appears to represent a new microglial phenotype, inconsistent with the well-described phenotypic transformation that microglia typically undergo in response to CNS injury.

Collectively, these studies represent new insight and provide future directions into neurobiological alterations occurring in the mPFC after exposure to chronic psychological stress. This includes identification of neurons which are chronically activated and activation of microglial cells, as well as the first report of the stress-induced change to microglial morphology. These results highlight the importance of the mPFC in responding to stress. The mPFC mediates some of the highest cognitive tasks and decision making processes, and its capacity to do this is compromised by exposure to stress. A growing body of research shows that these impairments are correlated with morphological changes in mPFC neurons in layer II/III. The results presented in this thesis further highlight the changes to neurons and microglia in these layers after exposure to chronic psychological stress.
**Future recommendations**

Based on the findings presented in this thesis, there are several opportunities for further investigation, such as investigating changes to microglial cell morphology across the mPFC. A logical progression of these studies would involve investigation of microglial cell morphology across the entire breadth of the mPFC, and along its rostral-caudal axis. Changes in microglial cell morphology associated with changes in either neuronal activation or spine morphology also warrant further investigation, and could initially be investigated utilising double-labelling techniques. In addition, further investigation into the behavioural correlates of these cellular changes is required. Whilst this body of work suggests that microglial activation in responsible for some of the neuronal activation associated with exposure to stress, as well as stress induced deficits to working memory, there is scope to extend this work further. This could focus on the mechanisms involved in microglial activation and morphological alterations associated with exposure to chronic stress, and whether pharmacological inhibition of these changes improves outcomes on neuronal and behavioural modifications.

Further work towards understanding the neuroinflammatory hypothesis of depression could include a focus on the role of pro-inflammatory cytokines and stress-related neurotransmitters, in order to elucidate the relationship between neuronal and microglial cell activation by chronic stress. Gaining an understanding of the interactions between these cell types, rather than studying the behaviour of each in isolation, will provide a greater insight into possible dysfunction underlying stress-related psychopathology.
Neuroinflammation mediated by glucocorticoids

Glucocorticoids are largely considered to be anti-inflammatory, and the mechanisms underlying these effects in the periphery are well understood and even exploited clinically in a number of therapies (Barnes 1998; Rhen & Cidlowski 2005). Recently, however, this view has been challenged, with glucocorticoids appearing to have a pro-inflammatory effect in the CNS under certain conditions (Szczepanik & Ringheim 2003). Whilst basal levels of glucocorticoids, and levels reached during the physiological response to an acute stressor are largely anti-inflammatory, high stress or prolonged secretion of glucocorticoids increase measures of cellular inflammation. Investigations into the bi-directional communication between the immune and central nervous systems have shown that prior exposure to stress and glucocorticoids can ‘prime’ the immune system to intensify its response during subsequent exposures to a stressor (Maier 2003). For example, rats exposed to chronically administered high dose corticosterone experience increased damage to the neurons of the hippocampus following an excitotoxic lesion, and show accelerated and exaggerated microglial activation (Sorrells & Sapolsky 2007). Animals exposed to chronic unpredictable stress show enhanced microglial activation following direct injection of lipopolysaccharide, a bacterial endotoxin, to the PFC (de Pablos et al. 2006). This ‘stress priming’ of the immune response appears to be mediated by glucocorticoids, as blockade of glucocorticoid receptors inhibits this effect. As part of the neuroinflammatory process, activated microglia produce a number of pro- and anti-inflammatory mediators, including cytokines, nitric oxide and complement factors.
Central proinflammatory cytokines increased after stress: Challenging the view that glucocorticoids are immunosuppressive.

Evidence suggests that microglia are the cellular source of the pro-inflammatory cytokine IL-1β in the CNS following stress (Blandino et al. 2006). Further, results from a number of studies suggest that levels of pro-inflammatory cytokines, both peripherally and centrally, are increased with exposure to stress (Maes et al. 1998; Goebel et al. 2000). This occurs in the absence of any injury. Blocking the activity of IL-1β using a receptor antagonist reduces endocrine (monoaminergic and glucocorticoids) and reverses behavioural effects of stressor exposure (Maier & Watkins 1998). Additionally, the central administration of IL-1β replicates some of the behavioural outcomes of exposure to stress, including learning deficits and learned helplessness (Bluthe et al. 1996; Maier & Watkins 1998). These findings provide evidence that IL-1β is an important mediator of the response to stress, and it is thus of interest to explore the mechanisms by which stress regulates production of IL-1β in the CNS.

Stress priming of the neuroinflammatory response to infection mediated by microglia

The hypothalamic increase in IL-1β may occur as a result of direct action on microglia. Systemic administration of a microglial inhibitor (minocycline) blocks the IL-1β response to footshock in the hypothalamus (Blandino et al. 2009). This is mediated by norepinephrine at adrenergic receptors on microglia. In the same study, minocycline administration had no effect on behavioural response in a basic conditioned fear paradigm, and has been previously shown not to have any behavioural effects in the forced swim test. This suggests that the actions on microglia are unlikely to be the result of any nonspecific cognitive
alterations produced by the drug (such as sedation). The cross sensitization between the neuroinflammatory sequelae of stress and pro-inflammatory immune activation has been well characterised. Acute and chronic stressors sensitize the neuroinflammatory response to both peripheral and central immune challenges. For example, exposure to chronic unpredictable stress potentiates the increase in hippocampus and mPFC expression of pro-inflammatory mediators produced by a peripheral injection of LPS (Munhoz et al. 2006). The activation of microglia could provide a mechanism by which certain stressors elicit priming-like effects towards later immune challenge. Further work is required to assess differences in this effect for acute and chronic stressors, and to assess associated changes in neuronal populations.

**Neuroinflammation and regulation of glutamate uptake by microglial cells**

Several lines of evidence implicate glutamate as having a role in stress and depression. Glutamate is the primary excitatory neurotransmitter and mediates complex cognitive tasks, including learning and memory. Glutamate neurotransmission is tightly controlled, as excessive levels are neurotoxic. The concentration of glutamate in the synaptic cleft is efficiently controlled by a family of high affinity glutamate transporters which are most abundant on astroglia. Glutamate is increased in several brain regions in both animal models of chronic stress, and in people with clinical depression (Moghaddam et al. 1994). Additionally, the well-known antidepressant effects of ketamine are due to its action as a NMDA receptor antagonist (Berman et al. 2000). It has been suggested that the dendritic remodelling caused by stress is mediated by a rise in extracellular glutamate, and subsequent over-stimulation of NMDA receptors (Sorrells & Sapolsky 2007; Sorrells et al. 2009). Interestingly, treatment with the antidepressant SSRIs citalopram and paroxetine
attenuate an evoked increase in glutamate release (Langman et al. 2006). The extrasynaptic glutamate resulting from exposure to chronic stress may be excitotoxic, and thus deleterious to neuronal function. Microglia possess glutamate, and have an ability to sense its synaptic release (Takeuchi et al. 2006). The inflammatory environment established by activated microglia may explain the alterations in glutamatergic neurotransmission observed during stress and depression. Whilst glutamate-mediated excitotoxicity in neurological and psychiatric disorders has been widely studied, the influence of pro-inflammatory mediators on glutamate uptake has only recently been described. It is thought that pro-inflammatory mediators released from activated microglia during stress act to reduce the efficiency of astrocytic uptake of glutamate, whilst simultaneously increasing levels of extra cellular glutamate by increasing the rate of its synthesis via the enzymatic pathway specific to microglia (Barger & Basile 2001; Tilleux & Hermans 2007). Pharmacological blockade of IL-1β or its receptor attenuate these effects, allowing normal excitatory amino acid removal of glutamate by astroglia and reducing levels of extra cellular glutamate. As microglia are thought to be the primary source of IL-1β released during stress, they may be in control of the potentially neurotoxic effects of excess glutamate release during stress, and provide a possible mechanistic link between neuroinflammation and excitotoxicity which frequently co-exist in stress related disorders.

**Final comments**

The results presented in this thesis have advanced our knowledge of the responses of neurons and microglia in the mPFC following exposure to chronic psychological stress. In particular, the involvement of microglia in the neural response to stress offers a completely novel way of potentially modulating the adverse outcomes associated with exposure to
chronic stress. It is hoped that these results, in combination with the findings of others, will one day allow the development of superior treatments for stress-related psychological disorders which are rapidly increasing in frequency and morbidity, and are heading towards being a leader in the global burden of disease.
REFERENCES


Mathers CD, Lopez AD, Murray CJL. 2006. The Burden of Disease and Mortality by Condition: Data, Methods, and Results for 2001. In: Lopez AD, Mathers CD, Ezzati M,


Uylings HB, Groenewegen HJ, Kolb B. 2003. Do rats have a prefrontal cortex? Behav Brain Res 146:3-17.


