The impact of stress on glia:

A characterisation of the chronic stress-induced density and morphological alterations observed in astrocytes and microglia

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Declaration

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__________________________  ______________________
Ross Tynan                   Date
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Metaphorically, I think that for me, the PhD process has been much like an intense roller coaster ride. It most certainly has had its ups and downs, twists and bends, and a number of loops along the way. However, over the course of this ride, I have met some of the most amazing and intelligent people, and it is with great pleasure that I now have the opportunity to acknowledge the assistance that they have provided me, to get to where I am today.

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Abstract

Stress is a ubiquitous sensation that everyone has experienced at some stage in their lives. Whilst in the short term, our response to stress is often essential and indeed sometimes even beneficial, excessive or prolonged exposure can be quite damaging. It is therefore not surprising that stress has been continuously associated with the development and/or exacerbation of a number of both pathological and psychopathological conditions. For instance, stress has frequently been implicated in the development of depression. Whilst the precise aetiology of depression remains elusive, a number of neurobiological models have been devised in an attempt to better our understanding. One of the most prominent models in recent literature is the neuroinflammatory model, which suggests that depression is associated with a dysregulation of inflammatory processes. Within the brain, the primary cells responsible for both responding to and synthesising pro-inflammatory molecules are a class of cells known as glia. The primary focus of the work presented within this thesis is to empirically investigate whether chronic stress, a common antecedent to depression, can induce significant perturbation to two of the primary subtypes of glial cells, astrocytes and microglia.

Initially (Chapter Two), it was necessary to characterise both the behavioural and physiological response to the stressor used in the current investigations, chronic restraint stress. Animals exposed to chronic restraint stress exhibited a number of physiological responses to stress, including a sustained reduction in weight gain and a significant elevation in core body temperature. Furthermore, they manifested a number of behaviours typically used to indicate depression in animal models, such as a significant stress induced anhedonia (as indicated by a decrease in sucrose preference), and also a learned helplessness like behaviour (as indicated by a significant reduction in struggling behaviour during restraint). The results of these preliminary investigations verified the effectiveness of the restraint
protocol used to induce behavioural changes consistent with what is commonly reported within the literature.

The experiments described in Chapter Two also demonstrate, using immunohistochemistry, that chronic stress could induce significant modulation of microglia. Specifically, it was shown for the first time, that ionized calcium adaptor protein-1 (Iba1), a protein marker constitutively expressed by microglia, was significantly increased following exposure to chronic stress. The observed increase occurred in a number of brain regions that have been previously identified as crucial in regulating the stress response, such as the medial prefrontal cortex, the hippocampus, periaqueductal grey, bed nucleus of the stria terminalis and the amygdala. While this increase in cell density is consistent with a shift in the activation status of the cell, we could find no appreciable change in the levels of major histocompatibility complex II, a protein marker often associated with microglial activation. Nevertheless, these findings provide a novel insight into the changes associated with chronic stress, clearly indicating that microglia are perturbed, and may suggest that the cell plays a vital role in controlling and/or adapting to stress.

In Chapter Three, we further investigated microglia, by evaluating changes in their activity when treated with commercially available antidepressants. Specifically, we sought to establish the relative anti-inflammatory potency of both selective serotonin and serotonin norepinephrine reuptake inhibitors (SSRI; SNRI). To do this, the study assessed the capacity of five SSRIs (fluoxetine, paroxetine, sertraline, citalopram, fluvoxamine) and one SNRI (venlafaxine) to suppress microglial activity in response to the inflammatory stimulant lipopolysaccharide (LPS). To compare the ability of antidepressants to suppress microglial activity, we measured the microglial production of the two pro-inflammatory molecules tumour necrosis factor-α (TNF-α) and nitric oxide (NO), at both 4 and 24 hours post LPS stimulation. Our results indicated that whilst the SNRI venlafaxine showed negligible anti-
inflammatory potency, all SSRIs significantly attenuated both TNF-α and NO production. In terms of an underlying mechanism, we found evidence to suggest that cAMP signalling may be involved in regulating the observed anti-inflammatory response. These findings highlight the sensitivity of microglia to antidepressant treatment, and question whether antidepressants owe at least some of their therapeutic effectiveness to their ability to suppress microglial activity.

In addition to the observed changes in microglia, we found that chronic stress could also induce substantial alterations to astrocytes, one of the primary glial cell subtypes within the CNS. Using three dimensional structural remodelling of glial fibrillary acidic protein positive (GFAP⁺) astrocytes, our results showed that chronic stress significantly decreased astrocyte cell volume, process length and the complexity of the branching of the cells processes. These changes were observed with coinciding decreases in the density of GFAP⁺ immunoreactive material and the number of GFAP⁺ cell counts. However, as there were no corresponding decreases observed in the total number of cells (as indicated by Nissl stain), or in the number of S100β positive cells, it is more likely that the observed decrease in GFAP⁺ cell counts reflects a loss of cell phenotype, rather than loss of the cell. Despite this, the observed decrease in the size of the cell could have profound implications, particularly given many diverse functional roles recently discovered in astrocytes.

Taken together, the experiments presented within this thesis clearly demonstrate that chronic stress can cause significant perturbation to glial cells. Specifically, the findings reported show that chronic stress can induce profound alterations to glial cell structural morphology, density, and their expression of cellular phenotype. In addition, evidence is presented to demonstrate that glial functioning can be substantially altered with antidepressant treatment. The relationship between stress and glia has a number of vast implications. For instance, they may indicate that the cells have vital functional roles in both responding to and adapting to
stress exposure. In addition, the evidence presented highlight glia as a potential target for the development of future medications designed for the treatment of stress and stress related conditions.
Publications

The work in this thesis describes alterations in both microglial and astroglial cell populations within the central nervous system as a consequence of chronic stress exposure. The thesis is divided into three accepted publications:


Additional Publications


Conference Proceedings

The research within this thesis has been personally presented at the following national and international conferences:


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ANCOVA  analysis of covariance
ANOVA  analysis of variance
AUC   area under curve
BBB   blood brain barrier
BNST   bed nucleus of the stria terminalis
BLA   basolateral amygdala
BSA   bovine serum albumin
cAMP   cyclic adenosine monophosphate
CeA   central amygdala
CA3   CA3 region of the hippocampus
dL1b   cluster of differentiation 11b
CNS   central nervous system
dLPFC   dorsolateral prefrontal cortex
GFAP   glial fibrillary acidic protein
HPA axis  hypothalamic pituitary adrenal axis
Iba1   ionized calcium binding adapter protein 1
IC50   inhibitory concentration of 50%
IL   infralimbic cortex
IL-1β   interleukin-1β
Ir   immunoreactive
LPS   lipopolysaccharide
MAOI   monoamine oxidase inhibitor
MeA   medial amygdala
MHC-II  major histocompatibility complex II
mPFC   medial prefrontal cortex
NAcC   nucleus accumbens core
NAcS   nucleus accumbens shell
NO   nitric oxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>OFC</td>
<td>orbitofrontal cortex</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PL</td>
<td>prelimbic cortex</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SNRI</td>
<td>serotonin and noradrenaline reuptake inhibitor</td>
</tr>
<tr>
<td>SPFT</td>
<td>sucrose preference test</td>
</tr>
<tr>
<td>TCA</td>
<td>tricyclic antidepressant</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>TRP</td>
<td>tryptophan</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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Chapter One

General Introduction
Stress

Stress…The number 1 killer! This is a phrase commonly sensationalised in media such as newspapers and current affair programs. While this statement may seem dramatic, stress is well recognised to be a major risk factor in the development and/or exacerbation of a number of both pathological and psychopathological conditions. Specifically, stress has been shown to cause or exacerbate anxiety (Maes et al., 1998), depression (Caspi et al., 2003), type II diabetes (Pouwer, Kupper, & Adriaanse, 2010), coronary heart disease (Rozanski, Blumenthal, & Kaplan, 1999), post-traumatic stress disorder (Mueser et al., 1998), and even the common cold (S. Cohen, Tyrrell, & Smith, 1991). Given these associations, it is not surprising that the effects of stress represent a substantial economic and psychological burden, with the symptoms of stress amongst the most heavily medicated in contemporary society.

While stress represents a sensation that almost all adults have experienced and intuitively understand, the scientific definition of the concept of ‘stress’ has been changed substantially since it was first introduced. One of the pioneers within the stress field, Hans Selye, was amongst the first to attempt to study and conceptualize the physiology of stress. Selye, often hailed as ‘the father of stress research’, authored or co-authored over 1700 publications on stress (Szabo, 1985). In his work, Selye defined stress as ‘the non-specific response of the body to any demand’ (H. Selye, 1998). In line with this theory, it was proposed that the body manifests an integrated set of responses, such as sympathomedullary and hypothalamic-pituitary-adrenal axis activation, which reorient almost all of the organism’s psychological and physiological resources to allow it to cope with stress (Day, 2005).

Despite extensive research, there remains ongoing controversy over what constitutes stress, and exactly how it should be defined (Day, 2005; McEwen & Wingfield, 2010). However, it
is now widely accepted that stress represents a perceived (real or imagined) threat or disturbance, that has the capacity to overwhelm homeostatic mechanisms of the internal milieu, resulting in the coordinated physiological activation of central, peripheral and autonomic nervous systems, in combination with both neuroendocrine and immunological pathways (McEwen, 2000). Within the scientific literature, the term stress is generally separated into a causative event referred to as a stressor (any stimulus that threatens homeostatic balance), and the bodily response, referred to as the stress response (innate mechanisms to restore homeostasis) or simply stress (Day, 2005).

**Stressors**

Stressors can be defined as any stimulus, environmental condition, biological agent or event that induces a stress response. Within the scientific literature, there appears to be somewhat of a dichotomy in the types of stressors. For instance, stressors can be loosely categorised on the basis of those that impose immediate physical disturbance, and those that are psychological in nature. Physical stressors, also referred to as systemic (Hans Selye, 1950), interoceptive (Mayer, Naliboff, Chang, & Coutinho, 2001) or homeostatic stressors (Romero, Dickens, & Cyr, 2009), typically involve an immediate disturbance to tissue integrity (e.g. extreme cold, heat, starvation). Whereas psychological stressors, which are commonly referred to as emotional (Dayas, Buller, & Day, 1999) or psychogenic (Hymie Anisman, Hayley, Kelly, Borowski, & Merali, 2001), are often regarded as stressors that provide a threat or emotional disturbance (e.g. fear, restraint, bereavement, loss, divorce, marital discord, financial difficulties). The impact that these stressors have on an organism can vary considerably, dependent upon factors such as novelty, controllability, frequency, temporal proximity, predictability, intensity and the duration of the stressor exposure (Seligman, 1972). Furthermore, there are a number of individual differences in the manner in which
people respond to stress, which is largely governed by personality, previous exposure to the stressor, genetic predispositions, perception of control, the type and magnitude of the stressor, all factors which can considerably modulate the stress response (Adell, Casanovas, & Artigas, 1997; Goldstein, 1973; Keay & Bandler, 2001; Vollrath, 2001).

**The Stress Response**

Psychological stress has the capability to be both beneficial (e.g. providing the requisite molecular substrates to escape or deal with threatening situations), and harmful, particularly in instances where exposure to stress is prolonged or uncontrolled. Exposure to acute stress can provoke a number of vital adaptive changes to allow the organism to effectively react and combat the stressor. This type of response to stress, originally coined by Cannon (1929) as the *fight-flight response*, is an emotional and visceral reaction that prepares an organism for either fighting, or fleeing, by mobilising all available energy resources (e.g. increasing heart rate, dilating pupils, piloerection), and inhibiting non-essential processes (e.g. digestion, sexual arousal). This type of response to stress has obvious adaptive advantages, effectively increasing the chance of survival by redirecting metabolic resources and inducing behavioural changes so that the organism may overcome the adverse effects of the stressor. However, the effects of stress are typically detrimental when exposure is prolonged, excessive or particularly traumatic. Repeated stress exposure is often associated with a number of neuroendocrine, immunological and behavioural alterations, which may fundamentally change underlying stress neurocircuitry and the subsequent response to stress (Chung, Martinez, & Herbert, 2000). Consequent maladaptive alterations may then predispose an individual to a range of both pathological and psychopathological conditions.

Traditionally, the physiological response to stress has been described in terms of co-activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal
(HPA) axis (Chrousos, 1995). The former acts rapidly through the release of catecholamines (such as adrenaline), and provides the organism with immediate energy mobilization required to effectively combat the stressor, and is a vital underlying neural process regulating the ‘fight or flight’ response (J. J. Radley & Morrison, 2005). The HPA axis forms part of the neuroendocrine system, and is often referred to as the slower acting stress system (Tsigos & Chrousos, 2002). In response to stress, afferent neurons compile information and send it to the paraventricular region of the hypothalamus, conveying information regarding the type and magnitude of the stressor (Smythe, Murphy, Timothy, & Costall, 1997). This process initiates a sequence of events which ultimately leads to glucocorticoid release, the major stress hormones (cortisol in humans and corticosterone in rats) from the adrenal cortex. The actions of these stress induced mediators are highly pleiotropic, but are probably best recognised for their ability to directly influence the activity of peripheral leukocytes (Chrousos & Gold, 1992), and neurons of the central nervous system (Joels, 2011). Increasingly, however, it is recognised that the stress response can also result in the peripheral and central production of pro-inflammatory molecules such as the pro-inflammatory cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α (Kiecolt-Glaser et al., 2003), and also significantly modify the activity of glia (Banasr & Duman, 2008; Czeh, Simon, Schmelting, Hiemke, & Fuchs, 2006; Sugama, Fujita, Hashimoto, & Conti, 2007). These recent observations appear at odds with the traditional stress ideology (Munck, Guyre, & Holbrook, 1984), which suggested an immunosuppressive nature of stress hormones, and would indicate that glia may play an important role in controlling and adapting to stress.

**Animal models of stress**

Animal models are commonly used to provide insight into the mechanisms involved in responding to stress (McEwen, 2000). As the behaviour and neurophysiological factors
associated with stress in animals closely resemble those found in humans, animals can be used as a valid and reliable analogue, suitable for measuring underlying factors (Rodgers, Cao, Dalvi, & Holmes, 1997). The use of animal models in stress research has a number of potential benefits for enhancing our understanding of stress in humans. For instance, they provide a model to minimise confounding factors attributed to individual variation by using inbred strains. Furthermore, medication regimes can be systematically manipulated to empirically evaluate their effectiveness as an intervention. But perhaps one of the most valuable features is that they provide a method to permit systematic evaluation of the underlying neurological changes, and the ability to examine the brain pathways that generate the stress response.

**Brain regions that modulate the stress response**

The brain is a key organ responsible for integrating sensory information regarding stressors, determining what is stressful, and orchestrating both the behavioural and physiological response (McEwen, 2000). In addition to the HPA axis, there have been a number of brain regions specifically identified as crucial for coordinating, initiating and regulating the response to stress. For instance the amygdala, a region often associated with emotional regulation (Joels, Fernandez, & Roozendaal, 2011), is considered to play a crucial role in evaluating whether sensory stimuli merit the initiation of a stress response (Roozendaal, McEwen, & Chattarji, 2009). This region possesses reciprocal connections to regions such as the periaqueductal grey (Krettek & Price, 1978), the hippocampus (Pitkanen, Pikkarainen, Nurminen, & Ylilnen, 2000) and the prefrontal cortex (Banks, Eddy, Angstadt, Nathan, & Phan, 2007), where it can modulate the ability of such regions to elicit a co-ordinated behavioural and physiological response to stress (Keay & Bandler, 2001). The hippocampus, widely recognised for its role in memory consolidation and learning (Squire, 1992), is
perhaps the most extensively investigated brain region in relation to stress (Jacobson & Sapolsky, 1991). Evidence linking stress and the hippocampus include the particularly high density of glucocorticoid receptors within this region allowing modulation of the HPA axis (J.J. Radley & Sawchenko, 2011), in combination with the deficit in hippocampal dependent functions (e.g. learning, working memory) as a consequence of stress exposure (Lupien & Lepage, 2001). Other brain regions that have been implicated in the initiation, modulation or regulation of stress responses include the nucleus accumbens (Campioni, Xu, & McGehee, 2009), bed nucleus of the stria terminalis (Crane, Buller, & Day, 2003), medial, central and basolateral amygdala (Davis, 1992; D. L. Walker, Toufexis, & Davis, 2003), ventral tegmental area (Rodaros, Caruana, Amir, & Stewart, 2007) and the periaqueductal grey (Keay & Bandler, 2001). Collectively, each of these regions have been empirically demonstrated to possess specific functional roles in initiating and/or modulating the stress response.

Recent investigations have also highlighted the crucial role of the medial prefrontal cortex in the mediation of the stress response (Diorio, Viau, & Meaney, 1993; J. J. Radley, Arias, & Sawchenko, 2006). Globally, the prefrontal cortex (PFC) is well recognised as a brain region that subserves our higher-order cognitive functions, such as attention, decision making, executive function, working memory and goal directed behaviour (E. K. Miller & J. D. Cohen, 2001). In the rat brain, the PFC lies in the frontal lobe, rostral to motor and premotor cortices, and is composed of three functionally and anatomically distinct areas, the orbitofrontal (OFC), dorsolateral (dLPFC) and medial prefrontal cortex (mPFC; Krettek & Price, 1977). Of these regions, the OFC and mPFC have extensive reciprocal connections to limbic structures including the hippocampus, amygdala and hypothalamus (Drevets, Price, & Furey, 2008). The mPFC is further subdivided into three cytoarchitecturally distinct subregions, the anterior cingulate (ACg), prelimbic (PL), and the infralimbic (IL) cortices
(Groenewegen & Uylings, 2000). Whilst the mPFC is widely considered as one of the most evolved regions (Earl K Miller & Jonathan D Cohen, 2001), it is particularly sensitive to stress exposure. Consistently, neuromodulatory changes that occur in response to chronic stress, and even in some instances acute stress, have been demonstrated to markedly impair PFC dependant function (Arnsten, 2009). But perhaps the most remarkable illustration of the sensitivity to stress within this region is given by the significant structural remodelling that occurs in response to chronic stress (e.g. Cook & Wellman, 2004; J. J. Radley, Arias, et al., 2006).

**Stress induced neural plasticity**

The brain is a sensitive and dynamic organ, which is receptive to its intrinsic and extrinsic environment. In response to stressor stimulation, the brain can undergo structural and functional changes (often referred to as neural plasticity), which have the potential for both adaptive and maladaptive consequences (McEwen, 2007). In a seminal investigation, Watanabe, Gould and McEwen (1992) were amongst the first to demonstrate that stress could provoke profound neuronal alterations, by showing that chronic restraint stress induced significant atrophy of apical dendrites in the CA3 region of the hippocampus. The dendritic retraction of pyramidal neurons in the hippocampus has consistently been reproduced using various stress protocols, including the exogenous administration of corticosterone, the primary stress hormone in rats (Magariños, McEwen, Flügge, & Fuchs, 1996; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000; Woolley, Gould, & McEwen, 1990). More recently, researchers have extended upon these initial findings, by showing that chronic stress can induce a 20-35% retraction of the apical dendrites of pyramidal neurons in the mPFC (Cook & Wellman, 2004; J. J. Radley, Rocher, et al., 2006; J. J. Radley et al., 2004). Interestingly, Brown et al. (2005) showed that even daily injections of vehicle resulted in
significant retraction of apical dendrites within the mPFC, albeit to a lesser degree, nevertheless highlighting the sensitivity to even minor stress within this region. Whilst the functional significance of these structural alterations in both hippocampal and mPFC regions remains uncertain, recent evidence has begun to emerge demonstrating significant correlations with impairments in cognitive capacity (Liston et al., 2006), which may indicate that these changes have many diverse and wide-spread implications.

**Clinical relevance of Stress**

Whilst stress forms are a necessary and vital component of the human psyche, excessive or prolonged exposure can be detrimental. Research has consistently demonstrated a relationship between chronic stress and a number of physical diseases including cancer (Thaker, Lutgendorf, & Sood, 2007), autoimmune disease (Stojanovich & Marisavljevich, 2008), diabetes (Pouwer et al., 2010) and coronary heart disease (Rozanski et al., 1999). Furthermore, the immunological, neuroendocrine and behavioural alterations that occur as a consequence of chronic stress are often suggested to play a key role in the development of mood related disorders such as depression. Whilst some remain cautious about the contribution of stress in the etiology of depression (Baune, 2009), it is commonly well-supported, with many suggesting chronic stress as a major cause of depression (Bartolomucci & Leopardi, 2009; Hammen, 2005; Rydmark et al., 2006; C. C. Tennant, Palmer, Langeluddecke, Jones, & Nelson, 1994).

In those who are susceptible to depression, stress may serve as a trigger initiating the onset of an episode, cause an individual in remission to relapse, or exacerbate an existing condition (Sheldon Cohen, Janicki-Deverts, & Miller, 2007; C. Tennant, 2002). Furthermore, even in individuals without any genetic or biological predisposition, stressful life events are known to increase the susceptibility to developing major depression (Joels, Karst, Krugers, &
Lucassen, 2007). As a consequence of the inexorable linkage of stress exposure and depression, empirically evaluating the effects of chronic stress can provide a greater understanding of the complexities associated with the pathophysiology of the disorder.

**Depression**

Depression is a major public health concern, which is reported to affect approximately 120 million people worldwide, and is the number one cause of non-fatal disease burden (World Health Organisation [WHO], 2004; F. R. Walker, 2012). Given the high prevalence of the disorder, it is not surprising that depression represents a substantial economic concern, with statistics indicating the direct and indirect costs amount to $1.8 billion in Australia, and $44 billion in the United States alone (Greenberg & Birnbaum, 2005; Greenberg et al., 2003; Hu, 2004; Lim, Sanderson, & Andrews, 2000; F. R. Walker, 2012).

Depression is far more than unhappiness, it is a clinical disorder that has a number of clearly defined symptoms. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR 2000), depression can be defined as a mood state characterized by anhedonia (decreased interest in pleasure), and is typified by symptoms such as suicidal ideation, irritability, deficits in cognitive capacity, apathy and a general lack of energy. Individuals who are living with depression commonly experience feelings of pessimism, despondency and sadness, which may continue their entire lives without suitable intervention (Moussavi et al., 2007). Given the pervasive and sometimes life-threatening nature of the disorder, combined with the economical and psychological burden it causes individuals living with the condition and society at large, finding effective methods of intervention is paramount.

Current clinical guidelines recommend that individuals with moderate to severe depression be treated with a combination of psychological and pharmacological therapy (Ellis, 2004). The first-choice pharmacological treatment options are classes of antidepressants known as either
selective serotonin reuptake inhibitors (SSRI) or serotonin noradrenalin reuptake inhibitors (SNRI). These classes of antidepressants have largely supplanted tricyclic (TCA) and monoamine oxidase inhibitors (MAOI) as the antidepressant of choice, as they substantially reduced the side-effect profile, increased the safety and tolerability of short and long term usage, whilst maintaining an equivalent level of clinical efficacy (Anderson, 2000; Dechant & Clissold, 1991; Murdoch & McTavish, 1992). These improvements are often credited to the fact that SSRIs and SNRIs were the first rationally designed psychiatric medications, which have a highly specific mechanism of action (Lane, Baldwin, & Preskorn, 1995). Whilst the mechanism of action involved is extremely complex, the underlying principle of the drugs is relatively simple, in that they aim to increase the bioavailability of the neurotransmitters serotonin and noradrenaline (Stahl, 2009). The drugs achieve this by binding to the presynaptic serotonergic and/or noradrenergic transporters, for which they have a high affinity, where they inhibit neurotransmitter reuptake from the synaptic cleft (Benmansour et al., 1999). This increases monoamine levels in the synapse available to bind and stimulate post-synaptic receptors, thereby boosting synaptic transmission. There are six principal agents within the class of SSRIs (fluoxetine, sertraline, citalopram, escitalopram, paroxetine and fluvoxamine), which as there name would suggest, all share the pharmacological property of serotonin reuptake inhibition. Despite this similarity, each of these agents can be distinguished through a number of unique secondary actions on muscarinic, adrenergic and cholinergic receptors (Owens, Morgan, Plott, & Nemerooff, 1997). This differentiation allows a more sophisticated approach to their prescription, by matching individual drug profiles to the specific symptoms of the patient.
**The monoamine theory of depression**

The putative function of the SSRI antidepressants is consistent with the *monoamine hypothesis*, one of the most prominent theories used to describe the pathophysiology of depression. This theory suggests that depression is the result of a deficit or imbalance in centrally available monoamine levels (Hindmarch, 2002; Mulinari, 2012). In accordance with this theory, antidepressants are primarily thought to exert their therapeutic influence by augmenting monoamine levels (Hyttel, 1993). This theory arose in the 1950s following the serendipitous discovery that the symptoms of severe depression could be effectively treated using two structurally unrelated classes of compounds (now commonly known as MAOI and TCA) that both, amongst other things, elevated levels of the monoamines noradrenalin and serotonin. The monoamine theory generated credence following the manufacture of SSRIs, as these drugs maintained an equivalent level of clinical efficacy in treating depression, despite specifically targeting serotonergic transporters, whilst having little to no effect on other transporters, receptors or enzymes influenced by predecessor antidepressants (Hyttel, 1994).

Whilst one cannot question the heuristic value of the monoamine hypothesis, nor the impetus it provided for research into the neurobiology of depression, over the past 50 years the model has been the subject of contentious debate and received a vast amount of criticism. A number of limitations have caused many to question whether the theory can provide a complete neurobiological account of the underlying mechanisms involved in depression (Hindmarch, 2002; Hinz, Stein, & Uncini, 2012; Kasper & Hamon, 2009; J. R. Lacasse & J. Leo, 2005).

Amongst the most commonly reported criticisms of the monoamine hypothesis are those directed towards the lag time between the commencement of treatment and the clinical attenuation of symptoms (Hirschfeld, 2000). Much of the empirical evidence suggests that clinically, in those that do show a therapeutic response to SSRI or SNRI treatment, the attenuation/amelioration of depression symptoms typically take a few weeks to show an
effect (Andrews & Nemeroff, 1994; Nierenberg et al., 2000; Papakostas et al., 2007). Despite this delayed onset in the clinical attenuation of symptoms, preclinical research has demonstrated that the elevation in serotonin occurs within minutes (Auerbach, Lundberg, & Hjorth, 1995; Felton, Kang, Hjorth, & Auerbach, 2003). This highlights a profound discrepancy between the function of antidepressants and the traditional monoamine theory. In accordance with the theory, attenuation of symptoms should occur coincident to elevations in monoamines. However, the findings clearly illustrate that elevations in central monoamine levels precedes obvious signs of clinical improvement. Advocates of the monoamine theory, however, have attempted to justify the delay in clinical efficacy by suggesting that it is a consequence of presynaptic autoreceptor desensitization and/or post-synaptic receptor downregulation (Lopez-Munoz & Alamo, 2009; Nakajima, Suzuki, Watanabe, Kashima, & Uchida, 2010). Inhibition of the presynaptic transporter floods the synapse with serotonin, which not only increases the levels available to bind and stimulate post-synaptic receptors, but also increases the amount available to activate presynaptic autoreceptors, which act as a feedback mechanism preventing production of serotonin. However, it is suggested that elevated levels of serotonin for a sustained period promote a desensitization of presynaptic autoreceptors, and/or downregulate the number of post-synaptic receptors, thereby increasing the serotonin to receptor ratio (Stahl, 2009). The time taken for the receptor adaptation is consistent with the delayed onset in the therapeutic effects of antidepressants.

One further criticism of the monoamine hypothesis comes from the lack of consistency in research attempting to induce depression by depleting monoamine levels (J.R. Lacasse & J. Leo, 2005). Centrally available monoamine levels can be empirically altered by depleting the body of their precursor amino acids. For instance, serotonin levels can be experimentally lowered using the tryptophan (TRP; a serotonin precursor) depletion method, in which subjects are given a TRP free drink rich in other amino acids, which compete with TRP to
cross the blood brain barrier, thereby restricting TRP entry into the brain (Bell, Abrams, & Nutt, 2001). In line with the monoamine theory, if depression is caused by a deficiency in monoamine levels, experimentally decreasing levels should induce depression symptoms. In a number of reviews of the literature, however, research has shown that monoamine depletion induced alterations to mood are highly dependent upon the health of the sample population tested, with patients genetically predisposed or currently drug-free in remission after an episode of depression most vulnerable to depletion induced mood change (Booij, Van der Does, & Riedel, 2003; Ruhé, Mason, & Schene, 2007). The primary conclusion from research in this area is that depleting monoamine levels can induce significant perturbation to mood, yet can neither prove, nor disprove, that disturbances to centrally available monoamine levels play a causal role in the development of mood related disorders.

Upon close scrutiny of the evidence directed against the monoamine hypothesis, it does appear that the criticisms have largely been resolved by slight reformulations of the theory. Nevertheless, they have provided motivation to develop novel conceptual frameworks in attempt to better our understanding of the pathophysiology of depression. Recent developments have suggested that depression may involve alterations in glutamatergic neurotransmission (Kugaya & Sanacora, 2005) or alternatively decreases in neurogenesis (Duman, Heninger, & Nestler, 1997), but perhaps the most promising and well-supported of the contemporary models is the inflammatory theory of depression (Smith, 1991).

**The Inflammatory hypothesis of depression**

The role of inflammation in the pathophysiology of depression has been increasingly recognised since Smith’s (1991) seminal publication “The macrophage theory of depression”, and the highly influential contributions to the theory in the early 1990s by Maes and colleagues (Maes, 1993, 1995; Maes, Meltzer, et al., 1995). The inflammatory theory, also
referred to as the cytokine hypothesis of depression (Maes et al., 2009), has a number of converging lines of evidence which all indicate the vital role of inflammatory processes in the development and maintenance of depression. A significant body of this evidence comes from investigations involving cytokines, which are a family of pleiotropic immunomodulatory signalling proteins that are secreted by immunological cells, and play a crucial role in mediating inflammation (Li, Soczynska, & Kennedy, 2011). Systemic and central administration of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-2 have been shown to induce sickness behaviours characteristic of depression, such as social withdrawal, anhedonia, activation of the HPA axis and fatigue (H. Anisman, Kokkinidis, & Merali, 2002; H. Anisman, Merali, Poulter, & Hayley, 2005; Dantzer, 2001; Dantzer et al., 2007). Whilst some have taken the considerable overlap in the behavioural phenotypes induced by sickness and depression to indicate that they could share the same underlying mechanisms (Yirmiya, 1996), others have suggested that the parallel between the symptoms could represent a potential bias, and that it is important to delineate cytokine induced sickness from cytokine induced depression (Dantzer, O’Connor, Freund, Johnson, & Kelley, 2008). Dantzer et al. (2008) suggests that for the behavioural alterations induced by cytokines to be considered depression-like, the behaviours should: (1) be pharmacologically validated by demonstrating a response to antidepressant treatment; and (2) be behaviourally validated by occurring in the absence of any obvious motor impairments. In line with this position, there is preclinical evidence that chronic antidepressant treatment are effective at attenuating cytokine induced alterations to behaviour, including improvements on measurements of anhedonia and social exploration (Castanon, Bluthe, & Dantzer, 2001; Shen, Connor, Nolan, Kelly, & Leonard, 1999; Yirmiya et al., 1999). Furthermore, Frenois et al. (2007) demonstrated that 24 hours after lipopolysaccharide (LPS) injection, a potent stimulant of pro-inflammatory cytokines, animals still displayed depression-like behaviour in
the forced swim, tail suspension and sucrose preference tests, despite no decrease in general motor activity. Taken together, the results from preclinical research are strongly supportive of the involvement of inflammatory processes inducing behaviour consistent with depression.

Whilst the research using animal models is intriguing, perhaps the most compelling evidence of the involvement of inflammatory processes in the development of depression comes from research on humans. It has been consistently demonstrated that the exogenous administration of pro-inflammatory cytokines can induce a number of behavioural alterations consistent with the symptoms of major depression. A significant body of this research comes from the observation that up to 70% of patients undergoing immunotherapy for the treatment of cancer or intractable viral diseases, which typically involves the exogenous administration of recombinant cytokines IL-2 or interferon-α (IFN-α), begin to manifest symptoms of depression at a clinically diagnosable level (Bonaccorso et al., 2002; Bonaccorso et al., 2001; Maes, 2001; Niiranen et al., 1988). Typically, the onset of these symptoms become apparent within days of commencing immunotherapy, and frequently dissipate shortly after the cessation, highly suggestive of cytokines playing a causal role in the mediation of the depression symptoms (Capuron & Miller, 2011). A somewhat striking example of the temporal aspects of this effect was demonstrated in Reichenberg’s et al. (2001) influential study, where it was shown that injecting healthy volunteers with the cytokine-inducer LPS produced a transient depressed mood four hours after injection, which disappeared a few hours later when the cytokine levels had normalised. Importantly, the evidence clearly demonstrates that the depression symptoms provoked by either exogenous cytokine administration or the cytokine-inducer LPS, can be effectively prophylaxed or treated with conventional antidepressants (Capuron et al., 2002; McNutt et al., 2012; Musselman et al., 2001). As such, it is quite clear from the available data that the symptoms of depression can
be induced by cytokines, and that these symptoms can be attenuated through the use of antidepressant treatment.

In further support of the link between depression and inflammatory disturbance, clinical research has shown that individuals who suffer from depression commonly exhibit elevations in a number of inflammatory biomarkers. Amongst the first to empirically demonstrate this relationship, Maes et al. showed that the plasma concentrations of IL-1 and IL-6 were increased in individuals with depression, with cytokine levels correlating with the severity of the condition (Maes, Bosmans, & Meltzer, 1995; Maes et al., 1993). With the exception of some inconsistencies (Brambilla, Monteleone, & Maj, 2004; Rothermundt et al., 2001), these findings are commonly well supported, with a number of recent meta-analyses demonstrating significant elevations in IL-1β, IL-6, TNF-α, IFN-γ and C-reactive protein in individuals with major depression (Dowlati et al., 2010; Howren, Lamkin, & Suls, 2009; Liu, Ho, & Mak, 2012; Zorrilla et al., 2001). In congruence with these observations, the prevalence of major depression is higher in individuals afflicted with diseases in which inflammation is recognised as a major contributing factor (Iosifescu, 2007; Smith, 1991). For instance, a high co-morbidity of depression is commonly identified with cardiovascular disease (Grippo & Johnson, 2009), multiple sclerosis (Gold & Irwin, 2009), rheumatoid arthritis (Uguz, Akman, Kucuksarac, & Tufekci, 2009) and asthma (Van Lieshout, Bienenstock, & MacQueen, 2009), with evidence showing patients suffering from these conditions are up to three times more likely to manifest symptoms of major depression relative to the general population.

In addition to the well-established linkage of peripheral inflammation with depression, evidence is beginning to emerge regarding corresponding changes within the brain. At the mRNA level, research conducted on post-mortem brain tissue describes how the whole pro-inflammatory cytokine network is up-regulated in individuals with depression (Shelton et al., 2010). Furthermore, evidence from functional neuroimaging and post-mortem counting
studies consistently demonstrate a profound reduction in the cortical volume and a reduction in cell number within the brain of individuals with a life-history of depression, a known consequence of prolonged neuroinflammation (Campbell & MacQueen, 2006; Koolschijn, van Haren, Lensvelt-Mulders, Hulshoff Pol, & Kahn, 2009; Rajkowska, 2002). One mechanism that may be underlying such effects that is increasingly recognised is the ability of peripheral cytokines to modulate CNS function. Whilst they are not able to passively cross the blood brain barrier (BBB) directly, they can access the brain by stimulation of the vagal nerve, active transportation across the BBB, passive transport at circumventricular sites of the BBB, or by binding to surface proteins on cerebrovascular endothelium where they induce a host of secondary messengers (Kronfol & Remick, 2000). This capacity of the peripheral immune system to exert immunomodulatory properties within the brain was recently demonstrated by Qin et al. (2007), who showed that systemic injection of LPS caused a rapid and sustained elevation in central levels of the pro-inflammatory cytokine TNF-α. But perhaps more important than the peripheral activation is that the brain itself actively synthesizes cytokines, which are primarily secreted by subtypes of a class of CNS glia cells known as astrocytes and microglia (Kronfol & Remick, 2000).

**Antidepressants possess the capacity to inhibit inflammation**

The compelling body of evidence implicating inflammatory processes in the etiology of depression has generated interest into investigating potential anti-inflammatory properties of antidepressants (F. R. Walker, 2012). Interestingly, the idea that antidepressants (specifically, the MAOI) could exert anti-inflammatory activity was described as early as 1959 (Setnikar, Salvaterra, & Temelcou, 1959), a finding that predates the inception of the monoamine hypothesis. In terms of the anti-inflammatory capacity of SSRI antidepressants, evidence began to emerge from studies involving peripheral immune cells in the 1990s, after the
influential findings of Martensson and Nassberger (1993), who demonstrated that both fluoxetine and citalopram could inhibit mitogen stimulated lymphocyte proliferation. Extending on these initial findings, it was subsequently demonstrated by Xia et al. (1996), that the SSRI citalopram significantly inhibited the release of IL-1β and TNF-α from stimulated monocytes that had been isolated from healthy volunteers. Further evidence of the anti-inflammatory properties of SSRIs comes from the research of Maes et al. (1999), who demonstrated that the SSRI sertraline not only inhibited LPS stimulated whole blood secretion of IFN-γ, but also significantly increased the secretion of IL-10, a known negative immunoregulatory cytokine. With the exception of one study where the SSRI fluoxetine was shown to have no anti-inflammatory effect on stimulated whole blood preparations (Kubera et al., 2004), the results are generally supportive of the anti-inflammatory nature of antidepressants.

Recent evidence has begun to emerge indicating that the anti-inflammatory effects of antidepressants on peripheral cells also extend to microglia, the innate immune effector cells within the central nervous system. These findings are particularly interesting, as microglia are both morphologically and functionally distinct from the cells that mediate the anti-inflammatory effects of antidepressants in the periphery (Graeber, 2010; Wake, Moorhouse, Jinno, Kohsaka, & Nabekura, 2009). From in vitro investigations, there is now independent data indicating that the SSRIs fluoxetine, paroxetine and sertraline can significantly inhibit the ability of murine microglia to secrete the free radical nitric oxide (NO), and the pro-inflammatory cytokine TNF-α (Hashioka et al., 2007; Horikawa et al., 2010; Hwang et al., 2008). However, there are some discrepancies within the literature, with some indicating that the SSRI fluoxetine can actually induce an increase in microglial pro-inflammatory cytokine production (Ha et al., 2006). These differences in the observed results most likely reflect variations in experimental protocols, such as the concentration and type of antidepressant
used, the duration of antidepressant exposure, and the impact on cellular viability assessments. Furthermore, the differences in experimental protocol prevent sufficient grounds for comparison of the relative anti-inflammatory potency for each of the antidepressants. To address these concerns, research within the current thesis conducted a comprehensive comparative analysis of the relative anti-inflammatory potency of SSRI and SNRI antidepressants (Tynan et al., 2012). The results from this research demonstrated whereas the SNRI exerted very little anti-inflammatory potency, all SSRIs were extremely potent inhibitors of inflammation. The results from this research raises the important question, to what extent do SSRIs owe their therapeutic effectiveness as an antidepressant, to their ability to inhibit microglial inflammation.

**Glia: The CNS innate immune cells**

Within the CNS, the primary cells responsible for responding to and propagating inflammatory stimuli are a class of cells known as glia. Glia have often been referred to as the most abundant cell population within the CNS, with some even suggesting that they outnumber neurons 9 to 1 (Erik M Ullian, Sapperstein, Christopherson, & Barres, 2001). Recent observations, however, are much more conservative, indicating that the ratio of glia and neurons is closer to 1 to 1 (Azevedo et al., 2009; Wang & Bordey, 2008). The name glia, also known as neuroglia, is derived from the Latin word nerve and the Greek word glue, with this title providing an insight into what was originally considered as the functional role of the cell type. Traditionally, it was thought that the primary function of glia was as a passive support cell, providing the brain with structural integrity, or alternatively, acting as the brains glue (Volterra & Meldolesi, 2005). They assumed this role firstly due to their widespread distribution throughout the cortex, and secondly and perhaps most importantly, that they differed from neurons in that they were deemed electrically inexcitable (Kettenmann &
Verkhratsky, 2008). As a consequence of the lack of excitability, glia have traditionally been neglected in scientific enquiry, in favour of research investigating neurons. Recently, however, with the emergence of new discoveries regarding vital functional roles of glia, we are beginning to understand that the cell type is far more complex than originally considered.

Glial cells are commonly divided into four main sub-categories on the basis of their differing morphological structure and function. Firstly, oligodendrocytes, which are present in the white matter, form myelin sheaths that surround axons and assist in signal propagation, increasing the speed of neuronal conduction by reducing the amount of ion leakage at inter-nodal regions (Laming et al, 2000). Secondly, Schwann cells, which have a very similar function but in the peripheral nervous system (Bunge, 1993). A third type of glial cell is the astrocyte, which have traditionally been recognised for their metabolic support properties, providing neurons with both nutrients and structural integrity (Volterra & Meldolesi, 2005). The final major sub-category of glial cells are known as microglia, which have long been considered as the immunoeffector cells of the CNS, playing a vital role in host defence (Kreutzberg, 1996).

**Microglia**

Microglial cells have a considerable history in the scientific literature, with the initial detection of the cell almost a century ago by del Rio-Hortega (1932). For an extended period of time, microglial cells have been widely recognised for their role in host defence, representing the frontline against invading pathogens and pathology (Kreutzberg, 1996). Whilst the contemporary view of microglial function has changed considerably, their precise role within the CNS remains to be elucidated, possibly due to the phenotypic and functional plasticity of the cell. Increasingly, however, microglia have become recognised as a critical mediator in the maintenance of homeostasis and also established as vital in the breakdown of
neural debris from degenerating neurons (Ransohoff & Perry, 2009), the repairing of damaged cells (Minghetti & Levi, 1998), programmed cellular atrophy (apoptosis), and as an essential molecular substrate required for neurogenesis (Block, Zecca, & Hong, 2007). Increasing evidence also suggests that microglia are critically involved in the aetiology of a number of neurodegenerative diseases such as Alzheimer’s (Ager et al., 2010; Akiyama et al., 2000) and Parkinson’s disease (Klegeris, McGeer, & McGeer, 2007; Qian, Flood, & Hong, 2010), with recent evidence also suggesting a role in the development of a number of psychopathological disorders, including depression (Leonard, 2007; McNally, Bhagwagar, & Hannestad, 2008).

Due to their involvement in inflammatory processes, microglial cells are commonly referred to as the immune-effector cells of the brain (Gehrmann, Matsumoto, & Kreutzberg, 1995). Despite this appellation, under non-pathological conditions microglia are readily distinguishable from peripheral macrophages, as they lack the constitutive expression of a number of surface antigens typically found on macrophage cells (e.g. MHC-II). They are also morphologically distinct, as in the healthy brain, resident microglial cells commonly manifest a downregulated quiescent phenotype, characterised by a relatively small soma, with a ramified morphology consisting of numerous finely branched processes (Kreutzberg, 1996). As microglia are not considered to be immunologically active cells while in this quiescent state, they are often erroneously referred to as resting microglia. Due to the widespread distribution of microglial cells throughout the CNS however, it is suspected that there functional role in the quiescent state is active surveillance of the intrinsic micro-environment, monitoring for ‘danger’ signals such as purines (ATP, UTP), pro-inflammatory cytokines, glutamate receptor agonists, and cell necrosis factors (de Haas, Boddeke, & Biber, 2008). This role is consistent with recent evidence obtained using two-photon microscopy, which demonstrated that the processes of quiescent microglia continuously palpate within the local
extracellular environment (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Quantification of the area covered by microglial processes using time-lapse recordings estimate that microglia can potentially scan the entire CNS extracellular space every few hours. Subsequent studies have revealed that these movements are preferentially directed towards the synapse, which strategically places them in a position to both monitor and modulate synaptic activity (Tremblay, Lowery, & Majewska, 2010; Wake et al., 2009). Therefore, whilst the precise role of resident microglia is still often debated, it is generally accepted that microglial cells are not functionally silent whilst in their quiescent state, but actually have a number of diverse functional roles.

Microglial cells respond to stimulatory signals within the local micro-environment by becoming activated, which is often characterised by a stereotypic functional and morphological transformation (Thameem Dheen, Kaur, & Ling, 2007). Activated microglia possess many macrophage-like properties, such as the ability to engage in phagocytosis, antigen presentation and the capacity to synthesize various pro-inflammatory cytokines. When activated, they are also known to rapidly proliferate through the mitosis of resident microglial cells, a process dependant on mitogens such as the glycoprotein colony stimulating factor. The response of microglia is extremely rapid, frequently occurring prior to the reaction of all other cell types within the brain (Kreutzberg, 1996). As illustrated in Figure 1, activation typically occurs in a graded sequence, ranging from the quiescent state, to an activated amoeboid state, with a number of intermediary stages with varied phenotypes and functional properties. The initial stages of activation involves immunophenotypic changes, including a shortening and thickening of the cells processes, with a corresponding increase in the size of the cell soma. This morphological transformation represents an intermediary activation step, whereby microglia are often referred to as possessing a hypertrophic state. At this intermediary stage of activation, microglia are not actively engaged in phagocytic
activity, but are essentially in a position primed for an immunological response (Kreutzberg, 1996). If the problem stimulating activation of microglia is resolved, the cells either return to their quiescent state, or initiate an apoptotic lineage for the cell. Further stimulation whilst in a hypertrophic state however, causes microglia to transform into an amoeboid or a reactive state. This involves further alterations to the cell morphology, such that the processes are retracted, and the soma size increases. Whilst in this state, microglia are capable of releasing a number of cytotoxic molecules such as reactive oxygen species (ROS) and pro-inflammatory cytokines to effectively address the problem causing activation.

**Figure 1 – Immunophenotypic and functional changes associated with microglial activation.**
The blue box shows an illustration of a quintessential ramified microglial cell, as commonly observed in an adult rat brain. Cells in this state are typified by their long and extensive processes, and a relatively small soma size. In response to stimulation microglial cells become activated, which is associated with both functional and immunophenotypic changes, as illustrated in the green box. The microglia illustrated in the green box shows a cell in the intermediary stage of activation known as hyper-ramified (or hypertrophic). In this state, the cell has more extensive processes, and a slightly enlarged soma size, and is also capable of synthesising various pro-inflammatory molecules. The schematic diagram in the red box show microglial changes associated with activation. Initially, stimulation of the cell causes microglia to enter a hyper-ramified state. In response to further stimulation, the cell transforms into a reactive state, whereby the processes are further shortened.
and thickened, increasing the capacity of the cell motility. Further stimulation induces an amoeboid or phagocytic phenotype, where the cell retracts all processes, the soma size is increased, and the cell has the capacity to release pro-inflammatory cytokines and reactive oxygen species. Stence et al. (2001) suggested that the cell can be further differentiated in the phagocytic state, into what Stence referred to as transitional (T-Stage), motile (M-Stage) and locomotor (L-Stage) microglia, as depicted on the far right of the red box. Image from Beynon and Walker (2012).
One of the most common methods used to evaluate microglial cells is immunohistochemistry. Immunohistochemical techniques allow the detection of specific cells at the single-cellular level, thus represent an important tool for investigating the location and distribution of cells, as well as cellular morphology and density. Amongst the most common molecular markers used for the detection of microglia are cluster of differentiation 11b (CD11b) and ionized calcium adaptor protein-1 (Iba1). Within the parenchyma, both of these prototypical markers are constitutively expressed and highly specific of microglia, with no evidence suggesting immunolabelling of astrocytes, neurons or oligodendrocytes (Imai & Kohsaka, 2002; Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). Microglial are also known to express a number of specific markers dependent upon their activity state, such as the expression of major histocompatibility complex II (MHC-II), a marker frequently present on activated microglia (Block et al., 2007). Whilst the presence of these markers have been utilized for a variety of different microglial investigations, recent research has shown them to be useful for evaluating microglial alterations as a consequence of stress.

**The impact of stress on microglia**

Increasing evidence has demonstrated that exposure to both acute and chronic stress can induce an inflammatory response within the CNS. In a seminal investigation, O’Connor (2003) was amongst the first to clearly illustrate such a relationship, where it was shown that acute exposure to inescapable shock induced a significant increase in the release of the pro-inflammatory cytokine IL-1β. Subsequent research directly implicated microglia in mediating this response, as it was shown the stress induced elevations in IL-1β were substantially attenuated following pre-treatment with minocycline (Blandino, Barnum, & Deak, 2006), a well-recognised inhibitor of microglia (Carty, Wixey, Colditz, & Buller, 2008). Indeed, these results align considerably well with recent observations showing that exposure to acute stress
can promote morphological activation of microglia (Sugama et al., 2007; Sugama, Takenouchi, Fujita, Conti, & Hashimoto, 2009). There is also evidence to suggest that these stress induced effects on microglia may be regionally specific, with the degree of change largely dependent upon the magnitude of the stressor exposure (Blandino et al., 2009; Frank, Baratta, Sprunger, Watkins, & Maier, 2007; Sugama et al., 2007). The research presented within the current thesis significantly expands on this literature, by demonstrating that exposure to chronic stress induces profound changes to the density and morphology of microglia in a number of specific brain regions often implicated in both responding to and controlling stress, and further implicates microglia as a prominent regulator of stress within the CNS.

**Astrocytes**

Astrocytes are ubiquitously distributed throughout the CNS, representing the most abundant cell type within the human brain (Sofroniew & Vinters, 2010). Whilst they are devoid of the axon and dendrites present on neurons, they possess a number cellular extensions known as processes, which give rise to their characteristic star-shaped morphology (Laming et al., 2000). Traditionally, astrocytes have been referred to as a homogenous population of cells (Czéh & Di Benedetto, 2012). Despite this appellation, they are typically classified into three primary subtypes based upon differing morphology and anatomical location. Firstly, radial glia, represent a form of glia particularly abundant during development that are widely known to retract their processes and transform into star-shaped astrocytes during maturation in the perinatal period (Hunter & Hatten, 1995). The second commonly identified subtype are fibrous astrocytes, which are typically found in white matter and are characterised by their long, thin and unbranched cellular processes, of which, the end-feet surround the Nodes of Ranvier. The third and most prevalent subtype is known as protoplasmic astrocytes, which
are primarily found within the grey matter and exhibit shorter, but substantially more complex branching of tertiary processes, which surround the synapse and whose end-feet cover the outer surfaces of blood vessels (Nishiyama, Yang, & Butt, 2005). While these subcategories are widely recognised and reported within the literature, some are beginning to argue that they are insufficient, particularly given the recent discoveries suggesting far greater morphological and functional diversity within the cell type (Wang & Bordey, 2008).

The understanding of astroglial functioning has evolved considerably in recent years, with the cell now recognised to be critically involved in regulating many central processes. Traditionally, astrocytes were thought to be merely passive non-excitatory support cells, providing an inert scaffolding for neuronal networks (Volterra & Meldolesi, 2005). In recent years, however, there is increasing evidence to suggest that they do not simply support neurons, but are instead actively involved in modulating and/or contributing to neuronal activity. It is now widely-recognised that through a calcium (Ca\(^{2+}\)) dependent mechanism, astrocytes are capable of extensive intercellular communication with both neighbouring astrocytes and neurons (Alfonso Araque, 2008; Pasti, Volterra, Pozzan, & Carmignoto, 1997). This bidirectional communication between astrocytes and neurons has led many to refer to them as the tripartite synapse (A. Araque, Parpura, Sanzgiri, & Haydon, 1999; Perea, Navarrete, & Araque, 2009). Indeed, astrocytes are in a strategic position to elicit strong neuromodulatory actions, with a recent calculated estimate suggesting that each astrocyte has the potential to make direct contact with over 100,000 synapses (Bushong, Martone, Jones, & Ellisman, 2002). In addition to these substantial advancements in our understanding of astroglial functioning, a number of further findings have begun to bring astrocytes into scientific prominence. For instance, it is now widely accepted that astrocytes are critically involved in the regulation of ion concentrations in the extracellular matrix (Panickar & Norenberg, 2005), play a crucial role in synaptogenesis and maintenance of synaptic integrity.
(Hashioka et al., 2007; E.M. Ullian, Christopherson, & Barres, 2004), are the primary cell responsible for uptake of excess glutamate preventing cytotoxicity (Pellerin & Magistretti, 1994), and also are capable of storing and releasing glutamate via a Ca\(^{2+}\) dependent mechanism in what is often referred to as gliotransmission (Parpura & Zorec, 2010).

The detection of astrocytes within the brain dates back over 150 years following the pioneering cytochemical studies of Virchow, Golgi and Cajal, who demonstrated the existence of non-neuronal cells within the brain (for historical review see Kettenmann & Verkhratsky, 2008). Since this time, a number of specific antigenic markers have been routinely used to detect astrocytes within the CNS. Amongst the most common markers are glial fibrillary acidic protein (GFAP) and S100β. GFAP is a major intermediate filament protein found within astrocytes crucial for maintaining the cells structure and organisation of cytoplasm, and is often considered a marker of mature astrocytes (Herrmann & Aebi, 2004). S100β, the most common of the S100 family of calcium binding proteins, has also been used extensively as a marker of astrocytes. In recent years, some have begun to criticize the usage of these markers, with claims that they are unable to completely label the cell (Bushong et al., 2002). Nevertheless, these markers are still widely used and accepted, and in combination with advancements in the sensitivity of modern microscopes and the increased sophistication of contemporary image analysis software, the usage of these markers allow a comprehensive characterisation of astrocytes.

**The impact of stress on astrocytes**

Within the past decade, evidence has begun to emerge indicating that stress can generate significant perturbation of astrocytes. This association was first detected in clinical research, where evidence from post-mortem PFC tissue of individuals with a life-history of depression consistently exhibit marked reductions in astrocyte number (Rajkowska, 2000, 2002;
Rajkowska & Miguel-Hidalgo, 2007). More recently, this stress-induced deficit in astrocytes has been demonstrated in preclinical research. Amongst the first to illustrate such an effect, Czeh et al. (2006) was able to demonstrate using unbiased stereological assessments that exposure to chronic psychosocial stress induced a significant reduction in the density of astrocytes within the hippocampus. The subsequent work of Banasr and Duman (2008) significantly extended upon these initial findings, by not only showing that chronic stress induces similar reductions of astrocyte number within the mPFC, but also highlighting the critical role of astrocytes in mediating behaviour, as specific pharmacological ablation of astrocytes within the mPFC induced a number of depression-like behavioural changes consistent with those observed following exposure to chronic stress. In the only other preclinical studies that have evaluated the impact of chronic stress on astrocytes, Ye et al (2011) observed a significant reduction in the astrocyte specific protein GFAP using western blot analysis, whilst Imbe et al (2012) demonstrated that chronic stress induced a decrease in GFAP immunoreactive material in the PAG. A critical review of the current literature clearly indicate a common theme connecting previous research investigating astrocytes and chronic stress, which is the collective focus on reductions in astrocyte number. While such changes are obviously important, with the compelling body of evidence now showing stress-induced plasticity in both microglial (Tynan et al., 2010) and neuronal networks (J. J. Radley & Morrison, 2005), there is reason to believe that astrocytes may also undergo structural remodelling as a consequence of stress. Given the intimate linkage between astrocyte morphology and function (Volterra & Meldolesi, 2005), combined with the extensive bidirectional communication with surrounding neurons, changes in astrocyte structure could potentially induce profound changes in the influence of the cell on neuronal activity. Despite this, to date, there have been no extensive investigations into the changes in astroglial morphology following exposure to stress.
**Experimental Aims and Hypothesis**

Within the past century, there has been considerable evolution in our understanding of the role of glia within the CNS, with the cells now recognised to possess many diverse and complex functions. The overall aim of the research undertaken within this thesis is to expand on the current knowledge base, by examining glial alterations as a consequence of exposure to chronic stress. Specifically, the research aims to investigate: (1) stress-induced changes in the density and morphology of both microglia and astrocytes; (2) identify how these changes relate to alterations in behaviour; and (3) how receptive these changes are to intervention.

*Experiment 1. Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions.*

The research undertaken in the first study had two primary aims. Firstly, to verify the efficacy of the restraint stress protocol to induce behavioural change consistent with that commonly observed following exposure to chronic stress. Secondly, to investigate regionally specific morphological and density alterations in microglia as a consequence of chronic restraint stress exposure. It was hypothesized that:

a) Chronic restraint stress will induce a significant behavioural response, as shown by a decrease in struggling behaviour; and a significant decrease in sucrose preference

b) Chronic restraint stress will induce a profound physiological response, as shown by a significant decrease in the rate of weight gain and a stress-induced elevation in core body temperature

c) Exposure to chronic restraint stress will be associated with a significant increase in Iba1 immunoreactivity in all stress responsive brain regions.

d) The stress induced alterations in microglia will be moderate, with no corresponding stress-induced changes in antigen presentation
Experiment 2. A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia.

Utilizing a microglial in-vitro experimental design, in the second study we established the relative anti-inflammatory potency of commercially available SSRI and SNRI antidepressant compounds, and the mechanism regulating the observed anti-inflammatory activity. It was hypothesized that:

a) All antidepressants would exert anti-inflammatory properties, as indicated by a significant decrease in the LPS stimulated microglial production of TNF-α and NO

b) The concentrations at which antidepressants exert their anti-inflammatory actions will coincide with the levels achievable within the brain following concentrations standardly prescribed in the treatment of depression

c) Each of the antidepressants measured will differ in their anti-inflammatory potency

d) Anti-inflammatory activity of antidepressants will be regulated by β-adrenoceptor activation

e) Anti-inflammatory activity of antidepressants will be regulated by augmenting intracellular levels of cAMP
Experiment 3. Chronic stress induces profound structural remodelling of astrocytes within the prefrontal cortex: A characterization of the relationship between astrocyte morphology and density.

In the final study, we investigated the influence of chronic stress on astrocytes. To examine the stress-induced alterations, we created high-resolution 3D reconstructions of astrocytes, taken from GFAP immunolabelled mPFC tissue of animals exposed to chronic stress. In addition, we also investigated stress induced changes in the expression of S100β, a marker commonly associated with astrocyte distress, to determine its relationship with astrocyte remodelling and density. It was hypothesized that:

a) Chronic stress would significantly reduce astrocyte density in the mPFC

b) Chronic stress would induce significant alterations to the morphology of astrocytes within the mPFC

c) The intracellular and extracellular expression of S100β protein would be elevated in chronic stress exposed animals

d) Elevations in S100β would be significantly correlated with stress-induced changes in both astrocyte morphology and density
Chapter Two

Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions
CHAPTER 2 - Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions

Ross Tynan, Sundresan Naicker, Madeleine Hinwood, Eugene Nalivaiko, Kathryn Buller, David Pow, Trevor Day and F. Rohan Walker.

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Statement of Author Contributions to Manuscript

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<thead>
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<tbody>
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<td>Ross Tynan</td>
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Named Series: Biology of Microglia

Chronic stress alters the density and morphology of microglia in a subset of stress-responsive brain regions

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A B S T R A C T

The current study, in parallel experiments, evaluated the impact of chronic psychological stress on physiological and behavioural measures, and on the activation status of microglia in 15 stress-responsive brain regions. Rats were subjected, for 14 days, to two 30 min sessions of restraint per day, at random times each day. In one experiment the effects of stress on sucrose preference, weight gain, core body temperature, and struggling behaviour during restraint, were determined. In the second experiment we used immunohistochemistry to investigate stress-induced changes in ionized calcium-binding adaptor molecule-1 (Iba1), major histocompatibility complex-II (MHC-II), and Ki67. We also investigated cellular proliferation in these regions using Ki67 labelling, to check for the possibility of microglial proliferation. Collectively, the results we obtained showed that chronic stress induced a significant increase in anhedonia, a decrease in weight gain across the entire observation period, a significant elevation in core body temperature during restraint, and a progressive decrease in struggling behaviour within and over sessions. With regard to microglial activation, chronic stress induced a significant increase in the density of Iba1 immunolabelling (nine of 15 regions) and the number of Iba1-positive cells (eight of 15 regions). Within the regions that exhibited an increased number of Iba1-positive cells after chronic stress, we found no evidence of a between group difference in the number of MHC-II or Ki67 positive cells. In summary, these results clearly demonstrate that chronic stress selectively increases the number of microglia in certain stress-sensitive brain regions, and also causes a marked transition of microglia from a ramified resting state to a non-resting state. These findings are consistent with the view that microglial activation could play an important role in controlling and/or adapting to stress.

1. Introduction

There is increasing evidence that acute psychological stress can trigger inflammation of neural tissue, including brain tissue. Amongst the first studies to describe stress-induced neuroinflammation in the brain was that of O’Connor et al. (2003), who demonstrated that acute exposure to inescapable shock triggered production of the pro-inflammatory cytokine interleukin (IL)-1β in the brain. Subsequently, Blandino et al. (2006) demonstrated that pre-treatment with minocycline substantially attenuated this increase in IL-1β and, because minocycline inhibits microglial activation (Carty et al., 2008), this was taken as evidence that microglia play a central role in mediating the neuroinflammatory effects of stress. Consistent with this conclusion, there have been several...
reports that acute stress promotes the morphological transition of microglia from a ramified-resting state to an activated state, as characterised by an increase in the density of immunoreactive material for a microglial specific activation marker (Sugama et al., 2007, 2009). Moreover, there is evidence that this response may be regionally specific and vary in magnitude over time (Blanpain et al., 2005; Frank et al., 2007).

Microglial cells are functionally and morphologically dynamic. Microglia have a widespread distribution and, because of this, it has been proposed that their functional role is to actively scan the micro-environment for 'danger' signals such as purines (e.g. adenosine triphosphate), pro-inflammatory cytokines, glutamate receptor agonists, and cell necrosis factors (de Haas et al., 2008). Upon detection of a 'danger' signal within the micro-environment, microglia rapidly alter both their structure and function in a manner dependent on the type of stimulus, the intensity of stimulation, and the local environment in which exposure occurs (Ransohoff and Perry, 2009). Morphologically, microglia are characterised by a number of different phenotypes including ramified or resting, and then at different stages of activation as: hyper-ramified; reactive; or phagocytic (Streit et al., 1999). Depending on their phase of activation, microglia can produce an array of pro-inflammatory and cytotoxic molecules, and in some instances can become antigen presenting cells with many macrophage like properties, such as the ability to phagocytose pathogens and cellular debris (Chew et al., 2006). When activated, microglial cells are also known to proliferate, primarily through mitosis of resident microglial cells (Streit, 2006). Whilst the activation of microglia is often considered a necessary and beneficial immunological response to danger signals within the micro-environment (Kimura et al., 2005; Neumann et al., 2009), excessive and/or prolonged activation has been associated with the development of a number of different pathologies (Klegeris et al., 2007; Streit, 2004).

Presently, the extent to which chronic stress is capable of altering microglial status is largely unknown. In the only study that has examined this phenomena, Nair and Bonneau (2006), using flow cytometry, observed that the number of cortical microglia in mice was significantly increased after 4 days of restraint stress. This study was the first to clearly suggest that chronic stress could substantially modify microglia. Nair and Bonneau's approach, however, did not permit questions regarding regional specificity to be addressed, a relevant issue given the existence of stress-specific neural circuitry (Ulrich-Lai and Herman, 2009). In particular there is a rich literature describing the involvement of the medial prefrontal cortex (Radley et al., 2006), nucleus accumbens (Campioni et al., 2009), bed nucleus of the stria terminalis (Crané et al., 2003), hypothalamus (Herman et al., 2002), amygdala (Dayas et al., 2001), hippocampus (Jacobson and Sapolsky, 1991), periaqueductal gray (Keay and Bandler, 2001), and the ventral tigmental area (Rodaros et al., 2007), in the stress response. As such, the primary aim of the present study was to evaluate, using immunohistochemistry, changes in microglial activation status in these stress-responsive brain regions after exposure to chronic restraint stress. Specifically, we examined changes in the density of ionized calcium-binding adaptor protein-1 (iba1), a constitutively expressed microglial specific marker that is upregulated as a result of activation (Imai and Kohsaka, 2002), as well as major histocompatibility complex-II (MHC-II), a marker that is frequently present on activated microglia (Black et al., 2007). Additionally, as microglia are known to be capable of proliferating once activated, we examined ki67 immunoabelling in the same anatomical regions in which iba1 and MHC-II were assessed. Ki67 is expressed by cells actively going through the mitotic phase of cell division (Kee et al., 2002) and has been widely used to determine the proliferation rate of microglial cells (Klein and Roggen Dorf, 2001). To document the efficacy of our chronic stress paradigm we determined, in a parallel group, the effects of stress on animals' weight gain, core body temperature, struggling behaviour during restraint, and sucrose preference, the latter providing a guide to the potential development of anhedonia.

2. Methods

2.1. Animals

All experiments used adult male Sprague–Dawley rats obtained from the Animal Services Unit at the University of Newcastle. Animals were maintained in a temperature (21 ± 1°C) and humidity controlled environment with food and water available ad libitum. Lighting was on a 12:12 h reverse light–dark cycle (lights on 19:00 h) with all procedures conducted in the dark phase under low level red lighting (40 Lux). All animals were allowed to acclimate to single housing for a minimum of seven days prior to start of the experiment. All experiments were approved by the University of Newcastle Animal Care and Ethics Committee, and conducted in accordance with the New South Wales Animals Research Act and the Australian Code of Practice for the use of animals for scientific purposes.

2.2. Experimental design

To facilitate the assessment of the impact of chronic stress on microglial activation as well as physiological and behavioural parameters, the current study consisted of two experiments, both of which used an identical treatment protocol. Although the experiments could have been combined, we chose not to do so because implantation of the temperature transmitter involved invasive surgery. This would certainly trigger a degree of inflammation, which could have potentially changed the activation status of microglia giving rise to a risk of confounding our results. At the start of both experiments, animals were randomly allocated to either the stress or the handled control group. The first experiment (n = 7 per group) involved verifying the efficacy of the stressor. Specifically, we assessed the animals’ hyperthermic and behavioural (struggling) responses to restraint, as well as the development of anhedonia. Additionally, animal weights were recorded, with baseline weights measured prior to the stress protocol, and then every 4 days throughout the duration of the protocol. All of these measures have previously been shown to be sensitive to the effects of stress (Banasar and Duman, 2008; Beig et al., 2009; Grissom et al., 2008; Klank et al., 2006). In the second experiment (n = 5 per group) animals were euthanased for immunohistochemical analysis 24 h after the final stress session.

2.3. Chronic restraint protocol

Prior to the commencement of the experiment, we ran a series of pilot studies in order to determine the most effective protocol at inducing a significant stress response. From these initial investigations, we chose to use 2 × 30 min of randomly administered restraint sessions per day, as this protocol consistently induced a significant stress response. Control animals were handled twice daily in a separate room at an equivalent time. The restraint used in the current study has been described previously (Walker et al., 2009); it was constructed of a fine gauge wire mesh (0.6 mm diameter; 6.5 × 6.5 mm grid), secured with butterfly clips, and designed in accordance with the size of the rat. Restraints were painted matt black to reduce reflection and to increase the contrast ratio between the rat and the restraint during filming. All animals were restrained in their home cages.
2.4. Handling protocol for controls

The restraint procedure involved removing the animal from their home cage, inserting them into the restraint device, and then placing them back in their home cage for 30 min before releasing them. The handling procedure for control animals was designed to emulate this, minus the restraint. Thus, each animal was individually removed from their home cage, held for approximately 1 min, and then returned to their home cage.

2.5. Experiment 1

2.5.1. Core body temperature response to stress

To measure core body temperature, telemetric transmitters were aseptically implanted into the peritoneal cavity (TALOSA-F40, Data Sciences International, St. Paul, MN, USA). Prior to surgery, all animals prophylactically received carprofen (5 mg/kg s.c.) and were then anaesthetized with ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.). After surgery animals were allowed one week to recover.

The implanted telemetric probes transmitted information to receive plates located directly beneath the animals’ home cage. The receiver plates then relayed this information to a PC running DSI Dataquest A.R.T. (version 4.1) software which determined the mean temperature over a specified time window by calculating the average temperature over 20 sub-segments of the window length (DSI, 2007). This procedure also automatically discarded outliers.

Prior to recording, animals were allowed to acclimatize to the experimental room for 16 h. Core body temperature was then measured from 9:00-17:00 h on days 1, 7, and 14 of the stress protocol. During this time, animals were exposed to either two sessions of restraint stress or two sessions of handling, depending on their respective group allocation. Core body temperature measurements were acquired once every 30 s over the 8 h period. Temperature data was analysed using two approaches. The first involved calculating the area under the curve (AUC) values during restraint periods and the hour immediately following, using the trapezoidal method (Prism 4.02, GraphPad Software Inc.). The second approach involved calculating the Δ change in temperature from a baseline period, which was defined as the average temperature from a 5 min window immediately preceding restraint or handling.

2.5.2. Effect of stress on sucrose preference

In the same cohort of animals that received probe implantation, animals underwent a two bottle sucrose preference test (SPFT). The SPFT was conducted in the home cage; this involved providing two bottles simultaneously, one containing 200 ml of 1% sucrose solution, and the other containing 200 ml of normal drinking water. To minimize neophobia and habituate animals to the sucrose solution, animals were given three 24 h exposure periods to a 1% sucrose solution in their drinking water. After habituation, a 24 h baseline measurement of sucrose preference was taken prior to the commencement of the treatment protocol. Animals were subsequently assessed using the SPFT 24 h after the cessation of the treatment protocol. Total consumption was determined by weighing the bottles before and after the SPFT and calculating the difference. Preference was then subsequently determined by taking the relative percentage of sucrose consumed with respect to the total volume of fluid consumed.

2.5.3. Struggling behaviour during stress

On days 1, 2, 4, 7, and 14 of the restraint stress procedure, animals’ behaviour whilst in restraint were recorded to DVD via overhead infrared cameras. Video files were subsequently analysed by a trained observer to quantify the duration of struggling behaviour for each min during the initial 5 min of the restraint session. Struggling was operationally defined as any attempt to rotate in either direction, move forward, backwards or turn in the restraint (Walker et al., 2009).

2.6. Experiment 2

2.6.1. Tissue preparation

All animals were deeply anaesthetized (sodium pentobarbital, 80 mg/kg i.p.) and transcardially perfused 24 h after cessation of the final stress episode. Tissue was cleared with 300 ml of a 0.1 M phosphate buffer (PB) solution containing 2% sodium nitrite (pH 7.40), and then subsequently fixed using 400 ml of 4% paraformaldehyde (pH 9.60) in 0.1 M PB. Brains were extracted and placed in the same fixative solution for 2 h, and then blocked at the base of the midbrain before being placed in a 12.5% sucrose solution in 0.1 M PB (pH 7.40; 4 °C) overnight for cryoprotection. Serial coronal sections were then sliced on a freezing (−25 °C) microtome (Leica SM2000R) at 30 μm intervals. Sections were then divided into a one-in-six series and stored in an anti-freeze solution (4 °C) until required for immunoperoxidase or immunofluorescent labelling.

2.6.2. Immunoperoxidase labelling for Iba1 and MHC-II

For Iba1 immunolabelling, a series of sections from all animals in both treatment conditions were processed simultaneously. MHC-II was subsequently analysed in each of the stress-responsive regions demonstrating a significantly increased amount of Iba1-positive cells or immunoreactive material. Sections were rinsed with 0.1 M PB and then endogenous peroxidases were destroyed in 0.1 M PB containing 3% hydrogen peroxide. Non-specific binding was blocked with 3% normal horse serum. The sections were then incubated with the primary antibodies (anti-rabbit Iba1, Wako, 1:10,000; anti-mouse MHC-II, Serotec, 1:500) in 0.1 M PB containing 1% horse serum, 0.1% bovine serum albumin (BSA) and 0.3% Triton-X for 72 h at 4 °C. Sections were then rinsed, incubated in the corresponding secondary antibody (Amersham donkey anti-rabbit, 1:400; Amersham donkey anti-mouse, 1:400) in phosphate buffered horse serum for 2 h, rinsed, incubated in 0.1% extravadin peroxidase for 1 h, and then rinsed again. The reaction was detected under a microscope after applying 2% nickel sulphate in 0.1 M PB containing 0.05% 3,3'-diaminobenzidine, and was stopped and rinsed with 0.1 M PB once optimal staining with minimal background labelling had been achieved. Sections were then mounted onto chrome alum coated slides, dehydrated using a series of graded alcohols (70%, 95%, 100%, absolute), cleared in xylene and coverslipped with ultramount (Fronine Laboratory Supplies).

2.6.3. Immunofluorescent labelling for Iba1 and Ki67

Sections from both groups from all animals were processed simultaneously. Initially, we attempted to fluorescently double label sections to determine the extent of co-localisation of Iba1 (Wako, 1:1000, donkey anti-rabbit) and Ki67 (Neomarkers, 1:100, donkey anti-rabbit). As both primary antibodies were derived from the same species, we attempted a serial labelling procedure which incorporated fragment antigen binding (Fab fragment) following the addition of the first primary to minimize cross-reactivity. The results indicated, however, that this did not effectively prevent cross-reactivity, regardless of the concentration of Fab fragments or rabbit serum used. As an alternative, we replaced the anti-rabbit Ki67 with an anti-goat Ki67 (Santa Cruz, 1:100). However, the resultant labelling was clearly not restricted to the nucleus, as Ki67 should be (Schoelgen and Gerdes, 2000). Consequently, we instead labelled our sections for Ki67 only, using the original donkey anti-rabbit antibody (Neomarkers).

Sections were rinsed three times in 0.1 M phosphate buffered saline (PBS; pH 7.40), followed by 1 h incubation in 3% BSA and 0.3% Triton-X. Sections were then transferred into a 1% BSA and 0.1% Triton-
X solution containing the anti-rabbit Ki67 (Neomarkers, 1:100) primary antibody and incubated overnight at 4 °C. Sections were next rinsed three times in 0.1 M PBS, and then incubated in a 1% BSA and 0.1% Triton-X solution containing a fluorescent donkey anti-rabbit secondary antibody (AlexaFluo-488, Invitrogen, 1:400) for 2 h. Following incubation, sections were rinsed, mounted on chrome alum coated slides, and coverslipped with Gelvatol.

2.6.4. Analysis of Iba1 and MHC-II immunolabelling data

Results were analysed by an independent trained observer, blind to experimental treatment conditions. Images from multiplerostro-caudal levels were taken using an Olympus BX51 microscope fitted with an Olympus DP71 camera and an Olympus UPlanFi objective (10×/0.30). The images were processed using DP Manager software (Version 3.1.1.208; Olympus Corporation) and stored at a resolution of 4086 × 3072 pixels (1 pixel = 0.429 μm² at 100× magnification). A rat brain atlas (Paxinos and Watson, 2005) was used to identify the anatomical location for each of the 15 regions of interest for Iba1 immunolabelling: infralimbic medial prefrontal cortex (IL, six sections, bregma +3.70 to +2.50 mm), prelimbic medial prefrontal cortex (PL, six sections, bregma +3.70 to +2.50 mm), anterior cingulate cortex (ACC, three sections, bregma +3.20 to +2.20 mm), dorsal bed nucleus of the stria terminalis (DBNST, two sections, bregma −0.26 to −0.40 mm), ventral bed nucleus of the stria terminalis (vBNST, two sections, bregma −0.26 to −0.40 mm), nucleus accumbens core (NAcc, three sections, bregma +2.76 to +2.28 mm), nucleus accumbens shell (NAcS, three sections, bregma +2.76 to +2.28 mm), basolateral amygdala (BLA, two sections, bregma −2.12 to −0.64 mm), central amygdala (CeA, two sections, bregma −2.12 to −2.64 mm), medial amygdala, (MeA, three sections, bregma −1.80 to −2.64 mm), CA3 region of hippocampus (CA3, three sections, bregma −1.80 to −2.56 mm), paraventricular nucleus of the hypothalamus (PVN, three sections, bregma −1.60 to −2.12 mm), dorsal periaqueductal gray (dPAG, one section, bregma −5.30 mm), lateral periaqueductal gray (lPAG, two sections, bregma −5.30 to −5.88 mm), and the ventral tegmental area (VTA, two sections, bregma −5.30 to −6.04 mm). In each of the aforementioned regions, left and right hemispheres were recorded independently to assess inter-hemispheric asymmetries.

Both the number of cells and the total density of immunoreactive material in the aforementioned regions were determined using Metamorph software (Version 7.1.3.0; Molecular Devices). Both cell number and density assessments were obtained using thresholding operations where only pixels within a given range of colour intensity and size were included for analysis (see Fig. 1). For cell counting, the following colour thresholding parameters were used: hue, 0–254; saturation, 9–255; intensity, 3–121. Additionally, thresholded material was size filtered to include only clusters of pixels 100–3000 in number, to restrict the semi-automated counts to cell bodies. This approach was highly correlated with manual counts of microglial cell bodies (r = 0.94). When determining the density of immunoreactive material, the colour threshold parameters were set to: hue, 0–255; saturation, 0–255; intensity, 4–150. These parameters resulted in the cell body and the associated processes, as opposed to just the cell body, being thresholded. The density of immunoreactive material was then determined by measuring the relative percentage of all thresholded material within the given region. To obtain an estimate of the size of each of the Iba1-positive cells, the total number of Iba1-positive cell bodies counted was subsequently divided by the percentage of thresholded material.

2.6.5. Analysis of Ki67 immunolabelling data

Ki67 immunolabelling was only performed in brain regions in which there was a significant increase in Iba1-positive cell number, or the density of Iba1 immunoreactive material. Similar to our analysis of Iba1 and MHC-II immunolabelled tissue, images were taken at 100× magnification using an Olympus BX51 microscope fitted with an Olympus DP71 camera and stored at a resolution of 4086 × 3072 pixels. For immunofluorescent images, a halogen light source was produced through an Olympus U-RFL-T burner. Captured images were imported into Paintshop Pro (Version 5.01, Jasc Software). Manual counting of Ki67 positive cells involved inserting a transparent layer above the image, onto which 2 mm dots were digitally placed directly over Ki67 positive cells. A semi-transparent digital grid was then overlaid on the region of interest and every fourth grid cell manually counted, resulting in 25% of the total region of interest being quantified. This process created a dot plot which was subsequently saved as a separate.tif file, imported into Metamorph and analysed in a manner identical to that used for the quantification of Iba1-positive cells. To verify the validity of this approach, several randomly selected regions were counted in full (100%), and these total counts were then correlated with the 25% counts. The results of this comparison revealed a strong correlation for handled control tissue (r = 0.969) and for stress tissue (r = 0.951) between the 100% and 25% count approaches.

2.7. Statistics

All data were analysed using the Statistical Package for Social Sciences (version 16). Body weight data was analysed using a repeated measures analysis of covariance with ‘Stress Group’ as the between subjects variable (two levels: stress, control) and ‘Time’ as the within subjects variable (five levels: days 1, 5, 9, 13, 17). Baseline weight was used as the covariate and planned compari-
sons were used to investigate the interaction between ‘Stress Group’ and ‘Time’ using the Holm correction to control the family-wise error rate. Two procedures were used to analyse core body temperature, area under the curve (AUC) and Δ change in temperature from baseline. Both of which were analysed using a repeated measures analysis of variance (ANOVA) with ‘Stress Group’ as the between subjects variable (two levels: stress, control) and ‘Time’ as the within subjects variable (six levels: day (d)1 session (s)1, d1s2, d7s1, d7s2, d14s1, d14s2). Sucrose preference data was analysed using an independent sample t-test with ‘Stress Group’ as the between subject variable (two levels: stress, control). Struggling behaviour during stress exposure was analysed using a repeated measures ANOVA with ‘Time’ of measurement (five levels: Min 1–5) and ‘Day’ of measurement (five levels: days 1, 2, 4, 7, 14) as within subject variables. Tukey’s HSD post hoc analysis was used to explore any significant within group differences in ‘Time’ and ‘Day’. Immunohistochemical data was analysed using independent sample t-tests with ‘Stress Group’ as the between subject variable (two levels: stress, control). Prior to analysis, ‘rostro-caudal Level’ and ‘Hemisphere’ were analysed using repeated measures ANOVA and independent sample t-tests, respectively. Immunofluorescent data were analysed using independent sample t-tests. The α criterion for all statistical analysis was $p < 0.05$.

3. Results

3.1. Effects of stress on body weight

The body weights of both stress and control groups were recorded every 4 days, for the duration of the protocol. The analysis of this data revealed a significant ‘Time’ by ‘Stress Group’ interaction, $F(3,33) = 5.36, p = 0.004$. Subsequent a priori comparisons indicated that the stress group weighed significantly less than the control group on days: 9, $t(12) = 2.762, p = 0.017$; 13, $t(12) = 3.314, p = 0.006$; and 17, $t(12) = 2.845, p = 0.015$. Average body weight (±SEM) data are shown in Fig. 2.

3.2. Core body temperature response to stress

Core body temperature was recorded from 5 min before through to 60 min after each of the restraint sessions conducted on days 1, 7, and 14, or at a corresponding time in the case of controls (Fig. 3a and b). Mean ambient room temperature in which stress-induced temperature changes were recorded on day 1 ($M = 18.9 \degree C, SE = 0.002$), day 7 ($M = 19.1 \degree C, SE = 0.003$), and day 14 ($M = 19.3 \degree C, SE = 0.003$) of the stress protocol. Analysis of AUC data for all of the sessions across the three days showed that
there was not a significant interaction between ‘Stress Group’ and
‘Time’, but there was a significant main effect of ‘Stress Group’,
\( F(1,12) = 49.211, p < 0.001 \). The second approach used to analyse
temperature data was to determine the magnitude of change from
baseline. This analysis indicated that there was no significant inter-
action between ‘Stress Group’ and ‘Time’. Further, there was no sig-
nificant main effect for ‘Time’, suggesting the degree of
temperature change was equivalent across assessment days. How-
ever, a significant main effect was found for ‘Stress Group’,
\( F(1,12) = 52.076, p < 0.001 \), indicating that animals subjected to re-
straint stress experienced a significant increase in temperature,
relative to controls, on all of the assessment days (Fig. 3c).

3.3. Effect of stress on sucrose preference

Sucrose preference data were analysed by first calculating the
relative percentage of sucrose consumed as a function of the total
fluid consumed using the formula: \([\text{sucrose consumed} / \text{sucrose}
\text{ consumed + water consumed}] \times 100\). To account for pre-existing
differences in sucrose preference, the relative percentage of su-
crose preference for the post-stress recordings were subsequently
adjusted relative to the percentage of each animal’s baseline mea-
surement. As illustrated in Fig. 4, animals in the stress treatment
condition demonstrated a significant reduction in their sucrose
preference relative to controls 24 h after the final restraint session,
\( t(11) = 2.22, p = 0.024 \).

3.4. Struggling behaviour during stress

The duration of struggling behaviour during the initial 5 min of
restraint was quantified on days 1, 2, 4, 7, and 14 of the restraint
protocol. The results indicated a significant interaction between
‘Time’ within a session and the ‘Day’ of session, \( F(16,100) = 1.809, p = 0.04 \). This interaction was explored using Tukeys HSD post hoc test, which revealed that animals subjected to restraint strugg-
gled significantly more during their first restraint session than in
all subsequent sessions (\( p < 0.05 \) in all cases). The analysis also
indicated that, within each of the restraint sessions, animals strugg-
gled significantly more at the beginning and progressively less
thereafter (\( p < 0.05 \) in all cases). The average duration of struggling
behaviour across each of the stress sessions is illustrated in Fig. 5.

3.5. Effects of stress on Iba1 immunolabelling

Initially, we evaluated whether lateralisation of each ‘Hemi-
sphere’ or ‘rostral-caudal Level’ should be incorporated into our
analysis within each of the 15 regions in which we assessed
changes in Iba1 immunoreactivity. These analyses indicated that
there were neither inter-hemispheric nor rostro-caudal differ-
ences. Accordingly, we report only group means that have been
collapsed across rostro-caudal level and hemisphere.

The first method used to evaluate microglial status was to count
the number of Iba1-positive cells in 15 regions commonly identi-
ﬁed as stress-responsive. As summarised in Table 1, independent
sample t-tests showed that, relative to controls, animals subjected
to the chronic stress protocol had significantly higher numbers of
Iba1-positive cells in the: IL, \( t(8) = 2.49, p = 0.038 \); PL, \( t(8) = 2.90, p = 0.020 \); ACg, \( t(8) = 3.70, p = 0.006 \); NAc, \( t(8) = 2.25, p = 0.041 \);

![Fig. 4. Mean (±SEM) percentage change in sucrose preference relative to baseline for chronically stressed and handled control animals 24 h after the cessation of the treatment protocol. Significant difference between groups (\( p < 0.05 \)).](image-url)

| Table 1 Mean (±SEM) Iba1-positive cell counts for handled control and chronically stressed animals across the 15 stress-responsive regions investigated in tissue harvested 24 h after the cessation of the treatment protocol. |
|-----------------------------------------------|---------------|
| **Control** | **Stress** |
| Medial prefrontal cortex IL | 292 (11) | 330 (11)* |
| PL | 462 (13) | 544 (25)* |
| ACg | 294 (7) | 342 (10)* |
| Nucleus accumbens NAcB | 329 (18) | 400 (23)* |
| NAcS | 302 (19) | 358 (15)* |
| Bed nucleus of the stria terminals dBNST | 340 (11) | 381 (9)* |
| vNST | 172 (12) | 187 (15) |
| Hypothalamus PVN | 140 (9) | 156 (9) |
| Amygdala BLA | 229 (17) | 257 (21) |
| MeA | 178 (10) | 209 (15) |
| CeA | 334 (19) | 379 (30) |
| Hippocampus CA3 | 119 (3) | 154 (7)* |
| Periaqueductal gray dPAG | 67 (9) | 74 (9) |
| IPAG | 304 (37) | 437 (37)* |
| Ventral tegmental area VTA | 119 (12) | 152 (28) |

* Significant difference between groups (\( p < 0.05 \)).
Table 2

Mean (±SEM) density of Iba1-positive immunoreactive material for handled control and chronically stressed animals in each of the 15 stress-responsive regions investigated in tissue harvested 24 h after the cessation of the treatment protocol.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial prefrontal cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>6.83 (0.21)</td>
<td>9.68 (0.68)</td>
</tr>
<tr>
<td>PL</td>
<td>6.12 (0.40)</td>
<td>9.42 (1.03)</td>
</tr>
<tr>
<td>AcG</td>
<td>7.86 (0.57)</td>
<td>11.49 (1.17)</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaC</td>
<td>7.61 (0.55)</td>
<td>10.92 (1.13)</td>
</tr>
<tr>
<td>NaC stress</td>
<td>7.38 (0.57)</td>
<td>10.05 (0.80)</td>
</tr>
<tr>
<td>Bed nucleus of the striae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>telencephali</td>
<td>11.13 (0.36)</td>
<td>13.53 (0.26)</td>
</tr>
<tr>
<td>vBST</td>
<td>16.36 (1.42)</td>
<td>17.89 (0.94)</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/VN</td>
<td>16.52 (0.57)</td>
<td>19.59 (1.38)</td>
</tr>
<tr>
<td>Amygdala</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLa</td>
<td>4.97 (0.45)</td>
<td>7.34 (1.18)</td>
</tr>
<tr>
<td>MeA</td>
<td>7.40 (0.63)</td>
<td>10.29 (1.02)</td>
</tr>
<tr>
<td>CeA</td>
<td>8.38 (0.64)</td>
<td>11.81 (1.73)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td>6.87 (0.44)</td>
<td>11.03 (0.95)</td>
</tr>
<tr>
<td>Periaqueductal gray</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBPG</td>
<td>14.15 (2.43)</td>
<td>15.84 (1.28)</td>
</tr>
<tr>
<td>IPAG</td>
<td>7.90 (0.88)</td>
<td>12.14 (1.29)</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VTA</td>
<td>11.05 (0.31)</td>
<td>13.22 (1.58)</td>
</tr>
</tbody>
</table>

*p Significant difference between groups (p < 0.05).

4. Discussion

In the present study, two weeks of unpredictable restraint stress produced significant physiological and behavioural changes, notably reduced weight gain and anhedonia, that are commonly regarded as characteristic of exposure to chronic psychological stress. Importantly, these changes were accompanied by signs of microglial activation in the brain. This is consistent with past evidence that exposure to stress can trigger a CNS neuroinflammatory response involving microglia (Blavodino et al., 2009; Johnson et al., 2005; Nair and Bonneau, 2006). However, the present study significantly extends previous reports by demonstrating that this microglial activation, as indexed through alterations in microglial morphology, displays evidence of regional specificity in the brain. In particular, we examined 15 brain regions previously implicated in the initiation, control and/or expression of stress responses, and observed a significant alteration in only nine of these: AcG, IL, and PL medial prefrontal cortex (mPFC), NaC, NaC stress, MeA, dBST, CA3, and IPAG. We believe that this outcome supports the view that regionally selective microglial activation may play an important role in the control of stress responses, and/or adaptation to stress, and may therefore constitute a novel target for ameliorating the mental and physical consequences of excessive psychological stress.

4.1. Significance of the behavioural and physiological response findings

When an animal is repeatedly exposed to the same stressor, their response to it may decline over time, an effect referred to as habituation (Rankin et al., 2009). Indeed, there is particularly good evidence that this occurs for some stress indices, such as glucocorticoid secretion (Dallman, 2007). Although we did not monitor glucocorticoid responses, our findings suggest that habituation did not occur in response to the chronic restraint procedure deployed in the current study. Specifically, as has been shown in previous studies, the weight reduction observed in stressed animals persisted throughout the entire protocol (Depke et al., 2008; Ottenweller et al., 1992). Furthermore, a significant elevation in core body temperature occurred during all monitored restraint sessions (Hashimoto et al., 2001; Ootsuka et al., 2008), with no evidence of habituation over the 14 days of the protocol. And finally, we also observed a significant decrease in sucrose preference even after the cessation of the stress protocol, an effect indicative of anhedonia (Bekris et al., 2005; Rygula et al., 2005). Interestingly, our data does show that the duration of struggling behaviour during restraint episodes decreased over time. While this effect could be taken to indicate that habituation to the stressor has occurred, an alternative interpretation is that it is consistent with the development of learned helplessness, as originally defined by Seligman (1975), and is therefore in keeping with our evidence of the development of anhedonia.

4.2. Iba1 as a marker of activated microglia

Ionomized calcium-binding adaptor protein-1 (Iba1), also known as allograft inflammatory factor-1 (ALF1), is a specific marker of microglia (Liu et al., 2007). Iba1 co-localises with filamentous actin, modulating the membrane ruffling that occurs during phagocytosis (Imai and Kohsaka, 2002). In immunohistochemical studies of brain tissue, Iba1 has been observed in microglia, but never astrocytes, oligodendrocytes or neurons (Imai et al., 1996).
Figure 6. Low (LHS, scale bar = 100 μm) and high power (RHS, scale bar = 50 μm) examples of Iba1 immunolabelled sections taken from nine brain regions where chronic stress elicited significant increases in Iba1-positive immunoreactive material relative to handled controls. Images are from the infralimbic cortex (IL, bregma +3.70 mm), prelimbic cortex (PL, bregma +3.70 mm), anterior cingulate cortex (ACg, bregma +2.70 mm), nucleus accumbens core (NACc, bregma +2.20 mm), nucleus accumbens shell (NACs, bregma +2.20 mm), dorsal bed nucleus of the stria terminalis (dBNST, bregma −0.30 mm), medial amygdala (MeA, bregma −1.80 mm), hippocampus (CA3, bregma −2.12 mm), and the lateral periaqueductal gray (IPAG, bregma −5.60 mm).
Table 3
Estimated mean (±SEM) cell size of Iba1-positive microglial cells for handled control and chronically stressed animals 24 h after the cessation of the treatment protocol.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial prefrontal cortex</td>
<td>42.8 (12)</td>
<td>34.5 (1.9)*</td>
</tr>
<tr>
<td>IL</td>
<td>76.5 (3.9)</td>
<td>59.4 (4.1)*</td>
</tr>
<tr>
<td>PL</td>
<td>38.2 (2.6)</td>
<td>30.7 (2.3)</td>
</tr>
<tr>
<td>ACg</td>
<td>43.7 (2.2)</td>
<td>36.2 (2.2)</td>
</tr>
<tr>
<td>NAcc</td>
<td>41.2 (1.3)</td>
<td>37.6 (2.7)</td>
</tr>
<tr>
<td>NACs</td>
<td>10.6 (0.4)</td>
<td>10.4 (0.4)</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminals</td>
<td>24.3 (0.7)</td>
<td>20.6 (0.9)*</td>
</tr>
<tr>
<td>DGNSST</td>
<td>17.51 (0.80)</td>
<td>14.12 (0.57)*</td>
</tr>
<tr>
<td>Perguesticulate gray</td>
<td>10.7 (0.8)</td>
<td>11.3 (0.7)</td>
</tr>
</tbody>
</table>

* Significant difference between groups (p < 0.05).

Table 4
Mean (±SEM) number of Ki67 positive immunolabelled cells for handled control and chronically stressed animals 24 h after the cessation of the treatment protocol.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial prefrontal cortex</td>
<td>85 (8)</td>
<td>107 (4)</td>
</tr>
<tr>
<td>IL</td>
<td>101 (11)</td>
<td>111 (7)</td>
</tr>
<tr>
<td>PL</td>
<td>74 (3)</td>
<td>65 (8)</td>
</tr>
<tr>
<td>ACg</td>
<td>67 (9)</td>
<td>57 (4)</td>
</tr>
<tr>
<td>NAcc</td>
<td>65 (6)</td>
<td>53 (4)</td>
</tr>
<tr>
<td>NACs</td>
<td>40 (4)</td>
<td>36 (3)</td>
</tr>
<tr>
<td>Bed nucleus of the stria terminals</td>
<td>112 (17)</td>
<td>129 (15)</td>
</tr>
<tr>
<td>dNST</td>
<td>30 (3)</td>
<td>28 (2)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>163 (42)</td>
<td>157 (25)</td>
</tr>
</tbody>
</table>

Furthermore, in brain tissue, Iba1 is expressed only weakly by ramified microglia, but strongly by activated microglia (hyper-ramified, reactive and amoeboid), which is why Iba1 is routinely used to map alterations in microglial activation status (Imai and Kohsaka, 2002).

4.3. Interpretation of observed changes in Iba1 immunolabelling

Thresholding is one of the procedures commonly used to quantify changes in immunoreactive material (Abbadi et al., 1996; Romero-Sandoval et al., 2008; Shapiro et al., 2008); in particular, it has now been used a number of times to quantify changes in the immunolabelling of microglia specific markers after exposure to stress (Sugama et al., 2004, 2009). In brief, it involves converting acquired colour images to greyscale, further converting these into a binary image with all values above or below a predefined set point converted to black and white, respectively, and then quantifying the proportion of black material. Although a simple technique, interpretation of the results is not straightforward, as there is no direct relationship between the amount of material quantified and the number of cells within the detection field. Because microglia can rapidly proliferate (Dailey and Waite, 1999), and significantly alter their morphology, a between group difference in thresholded material could reflect a change in cell number, a change in cell morphology, or both. Thus, to obtain a clearer profile of microglial alterations, cell number, in addition to the ratio of immunoreactive material to cell number, should be determined. In the present study we: (1) counted the total number of Iba1-positive cell bodies; (2) the total amount of immunoreactive material; and (3) if either of these were significant, we obtained an estimate of cell size by dividing the total number of cells by the percentage of immunoreactive material.

In regard to the number of Iba1-positive cells in animals subjected to stress, we found increased numbers within the ACg, IL, PL, NAcc, NACs, dNST, CA3, and the IPAG. There are two possible explanations for this finding. Firstly, it may have been the case that exposure to chronic stress increased the expression of Iba1, so that cells that were previously present but below the detection limit became detectable. Alternatively, the increased numbers of Iba1-positive cells could be the result of cell proliferation, as microglia can proliferate rapidly (within 45 min) once activated (Dailey and Waite, 1999). To investigate the latter possibility, we immunolabelled our tissue for Ki67, a nuclear protein that is expressed during mitosis (Scholzen and Gerdes, 2000) and has previously been used to identify mitotically active microglia (Klein and Roggendorf, 2001). We found that although there were significant numbers of mitotically active cells in both treatment groups, a finding consistent with previous results (Banas et al., 2007; Kee et al., 2002) there were no between group differences. This suggests that the increased number of Iba1-positive cells observed in chronically stressed animals is likely the result of an increased Iba1 expression rather than cell proliferation. However, two caveats apply to this conclusion. First, because we were unable to double label for Iba1 and Ki67, we cannot rule out the possibility that mitotically active cells in stressed animals corresponded to a different population from those seen in controls. Second, failure to obtain evidence of cell proliferation 15 days after the start of the stress protocol does not mean that it could not have occurred at an earlier time point in the protocol; as such, it would be profitable for future studies to collect tissue samples from parallel groups throughout the protocol to determine if microglial proliferation occurs at earlier time points.

A number of different techniques have been used to estimate cell size in immunolabelled sections, including 3D stereological estimation (Dorph-Petersen et al., 2001) and shell analysis (Vega et al., 2004). However, a common approach is to divide the number of cells present within a field by the relative proportion of immunoreactive material (Sugama et al., 2007). In the current study this approach yielded results indicating that Iba1-positive cells from chronically stressed animals were significantly smaller than those of controls in the IL, PL, CA3, and MeA. This reduced cell size is in fact consistent with the morphological alterations typically observed in reactive microglia; specifically, a decrease in the length of processes and a decrease in cell arborisation (Stoll et al., 1998). However, we did not observe an increase in numbers of MHC-II-positive cells, a protein most commonly associated with phagocytic microglia (Aloisi, 2001). As such, we believe that it is reasonable to conclude that Iba1-positive microglia in the PL, IL, CA3, and MeA had not transitioned to this final activation state.

A particularly intriguing suggestion that arises from the present findings is that there are likely to be regional differences, even between stress-sensitive brain regions, in the impact of chronic stress upon microglia. Accordingly, significant microglial activation, as assessed on the basis of changes in Iba1 immunolabelling, was seen in only 9 of the 15 stress-sensitive brain regions examined, while reductions in microglial cell size were seen in just four of those nine regions. The basis of these differences is unclear. One possibility is that, rather than absolute differences in sensitivity to the impact of chronic stress, the responses of microglia in differ-
ent regions may simply develop at different rates, or endure for differ-
ent periods. In this regard it is pertinent to note in passing that,
even if a microglial response resolves within a fixed period of time,
the event may still provide the basis of continuing functional changes in that region via mechanisms such as a residual trophic effect on neuronal signalling (Alexander et al., 2009). But whether these regional differences reflect absolute differences in the sensi-
tivity of microglia to chronic stress, or differences in the time course of microglial responses, they still require explanation. Regionally specific differences in neural inputs or receptors for endocrine signals merit consideration in this regard and, indeed, Blandino et al. (2006, 2009) have recently provided evidence that stress-induced release of cytokines by hypothalamic microglia can be modulated by catecholamines and glucocorticoids, the latter already being well known as modulators of microglial function (Nair and Bonneau, 2006).

With regard to a broader interpretation of the functional significance of the present findings, it is of course well known that all nine of the regions found to display signs of microglial activation play a crucial role in the initiation and/or modulation of responses to psy-
chosocial stress. The amygdala, for example, is thought to play a critical role in evaluating whether particular sensory inputs merit the initiation of a stress response (Roozendaal et al., 2009) while also projecting directly to structures, such as the PAG (Krettek and Price, 1978), to modulate the ability of such regions to elicit co-ordinated behavioural and physiological responses to stress (Kaye and Bandler, 2001). Likewise, the IL and PL are capable of strongly modulating the activity of a wide variety of sub-cortical structures, including the BNST and the NAC, that contribute to the control of the stress re-
sponse (Fasucci et al., 2007; Spencer et al., 2005).

5. Conclusions

In recent years, several research groups have described the abil-
ity of acute stress to induce changes in microglial status (Blandino et al., 2009; Frank et al., 2007; Sugamura et al., 2007). Despite the fact that these studies have contributed substantially to the knowledge base, it is widely recognised that it is, in most instances, exposure to chronic rather than acute stress that is a major risk factor in the emergence of a variety of stress-linked psychopathologies (Hu-
man, 2006; Rygula et al., 2005). As such, in the current study, we wished to determine how chronic stress influences regional microglial activation status. To do this, we exposed adult male rats to 14 days of unpredictable restraint stress. To evaluate the efficacy of this intervention, we monitored core body temperature, body weight, sucrose preference and struggling behaviour, all of which were significantly perturbed as a result of chronic stress. These re-
sults are consistent with the findings of numerous other studies investigating the behavioural and physiological consequences of chronic stress. Coincident with these alterations, we have found evidence to suggest that in several stress-responsive regions, chronic stress appears to have provoked a significant alteration in the phenotype of microglia, causing the transition from a resting to an activated phenotype. These findings are consistent with the view that microglial activation could play an important role in controlling stress responses.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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Chapter Three

A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia
**CHAPTER 3 – A Comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia**

Ross Tynan, Judith Weidenhofer, Madeleine Hinwood, Murray Cairns, Trevor Day and F. Rohan Walker.

Brain Behavior and Immunity, 24, (2010), p 1058-1068

**Statement of Author Contributions to Manuscript**

<table>
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<tr>
<th>Author</th>
<th>Description of contribution to manuscript</th>
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<tr>
<td>Ross Tynan</td>
<td>Designed and executed the study. Analysed and interpreted the data. Wrote the manuscript.</td>
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<td>Judith Weidenhofer</td>
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<td>Madeleine Hinwood</td>
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<td>Aided in data interpretation. Corrected the manuscript.</td>
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<td>Designed the study. Interpreted the data. Co-the manuscript.</td>
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A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia

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A B S T R A C T

Selective serotonin and serotonin norepinephrine reuptake inhibitors (SSRI; SNRI) are the first choice pharmacological treatment options for major depression. It has long been assumed that the primary therapeutic mechanism of action of these drugs involves the modulation of monoaminergic systems. However, contemporary investigations have revealed that depression is linked with inflammation, and that SSRI/SNRIs possess significant anti-inflammatory actions. While these anti-inflammatory properties initially only related to work undertaken on cells of the peripheral immune system, it has recently become apparent that these drugs also exert anti-inflammatory effects on microglia, the principal cells within the CNS that regulate and respond to inflammatory factors. The aim of the current study was to compare SSRI/SNRIs in terms of their anti-inflammatory potency, and to determine the specific mechanisms through which these effects are mediated. Accordingly, the current study evaluated the ability of five different SSRIs (fluoxetine, sertraline, paroxetine, fluvoxamine and citalopram) and one SNRI (venlafaxine) to suppress microglial responses to an inflammatory stimulus. Specifically, we examined their ability to alter tumour necrosis factor-α (TNF-α) and nitric oxide (NO) production after 4 and 24 h stimulation with lipopolysaccharide. Our results indicated that the SSRIs potentially inhibited microglial TNF-α and NO production. We then investigated whether these effects might involve either α-adrenoceptor or cAMP signalling. Using the protein kinase A inhibitor Rp-CAMPS, we found evidence to suggest that cAMP signalling is involved in regulating the anti-inflammatory response. These findings suggest that antidepressants may owe at least some of their therapeutic effectiveness to their anti-inflammatory properties.

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1. Introduction

At present, it is recommended that individuals with moderate to severe depression be prescribed either a selective serotonin or a serotonin–norepinephrine reuptake inhibitor (SSRI; SNRI) (Ellis, 2004). The long-standing theoretical justification for using such drugs to treat depression is provided by the monoamine theory which, in its original form, held that depression arises from a deficit in brain monoamines (Heninger et al., 1996). However, there is now a growing consensus that this theory is insufficient to explain the therapeutic effects of antidepressants (Heninger et al., 1996; Lacasse and Leo, 2005). In this regard, it is notable that many recent clinical (Hickie and Lloyd, 1995; Shelton et al., 2011) and pre-clinical studies (Hinwood et al., 2011; Tynan et al., 2010; Wohleb et al., 2011) have provided evidence that depression may involve inflammatory processes within the brain. Evidence of this type has now been used as the foundation of the neuroinflammatory theory of depression (Maes et al., 2009; Wager-Smith and Markou, 2011). An important question that arises in light of this theory is whether drugs currently prescribed on the basis of their ability to alter monoamine reuptake might, in fact, owe at least some of their effectiveness as antidepressants to their ability to inhibit inflammatory activity in the brain.

Numerous studies have found that depression is associated with elevated levels of pro-inflammatory (PI) cytokines (Dowlati et al., 2010; Howren et al., 2009), and an increased rate of single nucleotide polymorphisms within pro-inflammatory genes (Baune et al., 2010; Cerri et al., 2009, 2010; Galecki et al., 2010; Hong et al., 2005; Hwang et al., 2009; Wong et al., 2008). Further suggesting that PI cytokines contribute causally to the disorder are studies that have shown antidepressant treatment normalises pro-inflam-
matory cytokine elevations in patients with major depression (recently reviewed by Hannestad et al., 2011; Janssen et al., 2010). Aligning with these clinical reports are in vitro studies, demonstrating that SSRIs can inhibit the inflammatory responses of human and rodent peripheral immune cells (Martenson and Nassberger, 1993; Saccard et al., 1994; Xia et al., 1996b). The ability of SSRIs and SNRIs to reduce inflammation in the periphery is intriguing, but does this translate to an action in the brain that might be relevant to the treatment of depression? Evidence of peripheral anti-inflammatory actions does not necessarily constitute evidence of an anti-inflammatory action in the brain, as the cells that are primary to inflammatory processes in the brain (microglia) are functionally quite distinct from the immune system cells that mediate the anti-inflammatory effects of SSRIs and SNRIs in the periphery (Graeber, 2010; Wake et al., 2009). Recent studies, however, have provided tantalising evidence that at least some SSRIs can alter certain aspects of the microglial inflammatory response. In particular, it has been reported that fluoxetine, paroxetine and sertraline can modulate the ability of murine microglia to produce the pro-inflammatory tumour necrosis factor-α (TNF-α) and the free radical nitric oxide (NO) (Hashioka et al., 2007, 2009; Horikawa et al., 2010; Hwang et al., 2008). However, these effects have been variable, with both SSRI-induced decreases (Ha et al., 2006), and decreases (Hashioka et al., 2007) in microglial TNF cytokine production being reported. Nevertheless, it is plausible that these differing results arise from variations in factors such as the concentrations of drugs used and their impact on cellular viability measurements, and also the length of time that microglia are exposed to these drugs. To address these concerns, it would be preferable to compare a range of antidepressants in a single study.

Accordingly, the experiments undertaken in the current study sought to establish the relative anti-inflammatory potency, as determined by their IC_{50} of six antidepressants: five SSRIs (fluoxetine, sertraline, paroxetine, fluvoxamine, and citalopram) and one SNRI (venlafaxine). With respect to endpoints, we evaluated the ability of each of these drugs, to alter the production of TNF-α and NO, and 24 h after stimulation with lipopolysaccharide (LPS). TNF-α was chosen as a primary dependent variable as (i) depressed patients exhibit increased levels of TNF-α (Dowlati et al., 2010); (ii) TNF-α release dynamics in relation to other PI cytokines (i.e. IL-6, IL-1β) have been extensively studied in response to LPS stimulation (Henn et al., 2009) and; (iii) because TNF-α release has been routinely evaluated in other studies that have examined the anti-inflammatory effects of antidepressants (Ha et al., 2006; Hwang et al., 2008). NO was chosen on the basis that excessive free radical production has been hypothesised to play a role in the pathogenesis of depression (Maes et al., 2009), and it has been the most routinely assessed PI molecule in studies examining the anti-inflammatory effects of antidepressants (Ha et al., 2006; Hashioka et al., 2007; Horikawa et al., 2010; Lu et al., 2010). Finally, we chose LPS to stimulate microglia as this is the most extensively used and characterised method for inducing the release of PI molecules (Sebire et al., 1993), and the reduction of LPS-stimulated PI molecule release effectively represents the benchmark procedure for evaluating the anti-inflammatory efficacy of putative anti-inflammatory compounds (Innamorato et al., 2008). To ensure any observed actions were not secondary to an effect on cell viability, we screened for cytotoxicity using both a resazurin-based and a dye exclusion based test, the latter being more common but less sensitive than the former. Because the precise mechanisms through which antidepressants exert their effects on inflammatory processes remain uncertain, we investigated two proposed candidate mechanisms (Hashioka et al., 2009). Firstly, we evaluated if the anti-inflammatory effects occurred through a β-adrenoceptor linked mechanism, by co-incubating antidepressants with the general β-antagonist propranolol. Secondly, we examined the influence of intracellular levels of cyclic-adenosine-monophosphate (cAMP) by inhibiting adenylyl cyclase activity with SQ-22536, and also inhibition of the cAMP-dependent protein kinase A (PKA) with Rp-cAMPS. The results of this study demonstrate that SSRIs, but not the SNRI venlafaxine, potently inhibit LPS-induced microglial TNF-α and NO production, but have the potential to be both pro- and anti-inflammatory in a time- and concentration-dependent manner. We also observed evidence to suggest that these effects may be partially mediated via a PKA-dependent mechanism. In summary, the results of the current study add to the body of knowledge concerning the pharmacological properties of these drugs, and may in the future prove useful to improving treatment outcomes for those placed on antidepressant treatment.

2. Method

2.1. Microglial cell culture

All experimental analysis utilised the immortalised murine microglial cell line BV-2, gifted from Prof. Samuel Breitbart. The BV-2 cell line was chosen due to their consistent and reliable reproducibility of many of the functional and morphological properties displayed by primary microglia in vivo (Blasi et al., 1990; Henn et al., 2009). Furthermore, the cell line is commonly used as a substitute for primary microglia due to their high level of purity in culture (Ij et al., 2009). BV-2 cells were maintained in Dulbecco's modified eagle medium (DMEM, Thermo-scientific) supplemented with 2 mM l-glutamine, 10% foetal bovine serum (Sigma–Aldrich) and 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer. The broad spectrum antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the cell culture throughout the growth phase, but removed prior to experimental manipulation. Cultures were maintained in 175 cm² filtered cap cell culture flasks (Greiner Bio-One) in a series II water-jacketed CO₂ incubator (ThermoForma) set at 37 °C with 5% CO₂. Cellular growth was monitored daily, and culture medium changed every 3 days. The purity of the cell line was verified by positive immunostaining with Iba1, a cell surface protein selectively and constitutively expressed by microglial cells (Imai and Koshaka, 2002). Our results indicated a high level of purity, with greater than 99% of all nucleated cells positively expressing the Iba1 protein (see Fig. 1).

2.2. Reagents

Fluoxetine hydrochloride (PCCA), sertraline hydrochloride (Sigma–Aldrich), paroxetine hydrochloride hemihydrate (Sigma–Aldrich) and minocycline hydrochloride (Sigma–Aldrich) were initially dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich) to make 100 mM stock solutions. Venlafaxine hydrochloride (Sigma–Aldrich) and fluvoxamine maleate (Sigma–Aldrich) were initially dissolved in double distilled tissue culture water (ddH₂O, Sigma–Aldrich), at a concentration of 100 and 12.5 mM, respectively. Citalopram hydrobromide (Sigma–Aldrich) stock solution was made using molecular grade ethanol (EtOH) to a concentration of 12.5 mM. Lipopolysaccharide (LPS, Salmonella enterica, Sigma–Aldrich) was diluted to an initial stock solution of 1 mg/ml in phosphate buffered saline (PBS, In Vitrogen). Norepinephrine bitartrate salt monohydrate (Sigma–Aldrich), serotonin hydrochloride (Sigma–Aldrich), propranolol hydrochloride (Sigma–Aldrich), SQ-22536 (Sigma–Aldrich) and Rp-Adenosine 3',5'-cyclic monophosphate (Rp-cAMPS, Sigma–Aldrich) were initially dissolved in tissue culture ddH₂O to a stock concentration of 100 mM. In all cases, subsequent dilutions to working concentrations were made using cell culture media. Importantly,
the proportion of the initial solvent in the highest working concentration used in the study was maintained across all subsequent concentrations of drug to ensure that any observed differences were not the consequence of differing levels of solvent.

2.3. Experimental procedure

All experiments followed the same basic protocol. Microglial cells were grown to 80% confluency in tissue culture flasks and then detached from the growth flask by the addition of Trypsin (0.5%). Trypsin activity was then quenched by addition of an equal volume of cell culture medium and the cell suspension was removed and centrifuged (800g, 5 min, room temperature). After centrifugation the supernatant was discarded, the pellet resuspended in 8 mL of fresh medium, and 10 μL of the cell solution then removed and added to 10 μL of Trypan blue (0.4%). Stained cells were then counted using an automated cell counter (Countess; Invitrogen). Viability of cultures was routinely above 95%. In 200 μL of medium, 2.5 × 10^4 cells were seeded into each well of a 96-well plate and then returned to the incubator for 24 h to allow cellular adherence prior to any further intervention. At concentrations outlined in the result section, drugs of interest (flutaxetine, sertraline, paroxetine, fluvoxamine, venlafaxine, nortriptyline, sertraline, propranolol, SQ-22536 or Rp-cAMPS) were then added to the cells in the presence of 10 ng/mL of LPS, with five replicates for each concentration and two replicates for each plate, with the average between replicate error reported. LPS and antidepressants were added at the same time in order to align with the approach commonly used by other research groups investigating the anti-inflammatory effects of antidepressants (Hwang et al., 2008; Liu et al., 2011; Obuchowicz et al., 2006). Cells were then returned to the incubator for a further 4 or 24 h period. Post-incubation, cell culture supernatants were harvested and either stored at −20°C for subsequent analysis of TNF-α, or processed immediately for the detection of NO.

2.4. Measurement of TNF-α and nitric oxide

TNF-α was quantified using a Sandwich ELISA (R&D systems). The procedure was performed according to the manufacturer’s instructions. Absorbance was read at 450 nm with a wavelength correction of 570 nm using a VersaMax microplate reader (Molecular Devices). NO accumulation was quantified using the Griess reagent (Sigma-Aldrich) assay. Due to interference of the pH indicator phenol red with this reagent, phenol red free media was substituted for all experiments involving the Griess reaction. After 24 h incubation, 50 μL of cell culture supernatant was mixed with an equal volume of Griess reagent. The absorbance of the solution was then read at 540 nm after a 15 min incubation period at room temperature.

2.5. Immunohistochemistry

Cells were seeded onto 8-well Lab-Tek permox chamber slides (Nunc, Thermo Scientific) and left to incubate overnight to allow cell adherence. Plates were then rinsed with 0.1 M PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and then rinsed in 0.1 M PBS three times for 5 min. Cells were then incubated in 0.1 M PBS containing 3% bovine serum albumin (BSA) with 0.3% Triton X for 1 h, followed by overnight incubation at 4°C with the Iba1 primary antibody (anti-rabbit Iba1, Wako, 1:1000) in 0.1 M PBS supplemented with 1% BSA with 0.1% Triton X. Cells were then rinsed three times in 0.1 M PBS, and then incubated in a 1% BSA and 0.1% Triton-X solution containing a fluorescent donkey anti-rabbit secondary antibody (AlexaFlour-488, Invitrogen, 1:400) for 2 h, and then rinsed in the dark with 0.1 M PBS for 30 min. Cells were then fluorescently stained using the nuclei-specific marker DAPI (1:1000) in 0.1 M PBS for 5 min. After incubation, cells were rinsed in 0.1 M PBS and coverslipped with gelvatol.

2.6. Cellular viability

We evaluated the influence of all drugs on cellular viability with the resazurin-based PrestoBlue reagent (Invitrogen). Metabolically active cells are capable of reducing the PrestoBlue reagent, with the colorimetric changes used as an indicator to quantify the viability of cells in culture. BV-2 cells were seeded into 96-well plates at a density of 2.5 × 10^4 per well, in 200 μL of cell culture medium, and incubated for 24 h to allow cell adherence. Cells were then incubated in the presence or absence of each of the drug treatments for either a 4 or 24 h period, with untreated cells and cell culture medium alone used as positive and negative controls respectively. Cell viability was measured according to the manufacturer’s instructions. Briefly, the PrestoBlue solution (22 μL) was added into each well after 2 h incubation for our 4 h treatment condition, and after 22 h incubation for the 24 h treatment condition; plates were then placed back into the incubator for a further 2 h incubation, after which absorbance was measured at wavelengths 570 nm excitation and 600 nm emission.

In a subset of cases, we also used the Trypan blue exclusion assay to assess the effect of drugs on cell viability. Briefly, this involved incubating cells with a 100 μM antidepressant solution for 24 h. Cell supernatant was then removed, 200 μL of trypsin added to each well, and the plate placed back into the incubator for 3 min. Cells were then aspirated, added to 400 μL of cell culture medium, and centrifuged (800g, 5 min). Supernatant was then re-
moved and the cells resuspended in 500 μL of cell culture medium. Viability was then assessed by combining a 50% cell solution with a 50% Trypan blue solution (0.4%), with 10 μL of the resulting mixture being placed onto a counting chamber slide and read using an automated cell counter. As a positive control, subsets of cells were treated with 2 M HCl for 1 h to kill the cells; this confirmed that the counting parameters used by the automatic cell counter were appropriately calibrated to detect dead cells.

2.7. Data analysis

All raw data was transformed using Graphpad Prism (V4.02, Graphpad Software Inc.) so that 100% was the range defined by the distance between LPS + vehicle and unstimulated cell groups. The LPS + vehicle value was set to 100%, and changes in TNF-α and NO were expressed relative to this transformation. All inferential analyses were performed using SPSS v.19 (IBM). Significant differences were defined as any statistic where p < 0.05.

2.7.1. Comparison of drug concentrations

The ability of the antidepressants to alter TNF-α and/or NO production was assessed using multivariate ANOVAs, with Dunnett’s post-hoc test, using the LPS + vehicle group as the comparator. Tukey’s post-hoc analysis was also used where appropriate.

2.7.2. Calculation of the IC₅₀

Estimates of the drug concentration required to reduce the maximum response of cells to LPS + vehicle by 50%, was calculated using Graphpad Prism. This involved firstly taking a logarithmic transformation of the concentrations of antidepressants used in the current study, and then subsequently fitting the raw TNF-α data to a sigmoidal concentration response curve.

3. Results

3.1. Optimising the concentration of LPS

For our initial investigations, we sought to establish: (1) whether LPS at the concentration examined (0.1, 1, 10 and 100 ng/mL) influenced the viability of the cells; (2) the optimal concentration of LPS required to stimulate microglial pro-inflammatory activity (evidenced by TNF-α and NO expression); and (3) the time course of these changes.

![Graph showing cell viability over time](image)

**Fig. 2.** Optimisation of both the concentration of LPS and the duration of exposure required to elicit BV-2 pro-inflammatory activity, and determination of the concentration of minocycline required to attenuate it. TNF-α - Cells were left untreated or treated with LPS (0.1–100 ng/mL) for either 4 h (●) or 24 h (○). Mean (±SEM) levels of TNF-α were subsequently quantified using ELISA. “Indicate where TNF-α levels were significantly different from untreated cells (p < 0.05); NO - Cells were left untreated (●) or treated with 10 ng/mL LPS (○) for 4, 6, 8, 12 or 24 h. Mean (±SEM) production of NO was determined using the Griess reaction assay. *Indicate where NO levels were significantly different from untreated cells (p < 0.05). MINO – BV-2 cells were co-incubated with 10 ng/mL LPS and a series of increasing concentrations of minocycline (0–100 μM) for 24 h. Levels of NO were subsequently quantified using the Griess reagent assay. Data are represented as the mean (±SEM) percentage of NO production relative to LPS + vehicle treated cells (assigned a value of 100%).”

3.1.1. Cell viability

PrestoBlue data showed that LPS, regardless of concentration or period of incubation, had no effect on cell viability compared to that of untreated cells (4 h, F (5, 34) = 1.490, p = 0.22; 24 h, F (5, 34) = 0.30, p < 0.91).

3.1.2. LPS-induced TNF-α production

As shown in Fig. 2, the results of a two-way ANOVA indicated that there was no significant interaction between incubation length and LPS concentration, with only a main effect for LPS concentration (F (4, 60) = 585.80, p < 0.01). The fact that longer incubations did not result in more TNF-α accumulation most likely reflects the fact that maximal LPS stimulation of TNF-α release in this system is rapid (<4 h), and is coupled with processes that result in the breakdown of TNF-α within 24 h. Tukey’s post-hoc analysis indicated that TNF-α increased in a concentration-dependent manner, with higher concentrations of LPS associated with greater production of TNF-α (in all cases p < 0.01). No difference was observed in the levels of TNF-α produced by 10 ng/mL vs. 100 ng/mL of LPS (p = 0.84). As 10 ng/mL of LPS gave the maximal response, we utilised this concentration for all subsequent experiments.

3.1.3. LPS-induced NO production

Using 10 ng/mL of LPS, we assessed NO production at 4, 6, 8, 12 and 24 h. Analysis of this data indicated that the duration of incubation had a significant effect on NO production (F (9, 70) = 752.57, p < 0.01), with post-hoc analysis showing that NO increased in a time-dependent manner. No significant difference was found between LPS treated cells and unstimulated controls after 4 h of incubation. NO was detectable at a significant level compared to untreated cells after 6 h, and reached maximum levels by 24 h (in all cases p < 0.05; see Fig. 2). Cells were then co-incubated with 10 ng/mL of LPS and 12 concentrations (0, 0.1, 1, 2.5, 5, 10, 15, 20, 25, 35, 50, 75 and 100 μM) of minocycline, a known inhibitor of microglial activation. As illustrated in Fig. 2, minocycline treatment was associated with a significant change in the LPS-induced production of NO, with a main effect of treatment (F (12, 57) = 9.34, p < 0.01). Dunnett’s post-hoc analysis indicated that all concentrations of minocycline above 15 μM significantly reduced production of NO (in all cases p < 0.05).

3.2. Antidepressants exhibit both pro- and anti-inflammatory effects

Cells were co-incubated with LPS and one of 12 possible concentrations (0, 0.1, 1, 2.5, 5, 10, 15, 20, 25, 35, 50, 75 and
Table 1 – Anti-inflammatory effects of antidepressants on LPS-induced microglial TNF-α production at 4 h.

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The table indicates where there were significant positive (+), negative (−) or no significant (ns) deviation in the levels of TNF-α production, when compared to levels of LPS + vehicle treated cells (all significant statistics were p < 0.05). As measured using PrestoBlue, the grey boxes indicate where the viability of cells were significantly influenced by antidepressant treatment (p < 0.05).

100 µM) of six different antidepressants (fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram or venlafaxine). NO was only determined at 24 h as there was no detectable NO after 4 h of incubation.

3.2.1. Antidepressants impact on cell viability

Data from the Trypan blue exclusion test indicated that after 24 h there was no significant difference in the viability of cells treated with 100 µM of each of the six antidepressants, when compared to untreated cells (in all cases p > 0.05, data not shown). However, a clear reduction in cellular viability was detected using PrestoBlue, which indicated that the incubation of BV-2 cells with antidepressant for 24 h significantly lowered the viability of the cells, particularly at concentrations above 35 µM (see Table 2). PrestoBlue data also showed an antidepressant effect on the cellular viability after 4 h, however, this was only observed in the 100 µM concentration of fluoxetine, sertraline, paroxetine and fluvoxamine (in all cases p < 0.05), with all other drugs and concentrations not significantly different from untreated cells (in all cases p > 0.05; see Table 1). Due to the discrepancy in the findings obtained with these two measures, we chose to use for all subsequent statistical analysis data that were not influenced by viability issues according to the PrestoBlue results, as this was the more sensitive measure.

Table 2 – The pro and anti-inflammatory effects of antidepressants on LPS-induced microglial TNF-α and nitric oxide production at 24 h.

<table>
<thead>
<tr>
<th>µM</th>
<th>FLX</th>
<th>SERT</th>
<th>PARO</th>
<th>FLV</th>
<th>CIT</th>
<th>VEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>+</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
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<td>1</td>
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<td>2.5</td>
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<td>+</td>
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</tbody>
</table>

The table indicates where there was significant positive (+), negative (−) or no significant (ns) deviation in the levels of TNF-α and NO production, when compared to levels of LPS + vehicle treated cells (all significant statistics were p < 0.05). As measured using PrestoBlue, the grey boxes indicate where the viability of cells were significantly influenced by antidepressant treatment (p < 0.05).

3.2.2. Antidepressants effects on TNF-α production after 4 h

As illustrated in Fig. 3, all antidepressant treatments were associated with significant changes in the LPS-induced production of TNF-α, with a main effect of antidepressant treatment after 4 h of incubation: fluoxetine (F(11, 48) = 181.73, p < 0.01); sertraline (F(11, 48) = 101.60, p < 0.01); paroxetine (F(11, 48) = 214.64, p < 0.01); fluvoxamine (F(11, 48) = 23.01, p < 0.01); citalopram (F(12, 52) = 73.38, p < 0.01); and venlafaxine (F(12, 52) = 10.47, p < 0.01). After 4 h incubation, all six drugs reduced LPS-induced TNF-α production (see Fig. 3 and Table 1 for details of post-hoc comparisons).

3.2.3. Antidepressants effects on TNF-α production after 24 h

Within the viable range of data at 24 h, the results indicated a significant main effect of antidepressant treatment for: fluoxetine (F(8, 36) = 445.79, p < 0.01); sertraline (F(7, 32) = 68.24, p < 0.01); paroxetine (F(7, 32) = 92.72, p < 0.01); fluvoxamine (F(8, 36) = 390.31, p < 0.01); citalopram (F(9, 40) = 39.81, p < 0.01); and venlafaxine (F(9, 40) = 88.88, p < 0.01). As illustrated in Fig. 4 and Table 2, lower concentrations (0.1–5 µM) of fluoxetine, sertraline, paroxetine, fluvoxamine and venlafaxine were all associated with moderately elevated levels of TNF-α (in all cases p < 0.01). However, at higher concentrations all antidepressants, with the exception of venlafaxine, reduced the production of TNF-α (in all cases p < 0.05).

3.2.4. Antidepressants effects on NO production after 24 h

Within the viable range, LPS-induced NO production was also significantly influenced by antidepressant treatment, but only by a subset of the compounds tested: fluoxetine (F(8, 34) = 259.10, p < 0.01); sertraline (F(7, 26) = 57.46, p < 0.01); and paroxetine (F(7, 28) = 224.35, p < 0.01). As illustrated in Fig. 4 and Table 2, these antidepressants decreased NO production in a concentration dependent manner. Fluvoxamine, citalopram and venlafaxine did not significantly inhibit NO production within the viable range.

3.2.5. The half maximal inhibitory concentration (IC50) for the SSRIs on TNF-α production

Using the viable 4 h data, we calculated the IC50 to compare the relative ability of the five SSRIs antidepressants to inhibit LPS-induced microglial TNF-α production. An IC50 value for venlafaxine could not be determined, as venlafaxine exhibited only minimal capacity to inhibit TNF-α production, and therefore undertaking the curve fitting procedure was not appropriate. The IC50 is reported in Table 3 along with the curves corresponding hill slopes. We did not calculate IC50 values for the 24 h data because of both...
the stimulation observed at low concentrations and the low levels of cellular viability at higher concentrations resulted in an inability to accurately fit a curve to the data.

3.3. Anti-inflammatory potency of serotonin and norepinephrine (NE)

SSRIs and SNRIs are widely recognised to have high affinity for the serotonin and NE transporters, respectively (Owens et al., 1997). Importantly, even though SSRIs are recognised to have much higher affinity for the serotonin transporter, they do bind to the norepinephrine transporter (Owens et al., 1997), and restrict NE reuptake. As such, we wished to examine whether saturating levels of the respective natural ligands for these transporters (i.e. serotonin and NE) influenced the cells’ production of TNF-α.

3.3.1. Anti-inflammatory effects of serotonin

Incubation of LPS stimulated microglia with serotonin (0.1 mM-10 μM) for 4 h was observed to significantly alter TNF-α production ($F(6, 28) = 3.28, p < 0.05$; see Fig. 5). Dunnett’s post-hoc analysis, however, failed to show any specific difference between LPS
Table 3
The half maximal inhibitory concentration (IC₅₀) and hillslopes for the SSRIs on TNF-α production.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀</th>
<th>95% Confidence interval</th>
<th>Hillslope</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sertraline</td>
<td>6.6</td>
<td>(4.1–10.8)</td>
<td>-1.7</td>
<td>-3.0 to -0.5</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>7.8</td>
<td>(7.2–8.6)</td>
<td>-4.4</td>
<td>-5.7 to -3.2</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>8.3</td>
<td>(6.9–9.9)</td>
<td>-2.7</td>
<td>-3.8 to -1.6</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>9.7</td>
<td>(7.5–12.6)</td>
<td>-1.8</td>
<td>-2.6 to -1.0</td>
</tr>
<tr>
<td>Citalopram</td>
<td>18.3</td>
<td>(15.7–21.4)</td>
<td>-2.0</td>
<td>-2.7 to -1.3</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>ND³</td>
<td>ND³</td>
<td>ND³</td>
<td>ND³</td>
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</tbody>
</table>

IC₅₀ values are reported in μM. ND³ = Not determined.

Fig. 5. The anti-inflammatory effects of serotonin and norepinephrine. 5HT – BV-2 cells were co-incubated with 10 ng/mL LPS and increasing concentrations of serotonin for 4 h. NE – BV-2 cells were co-incubated with 10 ng/mL LPS and NE for 4 h. The curve fit used to determine the IC₅₀ for NE is shown as a solid line, along with the corresponding R² value. The IC₅₀ for NE was equal to 50 nM (CI = 0.03–0.07). NE + PROP – 15 μM of NE was co-incubated with 10 ng/mL of LPS and the general β-adrenoceptor antagonist propranolol for 4 h. For all panels, TNF-α levels were quantified using ELISA. Data represents mean ± SEM percentage of TNF-α relative to the levels produced by LPS + vehicle (assigned a value of 100%). For serotonin and NE panels, *Indicate where TNF-α production was significantly different from LPS + vehicle treated cells (p < 0.05). For the NE + PROP panel, *Indicate where TNF-α production was significantly different from LPS + NE treated cells (p < 0.05).

3.3.2. Anti-inflammatory effects of NE

Cells were incubated with NE (0.0001–100 μM) and LPS (10 ng/mL) for 4 h. The analysis of this data indicated that NE significantly altered TNF-α production, (F (7, 32) = 97.34, p < 0.01; see Fig. 5). Post-hoc analysis using Dunnett’s test indicated that NE reduced TNF-α production at all concentrations of NE greater than 0.01 μM (in all cases p < 0.01). We additionally determined that the IC₅₀ for NE was 50 nM (CI = 30–70 nM).

3.3.3. Inhibition of the NE anti-inflammatory effects with propranolol

To confirm that the anti-inflammatory effect of NE was β-adrenoceptor mediated, we incubated increasing concentrations of the non-specific β-adrenoceptor antagonist propranolol (0, 0.0001, 0.001, 0.01, 0.1, and 10 μM), with a fixed concentration of NE (15 μM) and LPS (10 ng/mL). The results of a one-way ANOVA indicated that propranolol significantly changed the anti-inflammatory profile of NE (F (7, 26) = 140.11, p < 0.01; see Fig. 5). Dunnett’s post-hoc analysis indicated that all concentrations of propranolol used in the current study significantly decreased the anti-inflammatory activity of NE (in all cases p < 0.01). The impact of propranolol also occurred in a concentration-dependent manner, with concentrations 0.1, 1 and 10 μM of propranolol completely blocking the anti-inflammatory effect of NE, with no statistically significant difference from LPS + vehicle treated levels of TNF-α.

3.4. The influence of β-adrenoceptor blockade on the anti-inflammatory effect of the SSRIs

To determine if the SSRIs exert their anti-inflammatory actions through a β-adrenoceptor mediated mechanism, similar to NE, a fixed concentration of fluoxetine, sertraline, paroxetine, fluvoxamine (all 10 μM), and within ±3 μM of each drug’s IC₅₀ and citalopram (20 μM), also within ±3 μM of the drug’s IC₅₀, were co-incubated with 10 ng/mL of LPS and propranolol (0.01, 0.1 and 1 μM). TNF-α production was quantified after 4 h of co-incubation. The analysis indicated that there was no significant effect of propranolol on TNF-α production for any antidepressant.

3.5. The influence of intracellular cAMP on the anti-inflammatory effect of the SSRIs

There is some evidence to suggest that increased intracellular cAMP decreases PI cytokine production, and that antidepressants may exert their immunomodulatory actions through this mechanism (Hashioka et al., 2007). To investigate this hypothesis, we targeted two points within the cAMP signalling pathway: (1) the production of cAMP with the adenylate cyclase inhibitor SQ-22536; and (2) the inhibition of the cAMP-dependent protein
kinase A (PKA) using Rp-Adenosine 3’5’-cyclic monophosphothioate triethylammonium salt (Rp-cAMPs).

3.5.1. Inhibition of adenylate cyclase

To evaluate the role of adenylate cyclase, we co-incubated each of the SSRIs (fluoxetine, sertraline, paroxetine, fluvoxamine at 10 μM and citalopram at 20 μM) with 10 ng/mL LPS and SQ-22536 (0, 0.1, 1 and 10 μM). A one-way ANOVA showed an effect for citalopram (F (3, 12) = 3.81, p < 0.05). Dunnett’s post-hoc analysis indicated that at a concentration of 0.1 μM, SQ-22536 significantly decreased the level of TNF-α production in cells co-incubated with antidepressant and LPS (p < 0.05). All other analyses were not significantly different from antidepressant treatment alone.

3.5.2. Influence of Rp-cAMPs, a putative inhibitor of PKA

In this experiment we co-incubated each of the SSRIs (fluoxetine, sertraline, paroxetine, fluvoxamine at 10 μM and citalopram at 20 μM) with LPS and Rp-cAMPs (0, 0.01, 0.1 and 1 μM). One-way ANOVAs indicated that there was a significant effect of Rp-cAMPs treatment for: fluoxetine (F (3, 12) = 5.11, p < 0.05); sertraline (F (3, 12) = 4.20, p < 0.05); and fluvoxamine (F (3, 12) = 12.90, p < 0.01). The results of Dunnett’s post-hoc analysis are illustrated in Fig. 6.

4. Discussion

It has long been thought that SSRIs and SNRIs exert their therapeutic effects via their blockade of presynaptic transporters. However, for at least two decades, it has been recognised that the SSRI/SNRIs also possess some immunomodulatory effects. The evidence for this originally related only to cells of the peripheral immune system, but recently it has been discovered that antidepressants can also alter the inflammatory potential of microglia. The aim of the current study was to extend these findings by examining the relative ability of commonly prescribed SSRI and SNRI antidepressants to modulate LPS-driven microglial production of the pro-inflammatory cytokine (TNF-α) and the free radical nitric oxide (NO). We found that all the antidepressants tested were able to reduce TNF-α and NO production, although venlafaxine’s efficacy was marginal. In an effort to identify the mechanism through which these SSRIs might exert these effects, we then examined the impact of: (1) blockade of β-adrenoceptors; (2) blockade of adenylyl cyclase; or (3) inhibition of the cAMP dependent protein kinase A (PKA). These experiments indicated that PKA antagonism was capable of partially attenuating the anti-inflammatory effect of the SSRIs. The findings from the current study highlight how similar the immunomodulatory effects of the SSRIs are, a finding consistent with similar mechanisms of action. Furthermore, given the compelling body of evidence now indicating that depression has a strong inflammatory component, these findings provide a platform for future studies to evaluate the extent to which antidepressants might owe at least some of their therapeutic efficacy to their anti-inflammatory properties.

Recently the SSRIs fluoxetine, sertraline, paroxetine and fluvoxamine have been independently investigated in terms of their immunomodulatory properties on microglia. However, it has been difficult to draw firm conclusions from these studies. For instance it has been shown that SSRIs can have no effect (Horikawa et al., 2010), increase (Ha et al., 2006) or decrease levels of TNF-α production (Hashioka et al., 2007). In large part, this discrepancy appears to be due to heterogeneous experimental conditions, with differences in the type of stimulus used (e.g. LPS, IFN-γ), the antidepressant concentration and the time course chosen. Ultimately, this situation makes it difficult to directly align, assess and compare findings between different experiments. Clearly, however, given the putative role of inflammatory processes in depression, determining how comparable the immunomodulatory properties are for each of the SSRIs would be of considerable benefit.

4.1. Antidepressant impact on cellular viability

Prior to evaluating the immunomodulatory actions of the antidepressants, we investigated whether they impacted on cellular
viability using both a resazurin based test (PrestoBlue), and a dye exclusion based test (Trypan blue). Interestingly, the results from these two tests differed considerably. Consistent with Hashioka et al. (2007), the dye exclusion procedure indicated that after 24 h of incubation, none of the antidepressants at their highest concentration (100 µM) influenced cellular viability. In contrast, the resazurin based test showed that the antidepressants significantly altered viability. Specifically, after 4 h incubation, four of the six antidepressants evaluated were observed to reduce cellular viability but only at the highest concentration evaluated (100 µM). In contrast, at 24 h, all the antidepressants evaluated reduced viability at concentrations above 35 µM. It is possible that the health of the cells at concentrations less than 35 µM were also compromised, although statistically this could not be ascertained. The most likely explanation of the difference in viability between the two assays is the greater sensitivity of the resazurin assay, a fact relating to its ability to detect functional differences in living cells vs. the dye exclusion assay that is only sensitive to dead cells.

One issue to consider with respect to the viability results is that the difference between the resazurin and dye exclusion tests may have been due to the antidepressants interfering with the reduction of the dye without actually influencing cellular viability. If this occurred it may have artificially lowered the index of viability. Experimentally, however, this possibility is difficult to assess as it is not clear how generalisable results would be when examining the ability of antidepressants to modify the activity of synthetic reducing agents. Despite the issues with viability observed at the 24 h timepoint, the 4 h results were largely unaffected and clearly demonstrate the differing anti-inflammatory capacities for each of the antidepressants.

4.2. Relative anti-inflammatory potency of SSRIs

In the current study, we evaluated the anti-inflammatory activity of five SSRIs and one SNRI. After 4 h of co-incubation of antidepressants with LPS, we observed a substantial reduction in the levels of TNF-α for all antidepressants. However, considerable differences existed between each of the compounds examined. Most notably, the SNRI venlafaxine was substantially less potent, only demonstrating anti-inflammatory activity at concentrations of 75 µM or higher. In contrast, all SSRIs had significant anti-inflammatory activity at concentrations 5 µM or above, with the exception of citalopram, which was only significant at concentrations above 10 µM. The 24 h TNF-α data largely paralleled the 4 h results, apart from two notable differences. Firstly, venlafaxine was no longer effective at inhibiting TNF-α production. Secondly, and perhaps the most intriguing, was the pro-inflammatory effect observed at low concentrations (5 µM or less) of fluoxetine, sertraline, paroxetine, fluvoxamine and venlafaxine, all of which were associated with significant elevations (approximately 10–15%) in TNF-α production. While the mild pro-inflammatory effect is notable, the ability of antidepressants to promote the release of inflammatory products has been previously described. Specifically, Ha et al. (2006) observed that microglia exposed to 1 μM of fluoxetine increased nitric oxide release, and further identified that this effect was associated with an increase in upstream signalling mediators of nuclear factor kappa-light-chain-enhancer of activated B cells. More recently, in an in vivo study, MacGillivray et al., 2011 demonstrated that rats treated chronically with fluoxetine exhibited a significant enhancement in microglial activity, as characterised by the density is OX42 immunolabelling. Given these observations it seems very probable that antidepressants possess the capacity to be both pro- and anti-inflammatory, an effect that appears to be dependent upon both the length of exposure and the concentration of antidepressant.

To examine the relative potency of the SSRIs, we calculated IC₅₀ values for each. This analysis revealed that sertraline exhibited the greatest potency and citalopram the least, with IC₅₀ values ranging between 6 and 18 µM. Obviously, a critical issue is whether these concentrations are achievable at concentrations which are standardly prescribed in the treatment of depression. In this regard there are two pertinent studies, both of which have utilised magnetic resonance spectroscopy to determine the concentration of fluoxetine and fluvoxamine within the human brain of patients undergoing antidepressant treatment (Bolo et al., 2000; Henry et al., 2005). These studies indicated that the brain concentration of fluoxetine and fluvoxamine, after a minimum of three weeks of treatment, ranged between 12 and 25 µM. Importantly, these concentrations coincide with the same concentrations at which these compounds exerted their anti-inflammatory actions within the current study.

Determining how well the in vitro findings align with the clinical efficacy of each of the antidepressants is challenging. To our knowledge only one study has directly compared the efficacy of new generation antidepressants (SSRIs, SNRIS, and atypical antidepressants) (Cipriani et al., 2009). Cipriani et al.'s study analysed the results of 117 separate studies, including nearly 26,000 individuals that had been assigned to 12 different new generation antidepressants (including all the antidepressants evaluated in the current study). The authors found clear differences in efficacy between the antidepressants included in the study, demonstrating that venlafaxine and sertraline were significantly more efficacious than fluoxetine, fluvoxamine, and paroxetine. These observations align partially with the results in the current study, inasmuch that sertraline was found to possess the lowest IC₅₀ of the SSRIs. Venlafaxine on the other hand was found to exhibit minimal anti-inflammatory activity. Obviously, this could be taken to indicate that antidepressants do not owe any of their therapeutic effect to their anti-inflammatory properties. However, venlafaxine may still indirectly exert anti-inflammatory actions in vivo, as it is known to elevate NE, and we, in the current study, and others, have shown that NE is potently anti-inflammatory (Dello Russo et al., 2004).

Activated microglia produce the free radical NO, which is synthesised by inducible nitric oxide synthase (iNOS or NOS2) in response to inflammatory stimuli such as LPS. Taking into account only the data unaffected by viability issues, fluoxetine, sertraline and paroxetine potently inhibited NO production. In contrast, the level of inhibition exerted by fluvoxamine, citalopram and venlafaxine was modest. Interestingly, the inhibition of NO by these SSRIs did not follow the same gradual response curve observed for TNF-α inhibition, rather appearing as a step function (see Fig. 4). Of note was the fact that while all the SSRIs inhibited TNF-α production, only fluoxetine, sertraline and paroxetine reduced NO production. This suggests that the anti-inflammatory actions of SSRIs involve at least two different mechanisms, possibly reflecting structural differences in the compounds’ molecular composition.

4.3. Determining the mechanism of action

In an attempt to understand the mechanism of action through which the SSRIs exerted their anti-inflammatory properties, we first decided to examine the effect of exposing microglia to saturating concentrations of serotonin or NE. We used this approach, as serotonin and NE bind to many of the same targets as the SSRIs, and a finding that either ligand exerted anti-inflammatory actions would assist in reducing the pool of candidate mechanisms. Our results indicated that serotonin had very limited anti-inflammatory effects, and certainly not of sufficient magnitude to explain those observed following SSRI treatment. Noradrenaline, however, was found to potently inhibit LPS-induced TNF-α production, even
at concentrations as low as 100 nM. In line with existing literature (Dello Russo et al., 2004), we observed that this was a β-adrenoceptor-mediated phenomenon, as blockade with the general β-agonist propranolol attenuated the anti-inflammatory effect of NE. Given this relationship, it was of interest to examine whether blocking the β-adrenoceptors using propranolol could alter the anti-inflammatory effects of SSRIs. However, our results clearly show that the ability of SSRIs to reduce TNF-α production is not altered by the presence of propranolol, suggesting that the anti-inflammatory actions of the SSRIs are not driven by a β-adrenoceptor-mediated mechanism.

Increased levels of intracellular cyclic adenosine monophosphate (cAMP) have been shown to exert anti-inflammatory effects (Yoshikawa et al., 1999, 2002). As SSRIs have been shown to alter cAMP, we next examined whether interfering with intracellular cAMP altered the anti-inflammatory profile of the SSRIs. We did this firstly by blocking adenylate cyclase, the rate-limiting enzyme responsible for converting ATP to cAMP; and secondly, by blocking protein kinase A (PKA), a cAMP-dependent kinase. These experiments involved co-incubating fixed concentrations of each of the five SSRIs with increasing concentrations of the adenylate cyclase inhibitor SQ-22536, or the PKA inhibitor Rp-cAMPS. Our results indicated that adenylate cyclase inhibition did not clearly reverse the anti-inflammatory effects of antidepressants. In contrast, with Rp-cAMPS at 0.01–1 μM for fluoxetine and fluvoxamine and 0.1 μM for sertraline we observed a consistent and moderate (~10–15%) increase in TNF-α production. Previously, only one study examining fluvoxamine has assessed the impact of SQ-22536 and Rp-cAMPS treatment (Hashioka et al., 2007). The authors of this study observed that both interventions attenuated the anti-inflammatory effect of fluvoxamine. It is difficult to reconcile the difference between our results and those reported previously. It is worthwhile noting that the prior report regarding the effect of both SQ-22536 and Rp-cAMPS on fluvoxamine was observed at a time point and at a drug concentration that we found caused decreased cellular viability. Nevertheless, combining our own results with those reported previously for fluvoxamine, it would appear that PKA may be partially involved in regulating the anti-inflammatory effect of antidepressants.

4.4. Limitations

In the current study we chose to evaluate the ability of antidepressants to reduce the level of TNF-α release from maximum levels of LPS stimulation. It would be useful for future studies to examine how effective the antidepressants used in the current study are at reducing sub-maximal levels of P1 cytokine and free-radical release, and further determine the degree to which the results generalise to other pro-inflammatory stimuli. Additionally, in order to achieve consistency with other studies in the field (Hwang et al., 2008; Liu et al., 2011; Obuchowicz et al., 2006), we chose to examine the effects of antidepressants on LPS-stimulated microglia, with the cells exposed to the antidepressant and the stimulant at the same time. It would be of considerable interest to undertake a comparable study with the microglia being exposed to the antidepressants prior to and following stimulation with LPS. Antidepressant treatment prior to LPS stimulation may be considered to more accurately model the ability of antidepressants to restrict a future inflammatory insult, whereas when administered post-LPS it may model the ability to quench an already initiated response. Finally, further characterisation of our finding regarding the effect of Rp-cAMPS on LPS stimulated TNF-α release is warranted. Specifically, it would be useful to examine the IC50 response shapes, using a log concentration of antidepressant in the presence of one or two concentrations of Rp-cAMP, to determine unequivocally whether Rp-cAMP is genuinely acting as an antagonist in this experimental system.

5. Conclusions

To our knowledge, the results of this study are the first to directly compare the immunomodulatory effects of the most commonly prescribed SSRI and SNRI antidepressants. From the results of this study, we observed four distinct phenomena of interest. Firstly, our results demonstrated that all SSRIs substantially attenuated LPS-induced TNF-α production, an effect that was observed at concentrations known to occur within the brain with conventional pharmacological treatment. Secondly, we found evidence that longer durations of exposure (24 h) to low concentrations (~5 μM) of antidepressant created a situation in which the SSRIs were moderately pro-inflammatory. This finding is of interest as it unifies existing literature, demonstrating that under certain conditions, antidepressants possess the capacity to exert both pro- and anti-inflammatory immunomodulatory properties. Thirdly, we observed that higher concentrations of antidepressant were associated with significant decreases in cellular viability. Within this context, antidepressants have previously been assessed for their capacity to induce apoptosis of human lymphocytes (Xia et al., 1996a), an effect that has been independently investigated for its therapeutic potential to treat cancer (Amit et al., 2009). Finally, in terms of understanding the mechanism through which the SSRIs exerted their immunomodulatory actions, we examined several pathways. The most promising evidence implicates PKA, as inhibition of this kinase with Rp-cAMPS attenuated the anti-inflammatory response of the majority of antidepressants. Our results indicate, however, that the role of PKA in the anti-inflammatory effects of the SSRIs is partial, and other mechanisms are clearly at work. Overall, these results indicate that striking the right balance between dosage and desired therapeutic outcome could be challenging, and highlight these issues for future clinical investigation.

SSRIs represent the first choice for the pharmacological treatment of moderate to severe depression (Mant et al., 2004). For several decades, the primary theoretical justification for the use of these drugs has been the monoamine theory. Increasingly, however, inflammation is being considered as a major factor in the emergence and maintenance of depression. This proposal appears to fit exceedingly well with data indicating that many antidepressants possess significant anti-inflammatory actions. The problem, however, is that SSRIs have been optimised not for their anti-inflammatory capacity, but for their effect on neurotransmitter reuptake. The challenge now is to continue to build the pharmacological evidence for the immunomodulatory properties of these drugs, with a view to providing a solid platform on which to base future clinical research trials.

Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

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Chapter Four

Chronic stress induces profound structural remodelling of astrocytes within the prefrontal cortex: A characterization of the relationship between astrocyte morphology and density
CHAPTER 4 - Chronic stress-induced disruption of the astrocyte network is driven by structural atrophy and not loss of astrocytes

Ross Tynan, Sarah B Beynon, Madeleine Hinwood, Sarah J Johnson, Michael Nilsson, Jason J Woods, Frederick R Walker

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**Statement of Author Contributions to Manuscript**

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Chronic stress-induced disruption of the astrocyte network is driven by structural atrophy and not loss of astrocytes

Ross J. Tynan · Sarah B. Beynon · Madeleine Hinwood · Sarah J. Johnson · Michael Nilsson · Jason J. Woods · Frederick R. Walker

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Abstract Chronic stress is well recognized to decrease the number of GFAP+ astrocytes within the prefrontal cortex (PFC). Recent research, however, has suggested that our understanding of how stress alters astrocytes may be incomplete. Specifically, chronic stress has been shown to induce a unique form of microglial remodelling, but it is not yet clear whether astrocytes also undergo similar structural modifications. Such alterations may be significant given the role of astrocytes in modulating synaptic function. Accordingly, in the current study we have examined changes in astrocyte morphology following exposure to chronic stress in adult rats, using three-dimensional digital reconstructions of astrocytes. Our analysis indicated that chronic stress produced profound atrophy of astrocyte process length, branching and volume. We additionally examined changes in astrocyte-specific S100β, which are both putative astrocyte marker and a protein whose expression is associated with astrocyte distress. While we found that S100β levels were increased by stress, this increase was not correlated with atrophy. We further established that while chronic stress was associated with a decrease in astrocyte numbers when GFAP labelling was used as a marker, we could find no evidence of a decrease in the total number of cells, based on Nissl staining, or in the number of S100β+ cells. This finding suggests that chronic stress may not actually reduce astrocyte numbers and may instead selectively decrease GFAP expression. The results of the current study are significant as they indicate stress-induced astrocyte-mediated disturbances may not be due to a loss of cells but rather due to significant remodeling of the astrocyte network.

Keywords Astrocyte · Chronic stress · GFAP · S100β · Infralimbic prefrontal cortex

Introduction

Exposure to chronically stressful situations is recognized to be one of the most significant risk factors in the emergence of clinically diagnosable depression [25]. While the neuronal disturbances induced by exposure to chronic stress have been extensively characterized, particularly within susceptible forebrain regions such as the medial prefrontal cortex (PFC, [42, 43]), considerably less is known about the effect of stress on glia.

Using an animal model of chronic stress, our research group has recently demonstrated that in addition to eliciting a substantial increase in anhedonic-like behaviour [57] and producing a deficit in working memory [26], chronic stress led to pronounced remodelling of microglia within the ventromedial PFC [27]. Specifically, the results from these studies revealed that chronic stress significantly increased
the internal complexity of microglia, by enhancing ramification (cellular branching) without altering the overall area occupied by the cell. These findings not only suggest the involvement of microglial ramification in mediating stress-induced behavioural effects, but also clearly demonstrate the potential of chronic stress to significantly modify glial morphology within the PFC.

Like microglia, astrocytes are also responsive to the effects of chronic stress. In one of the first studies to directly examine this relationship, Czeh et al. [13] revealed that tree shrews exposed to 5 weeks of chronic psychosocial stress exhibited significantly reduced numbers of glial fibrillary acidic protein (GFAP) positive astrocytes in the hippocampus. This study was important not only because the work was done in a close analogue of primates, but also because of the use of unbiased stereological assessments (based on the optical fractionator method), which currently represents the highest standard of evidence for determining changes in cellular density. In a seminal investigation that followed, Banaas and Duman [4] significantly expanded upon these findings, by firstly demonstrating that chronic unpredictable mild stress could induce a reduction in astrocyte (GFAP⁺) density within the rat PFC; and secondly, by highlighting the crucial role of astrocytes in mediating behaviour, by showing that specific pharmacological ablation of astrocytes induced depressive-like behaviour similar to that observed following exposure to chronic stress. The translational significance of these results is considerable, as they are consistent with findings demonstrating that the number of glia within the PFC of post-mortem tissue taken from individuals with a life history of depression is significantly reduced (succinctly reviewed by [45–47]). Together, these studies suggest that a reduced number of GFAP⁺ astrocytes within the PFC may drive the emergence of stress-linked behavioural changes and could under certain circumstances contribute to the emergence of mood disturbances.

One potentially important caveat to the proposed link between astrocyte cell loss and the emergence of cognitive disturbance is the reliance on GFAP as an immutable marker for astrocytes. There have now been several studies have shown that certain conditions can dramatically increase GFAP [49, 60] or produce a specific decrease in GFAP without any appreciable change in cell number [22]. In these situations, density assessments based on GFAP would indicate that a change in cell number has occurred, when in fact all that may have changed are the expression of the protein upon which density estimates were based. Whether the reported decrease in astrocyte numbers following stress represents a bona fide reduction in cell number has yet to be directly confirmed.

The protein S100β is widely recognized to be associated with astrocyte distress, and is particularly concentrated in astrocytes within the central nervous system (CNS). Due to this property, S100β has been extensively used as a surrogate marker of astrocytes [32, 50]. However, S100β has also been identified to possess a number of distinct functional roles. For instance, elevated circulating levels of S100β have been commonly observed in a variety of neurodegenerative conditions such as multiple sclerosis [39] and Alzheimer’s disease [58], and more recently in individuals with depression [2, 51]. Due to its detection in neurodegenerative disease, it was originally proposed that the elevations in S100β were the consequence of damaged cells within the CNS (see [34] for review). It is now well recognized that S100β can be actively released, exerting protective neurotrophic effects at low concentrations [29] and potentially harmful effects at higher concentrations, by increasing cyclooxygenase-2 expression and free radical release [8, 28, 29]. To date, the expression of S100β in response to chronic stress has not been characterized.

While changes in astrocyte density are obviously important, there is reason to believe that chronic stress may also drive significant structural remodelling of astrocytes. Astrocytes are exquisitely sensitive to changes in their extracellular environment [1], and like microglia, their structure is intimately linked with their function [33, 35]. Such remodelling may be significant given that each astrocyte is known to be capable of making contact with, and modulating the activity of, many thousands of individual synapses [10]. However, no studies to our knowledge have yet examined whether astrocyte morphology can be disturbed by exposure to chronic stress.

The primary aim of the current study was to assess how astrocyte morphology is altered by exposure to chronic stress. We have addressed this issue by creating high-resolution three-dimensional reconstructions of astrocytes, from each of the distinct anatomical layers within the PFC, and quantitatively examined changes in their structure. We have chosen to examine changes in astrocytes across layers, as it is well recognized that there are distinct afferent and efferent inputs into each layer [14], and such a laminar analysis will give insight into which functions and brain regions are likely to be most influenced (if at all). We have assessed changes in GFAP density using stereology, exhaustive counting and thresholding-based assessments and have undertaken an independent assessment of cell density using Nissl-based stereological counting and counts of S100β⁺ cells. Finally, given findings indicating that circulating S100β is elevated in depression, and its dose-dependent Jekyll and Hyde properties within the CNS, we examined the relationship between S100β and astrocyte remodelling and density within the PFC.

Materials and methods

Animals

All experiments used adult male Sprague–Dawley rats (70–day old at the commencement of the experiment;
Experimental procedure

Animals were randomly allocated into either a chronic stress (n = 10) or handled control (n = 10) condition, with both treatments consisting of a 21-day experimental protocol. The chronic stress procedure involved restraining animals as described previously [26, 27]. This model was chosen because it produces robust decreases in dendritic length and spine density, particularly within the PFC, that does not occur when shorter exposure periods are utilized [1, 4, 32, 43].

In brief, animals were placed within wire mesh (0.6 mm diameter, 6.5 x 6.5 mm grid) restrainers secured with butterfly clips for 6 h each day of the 21-day protocol. The handling procedure for control animals was designed to emulate the stress protocol, minus the restraint. Thus, each animal was individually removed from their home cage, held for approximately 2 min, and then returned to their home cage, twice each day throughout the 21-day protocol. Furthermore, for the duration of the 6-h stress period, animals in the handled control condition had their food and water access removed.

Tissue preparation

All animals were deeply anaesthetized (sodium pentobarbital, 80 mg/kg i.p.) and transcardially perfused via the ascending aorta 24 h after cessation of the final stress episode. Tissue was cleared with 300 ml of 0.1 M phosphate buffer (PB) solution containing 2 % sodium nitrate (pH 7.40), and then subsequently fixed using 400 ml of 4 % paraformaldehyde in 0.1 M PB. Brains were extracted and placed in the same fixative solution for 16 h, and then blocked at the base of the midbrain before being placed in a 12.5 % sucrose solution in 0.1 M PB (pH 7.40, 4 °C) overnight for cryoprotection. Serial coronal sections were sliced using a freezing (−25 °C) microtome (Leica SM 2000R) at 30-μm intervals. Sections were divided into a 1-in-6 series and stored in anti-freeze solution (4 °C) until required for immunoperoxidase or immunofluorescent labelling.

Immunoperoxidase labelling

For all immunohistochemistry, sections from both stress and control groups were processed simultaneously to avoid any possible artefacts as a consequence of the labelling procedure. Sections were rinsed with 0.1 M PB before the endogenous peroxidases were destroyed in 0.1 M PB containing 1 % H2O2, with non-specific binding then blocked by incubation in 0.1 M PB solution containing 3 % normal horse serum. Sections were then incubated with a rabbit polyclonal antiserum directed against GFAP (1:5,000; Millipore) or S100β (1:4,000; Sigma-Aldrich) in phosphate buffered horse serum (PBH) for 72 h at 4 °C. Sections were then washed in 0.1 M PB, incubated with a biotinylated donkey anti-mouse or -rabbit secondary antibody (1:300, Jackson Immunoresearch) in PBH for 2 h, rinsed, and then incubated in 0.1 % extravidin peroxidase (1:1,000; Sigma-Aldrich) for 1 h before being rinsed again. The immunolabelling was then developed using a nickel-enhanced 3,3′-diaminobenzidine solution, which was closely monitored under a microscope until optimal staining with minimal background labelling was achieved (5 min). Sections were then mounted onto chrome alum-coated slides, dehydrated using a graded series of ethanol (70 %, 95 %, 100 %, absolute), cleared in xylene and coverslipped.

Immunofluorescent labelling

As for immunoperoxidase, all immunofluorescently labelled sections were processed simultaneously to avoid any possible non-specific differences as a consequence of the labelling procedure. Initially, sections were rinsed three times in 0.1 M phosphate buffered saline (PBS; pH 7.40), followed by a 1-h incubation in 0.1 M PBS containing 3 % bovine serum albumin (BSA) and 0.3 % Triton X-100. Sections were then transferred into a 1 % BSA and 0.1 % Triton X-100 solution containing appropriate primary antibodies (anti-mouse GFAP, 1:200, Millipore; anti-mouse S100β, 1:4,000, Sigma-Aldrich; or anti-rabbit GFAP, 1:100, Millipore) and incubated overnight at 4 °C. Sections were next rinsed three times in 0.1 M PBS, and then incubated in a 1 % BSA and 0.1 % Triton X-100 solution containing appropriate fluorescent secondary antibodies (anti-mouse AlexaFluor-488 or anti-rabbit AlexaFluor-594, 1:400, Invitrogen). To determine the extent of co-localization, sections were double labelled using a sequential labelling process. After processing was complete, sections were rinsed, mounted onto chrome alum-coated slides, and coverslipped.
Fig. 1 The anatomical locus of the medial prefrontal cortex within the rat brain. The left panel of images shows the prefrontal cortex from both coronal (upper) and sagittal (lower) views, respectively. The image on the right shows a coronal section, with the inset image illustrating the relative cortical depths of each layer within the IL cortex (ML midline, PrL prelimbic cortex, Fmi forceps minor). Adapted from Paxinos and Watson [38].

Analysis of the infralimbic cortex

All analysis was conducted by an independent trained observer, blind to experimental conditions. To prevent identification of the experimental treatments, all tissues were assigned a random numerical coding, which was not broken until all processing were complete. For both immunoperoxidase and immunofluorescent labelled tissues, all sections were obtained from rostrocaudal levels within the range of approximately +2.20 and +3.20 mm from Bregma, according to the Paxinos and Watson rat brain atlas [38]. For initial observations, changes in GFAP immunoreactivity were investigated with regard to the total infralimbic (IL) cortex. Subsequent analysis involved separating this region into layers, which was achieved by firstly assigning the distance from the pial surface to the underlying white matter of the forceps minor a value of 100%. This value was then divided into cortical layer depths previously established for the Sprague-Dawley rat [19], and used in many recent publications [26, 36], with the width of each layer defined as I (17.8%), II (27.9%), III (46.6%), V (73.0%) and VI (100%), as shown in Fig. 1.

Acquisition of images for three-dimensional reconstructions of astrocytes

Digital reconstructions of astrocytes were based on confocal images of GFAP-labelled tissue acquired using a computer-assisted morphometry system. This consisted of a Nikon Eclipse 80i confocal microscope equipped with a motorized z-stage, which was connected to a Dell PC running EZ-C1 confocal microscope software (Gold Version 3.80; Nikon Corporation). Confocal Stacks (50–100 images separated by a 0.25-μm z-axis step size) of GFAP-immunolabelled tissue were acquired using a Nikon Plan VC 100× objective (oil immersion, N.A. 1.4; see Fig. 2a, b). Each image was taken at a resolution of 1,024 × 1,024 pixels, with the pixel dwell time set to 3.36 μs, giving a rate of 0.185 z-planes per second. Additional settings including gain, offset and pinhole size were optimized prior to imaging, with these settings held constant for all subsequent analysis. To be included for reconstruction, cells needed to fulfil each of the following criteria: (1) the cell must be located within layers II, III, V or VI of the IL cortex; (2) the entire cell must reside completely within the z-depth of the section; and (3) each cell must have intact astrocyte processes unobscured by neighbouring cells or background immunolabelling. Layer I of the IL PFC was excluded from analysis due to the high density of GFAP+ cells within the layer preventing reliable reconstruction of single cells (see Fig. 2d). Astrocytes were also excluded from analysis if they were surrounding blood vessels or capillaries. From each animal three cells were pseudo-randomly selected for reconstruction from within the IL PFC, with one cell representing the top, middle and bottom of each of the four measured IL PFC layers. This produced a total of 240 reconstructed cells (120 stress, 120 controls) comprised 60 cells from each layer (30...
stress, 30 controls). Figure 2c shows the distribution of scan sites recorded within the IL PFC.

Image processing of acquired images and digital reconstruction

Prior to reconstruction, all raw images collected were attenuation corrected using AutoDeblur (Version X2; AutoQuant X, Media Cybernetics Inc.), to minimize any loss in colour intensity as a consequence of photobleaching. This process was then followed by a 3D blind deconvolution (10 iterations) to compensate for optical aberrations and enhance contrast and resolution, producing a greyscale image that was saved in a tiff format. Finally, all images were processed in NeuronStudio, a software package that digitizes deconvolved confocal laser scanning microscopy stacks and creates a 3D reconstruction suitable for morphometric analysis. The software initially detects the medial axis of the cell by connecting chains of voxels. The diameter is then determined using a Rayburst sampling algorithm, where rays are sent from the centre of a lighted structure (medial axis), until they reach a given exit.
Density estimates of GFAP\(^+\) immunoreactive material

To further investigate stress-induced changes, the immunofluorescently labelled tissue was processed to determine the total density of GFAP\(^+\) immunoreactive material within the IL PFC. A low magnification (10\(\times\), N.A. 0.45) confocal stack (1,024 \(\times\) 1,024 resolution, step size 5 \(\mu\)m) of images was acquired using a Nikon Eclipse 80i confocal microscope with EZ-C1 software (Gold Version 3.80). A maximum intensity projection of the image stack was then produced using Neurolucida (Version 10.30.1, 64 bit; MBF Biosciences), with the percentage of immunoreactive material then quantified as described previously [57]. In brief, this involved a thresholding procedure using MetaMorph software (version 7.5.4.0; Molecular Devices), whereby only pixels within a predetermined range of colour intensity were included for analysis. The colour threshold was set so that all cell bodies and associated processes were thresholded, with the density of immunoreactive material then determined by quantifying the percentage of thresholded material within the region of interest (ROI), in this case being the IL PFC. Subsequently, the same thresholding procedure was used to investigate the density of GFAP within each layer of the IL PFC. Five ROIs were made (one for each layer) within the IL PFC corresponding to the predetermined size of each layer. Finally, a separate set of cells from the primary motor cortex, a region not known to display astrocyte changes in response to stress, were also processed for comparison to those in the IL PFC.

Stereological estimates of GFAP\(^+\) astrocytes and total cell numbers within the IL PFC

The number of GFAP\(^+\) astrocytes within the IL PFC was quantified by systematically sampling the tissue using StereoInvestigator software (version 8.27, MBF Bioscience, Williston, Vermont). Live video images of immuneroxidase-labelled GFAP\(^+\) cells were viewed using a Zeiss Axioskop photomicroscope equipped with a MAC 6000 XYZ computer-controlled motorized stage with joystick and focus control (Ludl Electronic Products), a Q Imaging video camera (MBF Biosciences), and a PC running Windows 7 (Dell Australia). Initially, the perimeter of the IL PFC was traced at a low magnification (4\(\times\), N.A. 0.1), with the boundaries of the individual layers demarcated at sizes previously defined [Layer I (17.8 \%), II (27.9 \%), III (46.6 \%), IV (73.0 \%) and VI (100 \%)]. Cells were then counted over three separate sections (inter-section interval of 6) at a high magnification (40\(\times\), N.A. 0.65). Counting frames (90 \(\times\) 90 \(\mu\)m) were systematically sampled within a grid with constant dimensions (125 \(\times\) 125 \(\mu\)m), yielding approximately 300–320 sampling sites per animal. Due to shrinkage of the tissue after immunohistochemical processing and coverslipping procedures, the average measured tissue thickness was 22.6 \(\pm\) 0.5 \(\mu\)m. As such, we used an optical dissector of 20 \(\mu\)m with a 1-\(\mu\)m guard zone for all counting. GFAP\(^+\) astrocyte cells were counted only if their cell body came into focus within the height of the dissector probe, and were clearly within the perimeter of the counting frame without intersecting the exclusion lines. A similar procedure was used to investigate the total cell population, with stereological estimates of the number of cells in a series of six Nissl-stained tissue sections (inter-section interval of 6). On average, 20 sites were sampled for each section across a grid of 175 \(\times\) 200 \(\mu\)m, with randomly placed counting frames of 50 \(\times\) 50 \(\mu\)m. Cells were only included in the analysis if the nucleus came into focus within the height of the optical dissector (7 \(\mu\)m with a 5-\(\mu\)m guard zone).

Exhaustive counting of GFAP\(^+\) cells within the IL PFC

Using the same tissue sections and microscope as used for counting with the optical fractionator, we investigated the distribution of astrocyte cells within the IL PFC. A low magnification image of the IL PFC (10\(\times\), N.A. 0.25) was captured and then imported into Photoshop CS6 (version 13.0 \(\times\) 64, Adobe Systems Inc.). A transparent layer was added above each image, onto which 2-mm dots were digitally placed directly above each GFAP-positive cell. This process created a dot plot similar to that created using the optical fractionator method. This approach, however, allowed us to determine the precise XY location of each GFAP positive cell. Specifically, this was achieved by importing the dot plots into MetaMorph software (version 7.5.4.0; Molecular Devices) where a ROI corresponding to the IL PFC was overlaid, and the co-ordinates of all cells within this region were recorded using integrated morphometry analysis. Cellular co-ordinates were then
Intrasomal and extrasomal intensity of S100β and S100β+ cell counts

We next investigated the impact of chronic stress on both the intra- and extracellular expressions of S100β. Confocal stacks (50–100 images separated by a 0.25-μm z step size; 100× magnification) of tissue co-labelled with S100β and GFAP were acquired on a Nikon Eclipse microscope, which were then imported into Neurolucida (version 10.30.1, MBF Biosciences) to create a maximum intensity projection. The density of intrasomal S100β was then quantified by tracing a perimeter surrounding S100β positive cells, and recording the colour intensity using Metamorph (version 7.5.4.0; Molecular Devices). Extrasomal S100β expression was measured using a similar colour intensity procedure, whereby four equally sized square ROIs were systematically placed in fixed positions within each corner of the image. We also undertook an exhaustive account of S100β+ cells using the method described above for GFAP+ cells.

Data analysis

All data were analyzed using the Statistical Package for Social Sciences (SPSS, version 19; IBM). Evaluation of the threshold and intensity data from GFAP- and S100β-immunolabelled tissue was conducted using an independent sample t test with ‘Stress group’ as the between subject variable (two levels: stress, control). For all analyses involving multiple layers within the IL PFC, we conducted multivariate ANOVAs, with ‘Stress group’ (two levels: stress, control) and ‘layer’ as between subject variables (five levels: I, II, III, V and VI). We used the same variables for the morphometric analysis of the 3D astrocyte reconstructions, with the addition of a further between-subject variable ‘Sholl ring’ (12 levels: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 and 36). Planned comparisons between average morphometric values for each subject from stress (n = 10) and control (n = 10) groups were conducted. The relationship between S100β and astrocyte morphological parameters (process length, volume and number of branches) and astrocyte density (optical fractionator estimates) were determined using Pearson’s correlation. For all analyses, planned comparisons of simple effects and Tukey’s post hoc tests were used to identify the location of any significant differences both between and within groups. In all cases, ANOVA assumptions including the homogeneity of variance, homogeneity of regression and sphericity were satisfied. Data are expressed as mean values ± SEM for all experimental groups, with the α criterion set at p < 0.05.

Results

Changes in the IL astrocyte process length

Within each layer, process length was assessed by calculating and comparing to the total length of all cellular processes, and then further investigated by comparing the process length within each of the concentric Sholl spheres. Irrespective of treatment condition or layer, Sholl analysis showed that on average greater than 90% of all process length occurred within 18 μm of the cell origin. Animals exposed to chronic stress (M = 148.77 μm, SEM ± 6.05 μm) showed a significant reduction in the mean total process length relative to controls (M = 88.34 μm, SEM ± 3.20 μm), corresponding to an average of a 40.6% reduction in total process length, t (18) = 5.83, p < 0.01. We next investigated the stress-induced changes in the process length within each of the 3-μm Sholl spheres across each of the four layers measured and found relative to controls, chronic stress was associated with a significant decrease in process length in layer II, F (1, 18) = 22.58, p < 0.01; layer III, F (1, 18) = 8.55, p < 0.01; layer V, F (1, 18) = 16.63, p < 0.01 and layer VI, F (1, 18) = 43.28, p < 0.01. Details regarding areas of statistical significance from planned comparisons are illustrated in Fig. 3.

Changes in the IL astrocyte process volume

Within each layer, process volume was initially assessed by calculating and comparing the volume of all cellular processes, and then further investigated by comparing the process volume within each of the concentric Sholl spheres. Irrespective of treatment condition, Sholl analysis showed that on average greater than 99% of the total process volume was within 21 μm of the cell origin. Animals within the chronic stress condition (M = 10.09 μm³, SEM ± 0.43 μm³) showed a significant reduction in the mean total volume relative to controls (M = 17.97 μm³, SEM ± 1.14 μm³), corresponding to an average of a 56.0% reduction in total process volume, t (18) = 4.37, p < 0.01. We next investigated the stress-induced changes in the process volume within each of the 3-μm Sholl spheres across each of the four layers measured and found relative to controls, chronic stress was associated with a significant decrease in cell volume in layer II, F (1, 18) = 7.44, p < 0.02; layer III, F (1, 18) = 7.08,
Changes in the IL astrocyte branch number

Within each layer, the number of branches was assessed by calculating and comparing the number of bifurcating branch points, and then subsequently the number of bifurcations within each of the Sholl spheres measured. Irrespective of treatment condition, Sholl analysis indicated that bifurcations primarily occurred within the proximal aspect of the cell, with greater than 90% of all branches occurring within 12 μm of the cell origin. Animals exposed to chronic stress ($M = 4.94, \text{SEM} \pm 0.23$) showed a significant reduction in the mean number of branch points relative to controls ($M = 8.64, \text{SEM} \pm 0.36$), corresponding to an average of a 57.78% reduction in the number of branch points, showing the mean summation of process length from the entire cell. In all cases, asterisks indicate where planned comparisons indicated a significant difference ($p < 0.05$).

Effects of chronic stress on GFAP immunolabelling

To verify consistency with existing literature, we sought to establish whether chronic stress influenced the total GFAP immunoreactivity within the IL PFC. This was evaluated over 2 rostrocaudal levels (+2.20 and +2.70 from Bregma), however, as there were no significant rostrocaudal differences (all $p > 0.05$), we report only group means.
that have been collapsed across the rostrocaudal levels. Data from the thresholded images of the total IL PFC indicated significantly greater density of immunoreactive material in the control sections ($M = 11.38\%$) when contrasted with stress tissue ($M = 7.06\%$), corresponding to an average of a $37.95\%$ reduction in immunoreactive material, $t (18) = 4.95$, $p < 0.01$ (see Fig. 6). We then investigated the distribution of immunoreactive material, to determine if there were any differences in intensity as a function of layer in the IL PFC, and found a significant main effect of both layer, $F (4, 72) = 318.65$, $p < 0.01$, and experimental group, $F (1, 18) = 17.05$, $p < 0.01$, with a significant interaction between the two variables, $F (4, 72) = 10.11$, $p < 0.01$ (see Fig. 3). Tukey’s post hoc analysis indicated that the difference in layers was driven by layer I, which had a greater density of immunoreactive material when contrasted with all other layers ($p < 0.05$), irrespective of treatment condition. Planned comparisons demonstrated that across all layers, control animals had graph shows the mean total process volume of the entire cell. In all cases, asterisks indicate where planned comparisons indicated a significant difference ($p < 0.05$)

significantly greater intensity of immunoreactive material when contrasted with stressed animals (all $p < 0.05$; see Fig. 6). Finally, to assess the specificity of these observed changes, we also evaluated the density of immunoreactive material within the primary motor cortex, an area not commonly associated with stress, and found that there was no significant difference between controls and animals exposed to chronic stress in this region ($p > 0.05$).

Effects of chronic stress on the number of GFAP$^+$ cells

The total number of GFAP$^+$ astrocytes within the volume of IL PFC sampled was estimated using the optical fractionator method. The data indicated that within the overall IL PFC, there was a significant reduction in astrocyte number in animals exposed to chronic stress ($M = 7,360$, SEM $\pm 230$), when contrasted with the control condition ($M = 8,640$, SEM $\pm 328$), corresponding to an average of a $14.81\%$ reduction in the total number of astrocytes,
Fig. 5 Mean (±SEM) number of branch bifurcations in the GFAP+ cells of chronically stressed (white) and handled control (black) animals within each layer of the IL PFC. Within each figure, the inset graph shows the average number of branch points in the entire cell for each layer. In all cases, asterisks indicate where planned comparisons indicated a significant difference ($p < 0.05$).

Fig. 6 Stress-induced reduction in the density of GFAP+ immunoreactive material within each layer of the IL PFC. The inset figure shows the mean percentage of immunoreactive material within the total IL PFC. Asterisks indicate area of statistical significance ($p < 0.05$). The photomicrographs represent typical imaging of GFAP-labelled tissue taken from the brain of control and chronically stress-treated rats, respectively. In both images, the anatomical location of the IL PFC is overlaid. Scale bar 200 μm.

$t (16) = 2.94, p = 0.01$ (see Fig. 7). We next wanted to compare the cellular density within each layer, however, as the size and volume of each layer was not homogeneous, we first transformed and normalized the estimated counts to determine the number of cells/0.1 mm$^3$. From these transformations, we observed a significant main effect of
both layer, $F(4, 64) = 113.55, p < 0.01$, and experimental group, $F(1, 16) = 8.13, p < 0.01$, but no significant interaction ($p > 0.05$). Tukey’s post hoc analysis again indicated that the difference in layer was driven by a greater number of cells within layer I when contrasted with all other layers ($p < 0.01$), irrespective of treatment condition, with no significant within group differences in the other layers (all $p > 0.05$). Planned comparisons of between group differences indicated that the control group had a greater number of cells in layer I, $t(16) = 2.66$, $p = 0.017$; layer III, $t(16) = 2.48, p = 0.025$; and layer V, $t(16) = 3.050, p = 0.008$; with no significant differences in layer II or VI (in both cases $p > 0.05$; see Fig. 7).

Effect of chronic stress on the number of Nissl$^+$ cells

We next investigated whether the observed reduction in the number of GFAP$^+$ cells was accompanied by a corresponding decrease in the overall number of cells, as indicated by stereological counts of Nissl$^+$-stained cells. Though GFAP$^+$ cell numbers were decreased in the IL PFC after stress, total cell numbers remained unchanged when compared with control animals ($p > 0.05$; see Fig. 8).

Effect of chronic stress on the number of S100β$^+$ cells

In order to investigate whether the decrease in GFAP$^+$ cells without a concomitant loss of total cell numbers was due to a reduction in GFAP expression, an alternative marker was utilized for identification of astrocytes in the IL PFC. Cells positive for S100β were exhaustively counted across the entire IL PFC. No differences between stress and control groups were observed ($p > 0.05$) (See Fig. 8).

Fig. 7 Stress-induced reduction in the number of GFAP$^+$ astrocytes within each layer of the IL PFC. The inset figure shows the estimated total number of astrocytes within the IL PFC, as determined using the optical fractionator counting method. Asterisks indicate area of statistical significance ($p < 0.05$)

Fig. 8 Chronic stress did not change overall cell numbers in the IL PFC. A Nissl stain of all cells in the mPFC demonstrated no difference in cell numbers between control (black) and stress (white) groups. Cells positively stained for the astrocyte marker S100β also demonstrated no difference in population of the IL PFC. Values are expressed as $\pm$SEM

Effect of chronic stress on the distribution of GFAP$^+$ cells

We next investigated how chronic stress influenced the distribution of GFAP$^+$ astrocytes within the IL PFC. Precise $XY$ co-ordinates of all cells within the IL PFC were extracted from GFAP-immunolabeled tissue and were used to generate a heatmap of the cellular density (see Fig. 9). These figures clearly illustrate the nature of the stress-induced reduction in the density of cells as revealed by both our thresholding and stereological assessments. We then compared the density of cells in the dorsal and ventral aspects of each cortical layer by determining within group, within layer differences. This analysis indicated that there was a significantly greater density of cells within the dorsal region of layer VI in animals exposed to chronic stress, $t(18) = 2.41,$
Fig. 9 Heatmaps illustrating the density of GFAP+ cells within the IL PFC of both stress and control animals in both 3D (upper panels) and 2D (lower panels). The scale bar on the right indicates the density of cells within each square cell of the heat map grid. M medial, L lateral, D dorsal, V ventral.

$p = 0.029$, with no significant within layer differences for all other cortical layers measured. This result indicates that while there was a stress-induced overall decrease in the density of cells, the proportionate distribution of cells within each layer was minimally influenced by stress exposure.

Intra- and extrasomal intensity of S100β

As cellular atrophy is often indicative of cell distress, we investigated chronic stress-induced changes in the intra- and extrasomal expressions of S100β, a protein marker commonly associated with astrocyte stress. A general increase in both the intra- and extrasomal expressions of S100β was noted (Fig. 10a, b). When individual astrocytes from each group were ranked in order of S100β intensity, astrocytes from chronically stressed rats nearly always displayed greater S100β than their matched controls. However, our results indicated that relative to controls, chronic stress was associated with significant elevations in extrasomal $[t(18) = -3.25, p < 0.01]$ but not intrasomal $[t(15) = -0.92, p > 0.05]$ expression of S100β (Fig. 10c, d). In addition, no correlation between intra- and extrasomal levels of S100β and astrocyte morphology was observed, suggesting that S100β expression was increased independently of structural changes to PFC astrocytes.

Discussion

One of the most consistently identified environmental challenges recognized to disrupt PFC function is exposure to chronic stress [3, 21]. At a cellular level, the ability of stress to induce profound structural remodelling of neurons is well described [41, 43, 44, 53]. By comparison, much less is known about how chronic stress alters astrocytes within the PFC. In an effort to further our understanding of how astrocytes are altered by stress, we undertook a detailed neuroanatomical investigation to determine whether exposure to chronic stress could induce significant structural remodelling of astrocytes within the PFC. To investigate this question, we digitally reconstructed astrocytes using high-resolution three-dimensional images acquired using confocal laser scanning microscopy. The results from this research have revealed that astrocytes undergo profound structural remodelling, consistent with atrophy, as a result of exposure to chronic stress.

Using a label specific to the astrocyte protein GFAP, our analysis revealed that chronic stress produced a substantial decrease in three main morphological parameters of astrocytes, with an average of a $\sim 40\%$ reduction in process length, a $\sim 56\%$ decrease in process volume, and a $\sim 58\%$ decline in the number of process branches. Across each of the cortical layers examined, our analysis of these changes indicated that the magnitude of change was remarkably consistent. To our knowledge, this is the first demonstration of structural remodelling of astrocytes following exposure to chronic stress. Therefore, it is only possible to speculate about the overall significance of these findings. Nevertheless, given the profound level of atrophy, that the effect appears to be region-wide, and the recognized involvement of astrocytic processes in regulating
Fig. 10 Co-localization of GFAP and astrocyte-specific S100β immunoreactive material. The upper panel of photomicrographs represents maximum intensity projections of confocal image stacks taken from both control- (upper row) and stress-treated (lower row) rats. The tissue was co-labelled with S100β (left) and GFAP (centre), with a corresponding merge of the two images (right) illustrating a significant degree of co-localization. Within each of the S100β images, the inset figure shows a colour intensity profile for intra- and extrasmall S100β for the pictured astrocyte. Higher colour intensity

synaptic function, the influence could be considerable. Certainly, this result is complementary to existing knowledge concerning GFAP⁺ cell loss within the PFC and goes some way to providing a clearer picture of how chronic stress disrupts PFC function [9, 12, 62].

Previous findings have consistently reported the ability of chronic stress to reduce the number of GFAP⁺ cells [22, 47]. Therefore, it was of great interest to determine whether the stress protocol we used in the current study also produced these effects. Accordingly, we undertook several assessments examining changes in both GFAP⁺ immunoreactivity and the density of GFAP⁺ cells. In addition to these assessments, we also evaluated changes in cell density using two GFAP-independent methods. We undertook these latter procedures as there has been some research to suggest that GFAP expression can vary in a manner that is not dependent upon the viability of the cells, leading to a situation where GFAP-based stereological assessment may not provide a totally accurate estimate of changes in cell number [7, 23, 54].

In terms of the GFAP-dependent methods, we used both thresholding and unbiased stereological counting to quantify astrocytes. Consistent with previous findings [22, 47], the results of the thresholding analysis revealed that chronic stress induced a significant reduction in the quantity of GFAP immunoreactive labelling (~38%). Similarly, the results from our unbiased stereological assessment confirmed existing findings regarding a chronic stress-induced global reduction in the number of GFAP⁺ cells within the PFC [30]. Of note, these alterations appear to be reasonably circuit specific, as we could not find any evidence of equivalent alterations within the motor cortex, an area not traditionally recognized to be disturbed by exposure to chronic stress.
Exhaustive counting of GFAP+ cells across cortical layers indicated that irrespective of treatment, there was a significantly greater density of GFAP cells within layer I, with no difference in the density between all subsequent layers, giving rise to the characteristic L shape observed in the three-dimensional heat maps. When we evaluated the laminar distribution of GFAP+ cells, we found that the number of cells within each layer was homogenous, with few significant differences in density between the dorsal and ventral portions of each layer for either treatment condition. After chronic stress treatment, a region-wide decrease in the density of immunoreactive material across each of the cortical layers was revealed by our thresholding analysis. However, the results from our unbiased stereological analysis of cellular density indicated that statistically, reductions were only significant in layers I, III and V, with no significant change in either layer II or VI.

While the current study is the first, to our knowledge, to examine astrocyte remodelling following chronic stress, other research groups have identified similar changes in astrocyte density. Specifically, Banasr and Duman [4], using unbiased stereological assessment, demonstrated that chronic unpredictable mild stress significantly decreased the number of GFAP+ astrocytes within the PFC. These results also align with a decrease in GFAP+ cells reported to occur in the hippocampus [13]. Among the few other studies to have systematically quantified chronic stress-induced changes in astrocytes, Ye et al. [61] found a significant decrease in GFAP protein within the hippocampus using Western blot analysis; and Imbe et al. [30], using a thresholding approach, showed a stress-induced decrease in GFAP immunoreactive material in the periaqueductal grey. Based on this literature, several groups have proposed that the reduced number of astrocytes is directly linked to disruptions in cognitive behaviour, with many now focused on astrocyte loss as a primary driver of pathology [6]. Indeed, this proposal seems to be empirically well supported by the study of Banasr and Duman [5] who demonstrated that the application of the astrocyte specific toxin t-alpha-aminodipic resulted in astrocyte ablation in the PFC, and was sufficient to induce depressive-like behaviour, similar to that observed following exposure to chronic stress.

To confirm our finding that chronic stress had decreased the number of astrocytes within the PFC, we also assessed changes in cell density using GFAP-independent measurements. We utilized Nissl staining as our primary GFAP-independent method. The Nissl stain binds extra nuclear RNA, making it a ubiquitous and highly utilized approach for assessing changes in cell density [20, 40]. Interestingly, our assessment of cell density within the PFC using Nissl-based stereological counting indicated that there were no statistical differences between the density of cells in the stress and control groups. However, as Nissl stain is a non-specific cellular marker, it cannot be readily used to distinguish between neurons and different types of glia. Accordingly, it is possible that chronic stress reduced the number of astrocytes but caused a corresponding increase in another cell type. While a remote possibility, this consideration led us to undertake a count of S100β, which has frequently been used as a marker of mature astrocytes. As was the case with the Nissl counts, we could not find any statistical difference in the number of S100β+ cells within the PFC between the stress and control groups. Together, this set of results suggests that chronic stress, using the 21-day restraint stress model, does not reduce the numbers of astrocytes. A more likely scenario is that chronic stress reduces the expression of GFAP.

The ability of chronic stress to decrease GFAP expression but not astrocyte numbers helps resolve a longstanding paradox; specifically, evidence demonstrating the absence of a neuroinflammatory response in chronically stressed animals [26, 57]. To elaborate, if it was the case that chronic stress actually caused a 15–20 % reduction in the number of astrocytes, one may anticipate a robust inflammatory response within the same region. Previously, however, we have determined, using a model identical to that reported in the current study, that chronic stress did not cause an appreciable increase in the pro-inflammatory cytokine interleukin-1β, did not increase the expression of the antigen presentation marker MHC-II; did not increase the expression of CD68, a marker of microglial phagocytosis; nor did it increase the expression of apoptotic markers (activated caspase-3 or TUNEL) [27]. Moreover, microglia within the PFC became hyper-ramified rather than assuming a hypo-ramified state, which is associated with the pro-inflammatory state induced by injury [27]. These results which indicate that there is a negligible neuroinflammatory response within the PFC of stress animals interdigitates extremely well with the present finding that there is no major loss of cells within the PFC following stress.

For some time now, GFAP has been the most extensively used marker to identify astrocytes [11, 15, 22, 30]. Specifically, it identifies the intermediate filaments that comprise the cell’s cytoskeleton [55]. Whilst this feature makes GFAP an extremely useful and appropriate marker to investigate structural remodelling, it was clear that the immunolabelling and imaging procedure used in the current study did not perfectly resolve extremely fine GFAP+ processes. It may be possible to circumvent this issue using an approach such as stimulated emission depletion microscopy to achieve higher spatial resolution. Nevertheless, it is likely to be the case that our investigative approach has provided an underestimate of the real magnitude of stress-induced cytoskeletal atrophy. It is also important to draw attention to the fact that GFAP labelling
only identifies one aspect of the cell’s overall structure. Indeed, it is estimated that GFAP only accounts for approximately 15% of the astrocyte’s overall volume [10]. Accordingly, it is worthwhile to note that no definitive generalizations can be made with respect to changes in cytoskeleton to changes in the overall volume of the cell. It would, nevertheless, seem reasonable to hypothesize that the cytoskeleton and the overall area occupied by the cell within the central nervous system would be highly correlated.

In terms of understanding the changes induced by chronic stress, one of the most consistent findings has been the pronounced dendritic atrophy and dendritic spine loss within layers II/III of the PFC [31, 41, 48, 53]. These findings appear to be highly consistent with a raft of clinical imaging studies, which have consistently demonstrated that individuals with a significant life history of depression exhibit marked reductions in grey matter volume within the ventromedial PFC [16, 37]. While many have hypothesized that it is these neuronal alterations that contribute to the emergence of cognitive and mood disturbances seen in individuals exposed to chronic stress [17], the specific mechanisms that contribute to these neuronal changes have remained elusive. However, studies over the past several years have strongly suggested that disturbances in astrocytes may play a role in stress induced neuronal and cognitive disturbances [18].

Over the past decade, several research groups have provided evidence that astrocytes may play a central role in mediating the effects of stress on neuronal structure and function [12, 22, 47, 51]. Based on the available evidence, it had become widely accepted that chronic stress induced a significant reduction in astrocyte density and that this reduction was pivotal to the observed neuronal disturbances. Contrary to this established understanding, the results from the current study suggest that chronic stress may not induce a reduction in astrocyte numbers, but rather significantly lower the expression of the GFAP marker upon which prior density assessments have been predicated. The fact that astrocytes are not dying should not, however, be taken to indicate that there are not profound changes in this cell group. Indeed, in the present study we have provided evidence demonstrating that chronic stress results in widespread structural atrophy of the astrocyte. This atrophy is likely to be extremely important given that GFAP is a major cytoskeletal protein that facilitates the astrocytes ability to appropriately remodel itself in response to environmental signals [49, 55].

One intriguing candidate mechanism that may have contributed to the remodelling of astrocytes is S100β, a protein that is particularly concentrated within astrocytes. Elevated levels of S100β have been widely recognized in association with astrocyte distress [28]. However, recent observations suggest a much more sophisticated concentration-dependent role for this calcium binding globulin. For instance, at nanomolar concentrations, S100β has been shown to possess neurotrophic qualities, promoting neurogenesis, cell survival, growth and differentiation [29, 58]. Micromolar concentrations of S100β, however, have been associated with both glial and neuronal apoptosis, as a consequence of S100β-induced up-regulation of cyclooxygenase-2 and elevations in free radical release [8, 28, 29]. Accordingly, from a theoretical perspective, alterations in the expression of S100β represent a plausible pathway that may contribute to the observed changes in astrocytes. In the current study, we observed that extrasomal S100β was elevated within the PFC following exposure to chronic stress. These observed results align well with clinical research demonstrating elevated serum levels of S100β in individuals suffering from major depression [51, 52] and from one other study that has examined S100β in hippocampal homogenate [61]. Given the deleterious effects attributed to high levels of S100β, it was of interest that our analysis revealed that there was no significant relationship between levels of S100β and astrocyte remodelling. These results suggest that the mechanisms responsible for the structural atrophy of astrocytes are separate to those responsible for increasing S100β expression.

Clearly, identifying the signalling molecules responsible for driving astrocyte atrophy is now an issue of considerable interest. In the current study, we hypothesized that S100β may contribute to cellular atrophy, given that the protein is known to be released by astrocytes and has been found to be elevated in patients with depression. While we found exposure to chronic stress increased S100β within the PFC, we could find no statistical evidence of a relationship with astrocyte remodelling.

When the results of the current study are taken in conjunction with earlier work on microglia, it appears that chronic stress can exert a very significant influence on glial morphology. Specifically, chronic stress, at least within the PFC, appears to promote microglial hyper-ramification and astrogial atrophy. At this stage we can only speculate on what the broader significance of these remodelling events may be. Recently, however, it has been recognized that astrocytes and microglia have an important contribution to synaptic function. Indeed, it is recently been suggested that the concept of the synapse be expanded (the quadiartiparte synapse) to acknowledge the central role of both astrocytes and microglia to synaptic function [24, 56]. Although much more experimental work lies ahead to explore this concept, it is intriguing to consider the possibility that chronic stress influences neuronal function by disrupting glial interactions at the level of the synapse. Finally, the results from the current study call attention to the fact that strategies
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stereotaxical study in comparison with astrocytes. Glia 55: 1334–1347
Chapter Five

General Discussion
Overview

The primary aim of the body of research presented within this thesis was to investigate functional and morphological characteristics of glial cells following exposure to chronic stress, and to determine how receptive these changes were to intervention. Specifically, the research sought to establish whether microglia and astrocytes, two of the major subtypes of glial cells within the CNS, were significantly altered in response to stress. In a series of immunohistochemical investigations, evidence has been presented to suggest that both cell types are extremely sensitive to stress exposure. For instance, in both classes of cells, we observed significant stress induced alterations to the cells: (1) structural phenotype; (2) density throughout the brain; and (3) expression of various protein markers. We also found compelling evidence that conventional antidepressants have the capacity to suppress microglial functioning. Given the previous dearth of evidence evaluating the relationship between stress and glia, these findings provide a substantial and novel contribution to our understanding of the impact of chronic stress, and how it can modulate both the structure and functioning of the brain. This linkage has broad implications, as they not only indicate that glia may play a vital functional role in both responding to, and adapting to stress, but they also highlight glia as a potential target to consider in the development of future medications designed for the treatment of stress and stress related pathology.

Characterisation of the Stress Protocol

One of the key outcomes of the first study in Chapter Two was both the physiological and behavioural characterisation of the chronic restraint stress model utilized throughout all subsequent neuroanatomical investigations. Our results were consistent with a number of previous reports (Banasr & Duman, 2008; Beig, Baumert, Walker, Day, & Nalivaiko, 2009; Depke et al., 2008; Grippo & Johnson, 2009; A. K. Walker et al., 2009), in that chronic stress
induced both profound physiological alterations (decrease in weight gain, increase in core body temperature) and corresponding perturbations to the animal’s behaviour (decrease in sucrose preference, reduction in the time actively struggling during restraint sessions). Interestingly, the magnitude of the physiological response to stress, such as the elevation observed in core body temperature, was maintained at an equivalent level across the each of the various time points measured (i.e. the first, middle and final stress session). This was taken as evidence to suggest that: (1) the stressor was indeed inducing a stress response; and (2), that the bodily response to the stressor did not show any signs of weakening as a consequence of repeated exposure. Despite this, in terms of the animals’ behaviour, we observed a significant reduction in the duration of time that the animal spent struggling in the restraint, which was markedly reduced on each successive restraint session. Taken alone, this finding could be interpreted as either a habituation to the stressor, indicating that the restraint no longer induced an equivalent stress response; or alternatively, that the animal was manifesting learned helplessness, a type of behaviour that has traditionally been used as an indicator of depression in animal models (Seligman, 1972). Combined with the physiological evidence, one may consider this to be the latter. This viewpoint is further supported by the significant decrease in sucrose preference, a behavioural marker often used as a measure of anhedonia, an additional measure associated with depression in animal models (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). Taken together, the evidence clearly demonstrated that the chronic stress protocol utilized in the current experiments was sufficient to induce substantial alterations to both the animals’ physiology, and also their behaviour, consistent with what is commonly reported within the literature.
**Chronic Stress Induced Microglial Modulation**

Our immunohistochemical analysis in Chapter Two demonstrated that exposure to chronic restraint stress caused a significant alteration to microglia in a number of stress responsive brain regions. This was the first study to clearly demonstrate, using immunohistochemistry, that chronic stress could induce changes in microglia, and further, that these changes were regionally specific. The results demonstrated clear increases in both the density and number of microglial cells within the hippocampus, amygdala, medial prefrontal cortex, bed nucleus of the stria terminalis and the periaqueductal grey, all regions which have been reported to be critically involved in the regulation of the stress response. Interestingly, our research indicated that whilst the density of microglial cells was altered by stress exposure, the cells did not manifest any further signs of activation. For instance, we were unable to detect an appreciable increase in markers that are often associated with microglial activation, such as those expressed following infection or tissue injury. These results were taken to indicate, that whilst stress caused substantial modulation of microglia, the cells were at an intermediary stage of activation. This may indicate that stress primes microglial cells for a potential immune response, but most likely suggest that microglia play a functional role in both responding to, and adapting to stress exposure.

**Antidepressant Attenuation of Microglial Activity**

Given the recent rise in the prominence of the neuroinflammatory model of depression within the literature, the primary aim of the research presented in Chapter Three was to investigate the extent that antidepressants could suppress microglial activity, the primary cell type responsible for both synthesising and responding to inflammatory stimuli within the CNS. Specifically, we sought to evaluate the capacity of both SSRI and SNRI antidepressants to modulate the microglial production of TNF-α and NO. Our results demonstrated that whilst
the SNRI venlafaxine exhibited negligible anti-inflammatory properties, all SSRIs substantially attenuated LPS stimulated microglial production of both TNF-α and NO. This was the first study to directly compare the potency of all SSRIs, and indicated that while they each exhibit a similar anti-inflammatory profile, sertraline was the most potent, with citalopram the least. In terms of an underlying mechanism, our data supported the hypothesis that antidepressants may exert their anti-inflammatory activity by modulating intracellular levels of cAMP (Hashioka et al., 2007; Hashioka, McGeer, Monji, & Kanba, 2009), as inhibiting protein kinase A, a cAMP dependent kinase, substantially attenuated the anti-inflammatory effect. Finally, the results also highlighted the importance of using appropriate methods of control, particularly when conducting viability assessments, as there were significant differences in the data obtained from two commonly utilized techniques for assessing cellular viability. Consistent with previous research (Hashioka et al., 2007; Horikawa et al., 2010; Hwang et al., 2008), the study provided further evidence that microglial activity could be substantially modulated by antidepressants, prompting the question, to what extent do antidepressants owe their effectiveness as a therapeutic compound to their ability to modulate inflammatory processes within the brain.

**Chronic Stress Induced Astrocyte Modulation**

In addition to the profound impact of stress on microglia, in Chapter Four, we present evidence to show that chronic stress also induces profound structural remodelling of astrocytes. Using high-resolution three dimensional reconstructions of GFAP+ labelled mPFC tissue, we were able to demonstrate for the first time that chronic stress provokes a significant decrease in the length, volume and complexity of astrocyte cellular processes. We also found, consistent with previous findings (Banasr & Duman, 2008; Czeh et al., 2006), that chronic stress induces a significant decrease in the density of GFAP+ immunoreactive material, and
also the number of GFAP$^+$ cell counts. However, as we could find no significant change in total number of Nissl or S100β$^+$ labelled cell numbers, this difference most likely reflects a loss of astrocyte expression of GFAP, rather than a loss of the cell. Indeed, this interpretation is supported by the absence of any evidence of apoptosis or neuroinflammatory activity observed in our previous research using the same chronic stress protocol (Madeleine Hinwood et al., 2012; Tynan et al., 2010). Whilst this result may suggest that chronic stress does not induce astrocyte cell death, it remains clear that stress can induce significant structural atrophy. The observed alterations to astrocyte morphology could have profound implications for the functioning of the cell, and may impair its communication and modulation of neuronal activity.

**Future Research**

Investigations involving the relationship between stress and glia are still very much in the embryonic stages. The findings presented within this thesis have provided a foundation for a number of future questions surrounding this relationship to be explored. Perhaps the most logical next step would be to integrate the findings of the research presented within this thesis, by investigating the capacity of antidepressants to attenuate/reverse the alterations observed in both microglia and astrocytes, and the corresponding changes to the animal’s behaviour. Indeed, subsequent research within our laboratory has begun to address this question. Specifically, the research showed that minocycline, a tetracycline derivate well-recognised as a potent inhibitor of microglia not only substantially attenuated the stress induced changes to microglia, but also decreased the stress-induced deficit in a working memory dependent task (M. Hinwood, Morandini, Day, & Walker, 2011). The results of this subsequent investigation clearly demonstrate that inhibiting the stress induced alterations to microglia can have a profound influence on behaviour. What is yet to be determined, is if
antidepressants have a similar influence on suppressing microglial activity in vivo. The results of the in vitro pharmacological investigations presented in Chapter Three could be taken as evidence to suggest that antidepressants may also exert microglial attenuating actions in vivo. Furthermore, they provide a number of potential concentrations of antidepressants that could be assessed in an in vivo experiment. Further testing is required to evaluate whether the ability of antidepressants to suppress microglial activity in vitro translate into a similar effect in vivo, and if these changes correspond to significant changes in the animal’s behaviour.

**Final Comments**

Our understanding of stress has evolved considerably over the past century, yet despite substantial advances, there still remains much to learn. The outcomes of the current thesis provide some novel insight into the changes that occur within the brain following exposure to chronic stress. We have shown, in many cases for the first time, that glia are significantly perturbed by exposure to chronic stress. Interestingly, the manner in which stress exerts its influence on glia was not universal. For instance, we see an upregulation in a number of surface markers present on microglial cells, while we see a corresponding decrease in the markers present on astrocytes. We also found evidence to show that these changes are regionally specific, only occurring in a subset of stress responsive brain regions. This research has made significant progress in our understanding of how glial cells respond to chronic stress. The work presented substantially contributes to our understanding of what happens in the brain when an organism is stressed, and highlight the importance of considering glia in future research investigating the stress response, and in the development of pharmacotherapies for the treatment of stress and stress related disorders.
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