THE EFFECT OF INFLAMMATION ON THE CENTRAL 5-HT SYSTEM IN MOOD DISORDERS

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A philosopher once said "It is necessary for the very existence of science that the same conditions always produce the same results." Well, they do not.

Richard Feynman
The Character of Physical Law

Abstract

Inflammation appears to play a major role in the pathogenesis of Major Depression, and understanding the relationship between the immune system and mood disorders is a growing area of research. Patients undergoing cytokine therapy frequently develop depressive-like signs which cease upon termination of treatment. In addition, depressed patients often present with high levels of circulating pro-inflammatory cytokines. There are also significant behavioural correlates between inflammation-associated sickness behaviours and Major Depression, suggesting there is a bidirectional relationship between the immune system and central systems regulating behaviour. Both sickness behaviours in animals and cytokine-induced depression in humans can be reversed with antidepressants targeting the serotonergic (5-HT) system. Against this background, this thesis sought to investigate the role of inflammation in the development of certain specific characteristics of depressive-like states in animal models. Specifically, a single systemic endotoxin challenge (LPS) was used to model acute sickness behaviour and to study the behaviour and molecular effects of this challenge on the 5-HT system. These studies indicated that a single systemic challenge changed the function of the 5-HT system, as well as increasing expression levels of a number of 5-HT-related genes, and markers of inflammation in the CNS. A rodent chronic stress paradigm was employed to investigate whether these changes also occur in animals displaying depressive-like signs. Chronically stressed mice were shown to display significant features of rodent depression; decreased sucrose preference and increased forced swim immobility. These changes were also accompanied by increased expression of 5-HT-related genes and inflammatory markers within the CNS. Importantly, many of the molecular changes observed in LPS-induced sickness behaviour were conserved in the model of stress-induced Major Depression. In particular, increased TNF expression within the CNS was highlighted as a feature in both models. Novel anti-inflammatory agents were used to study the role of TNF in the development of sickness behaviours. Anti-TNF therapy, specifically the fusion protein etanercept, ameliorated sickness behaviour induced by LPS. Together, these data demonstrate that inflammation is capable of significantly changing the 5-HT system, and that stress *per se* is capable of inducing an inflammatory state within the CNS, which support the thesis that sickness and Major Depression are closely related at a molecular level.

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Abbreviations

5-HIAA:	5-Hydroxyindolacetic Acid
5-HT:	5-Hydroxytryptamine
ABC:	Avidin Biotin Peroxidase Complex
aCSF:	Artifical Cerebral Spinal Fluid
ACTH:	Adrenocorticotrophic Hormone
APP:	Acute Phase Protein
APR:	Acute Phase Response
BDI-II:	Beck Depression Inventory
BDNF:	Brain Derived Neurotrophic Factor
BOLD:	Blood-Oxygen Level-Dependant
CBF:	Cerebral Blood Flow
CCL2	Chemokine Ligand 2 (c-c motif)
CD1:	Crl:CD1(ICR) Mice
cDNA:	Complementary Deoxyribonucleic Acid
CNS:	Central Nervous System
CMS:	Chronic Mild Stress
COX:	Cyclo-oxygenase
CRH:	Corticotrophin Releasing Hormone
CRP:	C-Reactive Protein
CVO:	Circumventricular Organs
DAB:	Diaminobenzidine Hydrochloride
DBS:	Deep Brain Stimulation
dH_2O:	Distilled Water
DOI:	(+/-)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane
DOPAC:	3,4-Dihydroxy-Phenylacetic Acid
DRN:	Dorsal Raphe Nucleus
DSM-(IV):	Diagnostic and Statistical Manual (4^{th}
ECT:	Ed.)
fMRI:	Electroconvulsive Therapy
FST:	Functional Magnetic Resonance
GABA:	Imaging
GR:	Forced Swim Test
h:	Gamma Amino Butyric Acid
HPA:	Glucocorticoid Receptor
RNA:	Hours
RT:	Hypothalamic Pituitary Adrenal
RT-PCR:	Ribonucleic Acid
SAA:	Room Temperature
SARI:	Reverse Transcription PCR
	Serum Amyloid-A
SERT:	Serotonin Antagonist and Reuptake
SNP:	Inhibitor
SNRI:	5-HT Transporter
	Single Nucleotide Polymorphism
SPECT:	Serotonin and Noradrenaline Reuptake Inhibitor
SSRI:	Single Photon Emission Computed
TCA:	Tomography
TDO:	Selective Serotonin Reuptake Inhibitor
TGF:	Tricyclic Antidepressant
TLR:	Tryptophan-2,3-dioxygenase
TMS:	Transforming Growth Factor
TNFα:	Toll-like receptor
TNF-R	Transcranial Magnetic Stimulation
TRP:	Tumor Necrosis Factor α
HPLC:	High-Pressure Liquid Chromatography
HVA:	Homovanillic Acid
IFN(γ):	Interferon-(γ)
i.p.:	Intraperitoneal
i.v.:	Intraveinous
IDO:	Indoleamine-2,3-Dioxygenase
IL-:	Interleukin
kg:	Kilogram
LDF:	Laser-Döppler Flowmetry
LPB:	LPS Binding Protein
LPS:	Lipopolysaccharide
mg:	Milligram
ml:	Millilitre
MRI:	Magnetic Resonance Imaging
mRNA:	Messenger RNA
MRS:	Magnetic Resonance Spectroscopy
NDRI:	Noradrenaline and Dopamine Reuptake Inhibitor
NFkB	Nuclear Factor kB
NMDA:	N-methyl-D-aspartate Receptor
OCT:	Optical Coating Compound
OVLT:	Organum vascularum of lamina terminalis
PAMP:	Pathogen Associated Molecular Pattern
PB:	Phosphate buffer
PBS:	Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PET:	Positron Emission Tomography
PFA:	Paraformaldehyde
PFC:	Prefrontal Cortex
phMRI	Pharmacological MRI
PVN:	Paraventricular Nucleus
qPCR:	Quantitative PCR

VNS:	TNF Receptor
µl:	Tryptophan
WDS:	Vagal Nerve Stimulation
WHO:	Microlitre
	Wet-dog Shakes
	World Health Organization

Chapter 1: General Introduction

The aim of this thesis is to explore the mechanisms involved in the development of sickness behaviour and depression in rodents. In particular, I sought to investigate the similarities and differences, at a molecular level, in lipopolysaccharide (LPS)-induced sickness behaviour and a model of stress-induced depression. Sickness behaviour is defined as a co-ordinated set of behavioural adaptations which develop in response to a systemic infection and immune system activation, in man and in other animals. Symptoms include lethargy, sleep disturbances, loss of appetite and loss of interest in previously pleasurable activities (anhedonia), behaviours which are also characteristic of periods of Major Depression. The mechanism by which the immune system, in particular systemic cytokines, communicates with the CNS in order to initiate these behaviours is currently unknown. The 5-HT system is strongly implicated and selective serotonin reuptake inhibitors (SSRIs), the most common class of antidepressant therapy, have been shown to reverse sickness behaviours. However, it is not clear whether the response to SSRIs represents an allostatic or a homeostatic response in this context (Figure 1.1). To our knowledge, no studies have compared the molecular changes in the 5-HT system in models of sickness behaviour and depression. This introduction seeks to give an overview of both the molecular milieu involved in the systemic immune response to infection, as well as the central mechanisms involved in co-ordinating mood.

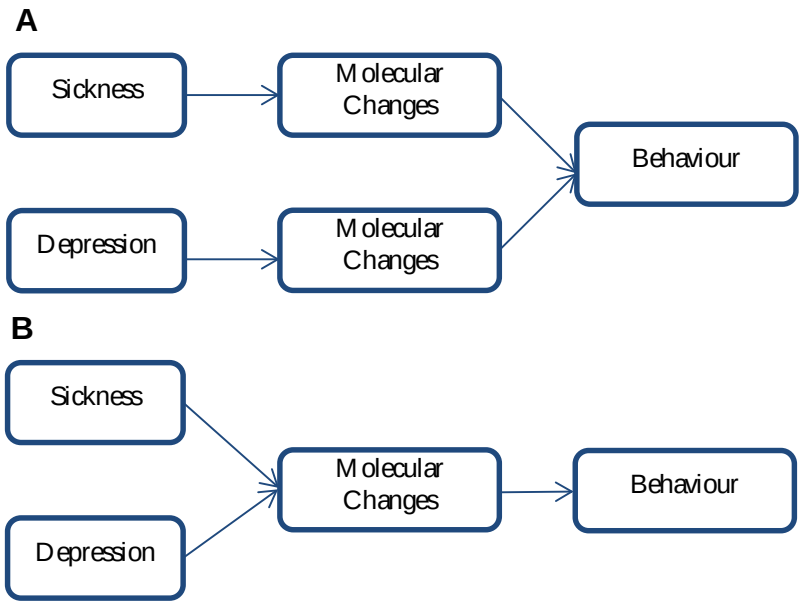


Figure 1.1 Potential convergence of sickness behaviour and depression. The similarities between sickness behaviour and depression are clear in terms of mood. However, current research has not shown whether divergent molecular changes result in similar behaviour (A), or whether similar behaviours are the result of similar molecular changes (B).

1.1 The Anatomy of Mood

A major problem with the study of depression is whether it represents a global disease affecting whole neurotransmitter systems or whether specific anatomical regions change, either in receptor expression or neuronal function. Current therapies, targeting either one neurotransmitter, in the case of serotonergic drugs, or one region, in the case of targeted magnetic therapy may result in suboptimal outcomes when used alone. It is possible that changes in mood affect receptor expression within specific regions and that more targeted and combination therapies may produce a more effective response to treatment. For the purposes of this thesis, the serotonergic system will be the main focus, as it is thought to underlie depression and regulate mood. This neurotransmitter system originates in the raphe nucleus. Neurons in the upper raphe nuclei project to the rest of the brain while neurons of the lower raphe nuclei project to the cerebellum (Dahlstroem *et al.*, 1964; Dahlstrom and Fuxe, 1964). These neurons can have diverse functional effects dependent upon the receptor expression on the target neuron, which may vary significantly by region. A number of different brain regions appear to regulate mood in both rodents and people, these will be discussed below.

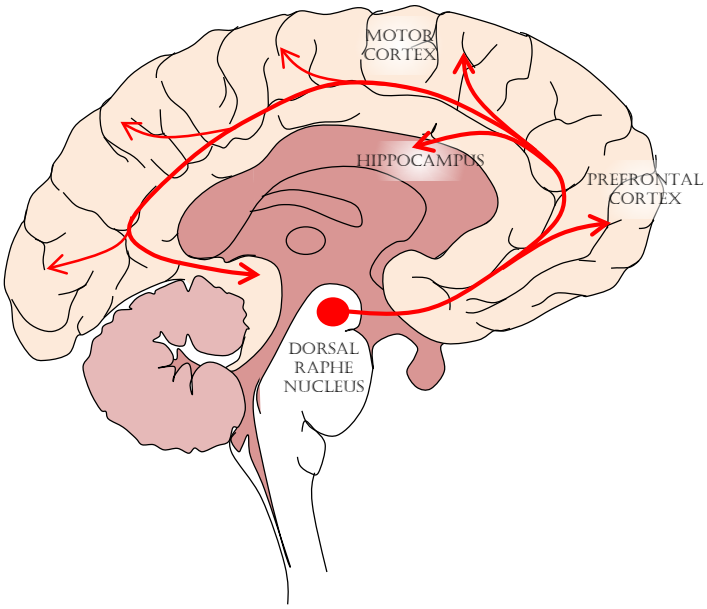


Figure 1.2 Schematic of the origins of the serotonergic system. 5-HT neurons originate in the raphe nucleus where they project to a number of different brain regions.

1.1.1 The Raphe

The origins of the 5-HT system within the CNS are known to be the raphe nuclei. There are a number of different nuclei, organised according to anatomical location, however, most literature studying the role of these nuclei in depression focus on the midbrain region, specifically on the dorsal raphe nucleus. This region contains over half of all serotonergic neurons (Dahlstrom and Fuxe, 1964) and will therefore, for the purposes of this introduction, be referred to as the raphe. Despite the high proportion of 5-HT neurons originating in this region, and the theory that 5-HT plays a crucial role in malaise, this region remains under-investigated in terms of changes during sickness behaviour.

A recent study has used a large cohort, including patients with both bipolar and unipolar depression, as well as taking into account manner of death, i.e. suicide versus non-suicide, to investigate anatomical changes in the raphe in affective disorders. Morphometric analysis demonstrated that the raphe showed an overall decrease in area in patients with unipolar Major Depression but that suicide victims, irrespective of disease, showed an increase in raphe size (Matthews and Harrison, 2012). They also demonstrate that serotonergic cell size is decreased and cell density is increased in suicide victims but remains unchanged in non-suicidal, depressed patients (Matthews and Harrison, 2012). This work is confirmed by other studies showing that no change occurs in cell number or morphometry in the raphe in non-suicidal, depressed patients (Hendricksen *et al.*, 2004; Syed *et al.*, 2005). However, work in the latter studies did not examine overall raphe area. The discrepancy between overall cell number and area found by Matthews and Harrison (2012) may reflect changes in non-neuronal components such as glia, however, the contribution of cells such as glia to these pathologies is yet to be tested.

The effect of inflammation on the raphe *in vivo* or in patients is a neglected area. In organotypic slice cultures, application of LPS or pro-inflammatory cytokines such as TNF or IFNγ reduced the number of serotonergic neurons within the raphe independent of

microglial activation (Hochstrasser *et al.*, 2011). Work using rats challenged with systemic LPS show neuronal activation in the raphe (Kopf *et al.*, 2011). Increased transcription factors and changes in cell morphology in slice culture studies suggest that the raphe responds to inflammation by regulating cell survival. However, cell death within this region would result in a global reduction in 5-HT production and this does not occur during sickness (O'Connor *et al.*, 2009). It is possible that neuronal reactivity seen after LPS is an attempt to promote cell survival during episodes of inflammation but that this may not always be successful. The discrepancy between 5-HT cell death and lack of change in 5-HT release in inflammation may be the result of compensatory pre- and post-synaptic mechanisms which require further attention.

1.1.2 The Limbic System

In terms of depression and mood the limbic system is an essential starting point for researchers. It consists of a variety of structures including the hippocampus, hypothalamus and amygdala; all regions shown to be involved in regulating emotion and behaviour (Papez, 1937).

1.1.2.1 The Hippocampus

The hippocampus has been shown to be significantly smaller in patients with Major Depression in a number of studies (Bremner *et al.*, 2000; Frodl *et al.*, 2002; Lange and Irle, 2004). These patients frequently show deficits in learning and memory, and reductions of up to 20% in hippocampal volume in some studies seems to mechanistically support these changes (Sheline *et al.*, 1996). These studies also indicate that the atrophy seen in Major Depression continues to evolve, despite years in remission and free of antidepressant drugs (Sheline *et al.*, 1996). Current literature is in conflict regarding whether hippocampal atrophy is the cause, or the result, of depressed mood. Decreases in 5-HT neurotransmission, a hallmark of depression, can inhibit hippocampal neurogenesis (Brezun and Daszuta, 1999), implying that the decrease in 5-HT combined with stress-related loss of neurons, could be the initiator of hippocampal atrophy. Thus there are broadly two theories describing the potential role of the hippocampus in

depression; either stress and early life events increase circulating factors such as cytokines which reduce hippocampal volume and function via unknown mechanisms thus leading to depression, or hippocampal neurogenesis is dysfunctional, or just decreased, and results in depression.

It is known that LPS inhibits hippocampal neurogenesis (Ekdahl *et al.*, 2003). The mechanisms by which this decreased neurogenesis occurs are unknown, but they are inhibited by anti-inflammatory agents. Minocycline, in particular, is known to inhibit LPS-induced decreases in neurogenesis, possibly via inhibition of microglial proliferation or matrix metalloproteinases (Machado *et al.*, 2009). The influenza virus has been shown to significantly alter spine density and neuronal morphology in the hippocampus, suggesting direct effects on the neurons (Jurgens *et al.*, 2012). The same group has also shown that environmental enrichment decreases this detrimental effect (Jurgens and Johnson, 2012). These data suggest that potentiating neuronal activity by facilitating environmental interactions and forcing cognitive function could enhance the activity of the brain and make it 'better prepared' for an immune response. This theory is strengthened by work showing an up-regulation of brain-derived neurotrophic factor (BDNF), as well as fraktalkine (CXCL-3) - an anti-inflammatory chemokine, in the hippocampi of mice with enriched environments (Jurgens and Johnson, 2012). Finally, microglia have been shown to have a decreased inflammatory response after electroshock therapy (Jinno and Kosaka, 2008), which is frequently used in treatment-resistant depression. It seems possible, therefore, that the effects of inflammation within the hippocampal regions could be cumulative over time and influence the development of depression in susceptible individuals. This theory is yet to be tested in either models of inflammation or depression.

1.1.2.2 The Amygdala

The data regarding the amygdala is considerably more consistent in terms of morphological changes during depression than the hippocampus, it is thought to be considerably more involved in emotional processing than the hippocampus. The

amygdala is a collection of nuclei at the base of the brain that, in complex mammals, regulates emotional memory and fear processing (Swanson and Petrovich, 1998). An important feature of the aforementioned study by Lange and colleagues in the hippocampus, was that hippocampal volume decreased but amydala volume increased (Lange and Irle, 2004). Volumetric analysis studies of this brain area, such as the one performed by Lange and Irle, should be considered carefully before strict conclusions are drawn. Meta-analysis has recently shown that amygdala volume changes in depression are medication dependant (Hamilton *et al.*, 2008). Unmedicated depressed patients showed significantly decreased volumes, and medicated depressed patients showed significantly increased volumes, compared to controls, suggesting that antidepressants may play a role in neurogenesis in this region. In terms of activation during imaging studies, the amygdala consistently shows increased activation to emotional stimuli in depressed patients compared to healthy controls (Monk *et al.*, 2008; Yang *et al.*, 2010). Work from some of these groups have also correlated 5-HT-related genotypes (e.g. different 5-HT transporter alleles) with altered amygdalal function suggesting that 5-HT genotype significantly affects how you process emotional information (Lau *et al.*, 2009).

Engler and colleagues (2011) have demonstrated a role for the amygdala in the response to systemic inflammation, and perhaps therefore sickness behaviour, by showing that LPS can activate noradrenergic neurons as well as cause an increase in IL-1β, TNF and IL-6 in this region. The same group has compared gram-negative and gram-positive bacteria and have shown that sickness behaviour in these animals was the same in response to both pathogens but the amygdaloid response was different (Prager *et al.*, 2012). While this suggests the amygdala is likely to be a critical relay point for peripheral immune afferents, the exact mechanisms have not been proposed. However, it may be as a result of changes in vesicular immune cells within this region. Depressed patients have also been shown to have reduced numbers of astrocytes in the amygdala (Altshuler *et al.*, 2010). Considering recent evidence suggesting that astrocyte-microglial communication can regulate neuronal excitability (Pascual *et al.*, 2012) it would seem reasonable to assume that this

communication could become dysregulated in both sickness and depression, to result in the consequent changes in mood.

1.1.2.3 The Hypothalamus

The hypothalamus is the first step in the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for mediating the stress response and maintaining homeostasis. It receives input from the vagus nerve via the nucleus tractus solitaris and this, in turn can be stimulated by peripheral cytokines. During both stress and inflammation the hypothalamus initiates a cascade of stress hormone responses. These begin with the release of corticotrophin releasing hormone (CRH) from neurons within the hypothalamus and subsequently adrenocorticotrophin (ACTH) release from the pituitary gland. ACTH is carried via the blood to the adrenal cortex where it stimulates cortisol release in humans; corticosterone release in rats and mice. These hormones act on glucocorticoid receptors and will be discussed in a later section. Production of cortisol is terminated by negative feedback throughout the HPA axis. A common way of testing hyperactivity of the HPA axis is using the dexamethasone suppression test. Dexamethasone, a synthetic corticosteroid, should reduce the amount of cortisol circulating by influencing the negative feedback loop required to suppress excess cortisol production. Patients with an overactive HPA axis will show dexamethasone non-suppression. This is frequently seen in depressed patients (Maes *et al.*, 1995), reflecting dysregulation in the HPA axis. This increase in HPA axis activity is also seen during sickness and the role this potentially plays in the development of behaviour will be discussed further below.

1.1.3 The Prefrontal Cortex

While is seems self-evident that changes in the limbic system can regulate mood and depression, there are a number of other brain regions which may significantly contribute to the pathophysiology of Major Depression. The prefrontal cortex (PFC) is loosely defined as the areas of the brain immediately before the motor and pre-motor areas. It is an extremely diverse region not only responsible for direct changes in mood, but also

known to regulate neurotransmitter systems responsible for reward, such as the dopaminergic system (Miller *et al.*, 2002). Both inhibitory and excitatory 5-HT receptors are expressed in the prefrontal cortex (Lambe *et al.*, 2011). A particular function of these relay receptors in this region is emotional regulation. Patients with Major Depression show decreased PFC activity, when compared to healthy controls, when they are asked to try to 'suppress feelings of sadness' (Beauregard *et al.*, 2006). The connectivity of the PFC has been shown to be important in regulating different responses to emotion. Connections to regions such as the amygdala and hippocampus suggest the PFC can discriminate between external and internal emotional cues (Price, 1999). The combination of these anatomical localizations (Figure 1.3) suggests that dysregulation of the activity in the PFC would result in an inability to down-regulate subjective emotionality in response to external emotional stimuli (Beauregard *et al.*, 2006). Despite changes in activity in the PFC, and the large number of 5-HT receptors present in this region, few studied have investigated the potential for change in this region during sickness.

Inflammation has been shown to induce changes in the PFC, both pro- and anti-inflammatory. A peripheral injection of a potent inflammogen such as caragennan induces microRNA changes in the PFC, specifically ones reflecting an increase in pro-inflammatory factors within this region (Poh *et al.*, 2011). Anti-inflammatory inputs may come from the vagus, studies have shown that the PFC is an important relay station for vagal afferents and efferents (Thayer, 2009). Finally, stress is a known vulnerability factor for the development of depression and stressed individuals have been shown to have atrophy in their PFC (Arnsten, 1998). Recent data have shown that stress can alter the response of the PFC to inflammatory mediators, potentially enhancing stress mediated cell death (de Pablos *et al.*, 2006). These data present a strong case for a heightened immune response within the PFC, compared to other regions discussed thus far, and that this change in immune status may result in altered mood. The mechanistic links between these two concepts are currently speculative, but increases in cytokines within the CNS during a systemic infection may modulate 5-HT signalling in the PFC.

Considering the data justifying links between depression and sickness, it would seem timely to study this region in models of both diseases.

1.1.4 The Motor Cortex

The motor cortex is not an area of the brain traditionally associated with Major Depression. Nevertheless, a number of groups have shown that motor cortex excitability is changed in depression. Specifically, antidepressant treatment has been shown to increase motor cortex excitability, and vagal nerve stimulation has also been shown to enhance neuronal activity in this area (Manganotti *et al.*, 2001; Herwig *et al.*, 2002). In excitability studies, depressed patients showed lateralization in terms of neuronal excitability in the motor cortex, with considerably reduced potentials in the left hemisphere (Maeda *et al.*, 2000). The authors speculate that depressed subjects show either considerably reduced glutamatergic activity or enhanced GABA activity in the left hemisphere, thus inputs into the motor cortex will be reduced. Conversely, other studies have shown that transcranial magnetic stimulation in patients with unipolar depression results in an increase in inhibition in the motor cortex (Bajbouj *et al.*, 2007). Both vagal nerve stimulation and transcranial stimulation studies should be interpreted with caution as data can be highly influenced by both frequency and amplitude of stimulation (Lomarev *et al.*, 2002). Despite these discrepancies, the data emerging on depressed patients in a number of stimulation paradigms strongly support a role for the motor cortex in depression.

In terms of the inflammatory response, little data exists specifically regarding the motor cortex. One study has demonstrated that viral infections may cause neuroinflammation in the motor cortex via motor neurons in the periphery (Wong *et al.*, 2008) and maternal inflammation increases the number of pyknotic cells in the motor cortex of the developing offspring (Golan *et al.*, 2005; Stolp *et al.*, 2011). These studies, and those above in depressed patients, indicate a role for this region in both depression and sickness and yet this region currently commands very little presence in the literature.

Finally, it is important to note that work studying this, and other brain regions, in depressed patients has often been hampered by small sample size and consistency within patient groups. The main problem lies with cause of death, specifically suicide victims; while depressed patients frequently commit suicide, suicide is not necessarily a reflection of unipolar depression. Patients may have complications such as bipolar depression or other personality disorders such as schizophrenia. The ability to parse these diseases mechanistically is crucial to our understanding of depression. The use of animal models of both depression and sickness allow for a uniform cohort of tissue, where the spectrum of signs is very narrow and may provide more insight into the mechanisms of these diseases than human studies.

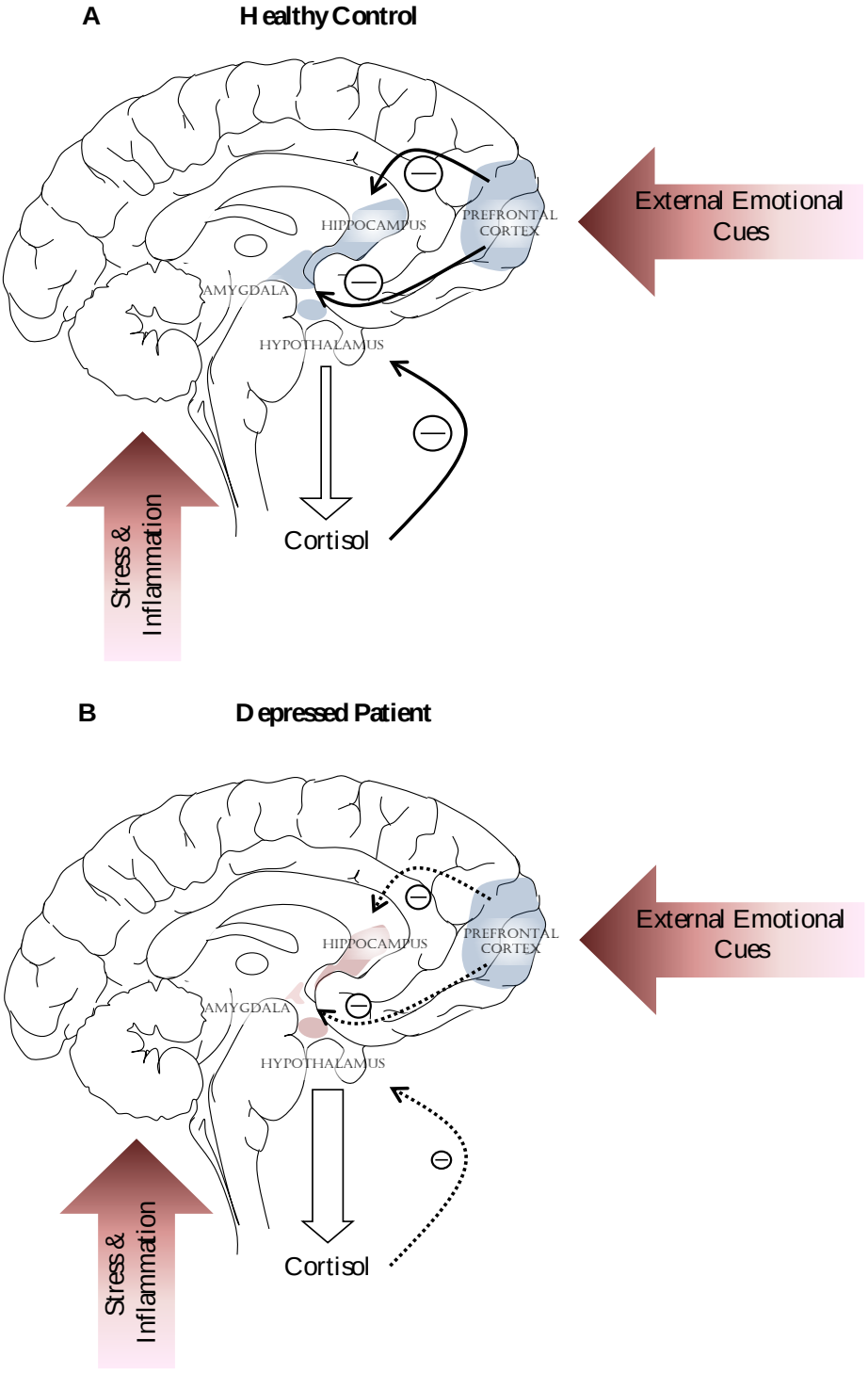


Figure 1.3 Simplified broad overview of some of the potential anatomical changes associated with depression. In normal healthy controls (A) emotional cues are processed by the prefrontal cortex and information is relayed to the hippocampus and amygdala for the appropriate storage or response, respectively. In depressed patients (B) the prefrontal cortex is frequently underactive so signals to the hippocampus and amygdala may elicit inappropriate responses. The stress response, elicited from either an external cue or from internal inflammation may also elicit excess cortisol secretion in depressed patients because of a hyperactive hypothalamic-pituitary-adrenal axis. Note also amygdala and hippocampal volume are reduced in some depressed patients.

1.2 The Molecular Basis of Mood: The Role of the 5-HT Receptor

Research is beginning to approach the study of depression and mood using both genetic and behavioural correlates; taking specific changes in receptor expression levels, or specific polymorphisms, into account when investigating behaviour – mainly depression. Despite this, links to sickness behaviour still remain relatively understudied in terms of changes in receptor expression. The genetics of mood and mood disorders are complex and require a basic background knowledge about the different 5-HT receptors, their location and their contribution to emotional processing. The use of receptor knockout animals is becoming increasingly popular in the study of the 5-HT system, therefore a comprehensive table of receptor knockouts currently in use and their response to stress has also been included. However, the reliance on one species for this work is a serious limitation considering the following introductory text. It is quite clear that there are strain and species differences in the molecular mechanisms that underpin mood and these have been largely ignored by the current literature.

1.2.1 5-HT_1 Receptors

5-HT_1 receptors represent the most abundant 5-HT receptors in the brain (Barnes and Sharp, 1999) and comprise 1A, 1B, 1D, 1E and 1F. The main 5-HT_1 receptors of interest in depression appear to be 1A and 1B.

5-HT_{1A} receptors are anatomically distributed in the raphe and the hippocampal formations (Chalmers and Watson, 1991). These receptors can be agonised by the specific ligand 8-OH-DPAT and have been shown to be both presynaptic autoreceptors (mainly in the raphe) as well as postsynaptic inhibitory receptors. Early studies indicate that the gene encoding the 5-HT_{1A} receptor is not mutated in any mood disorders, suggesting that genetic abnormalities in this receptor do not contribute to the development of depression (Xie *et al.*, 1995). They may, however, contribute to anxiety. 5-HT_{1A} knockout animals have been shown to have an anxiogenic phenotype (Ramboz *et al.*, 1998). While anxiety and depression represent distinct behavioural phenotypes, they may be linked in that stress seems to impact on both behaviours. High anxiety phenotypes could be thought to

be more susceptible to stress, and it is known that stress can result in behavioural depression. The role of specific 5-HT receptors in mediating either anxiety or depression, and whether stress mediates the development of depression and anxiety through changes in specific receptors, is currently unclear. Based on evidence from knockout animals, it is possible to hypothesise that changes in 5-HT_{1A} expression may result in increased susceptibility to stress induced depression. Despite the evidence from animal models to suggest that there is very little contribution of this receptor to depressed mood, PET imaging studies have shown it has a decreased binding potential in depressed patients compared to healthy controls (Drevets *et al.*, 1999).

There is evidence to suggest that 5-HT_{1B} knockouts exhibit a higher sensitivity to 5-HT_{1A} agonists (El-Khodor *et al.*, 2004), possibly indicating some kind of genetic compensation, i.e. 5-HT_{1B} expression is ablated and expression of 5-HT_{1A} is up-regulated as a consequence. 5-HT_{1B} receptors appear to be distributed largely in the basal ganglia, amygdala and hippocampus, but there is also some evidence to suggest that they are expressed at low densities in the forebrain (Pazos and Palacios, 1985). Functionally, they appear to inhibit neurotransmitter release but there is minimal evidence for a role in depressive-like behaviour. O'Neill and Conway (2001) have shown that both 5-HT_{1A} and 5-HT_{1B} agonists increase the amount of time spent swimming in the forced swim test. Interestingly, recent work has shown that pro-inflammatory cytokines such as TNF, specifically within the PFC region, can up-regulate a protein called p11, which is capable of increasing the cell surface expression of 5-HT_{1B} receptors (Warner-Schmidt *et al.*, 2011). Since there is currently little evidence to support a role for this receptor in depression these results should be considered with caution.

The role of 5-HT_{1E} and 5-HT_{1F} are largely understudied owing to the lack of specific pharmacological agents to target them. 5-HT_{1D} receptors have also received little attention, but research regarding its role in the CNS demonstrates that it may regulate vasoconstriction (Hamel *et al.*, 1993), but little research has confirmed this theory.

1.2.2 5-HT_2 Receptors

The 5-HT_2 subclass of receptors appears to play a more complex role in the development of mood disorders and in the processing of emotion than 5-HT_1 receptors. The group consists of the 2A, 2B and 2C receptors. Until recently the 5-HT_{2B} receptor was thought to play a minor role in the regulation of mood, owing to its relatively low abundance compared to 2A and 2C (Bonhaus *et al.*, 1995). However, recent evidence has suggested that pharmacological or genetic inhibition of the 5-HT_{2B} receptor can have long term implications for antidepressant action (Diaz *et al.*, 2012).

5-HT_{2A} receptors are largely limited to the forebrain, with the majority being in the prefrontal areas of the brain. As excitatory receptors they facilitate neurotransmission. The contribution of this receptor subtype to the development of depression and regulation of mood is controversial, with papers showing both an increase (Bhagwagar *et al.*, 2006) and a decrease (Mintun *et al.*, 2004) in receptor expression in the brains of depressed patients. There are also a number of 5-HT_{2A} polymorphisms which affect mood. Work studying one of these polymorphisms, which is reported to change the binding potential of the 5-HT_{2A} receptor, showed that in the face of stress-induced depression (bipolar depressed patients were subjected to three days of total sleep deprivation), patients with one particular variant showed a significantly better response to antidepressant treatment (Benedetti *et al.*, 2008) than their non-SNP counterparts. Despite this, treatment efficacy – at least with standard SSRI therapy - has not been shown to be associated with altered 5-HT_{2A} genotype (Andre *et al.*, 2010). The difficulty in interpreting these studies comes from the different cohorts of depressed patients (bipolar *vs* unipolar depression), as well as the different ligands used in positron-emission tomography (PET) studies. Post-mortem studies are also confounded by the caveat discussed above suggesting that while many depressed patients are suicidal, not all suicide victims are depressed. The literature currently lacks a long term pre- and post-mortem study of mood, antidepressant response and 5-HT_{2A} genotype.

In light of the above findings, it might be assumed that 5-HT_{2A} receptor knockouts would have altered mood, specifically in terms of depressive-like behaviour. In fact this does not seem to be the case. Despite links to depression, 5-HT_{2A} knockouts appear to be similar to 5-HT_{1A} knockouts and show a high-anxiety phenotype (Weisstaub *et al.*, 2006). Interestingly, the link between 5-HT_{2A} receptors and depression may be indirect, in a similar way to that argued for 5-HT_{1A} receptors. Psychosocial stress in rodents has been shown to increase 5-HT_{2A} mediated behaviour as well as the expression of 5-HT_{2A} receptors (Gorzalka *et al.*, 1998; Amano *et al.*, 2007). Since it has previously been suggested that increased 5-HT_{2A} receptor activity or expression in the PFC could result in changes in the capacity to process external emotional cues, it is possible that stress may contribute to behavioural depression in this manner in humans. Zhang and colleagues (2001) are one of the few studies showing that peripheral inflammation (a forepaw carrageenan injection) in rodents is capable of up-regulating the 5-HT_{2A} receptor in the brain. If the links between inflammation and depression are to be recognized, these studies, combined with high 5-HT_{2A} receptor expression in the PFC, and what is known about that region in particular, highlight this as an unmet area of research. Systemic inflammation, via a number of different routes, clearly has the potential to affect 5-HT_{2A} receptor expression in the CNS and yet studies in models of sickness and depression have not thus far investigated this.

For a number of years the roles of the 5-HT_{2A} and 5-HT_{2C} receptors were hard to distinguish because commonly used agonists such as meta-cholorphenylpiperazine (mCPP) often targeted both receptors simultaneously. The 5-HT_{2C} receptor is largely post-synaptic and excitatory in nature and is highly expressed in a number of brain regions, though particularly in the limbic system, the hippocampus, hypothalamus, amygdala and other areas important in the regulation of mood (Mengod *et al.*, 1990). Agonism of this receptor results in a general depression in activity, specifically hypolocomotion and hypophagia (Koek *et al.*, 1992), activities not known to be directly regulated by 5-HT, suggesting this receptor may mediate effects by modulation of other neurotransmitter systems. 5-HT_{2C} knockout mice appear to be hyper-responsive to

chronic stress, showing more severe changes in behaviour and body weight to stressors than wild-type littermates (Chou-Green *et al.*, 2003). This altered stress response appears to be mediated by the paraventricular nucleus (PVN) of the hypothalamus, a significant relay station for the actions of stressors. Stimulation of PVN neurons results in the release of vasopressin, among other stress hormones, which cause physiological effects such as increased heart rate and blood pressure. 5-HT_{2C} receptors appear to be abundant in the PVN (Clemett *et al.*, 2000) and thus it could be hypothesized that receptor knockout mice lack the inhibition of these receptors on the output of stress hormones and therefore display an exaggerated stress response. It is well known that repeated exposure to stressors is capable of changing the expression of 5-HT receptors (Marti and Armario, 1998) and therefore this seems like a reasonable mechanism for the effects of stress on this particular receptor.

Interestingly, these studies are complemented by work on cytokines and the 5-HT_{2C} receptor. IFN therapy has been shown to alter RNA editing of this receptor resulting in a variant with reduced 5-HT binding capacity (Yang *et al.*, 2004). These findings could suggest that behavioural depression during sickness and cytokine therapy is the result of HPA axis hyperactivity caused by a lack of 5-HT_{2C} inhibition in the PVN. Together, these data advocate the theory that both stress and inflammation are capable of changing the expression and function of the 5-HT_{2C} receptor and that this can have detrimental effects on behaviour. Once again, this highlights a lack of direct comparison in the literature between two diseases or models which represent similar pathologies.

1.2.3 5-HT_3 and 5-HT_4 Receptors

A role for the 5-HT_3 receptor in mood and mood disorders has only recently been studied. This receptor is the only ion channel of the 7 subtypes of 5-HT receptor and therefore is associated with more instant responses, resulting in changes in membrane potential, rather than more complex second messenger pathways. Early studies have shown that 5-HT_3 receptors are found in a number of brain regions; areas of the brainstem associated with the vagal vomiting reflex as well as regions of the hippocampus. It is in the latter that

they have been found to modulate inhibitory neurotransmission within the limbic system (Tecott *et al.*, 1993). This role in inhibitory modulation has been confirmed by data showing that 5-HT_3 agonism results in attenuated responses to imipramine and fluoxetine in the forced swim test (Nakagawa *et al.*, 1998).

The distribution of the 5-HT_4 receptor appears to be largely mesolimbic (Mengod *et al.*, 1996), suggesting a role in emotional processing. 5-HT_4 receptors are GPCRs and coupled to the cAMP system. The second messenger cascade results in phosphorylation of a number of different receptors, enabling 5-HT_4 receptor activation to modulate neurotransmitter release. In fact, 5-HT_4 receptors have been shown to alter levels of striatal dopamine (Steward *et al.*, 1996) as well as acetylcholine (Consolo *et al.*, 1994). These changes in neurotransmitter release may result in indirect changes in behaviour since studies using direct application of 5-HT_4 antagonists have not been shown to result in a decrease in dopamine-mediated behaviours (Reavill *et al.*, 1998). Overall, however, the role of 5-HT_4 receptors in behaviour remains unclear. 5-HT_4 knockout animals are a relatively recent development so extensive behavioural testing has not been carried out. Compan and colleagues (2004) have shown that 5-HT_4 receptor null mice have a decreased open field activity at baseline levels and that they also have a reduced hypophagia in response to restraint stress. These changes appear to be uncoupled from HPA axis activation since these mice have a robust cortisol response. This lack of response to stress in 5-HT_4 knockout mice advocates a role for aberrant 5-HT_4 signalling in stress-mediated depression. In support of this hypothesis, two mouse models of depression, olfactory bulbectomy and glucocorticoid mutants, show increased 5-HT_4 receptor binding (Licht *et al.*, 2010). In Flinder's sensitive rats, treatment with antidepressant paroxetine results in decreased 5-HT_4 receptor binding (Licht *et al.*, 2009). Recent work in 5-HT transporter (SERT) overexpressors and knockouts has shown a link between SERT expression and 5-HT_4 binding (Jennings *et al.*, 2011). Since the target of a large number of commercial antidepressants is SERT it could be postulated that a decrease in 5-HT levels, as is extant in depression and SERT overexpressing mice,

results in increased 5-HT$_4$ binding and that this increase is reversed by antidepressant treatment.

It should be noted here that the 5-HT$_5$ receptor has been shown to exist in two isoforms, 5-HT$_{5A}$ and 5-HT$_{5B}$, of which only the A isoform has been shown to exist in humans (Nelson, 2004). There are currently no known selective ligands for this receptor subtype and almost nothing is known about its physiology or role in mood. Therefore, details of this receptor will not be included in this thesis.

1.2.4 5-HT$_6$ Receptors

This receptor subtype is one of the few that is almost exclusively located in the CNS. Specifically, 5-HT$_6$ neurons appear in the striatum and hippocampus in both rodents and humans (Ruat *et al.*, 1993). Knockout animals show few behavioural differences to their wild-type littermates, they habituate normally to a novel environment, but show a slight increase in anxiety-related behaviours in the elevated plus maze (Tecott *et al.*, 1998). Recent work using the 5-HT$_6$ agonist SB27104 has indicated potential for a role for this receptor in depression. Using the mouse tail suspension test, Svenningsson and colleagues (2007) demonstrated that agonism of this receptor combined with the antidepressant fluoxetine, resulted in a more potent antidepressant effect than fluoxetine alone. However, an antagonist at the same receptor, SB399885, caused decreased immobility in the forced swim test – also suggesting antidepressant activities. These results suggest that, like the 5-HT$_4$ receptor, 5-HT$_6$ might be sensitive to the levels of synaptic 5-HT, perhaps in a dose-dependent manner. Also, much like the 5-HT$_4$ receptor, very little evidence links the 5-HT$_6$ receptor with inflammation or stress.

Receptor	Phenotype/Test	Response to Stress	Reference
5-HT$_{1A}$	**Anxious**: ↓ open field activity, ↑ closed arm of plus maze, decreased immobility in forced swim	No obvious phenotype: *novel environment & intruder*	(Ramboz *et al.*, 1998; Groenink *et al.*, 2003)
5-HT$_{1B}$	**Aggressive:** n/c open field, n/c forced swim, ↑ aggression resident intruder	Increased autonomic response: *novel environment & intruder*	(Ramboz *et al.*, 1996; Groenink *et al.*, 2003)
5-HT$_{2A}$	NA	NA	
5-HT$_{2C}$	**Tranquil**: ↑ open field activity, ↑	Hyperphagia: *novel*	(Chou-Green *et al.*,

	novel object exploration, ↑ open arm of plus maze	*environment* & *restraint*	2003; Heisler *et al.*, 2007)
5-HT$_3$	**Slightly anxious**: ↓ open field (males only), n/c forced swim	Decreased ACTH: *restraint*	(Bhatnagar *et al.*, 2004; Bhatnagar *et al.*, 2004)
5-HT$_4$	**Tranquil**: ↓ open field activity	Decreased stress-induced hypo-phagia: *restraint*	(Compan *et al.*, 2004)
5-HT$_6$	**Slightly anxious**: n/c open field, ↑ closed arm of plus maze	NA	(Tecott *et al.*, 1998)
5-HT$_7$	**Tranquil**: n/c open field, decreased immobility in forced swim, decreased immobility in tail suspension	NA	(Sarkisyan *et al.*, 2010)

Table 1.1 Overview of 5-HT receptor knockouts and their phenotypes. ↑ indicates greater activity than wild-type littermates; ↓ indicates less activity than wild-type littermates; n/c indicates no change from wild-type littermates. NA indicates the animals have not yet been tested or developed.

1.3 Treatment of Mood Disorders

According to the WHO, depression is the 4th leading cause of disability worldwide and antidepressant therapies are often found in the top 10-20 globally prescribed drugs. They are used to treat standard Major Depression, as well as post-traumatic stress disorder, post-partum depression and generalized anxiety. Current treatment strategies are based on rational drug design of pharmacologics which specifically inhibit reuptake of catecholamines. The first prescribed drug of this class of antidepressants was the little known indalpine (Shopsin *et al.*, 1976), which was withdrawn from the market within 10-years and rapidly replaced by its rival, fluoxetine (Wong *et al.*, 1974). Fluoxetine, a selective serotonin reuptake inhibitor, and drugs with similar mechanisms of action, have been in circulation for almost 20 years with very few modifications despite relatively poor clinical efficacy. Changes in the way we view depression as a disease, and new insights into the mechanisms which may underlie it, could result in the development of novel antidepressants.

1.3.1 Current Treatment Strategies

There are a number of classes of antidepressant therapy. Drugs targeting serotonin, either alone or in combination with other targets, occupy more than 50% of the current market (Figure 1.4). Selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs), block catecholamine transporters and prevent reuptake into the presynaptic terminal. Serotonin antagonist and reuptake inhibitors (SARI) combine reuptake inhibitors with serotonin receptor antagonists, such as blockers of the 5-HT_{2A} receptor. The rest of the market is occupied by drugs targeting other catecholamines. Noradrenaline-dopamine reuptake inhibitors (NDRIs) act in the same manner as SSRIs but work at the noradrenaline and dopamine transporters. Tri- and tetra-cyclic antidepressants are an early class of antidepressant, still used in some cases, which work as broad inhibitors of catecholamine reuptake.

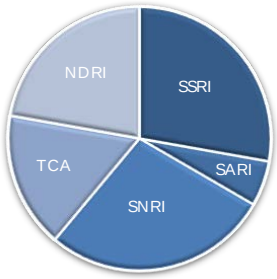


Figure 1.4 Representative diagram of the relative market position of the top 10 antidepressants prescribed in the US today. NDRI: noradrenaline-dopamine reuptake inhibitor, SARI: serotonin antagonist and reuptake inhibitor, SNRI: serotonin-noradrenaline reuptake inhibitor, SSRI: selective serotonin reuptake inhibitor and TCA: tri/tetracyclic antidepressant[1].

SSRIs are the most frequently prescribed antidepressant class because of their favourable side-effect profile and relatively low toxicity (Bartholow, 2010). Despite this description, studies have shown that more than 50% of patients on SSRIs report side effects that significantly impact on their lives (Hu *et al.*, 2004). These include changes in weight, sleep and appetite, as well as sexual dysfunction, and frequently result in discontinuation of treatment (Hu *et al.*, 2004). SSRIs such as sertraline, fluoxetine and citalopram suffer from a number of problems other than side effects; the main one being lack of efficacy. Extensive meta-analysis of the literature between 1987-2000 led Kirsch to conclude that the effect of antidepressants, especially fluoxetine, could not be distinguished from placebo in 6 out of 10 clinical trials (Kirsch *et al.*, 2002). This was followed more recently by a study suggesting that while antidepressant trials showed *statistical* significance, they did not have *clinical* significance, the measure by which national health organizations judge the efficacy of drugs (Kirsch *et al.*, 2008).

One of the problems with clinical significance may be the broad range of symptoms reported by patients with depression, and the interpretation of these symptoms by the clinicians who treat them. Many patients with unipolar depression often present with comorbid anxiety; however, this is not always the case and current antidepressant therapy does not distinguish between these two conditions. Investigation into the aetiology of depression and anxiety, and their differences at the molecular level, may go

[1] Data available from *Drug Topics* combined with data from Greenblatt, D. J., J. S. Harmatz and R. I. Shader (2011). "Psychotropic drug prescribing in the United States: extent, costs, and expenditures." *J Clin Psychopharmacol* **31**(1): 1-3.

some way to rectify the difficulties in treating unipolar depression. Studies have suggested that these patients may be more successfully treated with the dual-action SNRIs than the single-action SSRIs (De Nayer *et al.*, 2002; Sussman, 2003). Common SNRI venlafaxine has been shown to be more effective than SSRIs in a number of trials, demonstrating a greater ability to take patients through to remission (Stahl *et al.*, 2002). The main problem with dual action SNRIs is their targeting of two very broad neurotransmitters. This gives drugs such as venlafaxine a relatively poor side-effect profile, adding cardiovascular symptoms to those of nausea, headaches and dizziness already experienced by patients on SSRIs.

NDRIs are a more recent class of antidepressants and are most often used when treating residual, SSRI/SNRI-resistant forms of depression. In a similar manner to the SNRIs, NDRIs are thought to be responsible for ameliorating specific symptoms of depression, the most common of which is fatigue. One study demonstrated that up to 70% of patients taking fluoxetine continued to exhibit signs of fatigue after low-mood had abated (Fava, 2003). The most common additive therapy for this is the NDRI bupropion, which has been shown to be significantly more effective than SSRIs in resolving this particular aspect of depression (Papakostas *et al.*, 2006). The additive effect results in an increase in three different neurotransmitters – serotonin, dopamine and noradrenaline. The most common side effect of this type of therapy is seizures, with the highest dose of additive buproprione (600mg) causing the risk of seizures to be around 2%, four times higher than any other antidepressant therapy (Pisani *et al.*, 2002).

The broad range of action seen with additive therapy also blights the oldest class of antidepressants; TCAs. TCAs target a number of different receptors and transporters, producing a broad range of effects. For this reason they are infrequently prescribed and have been largely overtaken by the newer SSRIs, SNRIs and NDRIs.

Despite these problems, the use of antidepressants continues to rise, possibly due to the destigmatization of mental illness. However, the lack of efficacy means that both novel

treatment strategies, as well as new insights into disease mechanisms, are becoming more important.

1.3.2 Novel Treatment Strategies

Blier recently commented that the '*well of novel antidepressants*' was running dry (2010). He points out that a number of pharmaceutical companies have withdrawn from the antidepressant market recently, leaving the way open for the scientific community to introduce both novel mechanisms and novel therapies. Becoming increasingly popular are treatments that target localized brain regions using magnets, as well as broad therapies targeting peripheral systems now thought to contribute to mood, such as the immune system.

An emerging treatment strategy is vagal nerve stimulation (VNS). Originally used in treatment resistant epilepsy, the potential for VNS in depression mirrors that of the original antidepressants. It was noted that patients receiving VNS showed mood elevation, even with no depressive symptoms (Handforth *et al.*, 1998). Despite this promising start, trials have shown only around 10% of treatment resistant depressants responded to VNS (Rush *et al.*, 2005).

Other similar strategies include electroconvulsive therapy (ECT), transcranial magnetic stimulation (TMS) and deep brain stimulation (DBS). ECT has been used as a treatment for psychiatric disorders for almost 80 years but is only recently enjoying a resurgence for treatment-resistant depression. ECT involves application of electric currents to the scalp whilst anaesthetised, to induce seizure-like activity in the CNS. It has been shown to induce significant increases in 5-HT receptors within the brain (Nowak and Dulinski, 1991; Hayakawa *et al.*, 1994). Interestingly, HPA axis dysregulation, a common feature of depressed patients, and one possibly linked to an underlying immune system activation, has been shown to be corrected using ECT (Yuuki *et al.*, 2005). TMS uses magnetic fields to induce weak electric currents within the brain and is considerably less invasive than ECT. The first study into the use of TMS in depression showed considerable improvements in standard depression-scales after treatment (Kolbinger *et al.*, 1995).

Unlike ECT, however, TMS can be directed to specific brain regions, but considerable research still needs to be undertaken to find the optimal region for stimulation in depressed patients. Finally, DBS is considerably more invasive than both TMS and ECT, involving implantation of electrodes within the brain of the patient. Much like TMS, there is ongoing controversy regarding which brain area to stimulate. Stimulation of the substantia nigra, as occurs in Parkinson's patients, results in profound changes in mood in both directions, with episodes of mania as well as episodes of depression (Castelli *et al.*, 2006; Wang *et al.*, 2009). The use of stimulation paradigms such as the above are emerging therapies for treatment resistant depression and, possibly in combination with the standard pharmacotherapy, may also provide some novel insights into disease mechanisms.

Interestingly, anti-inflammatory drugs are becoming a popular focus in the study and treatment of depression. Studies in 2006 combined fluoxetine with salicylic acid and showed a shortened treatment efficacy onset time (Mendlewicz *et al.*, 2006), suggesting cyclo-oxygenase involvement. Indeed, in the same year cyclo-oxygenase inhibitor celecoxib was also used as a novel treatment strategy in depression. In combination with standard antidepressant reboxetine, Müller and colleagues (2006) have shown that celecoxib significantly improves the mood of patients experiencing acute episodes of depression. Inhibition of COX-2 to modulate inflammation, is based on work showing that the febrile response to intraventricular LPS is mediated via COX-2 in the brain endothelium (Cao *et al.*, 1999). COX-2 inhibition is also known to reduce inflammatory markers within the hippocampus (Casolini *et al.*, 2002) as well as enhance glucocorticoid receptor function (Hu *et al.*, 2005), both problems that occur in depression. Finally, COX-2 inhibition reduces the neurotoxicity of quinolinic acid, an NMDA agonist thought to be involved in excitotoxicity in depression (Kalonia *et al.*, 2010).

A number of studies have demonstrated a significant link between systemic cytokines and mood disorders (Maes *et al.*, 1997; Himmerich *et al.*, 2008). Recently, the evidence for a role for the pro-inflammatory cytokine TNF in depression has become more convincing,

with both positive anti-TNF drug trials (Hider *et al.*, 2009), as well as newly uncovered polymorphisms (Cerri *et al.*, 2010), significantly contributing to mood. A large number of studies into the exact role of TNF in mood have come about through the use of TNF and TNF receptor knockout animals. Knockouts for total TNF, TNF-R1 or TNF-R2 demonstrate overall decreased activity within an open field (Baune *et al.*, 2008). Animals with no functional TNF-R1 show decreased immobility in the forced swim test (Simen *et al.*, 2006; Kaster *et al.*, 2012), with similar results for the TNF-R2, suggesting an 'anti-depressant' phenotype. The use of anti-TNF therapy in patients with psoriasis has resulted in an increase in mood independent of physical improvements (Tyring *et al.*, 2006). These data, combined with the paucity of positive-affect after anti-IL-1 therapy (Genovese *et al.*, 2004), suggest that TNF is a potential target in inflammatory-related mood disorders such as those occurring during cancer chemotherapy. However, work thus far has failed to study the effects of these drugs in basic models of either sickness or depression in animals, leaving their efficacy in idiopathic depression or sickness behaviour untested. Despite this, these latter results indicate that anti-inflammatory drugs have therapeutic potential in affective disorders. Their advantage over more invasive techniques such as DBS and ECT is their global mechanism of action, which requires less knowledge regarding the functional anatomy of depression and may even provide insight into the role the immune system has in regulating mood.

1.4 Imaging in Depression

The use of novel drugs such as TNF inhibitors to treat depression suggests there is a convincing inflammatory component to the disease. However, work to date has failed to determine to what extent this inflammatory component affects the way we currently study depression in humans. It is possible that many of the imaging modalities used to study depression, fMRI in particular, may be significantly affected by systemic inflammation. Considering the extant behavioural link between sickness behaviour and depression, it is not unreasonable to assume that sickness may affect the way we study

data from imaging work. This is a considerable area of research which remains under investigated.

The anatomical locations of brain regions thought to be important in Major Depression have frequently been uncovered using functional imaging (Beauregard *et al.*, 1998). Recent advances in physics and modelling techniques has meant that the circuits controlling mood can be studied extensively in both healthy controls and patients with affective disorders. The variety of imaging becoming available is allowing for more and more complex biological questions to be posed. Imaging techniques currently available include PET, SPECT, MRS, fMRI and phMRI. Whilst PET and SPECT have been used to show changes in receptor binding (Meyer *et al.*, 2001) in depressed patients, their use in mood disorders is relatively limited and therefore they will not be discussed in this introduction.

1.4.1 fMRI and Mood

Functional imaging takes advantage of the ferromagnetism of deoxyhaemoglobin to image blood flow to specific areas of the brain, and subsequently use this as a correlate of neuronal activity, often whilst undertaking specific tasks (Logothetis, 2008). Often tasks involving emotional processing or response paradigms require patients to spend an hour or more in the scanner (Levesque *et al.*, 2011), and practically this is relatively untenable with radiotracers used in PET and SPECT work. Of specific interest to researchers in the field of Major Depression is the question of whether a brief episode of sadness activates the same brain regions as those activated in depression. Studies have started by showing that resting state connectivity, specifically between the left and right caudate nuclei, is fundamentally different between healthy controls and depressives (Kenny *et al.*, 2010), however, such studies fail to show whether the observed changes result in different capacity to process emotion. Beauregarde and colleagues (1998) have shown that transient sadness, such as that experienced when watching an emotional film clip, activates the same brain regions as the more malignant sadness experienced by depressed patients. However, this activity is considerably higher in the PFC, supporting an earlier

hypothesis that a dysfunctional PFC results in problems processing external emotional cues. This hypothesis is supported by meta-analysis showing that 75% of imaging studies looking at cerebral blood flow and metabolism (covering those using PET, SPECT and fMRI) in depressed patients show decreased function in the PFC compared to healthy controls, but no significant changes in any other regions (Ketter *et al.*, 1996).

Recent studies have applied a more comprehensive approach, combining methods such as fMRI, electroencephalic measures, pharmacological stimuli (phMRI), as well as behavioural information. This has allowed for correlation between perceived measures of sadness and brain activation (Raz *et al.*, 2012), and allowed researchers to use this compound approach as a way of predicting the future development and heritability of depression. For example, children of mothers with varying degrees of emotional depression, have been imaged at an early age and show a heightened response to sad stimuli early in life, possibly suggesting a predilection for the development of Major Depression later in life (Levesque *et al.*, 2011). While Levesque's study lacks a longitudinal aspect to show whether these changes result in depression, work correlating fMRI output to a direct measure of depression, has been more promising. Zhang and colleagues (2011) have used a personalized strategy, showing participants both neutral and 'attachment' faces – in this case their mother; changes in brain activity in the PFC seen when processing 'attachment' can be directly correlated to Beck Depression Inventory (BDI-II) and, the authors hypothesize, can be used as a predictor of depression.

Studying affective disorders in animals is challenging. The use of behavioural outcomes such sucrose preference and forced swim may result in an indication of depressive mood but are often used as stand-alone studies without the correlation of neuronal activity and brain mapping. fMRI in awake animals is becoming increasingly popular (Ferris *et al.*, 2011), mainly in larger preclinical models such as monkeys, a species particularly amenable to training. However, it is still only ethically viable to run short paradigms such as maternal affection (Febo *et al.*, 2005) and conditioned fear (Ferris *et al.*, 2011), both of which last 30 minutes or less and require a short baseline period followed by a short

exposure period. These fMRI experiments can be coupled with physiological methods such as non-invasive ECG and blood pressure monitoring. Awake fMRI can provide broad anatomical differences in function upon stimulation but cannot distinguish between neurotransmitter systems unless it is combined with a pharmacological challenge. phMRI is becoming an important tool in the study of specific neurotransmitter systems in rodents, enabling researchers to manipulate a number of different modalities to study a single system. This will be discussed more extensively later in this thesis (*see* 2.1.1.1 The Use of phMRI).

The application of these novel multimodal approaches, including phMRI and microdialysis, to the study of depression and sickness in animals allows a more comprehensive overview of the mechanisms involved. They also allow for confounds such as sickness and stress, and the subsequent development of sickness behaviours, to be studied in more detail.

1.5 Stress and Depression

Stress is now one of the most significantly reported causes of depression and is experienced by the majority of the population at least once in their lives. Longitudinal studies in both animals and humans have suggested that stress early in life can increase the risk of experiencing psychopathology, usually depression, later in development (Heim *et al.*, 1997; Newport *et al.*, 2002; Gutman and Nemeroff, 2003). In human twin studies, stressful early life events such as the loss of a parent, can predict the development of Major Depression with a high degree of accuracy (Kendler *et al.*, 1993). As with all translational research, human studies show considerably more variation than animal studies due to the subjectivity of what constitutes a 'stressful life event'. In animals, early adverse events can be very specifically introduced and controlled.

Some of the earliest models of early life stress employed very simple techniques. Levine (1957) noted that animals handled every day until post-natal day 20 were, contrary to popular opinion at the time, more resistant to adult stress than their non-handled peers. Studies since have shown that when tested as adults these animals do not show any

significant signs of depression (Ruedi-Bettschen *et al.*, 2006; Maniam and Morris, 2010). This may be due to the relatively short handling period (3-15 minutes) which appears to stimulate an increased maternal instinct upon the pups' return to the nest. In fact Maniam and colleagues compared two early life stress models; early handling and maternal separation. In the latter, pups are separated from the dams for longer periods – up to 3 hours at a time (Maniam and Morris, 2010). In these separation paradigms, the nurturing instinct of the dam appears to be suppressed and less care is given to the pup upon return to the nest. This approach provides more construct validity in terms of human depression. Prospective studies linking documented cases of childhood neglect with corresponding adult mental health status have found that there is a significant link between the two events (Horwitz *et al.*, 2001). These, and animal studies, demonstrate that early life stress can have a significant impact on adult neurochemistry, but also that adult events can be precipitant to the development of depression.

Adult stress is often studied retrospectively in humans. Work in Afghan refugees has shown that current life stress positively correlates with depressive symptoms (Mghir *et al.*, 1995; Thabet *et al.*, 2004). Kendler and colleagues (1999) note that there is a significant relationship between stressful life events and the onset of Major Depressive episodes. In humans, this is confounded by genotype. The study by Kendler employed a broad range of individuals including pairs of mono- and di-zygotic twins. They found that patients with a genetic predisposition for depression (e.g. long *vs* short 5-HT transporter promoter) were more prone to put themselves into 'high-risk situations' with stress a more likely outcome. These confounds make studying stress in humans inconsistent and challenging.

Studies in non-human primates often show construct validity, i.e. they mimic the human condition, by studying social stress (Shively *et al.*, 1997; Shively *et al.*, 1997). Social stress is the most common stressor experienced by humans (Almeida, 2005) and is frequently associated with the development of depression (Monroe *et al.*, 2009). Willard and Shively (2012) have shown that cynomolgus monkeys show a naturally occurring stress-induced

depression. The hierarchy of monkey social life results in social subordinates who have naturally high levels of cortisol and a retarded physiological stress response. This high-stress status results in an observable behavioural depression, characterized by social isolation and lack of 'interest' (Shively *et al.*, 2005; Willard and Shively, 2012), a phenomenon which has been seen to be associated with the human depressive syndrome.

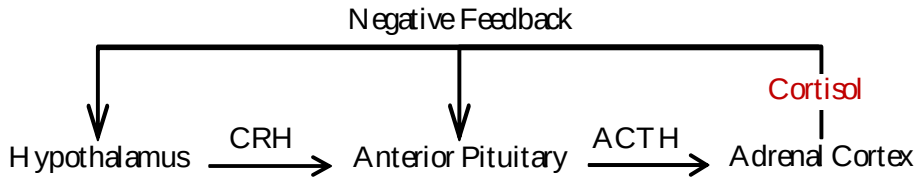
The anthropomorphism inherent in working with primate social groups is not present in induced rodent models of depression. Therefore, while modelling stress in rodents is physically easier it comes with significant social and ethical considerations, and data is much harder to interpret. Rodent models of stress are the most frequently used to investigate its effects on the development of affective disorders. The chronic stress model is one of the most frequently used to induce a depressive-like state and details will be discussed later in this thesis (*see* 4.1.1). Currently, a number of different stressors are employed during chronic stress to induce a type of 'depression' in rodents, including predator stress, restraint stress as well as forced swimming and tail suspension. The degree of depressive-like behaviour is mainly assessed by a decrease in the consumption of sweet solutions, a sign indicative of anhedonia, a specific aspect of depression. These models have been shown to induce a relatively rapid, if transient, change in mood and have been shown to have both face and predictive validity, at least in terms of anhedonia. Specifically, chronic stress causes rodents to develop many of the same symptoms as humans – changes in sleep and appetite, weight loss, anhedonia – and that the use of antidepressants has been shown to reverse these depressive-like symptoms (Strekalova *et al.*, 2006). While these data indicate that the chronic stress model may be a robust way of investigating affective disorders in rodents, there is considerable incongruity between the groups currently employing stress-based models of depression, resulting in a number of problems. The main issue with stress-induced depressive-like behaviour in rodents is its comparative inconsistency; approximately 50% of rodents fail to show a depressive phenotype at the end of the stress, but this aspect of the research is infrequently reported (Strekalova *et al.*, 2011). There may be key differences both socially and mechanistically, between the rodents which fail to show anhedonic signs, and those that do. These

differences could be crucial to our understanding of the susceptibility of humans to the development of depression but the changes which underlie them are currently unclear.

1.5.1 Hypothalamic-Pituitary-Adrenal Axis

Both rodent and non-human primate models of depression, as well as stressed human patients, have shown that stress significantly increases levels of stress-hormones such as cortisol. Cortisol is present only in humans, and is released from the zona fasciculata of the adrenal glands acting as the major stress hormone, whereas in rodents the major stress hormone is corticosterone. The effects of corticosterone in rodents, i.e. increased heart rate, blood pressure, etc. mimic those of cortisol in humans but the pathways regulating both hormones are different between species. In humans corticosterone is the precursor to aldosterone and regulates blood sodium and water content.

Levels of cortisol are regulated by the HPA axis and dysregulation of this system is a common feature among depressed patients (Yuuki *et al.*, 2005). The HPA axis controls the response of the body to stress and is present in all mammals. It is a feedback loop that exists between the hypothalamus and the adrenal medulla which regulates the release of cortisol.



Both emotional stimuli and physiological stimuli can affect the hypothalamus and its outputs. The purpose of this axis is to maintain homeostasis during episodes of stress, specifically to shift long-term metabolic processes towards ones critical for survival, e.g. gluconeogenesis. Stimulation of the hypothalamus, either via cortical efferents or via circulating hormones, results in the release of neuropeptides into the portal blood system. The neurosecretory terminals within the paraventricular nucleus of the hypothalamus

secrete corticotropin-releasing hormone (CRH) and vasopressin (Rang, 2007). These, in turn, act on the anterior lobe of the pituitary and cause the release of adrenocorticotropic hormone (ACTH) which is released into the circulatory system. ACTH acts on the adrenal medulla, stimulating glucocorticoid release.

Glucocorticoids bind to the glucocorticoid receptor (GR), an intracellular receptor which initiates transcription upon activation. Gene transcription takes the form of transactivation, i.e. active transcription of genes, or transrepression, where a cortisol bound GR can interfere with the activation of other transcription factors such as the pro-inflammatory NFkB (Ray and Prefontaine, 1994). Dhabhar has suggested that GR activation results in redistribution of immune cells (Dhabhar *et al.*, 2012). Initial periods of acute stress will result in mobilization of immune cells from reserve pools, such as the spleen, into the blood and from here they become redistributed to peripheral organs such as the skin (Dhabhar, 2003). In terms of stress and immunity this is a considerably overlooked area of research: immediate or acute stressors are likely to cause an increase in circulating cytokines and immune cells, where longer term stressors are likely to cause a decrease. It is also an important consideration when studying animal models of stress and immune function – that animals are frequently moved between rooms prior to testing. This translocation may induce short term changes in stress hormones and immune function, and this may confound any sensitive measures of immunity.

Depressed patients frequently show high levels of cortisol, indicative of HPA axis hyperactivation (Maes *et al.*, 1995; Alesci *et al.*, 2005). Pariante and Lightman (2008) have likened this phenomenon as akin to the chicken and egg quandary; it is currently unknown as to whether early life stress causes permanent changes in the HPA axis which make it hypersensitive, or whether fundamental genetic changes in the HPA axis make individuals hypersensitive to stressful events. Studies in animals as well as humans have shown that early life trauma, for example prolonged maternal separation, will result in persistent changes in HPA axis activity and a heightened cortisol response later in life (Heim *et al.*, 1997; Sanchez *et al.*, 2001). On the other side of the chicken/egg debate, GR

polymorphisms have been shown not only to be associated with the development of depression, but also with a more rapid response to treatment (van Rossum *et al.*, 2006). Combining the two theories, Oberlander and colleagues (2008) have shown that maternal mood can affect foetal HPA axis activation. Specifically, unmedicated depressed mothers were more likely to differentially methylate part of the GR gene of their offspring than those who were medicated or healthy controls. This altered methylation state correlated with changes in HPA axis activation, more precisely increased cortisol production in novel environments, at a young age (3 months) showing that HPA axis activation is a homeostatic mechanism.

The role of cortisol in regulating mood has been confirmed by studies using synthetic corticosteroids. Early studies using cortisone to treat rheumatoid arthritis noted that even low doses resulted in *some psychic changes* (Boland and Headley, 1950). From these studies to the present day, cortisol and synthetic compounds have been known to induce an *inappropriate sense of wellbeing* (Swinburn *et al.*, 1988), but these effects are seldom studied and given the umbrella terms 'steroid euphoria' or 'steroid psychosis' with no thought given to the mechanisms. While the mechanisms may be poorly understood, steroid euphoria reinforces the theory of HPA axis dysregulation as a homeostatic mechanism, rather than a cause, in depressed patients. It is possible that the initiation of the depressive-like state in the CNS may increase the activity of the HPA axis to release cortisol and improve mood. However, this theory is complex and yet to be tested in either rodents or humans. Considering the links hitherto suggested between the immune system and depression in humans, there is a consistent paucity of data regarding the role of stress and subsequent changes in the immune system on mood. Current models of depression are mainly used to test novel anti-depressant therapies and have yet to be used to study immune system function. Combinations of immune system stimulation and stress could result in novel insights into the mechanisms underlying stress-induced changes in mood.

1.6 Origins of the Cytokine Hypothesis of Depression

Steroid eurphoria is merely one of the reasons for the resurgence in interest in the immune system and its role in mood. Recent studies have shown improvements in mood in patients receiving anti-TNF therapy for psoriasis, and other work using novel anti-inflammatory agents, represent a growing body of evidence supporting the theory of depression having an inflammatory component. Depressed patients exhibit higher levels of circulating cytokines (Maes *et al.*, 1995; Maes *et al.*, 1997; Alesci *et al.*, 2005), and patients with high levels of circulating cytokines are prone to develop depression-like symptoms (Horikawa *et al.*, 2003). It is also important to note that the original hypothesis for the cause of depression states that it is the result of decreased monoamines, specifically of 5-HT, and new evidence has begun connecting the 5-HT system with inflammation. Whether the changes in the central 5-HT system are the result of systemic inflammation, or that the systemic inflammation is the result of changes in the CNS, remains unclear.

1.6.1 An Introduction to the Host Response to Pathogens

The response of the body to pathogenic insult begins with the invasion of the pathogen. This occurs when foreign organisms, such as bacteria or viruses, breach the physical barriers presented by an epithelium. Pathogens express specific molecular patterns on their cell surface known as PAMPs (pathogen-associated molecular patterns) which are recognised by PAMP recognition receptors on circulating sentinel cells such as macrophages and dendritic cells. This activation results in the up-regulation of both cytokines and chemokines (communicatory molecules produced by immune cells) and the activation of downstream pathways. These pathways can result in vascular changes, an influx of activated immune cells, and the production of inflammatory mediators.

Experimentally, the innate immune system can be activated to study immunity and sickness behaviour in a number of ways, including bypassing sentinel cells by direct injection of cytokines. However, the most relevant and frequently used model of an inflammatory challenge is systemic injection of bacterial endotoxin - LPS. LPS is

recognized in the circulation by free LPS-binding protein (LPB), an acute phase protein produced by the liver and released into the circulation. LPS/LPB complexes are then recognized by toll-like receptors 2 and 4 (TLR-2 and -4) on the cell surface of macrophages. This binding requires co-activation by either cell surface, or circulating, CD14 – an effector molecule required for bringing LPS/LBP/TLR-4 into contact with the intracellular protein MD-2 (Palsson-McDermott and O'Neill, 2004). This final complex brings together a number of intracellular adaptor proteins capable of uncoupling cytoplasmic transcription factors such as NFkB from their inhibitors and causing their translocation to the nucleus for transcription of inflammatory genes (Palsson-McDermott and O'Neill, 2004). This communication system is largely peripheral and, without severe damage to CNS tissue, there is only inconsequential movement of immune cells into the brain. Therefore, any behavioural changes elicited in response to a peripheral immune challenge are as a result of communication pathways between the periphery and the brain, which will be discussed below.

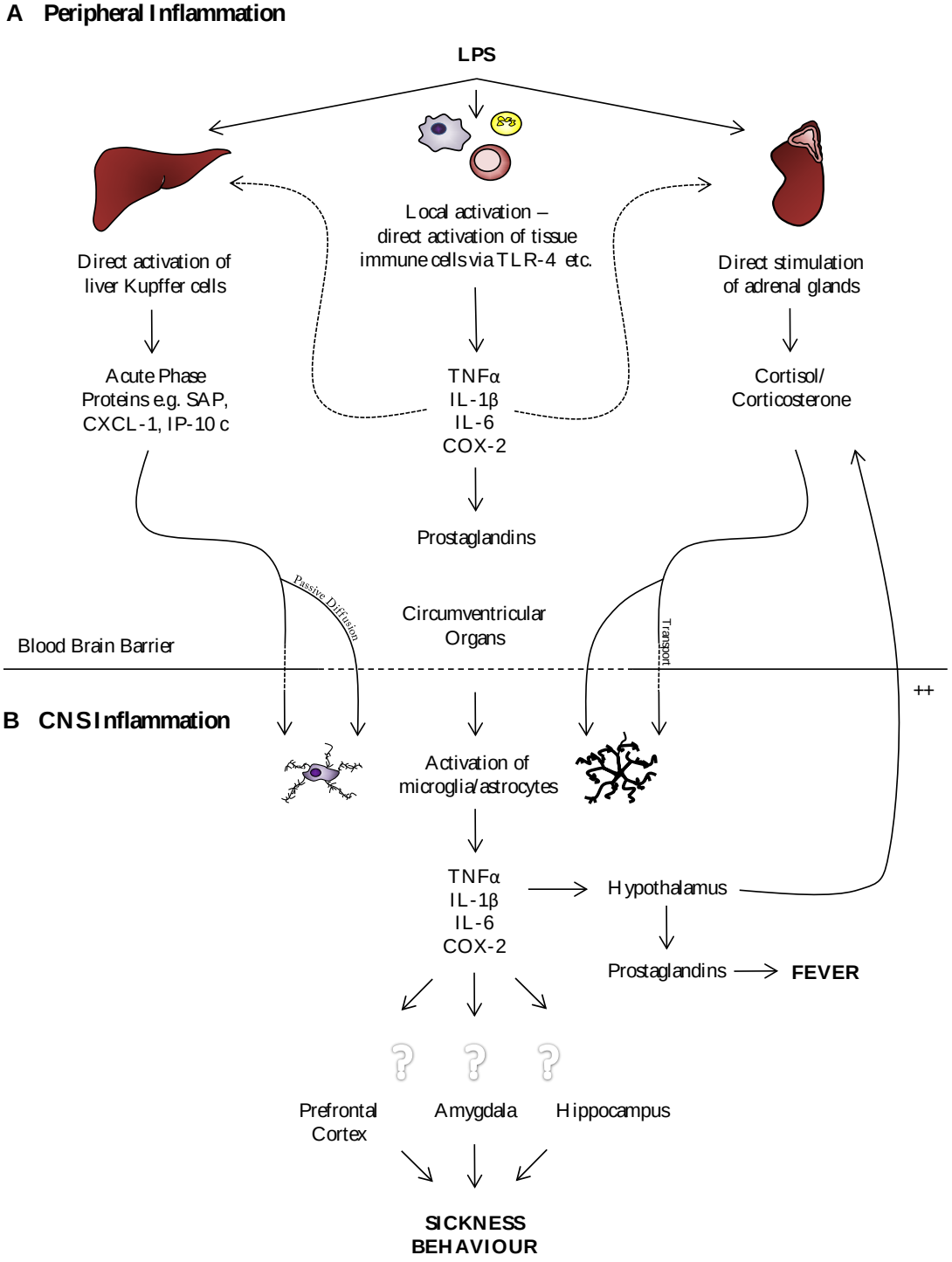


Figure 1.5 Schematic representing the possible pathways from immune system activation to sickness behaviour. (A) Invasion of pathogens such as bacteria activate circulating immune cells. Production of cytokines by these cells results in a number of different pathways being initiated: (i) activation of liver Kupffer cells and production of acute phase proteins; (ii) stimulation of cells in the adrenal glands and production of cortisol and (iii) downstream production of prostaglandins. (B) These messages are communicated via the circumventricular organs and via the CNS endothelium. Within the CNS microglia and astrocytes propagate the inflammatory signal resulting in local production of prostaglandins and fever. Further, cytokines have as yet unknown effects on specific brain regions, the net effect of which is sickness behaviour.

1.6.2 A Brief History of Sickness Behaviour

Sickness behaviour is defined as a set of behavioural adaptations instigated during episodes of inflammation. The first studies into sickness behaviour were made unwittingly by Neal Miller in the mid-1960s. Studying motivational states in rats, Miller postulated that bacterial endotoxin (LPS) would change behaviour, but predicted it would be unidirectional. In fact the animals' response to endotoxin was based on the motivation under scrutiny. For example, the rats pressed levers less frequently for rewards such as sweet drinking liquid or food, even when starved, but more frequently to generate rest periods when forced to walk on a motorized wheel. This led Miller to conclude that endotoxin was inducing a motivational state change which rearranged the hierarchy of existing behaviours in order to cope with a 'survival' situation (Miller, 1964). Interestingly, he found that central injection of endotoxin, into the 'feeding and drinking' area of the lateral hypothalamus did not induce any of these changes. He concluded that an intraperitoneal injection of endotoxin was producing 'factor X' in around 30-40 minutes which was capable of rearranging motivational states (Holmes and Miller, 1963).

The use of the terms such as 'motivation' and 'operant response' put the study of sickness behaviour firmly into the realm of psychology. It was not until the 1980s that the disciplines of psychology and immunology were combined by the research community. Prior to 1979 a number of different molecules, variously termed *lymphocyte activating factor, endogenous pyrogen* and *leukocyte endogenous mediator* were suspected of being at the root of immune communication between cells. A consensus was reached at the Second International Lymphokine Workshop (1979) that these molecules were one and the same and they were grouped and re-named *interleukin (IL)-1*. Experiments with this newly isolated recombinant cytokine in 1986 by Besedovsky showed that it was capable of eliciting the release of ACTH from the hypothalamus, indicating that IL-1 was capable of communicating with the CNS (Besedovsky *et al.*, 1986). However, echoing the original work of Miller, McCarthy and colleagues demonstrated that central administration of IL-1 to the satiety centres of the hypothalamus did not induce anorexia

in the same manner as a peripheral administration (McCarthy *et al.*, 1986). Once again, suggesting that the behavioural effects of inflammation are transduced in the periphery.

These papers led Hart to review the available literature and bring together the fields of psychology and immunology in an overview of the 'Biological Basis of the Behaviour of Sick Animals' (Hart, 1988). Despite this, the term 'sickness behaviour' was not officially used in print until the early 90's when Dantzer and colleagues were studying the behavioural effects of subpryogenic doses of IL-1 on exploration and feeding, and described the reduced capacity of animals to undertake these actions as *sickness behaviour*.

1.6.3 Sickness Behaviour and Depression

Loss of appetite, lethargy, sleep disturbances and anhedonia are all symptoms experienced by chronically ill patients. They also appear on the DSM-IV classification of Major Depression (APA, 2000). Despite these similarities, it was only around 15 years ago that mechanistic links began to be drawn between Major Depression and sickness (Maes, 1995). Maes' group was particularly interested in immune cell populations and their behaviour in depressed patients. Work with peripheral blood mononuclear cells (lymphocytes, monocytes and macrophages) from patients with depression showed that they produced significantly more IL-1β and IL-6 when challenged than the cells from control patients (Maes *et al.*, 1991). The authors even went as far as classifying depression, Major Depression and melancholia based on the behaviour of their lymphocyte populations and the cytokines that they produced (Maes, 1995). However, these data were largely based on *in vitro* assessments and are viewed with scepticism by others in the field (Dantzer and Kelley, 2007).

Maes' work approaches the subject of depression and immunity in a relatively unidirectional manner. They hypothesize that depression can cause changes in immunity and immune function but do not speculate on the area of immune activation and how it might contribute to the development of depression. Since patients on IFNα therapy frequently develop depression (Horikawa *et al.*, 2003), it is not unreasonable to assume

that this communication can be bidirectional, i.e. that while the state of the CNS can affect the immune system, the state of the immune system can also affect the CNS.

With this in mind, the first work suggesting the 'macrophage theory' of depression was written by Smith in the early 90's (Smith, 1991). A crucial point raised by Smith in this original article was that the Diagnostic and Statistical Manual for Mental Disorders (DSM) at the time stated that a diagnosis of Major Depression could not be made if '*it is established that an organic factor initiated and maintained the disturbance*'. This point fundamentally separated the brain from the body by requiring that Major Depression, and all its symptoms, could only occur if they were initiated from within the brain. This argument seems particularly illogical since a number of papers extant at the same time as the DSM-III-R showed that circadian cortisol levels were disrupted in depression and that antidepressants re-established their natural rhythms (Linkowski *et al.*, 1987; Jarrett *et al.*, 1990). Since both circadian cortisol levels and the taking of antidepressants both represent processes fundamentally associated periphery, the conclusions drawn by the DSM-III seem somewhat paradoxical. In an original and seminal study, McDonald and colleagues (1987) demonstrated that 63% of patients administered IFN-α as part of a treatment regime for lung cancer went on to become psychiatric inpatients. Despite these early observations, the idea of the immune system causing changes in mood was seen only as a side effect of cytokine therapy (Renault *et al.*, 1987), not a possible fundamental cause or as a new approach to study psychiatric disease. It was not until nearly 10 years after Smith's initial macrophage theory that Miller's group suggested that cytokines may play an important role in mediating mood (Capuron *et al.*, 2002).

Until this time, the link between depression and inflammation was only studied in humans. Now the immune system was considered capable of subjugating the brain, research began to use animal models to study behaviour during sickness. Dantzer proposes that sickness behaviour and behavioural depression represent distinct phenomena (Dantzer *et al.*, 2008). However, if it is assumed that the original works by Hart (1988) suggesting that sickness is simply an altered motivational state are correct,

then it is not unreasonable to assume that similar mechanisms may underlie both short term behaviours for sickness and long term behaviours in depression, if the behavioural outcomes are broadly similar. To date, models of depression and models of sickness have rarely overlapped and work comparing them is scarce. The assertions made by Dantzer regarding the fundamental differences between sickness and depression may be answered with a direct comparison of the two diseases.

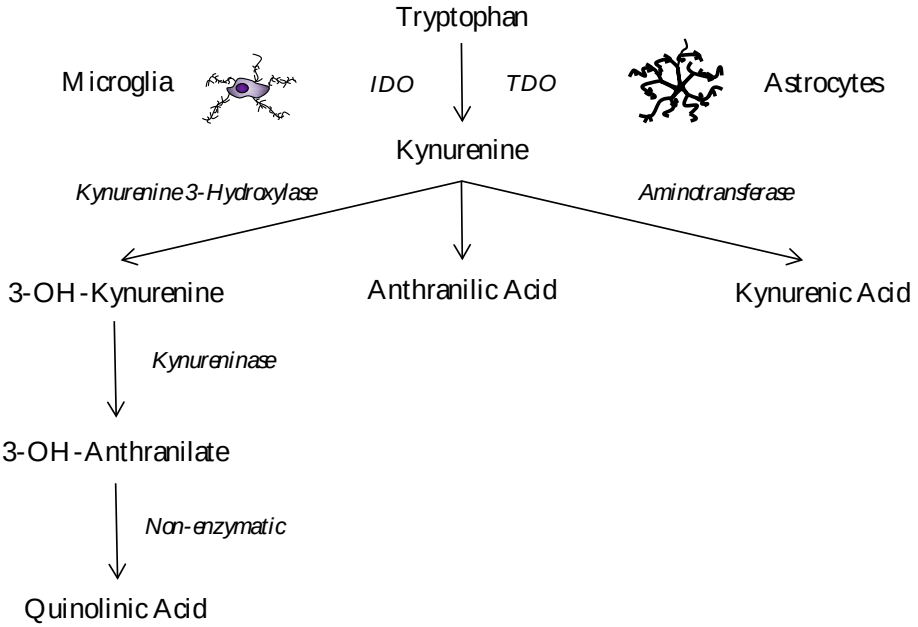


Figure 1.6 Simplified schematic of tryptophan metabolism. Tryptophan is an essential amino acid and the principal component required for synthesizing 5-HT. Breakdown of tryptophan can occur in a number of different cell types, including neurons, endothelial cells, astrocytes and microglia. The enzymes indoleamine 2,3-dioxygenase and tryptophan dioxygenase break down tryptophan into kynurenine. Kynurenine is further broken down into 3-hydroxykynurenine, anthranilic acid or kynurenic acid. Neither neurons or astrocytes express appreciable amounts of kynurenine 3-hydroxylase, therefore the production of 3-hydroxykynurenine, and later of quinolinic acid in the CNS, is principally in microglia.

1.6.4 Cytokines and the 5-HT System

Patients on immunomodulatory therapy who have been shown to develop symptoms of depression, also have low levels of the 5-HT precursor tryptophan (Capuron *et al.*, 2003), leading to the conclusion that the two systems may be linked. Mechanistically, there are a number of theories regarding the role of cytokines in the modulation of the 5-HT system. The principle theory involves indoleamine 2,3-dioxygenase (IDO), a catabolic enzyme which breaks down tryptophan into kynurenine (Figure 1.6). Cytokines such as TNF have been shown to increase the activity of this enzyme (Fujigaki *et al.*, 2006). Given that

tryptophan is the precursor for 5-HT, and if it is assumed that the 5-HT hypothesis of depression is correct, it could be argued that production of pro-inflammatory cytokines should result in decreased 5-HT. However, studies in animals have shown that while a systemic inflammatory challenge increases levels of kynurenine, there is no significant decrease in brain 5-HT (O'Connor *et al.*, 2009; O'Connor *et al.*, 2009)). Research thus far has aimed at studying the potential inflammatory mediators responsible for this increase in kynurenine, very little work has focused on other potential mechanisms for the subjugation of the 5-HT system by inflammation.

Within the CNS, production of pro-inflammatory cytokines, such as TNF, is largely by the resident macrophages – the microglia. Microglia can be activated by the same signals as tissue macrophages in the periphery, e.g. via TLR-4 and others in the case of bacteria or LPS. They also express significant amounts of IDO. Studies in cultured microglia reveal that stimulation with LPS results in JNK-dependant activation of IDO (Wang *et al.*, 2010). While astrocytes and neurons also express IDO, only microglia have been shown to be capable of producing significant levels of the end product; quinolinic acid (Guillemin *et al.*, 2005). Quinolinic acid is a relatively potent NMDA receptor agonist (Misztal *et al.*, 1996), and it may have a role in excitatory neurotransmission as well as excitotoxicity. Recent work by Steiner and colleagues has demonstrated that microglial quinolinic acid is increased in patients with both bipolar and unipolar depression, and is also increased in a region-specific manner (Steiner *et al.*, 2011). Depressed patients showed high levels of microglial quinolinic acid in brain areas such as the anterior cingulate. This region is associated with both high NMDA expression and a role in emotional processing. In fact quinolinic acid lesions of the cingulate cortex have been shown to decrease reward based learning (Bussey *et al.*, 1997).

The metabolism of kynurenine to quinolinic acid only occurs in microglia, whereas the conversion of kynurenine to kynurenic acid occurs in both astrocytes and microglia. Astrocytes also metabolise tryptophan using an IDO analogue, TDO (tryptophan 2,3-dioxygenase). While IDO is regulated by pro-inflammatory cytokines, TDO is regulated by

cortisol (Rubin, 1967). The end-product, kynurenic acid is an endogenous antagonist at the NMDA receptor. Therefore, kynurenine metabolism is self-regulating, producing substances capable of agonising and antagonising excitotoxic pathways. However, in excess, quinolinic acid is capable of causing apoptosis in astrocytes (Guillemin *et al.*, 2005). This data suggests that there may be an imbalance in depression, overproduction of quinolinic acid by microglia results in astrocyte cell death, thus there is less kynurenic acid to antagonise any excitotoxic effects that the quinolinic acid might have on neurons. Again, studies directly comparing models of stress-induced depression, where cortisol should be high independent of cytokines, and inflammation-induced depression, may allow some of these mechanistic questions to be answered.

1.7 How the Immune System Communicates

The majority of research on the brain-immune axis to date has focused on the pathway of communication between the brain and liver. Studies in ischemic animals have shown that brain injury results in a peripheral up-regulation of inflammatory molecules in the liver before there is appreciable central inflammation (Chapman *et al.*, 2009). This efferent communication pathway has been attributed to a number of different mechanisms including autonomic nervous system activation and the circulation of microparticles (for review see Anthony *et al.* (2011)). However, the afferent pathway, and specifically communication between the peripheral and CNS acute phase responses, is less well understood and is vital to the understanding of how peripheral immune challenges cause sickness behaviour.

Systemic immune challenges, such as those produced by intraperitoneal injection of LPS, must be communicated around the body in order for the immune system to mount an effective defence. Cytokines are released from the origin of infection, in the case of animal models of inflammation – the peritoneum, and diffuse through vascular fenestrations into the systemic circulation. Once in the circulation, cytokines such as TNF and IL-1 induce the acute phase response (APR).

The primary site of APR initiation is the liver. The liver has the largest population of resident macrophages, Kupffer cells, in the body. Activation of Kupffer cells by circulating cytokines results in the production of acute phase proteins (APPs) such as C-reactive protein (CRP), serum amyloid-A (SAA) and many of the complement proteins. Together these proteins help destroy pathogens by a number of mechanisms, including opsonisation and direct cell lysis. These processes prevent persistent production of cytokines and chemokines by activated macrophages and enable the body to 'switch off' the APR after the pathogen has been eliminated.

It has already been established that systemic administration of cytokines induces changes in behaviour (Kent *et al.*, 1992). The intact blood-brain barrier lacks the fenestrations of the peripheral vasculature. Cytokines are relative small proteins, approximately 15kD, and therefore they cannot cross the blood-brain barrier by passive diffusion (Banks *et al.*, 1989), suggesting that communication between the brain and the immune system must occur by other mechanisms. The blood-brain barrier exhibits physiology distinct from capillary networks elsewhere in the body. Endothelial cells in the CNS vasculature are connected by tight junctions and surrounded by pericytes, both of which prevent the existence of the standard *fenestrae* which occur between endothelial cells elsewhere in the body and which allow diffusion of larger molecules such as proteins in and out of blood vessels. At the circumventricular organs (CVO), the CNS is in close contact with the circulation via more highly-fenestrated endothelial cells than exist in the standard blood-brain barrier. This close proximity allows diffuse cytokines to come into contact with microglia. In a similar manner to Kupffer cells, microglia are capable of producing an APR and producing not only APPs but also cytokines (Van Dam *et al.*, 1995; Eriksson *et al.*, 2000). This leads to a central inflammatory cascade similar to that seen in peripheral tissues. The main CVO of interest in sickness behaviour is the organum vasculosum of lamina terminalis (OVLT). This is in close communication with the hypothalamus and is at least partially responsible for the mediation of the febrile response produced by a number of pro-inflammatory cytokines (Murakami *et al.*, 1990; Nakamori *et al.*, 1994).

Inhibition of the CNS APR is a mechanism of abrogating sickness behaviour and is enjoying a resurgence as a potential novel approach to antidepressant therapy. Non-selective COX inhibition has been shown to attenuate sickness behaviours (Teeling *et al.*, 2010), suggesting it is pivotal in the mediation of the behavioural response to inflammation, and celecoxib has already been mentioned as a potential novel antidepressant (Muller *et al.*, 2006). A single systemic injection of LPS is known to induce PGE_2 biosynthase, a downstream enzyme from COX-2, in a number of different cell types including CNS microglia (Murakami *et al.*, 2000), showing that systemic inflammation can cause central prostaglandin up-regulation. Inhibition of COX is associated with a number of unwanted side effects, mainly through actions in the stomach. Recent pharmacological developments have therefore aimed to specifically inhibit COX-2 or to inhibit enzymes such as PGE_2 synthase. PGE_2 synthase knockout mice do not exhibit fever in response to a systemic inflammatory challenge (Saha *et al.*, 2005). The capacity of COX inhibitors such as celecoxib to inhibit the behavioural response to both chronic stress (Guo *et al.*, 2009), and to a surgical model of depression in rodents (Myint *et al.*, 2007) suggests it plays an important role in mood regulation. The work on COX-2 inhibition is made possible through selective and non-selective inhibitors and allows for the dissection of the role of this prostanoid in sickness behaviour and depression. Novel anti-inflammatory therapies such as those targeting TNF may provide more insight into how other aspects of the inflammatory cascade could contribute to changes in mood.

1.7.1 Neural Communication

As well as direct cytokine communication, there are neural mechanisms which could mediate some of the behavioural aspects of systemic inflammation. Cytokine production in the liver is capable of activating vagal afferents (Blatteis and Sehic, 1997). Subdiaphragmatic vagotomy is the standard method for investigating the role of the vagus in the response to peripheral inflammation. However, results from these studies are contradictory. Sehic and colleagues (1996) showed that the febrile response to a

peripheral inflammatory challenge is absent in animals with bilateral vagotomy whereas Dantzer's group stated that vagotomy only reduces the behavioural effects of peripheral inflammation (Konsman *et al.*, 2000). Contrary to both sets of data, *stimulation* of the vagus in depressed patients, and epileptics, was found to significantly improve mood (Rush *et al.*, 2000). Peripheral blockade of nicotinic receptors will blunt the effects of neuroprotective compounds suggesting that there is a specificity in the efferent pathways after CNS injury (Ottani *et al.*, 2009). However, in experiments we have performed with muscarinic inhibitors scopolamine and met-scopolamine to investigate the role of muscarinic receptors in the hepatic response to brain injury, muscarinic inhibition did little to modify the peripheral response after brain injury. By comparing the systemic immune responses in animal models of both depression and sickness it may be possible to rectify some of these discrepancies.

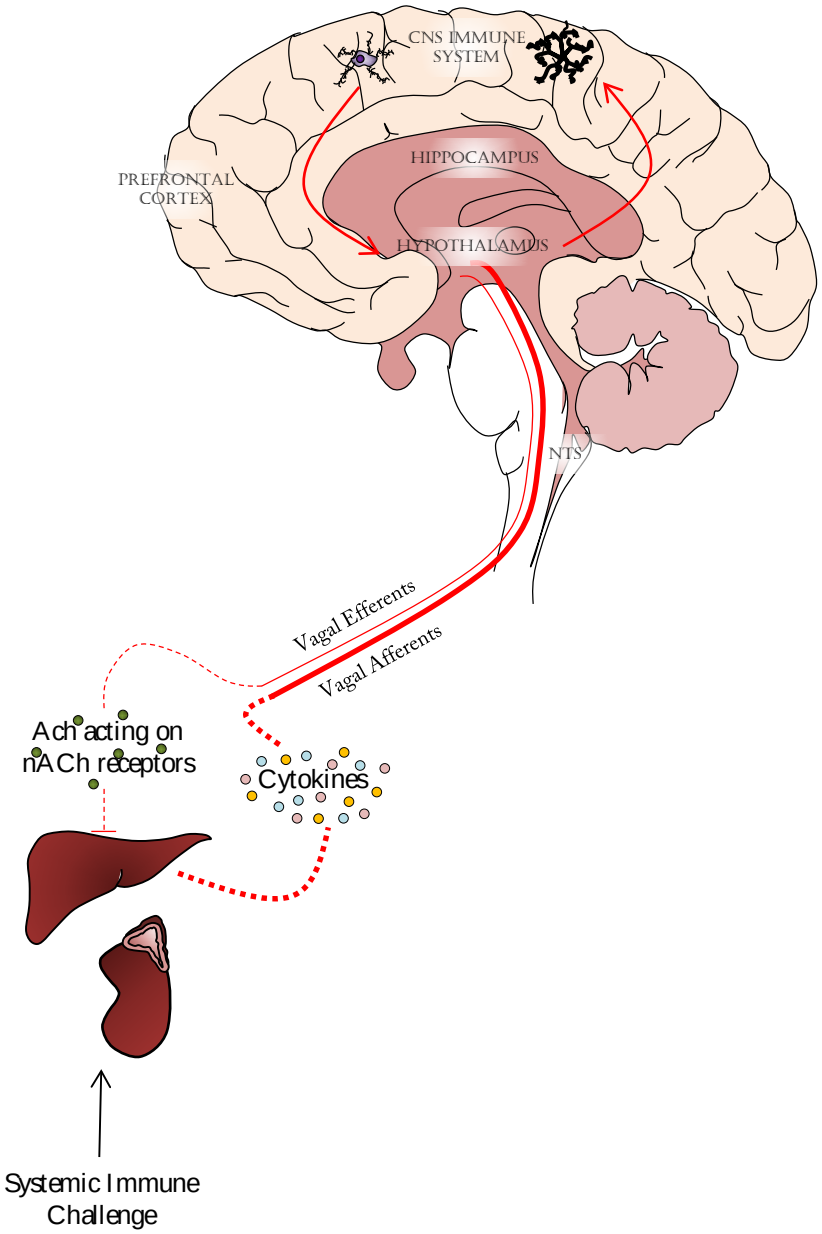


Figure 1.7 Schematic overview of the role of the vagus nerve in neural communication between the immune system and the brain. Systemic cytokines released by the liver stimulate vagal afferents allowing the CNS to be aware of the status of the immune system. Vagal efferents release ACh into the systemic circulation which is also capable of regulating the function of the immune system.

1.8 The Advantages of Studying Affect and the 5-HT System in Animals

Mechanistically, approaching depression in human patients is challenging. The spectrum of disease presentation, subjectivity in terms of diagnosis and drug response means that very large cohorts must be studied before any conformity occurs. The use of inbred rodent strains enables not only the use of considerably smaller cohorts, especially for large effect sizes, but also the use of a number of different strains and species to check for consistency and validity. The behavioural correlates between sickness and depression are well characterized; anhedonia, sleep disturbances, weight loss and appetite changes are all associated with Major Depression, long term inflammatory disease and acute sickness. As discussed above, current theories suggest that sickness behaviour and behavioural depression are different aspects of the same disease process (Dantzer *et al.*, 2008). By studying sickness at different times post-challenge, and using a number of different approaches, it may be possible to either differentiate between these processes or to demonstrate their similarity.

The advantages of using rodent models of sickness and depression to answer these questions are numerous. Firstly, rodent models of both sickness and affective disorders offer the capacity to use a multi-modal approach to obtain a more comprehensive view of the functional outcomes and underlying mechanisms. While behavioural studies may reflect specific changes in a correlate of mood, inconsistencies between laboratory settings and behavioural practices mean that confirmation using other techniques is advisable. Invasive techniques such as *in vivo* microdialysis allow for the measurement of CNS levels of neurotransmitters that can be correlated with final tissue measurements of neurotransmitter levels, or with fMRI imaging data. Specific brain regions can be removed to determine microenvironmental changes in transmitter levels or receptor expression. These techniques provide a considerably broader spectrum of information regarding many of the unexplored mechanisms which may underpin sickness behaviour and depression.

While there is compelling evidence linking the immune system to the development of affective disorders the exact mechanisms by which this occurs are not currently clear. Novel anti-inflammatory agents, specifically those targeting TNF, are already being shown to have significant mood altering properties (Tyring *et al.*, 2006) - which clearly demonstrate their potential as anti-depressant drugs. By understanding the mechanisms by which these drugs produce their effects it may be possible to elucidate the role that specific cytokines have in mediating depressive-like behaviour. These hitherto unexplored aspects of the field provide a potential mine of information regarding both disease mechanisms and new therapeutic targets and, therefore, represent a unique perspective on a timeless problem – why you feel miserable when you get sick.

1.9 Aims

The principal aims of this thesis are as follows:

- To determine the effect of systemic inflammation on the integrity of the central 5-HT system.
- To compare the molecular profile, in relation to the 5-HT system and cytokine expression, in validated models of sickness behaviour and models of depression.
- To discover the temporal and spatial extent of the changes in the 5-HT system and cytokine expression in relation to altered behaviour in the models of sickness behaviour and depression. More specifically to study whether there are changes in particular brain regions – looking at the prefrontal cortex, motor cortex, hippocampus and dorsal raphe.
- To discover whether anti-TNF therapy ameliorates sickness behaviour in mice.

Chapter 2: Changes in the Rat 5-HT System after Systemic Inflammation

Current studies of the 5-HT system in humans are limited to non-invasive positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) techniques (Rhodes *et al.*, 2007). While these studies allow us to investigate the potential role of 5-HT in regulating mood, the use of these techniques in humans as a direct correlate of neuronal activity is confounded by the indirect nature of the signal. In human studies, it is also not possible to follow up these non-invasive techniques with invasive molecular biology that would allow further insight into the neurobiology of mood and the 5-HT system. Animal studies of the 5-HT system are permitted to be more invasive and thus most of our knowledge about this system stems from this type of work. Moreover, studies using imaging modalities similar to those employed in humans can be followed up either with subsequent, or with concomitant, molecular techniques such as microdialysis (Schwarz *et al.*, 2004). This combination of techniques would allow unanswered questions, such as the effect of pathology on the fMRI signal, to be more extensively examined.

2.1.1.1 The Use of phMRI

Pharmacological MRI (phMRI) is a novel technique which uses the administration of pharmacological agents to reduce or enhance specific neurotransmitters, whilst concomitantly running fMRI in order to determine how these drugs change the fMRI signal. Whilst this field is relatively new, the term phMRI was only coined in 2008 (Rauch *et al.*), has largely focused on dopamine-related signals. This is inconsistent with the fact that the mainstay dopaminergic drug is the anti-Parkinson's Levodopa, with annual sales of around $100 million, whereas the SSRI Prozac (fluoxetine) has annual sales of approximately $250 million[2]. This discrepancy in preclinical research has allowed the field to remain relatively open for the study of 5-HT in phMRI paradigms.

[2] Sales of Carbidopa/Levodopa for Parkinson's $109m *vs* sales of Fluoxetine $206m for 2010/11 according to EvaluatePharma.com

It should be noted that BOLD fMRI is not a direct measure of neuronal activity, rather it is an indirect measure of activity-driven changes in blood flow. Initial studies merely aimed to achieve higher resolution than PET imaging. Silva and colleagues (1995) showed that it was possible to achieve better resolution during amphetamine stimulation using fMRI than using a standard labelled glucose PET scan. Work by Preece and colleagues has demonstrated that it is possible to use this high resolution technique, combined with pharmacological agents that target 5-HT release such as fenfluramine, to image the serotonergic system with a high degree of accuracy (Preece *et al.*, 2009). Another advantage of phMRI over PET, especially in preclinical 5-HT imaging, is the ability to run it concomitantly with other techniques to confirm data. The activation maps produced using a 5-HT agonist have been confirmed by measuring neuronal activation (Stark *et al.*, 2006), showing that this technique is a valid measure of 5-HT activation. It is also possible to run a number of techniques concurrently, as in the studies by Schwarz *et al.* (2004), where fMRI data was correlated with *in vivo* microdialysis to confirm that signal was related to neurotransmitter release. The same group have used a complex set of experiments using both 5-HT and dopaminergic drugs to demonstrate functional connectivity between neurotransmitter circuits (Schwarz *et al.*, 2007).

Finally, such studies can be used to investigate both pharmacology and pathology. Having established that it is possible to 'release' 5-HT in the brain using an agent such as fenfluramine, it is possible to compare activation maps before and after the application of agonists and antagonists. It is also possible to use the same basic methodology to study disease using both control *vs* depressed or medicated *vs* unmedicated. Human studies often come with a caveat – it is possible to infuse pharmacological agents into preclinical animal models whilst in the magnet, however, studies in patients and controls tend to be strictly before and after and are rarely *in situ*. This means that imaging the direct and immediate actions, if any, of drugs such as SSRIs and fenfluramine on the 5-HT system is almost impossible. Despite this confound, a number of studies have shown that specific activation maps in depressed patients correlate well with treatment outcome (Kalin *et al.*, 1997). Davidson and colleagues showed decreased activity in the PFC in response to

emotional faces which was reversed after treatment with the SSRI venlafaxine (Davidson *et al.*, 2003). Work such as this has given rise to the possibility of characterising, and therefore treating, depression in a personalized manner using an endophenotypic approach (Hasler and Northoff, 2011). However, as always, work is hampered by the lack of very specific agonists and antagonists to unpick the molecular biology underlying these disorders.

Thus, it can be argued that these signals provide a broad readout of composite activity within heterogenous neuronal populations that has an approximate functional specificity, allowing it to be a suitable modality for the investigation of the functional consequences of pharmacological interventions. We have previously demonstrated that it is possible to measure responses of the 5-HT system using BOLD phMRI and specific 5-HT releasing drugs such as fenfluramine (Preece *et al.*, 2009). In human studies, both 5-HT depletion and 5-HT augmentation through citalopram treatment (Anderson *et al.*, 2008; Murphy *et al.*, 2009) have demonstrated region-specific alterations in BOLD responses to emotional processing tasks. Together, these studies reinforce the concept that it is possible to measure the functional effects of 5-HT modulation using BOLD phMRI.

A risk factor that has received little attention in gene-environment interaction studies, favoured recently in the study of depression, is infection. There is considerable evidence of high co-morbidity between infection and depression. There are a number of studies showing that specific chemical inflammatory agents both evoke neuropsychiatric symptoms and modulate brain 5-HT (O'Connor *et al.*, 2009). For example, chronic treatment with cytokines such as interferon-α (IFNα), as is implemented in hepatitis and some types of cancer, results in 25% of patients suffering from a depressive episode where they have no family history of the disease (Horikawa *et al.*, 2003). This interaction between 5-HT and the immune system represents a bidirectional conversation – clinically depressed patients with no obvious systemic infection often present with high levels of circulating cytokines (Levine *et al.*, 1999; Alesci *et al.*, 2005). Despite this link between the immune system and the 5-HT system, technical limitations have failed to advance our

knowledge regarding the functional outcomes of systemic inflammation. Paradigms such as phMRI provide us with the opportunity to study mechanisms of infection related 5-HT changes thus far unexplored.

While phMRI may enable conclusions to be drawn regarding regional neuronal activation in response to a specific stimulus, the exact correlate of the BOLD response is currently unknown (Martin and Sibson, 2008). If it is assumed that there is a direct correlation between the vasculature and the BOLD response, it is important to determine whether the stimuli that is being measured with fMRI does not affect the vasculature. Inflammatory stimuli such as LPS are known to affect the contractility of the blood vessels (Gunnett *et al.*, 1998), and yet studies of inflammation thus far have not investigated whether the CNS vasculature responds in the same way in the sick and healthy animals. These caveats are important to consider when investigating the mechanisms of sickness behaviour using functional imaging paradigms.

The Sibson laboratory has used phMRI to show that it is possible to image the 5-HT system (Preece *et al.*, 2009). Administration of fenfluramine results in region-specific changes in BOLD response. While it is possible to image the 5-HT system with some degree of accuracy, the effect of CNS pathology, even something as benign as the common cold, is unknown. This particular imaging paradigm in rodent models allows us to look at functional outcomes during systemic inflammation, and to correlate circulating factors with changes in the CNS response. Many factors produced during a systemic infection could result in vascular changes that affect the BOLD signal. It is possible to answer some of these questions using a systemic LPS challenge in a pre-clinical, 5-HT-releasing BOLD paradigm such as that used by Preece and colleagues (2009), and this will be the focus of this chapter.

2.2 Aims of this Chapter

The aims of this chapter are to determine how systemic inflammation affects the structure and function of the 5-HT system. Using novel techniques such as phMRI, the effect of a single systemic LPS injection on 5-HT activity will be studied. The direct

correlate of the BOLD signal is not yet known and therefore work using techniques such as *in vivo* microdialysis and cerebral blood flow monitoring, will be carried out in order to determine whether any signal changes are the result of changes in release mechanisms or changes in neurovascular coupling. Post-synaptic changes in 5-HT receptor and transporter expression will also be investigated, as these have the potential to change the BOLD response to 5-HT.

2.3 Materials and Methods

2.3.1 Animals

Animals were provided with food and water *ad libitum* and all procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. All animals were treated under PIL 30/7863 and PPL 30/2524 and 30/2620. Adult male Sprague-Dawley inbred rats were obtained from Harlan at c.200g and housed under standard conditions for at least 1 week prior to experimentation to reduce the confound of stress.

2.3.2 Drug Administration

LPS (*E.Coli* 0111:B4; Sigma-Aldrich, UK) was dissolved in sterile 0.9% saline and administered to awake animals as an intraperitoneal (i.p.) dose of 0.5mg/kg 6 hours prior to administration of fenfluramine. Dose and time regime were based on previous studies from the Sharp laboratory (Josie Raley, unpublished), as well as published studies (Wohleb *et al.*, 2012). Control animals received an equivalent i.p. volume of sterile saline (0.1ml). Fenfluramine was administered to anaesthetised animals as an intraveinous (i.v.) dose of 10mg/kg via a tail cannula. Control animals received an equivalent volume (c.0.1ml) sterile saline. Dose and timing of fenfluramine was based on previous studies from the Sharp laboratory (Josie Raley, unpublished), as well as published studies (Gonzalez *et al.*, 2003). MDL100907 (Sigma Aldrich, UK), a selective 5-HT_{2A} antagonist, was administered as a single i.p. dose of 0.5mg/kg 15 minutes prior to administration of fenfluramine. Control animals received an equivalent volume (c.0.1ml) sterile saline. Dose and timing of MDL100907 was based on previous studies from the Sharp laboratory (Boothman *et al.*, 2006).

2.3.3 Anaesthesia

Animals were anaesthetized using 2% isoflurane (Rhodia Organique Fine Ltd., Bristol, UK) in a 70:30 N_2O:O_2 mix at approximately 1.5l/min. A tracheotomy was performed by exposing the trachea through the muscles of the neck and inserting and fixing a tracheal tube through a small hole. Wounds opened during surgery were subsequently closed

using sutures and covered in gauze to reduce heat loss. Gases were administered using the tracheal tube via a ventilator set to 60 breaths/min, levels were adjusted according to blood-gas measurements.

2.3.4 Surgical Placement of Femoral Vein and Artery Lines

In order to monitor blood gases to prevent hypercapnia interfering with experiments, and to administer drugs, cannulae were inserted into the femoral artery and vein. Vessels were exposed via the dorsal surface of the leg and cannulae inserted and tied in place. Wounds opened during surgery were subsequently closed using sutures and covered in gauze to reduce heat loss. Blood gases were monitored throughout the procedure to ensure maintenance of homeostasis.

2.3.5 Magnetic Resonance Imaging and Analysis

Here, magnetic resonance imaging was kindly performed with the assistance of Dr Chris Martin and Dr Josie Raley of the (Grey Institute for Radiation, Oncology and Biology, Oxford). Original dose response studies (data not shown) performed by Dr Josie Raley showed significant areas of activation in the motor cortex, nucleus accumbens and dorsal raphe nuclei, according to contiguent cluster analysis. These areas therefore remained the focus for both phMRI and subsequent microdialysis and CBF studies. Animals (n = 5/6 per group) were placed in a quadrature birdcage radiofrequency coil (internal diameter 5 cm) with an integral stereotaxic frame. Electrocardiogram (ECG) was monitored throughout and body temperature maintained at 37°C. MRI data were acquired using a 7T magnet (Magnex Scientific, Abingdon, UK), and a Varian Inova spectrometer (Varian Inc, Palo Alto, USA). Sets of five coronal images spanning the rat forebrain were acquired using a T2*-weighted FLASH sequence (TE = 25 ms, TR = 500 ms, 40° flip angle, field of view 80 x 40 mm, matrix size 256 x 128, slice thickness 1.5 mm). Dynamic updating of first order shims was used for each slice to maximize fMRI sensitivity 18,19. Each set of five images was acquired over a 2 min time period. Anatomical images of the same five coronal slices were acquired using a T2-weighted fast spin echo sequence (TR = 3 s, TE = 45 ms, field of view 30 x 30 mm, matrix size 128 x 128). Images were acquired

continuously throughout the experimental protocol, which consisted of 15 min of baseline imaging, followed by a bolus i.v. administration of either fenfluramine (10 mg/kg i.v.) or vehicle (as per 2.3.2), and a further 85 min of image acquisition.

Analysis of the fMRI datasets was carried out using AFNI (http://afni.nimh.nih.gov/afni/) and FEAT (http://www.fmrib.ox.ac.uk) software packages. Datasets were corrected for any motion artefacts using AFNI, and spatial smoothing performed in FEAT using a Gaussian kernel of FWHM 1 mm. Statistical analysis was performed using a general linear model within FEAT. The design matrix consisted of a simple temporal model of the expected response, with 0 specifying the baseline period and 1 or -1 specifying signal increases or decreases during the post-drug period respectively. Activation maps were generated as follows: a Z statistic threshold of $p<3.5105$ was used to define contiguous clusters, and each cluster's estimated significance level compared with the cluster probability threshold of $p<0.05$. A colour-coded z-statistic map was then overlaid onto the corresponding phMRI gradient echo image. With reference to the anatomical images and a stereotaxic atlas (Schwarz *et al.*, 2006), regions of interest (ROI) were manually defined on the functional datasets for anterior motor cortex, nucleus accumbens and dorsal raphe nucleus. The average BOLD signal intensity changes across all pixels over time within these ROIs was determined, expressed as percentage change from baseline signal intensity, and plotted as a function of time.

2.3.6 Laser-Döppler Flowmetry (LDF)

LDF experimental design and analysis was performed with the kind assistance of Dr Chris Martin (Grey Institute for Radiation, Oncology and Biology). In order to maintain continuity, animals were set up for microdialysis and LDF was carried out in the contralateral hemisphere. Animals received a single dose of either LPS or saline (as per 2.3.2) and were placed in a stereotaxic frame (Kopf Instruments), and a region of skull overlying the right anterior motor cortex was thinned to translucency using a dental drill. A laser Doppler probe (Perimed Probe 403, Jarafalla, Sweden) was positioned above the visible cortical surface. Carbon fibre stimulating electrodes were inserted through burr

holes made in the skull overlying the contralateral cortex and advanced over the cortical surface to a position overlying the anterior motor cortex. Electrical stimuli were delivered to the contralateral cortex and recordings of stimulus evoked cerebral blood flow changes were recorded using the laser Doppler probe. For each laser Doppler experimental session, 2 second stimuli of 5, 10, 20, 30 and 40 Hz were presented in a randomized sequence (10 repeats at each frequency) with a stimulus pulse width of 0.3ms and an inter-stimulus interval (ISI) of 25 seconds. All stimulus presentation was controlled through a 1401 plus (CED Ltd., UK) running custom-written code.

2.3.7 Stereotaxic Implantation of Microdialysis Probes

Microdialysis probes were purchased from Royem Scientific with a semi-permeable PES membrane tip (3mm effective tip length; 40,000 MW cut-off). Animals were placed in a stereotaxic frame (Stoetling Co., USA), the skull was exposed and a burr-hole drilled above the motor cortex (A/P +2.2mm and M/L +2.8mm). The probe was positioned in the motor cortex (D/V -1mm) and perfused with artificial cerebral spinal fluid (aCSF; 140mM NaCl, 3mM KCl, 1.2mM Na_2HPO_4, 0.27mM NaH_2PO_4, 1mM $MgCl_2$, 2.4mM $CaCl_2$ and 7.2mM glucose) at a flow rate of 2µl/min (CMA402, CMA Microdialysis, Sweden). The probe was perfused for 1.5-2 hours *in situ* for collection of baseline dialysate samples at 20 minute intervals for approximately 3 hours. This was followed by an intraveinous injection of fenfluramine (10mg/kg) and 3 hours of subsequent samples.

2.3.8 High Pressure Liquid Chromatography (HPLC)

HPLC was performed with the kind assistance of Dr Michael Stratford (Ludwig Institute for Cancer Research, Nuffield Department of Clinical Medicine, Oxford). The samples were analyzed using HPLC with electrochemical detection (HPLC-EC) and separated with an ACE microsorb column (C18, 3µm, 125 x 3 mm + ACE C18 guard, 10 x 3 mm run at 35°C). Samples were carried by an eluent (12.5% MeOH, 130 mM NaH_2PO_4, 0.85 mM Na_2EDTA, 0.1 mM 1-octanesulphonic acid, pH3.55) pumped with a flow rate of 0.6ml/min (Waters 2695 HPLC Pump). Samples were detected using a glassy carbon electrode held at +0.75V (Dionex ED40). The dialysate content was determined with

reference to daily calibrated standard solutions. These external standards indicated the peak retention times and peak height against perchloric acid (5pmol 5-HT, 5-HIAA, DOPAC and HVA in 0.06M perchloric acid). The chromatograms were displayed and analyzed using Waters Empower 2 software.

2.3.9 Tissue Collection for qPCR

Animals were surgically anaesthetized using approximately 0.1ml pentobarbitone (i.p.). Tissue was perfused with sterile, cold 0.9% saline at 20ml/minute for around 2 minutes. Brain tissue for PCR and autoradiography were collected from two separate groups of animals, both receiving one i.p. dose of LPS or saline (as per 2.3.2) 6 hours prior to perfusion. Brain regions were collected as per **Table 2.1**.

Region	Dissection Instructions
Prefrontal Cortex	Region immediately posterior to the olfactory bulbs but posterior to the joining of the corpus callosum. Less than 2mm in a mouse, less than 4mm in a rat
Motor Cortex	Region within 2mm slice immediately posterior to the prefrontal cortex. Take a punch from the cortex immediately adjacent to the central fissure
Striatum	Region in the same slice as the motor cortex. Take a punch from the area below the corpus callosum
Hippocampus	Region comprising ventral hippocampus. Remove cerebellum and slice 2mm (mouse) or 4mm (rat) anterior to the appearance of the cerebral aqueduct. Hippocampi are easily removed from the edge of the slice
Raphe	Region comprising <1mm^2 in mice and c.1mm^2 in rats immediately below the cerebral aqueduct

Table 2.1 Dissection instructions for removing specific regions of interest in both rat and mouse brains.

2.3.10 mRNA Extraction from Brain Tissue

Approximately 10mg of tissue was collected from the regions of interest. Tissue samples were snap frozen on dry ice for subsequent RNA extraction. RNA was extracted using the Qiagen RNEasy Mini Kit according to manufacturer's instructions. Briefly, tissue samples were disrupted in lysis buffer using a pestle before passing through a QiaShredder spin column to homogenize and shear genomic DNA. RNA was precipitated from the homogenate using ethanol and was passed through an RNEasy spin column and washed. Total RNA was eluted using 25µl RNAse free water. RNA quality and quantity were analyzed using a NanoDrop 1000 Spectrophotometer (Thermo, UK).

2.3.11 mRNA Extraction from Liver Tissue

Approximately 10mg of liver tissue was collected from animals immediately after perfusion with 0.9% saline and snap frozen on dry ice. RNA was extracted from tissues (see section 2.3.10)

2.3.12 cDNA Synthesis by RT-PCR

50ng total RNA was diluted in 10µl nuclease-free dH_2O and added to 10µl of mastermix (*see* Table 2.2). This reaction was run according to the manufacturer's instructions (Applied Biosystems, UK; *see* Table 2.3). Final reactions of 20µl were diluted with 80µl water to a final concentration of 5ng/µl.

Component	Volume (µl)	Final Concentration
10X RT Buffer	2	1X
25X dNTP Mix (100mM)	0.8	4mM
10X RT Random Primers	2	1X
Multiscribe™ Reverse Transcriptase	1	2.5U/µl
RNAse Inhibitors	1	0.4U/µl
Nuclease-Free dH_2O	3.2	-

Table 2.2 Components of cDNA synthesis mastermix .

Temperature (°C)	Time (mins)	Function
25	10	Extension of primers
37	120	Reverse transcription
85	5	Inactivation of transcriptase
4	∞	Storage

Table 2.3 Thermal cycling conditions for reverse transcription reaction.

2.3.13 qPCR

qPCR was run with primers detailed in **Table 2.5**. Primers were designed specifically by Primer Design Ltd. to work with the Roche LightCycler 480 using a SYBR green-based assay. Briefly, primers and mastermix were combined with cDNA according to Table 2.4 and run according to Table 2.3. See Table 2.5 for details of primer sequences used in this chapter. SYBR green binds to double stranded DNA, so each round of reverse transcription exponentially increases the amount of fluorescence. Run efficiency was determined using the Pfaffle method (Pfaffl, 2001). This requires an internal standard curve composed of combined cDNA samples. This standard curve allows determination of the efficiency of both the gene of interest reaction and the housekeeping gene reaction. This method allows the accurate normalization of cDNA input (Peirson *et al.*, 2003). Fluorescence was detected using a FAM-based laser. Once cDNA input levels were normalized, data was further normalized to a baseline value of '1' in control animals within each region giving final values relative to controls within each brain area.

Component	Volume (µl)
Precision 2X qPCR Mastermix	10
Primers	1
cDNA (25ng)	5
Nuclease-free dH_2O	4

Table 2.4 Components of qPCR mastermix.

Marker	Forward Primer	Reverse Primer	Amplicon Size (nt)
5-HT2a	TCTTTGGACTACAGGATGATTCG	TGATGGTTAGGGGGATGAAAAAT	114
5-HT6	CAATGTTGCTTGTGTAGTGTGG	TGAGAGAAAGGGATGAGGACAG	102
SERT	AAGGAGATGCTGGGCTTCA	GGGTGGGCTCATCAGAAAAC	108
5-HT1a	TGTTGCTCATGCTGGTTCTC	GTCCGTTCAGGCTCTTCTTG	147

Table 2.5 Primer sequences for qPCR.

2.3.14 Tissue Collection for Autoradiography

Animals were surgically anaesthetized and transcardially perfused with 0.9% saline at 20ml/min for approximately 5 minutes. Brains were removed whole and snap frozen on isopentane at -78^{o}C. Tissue was cut at 12µm using a standard cryostat (Leica, UK) onto charged SuperFrost Plus slides (Fisher, UK) and allowed to air dry briefly (2-5 minutes) at room temperature before storage at -20^{o}C.

2.3.15 Autoradiography

Slides were brought to room temperature for 20 minutes prior to incubating in Tris-HCl Buffer (50mM Tris-HCl) for 20 minutes. Adjacent slides were incubated with 300µl either 2nM [^{3}H]Ketanserin or 2nM [^{3}H] with 10µM methylsergide. Hydrophobic coverslips were placed to prevent evaporation and slides were incubated for 3 hours at room temperature. Slides were washed 2 x 10 minutes in Tris-HCl at 4^{o}C, followed by 2 x 5 minutes dH_2O at RT and then air dried in a cold air stream overnight. Sections were then placed in a cassette with Kodak® BioMax® MR film and exposed for approximately 6 weeks.

2.3.16 (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) induced wet dog shake behaviour

Animals received a single dose of LPS as with previous experiments and were allowed to recover for c.6 hours. On or around the 6 hour time point animals received a single dose of DOI (1mg/kg s.c.) and were observed for 20 minutes, during which the number of wet dog shakes (WDS) were counted. WDS behaviour constituted anything from a head

twitch to a full body shake and data are presented as total WDS over 20 minutes, as is standard in the literature (Kugaya *et al.*, 1996; Kouhata *et al.*, 2001).

2.3.17 Statistics

Statistical analysis was carried out using GraphPad Prism and InVivoStat software. Tests included standard Student's T-test and one-way and two-way repeated measures ANOVA with post-hoc tests as appropriate. Post-hoc analysis was either Bonferroni or Tukey. InVivoStat analysis allowed for unstructured co-variance within a repeated measures ANOVA and was used to analyse qPCR data. This allowed for data variance to be different within the repeated measure, specifically the brain region. Data are presented as mean ±SEM. Data was considered statistically significant with a $p<0.05$.

2.4 Results

2.4.1 A single LPS challenge significantly alters BOLD fMRI signal in a number of brain regions

Sickness is known to result in alterations in mood (Dantzer *et al.*, 2008), however, the mechanisms remain largely unexplored. The 5-HT system is known to drive changes in mood. This experiment sought to use newly developed phMRI techniques (Preece *et al.*, 2009) to study the functional outcomes of systemic inflammation on fenfluramine evoked 5-HT release. Animals received a single dose of LPS (0.5mg/kg) or vehicle (c.0.1ml saline) 6 hours prior to BOLD fMRI and a single dose fenfluramine (10mg/kg i.v.) whilst in the magnet. Pre-treatment with LPS significantly reduced the BOLD response to fenfluramine across all brain regions ($p<0.0001$; Figure 2.1) compared to saline treated animals.

The first 30 minutes of post-fenfluramine signal were analysed by one-way ANOVA in order to maximize signal:noise. In the analysis of the first 30 minutes post-fenfluramine injection there was a significant main effect of treatment (all regions $p<0.0001$; MCx $F_{2,11}=72.75$, NAc $F_{2,11}=63.09$, DRN $F_{2,11}=117.7$). Bonferroni post-hoc comparisons showed further differences between LPS-treated animals given fenfluramine and the control saline-saline group in all regions (cortex and DRN $p<0.01$ Figure 2.1A; nucleus accumbens $p<0.0001$ Figure 2.1C). These data suggest some residual effects of fenfluramine, although from the time course plots these effects appear minimal with the BOLD responses to fenfluramine being largely abolished (Figure 2.1A-C).

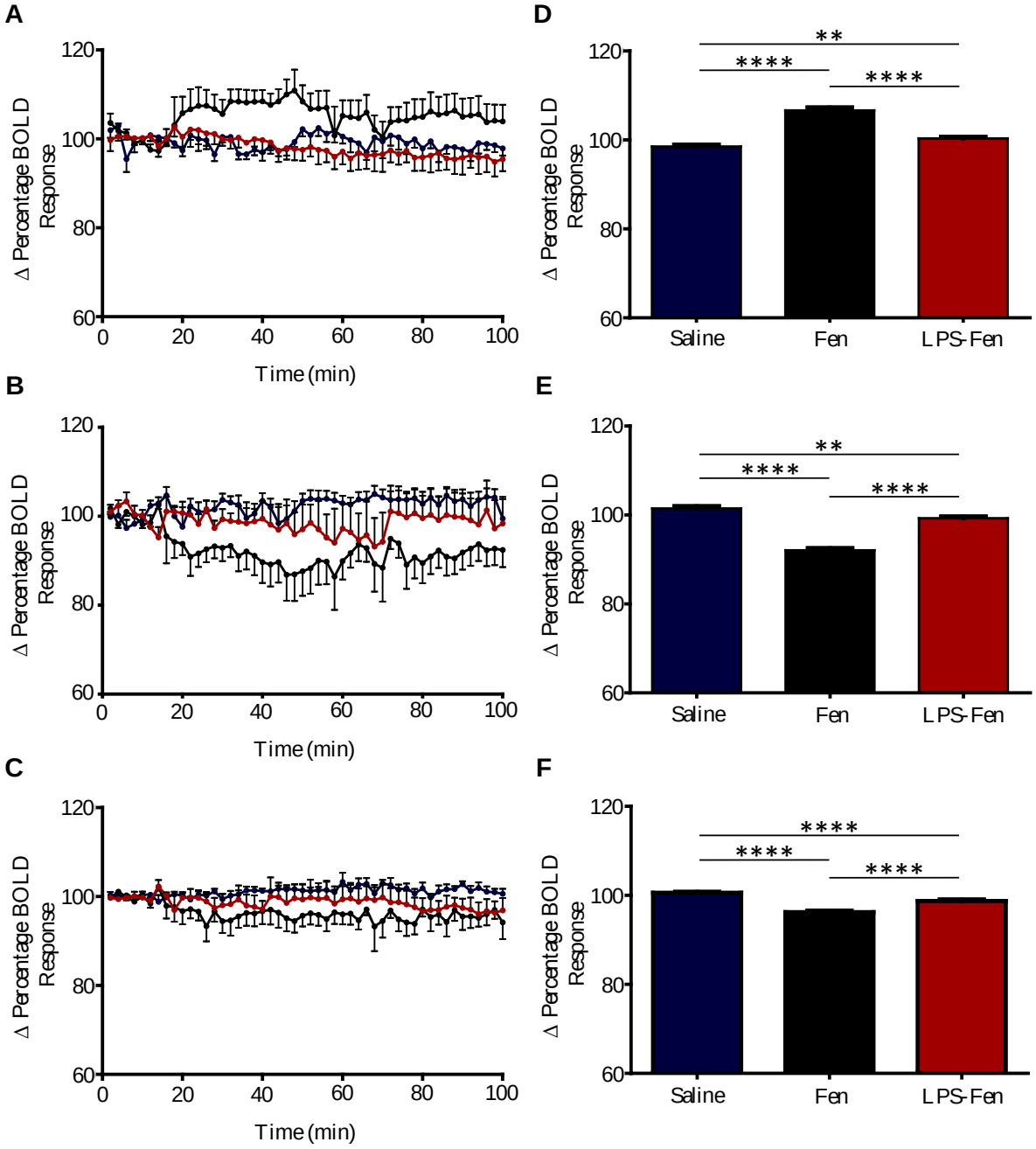


Figure 2.1 Time course plots showing the change in BOLD signal intensity in a number of brain regions. Data show change in BOLD in (A) motor cortex, (B) nucleus accumbens and (C) dorsal raphe nucleus following administration of saline (i.v.), fenfluramine (10 mg/kg i.v.) or fenfluramine (i.v.) preceded by LPS (0.5mg/kg i.p.) 6h before fenfluramine administration. Data represent mean ±SEM (n=4). Fenfluramine or saline was administered at t = 15 minutes. (D-F) Bar graphs showing mean of the signal intensity over the first 30 minutes after fenfluramine or saline administration. Data are mean ± SEM, n=4, **p<0.01 and ****p<0.0001.

2.4.2 BOLD-fMRI changes after LPS challenge are not caused by neurovascular uncoupling

In order to determine whether the basic unit of communication between the brain and the systemic circulation was functioning correctly after a systemic immune challenge we used laser-Döppler flowmetry (LDF). In order to maintain continuity these experiments were carried out concomitantly with the *in vivo* microdialysis experiments (2.4.3). Cranial windows were created in the contralateral hemisphere to the microdialysis probes (placed in the motor cortex as per 2.3.6) and stimulating electrodes were placed in the anterior cortical area. Blood flow was measured using LDF over a variety of stimulation amplitudes (5-40Hz) in animals that had been pre-treated with saline (Figure 2.2A) or LPS (Figure 2.2B) 6 hours previously. At low stimulation frequencies (5-10Hz) saline treated animals showed an average 2% change in blood flow, not significantly different from animals challenged with LPS where the change in blood flow was 3%, on average. At higher stimulation frequencies (20, 30 and 40Hz, respectively), saline treated animals showed a linear increase in cerebral blood flow, on average 15% , 20% and 25% increase above zero. LPS animals showed a similarly linear increase in CBF upon stimulation at these frequencies, with an average of 12.5%, 15% and 17.5% increase above zero. While area under the curve analysis shows there is a significant main effect of stimulation frequency (one-way ANOVA $p<0.001$; $F_{4,25}=9.531$) there are no significant differences between LPS and saline treated animals at any stimulation frequency in post-hoc tests (Figure 2.2C).

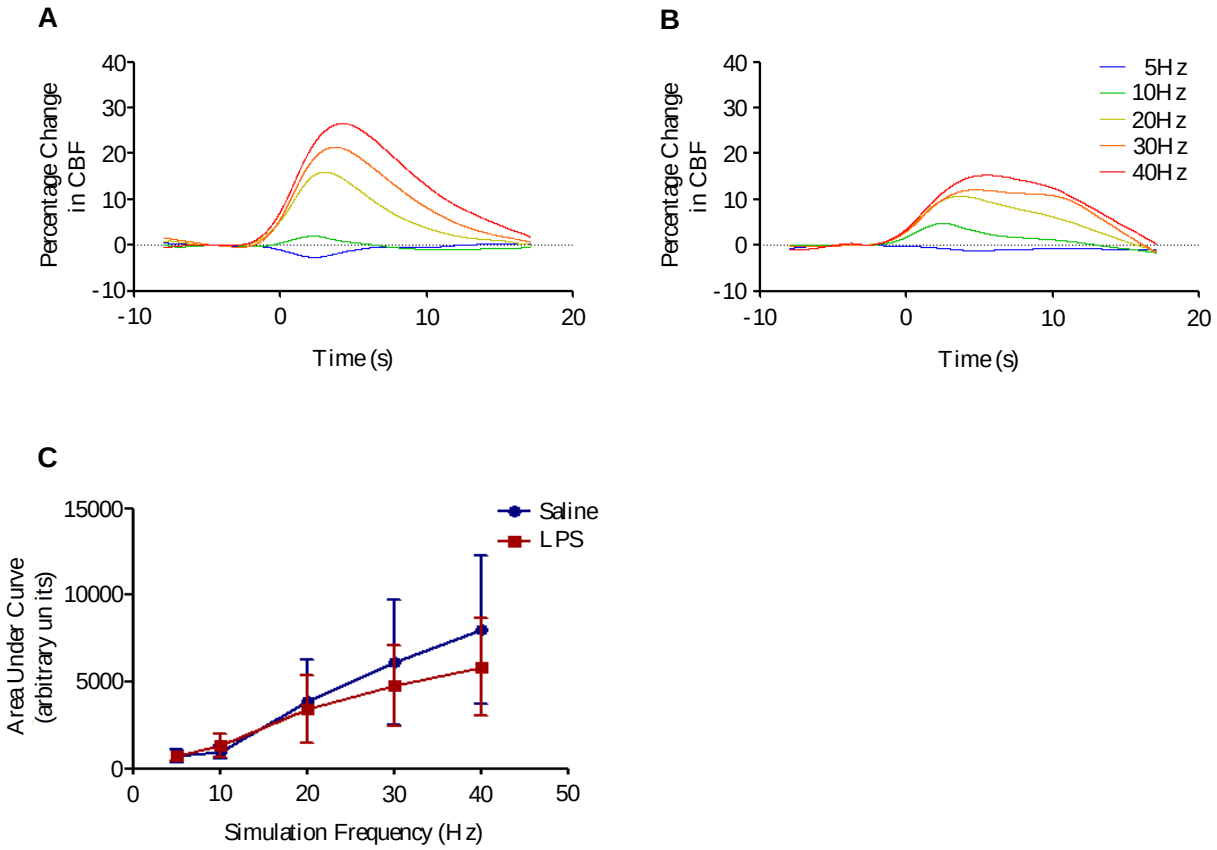


Figure 2.2 Effect of LPS treatment on neurovascular coupling and functional hyperaemia. Time course of cerebral blood flow (CBF) changes recorded in primary somatosensory cortex in response to electrical stimulation of the contralateral somatosensory cortex in (A) saline treated and (B) LPS treated animals (n = 3 per group). Stimuli were delivered using carbon fibre electrodes positioned overlying the somatosensory cortex and the stimulus evoked CBF changes were recorded using laser doppler flowmetry probe positioned over the corresponding contralateral cortex. Stimuli consisted of a 2 s train of 0.3 ms 1.5 mA pulses at one of 5 frequencies (5, 10, 20, 30 and 40 Hz). (C) CBF responses over the range of stimulation frequencies were quantified by determining area under the curve for the mean response to each condition for each animal; no significant difference was observed for the saline and LPS conditions.

2.4.3 Peripheral inflammation does not result in reduced 5-HT release or altered 5-HT metabolitess

Changes in BOLD response during episodes of inflammation (Figure 2.1) have been shown to be due to mechanisms other than neurovascular uncoupling (Figure **2.2**). One such possibility is presynaptic changes in release mechanisms. To determine whether this was the case *in vivo* microdialysis was carried under the same experimental conditions as the fMRI experiments. This included the same surgical procedure (*see* 2.3.3 and 2.3.4) as well as the same drug treatment regime for LPS and saline challenges (*see* 2.3.2). Some of the largest changes in BOLD response were observed in the motor cortex and therefore this region was chosen for microdialysis experiments. CSF was allowed to perfuse for approximately 2 hours prior to fenfluramine challenge, CSF samples were taken every 20 minutes. Those for the first hour were not included in the analysis due to the high levels of 5-HT in blood surrounding the site of surgery (data not shown). Data acquired during the second hour were averaged to 100 and considered as baseline values. Absolute values for baseline 5-HT were 2.90±0.041pmol in saline animals and 2.96±0.028pmol in LPS treated animals (data not shown).

Administration of fenfluramine (10mg/kg) resulted in a significant increase in 5-HT release approximately 40 minutes after injection in saline treated animals (t-test $p<0.01$; $t_8=3.763$ blue line Figure 2.3A), averaging 800% of baseline values. Animals challenged with 0.5mg/kg LPS 6 hours prior to fenfluramine administration also showed a significant 5-HT release in response to fenfluramine ($p<0.001$; $t_8=7.245$; red line Figure 2.3A), averaging 700% of baseline values. These peak 5-HT release values did not differ significantly between animals challenged with saline or LPS.

Having studied release mechanisms, it was important to investigate whether 5-HT metabolism had been affected by the immune challenge. 5-HIAA and HVA are metabolites of 5-HT (Figure 1.6). DOPAC levels reached a peak of 125% in animals receiving a saline injection prior to fenfluramine but this did not achieve significance from baseline values (blue line Figure 2.3B). Animals receiving LPS showed no such peak

in DOPAC levels after fenfluramine stimulation (red line Figure 2.3B). 5-HIAA levels in saline and LPS treated animals reached an average of 125% of baseline values 40 minutes after fenfluramine administration, these were not significantly different from each other, or from baseline values (Figure 2.3C). HVA values peaked at around 135% of baseline values in animals challenged with both saline and LPS prior to fenfluramine administration (Figure 2.3D). These values were not significantly different from baseline or each other.

Finally, sectioning of a number of microdialysis brains post-mortem enabled confirmation of probe placement in the motor cortex at the co-ordinates required (Figure 2.3E, *see* 2.3.7 for co-ordinates).

2.4.4 Systemic LPS causes an up-regulation of 5-HT$_{2A}$ receptor mRNA at 6 hours Post-Challenge

Differential expression of a number of 5-HT genes has been thought to contribute to the regulation of mood (Mann *et al.*, 1986; Serretti *et al.*, 2007). Taking this into consideration, we selected a number of candidate genes (5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{6}$ and the 5-HT transporter - SERT) in order to study whether our systemic immune challenge affected their expression. Expression of SERT doubled in the striatum after an LPS challenge but failed to increase in any other region (prefrontal and motor cortices, hippocampus and raphe). This change in mRNA expression was indicative of a lack of significant effect of LPS, but did show an effect of brain region and an interaction between brain region and treatment (RM-ANOVA; brain region $F_{4,24}=15.55$ $p<0.001$; LPS:brain region $F_{4,24}=15.55$ $p<0.001$ Figure 2.4A). 5-HT$_{2A}$ expression showed a significant main effect of treatment (LPS $F_{4,24}=1.705$ $p<0.001$; Figure 2.4B) but no interaction between treatment and brain region. Bonferoni post-hoc comparisons showed a significant increase in the prefrontal and motor cortices ($p<0.05$ Figure 2.4B). Mean striatal 5-HT$_{2A}$ expression was higher than control animals but did not reach significance ($p=0.06$; Figure 2.4B). 5-HT$_{2A}$ expression did not significantly change in the hippocampus or raphe compared to saline treated controls. 5-HT$_{6}$ expression did not

increase in animals challenged with LPS compared to control animals in any region investigated (Figure 2.4C). 5-HT_{1A} expression not increase in animals challenged with LPS compared to control animals in any region investigated (Figure 2.4D).

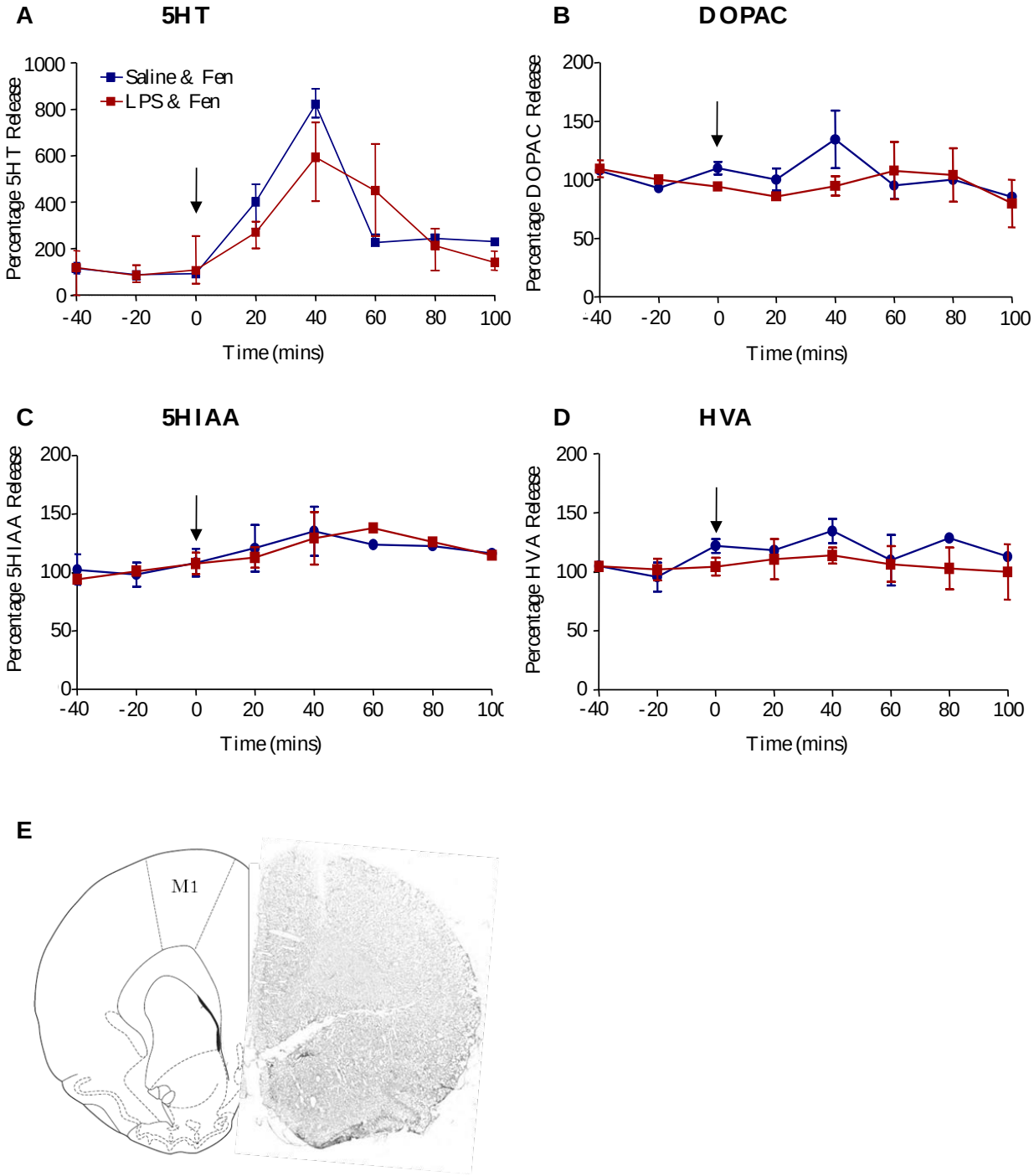


Figure 2.3 Percentage fenfluramine-induced 5-HT and metabolite release in the motor cortex of control and LPS pre-treated rats. Fenfluramine (10mg/kg) was administered at t=0 (arrow) to animals that had received an i.p. injection of saline (blue line) or LPS (0.5mg/kg i.p.; red line) 6 hours prior to t=0. (A) 5-HT; (B) 3,4-dihydroxyphenylacetic acid (DOPAC); (C) 5-hydroxyindoleacetic acid (5-

HIAA) and (D) homovanillica acid (HVA). Microdialysis probes were placed in the primary motor cortex (E). Data represent percentage release calculated against baseline. Points represent mean ± SEM.

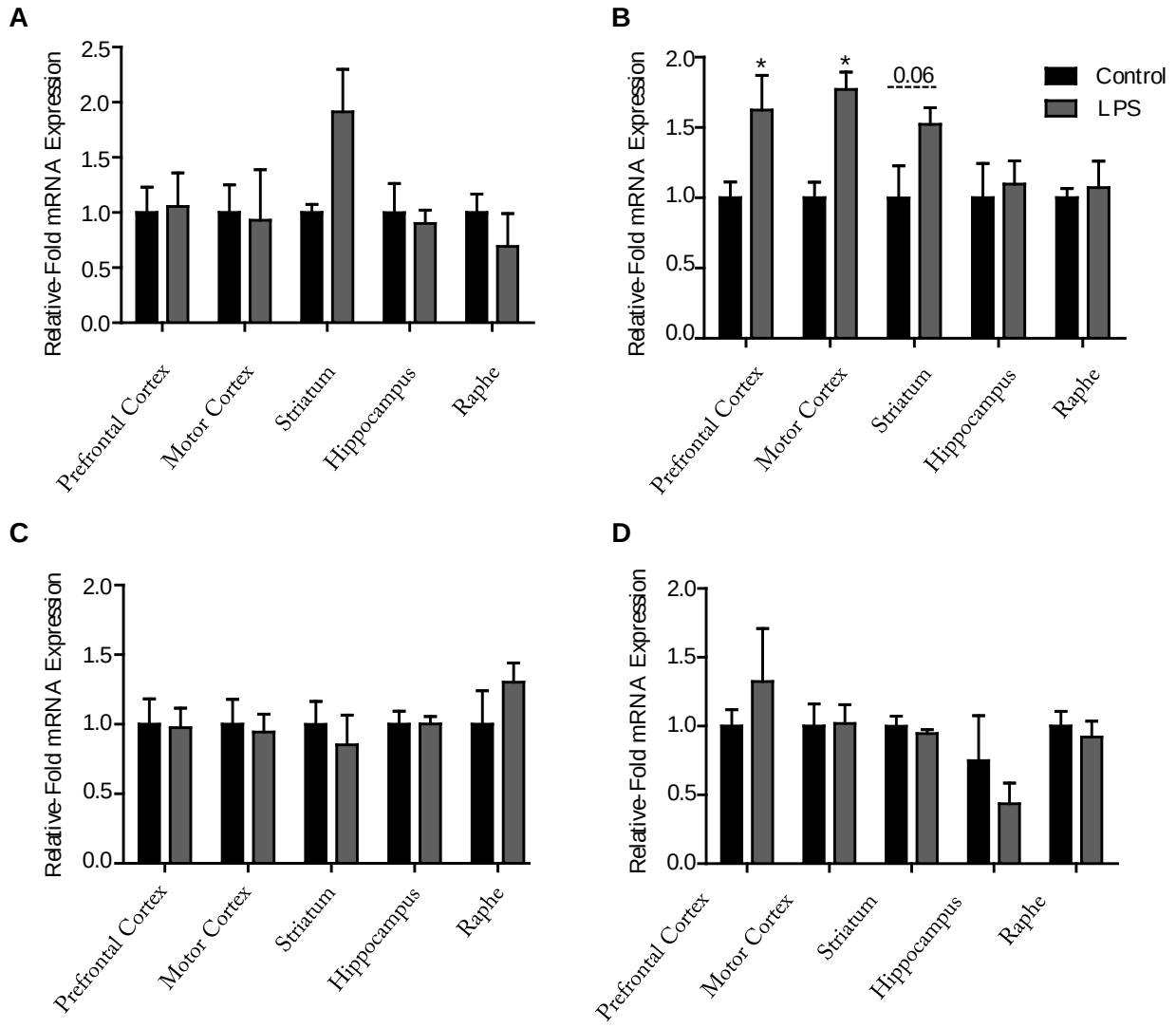


Figure 2.4 5-HT-related gene expression in the rat brain 6 hours after systemic LPS challenge. mRNA expression of (A) SERT; (B) 5-HT_{2A}; (C) 5-HT_6 and (D) 5-HT_{1A} were normalised to the housekeeping gene GAPDH and further normalized to control animals within each region. Animals received one dose of i.p. LPS (0.5mg/kg) and were allowed to recover for 6 hours prior to tissue removal. Data are mean ±SEM (n=4), *$p<0.05$.

2.4.5 Autoradiography

Changes in receptor mRNA do not necessarily reflect changes in protein expression. Data indicated that systemic immune challenge resulted in up-regulation of 5-HT_{2A} receptor mRNA (Figure 2.4B). Another cohort of animals was challenged in a similar manner to those for qPCR studies and brain tissue was removed and cryosectioned for autoradiographic analysis using ^{3}H-ketanserin, a 5-HT_{2A} ligand. Expression of the 5-HT_{2A} receptor is largely restricted to the prefrontal areas of the brain, therefore cryosections did not extend beyond the start of the hippocampus (Lopez-Gimenez *et al.*, 1997). Radioactivity was measured using MCID analysis software in the cingulate cortex and frontal cortex as per Figure 2.5A. 5-HT_{2A} expression was measured in saline (Figure 2.5B) and LPS (Figure 2.5C) brains and was found to be significantly affected by LPS but not by the brain region studied (RM-ANOVA; LPS $F_{1,8}$=5.4 $p<0.05$; region $F_{1,8}$=1.38 $p=0.2$ LPS:region $F_{1,8}$=0.19 $p=0.66$), post-hoc analysis showed that LPS caused 5-HT_{2A} expression to be significantly increased in the cingulate cortex (Bonferroni post-hoc; $p<0.05$).

2.4.6 Systemic LPS affects specific behaviours via the 5-HT_{2A} receptor

While changes in 5-HT_{2A} receptor expression indicate that LPS is directly affecting the serotonergic system, they do not show whether these effects mediate any of the behavioural aspects of sickness behaviour. With this in mind, 5-HT_{2A} agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; 1mg/kg subcutaneous) was administered in the presence and absence of systemic inflammation. Subcutaneous DOI in laboratory animals elicits very specific behavioural responses, in rats these are known as wet dog shake (WDS) behaviour. Analysis shows a significant main effect of DOI (two-way ANOVA; $p<0.001$; $F_{1,20}$=124.3) a significant main effect of LPS ($F_{1,20}$=38.17) and a significant interaction (LPS:DOI $p<0.001$; $F_{1,20}$=29.31). Post-hoc analysis (Bonferroni) demonstrates that administration DOI significantly increases the number of WDS in saline treated animals ($p<0.001$; Figure 2.6), and that administration of a single dose of

LPS 6 hours prior to DOI significantly reduced the number of observable WDS compared to saline injected animals receiving DOI ($p<0.001$; Figure 2.6).

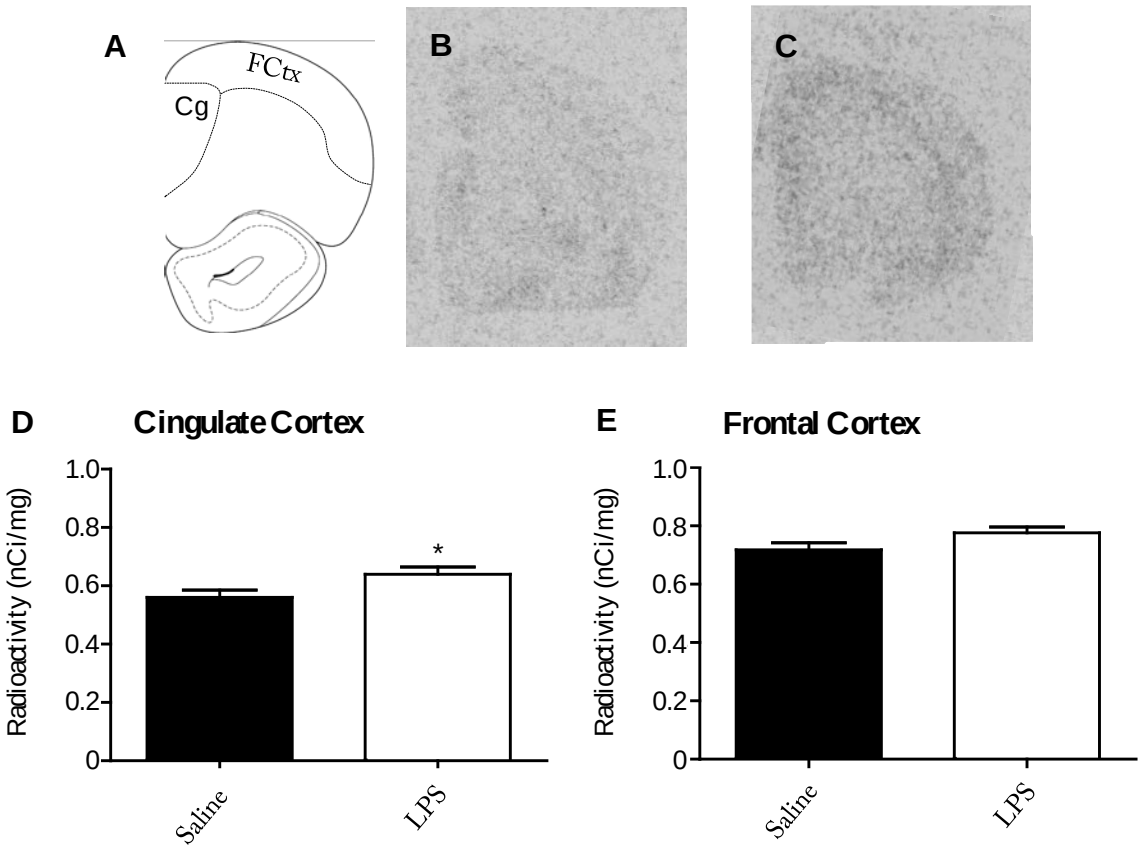


Figure 2.5 5-HT_{2A} protein expression in the rat brain 6 hours after an LPS challenge. Animals received a single i.p. dose of LPS 6 hours prior to sacrifice, brains were sectioned coronally and frontal regions were subjected to ^{3}H-ketanserin autoradiography. The cingulate cortex and frontal cortex were defined (A) and analysed using MCID Core Software. Representative photomicrographs from film autoradiograms from (B) saline and (C) LPS animals. Analysis shows data from (D) cingulate cortex and (E) frontal cortex. Data are mean ±SEM (n=3), *$p<0.05$.

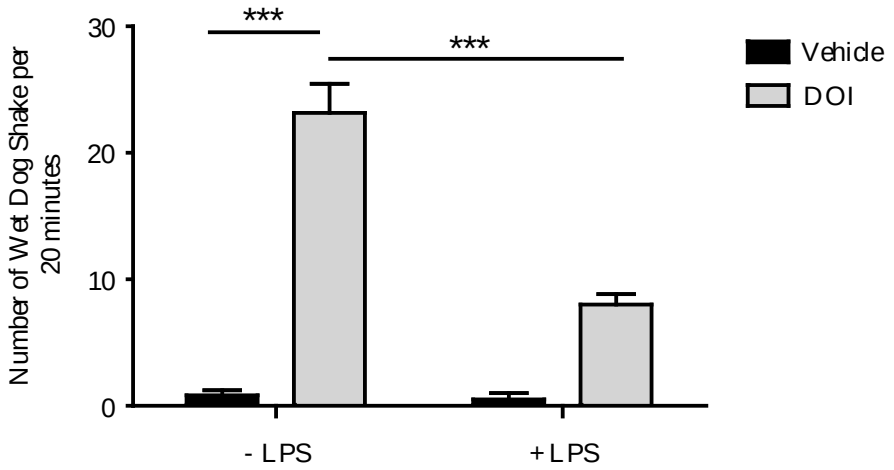


Figure 2.6 (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) induced wet dog shake behaviour in the presence and absence of systemic inflammation. Animals received a single i.p. dose of LPS (0.5mg/kg) or saline (c.0.1ml) and were allowed to recover for 6 hours prior to a single s.c. dose of DOI (1mg/kg) or saline. WDS behaviour was counted manually over a 20 minute period. Data represent mean ±SEM (n=6), ***$p<0.001$.

2.4.7 5-HT_{2A} antagonism mimics LPS in BOLD paradigms

Work from this chapter has demonstrated a significant role for the 5-HT_{2A} receptor in sickness behaviour (sections 2.4.4, 2.4.5 and 2.4.6). It was therefore important to discover whether the changes in behaviour and receptor expression resulted in changes in the BOLD response, in a similar manner to those obtained in response to LPS. Animals were pre-treated with either the selective 5-HT_{2A} receptor antagonist MDL100907 or an equivalent volume of saline (c.0.1ml) 15 minutes prior to administration of a fenfluramine challenge, as in previous sections (see 2.4.1). In animals pre-treated with the MDL100907, the BOLD response to fenfluramine was attenuated compared to untreated animals in all brain regions studied (Figure 2.7).

In the cortex, analysis of the first 30 min post-fenfluramine (or saline) administration showed a significant main effect of treatment (one-way ANOVA; $p<0.001$; $F_{3,15}=39.33$ Figure 2.7A), with post-hoc analysis (Bonferroni) showing the response to fenfluramine being completely abolished with MDL100907 pre-treatment ($p<0.001$; Figure 2.7A). No significant differences were found between the MDL100907-fenfluramine group and either the MDL100907 or saline alone groups.

In the DRN there was also a significant main effect of treatment ($p<0.001$; $F_{3,15}=120.5$) and post-hoc analysis showed that the fenfluramine-only group showed a significantly greater negative BOLD response to fenfluramine compared to all other groups ($p<0.0001$; Figure 2.7C). Although only minor differences in the BOLD response time course were evident between the MDL100907-fenfluramine group and the two control groups in the DRN (Figure 2.7C&E), these differences reached significance in the analysis of the first 30 min post-fenfluramine suggesting some residual effect of fenfluramine ($p<0.001$; Figure 2.7C&E).

Data in the nucleus accumbens showed a significant main effect of treatment ($p<0.001$; $F_{3,15}=62.53$) with post-hoc tests showing data in contrast to the cortex and DRN, with relatively little difference in the BOLD response of the nucleus accumbens to fenfluramine in the MDL100907-fenfluramine group compared to the fenfluramine

group, although this reached significance in post-hoc analysis of the first 30 minutes post-fenfluramine injection ($p<0.05$; Figure 2.7B&D). At the same time, the MDL100907-fenfluramine group showed highly significant differences to both the saline only group ($p<0.0001$; Fig. 2F) and the MDL100907-saline group ($p<0.0001$;Figure 2.7B&D) confirming effects of 5-HT_{2A} antagonism on the BOLD response to fenfluramine in the nucleus accumbens.

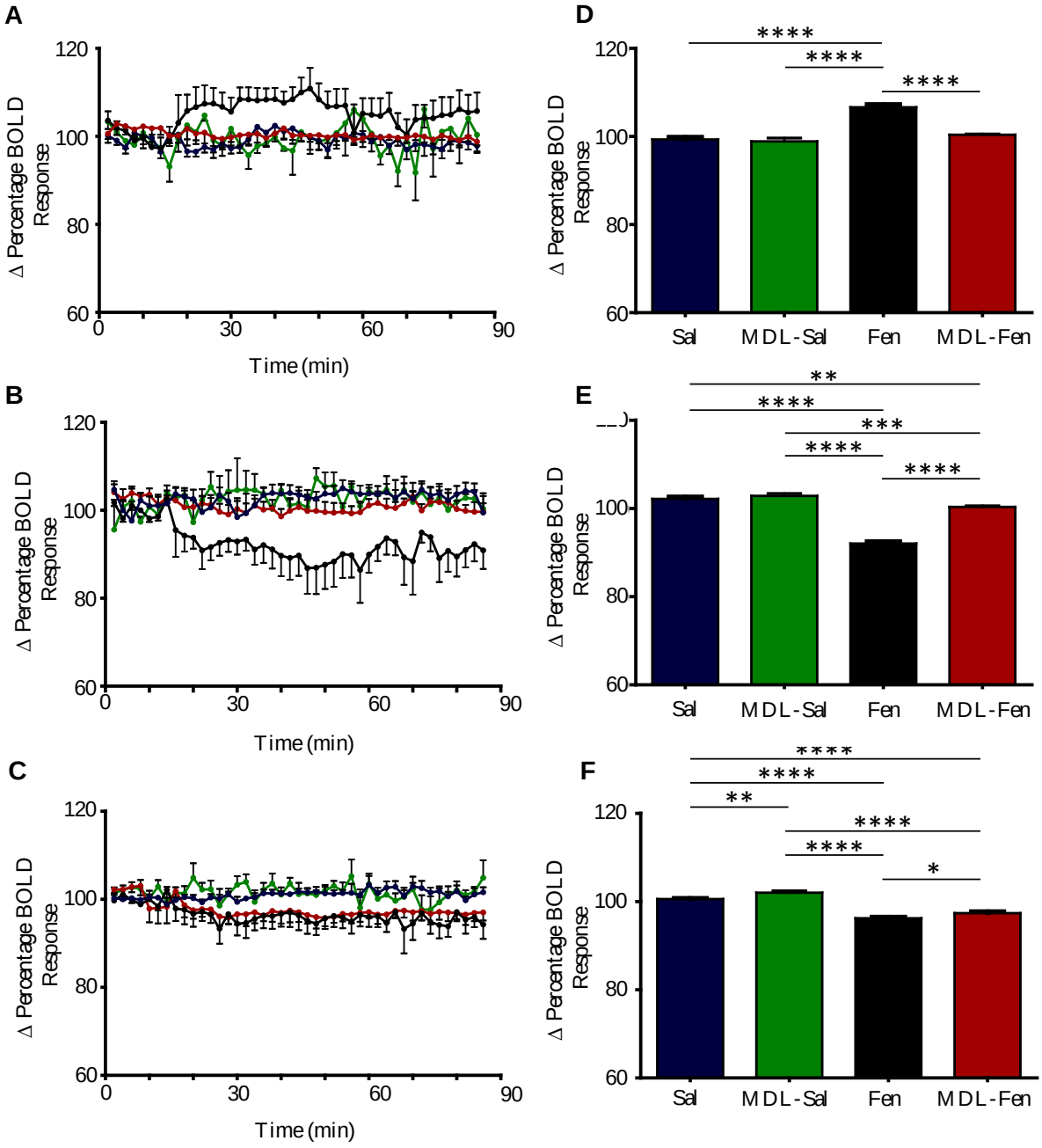


Figure 2.7 Time course plots showing the change in BOLD signal intensity after treatment with selective 5-HT$_{2A}$ antagonist MDL100907. BOLD response is shown in (A) motor cortex, (B) dorsal raphe nucleus and (C) nucleus accumbens following i.v. administration of saline or fenfluramine (10 mg/kg). In two groups animals were pre-treated with the selective 5-HT$_{2A}$ receptor antagonist MDL100907 (0.5mg/kg i.p.) 15min prior to i.v. fenfluramine (MDL-fen) or saline (MDL-sal) injection. Data represent mean ± SEM (n=3-5 per group). Fenfluramine or saline was administered i.v. at t = 15min on the timecourse plots (MDL100907 was administered at t = 0 min relative to these plots). (D-F) Bars represent mean ± SEM of the signal intensity over the first 30 min after fenfluramine or saline administration. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

2.5 Discussion

The purpose of this study was to investigate the functional effects of systemic inflammation on the 5-HT system. Challenge with LPS significantly altered fenfluramine-induced BOLD-fMRI responses in a number of brain regions, including the nucleus accumbens, motor cortex and dorsal raphe nucleus. The change in BOLD-fMRI was not caused by either altered 5-HT release, as measure by *in vivo* microdialysis, or by disruption of neurovascular coupling mechanisms, as measured by stimulated cerebral blood flow. qPCR studies of several brain regions (including the prefrontal and motor cortices, striatum, hippocampus and raphe) indicated an increase in 5-HT_{2A} receptors 6 hours post-LPS challenge compared to control animals, but no changes in 5-HT_{1A}, 5-HT_6 or SERT mRNA. Increases in 5-HT_{2A} expression in response to LPS were confirmed by autoradiography of the receptor. Further studies show that 5-HT_{2A} mediated behaviours, such as those induced by agonist DOI, are also decreased in the presence of systemic inflammation. Finally, changes in BOLD function in response to fenfluramine were abrogated by pre-treatment with 5-HT_{2A} antagonist MDL100907. These results suggest that function in the 5-HT system is altered by systemic inflammation and that this effect may be mediated by changes in 5-HT_{2A} receptor expression.

The initial findings in this chapter, that phMRI-measured brain responses to the 5-HT releasing agent fenfluramine were markedly reduced by prior administration of the systemic inflammatory agent LPS, indicate that systemic inflammation can significantly affect neuronal function. These data highlight the novel use of phMRI to study central neurotransmitter systems, and also the potential confound of systemic disease in the interpretation of human fMRI data. Our laboratories have recently reported that the 5-HT releasing agent fenfluramine elicits a pattern of BOLD signal changes across several brain regions in the rat (Preece *et al.*, 2009). This pattern is specific to 5-HT and does not occur in animals pre-treated with p-chlorophenylalanine, a 5-HT depleting agent. This type of experimental paradigm has been replicated in humans using citalopram (McKie *et al.*, 2005). While these measures show a functional change in the 5-HT system, studies so

far have failed to exploit phMRI and use specific antagonists and agonists to further dissect the mechanisms.

Changes in the 5-HT-mediated BOLD signal could have been caused by a failure of 5-HT release. The use of *in vivo* microdialysis enabled the measurement of 5-HT in the motor cortex, an area where the changes in BOLD response were most profound. These data demonstrated that LPS administration did not alter either basal extracellular 5-HT or its metabolite 5-HIAA in cortex, nor fenfluramine-evoked release of cortical 5-HT. Previous data from our laboratories has demonstrated similar increases in 5-HT in response to fenfluramine using microdialysis techniques (Series *et al.*, 1994). This suggests that changes in the BOLD response to fenfluramine are not mediated by pre-synaptic release mechanisms, but perhaps by post-synaptic modifications.

In our hands, the mRNA of a number of 5-HT related genes was significantly increased in mice in response to LPS (see 3.3.7). With this in mind we sought to determine whether the significant changes observed in the mouse were also reflected in the rat. Interestingly, the data indicate that little change occurs in the 5-HT transporter (SERT), or the 5-HT_{1B} and 5-HT_6 receptors in any of the regions studied. However, 5-HT_{2A} mRNA expression significantly increased in the prefrontal cortex after LPS administration. There is much evidence that links 5-HT_{2A} receptors to depression and a decrease in 5-HT_{2A} receptor expression or function would be in keeping with the general view that decreased 5-HT function is an important depression vulnerability factor (Anisman *et al.*, 2008). However, data regarding the role of 5-HT_{2A} receptors in depression are currently contradictory. For example, some post-mortem studies of depressed suicide victims report decreased 5-HT_{2A} receptor binding (Rosel *et al.*, 2000), whilst others show an increase (Pandey *et al.*, 2002). Positron emission tomography studies have also investigated the 5-HT_{2A} receptor in depressed patients, but findings to date are also inconsistent (Meyer *et al.*, 2001; Bhagwagar *et al.*, 2006). 5-HT_{2A} agonists have been proposed as a strategy to relieve depression and to trigger molecular effects linked to antidepressant action – mainly via

actions on dopamine (Berg *et al.*, 2008). However, 5-HT_{2A} antagonists have been shown to have antidepressant augmenting properties (Werneck *et al.*, 2009).

From a functional point of view, the specific 5-HT_{2A} agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) induces very specific behaviours in animals, and is therefore an extremely relevant tool for studying the role of the 5-HT_{2A} receptor. Data here show that a peripheral inflammatory challenge reduces the number of DOI-induced 'wet dog shakes' (WDS). Kouhata and colleagues have shown similar decreases in WDS behaviour in rats after administration of peripheral LPS (Kouhata *et al.*, 2001), albeit with different dosing regimens. Original studies show that this behaviour is not 5-HT_{2A} specific, but rather more generalized, being elicited in response to 5-hydroxytryptophan (5-HTP) and carbidopa, thyrotrophin-releasing hormone and after morphine withdrawal (Bedard and Pycock, 1977). Data in this chapter show that there is an increase in 5-HT_{2A} receptor expression after LPS, but that still 5-HT_{2A} mediated behaviours are decreased. Although these data appears contradictory there are a number of potential explanations. Work in diabetic rats has shown that 5-HT_{2A} receptor expression increases in pathology but that this is accompanied by a decrease in receptor transduction efficacy (Jackson and Barohn, 1998). It is therefore reasonable to assume that the increases in receptor number observed here may not result in significant increases in 5-HT_{2A} response – perhaps the opposite. It is possible to study receptor pharmacokinetics *in vitro* but it is beyond the scope of this thesis to do so.

Whilst it is not possible to study receptor pharmacokinetics, it is possible to study the effects of 5-HT_{2A} antagonism *in vivo*. The theory thus far is that increased expression in specific brain regions leads to a ceiling effect, rendering the receptors essentially useless – possibly by lowering their transduction efficiency. It would therefore seem reasonable to assume that pre-treatment of animals with a selective 5-HT_{2A} antagonist would result in similar functional outcomes – i.e. a decrease in stimulated response. Pre-treatment with the selective 5-HT_{2A} antagonist MDL100907 effectively blocked the BOLD response to fenfluramine in both cortex and DRN, but in the nucleus accumbens attenuation of the

BOLD response was only partial. These findings suggest a strong involvement of the 5-HT_{2A} receptor in the BOLD response to increased 5-HT, at least in the cortex and DRN. Previous studies have detected non-5-HT-mediated changes in c-Fos response to fenfluramine in the caudate nucleus (Javed *et al.*, 1998), and other studies report that fenfluramine releases catecholamines at higher doses (Balcioglu and Wurtman, 1998). We have previously demonstrated evidence for a feedback loop from the anterior cortex to the DRN involving cortical glutamatergic neurones that synapse onto local GABAergic neurons in the DRN (Varga *et al.*, 2001), which is likely mediated by 5-HT_{2A} receptors in cortex (Boothman *et al.*, 2003; Sharp *et al.*, 2007). Thus, the fenfluramine-evoked decrease in BOLD signal observed in the DRN might, in part, be triggered by cortical 5-HT_{2A} receptors, which excite cortico-raphe projections leading to a reduction in neuronal activity in the DRN through activated local GABAergic neurons. The fenfluramine-evoked BOLD decrease observed in nucleus accumbens may reflect activation of local 5-HT_{2A} receptors on inhibitory GABA neurons (Ward and Dorsa, 1996), although there is likely a contribution from additional mechanisms.

It should be noted that the BOLD responses measured herein may not be entirely neurally mediated. There is much evidence to suggest that certain 5-HT receptors, including 5-HT_{2A} receptors, are expressed on the microvasculature of the CNS (McKie *et al.*, 2005). Whilst the cellular location and function of these receptors remains unclear, there is evidence to suggest that 5-HT_{2A} receptors mediate both vessel contraction (Watts, 2002) and dilation (De Clerck *et al.*, 1985) in the brain. Although there is a report suggesting that 5-HT_{2A} receptor blockade inhibits CBF in a model of cortical spreading depression (Gold *et al.*, 1998), it was noted that factors such as vascular size and presence of the blood brain barrier may affect whether 5-HT dilates or contracts arterioles. Nevertheless, some of the observed effects of 5-HT_{2A} blockade may reflect modulation of these direct vascular actions of 5-HT on 5-HT_{2A} receptors. To this end, a model that we have previously used to study neurovascular coupling was used to verify that functional hyperaemic responses remained intact in LPS treated animals (Martin *et al.*, 2006). Although there was some variation in responses between LPS- and saline-treated

animals, these experiments demonstrated that it was still possible to elicit robust haemodynamic responses to increased neuronal activity in the LPS treated animals. Previous studies have shown that endotoxin-induced inflammation significantly alters CBF and disrupts autoregulation (Rosengarten *et al.*, 2008). Moreover, it has been hypothesised that systemic endotoxin causes a direct uncoupling of the cerebral microvasculature from its neuronal input, but that this is not caused by inflammation-induced oedema (Rosengarten *et al.*, 2008). In contrast, data from this chapter show that, in the cortex at least, neurovascular coupling mechanisms appear to remain intact during the acute phase of systemic inflammation.

Finally, we have shown elsewhere (*see* Chapter 3; p101-103) that behavioural responses to LPS and consequent changes in receptor expression, persist beyond 6 hours in a mouse model of sickness. However, in the rat model, even changes in 5-HT_{2A} expression, which were significant at 6 hours post challenge, do not continue to be so at 24 or 48 hours after administration of LPS (data not shown). Studies of inflammatory cytokines in the periphery after a single LPS challenge suggest that, in the rat, they do not persist much beyond 6-12 hours.

2.6 Conclusion

The data from this chapter have shown that systemic inflammation is capable of abrogating the BOLD fMRI response to stimulated 5-HT release. The mechanisms underlying the effects of systemic inflammation have been shown to not be pre-synaptic in origin, either through compromised 5-HT release or activated brain IDO, and that they do not reflect a disruption of neurovascular coupling – something which would significantly affect the BOLD response. 5-HT_{2A} receptor expression increased in response to LPS in frontal brain regions, specifically in areas associated with mood and depression in humans. The pattern of LPS-induced effects is strikingly similar to those observed with a specific 5-HT_{2A} receptor antagonist. When combined with data from studies using LPS in the presence of 5-HT_{2A} agonist DOI, these findings suggest that the effects of systemic inflammation on central 5-HT function reflect, at least in part, modulation of 5-HT_{2A}

signalling pathways in regions of the brain known to regulate mood. While these data show that inflammation is capable of regulating the 5-HT system, they do not have sufficient scope to determine whether depression may result in inflammation. The chicken/egg debate as to whether inflammation causes depression or whether the reverse is the case, is outside the scope of this chapter, but chapters later in the thesis may begin to address it.

Chapter 3: Changes in the 5-HT System During Systemic Inflammation in the Mouse

Work thus far has demonstrated that the 5-HT system is functionally adaptable during episodes of immune challenge. Both 5-HT receptor expression, and the functional output from this system, are subject to change when an animal is challenged with an inflammatory agent such as LPS. While new strains of transgenic rat are more widely available there is simply not the range of transgenic lines that there is for mice. Anecdotal evidence also suggests that rats appear more 'stoic' in the face of sickness than mice, although few reports have directly compared the species' behavioural response to a peripheral immune challenge. Recent work has shown that an LPS challenge in rats produces a significantly different inflammatory profile from mice (Schmidt *et al.*, 2012), which supports studies from the late 80's showing that the inflammatory profile in rats (as measured by oedema to an inflammatory challenge) is temporally different to that produced in mice (Tarayre *et al.*, 1989). It was therefore important, for this thesis, to determine whether some of the molecular changes in the 5-HT system that were observed in rats, were also conserved in mice.

In the rat, only acute time points were studied. However, Dantzer (2008) suggests that sickness behaviour is the acute response to an infection and any behavioural changes observed after 12 hours represent behavioural depression. Thus it is important to discover how the molecular profile of the 5-HT system changes with time, and whether there are molecular correlates with this switch from sickness behaviour to behavioural depression. Mechanistically, Dantzer separates sickness behaviour and depression but sickness behaviour is an immediate motivational state that mimics depression in a number of ways and could, in fact, be considered behavioural depression with a febrile component. Recent work by Painsipp and colleagues (2011) has demonstrated that LPS has the capacity to induce long term behavioural changes, up to 28 days post-challenge. However, this is one of the few studies to investigate the longer term changes associated with LPS and generally doses of between 0.1-0.5mg/kg are assumed not to result in long term depression (Cunningham *et al.*, 2009) and so are rarely used in studies lasting

longer than 6-8 hours. Interestingly, Painsipp also noted that the peak inflammatory response coincides with the initial behavioural depression, and that this has waned before 28 days. This suggests that inflammation may induce changes in cognitive systems associated with motivation, and that these changes persist up to 28 days later. Thus far, this concept has remained unexplored in the literature.

The study of the 5-HT system and its role in motivation and mood began in the early 1960's and 70's with the discovery of the anti-depressant properties of imipramine and iproniazid (*see* 1.3) as well as studies of 5-HT metabolites in the CSF and blood of depressed patients (Asberg *et al.*, 1976). It was found that low levels of 5-HT contributed to low mood but contributions to the field since this time have been confounded by the variety of symptoms presented by depressed patients (Schatzberg, 2002). If it is assumed that sickness behaviour and depression may represent similar motivational states, at least in terms of neurotransmitters, it could also be assumed that similar changes in 5-HT should be present. Despite the obvious connections, studies using LPS as an immune challenge have failed to find any global changes in brain 5-HT (O'Connor *et al.*, 2009). In fact, work using *in vivo* microdialysis has shown that regional levels of 5-HT actually increase during episodes of inflammation (Linthorst *et al.*, 1995). These data highlight unanswered questions in the current literature regarding the role of 5-HT in sickness behaviour, and specifically within selected anatomical regions, and whether other mechanisms may exist to explain the behavioural changes which occur during episodes of inflammation.

It has been established that peripheral inflammatory stimuli cause behavioural changes in both normal and transgenic mice (Bhatnagar *et al.*, 2004; Pitychoutis *et al.*, 2009), yet the molecular mechanisms that underlie these behavioural changes are largely unknown. The current theory states that peripheral cytokines increase IDO activity within the brain, which reduces the availability of tryptophan, a precursor for 5-HT synthesis (Maes *et al.*, 2007). These data suggest that IDO decreases tryptophan availability for the synthesis of 5-HT, however, the depletion of tryptophan using dietary methods has not been shown to

significantly induce depression (Barr *et al.*, 1997). This, in combination with recent data showing that tryptophan depletion and IDO activity are independent of each other (Hughes *et al.*, 2012), indicate that other mechanisms may be responsible for behavioural changes during sickness.

It is also possible that altered levels of receptors contribute to the pathology of both sickness behaviour and depression. Suicide victims have been shown to have high levels of 5-HT_{1A} receptor expression in the raphe nucleus (Stockmeier *et al.*, 1998), as well as high numbers of 5-HT_{2C} receptors in the prefrontal cortex (Niswender *et al.*, 2001). Animal models of depression also frequently show increased receptor expression levels, even when the genetic manipulation is in an unrelated gene. Pang and colleagues (2009) studied the R1/6 transgenic model of Huntington's after observing depression-like behaviour, and found increased levels of 5-HT_{1B} and 5-HT_{2A} receptors in the hippocampus and cortex. The high DPAT sensitivity (HSD) rat displays a higher sensitivity to the 5-HT_{1A} agonist, 8-OH-DPAT, than its wild type counterparts (Knapp et al., 1998) and is frequently used as a model of depression, displaying high rearing latency and immobility in forced swim and open field tests. These studies clearly show that altered receptor expression is present in disorders where mood is changed, but have not, as yet, shown differences after induction of behavioural depression, as might occur during stress or inflammation.

Altered receptor expression may contribute significantly to altered mood, considering the neuronal circuitry controlling affective behaviours (*see* 1.1). Another likely explanation is altered receptor function. Studies by Tuomisto and Tukiainen (1976) demonstrated that patients with depression showed decreased capacity in the platelet 5-HT transporter (SERT). This finding was followed by data showing that response to antidepressant treatment positively correlated with restoration of SERT function in platelets (Tuomisto *et al.*, 1979). More recently, a high frequency allele of the 5-HT_3 receptor has been associated with the development of Major Depression, and that genetic alterations caused the receptor kinetics to differ considerably (Krzywkowski *et al.*, 2008). 5-HT_{2A} receptors

in depressed patients show similar changes in kinetics, in both the hippocampus and prefrontal cortex (Khait *et al.*, 2005), indicating that some of these changes may show regional specificity.

In terms of CNS inflammation, little literature currently exists regarding the extent to which this persists after a peripheral inflammatory challenge, and the effect it has on the 5-HT system. The CNS inflammatory response will largely be propagated by microglia and astrocytes, and some evidence links both to the 5-HT system. The 5-HT_7 receptor is the only 5-HT receptor to have been successfully found on microglia and has also been found to be intimately linked to cytokine release (Mahe *et al.*, 2005). While cultured microglial cells will be less relevant to the clinical situation than *in vivo* studies, they do show a reduced response to the pro-inflammatory cytokine interferon-γ when pre-incubated with anti-depressants such as fluoxetine and citalopram, suggesting they express SERT (Hashioka *et al.*, 2007; Liu *et al.*, 2011). There is currently no available literature confirming the expression of the transporter on microglial cells, suggesting that either these experiments are yet to be performed, or that anti-depressants may bind to other cell surface receptors. In contrast, the 5-HT transporter is well characterized on astrocytes (Inazu *et al.*, 2001), but has been shown to be genetically distinct from the neuronal transporter. This version of the transporter allows astrocytes to both take up, package and release 5-HT (Watson, 2009). Despite these data, work on the role of either of these immune cells in the propagation of the CNS immune response, and the contribution of this to changes in behaviour, are unclear.

Data from the Sharp laboratory has previously shown using *in situ* hybridization that a peripheral immune challenge is capable of up-regulating the 5-HT_{2A} receptor in the CNS of mice (Qin Xie, unpublished). Considering extant studies on changes in this receptor in depressed patients, these data contribute positively to the theory of inflammation as a vulnerability factor in depression. Changes shown in data from the Sharp lab were largely in the pre-frontal areas of the brain, regions already known to be associated with mood.

Changes in other receptors, and specifically regional changes in these receptors, are yet to be studied extensively, in models of either depression or sickness.

3.1 Aims of this Chapter

Considering work showing differences in the temporal profiles between the immune response in rats and mice (Tarayre *et al.*, 1989), it is important to determine whether the effects observed in the previous chapter have a temporal component, i.e. do they last past the peak of inflammation at 6 hours. The aims of this chapter will therefore be to study behaviour at longer time points, as well as to determine whether there are any molecular changes in inflammation, or the 5-HT system, which are associated with continuing behavioural depression *vs* acute sickness behaviour.

3.2 Materials and Methods

3.2.1 Reagents

All reagents were purchased from commercial suppliers unless otherwise stated in the text.

3.2.2 Animals

Animals were provided with food and water *ad libitum* and all procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. All animals were treated under PIL 30/7863 and PPL 30/2524 and 30/2620. Adult male CD1 outbred mice were obtained from Harlan at c.8 weeks of age and housed under standard conditions. CD1 mice were used as an extension of work from the Sharp laboratory. All animals were allowed to acclimatize for 1 week prior to the onset of experiments to account for the effects of transport stress.

3.2.3 Injection Procedure

Animals received a single intraperitoneal injection of LPS or an equivalent volume of saline (c.0.1ml) at c.8am and were allowed to recover for 6, 24 or 48 hours prior to behavioural testing or sacrifice. The dose of 0.5mg/kg was based on previous work from the Sharp laboratory (Qin Xie, unpublished), as well as published work on sickness behaviour and immunity (Wohleb *et al.*, 2012). Timing of experiments was also designed based on the previous work from the Sharp laboratory.

3.2.4 Tissue Collection

Animals were surgically anaesthetized with 0.1ml pentobarbitone. Blood was removed by cardiac puncture. Peripheral tissues were perfused with 0.9% saline at 20ml/minute for 1-2 minutes. Whole liver lobes were bisected and snap frozen on isopentane on dry ice (approximately -78^{o}C). Brains were removed for molecular biology and dissected into regions for qPCR. Specific regions included the prefrontal cortex, motor cortex, striatum and hippocampus. Tissue was dissected as per instructions in chapter 2 (*see* 2.3.9).

3.2.5 Neutrophil Immunohistochemistry

Animals were surgically anaesthetized with pentobarbitone and transcardially perfused for approximately 1 minute with a solution of heparinized saline (0.9%), followed by 5 minutes of 4% paraformaldehyde (PFA). Liver tissue was removed and post-fixed in PFA for 24 hours prior to embedding in OCT-embedding media (Fisher Scientific, UK) at -20^{o}C. Frozen tissue was cut at 12µm using a Leica cryostat (Leica Microsystems, UK) onto gelatin-coated slides (made in house). Neutrophils were identified using light-microscopy immunohistochemistry. Briefly, sections were quenched in a 3% hydrogen peroxide/methanol solution to reduce endogenous peroxidase activity. Endogenous biotin was reduced using an avidin-biotin blocking kit (Vectorlabs, UK) and non-specific binding was blocked using 10% rat serum. Primary antibody MBS (1:10,000; made in house) against whole mouse neutrophils was applied to tissue sections at room temperature for 2-3 hours. Streptavidin-conjugated secondary rat anti-mouse antibody (1:100; Vectorlabs, UK) was applied at room temperature for 1 hour and the signal was amplified using an avidin-biotin complex kit (ABC Kit, Vectorlabs, UK). Visualization was achieved using a diaminobenzidine and hydrogen peroxide solution (DAB; Sigma-Aldrich, UK). Sections were counterstained for visualization of nuclei with haematoxylin. Neutrophil numbers were quantified using a standard 1mm^{2} grid at x20 magnification on a standard Leica microscope.

3.2.6 Molecular Biology

RNA extraction and quantitative PCR were performed as in previous chapters (*see* 2.3.10, 2.3.12 and 2.3.13). RNA was extracted from the prefrontal and motor cortices, striatum, hippocampus and dorsal raphe nucleus and qPCR was performed using primers for a number of different genes (*see* Table 3.1).

Marker	Forward Primer	Reverse Primer	Amplicon Size (nt)
TNFα	GCCTCCCTCTCATCAGTTC TAT	TTTGCTACGACGTGGGCTA	94
IL-1β	CAACCAACAAGTGATATTC TCCAT	GGGTGTGCCGTCTTTCATTA	127
COX-1	TAAGAAAGTTAGAAGTGGT TTTGTCT	CACACCCTACCCTATAATAT AAAGTT	126
IDO	TGCTTACTCTCTTTTCCCTT CC	CATCAGACCTGGTGCTTCA	87
SERT	TGCCTTTTATATCGCCTCC TAC	CAGTTGCCAGTGTTCCAAGA	127
$5HT_{1A}$	AACCAGTTTTGTGTCCTCT CA	AGCACCTAAATAATTTTCTTC TCTGG	115
$5HT_{1B}$	GTCCTGCTGGTTGCTTTGT	TCCGATACACCGTAGCGATT	82
$5HT_{2A}$	CAGGCAAGTCACAGGATAG C	TTAAGCAGAAAGAAAATCCC ACAG	93
$5HT_{2B}$	CCGCTTTGGCAGTTTTATG G	CTGTAAAGTGTGAATGGTGA GAAA	100
$5HT_{2C}$	TCTCCCTTCCTTCCGTATT CC	ACATCAACTTTTCCACATTCA CAA	95
$5HT_{6}$	CCTGACCCTCGGCATCCTG	GGTTCATGGTGCTATTACAG TATCC	149

Table 3.1 Primer sequences for qPCR.

3.2.7 Behavioural Testing

All behavioural testing was carried out at 80 Lux during the light phase of the animals' light-dark cycle. Animals were allowed to acclimatize in the room in which testing was carried out for at least 1 hour prior to the onset of the test to decrease the effects of brief transport stress. Testers were blind to treatment group.

3.2.7.1 Locomotor Activity Testing

Spontaneous horizontal locomotor activity was measured using a photobeam activity system (PAS System, SanDiego Instruments, San Francisco). 16 x 16 passive infrared beams measure spontaneous activity in 10-minute bins across 2 hours. Animals' baseline horizontal locomotor activity was measured 3 days prior to testing at the same time of day as testing procedures were due to be carried out. Animals received injections of either

saline or LPS at 6, 24 or 48 hours prior to testing and were allowed to acclimatize in the locomotor activity room for 1 hour prior to testing.

3.2.7.2 Open Field Testing

The open field is one of the most common behavioural tests used when studying animal psychology. The test has the capacity to measure locomotor activity, exploratory activity and anxiety. It has been known for some time that exploration is a distinct feature of higher animals. When placed in a novel environment the animals have to reconcile the conflict between the impulse to avoid dangerous situations and the need to explore with respect to escape routes (Berlyne, 1966). As a rule, when placed in a novel environment animals will initially undertake risk assessing behaviour by doing a full circuit from their 'home base', this is followed by more extensive excursions as the animals habituate to the space. The number of parameters' monitored is dependant upon the researcher and the aspects of behaviour they wish to observe. For example, animals with a unilateral lesion may be monitored for turning activity where animals which have been challenged amphetamine may only be monitored for locomotor activity and stereotypy. The flexibility of this test leaves results somewhat open to interpretation but for assessment of basic motivation it is standard.

Open field testing was carried out as described previously (Deacon *et al.*, 2007; Jiang *et al.*, 2008). The open field consisted of a rectangular wooden box 20cm x (30cm x 50cm; Figure 3.1), the mice were placed into a corner and allowed to explore for 3 minutes. During this time the number of exploratory rears were counted manually. Speed and distance were quantified *post-hoc* from digital recordings using AnyMaze software (Stoetling Co., UK).

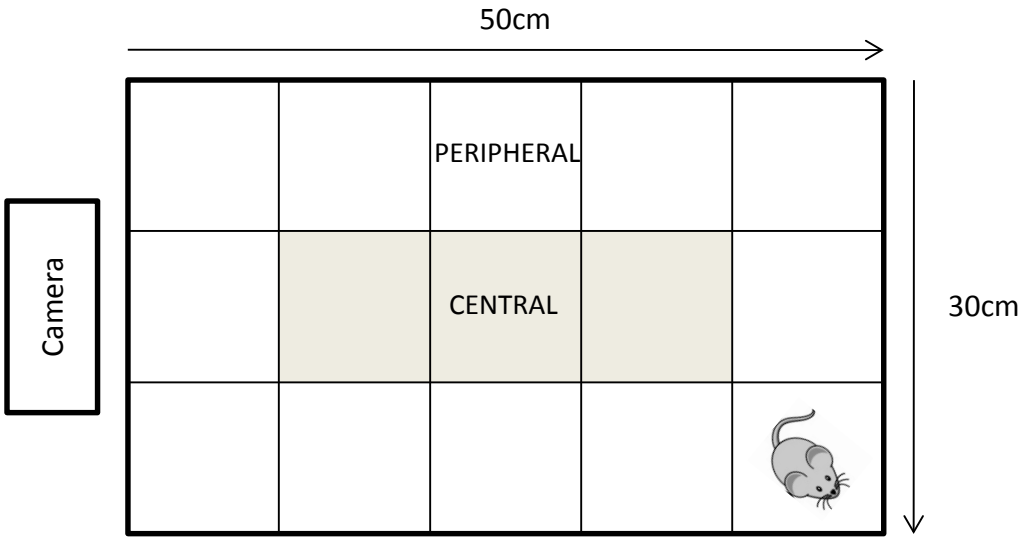


Figure 3.1 Schematic of open field apparatus. Box was black with markings delineating specific 10 x 10 squares. Animals were placed with their nose towards the far right corner of the box (in relation to the camera) and the timer was started immediately.

3.2.8 Cortisol HPLC

Blood was taken via cardiac puncture immediately before perfusion and stored in heparinized vials prior to centrifugation (10k rpm, 10 minutes, 4^{o}C), plasma was removed and immediately stored at -20^{o}C. Cortisol was analysed with the assistance of Dr Michael Stratford (Ludwig Institute for Cancer Research, Nuffield Department of Clinical Medicine, Oxford) using HPLC coupled with mass spectroscopy, based on the principles from Marwah *et al*. (2001). Briefly, plasma samples were diluted 1:1 with distilled water and applied to 2ng of internal standard (5-pregnen-3b-ol-20-one-16a-carbonitrile). Diethyl ether was added to separate organic compounds into a water-free layer. Samples were vortexed and centrifuged (5 minutes, 1500G) to fully separate solvent and aqueous layers. Solvent layers were removed and dried using a heated vacuum centrifuge. Organic residues were dissolved in 100µl eluent A (see below) and applied to columns. Separations were carried out using a Waters 2695 separations module (Waters, Elstree, UK) with an ACE C18 3µm, 100 x 2.1mm column (Hichrom, Reading) maintained at 35^{o}C. The specific eluents were: (A) 2 mM acetic acid and (B) acetonitrile, with a linear gradient of 30-75% of B over 8 min. The flow rate was 0.25ml/min. The eluent was monitored using a Waters Micromass ZQ mass detector using positive electrospray ionization in single ion mode, and Waters Empower 2 software. The mass detector

employed the following conditions: capillary voltage, 2.7 kV; source temperature, 125°C; desolvation temperature, 475°C; desolvation gas flow, 575L/h; cone gas flow, 80L/h. Corticosterone was monitored at m/z 347.1 (M+H), cone voltage 20V. The internal standard CA4 was monitored at /z 302.1, cone voltage 35V.

3.2.9 Neurotransmitter HPLC

Half brains were snap frozen and bisected at the approximate position of Bregma. Cerebella were removed and quarter brains were weighed and homogenized in 0.06% perchloric acid at 10µl/mg tissue. Samples were analyzed using HPLC with electrochemical detection and separated with an ACE microsorb column (C18, 3µm, 125 x 3 mm + ACE C18 guard, 10 x 3 mm run at 35 °C). Samples were carried by an eluent (12.5% methanol, 130 mM NaH_2PO_4, 0.85 mM Na_2EDTA, 0.1mM 1-octanesulphonic acid, pH3.55) pumped with a flow rate of 0.6 ml/min (Waters 2695 HPLC Pump). Samples were detected using a glassy carbon electrode held at +0.75V (Dionex ED40). The dialysate content was determined with reference to daily-calibrated standard solutions in 0.06M perchloric acid (5pmol 5-HT, 5-HIAA, dopamine, DOPAC and noradrenaline). Chromatograms were displayed and analyzed using Waters Empower 2 software.

3.2.10 Statistics

Statistical analysis was carried out using GraphPad Prism and InVivoStat software. Tests included standard Student's t-test and one-way and two-way repeated measures ANOVA with post-hoc tests as appropriate. Post-hoc analysis was either Bonferroni or Tukey. InVivoStat analysis allowed for unstructured co-variance within a repeated measures ANOVA and was used to analyse qPCR data. This allowed for data variance to be different within the repeated measure, specifically the brain region. Data are presented as mean ±SEM. Data was considered statistically significant with a $p<0.05$.

3.3 Results

3.3.1 The systemic response to LPS

Systemic inflammation can be characterized by a significant immune response within the liver. In order to determine the degree to which a single LPS injection causes a state of systemic inflammation, livers were removed at 6, 24 and 48 hours post-LPS and processed for qPCR of TNF mRNA, as well as for immunohistochemistry of infiltrating leukocytes. Two-way ANOVA analysis of TNF mRNA showed significant main effects of LPS treatment and time, as well as an interaction (LPS $p<0.001$ $F_{1,30}=24.17$; time $p<0.001$ $F_{2,30}=16.34$; LPS:time $p<0.001$ $F_{2,30}=16.25$). Specifically, TNF mRNA increased 10,000-fold in the liver after 6 hours of LPS treatment, a significant increase compared to control animals (Bonferroni post-hoc $p<0.001$; Figure 3.2A). At 24 hours (c.10-fold) and 48 hours (c.100 fold) post-challenge mean TNF appeared to remain elevated, but did not reach significance compared to controls (Figure 3.2A). This increase in cytokines results in the induction of acute phase proteins and chemokines within the liver, and the subsequent recruitment of neutrophils which can be used as a positive marker for a systemic inflammatory response.

Analysis revealed significant effects of LPS across time on the numbers of infiltrating neutrophils (LPS $p<0.001$ $F_{1,30}=260$; time $p<0.001$ $F_{2,30}=146.9$; LPS:time $p<0.001$ $F_{2,30}=152.9$). Significant numbers of infiltrating neutrophils were found in the livers of LPS treated animals compared to saline treated animals at both 6 hours (Bonferroni post-hoc $p<0.001$) and 24 hours ($p<0.01$; Figure 3.2B) post-challenge. At 48 hours post-LPS the number of PMNs in the livers of LPS treated animals did not differ significantly from controls (Figure 3.2). PMNs were quantified using positive neutrophil staining under light microscopy, examples of tissue from both vehicle (Figure 3.2C) and LPS treated (Figure 3.2D) animals are shown.

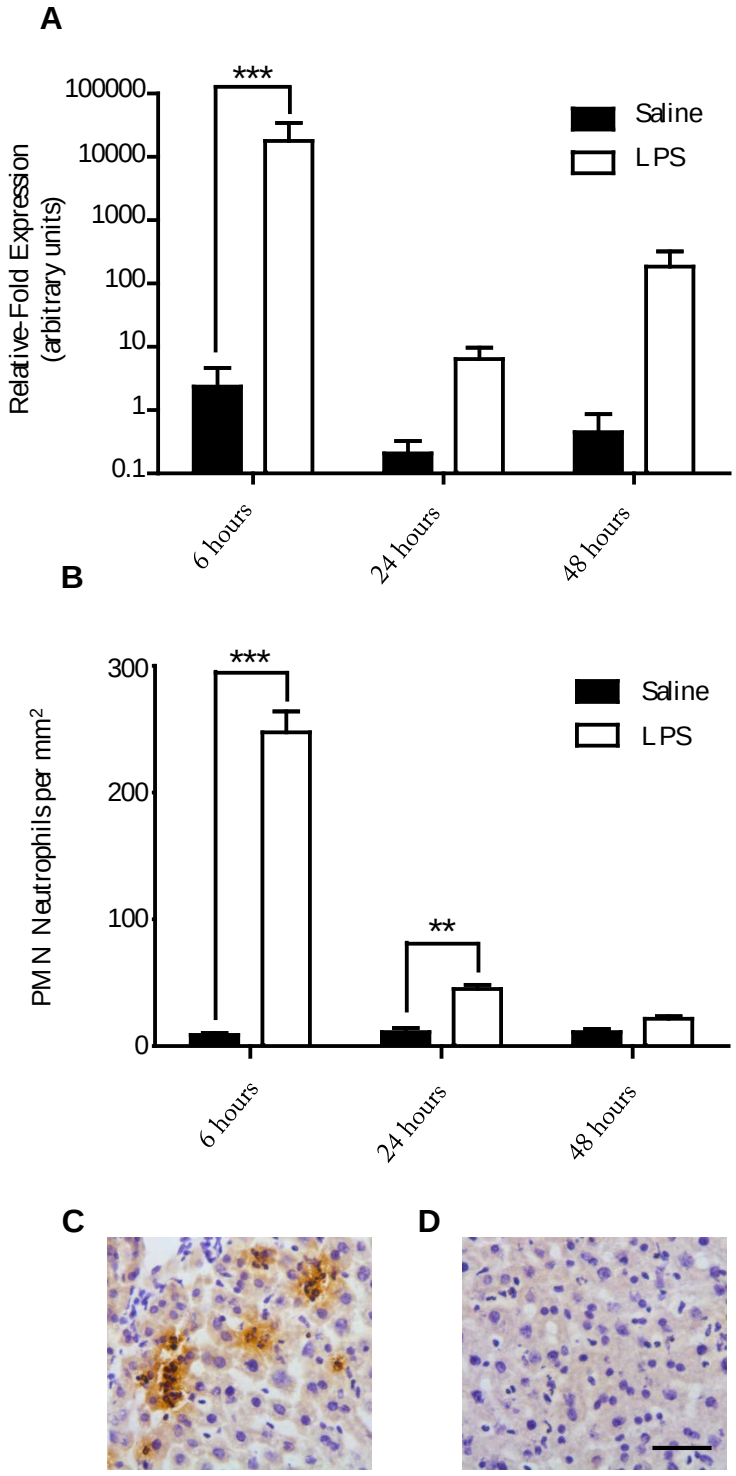


Figure 3.2 Systemic inflammatory response to a single injection of LPS. Animals received a single dose of LPS (0.5mg/kg) and were allowed to recover for 6, 24 or 48 hours prior to tissue collection. Liver tissue was analysed for (A) TNF mRNA and fixed livers were stained for neutrophils and MBS-positive cells were quantified (B). Representative photomicrographs of a control liver (C) and an LPS injected animal (D), arrows indicate positive cells. Data are mean ±SEM, n=6, **p<0.01 and ***p<0.001. Scale bar represents 50µm.

3.3.2 Systemic LPS causes depressed locomotor behaviour not detectable in open field

Peripheral inflammation is known to cause behavioural changes in rodents with cytokine levels naturally peaking between 4-12 hours post-challenge. Animals were challenged with 0.5mg/kg LPS i.p. and allowed to recover for 6, 24 and 48 hours and were studied using both a standard 3-minute open field test as well as a more extensive 2-hour locomotor activity study.

In terms of locomotor activity, baseline values were found to be similar across all animals. Activity was initially high with an average of 350 beam breaks per 10 minute bin, and gradually decreased to 30 per 10 minutes at the end of 2 hours (black line; Figure 3.3A, Figure 3.4A and Figure 3.5A). Animals were then randomly assigned to either saline or LPS groups, and to 6, 24 and 48 hour survival times. At no time point after a saline challenge was the activity in the locomotor activity box different from baseline values (blue line; Figure 3.3A, Figure 3.4A and Figure 3.5A).

At 6 hours post-LPS challenge, animals moved around significantly less from the onset of the test. The first 10 minute bin showed an average of 350 beam breaks in saline challenged controls and 120 in LPS challenged animals, which gradually declined to zero (less than baseline) across time (red line; Figure 3.3A). Analysis demonstrated that animals moved significantly less across time, that LPS treatment affected movement and that there was a significant interaction between LPS treatment and time, specifically that LPS treated animals moved less than saline-treated controls (RM-ANOVA time $p<0.001$ $F_{11,110}=26.6$; LPS $p<0.01$ $F_{1,10}=8.49$; time:LPS $p<0.001$ $F_{11,110}=6.40$; Figure 3.3A). At 24 hours post-LPS, animals showed an average of 250 beam breaks in the first 10 minutes compared to an average of 350-400 for control and baseline animals, which gradually decreased across time reaching baseline values between 80 and 90 minutes (red line; Figure 3.4A). Whilst analysis at 24 hours post-LPS showed no significant effect of LPS, there was an interaction between LPS treatment and time (time $p<0.001$ $F_{11,110}=26.9$; LPS $p=0.07$ $F_{1,10}=3.84$; time:LPS $p<0.001$ $F_{11,110}=5.09$; Figure 3.4A). At 48 hours post-

LPS animals showed an average of 270 beam breaks during the first 10 minutes, compared to 350 for saline and baseline values (red line; Figure 3.5A). Movement gradually decreased across time, in a manner similar to animals studied at 24 hours, reaching baseline levels at approximately 90 minutes. Analysis showed that activity significantly varied across time and that this was affected by LPS treatment (time $p<0.001$ $F_{11,110}=28.23$; LPS $p<0.05$ $F_{1,10}=7.65$; time:LPS $p<0.05$ $F_{11,110}=1.92$; Figure 3.5B).

Open field activity was defined as exploratory rears (both free standing and against a wall), as well as speed and distance over 3 minutes. Saline injected animals consistently showed an average of 45 exploratory rears, an average speed of 0.1m/s and 190m/3 minutes at all time points. At 6 hours post-LPS animals showed significantly decreased activity in all aspects of the open field studied. Specifically, Student's t-tests revealed exploratory rears decreased from an average of 45 to an average of 20 ($p<0.001$; Figure 3.2C), distance decreased from an average of 190m to an average of 140m ($p<0.01$; Figure 3.2D) and speed decreased from an average of 0.1m/s to 0.07m/s ($p<0.01$; Figure 3.3E) in LPS-treated animals when compared to saline injected controls. These changes were not observed at 24 and 48 hours post-challenge (Figure 3.4C-E; Figure 3.5C-E). Overall analysis of rearing behaviour showed that there was an effect of time, an effect of LPS and a significant interaction but that changes were only significant at 6 hours post-LPS (time $p<0.05$ $F_{2,30}=3.64$; LPS $p<0.001$ $F_{1,30}=20.27$; time:LPS $p<0.05$ $F_{2,30}=10.91$). Speed and distance showed similar results.

3.3.3 Systemic LPS causes corticosterone release at 6 hours but not 24 or 48 hours after challenge

Systemic inflammation is known to regulate circulating corticosterone levels in rodents (Zacharowski *et al.*, 2006). Animals received a single i.p. dose of LPS (0.5mg/kg) or saline (0.1ml) and were allowed to recover for 6, 24 or 48 hours prior tissue collection. Two-way ANOVA of adrenal corticosterone levels shows group significance (time $p<0.01$ $F_{2,17}=9.45$; LPS $p=0.4$ ns $F_{1,17}=0.69$; LPS:time $p<0.05$ $F_{2,17}=3.79$), and post-hoc analysis

shows that corticosterone levels were increased significantly at 6 hours post-LPS (Bonferroni post-hoc $p<0.05$; Figure 3.6A) compared to control saline injected animals. 24 and 48 hours post-LPS levels of corticosterone in the adrenal glands were not significantly different from baseline levels. Analysis of blood cortisol showed an overall effect of time (two-way ANOVA $p<0.05$ $F_{2,17}=16.6$) but no overall effect of LPS and no interaction (LPS $p=0.1$ $F_{1,17}=2.86$; LPS:time $p=0.06$ $F_{2,17}=3.3$). Post-hoc analysis showed values were significantly different from control animals 6 hours after LPS treatment ($p<0.01$; Figure 3.6B), but again, was not different from controls at 24 and 48 hours after LPS.

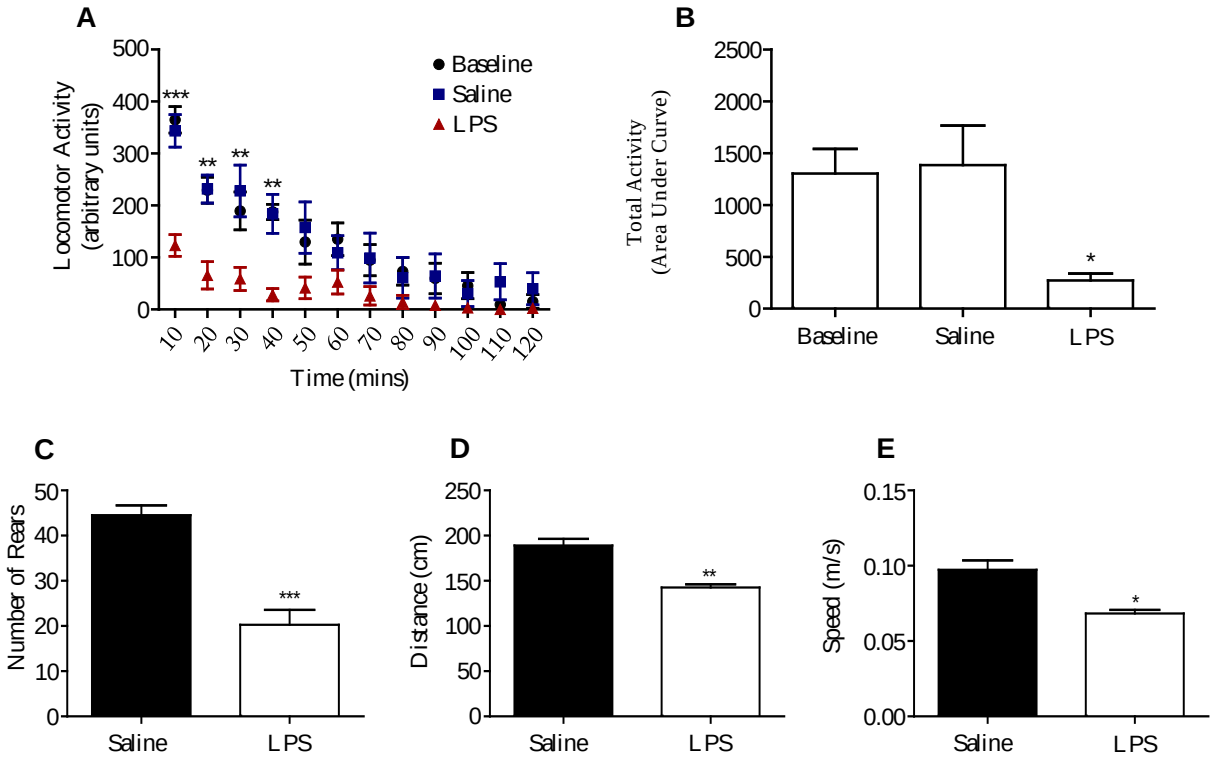


Figure 3.3 Locomotor and open field activity 6 hours post-LPS. Locomotor activity (A) was studied using a beam-break system for 2 hours, at 6 hours post-LPS (0.5mg/kg i.p.). Open field activity in the form of (C) exploratory rears; (D) distance and (E) speed were measured over 3-minutes at 6 hours post-LPS. Data are mean ±SEM (n=6), *$p<0.05$; **$p<0.01$ and ***$p<0.001$.

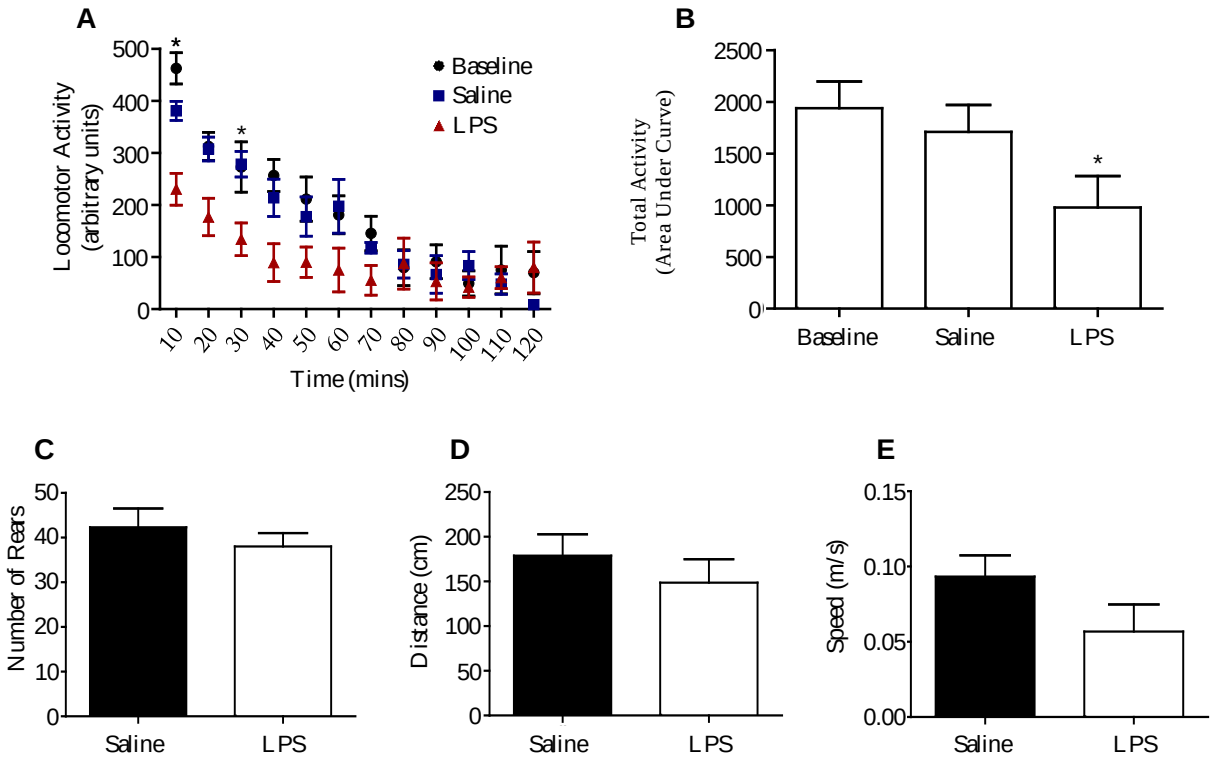


Figure 3.4 Locomotor and open field activity 24 hours post-LPS. Locomotor activity (A) was studied using a beam-break system for 2 hours, at 24 hours post-LPS (0.5mg/kg i.p.). Open field activity in the form of (C) exploratory rears; (D) distance and (E) speed were measured over 3-minutes at 24 hours post-LPS. Data are mean ±SEM (n=6), *p<0.05.

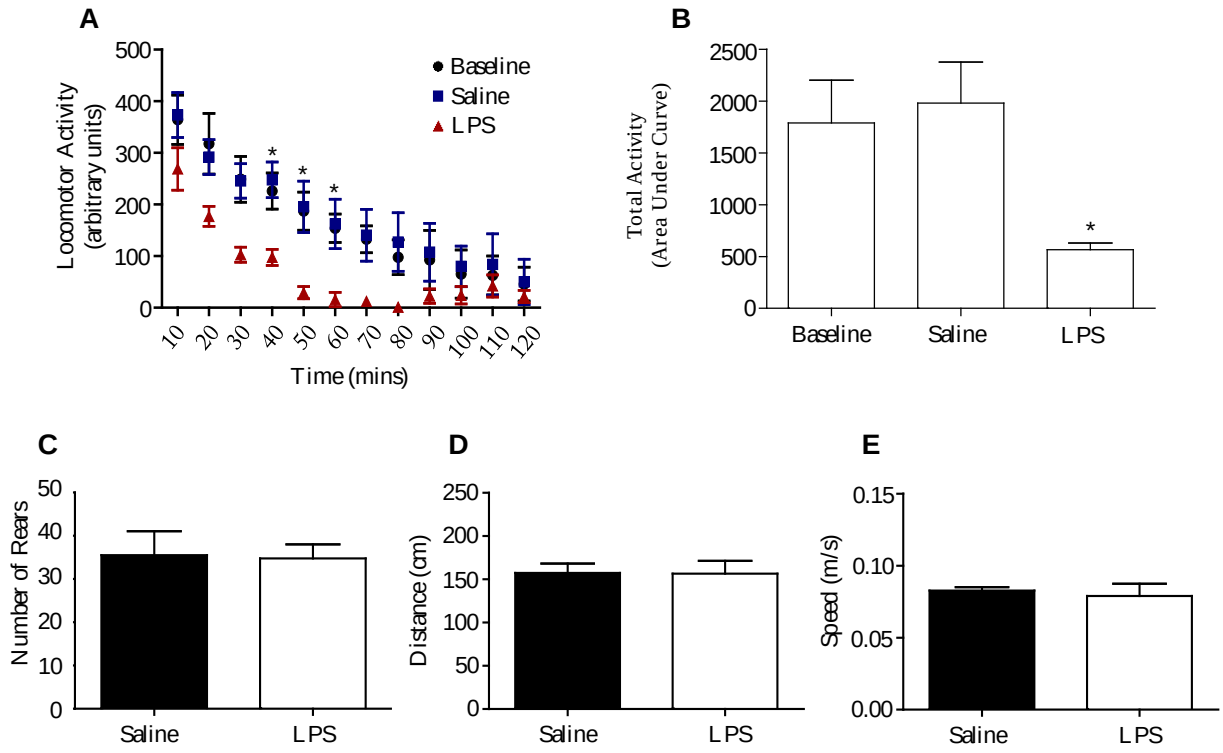


Figure 3.5 Locomotor and open field activity at 48 hours post-LPS. Locomotor activity (A) was studied using a beam-break system for 2 hours, at 24 hours post-LPS (0.5mg/kg i.p.). Open field activity in the form of (C) exploratory rears; (D) distance and (E) speed were measured over 3-minutes at 24 hours post-LPS. Data are mean ±SEM (n=6), *p<0.05.

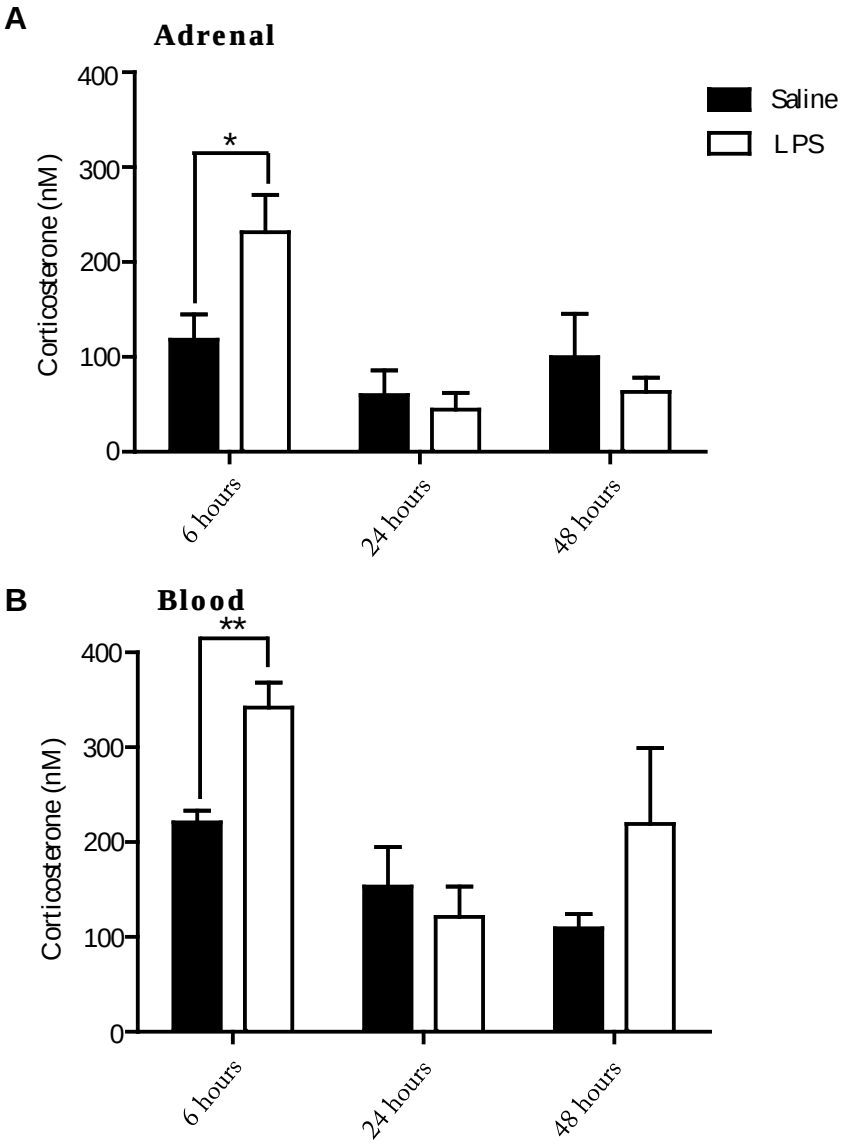


Figure 3.6 Adrenal and blood corticosterone levels after an LPS challenge. Animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to recover for 6, 24 or 48 hours post-challenge. (A) Plasma and (B) adrenal glands were collected and analysed for corticosterone by HPLC, adrenal corticosterone levels are adjusted for tissue weight. Data are mean ±SEM, n=6, *$p<0.05$ and **$p<0.01$.

3.3.4 Systemic inflammation significantly up-regulates IDO mRNA in the raphe at 6 hours post-LPS

Work by Dantzer and colleagues has demonstrated that systemic inflammation up-regulates the activity and mRNA expression of IDO at 24 hours post-LPS (O'Connor *et al.*, 2009) but no other time points were studied. Here, animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to survive for 6, 24 or 48 hours. Tissue was collected fresh from the prefrontal and motor cortices, striatum, hippocampus and dorsal raphe nuclei and analysed for mRNA expression of IDO. Preliminary studies showed that injection stress (0.1ml saline) did not significantly change any mRNA expression studied in this chapter (example TNF data shown; Figure 3.7); therefore all animals were compared to naïve controls.

IDO mRNA expression was significantly affected by both treatment and brain region, and these effects showed an interaction (Two-way, RM-ANOVA LPS: $p<0.05$ $F_{3,20}=4.79$; brain region: $p<0.001$ $F_{4,76}=9.94$; LPS:brain region $p<0.01$ $F_{12,76}=3.02$). Post-hoc tests (Bonferroni) showed that at 6 hours post-LPS, mean IDO mRNA expression was higher than control animals in the motor cortex and dorsal raphe, but these changes only reached significance in the raphe (motor cortex $p=0.07$, raphe $p<0.05$; Figure 3.8). In the prefrontal cortex, striatum and hippocampus there was no appreciable change in IDO mRNA at 6 hours after LPS, compared to control animals. At 24 hours post-LPS there were similar changes in mean mRNA levels in the motor cortex and the raphe compared to control animals, as were seen at 6 hours, but once again these did not reach significance (motor cortex $p=0.071$, raphe $p=0.082$; Figure 3.8). IDO mRNA in the prefrontal cortex showed a slight, but not significant, decrease at 24 hours post-LPS ($p=0.183$; Figure 3.8). The striatum and hippocampus showed no significant change in IDO mRNA at 24 hours post-LPS, compared to controls. At 48 hours post-LPS there appeared to be a decrease in mean IDO mRNA expression in the hippocampus and raphe, compared to control animals, but this did not reach significance ($p=0.195$ and $p=0.103$;

Figure 3.8). In all other brain regions IDO mRNA was not appreciably different from control levels at 48 hours post-LPS.

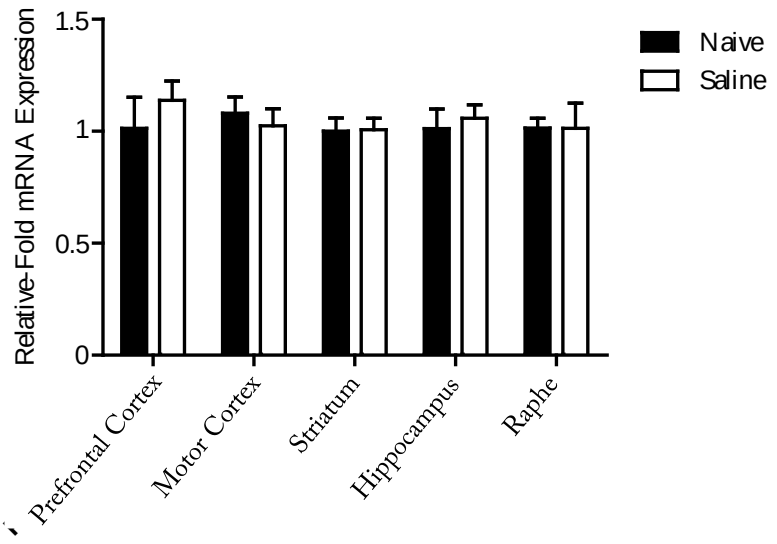


Figure 3.7 TNF expression in the CNS post-injection. Animals received a single i.p. dose of saline (0.1ml) and were allowed to recover for 6 hours post-challenge to compare to completely naïve controls. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for TNF was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6.

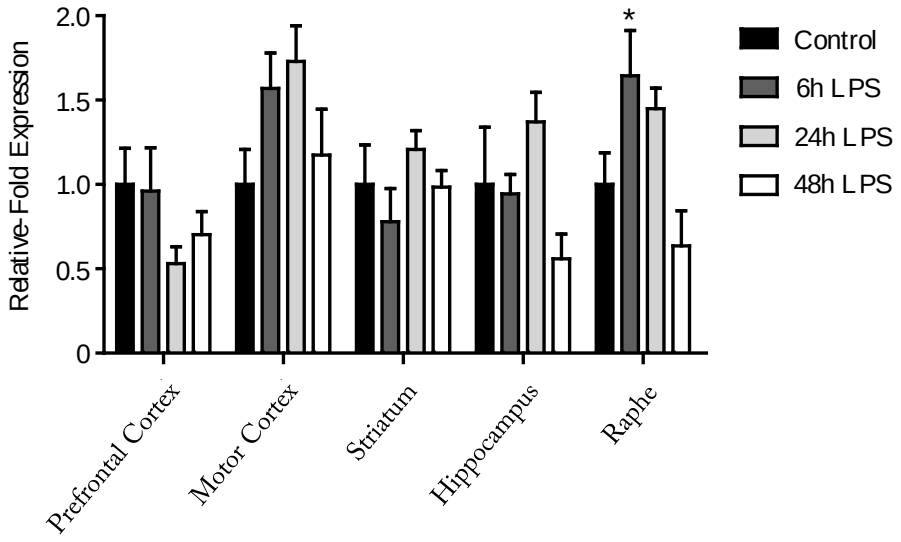


Figure 3.8 CNS IDO mRNA after a systemic inflammatory challenge. Animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to recover for 6 (dark grey bars), 24 (light grey bars) or 48 hours (open bars) post-challenge. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for IDO was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6, *$p<0.05$.

3.3.5 Systemic inflammation up-regulates CNS mediators of inflammation

Having established the basic inflammatory profile of peripheral inflammation post-LPS it was important to establish whether, if at all, this resulted in a substantial inflammatory response in the CNS. With this in mind, brain regions of interest in mood regulation were microdissected and analysed by qPCR for the two primary pro-inflammatory cytokines; TNF and IL-1β, as well as cyclooxygenase (COX). COX exists in two isoforms, constitutive COX-1 which exists in almost all mammalian cells, and inducible COX-2 which is up-regulated during episodes of inflammation. Repeated measures analysis with unstructured co-variance allows for different levels of variation between brain regions whilst still using this as a repeated measure.

There was a significant main effect of both LPS and brain region on TNF mRNA expression, as well as a significant interaction (two-way, RM-ANOVA LPS: $p<0.001$ $F_{3,20}=20.87$; brain region: $p<0.001$ $F_{4,76}=7.22$; LPS:brain region $p<0.05$ $F_{12,76}=1.90$). Post-hoc analysis (Bonferroni) shows that TNF mRNA is up-regulated in all brain regions studied at 6 hours post-challenge when compared to control animals (dark grey bars: prefrontal cortex $p<0.01$, motor cortex $p<0.05$, striatum $p<0.05$, hippocampus $p<0.05$, raphe $p<0.05$;

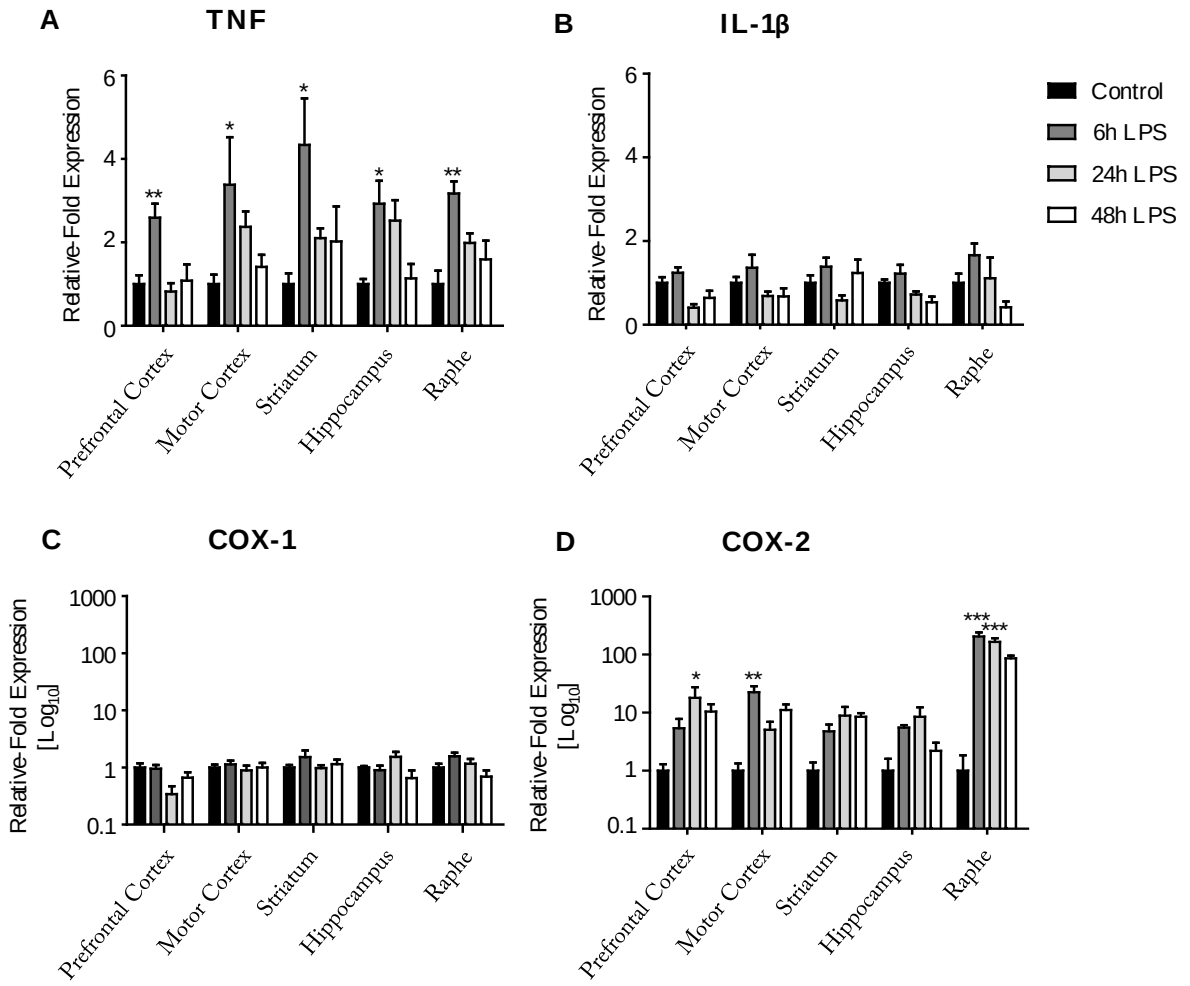


Figure 3.9A). In the motor cortex, striatum and hippocampus mRNA levels of TNF remained higher than control animals at 24 hours after LPS, but the differences were not significant. In the prefrontal cortex and raphe, TNF mRNA levels were not different from control animals (light grey bars;

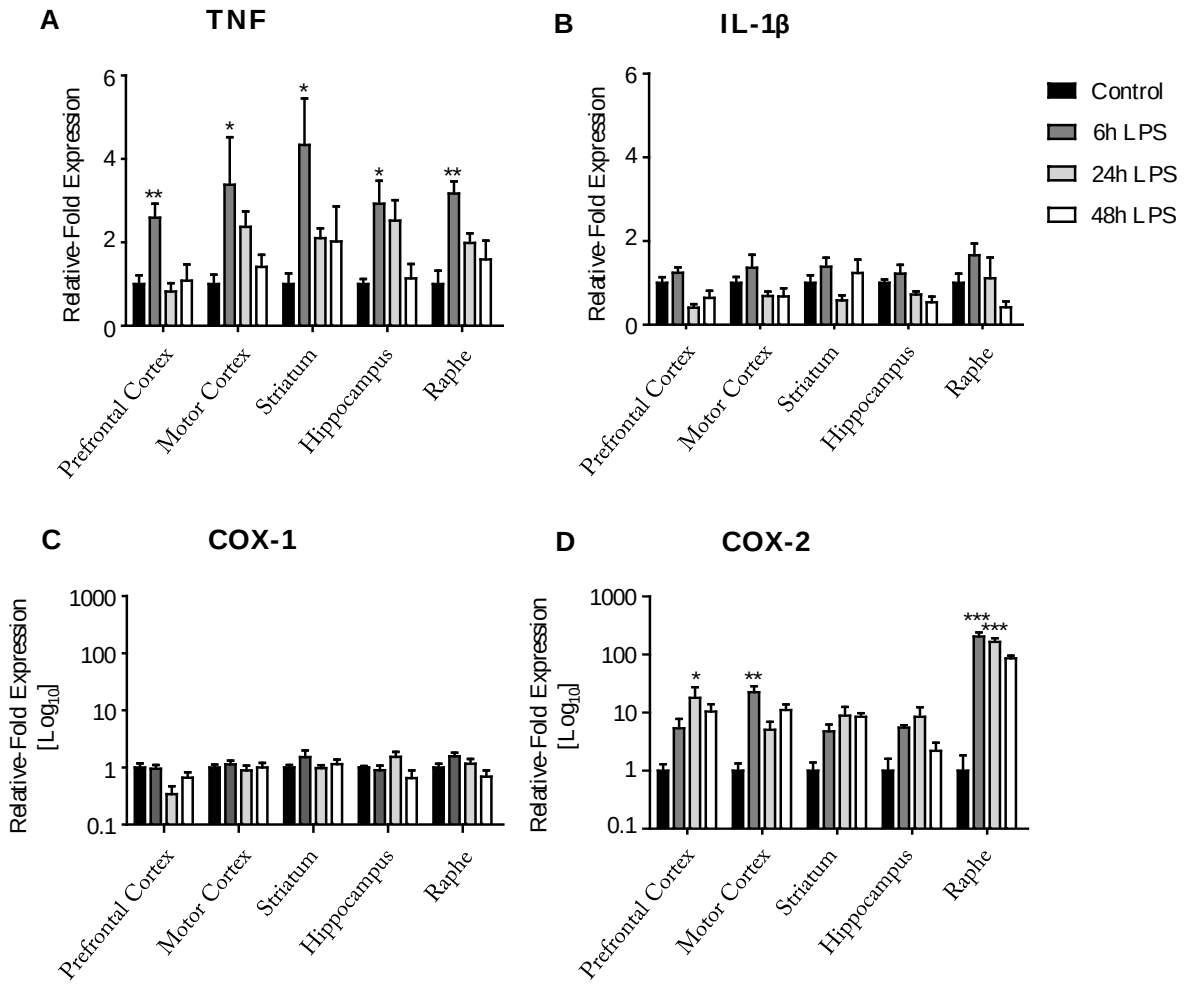


Figure 3.9). At 48 hours post-LPS, TNF mRNA was not significantly different from control animals in any brain region studied (open bars;

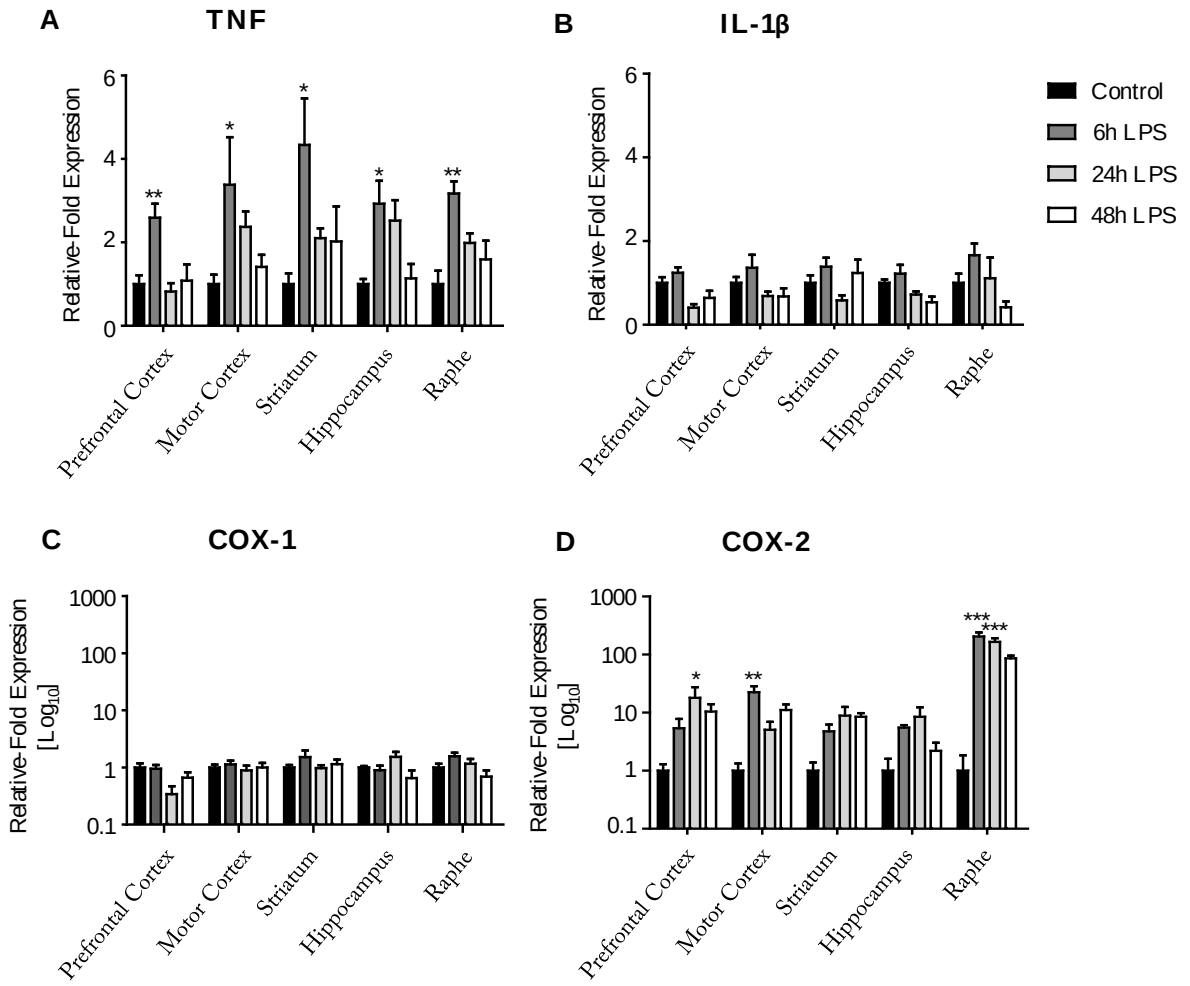


Figure 3.9).

While IL-1β levels do not appear to be different between time points, there is a significant effect of LPS treatment. Despite this, there is no variation in IL-1β mRNA across brain regions and LPS treatment does not affect mRNA expression differently in different brain regions (LPS: $p<0.001$ $F_{3,20}=12.64$; brain region: $p=0.34$ $F_{4,76}=1.92$; LPS:brain region $p=0.34$ $F_{12,76}=1.16$). IL-1β mRNA levels appeared to be lower than controls at 24 hours post-LPS in the prefrontal cortex but this change was not significant. At no other time point in any brain region studied was IL-1β mRNA significantly different in LPS-treated animals compared to control animals (

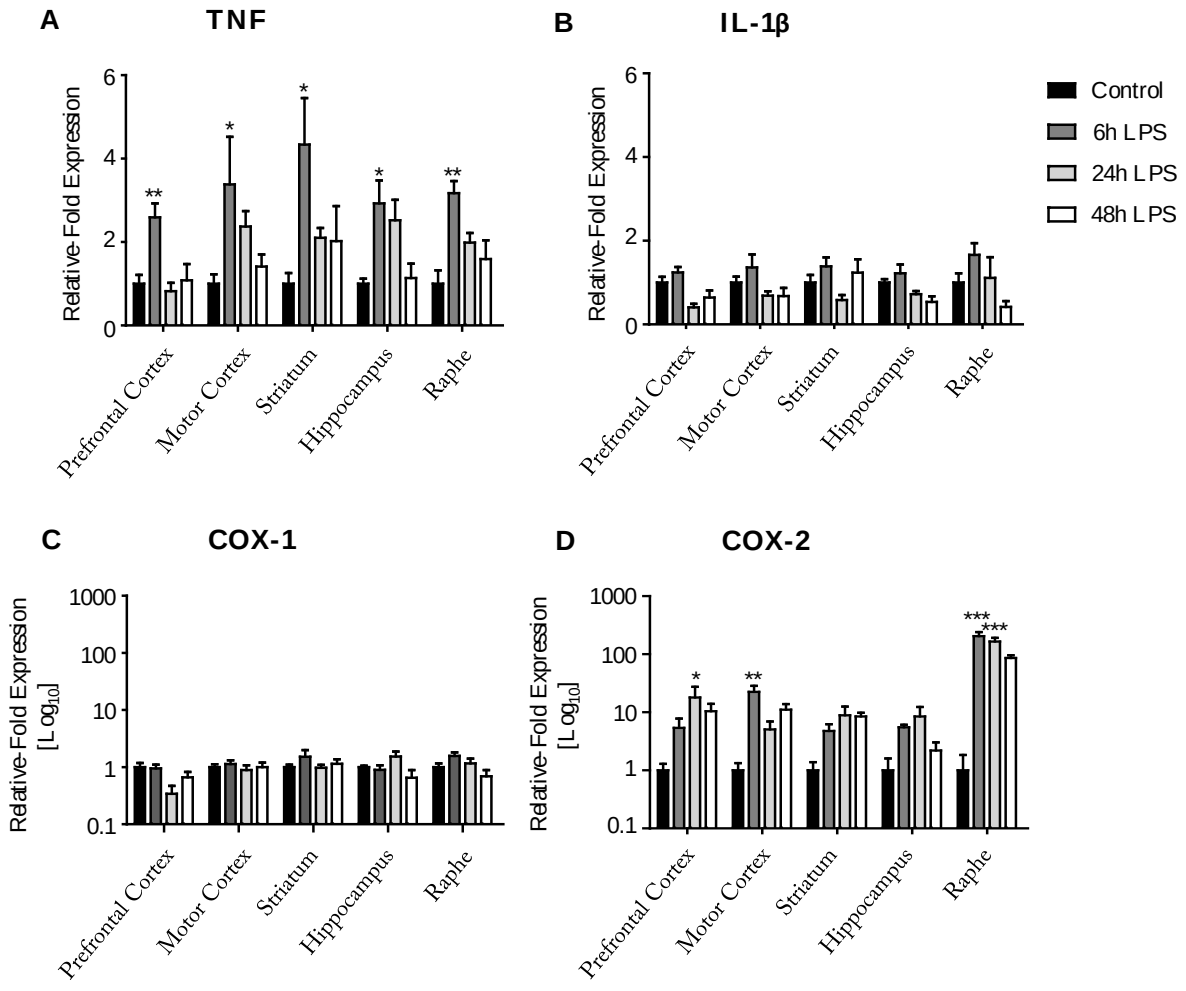


Figure 3.9B). The statistical significance of the overall LPS effect, and the lack of significance in post-hoc comparisons, may reflect an underpowered experiment.

COX-1 mRNA was significantly affected by LPS treatment and this effect was different in different brain regions (LPS: $p<0.001$ $F_{3,20}=15.99$; brain region: $p<0.002$ $F_{4,76}=4.93$; LPS:brain region $p<0.008$ $F_{12,76}=2.66$). However, post-hoc tests (Bonferroni) showed

that mRNA levels did not vary from baseline across any brain region at any timepoint compared to control animals. Much like IL-1β levels this may reflect lack of statistical power.

Analysis of COX-2 mRNA expression showed that there was a significant main effect of LPS treatment, a significant effect of brain region and a significant interaction between LPS treatment and brain region (LPS: $p<0.001$ $F_{3,20}=26.43$; brain region: $p<0.001$ $F_{4,76}=54.77$; LPS:brain region $p<0.001$ $F_{12,76}=14.22$). Specific post-hoc tests show that COX-2 was significantly up-regulated at 6 hours post-LPS in the motor cortex ($p<0.05$) and the raphe ($p<0.001$;

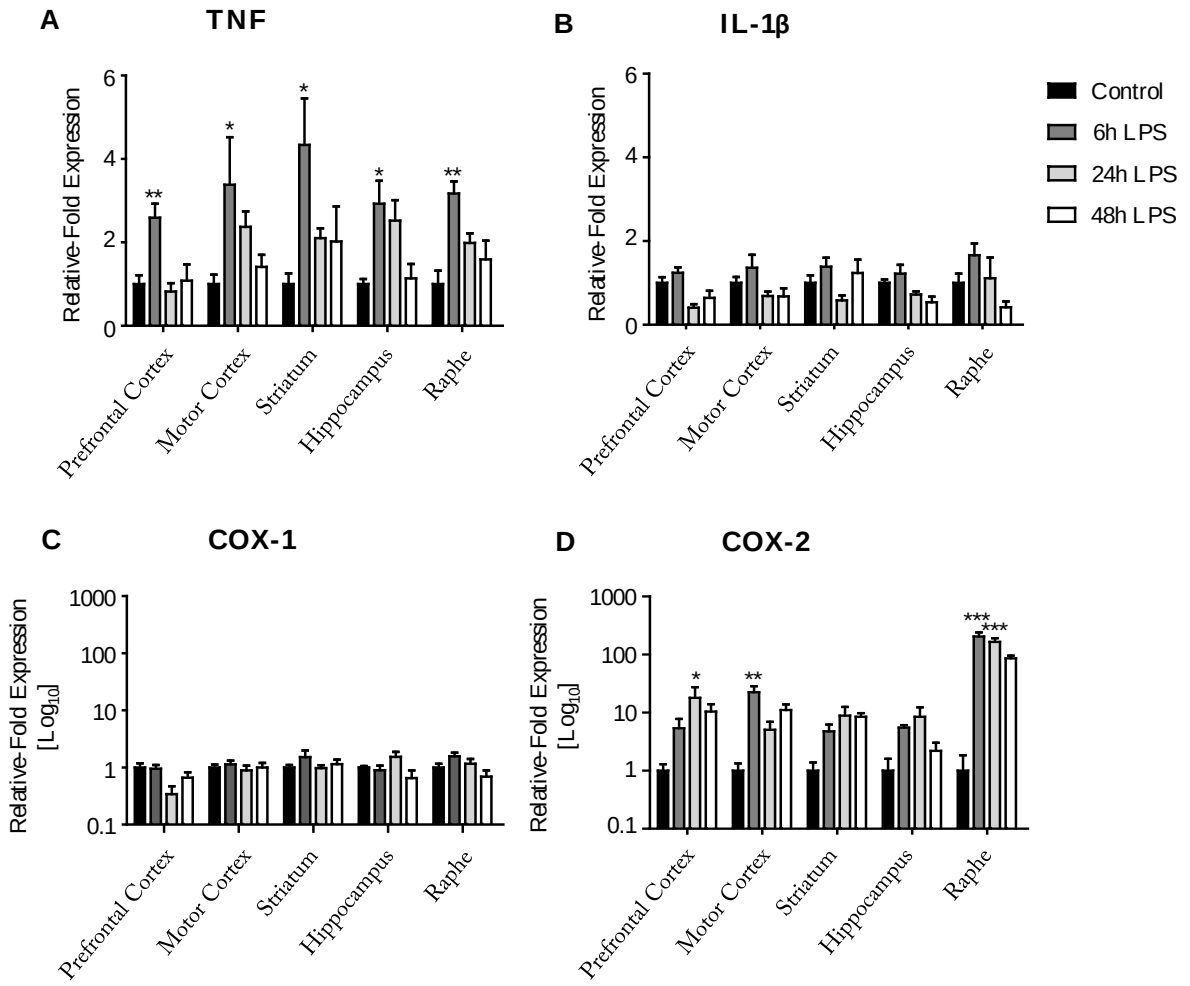


Figure 3.9D), although all other areas also showed a tendency to levels higher than controls (prefrontal cortex p=0.06, striatum p=0.076, hippocampus p=0.08). At 24 hours post-LPS, COX-2 mRNA levels were significantly elevated in the pre-frontal cortex ($p<0.01$) and the raphe ($p<0.001$;

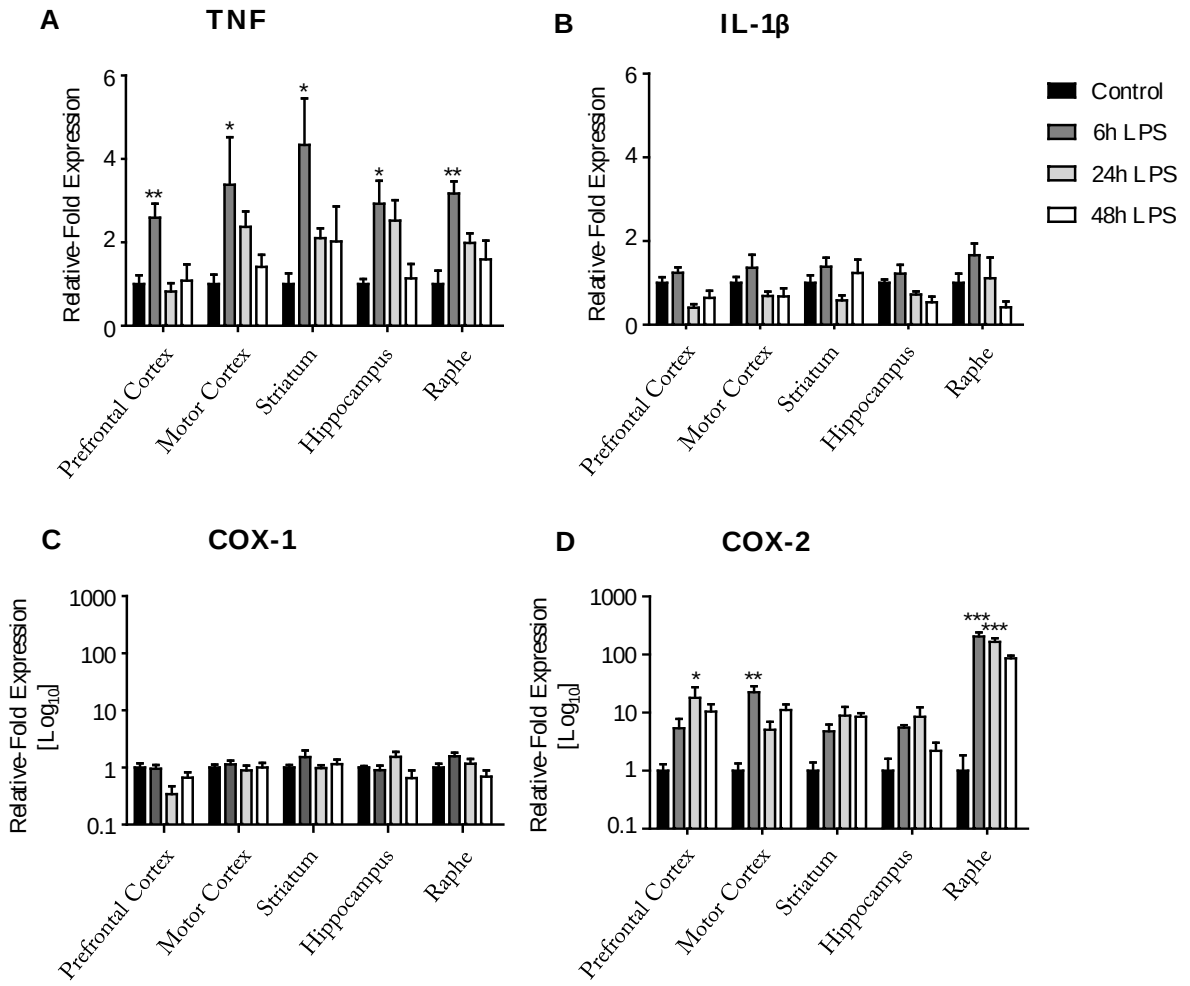


Figure 3.9D). While other areas showed a trend towards higher COX-2 mRNA compared to controls at 24 hours post-LPS, these increases did not reach significance. At 48 hours mean COX-2 mRNA levels remained greater than controls in all brain areas but still not to a degree which was significant (

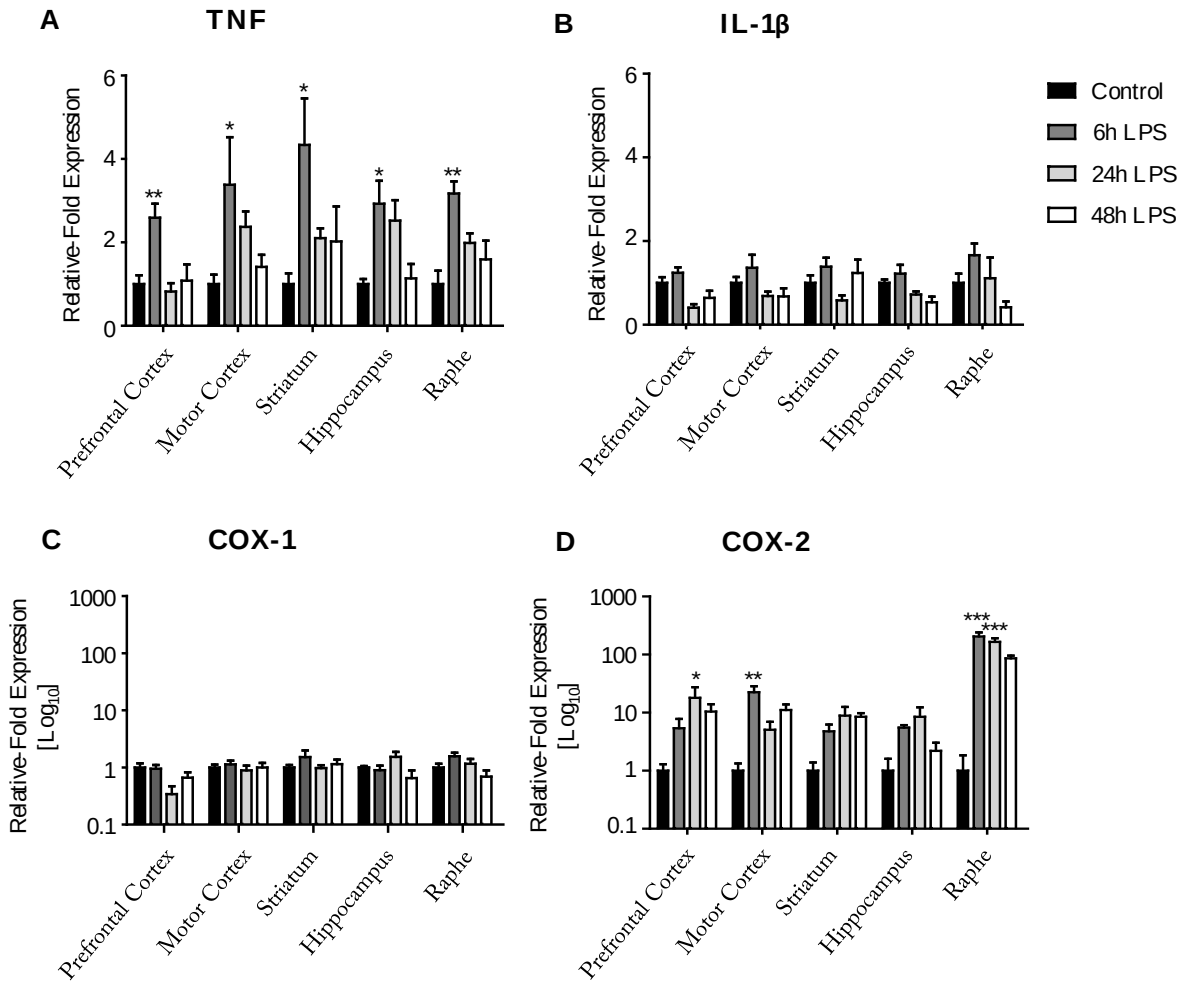

A
TNF
B
IL-1β
Control
6h LPS
24h LPS
48h LPS
Relative-Fold Expression
Relative-Fold Expression [Log10]
Prefrontal Cortex
Motor Cortex
Striatum
Hippocampus
Raphe
C
COX-1
D
COX-2

Figure 3.9D).

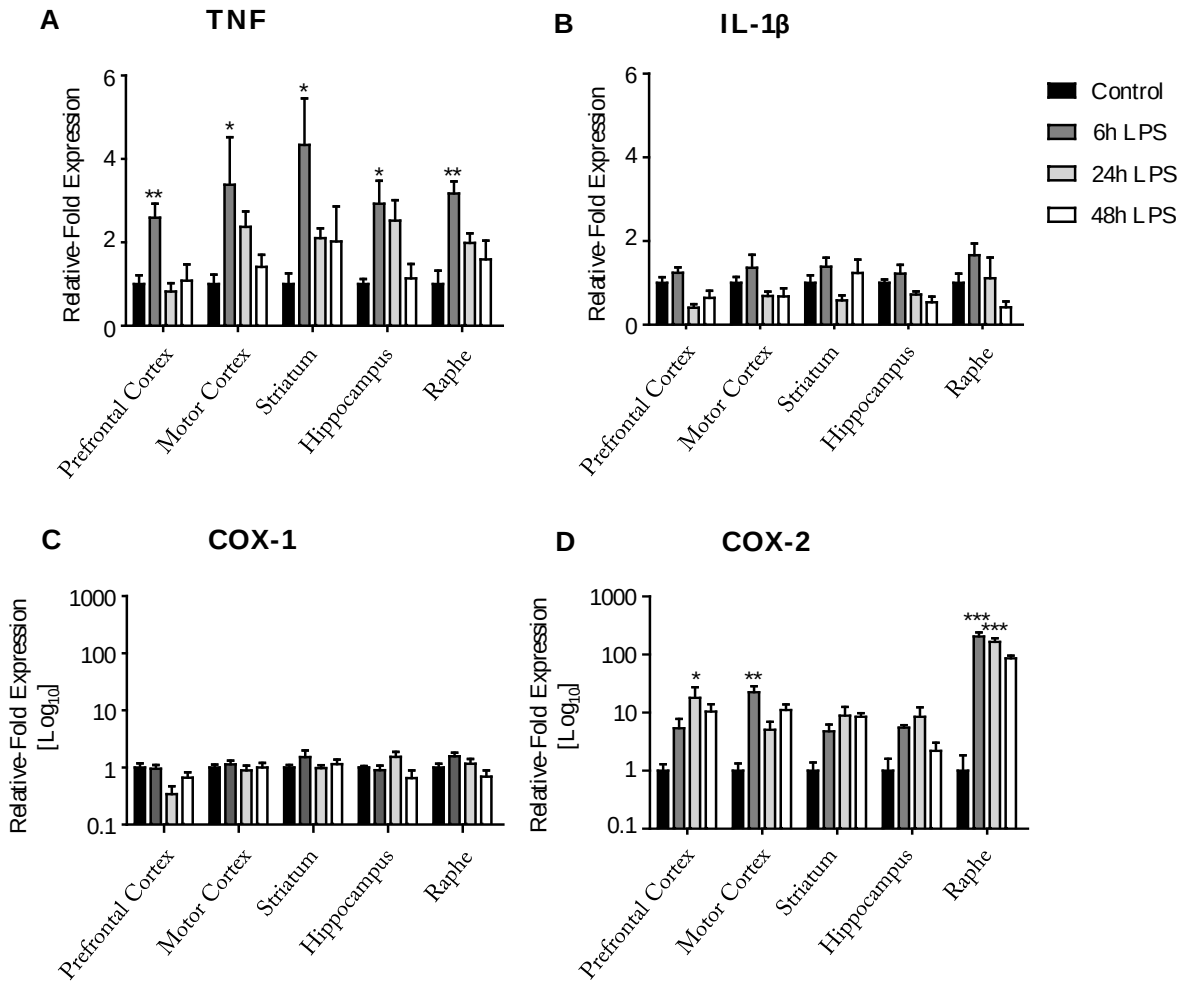


Figure 3.9 CNS cytokines after a systemic inflammatory challenge. Animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to recover for 6 (dark grey bars), 24 (light grey bars) or 48 hours (open bars) post-challenge. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) TNF; (B) IL-1β; (C) COX-1 and (D) COX-2 was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6, *p<0.05, **p<0.01 and ***p<0.001.

3.3.6 LPS does not change total brain 5-HT

The IDO theory of sickness behaviour suggested by Dantzer should result in reduced levels of 5-HT (O'Connor *et al.*, 2009). Here, total brain 5-HT and its metabolite 5-HIAA were measured in fore and hindbrain tissue at 6, 24 and 48 hours after an LPS challenge (0.5mg/kg) or an equivalent saline control (c.0.1ml). The ratio of 5-HT:5-HIAA has been used in other studies as a measure of 5-HT turnover (Godbout *et al.*, 2008) and was therefore applied in this study to determine the effect of LPS on 5-HT metabolism.

In forebrain regions at 6 hours post-LPS 5-HT was slightly higher than baseline but overall LPS had no effect on 5-HT, nor did time (two-way ANOVA; Figure 3.10). Absolute 5-HIAA levels were affected by LPS but there was no significant differences across time (LPS $p<0.05$ $F_{1,30}=5.47$; time $p=0.21$ $F_{2,30}=1.64$; LPS:time region $p=0.38$ $F_{2,30}=0.99$; Figure 3.10). No net change in 5-HIAA:5-HT ratio was evident, therefore, in LPS treated animals compared to controls. In a similar manner, hindbrain regions were affected by LPS but not differently across time (LPS $p<0.01$ $F_{1,30}=25.39$; time $p=0.42$ $F_{2,30}=0.90$; LPS:time region $p=0.49$ $F_{2,30}=0.71$; Figure 3.10 Figure 3.10). This discrepancy between 5-HT and 5-HIAA levels caused the 5-HIAA:5-HT ratio in LPS animals to be increased relative to controls, but this did not reach significance.

In both forebrain and hindbrain regions at 24 and 48 hours post-LPS there was no significant change in either 5-HT or 5-HIAA levels, and hence 5-HT turnover, compared to saline injected animals.

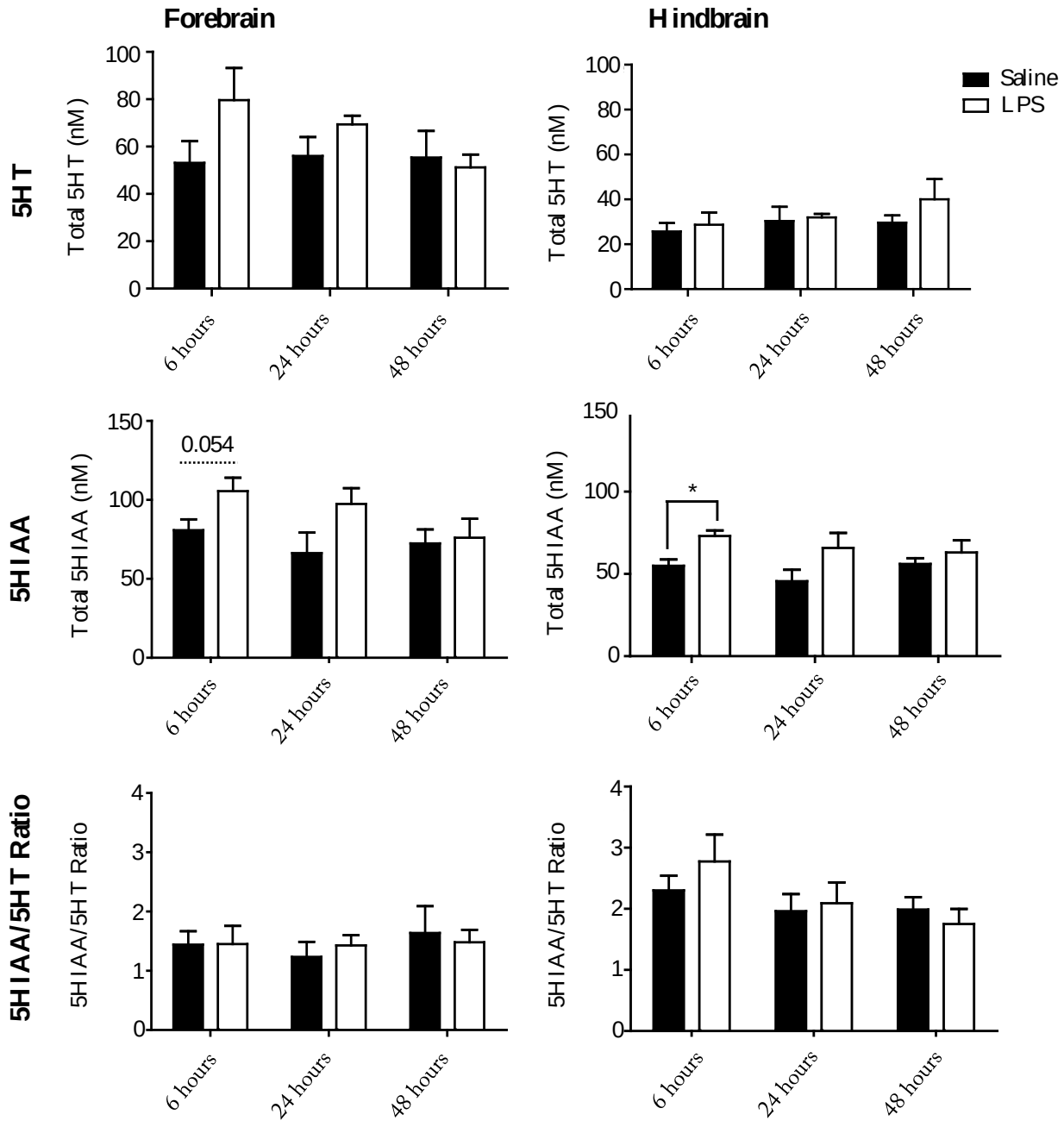


Figure 3.10 Brain 5-HT and metabolite levels after a single i.p. injection of LPS. Fore and hind-brains were removed whole and analysed by HPLC for 5-HT and 5-HIAA levels. Concentrations are normalized for weight of tissue. 5-HIAA/5-HT ratio is expressed as arbitrary units. Data are mean ±SEM, n=6, *p<0.05.

3.3.7 LPS does not alter CNS levels of noradrenaline, dopamine or dopamine turnover

As well as absolute brain 5-HT, CNS levels of dopamine and noradrenaline were measured. Animals were challenged with 0.5mg/kg LPS or an equivalent volume of saline (0.1ml) and allowed to recover for 6, 24 or 48 hours before fore and hindbrain regions were collected. Dopamine, DOPAC and noradrenaline were analysed by HPLC. Relative turnover of dopamine was determined by a ratio of dopamine:DOPAC, as in 3.3.6.

Forebrain dopamine levels were, on average, 1500nM in both saline and LPS injected animals. Absolute levels of dopamine in the forebrain did not change significantly between saline and LPS, or at different time points (two-way ANOVA; Figure 3.11). Hindbrain absolute dopamine was, on average, 250nM and was not significantly different between saline and LPS animals, nor at different time-points post-challenge.

Absolute levels of DOPAC in the forebrain were affected by LPS but not differently across time (LPS $p<0.01$ $F_{1,30}=14.06$; time $p=0.32$ $F_{2,30}=1.2$; LPS:time region $p=0.44$ $F_{2,30}=0.84$; Figure 3.11). This discrepancy between DOPAC and dopamine did not result in a significant change in dopamine turnover at any time after LPS when compared to saline-challenged animals (Figure 3.11).

Noradrenaline levels in saline animals were, on average, 1400nM in both fore and hindbrain regions in saline treated animals (Figure 3.12). LPS treatment did not change the levels in either brain region after 6, 24 or 48 hours of treatment compared to control animals (Figure 3.12).

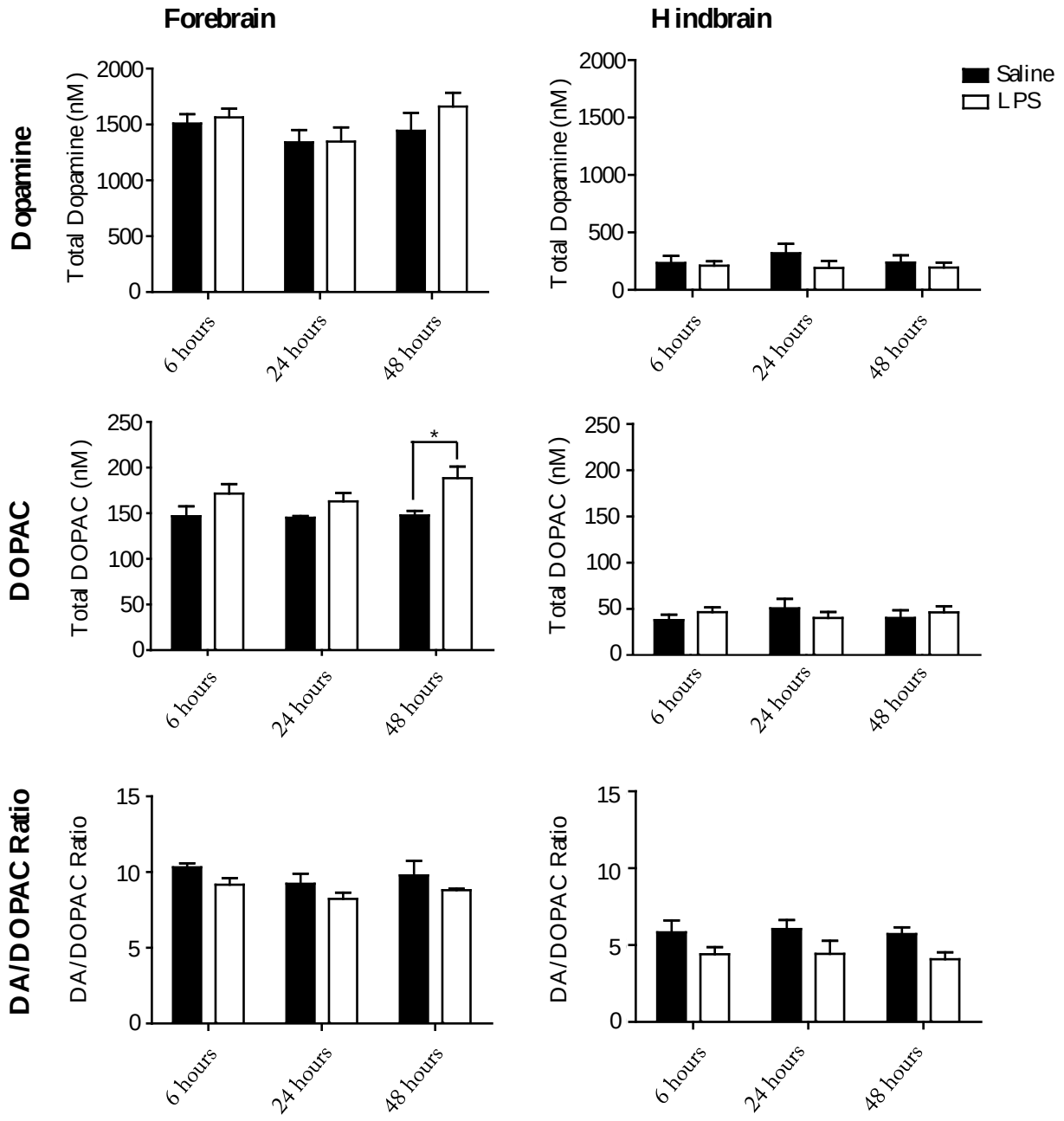


Figure 3.11 Brain dopamine and metabolite levels after a single i.p. injection of LPS. Fore and hind-brains were removed whole and analysed by HPLC for dopamine and DOPAC levels. Concentrations are normalized for weight of tissue. Dopamine/DOPAC ratio is expressed as arbitrary units. Data are mean ±SEM, n=6, *$p<0.05$.

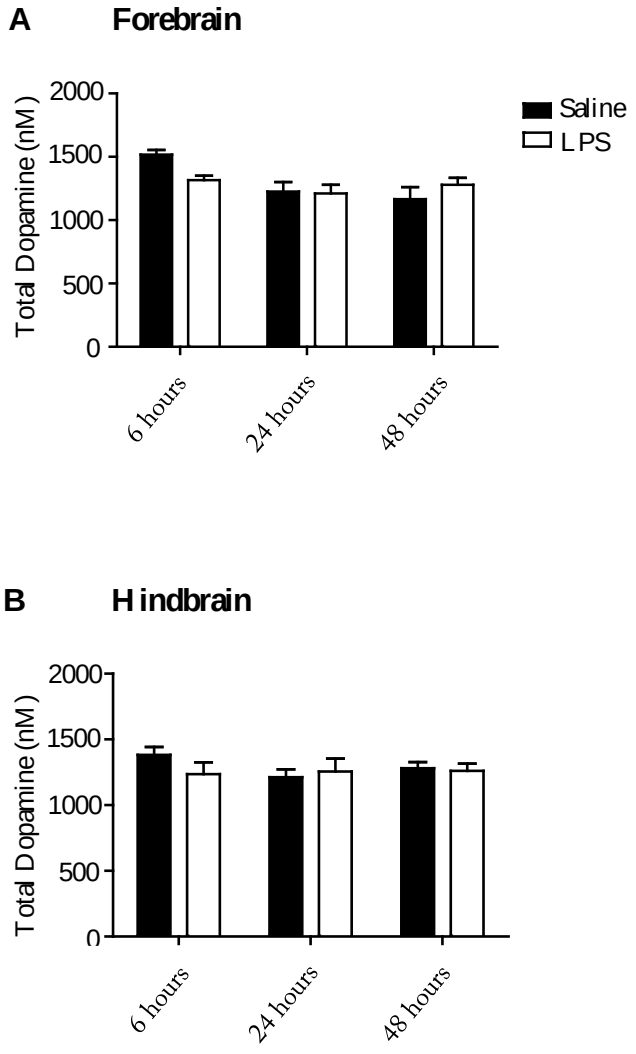


Figure 3.12 Brain noradrenaline levels after a single i.p. injection of LPS. (A) Fore and (B) hindbrains were removed whole and analysed for noradrenaline by HPLC. Concentrations are normalized for weight of tissue. Data are mean ±SEM, n=6.

3.3.9 Systemic LPS alters the expression of 5-HT-related genes

The anatomy of the 5-HT system is complex, and the regulation of the receptors within it even more so. The role of inflammation in the regulation of these receptors has thus far been neglected. Here, experiments sought to determine whether a single systemic inflammatory challenge altered some of the 5-HT related genes associated with changes in mood.

Work by others has shown that 5-HT_{1A} and 5-HT_{1B} receptors may mediate some depressive-like behaviours in animals (O'Neill and Conway, 2001), however, little evidence exists to link these receptors with inflammation. Analysis of the effects of LPS on 5-HT_{1A} expression in the CNS suggests that there is a significant main effect of LPS, a significant main effect of brain region but that LPS does not affect expression differently within different regions (Two-way, RM-ANOVA LPS: $p<0.001$ $F_{3,20}=25.72$; brain region $p<0.001$ $F_{4,80}=7.59$; LPS:brain region $p=1.59$ $F_{12,80}=0.159$). Specifically, post-hoc analysis shows that there were significant differences in expression in the prefrontal cortex at 6 hours post-LPS (Bonferroni post-hoc $p<0.05$; Figure 3.13A) but did not change 5-HT_{1A} mRNA in any other region at this time, compared to control animals. 5-HT_{1A} receptor expression remained up-regulated in the prefrontal cortex, at both 24 and 48 hours post-LPS ($p<0.05$), compared to controls (Figure 3.13A). However, whilst no change in 5-HT_{1A} mRNA in any other region was seen at 24 hours after LPS administration, a significant increase was found in the motor cortex ($p<0.05$) and striatum ($p<0.01$; Figure 3.13A) compared to controls.

5-HT_{1B} receptor expression was significantly affected by LPS treatment, and was different across brain regions, and showed an interaction (LPS: $p<0.05$ $F_{3,20}=5.15$; brain region $p<0.001$ $F_{4,80}=15.28$; LPS:brain region $p<0.01$ $F_{12,80}=2.76$). However, post-hoc tests (Bonferroni) show that expression of the 5-HT_{1B} receptor does not change significantly in any brain region studied at any time point after LPS, compared to control animals (Figure 3.13B). This may reflect a lack of power when studying this particular gene.

5-HT_2 receptors have long been thought to play an important role in mood regulation (Deakin, 1988; Yates *et al.*, 1990), but, like 5-HT_1 receptors, are only recently being investigated in terms of peripheral inflammation (Zhang *et al.*, 2001). In this study, 5-HT_{2A} expression was significantly affected by LPS treatment, showed significant variation across brain regions and was also significantly different in different brain regions (LPS: $p<0.001$ $F_{3,20}=286.83$; brain region $p<0.001$ $F_{4,80}=11.29$; LPS:brain region $p<0.001$ $F_{12,80}=11.73$). Post-hoc analysis (Bonferroni) showed that after 6 hours of LPS treatment was not different to controls in any region studied, although there was a trend to increase in the pre-frontal and motor cortices (Figure 3.14A). At 24 hours post-LPS, all regions appeared to show higher levels of 5-HT_{2A} receptor expression than controls, but this only reached significance in the raphe ($p<0.05$; Figure 3.14B). By 48 hours after LPS, 5-HT_{2A} receptor expression was significantly increased in all brain regions studied compared to control animals (prefrontal cortex $p<0.001$, motor cortex $p<0.001$, striatum $p<0.001$, hippocampus $p<0.001$, raphe $p<0.01$; Figure 3.14A).

5-HT_{2B} receptor expression appeared to peak acutely at 6 hours post-LPS. There was a significant main effect of LPS and a significant main effect of brain region but no interaction between the two factors (LPS: $p<0.001$ $F_{3,20}=25.49$; brain region $p<0.01$ $F_{4,80}=3.78$; LPS:brain region $p=1.66$ $F_{12,80}=0.107$). The peak expression at 6h occurred in the hippocampus (Bonferroni post-hoc $p<0.01$) and raphe ($p<0.01$) but did not change significantly in the other brain regions investigated, compared to control animals (Figure 3.14B). At 24 hours post-LPS, 5-HT_{2B} expression was no longer different from control animals (Figure 3.14B) in any brain region, and the same was found at 48 hours post-LPS (Figure 3.14B).

Analysis of 5-HT2C expression showed a significant main effect of LPS, a very nearly significant main effect of brain region and a significant interaction (LPS: $p<0.01$ $F_{3,20}=8.75$; brain region $p=0.053$ $F_{4,80}=2.45$; LPS:brain region $p<0.001$ $F_{12,80}=5.60$). 5-HT_{2C} expression peaked 6 hours after LPS in the raphe (Bonferroni post-hoc $p<0.05$), and almost reached significance in the motor cortex ($p=0.07$). 5-HT_{2C} mRNA levels

appeared to be higher at 6 hours post-LPS in all other brain regions, compared to controls, but these values were not significant compared to controls (Figure 3.14C). At 24 and 48 hours post-LPS, 5-HT_{2C} mRNA expression was not significantly different from control levels in any brain region (Figure 3.14C), although at 24 hours 5-HT_{2C} expression showed a trend towards increased levels in the hippocampus and raphe in LPS-treated animals, but in all other brain regions studied mRNA expression was not different from control values (Figure 3.14C).

5-HT_6 receptors are currently under investigation as potential novel targets in depression (Kulkarni and Dhir, 2009). Receptor expression in this experiment was different after treatment, across brain regions and showed an inclination to vary in different brain regions after LPS treatment (LPS: $p<0.001$ $F_{3,20}=624.65$; brain region $p<0.001$ $F_{4,80}=50.14$; LPS:brain region $p<0.001$ $F_{12,80}=24.69$). After 6 hours of LPS challenge, mean 5-HT_6 mRNA expression was greater than control values in all areas, but only reached significance in the prefrontal cortex (Bonferroni post-hoc $p<0.01$; Figure 3.15A). No significant changes were found after 24 hours of LPS challenge, although 5-HT_6 expression showed a trend towards increased levels in the prefrontal and motor cortices compared to controls (Figure 3.15A). At 48 hours post-LPS challenge, 5-HT_6 expression was significantly increased, compared to control animals, in the prefrontal cortex ($p<0.05$), motor cortex ($p<0.05$) and hippocampus ($p<0.001$; Figure 3.15A).

The 5-HT transporter (SERT) plays a significant role in depression as the target for many antidepressant drugs. Much like many of the previous receptors, the transporter is significantly affected by LPS and is affected differently in different brain regions (LPS: $p<0.01$ $F_{3,20}=7.93$; brain region $p<0.001$ $F_{4,80}=36.59$; LPS:brain region $p=1.59$ $F_{12,80}=17.92$). Post-hoc analysis (Bonferroni) showed that at both 6 and 24 hours after LPS treatment there were no significant changes in SERT expression, although trends towards elevated expression were seen in several brain regions (Figure 3.15B). By 48 hours after LPS challenge SERT expression significantly increased in the prefrontal

cortex ($p<0.001$), motor cortex ($p<0.001$), striatum ($p<0.01$) and hippocampus ($p<0.05$) but expression in the raphe was not significantly different from controls (Figure 3.15B).

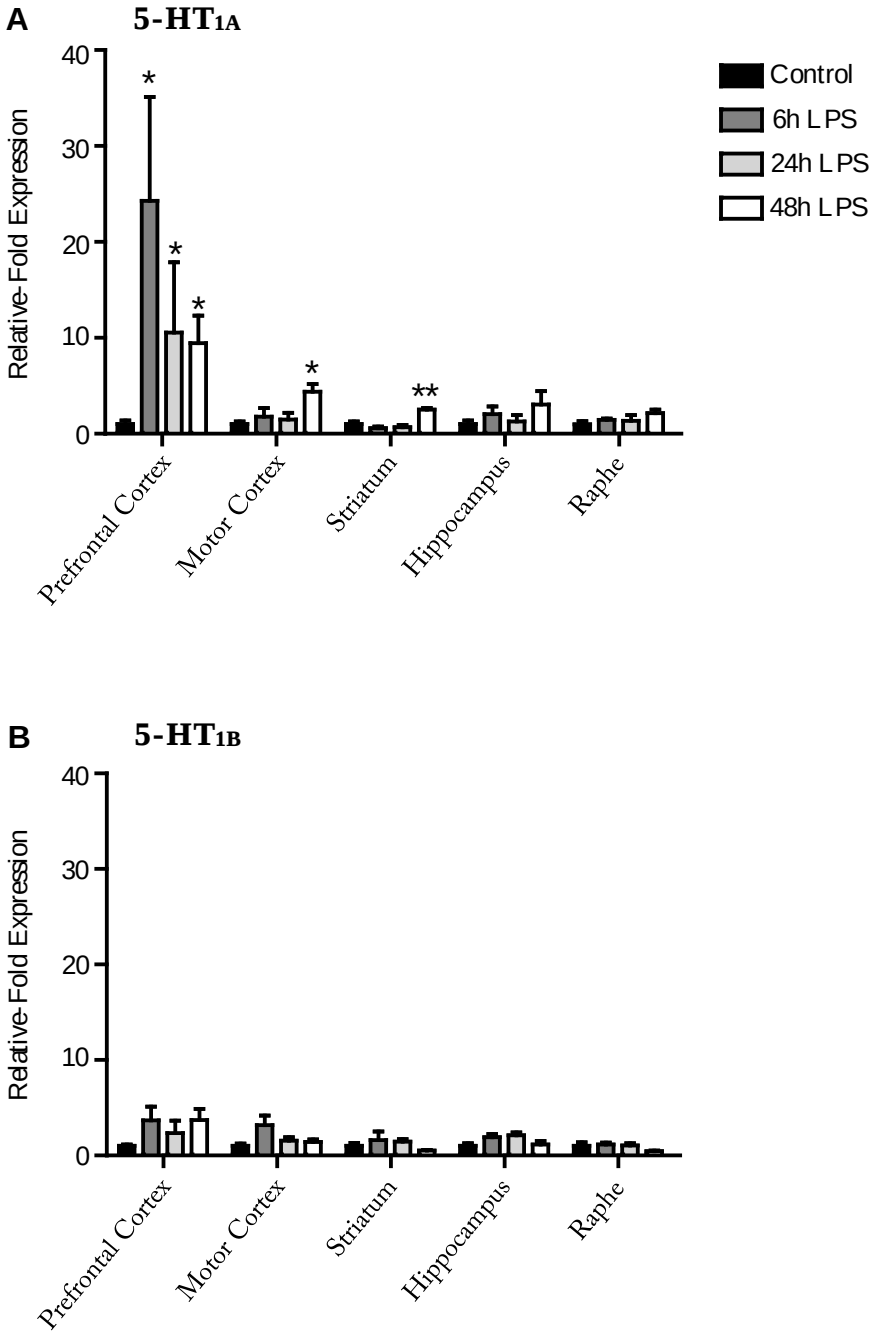


Figure 3.13 5-HT$_1$ receptor expression after a systemic inflammatory challenge. Animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to recover for 6 (dark grey bars), 24 (light grey bars) or 48 hours (open bars) post-challenge. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) 5-HT$_{1A}$ and (B) 5-HT$_{1B}$ was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6, *p<0.05, **p<0.01 and ***p<0.001.

5-HT$_{2A}$ **5-HT$_{2B}$**

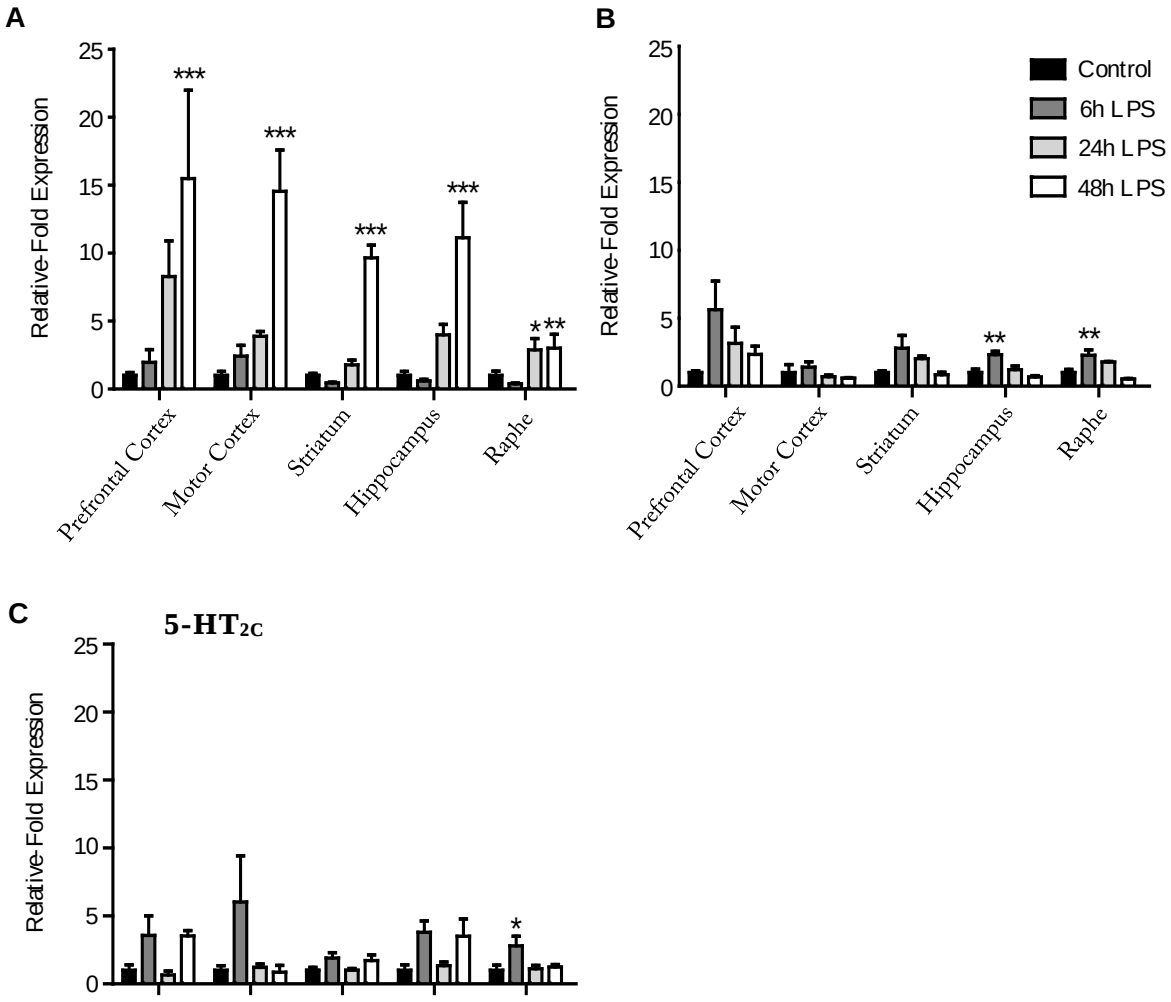


Figure 3.14 5-HT$_2$ receptor expression after a systemic inflammatory challenge. Animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to recover for 6 (dark grey bars), 24 (light grey bars) or 48 hours (open bars) post-challenge. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) 5-HT$_{2A}$; (B) 5-HT$_{2B}$ and (C) 5-HT$_{2C}$ was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6, *p<0.05, **p<0.01 and ***p<0.001.

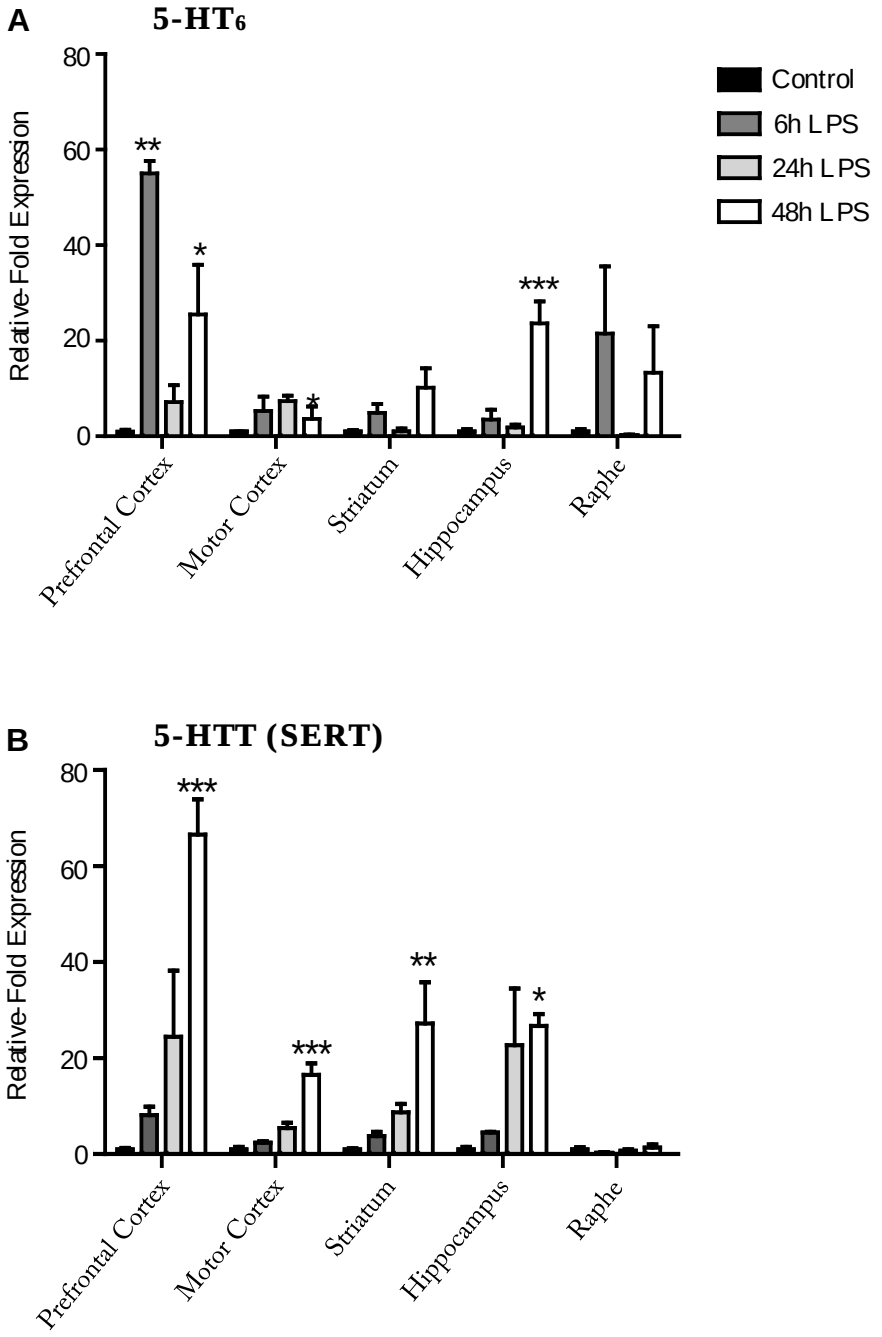


Figure 3.15 5-HT$_6$ receptor and transporter expression after a systemic inflammatory challenge. Animals received a single i.p. dose of LPS (0.5mg/kg) and were allowed to recover for 6 (dark grey bars), 24 (light grey bars) or 48 hours (open bars) post-challenge. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) 5-HT$_6$ and (B) the 5-HT transporter was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6, *p<0.05, **p<0.01 and ***p<0.001.

3.4 Discussion

The purpose of these initial studies was to establish the effects of peripheral inflammation on the central serotonergic system, both in terms of molecular alterations and functional changes. Initial studies in mice involved determining the immediate effects of LPS on both exploratory and locomotor behaviours. A single systemic LPS challenge resulted in decreased exploratory open field behaviour at 6 hours. At 24 and 48 hours post-challenge exploratory behaviour in the open field was not distinguishable from controls. However, when studying locomotor activity over 2 hours, it is still possible to discriminate sick animals from healthy controls at 24 and 48 hours post-injection. Mirroring the open field behaviour, corticosterone levels increased in the peak inflammatory period, and decreased by 24 hours to baseline levels. In parallel with the longer term changes in behaviour, differences in the mRNA expression profiles of a number of inflammatory genes, as well as 5-HT-related genes, changed at a number of time points. While these mRNA expression studies frequently showed few significant differences in 5-HT gene expression after inflammation (e.g. 5-HT_{2B}), they did highlight increases in CNS TNF expression during peak systemic inflammation, as well as changes in both 5-HT_{2A} and SERT expression, both genes pertinent to the study of depression.

Work studying behavioural depression often uses the forced swim test (FST) as a consistent measure of both depression-like behaviour, and antidepressant treatment (Slattery and Cryan, 2012). The ethics and practicalities of exposing animals with a fever to a wet environment mean that while this test may be extremely sensitive, it is not necessarily practical in studies of sickness behaviour. Viana and colleagues (2010) use a similar test, tail suspension, to demonstrate that at 6 and 24 hours, animals show a significantly decreased inclination to move compared to controls. This is mimicked in experiments in this chapter using a basic locomotor activity study. Over 2 hours, animals receiving LPS were significantly less active than those receiving a saline challenge. Furthermore, work from this chapter has demonstrated that these changes persist to 48 hours, while those in the study by Viana do not. This may be reflective of differences in

strain. The CD1 mice used in this chapter have been shown to have a different behavioural and inflammatory profile, when compared to standard C57/Bl6 mice (Painsipp *et al.*, 2011). The latter work by Painsipp and colleagues studies behaviour further from the challenge than in this chapter, finding differences in forced swim activity 28 days post-LPS.

Current literature studying behavioural changes in response to inflammatory challenges often focus on the role of specific cytokines in the CNS. Two major pro-inflammatory cytokines are TNF and IL-1. A paper by Kelley's group in the early 1990s suggested that the only role for TNF in sickness behaviour was to compensate for dwindling IL-1 (Bluthe *et al.*, 1991). In apparent contradiction to this, work from this chapter has shown that a systemic challenge with LPS results in significant up-regulation of CNS TNF, with no significant elevations of IL-1. The role of TNF as a mediator of sickness behaviour is evident in studies using anti-TNF drugs to combat this (Jiang *et al.*, 2008). Furthermore, studies in depressed patients have begun to investigate the potential of inhibiting cytokines in treatment-resistant depression, clearly indicating a positive role for cytokines such as TNF in regulating mood (Miyaoka *et al.*, 2012). TNF receptor knockout animals show a distinctly different behavioural phenotype, specifically an antidepressant phenotype in the forced swim, compared to littermate controls (Simen *et al.*, 2006). The mechanisms by which TNF mediates changes in mood and its potential role in both sickness and depression, are yet to be explored.

Cyclooxygenase (COX) is also emerging as a potential target in terms of novel antidepressant therapy. Depressive-like behaviour in rodents, as induced by olfactory bulbectomy or chronic stress, can be attenuated by inhibition of COX-2 using celecoxib (Myint *et al.*, 2007; Guo *et al.*, 2009). These papers highlight the potential of COX-2 to mediate behaviour, at least in models of depression. Work from this chapter has shown that peak inflammation at 6 hours post-LPS is associated with increased levels of brain COX-2. Interestingly, work in models of sickness has shown conflicting data in terms of the relative importance of COX-1 and COX-2. Studies by Teeling and colleagues have

shown that, in terms of inflammation-induced behavioural changes, inhibition of COX-1 is important (Teeling *et al.*, 2010). In contrast, work by Carey *et al.* (Carey *et al.*, 2005) has shown that COX-2 knockout animals have a better survival and less pronounced 'sickness' than COX-1 knockouts. Non-steroidal anti-inflammatory drugs, which primarily target COX-1, are used to reduce fever during periods of sickness. Considering the hypothesis put forward in this thesis, that sickness behaviour is simply behavioural depression with a febrile component, studying the exact roles of COX-1 *vs* COX-2 in rodent models of depression *vs* rodent models of sickness would seem timely.

Initial studies in the Sharp lab (Qin Xie, unpublished data) aimed to recapitulate work by Dantzer and colleagues indicating central changes in indoleamine-2,3-dioxygenase (IDO) mRNA and activity (O'Connor *et al.*, 2009). Data from the Sharp lab (unpublished), and data from this chapter, indicate that LPS does not significantly alter the expression of IDO mRNA in any brain region studied. Although increases in the dorsal raphe nucleus and the motor cortex of up to 2-fold were observed, these were not significant at either 6 or 24 hours post-administration. These data fail to support findings by O'Connor *et al.*, who showed a 5-fold increase in IDO mRNA after 7-days of inflammation induced by LPS. Further work from the Sharp lab indicate that IDO activity, as measured by kynurenine output, is also not changed post-challenge (data not shown), suggesting that other mechanisms may be responsible for sickness behaviours observed during inflammation.

The role of IDO in the breakdown of tryptophan suggests that any circulating factors altering the activity of this enzyme, should alter overall 5-HT metabolism (Gjerris *et al.*, 1987; Sullivan *et al.*, 2006; Barton *et al.*, 2008). In view of the above data showing little IDO response to systemic inflammation, it is vital to also investigate changes in 5-HT metabolism under similar circumstances. Work in the literature suggests that systemic inflammation is capable of increasing the turnover of 5-HT (Dunn, 1992; Linthorst *et al.*, 1996; Pitychoutis *et al.*, 2009). Interestingly, the work by Dunn (1992) used a number of different doses of LPS, showing increases in 5-HT turnover at low doses but no change at

higher doses in some brain regions. Considering the monoamine theory of depression, an increased turnover of 5-HT could seem counterintuitive. However, turnover must be considered as the relative difference between synthesis and release/breakdown, with this in mind it could then be assumed that while more is made, more is also broken down. The change in rate of this process may result in altered levels of neurotransmitter being available at the synapse, rather than total 5-HT levels. Work in this chapter showed slight increases in 5-HIAA in both fore and hindbrain regions at 6 and 24 hours after an LPS injection, however these values did not reach significance and, when 5-HT values were taken into account, did not seem to represent a change in 5-HT turnover. Discrepancies between the work in this chapter and those in the published literature make interpretation of these results challenging. Recent work by Hughes and colleagues (2012) has suggested that changes in tryptophan occur independently of changes in IDO and inflammatory cytokines so it is possible that the IDO theory may represent a dead-end and other mechanisms may play a more important role in mediating sickness behaviour.

3.4.1 Regulation of the 5-HT$_{2A}$ receptor and its role in sickness behaviour

Dantzer and colleagues (2008) have put forward the hypothesis that behaviour, specifically decreases in open field and forced swim behaviours, observed immediately after an inflammatory challenge is defined as 'sickness' and behaviour observed more than 24 hours later is 'depression'. Work from this chapter has demonstrated that it is not possible to see both sickness and depression using a standard open field, a test frequently used because of its ability to rapidly assess very basic emotional status (Denenberg, 1969), it is only possible to see acute sickness. However, it is possible to observe the behavioural changes considered as 'depression', using a longer locomotor activity study across two hours. This decrease in general locomotor activity at 48 hours after an LPS challenge has received little attention in terms of the molecular biology of the 5-HT system, and may, in fact, reflect more persistent molecular changes, as discussed below.

Study of a number of 5-HT related genes in the mouse revealed some significant changes both during, and for a period after, a brief episode of inflammation. However, these

results should be interpreted with caution. Overall Two-way, RM-ANOVA analysis showed that in several genes there was an effect of LPS treatment but post-hoc tests revealed no specific changes. This suggests that these experiments were underpowered and that when considering future experiments a larger n would be prudent. However, in spite of this a number of genes did show significance in post-hoc test. 5-HT_2 genes were differentially regulated across time with the most significant changes being observed in 5-HT_{2A}. In this study, treatment with LPS caused a significant increase in 5-HT_{2A} receptor expression in a number of brain regions. The involvement of the 5-HT_{2A} receptor in depression is currently controversial. Studies from the late 80s indicate that receptor expression increases in patients with suicidal depression (Stanley and Mann, 1983; Mann *et al.*, 1986; Stanley *et al.*, 1986), while further data suggested that this increase may be non-neuronal (Serres *et al.*, 1999). The development of modern imaging techniques has produced conflicting data, showing that receptor expression decreases in untreated patients with Major Depression (Mintun *et al.*, 2004), but that the affective symptoms of some psychiatric disorders may be caused by increased 5-HT_{2A} expression (Soloff *et al.*, 2007). Studies of 5-HT_{2A} polymorphisms yield similarly contradictory data, showing both associations between polymorphisms and depression, as well as a lack of association (Serretti *et al.*, 2007). In terms of inflammation, and as a consequence sickness behaviour, increases in 5-HT_{2A} receptor expression have been seen in models of inflammatory pain, as well as in previous chapters in this thesis, indicating that systemic inflammation is capable of regulating this response *in vivo* (Zhang *et al.*, 2001).

The mechanisms by which changes in receptor expression occur were beyond the scope of this thesis; however, hypotheses can be generated based on existing research. Studies linking the 5-HT_{2A} receptor with inflammation have suggested that its activation may result in the down-regulation of inflammatory cytokines such as IL-6 (Yu *et al.*, 2008). Furthermore, studies in cultured glioma cells have demonstrated an inhibition of inducible nitric oxide synthase (iNOS) by 5-HT_{2A} agonists (Miller and Gonzalez, 1998). Work by Zhang and colleagues (2001), mentioned above, demonstrated that the up-regulation of 5-HT_{2A} after inflammation was not co-localized with 5-HT, indicating that it

was not in serotonergic neurons. At the same time, 5-HT_{2A} receptors have been shown to be present on astrocytes (Wu *et al.*, 1999), but not microglia. Therefore, increased 5-HT_{2A} receptor expression after a peripheral inflammatory stimulus could potentially occur in non-neuronal cells for the purposes of potentiating an anti-inflammatory response in the CNS.

Linking the inflammation-mediated changes in 5-HT_{2A} expression to behaviour - it has been suggested elsewhere that prefrontal 5-HT_{2A} expression mediates a 'top-down' inhibition of the amygdala, specifically in response to fearful situations (Fisher *et al.*, 2009). Fisher *et al.* showed that a higher number of 5-HT_{2A} receptors within the prefrontal area reduce the response to threat in humans. Novel environments could be considered to be threatening to rodents; the novelty induces a partially fearful response resulting in exploratory behaviour to determine the threat level. Blockade of the 5-HT_{2A} receptor has been shown to decrease response to novelty in rabbits (Aloyo and Dave, 2007). Taken together these data could be used to explain a potential link between the behaviour and molecular biology observed in this chapter.

3.4.2 The 5-HT transporter and its role in sickness behaviour

Of further note in this chapter are the significant increases in SERT expression in all brain regions studied. More than the 5-HT_{2A} receptor, increased SERT expression in depressed patients may explain depressive symptoms by means of 5-HT sequestration. This hypothesis is supported by studies showing that individuals with low-expression variants of SERT are more susceptible to post-traumatic stress associated depression (Kilpatrick *et al.*, 2007). *In vitro* data indicate that treatment of cultured astrocytes with cytokines such as TNFα increases the expression of SERT (O'Connor *et al.*, 2008). However, double *in situ* hybridization and immunohistochemistry failed to co-localize the SERT signal with a marker for astrocytes at any time after inflammation (data not shown). Studies in an LPS-model of sickness behaviour have shown that inflammation is capable of increasing the activity of SERT, and that inflammation in a SERT knockout animal does not result in significant behavioural despair (Zhu *et al.*, 2010). The latter

should be considered carefully since the baseline behavioural phenotype of the SERT knockout mouse is complex and deletion of this gene may be anxiogenic (Kalueff *et al.*, 2007). The introduction of complex sickness behaviours in a model where the behavioural phenotype is already confusing may be difficult to interpret. In a model of chronic stress, an inflammatory challenge has been shown to inhibit the effects of fluoxetine in reversing behavioural depression (Wang *et al.*, 2011). These data further indicate that regulation of SERT by inflammatory stimuli may play a significant role in both depression, and antidepressant treatment resistance.

3.5 Conclusion

Overall this chapter has begun to explore the potential mechanisms of sickness behaviour, specifically those relating to the CNS inflammatory response and the central 5-HT system. The results from this chapter indicate that a single pro-inflammatory challenge is capable of decreasing exploratory behaviour up to 48 hours later, a time largely unstudied because sickness behaviour was thought to have abated. A single LPS injection also results in up-regulation of both peripheral and central pro-inflammatory cytokines, specifically TNFα. 5-HT_{2A} receptor expression increased in response to a single LPS challenge, data which is in accord with the previous chapter focusing on the contribution of this receptor to changes in signalling in the rat. However, in contrast to data in the rat, CD1 mice show a significant increase in other genes such as the 5-HT transporter. These changes in receptor expression persist up to 48 hours post-challenge and may be reflective of the mechanisms underlying the persistent changes in behaviour. Dantzer and colleagues (2008) suggest that any behavioural changes that persist beyond the peak of inflammation represent behavioural depression rather than sickness behaviour, but this thesis seeks to determine whether sickness is, in fact, behavioural depression with a febrile component. Despite this theory, and the conclusions of Dantzer, no direct comparison of the two models has yet been undertaken.

Chapter 4: Chronic Stress as a Model of Depression

Research into affective disorders such as depression, is currently hampered by the lack of a truly indicative animal model. Chronic stress in animals results in a 'depression-like' state characterized by anhedonia, loss of appetite and altered activity patterns which are reversible by antidepressants, giving this model both face and predictive validity as a model of depression. Work in previous chapters has used a single endotoxin challenge, a model which has not been validated for research into 'depression', as the changes seen are often considered too transient. However, as demonstrated in chapters 2 and 3, inflammation is capable of inducing both short term functional changes in the serotonergic system, as well as longer term behavioural changes. These changes are often accompanied by specific regional and temporal changes in 5-HT receptor gene expression. In order to validate the hypothesis that sickness behaviour is merely behavioural depression with a febrile component, it is important to compare and contrast models of both sickness and depression. Thus far this thesis has studied models of acute sickness using a single LPS challenge, here, consistent aspects of change such as behaviour and changes in 5-HT_{2A} expression, will be studied in a validated rodent model of depression.

4.1.1 The Chronic Stress Model

The use of chronic stress as a way of inducing a depression-like state in animals was introduced in the early 1980s by Katz and colleagues, using a rat model that involved 21 days of randomly applied stressors (Katz *et al.*, 1981). These stressors resulted in significant changes in open field behaviour but also, co-incidentally, a failure to increase liquid consumption when saccharin was introduced to their drinking water. This was thought to reflect a failure of the hedonic impact of the sweetened solution and, thus, was defined as 'hedonic deficit' or *anhedonia*. Since anhedonia was (at the time of DSM-II), and remains (now using DSM-IV), a key symptom of melancholia, or depression, it was considered critical to be able to model it in animals. Using Katz' model, Willner and colleagues successfully demonstrated that anhedonia, as defined by a decrease in sucrose

preference, could be induced by 5-9 weeks of chronic mild stress (CMS) and this could be attenuated using tricyclic antidepressants (Willner *et al.*, 1987). The use of antidepressants such as citalopram and imipramine in this model of CMS is particularly important; it not only provides predictive validity for the model, but also links it to rodent models of sickness, where antidepressants have been shown to reverse behavioural symptoms (Yirmiya *et al.*, 2001).

The development of CMS has taken many years and is still not consistent across laboratories (Willner, 2005). The term chronic mild stress covers an array of stressors including the introduction of a predator, random bright light episodes, orbital shaking, cage-mate swapping, social isolation, introduction of aggressive cage-mates, mild food and water deprivation, white noise and restraint, to name but a few. Recently, the use of severe stressors such as foot-shock, extreme food and water deprivation and extreme temperature exposure has been largely eliminated from chronic stress paradigms. Work by Matthews and colleagues contributed to this reduction by showing that animals are more likely to habituate to extreme stressors (Matthews *et al.*, 1995). Habituation is also a problem when stressors are applied repeatedly or predictably (Muscat and Willner, 1992). Therefore, more recent incarnations of the CMS approach have used unpredictable and unrepeated stressors, or stressors repeated in an unpredictable manner. These studies have consistently, with a few exceptions (Murison and Hansen, 2001), reported a decrease in sucrose preference to below 65%. This level is set because control animals in all paradigms never show a preference for sweetened solutions of less than 65%.

4.1.2 Stress and Inflammation

As for systemic inflammation, stress results in the production of the glucocorticoid cortisol, or corticosterone in animals. Glucocorticoids are an important regulatory feedback mechanism for the immune system and act via the glucocorticoid receptor (GR) to repress the expression of pro-inflammatory cytokines, and enhance the expression of anti-inflammatory cytokines (Newton, 2000). Recent studies in humans have demonstrated a link between stressful life events and immune dysregulation (Cohen *et al.*, 2012), suggesting that circulating cortisol could significantly change the response to

inflammatory stimuli. However, data demonstrating a role for corticosterone in the response to CMS has been largely varied. Previous work has shown that CMS causes an increase (Silberman *et al.*, 2002), a decrease (Paternain *et al.*, 2011) and no change at all (Azpiroz *et al.*, 1999) in corticosterone levels.

The story supporting cytokines and stress is equally varied. If it is assumed that stress increases cortisol and cortisol suppresses pro-inflammatory cytokines then the use of the CMS model should result in decreased circulating levels of cytokines such as IL-1β and TNF. Despite this assumption, Grippo and colleagues (2005) demonstrated that CMS increases cortisol but also increases circulating TNF and IL-1β. Conversely, others have shown that CMS decreases peripheral cytokine levels (Mormede *et al.*, 2002). The latter work, however, showed that while CMS decreases peripheral cytokines, levels of central pro-inflammatory cytokines such as IL-6, continue to rise. The authors suggest that central, not circulating, cytokines are responsible for the behavioural changes seen in models of CMS. These questions need the application of a consistent model of stress and stress-induced depression for convincing answers.

4.1.3 Species and Strain Differences in Inflammation and CMS

Use of the CMS model has shown that corticosterone will go up in rats (Ayensu *et al.*, 1995) but remain unchanged in mice (Azpiroz *et al.*, 1999). Other studies have indicated that social interactions can change the response to stress in the different sexes (Westenbroek *et al.*, 2003), whilst, recent data have shown that social status can affect susceptibility to developing a depression-like state in mice (Strekalova *et al.*, 2011). With these data in mind it is important to consider basic differences between animals when establishing a model of CMS.

Similarly confounding data exists when studying the immune response. CD1 mice are an outbred strain that are frequently used in studies of sickness behaviour and have been shown to have a significantly enhanced immune response; including high leukocyte reactivity and cytokine production, when compared to other strains of laboratory mouse (Brummer *et al.*, 2005). This may be advantageous when using a simple LPS model of

sickness behaviour and depression, as it results in a robust production of cytokines in response to the immune challenge. Anecdotal evidence suggests that CD1 mice are 'highly aggressive' compared to other commonly used strains such as Balb/c or C57/Bl6 and thus may be unsuitable for the CMS paradigm (Miczek *et al.*, 2001). C57/Bl6 mice are an inbred strain and form the background for a number of different transgenic lines. This allows flexibility when studying the effects of specific genes, but also allows more aggressive strains such as the CD1 to be used as resident intruders. For the work described in this chapter, the experimental set up has been extensively reviewed (Strekalova *et al.*, 2011) to determine optimal CMS procedure, immunological challenge and strain.

4.2 Aims of this Chapter

The overall aim of this chapter is to determine whether the changes observed in a rodent model of sickness are conserved in a validated model of depression. This particular rodent model of depression suffers from largely unreported segregation of behaviours (Strekalova *et al.*, 2011); mice subjected to chronic stress are either 'susceptible' or 'resilient'. With this in mind, it is important to determine the behavioural profile of *all* mice subjected to stress, and to further determine whether this model has predictive validity with the use of prophylactic antidepressant therapy.

To confirm the overall theory of this thesis, that sickness behaviour represents behavioural depression with a febrile component, it is important to also study aspects of the CNS which have changed in a model of sickness. Specifically, CNS 5-HT_{2A} and SERT expression, as well as levels of CNS cytokines in mice subjected to chronic stress. If behavioural depression is considered to be sickness behaviour without fever, it could be hypothesized that induction of fever *during* an episode of depression could result in cumulative behavioural changes. To study this, a low dose of LPS will be introduced late in the chronic stress procedure, and animals will be studied as above.

4.3 Materials and Method

All chronic stress work was carried out in collaboration with Dr Tatyana Strekalova at the University of Lisbon, Portugal. For baseline chronic stress studies data were collected from a number of different studies using the same stress protocol with different anti-depressant treatments. For the LPS study a single chronic stress procedure and LPS treatment was carried out.

4.3.1 Animals & Drug Treatments

Animals were provided with food and water *ad libitum* and housed under a reversed light-dark cycle (lights on: 21:00). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals and with approval by the local governmental bodies for animal care and welfare. Male C57Bl/6J inbred mice were obtained from Charles River (Suzfeld, Germany) at 14 weeks of age and house singly for 10-14 days prior to, and for the duration of, the experiment. Male CD1 outbred mice were obtained from Charles River (Germany) at 14 weeks of age and housed in groups of 5 prior to the onset of experiments. Male Wistar rats were obtained from the Gulbenkian Institute of Science (Oeiras, Portugal) at 2-5 months of age and were housed in groups of 5 prior to the onset of experiments. Animals received citalopram or imipramine via drinking water from before the onset of chronic stress. Citalopram (15mg/kg/day) and imipramine (7mg/kg/day) solutions were changed daily.

4.3.2 Chronic Stress

The chronic stress procedure used in this chapter has recently been validated by Dr Strekalova and considerably shortens the time required to induce anhedonia in comparison to previous models (Strekalova *et al.*, 2011). Where drug treatment was used, drugs were introduced prophylactically before the introduction of the chronic stress procedure. Briefly, the model uses a 10-day chronic stress procedure comprising night time rate exposure and day time application of two of three stressors. The stressors include social defeat, restraint stress and tail suspension. Between 18:00 and 11:00 animals were exposed to rats (Strekalova *et al.*, 2006). Between 11:00 and 18:00 the

animals received two of either social defeat with an aggressive CD1 male for 30 minutes (Buwalda *et al.*, 2012), restraint stress for 2 hours (Strekalova and Steinbusch, 2010) or tail suspension for 40 minutes (Strekalova *et al.*, 2006). The stressors were applied with an inter-session interval of approximately 4 hours.

4.3.3 Physiological and Behavioural Testing

Unless otherwise stated, all behavioural testing was carried out under the animals' dark phase in order to prevent confounds of inducing activity during the sleep cycle. The schedule of stress, drug treatment and behavioural testing is outlined in Figure 4.1. Testers were not blind to stress or treatment.

4.3.3.1 Body Weight

Animals were weighed at day -14 to day -7 and continually throughout the chronic stress procedure.

4.3.3.2 Sucrose Preference

The consumption of sugary and fatty foods has been known for some time to be linked to reward circuits in the brain (Levine *et al.*, 2003). Original studies by Richter and colleagues (1938) showed that rats will naturally self-administer sugary and salty foods. Recent studies have shown that these foods will alter neurotransmitter systems in a feed-forward manner, increases in CNS peptides will result in an increased 'need' for the same sugary and salty foods. Both physiological and pharmacological manipulations of animals have been shown to alter preference for a drinking solution of sucrose and water (Willner *et al.*, 1987). This reduction in the apparent 'enjoyment' of a sweet solution has been deemed a measure of hedonic deficit and, as such, as an indicator of a depressive-like state in rodents. Here, sucrose preference was determined during an 8 hour period where animals were given a choice between two bottles, one containing regular drinking water and one containing a 1% sucrose solution. Testing begins during the dark phase of the cycle where animals are most likely to be active and drinking. To prevent location bias, the positions of the bottles were switched 4 hours after the onset of testing. No previous food or water deprivation was applied before the test as these have been shown to

confound outcomes. Bottles were weighed immediately before the onset of testing and again at the end, allowing calculation of total liquid intake as well as intake of water and sucrose solutions. Sucrose preference was calculated using the formula:

$$\text{Percentage Sucrose Preference} = \left[\frac{\text{Volume (sucrose solution)}}{\text{Volume (sucrose solution)} + \text{Volume (water)}} \right] \text{x100}$$

Sucrose preference of <65% was considered an indication of anhedonia based on data indicating that no control mice ever exhibit less than 65% sucrose preference (Strekalova *et al.*, 2011). After 10 days of CMS, animals were assigned to either the stress susceptible or the stress resilient groups based on this criterion.

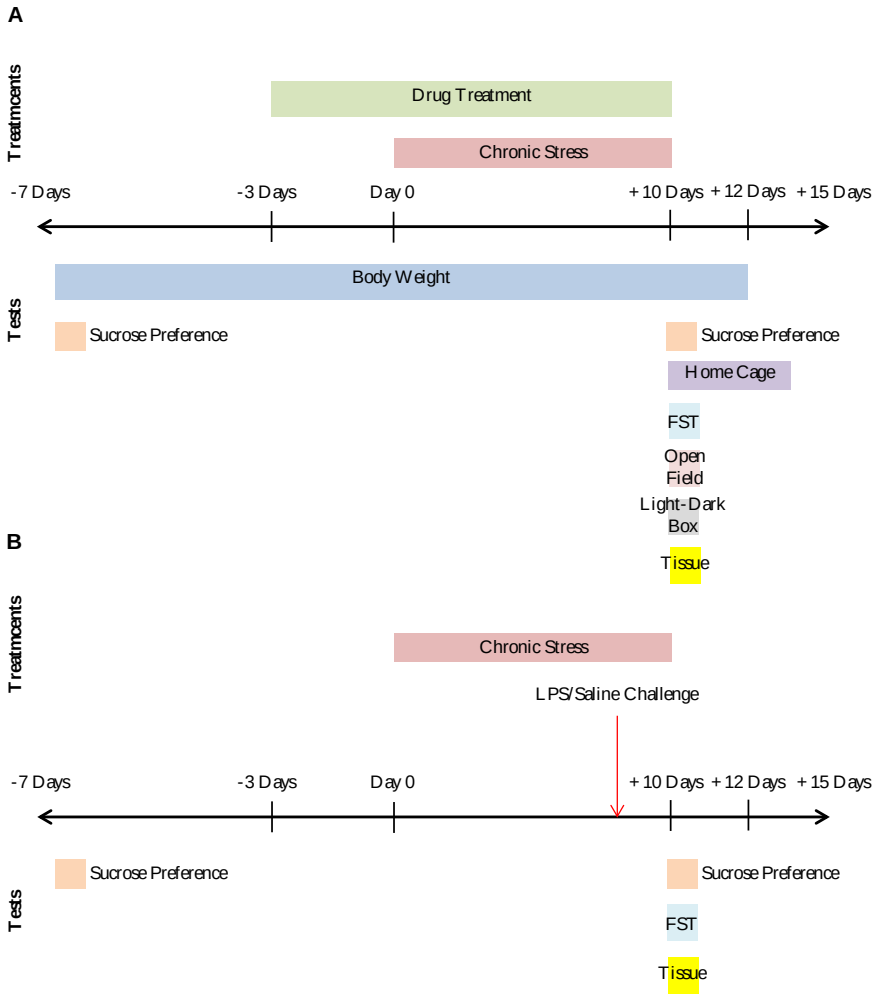


Figure 4.1 Timeline of the chronic mild stress paradigm. Schedule includes behavioural testing both before and after the induction of chronic stress as well as drug treatment before and during the stress procedure. (A) Testing procedure during chronic mild stress treated with anti-depressant and (B) testing procedure combined with an LPS challenge (0.1mg/kg) at day 9 of the chronic stress procedure.

4.3.3.3 Forced Swim Test (FST)

The forced swim test (FST) was originally developed by Porsolt in the late 1970's as a method for measuring behavioural despair (Porsolt *et al.*, 1977). Animals are placed into a tank of water from which there is no obvious escape. This will initially result in an escape reflex where the animal swims frantically to find an escape route, followed by a period where the animal moves only to keep its head above water. Porsolt concluded that this immobility is the result of behavioural despair in which the animal '*has learned that escape is impossible and resigns itself to the experimental conditions*'. In terms of depression, this test was found to be particularly sensitive to antidepressant treatment (Porsolt *et al.*, 1978). The only drugs to mimic antidepressant effects in the FST are psychomotor stimulants such as amphetamine, which increase locomotion. However, the simple expedient of coupling the FST with a locomotor task such as the open field would reveal these differences; amphetamine would increase locomotion where antidepressants would decrease locomotion. An important aspect of FST behaviour not present in the open field is the lack of habituation. There was speculation at one point that the FST was simply a learned passivity (West, 1990), where animals learned swiftly that they must simply become passive, conserve energy and wait to be removed from the tank. Studies using repeated and single exposures to the FST suggest that this is not true (O'Neill and Valentino, 1982). A number of significant modifications to the original FST have been made in recent years including consistent monitoring of water temperature to avoid hypothermic effects, as well as changes in water depth to avoid wall and floor stimulated escape behaviours (Detke and Lucki, 1996). This modified FST is the most commonly used antidepressant test currently available. Here, mice exhibiting decreased latency to float and an increased duration of floating are considered as being in a depressive-like state.

Mice were placed into a transparent pool (20 x 35 x 15cm) lit with red light and filled with warm water (30°C, to a depth of 9.5 cm) for 2 minutes. Floating behaviour was defined as the absence of directed movements of animals' head and body, and was measured by visual observation that was validated previously by automated scoring with CleverSys

software (CleverSys, VA, US; Malatynska et al., 2012). Latency to begin floating was scored as time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 seconds, irrespective of the duration of the first floating episode. The total time spent floating was scored for the entire duration of the test using post-test video footage.

4.3.3.4 Open Field

Animals are stimulated by novelty. The conflict between the need to investigate a novel object or environment and the impulse to avoid potential danger must be resolved in the mind of the animal. Therefore, the specific 'mental state' of the animal when it is introduced to a new environment could potentially be evaluated by the degree to which said environment is explored. For example, it has been shown previously that chronic stress is capable of reducing the exploratory behaviour of standard laboratory mice (Strekalova *et al.*, 2004). Therefore, apathy towards a novel object or environment may be considered an indicator of a depressive-like state; the novel space presents no immediate danger therefore they do not feel the 'urge' to explore it. By combining locomotor activity, escape behaviours and exploratory behaviours it is possible to build up a relatively subjective view of the motivational status of the animal. Here, anxiety and locomotor activity were initially monitored over a short period of time using an open field. Animals were introduced into a novel Perspex cage (20 x 20 x 10 cm) filled with fresh sawdust. Exploratory rearing activity was counted *in situ* under bright lighting conditions (600 Lux) for 5 minutes.

4.3.3.5 Monitoring of Home-Cage Activity

Home-cage activity was monitored in animals using the SAMAB (system for automated measurement of behaviour) system (Technoplast, Reggio Emilia, Italy). Infrared beams monitored horizontal activity in the home-cage environment, allowing animals' access to food and water ad libitum and also allowing normal behaviour to be monitored over a number of days.

4.3.3.6 Light-Dark Box

Anxiety was measured using the light-dark box based on the assumption that brightly lit open spaces make animals inherently nervous and therefore an anxiogenic paradigm is more likely to cause them to spend increased amounts of time in the dark box where they feel less exposed. Animals were introduced into a dark plexiglass box (15 x 20 x 10 cm) connected by tunnel to a standard plexiglass box (15 x 20 x 10 cm) lit at 50 Lux. Animals were introduced into the dark box and latency to enter the light box was timed. Number of transitions between light and dark zones was measured during a 5 minute period.

4.3.4 RNA Extraction and qPCR

RNA was extracted and converted to cDNA as previously described (*see* 2.3.9, 2.3.10 and 2.3.12). The primers for qPCR are detailed in Table 3.1.

4.3.5 Cortisol Analysis

Plasma corticosterone was analysed as described previously (*see* 3.2.8).

4.3.6 Immunohistochemistry

Immunohistochemical analysis of brain tissue was kindly performed with the assistance of Dr Alexander Revishchin based on our previously published methods (Couch *et al.*, 2011). Briefly, brain tissue was perfuse-fixed in 4% paraformaldehyde (PFA) and post-fixed in PFA followed by 30% sucrose. Tissue was cryosectioned at 40µm onto gelatin coated slides and stained for activated microglia. Sections were blocked with 10% normal donkey serum and stained with anti-Iba-1 (AbCam, ab5076, Cambridge, UK) antibody. Secondary biotinylated donkey-anti-goat antibody was used for standard visualization procedures followed by amplification using Vectorlabs avidin biotin kit (ABC Kit, Vectorlabs, UK). Visualization was performed using 3'3'-diaminobenzidine hydrochloride. Immunostaining was studied using a light microscope Olympus IX81, computer-controlled Märzhäuser motorized stage and digital camera Olympus DP72. Cell counting was carried out in a predefined prefrontal area using "Cell" software (Olympus Soft Imaging Solution GmbH).

4.3.7 Statistics

Statistical analysis were performed using GraphPad Prism and InVivoStat software using one-way and two-way ANOVA and Student's t-tests as appropriate. Area under curve analysis was applied to SAMAB data, followed by a standard Student's t-test between groups. Data are presented as mean ±SEM.

4.4 Results

4.4.1 Chronic stress changes physiology and behaviour in a subgroup of mice

Studying affect in rodents is challenging. There are a number of behaviours which are considered stereotypical and are therefore used to determine whether the animals are behaving 'normally'. A measure of hedonic deficit, the sucrose preference test, aims to uncover how much 'enjoyment' the animals are getting out of life. Control animals who have undergone no procedures always show a sucrose preference of >65% (Strekalova *et al.*, 2011). Animals in the studies presented here were subjected to 10 days of chronic stress, specifically a combination of predator stress, restraint stress, tail suspension stress and resident intruder stress. In control groups, where no stress was administered, but the same housing conditions were maintained, no animals showed a sucrose preference of <65% (

Figure **4.2**). For the purposes of analysis, sucrose preference level was defined as the dependent variable and named 'group' in all analyses.

In the current 10-day chronic stress paradigm, repeated measures two-way ANOVA revealed an overall effect of stress, sucrose preference level (group) and an interaction between stress and group ($p<0.001$ stress $F_{1,38}=22.81$; group $F_{2,38}=35.47$; stress:group $F_{2,38}=31.54$). Post-hoc analysis showed a significant decrease in sucrose preference (<65%) in a subgroup of mice (Bonferroni post-hoc; $p<0.001$) in a subgroup of chronically stressed animals (

Figure **4.2**). These animals were then designated as 'stress susceptible' for the remainder of the behavioural tests. At no point did any control animals display a sucrose preference of <65%. Animals which had undergone stress, but still showed a robust sucrose preference, were deemed to be 'stress-resilient'.

The forced swim test (FST) is a recognized measure of depressive-like behaviour in rodents because of its capacity to distinguish drugs with antidepressant action (Cryan *et al.*, 2005). One-way ANOVA of latency to begin floating shows an overall effect of group ($p<0.001$ $F_{3,26}=16.83$) and post-hoc analysis further showed latency to begin floating was significantly decreased in stress-susceptible animals compared to both control animals (Bonferroni post-hoc; $p<0.001$) and stress-resilient animals ($p<0.05$ Figure 4.3C). Complementary results were seen with total time spent floating which was significantly affected by sucrose preference (one-way ANOVA; $p<0.001$ $F_{3,26}=16.56$) and post-hoc analysis showed that stress-susceptible animals spent more time floating compared to controls ($p<0.001$) and compared to stress-resilient animals ($p<0.01$ Figure 4.3B).

In the novel cage test the total number of exploratory rears, a natural behaviour observed in all laboratory rodents, was counted under bright lighting conditions for 5 minutes. Bright lighting conditions have been shown to be a stressful environment and therefore increased rearing is seen as a measure of anxiety. Rearing behaviour was shown to be significantly affected by overall group (one-way ANOVA; $p<0.001$ $F_{3,26}=21.23$), further post-hoc analysis showed that rearing was significantly increased compared to control animals in both susceptible (Bonferroni post-hoc; $p<0.001$) and stress-resilient mice ($p<0.01$ Figure 4.3D). Finally, in terms of basic physiology, group had an overall effect on body weight (one-way ANOVA; $p<0.05$ $F_{3,26}=4.489$) with all stressed animals showing a significant decrease in body weight compared to controls (Bonferroni post-hoc; $p<0.05$ Figure 4.3A).

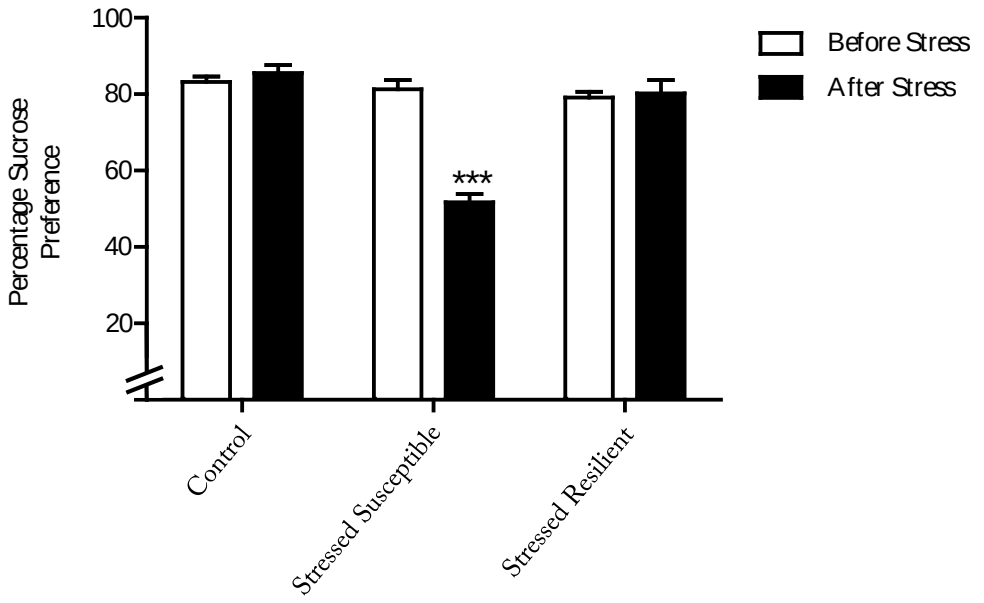


Figure 4.2 Sucrose preference in control animals and animals after 10-days of chronic stress. Sucrose preference was decreased to <65% in a subgroup of animals after undergoing the chronic stress paradigm. Animals were subjected to 10 days of chronic stress followed by a two-bottle sucrose preference test over 12 hours. Data are mean ±SEM n=6+. ***p<0.001 compared to pre-stress animals.

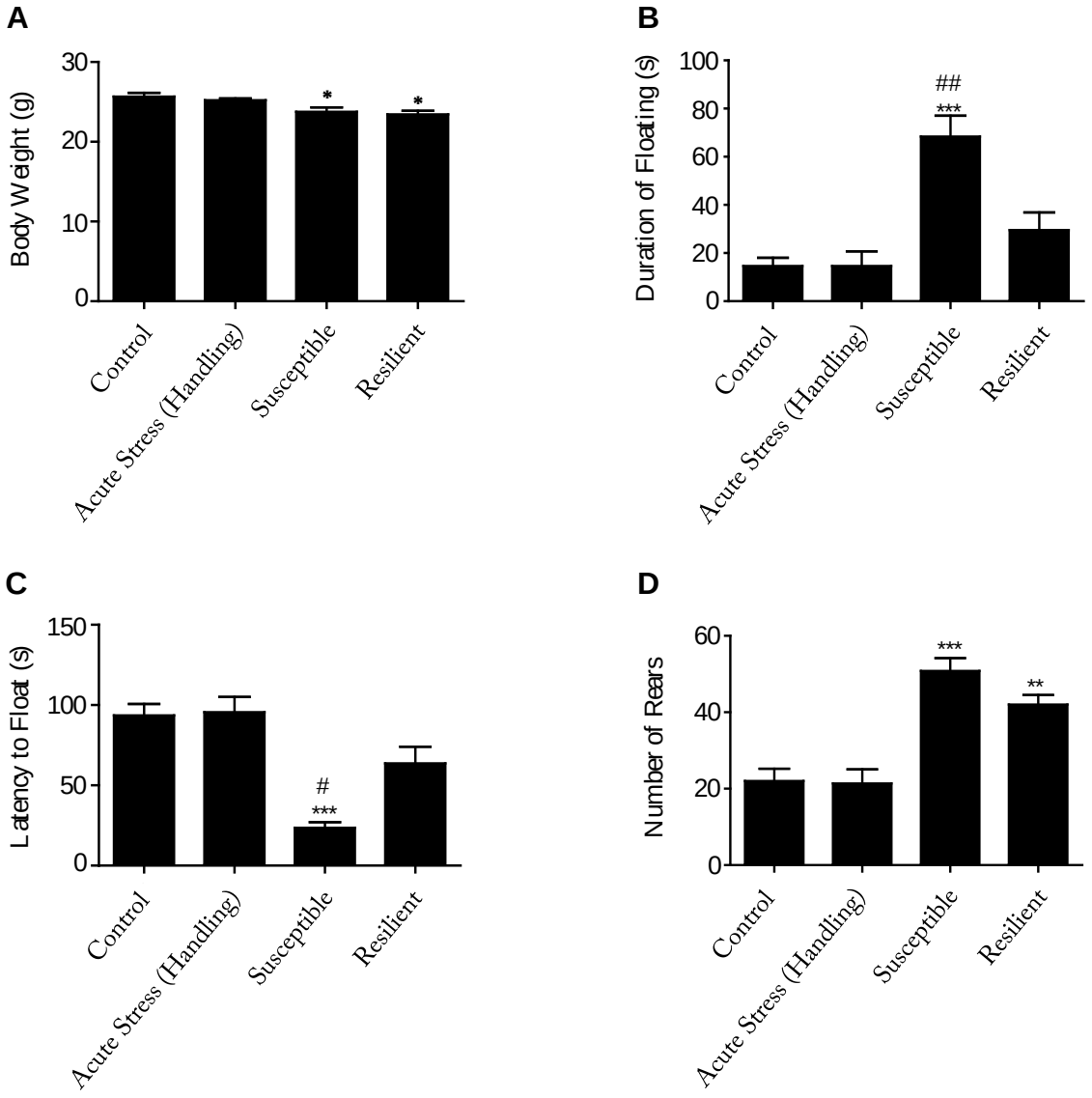


Figure 4.3 Measurements of body weight, forced swim behaviour and novel cage exploration in control and stressed animals. Chronic stress causes a decrease in body weight (A) in susceptible and resilient groups compared to non-stressed control mice. (B) Duration of floating in the forced swim test was significantly longer in susceptible mice than in control and resilient animals. (C) Latency to float in the forced swim test was significantly shorter in susceptible mice than in control and resilient groups. (D) In comparison to control mice, the number of rearings in the novel cage test was significantly increased in susceptible mice and to lesser extent, in resilient animals. Data are mean ±SEM n=6+. *=$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control animals. #=$p<0.05$ and ##=$p<0.01$ compared to resilient animals.

4.4.2 Chronic stress causes anxiety independent of depressive-like behaviour

Changes in locomotor activity observed in both the open field in terms of rearing behaviour and in the forced swim test, may be affected by anxiety. It is also important to determine whether stress induces depression independently of anxiety. While anxiety and depression are frequently comorbid in depressed patients, anxiety is also an independent disorder. To test this, animals were introduced into the light-dark box. The lit area was maintained at 50 Lux and as such is a brightly lit open space, a situation designed to induce anxiety, this space is, therefore, less likely to be frequented by animals already in a high-anxiety state. 10 days of chronic stress resulted in significant differences in latency to leave the dark box (one-way ANOVA; $p<0.05$ $F_{2,20}=4.402$; Figure 4.4A). Specifically, that both stress-susceptible and stress resilient animals showed an increase in time taken to leave the dark area (Bonferroni post-hoc; $p<0.05$ stress-susceptible and stress-resilient) compared to control animals. Consequently, they also spent significantly less time in the lit area (one-way ANOVA; $p<0.05$ $F_{2,20}=5.857$) with both susceptible ($p<0.01$) and resilient ($p<0.05$; Figure 4.4B) animals showing a decreased amount of time in the lit area in post-hoc analysis when compared to controls. Finally, group analysis showed that there was no overall difference in transitions between the lit and dark areas (one-way ANOVA; $p=0.06$ $F_{2,20}=3.244$) but post-hoc analysis did reveal and a decreased number of transitions between the light and dark zones in stress-resilient animals when compared to controls ($p<0.05$; Figure 4.4C).

4.4.3 Chronic stress alters home-cage activity in stress susceptible mice

As well as measuring behaviour in novel and challenging environments such as the open field and the forced swim test, the SAMAB system enables monitoring of animals in their home cage over a number of days. This enables the measurement of activity not only during the dark phase (09:00-21:00), when all other testing was taking place, but also during the light phase (21:00-09:00), when the animals would normally be resting. This system was used to measure activity for 3 days before and 3 days after the 10 days of chronic stress. The data were averaged across the 3 days to give an overall day/night

activity graph. Control animals were inactive during the light phase of the light/dark cycle followed by an increase in activity peaking at the start of the dark phase and gradually declining across 12 hours (Figure 4.5A). Activity in the home cage environment prior to stress in stress susceptible animals did not appear different to control animals. Repeated measures analysis of activity over time indicates that both stress and time have a significant effect on activity, but that these factors do not interact (stress $p<0.001$ $F_{5,84}=17.47$; time $p<0.001$ $F_{7,588}=75.97$; stress:time $p=0.18$ $F_{35,588}=1.213$). After the onset of stress, susceptible animals were significantly more active during the dark phase of the light/dark cycle (Bonferroni post-hoc $p<0.01$ between 09:00-18:00; Figure 4.5B) than all other groups. This increase in activity was not observed in animals that had undergone the stress procedure, but had a high sucrose preference (stress-resilient animals), whose light/dark activity patterns were similar to those of control animals (Figure 4.5C).

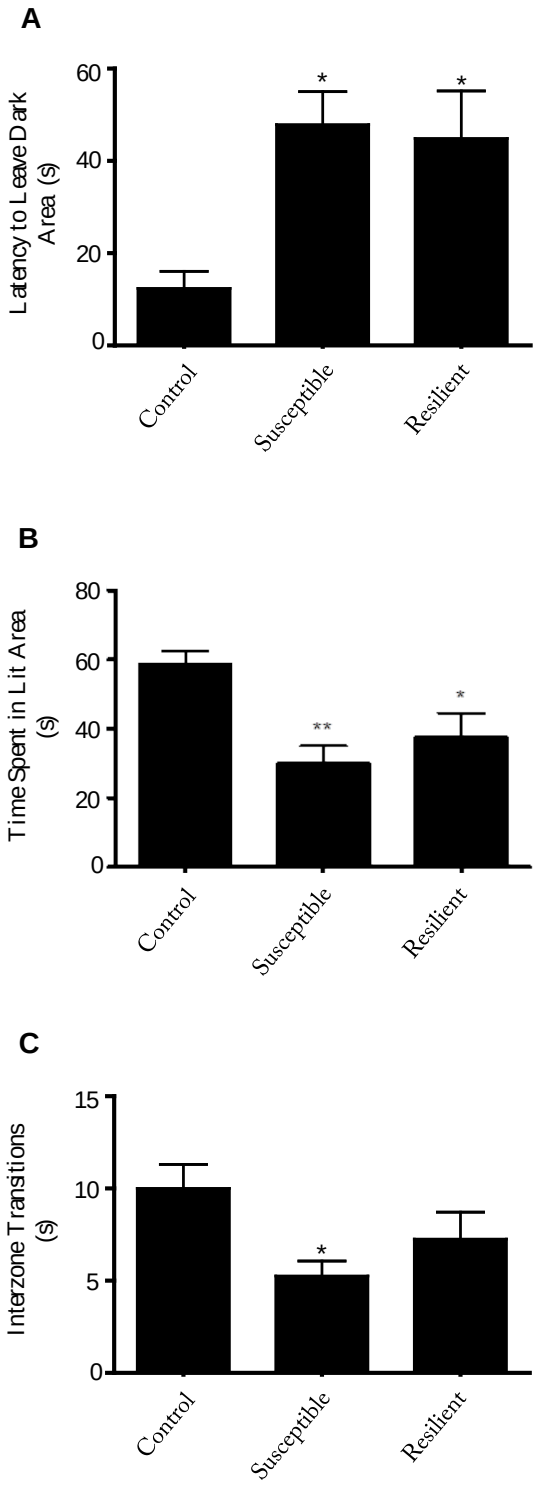


Figure 4.4 Light-dark box activity in control and stressed animals. 10 days of chronic stress causes (A) an increased latency to leave the dark area in the light-dark box; (B) an increased time spent in the dark box and (C) a decreased number of transitions between the light and dark areas. Data are mean ±SEM n=6+. *=$p<0.05$ and **$p<0.01$ compared to control animals.

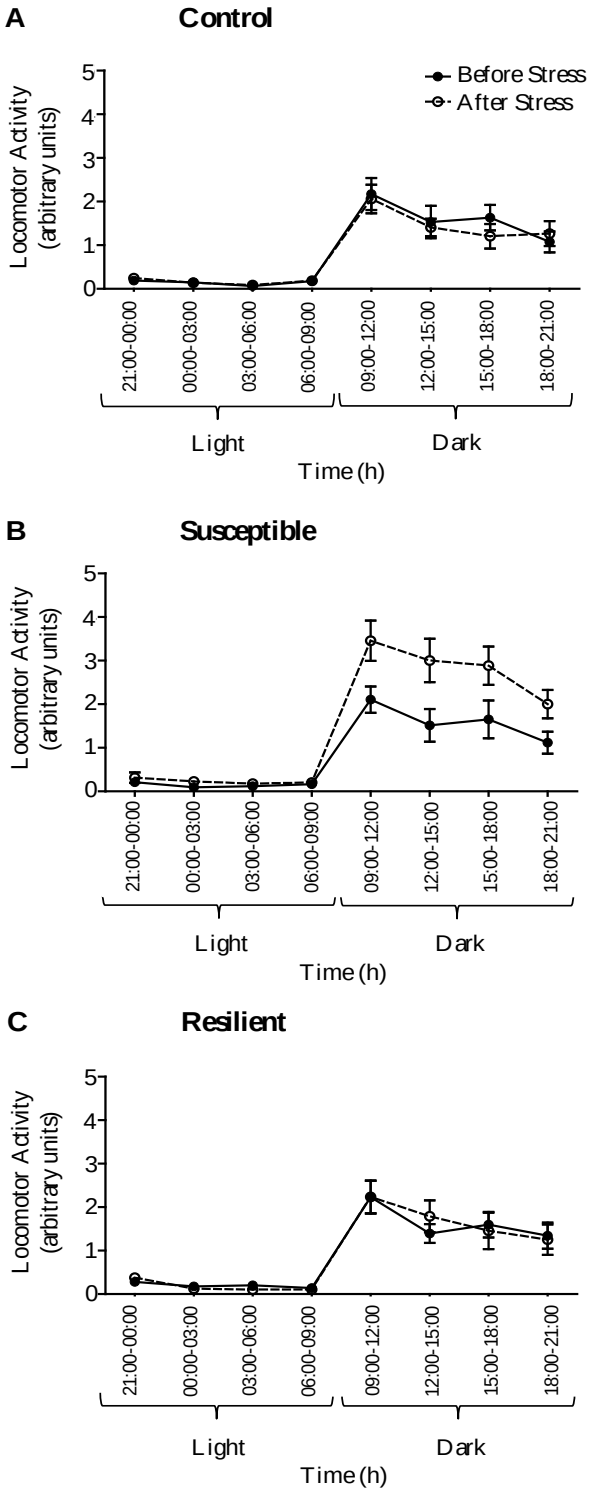


Figure 4.5 Home-cage activity in animals before and after stress. Animals were subjected to chronic stress for 10 days and separated into susceptible and resilient subgroups according to sucrose preference at this time. Locomotor activity was quantified using the SAMAB system in the home-cage environment in (A) control animals; (B) stress-susceptible animals and (C) stress-resilient animals. Data was acquired as locomotor counts/h in arbitrary units and binned into 3 hour groups. Baseline and measurement data was acquired across 3 days. Data are mean ±SEM, n=6+.

4.4.4 Cortisol, but not systemic cytokines, increase after chronic stress

Studies have shown that depressed patients often have elevated levels of circulating pro-inflammatory cytokines (Maes *et al.*, 1997). In order to determine the degree to which chronic stress induces an inflammatory phenotype, the levels of the pro-inflammatory cytokines TNFα and IL-1β, as well as the acute phase protein CXCL-1, were measured in the livers of chronically stressed animals. Stress did not significantly increase levels of hepatic TNF compared to controls. Furthermore, citalopram administration also had no effect on this cytokine (Figure 4.6A). Analysis of IL-1β levels showed that there was an overall effect of stress (one-way ANOVA $p<0.001$ $F_{2,18}=15.88$) with post-hoc analysis revealing a tendency to increase after stress alone (Bonferroni post-hoc; $p=0.07$; Figure 4.6B), an effect that was significantly augmented by pre-treatment with citalopram ($p<0.001$; Figure 4.6B). The neutrophil chemoattractant CXCL-1 was significantly affected by stress-group (one-way ANOVA $p<0.05$ $F_{2,18}=5.867$), although this may be due to the potent effect of citalopram, but it was not significantly up-regulated in the stress alone group when compared to control animals. However, it did increase in animals that had been treated with citalopram prior to and during the stress procedure (Bonferroni post-hoc; $p<0.05$; Figure 4.6C).

Stress is known to up-regulate corticosterone (Dallman *et al.*, 2004) and, in humans, depressed patients often show dexamethasone non-suppression, indicating an overactive HPA axis and extremely high levels of circulating cortisol. Here, circulating levels of corticosterone in control animals were, on average, 10nM. Circulating corticosterone significantly changes across groups (one-way ANOVA; $p<0.01$ $F_{3,29}=5.653$), and that it was significantly increased in stress susceptible (Bonferroni post-hoc; $p<0.01$) and stress resilient ($p<0.01$) animals compared to controls (Figure 4.7). Animals receiving anti-depressant treatment both before and during the stress procedure also had significantly higher levels of circulating corticosterone than controls ($p<0.05$). No significant differences were found between stress susceptible, stress resilient and antidepressant treated animals.

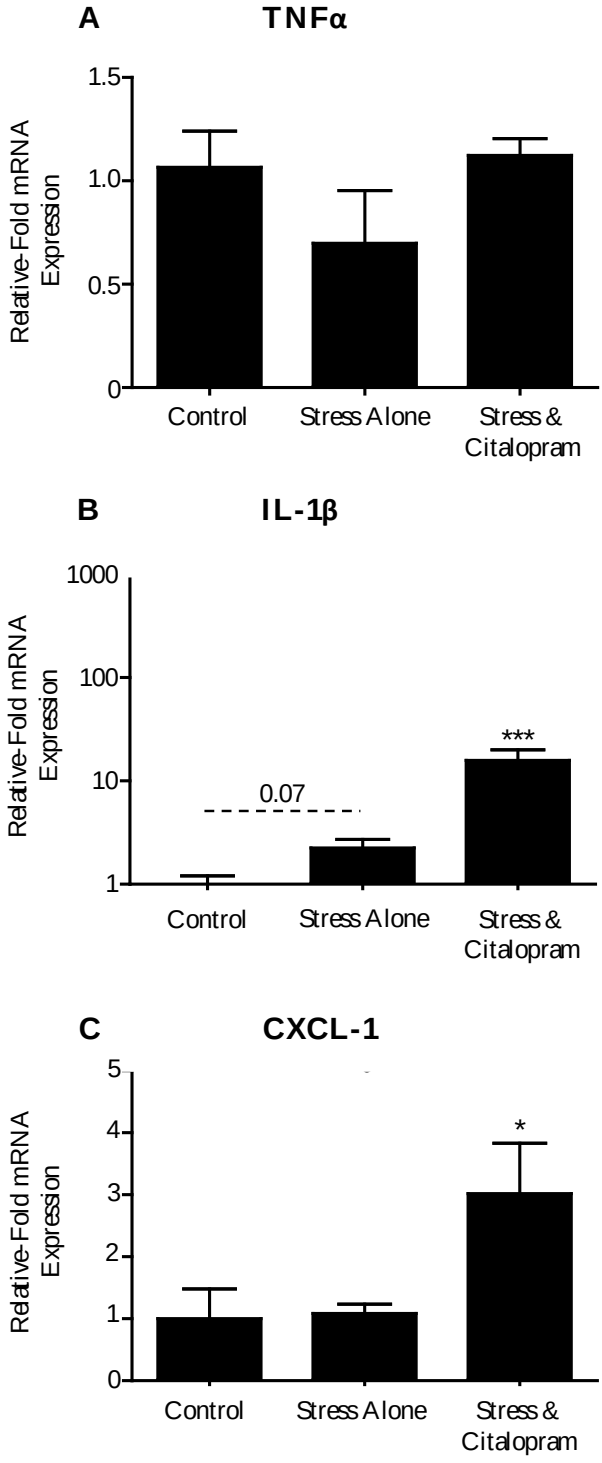


Figure 4.6 Cytokine mRNA in the liver after untreated and treated chronic stress.mRNA levels of (A) TNFα; (B) IL-1β and (C) CXCL-1 mRNA were measured by qPCR in the livers of animals after either 10 days of chronic stress or 10 days of chronic stress treated concomitantly with citalopram. Data are relative-fold expression normalized to GAPDH. Bars are mean ±SEM (n=5), $p<0.05$ and ***$p<0.001$ compared to control animals.

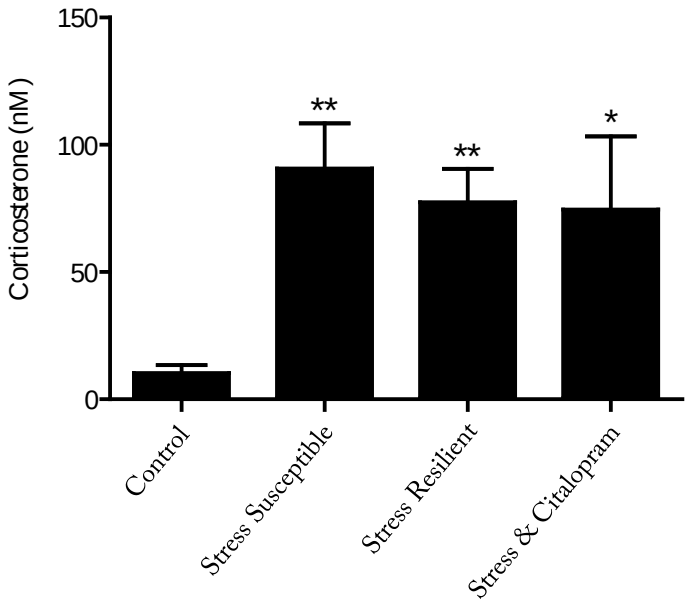


Figure 4.7 Corticosterone concentration in plasma in control and stressed animals. Plasma samples were taken after 10 days of chronic stress either with or without anti-depressant treatment. Corticosterone levels were assessed by HPLC and are presented as absolute concentrations in plasma. Data are mean ±SEM (n=6); *$p<0.05$ and **$p<0.01$.

4.4.5 CNS cytokines, COX-1 and IDO are affected by chronic stress

Stress induces corticosterone release in animals and this is generally considered to down-regulate the expression of pro-inflammatory cytokines such as TNF, and up-regulate the expression of anti-inflammatory cytokines such as IL-10. However, stress is also known to exacerbate CNS lesions (Stein-Behrens *et al.*, 1994). Whether this exacerbation is via the direct action of glucocorticoids or via the induction of CNS cytokines is thus far unknown and very few studies have investigated the CNS inflammatory profile after chronic stress. Therefore, this study aimed to look at the induction of TNFα and IL-1β mRNA in a number of key brain regions studied in previous chapters. Specifically, the prefrontal and motor cortices, striatum, hippocampus and dorsal raphe nucleus. Animals were subjected to 10 days of chronic stress, followed by a panel of behavioural tests in order to separate stress susceptible and stress resilient animals from each other.

Despite no evidence of a peripheral increase in TNF expression after stress (*see* 4.4.4), brain TNF appeared to change in response to chronic stress. However, ANOVA revealed no significant effects of either stress-group or brain region, or an interaction, on TNF expression after 10 days of chronic stress (two-way, RM-ANOVA group $p=0.06$ $F_{3,20}=3.06$; brain region $p=0.69$ $F_{4,80}=0.55$; group:brain region $p=0.22$ $F_{12,80}=1.34$). Despite the lack of significant main effects, it is still important to consider specific comparisons within the factor levels, but it is important to remember these results should be considered with caution. Post-hoc analysis (Bonferroni) shows that TNF mRNA significantly increased in the chronic stress paradigm in both the prefrontal cortex and raphe nucleus in stress susceptible animals (prefrontal cortex $p<0.05$, raphe $p<0.05$; Figure 4.8A). TNF levels appeared to show increases in other brain areas in stress susceptible animals, such as the motor cortex, but these increases were not statistically significant. Stress resilient animals appeared to show a slight increase in TNF in the striatum, but in no region were the changes in TNF expression significantly different from control animals. Similarly, the levels of expression of TNF were unchanged in all brain regions, after treatment with antidepressant.

Chronic stress also significantly affected IL-1β expression, although there was no main effect of brain region and no interaction (group $p<0.001$ $F_{3,20}=16.32$; brain region $p=0.15$ $F_{4,80}=1.73$; group:brain region $p=0.50$ $F_{12,80}=0.95$). Post-hoc tests (Bonferroni) showed that there was significantly increased IL-1β expression in the motor cortex (susceptible $p<0.05$, resilient $p<0.01$), striatum (susceptible $p<0.001$, resilient $p<0.01$) and hippocampus (susceptible $p<0.05$, resilient $p<0.01$) in all stressed animals (Figure 4.8B). In the raphe, however, significant increases in IL-1β were only seen in stress susceptible animals compared to controls ($p<0.05$; Figure 4.8B). Animals treated with antidepressant prior to, and during, the stress procedure showed no significant elevations in IL-1β in any brain region (Figure 4.8B) compared to control animals.

COX inhibitors have been shown to be a potential novel anti-depressant therapy and been shown to be effective in models of chronic stress (Guo *et al.*, 2009). With this in mind it was considered important to study the levels of COX in the CNS. Both the constitutive (COX-1) and the inducible form of COX (COX-2) were studied but there was no significant expression of COX-2 in any brain region at any time (data not shown). Analysis of COX-1 expression showed a significant main effect of group, a significant main effect of brain region and a significant interaction (group $p<0.01$ $F_{3,20}=10.10$; brain region $p<0.001$ $F_{4,80}=13.38$; group:brain region $p<0.05$ $F_{12,80}=2.40$; Figure 4.8C). More detailed analysis shows an increase in COX-1 mRNA in stress susceptible animals in all brain regions. However, this only reaches significance in the prefrontal cortex, striatum and dorsal raphe (Bonferroni post-hoc prefrontal cortex $p<0.001$; striatum $p<0.01$; raphe $p<0.01$). In stress resilient animals there was also a significant increase in COX-1 mRNA in the prefrontal cortex ($p<0.001$), the striatum ($p<0.001$) and the raphe ($p<0.05$), with an increase, but not to significance, in the motor cortex and hippocampus (Figure 4.8C). Finally, imipramine-treated stressed animals showed a significant increase in COX-1 mRNA in the prefrontal cortex ($p<0.001$), but while there was a slight increase in all other brain regions this did not reach significance (Figure 4.8C).

IDO expression was significantly affected by stress, showed a different pattern of expression across brain regions and showed a significant interaction between group and

brain region (group $p<0.05$ $F_{3,20}=4.18$; brain region $p<0.001$ $F_{4,80}=7.27$; group:brain region $p<0.01$ $F_{12,80}=2.20$; Figure 4.8D). Post-hoc analysis showed no significant differences in any stress regime in any brain region with the exception of stress susceptible animals, which showed a significant increase in IDO mRNA in the raphe compared to controls (Bonferroni post-hoc $p<0.001$).

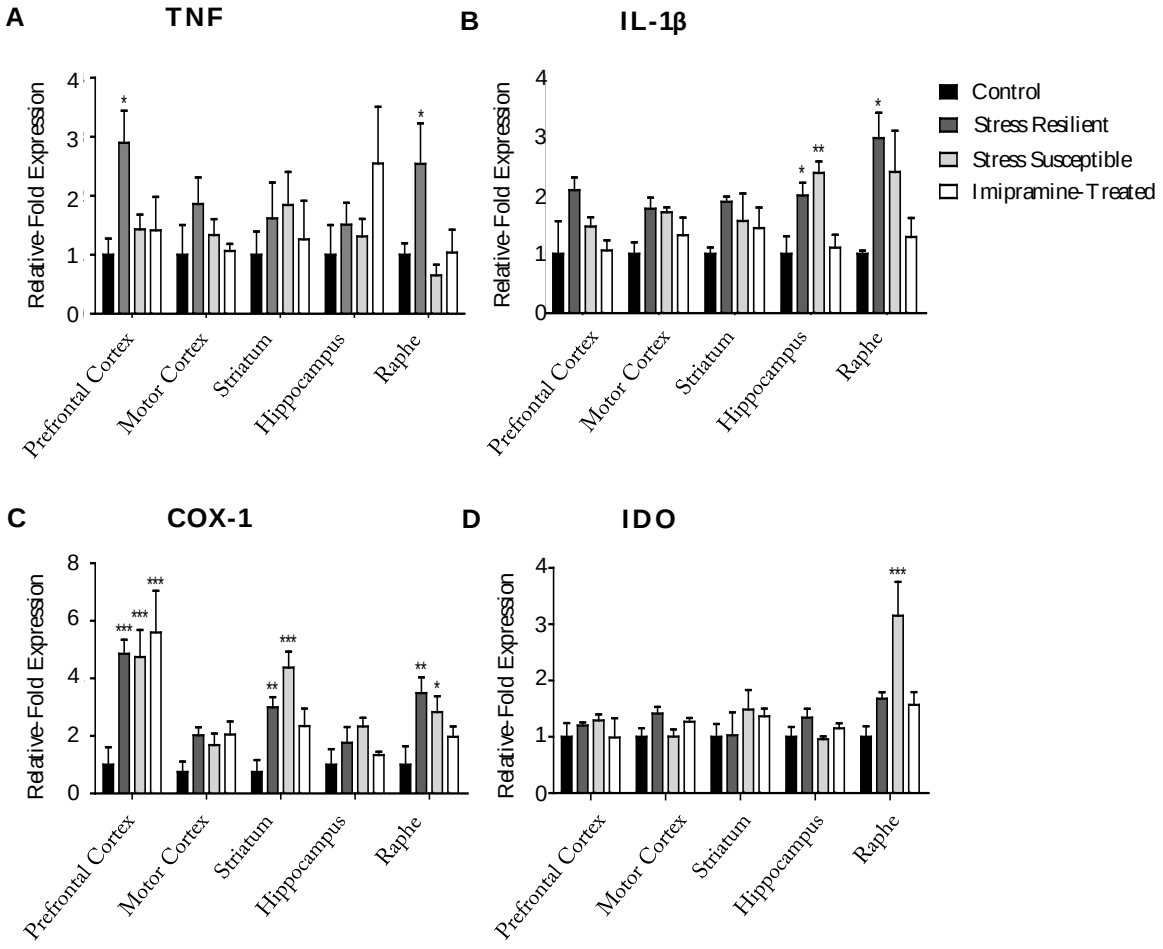


Figure 4.8 CNS cytokines, COX-1 and IDO after chronic stress. Animals were subjected to 10 days of chronic stress in the presence or absence of anti-depressant imipramine. Based on behaviour animals were separated into low sucrose preference (dark grey bars) or normal sucrose preference (light grey bars) after stress. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) TNF; (B) IL-1β; (C) COX-1 and (D) IDO were examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=4, *p<0.05, **p<0.01 and ***p<0.001 when compared to controls within each region.

4.4.6 Stress susceptible mice show an increase in reactive microglia only in the prefrontal cortex

Peripheral inflammatory cytokines, as well as central cytokines, will affect the inflammatory status of the brain and this will be indirectly reflected in the number of activated microglia. Here, a number of areas were defined according to Paxinos and Watson to study microglial numbers throughout the brain. At the front of the brain an area was predefined as 'prefrontal cortex', in order for it to be associated with the area removed for PCR in other sections within this chapter (Figure 4.9A). The PFC is a critical relay station for the processing of external emotional or stress based cues (Miller *et al.*, 2002) and has been shown in this chapter, and in previous chapters, to show a marked inflammatory response to a systemic LPS challenge which is not reflected in other areas in the brain. In the same sagittal sections of tissue it was possible to study both the dorsal striatum and the lateral periacqueductal grey (PAG) area. Microglia were quantified in these regions to determine whether there was any significant difference in microglial numbers between stress susceptible and stress resilient animals. Control animals in the dorsal striatum and PAG did not show significantly different numbers of microglia to stressed mice and therefore data were not included.

Control animals showed an average of 330 Iba-1-positive microglia per mm^2 in the PFC (Figure 4.9B). Lack of control counts for the dorsal striatum and lateral PAG made analysis challenging. Microglial number vary across brain regions therefore a simple one-way ANOVA within the PFC was considered appropriate. This analysis showed there was variation between the groups ($p<0.05$ $F_{2,15}=4.308$) with post-hoc analysis showing that chronic stress significantly increased the number of Iba-1-positive microglia in the prefrontal cortex in stress susceptible animals (Bonferroni post-hoc; $p<0.05$; Figure 4.9B), whilst stress resilient showed no significant differences from control animals. In the dorsal striatum there was an average of 330 Iba-1-positive microglia per mm^2 in both stress-susceptible and stress-resilient mice, showing no difference between the means of the two groups. The lateral PAG showed an average of 210 Iba-1-positive microglia per

mm^2 in stress-susceptible mice, which was not different from counts in stress-resilient mice.

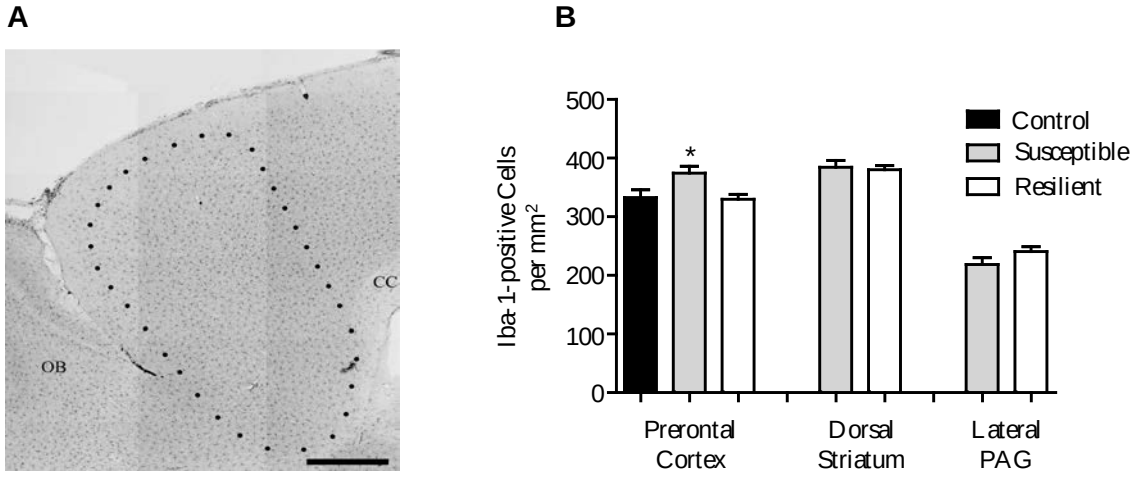


Figure 4.9 Iba-1 reactive microglia in the prefrontal cortex of control and stressed animals. Animals were subjected to either handling stress prior to sacrifice, acute (overnight with predator) stress or chronic stress for 10 days. Susceptible and resilient animals were separated according to sucrose preference at day 10. Brain tissue was fixed, cut sagitally and stained for the microglial activation marker Iba-1 and cells were counted across a 1mm^2 grid over serial sections in each animal. (A) Micrograph and outline of area counted within each brain. (B) Mean Iba-1 counts across serial sections. Scale bar is 100µm. Data are mean ±SEM, n=5+. *$p<0.05$ compared to control animals.

4.4.7 Chronic stress alters the CNS expression of 5-HT$_{2A}$ and SERT

Changes in 5-HT$_{2A}$ and SERT have already been demonstrated in an inflammatory model of sickness behaviour (*see* 3.3.7); it was therefore important to determine whether similar changes occur in a mouse model showing depressive-like symptoms. Animals were challenged with 10 days of chronic stress followed by a number of behavioural tests to separate stress susceptible and stress resilient animals. Tissue was removed after behavioural testing to study gene changes by PCR in the prefrontal and motor cortices, striatum, hippocampus and raphe. Data were normalized to control animals.

Overall analysis of 5-HT$_{2A}$ expression showed a significant main effect of stress, a significant effect of brain region and a significant interaction between stress-induced changes and brain region (Two-way, RM-ANOVA stress $p<0.001$ $F_{3,12}=26.62$; brain region $p<0.001$ $F_{4,48}=55.43$; stress:brain region $p<0.001$ $F_{12,48}=9.19$). More specifically, chronic stress resulted in a significant 3-fold increase in 5-HT$_{2A}$ expression in the prefrontal cortex in all stressed animals (Bonferroni post-hoc; susceptible $p<0.01$, resilient $p<0.05$ and imipramine-treated $p<0.05$; Figure 4.10A), compared to controls. Elsewhere, susceptible animals showed an increase in receptor expression in the striatum ($p<0.05$) compared to control animals and also showed a trend towards an increase in the raphe ($p=0.062$; Figure 4.10A). Resilient animals did not show any changes in receptor expression anywhere other than the prefrontal cortex, nor did animals receiving imipramine prior to, and during, stress, when either group was compared with control animals.

SERT expression was significantly affected by stress but not different across brain regions, there was, however, a significant interaction between stress and brain region (stress $p<0.001$ $F_{3,12}=28.17$; brain region $p=0.29$ $F_{4,48}=1.28$; stress:brain region $p<0.001$ $F_{12,48}=8.47$). Post-hoc analysis demonstrated that SERT increased significantly in stress susceptible animals in the prefrontal cortex (Bonferroni post-hoc; $p<0.001$), striatum ($p<0.05$) and hippocampus ($p<0.05$; Figure 4.10B). These animals showed no significant changes in the raphe or motor cortex compared to controls. Stress resilient animals showed no significant changes in SERT expression compared to control animals, neither

did animals receiving imipramine prior to, and during, the stress procedure (Figure 4.10B)

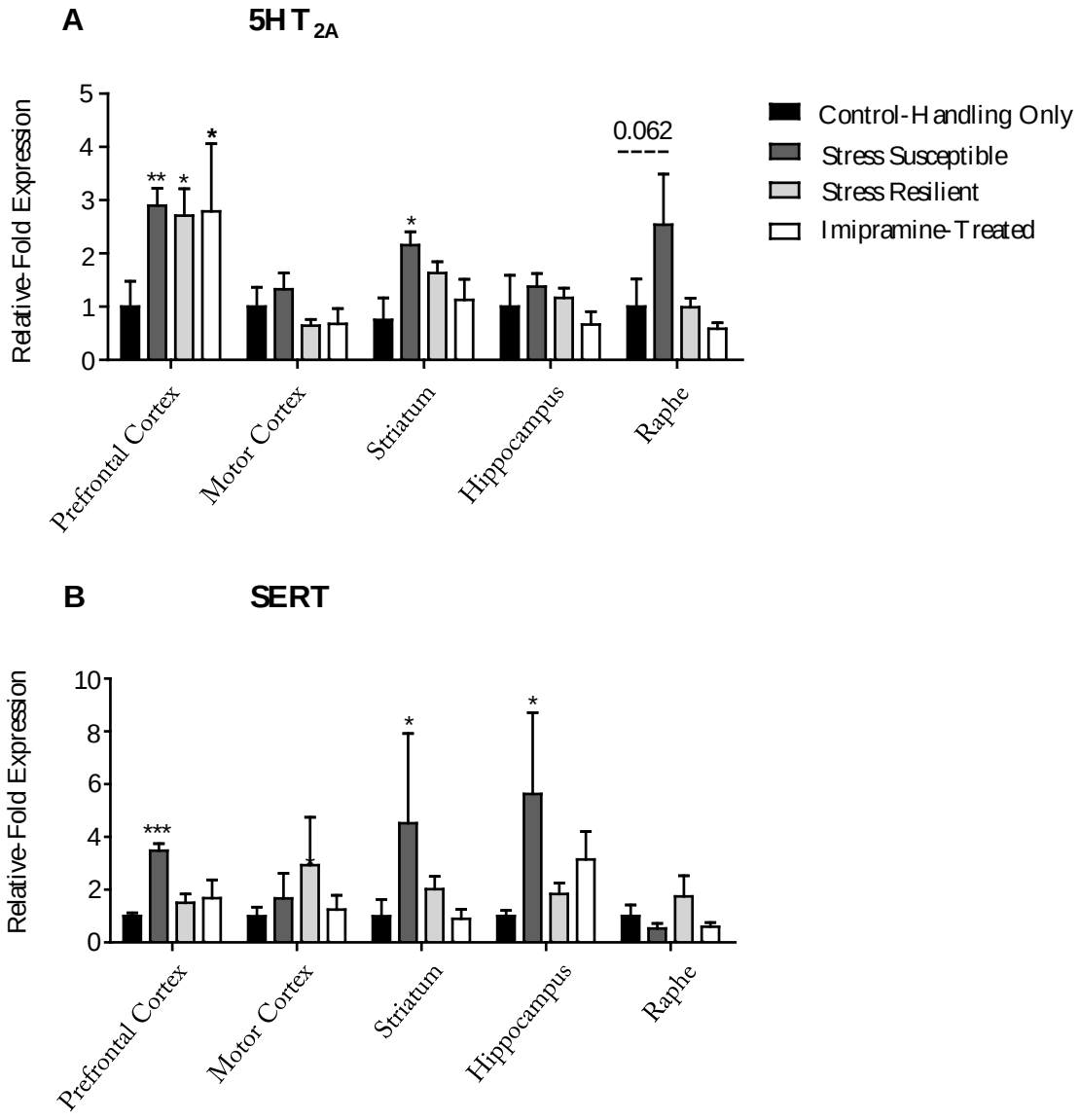


Figure 4.10 5-HT$_{2A}$ receptor and SERT mRNA expression after chronic stress. Animals were subjected to 10 days of chronic stress in the presence or absence of anti-depressant imipramine. Based on behaviour animals were separated into low sucrose preference (dark grey bars) or normal sucrose preference (light grey bars) after stress. Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) 5-HT$_{2A}$ and (B) SERT was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=6, *p<0.05,**p<0.01 and ***p<0.001.

4.4.8 LPS does not significantly exacerbate stress-induced changes in sucrose preference

Low dose LPS (0.1mg/kg) given 24 hours prior to testing does not significantly alter baseline behaviour (Cunningham *et al.*, 2009). However, it was hypothesized that if stress naturally increases pro-inflammatory cytokines, stimulation of the system with an inflammatory challenge may significantly alter behaviour. All animals showed a preference of >65% for a 1% sucrose solution prior to testing (data not shown). Control animals, and animals injected 24 hours prior to testing with 0.1mg/kg LPS, maintained a sucrose preference of >65% and were not significantly different from each other (Figure 4.11). After 10 days of chronic stress there was a significant main effect of stress on sucrose consumption but not of LPS and there was no interaction between stress and LPS (two-way ANOVA; stress $p<0.001$ $F_{1,54}=16.62$; LPS $p=0.28$ $F_{1,54}=1.182$; stress:LPS $p=0.41$ $F_{1,54}=0.689$). Post-hoc tests showed that after 10 days of chronic stress and a single i.p. dose of saline, animals showed <65% preference for a sucrose solution, which was significantly different from controls (Bonferroni post-hoc $p<0.05$; Figure 4.11). Animals undergoing 10 days of chronic stress and a single i.p. dose of LPS (0.1mg/kg) 24 hours prior to testing also showed a decrease in sucrose preference compared to controls ($p<0.001$; Figure 4.11). Whilst there appears to be a further decrease between stressed animals and stressed animals receiving LPS, this difference is not significant ($p=0.192$). Since samples sizes are unequal across groups post-hoc tests should be considered with caution.

4.4.9 LPS may exacerbate behavioural changes induced by chronic stress

Data from the Sharp laboratory (Qin Xie, unpublished data) and data from this thesis have demonstrated that endotoxin causes short-term behavioural changes (*see* 3.3.2). Many of these behavioural changes are generally agreed to have abated by 24 hours post-challenge, while elevations in cytokine levels persist (Cunningham *et al.*, 2005). Here we used a subpyrogenic dose-time regime (0.1mg/kg 24 hours prior to the end of stress; *see* Figure 4.1) to study whether low levels of circulating cytokines can influence stress-like behaviours. In the FST, control and LPS alone animals showed similar values in both

latency to float and total time spent floating. Analysis showed that both stress and LPS had a significant effect on floating behaviour but that there was no interaction between factors and therefore all results should be considered with caution (two-way ANOVA; stress $p<0.001$ $F_{1,43}=21.46$; LPS $p<0.05$ $F_{1,43}=5.495$; stress:LPS $p=0.19$ $F_{1,43}=1.76$). In post-hoc tests chronic stress significantly decreased latency to float compared to controls ($p<0.05$; Figure 4.12A), as did chronic stress combined with LPS ($p<0.001$). Using multiple pairwise comparisons (Bonferroni post-hoc), LPS combined with stress is significantly different from stress alone group ($p<0.05$), however, as there is no interaction between these factors this result should be interpreted with caution.

Total duration of floating behaviour was only significantly affected by stress, not LPS, and there was no interaction between factors (two-way ANOVA; stress $p<0.01$ $F_{1,43}=9.654$; LPS $p=0.17$ $F_{1,43}=1.922$; stress:LPS $p=0.32$ $F_{1,43}=0.99$). In post-hoc tests, the combination of chronic stress and LPS is the only factor which significantly increases the total time spent floating compared to any other group in the FST (Bonferroni post-hoc; $p<0.05$ Figure 4.12B). While this suggests that LPS and stress significantly affect this behaviour, the lack of interaction makes these results difficult to interpret.

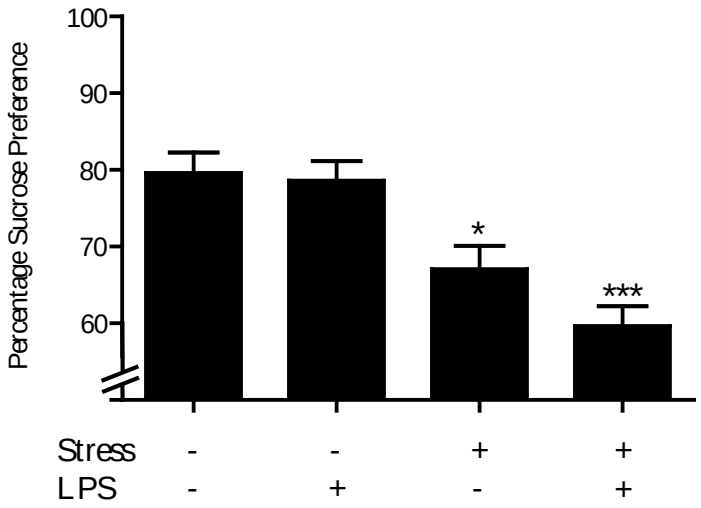


Figure 4.11 Sucrose preference in control, stressed and LPS treated animals. Animals were subjected to 10 days of chronic stress followed by a two-bottle sucrose preference test over 12 hours. All animals showed >65% preference at baseline (data not shown). Neither control animals, nor animals receiving a single i.p. dose of LPS (0.1mg/kg at t-24) showed a decrease in sucrose preference. Both chronic stress and chronic stress with a systemic immune challenge, significantly reduced sucrose preference. Data are mean ±SEM n=6; *$p<0.05$ and ***$p<0.001$ compared to control animals.

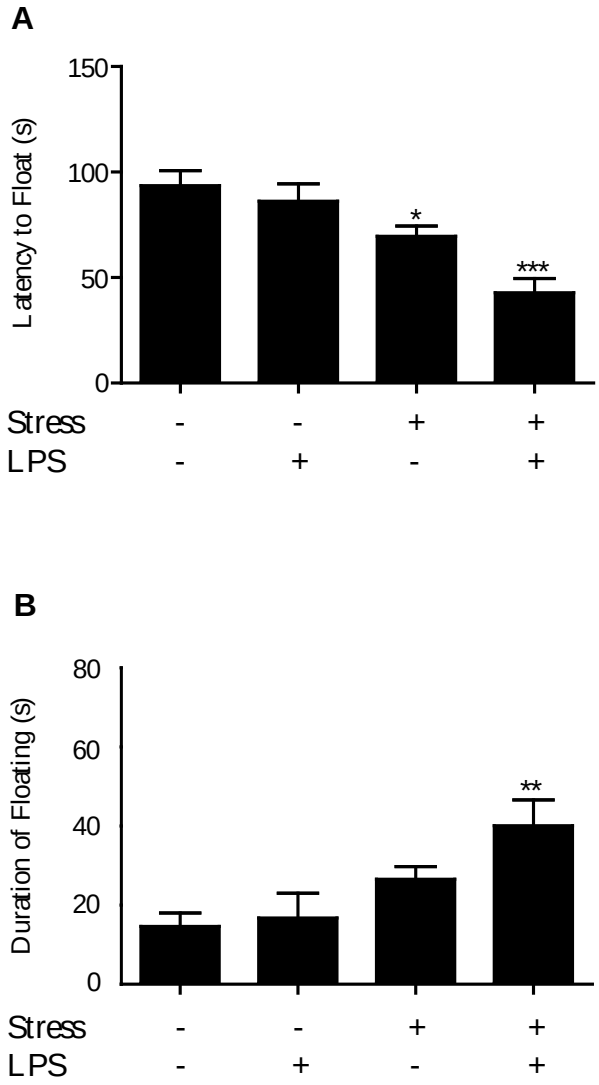


Figure 4.12 Measurements of forced swim behaviour in control, stressed and LPS animals. Chronic stress and LPS, as well as a combination of these factors, changes latency to float (A), as well as duration of floating (B) in the forced swim tests. Animals were subjected to 10 days of chronic stress followed by a low-dose inflammatory challenge (0.1mg/kg LPS) on day 9, behavioural testing took place on day 10. Data are mean ±SEM n=8+. *=$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control animals.

4.4.10 The effect of chronic stress and LPS on corticosterone and liver cytokines

Systemic inflammation has been shown to increase circulating cytokines, and stress is known to decrease pro-inflammatory cytokine expression via glucocorticoid induction (Propes and Johnson, 1997). The levels of pro-inflammatory cytokines present 24 hours after injection of 0.1mg/kg endotoxin should be relatively low (Cunningham *et al.*, 2009).

In this experiment, both LPS and stress had a significant effect on TNF expression, furthermore there was a significant interaction between the factors (two-way ANOVA; stress $p<0.01$ $F_{1,18}=9.259$; LPS $p<0.001$ $F_{1,18}=22.07$; stress:LPS $p<0.05$ $F_{1,18}=6.472$). At 24 hours after LPS injection (0.1mg/kg), a 5-fold increase in hepatic TNF was found to be significantly elevated when compared to controls (Bonferroni post-hoc; $p<0.001$; Figure 4.13A). As previously (*see* Figure 4.6), chronic stress did not up-regulate TNF mRNA in the liver. Chronic stress and LPS, combined, appeared to increase levels of TNF mRNA compared to controls, but this change is not significant (Figure 4.13A).

IL-1β mRNA expression was affected by stress and LPS but there was no significant interaction between the two factors (two-way ANOVA; stress $p<0.001$ $F_{1,18}=15.56$; LPS $p<0.01$ $F_{1,18}=12.61$; stress:LPS $p=0.07$ $F_{1,18}=3.711$). IL-1β expression was slightly higher in stress- and LPS-alone animals, but in neither case are they significantly different from controls (Figure 4.13B). The combination of 10 days of chronic stress and a low-dose LPS challenge on day 9 resulted in a significant 6-fold increase in hepatic IL-1β ($p<0.001$; Figure 4.13B).

The effect of group on hepatic CXCL-1 expression was significant for both stress and LPS, and they showed a significant interaction (two-way ANOVA; stress $p<0.001$ $F_{1,18}=34.23$; LPS $p<0.001$ $F_{1,18}=30.79$; stress:LPS $p<0.001$ $F_{1,18}=37.70$). Post-hoc analysis showed a significant increase after an LPS-challenge ($p<0.001$; Figure 4.13C), but no significant increase in stressed animals or in animals which had undergone stress and LPS in combination (Figure 4.13C).

Plasma corticosterone levels increased after chronic stress and were not affected by antidepressant treatment (*see* Figure 4.7). High dose (0.5mg/kg) LPS has been shown to increase corticosterone levels elsewhere in this thesis (*see* 3.3.3). Control animals had an average of 10nM corticosterone. Analysis showed that both stress and LPS had a significant effect on corticosterone levels, and that there was a significant interaction between these factors (two-way ANOVA; stress $p<0.05$ $F_{1,25}=4.605$; LPS $p<0.01$ $F_{1,25}=9.355$; stress:LPS $p<0.05$ $F_{1,25}=6.659$) More specifically, data showed that administration of 0.1mg/kg LPS significantly increased circulating corticosterone, to an average of 90nM (Bonferroni post-hoc; $p<0.01$; Figure 4.14). Following 10 days of chronic stress and 10 days of stress in combination with a 0.1mg/kg LPS challenge on day 9, elevated circulating corticosterone levels (100nM) were also found, and were significantly higher than controls (stress alone $p<0.01$, stress and LPS $p<0.01$; Figure 4.14). At no point were stressed or LPS treated animals different from each other.

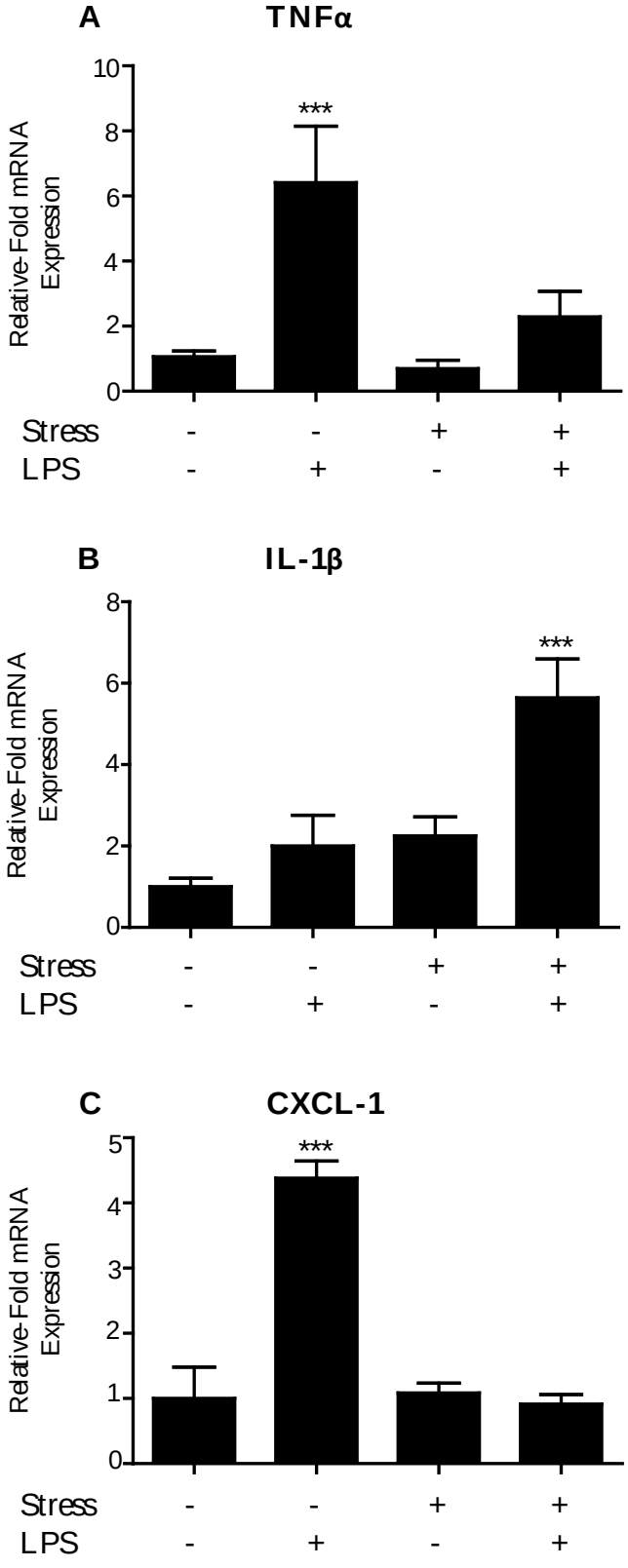


Figure 4.13 Cytokine mRNA in the liver after untreated and treated chronic stress or LPS treatment.mRNA levels of (A) TNFα; (B) IL-1β and (C) CXCL-1 mRNA were measured by qPCR in the livers of animals after either 10 days of chronic stress, 10 days of chronic stress treated concomitantly with citalopram or an acute LPS challenge (0.5mg/kg; 6 hours). Data are relative-fold expression normalized to GAPDH. Bars are mean ±SEM (n=4+), $p<0.05$ and ***$p<0.001$ compared to control animals.

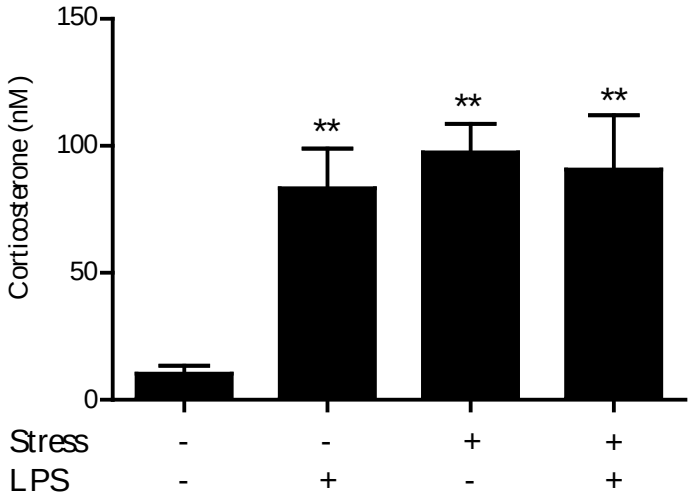


Figure 4.14 Corticosterone concentration in plasma after chronic stress and LPS. Plasma samples were taken after 10 days of chronic stress either with or without anti-depressant treatment or with an additional LPS challenge 24 hours prior to sample collection. Corticosterone levels were assessed by HPLC and are presented as absolute concentrations in plasma. Data are mean ±SEM (n=4+); **p<0.01 compared to control animals.

4.4.11 LPS does not exacerbate chronic stress-induced changes in 5-HT$_{2A}$ and SERT expression

Earlier studies in this thesis (*see* 3.3.7), as well as work from this chapter (*see* Figure 4.10) have demonstrated that both LPS and chronic stress are capable of changing the expression of the 5-HT$_{2A}$ receptor and SERT. After demonstrating that LPS is capable of exacerbating behaviours induced by chronic stress (*see* Figure 4.12), it was important to determine whether receptor expression was also cumulatively increased, or whether, like corticosterone (*see* Figure 4.14), stress and LPS combined did not affect receptor expression. Animals received 10 days of chronic stress and were challenged on day 9 with 0.1mg/kg LPS (i.p.). The addition of both stress and LPS into the model requires a more complex analysis with stress and LPS and treatment factors and brain region as a repeated factor. The general linear model applied to the earlier data remains, with unstructured co-variance, but with the added capacity of determining whether stress and LPS interact with each other. The number of possible interactions makes reporting this data rather excessive, therefore only significant values will be reported.

Analysis shows that there was only a significant main effect of brain region on 5-HT$_{2A}$ expression, as well as a significant interaction between brain region, stress and LPS challenge (RM-ANOVA brain region $p<0.001$ $F_{4,48}=16.20$; stress:LPS:brain region $p<0.01$ $F_{4,48}=4.96$). Post-hoc analysis shows 5-HT$_{2A}$ receptor expression appeared to increase after a single LPS injection in the prefrontal cortex, motor cortex, striatum and hippocampus, compared to controls, but was only significantly different in the hippocampus (Bonferroni post-hoc $p<0.01$; Figure 4.15A). There was no difference, significant or otherwise, in 5-HT$_{2A}$ levels in the raphe compared to controls (Figure 4.15A). In a similar manner, after 10 days of chronic stress, 5-HT$_{2A}$ mRNA appeared to be elevated in the prefrontal and motor cortices, as well as the hippocampus, but again, only reached significance in the latter when compared to control animals ($p<0.01$; Figure 4.15A). Chronic stress did not change receptor expression in either the striatum or the raphe. In the CNS of animals challenged with 10 days of chronic stress and a dose of LPS, 5-HT$_{2A}$ receptor expression was not different from controls in any region except the

hippocampus, where it showed an increase of a similar magnitude to stress- and LPS-alone ($p<0.05$; Figure 4.15A).

SERT expression showed the same main effects as the 5-HT_{2A} receptor but significantly more interactions. Specifically, there was a main effect of brain region and interactions between brain region and stress, brain region and LPS challenge and all three factors (ANOVA; brain region $p<0.001$ $F_{4,48}=22.23$; stress:brain region $p<0.001$ $F_{4,48}=15.46$; LPS:brain region $p<0.001$ $F_{4,48}=6.42$; stress:LPS:brain region $p<0.01$ $F_{4,48}=12.32$). Further analysis showed that SERT expression in the prefrontal and motor cortices after a single LPS challenge appeared to be higher than controls, but did not reach significance (Bonferroni post-hoc prefrontal cortex $p=0.081$, motor cortex $p=0.072$; Figure 4.15B). No other brain regions studied showed any change in SERT mRNA compared to controls after a single dose of LPS. 10 days of chronic stress did not change SERT expression in the prefrontal cortex, motor cortex or the raphe compared to controls, but led to significantly higher expression in the striatum and hippocampus (striatum $p<0.001$, hippocampus $p<0.01$; Figure 4.15B). Stress and LPS combined resulted in a slight, but non-significant, elevation in SERT mRNA in the motor cortex and striatum, and no change in any other brain region (Figure 4.15B).

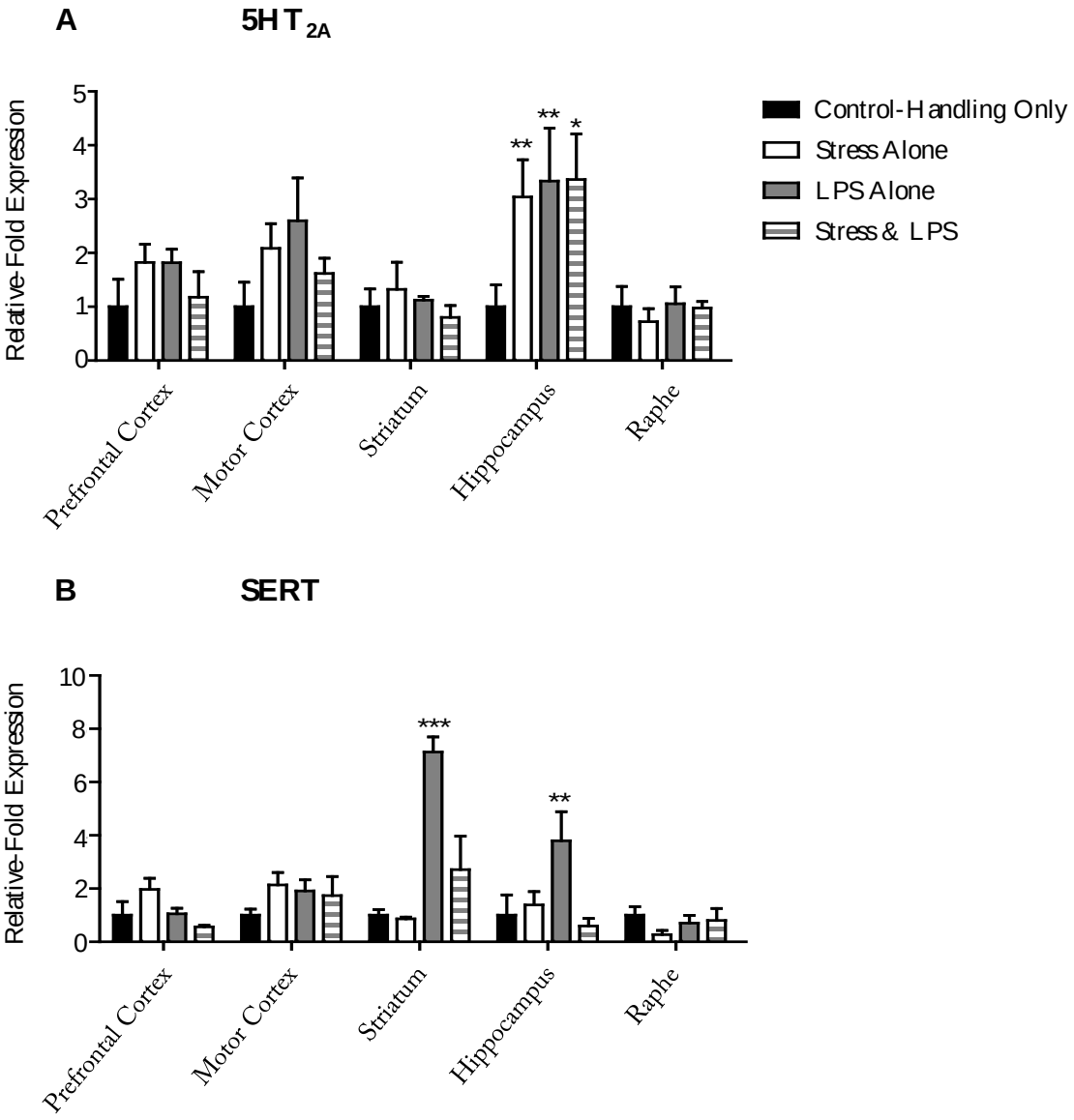


Figure 4.15 5-HT_{2A} receptor and SERT mRNA expression after chronic stress. Animals were subjected to 10 days of chronic stress (open bars) or a single dose of LPS (0.1mg/kg i.p. for 24 hours) Tissue was dissected to compare the prefrontal and motor cortices, striatum, dorsal hippocampus and raphe. mRNA for (A) 5-HT_{2A} and (B) SERT was examined by qPCR. Values are expressed as normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ±SEM, n=4, *$p<0.05$,**$p<0.01$ and ***$p<0.001$.

4.5 Discussion

In this study, using a chronic stress model in inbred mice, we have discovered that our animals segregate into two groups that can be described as resilient or susceptible. All animals displayed anxiety-like behaviours, but only a subset of susceptible animals developed an anhedonic phenotype and exhibit depression-like behaviour. We have explored the molecular profile of these subtypes in the brain and in the periphery. The behavioural changes are reflected by distinct changes in molecular biology, specifically changes in CNS cytokines and 5-HT-related genes. Susceptible animals exhibit increases in TNF expression in the prefrontal cortex, but resilient animals do not. All animals exhibited similar changes in cortisol and $5HT_{2A}$ expression, but the susceptible animals displayed greater increases in SERT expression. Thus, at a molecular level, for depression-like behaviours which are neurophysiologically uncoupled from anxiety-like behaviours the expression of TNF and SERT seem to be important determinants. In animals susceptible to chronic stress the number of Iba-1 positive microglia were also increased in the prefrontal cortex. Moreover, we have challenged stressed animals with a low dose of LPS (0.1mg/kg) 24 hours prior to behavioural testing and found that this has the tendency to further increase behavioural changes resulting from stress alone. However, these data are not accompanied by compound changes in cytokines expression in the CNS, nor by changes in receptor expression. These latter data suggest that inflammation may be capable of exacerbating the behavioural changes caused by stress but that the molecular origin of these changes is as yet unknown.

One of the major problems with modelling affective disorders such as depression in rodent models is the inter-individual variation that is encountered and can often appear dichotomous. Our approach here has been to recognise the existence of this variation and seek to explore the underpinning molecular mechanism. Others have found increases in depression-like behaviours similar to those we found (decreased sucrose consumption, increased floating in the FST), as well as 'anomalous' anxiolytic behaviours in response to novel environments (Mineur *et al.*, 2006; Schweizer *et al.*, 2009). Our experiments with the light/dark box revealed that susceptible and resilient animals performed in a similar manner,

showing stress is capable of inducing anxiety in all animals. However, the significant differences in the home cage and forced swim test are consistent with a subgroup with depression-like signs. In light of these data, and taking into account our resilient animals' increased exploratory behaviour, it seems reasonable to assume that chronic stress is capable of inducing depression-like behaviours which are neurophysiologically uncoupled from anxiogenic behaviours. Unfortunately during the latter experiments using LPS as a post-stress challenge, it was not possible to include an anxiety test. Data from our laboratories (Qin Xie, unpublished) suggests that a high dose of LPS only induces very low level anxiety, as demonstrated by the elevated plus maze, and that this does not persist to 24 hours. It could be assumed that if stress induced anxiety in all animals, and that LPS is not anxiolytic, that a combination would also result in anxiety in all animals.

The data here using an LPS challenge in both the sucrose preference test and the forced swim test shows that LPS has a tendency to exacerbate stress induced changes. Unfortunately, these tests showed no interaction at the level of stress and LPS but significances did appear between stressed animals and stressed animals treated with LPS. Work studying the immune response after a stressful event has suggested that stress 'primes' it for any impending further stress, such as an immune insult (Barnum *et al.*, 2012). It could be that this behavioural response is a reflection of the change in immune status. Specifically, that an additional LPS challenge has made the animal more vulnerable to disease and predation so it has become more physically inactive. However, as ventured previously, the lack of interaction in these experiments may reflect a lack of statistical power and therefore any behavioural data should be interpreted with caution.

Stress is well known to increase circulating cortisol and there have been numerous studies that have examined the association between cortisol levels and anxiety disorders and depression. Depressed patients frequently show dexamethasone non-suppression, suggesting hyperactivity of the HPA axis (Coppen *et al.*, 1983). In our studies, 10 days of chronic stress results in around a 7-fold increase in serum corticosterone. Others have shown similar increases after stress in both susceptible and resilient animals (Goshen *et al.*, 2008; Li *et al.*,

2008). However, the absence of a clear difference in the susceptible versus the resilient animals indicates that cortisol levels are not a useful discriminator.

Despite the failure of cortisol levels to be a good discriminator of resilient and susceptible animals, we discovered effective markers by examining the 5HT system and inflammation-related transcripts in the CNS. We have found up-regulated levels of SERT mRNA in the prefrontal cortex of mice susceptible to stress-induced anhedonia that were not found in the resilient animals. Mouse models of social defeat have also shown changes in SERT mRNA, albeit in different brain regions (Filipenko *et al.*, 2002). The differences in these studies may reflect structural and temporal specificity of the serotoninergic response to stress over time. However, overall a change in SERT expression within the CNS in both chronic stress and depression is supported elsewhere (Murrough *et al.*, 2011; Tordera *et al.*, 2011; Kohut *et al.*, 2012; Nikolaus *et al.*, 2012). It is possible that during the first stages of depression, or experimental induction of a depressive-like state in animal models, SERT function is increased because of enhanced serotonin release, but longer-lasting over-expression of SERT in susceptible individuals results in lower serotonin activity at later time points. Interestingly, an LPS challenge combined with stress does not result in this potentially compensatory increase in SERT expression. Data from this chapter, and previous chapters, has demonstrated that LPS and stress are both capable of increasing SERT expression levels, however, the combination does not seem to result in additive expression but rather in suppression. This may be the result of other, as yet uninvestigated factors which may be produced after the combination of stress and LPS such as changes in glucocorticoid receptor expression.

Similar results have been shown for the 5-HT_{2A} receptor. While this chapter demonstrated a significant up-regulation of 5-HT_{2A} receptor mRNA in the prefrontal cortex of both susceptible and resilient animals, as well as a significant up-regulation in the striatum of susceptible animals, and previous chapters have shown increases in LPS animals, there was no additive effect but rather no change at all in most cases. It is widely accepted that 5-HT_{2A} receptors are involved in the pathogenesis of depression and action of atypical antidepressants (Kirino, 2012). Down-regulation of 5-HT_{2A} receptor

expression by mirtazapine and mianserin was shown to contribute to their antidepressant effects (Davis and Wilde, 1996; Golden *et al.*, 2002). Here altered 5-HT_{2A} expression was not restricted to the susceptible animals and thus could not be described as discriminating in the same way as SERT. However, the data do support the use of selective 5-HT_{2A} modulators for mood disorders.

Previously, neuronal SERT expression has been shown to be dose- and time-dependently stimulated by IL-1β and TNF-α p38 MAPK-linked pathways (Zhu *et al.*, 2010). Moreover, selective p38α MAPK deletion in serotonergic neurons results in stress resilience in a model of depression (Bruchas *et al.*, 2011). Against this background, elevated expression of SERT and 5-HT_{2A} generally paralleled enhanced expression of TNF-α and IL-1β, this relationship was most consistent for the prefrontal cortex, but was detected in other brain structures also. However, there were clear differences in the regional expression profile and TNF expression was linked to depression-like behaviour in the susceptible, and not the resilient, animals.

Over the years most attention has focused on the role of central IL-1β expression in the generation of behavioural changes, and there is no doubt that the manipulation of central IL-1β signalling pathways can profoundly affect whether or not altered behaviour occurs after chronic stress (Goshen and Yirmiya, 2009). However, anti-IL-1β drugs such as anakinra have shown little success in combating inflammation-associated depression in patients with chronic inflammatory disorders where anti-TNF drugs have (Tyring *et al.*, 2006; Jiang *et al.*, 2008). While in animal models complete ablation of TNF does not result in such a robust antidepressant phenotype (Yamada *et al.*, 2000), it could be hypothesized that signalling via the TNF receptor, and not the presence of TNF, is the critical factor in mood regulation. Tynan and colleagues have shown that SSRIs can significantly inhibit the production of both TNF and nitric oxide in cultured microglia treated with LPS (Tynan *et al.*, 2012). Although the target of the SSRIs and the specific effects of microglial-derived TNF on the brain serotonin microenvironment are unclear, it is becoming increasingly obvious that TNF plays a significant role in mood regulation.

Our study also revealed a general increase in COX-1 mRNA which appeared to be independent of whether the animals were resilient or susceptible. Susceptible mice did show significant elevation of COX-1 expression in the raphe, showing a general overlap between the expression profiles of both proinflammatory cytokines and SERT/$5HT_{2A}$. Selective inhibition of COX-1 in depressed patients has been shown to decrease depressive symptoms (Savitz *et al.*, 2009). Recent data have shown that in repeated social defeat models, mice lacking COX-1 do not develop an anhedonic-phenotype (Tanaka *et al.*, 1998). We have previously demonstrated that COX-2 is up-regulated by long-term chronic stress (Strekalova *et al.*, 2011) and in various pathologies, over-expression of COX-1 was shown to precede the up-regulation of COX-2 (Yamaguchi and Okada, 2009; Dargahi *et al.*, 2011). In this particular chronic stress variant, no significant up-regulation of COX-2 was found (data not shown). The elevation of COX-1 in our animals supports the use of NSAIDS for depression, but it does not represent a biomarker for anhedonic behaviours.

Of all the genes studied here, perhaps the most surprising result was the lack of an IDO response. To date, studies of the role of inflammation in depression have largely accepted the theory that cytokines increase IDO activity and decrease 5-HT levels. However, the degree of cytokine induction observed in most depressed patients is relatively small compared to that achieved with an endotoxin challenge. Recent work by Hughes and colleagues has shown that the increase in cytokines in depressed patients occurs independently of changes in plasma tryptophan (Hughes *et al.*, 2012). Data here show that IDO expression was altered in the raphe, along with IL-1β, and it is possible that local increases in IDO activity may be contributing to altered 5HT neurotransmission.

The principal source of TNF in both the uninflamed and inflamed brain appears to be from microglia (Stellwagen and Malenka, 2006). Thus, given the up-regulation of TNF in the susceptible animals we were keen to discover whether the microglia population was altered in this set of animals. The number of Iba-1-positive microglia was indeed up-regulated in the prefrontal cortex, but not in the striatum or the lateral periaqueductal grey. We suspect that the changes in activation and number of microglia in the prefrontal

cortex, in susceptible animals, reflect a TNF-associated remodeling process that is likely to be more marked in the susceptible animals than in the resilient animals. This is, at least partly, confirmed by studies showing positive correlations between chronic stress, increased microglial activity and losses in working memory (Hinwood *et al.*, 2012).

4.6 Conclusion

Data from this chapter show that changes in behaviour are reflected by changes in molecular biology, specifically changes in CNS cytokines and 5-HT-related gene changes which are similar to those from previous chapters. However, changes in behaviour which appear to be additive in stressed animals challenged with LPS, are not necessarily additive in their molecular biology profile. The changes observed in both pro-inflammatory cytokines and 5-HT gene expression, reflect the behavioural outcomes occurring in different susceptible and resilient animals. Specifically, gene expression profiles are different between animals susceptible to stress-induced anhedonia, and those only suffering from anxiety after stress. Chronic stress induces a more potent inflammatory profile in animals susceptible to the development of anhedonia. It is possible to conclude that chronic or mild stress causes the immune system to be hyperactive. Work from this chapter suggests that while stress may not always change the baseline cytokine profile, it makes the organism considerably more sensitive when challenged with a minor infection. In the context of survival it could be considered important to maintain skin and peripheral immunity at high levels in stressful situations, since bacterial infections are likely to make an animal more vulnerable to predation. In terms of persistent inflammatory diseases, such as psoriasis, stress is likely to make prognosis significantly worse.

Work thus far has demonstrated significant increases in the CNS expression of the cytokine tumor necrosis factor (TNF) in the CNS of animals exposed to both single injections of LPS, as well as chronic stress. In particular, chronically stressed mice susceptible to the development of anhedonia express higher CNS TNF than those which appear to be resilient to the effects of stress. These increases are independent of any brain pathology, indicating significant peripheral-CNS immune communication. The role of TNF in mood is largely unknown, however long term inflammatory diseases such as psoriasis and rheumatoid arthritis are often associated with episodes of depression. In particular, the degree of stress and stress-induced depression in these diseases is considerably higher than in patients with non-inflammatory disease, or healthy controls (Mancuso *et al.*, 2006). Studies using anti-TNF therapy in these patients has shown that there are significant improvements in their symptomatology well before histological and physical improvements (Tyring *et al.*, 2006). This has been attributed to the effect of TNF neutralization on mood. Work from previous chapters of this thesis has demonstrated that there is significant up-regulation of TNF in the CNS of animals challenged with LPS, as well as in those animals susceptible to stress-induced depression. These data indicate a possible link between TNF and mood which has thus far not been extensively explored.

5.1.1 TNF and anti-TNF Therapy

TNF is a stable membrane bound homotrimer, with each protomer comprising 157 amino acids at 17kDa. The N-terminal tail has a 35 amino acid region which anchors it to the membrane (mTNF). Whilst the membrane bound form is capable of cell signalling it is often termed a 'pro-protein' because of its capacity to be cleaved further and remain functional. Tumour necrosis factor-α converting enzyme (TACE) cleaves mTNF at specific residues to release the ectodomain known as sTNF.

TNF signalling is via two receptors; TNF receptor 1 (TNF-R1, CD120a or p55) and TNF receptor 2 (TNF-R2, CD120b or p75; Wajant *et al.*, (2003)). mTNF is more capable of fully activating TNF-R2 and sTNF is more capable of fully activating TNF-R1 (Figure 5.1).

TNF-R1 is also known as a death receptor, indicating that its subcellular machinery is intimately connected to proteins that initiate apoptosis (Wajant *et al.*, 2003). Conversely, TNF-R2 is linked to a number of anti-apoptotic factors such as c-IAP (Wajant *et al.*, 2003). TNF receptor intracellular signalling mechanisms are also capable of activating a nuclear factor-k B/c-Jun pathway that leads to gene transcription promoting cell survival and proliferation. The exact mechanisms by which cells 'choose' to activate one pathway or the other are currently speculative. It is known that cells in culture are more liable to cytotoxicity upon TNF stimulation than during *in vivo* situations (Kettelhut *et al.*, 1987), however, the methodological differences between these stimulation paradigms make these data difficult to interpret.

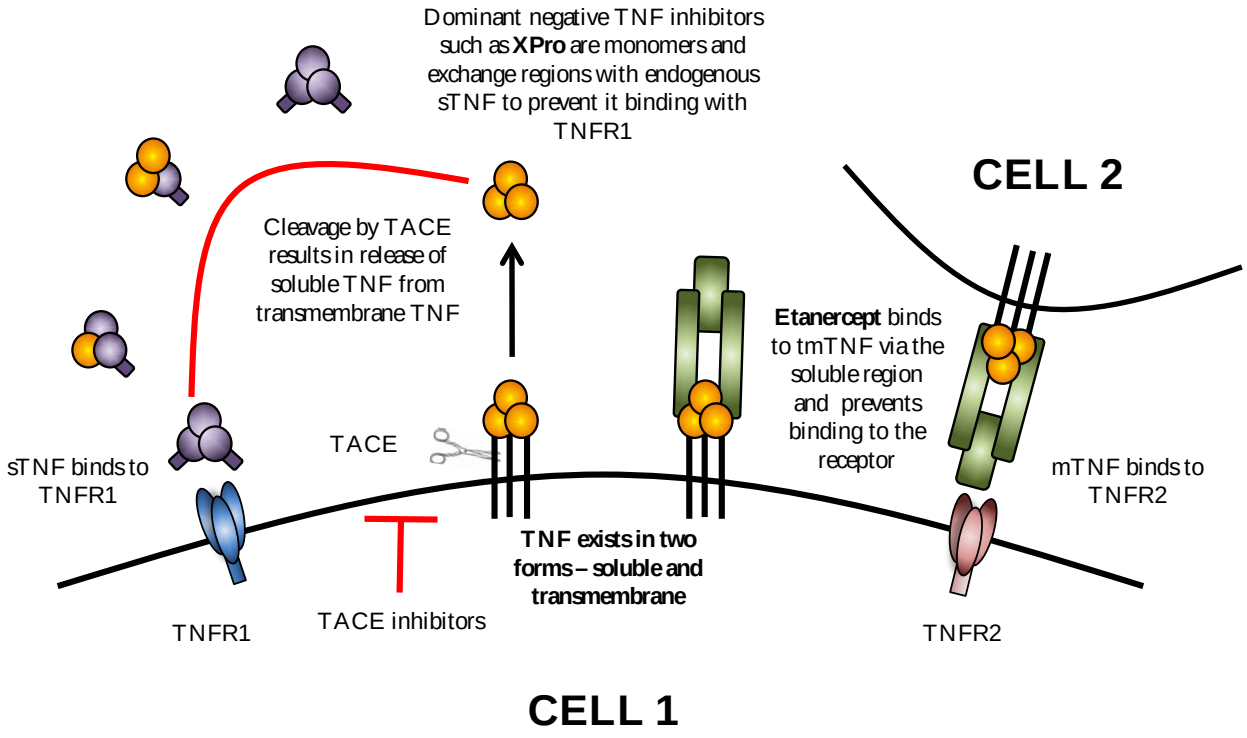


Figure 5.1 Schematic outlining the production and signalling of TNF. TNF exists as a membrane bound homotrimer (mTNF) and is cleaved by TACE into a soluble form (sTNF). mTNF is involved in paracrine signalling between cells where sTNF is involved in exocrine cytokine communication. Drugs such as etanercept and infliximab prevent sTNF and mTNF binding directly to their relevant receptors where more novel compounds such as the dominant negative inhibitor XPRO®1595 prevent sTNF from taking effect.

The mechanisms by which the systemic circulation stimulates central production of TNF are somewhat speculative. The source of CNS TNF is the brain's resident macrophage population – the microglia. While microglia express TLR-4, direct stimulation by LPS is unlikely since only small amounts of LPS are capable of crossing the blood brain barrier (Banks and Robinson, 2010). Transport of peripheral TNF is more likely. Studies have shown that radiolabelled albumin (c.60kDa) does not cross the intact blood brain barrier but that TNF (c.50kDa) does (Osburg *et al.*, 2002). Osburg and colleagues (2002) demonstrated that this transport is mediated by TNF-R2, suggesting tmTNF may be largely responsible for immune-brain communication at the level of the vasculature. Within the CNS parenchyma, sTNF and mTNF have been shown to have important diverse functions in neurocognitive processes. Aged rats express high levels of TNF-R1, and infusion of anti-TNF drugs into the brains of these animals decreases age-induced changes in microglial activation, as well as improving cognitive function in the Morris water maze (Sama *et al.*, 2012). Work in human elderly and depressed patients has been contradictory. Adult euthymic depressed patients have been shown to have elevated levels of both TNF-R1 and TNF-R2 (Grassi-Oliveira *et al.*, 2009), whereas depressed elderly patients only show elevations in TNF-R2 (Diniz *et al.*, 2010).

TNF-R1 and TNF-R2 are expressed by different cell types. Specifically, TNF-R1 is expressed constitutively on all cells, where TNF-R2 is up-regulated during inflammation on haemopoetic cells, i.e. inflammatory immune cells such as monocytes and macrophages, as well as on endothelial cells (McCoy and Tansey, 2008). The downstream pathways of both receptors are complex and can lead to the activation of an array of subcellular machinery. This can include p38-MAPK (mitogen-activated protein kinase), an enzyme expressed in both astroglia and neurons which has received particular attention within CNS research recently. Expression of TNF receptors on astrocytes and neurons results in TNF-mediated signal transduction that up-regulates p38 MAPK activity. In neurons this can result in an increased release of glutamate (Kawasaki *et al.*, 1997). TNF has also been shown to stimulate glutamate release from astrocytes (Bezzi *et al.*, 2001) and microglia (Takeuchi *et al.*, 2006). Increased levels of glutamate can result in excessive intracellular calcium, leading to activation of calcium-dependent lipases and

up-regulation of reactive oxygen species, both of which can damage the cytoskeleton and result in cell death (Manev *et al.*, 1989). Patients with bipolar depression have been shown to have increased markers of excitotoxicity in the frontal cortex, suggesting this may contribute to disease progression (Rao *et al.*, 2010). Similar data have not yet been demonstrated in patients with unipolar depression. However, the use of minocycline as an adjunct therapy in unipolar depression (Miyaoka *et al.*, 2012) may demonstrate a role for p38 MAPK activity and excitotoxicity, since this is where minocycline exerts its predominant effects (Cui *et al.*, 2008). It is possible that a combination of low level inflammation in states of Major Depression induces excitotoxicity not only via production of quinolinic acid and excessive NMDA receptor stimulation, but also by stimulation of glutamate release and inhibition of glutamate reuptake.

Currently, anti-TNF therapy is used mainly for inflammatory diseases such as arthritis (including rheumatoid, juvenile and psoriatic), psoriasis, Crohn's disease, ulcerative colitis and ankylosing spondylitis (Sfikakis, 2010). A number of 'off-label' uses include other inflammatory diseases such as acne, nephropathy and COPD (Sfikakis, 2010). Evidence from clinical trials using etanercept (Enbrel®), an anti-TNF drug, in psoriasis patients demonstrated that the treatment caused significant improvements in mood (Tyring *et al.*, 2006). This study used both the Beck depression index and the Hamilton depression scoring system to show that improvements in mood were independent of physical improvement. Despite this, and a number of other studies, a PubMed search for 'TNF and sickness behaviour' will return approximately half the number of papers as one for 'IL1 and sickness behaviour'[3].

One potential reason for the interest in IL-1β over TNF is the ready availability of a naturally occurring antagonist. Mimicking the IL-1 receptor antagonist (IL-1ra) has been shown to be important in reducing the effects of post-stroke immunodeficiency (Smith *et al.*, 2012) as well as the symptoms of rheumatoid arthritis (Niu *et al.*, 2011). The IL-1ra mimic anakinra (Kineret®) has been used in a number of clinical trials for rheumatoid

[3] PubMed search July 2012 "sickness behaviour" AND TNF: 11 citations, "sickness behaviour" AND IL-1: 29 citations

arthritis but, even in combination with etanercept, has been shown to be less effective than standard anti-TNF therapy (Genovese *et al.*, 2004). High levels of endogenous IL-1ra have been shown to be associated with Major Depression (Maes *et al.*, 1995; Maes *et al.*, 1997). Studies in patients trialling anakinra for rheumatoid arthritis resulted in the onset of depression with treatment, and the cessation of symptoms with the cessation of anakinra (Jonville-Bera *et al.*, 2011).

Despite the relatively poor efficacy of anti-IL-1 drugs and their mood-related side effects they still appear more popular than anti-TNF therapy. One reason for this could be the immunological side effects related to anti-TNF drugs. The current major anti-TNF drugs in use are etanercept and infliximab. Etanercept is a fusion protein – a soluble combination of TNF-R2 and human IgG1 – which 'mops up' circulating TNF, whether it is mTNF or sTNF. Infliximab (Remicade®) is a monoclonal antibody against TNF which binds to the trimeric structure and stops it binding correctly to the TNFRs. While these drugs have been shown to be effective in a number of inflammatory diseases, their use is hampered by often severe side effects. These can include sepsis and demyelinating disease as well as lymphoma and heart problems (Scheinfeld, 2004). Interestingly, specific inhibitors of TACE are becoming more popular as a potential to limit the amount of circulating sTNF without causing significant immunosuppression (DasGupta *et al.*, 2009).

A novel anti-TNF drug has recently become available. Xencor have developed XPRO®1595 (XPRO), a dominant negative inhibitor of TNF (Steed *et al.*, 2003). Dominant negative inhibitors act by recombining with the native form of sTNF, resulting in a number of heterotrimers which cannot bind effectively to their receptors (Figure 5.1). This provides a novel mechanism of action; while other drugs bind TNF in all forms, XPRO only prevents sTNF from binding to its receptor, allowing mTNF to continue functioning.

5.1.2 TNF and Mood

The potential of TNF to contribute to mood disorders is characterized by the basal 'antidepressant' phenotype of TNF and TNF receptor knockout mice (Simen *et al.*, 2006). The expression of these receptors is widespread throughout the brain. TNF-R1 and TNF-R2 are both present on microglia, whereas astrocytes and oligodendrocytes primarily express TNF-R2 (Dopp *et al.*, 1997). Studies in neurons show TNF-R1 expression in the hippocampus as well as other minor areas (Yang *et al.*, 2002). This hippocampal location appears to contribute little to either structural integrity or to function, as TNF-R1 knockout mice appear to have no changes in gross brain morphology or in hippocampal-dependent learning and memory tasks such as the Morris water maze (Bruce *et al.*, 1996). Targeted deletion of TNF-R2 resulted in increased sucrose consumption after a period of water deprivation. Complete ablation of TNFα does not result in such a robust antidepressant phenotype (Yamada *et al.*, 2000). It could be hypothesized that signalling via the TNF receptor, and not the presence of TNF, is the critical factor in mood regulation since signalling could still be achieved via TNFβ in TNFα knockout animals.

The successful use of infliximab in the elevation of rheumatoid arthritis-induced depression shows that preventing TNF signalling pathways from becoming active can directly change mood (Ertenli *et al.*, 2012). Central inflammation has also been shown to cause malaise. Experiments from our own laboratory have demonstrated that inhibition of peripheral TNF using etanercept significantly reduced the sickness behaviour associated with a central IL-1 injection (Jiang *et al.*, 2008). New data also indicate that inhibition of signalling and changes in mood could work both ways. Tynan and colleagues have demonstrated that in cultured microglia treated with LPS, SSRIs can significantly inhibit the production of both TNF and nitric oxide (Tynan *et al.*, 2012), suggesting that their mood-enhancing ability may, at least in part, be due to their anti-inflammatory properties.

Thus far, this thesis has demonstrated that there are significant similarities between rodent models of sickness, and models of depression. Anti-TNF therapy has been shown to significantly elevate mood in patients with inflammation-associated depression, but

has yet to be tested in a basic model of sickness. If anti-TNF therapy is capable of reversing the behavioural changes associated with LPS it will confirm a role for TNF in the development of sickness behaviour, as well as paving the way for testing such drugs in both models of depression and in depressed patients.

5.2 Aims of this Chapter

The aims of this chapter are to explore the role of TNF in sickness behaviour, using prophylactic anti-TNF drugs followed by a single i.p. challenge of LPS. Sickness behaviour will be assessed using open field, burrowing and locomotor activity tests to study instinctive exploratory behaviours that may be affected by sickness. Two anti-TNF therapies will be investigated; the fusion protein etanercept and the novel dominant-negative inhibitor XPRO. By using a drug which targets both forms of TNF (soluble and membrane), as well one that only targets the soluble form of TNF, it may be possible to determine which form contributes to the development of sickness behaviour. These studies will be confirmed using a transgenic mouse line (tmTNFΔ/Δ) which has a mutation in TACE. tmTNFΔ/Δ mice do not have functional TACE, and therefore cannot produce sTNF. These mice will be challenged with LPS and assessed using similar behavioural tests as previously, to evaluate whether they develop sickness behaviours. Finally, studying 5-HT and inflammation-associated genes in the CNS, as in previous chapters, will enable conclusions to be drawn regarding the role of TNF, and anti-TNF therapy, in any changes which may occur during episodes of sickness and sickness-associated depression.

5.3 Materials and Methods

All reagents were purchased from commercial suppliers unless otherwise stated in the text.

5.3.1 Animals

Animals were provided with food and water *ad libitum* and all procedures were carried out in accordance with guidelines approved by the Danish Animal Inspectorate (Journal Number: 2011/561-1950). Adult male C57/Bl6 inbred mice were obtained from Taconic (Ry, Denmark) at c.8 weeks of age and housed under standard conditions. tmTNFΔ/Δ mice were kindly provided by Dr Fabienne Tachinni-Cottier (University of Lausanne) and housed as above. Animals were genotyped at 3 weeks of age and used between 6-10 weeks. All animals were allowed to acclimatize for 1 week prior to the onset of experiments.

5.3.2 Injection Procedure

Etanercept (Enbrel, Amgen) and XPRO®1595 (XPRO, Xencor) were kindly provided by Dr David Szymkowski (Xencor, California, USA). XPRO (100mg/ml) and etanercept (50mg/ml) were packaged as liquids in sterile. All drug and LPS concentrations were calculated to give animals an injection with a volume of not more than 0.1ml. Animals received a single injection of either vehicle (saline), etanercept (10mg/kg) or XPRO (10mg/kg) i.p. approximately 24 hours prior to injection with LPS (0.5mg/kg). Doses of drugs were selected according to previous studies (Zalevsky *et al.*, 2007) and dose of LPS was used to match other studies in this thesis. Throughout the rest of this chapter, the drug injection regime will be separated by an oblique line, for example animals receiving etanercept 24 hours prior to LPS will be denoted as etanercept/LPS. The injection immediately prior to testing will always be LPS or saline and will be referred to as the 'challenge'. Further dose response studies were carried out using 0.5mg/kg and 0.05mg/kg LPS.

5.3.3 Behavioural Testing

Animals were allowed to acclimatize in the room in which testing was carried out for at least 1 hour prior to the onset of the test. Behavioural tests were carried out between 2-48 hours post-LPS; details are specified below. Testers were blind to treatment group.

5.3.3.1 Open Field Testing

Animals were observed for 3 minutes in the open field as in section 3.2.7.2.

5.3.3.2 Locomotor Activity Testing

Animals were tested for longer periods of time using locomotor activity monitoring systems as in 3.2.7.1.

5.3.4 Molecular Biology

RNA extraction and quantitative PCR were performed as in previous chapters (*see* 2.3.9, 2.3.10, 2.3.12 and 2.3.13). RNA was extracted from the prefrontal and motor cortices, striatum, hippocampus and dorsal raphe nucleus and qPCR was performed using primers for pro-inflammatory cytokines TNFα and IL-1β, as well as the 5-HT_{2A} receptor and SERT (*see* Table 3.1).

5.3.5 Neutrophil Immunohistochemistry

Animals were surgically anaesthetised with pentobarbitone and transcardially perfused for approximately 1 minute with a solution of heparinized saline (0.9%), followed by 5 minutes of 4% paraformaldehyde (PFA). Liver tissue was removed and post-fixed in PFA for 24 hours prior to embedding in OCT-embedding media (Fisher Scientific, UK) at -20^{o}C. Frozen tissue was cut at 12µm using a Leica cryostat (Leica Microsystems, UK) onto gelatin-coated slides (made in house). Neutrophils were identified using light-microscopy immunohistochemistry. Briefly, sections were quenched in a 3% hydrogen peroxide/methanol solution to reduce endogenous peroxidase activity. Endogenous biotin was reduced using an avidin-biotin blocking kit (Vectorlabs, UK) and non-specific binding was blocked using 10% rat serum. Primary antibody MBS (1:10,000; made in

house) against whole mouse neutrophils was applied to tissue sections at room temperature for 2-3 hours. Streptavidin-conjugated secondary rat anti-mouse antibody (1:100; Vectorlabs, UK) was applied at room temperature for 1 hour and the signal was amplified using an avidin-biotin complex kit (ABC Kit, Vectorlabs, UK). Visualization was achieved using a diaminobenzidine and hydrogen peroxide solution (DAB; Sigma-Aldrich, UK). Sections were counterstained for visualization of nuclei with haematoxylin. Neutrophil numbers were quantified using a standard $1mm^2$ grid at x20 magnification on a standard Leica microscope.

5.3.6 Cortisol HPLC

Blood was taken via cardiac puncture immediately before perfusion and stored in heparinized vials prior to centrifugation (10k rpm, 10 minutes, 4°C), plasma was removed and immediately stored at -20°C. Cortisol was analysed using HPLC (*see* 3.2.8).

5.3.7 Neurotransmitter HPLC

Half brains were snap frozen and bisected at the approximate position of Bregma. The cerebella were removed and quarter brains were weighed and homogenized in 0.06% perchloric acid at 10µl/mg tissue. Samples were analyzed using HPLC with electrochemical detection and separated with an ACE microsorb column (C18, 3µm, 125 x 3 mm + ACE C18 guard, 10 x 3 mm run at 35 °C). Samples were carried by an eluent (12.5% methanol, 130 mM NaH_2PO_4, 0.85 mM Na_2EDTA, 0.1mM 1-octanesulphonic acid, pH3.55) pumped with a flow rate of 0.6 ml/min (Waters 2695 HPLC Pump). Samples were detected using a glassy carbon electrode held at +0.75V (Dionex ED40). The dialysate content was determined with reference to daily-calibrated standard solutions in 0.06M perchloric acid (5pmol 5-HT, 5-HIAA, dopamine, DOPAC and noradrenaline). Chromatograms were displayed and analyzed using Waters Empower 2 software.

5.3.8 Iba-1 Immunohistochemistry

Iba-1 immunohistochemistry was performed with the assistance of Maria Gammelstrup and Maria Ormhøj at the University of Southern Denmark. Brain tissue was extracted

using the same methodology as 5.3.5. Tissue was stored in a 1% PFA solution until sectioning. Brains were sectioned using VT1000S Vibratome (Leica, Germany) at 60μm and collected free floating in in cryoprotectant. Cryoprotectant was a $NaPO_4$ buffer base containing 1% polyvinylpyrrolidone (Sigma Aldrich) and 30% sucrose diluted in ethyleneglycol (Merck). Tissue was stored at -20°C when not being used for immunohistochemistry. Sections were adjusted to room temperature in PBS and blocked for endogenous peroxidase activity using a 1% hydrogen peroxide/methanol solution for 30 minutes. All wash steps were performed with 1% triton-X (Sigma-Aldrich) in PBS. Blocking of alternate binding sites was performed with 10% foetal bovine serum in PBS for 30 minutes, followed by incubation with primary antibody (1:600; rabbit anti-Iba-1; Wako) for 48 hours at 4°C. Secondary antibody biotinylated anti-rabbit (1:200) was applied for 1 hour at room temperature and amplified using horseradish peroxidase-conjugated streptavidin (Amersham) for 1 hour at room temperature. Immunostaining was visualized with DAB as previously (see 3.2.5). Sections were mounted on gelatine coated glass slides and allowed to air dry overnight at 4°C. Counterstaining was performed by immersion in a toluidine blue solution for 30 minutes followed by dehydration in increasing concentrations of ethanol and xylene. Slides were cover-slipped using DePeX mounting medium (Dako). Microglial activation was quantified by the ramification of microglia at x60 magnification. A specific threshold was applied to images and staining intensity was quantified objectively and presented as arbitrary units per area. This evaluation technique is based on that of Tynan *et al.* (2010).

5.3.9 Statistics

Statistical analysis was carried out using GraphPad Prism and InVivoStat software. Data are mean ±SEM. Tests included standard Student's T-test and one and two-way ANOVA, with post-hoc tests as appropriate. Data was considered statistically significant with a $p<0.05$.

5.4 Results

5.4.1 Anti-TNF therapy does not significantly improve behaviour after 0.5mg/kg LPS

In order to determine whether anti-TNF therapy has a significant impact upon LPS induced sickness behaviour, a panel of behavioural tests were run. Animals received 10mg/kg of either etanercept, XPro or vehicle on day 1, followed by a single dose of LPS (0.5mg/kg) or saline (0.1ml) on day 2, followed by behavioural testing between 2-6 hours later. TNF knockout animals have been previously shown to have alterations in standard open field behaviour (Baune *et al.*, 2008) and therefore, this test was used to determine the effect of anti-TNF therapy on the response to LPS.

Open field data indicated that the drugs themselves had no significant effect on behaviour (Figure 5.2 & Figure 5.3). LPS had a significant effect on rearing behaviour where anti-TNF treatment did not, furthermore there was no interaction between the two (two-way ANOVA; anti-TNF $p=0.65$ $F_{2,42}=0.57$; LPS $p<0.001$ $F_{1,42}=89.81$; anti-TNF:LPS $p=0.56$ $F_{2,42}=0.418$). This is reflected in post-hoc analysis where all LPS treated animals showed significantly fewer rears than the non-treated controls (Bonferroni post-hoc; $p<0.001$ for all groups; Figure 5.2A). Speed showed similar data with an overall effect of LPS but no effect of anti-TNF therapy and no interaction (anti-TNF $p=0.09$ $F_{2,42}=2.459$; LPS $p<0.001$ $F_{1,42}=35.79$; anti-TNF:LPS $p=0.76$ $F_{2,42}=0.277$). Post-hoc analysis also showed similar results to rearing behaviour with LPS significantly decreasing speed irrespective of treatment ($p<0.001$ for all groups; Figure 5.2B). Total distance travelled also reflected the previous data with an overall effect of LPS but no effect of anti-TNF therapy and no interaction (anti-TNF $p=0.15$ $F_{2,42}=0.984$; LPS $p<0.001$ $F_{1,42}=28$; anti-TNF:LPS $p=0.69$ $F_{2,42}=0.372$). Furthermore, post-hoc analysis showed that LPS significantly decreased total distance travelled when compared to control animals ($p<0.001$ vehicle and etanercept treated groups; $p<0.01$ XPRO treated group Figure 5.2C)

Burrowing is a normal rodent behaviour and alterations in burrowing activity have been previously shown to be an extremely sensitive, non-selective indicator of injury or disease (Cunningham *et al.*, 2009; Deacon, 2009), and was therefore used to determine the effects of anti-TNF drugs on LPS-induced behavioural changes.

Analysis of burrowing data shows that injection stress has no effect on burrowing activity irrespective of the type of injection. More specifically there was no effect of anti-TNF, no effect of saline and no interaction (repeated measures two-way ANOVA; anti-TNF $p=0.89$ $F_{2,12}=0.225$; saline $p=0.052$ $F_{1,12}=5.582$; anti-TNF:saline $p=0.80$ $F_{2,12}=0.110$; Figure 5.3). LPS injection had a significant effect on burrowing behaviour where treatment did not (anti-TNF $p=0.62$ $F_{2,12}=0.502$; LPS $p<0.001$ $F_{1,12}=256.7$; anti-TNF:LPS $p=0.59$ $F_{2,12}=0.50$) A marked decrease in burrowing behaviour was observed after a single i.p. injection of LPS ($p<0.001$ for all groups; Figure 5.3B) compared to saline and drug controls, which again was not ameliorated by anti-TNF therapy.

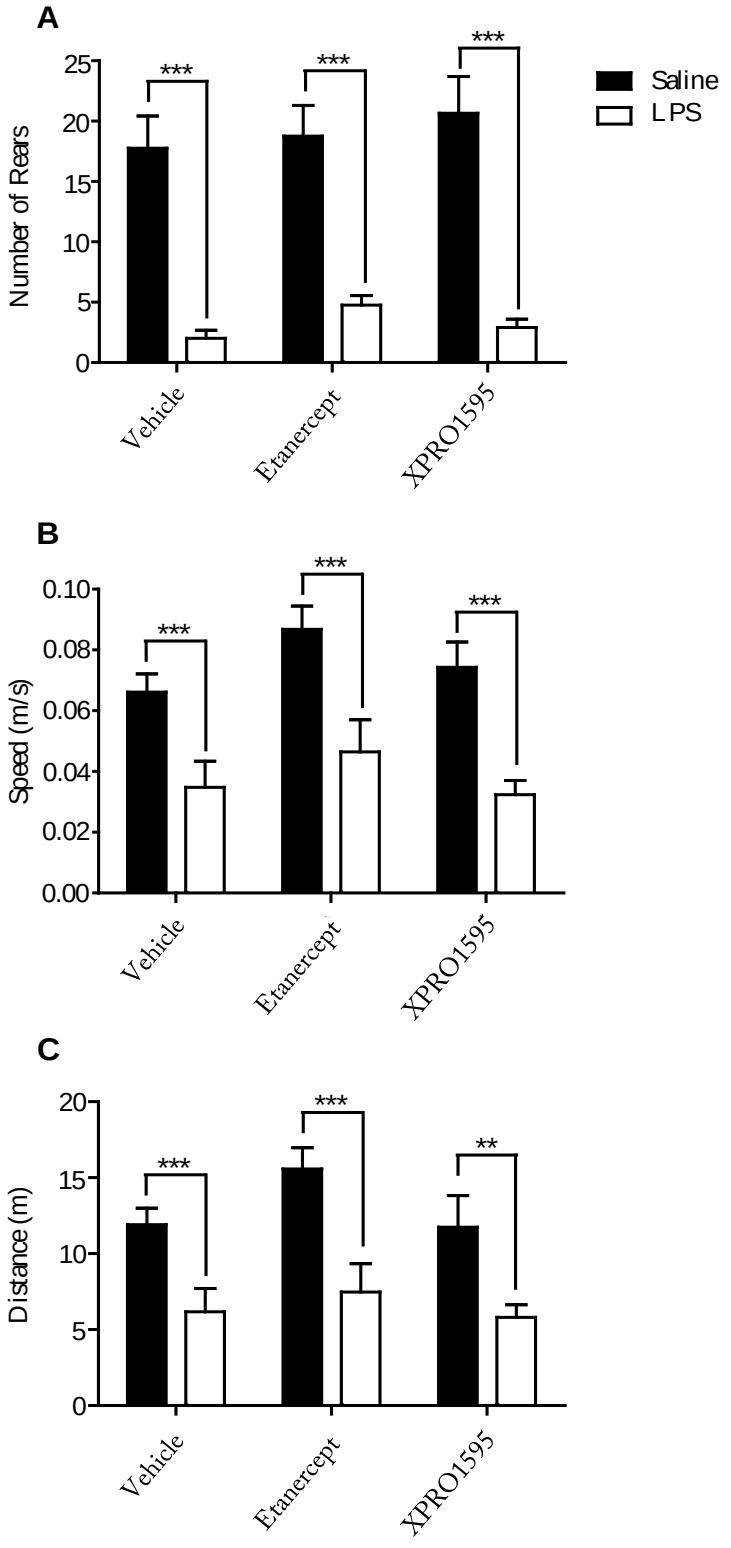


Figure 5.2 Open field behaviour after a single dose of anti-TNF therapy and a single LPS challenge. Open field behaviour measuring (A) total number of exploratory rears; (B) maximum speed and (C) total distance covered during 3 minutes. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Behaviour was carried out approximately 4 hours post-LPS. Data are mean ±SEM, n=5, ***$p<0.001$ and **$p<0.01$.

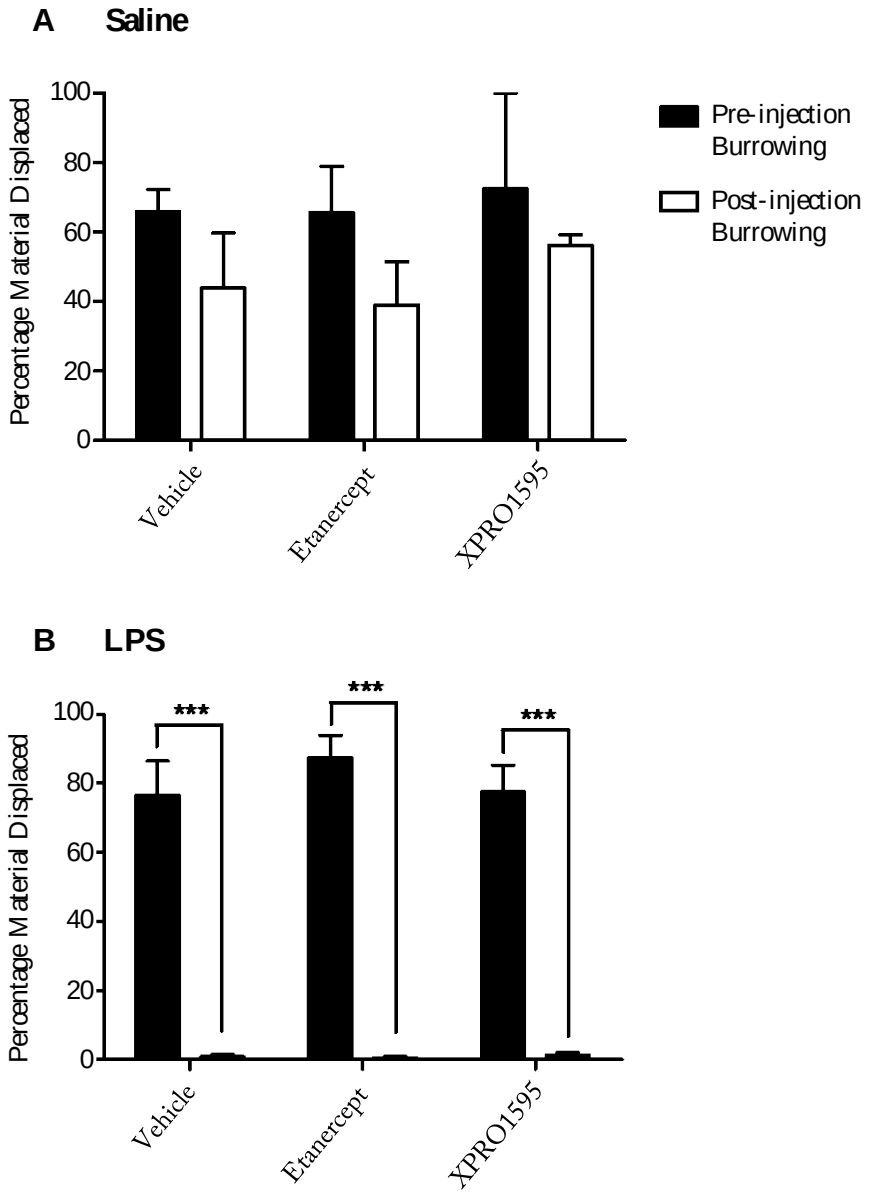


Figure 5.3 Burrowing behaviour after a single dose of anti-TNF therapy and a single LPS challenge. Burrowing behaviour before (closed bars) and after (open bars) injections in animals that had received a single dose of anti-TNF therapy followed by (A) saline or (B) LPS. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Behaviour was carried out approximately 4 hours post-LPS. Data are mean ±SEM, n=5, ***$p<0.001$.

5.4.2 Anti-TNF therapy does not significantly reduce the expression of cytokine mRNA in the liver

Behavioural data indicated that anti-TNF therapy had little impact on LPS-induced sickness behaviour. It is possible that the levels of TNF induced by this dose of LPS were too high to be overcome by standard doses of anti-TNF drugs. A single dose of LPS is known to up-regulate TNF and IL-1β in the liver, as well as increasing the number of hepatic neutrophils. These aspects of peripheral inflammation were, therefore, used to study the effect of systemic anti-TNF on the basic immune response. LPS had a significant effect on TNF expression where anti-TNF therapy did not, there was also no significant interaction between LPS and anti-TNF therapy (two-way ANOVA; LPS $p<0.001$ $F_{1,18}=24.39$; anti-TNF $p=0.23$ $F_{2,18}=1.574$; LPS:anti-TNF $p=0.52$ $F_{2,18}=0.678$). Post-hoc tests reveal that LPS significantly up-regulated the expression of hepatic TNF in all animals compared to saline injected controls, albeit to a different degree of significance depending on drug (Bonferroni post-hoc; vehicle $p<0.05$; etanercept $p<0.001$; XPRO $p<0.05$; Figure 5.4A).

LPS also had a significant effect on IL-1β expression in the liver, with no interaction and no effect of anti-TNF therapy (two-way ANOVA; LPS $p<0.05$ $F=_{1,18}=8.05$; anti-TNF $p=0.40$ $F_{2,18}=0.959$; LPS:anti-TNF $p=0.42$ $F_{2,18}=0.90$) Although hepatic IL-1β mRNA appeared to be higher in LPS/vehicle treated animals than in saline/vehicle treated animals, this did not reach significance in post-hoc tests. However, LPS induced a significant increase in liver IL-1β in etanercept treated animals when compared to drug-along controls (Bonferroni post-hoc; $p<0.05$; Figure 5.4B).

Neutrophils are recruited to the liver by cytokines such as TNF immediately after an inflammatory insult (Campbell *et al.*, 2003). Neutrophil numbers were increased in animals receiving a single i.p. dose of LPS but were not significantly affected by anti-TNF therapy (two-way ANOVA LPS $p<0.001$ $F_{1,30}=775.5$; anti-TNF $p=0.27$ $F_{2,30}=1.349$; LPS:anti-TNF $p=0.17$ $F_{2,30}=1.873$). Specifically, LPS injection significantly increase neutrophil numbers in all animals (Bonferroni post-hoc; $p<0.001$ for all drugs) and were not significantly decreased in animals receiving anti-TNF therapy (Figure 5.4C-E).

5.4.3 Corticosterone synthesis is up-regulated by LPS and not attenuated by anti-TNF therapy

Since it is acknowledged that corticosterone and cytokines operate a complex interactive feedback system between each other (Zuckerman and Bendele, 1989), it was important to discover whether anti-TNF therapy affected corticosterone synthesis. There was a significant main effect of LPS on corticosterone expression but no main effect of anti-TNF therapy and no interaction (two-way ANOVA; LPS $p<0.01$ $F_{1,18}=9.089$; anti-TNF $p=0.8$ $F_{2,18}=0.255$; LPS:anti-TNF $p=0.94$ $F_{2,18}=0.054$). Post-hoc tests show that while LPS significantly affected corticosterone, specific adrenal corticosterone levels were not significantly elevated in any group (Figure 5.5A).

In order to determine that adrenal and plasma corticosterone were regulated in the same manner by the drugs, plasma corticosterone levels were also measured, as shown in Chapter 3. In a similar manner to the adrenal glands, LPS had a significant effect on corticosterone levels, where anti-TNF therapy did not, nor was there any interaction (two-way ANOVA; LPS $p<0.001$ $F_{1,18}= 32.09$; anti-TNF $p=0.21$ $F_{2,18}=1.659$; LPS:anti-TNF $p=0.75$ $F_{2,18}=0.283$). LPS up-regulated corticosterone levels in all groups, and these were not attenuated by any prophylactic anti-TNF therapy (Bonferroni post-hoc; vehicle $p<0.05$; etanercept $p<0.01$; XPRO $p<0.05$; Figure 5.5B).

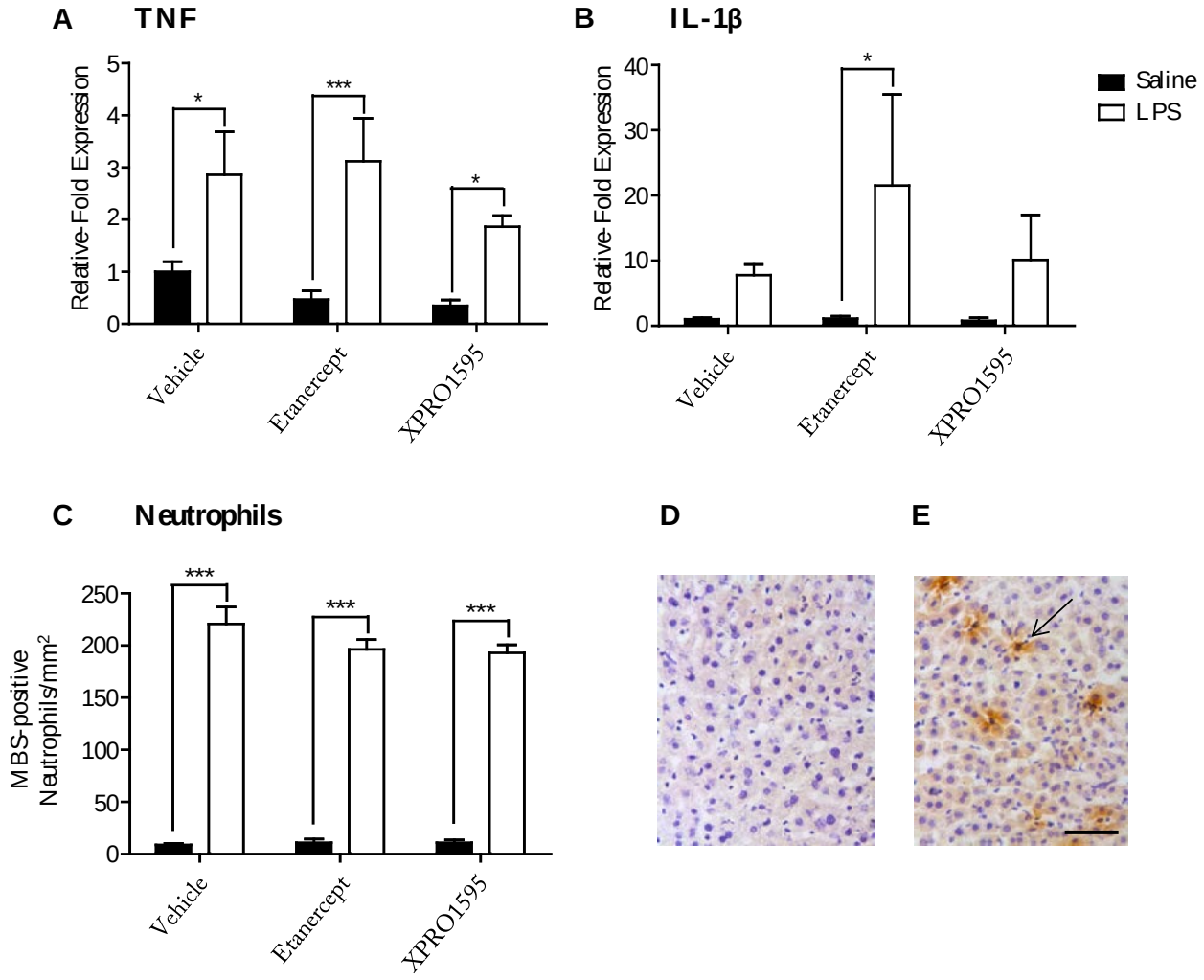


Figure 5.4 The inflammatory profile in the liver after a single dose of anti-TNF and a single dose of LPS. Liver mRNA of (A) TNF and (B) IL-1β as measured by relative qPCR. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Tissue was removed between 2 and 6 hours post-LPS, mRNA is expressed relative to housekeeping gene GAPDH. Hepatic neutrophils were assessed by immunohistochemistry using MBS and developed with diaminobenzidine (DAB; brown staining) and were counted at x20 magnification (C). Representative photomicrographs of (D) vehicle/etancercept/XPRO treated animals and (E) vehicle/etanercept/XPRO and LPS treated animals (arrow indicates MBS-positive neutrophil). Data are mean ±SEM, n=6, *$p<0.05$ and ***$p<0.001$. Scale bar represents 50µm.

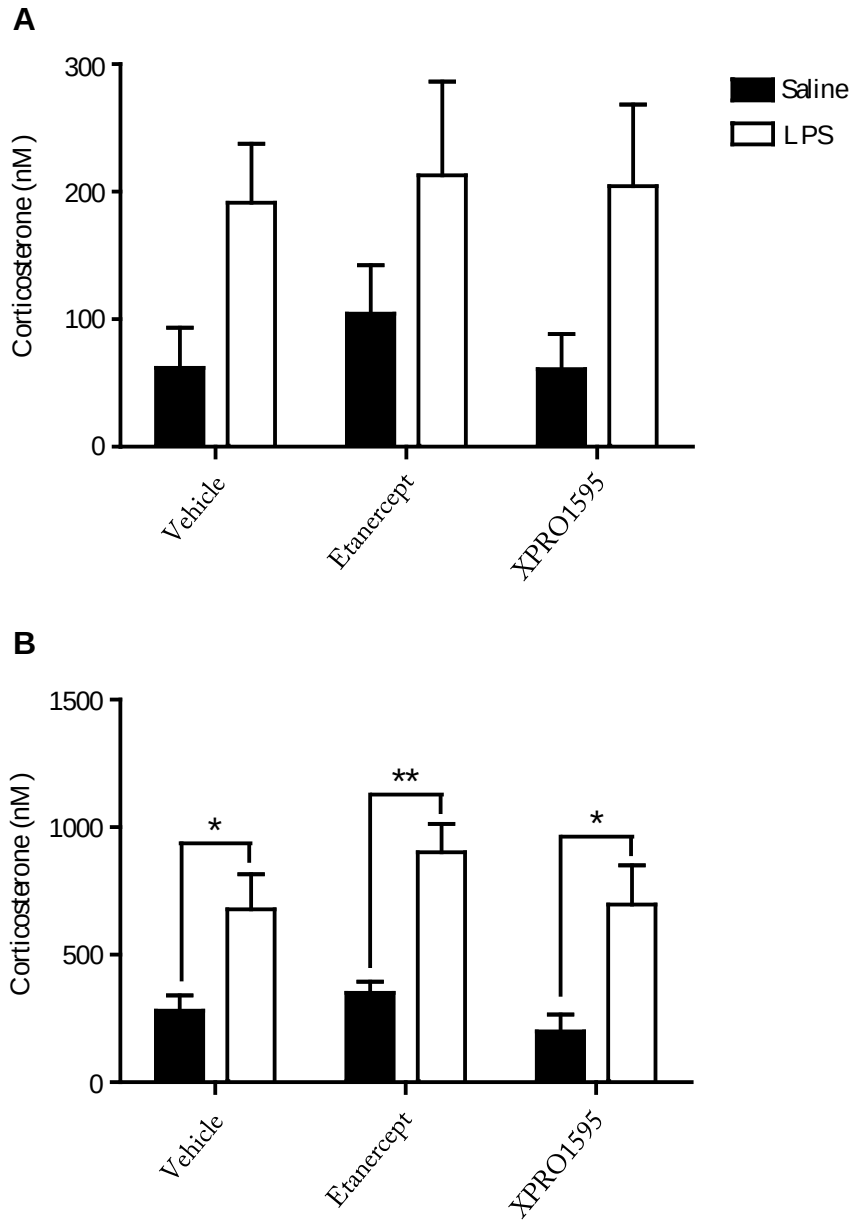


Figure 5.5 Corticosterone levels after a single dose of anti-TNF therapy and a single dose of LPS.(A) Adrenal and (B) blood levels of corticosterone in animals receiving a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Tissue was removed between 2 and 6 hours post-LPS. Data are mean ±SEM, n=10, *$p<0.05$ and **$p<0.01$.

5.4.4 Anti-TNF therapy does not decrease central cytokine mRNA expression

The mechanisms by which systemic inflammation is communicated to the brain are still largely speculative. Therefore, whilst anti-TNF therapy failed to reduce the up-regulation of systemic cytokine RNA, the nature of the inhibition used may mean that communication to the CNS, and thus up-regulation of CNS cytokines, may have been interrupted. A single i.p. injection of 0.5mg/kg LPS resulted in approximately a 10-fold higher expression of TNF in all brain areas studied, (prefrontal and motor cortices, hippocampus $p>0.05$, striatum $p=0.07$, raphe $p=0.08$; Figure 5.6), possibly due to an underpowered experiment. Etanercept/LPS animals did not show any reduction in TNF mRNA expression. In fact, in these animals, TNF was significantly up-regulated in the prefrontal cortex ($p<0.01$), striatum ($p<0.05$) and hippocampus ($p<0.05$) and increased in the motor cortex and raphe compared to control animals receiving only vehicle after etanercept (Figure 5.6). Finally, XPRO/LPS resulted in 10-fold higher TNF expression across all regions, compared to animals receiving a saline challenge, this increase only reached significance in the prefrontal cortex ($p<0.05$; Figure 5.6). Thus, in all cases, LPS induced a central increase in TNF expression that was not abrogated by anti-TNF therapy.

IL-1β mRNA was between 5- and 10-fold higher in vehicle/LPS animals compared to vehicle/saline, in all brain regions studied although this does not reach significance in any region (prefrontal cortex $p=0.058$ and raphe $p=0.051$). Etanercept/LPS animals did not show any lessening of the LPS-induced changes in IL-1β mRNA, with similar fold increases across all brain regions as animals receiving vehicle (Figure 5.6). The higher expression levels of IL-1β mRNA reached significance in the prefrontal cortex ($p<0.05$) and striatum ($p<0.05$), compared to animals receiving etanercept/saline. In XPRO/LPS treated animals, IL-1β mRNA appeared to be higher in all brain regions studied, compared to XPRO/saline, but did not achieve significance in any region (Figure 5.6). Thus, in a similar manner to TNF, CNS IL-1β mRNA appeared to be up-regulated by LPS in all regions studied, but in no region was this induction reduced by anti-TNF therapy.

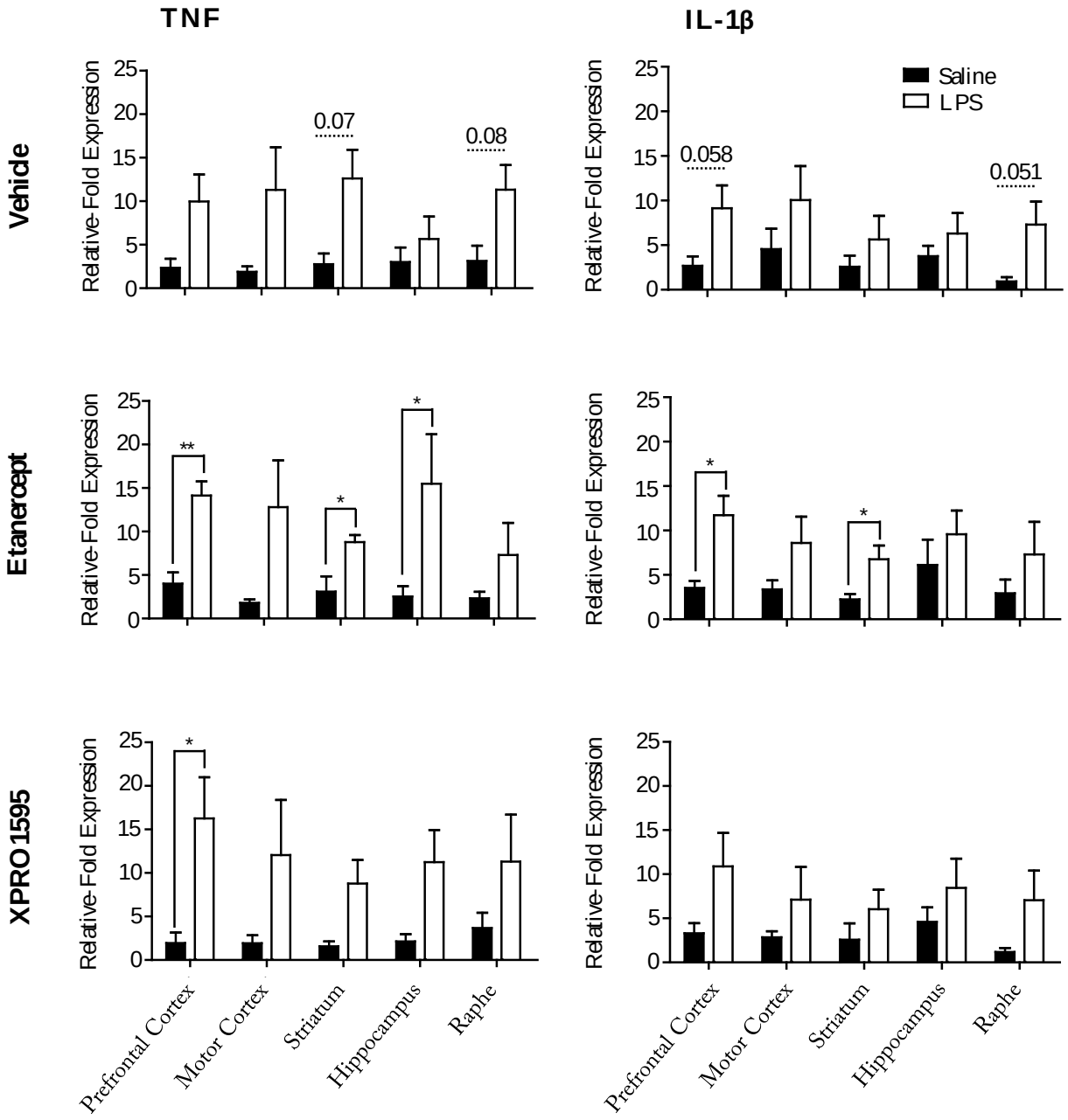


Figure 5.6 The inflammatory mRNA profile in the brain after a single dose of anti-TNF and a single dose of LPS. Brain mRNA of (A) TNF and (B) IL-1β as measured by relative qPCR. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Tissue was removed between 2 and 6 hours post-LPS, data are normalised to the housekeeping gene GAPDH and expressed as relative-fold compared to controls. Data are mean ±SEM, n=4.

5.4.5 5-HT turnover is increased by LPS but unaffected by anti-TNF therapy

The contribution of 5-HT to the development of low mood is well known but whether there is a decrease in total 5-HT, 5-HT metabolism or receptor signal transduction, is yet to be determined. 5-HT is metabolised to, among other metabolites, 5-HIAA and changes in these substances may indicate a change in 5-HT metabolism. As discussed in chapter 3, 5-HIAA/5-HT ratio is considered to be a measure of this turnover. Here, both 5-HT and metabolite 5-HIAA levels were determined in gross fore and hindbrain areas during peak systemic inflammation (2-6 hours after 0.5mg/kg LPS) in the presence and absence of anti-TNF therapy (etanercept and XPRO).

Absolute 5-HT levels in the forebrain region were affected by LPS but not by anti-TNF therapy and furthermore, there was no interaction between LPS and anti-TNF therapy (two-way ANOVA; LPS $p<0.05$ $F_{1,18}=5.950$; anti-TNF $p=0.06$ $F_{2,18}=3.349$; LPS:anti-TNF $p=0.19$ $F_{2,18}=1.769$). Post-hoc analysis shows that only in animals receiving XPRO/LPS, were total 5-HT levels significantly different from control animals (Bonferroni post-hoc; $p<0.05$; Figure 5.7). Forebrain 5-HIAA showed a similar pattern to 5-HT, where absolute 5-HIAA levels were significantly affected by LPS but not by anti-TNF therapy (LPS $p<0.001$ $F_{1,18}=16.36$; anti-TNF $p=0.85$ $F_{2,18}=0.156$; LPS:anti-TNF $p=0.29$ $F_{2,18}=1.297$; Figure 5.7). Specific post-hoc comparisons revealed increases in 5-HIAA in LPS treated animals receiving either vehicle or XPRO, when compared to their respective controls (Bonferroni post-hoc; $p<0.05$; Figure 5.7). 5-HT turnover in the forebrain, as measured by 5-HT:5-HIAA ratio, was affected by LPS but not by anti-TNF therapy (LPS $p<0.05$ $F_{1,18}=6.059$; anti-TNF $p=0.2$ $F_{2,18}=1.760$; LPS:anti-TNF $p=0.7$ $F_{2,18}=0.353$). However, post-hoc analysis failed to show any significant differences in 5-HT turnover between saline and LPS injected animals (Figure 5.7).

In hindbrain regions, no treatment – either LPS or anti-TNF – had any effect on total 5-HT. However, total 5-HIAA in this region was significantly affected by LPS challenge, but not by anti-TNF treatment (two-way ANOVA; LPS $p<0.001$ $F_{1,18}=62.99$; anti-TNF $p=0.11$ $F_{2,18}=2.419$; LPS:anti-TNF $p=0.18$ $F_{2,18}=1.830$). Specifically, injection of LPS increased total hindbrain 5-HIAA irrespective of pre-treatment (Bonferroni post-hoc; vehicle

$p<0.05$; etanercept $p<0.001$; XPRO $p<0.001$). This discrepancy between 5-HT and 5-HIAA levels resulted in a significant increase in 5-HT turnover in animals challenged with LPS, which was not affected by anti-TNF therapy (LPS $p<0.001$ $F_{1,18}=24.72$; anti-TNF $p=0.16$ $F_{2,18}=2.013$; LPS:anti-TNF $p=0.50$ $F_{2,18}=0.709$). Post-hoc analysis shows that there was a significant increase in 5-HT turnover in all animals receiving LPS, regardless of their prior treatment (vehicle $p<0.01$; etanercept $p<0.001$, XPRO $p<0.001$; Figure 5.7), when compared to animals challenged with saline.

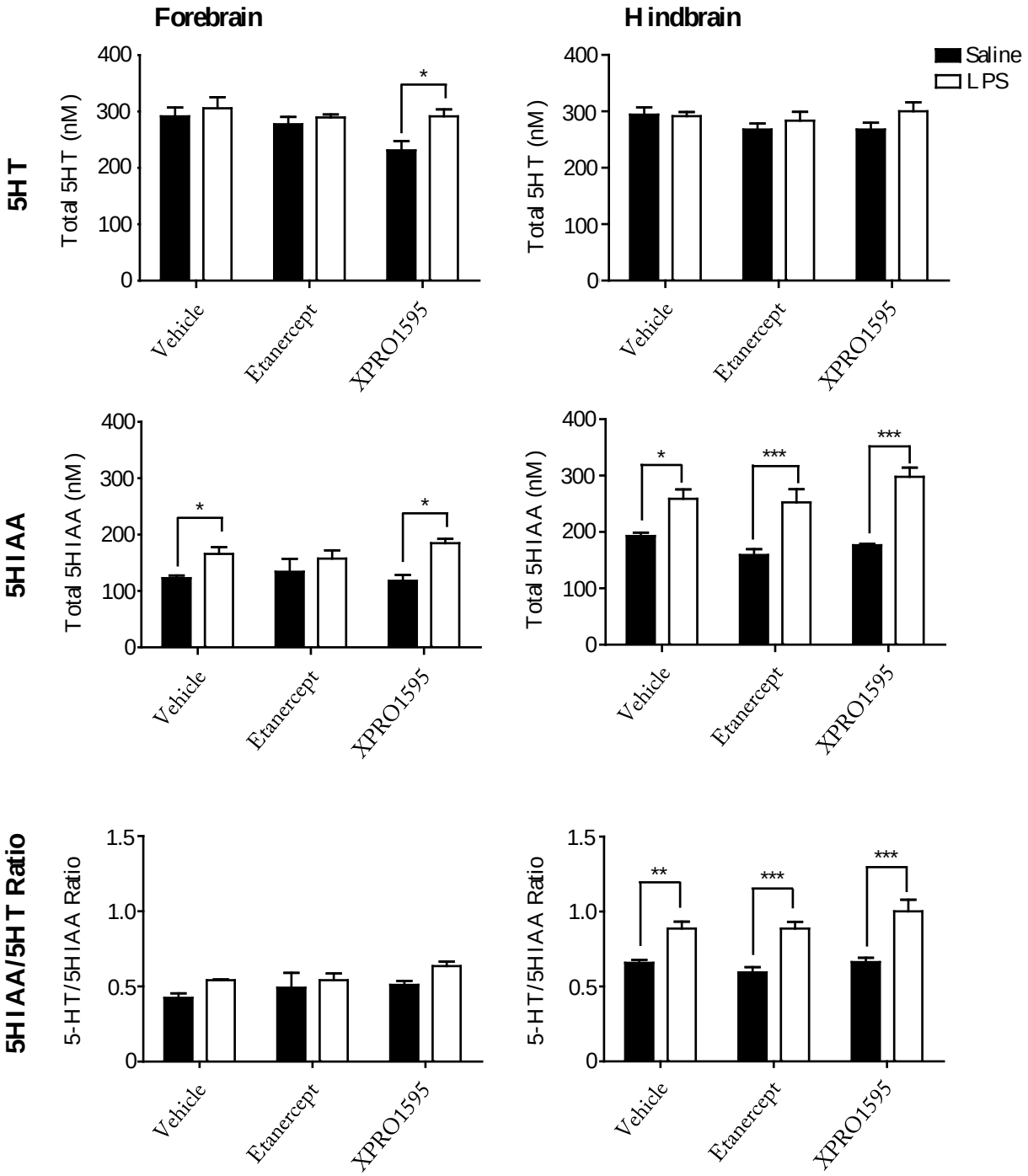


Figure 5.7 Absolute levels of 5-HT and 5-HIAA after a single dose of anti-TNF therapy and a single LPS challenge. Fore and hindbrain levels of 5-HT and 5-HIAA, as well as a ratio of 5-HIAA/5-HT, as measured by HPLC. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Tissue was removed between 2 and 6 hours post-LPS. Data are mean ±SEM, n=6, *p<0.05, **p<0.01 and ***p<0.001.

5.4.6 Microglia are morphologically activated by systemic LPS

The primary source of cytokines in the brain is the microglial population. Previous chapters from this thesis have shown an increase in microglial number after stress (Figure 4.9), specifically in the prefrontal cortex. At the same time, other studies have shown that a systemic inflammatory challenge increases microglial activation (Qin *et al.*, 2007). Here, the brief inflammatory challenge did not change absolute microglial numbers (data not shown), however, as in Qin *et al* (2007) the microglia became visibly denser, with larger cell bodies.

In the prefrontal cortex, densitometric analysis using ImageJ showed a significant effect of LPS on staining intensity, but no effect of anti-TNF therapy (two-way ANOVA; LPS $p<0.001$ $F_{1,18}=20.65$; anti-TNF $p=0.32$ $F_{2,18}=1.204$; LPS:anti-TNF $p=0.49$ $F_{2,18}=0.783$; Figure 5.8A). Post-hoc analysis showed that this increase was only significant in XPRO/LPS animals (Bonferroni post-hoc; $p<0.01$; Figure 5.8A), compared to XPRO/saline controls. Neither etanercept nor XPRO significantly decreased the degree of microglial activation.

Studies in human post-mortem tissue have also demonstrated microgliosis in the hippocampus of suicide victims, suggesting a possible role for inflammation within this region (Steiner *et al.*, 2008). Furthermore, studies of sickness behaviour in rodents have shown significant inflammation in the hippocampus, and that attenuation of inflammation in this region could contribute to the resolution of malaise (Henry *et al.*, 2008). Here, in the CA1 region of the hippocampus, microglial cell body density was significantly affected by LPS, but not by anti-TNF (LPS $p<0.001$ $F_{1,18}=26.01$; anti-TNF $p=0.68$ $F_{2,18}=0.377$; LPS:anti-TNF $p=0.66$ $F_{2,18}=0.416$). Specifically, LPS significantly increased Iba-1 staining intensity in LPS-challenged animals irrespective of prior treatment with anti-TNF therapy (vehicle/LPS $p<0.05$, etanercept/LPS $p<0.05$, XPRO/LPS $p<0.01$; Figure 5.8).

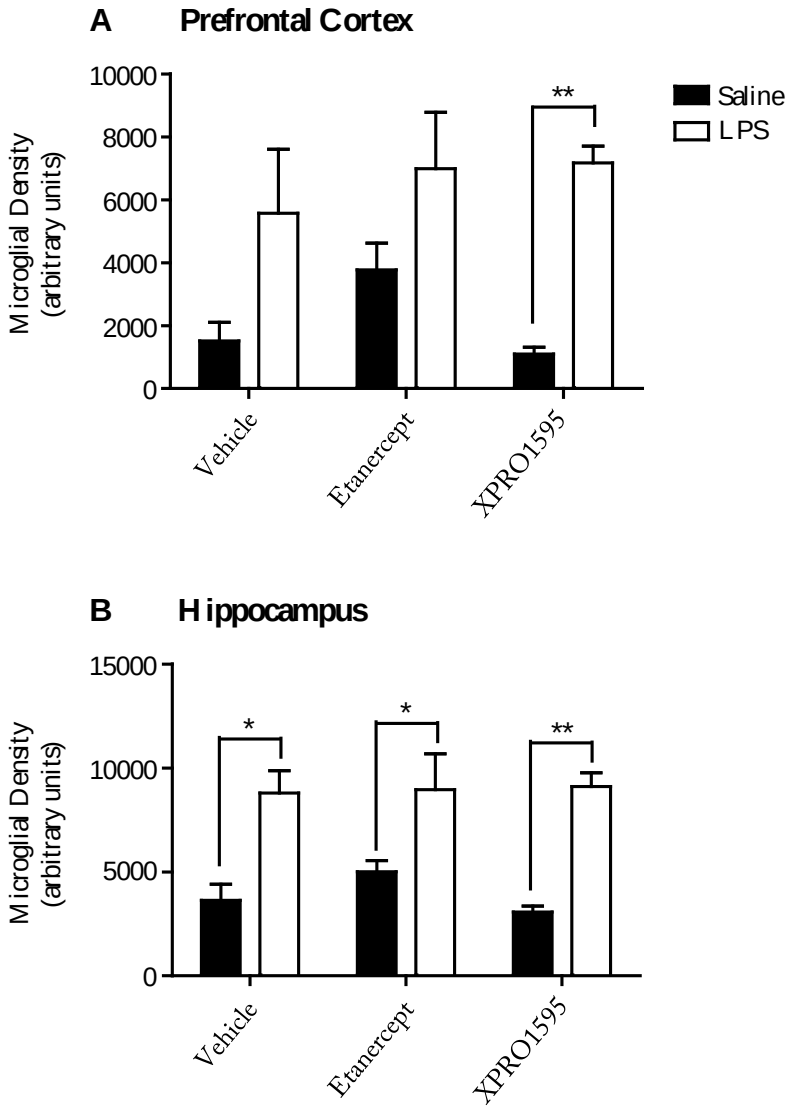


Figure 5.8 Microglia in the prefrontal cortex and CA1 region of the hippocampus after a single dose of anti-TNF therapy and a single dose of LPS. Microglia were stained for Iba-1 and counted in the prefrontal cortex (A) and hippocampus (B). Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Tissue was removed between 2 and 6 hours post-LPS. Data are mean ±SEM, n=6, *$p<0.05$ and **$p<0.01$.

5.4.7 Neither LPS nor anti-TNF therapy significantly alter the expression of 5-HT$_{2A}$ or SERT in C57/Bl6 mice

Previous chapters have shown that systemic inflammation is capable of causing increases in both SERT and 5-HT$_{2A}$ expression in the CNS of both stressed and LPS-challenged mice (Figure 3.14 and Figure 3.15). In order to test whether this change was applicable in a different strain of mouse, and to test whether anti-TNF therapy could alter any changes that occurred, qPCR was performed on microdissected brain regions.

5-HT$_{2A}$ receptor expression in vehicle treated animals challenged with LPS varied by only 0.5-fold around control values of 1 and was not, therefore, significantly different from animals challenged with saline (two-way RM-ANOVA; Figure 5.9). Similar results occurred in animals pre-treated with either etanercept or XPRO showing no significant change in animals receiving LPS from those challenged with saline. The standard error of the measurements in these animals was also particularly high (Figure 5.9), which may explain the lack of effect of LPS.

SERT expression showed similar patterns to 5-HT$_{2A}$ receptor expression, although the standard error in these data was less than for 5-HT$_{2A}$. Expression in animals treated with vehicle prior to challenge with either LPS or saline varied by less than 0.5-fold in all brain regions (two-way RM-ANOVA; Figure 5.9B). The same pattern of receptor expression occurred in animals pre-treated with etanercept or XPRO, with less than 0.5-fold variation between animals challenged with LPS or saline (Figure 5.9).

Thus, despite similar elevations after a chronic stress paradigm, C57/Bl6 mice appear to be less sensitive to the effect of systemic LPS on the central 5-HT system than CD1 mice.

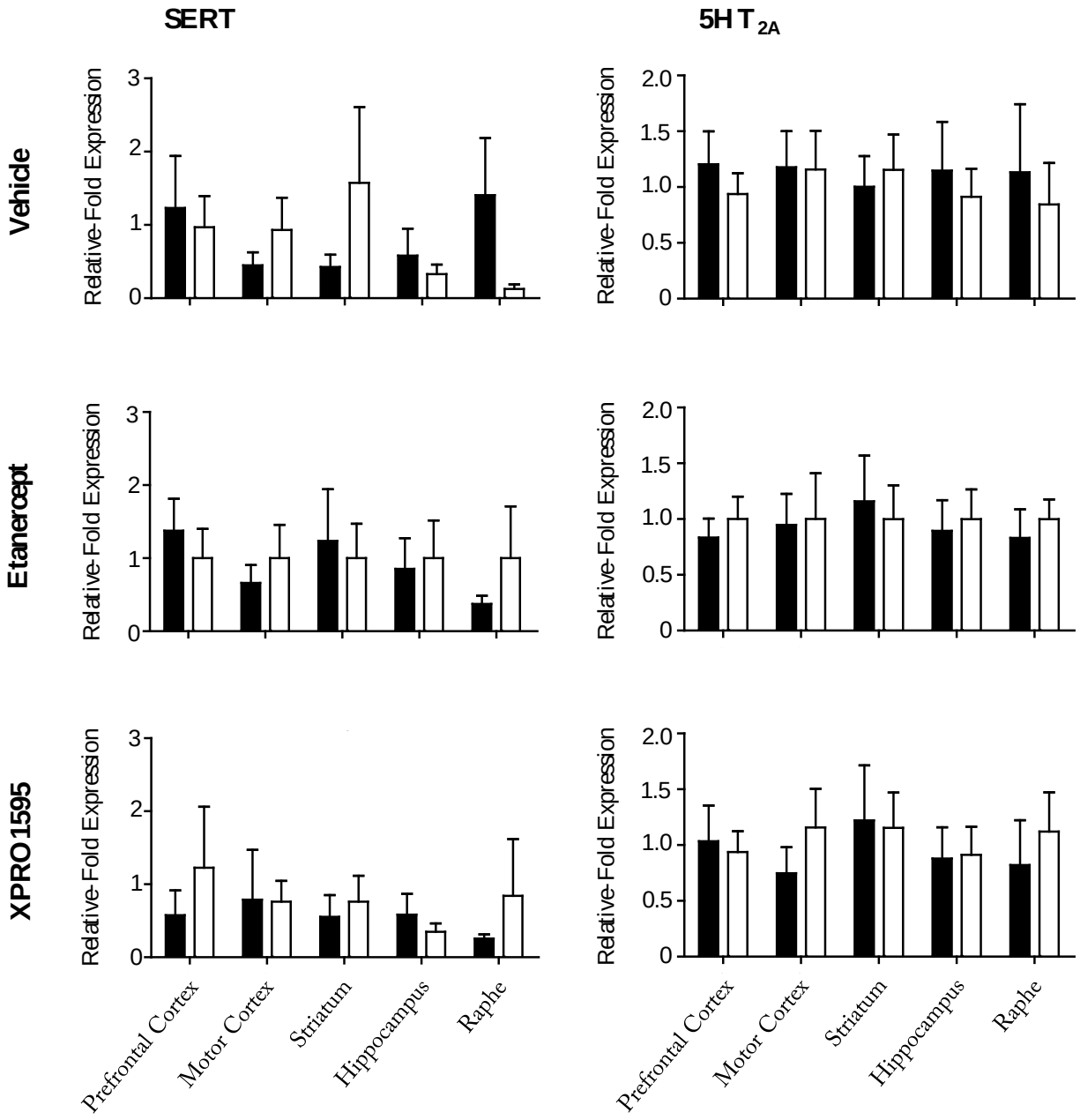


Figure 5.9 The SERT and 5-HT$_{2A}$ mRNA profiles in the brain after a single dose of anti-TNF and a single dose of LPS. Brain mRNA of (A) SERT and (B) 5-HT$_{2A}$ as measured by relative qPCR. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPRO (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Tissue was removed between 2 and 6 hours post-LPS, data are normalised to the housekeeping gene GAPDH and expressed as relative-fold compared to controls. Data are mean ±SEM, n=6.

5.4.8 LPS results in long-term behavioural changes not recoverable by anti-TNF therapy

Studies earlier in this thesis indicated that a single challenge with a relatively high dose of LPS was capable of inducing long-term behavioural changes in CD1 mice (*see* 3.3.2). It is possible that persistent TNF expression may underlie these changes and that these sickness behaviours may be ameliorated by anti-TNF therapy. Animals were pre-treated with either vehicle, etanercept or XPRO followed by a challenge with LPS 24 hours later. Recovery was for 6, 24 or 48 hours before locomotor activity was assessed. At no point were baseline values (in any drug treatment group) different from vehicle-saline animals (data not shown). Baseline animals showed an average of 500 beam breaks within the first 10 minutes, slowing down over 2 hours to an average of 100 by 60 minutes, which did not change between 60 and 120 minutes.

At 6 hours after challenge LPS had a significant effect on activity, irrespective of anti-TNF therapy, when compared to baseline levels (two-way RM-ANOVA; time $p<0.001$ $F_{11,132}=52.70$; treatment $p<0.001$ $F_{3,12}=50.0$; time:treatment $p<0.001$ $F_{11,132}=26.95$; Figure 5.10A). Animals receiving LPS, irrespective of pre-treatment, showed less activity in the first 10 minute bin than baseline values, with an average of 500 beam breaks *vs* an average of less than 100 in LPS treated animals. LPS treated animals in all treatment groups, slowed to zero movement by 40 minutes.

After 24 hours of treatment with LPS maintained its significant effect on activity over time, irrespective of treatment (two-way RM-ANOVA; time $p<0.001$ $F_{11,110}=41.50$; treatment $p<0.05$ $F_{1,10}=3.49$; time:treatment $p<0.001$ $F_{11,110}=2.87$; Figure 5.10).

At 48 hours post-LPS, all animals appeared to show decreased movement compared to baseline, although this seemed to be attenuated in etanercept treated animals. While treatment did not have a significant effect on overall activity over time (time $p<0.001$ $F_{11,110}=44.19$; treatment $p=0.06$ $F_{1,10}=3.15$; time:LPS $p<0.05$ $F_{11,110}=1.77$; Figure 5.10), there was an interaction, suggesting that the different anti-TNF therapies may have affected activity across time.

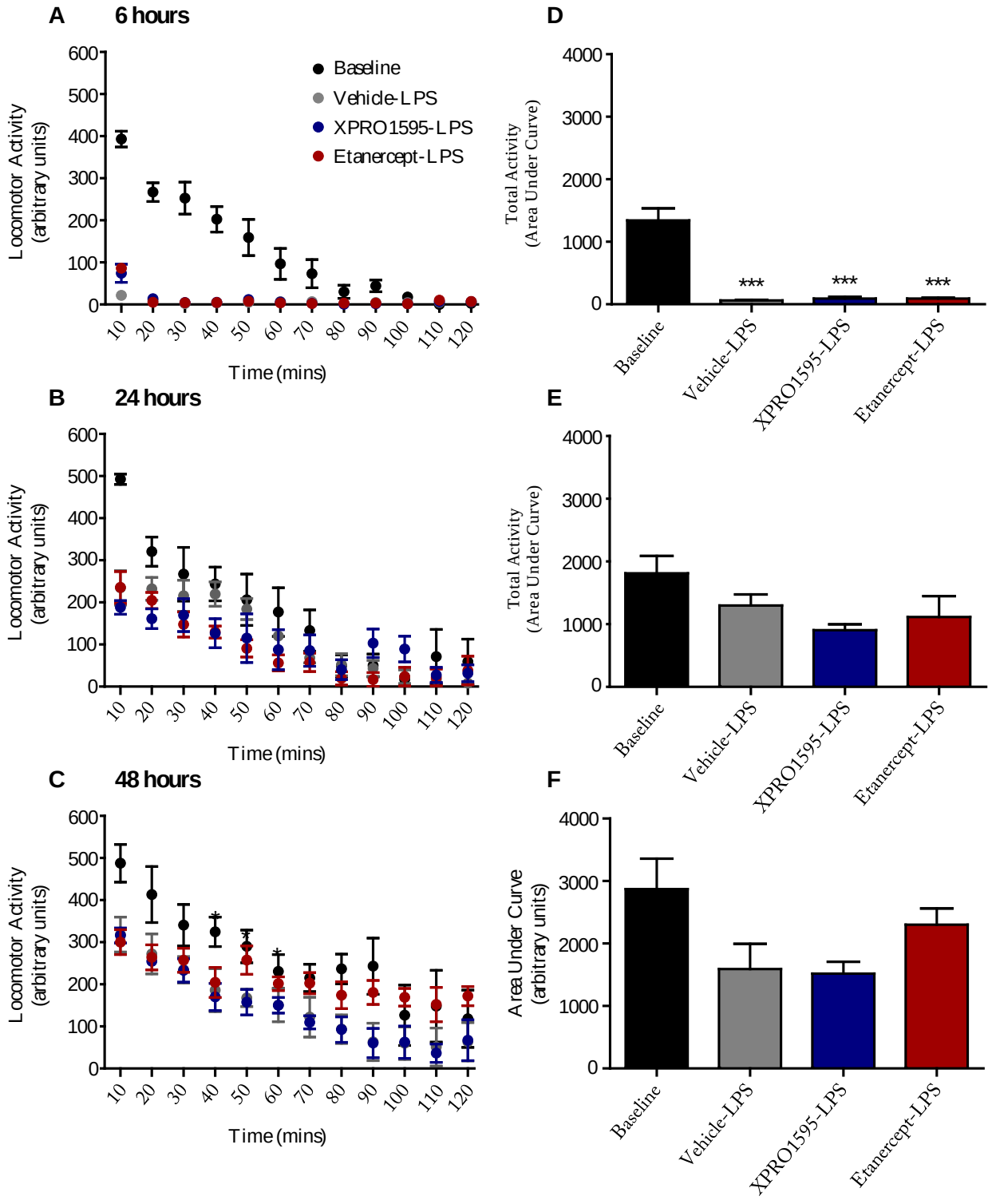


Figure 5.10 Locomotor activity across two hours in animals receiving a single dose of anti-TNF therapy followed by a single LPS or saline challenge. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS (0.5mg/kg) on day 2. Locomotor activity was measured over 2 hours at (A) 6 hours; (B) 24 hours and (C) 48 hours post-challenge. (D-F) Area under curve analysis of A-C. Data are mean ±SEM, n=4, ***$p<0.001$ compared to baseline measurements.

5.4.9 Anti-TNF therapy inhibits sickness behaviour induced by a low dose of LPS

0.5mg/kg is the dose of LPS used in most studies of sickness behaviour and immune challenge, in fact many studies use higher doses (O'Connor *et al.*, 2009). However, it is possible to induce sickness behaviour in animals with a significantly lower dose (Cunningham *et al.*, 2009). Here, a dose response study was carried out using the same dosing and testing protocol as used previously in this chapter, with doses of LPS at 0.5 and 0.05mg/kg. Control animals were treated with vehicle (saline), etanercept or XPRO followed by a saline challenge. Values between control groups did not differ from each other (data not shown) and therefore all control groups (saline, etanercept and XPRO treated animals challenged with saline) were averaged and post-LPS data are presented as a percentage of these controls.

At this short time point, in the open field, LPS (0.5mg/kg) reduced speed and distance to approximately 50% of control values, irrespective of treatment. Rearing behaviour was reduced to 10% of control values in vehicle/LPS and XPRO/LPS animals, but only to 50% of control values in etanercept/LPS animals. These differences resulted in an overall effect of treatment on rearing behaviour when compared to vehicle treated animals (one-way ANOVA; $p<0.05$ $F_{2,11}=4.62$), more specifically that etanercept increased rearing behaviour when compared to control animals (Bonferroni post-hoc; $p<0.05$; Figure 5.11).

At 0.05mg/kg, LPS reduced speed to approximately 75% of control values in vehicle treated animals. In etanercept/LPS animals speed was almost 100% of control values, whilst XPRO/LPS animals remained at approximately 75% of controls. Distance was reduced to around 60% of controls in vehicle/LPS treated animals. Etanercept/LPS animals travelled further than vehicle/LPS animals at 0.05mg/kg LPS. XPRO/LPS animals still showed a reduction in distance travelled, to an average of 80% of control values. However, overall treatment had no significant effect on speed or distance (one-way ANOVA; speed $p=0.60$ $F_{2,11}=0.322$; distance $p=0.11$ $F_{2,11}=2.828$; Figure 5.11). Rearing behaviour was reduced to 45% of control values in vehicle/LPS treated animals. Animals in the etanercept/LPS group reared significantly more than those in the

vehicle/LPS group ($p<0.01$; Figure 5.11), averaging 100% of control values. XPRO/LPS animals did not recover as well, showing around 60% of control rearing values.

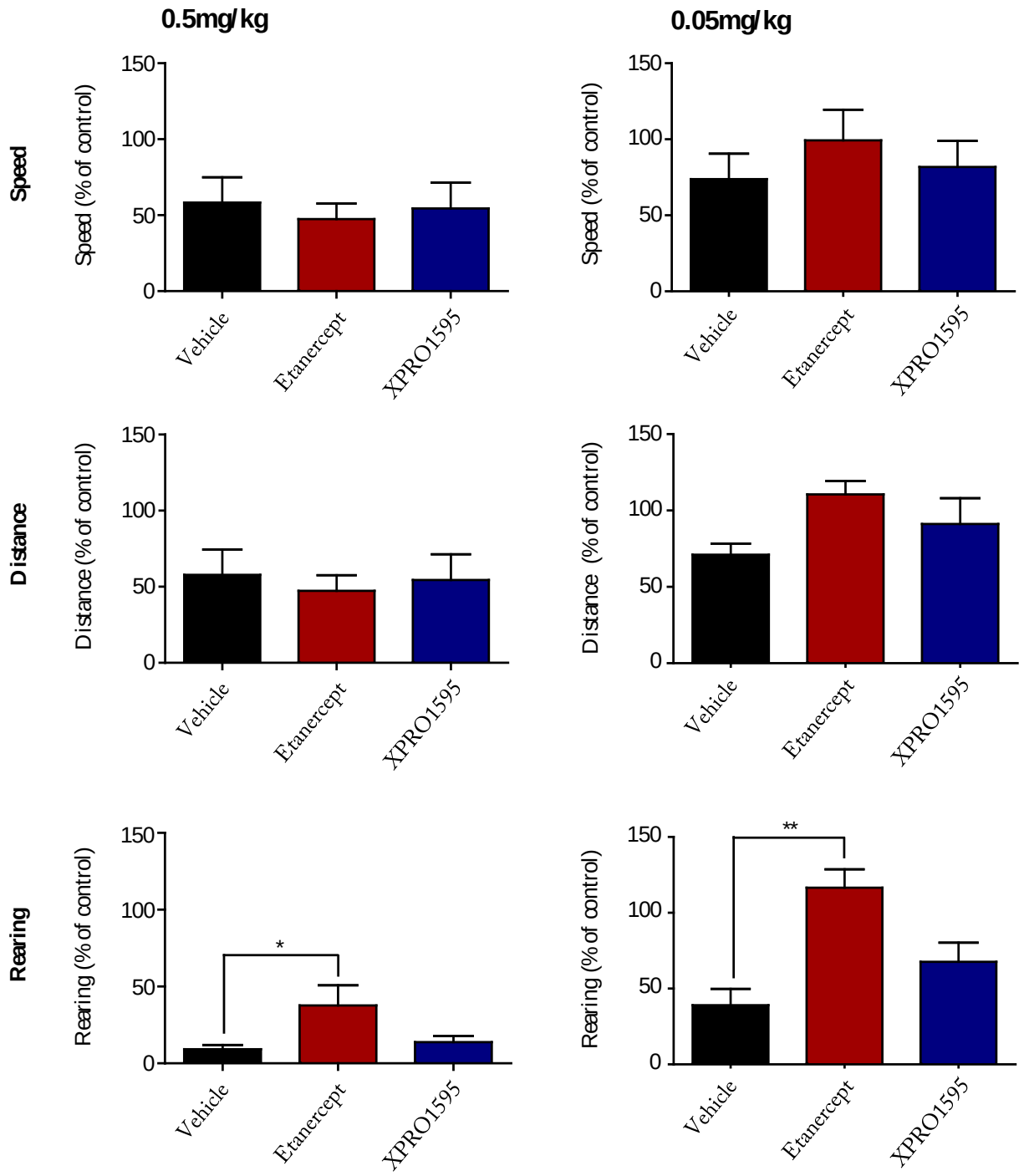


Figure 5.11 Changes in speed, distance and rearing in the open field at high and low-dose LPS in the presence and absence of anti-TNF therapy. Animals received a single dose of either vehicle (saline, c.0.1ml), etanercept (10mg/kg) or XPro (10mg/kg) i.p. on day 1, followed by a single dose of either saline (c.0.1ml) or LPS the following day. LPS doses were (A) 0.5mg/kg; (B) 0.1mg/kg; (C) 0.05mg/kg and (D) 0.01mg/kg. Open field behaviour was measured for 3 minutes between 2-6 hours post-injection. Data are mean ±SEM, n=12, *p<0.05 and **p<0.01.

5.4.10 Mice expressing an uncleavable membrane bound form of TNF show decreased horizontal activity in the open field after LPS

tmTNFΔ/Δ mice express an uncleavable form of mTNF, preventing them from producing sTNF (Ruuls *et al.*, 2001), in effect these are permanent XPRO mice. Animals receiving XPRO in previous open field studies, did not recover behaviours after LPS treatment to the same degree as those receiving etanercept. To confirm the theory that sTNF does not contribute significantly to sickness behaviour, animals transgenic for an uncleavable form of mTNF were treated with a single i.p. dose of LPS (0.5mg/kg) and allowed to recover for 6 hours prior to studying open field behaviour.

Despite visible differences between wild-type and tmTNFΔ/Δ rearing behaviour post-LPS, analysis showed that there were no significant main effects of either LPS or genotype on rearing behaviour in the open field after 6 hours of treatment (two-way ANOVA; all comparisons ns; Figure 5.12A). In terms of total distance travelled there was a significant main effect of LPS treatment but no effect of genotype (LPS $p<0.001$ $F_{1,14}=62.65$; genotype $p=0.06$ $F_{1,14}=4.189$; LPS:genotype $p=0.51$ $F_{1,14}=0.441$). Post-hoc analysis showed that both wild-type and tmΔ/Δ mice travelled significantly less after an LPS challenge than the saline challenged controls (Bonferroni post-hoc $p<0.001$ for both wild-type and tmΔ/Δ mice; Figure 5.12B). There were significant main effects of both LPS and genotype on speed in the open field, but no interaction between the factors (LPS $p<0.001$ $F_{1,14}=23.10$; genotype $p<0.05$ $F_{1,14}=4.650$; LPS:genotype $p=0.9$ $F_{1,14}=0.007$). In wild-type animals, post-hoc analysis showed that LPS significantly reduced total speed ($p<0.05$), with similar data from the tmTNFΔ/Δ mice ($p<0.01$; Figure 5.12C), with respect to saline-challenged animals.

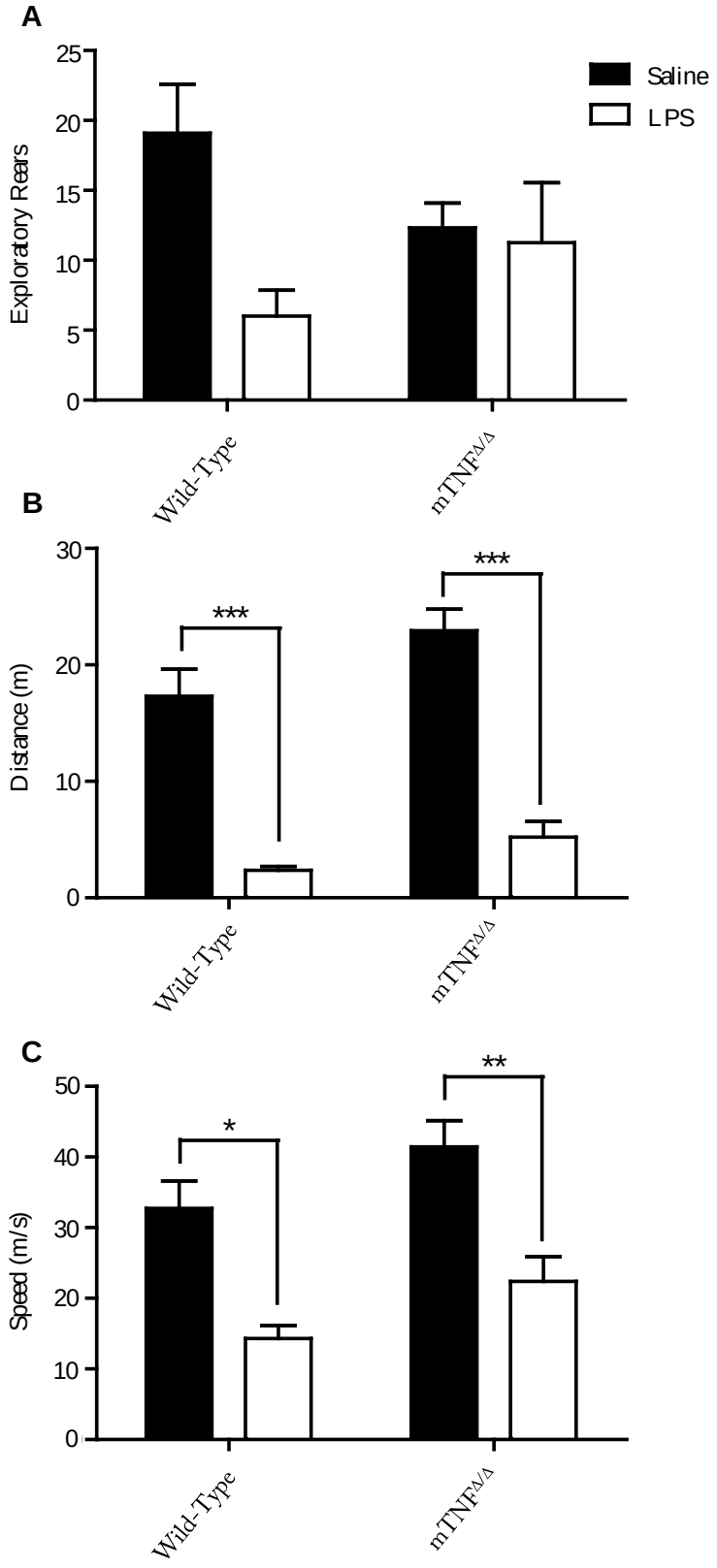


Figure 5.12 Open field behaviour in mTNFΔ/Δ mice. Animals received a single i.p dose of LPS (0.5mg/kg) and were allowed to recover for 6 hours before being subjected to a 3-minute open field where animals were analysed for (A) rearing; (B) distance and (C) speed. Data are mean ±SEM, n=4+, *p<0.05.

5.5 Discussion

The role of TNF in sickness behaviour is still contentious with studies in animal models of sickness and depression largely focusing on IL-1 as the main perpetrator. Despite this, anti-TNF therapy has promising effects in inflammatory diseases such as psoriasis (Tyring *et al.*, 2006) and psoriasis (Krishnan *et al.*, 2007). Surprisingly, the mechanisms underlying this phenomenon are largely underinvestigated. The aims of this chapter were to uncover the role of TNF in the development of sickness behaviour. Using anti-TNF drugs etanercept and XPRO it has been possible to demonstrate that a standard, if somewhat high, dose of LPS is not recoverable by anti-TNF therapy. By using a lower dose of inflammogen (0.05mg/kg) it is possible to induce sickness behaviour which is recoverable by prophylactic anti-TNF treatment. Etanercept and XPRO target different forms of TNF, etanercept targets the soluble (sTNF) and membrane bound (mTNF) forms, where XPRO only targets the soluble form.

Studies into the different roles played by the soluble and membrane bound forms of TNF have mainly been performed in transgenic animals, owing to a paucity of compounds selective of one relative to the other. Current therapies blocking both include etanercept, as well as antibody therapies such as infliximab and adalimumab. The relative efficacy of these drugs is speculative and appears to depend on their ability to successfully block mTNF. Traditional monoclonal antibody therapies such as infliximab, bind to mTNF and form very large complexes which may be cytotoxic (Kohno *et al.*, 2007). This does not occur with etanercept, from which the binding efficiency at mTNF is around 2-fold lower than some of the antibodies (Nesbitt *et al.*, 2007). This difference in signalling capacity significantly affects their ability to treat granulomatous diseases such as Crohn's, in which infliximab is successful and etanercept is not (Tracey *et al.*, 2008). Despite these differences, all of the above drugs bind to sTNF with the same affinity (Kohno *et al.*, 2007). These data, combined with the knowledge that etanercept is associated with significantly lower rates of infection than traditional antibody therapies (Furst *et al.*, 2006), suggest that selectively inhibiting sTNF would be advantageous.

In the studies presented here, at a relatively high dose of LPS, selective inhibition of sTNF using the dominant negative inhibitor XPRO made little difference to behaviour or the the inflammatory profile of the animals. To date this appears to be the first time anti-TNF drugs have been tested in a basic model of sickness. Initial studied into the basic inflammatory response in this chapter demonstrate no significant down-regulation of inflammatory cytokine mRNA or accumulation of liver neutrophils. The mechanisms of action of etanercept and XPRO are both considerably downstream of transcription, thus any effects on cytokine mRNA are likely to be via feedback mechanisms. Whilst other studies have demonstrated a change in cytokine mRNA using etanercept (So *et al.*, 2007), others have shown that treatment may take weeks to have effects on mRNA levels (Zaba *et al.*, 2007). The difference in these studied may reflect differences in treatment regime, species or tissue studied. Serum and liver cytokine protein assays would be of more use in future studies to determine whether the inflammatory cascade has been significantly attenuated by etanercept. Liver neutrophils are recruited by part of this pro-inflammatory cascade. However, they appear to rely largely on the expression of cell surface adhesion molecules and their accumulation after LPS treatment may be independent of TNF (Shaw *et al.*, 2009), confirming data from this chapter showing that neither anti-TNF therapy had any significant impact on hepatic neutrophils.

Further to having little or no effect on pro-inflammatory cytokines, neither anti-TNF drug had any effect on corticosterone release. This data is relatively unsurprising; treatment of mice with monoclonal anti-TNF antibodies has been shown to have no effect on LPS-stimulated corticosterone release elsewhere (Perretti *et al.*, 1993). Baseline levels of corticosterone appear capable of regulating cytokine release, but corticosterone released during an inflammatory episode appears to have little effect on cytokine production and vice versa (Perretti *et al.*, 1993). TNF-R1-deficient mice produce a corticosterone response to LPS that is indistinguishable from wild-type animals, suggesting that signalling through this receptor is not necessary for regulation of HPA axis activity (Hayley *et al.*, 2004). These data, and those presented within this chapter, are all in acute inflammatory challenges. In more persistent inflammatory disorders, such as rat rheumatoid arthritis models, inhibition of TNF using etanercept has been shown to

down-regulate cortisone converting enzymes (Ergang *et al.*, 2010). The sparse data currently available on the interactions between cortisol and TNF suggest that the short term response achieved in these experiments is not unexpected. However, if the study had been extended to investigate whether this dose of LPS persistently changed circulating TNF levels, it may have been possible to attenuate the cortisol response with an anti-TNF drug.

Data from this chapter also demonstrate no significant reversal of LPS-mediated sickness behaviours by either etanercept or XPRO. The incomplete lack of behavioural reversal is not unexpected with this dose. Anti-inflammatory drugs have been used with a higher dose of LPS and still only shown partial reversal (O'Connor *et al.*, 2009; Kang *et al.*, 2011). Henry *et al.* (2008) demonstrated a significant lowering of brain IL-1β levels after minocycline treatment but not plasma IL-1β levels, suggesting the inhibition of sickness behaviours was mediated by inhibition of CNS cytokines. Since data from this chapter does not demonstrate a significant lowering of brain cytokine levels it would may not be reasonable to assume improvements in behaviour. A brief dose response study demonstrated that at 0.05mg/kg LPS it was possible induce sickness behaviours visible in the open field which were not apparent in animals pre-treated with either etanercept or XPRO (Figure 5.11, **Error! Reference source not found.** and **Error! Reference source not found.**).

This dose response data show that animals treated with either etanercept or XPRO perform equally well in the open field post-LPS. Since XPRO selectively inhibits sTNF and prevents sickness behaviour in a manner similar to etanercept, it could be postulated that mTNF plays no particular role in sickness behaviour. This was, in part, confirmed using tmTNFΔ/Δ mice, who express an uncleavable form of mTNF. While these mice move around less in an open field than wildtype litter mates (non-significant; $p=0.4$), they also show no decrease in open field activity upon LPS challenge (Figure 5.12). A series of elegant experiments in an animal model of arthritis also confirmed that mTNF is less important in mediating inflammation. Kontoyiannis and colleagues (1999) showed that animals overexpressing TNF eventually develop arthritis but if these animals are

crossed with either TNF-R1 or TNF-R2 knockout animals two distinct phenotypes occur. The TNF-R1 crosses still produce a lot of TNF but the symptoms of inflammatory arthritis are significantly reduced, in contrast to the TNF-R2 crosses, which still develop arthritis. This suggests that TNF-R2 signalling is dispensable in terms of producing an inflammatory response. Since mTNF largely signals through TNF-R2 it could be hypothesized that sTNF is largely responsible for inducing inflammation.

Two recent papers show opposing views on the role of sTNF in depression-like behaviour. In human post-mortem tissue from patients with Major Depression, schizophrenia and bipolar disorder, Dean and colleagues (2012) demonstrated that mood disorder patients showed large increases in mTNF but not sTNF in a number of brain regions. In contrast, Bedrosian *et al* (2012) used a stress-based model of depressive-like behaviour in rodents to demonstrate that inhibition of sTNF using XPRO successfully reverses depression-induced forced swim behaviour. These conflicting data may represent differences in human *vs* rodent models or differences in the type of depression – idiopathic *vs* stress induced. To date, these papers represent the only available work on the role of sTNF in affective disorders, indicating that the studies begun in this chapter have the potential to significantly contribute to the field.

5.6 Conclusion

Data from this chapter demonstrate that 0.5mg/kg LPS produces sickness behaviour that is insurmountable by standard anti-TNF therapy. However, lower doses of LPS induced sickness behaviour that were successfully reversed by etanercept, but not XPRO. These data, combined with those from tmTNFΔ/Δ mice, showing normal development of sickness behaviours in response to LPS, suggest that soluble TNF is not essential for the development of sickness behaviour.

Surprisingly, changes in 5-HT receptor expression seen in previous chapters in CD1 mice after an endotoxin challenge, are not reflected in this cohort of C57/Bl6 mice. This may be the result of different housing conditions, different suppliers and different handling conditions. Increases in TNF in the CNS were consistent throughout the models - both

stress and endotoxin, as well as in different strains of mice, although at the higher dose of LPS, anti-TNF therapy failed to significantly release of this cytokine. Overall, the data in this chapter suggest that TNF plays a significant role in the regulation of mood, and that anti-TNF therapies such as etanercept should be seriously considered in the long-running effort to develop new antidepressants.

Chapter 6: General Discussion

The principal findings of this thesis are as follows:

- That systemic inflammation is capable of altering the functional output of the 5-HT system in the rat.
- That a systemic LPS challenge is capable of changing 5-HT receptor and transporter expression within the CNS in both rats and mice.
- That changes in 5-HT receptor expression occur within discrete areas of the brain, specifically that more profound changes often occur in the prefrontal cortex, hippocampus and dorsal raphe nucleus.
- That chronic stress is capable of inducing CNS inflammation, as shown by increased levels of TNF and IL-1β in the brain.
- That etanercept, an anti-TNF drug, is capable of recovering sickness behaviour associated with low-dose LPS.

Overall, this thesis has demonstrated that peripheral inflammation and chronic stress-induced anhedonia can produce similar changes in CNS cytokine expression and changes in the central 5-HT system. Importantly, the studies reported here have shown that a single systemic inflammatory challenge is capable of producing both functional and behavioural changes as well as persistent changes within the 5-HT system that last beyond what would be considered the normal duration of 'sickness behaviour' after LPS. This work also demonstrated that inducing 'depression-like' signs in rodents following chronic stress is associated with local microglial activation and neuroinflammation. Moreover, these inflammatory effects may be further exacerbated by a systemic immune challenge insufficient to cause behavioural alterations *per se*, but which, in combination with the stress, results in an exacerbated behavioural phenotype. Together, the data from this thesis show the importance of the immune system in regulating behaviour in distinct rodent models of mood disorder, and how the combination of stress and inflammation can synergise in a manner that is directly relevant to the human condition of Major Depression. For important technical reasons, different strains and species of rodent were

employed. Though there were clear differences between strains of mice, and between rats and mice, a number of themes were recurrent within the data generated, and the significance of these changes will be discussed below.

To date, studies of the role of inflammation in depression have largely accepted the theory that cytokines increase IDO activity and decrease 5-HT levels. However, the degree of cytokine induction in most depressed patients is relatively small compared to that achieved with an endotoxin challenge. Recent work by Hughes and colleagues (2012) has shown that the increase in cytokines in depressed patients occurs independently of changes in plasma tryptophan. While tryptophan depletion did occur in the patients studied, increases in IDO, or IDO activity, did not. This is in accord with work from this thesis, and within the Sharp lab (Qin Xie, unpublished data), where brain IDO mRNA and kynurenine levels were not significantly changed, but CNS turnover of 5-HT was increased. Those previous findings, combined with work described in this thesis, indicate that a number of different pathways potentially contribute to changes in mood during sickness and depression. Fundamental differences in the molecular changes associated with different moods may shed light on the mechanisms associated with the development of diseases such as Major Depression.

6.1 Depression *vs* anxiety

Studies have shown that increased 5-HT turnover, as demonstrated in models of sickness in this thesis, is also present in non-medicated, depressed patients (Barton *et al.*, 2008), and that this is reversed by SSRI treatment (Esler *et al.*, 2007). Barton and colleagues (2008) have shown that this change in neurotransmitter turnover is positively correlated with SERT genotype. Work described here has shown that inflammation is capable of increasing SERT expression, and work of others has shown that cytokines such as TNF can regulate SERT activity (Zhu *et al.*, 2006). In the chronic stress model of depression, used in this thesis, significant up-regulation of, not only TNF in the CNS, but also of SERT, was also found, specifically in the prefrontal brain areas. Taken together these studies suggest that local TNF production might play an important role in regulating both the expression and activity of SERT, and that this has a significant impact on mood.

Interestingly, studies on 5-HT transporter knockout and over-expressing mice highlight some puzzling results from this thesis. Animals deficient in the 5-HT transporter show conflicting results in terms of depressive-like behaviour (Barkus, 2012), but do consistently show an increased anxiety phenotype (Kalueff *et al.*, 2007). Conversely, mice over-expressing SERT show a decreased anxiety phenotype (Jennings *et al.*, 2006). Work from this thesis has demonstrated that it is possible to separate stressed mice into susceptible and resilient, based on the expression of a number of different markers. SERT expression is almost exclusively increased in animals showing a low sucrose preference. This finding indicates that the 5-HT transporter is crucial in mediating depressive behaviours, rather than anxiety behaviours, since both low- and high-sucrose preference animals demonstrate anxiety. An alternative interpretation of these results, considering the data from knockout animals, could be that SERT expression is increased as a compensatory mechanism to decrease anxiety during stressful situations. Since the stress paradigm used in these studies is considerable, it is possible that the neurocircuitry involved in depressive-like behaviours overrides that mediating anxiety-like behaviours. These studies provide interesting mechanistic insights into the role of SERT in depression and anxiety. Anxiety is often, but not always, comorbid with depression and by untangling the role of specific receptors in the pathogenesis of the two it may be possible to target specific patient groups with novel treatments.

SERT expression was also increased in the model of sickness behaviour used in this thesis. Unfortunately, anxiety, specifically using the light-dark box test, was not a behavioural outcome assessed in this model of sickness behaviour. However, previous work from the Sharp lab using the elevated plus maze, 6 and 24 hours after animals were challenged with LPS, suggests that systemic inflammation does not induce significant anxiety-like behaviour. The discrepancy between SERT expression and anxiety behaviour between the two models could reflect the homeostatic compensatory hypothesis suggested above. Increases in SERT in response to a single injection of LPS result in no significant anxiety outcomes, similar to the SERT over-expressing mice. Conversely, the longevity of the chronic stress paradigm results in an increased anxiety phenotype via mechanisms as yet unexplored. It would be interesting to test whether the anxiety

phenotype present in chronic stress animals is decreased or exacerbated by the addition of the LPS challenge used in chapter 4, as well as to study the effect of systemic inflammation in both SERT overexpressors and knockouts.

6.2 The CNS immune response in models of stress and inflammation

Depression is a complex multifaceted human disease, often diagnosed through counselling and reported changes in mood, both hard to replicate in rodent models. With this in mind, many researchers choose to dissociate symptoms in order to model them individually. The most common feature of depression modelled in animals is anhedonia; a *loss of interest in, or failure to derive pleasure from, previously enjoyable stimuli or activities* (APA, 2000). This translation to rodents is based on their relative preference for palatable sucrose solutions over plain drinking water, a feature mimicking hedonia, the loss of which is deemed to be anhedonia. Reduced consumption of sucrose solutions has been shown in both chronic stress models of anhedonia (Willner *et al.*, 1987; Muscat and Willner, 1992; Matthews *et al.*, 1995; Willner *et al.*, 1996) as well as after endotoxin challenge (Yirmiya, 1996; Yirmiya *et al.*, 2001). These data suggest that, in terms of face validity, it may be simpler to model depression in rodents using an endotoxin challenge.

Ethically, refining the current models of depression in rodents by using a single endotoxin challenge, rather than persistent chronic stress, would be advantageous. This thesis has demonstrated that there are recurrent themes in the two models, one of which is an increase in CNS cytokine expression, specifically TNF. Despite the low level of expression of pro-inflammatory cytokines induced by chronic stress, an induction of TNF is perhaps sufficient to describe the response as neuroinflammatory. The semantics of reporting the presence of inflammation in the CNS has always been difficult, owing to the absence of leukocyte recruitment in all but the most severe challenges to the CNS. Thus, the presence of microglial activation has, historically, been applied to define the presence of inflammation in the CNS. It is now clear that microglia can look *active*, i.e. amoeboid rather than stellate, in the absence of pro-inflammatory cytokine production (Perry *et al.*, 2007). Thus, in fact, inference of activation state from morphology is inconclusive and outdated. Recently, a number of different activation states have been recognised –

resting, M1 (pro-inflammatory), and M2 (phagocytic), which has led to a reclassification on this basis. M1 microglia make pro-inflammatory cytokines and are viewed as destructive, and M2 microglia make COX and TGFβ, and are considered reparative (Olah *et al.*, 2011). Here, in this thesis, production of TNF in the prefrontal cortex, and increased numbers of microglia are associated with the chronic stress paradigm, but a destructive M1-type phenotype has not been extensively investigated, therefore a neurodegenerative hypothesis cannot be concluded. These data are in accordance with other work suggesting that microglia-specific immunoreactivity increases during stress, but that this may not reflect a change in inflammatory status (Tynan *et al.*, 2010). Similar data have been found in post-mortem tissue from suicide victims (Steiner *et al.*, 2008), although, again, no significant conclusions regarding the inflammatory status of the microglia in these studies could be drawn. It seems likely that the new polarised view of microglial activation is also portraying an incomplete picture. Thus far in this thesis neuroinflammation has been used to describe the activation and proliferation of microglia and the production of TNF. However, the CNS pathology and cytokine profile does not represent, for example, that of a multiple sclerosis lesion and it is clear that the term neuroinflammation is being stretched to encompass a number of disparate phenomena, or perhaps a spectrum of inflammatory responses. In support of this, work from this thesis has shown that there is very little in terms of an inflammatory response in peripheral tissue to chronic stress when CNS cytokine levels are increased.

Increases in TNF expression within the prefrontal areas of animals receiving both a single i.p. injection of LPS and 10 days of chronic stress, suggest that the initial challenge and subsequent behaviours lead to similar outcomes, specifically regarding the expression of this cytokine. TNF expression in the CNS is an emerging market in terms of behavioural research. Both TNF and TNF receptor knock-out animals have been shown to have decreased learning and memory, as well as decreased open field activity (Baune *et al.*, 2008). In contrast, IL-1 deficient mice show no particular baseline behavioural phenotype (Bluthe *et al.*, 2000). Considering these studies, and in light of data from this thesis demonstrating that blocking TNF partly ameliorates sickness behaviour, the role of TNF, over IL-1β, in mediating behaviour should be reconsidered.

Anti-TNF therapy has been shown to be considerably more effective in treating the malaise associated with inflammatory diseases than anti-IL-1 (Genovese *et al.*, 2004), confirming the potential for TNF to play a more definitive role in sickness behaviour than IL-1. The promise of targeting a specific cytokine in cases of inflammation-induced depression, even one as early in the cascade as TNF, has promise over broad spectrum anti-inflammatories, which are likely to have severe side effects. The use of anti-TNF therapy in patients with inflammatory disorders has shown promising results, improving both mood and quality of life (Tyring *et al.*, 2006; Ertenli *et al.*, 2012). Despite this selectivity, anti-TNF therapy still has side effects, including increased risk of pneumonia and tuberculosis (Dewedar *et al.*, 2012). The study of the downstream mechanisms of TNF signalling, including the use of drugs targeting only the soluble form of TNF, may improve these outcomes.

The work from this thesis demonstrating that soluble TNF inhibitor XPRO is capable of partially ameliorating sickness behaviours, even if not as effectively as etanercept. When combined with recent studies in models of depression, suggest that targeting the soluble form of TNF may be advantageous in combatting inflammation induced changes in mood (Bedrosian *et al.*, 2012) these data suggest that more work studying the role of sTNF and mTNF in mood may be appropriate. Work by Hughes and colleagues (2012), suggesting that depression may represent low grade inflammation, combined with data from this thesis showing that XPRO successfully attenuates low-level inflammatory sickness behaviours, indicate that this therapy may be viable in many cases of depression where traditional therapy has failed. Considering the data from this thesis showing that there is an increase in TNF in the brains of chronically stressed animals, it would be important in future experiments to test whether this therapy is effective in that model of depression.

6.3 Chronic stress *vs* inflammation

While it important to validate therapies in a number of different models, it is also important to be able to reduce and refine models currently in use. The chronic stress model used in this thesis has been shown to result in behavioural alterations which persist from 7 to 21 days after the cessation of stress (Strekalova *et al.*, 2004). Painsipp

and colleagues (2011) have shown that a single i.p. injection of LPS is capable of inducing persistent changes in forced swim behaviour up to 28 days post-injection in two different strains of mice. These persistent changes in behaviour, in both the chronic stress model, and in the LPS model, have not yet been investigated. Work from this thesis has demonstrated that significant changes in the expression of CNS cytokines and 5-HT receptors persist with the behaviour, up to 48 hours after an LPS challenge and after 10 days of chronic stress.

One of the principal findings linking the chronic stress model and the LPS model is the increase in 5-HT_{2A} receptor expression in the prefrontal CNS. These studies are particularly important, as this receptor appears to be up-regulated in response to stressors, irrespective of whether the animals showed significant behavioural changes. It is possible that changes in 5-HT_{2A} receptor expression in the CNS are the result of homeostatic mechanisms, rather than pathology. Work *in vitro* has shown that 5-HT_2 agonist DOI significantly inhibits TNF-induced cytokine production (Yu *et al.*, 2008), data that is confirmed by preliminary neutrophil counts and pro-inflammatory cytokine production in the livers of animals from the DOI experiment presented in this thesis (Chapter 1; data not shown). Studies from other groups have suggested that 5-HT_{2A} receptors may also mediate an anti-inflammatory response in the glial population in the CNS, indicating that perhaps changes in expression of the receptor may be the result of inflammation and not the cause of mood changes (Wu *et al.*, 1999). The lack of change in 5-HT_{2A} mRNA after LPS in C57/Bl6 mice (Chapter 5; p176), compared to CD1 mice (Chapter 3; p103), may be the result of differences in response to a systemic inflammatory challenge between strains of mice. A number of simple, and as yet untried, experiments present themselves. If it is true that inflammation indirectly up-regulates 5-HT_{2A} expression, which then produces an anti-inflammatory response, 5-HT_{2A} knockout mice should be more susceptible to LPS-induced sickness behaviour. Similarly, chronic administration of a 5-HT_{2A} antagonist such as MDL100907 should also exacerbate sickness behaviours. These experiments were outside the scope of this thesis but would be an interesting way forward.

Another recurrent theme in both the chronic stress model and the inflammatory model, is the presence of high levels of corticosterone. The exact role of this endogenous steroid in regulating mood is unclear, although *steroid euphoria* is a common problem in patients on steroid therapy (Swinburn *et al.*, 1988). Data from this thesis have already suggested that pro-inflammatory cytokines may regulate central 5-HT_{2A} expression, and studies elsewhere have shown that cortisol is capable of increasing systemic expression levels of this receptor (Frere, 2012). Chronic stress, and as such corticosterone, have also been shown previously to regulate 5-HT_{2A}-mediated behaviours (Gorzalka *et al.*, 1999). Taken together these findings could suggest that while elevations in pro-inflammatory cytokines may result in homeostatic up-regulation of 5-HT_{2A}, and potentially an anti-inflammatory response in the CNS, elevations in corticosterone may potentiate the anti-inflammatory response by also up-regulating this receptor.

However, if corticosterone was a key factor in mediating behaviour, both in sickness and stress, then the above theory fails to explain the differences in behaviour exhibited by stressed animals and stressed animals receiving an LPS challenge, where the corticosterone response was not significantly different between the two groups. However, changes in corticosterone release may not necessarily reflect an increase in glucocorticoid receptor signalling. Work in depressed patients has shown that long term disease results in a decrease in sensitivity to cortisol (Miller *et al.*, 2005). It is, therefore, possible to speculate that stress results in decreased sensitivity to corticosterone and a single low dose of LPS has a much more potent pro-inflammatory mechanism of action than LPS *per se*. Experimentally, this is easy to test using binding assays for glucocorticoid receptors in stressed and LPS-treated animals.

A further interpretation of these data might be that the balance between corticosterone and pro-inflammatory cytokines may regulate the expression of central 5-HT_{2A} receptors, and any perturbations to this homeostatic balance results in changes in mood. Persistent stressors may result in permanent changes to the central 5-HT system and result in increased susceptibility to depression. The scope of this thesis did not allow for

longitudinal studies into the effects of stress on receptor expression, which would enable these questions to be answered.

Work from these latter studies indicating that behaviour may be exacerbated in animals receiving a single dose of LPS after 10 days of chronic stress has yielded some puzzling results. If the theories presented thus far regarding the potential role of pro-inflammatory cytokines to regulate the expression of central 5-HT receptors are to be substantiated, then the combination of stress and inflammation should have exacerbated these changes. In fact this combination largely returned 5-HT_{2A} receptor expression to baseline levels, suggesting that alternate mechanisms may be responsible for the changes in behaviour. Few studies have thus far used DOI in combination with chronic stress. The induction of wet-dog shake behaviour by DOI at baseline should, theoretically, be enhanced by chronic stress if 5-HT_{2A} receptor expression is indeed increased. Data from studies investigating the role of this receptor in sexual behaviour indicate that this may be true (Gorzalka *et al.*, 1998), although these studies failed to show baseline DOI behaviour. The use of this drug in the studies presented here combining stress and inflammation may indicate potential mechanisms underlying the paradoxical decrease in receptor expression in these animals. Furthermore, binding assays in all of these studies would determine whether the increase in receptor expression was indicative of increased binding, or whether it was a compensatory mechanism in response to decreased binding.

Finally, use of these two models may help us to answer the chicken/egg debate currently plaguing the depression literature. Work from the second and third chapters of this thesis has demonstrated that inflammation is capable of changing behaviour and the machinery of the 5-HT system. Work from the fourth chapter of this thesis has demonstrated that chronic stress is capable of inducing an inflammatory state. However, neither of these results help answer the question of whether inflammation precedes depression or whether the reverse is the case. To answer this question more longitudinal studies than are possible in rodents would need to be carried out. By studying the immune status of an animal over time it might be possible to say whether animals with a high basal inflammatory state may be more susceptible to the effects of chronic stress. Recent work

by Copeland and colleagues (2012; 2012) has begun to answer this question. In longitudinal studies of people over a period of >10 years they were able to demonstrate that emotional status significantly affected immune status. Patients demonstrating cumulative depressive episodes had higher levels of C-reactive protein in their blood than those who were more emotionally stable. In the second paper, Copeland and colleagues further contribute to the conclusions of this thesis by showing that generalized anxiety disorder does not necessarily co-relate with inflammation in the same way. These studies suggest that depression may precede inflammation but also that inflammation may be capable of potentiating a depressive state.

6.4 The role of the prefrontal cortex in regulating mood

In addition to the data above regarding increases in TNF and the 5-HT_{2A} receptor in prefrontal brain regions in models of inflammation and stress, imaging data from this thesis have also shown significant functional changes in this region. The prefrontal cortex has been shown to be associated with emotional processing (Miller *et al.*, 2002), with Miller suggesting that the PFC is responsible for us knowing the *rules of the game*. While this region was traditionally considered unique to primates, with specific emphasis being applied to emotional processing, the definitions were largely architectural. The delineation between regions was previously based on cortical layers thought to be present or not present between the various species. Uylings and colleagues (2003) suggest that definition should, in fact, be based on connectivity and physiology, which is broadly similar between primates and rodents. Although it is not necessarily possible to test emotional processing capacity in rodents, it must not be assumed that the prefrontal cortex plays no part in regulating mood in this species.

The imaging data from Chapter 2 (p59), showing that there are significant functional changes in the prefrontal cortex in response to fenfluramine, and that these are diminished following systemic LPS treatment, adds to the theory that changes in the 5-HT system in this region may result in changes in mood. This work confirms that of others in humans showing that patients challenged with typhoid show blunted emotional processing for 3-6 hours after the injection (Harrison *et al.*, 2009). Further work by the

same group has suggested that specific brain regions, mainly within the prefrontal area, may be recruited during inflammation-associated fatigue, in order to prioritise and maintain cognitive processing (Harrison *et al.*, 2009). The latter work specified the recruitment, or increased activity, within the prefrontal regions. It could be hypothesized that the increased 5-HT_{2A} expression in this area, observed throughout this thesis, may form part of this functional prioritization. Since 5-HT_{2A} receptors in the prefrontal cortex have been shown to be associated with threat-related activity (Fisher *et al.*, 2009; Fisher *et al.*, 2011), it could further be assumed that this reorganization may be in response to the individuals' diminished capacity to cope with threats when immunologically compromised. However, these theories are yet to be tested efficiently.

6.5 Limitations of the current studies

In the context of this thesis, validity is particularly important in determining whether the similarities and differences between inflammatory and stress-induced models of depression-like behaviour discussed above, are relevant in terms of modelling actual disease. Model validity has a number of different aspects, with regard to animal models of human disease; the three most important are face validity, construct validity and predictive validity.

Face validity is the ability of the model to mimic the signs of the human condition. The model of sickness behaviour used in this thesis accurately reproduces the human condition in many aspects. During sickness the mice were less prone to activity, less interested in exploring novel spaces and more inclined to social isolation. Similar studies have been conducted in humans, showing a general decrease in mood, an increase in anxiety, and decreases in learning and memory during sickness (Grigoleit *et al.*, 2011), suggesting the model of endotoxaemia used in these studies mimics sickness in humans. Whether this model can also be employed to mimic Major Depression in humans will be discussed below in the light of findings from this thesis.

Construct validity is whether the rationale of the model matches the human experience. In terms of the models used here, the evidence comparing sickness behaviour in rodents,

to sickness in humans (Grigoleit *et al.*, 2011), suggests that this model has good construct validity. In fact Grigoleit and colleages showed that an LPS challenge in humans has persistent effects on emotional behaviour for up to 24 hours, and that cortisol and certain pro-inflammatory cytokines peak 2-6 hours after administration of LPS, and return to baseline by 24 hours. These data are in line with the work presented in this thesis suggesting that the model of sickness behaviour used here is a valid model of human sickness-associated behaviour.

The model of chronic stress used to mimic anhedonia is more challenging to validate. Chronic stress in humans is often psychosocial in nature; resulting from long-term problems with relationships and other such daily life experiences. Alternatively, acute stress is often associated with actual trauma and threat; road traffic incidents for example. Interestingly, in humans, acute stress is more highly associated with an episode of Major Depression than chronic stress (Hammen *et al.*, 2009). This raises concerns regarding the use of a chronic stress model to generate depressive-like signs in rodents. Importantly, acute stress (in the form of overnight predator stress) does not induce significant behavioural changes. However, humans will continue to dwell on the experience of an acute stressor, in a manner that is perhaps unlikely in a rodent. As such, using rodent models to reproduce the long-term anxieties experienced by humans in response to an acute stressor is likely to be impossible. This makes the chronic stress model difficult to justify in terms of construct validity. Many of the stressors are clearly 'traumatic', and are, therefore, more akin to acute stressors in humans. It has been suggested that a more realistic model of human chronic stress would be more frequent, but physiologically milder, stressors (Willner, 2005). In fact, anecdotal evidence suggests that increasing the intensity of the stressors results in a paradoxical increase in sucrose consumption (Mariusz Papp, personal communication). Stressors, such as restraint and tail suspension could be considered 'life-threatening' experiences for a mouse, akin to a hostage situation in humans perhaps, whereas wet bedding is more an uncomfortable inconvenience and would perhaps model chronic stress in humans more accurately. If time and resources permitted, it would have been interesting to examine the CNS molecular profile associated with other, milder, chronic stress paradigms.

Finally, predictive validity determines whether the model predicts the outcome of specific criteria. For example, do the drugs used in the human condition work in the animal model, if so the model can be said to have predictive validity and can, therefore, be used to test potential future therapies. From the data presented here and elsewhere, it can be concluded that both the inflammatory model of sickness behaviour and the chronic stress model of anhedonia used in this thesis have good predictive validity. Animals treated with non-steroidal anti-inflammatory drugs show significant attenuation of the behavioural response to an LPS challenge (De La Garza *et al.*, 2004), indicating the behaviour is caused by inflammation. This inflammatory induced behaviour is also reversible by antidepressant drugs (Yirmiya *et al.*, 2001; Sammut *et al.*, 2002), suggesting a 'depressive-like' component to the behaviour. Similarly, the behavioural effects of chronic stress have also been shown to be reversed by antidepressants (Duncan *et al.*, 1998; Strekalova *et al.*, 2006; Rogoz *et al.*, 2008). The latter is confirmed in this thesis whereby antidepressant treatment prior to chronic stress significantly attenuates depressive-like behaviours. Work using the COX inhibitor, celecoxib, has also shown that inhibition of inflammation is capable of reversing behavioural changes induced by chronic stress (Guo *et al.*, 2009). Interestingly, both celecoxib and many of the antidepressants used in the above studies are only effective when administered chronically. This fits with the notion of delayed-treatment response, a known, and currently unexplained, facet of antidepressant treatment in humans (Stassen *et al.*, 1996). Since the placebo effect is unlikely to occur in animal models it seems likely that the effects of antidepressant treatment potentially rely on long-term changes in brain architecture, neurotransmitter or neuroendocrine systems. However, there is currently little literature to confirm this hypothesis.

6.6 Summary

This thesis has demonstrated that a single systemic inflammatory challenge has the capacity to induce persistent, significant changes in behavioural, neuroendocrine, neuroimmune and neurotransmitter systems. It has shown that some of the changes in these systems also occur in rodent models of depression. Using these correlates, it has

further been possible to dissociate stress susceptible 'depressed' animals from stress resistant ones. This work has shown that, on a background of stress, a single low-dose of endotoxin is capable of significantly exacerbating depressive-like behaviour. Finally, it has been demonstrated that anti-TNF therapy may be successful in ameliorating some of the behavioural effects induced by an LPS challenge. Together, these data increase our understanding of the neurobiological mechanisms underlying sickness behaviour and depression. However, while the molecular profiles of the 'on' mechanisms of sickness behaviour and MDD are closer than perhaps expected, it is clear that sickness behaviour resolves following infection or injury, whilst depression does not. In future studies, now that the similarities between the conditions are established, exploration of the molecular profile of sickness behaviour resolution in comparison to Major Depression might provide greater insight into 'off' mechanisms that may be absent, and therefore pathological, in depression.

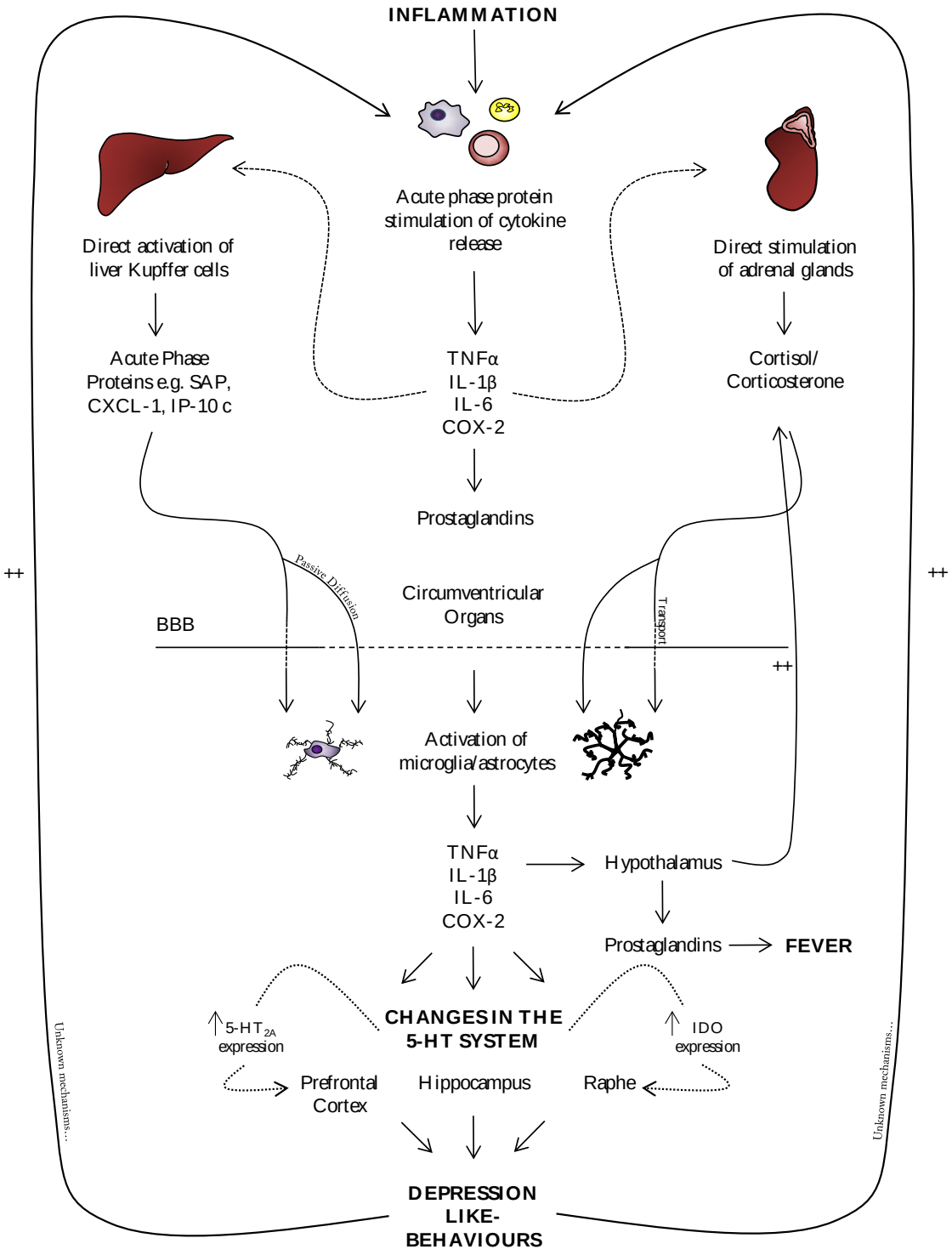


Figure 6.1 Overview of the potential interactions and similarities between sickness and depression. While this thesis has demonstrated that sickness behaviour is capable of inducing changes in the 5-HT system, and that stress and stress-induced depression are associated with an increase in the expression of pro-inflammatory cytokines, there remain many unanswered questions. The inflammatory state of the individual is clearly capable of affecting mood and mood is clearly capable of affecting the inflammatory state of the individual, however, the exact mechanisms by which these interactions occur remain elusive.

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Appendix I

Publications and presentations produced within the duration of this thesis but not specifically relevant to the work contained herein.

Publications

The following publications have come about as a result of collaborative work both within the department and university as well as internationally.

Alvarez-Erviti L., **Couch Y**., Richardson J., Cooper J.M., Wood M.J.: Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. *Neurosci. Res*. (2011) 69(4):337-42

Couch Y., Alvarez-Erviti L., Sibson N.R., Wood M.J., Anthony D.C.: The acute inflammatory response to intranigral α-synuclein differs significantly from intranigral lipopolysaccharide and is exacerbated by peripheral inflammation. *J Neuroinflammation* (2011) 8:166

Jagannath A., Butler R., Godinho S.H., **Couch Y**., Brown L.A., Flanagan K., Anthony D., Wood M., Ebeling M., Hossbach M., Steiner G., Wettstein J., Duffield G.E., Gatti S., Hankins M.W., Foster R.G., Peirson S.N.: Negative feedback by the inducible repressor SIK1 regulates light input to the circadian clock. (submitted 2012)

Brambilla R., **Couch Y**., Lambertsen K.: The Effect of Stroke on Immune Function. *Mol.Cell.Neurosci.* (2012 *in press*)

Presentations

Couch Y., Wood M. and Anthony D.C. The Inflammatory Properties of Alpha Synuclein. 7th FENS Forum of European Neuroscience, Amsterdam, Netherlands.

Couch Y., Anthony D.C., Ormhøj M., Clausen B.H., Martin N.A., Schmidt M-L., Deierborg T., Petit G., Finsen B., Szymkowski D.E., Lambertsen K.L. Anti-TNF therapy improves behavioural outcomes after pMCAO in mice. 7th International Symposium on Neuroprotection and Neurorepair, Potsdam, Germany.

Anthony D.C., Evans M., Losey P., **Couch Y**., Grau G. Endogenous microparticles initiate the acute phase response to brain injury. 10th European Meeting on Glial Cells in Health and Disease, Prague, Czech Republic.