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Multimodal magnetic resonance imaging

in animal models of depression

A thesis submitted to University of Dublin, Trinity College
for the Degree of Doctor of Philosophy by:

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March 2015

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And Trinity College Institute of Neuroscience
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Declaration

I declare that this thesis is submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College and has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work with the exception of the following:

- OB surgery and behaviour outlined in Chapter 3 were conducted by Dr. Valentina Gigliucci.
- ECS treatments outlined in Chapter 4 were carried out by Dr. Karen Ryan and Dr. Sinead O’Donovan.

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Shane Gormley

March 2015
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Summary

Depression is a debilitating and potentially fatal disorder for which the pathophysiology underlying the illness is still unknown. Aberrant glutamatergic neurotransmission and altered astrocyte function have recently been implicated as potential physiological markers related to depressive illness. The development of magnetic resonance imaging (MRI) as a clinical tool has allowed for the identification of a series of neuroimaging markers in depressed patients. However, the physiological processes which underlie these neuroimaging markers and their role in depressive illness is still poorly understood. Rodent models of depression have provided us with important insights into brain regions and cellular mechanisms which mediate depression-related behaviours and antidepressant response. To date, few animal models of depression have been characterised using in vivo MRI to establish if they exhibit brain structural or functional alterations akin to those reported in human depressed patients. Thus we sought to investigate if three animal models of depression exhibited any changes in brain structure or function relative to their respective controls. Furthermore, we sought to identify if any observed alterations in MR imaging markers in our animal models were associated with changes in astrocyte cell function.

The olfactory bulbectomised (OB) rat model is a well characterised animal model with known predictive validity. We report that OB is associated with an increase in lateral ventricular volume and a decrease in cortical T2 relaxation time. Chronic treatment with the tri-cyclic antidepressant imipramine and nitric oxide synthase inhibitor N\(^{\text{N}}\)-Nitro-L-arginine (L-NA) attenuated OB-related hyperactivity in the open field test. The antidepressant response of L-NA was associated with an attenuation of the OB-related decrease in cortical T2 relaxation time and an increase in lateral ventricular volume. Sub-acute dosing with N-methyl-D-aspartate (NMDA) receptor antagonist ketamine did not produce an antidepressant response and was not associated with changes in cortical T2 relaxation time or lateral ventricular volume. These data suggest that cortical T2 relaxation time may represent a state dependent marker in the OB rat model.

The Wistar-Kyoto (WKY) rat is a strain which endogenously expresses highly anxious and depressive-like behaviours and has previously been reported to exhibit alterations in the number of cells immunoreactive for astrocyte marker glial fibrillary acidic protein (GFAP) in brain sub-regions relative to out-bred comparator strains. Here we report that the depressive and anxiety-like behaviours exhibited by the WKY rat strain are associated with alterations in brain morphology including a decrease in hippocampal volume.
coupled with reduced resting state frontal cortical perfusion as assessed by MR arterial spin labelling (ASL) relative to the out-bred Wistar strain. Pre-limbic cortical GFAP positive cell number was positively correlated with blood perfusion in the WKY strain. The effects of both electroconvulsive stimulation (ECS) and chronic stress on behaviour, transcriptional and protein markers related to astrocyte function in the WKY strain were also assessed. ECS treatment produced a decrease in immobility in the forced swim test in the WKY rat strain and was associated with cognitive deficits in the passive avoidance task. Chronic immobilisation stress resulted in an increase in immobility in the WKY rat strain in the forced swim test which was coupled to altered brain mRNA expression of genes related to astrocyte function.

To further elaborate on the role of regional astrocyte dysfunction on behaviour in rodent models, the astrocyte specific toxin L-alpha aminoacidipic acid (L-AAA) was administered into the rodent brain. We report that L-AAA is associated with administration site specific changes in behaviour. Basolateral amygdala and pre-limbic cortical L-AAA administration result in an increase in depression-related behaviour, while basolateral amygdala administration was associated with more pronounced anxiety-like behaviour. No change in behaviour was detected following hippocampal L-AAA administration. We also report that pre-limbic cortical L-AAA administration is associated with a transient change in astrocyte cell function as assessed through GFAP positive cell number. Pre-limbic cortical L-AAA administration also resulted in a delayed increase in resting state cortical blood perfusion in the retrospleniial and visual cortices, sites distal from the administration site.

These experiments provide further evidence in support of a role for aberrant astrocytic dysfunction in depression and anxiety-related behaviours. Future studies in animal models are required to further develop potential neuroimaging markers associated with astrocytic dysfunction. Future studies combining multimodal in vivo MR imaging in animal models of depression with post-mortem immunohistochemical analysis should provide us with further insights into the cellular and molecular mechanisms which impact on the structural and functional imaging markers reported in the depressed clinical population.
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-isozasole-4-propionic acid</td>
</tr>
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<td>AQP4</td>
<td>Aquaporin 4</td>
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<td>ASL</td>
<td>Arterial spin labelling</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
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<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood-oxygen-level dependent</td>
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<td>Ca²⁺</td>
<td>Calcium</td>
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<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CBF</td>
<td>Cerebral blood flow</td>
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<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
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<td>CMS</td>
<td>Chronic mild stress</td>
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<td>DHK</td>
<td>Dihydrokainic acid</td>
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<td>DTI</td>
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<td>ECS</td>
<td>Electroconvulsive stimulation</td>
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<td>EAAT</td>
<td>Excitatory amino acid transporters</td>
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<td>EET</td>
<td>Epoxideicosatrienoic acids</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>EPM</td>
<td>Elevated plus maze</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FA</td>
<td>Fractional anisotropy</td>
</tr>
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<td>FLASH</td>
<td>Fast low angle shot</td>
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<td>Field of view</td>
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<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<td>Glial fibrillary acid protein</td>
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<td>Glutamate-aspartate transporter</td>
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<td>Glutamine</td>
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<td>Glutamate transporter 1</td>
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<td>Glu</td>
<td>Glutamate</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic pituitary adrenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>Iba-1</td>
<td>Ionized calcium-binding adapter molecule 1</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IP$_3$</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>K$^+$</td>
<td>Potassium</td>
</tr>
<tr>
<td>KAT II</td>
<td>Kynurenine aminotransferase II</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>L-AAA</td>
<td>L-alpha-aminoadipic acid</td>
</tr>
<tr>
<td>L-NA</td>
<td>N$^\text{N\textdegree}$-Nitro-L-arginine</td>
</tr>
<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
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<td>MSME</td>
<td>Multi-slice multi-echo</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>MTT</td>
<td>Mean transit time</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>Olfactory bulbectomy</td>
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<td>Na$^+$</td>
<td>Sodium</td>
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<td>N-methyl-D-aspartate</td>
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<td>Nitric oxide</td>
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<td>Neuronal nitric oxide synthase</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
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Chapter 1 – General introduction
1.1 Major depressive disorder

Major depressive disorder (MDD) is a debilitating and potentially fatal mood disorder which is the primary disabling condition in Europe at present with a prevalence of 7% (Wittchen et al., 2011). It is a highly heterogeneous disease which commonly presents with co-morbid anxiety and is linked to several somatic disorders including pain, insomnia, cardiovascular disease and gastritis (Sato and Yeh, 2013; Shorter and Tyrer, 2003; Topić et al., 2013). Risk factors for experiencing a depressive episode are diverse and include heritable genetic predisposition, early life experiences and environmental factors (Kendler et al., 2006, 2002). The World Health Organisation estimates that MDD is the third leading contributor to total disease burden worldwide and is predicted to be the leading contributor by the year 2030 (World Health Organization, 2008). Depressive symptoms range from moderate to severe and include depressed mood, anhedonia, low energy or fatigue, changes in appetite, suicidal ideation, irritability and restlessness and reduced ability to concentrate (American Psychiatric Association, 2000). At present there is a paucity of biological or neuroimaging markers specific to depressive illness and as such, diagnosis relies heavily on symptomatology rather than etiology (Mössner et al., 2007; Savitz and Drevets, 2009a). Given the complex nature of depressive illness, elucidating the neurophysiological processes underlying the disorder has proven difficult and partially as a result of this lack of understanding, tackling depression therapeutically is also problematic.

1.1.1 Current pharmacotherapies for major depression

National Institute for Health and Clinical excellence (NICE, 2009) guidelines recommend that drug therapy should only be initiated for patients with moderate to severe depression, due to the relative poor risk benefit ratio of most current antidepressant medications. The majority of currently prescribed antidepressant medications target the monoaminergic system, altering the synaptic availability of serotonin (5-hydroxytryptamine), noradrenaline and dopamine. These agents are based on earlier drugs which were serendipitously discovered to produce an antidepressant response. Current classes of drugs used to treat depression include selective serotonin reuptake inhibitors (SSRI) such as sertraline and fluoxetine, serotonin noradrenaline reuptake inhibitors (SNRI) such as venlafaxine and atypical agents such as mirtazapine (Berton and Nestler, 2006). While these medications possess a better side effect profile than older tri-cyclic antidepressants like imipramine there is little difference in their efficacy (Cipriani et al., 2009).
There are significant limitations associated with these current treatments. Rates of response to treatment are very low with only 30% of patients achieving remission following initial antidepressant treatment, while lack of initial response predicts a lower response and higher relapse rates following further treatment steps (Rush et al., 2006). In addition it takes several weeks following commencement of antidepressant therapy before an improvement in symptoms occurs and patients are at an increased risk of suicide during this period (Simon et al., 2006). Furthermore in contrast to the delay in therapeutic effect, it is known that these drugs produce acute effects on the brains monoaminergic system which suggests that alterations in the patient’s mood following antidepressant treatment may be reliant on changes in other brain neurotransmitter systems (Berton and Nestler, 2006).

1.1.2 Novel approaches for treating major depression

Considering the limitations of current antidepressant agents there is a clear unmet clinical need for novel pharmacological agents to provide faster onset of action and greater efficacy for the treatment of major depression. Unsurprisingly then, much work in recent years has focused on development of new antidepressant drugs. Triple reuptake inhibitors which increase the synaptic availability of serotonin, noradrenaline and dopamine, drugs which modulate the activity of the hypothalamic pituitary adrenal (HPA) axis and anti-inflammatory drugs have all reached phase 2 clinical trial stage for the treatment and adjunct treatment of depression (Fornaro, 2012). However one of the most exciting developments in the field in recent years was the discovery that ketamine, a dissociative anaesthetic that blocks the glutamate N-methyl-D-aspartate (NMDA) receptor, produces rapid antidepressant effects in patients with severe and treatment resistant depression [for review see (Machado-Vieira et al., 2009)].

Randomised control trial data shows that intravenous infusion of the non-competitive NMDA receptor antagonist ketamine at a subanaesthetic dose of 0.5mg/kg produced a rapid antidepressant response in depressed patients (Berman et al., 2000; Zarate et al., 2006). Furthermore these effects extend to treatment resistant patients and provide rapid relief from suicidal ideation (DiazGranados et al., 2010; Price et al., 2009). Despite these results there are still major limitations on the widespread use of ketamine in the treatment of major depression. Ketamine has relatively low bioavailability when administered orally and hence intravenous administration appears to be necessary to produce an antidepressant effect (Peltoniemi et al., 2012; Zarate et al., 2006). Ketamine is also associated with short lived dissociative side effects and the potential for abuse (Berman et al., 2000).
Targets downstream of the NMDA receptor have been investigated for antidepressant activity in animal models. Neuronal nitric oxide synthase (nNOS) is coupled to the NMDA receptor through the cell scaffold protein, post synaptic density 95 kDa (PSD-95). Activation of the NMDA receptor leads to calcium (Ca\(^{2+}\)) influx and subsequent Ca\(^{2+}\)-calmodulin dependent activation of nNOS leading to the production of nitric oxide (Guix et al., 2005). NOS inhibitors have been shown to possess antidepressant activity in pre-clinical models (Harkin et al., 1999; Wegener and Volke, 2010). In addition low doses of NOS inhibitor Nω-Nitro-L-arginine (L-NA) have been shown to augment the effects of antidepressant drugs in pre-clinical tests (Harkin et al., 2004). Recently, small molecular weight inhibitors which uncouple NOS from the NMDA receptor by interacting with the PSD-95 have been shown to exhibit sustained antidepressant activity following a single dose in pre-clinical models (Doucet et al., 2013). Hence molecular targets downstream of the NMDA receptor, on the nNOS pathway appear to possess good potential for the development of novel antidepressant agents and warrant further study [for review see (Doucet et al., 2012)].

1.1.3 Electroconvulsive therapy (ECT)

Despite these recent advances in the development of drugs to treat depression, in cases where rapid clinical response is required or where patients have failed to respond to pharmacotherapy, electroconvulsive therapy (ECT) is the most effective treatment option (Payne and Prudic, 2009). ECT has been shown to be the most effective antidepressant treatment available for severe depression (The UK ECT Review Group, 2003). Remission rates following ECT vary depending on the technique employed but several studies have reported remission rates of 80%, significantly higher than those reported following antidepressant drug administration. A course of ECT usually consists of undertaking three treatments weekly for a period of between 2 and 4 weeks [for review see (Lisanby, 2008)]. Although it is considered a safe technique, ECT is associated with a transient impairment in memory and cognitive function. The deficits in memory following ECT can be both anterograde (inability to form new memories) and retrograde (the loss of access to memory events that occurred in the past) [for review see (Fink, 2001)]. ECT induced deficits in executive function and episodic memory appear most pronounced in the days immediately following ECT with these variables returning to at least pre-treatment levels 15 days following treatment (Semkovska and McLoughlin, 2010). There are however conflicting reports on the duration of retrograde amnesia following ECT, with reports that retrograde amnesia immediately following ECT has recovered when patients were followed up 3 months later (Meeter et al., 2011), while
others report significant deficits in retrograde memory 6 months following treatment (Sackeim et al., 2007). Differences in ECT and in memory testing protocols employed may account for these apparent discrepancies. Efforts have been made to refine ECT in an attempt to minimise side effects associated with it. Brief pulse (BP) ECT commonly delivered with a pulse width of between 0.5-1ms has replaced the older sine-wave form delivery and has been shown to produce fewer cognitive side effects (Sackeim et al., 2008; Weiner et al., 1986). ECT can be administered via bilateral electrode placement or unilateral electrode placement, with unilateral placement reportedly associated with fewer cognitive side effects but decreased antidepressant efficacy (Sackeim et al., 1993). A recent innovation to reduce the side effect profile of ECT treatment is to reduce the pulse width even more to bring it closer to the optimal width for neuronal depolarisation and thus minimise the inefficient use of energy associated with brief pulse stimulation. This move to ultrabrief pulse (UBP) stimulation (<0.5ms) is associated with reduction in cognitive side effects (Loo et al., 2008; Sackeim et al., 2008). However the efficacy of UBP appears to be lower than that of BP ECT [for review see (Loo et al., 2012)].

The mechanism of action of ECT is still unknown. Pre-clinical models have shown that electroconvulsive stimulation (ECS), the rodent equivalent of ECT enhances monoaminergic transmission and increases the expression of neurotrophic brain-derived neurotrophic factor (BDNF) and astrocyte cell marker glial fibrillary acid protein (GFAP) in the hippocampus (O'Donovan et al., 2014a, 2012; Yoshida et al., 1998). It has also been shown to result in a transient increase in astrocytes positive for activation marker nestin, as well as microglial cells positive for activation markers major histocompatibility complex class 2 (MHC II) in the hippocampus and frontal cortex (Jansson et al., 2009). There is relatively little data in the preclinical literature investigating the effects of pulse width on cognitive outcomes in animal models. Such data could aid in our understanding of potential neurophysiological mechanisms underlying ECT induced cognitive deficits and antidepressant response.
1.2 The neurobiology of depression

One of the major stumbling blocks in the development of novel therapeutic agents to treat depressive disorder is a lack of understanding of the biological mechanisms which underlie depressive symptomatology and antidepressant response. There are several different theories which link observations of aberrant brain function and the mechanism of action of drugs with antidepressant activity to possible pathophysiological mechanisms which may underlie depressive illness.

1.2.1 The monoamine hypothesis of depression

The monoamine theory of depression is the oldest and most well established hypothesis on depression formulated to date. The theory suggests that lower concentrations of the monoamine neurotransmitters noradrenaline, serotonin and dopamine in the central nervous system results in depressive illness (Schildkraut, 1965). Much of the evidence for the monoamine hypothesis is pharmacological, with drugs like the tri-cyclic antidepressants and SSRIs which increase the synaptic availability of monoamines shown to produce antidepressant effects in depressed patients, while depletion of serotonin levels is associated with a rapid return of depressive symptoms (Delgado et al., 1999). However as a therapeutic response takes several weeks to develop following the commencement of therapy with these agents, it suggests that other neuronal adaptions may be the cause of the change in depressive state rather than the alteration in the concentrations of monoamine transmitters themselves (Doucet et al., 2012). This combined with the poor rates of response to monoaminergic drugs has led to the development of other theories as to the neurobiology of depressive illness and of antidepressant action.

1.2.2 A role for glutamate in depression

As glutamate is the primary excitatory neurotransmitter system in the brain and 85% of cortical synapses are thought to be glutamatergic, it is perhaps unsurprising that an alteration in glutamate neurotransmission has been implicated in depressive disorder [for review see (Sanacora et al., 2012)]. As discussed above, antagonists of the glutamatergic NMDA receptor have been shown to produce a rapid antidepressant response in animal models as well as in depressed patient cohorts. Monoamines are known to modulate glutamatergic neurotransmission in limbic structures important in emotional cognition suggesting a possible link between a glutamatergic hypothesis of depressive illness and the known antidepressant effects of current drugs which alter the synaptic availability of monoamines (Pralong et al., 2002). There is also physiological
evidence for alterations in glutamatergic function in depressive illness. Increased plasma levels of glutamate, glutamine and glycine have been reported in depressed patients relative to non-psychiatric controls, with increased plasma levels of glutamate positively correlated with increased severity of depression (Mitani et al., 2006). While decreases in plasma glutamate and inhibitory neurotransmitter gamma-aminobutyric acid (GABA) levels have been reported following antidepressant treatment (Kucukibrahimoglu et al., 2009). However the relationship between plasma concentrations of these neurotransmitters and brain concentrations is still unknown. Increased levels of glutamate have been reported in post-mortem prefrontal cortical tissue from depressed patients when compared to non-psychiatric controls after controlling for post-mortem interval (Hashimoto et al., 2007).

Magnetic resonance spectroscopy (MRS) provides us with the opportunity to assess in vivo concentrations of glutamate, GABA and glutamine in various brain regions of depressed patients and compare such levels with that of healthy controls. There are however several technical problems with the interpretation of such data. The majority of studies published do not have adequate spectral resolution to distinguish between glutamate, GABA and glutamine peaks and hence report a combined measure termed Glx which primarily relates to glutamate but also contains GABA and glutamine components. It is also not possible to distinguish between intracellular and extracellular quantities of these metabolites, further increasing the difficulty in interpretation of these data. Despite these limitations, MRS is an important tool in helping us identify whether regional changes in the concentration of these neurotransmitters in the brain are associated with depressive illness (Yüksel and Öngür, 2010). Several studies have shown reduced Glx levels in the dorsolateral prefrontal cortex and hippocampus regions in patients with major depressive disorder with increased levels reported in the dorsolateral prefrontal and cingulate cortices of patients with bipolar disorder. It has been suggested that these data may suggest an increase and decrease in glutamate metabolism during mania and depression respectively [for review see (Yildiz-Yesiloglu and Ankerst, 2006; Yüksel and Öngür, 2010)]. This finding of decreased Glx concentrations in certain brain regions seems to extend to MRS studies of childhood depression with reduced Glx concentrations reported in the anterior cingulate cortex of childhood patients with major depressive disorder (Mirza et al., 2004; Rosenberg et al., 2004). Hence there is ample evidence implicating a dysregulation in brain glutamate metabolism in depressive illness. As astrocytes are known to be the primary cell responsible for the metabolism of glutamate in the central nervous system (CNS), it has
been suggested that some of the alterations observed in glutamatergic metabolism in depressive illness may be related to aberrant astrocyte cell function (for review see Rajkowska and Stockmeier, 2013). Before further exploring the evidence supporting a role for astrocyte dysfunction in depressive illness, a brief introduction to the role of astrocytes in the CNS is provided.

1.2.2.1 Astrocytes

90% of cells in the human brain are glial cells which can be divided into four subtypes, astrocytes, microglia, oligodendrocytes and ependymal cells (He and Sun, 2007). Of these astrocytes are the most numerous and perform a variety of important functions in the CNS. Astrocytes can be divided into two subtypes based on their cellular morphology, protoplasmic astrocytes which predominate in grey matter and fibrous astrocytes which are abundant in white matter. Protoplasmic astrocytes are identified as having spherical nuclei with numerous thick processes, while fibrous astrocytes have ovoid nuclei and fewer, longer and thinner processes (Rajkowska & Miguel-Hidalgo 2007). They are linked to neighbouring astrocytes through gap junctions formed by connexin proteins to form a complex multicellular network. These astrocytic networks are thought to be of importance in rapidly dissipating potassium ($K^+$) and glutamate. They are excitable cells which exhibit regulated increases in intracellular Ca$^{2+}$ concentration in response to neurotransmitters and which may induce astrocytic neurotransmitter release. Astrocyte processes are in contact with practically all synapses and are vitally important in maintaining fluid, ion, pH, and neurotransmitter homeostasis in the synaptic cleft and astrocyte end feet cover the cerebrovasculature and form part of the blood brain barrier (BBB). Astrocyte synaptic and end foot processes are rich in aquaporin 4 (AQP4), these porous membrane structures allow water to passively diffuse between the intra and extracellular space and help regulate normal CNS fluid balance. They have high $K^+$ permeability and are important in regulating the extracellular $K^+$ concentration essential for maintaining neuronal excitability. They also possess several types of proton and bicarbonate transporters which are thought to be of importance in maintaining normal brain pH (for review see Sofroniew and Vinters, 2010)]. Astrocytes are involved in the regulation of synaptogenesis and synaptic plasticity, secreting molecules important in synapse formation, maturation and pruning (for review see Clarke and Barres, 2013)]. Astrocytes also provide important trophic support for neurons secreting neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), BDNF, nerve growth...
factor β (NGF- β) and vascular endothelial growth factor (VEGF) (Sofroniew and Vinters, 2010).

Astrocytes have an important role in regulating glutamate neurotransmission at the level of the synapse. Glutamate is synthesised from glutamine in the nerve terminal, once released from the pre-synaptic neuron it can bind to one of four main subtypes of glutamate receptor, the NMDA receptor, the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, as well as kainate and metabotropic glutamate receptors (mGLuR). Astrocytes possess specific transporters involved in the uptake and metabolism of glutamate from the synapse following release from the pre-synaptic neuron. This glutamate is then metabolised to glutamine by astrocytic enzyme glutamine synthetase, which is then released by astrocytes and taken up by neurons where it can be reconverted into either GABA or glutamate [see figure 1.2.2.1]. In humans these are the excitatory amino acid transporters (EAAT) 1 and 2, the equivalent transporters in the rodent brain are the glutamate-aspartate transporter (GLAST) and the glutamate transporter-1 (GLT-1) (Rajkowska and Stockmeier, 2013). In vitro work has shown that astrocytes can also modulate glutamatergic neurotransmission through release of astrocytic glutamate which inhibits the release of glutamate from the pre-synaptic neuron via a mGLuR dependent mechanism (Araque et al., 1998). Astrocytes also regulate the synaptic availability of D-serine, a co-agonist which binds to the glycine site of the NMDA receptor and is required for NMDA receptor activity, via this mechanism astrocytes can regulate NMDA receptor activation (Wolosker et al., 1999).
Figure 1.2.2.1: The glutamatergic synapse

Glutamate released from the pre-synaptic neuron, into the synaptic cleft, can activate AMPA, NMDA, kainate as well as pre and post synaptic mGLU receptors. Excess glutamate is taken up by astrocytic EAAT, metabolised to glutamine and then recycled back to the pre-synaptic neuron.

EAAT, excitatory amino acid transporter; Gln, glutamine; Glu, glutamate; GlnT, glutamine transporter
1.2.2.2 Astrocyte regulation of cerebrovascular tone

The level of blood supply to the brain at any given time is tightly controlled. The carotid arteries which supply blood to the brain branch out into smaller arteries and arterioles which become progressively smaller and eventually form cerebral capillaries. Blood flow within the brain is highly regulated to ensure maintenance of energy dependent processes and in order to clear the metabolic by-products of neuronal activity. The link between regional synaptic activity and the related increase in regional cerebral blood flow is termed functional hyperaemia (Girouard and Ladecola, 2006). Neurons, glia, endothelial cells, vascular smooth muscle and pericytes form a close anatomical arrangement referred to as the neurovascular unit [Figure 1.2.2.2a]. It is this neurovascular unit which provides the physiological basis for the moment by moment regulation of cerebrovascular tone and hence cerebral blood flow (Drake and Ladecola, 2007). As stated above astrocytic processes make extensive synaptic contacts, while astrocytic end feet cover capillaries in the CNS and form part of the BBB. Astrocytes are thus ideally positioned to regulate cerebrovascular tone in response to changes in neuronal activity. As such they are vitally important in ensuring that cerebral blood flow is sufficient to meet the metabolic demands of active neurons.

Figure 1.2.2.2a: Anatomical arrangement of the neurovascular unit

[adapted from (Drake & Ladecola, 2007)]
A schematic representation of the different ways in which glutamatergic neuronal activation can activate a series of metabolic pathways within astrocytes which can lead to the release of various vasoactive mediators from astrocytic end feet is depicted in figure 1.2.2.2b. Activation of astrocytic mGLU receptor at the astrocytic-neuronal synapse results in the activation of phospholipase C (PLC) and an inositol triphosphate (IP$_3$) mediated increase in intracellular Ca$^{2+}$ concentration. This increase in Ca$^{2+}$ concentration can be propagated to the astrocytic end feet through the action of Ca$^{2+}$ induced release of ATP on extracellular purinergic receptors. The increase in Ca$^{2+}$ concentration in the end feet activates phospholipase A$_2$ (PLA$_2$) and leads to the mobilisation of arachidonic acid (AA) from the cell membrane. AA can then be metabolised via a number of routes. It may serve as a substrate for cyclooxygenase-1 (COX-1) leading to the formation of prostaglandin E$_2$ (PGE$_2$) which can stimulate vasodilatory cyclic adenosine monophosphate (cAMP) production in vascular smooth muscle. Alternatively it may serve as a substrate for cytochrome p450 2C11 (CYP 2C11) leading to the formation of epoxyeicosatrienoic acids (EET) which are vasodilatory via their activation of calcium sensitive K$^+$ channels (K$_{Ca}$). Finally the AA may be released from the astrocyte and be metabolised within vascular smooth muscle by cytochrome p450 4A (CYP 4A) to form 20-hydroxyeicosatetraenoic acid (20-HETE). 20-HETE exerts a vasoconstrictory action on vascular smooth muscle through inhibition of K$_{Ca}$ channels. CYP 2C11 and CYP 4A function is sensitive to NO levels and hence diffusible NO concentration generated from nNOS which is coupled to NMDA receptors via PSD-95 may dictate whether a vasodilatory or vasoconstrictory response is elicited. NO inhibits CYP 4A activity and activates guanylyl cyclase (GC) leading to the generation of vasodilatory cyclic guanosine monophosphate (cGMP) [for review see (Attwell et al., 2010; Koehler et al., 2009)].
Figure 1.2.2.2b: Mechanisms of astrocytic regulation of cerebrovascular tone

20-HETE, 20-hydroxyeicosatetraenoic acid; AA, arachidonic acid; Ca\(^{2+}\), calcium; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX-1, cyclooxygenase-1; CYP 2C11, cytochrome p450 2C11; CYP 4A, cytochrome p450 4A; EAAT, excitatory amino acid transporter; EET, epoxyeicosatrienoic acids; eNOS, endothelial nitric oxide synthase; GC, guanylyl cyclase; Glu, glutamate; IP\(_3\), inositol triphosphate; K\(^+\), potassium; NMDA-R, N-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PLA2, phospholipase A\(_2\); PLC, phospholipase C; PGE\(_2\), prostaglandin E\(_2\); PSD-95, post synaptic density-95. [Adapted from (Koehler et al., 2009)].
1.2.2.3 Glial fibrillary acidic protein (GFAP)

GFAP, an 8-12nm long intermediate filament is a key component of the mature astrocyte cytoskeleton and was originally discovered as part of white matter plaques from multiple sclerosis patients in the 1970s. While GFAP is a characteristic protein for mature astrocytes in the CNS it is also transiently expressed by neuronal precursor cells during development and in peripheral glia such as Schwann cells and enteric glia (Middeldorp and Hol, 2011; Zhuo et al., 2001). Eight different isoforms of GFAP exist, which identify specific subgroups of astrocytes, these isoforms may have specific functional roles, however these have yet to be defined. GFAP has a functional role in several astrocyte cell processes including cell motility and proliferation, formation and maintenance of the BBB, maintenance of normal CNS myelination through astrocyte-oligodendrocyte interactions and formation of a glial scar in response to acute trauma (Middeldorp and Hol, 2011). In response to an insult astrocytes become activated, proliferate (gliosis) and increase expression GFAP [for review see (Burda and Sofroniew, 2014)]. The majority of the reported increases in GFAP immunostaining in response to acute injury are the result of an increase in GFAP expression from astrocytes in situ at the site of injury rather than as a result of proliferation or migration to the area of injury. A subset of astrocytes polarise towards the site of injury and elongate their processes in the direction of the lesion site, which also accounts for some of the reported increase in GFAP immunostaining in response to an acute injury (Bardehle et al., 2013). GFAP has been shown to be important in trafficking GLT-1 to the cell surface in response to protein kinase A stimulation (Hughes et al., 2004), while anchoring of GLAST to the plasma membrane of astrocytes has also been shown to be dependent on GFAP (Sullivan et al., 2007). Thus GFAP is important in modulating astrocytic regulation of glutamatergic signalling. GFAP immunostaining is routinely used in identification of astrocytes in the CNS, however the epitope to which most commercially available antibodies bind is unknown. Thus it is normally not possible to distinguish which GFAP isoform is stained [for review see (Eng et al., 2000; Middeldorp and Hol, 2011)].
1.2.3 Evidence implicating astrocytic dysfunction in depressive illness

There is a growing body of evidence, both clinical and pre-clinical, to suggest a role for glial dysfunction in depression [for review see (Rajkowska and Stockmeier, 2013)].

Early studies investigating cell densities in post-mortem tissue from depressed patients via stereological methods revealed decreases in cortical neuronal and glial cell packing densities in orbitofrontal, dorsolateral and subgenual prefrontal as well as the anterior cingulate cortices when compared to non-psychiatric controls (Cotter et al., 2001; Ongür et al., 1998; Rajkowska et al., 1999). Glial cell density and glia/neuron ratio were shown to be reduced in the amygdala of patients with MDD relative to controls (Bowley et al., 2002). An increase in the cell packing density of glial cells, pyramidal neurons and granule cell neurons was also reported in all hippocampal subfields as well as the dentate gyrus (Stockmeier and Mahajan, 2004). These differences were shown to be region specific with no difference in glial cell density reported in the sensorimotor or entorhinal cortices of depressed patients relative to non-psychiatric controls. However these studies did not identify the subtype of glial cells which accounted for these apparent differences in regional brain microstructure [for review see (Rajkowska, 2003)].

Further studies have reported that differences in GFAP immunoreactivity in the dorsolateral prefrontal cortex of depressed patients is related with age, with younger patients displaying more significant reductions in GFAP immunoreactivity relative to older patients (Miguel-Hidalgo et al., 2000). Further Western blot analysis confirmed this observation with patients under 60 years old at the time of death exhibiting a significant decrease in GFAP protein levels in the prefrontal cortex which was positively correlated with age at time of death (Si et al., 2004). In contrast elderly patients have been reported to exhibit increased expression of GFAP in the dorsolateral prefrontal cortex relative to age matched non-psychiatric controls (Davis et al., 2002). Thus GFAP protein levels in the frontal cortex of depressed patients are age dependent and may represent a marker for progression of the disease. This is in agreement with a report showing decreased mRNA expression of astrocytic gap junction proteins connexin 30 and 43 in the dorsolateral prefrontal cortex of post-mortem brain tissue from middle aged patients that committed suicide (Ernst et al., 2011). Decreased numbers of GFAP immunoreactive cells have also been reported in the CA1 and CA2 subfields of the hippocampus of depressed patients relative to non-psychiatric controls (Müller et al., 2001). Decreased protein levels of astrocytic glutamate transporters EAAT 1 and 2 have also been reported in the orbitofrontal cortex of depressed patients relative to non-
psychiatric controls (Miguel-Hidalgo et al., 2010). This is in agreement with a study by Choudary and colleagues (2005) which reports decreased mRNA expression of EAAT 1 and 2 as well as glutamine synthetase in the anterior cingulate cortex of depressed patients relative to non-psychiatric controls (Choudary and Molnar, 2005). Astrocyte dysfunction in depression does not appear to be restricted to cortical and limbic areas with microarray analysis of locus coeruleus tissue from depressed patients revealing decreased levels of mRNA expression for GFAP, S100B, EAAT 1 and 2, as well as astrocytic proteins AQP4 and gap junction proteins connexin 30 and 43 (Bernard et al., 2011). Reduced GFAP protein in the locus coeruleus in depressed patients relative to non-psychiatric controls has been confirmed via immunohistochemistry and Western blotting (Chandley et al., 2013).

There is also recent evidence to suggest that peripheral S100B levels may be a suitable biomarker for major depression related to astroglial dysfunction [for review see (Schroeter et al., 2013)]. S100B is a member of the S100-calcium-modulin-troponin superfamily and is also commonly used as an astrocytic marker but is less astrocyte specific than GFAP (Steiner et al., 2007). Increased levels of S100B have been reported in the serum of MDD patients when compared to age and gender matched controls (Schroeter et al., 2008). Decreased numbers of S100B immunoreactive astrocytes have been reported in the CA1 subfield of the hippocampus of depressed patients relative to non-psychiatric controls (Gos et al., 2013). The mechanisms mediating the apparent dysfunction in astrocytes in depression are not well understood, pre-clinical in vitro and in vivo work suggests that excessive levels of glutamate and glucocorticoids may play a role (Banasr et al., 2010; Unemura et al., 2012), however, it may be a marker for predisposition to depressive illness. There is also pre-clinical evidence which supports a role for astrocytic dysfunction in depression; a brief review of animal models of depression will follow.
1.3 Animal models of depression

Animal models of depression have been developed in order to screen novel compounds for antidepressant action, and to investigate neurochemical and physiological processes which may underlie depressive behaviours. The development of animal models of depression is problematic, as the pathophysiological processes and genetic risk factors underlying the human disorder and the mechanism of antidepressant action are currently not well understood. Animal models of human disease are normally validated through three sets of criteria [for review see (Nestler and Hyman, 2010)].

Construct validity refers to the etiological relevance of how the model was constructed relative to the human disease. This proves particularly difficult for animal models of depression as the genetic and environmental risk factors associated with depression in the human population are heterogenous. Rodents which have been bred for specific depressive-like behaviours are often used to model genetic risk for depression. Specific genetic mutations in mouse models have yielded important insights into genes associated with various neurotransmitter systems which modulate depressive-like behaviour [for review see (Cryan et al., 2002)]. Early life stress paradigms such as maternal separation are used to model stress related risk factors for the development of depression [for review see (Holmes et al., 2005)]. Chronic unpredictable stress is a well established procedure which reliably induces anhedonic-like behaviour in rodent models [for review see (Willner, 1997)]. Stress related paradigms have the advantage that animals can be subdivided into resilient and susceptible groups, allowing for the investigation of biological mechanisms underpinning these phenotypes.

Face validity refers to the accuracy with which the model reproduces anatomical, biological and behavioural markers which are evident in the human disease. It is not possible to accurately model all of the symptoms associated with a complex disorder like depression in a rodent model. However some criteria of the human disorder such as anhedonia and changes in weight, sleep and cognitive function can be modelled in rodents. Thus contemporary animal models attempt to relate behavioural and neurochemical observations induced by a variety of means in rodents as discussed above, to endophenotypes relevant to the human disorder. Decreased consumption of a palatable saccharin or sucrose solution following a manipulation has been interpreted as anhedonic-like behaviour in rodent models, while passive coping strategies in paradigms such as the forced swim test (FST) and two-way active avoidance have been interpreted as behavioural despair. These behaviours have been shown to be sensitive to antidepressant treatment (Deussing, 2006). Changes in physiological parameters such as
alterations in HPA axis function or neuroimaging markers in depression can also be modelled in animals.

Predictive validity is a measure of how the behavioural alterations presented in the model should be sensitive to currently used treatments for depression. Behavioural readouts for pharmacological response should ideally be reliable and reproducible between different laboratories. The most commonly used test to assess antidepressant response in rodent models is the FST (Cryan et al., 2002).

1.3.1 **Forced swim test (FST)**

The FST was originally designed as a rapid screen for antidepressant activity in novel compounds. It is based on the observation that animals who are faced with an inescapable stressor will desist from attempting to escape after a period of time. Once a rodent is placed in a large container of water from which they cannot escape they will initially vigorously attempt to escape, but will then cease escape orientated behaviours. The immobility seen in the FST has been reported to reflect "behavioural despair". Antidepressant treatments reduce the time the animal is immobile and increase escape orientated behaviours. Unlike the clinical setting however most antidepressant treatments are effective at reducing immobility in the FST after acute treatment. (Cryan et al., 2005; Porsolt et al., 1977a, 1977b). In the studies presented in this thesis a modified version of the FST with an increase in water depth from 18 to 30cm has been adopted. This modified FST has been shown to be more sensitive to the effects of antidepressants despite lower baseline immobility times (Cryan et al., 2005). Conversely, increased immobility in the FST is increasingly being used as a measure of depressive-like behaviour in animal models of depression. Immobility time in the FST has been shown to be increased by manipulations such as chronic restraint stress (Chiba et al., 2012), maternal deprivation stress (Lambás-Señas et al., 2009), exogenous administration of corticosterone (O'Donovan et al., 2014a) and cortical astrocytic ablation (Banasr and Duman, 2008). Hence increased immobility in the FST does appear to be related to alterations in the physiological processes similar to those reported in human depressed patients.

1.3.2 **Assessing anxiety-related behaviour in rodents**

Changes in anxiety-related behaviours in rodents often occur in tandem with changes in depressive-like behaviours. The open field test is a commonly used paradigm to assess locomotor activity and anxiety-related behaviour in rodents. The open field test relies on rodents innate preference for staying on the periphery of an open arena, behaviour
referred to as thigmotaxis. The latency to enter and the duration of time that the animal explores the inner zone of the open field are taken as a measure of anxiety. Rodents which exhibit anxiety-like behaviour will spend more time on the periphery, while a wide range of anxiolytic drugs lead to increased levels of exploration of the inner zone of the open field [for review see (Prut and Belzung, 2003)].

The elevated plus maze is another commonly used paradigm to assess anxiety-related behaviour in rodents. The apparatus consists of a maze elevated from the ground with a small central square from which four arms emanate. Two of these arms are enclosed and two are open. Rodents will tend to spend more time in the enclosed arms than the open arms. Administration of benzodiazepines or other anxiolytic drugs results in an increase in time spent exploring the open arms of the elevated plus maze [for review see (Carobrez and Bertoglio, 2005)].

1.3.3 Assessing memory function in rodents

Cognitive deficits have been reported in animal models of depression. Learning can be divided into three phases, acquisition, consolidation and retrieval. There is a wide variety of tests used to assess learning and memory in rodent models, many rely on different variations of maze learning tasks such as the Morris water maze or T or Y maze tasks [for review see (Paul et al., 2009)]. Performance in tasks of spatial memory in rodents have previously been shown to be hippocampal dependent (Broadbent et al., 2004). In the present thesis a variation on the novel object recognition task called the object displacement task is used to assess spatial memory in rodents (Griffin et al., 2009). This test is based on the observation that rodents are likely to explore novel objects more than an object which is familiar (Dere et al., 2007). In the object displacement task, the object itself is not novel, but a familiar object is instead moved to a novel location. The animal is trained to recognise the location of three objects in an arena and following a retention interval, reintroduced into the arena in which one of the objects has been moved. The level of exploration of the object in the novel location relative to the stationary objects is used to score the task. Increased exploration of the displaced object relative to the stationary is interpreted as learning the task, and changes in the ability of the rodent to recognise the displaced object induced following stress or treatment can thus be quantified. An advantage of this test is that it relies on the spontaneous exploration of novelty in rodents rather than an aversive stimuli such as swim stress in paradigms like the Morris water maze (Engelmann et al., 2006). Memory acquisition in this paradigm is dependent on NMDA receptor function, administration of NMDA receptor antagonist (±)-3-(2-carboxypiperazin-4-yl)propyl-1-
phosphonic acid (CPP) before, but not after the training phase has been shown to impair performance in this task. Performance in the task has also been shown to be related to increased expression of phosphorylated extracellular signal regulated kinase (ERK), a cell signalling protein downstream from the NMDA receptor in the dentate gyrus (Larkin et al., 2008).

The passive avoidance task is a test of contextual fear conditioned memory. The step through passive avoidance paradigm relies on rodents learning to associate the dark compartment of the passive avoidance apparatus with an aversive stimulus. Rodents are placed in a bright compartment and are free to move into an adjacent dark compartment. Upon entering the dark compartment a foot shock is administered. Following a retention period the rodents are returned to the bright compartment and latency to enter the dark compartment which should be contextually associated with the foot shock is used as a measure of performance in the task. Performance in the test is dependent on the hippocampus and the amygdala (Cahill and McGaugh, 1998; Holland and Bouton, 1999).

1.3.4 The olfactory bulbectomised (OB) rat

Bilateral olfactory bulbectomy (OB) in rodents produces a well characterised behavioural phenotype which is associated with alterations in endocrine and neurotransmitter levels similar to those reported in depressed human patients [for review see (Harkin et al., 2003; Kelly et al., 1997; Song and Leonard, 2005)]. OB results in a behavioural phenotype characterised by hyperactivity in a novel environment, deficits in learning and memory tasks including the eight arm radial maze, Morris water maze and operant tasks and increased aggressive behaviour. These behavioural changes are independent of loss of smell as peripherally induced anosmia does not result in OB like behaviour (Harkin et al., 2003). In addition bulbectomy is associated with altered physiological response to stressors, OB rats exhibit an attenuated increase in heart rate and body temperature when compared to sham-operated controls following open field exposure. Open field stress exposure is associated with increased neuronal activation as assessed via immunohistochemical mapping of expression for immediate early gene marker c-fos in the hippocampus, amygdala and paraventricular nucleus with a reduction in expression in the bed nucleus of the stria terminalis (BNST) (Roche et al., 2007). The olfactory bulbs project to the BNST and the amygdala, retrograde degeneration of these neuronal tracts and subsequent disinhibition of the amygdala following bulbectomy has been proposed as a primary mechanism for the behavioural change observed following bulbectomy (Song and Leonard, 2005). However other
reports indicate that changes in homecage nocturnal locomotor activity and basal body temperature occur in the first week following OB surgery, suggesting that increased amygdala stress responsivity in the model is not as a result of neurodegenerative processes which would require more time (Vinkers et al., 2009). The only magnetic resonance imaging (MRI) study carried out in the OB model reports increased signal intensity from the lateral ventricles of OB rats relative to sham-operated controls suggesting a possible increase in lateral ventricular volume (Wrynn et al., 2000).

The model has been shown to have good predictive validity, unlike most other tests for antidepressant activity in rodent models, OB-induced hyperactivity in the open field is sensitive to chronic, but not acute antidepressant treatment. All monoaminergic based therapeutically active antidepressants have been shown to attenuate hyperactivity in the open field test in the OB rat following chronic administration [for review see (Song and Leonard, 2005)]. In addition to attenuating OB-induced behavioural changes, chronic fluoxetine treatment has been shown to attenuate impaired physiological and neuronal activation in response to stress (Roche et al., 2007). Attenuation of OB induced hyperactivity in the open field following chronic imipramine treatment has been shown to persist for up to six weeks following the cessation of treatment, after which hyperactivity is again evident (Breuer et al., 2007). The requirement for chronic dosing to produce behavioural change in the model provides an opportunity to assess onset of antidepressant action for novel antidepressants in the model.

OB results in alterations in NMDA receptor density in the frontal cortex and amygdala (Robichaud et al., 2001; Webster et al., 2000), while chronic daily dosing with NMDA receptor antagonist MK-801 has been shown to attenuate OB-induced hyperactivity in the open field (Redmond et al., 1997). Riluzole, an agent which modulates glutamatergic neurotransmission, has been shown to exhibit an antidepressant like response in the OB model which is associated with a decrease in extracellular glutamate in the frontal cortex of OB rats (Takahashi et al., 2011). These data suggest altered glutamatergic signalling may be involved in the behavioural phenotype exhibited by the OB rat.

1.3.5 The Wistar-Kyoto (WKY) rat

The Wistar-Kyoto rat strain, originally bred as the normotensive control strain for the spontaneously hypertensive rat (SHR), has subsequently been proposed as a model of depression which endogenously expresses some of the behavioural, endocrine and neurotransmitter changes found in depressed human patients (Redei et al., 2001). It exhibits a complex and well characterised behavioural phenotype relative to out-bred
comparator strains. The WKY strain exhibits anxiety-like behaviour in a novel environment characterised by freezing behaviour and decreased exploration relative to Wistar (Malkesman et al., 2005; Nagasawa et al., 2012; Nam et al., 2014; Paré, 1994; Tizabi et al., 2010) and Sprague-Dawley strains (Burke et al., 2010; Nam et al., 2014; O'Mahony et al., 2011; Pardon et al., 2002). This is coupled with increased immobility time in the FST relative to Wistar (Getachew et al., 2010, 2008; Malkesman et al., 2007; Nagasawa et al., 2012; Paré and Redei, 1993; Paré, 1994; Redei et al., 2001; Tejani-Butt et al., 2003) and Sprague-Dawley strains (Burke et al., 2010; Carr et al., 2010; López-Rubalcava and Lucki, 2000; Nam et al., 2014; O'Mahony et al., 2011; Rittenhouse et al., 2002; Schaffer et al., 2010; Tejani-Butt et al., 2003). There is conflicting data on the strains performance in tests of cognition. Wyss and colleagues (2000) have reported that WKY rats were significantly slower at learning the Morris water maze task when compared to the Sprague-Dawley strain (Wyss et al., 2000). While Ferguson and colleagues (2004) reported decreased performance in the Morris water maze in the WKY strain relative to SHR but no difference relative to the Sprague-Dawley strain (Ferguson and Cada, 2004). Kyeremanteng et al (2014) reported no baseline differences between the WKY and Wistar strains in the Morris water maze task (Kyeremanteng et al., 2014). WKY rats have been shown to exhibit deficits in performance in a radial arm maze task of spatial working memory when compared to Wistar rats (Hernandez et al., 2003). In contrast the WKY rat strain does not show any deficits in a passive avoidance test and in fact have been shown to exhibit a greater passive avoidance response following an inescapable tail shock stress when compared to the Wistar strain (Paré, 1996a, 1993).

The model has been shown to have good predictive validity with immobility in the FST in the WKY rat strain shown to be sensitive to both conventional and novel antidepressant drugs including tri-cyclic antidepressants desipramine and imipramine (Jeannotte et al., 2009; Lahmame and Armario, 1996; Lahmame et al., 1997), noradrenaline and dopamine reuptake inhibitor nomifensine (Tejani-Butt et al., 2003), NMDA receptor antagonist ketamine (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012) as well as non-pharmacological treatments such as ECS (Kyeremanteng et al., 2014) and deep brain stimulation (Krahl et al., 2004). The WKY strain also exhibits aberrant HPA axis function, Rittenhouse and colleagues (2002) have reported that WKY rats show less corticosterone suppression following administration of exogenous glucocorticoid receptor agonist dexamethasone, relative to Wistar rats (Rittenhouse et al., 2002). Activation of the HPA axis of WKY rats also differs in its response to stress. WKY rats show a prolonged corticosterone response following an acute swim stress when
compared to Wistar rats, exhibiting elevated corticosterone levels 60 minutes following the cessation of the acute stress, while Wistar corticosterone levels return to baseline (De La Garza and Mahoney, 2004). This is in agreement with another report which found that adrenocorticotropic hormone (ACTH) levels in WKY rats remained elevated 60 minutes following a cold restraint stress while levels in Sprague-Dawley rats had returned to baseline (Pardon et al., 2003).

The WKY rat strain exhibits altered brain morphology relative to out-bred comparator strains, female WKY rats exhibit a smaller hippocampal volume when compared to female Wistar rats (Tizabi et al., 2010). Consistent with this is the finding that female Brown-Norway rats have a higher hippocampal wet weight and volume than age matched female WKY rats (Gilad and Gilad, 1981). Reductions in NMDA receptor binding in the nucleus accumbens, caudate putamen, the CA1 subfield of the hippocampus and the prefrontal cortex relative to the Wistar strain have been reported (Lei and Tejani-Butt, 2010; Lei et al., 2009). It has been suggested that decreased NMDA receptor activity along the nigro-striatal pathway may have a role to play in both the dopaminergic abnormalities in this strain as well as its lower locomotor activity (Lei et al., 2009). Gosselin and colleagues (2009) have reported a decrease in the number of GFAP positive astrocytes in the prefrontal cortex, basolateral amygdala and specific subfields of the hippocampus in the WKY strain relative to the Spraque-Dawley strain. As decreased GFAP expression has previously been linked to alterations in astrocytic glutamate reuptake, these data suggest possible glutamate metabolism abnormalities in this model (Gosselin et al., 2009).

1.3.6 Astrocytic dysfunction in animal models of depression

Several pre-clinical animal models of depression have been shown to exhibit alterations in astrocyte cell function. The flinders sensitive line (FSL) rat has been proposed as a selectively bred animal model which expresses some behavioural and neurochemical characteristics similar to those of human depressed patients [for review see (Overstreet et al., 2005)]. FSL rats have been shown to exhibit a reduced hippocampal GLAST protein expression relative to Sprague-Dawley rats. This is coupled with increased GFAP protein levels and decreased levels of D-serine, resulting in altered neuronal glutamatergic signalling (Gómez-Galán et al., 2012). This is the opposite to the reduction in sub-regional hippocampal GFAP immunoreactivity reported in the WKY rat, as discussed above (Gosselin et al., 2009). Sprague-Dawley rats selectively bred for increased learned helplessness behaviour show decreased expression of GLT-1 protein in all hippocampal subfields (Zink et al., 2010).
Rats subjected to chronic unpredictable stress exhibit a reduction in GFAP positive cell numbers as well as a decrease in GFAP mRNA expression in the pre-limbic cortex (Banasr and Duman, 2008; Banasr et al., 2010). Chronic unpredictable mild stress induced decreased hedonic behaviour, as assessed by sucrose preference, is associated with a decrease in GFAP protein levels and an increase in S100B levels in the rat hippocampus. The authors report that intra-hippocampal injection of BDNF attenuated chronic mild stress induced anhedonic behaviour while increasing GFAP and decreasing S100B protein levels. The method of BDNF delivery is however a confound to interpretation of these results (Ye et al., 2011). Male tree shrews exposed to a chronic psychosocial stress paradigm exhibit a decrease in GFAP positive cell number in the hippocampus which is attenuated by chronic fluoxetine administration (Czéh et al., 2006).

Furthermore it has been shown that central administration of drugs which alter astrocyte cell function is sufficient to produce a depressive phenotype in rodent models. Blockade of astrocyte gap junctions or GLT-1 in the prefrontal cortex is sufficient to induce depression-related behaviours in rodents (John et al., 2012; Sun et al., 2011). Inducing astrocytic dysfunction using the astrocytic toxin L-alpha-aminoadipic acid (L-AAA) in the prefrontal cortex has also been shown to produce a depressive phenotype in rodent models (Banasr and Duman, 2008; Domin et al., 2014; Lee et al., 2013). L-AAA is a glutamate analogue [see figure 1.3.6], present in the body as a normal product of lysine metabolism in mammalian cells. It is present in the rodent brain in the low to mid micromolar range with only mild regional variation and is thought to be primarily located in astrocytes (Guidetti and Schwarcz, 2003). Of interest micromolar concentrations of L-AAA have been shown to be sufficient to inhibit GLT-1, but not GLAST mediated glutamate uptake in cultured astrocytes suggesting a possible endogenous modulatory role for L-AAA in glutamatergic neurotransmission (Tsai et al., 1996).

In vitro work has shown that alpha aminoadipic acid is transported into astrocytes primarily through the Na⁺ dependent transport system, but may also be taken up through the chloride (Cl⁻) dependent cysteine/glutamate antiporter. It has minimal toxicity on neurons and the L-isomer exhibits higher and more selective astrocytic toxicity (Brown and Kretzschmar, 1998; Huck et al., 1984; Tsai et al., 1996). In vivo work has also shown that administration of L-AAA into the rodent brain produces a decrease in GFAP positive cell numbers in peri-lesion sites with no detectable damage to neurons (Banasr and Duman, 2008; Khurgel et al., 1996; Takada and Hattori, 1986).
Although the exact mechanism through which L-AAA exerts its gliotoxic effects are unknown, in vitro work implicates L-AAA induced increases in astrocyte intracellular Ca$^{2+}$ (Brown and Kretzschmar, 1998), competitive inhibition of glutamate transporters, and competitive inhibition of glutamine synthetase and Y-glutamylcysteine synthetase (McBean, 1994). L-AAA is primarily metabolised by astrocytic alpha-aminoadipic acid transaminase, but also inhibits other astrocyte transaminase proteins. L-AAA inhibits the production of neuroprotective metabolite kynurenic acid by astrocytic kynurenine aminotransferase II (KAT II), kynurenic acid acts as a non-competitive antagonist of the NMDA receptor, hence L-AAA may have effects on neuronal excitability (Guidetti et al., 1997). It has thus been proposed as a possible endogenous modulator of excitatory neurotransmission (Guidetti and Schwarcz, 2003). This extends to in vivo work, Wu and colleagues (1995) report that administration of L-AAA into the rodent hippocampus via a microdialysis probe dose dependently reduces extracellular kynurenic acid concentrations (Wu et al., 1995), while Chang and colleagues (1997) report that hippocampal KAT activity was reduced by 43% 20 days following a single high dose L-AAA injection (Chang et al., 1997). All of these mechanisms are likely to be involved in the gliotoxic mechanism of L-AAA. Its specificity for astrocytes means it is a potentially useful tool to study behavioural and physiological changes induced by regional astrocytic dysfunction in rodent models.

Thus there is a large body of evidence, both clinical and pre-clinical, implicating altered glutamatergic neurotransmission and astrocytic dysfunction in the pathophysiology of depressive illness. MRI is a medical imaging modality which allows us to non-invasively assess in vivo differences in the brain structure and function between depressed patients and non-psychiatric controls. Importantly it can be applied in equal measure to
the assessment of rodent brain structure and function in the pre-clinical setting. Thus MRI is a potentially powerful translational tool enabling us to further assess the face validity of animal models of depression and antidepressant action. Furthermore investigations into the physiological basis of neuroimaging markers in animal models may provide potentially useful data on possible pathophysiological processes associated with similar imaging markers in the clinical population. A brief review of MRI techniques and markers in depression will follow.
1.4 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a medical imaging technique used both clinically and pre-clinically to enable the acquisition of high quality images of the body's interior. MRI is based on the principle of nuclear magnetic resonance. The nuclei of certain atoms such as hydrogen possess a property referred to as spin. MRI utilises this property as living organisms are primarily composed of water molecules which contain two hydrogen nuclei. This property means that these hydrogen nuclei act as though they possess a small magnetic field. When placed in a strong magnetic field the magnetic moments of the majority of hydrogen nuclei will align in the direction of the applied magnetic field. This results in a small net magnetisation in the direction of the applied magnetic field [see figure 1.4a]. The hydrogen nuclei within the magnetic field resonate at a specific frequency which is dependent on the chemical environment of the nuclei and the strength of the applied magnetic field, this is called the Larmor frequency. Within MRI this falls within the radiofrequency (RF) range. When RF energy is transmitted at the Larmor frequency it is absorbed by the nuclei which then flip to a different plane [see figure 1.4b]. When the RF field is removed the nuclei return to their natural alignment within the magnetic field and release a photon which induces a small current in the receiver coil. By sending multiple RF ‘pulses’ while applying a gradient magnetic field, data can be obtained which, when integrated and converted through the use of a Fourier transformation, can be used to gather three dimensional information on the location of hydrogen nuclei. Its non-invasive nature coupled with the ability of MRI to acquire functional and structural data in a single session make it a powerful tool for gathering information on the brain which can be applied in both pre-clinical and clinical environments. This characteristic provides an opportunity for its use as a potentially powerful translational tool. (Berry and Bulpitt, 2009).
Figure 1.4: MRI signal generation

a) When hydrogen nuclei are placed in a strong magnetic field, a small majority of them will align in the direction of the applied magnetic field (the spin-up state) with the remainder aligned in the opposite direction (the spin-down state) resulting in a small net magnetisation in the direction of the applied magnetic field. b) When a 90 degree RF pulse is applied the net magnetisation vector is flipped to the xy plane and the phase of the spins is aligned. When the RF pulse is removed two forms of relaxation occur which emit T1 and T2 signals. c) T1 or spin-lattice relaxation involves the return to equilibrium and the restoration of the net magnetisation vector to the Z direction (i.e. the direction of the applied magnetic field). The T1 relaxation time is the time it takes for the signal to recover to approximately 63% of its initial value after flipping to the transverse plane. d) T2 or spin-spin relaxation results from the de-phasing of the spins. The T2 relaxation time is the time it takes the signal to decay to approximately 37% of its initial value after flipping to the transverse plane. [Adapted from http://www.cardiff.ac.uk/biosi/researchsites/emric/basics.html]
1.4.1 T1 and T2 relaxation time

The change back to the equilibrium state following the removal of the RF energy is referred to as saturation recovery. Longitudinal relaxation time (also referred to as spin-lattice relaxation) occurs exponentially as the sample recovers its longitudinal magnetisation vector with a constant T1. During T1 relaxation, energy is transferred from the excited nuclei to their surroundings, referred to as the lattice. T1 is calculated by the equation:

\[ M_z(t) = M_{z,\text{eq}} \left( 1 - e^{-t/T_1} \right) \]

Where \( t \) is the time since the RF pulse was stopped. It is the time taken for the signal to recover to \([1 - (1/e)]\) or 63% of its initial value following a 90° inversion pulse.

Spin-spin relaxation time occurs as the sample recovers its transverse magnetisation vector with a constant T2. During T2 relaxation there is no energy transfer but rather a loss of phase coherence due to the interactions of the nuclei’s individual magnetic moments. T2 is calculated by the equation:

\[ M_{xy}(t) = M_{xy}(0) e^{-t/T_2} \]

Again, \( t \) is the time since the RF pulse was stopped. It is the time taken for the signal to decay to \((1/e)\) or 37% of its initial value following flipping into the transverse plain. T2 relaxation generally occurs more rapidly than T1 relaxation. T1 and T2 occur independently and are intrinsic properties of the tissue being scanned, not normally modifiable except with the addition of a contrast agent (Bryan, 2010). The time between the application of the RF pulse and the acquisition is referred to as the echo time (TE); the time between the application of further RF pulses is referred to as the repetition time (TR). Image acquisitions with short TR and TE will result in T1 weighted images whereas those with long TE and TR will produce T2 weighted images. Tissues with longer T1 will appear hypointense (darker) on T1 weighted images, while tissues with longer T2 relaxation time appear hyperintense (brighter) on T2 weighted images (Moser et al., 2009).
1.4.2 Bolus-tracking Arterial Spin Labelling (ASL)

There are several different medical imaging techniques capable of assessing blood perfusion in the brain, most however require the administration of an exogenous tracer [for review see (Wintermark et al., 2005)]. Arterial spin labelling (ASL) perfusion imaging uses inflowing arterial blood water as a freely diffusible endogenous tracer to enable the measurement and quantification of cerebral blood flow (Williams et al., 1992). Inflowing water molecules within arterial blood are magnetically labelled in the neck via either adiabatic continuous inversion or repeated saturation pulses to invert or saturate the magnetisation of the hydrogen nuclei. Alteration in the tissue magnetisation in the imaging slice due to the labelled arterial blood is proportional to cerebral blood flow (Buxton et al., 1998). A control image in which inflowing arterial blood has not been labelled is then acquired, this is subtracted from the labelled image to provide a contrast between perfused and unperfused tissue. These images can provide a qualitative assessment of perfusion (Liu & Brown 2007). The non-invasive nature of the ASL procedure makes it ideal for longitudinal studies, and reduces potential concerns about the use of contrast agents in healthy volunteers.

In order for a quantitative assessment of perfusion, ASL data must be input into a theoretical model of the perfusion process. There are several different models which are commonly used to describe how the labelled arterial water diffuses within the tissue, based on different compartmental approaches [for review see (Martirosian et al., 2010)]. A recent non-compartmental approach involves the use of a series of continuous ASL acquisitions using a constant labelling duration, but variable labelling delays. The resultant data can be interpreted as a bolus of labelled arterial water flowing through the imaging plane. This bolus-tracking ASL technique allows for the calculation of mean transit time (MTT), capillary transit time (CTT) and signal amplitude parameters. MTT can be interpreted as the time it takes for the labelled water to reach the imaging plane while CTT can be interpreted as the diffusion of the labelled water within the imaging plane, both are inversely proportional to cerebral blood flow. The signal amplitude is directly proportional to the area under the signal time curve and is related to cerebral blood volume (this includes contributions from both intra and extravascular spaces) (Kelly et al. 2009; Kelly et al. 2010).
1) The inflowing arterial blood water is magnetically labelled, 2) an image of this magnetically labelled blood is taken at the imaging plane (the labelled image), 3) the inflowing arterial blood is not magnetically labelled and 4) an image is taken at the same imaging plane (the control image). Subtraction of the label from the control image generates a perfusion weighted image. By varying the delay between labelling and image acquisition, a time series of perfusion weighted images is acquired. Signal time curves for regions of interest can be constructed from this time series which are then fitted to a non-compartmental model of cerebral perfusion as previously described (Kelly et al., 2009). [Adapted from (Paxinos and Watson, 1998)]
1.4.3 Functional magnetic resonance imaging (fMRI)

fMRI uses the property of functional hyperaemia discussed above to map neuronal activation by detecting changes in energy consumption and blood flow in the brain via MRI. The most common fMRI modality currently in use is that which depends on the blood-oxygen-level dependent (BOLD) contrast signal (Shulman et al., 2004). BOLD signal is dependent on the interaction between the change in blood flow, blood volume and blood oxygen which is coupled to neuronal activation. It relies on the change in haemoglobin oxygenation resulting in the formation of paramagnetic deoxyhaemoglobin, decreased levels of deoxyhaemoglobin due to increased levels of localised blood flow in response to neuronal activation resulting in a change in contrast in a T2* weighted scan (Detre and Wang, 2002). Animal models have shown there is a high degree of spatial correlation between the BOLD signal and measures of cerebral blood flow, and it has been suggested that ASL may be superior to BOLD in tracking changes in neuronal activity (Silva, 2005). The bolus tracking ASL technique outlined above has been shown to be sensitive to changes in blood flow in the rodent somatosensory cortex in response to forepaw stimulation (Griffin et al., 2010). Furthermore this technique has also been shown to be sensitive to recreational drug 3,4-methylenedioxymethamphetamine (MDMA) induced increases in regional cerebral perfusion in rats in pharmacological MRI studies (Rouine et al., 2014, 2013). Given that astrocytes are integral in the regulation of the cerebrovascular changes in response to neuronal activation that form the basis of fMRI methods, it has been proposed that changes in fMRI signals are more closely associated with functional and metabolic activity in astrocytes than in neurons (for review see (Figley and Stroman, 2011)). Thus MRI is a powerful tool to non-invasively investigate the structure and function of the brain in healthy and depressed patients.
1.5 MRI in depression

The clinical neuroimaging literature suggests that there are several structural and functional brain changes which distinguish the brains of depressed patients from those of non-psychiatric controls. These will briefly be reviewed below [for changes in MRS parameters related to depression see section 1.2.2].

1.5.1 Brain structural alterations in depression

Recent meta-analyses of structural imaging studies in the clinical literature have implicated alterations in the volume of a number of brain regions in unipolar depressed patients relative to controls including a decrease in the volume of prefrontal, dorsofrontomedial, orbitofrontal and cingulate cortices and striatum (Arnone et al., 2012; Bora et al., 2012; Sacher et al., 2012). In contrast increased volume in the lateral ventricles has been reported (Kempton et al., 2011).

Reduced hippocampal volume in depressed patients is a recurrent finding in the literature (Arnone et al., 2012; Campbell et al., 2004; Kempton et al., 2011; Videbech and Ravnkilde, 2004). It has been suggested that major depression is more associated with decreased hippocampal volume while bipolar disorder is more associated with white matter hyperintensities and reduced corpus callosum density (Kempton et al., 2011). Although there have been previous meta-analyses which suggest that the degree of hippocampal volume loss is dependent on age of the patient and the disease duration (McKinnon et al., 2009), a more recent analysis of studies in first episode patients revealed significant hippocampal volume reductions, hence hippocampal volume may be a possible risk factor for depression, rather than a marker of disease progression (Cole et al., 2011). This is in agreement with a three year longitudinal study which reported no significant reduction in hippocampal or amygdala volume as assessed using a region of interest based approach over the course of the study, but did show that smaller baseline hippocampal volume was associated with a poorer clinical outcome (FrodI et al., 2008a). A recent meta-analysis suggests that lower right hippocampal volume is a predictor of poor antidepressant response (Fu et al., 2013). It should be noted that different results have been reported following whole brain voxel based morphometry analysis with a decrease in grey matter density in the hippocampus in patients with ongoing depression over a three year period which was not seen in controls, thus highlighting the importance of the method of analysis of structural data (FrodI et al., 2008b). Of interest, changes in hippocampal volume have been inversely correlated with markers of glucocorticoid receptor activation in peripheral plasma, suggesting that hippocampal volume changes seen in depression may be related to
changes in HPA axis function (FrodI et al., 2012). Changes in hippocampal volume are sensitive to antidepressant treatment with an increase in volume reported following traditional antidepressant (FrodI et al., 2008a) and ECT (Abbott et al., 2014) treatment. Reduced hippocampal volume in depressed patients correlates with reduced performance in Wisconsin card sorting task, a task of executive function (FrodI et al., 2006).

Data for the amygdala suggests that amygdala volume may be related to duration of disease with increased volumes reported at the early stages of the illness and reduced volumes reported following a greater number of depressive episodes (Lorenzetti et al., 2009). Amygdala volume does not appear to be a predictor of antidepressant response (FrodI et al., 2008a).

Abnormalities in white matter have also been reported in depression. Diffusion tensor imaging (DTI) is another structural modality which allows for an assessment of in vivo white matter microstructural integrity through measurement of the restriction in diffusion of water molecules in brain tissue (Moser et al., 2009). Several DTI studies in patients with unipolar depression have reported decreased frontal cortical fractional anisotropy, a measure of white matter integrity and fibre directionality [for review see (Sexton et al., 2009)]. This seems to be evident in both late life depression (Bae et al., 2006; Nobuhara et al., 2006) and in young adults (Li et al., 2007). The data to date suggest that altered fractional anisotropy values may be a marker of pre-disposition to depression or may occur early in the course of disease, as patients with first onset depression have also been shown to exhibit decreased FA values in frontal cortical regions such as the anterior cingulate cortex (Zhu et al., 2011) and the frontal gyrus (Ma et al., 2007). Of interest, levels of GFAP in the rodent brain following an acute injury have been shown to positively correlate with cortical FA values (Budde et al., 2011). Arnold and colleagues (2012) report increased FA with no change in volume in the amygdala of remitted middle aged depressed patients relative to healthy controls suggesting an increase in cell density in the region, possibly related to increased structural connectivity with other regions. It was not possible to assess if this occurred as a result of depression or predated the development of depressive symptoms (Arnold et al., 2012). This is in keeping with tractography analysis which suggests that first episode depressed patients exhibit increased cortico-limbic structural connectivity (Fang et al., 2012). The low number of studies employing DTI in unipolar depression combined with methodological differences between studies to date makes it difficult to draw conclusions from the current DTI literature. However, there has been a suggestion...
that depression is associated with a disruption in the pathways linking subcortical regions to the prefrontal cortex (Liao et al., 2013).

1.5.2 Brain functional alterations in depression

fMRI provides a way to map regional changes in cerebral blood flow at resting state and in response to the performance of tasks. Decreased resting state dorsolateral prefrontal cortex activation has been reported in depressed patients relative to controls (Alcaro et al., 2010). Furthermore decreased dorsolateral prefrontal cortical activation in depressed patients during the performance of an emotional processing task couples with an increase in amygdala activation (Siegle et al., 2007). Increased activity in the amygdala in response to negative stimuli is one of the most replicated findings in the literature [for review see (Savitz and Drevets, 2009b)]. In contrast to the dorsolateral prefrontal cortex, the orbitofrontal and subgenual anterior cingulate cortices show increased baseline brain activity in depressed patients [for review see (Hasler and Northoff, 2011)]. Increased resting state perfusion as assessed via ASL in the subgenual anterior cingulate cortex has been reported in treatment resistant depressed patients relative to controls (Duhameau et al., 2010). These alterations in resting state brain activity are sensitive to antidepressant treatment. Decreased resting state perfusion as assessed via ASL in the anterior cingulate cortex has been shown following antidepressant response to sleep deprivation in depressed patients (Clark et al., 2006). Hyper-reactivity in the amygdala in depression is attenuated following antidepressant treatment (Fu et al., 2004) and increased dorsomedial prefrontal cortical activity has been reported following antidepressant treatment (Savitz and Drevets, 2009b). Hence unlike some of the structural alterations discussed above, brain functional alterations appear to be state dependent markers of depressive illness.

The default mode network is a set of brain regions which are more active at rest, i.e. when a person is not performing a cognitive task. These include the orbital frontal cortex, the medial prefrontal/anterior cingulate cortex, the lateral temporal cortex, the inferior parietal lobe, the posterior cingulate and retrosplenial cortex and the hippocampus and parahippocampal cortex (Lu et al., 2012). Resting state functional connectivity MRI relies on BOLD contrast to map brain regions which show a spontaneous temporal alignment of activity. Hyperactivity in the default mode network during task performance in depressed patients has been reported, which has been interpreted as a possible MRI marker for rumination in depressed patients [for review see (Whitfield-Gabrieli and Ford, 2012)]. Hyper connectivity between the subgenual anterior cingulate and default mode network during rest has also been reported in
depressed patients (Berman et al., 2011; Greicius et al., 2007), while increased default mode network connectivity with the dorsolateral prefrontal cortex has been reported which is thought to underlie some aspects of the emotional dysregulation characteristic of depressive illness (Sheline et al., 2010). It has been suggested that resting state hyperactivity in cortical midline structures is mediated by alterations in regional glutamate and GABA metabolism (Alcaro et al., 2010). Translational approaches utilising animal models may serve to increase our understanding of the physiological basis of the functional and structural brain alterations discussed in depressed patients.

1.6 MRI in animal models of depression

As discussed above, work on animal models of depression has led to an increased understanding of the biological basis and neural circuits involved in the human disorder. Translational neuroimaging in rodents offers opportunities to further our understanding of the physiological basis of some of the commonly observed neuroimaging markers in depression. A limitation in the interpretation of animal MRI results is that animals are usually anaesthetised when undergoing MRI scans which may alter resting state brain function and perfusion. The choice of anaesthetic is important in this regard with α₂-adrenoceptor agonist medetomidine shown to be more suited to resting state functional connectivity studies than inhalable anaesthetic isoflurane. However for most other imaging acquisitions isoflurane is still the most commonly used anaesthetic [for review see (Hanusch et al., 2007; Hoyer et al., 2014)].

Several studies have reported structural alterations in the rodent brain in animal models of depression. Chronic stress paradigms have been shown to produce changes in rodent brain morphology. Structural MRI of ex vivo brains of Wistar rats which were subjected to a 10 day immobilisation stress, have shown an increase in lateral ventricular volume without any change in hippocampal volume or shape when compared to non-stressed controls (Henckens et al., 2015). However the lack of stress induced changes in hippocampal morphology reported may be related to severity of stressor and/or the ex vivo acquisition of structural data. Lee and colleagues (2009) report that 21 days of immobilisation stress results in a 3% reduction in baseline hippocampal volume which was not observed in control animals as assessed via in vivo MRI structural scans in a longitudinal study design. There was no change observed in anterior cingulate or retrosplenial cortical volumes reported (Lee et al., 2009). A multimodal imaging study by Delgado Y Palacios and colleagues (2011) reports that 8 weeks of chronic mild stress does not result in a change in hippocampal volume, changes in hippocampal shape were evident though. This was coupled with an increase in ventral hippocampal glutamate
levels as assessed by MRS in animals which were behaviourally anhedonic (Delgado Y Palacios et al., 2011). Increased caudate putamen volume has also been reported in animals who are behaviourally anhedonic following 8 weeks of chronic mild stress (Delgado Y Palacios et al., 2014).

Rodents have also been shown to have a default mode network similar to that seen in humans (Lu et al., 2012), and this has been shown to be sensitive to stress. 10 days of immobilisation stress has been reported to increase resting state functional connectivity within the rodent default mode, visual, and somatosensory networks (Henckens et al., 2015). Differences in resting state functional connectivity have been reported between two animal strains selectively bred for immobility behaviour in the FST. WKY rats bred for high immobility in the FST have been shown to exhibit decreased functional connectivity between the hippocampus and the somatosensory cortex and striatum, while exhibiting increased connectivity with the dorsal orbitofrontal cortex when compared to WKY rats bred for low immobility in the FST (Williams et al., 2014).

Decreased resting state cerebral blood volume has been reported in the habenula and thalamic regions of Sprague-Dawley rats bred for learned helplessness (LH) behaviour, the congenital LH rat, relative to its control, the non LH rat. Increased levels of functional connectivity between the frontal cortex and the dorsal raphe nucleus and between the hippocampus and the retrosplenial cortex were also reported in LH rats relative to non LH rats. These results increase the validity of this model as a model of treatment resistant depression as they show resting state functional connectivity deficits in brain circuits which have been implicated in human depression and antidepressant response (Gass et al., 2014). Resting state functional connectivity in the rodent is sensitive to antidepressant treatment, with ketamine treatment dose dependently increasing resting state functional connectivity between the frontal cortex and the hippocampus (Gass et al., 2013). Other brain regions which exhibit resting state hyperactivity in animal models of depression as assessed via post-mortem immunostaining for neuronal activation marker c-fos include the amygdala, hippocampus, hypothalamus, septum and locus coeruleus, while resting state hypoactivity has been reported in the prefrontal cortex, the dentate gyrus and the lateral septal nucleus [for review see (Alcaro et al., 2010)].

Proton MRS studies in three mouse lines selectively bred for HPA axis stress reactivity have revealed decreased N-acetylaspartate (NAA) levels in the dorsal hippocampus and prefrontal cortex of mice with high reactivity which is coupled with decreased levels of performance in hippocampal dependent spatial memory tasks when compared with mice with low HPA axis stress reactivity. Post-mortem analysis suggests that this change
in regional NAA levels is related to alterations in protein levels related to mitochondrial metabolism (Knapman et al., 2012). This is in agreement with data from Xi and colleagues (2011), who report that 6 weeks chronic unpredictable stress results in a decrease in dorsal hippocampal NAA levels which is related to impaired performance in the Morris water maze, both of which were attenuated by chronic treatment with SSRI escitalopram (Xi et al., 2011). 6 weeks of chronic mild stress has been shown to decrease glutamate and glutamine levels in the dorsal hippocampus, and decrease glutamine levels in the frontal cortex of Sprague-Dawley rats, interpreted as possible evidence of stress induced glial dysfunction in this model (Kumar et al., 2013).

The characterisation of animal models of depression by MRI has already provided us with important insights into potential environmental factors associated with, and physiological mechanisms which may underlie the observed changes in brain structure and function in depressed patients. However relatively few models have been characterised to date. Furthermore, no studies to date have investigated if changes in neuroimaging markers observed in animal models of depression are associated with changes in regional astrocyte cell function.
1.7 Aims and objectives of this thesis

The primary aim of this thesis was to investigate if depression-related behaviour in three animal models of depression was associated with changes in brain structure and function that could be measured by in vivo MRI. Studies were performed with the aim of improving the face validity of the models, and to provide potential neurophysiological correlates with imaging markers in the animal models which may be relevant to possible pathophysiological mechanisms underlying neuroimaging markers commonly observed in the depressed clinical population.

To this end MR imaging was performed in the olfactory bulbectomised rat model of depression, a well characterised model with known predictive validity which has been reported to exhibit alterations in glutamatergic signalling. Furthermore, the effects of chronic administration of tri-cyclic antidepressant imipramine and NOS inhibitor L-NA on behavioural and neuroimaging markers in the model were assessed. The effects of sub-acute administration of the NMDA receptor antagonist ketamine on behavioural and neuroimaging markers in the model were also assessed.

MR imaging was performed in the WKY rat model, a rat strain which presents with an anxious and depressive-like phenotype relative to out-bred comparator strains. Post-mortem assessment of GFAP positive cell numbers was undertaken to investigate if behavioural and neuroimaging markers present are related to changes in astrocyte cell function. Furthermore the effects of BP and UBP ECS treatment on behavioural and post-mortem markers of astrocyte function in the WKY rat model were assessed. Finally the effects of chronic stress on behaviour and post-mortem markers of astrocyte function in the WKY rat model were assessed.

MR imaging was performed to investigate if the change in behaviour following intracortical administration of astrocyte toxin L-AAA is associated with changes in neuroimaging markers. Furthermore behavioural change following L-AAA administration into the basolateral amygdala and the CA3 subfield of the hippocampus was assessed to further characterise the effect of astrocytic dysfunction in these regions on depressive and anxiety-like behaviour in rodents.
Chapter 2 – Materials and methods
2.1 Materials

2.1.1 Animals

Male Wistar rats
Male Wistar-Kyoto rats
Male Sprague-Dawley rats

2.1.2 Experimental treatments

L-2 Aminoadipic Acid
\textsuperscript{N\textsuperscript{ω}}-Nitro-L-arginine (L-NA)
Ketamine hydrochloride (100mg/ml)
Imipramine hydrochloride

2.1.3 ECS equipment

ECS pulse generator 57800
Padded ear clip electrodes

2.1.4 General laboratory chemicals

3,3'-diaminobenzidine (DAB)
\textbeta-mercaptopethanol
2-Propanol
Acrylamide
Ammonium Persulfate (APS)
Bis Acrylamide
Bovine Serum Albumin 96% (BSA)
Bromophenol Blue
Chromium (III) potassium sulfate (CrK(SO\textsubscript{4})\textsubscript{2})
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (EtOH)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Hydrochloric Acid (HCl)</td>
<td>BDH Chemicals</td>
</tr>
<tr>
<td>Isopentane</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Liquid nitrogen (N₂)</td>
<td>BOC</td>
</tr>
<tr>
<td>Magnesium Chloride (MgCl₂)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Medical oxygen (O₂)</td>
<td>BOC</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Normal Goat Serum (NGS)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>BDH Chemicals</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate (SDS)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sodium Dihydrogen Phosphate (NaH₂PO₄)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sodium Hydrogen Phosphate (Na₂HPO₄)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tetramethylethylene-Diamine (TEMED)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tissue-tek OCT compound</td>
<td>Sakura</td>
</tr>
<tr>
<td>Triton-X</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Trizma-HCl</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Trizma Base</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
Sucrose
Xylene

**2.1.5 General laboratory equipment**

Borosilicate glass mortar /pestle

Centrifuges : Heraeus Pico 17
  - Rotina 380R
  - Z216MK
  - Legend RT+

Cryostat

Dark box (LAS-3000)

Gyro rocker

Haemocytometer

Heating block

Micropipette puller (P-87)

Microscopes: CKX41
  - Axio Imager Z1

Peristatic pump

pH meter

Polyacrylamide gel electrophoresis chamber

Polymerase chain reaction (PCR) GeneAmp 9700

Power supply for electrophoresis chambers

Pulse controller and gene pulser

Roller mixer

Sigma Aldrich
Sigma-Aldrich
Fisher Scientific
Thermo Scientific
Hettich
Hermle
Sorvall
Leica
Fujifilm
Stuart
VWR
Grant
Sutter Instrument
Olympus
Carl Zeiss
Gilson
Mettler-Toledo
Atto Corporation
Applied Biosystems
Bio-Rad
Bio-Rad
Stuart
Scale (precision) Mettler-Toledo
Spectrophotometer “Nanodrop” Thermo Scientific
Vortex Ika
Water bath Grant
Western blotting transfer machine Atto corporation

2.1.6 Immunohistochemistry kits
Vectastain elite ABC kit Vector Laboratories

2.1.7 Western blotting reagents and antibodies
Anti-mouse IgG Sigma-Aldrich
Anti-rabbit IgG Amersham.
Dual colour molecular weight marker Biorad
Phosphatase inhibitor cocktail I and II Sigma-Aldrich
Polyvinylidene Millipore
Protease inhibitor cocktail Sigma-Aldrich
Re-blot plus Millipore

2.1.8 mRNA related materials
Nucleospin RNA II Macherey-Nagel
AB 1900 PCR plate Thermo Fisher Scientific
RNAse Zap Ambion
TaqMan® gene expression assays Applied BioSystems

2.1.9 Equipment for surgical procedures
Anaesthesia box Harvard Apparatus
Anaesthesia system with O₂ flow metre MSS
Dental drill equipped with a 0.7mm bit Silfradent
Fluovac system
Gauze swabs 100mm×100mm
Haemofibrine
Hamilton syringe (Model 7105KH)
IMF Fluosorber filter canister
Isoflurane vaporiser
Luer-Lock extension set (Posiflow)
Microcapillary tubes
Needles, hypodermic (26G) (Microlance)
Rat anaesthesia mask
Rat ear bars
Rat gas anaesthesia head holder
Savlon\textsuperscript{*} cream (cetrimide/chlorhexidine)
Skin staples
Stereotactic frame
Syringes, plastic (1 mL)
Tissue adhesive (Surgibond\textsuperscript{*})

2.1.10 Equipment for behavioural testing

Behavioural recording system (Ethovision 3.1)
Forced swim test water tank
Duplo Bricks
Open field circular arena (1.25m)
Passive avoidance and light-dark tests apparatus

Harvard apparatus
Vernon-Carus Ltd.
Specialities Septodont
Hamilton
Harvard apparatus
MSS
BD
Sigma-Aldrich
BD
Kopf Instruments
Kopf Instruments
Kopf Instruments
Novartis, Ireland
Ethicon Endo-Surgery
Kopf instruments
B-Braun
SMI AG

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2.1.11 General laboratory plastics

6-well plates                  Fisher Scientific
Containers (70 mL)             Sarstedt
Falcon tubes (15 mL), sterile  Sarstedt
Falcon tubes (50 mL), sterile  Sarstedt
Glass coverslips 22 mm x 50 mm Fisher Scientific
Microscope slides 76 mm x 26 mm Fisher Scientific
Microtome blades (<35 type)    Lab. Instr. & Supply
Microtubes (0.5 mL)            Sarstedt
Microtubes (1.5 mL)            Sarstedt
Microtubes (2 mL)              Sarstedt
Pasteur pipettes (3.5 mL)      Sarstedt
PCR tubes (0.2 mL)             Sarstedt
Pipette tips (10 μL)           Sarstedt
Pipette tips (200 μL)          Sarstedt
Pipette tips (1000 μL)         Sarstedt
Pipettes (5 mL), sterile       Sarstedt
Pipettes (10 mL), sterile      Sarstedt
Pipettes (25 mL), sterile      Sarstedt
Scalpels, disposable, sterile (Swann-Morton) Fisher Scientific

2.1.12 Anaesthetics

Isoflurane (Isoflo)            Abbott
Urethane                      Sigma-Aldrich
2.2 Methods

2.2.1 Animals

Male Wistar rats (175 - 250 g) were obtained from the Bioresources Unit, Trinity College Dublin. Male Wistar-Kyoto and Sprague-Dawley rats were obtained from Harlan UK. Animals were housed in medium-sized, hard-bottomed propylene cages with stainless steel lids. All animals were group housed unless otherwise stated and kept under standard housing conditions at a constant temperature (20 ± 2°C) and at standard lighting conditions (12 hour light:12 hour dark cycle, lights on from 0800 to 2000 hours). Food and water were available ad libitum. All experiments were carried out in accordance with the guidelines of the Animal Ethics Committee Trinity College Dublin and the European Council Directive 1986 (86/806/EEC).
2.2.2 Behavioural tests

2.2.2.1 Open field test
The open field test is a behavioural test used to assess locomotor activity and anxiety-like behaviour in rodents. The test was conducted in dim light and lasted 15 minutes. Animals were placed at the edge of a 1.25m diameter open field arena with 50cm high black walls and were free to roam, while their movement tracks were recorded using the image acquisition software Ethovision 3.1 (Noldus), which allowed later analysis of each animal’s movements. An experimenter observed each test session and recorded a number of behavioural parameters for each animal: frequency of rearing (wall assisted and free standing), frequency of grooming and total time grooming, and number of faecal boli excreted during test. The arena was cleaned with 70% ethanol between trials in order to remove olfactory cues. Ethovision software allowed the arena to be divided into an inner and outer zone. The total distance moved in the arena, distance moved in the inner zone of the arena and number of transitions between the inner and outer zones as well as time spent in each zone were recorded.

For OB experiments a mirrored circular open field arena with a 1m diameter was used. Stronger illumination was provided by floor lamps positioned in the room (200-250 lux) when testing for locomotor activity. Such conditions have previously been described as optimal for the determination of OB-related hyperactivity (Kelly et al., 1997; Mar et al., 2002). Floor lights were positioned to provide an equal dispersion of light and to avoid casting shadows in the open field. Each animal was placed in the centre of the open field and left free to explore the arena. The distance moved was recorded by a video camera placed above the arena and analysed by the Menu2020 software (HVS Image Ltd, UK) videotracking system. Each animal was exposed to the open field for a duration of 15 minutes. The arena was cleaned with 70% ethanol after each trial to remove olfactory cues. Analysis was conducted on the first 3 minutes of the open field session when OB rats exhibit the most hyperactivity. The bout speed parameter was calculated as distance moved during each bout of movement divided by the duration of each bout in seconds.
Figure 2.2.2.1: Image of an open field arena
2.2.2.2 Elevated plus maze

The elevated plus maze (EPM) is a test used to assess anxiety-like behaviour in rodents. The arena consists of a grey plus maze elevated 50cm from off the ground. Each arm measured 50cm × 10cm with a 10cm × 10cm central junction, 50cm high grey plexiglass surrounded two opposing arms and the other two remained open. The test was carried out in dim light and lasted 5 minutes. The animals were observed by an experimenter while their movement tracks were recorded using image acquisition software Ethovision 3.1 (Noldus). For each trial the animal was placed in the centre of the maze facing an open arm and the image recording was started. The arena was cleaned with 70% ethanol between trials in order to remove olfactory cues. Ethovision software allowed for the subdivision of the arena into open arms, closed arms and centre zones. The total distance moved in the arena, distance moved, number of entries and time spent in each zone were recorded.

Figure 2.2.2.2: Image of an elevated plus maze
2.2.2.3 Forced swim test

The forced swim test (FST) is used to assess depressive-like behaviour in rodents. The FST was performed as previously described (Porsolt et al., 1977b). 24 hours preceding the test animals were placed in a glass cylinder which is filled to a depth of 30cm with water at a temperature of 23 ± 1°C for 15 minutes. The next day rats were again placed in the cylinder under the same conditions for 5 minutes. Both sessions were recorded and time spent immobile was scored later by an experimenter blind to treatment/strain. Immobility was defined as the absence of any active escape orientated behaviour such as swimming, diving or climbing. Clean water was used for each animal. An increase in immobility was interpreted as increased depressive-like behaviour while a decrease in immobility indicated an antidepressant response.

2.2.2.4 Saccharin preference test

The saccharin preference test is used to assess hedonic behaviour in rodents by measuring their preference for a palatable saccharin containing solution. Rats were habituated to drinking from 2 equally accessible water bottles for 3 days, with total fluid consumption measured daily as change in bottle weight over a 24 hour period. On day 3 one water bottle for each animal was replaced with 0.1% saccharin solution. Saccharin preference was calculated by taking weight change in the saccharin bottle as a percentage of the total weight change of both bottles. Bottles were swapped daily to avoid side preference. Bottle weights were measured daily at 10hr.
2.2.2.5 Passive avoidance test

Rats were tested on a step-through inhibitory avoidance apparatus in which two compartments that are similar in size (40 cm x 20 cm x 22 cm; length x width x height) are divided by a partition with a guillotine door. The test was divided into habituation, training and retention phases and the protocol adapted from (Yildiz Akar et al., 2007). During the test the animal learns to associate a specific place with an aversive event (foot shock). Decreased latency to re-enter the dark side of the compartment is interpreted as impairment in fear conditioned memory. In the habituation trial, the rat was placed in the brightly lit start compartment (10W light) and the guillotine door was opened 30 seconds later. After the rat crossed over into the dark chamber, the latency was recorded, the door was closed and the rat left in the dark compartment for another 30 seconds. The rat was returned to its home cage for 15 minutes. In the training trial, the rat was placed in the brightly lit “start” compartment again and the guillotine door opened following a 30 second delay. Once the rat crossed over into the dark chamber, and latency recorded, the door was closed and a mild foot shock (1 mA, 3 seconds) delivered. The rat remained in the shock compartment for an additional 30 seconds to associate spatial cues with the shock. The initial latency to enter the dark (shock) compartment served as a baseline measure. Rats were placed in the light compartment 24h later and their latency to enter the dark compartment (previously associated with the foot shock) was measured as an index of inhibitory avoidance. Cut-off latency was 5 minutes. In the retention test session, the foot shock was omitted. The arena was cleaned with 70% ethanol between trials in order to remove olfactory cues.
2.2.2.6 Object displacement task

The object displacement task was performed to assess spatial recognition memory as previously described (Griffin et al., 2009) [see figure 2.2.2.6]. The arena used was the same as in the open field test, a 1.25m diameter black arena with 50cm high black walls. Objects were constructed from large plastic Lego® blocks which were similar in colour but each having a distinctive configuration. Objects were fixed to the floor of the open field equidistant from the walls and from each other. Animals were placed in the middle of the open field at the beginning of the training and test sessions to encourage exploration of the objects. Objects and the test arena were wiped down with 70% ethanol between sessions to remove any olfactory cues. The 15 minute open field test served to habituate the animals to the arena. 24 hours following the habituation session animals were re-introduced to the open field. Three distinct objects were positioned at fixed locations and spatial cues (cardboard triangle and square) were fixed to the walls of the open field. The animals were first subjected to a training period to allow them to learn the location of the objects. Each animal was allowed to freely explore the arena for three 5 minute sessions with 5 minute intervals. During the inter-session period animals were placed in a holding cage in the same room as the test. Following training animals were returned to their home cages. 24 hours later animals were re-introduced to the arena for a single 5 minute trial. In the testing period one of the objects was moved from its original position and placed in a novel position within the open field. A reviewer recorded time spent exploring each of the objects in the open field and the data were expressed as time exploring each object expressed as a percentage of total exploration time. The arena was cleaned with 70% ethanol between each session in order to remove olfactory cues.
3×5 minute training session 1×5 minute test session

24 hours

Figure 2.2.2.6: Object displacement paradigm schematic
2.2.3 Electroconvulsive stimulation (ECS) treatment

Animals were handled daily for one week prior to the start of treatment. Animals were then habituated to ear clip electrodes for one week. Ear clip electrodes were dampened with 0.9% saline solution and attached to the rat's ears. The rat was then placed in a tissue lined treatment box and the ear clip electrodes were then attached to the ECT unit (Ugo Basile, Italy). The charge was then applied rendering the animal unconscious. Parameters for BP ECS were: 0.5ms pulse-width; 100 pulses/s; 0.7 s duration; 75mA current, for UBP ECS the pulse-width was reduced to 0.3ms. "Sham-ECS treated" animals were identically handled to ECS treated animals but received no charge. The durations of the tonic and clonic phases of the seizure were recorded. Animals were then removed to a heated recovery cage and the duration of recovery was monitored.
2.2.4 Magnetic resonance imaging

All magnetic resonance imaging (MRI) was carried out on a dedicated rodent Bruker Biospec system (Bruker BioSpin, Germany) with a 7 Tesla magnet and a 30cm diameter bore, equipped with a 20cm actively-shielded gradient system. A pair of actively decoupled 12 cm Helmholtz transmit and 3 cm surface quadrature receive coils (Bruker BioSpin, Germany) were used for signal transmission and reception respectively. The machine was set up to a workstation running ParaVision 4.0 software (Bruker BioSpin, Germany) for data reconstruction and analysis.

2.2.4.1 Anaesthesia and animal preparation

Animals were anaesthetised using 5% isoflurane and maintained in an anaesthetised state at 1.5%. Animals were subsequently placed onto a custom-built fibreglass cradle and temperature was maintained using a warming surface controlled by a water pump-driven temperature regulator (SA Instruments Inc., Stony Brook, NY, USA). A mechanical ventilator (Ugo Basile, Comerio, VA, Italy) was used to deliver adequate inflowing gas to the facemask and the respiration signal was monitored using custom hardware and software (SA Instruments Inc., Stony Brook, NY, USA). Anaesthetic depth was controlled by maintaining respiration rate in the range of 60 to 75 breaths per minute. The receiver coil was placed over the skull of the animal and fixed with adhesive tape. The cradle was then inserted into the bore of the scanner. Accurate positioning was ensured by acquiring an initial pilot image using a fast gradient echo scan and a single-slice high contrast scan taken at the isocentre of the magnetic field. To maintain consistent positioning throughout all experiments, the animal was repositioned and scanned until the required slice was determined to be in the isocentre.

2.2.4.2 MRI sequences

Accurate positioning of the animal at the centre of the B0 magnetic field was ensured by performing an initial pilot high resolution anatomical scan (T2-weighted RARE; Rapid Acquisition with Relaxation Enhancement) using the following parameters: slice thickness=1.5 mm, repetition time (TR)=3134.511 ms, echo time (TE)=12 ms, RARE factor=8, RF flip angle=90°/180°, field of view (FOV)=3 x 3 cm, image matrix=128 x 128, total scan time= 50 s. This was compared to a rat brain atlas in order to ensure the imaging slice was centred at 2.2mm anterior to and then 4.3mm posterior to bregma to allow consistent imaging of the regions of interest (ROIs) (Paxinos and Watson, 1998).
A continuous arterial spin labelling (cASL) sequence was subsequently applied, as previously described (Kelly et al., 2009). Briefly, the sequence consisted of a 5 second preparation interval which contained the inversion pulse followed by snapshot fast low angle shot (FLASH) acquisition. The sequence was used to provide signal-time curves of the passage of a 3 second bolus through the region of interest. The following acquisition parameters were used: slice thickness = 2 mm, TR = 6.938 ms, TE = 2.63 ms, RF flip angle = 30°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 64. Six repetitions of each image type were acquired for signal averaging.

T1 relaxation times were calculated from a rapid acquisition with relaxation enhancement (RARE) with variable repetition time (RARE-VTR) image. The following acquisition parameters were used: slice thickness = 1.5 mm, TE = 25.3 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with varying repetition time (TR), using values of 300.0, 589.12, 942.3, 1396.1, 2032.0, 3103.1 and 8000.0ms.

T2 relaxation times were calculated from a multi-slice multi-echo (MSME) image. The following acquisition parameters were used: slice thickness = 1.5 mm, TR = 2000 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with varying echo time (TE), using values of 8.06, 16.12, 24.18, 32.24, 40.29, 48.35, 56.41, 64.47, 72.53, 80.59, 88.65 and 96.71ms. An echo train of 12 values was used to ensure signal was at noise level by the last echo times to ensure accuracy of T2 estimation. Only the central slice of the MSME scan was used for analysis of T2 relaxation times.

High resolution anatomical images were acquired using T2-weighted MR axial images were collected using a rapid-acquisition relaxation-enhanced (RARE) sequence. The following acquisition parameters: field of view (FOV) = 4.00 x 3.00cm, image matrix = 512 x 200, 64 x 0.5mm slices, repetition time (TR) = 6.26s, echo time (TE) = 36.00ms

Total scanning time was approximately 60 minutes.
2.2.4.3 MRI data analysis

2.2.4.3.1 Bolus tracking arterial spin labelling

Data were analysed using data acquisition and analysis software, Paravision (Bruker Biospin, Germany), and scripts written in Interactive Data Language (IDL; ITT Visual Information Systems, USA) software version 7.0. In addition to the inbuilt functions of IDL, use was also made of the Coyote IDL Library (Fanning Software Consulting, USA; downloaded from http://www.dfanning.com) to generate perfusion weighted maps. Changes in signal amplitude are represented on a colour scale adjacent to the perfusion weighted maps. Brighter colours on the signal amplitude scale indicate areas of increased cerebral blood volume (CBV) as signal amplitude is directly proportional to the area under the curve and hence to CBV. Brighter areas correspond to those regions of interest with highest CBV.

ImageJ (Rasband, USA) software was used to select ROIs for analysis with reference to a standard rat stereotactic atlas (Paxinos and Watson, 1998) [Figure 2.2.4.3.1]. Analysis of two brain sections at different levels along the coronal plane was carried out. The first coronal section (2.2mm anterior to bregma) comprised primary and secondary motor, somatosensory and pre-limbic cortex as well as striatum. The second coronal section chosen (4.3 mm posterior to bregma) comprised visual, auditory, parietal association and retrosplenial cortex in addition to thalamus and hippocampus.

Mean transit time (MTT), capillary transit time (CTT) and signal amplitude values were generated by fitting the non-compartmental model of cerebral perfusion to the experimental data (Kelly et al. 2009). The curve-fitting routine in Mathematica (Wolfram Research Inc, Version 5.1, Champaign, IL, USA) was used to calculate MTT and CTT from the first and second statistical moments of the signal-time curves respectively. The amplitude of the fitted curve is also reported as it is directly proportional to the area under the curve and therefore an estimate of regional cerebral blood volume (Kelly et al., 2010). MTT and CTT are inversely proportional to cerebral blood flow.
Figure 2.2.4.3.1: Regions of interest acquired for ASL analysis
2.2.4.3.2 Manual volume measurements

A single reviewer blinded to strain/treatment used MIPAV software to manually trace out regions of interest slice by slice along the coronal plane. Regions of interest analysed included both lateral and third ventricular volume, cortex, corpus striatum, midbrain, and hippocampus. The regions were defined with reference to anatomical landmarks set out in the rat brain atlas (Paxinos and Watson, 1998), these are summarised in Table 2.1 [adapted from (Vernon et al., 2010)]. Manual tracing of these regions along the coronal plane formed a three dimensional mask which could be then quantified using MIPAV tools. This was then normalised for brain size by calculating the relative percentage volume of each region in relation to total brain volume. To assess intra-rater reliability 5 brains were randomly selected and re-segmented, intraclass correlation coefficient (ICC) was then calculated for each region analysed. For all brain regions analysed ICC was greater than 0.9, suggesting reliable brain segmentation.

Table 2.1: Anatomical criteria for volume measurements

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Anatomical criteria for measurements</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>Defined with reference to corpus callosum, starting at the fornix above the olfactory tubules and finishing after the hippocampus splits into two separate hemispheres.</td>
<td>0.99</td>
</tr>
<tr>
<td>Corpus striatum</td>
<td>Defined with reference to corpus callosum, external capsule, anterior commissure and lateral ventricles.</td>
<td>0.98</td>
</tr>
<tr>
<td>Midbrain</td>
<td>Defined with reference to dorsal hippocampal formation.</td>
<td>0.97</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Defined with reference to the corpus callosum.</td>
<td>0.97</td>
</tr>
<tr>
<td>Ventrices</td>
<td>Including both lateral and third ventricles and defined from brain areas of intensive CSF contrast.</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Figure 2.2.4.3.2: Representative MR images depicting sample regions of interest for volumetric analysis
2.2.4.3.3 T1 and T2 analysis

Analysis of T1 and T2 relaxation times was performed on the RARE-VTR and MSME images respectively. All analysis was performed using the image sequence analysis (ISA) tool in the Bruker Paravision 4.0 Software package. Regions of interest selected using the ROI tool included, the pre-limbic cortex, the primary and secondary motor cortex, the striatum and the somatosensory cortex 2.2mm anterior to bregma and the cortex, hippocampus and thalamus 4.3mm posterior to bregma bilaterally. The same regions in each animal were used for the analysis of both T1 and T2 relaxation times.
2.2.5 Surgical procedures
Surgical procedures were performed in a dedicated surgery room, paying particular attention to hygiene and sterility.

2.2.5.1 L-AAA administration via stereotactic surgery
All surgical procedures were performed under isoflurane (IsoFlo, Abbott Animal Health, UK) anaesthesia with induction at a concentration of 2% and maintained at a concentration of 1.5-2% in 2 L/min O₂. Once anaesthetised the rats were fitted in a stereotactic surgery frame, with incisors placed into the tooth bar, and ear bars inserted into each ear canal in order to hold the head securely in place. Isoflurane was supplied via a nose mask, with waste gas being eliminated by a scavenger system. A test of the pedal reflex was used to ensure the animal was sufficiently anaesthetised. The head was shaved, prepared with surgical scrub (Betadine, Medlock Medical) and a midline sagittal incision was made with a scalpel to the skin overlying the skull. The dura mater was removed in order to expose bregma, from which all surgical co-ordinates were taken. Burr holes were drilled in the skull to facilitate bilateral injection. Behavioural phenotypic changes induced following toxin administration to three distinct brain regions were assessed [co-ordinates derived from (Paxinos and Watson, 1998), see figure 2.2.5.1]. Co-ordinates for the regions analysed are listed in Table 2.2 and depicted in figure 2.2.5.1. L-2-aminoadipic acid (Sigma, A7275-IG) was dissolved in saline solution (0.9% NaCl) to a final concentration of 25mg/ml, with pH adjusted to 7.4. Injections in the pre-limbic cortical region were achieved using long-shanked, volume-calibrated micro-capillary tubes (Sigma, P0549) pulled to achieve a tip size of 25-30 μm. Injections into the hippocampus and amygdala were performed using a Hamilton syringe (7105KH).

L-AAA or saline solution was injected bilaterally into the delivery region at a rate of 0.4μl per minute to a final volume of 2 μl per hemisphere. This dose was chosen based on previously published reports (Banasr and Duman, 2008). The capillary or syringe was left in situ for an additional two minutes at each injection site to prevent reflux, and was withdrawn slowly over a two minute period. Surgibond tissue adhesive was used to close the wound and topical antiseptic cream (Savlon*) was applied to the incision area. The time from initial anaesthesia to completing the procedure was approximately 40 minutes. Animals underwent a second surgery 24 hours later and received a second 2μl
dose per hemisphere of L-AAA or saline at the same co-ordinates. The animals recovery was monitored in home cages.

Table 2.2: Co-ordinates for administration of LAAA to specific brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Posterior/anterior</th>
<th>Lateral</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre limbic cortex</td>
<td>+3.2mm</td>
<td>±0.5mm</td>
<td>4mm</td>
</tr>
<tr>
<td>Basolateral Amygdala</td>
<td>-3mm</td>
<td>±4.6mm</td>
<td>8.6mm</td>
</tr>
<tr>
<td>Ca3 Hippocampus</td>
<td>-4.3mm</td>
<td>±4.2mm</td>
<td>4.1mm</td>
</tr>
</tbody>
</table>

Figure 2.2.5.1: Co-ordinates of stereotactic injections

[Adapted from Paxinos and Watson (1998)]
2.2.5.2 Bilateral olfactory bulbectomy (OB)

The surgical removal of both the right and left olfactory bulbs was performed as previously described (Kelly et al., 1997). Briefly, rats were anaesthetised with 3% isoflurane (IsoFlo, Abbott Animal Health, UK) in O₂, mounted on a stereotactic frame and maintained under anaesthesia with 1.5-2% isoflurane in 2 L/min O₂. The head was shaven, prepared with surgical scrub (Betadine, Medlock Medical) and a midline sagittal incision was made with a scalpel to the skin overlying the skull. The dura mater was removed in order to expose bregma, from which all surgical co-ordinates were taken. Two burr holes of 2 mm diameter were then drilled 7.5-8 mm rostral to bregma, and 2 mm lateral to the midline on each side. The olfactory bulbs were aspirated using a vacuum suction pump. After the operation, bleeding was controlled with haemostatic sponge (Haemofibrine, Specialities Septodont, France). For sham animals the procedure was identical and the dura mater was carefully pierced without aspirating the bulbs. Once the bleeding was contained the skin was closed with surgical staples and antiseptic cream was applied (Savlon®). Animals were returned to their home cage after recovery from anaesthesia. Analgesia (disprol® containing aspirin and saccharin in the drinking water) was provided and wet food was placed inside the cage for the first day post-surgery. The general appearance of the animals and their body weights were monitored daily. Rats were given 14 days recovery prior to any further treatment or behavioural tests and were handled daily throughout the recovery period to eliminate any aggressiveness that would otherwise arise.
2.2.6 Immunohistochemistry

2.2.6.1 Tissue preparation
For preparation of tissue for immunohistochemical analysis animals were transcardially perfused. Animals were anaesthetised using urethane at a concentration of 0.41g/ml. A small incision was made at the apex of the left ventricle and a gavage was inserted into the aorta to ensure complete perfusion of the systemic circulation. A right atrial incision was also made to prevent blood from re-entering the systemic circulation. Ice cold phosphate-buffered saline (PBS) was infused through the gavage over a 5 minute period followed by 4% paraformaldehyde over 10 minutes. The perfused brains were dissected free and post-fixed in 4% paraformaldehyde for 24 hr followed by immersion in a cryoprotectant sucrose solution (sucrose 30% w/v in PBS) for 48 hours. Brains were then snap frozen in isopentane cooled on dry ice. Brains were sliced, using a cryostat, into 30μm thick sections which were immediately transferred to storing solution (sucrose 30% w/v, ethylene glycol 30% v/v in PBS). Sections were stored at -80°C.

2.2.6.2 GFAP Immunofluorescence
Sections were defrosted and washed 3 times in PBS for 10 minutes and then incubated in blocking solution for 40 minutes at room temperature (10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS). Sections were washed in PBS once for 5 minutes and incubated overnight in either negative control solution (1% bovine albumin serum (BSA) and 0.1% Triton X-100 in PBS) or in primary antibody solution (negative control solution including rabbit polyclonal anti-GFAP 1:1000, DAKO) at 4°C. Sections were washed 3 times in PBS for 5 minutes and then incubated in the secondary antibody (1:1000 alexa 488nm fluorescent secondary anti-rabbit (Invitrogen) and 1% BSA in PBS) for 2 hours at room temperature. Sections were washed 3 times in PBS for 5 minutes and mounted onto gelatin soaked slides and any excess liquid was removed. Vectashield mounting medium containing DAPI (Vector laboratories) was applied and coverslips were placed on the slides and sealed with nail varnish. The sections were stored at 2-4°C until ready for use.

2.2.6.2 IBA-1 DAB immunohistochemistry
Sections were defrosted and washed 3 times in PBS for 10 minutes and then incubated in 0.75% H₂O₂ solution containing 5% methanol to quench endogenous peroxidises. Sections were washed 3 times in PBS for 5 minutes and then incubated in blocking solution for 30 minutes at room temperature (10% NGS and 0.1% Triton X-100 in PBS).
Sections were washed in PBS once for 5 minutes and incubated overnight in either negative control solution (2% NGS and 0.05% Triton X-100 in PBS) or in primary antibody solution (negative control solution including rabbit polyclonal anti-IBA-1 1:3000, DAKO) at room temperature. Sections were washed 3 times in PBS for 5 minutes and then incubated in the secondary antibody (Biotinylated goat anti-rabbit; Vectastain Elite ABC kit; Vector Laboratories) for 90 minutes. Sections were washed in PBS 3 times for 5 minutes and then incubated in avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories) for 90 minutes. Sections were washed in PBS 3 times for 5 minutes and then incubated in DAB (4mg/ml) solution for 7 minutes. Sections were washed twice in distilled H2O for 5 minutes and then twice in PBS for 5 minutes. Sections were then mounted onto gelatin soaked slides and allowed to air dry overnight. Sections were dehydrated using three increasing concentrations of ethanol (70%, 85%, 100%) and then cleared with xylene. Coverslips were then applied using DPX mounting medium. The slides were left to dry for 24 hours. Sections were imaged using the Cell-D software linked to an Olympus brightfield microscope.

2.2.6.4 GFAP imaging and cell counting
Image stacks of the regions of interest were acquired at 200x magnification using the Axiovert 200M microscope and Zeiss M Image software. Selected images were then imported into ImageJ for processing and quantification. Image stacks were converted into single images allowing for the quantification of cells throughout the thickness of the slice. GFAP positive cells were quantified using a method adapted from (Gosselin et al., 2009). GFAP immunoreactive cells were only quantified if they met three criteria: (1) Continuous labelling with at least three distinct processes, to prevent quantification of single processes transversally crossing the slice; (2) did not cross the limit of the acquired field; (3) was not involved in the formation of a blood vessel, as astrocytes surrounding vessels are often not individually distinguishable. The total number of GFAP positive cells to meet these criteria were counted in three 250µm by 250µm contours per region. The number of cells was then averaged and expressed as number of cells per mm². Counts were taken from the right and left hemisphere of each slice and averaged to give the final number of GFAP immunoreactive cells present.
Figure 2.2.6.4: Regions analysed for GFAP positive cell quantification

GFAP positive cell numbers were assessed in 1) retrosplenial cortex, 2) visual cortex, 3) CA1 hippocampus, 4) CA2 hippocampus, 5) CA3 hippocampus, 6) Dentate gyrus, 7) CA1 hippocampus, 8) CA2 hippocampus, 9) CA3 hippocampus, 10) dentate gyrus, 11) Basolateral amygdala, 12) primary motor cortex, 13) pre-limbic cortex.
2.2.7 Fresh tissue collection
Rats were sacrificed by decapitation. The brain was carefully removed from the skull and placed on a petri dish containing dry ice. Tissue samples of interest were retrieved and snap frozen on dry ice, and then stored at -80°C until use.

2.2.7.1 Total RNA extraction
Isolation of total RNA from dissected tissue was carried out using NucleoSpin® RNA II kits (Macherey-Nagel) as instructed in the manufacturer’s protocol. Prior to this all surfaces and equipment were wiped down with RNase away wipes, which degrades any RNAse present that might reduced the yield of total RNA being extracted. In brief, up to 30mg of tissue, that had been snap frozen on dry ice and stored at -80°C, was weighed out and placed into a lysis buffer mixture of 350μl RA1 buffer and 3.5μl β-mercaptoethanol. This tissue was then disrupted using a polytron, until there was a uniform mixture present. Following homogenisation, the lysate was placed in a Nucleospin Filter unit and filtered by centrifuging for 1min at 11,000g. 350μl of 70% ethanol was then added to the lysate and pipetted up and down several times until dissolution occurred. This lysate-ethanol mixture was loaded into a Nucleospin II column and centrifuged for 30 seconds at 11,000g. The column containing nucleic acid was retained and desalted by adding 350μl of a membrane desalting buffer and centrifuging for 1 minute at 11,000g. At this point DNase was added to the column and incubated for 15 minutes at room temperature to remove any DNA present in the column. This was then deactivated by passing 200μl of the “RA2” solution through the column by centrifuging the column for 30 seconds at 11,000g. The column was then washed on two occasions with the “RA3” buffer, 600μl centrifuged at 11,000g for 30 seconds and 200μl centrifuged for 2 minutes at 11,000g. The column was then placed in a nuclease free collecting tube and the total RNA present was eluted with 60μl RNase free water via centrifugation at 11,000g for 1 minute. The total RNA samples were then either stored at -80°C until ready for the next step or equalised immediately post extraction.

2.2.7.2 RNA equalisation
Total RNA samples, previously extracted and stored -80°C, were removed from the freezer, allowed to defrost slowly on ice and were kept on ice for the duration of this procedure. A spectrophotometer (Nanodrop ND-1000) was used to measure total RNA concentrations. The spectrophotometer was blanked with nuclease free H₂O. 1μl of each sample was placed on the pedestal and read, with results expressed as ng/μl. The purity
of the sample was judged using the A260/280. Once all of the concentrations were calculated, the samples were all equalised to the sample with the lowest concentration detected, using nuclease free H$_2$O.

2.2.7.3 Complimentary DNA synthesis
In brief, a master mix solution was made up using reverse transcription buffer, dNTPs, random primers, multiscribe reverse transcriptase and RNase free H$_2$O [Table 2.3] 10µl of RNA from each sample was added to 10µl of the master mix in micro tubes. These tubes were then placed in a thermocycler (Peltier Thermal Cycler- 200) and set to the following program: 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. When the cDNA synthesis was completed, each sample was diluted 1:5 with nuclease free H$_2$O (20µl cDNA + 80µl of H$_2$O), and samples were stored at -20°C until needed.

Table 2.3: Volumes of the components of a cDNA synthesis master mix

<table>
<thead>
<tr>
<th>Master Mix</th>
<th>Volume per sample (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT buffer</td>
<td>2</td>
</tr>
<tr>
<td>25x dNTPs</td>
<td>0.8</td>
</tr>
<tr>
<td>10x Random Primers</td>
<td>2</td>
</tr>
<tr>
<td>Multiscribe RT (50u/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease free H$_2$O</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>
2.2.7.4 Real-time polymerase chain reaction (PCR)

Target gene expression was assessed using a Taqman® gene expression assay, which contain specific target primers and FAM-labelled MGB target probes (Applied Biosystems). β-actin gene expression was used as an endogenous control between samples and was quantified using specific primers and a VIC-labelled MGB probe.

Table 2.4: List of rat primers used in QPCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Taqman® Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Rn00667869_ml</td>
</tr>
<tr>
<td>CD11b</td>
<td>Rn00709342_ml</td>
</tr>
<tr>
<td>CD40</td>
<td>Rn01423590_ml</td>
</tr>
<tr>
<td>eNOS</td>
<td>Rn02132634_ml</td>
</tr>
<tr>
<td>GDNF</td>
<td>Rn00755092_ml</td>
</tr>
<tr>
<td>GFAP</td>
<td>Rn00566603_ml</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Rn00568080_ml</td>
</tr>
<tr>
<td>GLAST</td>
<td>Rn00570130_ml</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Rn01483107_ml</td>
</tr>
<tr>
<td>iNOS</td>
<td>Rn00561646_ml</td>
</tr>
<tr>
<td>nNOS</td>
<td>Rn00583793_ml</td>
</tr>
<tr>
<td>S100B</td>
<td>Rn00566139_ml</td>
</tr>
</tbody>
</table>

In brief, diluted cDNA samples which had been previously synthesised and stored at -20°C were removed from the freezer and allowed to defrost on ice. Once defrosted each sample was added to an individual well on a fast PCR plate (Thermo Scientific). A master mix solution was made up with Taqman® fast Advanced Master Mix, the endogenous control primer, and the target primer of interest [Table2.5] This master mix was then
added to each well and the plate sealed with an optically clear plastic cover, before being pulse centrifuged for a few seconds to ensure all of the sample cDNA and master mix was at the bottom of the wells.

<p>| Table 2.5: Volumes of the components in each well of a PCR plate |
|---------------------------------|------------------|</p>
<table>
<thead>
<tr>
<th>PCR mix (per well)</th>
<th>Volumes (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA (diluted 1 in 5)</td>
<td>4</td>
</tr>
<tr>
<td>Fast Taq</td>
<td>5</td>
</tr>
<tr>
<td>Endogenous control</td>
<td>0.5</td>
</tr>
<tr>
<td>Target gene</td>
<td>0.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10</td>
</tr>
</tbody>
</table>

The sealed plate was inserted into Step One Plus real time PCR system (Applied Biosystems) and run on the following settings: 50°C for a period of 2 minutes, followed by 95°C for 20 seconds. This constituted the first step. The second step was repeated 40 times and consisted of a denaturation temperature of 95°C for 1 second, followed by a temperature of 60°C for 20 seconds, the annealing and extension phase, during which the fluorescence was read by the machine. During this second step, the double stranded DNA is denatured at 95°C for 1 second, as the temperature falls to 60°C, the target probe binds to the single stranded coda first, owing to its higher melting point in comparison to the target primers. This probe contains the FAM/VIC dye and a proprietary no-fluorescent quencher (NFQ) dye. This prevents the dye from emitting a fluorescence signal by fluorescence resonance energy transfer (FRET) technology (Applied Biosystems). When the temperature reaches 60°C the target primers anneal and the strand is extended at the 5' end by the nuclease activity of Taq polymerase. This displaces the FAM/VIC labelled probe which in turn causes the FRET between the dye and quencher to be broken, leading to the generation of a fluorescent signal being generated. Due to the specificity of the probe and the primers of the cDNA sequence, one fluorescent signal is generated for each new cDNA copy.

2.2.7.5 Real-time PCR analysis

Assessment of gene expression of all the real-time PCR was carried out using the AACT method (Applied Biosystems RQ software). This method is used to assess relative gene expression by comparing the gene expression of treatment samples to that of a control sample, rather than quantifying the exact copy number of the target gene. This allows
for fold difference to be assessed between samples of different treatments. The fold
difference is calculated using the cycle number (CT) difference between samples. To
measure this difference, the CT of the endogenous control (β-actin) is subtracted from
the CT of the target gene for each sample, which accounts for any variations in cDNA
quantity (termed ΔCT). The ΔCT of a control sample is subtracted from itself and all of
the other samples giving the ΔΔCT value (cycle difference corrected for endogenous
control). This can then be converted into a fold difference from which the related
expression change can be calculated.
2.2.8 Tissue preparation for Western blotting

Tissue samples stored at -80°C were removed and adjusted to ~30mg. This tissue was then added to 300µl of lysis buffer (50nM Tris HCl, 150nM NaCl, 1% Triton-X-100) that contained 1% phosphatase and 1% protease inhibitors. The samples were mechanically homogenised with a polytron in a 2ml round bottomed microtube. These homogenised samples were centrifuged at 11,000g at 4°C for 5 minutes and the supernatant removed. Protein concentration of this supernatant was determined using a bicinchoninic (BCA) assay and samples were equalised with lysis buffer. The equalised samples were then added to Laemmli sample buffer (1:1, 1M Tris-HCl [pH 6.8], 25% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 2% (w/v) bromphenol blue) and boiled at 60°C for 5 minutes proper to loading on gels for SDS-PAGE.

2.2.8.1 Protein quantification – BCA protein assay

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Protein content of samples used for western blot was quantified using this assay. A working solution of 2000µg/ml of bovine serum albumin (BSA) was prepared using lysis buffer stock as the diluent. A set of dilutions were prepared to give final protein concentrations of: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 µg/ml BSA. Next, 25µl of the dilutions and of the individual samples were pipetted in duplicate into the wells of a new 96-well plate. A BCA working solution was prepared by adding 1 part of the BCA working reagent B to 50 parts of reagent A. 200µl of this working solution was added to each sample/standard. The 96-well plate was then covered and incubated at 37°C for 30 minutes. The plate was subsequently cooled to room temperature and the absorbance read at 560nm using a microtitre plate reader (Elx800; Biotek, Germany). A standard curve was constructed by plotting the standards against the absorbance recorded. From this, the concentration of the samples were obtained and expressed as µg/ml of protein. The samples were equalised by adding the required volume of lysis buffer as a diluent.

2.2.8.2 SDS-PAGE

Sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was used on the equalised protein samples to separate them for subsequent analysis via western immunoblotting.

Polyacrylamide gels were divided in to two gels of distinct concentrations, a 12% separating gel (1.675ml distilled H₂O, 2ml Bis/acrylamide, 1.25ml Tris-HCl [pH8.8], 50µl
SDS, 25µl APS, 3µl TEMED per gel) and a 4% stacking gel (1.525ml distilled H₂O, 325µl Bis/acrylamide, 625µl Tris-HCL [pH6.8], 25µl SDS, 12.5µl APS, 4µl TEMED per gel). Two glass plates were cleaned with 70% ethanol and arranged 1mm apart using a spacer. The plates were placed on top of a rubber seal and held securely in place with a casting frame. The 12% separating gel was prepared and, after mixing, quickly pipetted between the two glass plates. A temporary layer of isopropanol was placed on top of this while it set to avoid dehydration of the gel. The gel was allowed to set for 30 minutes. Once the gel had solidified, the isopropanol layer was aspirated off and the gel washed with distilled H₂O. The 4% stacking gel was then pipetted on top of the separating gel. A 10-well comb was carefully inserted into the stacking gel and allowed to set for 30 minutes. Once set the gels were arranged in the gel rig. The upper and lower reservoirs were filled with electrode running buffer (125mM Trizma Base, 960mM glycine, 0.5% SDS). The well combs were carefully removed and 10µl of equalised sample was added to each well. 5µl of a molecular marker (BioRad dual colour) was added to each gel. At least one representative of each treatment group was added to each gel. The proteins were separated by applying a constant current of 33mA per gel for 60 minutes or until the blue dye from the sample buffer had run to the bottom of the gel.

2.2.8.3 Semi-dry transfer

Following electrophoresis, separated proteins were transferred from the polyacrylamide gel onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry blotter at a constant current of 100mA per gel for a period of 70 minutes. A sandwich consisting of six pieces of filter paper, a piece of PVDF membrane and the gel was created on the transfer rig. Two pieces of filter paper soaked in Anode buffer I (0.3M Tris-HCl, pH10.4, 10% methanol) were placed on the anode (+) plate of the semi-dry blotter and onto this a piece of filter paper soaked in anode buffer II (25mM Tris-HCl, pH 10.4, 10% Methanol) was placed. The PVDF membrane was first activated by submerging in methanol for 30 seconds followed by 2 minutes in distilled H₂O, before being added to the sandwich. A completed SDS-PAGE gel was removed from the glass plates and placed on top of the PVDF membrane at this point and then lastly three pieces of filter paper soaked in cathode buffer (25mM Tris-HCl, pH 9.4, 40mM Glycine, 10% Methanol) were placed on to the gel to complete the sandwich. Any bubbles that may have been present were removed by gently rolling a Pasteur pipette over the top of the sandwich. The cathode (-) plate was then placed on top of the sandwich and the current applied. In this set-up,
the proteins will migrate from the gel towards the anode plate and hence into the PVDF membrane.

2.2.8.4 Western immunoblotting

Following transfer of the proteins from the gel onto the PVDF membrane, the membrane was immediately blocked in 5% milk (Marvel®) in Tris-buffered saline-Tween 20 (TBS-T) for 1 hour on a rocker at room temperature. The membrane was then washed for 3 times for 10 minutes in TBS-T on a rocker at room temperature before being transferred to the primary antibody at the appropriate dilution (see Table below) and incubated overnight at 4°C on a rocker. The membrane was washed 3 times for 10 minutes in TBS-T and then the secondary antibody at the appropriate dilution (see Table below) was applied to the membrane for a period of 1 hour at room temperature on a rocker. Following this, the membrane was washed 3 times for 10 minutes in TBS-T. The membrane was then exposed to chemiluminescent solution (Millipore, Ireland) and developed on the Fujifilm Luminescent Image Analyzer LAS-3000. Following this step, the membrane was washed 3 times for 5 minutes in TBS-T and stripped in a striping solution for 10 minutes and then washed again 3 times for 5 minutes. At this point the blot was ready to be re-blocked and re-probed with another antibody. Protein bands were quantified using ImageJ software.

Table 2.6: Protein targets for Western immunoblotting

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>1 in 1000</td>
<td>1 in 5000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1 in 1000</td>
<td>1 in 5000</td>
</tr>
<tr>
<td>GFAP</td>
<td>1 in 5000</td>
<td>1 in 5000</td>
</tr>
<tr>
<td>S100B</td>
<td>1 in 1000</td>
<td>1 in 5000</td>
</tr>
</tbody>
</table>
2.2.9 Statistical analysis

Data are expressed as group mean with standard errors and were analysed using unpaired Student’s t tests or analysis of variance (ANOVA) where appropriate. If statistically significant results were found following analysis of variance, post-hoc comparisons were performed using Newman-keuls tests. Statistical analysis was carried out using GB-Stat v.10. Comparisons between two independent groups were carried out by Student’s t-test. Differences between groups were deemed significant when \( p < 0.05 \). Intra-class correlation (ICC) for investigation of intra-rater reliability of analysis of the structural data was carried out using IBM SPSS version 22.
Chapter 3 – The antidepressant response in the OB rat model of depression is associated with changes in MR imaging markers
3.1 Introduction

There is a growing body of evidence suggesting that alterations in glutamatergic signalling plays a role in the development of depressive illness [for review see (Sanacora et al., 2012)]. N-methyl-D-aspartate (NMDA) glutamate receptor antagonists such as ketamine have been shown to produce rapid antidepressant-like activity in human cohorts (Zarate et al., 2006) and in animal models (Gigliucci et al., 2013; Tizabi et al., 2012). Activation of the NMDA receptor is coupled to activation of neuronal nitric oxide synthase (NOS) leading to the production of nitric oxide (NO) (Guix et al., 2005). Treatment with NOS inhibitors such as 1-(2-tri-fluro-methyl-phenyl) imidazole (TRIM) and N"-Nitro-L-arginine (L-NA) has been shown to produce antidepressant like activity in rodent models (Doucet et al., 2013; Gigliucci et al., 2014, 2010). Thus the NMDA receptor and the downstream NOS signalling pathway appear to be promising leads for the development of novel antidepressant compounds. Most tests conducted to assess antidepressant activity in rodent models such as the forced swim test (FST) and tail suspension test respond to acute antidepressant administration [for review see (Cryan and Holmes, 2005)]. This is in contrast the effects of standard antidepressant treatments in the clinical population, in which an antidepressant response occurs only after weeks (Adell et al., 2005).

Bilateral olfactory bulbectomy (OB) in rodents produces a well characterised behavioural phenotype which is sensitive to antidepressant treatment. An advantage of the OB rat as a model of depressive-like behaviour is that hyperactivity in a novel environment induced following OB is attenuated following chronic, but not acute treatment with compounds which possess antidepressant activity [for review see (Harkin et al., 2003; Kelly et al., 1997; Song and Leonard, 2005)]. However although the OB rat shows good predictive validity as an animal model of depressive-like behaviour, questions remain as to the model's face validity. Hence in order to further elaborate on the anatomical and functional alterations induced following OB surgery we undertook a series of studies to investigate whether OB was associated with changes in magnetic resonance imaging (MRI) markers and to what extent such markers were affected by antidepressant treatment. The only other MRI study in the OB rat reported to date suggests that the OB rat may exhibit volumetric abnormalities including enlarged ventricular volume (Wrynn et al., 2000). Recent meta-analyses of structural imaging studies in the clinical literature have implicated alterations in the volume of a number of brain regions including increased ventricular volume, and a decrease in the volume of prefrontal,
dorsofrontomedial, orbitofrontal and cingulate cortex and striatum (Arnone et al., 2012; Bora et al., 2012; Sacher et al., 2012), as well as changes in the volume of the hippocampus (Videbech and Ravnkilde, 2004) and amygdala (Sacher et al., 2012) [see also (Kempton et al., 2011)]. Any similar alterations in regional brain volume in the OB rat would improve its validity as an animal model of depression.

The OB rat has previously been shown to exhibit alterations in glutamatergic signalling as evidenced by reduced NMDA receptor density in the frontal cortex and amygdala relative to sham operated controls (Robichaud et al., 2001; Webster et al., 2000). Furthermore chronic daily dosing with NMDA receptor antagonist MK-801 has been shown to attenuate OB-induced hyperactivity in the open field (Redmond et al., 1997), and riluzole an agent which modulates glutamatergic neurotransmission, has been shown to exhibit an antidepressant like response in the OB model which is associated with a decrease in extracellular glutamate in the frontal cortex of OB rats (Takahashi et al., 2011). We sought to investigate if chronic dosing with the NOS inhibitor L-NA produced an antidepressant response in the OB model. Chronic treatment with tri-cyclic antidepressant imipramine served as a positive control. Although in recent years, there have been major advances in our understanding of the neural circuits and brain structures implicated in depressive illness, there is still a paucity of biological and imaging markers which can serve as predictors or markers of therapeutic response to antidepressant treatments (Phillips et al., 2015). Multimodal scanning in animal models may provide us with leads on how such markers may present in the clinic. MR scans were acquired to assess if the antidepressant response following chronic drug administration in the OB rat model was associated with any detectable change in resting state blood perfusion as assessed via a bolus tracking arterial spin labelling (ASL) sequence or in brain structure via a high resolution anatomical scan. Scans to assess regional T1 and T2 relaxation times were also acquired, T1 relaxation times have previously been positively correlated with astroglial function (Cowley et al., 2012), while T2 relaxation times may be associated with microglial function (Justicia et al., 2008).

We have previously shown that 7 days treatment with nNOS inhibitor TRIM is sufficient to produce an antidepressant response in the OB rat model (Gigliucci et al., 2014). A second study was carried out to investigate whether sub-acute dosing with the NMDA receptor antagonist ketamine was sufficient to attenuate OB induced hyperactivity in the open field. 6 infusions of ketamine administered thrice weekly has previously been
shown to induce a prolonged antidepressant response in clinical cohorts (Murrough et al., 2013). We used a similar dosage schedule in the current study to investigate if ketamine administration was associated with a rapid antidepressant response in the model and if any such response was associated with changes in MR imaging markers.

3.1.1 Aims and objectives

The aim of the experiments described in this chapter was to assess whether altered behaviour induced following OB was associated with changes in MR imaging markers. A second aim of the present experiments was to assess if chronic dosing with NOS inhibitor L-NA produced an antidepressant effect in the OB rat comparable to chronic dosing with classical tri-cyclic antidepressant imipramine. We also sought to identify if the antidepressant response in the model was associated with any changes in MR imaging markers. A final aim was to investigate if sub-acute dosing with NMDA receptor antagonist ketamine was sufficient to produce an antidepressant response in the OB rat model of depression and again assess any changes in MR imaging markers associated with ketamine administration.
3.2 Methods

3.2.1 Characterisation of MR imaging markers associated with OB and the antidepressant effect of imipramine and L-NA

Male Sprague-Dawley rats (250-300g) were housed 4 per cage. Animals were habituated to the animal facility for at least one week prior to bilateral OB surgery. Animals were given a 2 week recovery period following surgery prior to the commencement of drug treatment. Imipramine was dissolved in maple syrup and administered orally once daily at a dose of 20mg/kg. L-NA was dissolved in maple syrup and administered orally once daily at a dose of 10mg/kg. Respective doses were chosen based on previous work which showed that they were sufficient to induce an antidepressant response in animal models (Breuer et al., 2009; Gigliucci et al., 2010). Animals were administered drugs for 17 days. 24 hours following the last drug administration animals were tested to assess the impact of drug treatment on OB-induced hyperactivity in the open field. 24 hours following the open field test MRI data was acquired. All behavioural readouts were performed in dedicated rooms by one of two experimenters. A separate cohort of animals was used to assess inflammatory marker expression 21 days following OB surgery. All procedures were conducted as outlined in the Methods section (See Chapter 2).

![Figure 3.2.1: Imipramine and L-NA study design](image)

MRI, magnetic resonance imaging; OB, olfactory bulbectomy; OF, open field.
3.2.2 Characterisation of MR imaging markers associated with sub-acute ketamine administration in the OB rat

Male Sprague-Dawley rats (250-300g) were housed 4 per cage. Animals were habituated to the animal facility for at least one week prior to bilateral OB surgery. Animals were given a 2 week recovery period following surgery prior to the commencement of drug treatment. Ketamine was administered at a dose of 10mg/kg by intraperitoneal (i.p.) injection. Dose was chosen based on previous work which showed that 10mg/kg was sufficient to produce an antidepressant response in rodents (Gigliucci et al., 2013). 6 doses of ketamine were administered in total. The first three were administered at 4 daily intervals beginning 18 days following OB surgery. Repeated open field tests were performed to determine if acute ketamine administration was associated with an antidepressant response in the OB model. There was no significant change in the bout speed parameter following ketamine administration at any of these time points. As such only the data from the final open field session is reported here, as it bears most relevance to the MRI data acquired. The final three doses were administered daily for the three days prior to the final open field test. The final dose was administered 24 hours prior to the open field test. 24 hours following the open field test MRI data was acquired. All behavioural readouts were performed in dedicated rooms by a single experimenter. All procedures were conducted as outlined in the Methods section (See Chapter 2).

Figure 3.2.1: Ketamine study design
MRI, magnetic resonance imaging; OB, olfactory bulbectomy; OF, open field.
3.3 Results

3.3.1 Characterisation of MR imaging markers associated with OB and the antidepressant effect of the tri-cyclic antidepressant imipramine and NOS inhibitor L-NA

The behavioural response of the OB rat to treatment with the tri-cyclic antidepressant imipramine or NOS inhibitor L-NA was assessed in the open field test. In addition a battery of MR neuroimaging markers associated with OB and with the antidepressant response were acquired.

3.3.1.1 Chronic treatment with L-NA and imipramine produce an antidepressant response in the open field test

Following imipramine treatment two way analysis of variance (ANOVA) of bout speed revealed an effect of OB \( [F_{(1,30)}=9.94985, p=0.0042] \) [Figure 3.3.1.1 (a)] and imipramine \( [F_{(1,30)}=12.8071, p=0.0013] \) [Figure 3.3.1.1 (a)]. Newman-keuls \textit{post hoc} comparisons revealed that the OB-vehicle group exhibited a significant increase in bout speed relative to the sham-vehicle group \( (p<0.01) \) which was attenuated by imipramine treatment \( (p<0.01) \).

Following L-NA treatment two way ANOVA of bout speed revealed an effect of OB \( [F_{(1,27)}=4.78924, p=0.0386] \) [Figure 3.3.1.1 (b)]. Newman-keuls \textit{post hoc} comparisons revealed that the OB-vehicle group exhibited a significant increase in bout speed relative to the sham-vehicle group \( (p<0.05) \) which was attenuated by L-NA treatment \( (p<0.05) \).
Figure 3.3.1.1: Chronic treatment with L-NA and imipramine produce an antidepressant response in the open field test

Imipramine (a) and L-NA (b) attenuate OB induced hyperactivity in the open field test. Data expressed as mean ± SEM (n=6-9). * p<0.05 relative to sham-vehicle group; ** p<0.01 relative to sham-vehicle group; + p<0.05 relative to OB-vehicle group; ++ p<0.01 relative to OB-vehicle group
3.3.1.2 L-NA and imipramine treatment are associated with changes in regional brain volumetrics in the OB rat

Following imipramine treatment, two way ANOVA of ventricular volume as % total brain volume revealed a significant effect of OB \([F_{(1,27)}=17.70094, p=0.0003]\) [Figure 3.3.1.2a (a)]. Two way ANOVA of hippocampal volume as % total brain volume revealed no significant effects of OB \([F_{(1,27)}=1.61704, p=0.2157]\) [Figure 3.3.1.2a (b)] or treatment \([F_{(1,25)}=0.4872, p=0.4872]\) [Figure 3.3.1.2a (b)].

Following L-NA treatment, two way ANOVA of ventricular volume as % total brain volume revealed a significant effect of OB \([F_{(1,25)}=20.36443, p=0.0002]\) [Figure 3.3.1.2a (a)] and an OB \(\times\) L-NA interaction \([F_{(1,25)}=5.10423, p=0.0341]\) [Figure 3.3.1.2a (a)]. Newman-keuls post hoc comparisons revealed that L-NA treatment was associated with an increase in ventricular volume as % total brain volume when compared to the OB-vehicle treated group \((p<0.05)\). Two way ANOVA of hippocampal volume as % total brain volume revealed no significant effects of OB \([F_{(1,25)}=0.05622, p=0.8148]\) [Figure 3.3.1.2a (b)] or treatment \([F_{(1,25)}=0.4083, p=0.7107]\) [Figure 3.3.1.2a (b)].
Figure 3.3.1.2a: L-NA and imipramine treatment are associated with changes in regional brain volumetrics in the OB rat

The effects of OB and of drug treatment were assessed on (a) ventricular volume and (b) hippocampal volume. Data expressed as mean percentage of total intracranial volume ± SEM (n=6-9). + p<0.05 vs. OB-vehicle group
Figure 3.3.1.2b: Representative images depicting changes in ventricular volume following OB and antidepressant treatment

Representative images depicting lateral ventricular volume in a) the sham-vehicle group, b) the OB-vehicle group, c) the OB-imipramine group, d) the OB-L-NA group. Arrows identify lateral ventricles.
3.3.1.3 L-NA and imipramine treatment are not associated with robust cerebral blood flow changes in the OB rat

Following imipramine treatment, two way ANOVA of mean transit time (MTT) and capillary transit time (CTT) in the third ventricular region revealed an OB x treatment interaction \(F_{(1,30)}=4.49529, p=0.0433\) [Table 3.3.1], \(F_{(1,30)}=4.55329, p=0.0421\) [Table 3.3.1] respectively.

Following L-NA treatment, two way ANOVA of MTT in the auditory cortex revealed an effect of treatment \(F_{(1,26)}=8.1583, p=0.0087\) [Table 3.3.1]. Newman-keuls post hoc comparisons revealed that the sham-L-NA treated group exhibited a decrease in MTT relative to the sham-vehicle group (\(p<0.05\)). Two way ANOVA of MTT in the hippocampus revealed an effect of treatment \(F_{(1,27)}=7.5285, p=0.0113\) [Table 3.3.1]. Newman-keuls post hoc comparisons revealed that the OB-L-NA group exhibited an increase in MTT relative to the OB-vehicle group (\(p<0.05\)). Two way ANOVA of signal amplitude in the thalamus revealed an effect of treatment \(F_{(1,27)}=5.01022, p=0.0347\) [Table 3.1]. Two way ANOVA of MTT in the third ventricle showed an OB x treatment interaction \(F_{(1,25)}=6.21206, p=0.0203\) [Table 3.3.1], CTT in the third ventricle also showed an OB x treatment interaction \(F_{(1,25)}=5.16806, p=0.0327\) [Table 3.3.1] while signal amplitude in the third ventricle showed an effect of treatment \(F_{(1,25)}=6.00613, p=0.0219\) [Table 3.3.1].
### Table 3.3.1: L-NA and imipramine treatment are not associated with robust cerebral blood flow changes in the OB rat

<table>
<thead>
<tr>
<th>Table 3.3.1: L-NA and imipramine treatment are not associated with robust cerebral blood flow changes in the OB rat</th>
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<tbody>
<tr>
<td><strong>Sham</strong></td>
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<tr>
<td>Auditor cortex</td>
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<td>Hippocampus</td>
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<td>Thalamus</td>
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<td>Third ventricle</td>
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<td>Visual cortex</td>
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<td>Parietal association cortex</td>
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<tr>
<td>Retrosplenial cortex</td>
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</table>

Data expressed as mean ± SEM (n=6-9). * p<0.05 vs. sham-vehicle group; + p<0.05 vs. OB-vehicle group;
3.3.1.4 OB and L-NA treatment are associated with changes in T1 and T2 relaxation times in the rodent brain

In the imipramine treated groups, two way ANOVA of T2 relaxation time in the hippocampus, the retrosplenial and visual cortices revealed an effect of OB \([F(1,30)=6.77393, p=0.0148]\), \([F(1,33)=8.66521, p=0.0065]\) and \([F(1,33)=11.47855, p=0.0021]\) \(\text{[Table 3.3.2]}\) respectively. Newman-keuls post hoc comparisons revealed that the OB-vehicle group exhibited a decrease in T2 relaxation time in both regions relative to the sham-vehicle group \((p<0.05)\).

In the L-NA treated group, two way ANOVA of T1 relaxation time in the auditory cortex and hippocampus revealed an effect of OB \([F(1,26)=4.36743, p=0.0479]\) \(\text{[Table 3.3.2]}\) and an effect of L-NA \([F(1,26)=9.84501, p=0.0046]\) \(\text{[Table 3.3.2]}\) respectively. Newman-keuls post hoc comparisons revealed that the OB-L-NA group exhibited an increase in T1 relaxation time in the hippocampus relative to the OB-vehicle group \((p<0.05)\). Two way ANOVA of T1 relaxation time in the thalamus revealed an effect of L-NA \([F(1,26)=19.4559, p=0.0002]\) \(\text{[Table 3.3.2]}\). Newman-keuls post hoc comparisons revealed that the sham-L-NA group exhibited an increase in T1 relaxation time relative to the sham-vehicle group \((p<0.01)\). Two way ANOVA of T1 relaxation time in the retrosplenial cortex revealed an effect of L-NA \([F(1,27)=10.21558, p=0.0039]\) \(\text{[Table 3.3.2]}\). Newman-keuls post hoc comparisons revealed that the OB-L-NA group exhibited an increase in T1 relaxation time relative to the OB-vehicle group \((p<0.05)\). Two way ANOVA of T1 relaxation time in the visual cortex revealed an effect of L-NA \([F(1,27)=8.35974, p=0.008]\) \(\text{[Table 3.3.2]}\) and an OB \(\times\) L-NA interaction \([F(1,27)=4.70417, p=0.0402]\) \(\text{[Table 3.3.2]}\). Newman-keuls post hoc comparisons revealed that the OB-L-NA group exhibited an increase in T1 relaxation time relative to the OB-vehicle group \((p<0.05)\).

Two way ANOVA of T2 relaxation time in the hippocampus, thalamus and visual cortex revealed an effect of L-NA \([F(1,26)=9.46226, p=0.0053]\), \([F(1,27)=23.30928, p<0.0001]\) and \([F(1,27)=6.01264, p=0.0219]\) \(\text{[Table 3.3.2]}\) respectively and an OB \(\times\) L-NA interaction \([F(1,26)=6.81991, p=0.0156]\) in the hippocampus \(\text{[Table 3.3.2]}\). Newman-keuls post hoc comparisons revealed that the OB-vehicle group exhibited a decrease in T2 relaxation time in the hippocampus relative to the sham-vehicle group and that the OB-L-NA group exhibited an increase in T2 relaxation in the hippocampus relative to the OB-vehicle.
group \((p<0.01)\). Newman-keuls \textit{post hoc} comparisons revealed that the sham-L-NA group exhibited an increase in T2 relaxation time in the thalamus relative to the sham-vehicle group \((p<0.05)\) and the OB-L-NA group exhibited an increase in T2 relaxation time relative to the OB-vehicle group \((p<0.01)\). Two way ANOVA of T2 relaxation time in the retrosplenial cortex revealed an effect of L-NA \([F_{(1,27)}=6.51873, p=0.0175]\) [Table 3.3.2] and an OB x L-NA interaction \([F_{(1,27)}=4.55079, p=0.0433]\). Newman-keuls \textit{post hoc} comparisons revealed that the OB-vehicle group exhibited a decrease in T2 relaxation time relative to the sham-vehicle group \((p<0.01)\) and that L-NA treatment attenuated this change \((p<0.05)\).
Table 3.3.2: OB and L-NA treatment are associated with changes in T1 and T2 relaxation times in the rodent brain

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Imipramine</td>
</tr>
<tr>
<td></td>
<td>T1 (ms)</td>
<td>T2 (ms)</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1813 ± 25.43</td>
<td>1842 ± 14.90</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>50.21 ± 0.35</td>
<td>50.24 ± 0.25</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1907 ± 19.90</td>
<td>1905 ± 16.61</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>52.90 ± 0.31</td>
<td>52.74 ± 0.21</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1705 ± 42.52</td>
<td>1702 ± 38.28</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>46.38 ± 0.21</td>
<td>46.67 ± 0.32</td>
</tr>
<tr>
<td>Third ventricle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1995 ± 68.60</td>
<td>1908 ± 61.51</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>66.17 ± 2.41</td>
<td>65.26 ± 3.23</td>
</tr>
<tr>
<td>Visual cortex</td>
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<tr>
<td>T2 (ms)</td>
<td>52.35 ± 0.31</td>
<td>52.48 ± 0.30</td>
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<tr>
<td>Parietal association</td>
<td></td>
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<tr>
<td>cortex</td>
<td>1880 ± 24.75</td>
<td>1895 ± 15.45</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>50.75 ± 0.27</td>
<td>50.96 ± 0.25</td>
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<tr>
<td>Retrosplenial cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1816 ± 25.35</td>
<td>1819 ± 17.55</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>49.25 ± 0.52</td>
<td>48.81 ± 0.36</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=6-9). * p<0.05 vs. sham-vehicle group; ** p<0.01 vs. sham-vehicle group; + p<0.05 vs. OB-vehicle group; ++ p<0.01 vs. OB-vehicle group
3.3.1.5 OB is not associated with changes in mRNA expression of NOS isoforms or microglial activation markers in either the frontal cortex or the hippocampus

Students t-test revealed that OB results in a significant increase in astrocytic marker glial fibrillary acidic protein (GFAP) \( [t=3.083; \text{df}=12; p=0.0095] \) \[Table 3.3.3\] in the frontal cortex relative to sham operated controls. OB was not associated with other significant changes in mRNA expression of any NOS isoform or in the microglial activation markers CD40 or CD11b observed in either the frontal cortex \[Table 3.3.3\] or in the hippocampus \[Table 3.3.4\] of OB rats relative to sham-operated controls.

Table 3.3.3: OB is not associated with changes in mRNA expression of NOS isoforms or microglial activation markers in the frontal cortex

<table>
<thead>
<tr>
<th>Target</th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>1 ± 0.117</td>
<td>1.152 ± 0.064</td>
</tr>
<tr>
<td>nNOS</td>
<td>1 ± 0.052</td>
<td>1.040 ± 0.110</td>
</tr>
<tr>
<td>iNOS</td>
<td>1 ± 0.155</td>
<td>2.082 ± 0.340</td>
</tr>
<tr>
<td>GFAP</td>
<td>1 ± 0.152</td>
<td>1.794 ± 0.208 **</td>
</tr>
<tr>
<td>CD40</td>
<td>1 ± 0.18</td>
<td>1.496 ± 0.226</td>
</tr>
<tr>
<td>CD11b</td>
<td>1 ± 0.084</td>
<td>1.148 ± 0.067</td>
</tr>
</tbody>
</table>

Table 3.3.4: OB is not associated with changes in mRNA expression of NOS isoforms or microglial activation markers in the hippocampus

<table>
<thead>
<tr>
<th>Target</th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>1 ± 0.092</td>
<td>1.100 ± 0.036</td>
</tr>
<tr>
<td>nNOS</td>
<td>1 ± 0.150</td>
<td>1.115 ± 0.187</td>
</tr>
<tr>
<td>iNOS</td>
<td>1 ± 0.458</td>
<td>3.289 ± 1.154</td>
</tr>
<tr>
<td>GFAP</td>
<td>1 ± 0.178</td>
<td>0.726 ± 0.036</td>
</tr>
<tr>
<td>CD40</td>
<td>1 ± 0.026</td>
<td>1.006 ± 0.084</td>
</tr>
<tr>
<td>CD11b</td>
<td>1 ± 0.300</td>
<td>0.651 ± 0.054</td>
</tr>
</tbody>
</table>

eNOS, endothelial nitric oxide synthase, GFAP, glial fibrillary acidic protein, iNOS, inducible nitric oxide synthase, nNOS, neuronal nitric oxide synthase

Data expressed as mean ± SEM \((n=5-6)\). ** \(p<0.01\) vs. sham-operated group;
3.3.2 Characterisation of MR imaging markers associated with sub-acute ketamine administration to the OB rat

Following on from the finding that NOS inhibitor L-NA produced an antidepressant response in the OB rat comparable to the tri-cyclic antidepressant imipramine, we sought to investigate if sub-acute dosing with NMDA receptor antagonist ketamine was sufficient to produce and antidepressant response as assessed in the open field test. We also wished to establish if repeated ketamine administration was associated with changes to MR neuroimaging markers.

3.3.2.1 Ketamine administration fails to produce an antidepressant effect as assessed in the open field test

Two way ANOVA of bout speed revealed an effect of OB \([F_{(i,3d)}=19.90374, \ p=0.0001]\) [Figure 3.3.2.1]. Newman-keuls post hoc comparisons revealed that the OB-vehicle group (\(p<0.05\)) exhibits an increase in bout speed relative to the sham-vehicle group.

Figure 3.3.2.1: Ketamine administration failed to produce an antidepressant effect as assessed in the open field test

Ketamine does not attenuate OB induced hyperactivity in the open field test. Data expressed as mean ± SEM (\(n=8\)). * \(p<0.05\) relative to sham-vehicle group.
3.3.2.2 Ketamine treatment does not alter the OB-induced increase in ventricular volume

Two way ANOVA of ventricular volume as % total brain volume revealed a significant effect of OB [F(1,29)=13.01975, p=0.0013] [Figure 3.3.2.2a (a)]. Newman-keuls post hoc comparisons revealed that OB was associated with an increase in ventricular volume as % total brain volume when compared to sham-vehicle treated controls (p<0.05) which was not attenuated by treatment with ketamine. Two way ANOVA of hippocampal volume as % total brain volume revealed no significant effects of OB [F(1,29)=1.46127, p=0.2376] [Figure 3.3.2.2a (b)] or treatment [F(1,29)=3.16943, p=0.0867] [Figure 3.3.2.2.1 (b)].
Figure 3.3.2.2a: Ketamine treatment does not alter the OB-induced increase in ventricular volume

The effects of OB and of drug treatment were assessed on (a) ventricular volume and (b) hippocampal volume. Data expressed as mean percentage of total intracranial volume ± SEM (n=7-8). * p<0.05 vs. sham-vehicle group;
Figure 3.3.2.2b: Representative images depicting changes in ventricular volume following OB

Representative images depicting lateral ventricular volume in a) the sham-vehicle group, b) the OB-vehicle group, c) the OB-ketamine group. Arrows identify lateral ventricles.
3.3.2.3 OB and ketamine treatment are associated with changes in cerebral blood perfusion

Two way ANOVA of CTT in the hippocampus revealed an effect of OB \( [F_{(1,28)}=5.36628, \ p=0.029] \) [Table 3.3.5] and an OB \( \times \) ketamine interaction \( [F_{(1,28)}=8.26193, \ p=0.0081] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited an increase in CTT relative to the sham-vehicle group (\( p<0.01 \)). Two way ANOVA of signal amplitude in the thalamus revealed an effect of OB \( [F_{(1,28)}=5.13148, \ p=0.0324] \) [Table 3.3.5] and an OB \( \times \) ketamine interaction \( [F_{(1,28)}=4.46037, \ p=0.0449] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited a decrease in signal amplitude relative to the sham-vehicle group (\( p<0.05 \)) which was attenuated by ketamine treatment (\( p<0.05 \)). Two way ANOVA of MTT in the third ventricle revealed an effect of OB \( [F_{(1,28)}=8.48764, \ p=0.0076] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited an increase in MTT relative to the sham-vehicle group (\( p<0.05 \)). Two way ANOVA of CTT in the visual cortex revealed an effect of ketamine \( [F_{(1,28)}=4.80563, \ p=0.0379] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited a decrease in signal amplitude relative to the sham-vehicle group (\( p<0.05 \)) which was attenuated by ketamine treatment (\( p<0.05 \)). Two way ANOVA of MTT in the parietal association cortex revealed an effect of OB \( [F_{(1,28)}=8.72586, \ p=0.0067] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited an increase in MTT relative to the sham-vehicle group (\( p<0.05 \)). Two way ANOVA of CTT in the parietal association cortex revealed an effect of OB \( [F_{(1,28)}=8.53471, \ p=0.0075] \) [Table 3.3.5], ketamine \( [F_{(1,28)}=6.92635, \ p=0.0146] \) [Table 3.3.5], and an OB \( \times \) ketamine interaction \( [F_{(1,28)}=6.04736, \ p=0.0215] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited an increase in CTT relative to the sham-vehicle group (\( p<0.01 \)) which was attenuated by ketamine treatment (\( p<0.01 \)). Two way ANOVA of CTT in the retrosplenial cortex revealed an effect of OB \( [F_{(1,28)}=5.98262, \ p=0.0218] \) [Table 3.3.5] and ketamine \( [F_{(1,28)}=4.36064, \ p=0.0471] \) [Table 3.3.5]. Newman-keuls *post hoc* comparisons revealed that the OB-vehicle group exhibited an increase in CTT relative to the sham-vehicle group (\( p<0.05 \)) which was attenuated by ketamine treatment (\( p<0.05 \)).
Table 3.3.5: OB is associated with changes in cerebral blood perfusion

<table>
<thead>
<tr>
<th>Structure</th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Ketamine</td>
</tr>
<tr>
<td><strong>Auditory cortex</strong></td>
<td>MTT (s)</td>
<td>1.844 ± 0.067</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.661 ± 0.149</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1030 ± 0.007</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td>MTT (s)</td>
<td>1.469 ± 0.065</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.245 ± 0.029</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1189 ± 0.007</td>
</tr>
<tr>
<td><strong>Thalamus</strong></td>
<td>MTT (s)</td>
<td>1.458 ± 0.052</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.240 ± 0.030</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1341 ± 0.009</td>
</tr>
<tr>
<td><strong>Third ventricle</strong></td>
<td>MTT (s)</td>
<td>1.425 ± 0.056</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.067 ± 0.072</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1747 ± 0.013</td>
</tr>
<tr>
<td><strong>Visual cortex</strong></td>
<td>MTT (s)</td>
<td>1.854 ± 0.130</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.503 ± 0.099</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1032 ± 0.006</td>
</tr>
<tr>
<td><strong>Parietal association cortex</strong></td>
<td>MTT (s)</td>
<td>1.510 ± 0.065</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.283 ± 0.038</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1096 ± 0.003</td>
</tr>
<tr>
<td><strong>Retrosplenial cortex</strong></td>
<td>MTT (s)</td>
<td>1.601 ± 0.077</td>
</tr>
<tr>
<td></td>
<td>CTT (s)</td>
<td>1.322 ± 0.062</td>
</tr>
<tr>
<td></td>
<td>Amplitude (a.u.)</td>
<td>0.1078 ± 0.003</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=7-8). * p<0.05 vs. sham-vehicle group; ** p<0.01 vs. sham-vehicle group; + p<0.05 vs. OB-vehicle group; ++ p<0.01 vs. OB-vehicle group.
3.3.2.4 OB and ketamine treatment are associated with changes in T1 and T2 relaxation times in the rodent brain

Two way ANOVA of T1 relaxation time in the auditory cortex revealed an effect of OB \( [F(1,29)=6.64711, p=0.0159] \) [Table 3.3.6] and an effect of ketamine \( [F(1,29)=20.43271, p<0.0001] \) [Table 3.3.6]. Newman-keuls post hoc comparisons revealed that the sham-ketamine group exhibited an increase in T1 relaxation time relative to the sham-vehicle group (p<0.05). Two way ANOVA of T1 relaxation time in the parietal association cortex revealed an effect of ketamine \( [F(1,29)=21.13002, p<0.0001] \) [Table 3.3.6]. Newman-keuls post hoc comparisons revealed that the sham-ketamine (p<0.01) exhibited an increase in T1 relaxation time relative to the sham-vehicle group.

Two way ANOVA of T2 relaxation time in the auditory cortex revealed an effect of OB \( [F(1,29)=23.34827, p<0.0001] \) and an OB x ketamine interaction \( [F(1,28)=5.96114, p=0.0217] \) [Table 3.3.6]. Newman-keuls post hoc comparisons revealed that the OB-vehicle group exhibited a decrease (p<0.01) in T2 relaxation time relative to the sham-vehicle group. Two way ANOVA of T2 relaxation time in the hippocampus revealed an effect of OB \( [F(1,28)=9.84501, p=0.0429] \) [Table 3.3.6] and an effect of ketamine \( [F(1,28)=5.01709, p=0.0342] \) [Table 3.3.6]. Two way ANOVA of T2 relaxation time in the thalamus, and parietal association and retrosplenial cortices revealed an effect of OB \( [F(1,29)=5.51757, p<0.0267], [F(1,29)=21.13002, p=0.0001] \) and \( [F(1,29)=6.31915, p=0.0185] \) [Table 3.3.6] respectively. Newman-keuls post hoc comparisons revealed that the OB-vehicle group (p<0.05) exhibited a decrease in T2 relaxation time in the parietal association cortex relative to the sham-vehicle group. Two way ANOVA of T2 relaxation time in the visual cortex revealed an effect of OB \( [F(1,28)=30.99468, p<0.0001] \) [Table 3.3.6] and an OB x ketamine interaction \( [F(1,28)=4.29056, p=0.0007] \) [Table 3.3.6]. Newman-keuls post hoc comparisons revealed that the sham-ketamine group (p<0.05) and the OB-vehicle group (p<0.01) exhibited a decrease in T2 relaxation time relative to the sham-vehicle group.
Table 3.3.6: OB and ketamine treatment are associated with changes in T1 and T2 relaxation times in the rodent brain

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (ms)</td>
<td>Ketamine (ms)</td>
</tr>
<tr>
<td><strong>Auditory cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1854 ± 25.29</td>
<td>1925 ± 14.64*</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>50.87 ± 0.33</td>
<td>50.13 ± 0.22</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1919 ± 20.62</td>
<td>1945 ± 21.79</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>53.01 ± 0.37</td>
<td>52.38 ± 0.20</td>
</tr>
<tr>
<td><strong>Thalamus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1668 ± 20.41</td>
<td>1692 ± 20.92</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>47.18 ± 0.18</td>
<td>47.00 ± 0.16</td>
</tr>
<tr>
<td><strong>Third ventricle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>2006 ± 84.93</td>
<td>2088 ± 52.52</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>69.24 ± 2.49</td>
<td>66.22 ± 1.51</td>
</tr>
<tr>
<td><strong>Visual cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1976 ± 18.95</td>
<td>1999 ± 7.062</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>53.25 ± 0.28</td>
<td>52.49 ± 0.16*</td>
</tr>
<tr>
<td><strong>Parietal association cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1894 ± 26.22</td>
<td>2017 ± 32.31**</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>51.14 ± 0.27</td>
<td>51.49 ± 0.26</td>
</tr>
<tr>
<td><strong>Retrosplenial cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (ms)</td>
<td>1842 ± 23.41</td>
<td>1876 ± 30.31</td>
</tr>
<tr>
<td>T2 (ms)</td>
<td>49.13 ± 0.64</td>
<td>49.63 ± 0.47</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=7-8). * p<0.05 vs. sham-vehicle group; ** p<0.01 vs. sham-vehicle group.
3.4 Discussion

3.4.1 Characterisation of MR imaging markers associated with OB and the antidepressant effect of imipramine and L-NA

The first aim of the present studies was to assess whether L-NA produced an antidepressant response in the OB rat model of depressive-like behaviour. Imipramine was used as a positive control. Secondly we sought to establish if the OB rat was associated with any trait or state dependent neuroimaging markers and to investigate changes in state dependent markers following an antidepressant response.

3.4.1.1 L-NA and imipramine treatment produce an antidepressant response in the OB rat

Chronic treatment with both L-NA and imipramine produced a comparable antidepressant response as assessed by an attenuation of hyperactivity in the open field in the OB rat model. These studies are the first to report that NOS inhibitor L-NA is effective at attenuating OB induced hyperactivity in the open field test. Imipramine was chosen as a positive control in these experiments as chronic treatment with this drug has previously been shown to attenuate OB-related behaviour (Song and Leonard, 2005). The results presented here are in accordance with the growing body of literature which suggests that inhibitors of nNOS, a downstream signalling molecule coupled to the NMDA receptor, possess antidepressant activity (Doucet et al., 2013; Gigliucci et al., 2010) and reinforce that NOS may serve as a novel target for the development of antidepressant drugs for use in the clinic (Dhir and Kulkarni, 2011). As L-NA is a non-selective inhibitor of NOS, it is not possible to determine whether its antidepressant effects are as a result of inhibition of inducible (iNOS), endothelial (eNOS) or neuronal (nNOS) isoforms of NOS. However we have previously shown that sub-acute dosing with selective nNOS inhibitor TRIM is sufficient to attenuate OB induced hyperactivity in the open field (Gigliucci et al., 2014). Furthermore as OB was not associated with a significant increase in either cortical or hippocampal iNOS mRNA expression, inhibition of iNOS is unlikely to be associated with the antidepressant response following chronic L-NA administration, hence the antidepressant-like effect of L-NA in the OB model are most likely mediated through nNOS inhibition.
3.4.1.2 L-NA and imipramine treatment are associated with changes in MR imaging markers in the OB rat

There was a non-significant trend for an increase in ventricular volume in OB rats relative to sham-treated controls (p<0.05 vs. sham-operated controls via students t-test). This is in agreement with findings from the only previous MRI study of the OB rat which reported increased signal intensity from the ventricular regions of the OB rat relative to sham-operated controls interpreted as an increase in ventricular volume (Wrynn et al., 2000). Increased lateral ventricular volume is also a common finding in depressed patients reported in the clinical neuroimaging literature (Kempton et al., 2011). In the OB rat model this change in ventricular volume is not accompanied by any significant change in hippocampal volume. Thus changes in ventricular volume reported here are most likely due to changes in thalamic or striatal volume in this model. This is in agreement with a previous study which reported no change in the volume of the dentate gyrus region of OB rats relative to sham-operated control animals (Jaako-Movits and Zharkovsky, 2005). Rather than attenuate the OB-induced increase in ventricular volume, chronic treatment with L-NA was associated with a significant increase in ventricular volume relative to OB vehicle treated groups while there was a non significant increase in ventricular volume in the OB-imipramine group relative to the OB-vehicle group. There is little data on the effects of antidepressant drugs on ventricular volume in the clinical neuroimaging literature. OB has previously been associated with a process of retrograde, anterograde and transneuronal neurodegeneration (Song and Leonard, 2005). Indeed it has been suggested that the OB may serve as a suitable model of neurodegenerative diseases such as Alzheimer’s due to the cognitive impairments reported in OB animals relative to sham operated controls (Yehuda and Rabinovitz, 2014a, 2014b). Alzheimer’s disease is also associated with an increase in ventricular volume and there have been reports linking cognitive improvement following drug treatment in the clinic to an increase in ventricular volume (Fox et al., 2005; Mueller et al., 2006). The physiology underlying these observations and the relevance to disease state remain unknown. The data presented here suggest that the OB rat may be a suitable model to explore possible pathophysiological processes associated with brain morphological differences reported in the neurodegenerative disease literature.

There was no significant effect of OB on cortical or limbic perfusion parameters acquired. Mean transit time (MTT) and capillary transit time (CTT) are inversely proportional to blood flow, while signal amplitude is proportional to relative cerebral
blood volume (Kelly et al., 2010). It is perhaps surprising that L-NA treatment was not associated with more robust changes in cerebral blood flow as assessed via MR ASL. As discussed previously L-NA is a non selective NOS inhibitor and as such inhibits all three isoforms of NOS. Decreases in vasodilatory NO resulting from eNOS and nNOS inhibition would be expected to result in a decrease in resting state blood perfusion in both sham and OB groups treated with L-NA. This was not however evident in the data. It is unlikely that the lack of changes in cerebral blood perfusion parameters presented here is related to a “wash out” of the drug, L-NA has previously been reported to exhibit an effective plasma half life of 4.2 days in rodents following oral administration (Dananberg et al., 1993). Thus it is more likely that the lack of detectable change in cerebral blood perfusion following L-NA administration is related to homeostatic compensatory mechanisms, possibly involving upregulation of the production of astrocytic vasodilatory arachidonic acid derivatives (Attwell et al., 2010).

OB was associated with a decrease in T2 relaxation times relative to sham-operated controls in cortical and limbic regions which was attenuated by L-NA treatment, there was also a significant negative correlation between T2 relaxation time in the retrosplenial (r= -0.5174; P=0.0057; n=27) and visual cortical regions (r= -0.4584; P=0.0162; n=27) and bout speed. Although it did not reach statistical significance there is also a trend for increased T2 relaxation time in these regions in the OB-imipramine treated group relative to the OB-vehicle treated group and again there was also a significant negative correlation between T2 relaxation time in the retrosplenial (r= -0.4152; P=0.0202; n=31) and visual (r= -0.4358; P=0.0143; n=31) cortical regions and bout speed. Thus it is possible that changes in cortical T2 relaxation times in the OB rat may represent a state dependent marker for antidepressant response in the model. The OB rat has previously been reported to exhibit an increased concentration of iron in the cortex relative to sham operated controls, chronic treatment with fatty acids reduced the concentration of iron in the cortex which was associated with behavioural change (Yehuda and Rabinovitz, 2014a). Brain iron concentrations have previously been reported to be increased in brain regions targeted by degenerative diseases like Alzheimer’s [for review see (Zecca et al., 2004)] and brain iron content has been shown to negatively correlate with T2 relaxation time (Vymazal et al., 1999). The mechanism through which antidepressant response in the model may be linked to brain iron content remains to be elucidated. From the data presented here, changes in T2 relaxation time do not appear to be related to inflammation as there were no significant
differences found in the expression of iNOS or markers of microglial activation CD40 and
CD11b in either the frontal cortex or the hippocampus, this does not however preclude
an inflammatory response in posterior cortex where imaging data was acquired.
Another possible explanation for the decrease observed in T2 relaxation time may be an
alteration in the packing density of cells in the brain of OB rats. Higher cell packing
density has previously been associated with decreased T2 relaxation time (Ding et al.,
2008). Such alterations may be expected given the previously discussed
neurodegenerative nature of the model. If this is the case attenuation of such changes
following antidepressant treatment may represent a protective effect of treatment
against neurodegeneration. Drug dependent differences in protection from
neurodegeneration induced following OB in mice have previously been reported.
Chronic treatment with tri-cyclic antidepressant amitriptyline but not SSRI citalopram
protected against cortical neurodegeneration following OB in mice (Jarosik et al., 2007).
Further post-mortem analysis is required to assess the pathophysiological mechanism
underlying the observed changes in T2 relaxation time.
3.4.2 Characterisation of MR imaging markers associated with sub-acute ketamine administration in the OB rat

The second aim of the present experiments was to establish if sub-acute dosing with NMDA receptor antagonist ketamine was sufficient to produce an antidepressant response in the OB rat model of depressive-like behaviour, and whether any such behavioural change was associated with changes in neuroimaging markers.

3.4.2.2 Sub-acute ketamine dosing fails to produce an antidepressant response in the OB rat

The sub-acute ketamine dosing regimen used in the present study did not produce an attenuation of OB induced hyperactivity in the open field. It would appear from the results of the present study that chronic dosing with ketamine may be required to elicit an antidepressant response in the OB rat model. Acute ketamine treatment has repeatedly been shown to be sufficient to produce an antidepressant response in other paradigms which test for antidepressant activity in rodents such as the FST (Autry et al., 2011; Engin et al., 2009; Gigliucci et al., 2010). While there are reports that this rapid antidepressant action following acute ketamine administration extends to other paradigms such as the sucrose preference test and novelty suppressed feeding test in the chronic mild stress model (CMS) (Li et al., 2011), others have shown that attenuation of changes in sucrose consumption following CMS require chronic ketamine administration (Garcia et al., 2009). Hence the detection of ketamine’s rapid antidepressant action may be test and model specific. Chronic treatment with NMDA receptor antagonist MK-801 has previously been shown to attenuate OB-induced hyperactivity in the novel open field while having no significant effect on activity in the home-cage environment (Redmond et al., 1997). OB is associated with alterations in resting state monoaminergic neurotransmitters including decreased baseline levels of serotonin in the hippocampus and amygdala and increased baseline levels of dopamine in the dorsal and ventral striatum (Masini et al., 2004; Van Der Stelt et al., 2005), while ketamine has previously been shown to dose dependently increase serotonin concentrations in the rodent prefrontal cortex through an AMPA dependent mechanism (Nishitani et al., 2014). Hence it is possible that the behavioural effects of agents which act on the glutamatergic system in the OB rat may be mediated through monoaminergic means. The acute antidepressant response of both ketamine and L-NA in the FST has been shown to be serotonin dependent (Gigliucci et al., 2013, 2010). Future studies are
required to assess if chronic dosing with ketamine is sufficient to produce an antidepressant response in the OB rat model.

3.4.2.2 Ketamine treatment is associated with changes in MR imaging markers in the OB rat

OB resulted in a significant increase in total ventricular volume which was not associated with any change in hippocampal volume. Ketamine treatment did not significantly alter ventricular volume or hippocampal volume in either sham operated or OB rats. This is in contrast to the increase in ventricular volume reported in response to both L-NA and imipramine treated. This may suggest that an increase in ventricular volume is a marker of antidepressant response in the model. There was however no correlation between bout speed and ventricular volume in any of the groups analysed in the present studies (data not shown). Hence the role that changes in brain morphology plays in antidepressant response in the OB requires further study.

There was a trend towards decreased cortical perfusion in the OB rat relative to sham operated controls in several brain regions, with statistical significance in the parietal association cortical region. This is in contrast with the imipramine/L-NA study which showed no effect of OB on perfusion related parameters. This may suggest that cortical perfusion changes are not a robust neuroimaging marker in the OB model. It is possible that differences in vehicle administration and animal handling (i.p. as opposed to oral administration) may also have a role in this apparent discrepancy. Deakin and colleagues (2014) have previously reported that acute intravenous ketamine infusion in patients is associated with changes in blood-oxygen-level dependent (BOLD) contrast in several brain regions which was shown to be dependent on ketamine induced glutamate release (Deakin et al., 2014). Similar effects on BOLD contrast have been observed in rodents following acute ketamine administration (Littlewood et al., 2006). Ketamine partially attenuated OB-induced changes in cortical blood perfusion, as scanning occurred two days following the final ketamine dose and ketamine has previously been shown to exhibit a plasma half life of approximately 1.3 hours following i.p. administration in rodents, any change in cerebral blood perfusion parameters is not related to the acute effects of ketamine administration (Veilleux-Lemieux et al., 2013). As these changes occur in the absence of any antidepressant response, this result casts further doubt on the utility of ASL measures of blood perfusion as a potential marker of antidepressant response in this model.
OB was again associated with a decrease in T2 relaxation time in several cortical regions relative to sham operated control animals. Thus cortical decreases in T2 relaxation time appear to be a robust neuroimaging marker following OB in rats. Furthermore as ketamine's lack of antidepressant response was also associated with no attenuation of cortical T2 relaxation times, it again suggests that decreased T2 relaxation time represents a state dependent rather than a trait marker in the OB rat. Further studies are required to elucidate the physiological mechanisms underlying these changes in T2 relaxation time.

3.5 Conclusion

We report that chronic dosing with NOS inhibitor L-NA produces an antidepressant-like effect in the OB model of depression which is comparable to that produced following chronic administration of tri-cyclic antidepressant imipramine. Sub-chronic dosing with NMDA receptor antagonist ketamine did not produce an antidepressant response in the OB model. Additional studies are required to establish if chronic dosing with ketamine produces an antidepressant response in the OB rat. We report here for the first time a series of neuroimaging markers associated with OB in rats. OB results in an increase in total ventricular volume, without any corresponding decrease in hippocampal volume. A decrease in cortical T2 relaxation time, possibly linked to increased cortical iron content is also a robust neuroimaging marker associated with OB. Furthermore behavioural change following chronic antidepressant treatment with imipramine and L-NA was associated with an increase in total ventricular volume and cortical T2 relaxation times. These changes were not evident following ketamine administration and as such may represent MR markers of antidepressant response in the OB rat model. Future studies may employ diffusion tensor imaging to investigate if the structural and T2 relaxation time changes reported here are associated with changes in brain microstructure and/or altered anatomical connectivity between different brain regions [for review see (Zhang et al., 2012)]. Longitudinal studies using such techniques could temporally map retrograde degeneration of nerves following bulbectomy, potentially providing further insight into the neural circuits associated with OB-related behaviour in rodents which may be of translational relevance for human neurodegenerative conditions and depression.
Chapter 4 – Strain related differences in behaviour are associated with differences in astrocytic and MR imaging markers
4.1 Introduction

There is a growing body of evidence suggesting the involvement of astrocytes in the neuropathology of psychiatric disorders (Sofroniew and Vinters, 2010). This is perhaps to be expected given their wide range of functions within the central nervous system (CNS) including the regulation of localised cerebrovasculature tone and blood flow, neurotransmitter and fluid homeostasis, as well as formation of the blood brain barrier (BBB) (Abbott et al., 2005; Koehler et al., 2009; Sanacora et al., 2012).

There is both pre-clinical and clinical evidence to suggest that aberrant astrocyte function is implicated in the pathophysiology of major depressive disorder (MDD). Decreased expression of glial fibrillary acidic protein (GFAP), a cytoskeletal marker for mature and reactive astrocytes, has been reported in the dorsolateral and orbital prefrontal cortices and the CA1 and CA2 subfields of the hippocampus in post-mortem brain tissue from depressed patients when compared to non-psychiatric, age matched controls [for review see (Rajkowska and Stockmeier, 2013)]. In addition several animal models of depressive-like behaviour have also been shown to exhibit reductions in expression of GFAP in the prefrontal cortex, hippocampus and amygdala and glial ablation has been found to be sufficient to induce depressive-like behaviours in animal models (Banasr and Duman, 2008; Leventopoulos et al., 2007).

Changes in astrocyte cell number and function may be related to the abnormal glutamate metabolism reported in some brain regions within depressed patients (Rajkowska and Miguel-Hidalgo, 2007). A recent meta-analysis shows decreased levels of glutamate in the anterior cingulate cortex and decreased levels of Glx (combined glutamate and glutamine signal) in several brain areas of depressed patients relative to non-psychiatric controls as assessed by in vivo magnetic resonance (MR) spectroscopy (Luykx et al., 2012).

MR imaging has allowed for the non-invasive acquisition of a set of neuroimaging markers associated with psychiatric illnesses. Depressive-illness is associated with brain structural abnormalities including increased lateral ventricular volume as well as decreased hippocampal, prefrontal cortical and amygdaloid volume [For review see (Kempton et al., 2011; Sacher et al., 2012)]. In addition functional neuroimaging studies have shown increased blood-oxygen-level dependent (BOLD) contrast signal (thought to be an indirect measure of neuronal activity) within the amygdala as well as decreased BOLD contrast signal in prefrontal cortical areas in response to negative stimuli.
(Hamilton and Etkin, 2012). However there is still a paucity of data regarding the biological mechanisms underlying such changes in brain structure and function and whether these are related to alterations in astrocyte function observed in post-mortem brains. Animal models can provide us with a useful tool to investigate the mechanisms which may underpin some of these MR neuroimaging markers (Denic et al., 2011).

The inbred Wistar-Kyoto (WKY) rat strain, originally bred as the normotensive control strain for the spontaneously hypertensive rat (SHR), has been proposed as a model of depressive-like behaviour as it endogenously expresses some behavioural, endocrine and neurotransmitter changes akin to those exhibited in depressed human patients (Nagasawa et al., 2012; Nam et al., 2014; Redei et al., 2001; Rittenhouse et al., 2002). We sought to investigate whether the WKY rat strain exhibited anatomical volumetric abnormalities or changes to cerebral blood perfusion in cortical and limbic regions of the brain as measured by MR imaging and if such changes are associated with astroglial GFAP immunoreactivity ex vivo. This model provides a unique opportunity to investigate if variations in regional brain volumetrics and blood flow are related to alterations in astrocytic function. We hypothesised that the decrease in GFAP positive cell numbers previously reported by Gosselin and colleagues (2009) in this model may result in detectable resting state changes in cerebral blood perfusion markers as assessed by bolus tracking arterial spin labelling (ASL) (Gosselin et al., 2009; Kelly et al., 2009). Furthermore MRI scans to quantify T1 and T2 relaxation times in cortical and subcortical structures were acquired. Increases in T1 relaxation times have previously been associated with increased GFAP immunoreactivity in the rodent brain in response to an acute insult (Sibson et al., 2008), in aged (Cowley et al., 2012) and in mutant (Kelly et al., 2014) animals. Decreases in T2 relaxation times have previously been associated with increased microglial activation (Blau et al., 2012).

There is some evidence to suggest that the WKY rat strain is an animal model of depressive-like behaviours which shows decreased sensitivity to the effects of selective serotonin reuptake inhibitor (SSRI) antidepressants on forced swim test (FST) behaviour when compared to other strains. This has led to the suggestion that the WKY strain may be a suitable model of treatment resistant depression (Lahmame and Armario, 1996; Lahmame et al., 1997). In severe and treatment resistant depression in the human population, electroconvulsive therapy (ECT) is the most effective treatment with higher rates of remission than pharmacotherapy (Eranti et al., 2007; Medda et al., 2009; The UK
ECT Review Group, 2003). It is however also associated with cognitive side effects including adverse effects on memory (Semkovska and McLoughlin, 2010). One change in the ECT procedure designed to minimise potential cognitive side effects associated with the treatment is to reduce the ECT pulse width from a brief pulse (BP) width, commonly in the range of 0.5-1.5ms to an ultrabrief pulse (UBP) width of 0.3ms. This brings the pulse duration closer to that of neuronal depolarisation, thus reducing stimulation of groups of neurons which have already depolarised (Sackeim, 2004). There is however conflicting evidence as to the efficacy of UBP ECT in depression, with one randomised trial reporting a high remission rate (77%) with a reduced cognitive side effect profile relative to BP ECT (Sackeim et al., 2008), while other trials have reported lower remission rates (Quante et al., 2011; Sienaert et al., 2009). In a recent review of the clinical literature Spaans and colleagues (2013) have suggested that further data must be acquired before the degree of efficacy of UBP ECT can be ascertained (Spaans et al., 2013).

Both BP and UBP electroconvulsive stimulation (ECS), the animal equivalent of ECT, have been shown to be effective in attenuating chronic corticosterone treatment induced increases in immobility time in the FST paradigm in male Sprague-Dawley rats. BP ECS was also found to attenuate chronic corticosterone induced decreases in GFAP expression in the frontal cortex and hippocampus of these animals (O'Donovan et al., 2014a). Repeated ECS treatment has previously been shown to be effective in reducing immobility in the FST in the WKY rat strain (Krahl et al., 2004; Kyeremanteng et al., 2014). However to our knowledge no study to date has assessed the effect of BP and UBP ECS treatments on the behavioural phenotype of the WKY rat strain, a genetic model with a predisposition to anxiety and depressive-like behaviours.
4.1.1 Aims and objectives

The aim of the experiments described in this chapter was to assess the relationship between behavioural, astroglial and neuroimaging markers in the inbred WKY and out-bred Wistar rat strains. We also sought to investigate if BP and UBP ECS treatments produced differential behavioural responses in the WKY and Wistar rat strains and if these responses were related to changes in brain GFAP protein levels. Finally we investigated whether stress-induced behavioural change in the WKY rat strain was related to changes in brain mRNA markers related to astrocyte function and/or GFAP protein expression.
4.2 Methods

4.2.1 Behavioural, MRI and GFAP immunohistochemical characterisation of the WKY rat strain

Adult male WKY and Wistar rats (200-250g) were housed 2-3 per cage. Animals were habituated to the animal facility for at least one week prior to behavioural testing. One behavioural test was performed daily as per figure 4.2.1. All behavioural readouts were performed in dedicated rooms by a single experimenter. All procedures were conducted as outlined in the Methods section (See Chapter 2). Two separate studies were conducted to characterise the behavioural phenotype and MRI markers exhibited in the WKY rat strain. The order of tests was chosen to minimise test interactions, by beginning with relatively low stress tasks (e.g. Open field, EPM) and incrementally introducing more stressful tasks (e.g. passive avoidance/FST). In study 2 animals were given a one week washout period following MRI before the object recognition test was performed. ASL and structural MRI data were pooled from both studies. T1 and T2 relaxation data from a frontal cortical section (2.2mm anterior to bregma) were acquired in study 1 and relaxation data from a hippocampal section (4.3mm posterior to bregma) were acquired from study 2. All post-mortem analyses were conducted on perfused tissue from study 1. A separate cohort of rats was used to assess strain related differences in baseline saccharin preference via the saccharin preference test (SPT).

Study 1

<table>
<thead>
<tr>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
<th>D6</th>
</tr>
</thead>
<tbody>
<tr>
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<td>EPM</td>
<td>FST</td>
<td>FST</td>
<td>MRI</td>
<td>Perfusion</td>
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</table>

Study 2

<table>
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</thead>
<tbody>
<tr>
<td>OF</td>
<td>PA 1</td>
<td>PA 2</td>
<td>FST</td>
<td>MRI</td>
<td>OR 1</td>
<td>OR 2</td>
</tr>
</tbody>
</table>

Figure 4.2.1: Study design

EPM, elevated plus maze; FST, forced swim test; MRI, magnetic resonance imaging; OF, open field; OR, object relocation task; PA, passive avoidance test.
4.2.2 The effects of BP and UBP ECS on the behavioural phenotype and brain GFAP protein expression in the WKY rat strain

Adult male WKY and Wistar rats (200-250g) were housed 3 per cage. Animals were habituated to the animal facility for at least one week before ECS treatments began. Animals from each strain were randomly assigned to BP, UBP and sham treated control groups. All cages contained one animal from each treatment group. ECS treatments were carried out as previously described (O’Donovan et al., 2012). Animals received ten ECS treatments which were administered every Monday, Wednesday and Friday between 9hr and 12hr over a period of three weeks. Parameters for BP ECS were: 0.5ms pulse-width; 100 pulses/s; 0.7 s duration; 75mA current, for UBP ECS the pulse-width was reduced to 0.3ms. Behavioural assessments began following a one day recovery period after the final ECS treatment. One behavioural test was performed daily as per figure 4.2.2. All behavioural readouts were performed in dedicated rooms by a single experimenter. All procedures were conducted as outlined in the Methods section (See Chapter 2). As before the order of tests was chosen to minimise test interactions. Tissue for post-mortem protein expression analysis was acquired 24 hours following the FST.

Figure 4.2.2: ECS study design

ECS, electroconvulsive stimulation; FST, forced swim test; OF, open field; OR, object relocation task; PA, passive avoidance test.
4.2.3 The effects of chronic restraint stress on the behavioural phenotype and astrocyte markers in the WKY rat strain

Adult male WKY rats (200-250g) were singly housed. Animals were habituated to the animal facility for at least one week before restraint stress began. Animals were placed in restrainers for two hours between 10hr and 14hr daily. The restrainers were placed parallel in cages with fresh bedding in a brightly lit room. Following the procedure animals were returned to their home cages. Starting on Day 7 one behavioural test was conducted daily as per figure 4.2.3. Behaviour was conducted 24 hours after the previous restraint stress. All behavioural readouts were performed in dedicated rooms by a single experimenter. All procedures were conducted as outlined in the Methods section (See Chapter 2), with the exception of the FST. 24 hours following the previous restraint stress session animals were subjected to a single 15 minute swim session, this session was recorded and the first 5 minutes were scored for immobility time by a trained experimenter blinded to treatment. Tissue for post-mortem protein expression and mRNA analysis was acquired 2 hours following the final restraint stress session.

Study 1 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10

OF EPM FST Sacrifice

2 hours daily restraint stress

Figure 4.2.3: Restraint stress study design

EPM, elevated plus maze; FST, forced swim test; OF, open field
4.3 Results

4.3.1 Behavioural, MRI and GFAP immunohistochemical characterisation of the WKY rat strain

4.3.1.1 The WKY rat strain exhibits depressive-like behaviour as assessed by the SPT and FST paradigms when compared to the Wistar strain

Student’s t-test revealed a significant decrease in average saccharin preference over three days \( t=3.779; \text{df}=30; \ p=0.0007 \) [Figure 4.3.1.1 (a)] in the WKY strain when compared to the Wistar strain. Student’s t-test revealed the WKY strain exhibited a significant increase in immobility time in the FST \( t=2.409; \text{df}=17; \ p=0.0276 \) [Figure 4.3.1.1 (b)] when compared to the Wistar strain.

![Figure 4.3.1.1: The WKY rat strain exhibits depressive-like behaviour as assessed by the SPT and FST paradigms when compared to the Wistar strain](image)

Strain dependent depressive-like behaviour was assessed via (a) Three day preference for a 0.1% saccharin solution and (b) immobility in the FST. Data expressed as mean ± SEM (n=9-10 for FST, n=16 for SPT). *** \( p<0.001 \); * \( p<0.05 \) vs. WIS
4.3.1.2 The WKY rat strain exhibits anxiety-like behaviour in the open field test when compared to the Wistar strain

Student’s t-test revealed no significant strain difference in movement in the total open field arena [t=0.1761; df=18; p=0.8622] [Figure 4.3.1.2 (a)]. Student’s t-test revealed a significant decrease in movement [t=2.281; df=18; p=0.0349] [Figure 4.3.1.2 (b)], and in time spent [t=2.171; df=18; p=0.0436] [Figure 4.3.1.2 (c)] in the centre zone of the open field arena in the WKY strain when compared to the Wistar strain. Student’s t-test revealed a significant increase in latency to enter [t=2.244; df=18; p=0.0377] [Figure 4.3.1.2 (d)] the centre zone of the open field arena in the WKY strain when compared to the Wistar strain. Student’s t-test revealed a significant decrease in total rears [t=7.205; df=18; p<0.0001] [Figure 4.3.1.2 (e)] in the WKY strain when compared to the Wistar strain. Student’s t-test revealed no significant strain difference in total time spent grooming [t=1.396; df=18; p=0.1796] [Figure 4.3.1.2 (f)].
Figure 4.3.1.2: The WKY rat strain exhibits anxiety-like behaviour as assessed in the open field test when compared to the Wistar strain

Strain dependent differences in locomotor activity and anxiety-like behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena (c) Total time in the inner zone of the OF arena (d) Latency to enter the inner zone of the OF arena (e) Total number of rears in the open field (f) Total time spent grooming. Data expressed as mean ± SEM (n=9-10). * p<0.05; *** p<0.001 vs. WIS
4.3.1.3 The WKY rat strain exhibits anxiety-like behaviour in the elevated plus maze when compared to the Wistar strain

Student’s $t$-test revealed a significant decrease in the number of entries into the open arms of the elevated plus maze in the WKY strain when compared to the Wistar strain [$t=2.532; df=17; p=0.0215$] [Figure 4.3.1.3 (a)]. No significant strain difference in number of entries to the closed arms was observed [$t=0.5790; df=17; p=0.5702$] [Figure 4.3.1.3 (b)]. Student’s $t$-test revealed a significant decrease in total distance moved in the elevated plus maze in the WKY strain when compared to the Wistar strain [$t=3.613; df=17; p=0.0021$] [Figure 4.3.1.3 (c)].

![Figure 4.3.1.3: The WKY rat strain exhibits anxiety-like behaviour as assessed in the elevated plus maze when compared to the Wistar strain](image)

(a) Total number of entries into the open arms of the elevated plus maze, (b) total number of entries into the closed arms of the elevated plus maze, (c) total distance moved in the elevated plus maze. Data expressed as mean ± SEM (n=9-10). * $p<0.05$; ** $p<0.01$ vs. WIS.
4.3.1.4 The WKY rat strain exhibits a deficit in spatial recognition memory as assessed by the object displacement task.

Two way analysis of variance (ANOVA) analysis revealed a significant interaction between strain x object \( F(15,8)=15.50377, p<0.001 \) [Figure 4.3.1.4 (a)] in the training session. Newman-keuls post hoc comparisons revealed no significant differences between the groups. Two way ANOVA analysis revealed a significant interaction between strain x object \( F(15,58)=15.50377, p<0.001 \) [Figure 4.3.1.4 (b)] in the test session. Newman-keuls post hoc comparisons revealed that the Wistar strain showed a significant \((p<0.01)\) increase in percentage of time spent exploring the displaced object C relative to stationary objects A and B, while the WKY strain showed significantly lower \((p<0.01)\) exploration of the object C relative to the Wistar strain.

![Graphs showing exploration time of objects A, B, and C for Wistar and WKY strains during training and testing phases.](image)

Figure 4.3.1.4: The WKY rat strain exhibits a deficit in spatial recognition memory as assessed by the object displacement task

Strain dependent differences in spatial recognition memory were assessed by the object displacement task. (a) Neither the Wistar or WKY strain showed any differences in exploration of any of the objects during the training session. (b) The Wistar rat strain showed a significant increase in exploration of the displaced object C during the test session. Data expressed as mean ± SEM \((n=9-10)\). ++ \( p<0.01 \) relative to % exploration time of stationary objects; ** \( p<0.01 \) relative to WIS % exploration time of displaced object C
4.3.1.5 There is no strain dependent difference in fear conditioned memory as assessed by the step-through passive avoidance test

Two way repeated measure ANOVA analysis revealed a significant effect of time [$F_{(1,39)}=284.1187, p<0.001$] [Figure 4.3.1.5] on latency to enter the shock compartment. Newman-keuls post hoc comparisons revealed that step-through latencies were significantly ($p<0.01$) increased during the retention trials when compared to latencies during the training session in both strains.

![Figure 4.3.1.5: There is no strain dependent difference in fear conditioned memory as assessed by the step-through passive avoidance test](image)

There were no strain dependent differences in fear conditioned memory. Data expressed as mean ± SEM (n=9-11). ** $p<0.01$ vs. Training session
4.3.1.6 The WKY rat strain exhibits regional brain volumetric differences when compared to the Wistar strain

Two way ANOVA revealed a significant effect of stain \([F(3,137)=5.18799, p=0.0243]\) [Figure 4.3.1.6], brain region \([F(3,137)=22796.82, p<0.0001]\) [Figure 4.3.1.6] and a strain \(\times\) brain region \([F(3,137)=6.16836, p=0.006]\) [Figure 4.3.1.6] interaction. Student’s \(t\)-test revealed a significant decrease in hippocampal volume in the WKY strain when compared to the Wistar strain \([t=8.247; df=35; p<0.0001]\) [Figure 4.3.1.6a (c)]. Student’s \(t\)-test revealed a significant increase in lateral \([t=6.222; df=36; p<0.0001]\) [Figure 4.3.1.6a (a)] and third ventricular volume \([t=7.776; df=36; p<0.0001]\) [Figure 4.3.1.6a (b)], and midbrain \([t=2.685; df=35; p=0.011]\) [Figure 4.3.1.6a (e)] volume in the WKY strain when compared to the Wistar strain. Student’s \(t\)-test revealed no significant strain differences in striatal \([t=1.042; df=35; p=0.3045]\) [Figure 4.3.1.6a (f)] or cortical \([t=0.813; df=35; p=0.4217]\) [Figure 4.3.1.6a (d)] volume.
Figure 4.3.1.6a: The WKY rat strain exhibits regional brain volumetric differences when compared to the Wistar strain

Bar graphs representing (a) lateral ventricular volume, (b) third ventricular volume, (c) hippocampal volume (d) cortical volume (e) midbrain volume and (f) striatal volume. Data expressed as mean percentage of total intracranial volume ± SEM (n=17-20).

*** p<0.001; * p<0.05 vs. WIS
Figure 4.3.1.6b: Representative images depicting regional brain volumetric differences in the WKY rat when compared to the Wistar strain

Representative MR images of Wistar and WKY rat brains. Arrows identify enlarged lateral ventricles (above right) and smaller hippocampus (below right) in the WKY strain relative to the Wistar rat strain.
4.3.1.7 The WKY rat strain exhibits decreased resting blood perfusion in a number of brain regions when compared to the Wistar strain

Student's t-test revealed that the WKY rat strain exhibited a significant increase in mean transit time (MTT) in the pre-limbic cortical \([t=2.937; \text{df}=26; p=0.0069]\) [Table 4.3.1], the primary motor cortical \([t=2.707; \text{df}=26; p=0.0118]\) [Table 4.3.1], the primary somatosensory cortical \([t=2.144; \text{df}=26; p=0.0415]\) [Table 4.3.1] and the striatal regions \([t=3.84; \text{df}=26; p=0.0007]\) [Table 4.3.1] analysed. Student's t-test revealed the WKY strain exhibited a significant increase in capillary transit time (CTT) in the pre-limbic cortical \([t=3.018; \text{df}=26; p=0.0056]\) [Table 4.3.1], the striatal regions \([t=3.208; \text{df}=26; p=0.0035]\) [Table 4.3.1] analysed. Student's t-test revealed that the WKY rat strain exhibited a significant decrease in signal amplitude in the pre-limbic cortical \([t=2.18; \text{df}=26; p=0.0385]\) [Table 4.3.1], the primary motor cortical \([t=2.606; \text{df}=26; p=0.015]\) [Table 4.3.1], the secondary motor cortical \([t=3.651; \text{df}=26; p=0.0012]\) [Table 4.3.1], the primary somatosensory cortical \([t=3.407; \text{df}=25; p=0.022]\) [Table 4.3.1], and the striatal regions \([t=3.129; \text{df}=24; p=0.0046]\) [Table 4.3.1] analysed.
Table 4.3.1: The WKY rat strain exhibits decreased resting blood perfusion in a number of brain regions when compared to the Wistar strain

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>WIS</th>
<th></th>
<th></th>
<th>WKY</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT (s)</td>
<td>CTT (s)</td>
<td>Amplitude (a.u.)</td>
<td>MTT (s)</td>
<td>CTT (s)</td>
<td>Amplitude (a.u.)</td>
</tr>
<tr>
<td>Pre-limbic cortex</td>
<td>1.611 ±  1.368 ± 0.1158 ± 0.007</td>
<td>0.081 0.054</td>
<td>*</td>
<td>1.967 ± 1.637 ± 0.09521 ± 0.007</td>
<td>0.090 0.071</td>
<td>0.081 0.054</td>
</tr>
<tr>
<td>Primary motor cortex</td>
<td>1.536 ±  1.341 ± 0.1110 ± 0.005</td>
<td>0.081 0.061</td>
<td>*</td>
<td>1.827 ± 1.477 ± 0.09262 ± 0.005</td>
<td>0.071 0.053</td>
<td>*</td>
</tr>
<tr>
<td>Secondary motor cortex</td>
<td>1.719 ±  1.450 ± 0.1261 ± 0.001</td>
<td>0.085 0.076</td>
<td>0.097 0.078</td>
<td>0.09473 ± 0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>1.47 ±   1.328 ± 0.1314 ± 0.007</td>
<td>0.055 0.059</td>
<td>0.085 0.082</td>
<td>0.100 ± 0.006</td>
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<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>1.434 ±  1.260 ± 0.1251 ± 0.005</td>
<td>0.050 0.037</td>
<td>0.085 0.068</td>
<td>0.09977 ± 0.006</td>
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</tr>
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</table>

Data expressed as mean ± SEM (n=13-14). *** p<0.001; ** p<0.01; * p<0.05 vs. WIS
Figure 4.3.1.7: The WKY rat strain exhibits decreased resting blood perfusion in a number of regions when compared to the WIS strain.

Representative cerebral blood volume (CBV) maps depicting CBV in the frontal coronal section analysed of a WKY rat when compared to a Wistar rat. Brighter colours indicate increased signal amplitude which is proportional to CBV.
4.3.1.8 The WKY rat strain exhibits a decrease in GFAP positive cell number in a number of brain regions analysed when compared to the Wistar strain

GFAP positive cell number were quantified in regions across three coronal sections at 2.2mm anterior to bregma, 3.3mm posterior to bregma and 4.3mm posterior to bregma.

4.3.1.8.1 Bregma +2.2mm

Student's t-test revealed the WKY strain exhibited a significant decrease in GFAP positive cell number in the pre-limbic cortex \( t=2.39; \ df=16; \ p=0.0295 \) [Table 4.3.2] with no change in GFAP positive cell numbers in the primary motor cortex \( t=0.3765; \ df=13; \ p=0.7126 \) [Table 4.3.2] when compared to the Wistar strain.

Table 4.3.2: The WKY strain exhibits decreased GFAP positive cell number in the pre-limbic cortex

<table>
<thead>
<tr>
<th></th>
<th>WIS</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary motor cortex</td>
<td>100 ± 8.27</td>
<td>95.17 ± 10.15</td>
</tr>
<tr>
<td>Pre-limbic cortex</td>
<td>190.2 ± 21.83</td>
<td>131.6 ± 11.22 *</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm\(^2\) ± SEM (n=7-9).
* \( p<0.05 \) vs. WIS
a) The WKY strain exhibits a decrease in GFAP positive cell number in the PLC region analysed, b) Representative images depicting decreased GFAP positive cell number in the PLC region of WKY rats. Data expressed as mean number of GFAP positive cells per mm² ± SEM (n=7-9) * p<0.05 vs. WIS
4.3.1.8.2 Bregma - 3.3mm

Student's $t$-test revealed the WKY strain exhibited a significant decrease in GFAP positive cell number in the CA1 [$t=3.131; \text{df}=17; p=0.0061$] [Table 4.3.3] and the CA3 subfields [$t=0.0432; \text{df}=17; p=0.0432$] [Table 4.3.3] of the hippocampus when compared to the Wistar strain.

**Table 4.3.3: The WKY strain exhibits decreased GFAP positive cell number in hippocampal subfields**

<table>
<thead>
<tr>
<th></th>
<th>WIS</th>
<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>287.6 ± 14.71</td>
<td>226.8 ± 12.80**</td>
</tr>
<tr>
<td>CA2</td>
<td>276.1 ± 19.57</td>
<td>228.8 ± 14.70</td>
</tr>
<tr>
<td>CA3</td>
<td>280.3 ± 21.17</td>
<td>225.3 ± 14.40*</td>
</tr>
<tr>
<td>Dentate</td>
<td>239.4 ± 21.43</td>
<td>208.5 ± 25.31</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm$^2$ ± SEM (n=9-10).
* $p<0.05$ vs. WIS; ** $p<0.01$ vs. WIS
4.3.1.8.3 Bregma - 4.3mm

Student's t-test revealed the WKY strain exhibited a significant decrease in GFAP positive cell number in the CA3 \[t=2.249; \text{df}=14; \text{p}=0.0411\] [Table 4.3.4] subfield of the hippocampus when compared to the Wistar strain.

Table 4.3.4: The WKY strain exhibits decreased GFAP positive cell number in hippocampal subfields

<table>
<thead>
<tr>
<th>Strain</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>Dentate</th>
</tr>
</thead>
<tbody>
<tr>
<td>WIS</td>
<td>225.8 ± 21.61</td>
<td>220 ± 14.88</td>
<td>267.1 ± 16.93</td>
<td>261.8 ± 17.79</td>
</tr>
<tr>
<td>WKY</td>
<td>206.7 ± 11.37</td>
<td>224.3 ± 15.21</td>
<td>218.1 ± 13.46*</td>
<td>250.4 ± 17.80</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm² ± SEM (n=6-10).

\* \text{p}<0.05 vs. WIS
4.3.1.9 Decreased GFAP positive cell number correlates with decreased cerebral blood perfusion in the pre-limbic and primary motor cortical regions of the WKY rat

There was a significant negative correlation revealed between GFAP positive cell number per mm$^2$ and MTT [$r = -0.7477; P=0.0129; n=10$] [Table 4.3.5] and CTT [$r = -0.7597; p=0.0108; n=10$] [Table 4.3.5] while the correlation between GFAP positive cell number per mm$^2$ and signal amplitude [$r = 0.3037; p=0.3937; n=10$] [Table 4.3.5] did not achieve significance in the WKY rat within the pre-limbic cortex. A correlation was not obtained between GFAP positive cell number per mm$^2$ and MTT [$r = 0.003632; P=0.9932; n=8$] [Table 4.3.5], CTT [$r = 0.1077; p=0.7997; n=8$] [Table 4.3.5] or signal amplitude [$r = 0.3885; p=0.3415; n=8$] [Table 4.3.5] in the Wistar strain in the pre-limbic cortex.

There was a significant negative correlation revealed between GFAP positive cell number per mm$^2$ and MTT [$r = -0.797; P=0.0179; n=8$] [Table 4.3.5] and CTT [$r = -0.8470; p=0.008; n=8$] [Table 4.3.5], while the correlation between GFAP positive cell number per mm$^2$ and signal amplitude [$r = 0.557; p=0.1515; n=8$] [Table 4.3.5] did not achieve significance in the WKY rat in the primary motor cortex. A correlation was not obtained between GFAP positive cell number per mm$^2$ and MTT [$r = 0.2982; P=0.516; n=7$] [Table 4.3.5], CTT [$r = 0.3348; p=0.4630; n=7$] [Table 4.3.5] or signal amplitude [$r = 0.4431; p=0.3194; n=7$] [Table 4.3.5] in the Wistar strain in the primary motor cortex.
Table 4.3.5: Decreased GFAP positive cell number correlates with decreased cerebral blood perfusion in the pre-limbic and primary motor cortical regions of the WKY rat.

<table>
<thead>
<tr>
<th></th>
<th>WIS</th>
<th>WKY</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT</td>
<td>CTT</td>
<td>Amplitude</td>
<td>MTT</td>
<td>CTT</td>
<td>Amplitude</td>
</tr>
<tr>
<td>Pre-limbic cortex</td>
<td>0.00363</td>
<td>0.1077</td>
<td>0.3885</td>
<td>-0.7477*</td>
<td>-0.7597*</td>
<td>0.3037</td>
</tr>
<tr>
<td>Primary motor cortex</td>
<td>0.2982</td>
<td>0.3348</td>
<td>0.4431</td>
<td>-0.797*</td>
<td>-0.8470**</td>
<td>0.557</td>
</tr>
</tbody>
</table>

Data express as r value (n=7-10). ** p<0.01; * p<0.05
4.3.1.10 The WKY rat exhibits a decrease in blood perfusion in the third ventricular region when compared to the Wistar strain.

Student’s t-test revealed the WKY strain exhibited a significant increase in MTT [t=2.171; df=23; p=0.0405] [Table 4.3.6] and a decrease in signal amplitude [t=2.409; df=25; p=0.0237] [Table 4.3.6] in the third ventricular region compared to the Wistar strain.

Table 4.3.6: The WKY rat exhibits a decrease in blood perfusion in the third ventricular region but not surrounding parenchymal regions when compared to the Wistar strain

<table>
<thead>
<tr>
<th></th>
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<th>WKY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT (s)</td>
<td>CTT (s)</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.686±</td>
<td>1.505±</td>
</tr>
<tr>
<td></td>
<td>0.076</td>
<td>0.064</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.646±</td>
<td>1.481±</td>
</tr>
<tr>
<td></td>
<td>0.070</td>
<td>0.087</td>
</tr>
<tr>
<td>Parietal association</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>1.699±</td>
<td>1.429±</td>
</tr>
<tr>
<td></td>
<td>0.089</td>
<td>0.095</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1.639±</td>
<td>1.451±</td>
</tr>
<tr>
<td></td>
<td>0.084</td>
<td>0.086</td>
</tr>
<tr>
<td>Thalamus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.383±</td>
<td>1.188±</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>0.056</td>
</tr>
<tr>
<td>Visual cortex</td>
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</tr>
<tr>
<td></td>
<td>1.780±</td>
<td>1.518±</td>
</tr>
<tr>
<td></td>
<td>0.108</td>
<td>0.086</td>
</tr>
<tr>
<td>Third ventricle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.421±</td>
<td>1.19±</td>
</tr>
<tr>
<td></td>
<td>0.082</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=13-14). * p<0.05 vs. WIS
4.3.1.11 The WKY rat strain exhibits an increase in T2 relaxation time in the hippocampus when compared to the Wistar strain

Student's t-test revealed that the WKY strain exhibited a significant increase in T2 relaxation time in the hippocampus [t=3.178; df=14; p=0.0067] [Table 4.3.7] when compared to the Wistar strain.

Table 4.3.7: The WKY strain exhibits an increase in T2 relaxation time in the hippocampus when compared to the Wistar strain

<table>
<thead>
<tr>
<th></th>
<th>WIS</th>
<th></th>
<th>WKY</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1 (ms)</td>
<td>T2 (ms)</td>
<td>T1 (ms)</td>
<td>T2 (ms)</td>
</tr>
<tr>
<td>Cortex</td>
<td>2174 ± 25.85</td>
<td>53.01 ± 0.12</td>
<td>2117 ± 31.84</td>
<td>53.84 ± 0.51</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>2176 ± 19.57</td>
<td>53.08 ± 0.20</td>
<td>2117 ± 38.75</td>
<td>54.70 ± 0.52 **</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1904 ± 16.64</td>
<td>49.03 ± 0.15</td>
<td>1828 ± 46.44</td>
<td>48.69 ± 0.44</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=7-9). ** p<0.01 vs. WIS
4.3.2 The effects of BP and UBP ECS on the behavioural phenotype and brain GFAP protein expression in the WKY rat strain

Following characterisation of behavioural, MR and immunohistochemical markers in the WKY rat strain, we investigated the effect of BP and UBP ECS treatment on the behavioural phenotype and brain GFAP protein expression in the WKY and Wistar rat strains.

4.3.2.1 UBP and BP ECS treatment decreases immobility time in the WKY rat strain in the FST paradigm

One way ANOVA of time spent immobile in the FST in the Wistar rat strain revealed no effect of ECS treatment when compared to sham ECS treated controls \([F(2,28)=0.20815, p=0.8134]\) [Figure 4.3.2.1 (a)]. One way ANOVA of time spent immobile in the FST in the WKY rat strain revealed an effect of treatment \([F(2,28)=3.89177, p=0.0332]\) [Figure 4.3.2.1 (b)]. Newman-keuls post hoc comparisons revealed that both BP and UBP ECS treatment resulted in a significant decrease in time spent immobile in the FST when compared to sham ECS treated controls \((p<0.05)\).

Figure 4.3.2.1: UBP and BP ECS treatment reduces immobility time in the WKY rat strain in the FST paradigm

ECS treatment dependent differences in immobility time were assessed using the FST paradigm in a) The Wistar and b) the WKY strain. Data expressed as mean ± SEM \((n=8-10)\). * \(p<0.05\) relative to the sham ECS treated group.
4.3.2.2 UBP ECS treatment results in increased anxiety-like behaviour in the open field test in Wistar rats.

One way ANOVA of distance moved in the total arena in the Wistar strain revealed an effect of ECS treatment \( F_{(2,30)} = 4.38222, \ p = 0.0221 \) [Figure 4.3.2.2 (a)]. Newman-keuls post hoc comparisons revealed that UBP ECS treated Wistar rats exhibited significantly lower distance moved in the total arena compared to sham ECS treated controls \( (p<0.05) \). One way ANOVA of distance moved in the inner zone in the Wistar strain revealed no effect of ECS treatment \( F_{(2,28)} = 1.7379, \ p = 0.1943 \) [Figure 4.3.2.2 (b)]. One way ANOVA of the number of faecal boli deposited during the test revealed no effect of ECS treatment \( F_{(2,28)} = 2.80374, \ p = 0.079 \) [Figure 4.3.2.2 (c)]. One way ANOVA of latency to enter the inner zone in the Wistar strain revealed a significant effect of ECS treatment \( F_{(2,29)} = 3.54838, \ p = 0.0428 \) [Figure 4.3.2.2 (d)]. Newman-keuls post hoc revealed that UBP ECS treated WIS rats exhibit significantly higher latency to enter the inner zone than sham ECS treated controls \( (p<0.05) \). One way ANOVA of total grooming time in the Wistar strain revealed an effect of ECS treatment \( F_{(2,29)} = 7.12822, \ p = 0.003 \) [Figure 4.3.2.2 (e)]. Newman-keuls post hoc comparisons revealed that both BP \( (p<0.01) \) and UBP \( (p<0.05) \) ECS treated Wistar rats exhibited significantly higher total time grooming when compared to sham ECS treated controls. One way ANOVA of total rears in the Wistar strain revealed no effect of ECS treatment \( F_{(2,30)} = 2.99852, \ p = 0.0661 \) [Figure 4.3.2.2 (f)].
Figure 4.3.2.2: UBP ECS treatment results in increased anxiety-like behaviour in the open field test in Wistar rats

The effect of BP and UBP ECS treatment on locomotor activity and anxiety-like behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena, (c) number of faecal boli excreted, (d) Latency to enter the inner zone of the OF arena, (e) total grooming time and (f) number of rears in the OF arena. Data expressed as mean ± SEM (n=8-10) ** p<0.01 relative to sham treated controls; * p<0.05 relative to sham treated controls
4.3.2.3 ECS treatment decreases locomotor and exploratory activity in the open field test in WKY rats

One way ANOVA of distance moved in the total arena in the WKY strain revealed an effect of ECS treatment \( [F(2,28)=23.11732, p<0.0001] \) \([\text{Figure 4.3.2.3 (a)}]\). Newman-keuls post hoc comparisons revealed that both BP and UBP ECS treated WKY rats exhibited significantly lower distance moved in the total arena compared to sham ECS treated controls (p<0.01). One way ANOVA of distance moved in the inner zone in the WKY strain revealed no effect of ECS treatment \( [F(2,27)=0.86571, p=0.433] \) \([\text{Figure 4.3.2.3 (b)}]\). One way ANOVA of the number of faecal boli deposited during the test revealed an effect of ECS treatment \( [F(2,28)=3.81298, p=0.0353] \) \([\text{Figure 4.3.2.3 (c)}]\). Newman-keuls post hoc comparisons revealed that UBP treated WKY rats exhibited a significant increase in the number of faecal boli deposited during the test when compared to sham ECS treated controls (p<0.05). One way ANOVA of latency to enter the inner zone in the WKY strain revealed no effect of ECS treatment \( [F(2,29)=1.80152, p=0.1843] \) \([\text{Figure 4.3.2.3(d)}]\). One way ANOVA of number of total grooming time in the WKY strain revealed no effect of ECS treatment \( [F(2,29)=0.8472, p=0.1821] \) \([\text{Figure 4.3.2.3 (e)}]\). One way ANOVA of total rears in the WKY strain revealed a significant effect of ECS treatment \( [F(2,30)=2.99852, p=0.0661] \) \([\text{Figure 4.3.2.3 (f)}]\). Newman-keuls post hoc comparisons revealed that both BP and UBP treated WKY rats exhibited a significant decrease in total rears when compared to sham ECS treated controls (p<0.01).
Figure 4.3.2.3: ECS treatment results in decreased locomotor and exploratory activity in the open field test in WKY rats.

The effect of BP and UBP ECS treatment on locomotor activity and anxiety-like behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the centre circle of the OF arena, (c) number of faecal bolii excreted, (d) Latency to enter the centre circle of the OF arena, (e) total grooming time and (f) number of rears in the OF arena. Data expressed as mean ± SEM (n=8-10) ** p<0.01 relative to sham treated controls; * p<0.05 relative to sham treated controls.
4.3.2.4 Both BP and UBP ECS treatment are associated with spatial memory deficits in the Wistar strain as assessed by the object relocation paradigm

Two way ANOVA of % time spent exploring objects in the Wistar strain during the training session revealed an effect of object \([F(2,86)=8.4196, \ p=0.0001]\) [Figure 4.3.2.4 (a)]. Newman-keuls post hoc comparisons revealed no significant differences. Two way ANOVA of % time spent exploring objects in the Wistar strain during the test session revealed a significant object x treatment \([F(4,92)=8.05561, \ p<0.0001]\) [Figure 4.3.2.4 (c)] interaction. Newman-keuls post hoc comparisons revealed that sham ECS treated Wistar rats exhibited significantly higher % time exploring the displaced object “C” \((p<0.01)\) relative to stationary objects “A” and “B”. Both BP \((p<0.01)\) and UBP \((p<0.05)\) groups exhibited significantly lower % time exploring the displaced object c relative to sham ECS treated Wistar controls. Two way ANOVA of % time spent exploring objects in the WKY strain during the training and test sessions did not reveal any significant change in % exploration time [Figure 4.3.2.4 (b) (d)]. Neither BP nor UBP ECS resulted in a significant change in % time exploring the displaced object c in the WKY strain.
Figure 4.3.2.4: Both BP and UBP ECS treatment are associated with spatial memory deficits in the Wistar strain as assessed by the object relocation paradigm.

The effect of both BP and UBP ECS treatment on spatial working memory in the Wistar and WKY strain was assessed via the object displacement paradigm. Neither Wistar (a) nor WKY (b) strains showed any difference in exploration of any object during the training session. (c) Sham ECS treated Wistar showed an increased exploration of the displaced object C, this was attenuated by both BP and UBP ECS treatment. (d) Sham ECS treated WKY rats showed no difference in exploration of any object during the test session. Data expressed as mean ± SEM (n=8-10). ++ p<0.01 relative to % exploration time of stationary objects; ** p<0.01 relative to sham treated control % exploration time of displaced object c; * p<0.05 relative to sham treated control % exploration time of displaced object c.
4.3.2.5 Both BP and UBP ECS treatment are associated with a deficit in fear conditioned memory response in the WKY but not the Wistar strain as assessed by the step-through passive avoidance test

Two way repeated measures ANOVA of latencies in the step-through passive avoidance trials within the Wistar strain showed an effect of time \( F(1,53) = 90.79793, p < 0.0001 \) [Figure 4.3.2.5 (a)]. Newman-keuls post hoc comparisons revealed that step-through latencies were significantly increased during the retention trials at 24h when compared to latencies during the training session in all three groups \( (p < 0.01) \) suggesting that neither BP nor UBP ECS treatment results in a deficit in passive avoidance behaviour in the Wistar strain. Two way repeated measures ANOVA of latencies in the step-through passive avoidance trials within the WKY strain showed an effect of time \( F(1,53) = 51.61355, p < 0.0001 \) and a treatment x time interaction \( F(2,53) = 5.06576, p = 0.0146 \) [Figure 4.3.2.5 (b)]. Newman-keuls post hoc comparisons revealed that step-through latencies were significantly increased during the retention trials at 24h when compared to latencies during the training session in the sham treated control \( (p < 0.01) \) and UBP \( (p < 0.01) \) groups but not the BP groups. Both UBP and BP ECS treatment resulted in a significant decrease in latency to enter the dark chamber of the passive avoidance apparatus relevant to sham treated controls \( (p < 0.01) \). This suggests that both UBP and BP ECS treatment result in a deficit in passive avoidance behaviour in the WKY strain.
Figure 4.3.2.5: Both BP and UBP ECS treatment are associated with a deficit in fear conditioned memory response in the WKY but not the Wistar strain as assessed by the step-through passive avoidance test.

The effect of both BP and UBP ECS treatment on fear conditioned memory in the Wistar and WKY strain was assessed via the step through passive avoidance task. The Wistar strain (a) exhibits a significant increase in latency during the retention trial which is effected by neither BP nor UBP ECS treatment. The sham treated control WKY group (b) exhibits an increase in latency during the retention trial, however both BP and UBP ECS treatment result in a significant decrease in latency indicating a deficit in fear conditioned memory in this task. Data expressed as mean ± SEM (n=6-10). ++ p<0.01 relative to sham treated control; ** p<0.01 relative to training latency.
4.3.2.6 BP and UBP ECS treatment are associated with changes in GFAP protein expression in the Wistar rat strain as assessed by Western immunoblotting

One way ANOVA revealed an effect of treatment \([F(2,27)=5.15828, P=0.0133]\) \([\text{Figure 4.3.2.6 (a)}]\) on the relative quantification of GFAP in the frontal cortex of the Wistar rat strain. Newman-keuls post hoc comparisons revealed significantly lower GFAP expression in both the BP and the UBP ECS treated group relative to the sham ECS controls \((p<0.05)\). One way ANOVA revealed no effect of treatment \([F(2,28)=2.31305, P=0.119]\) \([\text{Figure 4.3.2.6 (c)}]\) on the relative quantification of actin in the frontal cortex of the Wistar rat strain. One way ANOVA revealed an effect of treatment \([F(2,28)=6.09504, P=0.0067]\) \([\text{Figure 4.3.2.6 (a)}]\) on the relative quantification of GFAP in the hippocampus of the Wistar rat strain. Newman-keuls post hoc comparisons revealed higher GFAP expression in the UBP ECS treated group relative to the BP ECS treated group \((p<0.01)\). One way ANOVA revealed no effect of treatment \([F(2,28)=1.75455, P=0.1929]\) \([\text{Figure 4.3.2.6 (d)}]\) on the relative quantification of actin in the hippocampus of the Wistar rat strain.
Figure 4.3.2.6: BP and UBP ECS treatment are associated with changes in GFAP protein expression in the Wistar rat strain as assessed by Western immunoblotting

BP and UBP ECS treatment led to a decrease in expression of GFAP in the frontal cortex of Wistar rats relative to sham ECS treated controls (a). There was an effect of treatment on the expression of GFAP in the hippocampus of Wistar rats (b). There was no effect of ECS treatment on actin expression in either the frontal cortex (c) or hippocampus (d) of Wistar rats. Data expressed as mean ± SEM (n=9-10). + p<0.05 relative to BP ECS treated group; * p<0.05 relative to sham ECS controls
4.3.2.7 BP ECS treatment is associated with changes in actin protein expression in the WKY rat strain as assessed by Western immunoblotting

One way ANOVA revealed no effect of treatment [F(2,27)=1.41993, P=0.2606] [Figure 4.3.2.7 (a)] on the relative quantification of GFAP in the frontal cortex of the WKY rat strain. One way ANOVA revealed an effect of treatment [F(2,27)=4.58581, P=0.0197] [Figure 4.3.2.7 (c)] on the relative quantification of actin in the frontal cortex of the WKY rat strain. Newman-keuls post hoc comparisons revealed significantly higher actin expression in both the BP ECS treated group relative to the sham ECS controls (p<0.05). One way ANOVA revealed no effect of treatment [F(2,26)=0.18549, P=0.8319] [Figure 4.3.2.7 (b)] on the relative quantification of GFAP in the hippocampus of the WKY rat strain. One way ANOVA revealed no effect of treatment [F(2,27)=0.55379, P=0.5817] [Figure 4.3.2.7 (d)] on the relative quantification of actin in the hippocampus of the WKY rat strain.
Figure 4.3.2.7: BP ECS treatment is associated with changes in actin protein expression in the WKY rat strain as assessed by Western immunoblotting

Neither BP nor UBP ECS treatment led to a change in the expression of GFAP in the frontal cortex a) or the hippocampus b) of WKY rats relative to sham ECS treated controls (a). BP ECS treated led to an increase in the expression of actin in the frontal cortex of WKY rats relative to sham ECS treated controls. Neither BP nor UBP ECS treatment led to a change in the expression of actin in the hippocampus (d) of WKY rats. Data expressed as mean ± SEM (n=9-10). + p<0.05 relative to BP ECS treated group; * p<0.05 relative to sham ECS controls
4.3.3 The effects of chronic restraint stress on the behavioural phenotype and astrocyte markers in the WKY rat strain

Given that the WKY rat strain is known to be sensitive to stress, and as the ECS induced antidepressant response in the WKY rat strain was associated with a non significant increase in GFAP protein expression in the frontal cortex, we sought to investigate if chronic stress in the WKY rat strain resulted in increased depressive and anxiety-like behaviours and/or changes in brain GFAP protein expression.

4.3.3.1 10 days of restraint stress results in an increase in immobility in the FST in the WKY rat strain

Student’s t-test revealed that chronic restraint stress results in an increase in immobility time in the FST \( t=2.124; \ df=16; \ p=0.0496 \) [Figure 4.3.3.1 (a)] when compared to non-stressed WKY controls.

![Figure 4.3.3.1: 10 days of chronic restraint stress results in an increase immobility in the FST in the WKY rat strain](image)

Stress dependent differences in depressive-like behaviour were assessed using the FST paradigm. Data expressed as mean ± SEM (n=9). * \( p<0.05 \) vs. non-stressed control group.
4.3.3.2 10 days of restraint stress does not result in an increase in anxiety-like behaviour in the open field test in the WKY rat strain

Student's t-test revealed that chronic restraint stress results in a significant decrease in total distance moved in the open field arena \[t=2.973; \text{df}=16; p=0.009\] [Figure 4.3.3.2 (a)] when compared to non-stressed WKY controls. Student's t-test revealed that chronic restraint stress results in no significant change in total distance moved in the inner zone of the open field arena \[t=0.2857; \text{df}=15; p=0.779\] [Figure 4.3.3.2 (b)], or in number of rearings in the open field arena \[t=0.6565; \text{df}=15; p=0.5214\] [Figure 4.3.3.2 (c)] when compared to non-stressed WKY controls. Student's t-test revealed that chronic restraint stress results in a significant increase in total time spent grooming in the open field arena \[t=2.785; \text{df}=15; p=0.0139\] [Figure 4.3.3.2 (d)] when compared to non-stressed WKY controls.
Figure 4.3.3.2: 10 days of restraint stress does not result in an increase in anxiety-like behaviour in the open field test in the WKY rat strain

The effect of chronic restraint stress on locomotor activity and anxiety-like behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena (c) Total number of rears in the open field (d) Total time spent grooming. Data expressed as mean ± SEM (n=8-9). * p<0.05; ** p<0.01 vs. non-stressed control group
4.3.3.3 10 days of restraint stress does not result in an increase in anxiety-like behaviour in the elevated plus maze in the WKY rat strain

Student's t-test revealed that chronic restraint stress results in no significant change in entries into the open arms of the elevated plus maze \( t=0.1178; \ df=16; \ p=0.9077 \) [Figure 4.3.3.3 (a)] when compared to non-stressed WKY controls. Student's t-test revealed that chronic restraint stress results in a significant increase in entries into the closed arms of the elevated plus maze \( t=2.842; \ df=15; \ p=0.0124 \) [Figure 4.3.3.3 (b)] when compared to non-stressed WKY controls. Student's t-test revealed that chronic restraint stress results in no significant change in total distance moved in the elevated plus maze \( t=0.3335; \ df=16; \ p=0.7431 \) [Figure 4.3.3.3 (c)] when compared to non-stressed WKY controls.
Figure 4.3.3.3: 10 days of restraint stress does not result in an increase in anxiety-like behaviour in the elevated plus maze in the WKY rat strain
(a) Total number of entries into the open arms of the elevated plus maze, (b) total number of entries into the closed arms of the elevated plus maze, (c) total distance moved in the elevated plus maze. Data expressed as mean ± SEM (n=8-9). * p<0.05 vs. non-stressed control group
4.3.3.4 10 days of immobilisation stress results in altered mRNA expression of genes related to astrocyte function in the frontal cortex of the WKY rat

Student's t-test revealed that chronic restraint stress results in a significant decrease in S100B \( [t=2.170; \text{df}=16; \ p=0.0454] \) [Table 4.3.8] and glutamine synthetase mRNA expression \( [t=4.202; \text{df}=15; \ p=0.0008] \) [Table 4.3.8] and an increase in GDNF mRNA expression \( [t=2.228; \text{df}=15; \ p=0.0416] \) [Table 4.3.8] in the frontal cortex when compared to non-stressed WKY controls.

Table 4.3.8: 10 days of immobilisation stress results in altered mRNA expression related to astrocyte function in the frontal cortex at baseline

<table>
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</tr>
</thead>
<tbody>
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<td>1 ± 0.0505</td>
<td>0.9142 ± 0.0359</td>
</tr>
<tr>
<td>GLAST</td>
<td>1 ± 0.0616</td>
<td>0.8917 ± 0.0473</td>
</tr>
<tr>
<td>GFAP</td>
<td>1 ± 0.0842</td>
<td>0.9537 ± 0.0742</td>
</tr>
<tr>
<td>S100B</td>
<td>1 ± 0.0616</td>
<td>0.8261 ± 0.0512 *</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>1 ± 0.1330</td>
<td>0.4203 ± 0.0543 ***</td>
</tr>
<tr>
<td>GDNF</td>
<td>1 ± 0.1280</td>
<td>2.285 ± 0.5300 *</td>
</tr>
</tbody>
</table>

GDNF, glial cell line-derived neurotrophic factor, GFAP, glial fibrillary acidic protein, GLAST, glutamate-aspartate transporter, GLT-1, glutamate transporter type 1.

Data expressed as mean ± SEM (n=9) *** \( p<0.001 \); * \( p<0.05 \) vs. non-stressed control group
4.3.3.5 10 days of restraint stress results in a decrease in GDNF mRNA expression in the hippocampus of the WKY rat

Student’s t-test revealed that chronic restraint stress results in a significant decrease in GDNF mRNA expression [t=2.57; df=15; p=0.0213] [Table 4.3.9] in the hippocampus when compared to non-stressed WKY controls.

Table 4.3.9: 10 days of restraint stress results in no change in mRNA expression related to astrocyte function in the hippocampus of the WKY rat

<table>
<thead>
<tr>
<th>Target</th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLT-1</td>
<td>1 ± 0.0661</td>
<td>1.050 ± 0.0960</td>
</tr>
<tr>
<td>GLAST</td>
<td>1 ± 0.0625</td>
<td>1.031 ± 0.0650</td>
</tr>
<tr>
<td>GFAP</td>
<td>1 ± 0.0626</td>
<td>0.9088 ± 0.0351</td>
</tr>
<tr>
<td>S100B</td>
<td>1 ± 0.0436</td>
<td>1.039 ± 0.0886</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>1 ± 0.1030</td>
<td>0.9727 ± 0.1111</td>
</tr>
<tr>
<td>GDNF</td>
<td>1 ± 0.0784</td>
<td>0.7359 ± 0.0639 *</td>
</tr>
</tbody>
</table>

GDNF, glial cell line-derived neurotrophic factor, GFAP, glial fibrillary acidic protein, GLAST, glutamate-aspartate transporter, GLT-1, glutamate transporter type 1.

Data expressed as mean ± SEM (n=8-9)
4.3.3.6 10 days of restraint stress results in no change in GFAP protein expression in the frontal cortex or the hippocampus of the WKY rat as assessed by Western immunoblotting.

Student’s t-test revealed that chronic restraint stress results in no significant difference in GFAP protein expression in the frontal cortex \([t=0.4546; \text{df}=14; \ p=0.6563]\) [Figure 4.3.3.4 (a)] or the hippocampus \([t=0.8997; \text{df}=15; \ p=0.8997]\) [Figure 4.3.3.4 (b)] when compared to non-stressed WKY controls.

Figure 4.3.3.4: 10 days of restraint stress results in no change in GFAP protein expression in the frontal cortex or the hippocampus of the WKY rat as assessed by Western immunoblotting.

The effect of chronic restraint stress on GFAP protein expression was quantified in (a) the frontal cortex and (b) the hippocampus. Data expressed as mean ± SEM \((n=7-8)\)
4.4 Discussion

4.4.1 Behavioural, MRI and GFAP immunohistochemical characterisation of the WKY rat strain

The primary aim of the present study was to determine if the changes in the density of GFAP positive astrocytes previously reported in the WKY rat strain relative to the Sprague-Dawley rat (Gosselin et al., 2009) were also evident relative to the Wistar rat, the out-bred progenitor strain of the WKY rat. These studies also sought to investigate if changes in GFAP positive astrocyte density were associated with changes in MR imaging markers.

4.4.1.1 Performance deficits in hippocampal dependent OD learning task

As expected, the WKY rat strain exhibited depressive and anxiety-like behaviour when compared to the Wistar comparator strain in line with previous reports (Nagasawa et al., 2012; Nam et al., 2014; Tejani-Butt et al., 2003). Although the behavioural phenotype of the WKY strain is well characterised, one aspect that has not been extensively studied is its performance in tests of cognition. Although there are some previous reports that the strain exhibits deficits in working memory as assessed in the Morris water maze paradigm when compared to Sprague-Dawley out-bred rats (Wyss et al., 2000), others have reported no significant difference in performance in this test between the strains (Ferguson and Cada, 2004). We sought to further investigate if the WKY rat strain displays any deficits in memory related tasks when compared to the Wistar strain. WKY rats were subjected to an object displacement paradigm, a test of spatial learning mediated by the NMDA receptor and thought to be associated with the expression of phosphorylated extracellular signalling kinase (ERK) in the dentate gyrus (Griffin et al., 2009; Larkin et al., 2008). These data show that unlike the out-bred Wistar comparator strain the WKY strain does not learn the task. Given that decreased hippocampal volume has previously been correlated with memory loss in depressive patients (Hickie et al., 2005), it is possible that the decreased hippocampal volume reported in WKY rats here may be related to deficits in the performance of this task. However, a significant correlation between performance in this task and hippocampal volume measurements within the WKY rat strain was not observed (data not shown). In contrast the WKY rat strain did not show any deficit in the passive avoidance test. The passive avoidance test is thought to be a test of emotional memory, which is dependent on the hippocampus and the amygdala (Cahill and McGaugh, 1998; Holland and Bouton, 1999). This is in
agreement with previous studies which have reported that WKY rats show prolonged latency in the passive avoidance test when compared to other strains (Paré, 1996a, 1993).

### 4.4.1.2 Differences in regional brain volume in the WKY rat strain

Here, for the first time, the brain morphology of the WKY rat strain has been characterised by MR imaging relative to its out-bred progenitor strain, the Wistar rat. Consistent with previous reports of lower hippocampal volume in female WKY rats relative to sex matched Wistar rats, it was shown that male WKY rats exhibit a significant decrease in normalised hippocampal volume relative to the Wistar comparator strain. This is of interest when we consider that reduced hippocampal volume is one of the most robust imaging markers observed in depressive illness (Arnone et al., 2012; Kempton et al., 2011; Koolschijn et al., 2009) and is also thought to be indicative of poor antidepressant response (Frodl et al., 2008a). The cause of the decreased hippocampal volume observed in depressed patients is still unclear, but it appears to occur in the absence of major neuronal or glial cell loss, with several studies reporting significant increases in neuronal and glial packing density in several hippocampal subfields analysed (Cobb et al., 2013; Czéh and Lucassen, 2007; Stockmeier and Mahajan, 2004). These studies do not however differentiate between which glial cells are quantified. There is some evidence to suggest a reduction in GFAP immunoreactivity in the hippocampus in depressed patients. Semiquantitative assessment on GFAP immunoreactivity in post-mortem depressed patients has revealed decreased immunoreactivity in the CA1 and CA2 subfields of the hippocampus (Müller et al., 2001). Stress induced increases in glucocorticoids are also thought to play a role, perhaps through induction of dendritic remodelling (Musazzi et al., 2011).

Reduced hippocampal volumes have been reported in other animal models of depression, including in behaviourally depressed female macaques primates (Willard et al., 2013), prenatally stressed Rhesus monkeys (Coe et al., 2003), prenatally stressed female mice (Behan et al., 2011) and chronically stressed rodents (Lee et al., 2009). It has been suggested that reduced noradrenaline levels in the ventral hippocampus as reported by Scholl and colleagues (2010) may be related to the abnormal hippocampal morphology reported in the WKY strain as noradrenaline has previously been shown to promote neurogenesis in this region (Rizk et al., 2006; Scholl et al., 2010). Another possible cause of the morphological differences is the observation that WKY rats have
lower basal levels of brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus when compared to Wistar rats (Vinod et al., 2012). The decrease in GFAP positive astrocyte cell number presented above may also be a factor in the apparent difference in hippocampal volume. Glucocorticoid related excitotoxicity and dendritic remodelling may also play a role as unpublished results from our lab suggest that the WKY strain exhibits glucocorticoid receptor hypersensitivity. This is evidenced by increased stress induced mRNA expression of several glucocorticoid inducible genes including FK506 binding protein (FKBP5), glucocorticoid inducible leucine zipper (GILZ) and serum and glucocorticoid induced protein kinase (SGK1) relative to the Wistar rat strain (data not shown).

This is the first time to the authors knowledge that enlarged ventricles have been reported in this model. This is in contrast to data from Tajima and colleagues (1993), who have reported smaller ventricular volume and larger total brain volume in WKY rats when compared to the SHR strain, however it is likely that the hypertension exhibited in the SHR may be related to increased ventricular size in that strain (Tajima et al., 1993). Ventricular enlargement is another commonly reported finding of clinical neuroimaging in depression literature (Kempton et al., 2011). Increased ventricular volume has also been found in another animal model of depression, the olfactory bulbectomised rat (Gigliucci et al., 2014; Wrynn et al., 2000). The cause of these changes in ventricular volume in the clinical population and in these pre-clinical models remains unknown. Changes in ventricular volume do not appear to be directly related to changes in hippocampal volume as there is no significant correlation between the two in the WKY strain (data not shown).

The relatively small, but significant increase in midbrain volume within the WKY rat strain is of interest when we consider that increased midbrain volume has previously been associated with panic disorder in the clinical population (Fujiwara et al., 2011). This is in agreement with the growing body of literature suggesting that the WKY rat model is a suitable model of vulnerability to anxiety disorders (Jiao et al., 2011).

These results add significantly to the face and construct validity of the WKY rat strain as an animal model of anxiety and depression and suggest it is a suitable model for further studies investigating the biology underlying some of the most robust imaging markers observed in depressive disorders. One limitation of the region of interest based quantification technique employed in these studies is that it is only possible to analyse
well defined anatomical structures. Future studies may make use of voxel based
morphometry to provide whole brain unbiased information on grey matter volumes in
regions which cannot be easily defined manually such as the prefrontal cortex
(Ashburner and Friston, 2000). Furthermore diffusion tensor imaging (DTI) techniques
may enable us to gain further knowledge of strain related differences in brain
microstructure and how this may and how this is related to behavioural phenotype in
the WKY rat [for review see (Zhang et al., 2012)].

4.4.1.3 Decreased cerebral blood flow in the WKY rat strain
The data show that the WKY strain exhibits a trend for lower resting state blood
perfusion in all regions analysed relative to the Wistar strain expressed as increased CTT
and MTT. The most significant findings are of decreased blood perfusion in the pre-
limbic cortical and striatal regions analysed, both of which show a significant increase in
MTT and CTT times as well as a corresponding decrease in signal amplitude. This is in
agreement with a previous imaging study in WKY rats by Danker and colleagues (2009)
which showed decreased resting state blood perfusion in the medial prefrontal cortical
region relative to the SHR strain via ASL (Danker and Duong, 2007). It is noteworthy that
the medial prefrontal cortex in rodents is commonly regarded as analogous to the
dorsolateral prefrontal cortex in primates, although that is still a matter of debate
(Preuss, 1995). These data also show a decrease in perfusion in the striatal region
analysed. Perfusion changes in this region have also been found in depressive patients.

This is the first study comparing brain perfusion between the WKY rat model of
depression and its normotensive progenitor strain the Wistar rat. These data add to the
validity of the WKY rat strain as a model of depression as there is evidence to suggest
decreased resting state activity in the medial prefrontal cortex of depressed patients
(Savitz and Drevets, 2009b). Although there are relatively few studies assessing cerebral
blood flow via ASL in depression, it is of interest that several studies have reported
decreased resting state blood perfusion in the frontal cortex of depressed patients
relative to healthy controls via MR ASL (Ho et al., 2013; Järnum et al., 2011; Lui et al.,
2009). Decreased perfusion in the prefrontal cortex may be related to decreased
neuronal activity in these regions which are known to play an important role in the
neurocircuitry associated with depressive disorders [for review see (Price and Drevets,
2010)]. There is also evidence of decreased volume, perfusion and glucose metabolism
in the ventromedial striatum in depressed patients relative to healthy controls (Drevets,
2007). However it is difficult to compare the results of decreased striatal perfusion presented here with the human data, as the dorsal striatum in rodents is not divided up clearly into ventromedial and dorsolateral as in primates. Hence it is possible that the hypoperfusion observed in these regions may be related to the depressive-like phenotype exhibited in the WKY rat strain. Indeed decreased frontal cortical perfusion has previously been linked with negative depressive symptoms (Galynker et al., 1998).

The significant change in MTT and the corresponding decrease in signal amplitude reported in the third ventricular region are most likely related to the increased ventricular volume exhibited in the WKY strain as discussed above. The lack of any other significant change in perfusion in the hippocampal section suggests that changes recorded elsewhere are regionally specific and not as a result of a strain related difference in general cerebral perfusion.

Changes in cerebral blood flow (CBF) are commonly interpreted as a surrogate marker for changes in neuronal activity as cerebral blood flow is tightly regulated and linked to regional synaptic activity through a process referred to as functional hyperaemia (Drake and Iadecola, 2007). There is some evidence from the literature relating to animals models to suggest that decreased neuronal activation within the prefrontal cortex is associated with depressive-like behaviours, but findings to date are contradictory [for review see (Alcaro et al., 2010)]. However as both BOLD and ASL signals are dependent on changes in local cerebrovascular dynamics, and given that astrocytes directly control vascular tone, it is not unreasonable to suggest that rather than surrogate markers for neuronal activations, these may be surrogate markers of astrocytic function (Figley and Stroman, 2011; Koehler et al., 2009). Thus it is of interest that within the WKY strain there was a moderate negative correlation between perfusion values MTT and CTT and the number of GFAP positive cells found in the pre-limbic cortical and primary motor cortical regions. This may suggest that decreased GFAP positive astrocytes may be associated with decreased cortical perfusion within these regions. Further studies will have to be conducted to investigate if modulating glial activity in these regions leads to changes in localised perfusion parameters.

4.4.1.4 Increased T2 relaxation time in the hippocampus of WKY rats

Given that increases in GFAP immunoreactivity have previously been correlated with increases in T1 relaxation times of tissue, we hypothesised that the regional decreased GFAP immunoreactivity reported in the WKY rat strain may be detectable as reduced
regional tissue T1 relaxation times (Cowley et al., 2012). However, no significant changes in T1 relaxation time were observed in either the pre-limbic (data not shown) or the hippocampal regions which we have shown exhibit decreased GFAP immunoreactive cell number in the post-mortem brain. Previous studies have shown that both acute astrocytic activation following ischaemic insult, and chronic astrocytic activation observed in aged animals are associated with an increase in T1 relaxation time of tissue (Cowley et al., 2012; Sibson et al., 2008). However the exact mechanism by which astrocytic activation increases T1 relaxation time is as yet unknown. Thus it may be that T1 relaxation times are not a suitable MR marker for the detection of decreased astrocytic function and may instead only change in response to increased astrocytic activation.

A significant increase in hippocampal T2 relaxation time was detected in the hippocampus of WKY rats relative to the Wistar rat strain. Increased hippocampal T2 relaxation times have previously been reported in a number of temporal lobe epilepsy patient cohorts (Briellmann et al., 2002; Grunewald et al., 1994; Scott et al., 2003). Briellman and colleagues (2007) have reported that patients with both major depression and temporal lobe epilepsy did not exhibit a significant increase in hippocampal T2 relaxation time or decrease in hippocampal volume when compared to patients with temporal lobe epilepsy alone, hence depression does not appear to directly affect T2 relaxation times in these patients (Briellmann et al., 2007). Moreover, a single previous study has reported no significant change in T2 relaxation times in the hippocampus of drug resistant depressed patients relative to age matched controls (Mervaala et al., 2000). Increases in T2 relaxation times correlate with reduced cerebral blood volume in brain sub-regions in human controls (Anderson et al., 2005). However there was no correlation between T2 relaxation times and perfusion markers in any of the regions analysed in these studies (data not shown). Increased T2 may suggest oedema or changes in vascular permeability in the WKY rat relative to the Wistar rat (Qiao et al., 2001).

4.4.1.5 Decrease GFAP positive cell numbers in cortical and limbic regions in the WKY strain

These data show that the WKY rat strain exhibits significantly lower GFAP positive astrocyte cell number in the pre-limbic cortical and the CA3 subfield of the hippocampus relative to the Wistar rat strain. This is in agreement with data previously published by
Gosselin and colleagues (2009) which showed decreased GFAP positive cell numbers in the prefrontal cortex, basolateral amygdala and the CA3 subfield of the hippocampus in the WKY when compared to the Sprague-Dawley rat strain (Gosselin et al., 2009).

As previously stated changes in GFAP immunoreactivity in a number of brain regions have been reported in post-mortem studies of the brains of depressed patients (Rajkowska and Stockmeier, 2013). It should be noted that reductions in GFAP positive cell numbers may not represent reductions in total astrocyte cell numbers in these animals. GFAP is an important structural protein in mature astrocytes, but several isoforms of GFAP have been identified, which may represent subpopulations of astrocytic cells, not all of these isoforms will be stained by commonly used antibodies. The exact epitope to which the DAKO GFAP antibody used in these studies binds to is unknown (Middeldorp and Hol, 2011). GFAP has been shown to play an important role in the transport of astrocytic glutamate transporter type 1 (GLT-1) and anchoring of the glutamate-aspartate transporter (GLAST) to the membrane (Hughes et al., 2004; Sullivan et al., 2007). Hence changes in GFAP immunoreactivity are related to changes in the ability of astrocytes to control synaptic glutamate concentrations. Thus future studies may use MR spectroscopy to identify if the observed changes in GFAP positive cell numbers in these regions are correlated with changes in glutamate concentrations.
4.4.2 The effects of BP and UBP ECS treatment on behavioural phenotype and brain GFAP expression in the WKY rat strain

O'Donovan and colleagues (2014) have shown that both BP and UBP ECS treatment is effective in attenuating chronic corticosterone administration induced depressive-like behaviour, to date this is the only study to examine the effects of UBP ECS treatment in an animal model of depression (O’Donovan et al., 2014a). Following the behavioural, MRI and immunohistochemical characterisation of the WKY rat strain, we sought to investigate the effects of BP and UBP ECS treatment on the behavioural phenotype of this animal model of depressive-like behaviour.

4.4.2.1 Both BP and UBP ECS treatment reduce immobility time in the FST in the WKY rat strain

Both BP and UBP ECS treatment resulted in a decrease in immobility time in the FST to the same extent in the WKY strain. This is in agreement with previous studies which have shown that ECS treatment is effective at reducing immobility in the WKY rat strain, and that both BP and UBP ECS treatment are similarly effective at reducing immobility in the FST in an animal model of depressive-like behaviour (Krahl et al., 2004; Kyeremanteng et al., 2014; O’Donovan et al., 2014a). Whereas O’Donovan and colleagues (2012) have previously reported that BP, but not UBP ECS treatment resulted in a decrease in immobility in the FST in Sprague-Dawley rats relative to sham treated controls, in the present study neither BP nor UBP ECS treatment resulted in a significant decrease in immobility in the FST within the Wistar strain (O’Donovan et al., 2012). This may be related to previously reported differences in baseline immobility and antidepressant response between Sprague-Dawley and Wistar rats (Crespi, 2010).

4.4.2.2 Both BP and UBP ECS treatment are associated with increased anxiety-like behaviour in the open field

The open field test is commonly used in most behavioural batteries to test for differences in locomotor activity and in anxiety-like behaviour (Stanford, 2007). Within the Wistar strain UBP ECS treatment appears to be associated with more anxiety-like behaviours expressed as increased latency to enter the centre circle of the open field. ECS treatment has previously been reported to have no effects on locomotor activity (Nakamura et al., 2013). This may be related to the relatively stressful nature of ECS treatment. Unlike ECT, muscle relaxants and anaesthesia are not commonly used in ECS. Both BP and UBP ECS treated Wistar rats also exhibited increased time grooming, a
behaviour that has previously been reported in an open field test conducted 5 days following an acute stressor (Daniels et al., 2008). Decreased locomotor activity as assessed in the open field test has previously been reported in ECS treated animals 7 days post ECS treatment, this then returned to sham treated control levels 14 days following treatment. It should be noted that this was a sine wave electrical stimulus, unlike the square wave stimulus used in the present study (Calais et al., 2013). In the present study we cannot exclude the possibility that the change in latency to enter and number of entries to the centre circle here interpreted as anxiety-like behaviour are confounded by a change in locomotor activity following treatment. In the WKY strain both BP and UBP ECS treatment resulted in decreased exploratory behaviour in the open field. Again UBP ECS treatment produced more anxiety-like behaviour as evidenced by the increased number of faecal boli deposited by the UBP ECS treated group relative to sham treated controls.

4.4.2.3 Both BP and UBP ECS treatment produce cognitive deficits

As UBP ECS has been suggested to produce less cognitive side effects than BP ECS treatment in clinical cohorts, we wished to investigate the cognitive effects of these treatments in an animal model of depressive-like behaviour (Verwijk et al., 2012). Two tests of hippocampal dependent learning were conducted to determine whether UBP ECS administration results in less cognitive side effects when compared to BP ECS treatment. Both BP and UBP treatment resulted in a deficit in spatial working memory in the Wistar strain as assessed by the object displacement task. No effect of treatment was detected in the WKY strain as sham ECS treated controls did not learn the task. Future studies should reduce the difficulty of the task by reducing the retention period down from 24 hours to allow for treatment associated effects to be assessed in the WKY rat strain. Liu and colleagues (2012) have previously reported that ECS treatment does induce a deficit in spatial working memory in the WKY rat strain. They report that ECS induced cognitive deficits in the WKY rat strain as assessed in the Morris water maze paradigm are associated with increased hippocampal glutamate levels and Tau protein phosphorylation (Liu et al., 2012).

The opposite effects were seen in the step through passive avoidance test. Both BP and UBP treatment resulted in a significant decrease in latency to cross in the WKY strain, while neither treatment had an effect in the Wistar strain. This is of interest as WKY rats have previously been reported to have a more pronounced passive avoidance response
when compared to a number of other strains (Paré, 1993). These data suggest that the two strains may be exhibit differing susceptibility to the deleterious effects of ECS treatment, and that this may be related to the effects of ECS treatment on different parts of the hippocampus. In the Wistar strain cognitive deficits appear to be associated with impairment in dorsal hippocampal dependent memory more so than in ventral hippocampal dependent memory. Spatial memory is thought to be dependent on the dorsal hippocampus in rodents, which is analogous to the posterior hippocampus in primates. Fear conditioned tasks by contrast are thought to be more dependent on the ventral hippocampus in rodents which is analogous to the anterior hippocampus in primates. However retention of the contextual (spatially associated) fear required for the step through passive avoidance task employed in the present studies relies on both dorsal and ventral hippocampus. Cued fear conditioned paradigms are thought to be more dependent of the ventral hippocampus and amygdala, hence future studies may employ such a behavioural test to help further elucidate the hippocampal regions involved in the strain dependent cognitive deficits induced by ECS treatment [for review see (Fanselow and Dong, 2010)]. From the tests conducted in this study, both BP and UBP ECS treatment appear to induce cognitive deficits, however they differ by strain. It is possible that alternative ECS dosing regimens may produce differing effects.

4.4.2.4 BP and UBP ECS treatment alter the expression of cytoskeleton proteins in the frontal cortex and hippocampus of WKY and Wistar rats

BP and UBP ECS treatment resulted in a small but significant decrease in GFAP protein expression in the frontal cortical region of Wistar rats relative to sham treated controls. This was not seen in WKY rats where BP and UBP ECS treatment resulted in a non significant increase in GFAP protein expression. UBP ECS treatment resulted in significantly more GFAP protein expression than BP ECS treatment in the hippocampus of Wistar rats, however neither treatment produced a significant difference from sham treated controls. Neither ECS treatment had any effect on GFAP protein levels in the hippocampus of WKY rats relative to sham treated controls. There are conflicting reports in the literature as to the effects of ECS treatment on GFAP expression in the rodent brain. Ceresér and colleagues (2006) have previously reported decreased hippocampal GFAP protein levels following ECS treatment which persist for 7 days, however no change in cortical GFAP expression was recorded (Ceresér et al., 2006). Furthermore immunohistochemical quantification has previously shown that ECS treatment is not associated with any changes in glial or neuronal cell number in the hippocampus or
frontal cortex in a primate model (Dwork et al., 2009). Increased numbers of reactive astrocytes (astrocytes which express intermediate filament nestin) were reported in the prefrontal cortex, hippocampus and amygdala of rodents 2 hours following ECS treatment, this did not however persist to a subsequent four week time point (Jansson et al., 2009). One limitation of the current study is that GFAP protein was assessed for the whole hippocampus and frontal cortex thus making it impossible to discern if BP or UBP ECS treatment is associated with sub-regional specific changes in astrocyte activity or morphology. Hence further work is warranted to investigate the effects of ECS treatment on astrocyte cell number and morphology, to establish if changes in astrocyte activity are involved in the antidepressant response to ECS treatment.

Levels of the cytoskeleton protein actin were also quantified, as reductions in hippocampal actin protein have previously been reported up to four weeks following chronic ECS treatment in rodents (O’Donovan et al., 2014b). Here BP ECS treatment resulted in an increase in actin protein levels in the frontal cortex of WKY rats, with no significant change in hippocampal protein levels, while no ECS induced change in actin protein levels were detected in the Wistar rat strain. It should be noted that tissue in this study was taken nine days following the final ECS treatment, not four weeks as in the O’Donovan study which may account for the lack of change in Wistar actin protein levels. Actin is known to play an important role in synaptic plasticity and changes in actin protein levels have previously been reported following antidepressant treatment (Carboni et al., 2006; Matus, 2000). However as only BP ECS treatment led to a significant increase in actin protein levels in the frontal cortex, while both BP and UBP ECS treatment produced a similar antidepressant behavioural response in the WKY rat, actin protein levels are not a reliable marker of antidepressant response in the WKY rat.
4.4.3 The effects of chronic restraint stress on the behavioural phenotype and astrocyte markers in the WKY rat strain

Although it is known that the WKY rat strain exhibits heightened behavioural responsivity to stressors and exhibits a prolonged stress induced elevation in plasma corticosterone concentration relative to the Wistar strain (De La Garza and Mahoney, 2004; Paré, 1996b), the effect of stress on astroglial cell function in the strain has not been well studied. We sought to investigate the effects of 10 days of chronic restraint stress on the behavioural phenotype of the WKY rat and to assess if any changes in behaviour were associated with changes in mRNA expression of genes associated with astrocytic function and/or GFAP protein expression.

4.4.3.1 Chronic restraint stress induced changes in depressive and anxiety-like behaviour in the WKY rat is associated with changes in astrocyte related mRNA markers in the frontal cortex

The chronic restraint stress paradigm adopted in the present experiments was chosen as it has previously been shown to alter neurotransmitter levels and decrease serum BDNF concentrations in the WKY rat strain (O'Mahony et al., 2011). Chronic restraint stress resulted in an increase in immobility behaviour over the course of the first 5 minutes in a single 15 minute swim, in the absence of any significant change in anxiety-like behaviour as assessed in the open field test and the elevated plus maze. 21 days of chronic unpredictable stress has previously been shown to increase immobility in the FST in the WKY rat strain as well as reduce exploration in an open field test (Tejani-Butt et al., 1994). This is in agreement with previous work in rodent models which has shown that chronic restraint stress (6 hours per day for 28 days) in Wistar rats results in an increase in anxiety-like behaviour as assessed in the open field and an increase in immobility time in the FST (Chiba et al., 2012). However this is the first study to show that 10 days of chronic restraint stress is sufficient to induce an increase in immobility time in the FST in the WKY rat strain.

Chronic restraint stress resulted in a decrease in mRNA expression of calcium binding protein S100B and glutamine synthetase enzyme and an increase in glial cell-line derived neurotrophic factor (GDNF) in the frontal cortex of stressed WKY rats relative to non-stressed control animals. Glutamine synthetase is an astroglial enzyme responsible for the metabolism of glutamate to glutamine. Decreased levels of glutamine synthetase have previously been reported in the frontal cortex of depressed patients (Choudary - 174 -
and Molnar, 2005). Furthermore inhibition of glutamine synthetase in the frontal cortex has been shown to induce depressive-like behaviours in animal models (Lee et al., 2013). Hence it is possible that reductions in the expression of this enzyme are associated with the increase in depressive-like behaviours exhibited in the WKY rat following chronic restraint stress. S100B is a calcium binding protein expressed primarily in the central nervous system by astrocytes and oligodendrocytes. Decreased levels of S100B protein have previously been reported in the hippocampus of depressed patients (Gos et al., 2013), and the frontal cortex of Sprague-Dawley rats subjected to a 21 day chronic unpredictable stress paradigm (Luo et al., 2010). Furthermore increased serum levels of S100B have been suggested as a potential biomarker for mood disorders (Schroeter et al., 2008). At normal physiological concentrations S100B is neurotrophic, however at higher concentrations it exerts pro-inflammatory and toxic effects on neurons (Donato and Heizmann, 2010). The effect of central alterations in S100B protein levels on depressive-like behaviour is still unknown, however it is possible that the decreased mRNA expression of S100B reported here may be related to decreased trophic support for neurons in the frontal cortex, which may in turn impact on behaviour. The increase in GDNF in the frontal cortex is in contrast to this. GDNF has been suggested as a marker for activated astrocytes, with increased GDNF protein levels reported in the hippocampus of rats in a prion disease model thought to be associated with a protective effect against prion induced neuronal damage (Bresjanac and Antauer, 2000; Lee et al., 2006). This is in keeping with data from human post-mortem brain tissue, suggesting that patients with neurodegenerative Alzheimer’s disease exhibited increased frontal cortical protein levels of brain-derived neurotrophic factor (BDNF) (Durany et al., 2000). GDNF mRNA levels were significantly lower in the hippocampus of stressed WKY rats relative to non-stressed controls. GDNF protein levels have previously been reported to be decreased in the hippocampus of Sprague-Dawley rats which were subjected to a chronic unpredictable stress procedure (Liu et al., 2012). The data presented here is in line with a study by Michel and colleagues (2008) which reported significantly increased cortical GDNF protein concentrations in post-mortem tissue from depressed patients, while there was a non significant trend towards decreased hippocampal GDNF expression (Michel et al., 2008). Differential expression patterns of GDNF have also been reported in aged rats which show a significant increase in frontal cortical GDNF protein expression without any change in hippocampal protein expression (Matsunaga et al., 2006). The absence of any other changes in mRNA markers associated with astroglial cell...
function in the hippocampus suggests that astrocytes in the WKY strain respond to stress in a regionally dependant manner. The data reported here relate to changes in mRNA markers only, future studies will investigate if the change in mRNA gene expression for these markers is coupled with alterations in protein expression.

The lack of change in GFAP protein or mRNA expression in either the hippocampus or the frontal cortex in response to this chronic restraint stress paradigm is surprising. There have been previous reports that chronic stressors reduce GFAP positive cells and alter astrocyte morphology in the hippocampus in a tree shrew model (Czéh et al., 2006; Fuchs and Flügge, 2003). In addition decrease in GFAP protein levels in the periaqueductal grey matter have been detected in rodent models following chronic restraint stress (Imbe et al., 2012). In these reports the animals were subjected to stress for a longer period of time than in the 10 day restraint stress procedure described here. It is possible that a more prolonged or severe stress procedure is required in order to produce detectable differences in GFAP protein expression in these brain regions. Another possible explanation is that GFAP protein expression was measured for the whole hippocampus and frontal cortex. Thus the lack of detection of any significant changes in GFAP protein expression in these full regions does not preclude sub-regional specific changes in protein expression.
4.5 Conclusions

In these studies we report for the first time a number of MR imaging markers in a strain which is genetically predisposed to depressive and anxiety-like behaviours. The WKY rat strain exhibits decreased cortical and striatal perfusion relative to its out-bred progenitor strain the Wistar rat. Furthermore, cortical perfusion in the WKY rat strain is correlated with GFAP positive cell number. We also report on a number of brain volumetric abnormalities in the WKY rat strain relative to the Wistar including decreased hippocampal volume and increased ventricular volume that are similar to those seen in human depressed patients relative to non psychiatric controls. The WKY rat also exhibits an increase in T2 relaxation time in the hippocampus; further studies will be required to investigate if this is related to a change in BBB permeability in the WKY strain. These data add further validity to the WKY rat as a model of depressive and anxiety-like behaviour. They also suggest that the WKY rat may prove a suitable model to further investigate the underlying biological and molecular mechanisms which contribute to the MR imaging markers observed in human depressed patients. These data suggest that resting state blood perfusion may be a suitable surrogate marker for GFAP positive astroglial cell function. Further studies are required to investigate whether treatments which can attenuate depressive-like behaviour in the WKY rat are associated with an increase in GFAP positive cell number and blood perfusion markers in these regions.

We have also shown that chronic UBP ECS treatment is as effective as BP ECS treatment at reducing depressive-like behaviours in the WKY rat strain. While both BP and UBP reduce depressive-like behaviour and induce a deficit in the passive avoidance task to the same extent, neither significantly alters GFAP expression in the brains of WKY rats. Only BP ECS treatment is associated with a change in actin protein expression in the frontal cortex. As previously stated a limitation of immunoblotting is that it quantifies protein expression for a whole brain region, thus making it impossible to detect sub-region specific changes in expression of proteins of interest. Thus future studies should gather perfused tissue to enable immunohistochemical analysis of ECS induced changes in the WKY brain. Conducting MR imaging in the WKY rat strain following ECS treatment may be of interest to discern whether the antidepressant response exhibited in the WKY strain is related to changes in regional brain volumetrics or regional cerebral perfusion parameters.
Finally we have shown that the baseline depressive-like behaviour exhibited by the WKY rat strain is potentiated following chronic restraint stress and that this change in behaviour is associated with changes in mRNA expression of astroglial related markers. This is not however coupled with a decrease in frontal cortical or hippocampal GFAP protein expression. However the same limitations in protein quantification via immunoblotting discussed above apply here also. Hence future studies should investigate if chronic restraint stress is associated with sub-regional specific differences in brain GFAP protein expression. Conducting MR imaging following chronic stress may also be of interest to discern if the increase in depressive-like behaviour exhibited in the WKY rat strain is related to changes in regional brain volumetrics or regional cerebral perfusion parameters, and whether any such changes oppose those observed following antidepressant treatment.
Chapter 5 – Astrocytic toxin L-AAA results in administration site specific changes in rodent behaviour and is associated with changes in MR imaging markers.
5.1 Introduction

As previously discussed there is a growing body of evidence which implicates regional alterations in astrocyte function in the pathophysiology of depressive disorder [for review see (Rajkowska and Stockmeier, 2013)]. Several studies have reported that depressive-like behaviours in animal models are associated with changes in astrocyte cell number and function. Manipulations such as early life stress and chronic unpredictable stress, known to produce depressive-like behaviours are associated with decreased numbers of cells immunoreactive for astrocyte cytoskeletal protein glial fibrillary acidic protein (GFAP) in several brain regions (Banasr and Duman, 2008; Braun et al., 2009; Leventopoulos et al., 2007). Furthermore pharmacologically altering astrocyte function is sufficient to induce depressive-like behaviours in animal models (Banasr and Duman, 2008; Lee et al., 2013; Sun et al., 2011). These data all point to a role for localised alterations in astrocytic function in the development of mood disorders.

In the previous chapter we have shown that the depressive and anxiety-like behaviour exhibited by the Wistar-Kyoto (WKY) rat strain is associated with regional decreases in GFAP positive cell numbers. In order to further investigate the role that regionally altered astrocytic function has on behaviour in rodent models, we sought to characterise the behavioural change following administration of astrocyte specific toxin L-alpha amino adipic acid (L-AAA) into discrete brain regions involved in emotional cognition. L-AAA is a glutamate analogue which is transported into astrocytes primarily through the Na⁺ dependent transport system, but may also be taken up through the cysteine/glutamate antiporter (Brown and Kretzschmar, 1998). Significant and transient decreases in GFAP positive cell numbers have been reported in the vicinity of the injection site following L-AAA administration into the rodent brain (Khurgel et al., 1996). Furthermore administration of L-AAA into the rodent pre-limbic cortex, a brain region known to exhibit decreased numbers of GFAP positive cells in post-mortem tissue from depressed patients, has previously been shown to produce a depressive-like phenotype as assessed by a number of behavioural tests, including the forced swim test (FST) and the sucrose preference test (SPT) (Banasr and Duman, 2008). Administration of the tricyclic antidepressant imipramine has been shown to attenuate L-AAA induced increases in immobility time in the FST, indicating that this model has predictive validity (Domin et al., 2014).
In the previous chapter we reported that the WKY rat strain exhibits a decrease in the number of GFAP positive cells in the CA3 subfield of the hippocampus when compared to the Wistar strain, this is in agreement with previous work which has shown a decrease in GFAP positive cell numbers in WKY rats relative to the Sprague-Dawley strain (Gosselin et al., 2009). Decreased numbers of GFAP positive cells have previously been reported in the amygdala of post-mortem tissue from depressed patients as well as in animal models of depressive-like behaviour (Altshuler et al., 2010; Gosselin et al., 2009; Leventopoulos et al., 2007). Hence we sought to further elucidate the impact of astroglial dysfunction in these brain regions on depression and anxiety-related behaviours in a rodent model. To this end studies were undertaken to establish whether ablation of astrocytes in these regions via administration of astroglial toxin L-AAA was sufficient to induce depression and/or anxiety-related behaviours in Wistar rats.

Given that we have previously shown a positive correlation between GFAP positive cell number and blood perfusion in the pre-limbic cortical region of the WKY rat strain, we also sought to investigate if L-AAA induced astrocytic dysfunction produced a change in blood perfusion as assessed by magnetic resonance (MR) arterial spin labelling (ASL). Scans to assess T1 and T2 relaxation time data were also acquired to investigate if astrocytic dysfunction was associated with changes in these MR markers in surrounding tissues.

5.1.1 Aims and objectives

The aim of the experiments described in this chapter was to characterise the behavioural phenotype of Wistar rats following L-AAA administration into the pre-limbic cortex, the CA3 subfield of the hippocampus and the basolateral amygdala. A second aim of these experiments was to establish if astrocytic ablation in the pre-limbic cortex is related to changes in regional blood perfusion or T1 and T2 MR imaging markers.
5.2 Methods

5.2.1 Characterisation of the behavioural and MR marker changes induced following L-AAA administration into the pre-limbic cortex of Wistar rats

Adult male Wistar rats (200-250g) were housed 3 per cage. Animals were habituated to the animal facility for one week before L-AAA administration via stereotactic microinjection. To assess the effects of L-AAA administration in the pre-limbic cortex, animals were injected bilaterally with 2μl of L-AAA (25μg/μl) at co-ordinates 3.2mm anterior to bregma, 0.5mm bilateral, to a depth of 4mm; this was then repeated 24 hours later. Following this, one behavioural test was performed daily as per figure 5.2.1 (Study 1); animals were sacrificed by perfusion 24 hours following MRI scanning. All behavioural readouts were performed in dedicated rooms by a single experimenter. A second study was carried out to assess if the depressive-like behaviour induced by L-AAA administration into the pre-limbic cortex persisted two weeks following surgery. Following an 8 day recovery period one behavioural test was performed daily as per figure 5.2.1 (Study 2). A third study was carried out to investigate the effects of acute L-AAA administration into the pre-limbic cortex on MR imaging markers. This study was carried out as per figure 5.2.1 (Study 3). All procedures were conducted as outlined in the Methods section (See Chapter 2).

Study 1

D1 D2 D3 D4 D5 D6 D7
Surgery Surgery OF FST FST MRI Perfusion

Study 2

D1 D2 D10 D11 D12 D14
Surgery Surgery OF FST FST Perfusion

Study 3

D1 D2 D3 D4
Surgery Surgery MRI Perfusion

Figure 5.2.1: Pre-limbic cortical administration of L-AAA study designs

FST, forced swim test; MRI, magnetic resonance imaging; OF, open field.
5.2.2 Characterisation of the behavioural response to L-AAA administration into the amygdala and CA3 subfield of the hippocampus in the Wistar rat

Adult male Wistar rats (200-250g) were housed 3 per cage. Animals were habituated to the animal facility for one week before L-AAA administration via stereotactic microinjection. To assess the effects of L-AAA administration in the basolateral amygdala, animals were injected bilaterally with 2μl of L-AAA (25μg/μl) at co-ordinates 3mm posterior to bregma, 4.6mm bilateral, to a depth of 8.6mm; this was then repeated 24 hours later. Following this one behavioural test was performed daily as per figure 5.2.2; animals were sacrificed by perfusion 48 hours following the FST. To assess the effects of L-AAA administration in the CA3 subfield of the hippocampus, animals were injected bilaterally with 2μl of L-AAA (25μg/μl) at co-ordinates 4.3mm posterior to bregma, 4.2mm bilateral, to a depth of 4.1mm; this was then repeated 24 hours later. Following this one behavioural test was performed daily as per figure 5.2.2; animals were sacrificed by perfusion 48 hours following the FST. As before, all behavioural readouts were performed in dedicated rooms by a single experimenter. All procedures were conducted as outlined in the Methods section (See Chapter 2) with the exception of imaging GFAP stained sections. Imaging in these studies was performed on a leica SP8 confocal microscope.

Figure 5.2.2: Basolateral amygdala and CA3 subfield of the hippocampus administration of L-AAA study design

FST, forced swim test; OF, open field
5.3 Results

5.3.1 Characterisation of the behavioural effects of regional L-AAA administration

The first aim of the present experiments was to investigate the behavioural phenotype exhibited by animals following L-AAA administration into the pre-limbic cortex, the basolateral amygdala and the CA3 subfield of the hippocampus when compared to saline treated controls.

5.3.1.1 L-AAA administration into the pre-limbic cortex induces an increase in immobility in the FST paradigm

Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in an increase in immobility time in the FST \( t=2.143; \) \( df=18; \) \( p=0.046 \) [Figure 5.3.1.1].

![Figure 5.3.1.1: L-AAA administration into the pre-limbic cortex induces an increase in immobility in the FST paradigm](image)

Treatment dependent differences in depressive-like behaviour were assessed using the FST paradigm. Data expressed as mean ± SEM \( (n=10) \). * \( p<0.05 \) vs. saline treated control group
5.3.1.2 L-AAA administration into the pre-limbic cortex induces anxiety-related behaviour in the open field test

Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in no difference in total distance moved in the open field arena \([t=1.311; \text{df}=16; p=0.2085]\) [Figure 5.3.1.2 (a)] when compared to the saline treated control group. Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in no change in distance moved \([t=1.730; \text{df}=17; p=0.1017]\) [Figure 5.3.1.2 (b)] or in latency to enter \([t=1.285; \text{df}=16; p=0.2171]\) [Figure 5.3.1.2 (c)] the inner zone of the open field arena when compared to the saline treated control group. Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in a decrease in entries into the inner zone of the open field arena \([t=2.122; \text{df}=16; p=0.0498]\) [Figure 5.3.1.2 (d)] and an increase in total number of rears in the open field arena \([t=2.319; \text{df}=16; p=0.0339]\) [Figure 5.3.1.2 (e)] when compared to the saline treated control group. Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in no change in time spent grooming \([t=1.004; \text{df}=17; p=0.3293]\) [Figure 5.3.1.2 (f)] when compared to the saline treated control group.
Figure 5.3.1.2: L-AAA administration into the pre-limbic cortex induces anxiety-related behaviour in the open field test

Treatment dependent differences in locomotor activity and anxiety-related behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena, c) Latency to enter the inner zone, (d) Entries into the inner zone of the OF arena, (e) Total number of rears in the OF arena and (f) Total time spent grooming. Data expressed as mean ± SEM (n=9-10). * p<0.05 vs. saline treated control group
5.3.1.3 Pre-limbic cortical L-AAA administration induced increases in immobility time in the FST paradigm are transient

Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in no difference in immobility time in the FST 10 days post surgery [t=1.696; df=8; p=0.1283] [Figure 5.3.1.3] when compared to saline treated control group.

![Figure 5.3.1.3: Pre-limbic cortical L-AAA administration induced increases in immobility time in the FST are transient](image)

Treatment dependent differences in depressive-like behaviour were assessed using the FST paradigm. Data expressed as mean ± SEM (n=5)
5.3.1.4 Pre-limbic cortical L-AAA administration induced anxiety-related behaviour in the open field is transient

Student’s t-test revealed that L-AAA administration into the pre-limbic cortex results in no difference in total distance moved in the open field arena [t=0.1409; df=8; p=0.8914] [Figure 5.3.1.4 (a)], in distance moved in the inner zone of the open field arena [t=0.3044; df=8; p=0.7686] [Figure 5.3.1.4 (b)], in the number of rears in the open field arena [t=1.876; df=8; p=0.0975] [Figure 5.3.1.4 (e)] or in latency to enter [t=0.8220; df=8; p=0.4349] [Figure 5.3.1.4 (c)] and number of entries into the inner zone of the open field arena [t=0.9201; df=8; p=0.3844] [Figure 5.3.1.4 (d)] or in time spent grooming in the open field arena [t=0.04739; df=8; p=0.9634] [Figure 5.3.1.4 (f)] when compared to the saline treated control group 8 days post surgery.
Figure 5.3.1.4: Pre-limbic cortical L-AAA administration induced anxiety-related behaviour in the open field is transient.

Treatment dependent differences in locomotor activity and anxiety-related behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena, c) Latency to enter the inner zone, (d) Entries into the inner zone of the OF arena, (e) Total number of rears in the OF arena and (f) Total time spent grooming. Data expressed as mean ± SEM (n=5).
5.3.1.5 L-AAA administration into the pre-limbic cortex results in a transient decrease in GFAP positive cell number in the pre-limbic cortex relative to saline treated controls

Student’s t-test revealed that 2 days following L-AAA administration into the pre-limbic cortex a significant decrease in GFAP positive cell number per mm$^2$ in the pre-limbic cortical [t=3.267; df=12; p=0.0067] [Figure 5.3.1.5 (a)] [Table 5.3.1] region was detected. 5 days following L-AAA administration into the pre-limbic cortex, there was no significant change in GFAP positive cell number per mm$^2$ in any of the regions analysed [Table 5.3.2]

Table 5.3.1: Acute L-AAA administration into the pre-limbic cortex results in decreased GFAP positive cell number in the pre-limbic cortex relative to saline treated controls

<table>
<thead>
<tr>
<th>Region</th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-limbic cortex</td>
<td>226.7 ± 7.52</td>
<td>171.8 ± 12.53 **</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
<td>125.3 ± 15.77</td>
<td>148.2 ± 14.67</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>161.1 ± 7.40</td>
<td>168.4 ± 10.89</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm$^2$ ± SEM (n=7). ** p<0.01 vs. saline treated control group
Figure 5.3.1.5a: Acute L-AAA administration into the pre-limbic cortex results in decreased GFAP positive cell number in the pre-limbic cortex relative to saline treated controls

a) The L-AAA treated group exhibits a decrease in GFAP positive cell number in the pre-limbic cortical region, b) Representative images depicting decreased GFAP positive cell number in the pre-limbic cortical region of the L-AAA treated group 2 days following L-AAA administration. Data expressed as mean number of GFAP positive cells per mm$^2$ ± SEM (n=7) ** $p<0.01$ vs. saline treated control group
Table 5.3.2: There is no significant difference in GFAP positive cell number in the frontal cortex 5 days following L-AAA administration into the pre-limbic cortex

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-limbic cortex</td>
<td>171 ± 11.62</td>
<td>168.0 ± 11.94</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
<td>162.9 ± 11.62</td>
<td>171.2 ± 6.80</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>152 ± 15.39</td>
<td>141.3 ± 11.93</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm$^2$ ± SEM (n=8-10).

**Figure 5.3.1.5b:** There is no significant difference in GFAP positive cell number 5 days following L-AAA administration into the pre-limbic cortex

Representative images depicting no difference in GFAP positive cell number in the pre-limbic cortical region 5 days following L-AAA administration
5.1.1.6 L-AAA administration into the pre-limbic cortex is not associated with any qualitative difference in microglial activation as assessed by Iba-1 staining.

L-AAA administration into the pre-limbic cortex was not associated with any qualitative difference in microglial activation as assessed via Iba-1 immunostaining two or five days post L-AAA administration in the pre-limbic cortex [Figure 5.3.1.6a], the retrosplenial cortex or the visual cortex [Figure 5.3.1.6b].

Figure 5.3.1.6a: There is no qualitative difference in microglial activation in the pre-limbic cortex 2 or 5 days following L-AAA administration into the pre-limbic cortex as assessed via Iba-1 immunostaining. Arrows identify activated microglial cells at the injection site, evident by their altered cell morphology relative to resting state ramified microglial cells.
Figure 5.3.1.6b: There is no qualitative difference in microglial activation in the retrosplenial or visual cortices 5 days following L-AAA administration into the pre-limbic cortex as assessed via Iba-1 immunostaining.

Microglial cells in the retrosplenial and visual cortices exhibit resting ramified morphology in both saline and L-AAA treated groups 5 days following L-AAA administration into the pre-limbic cortex.
5.3.1.7 L-AAA administration into the basolateral amygdala induces an increase in immobility in the FST paradigm

Student's t-test revealed that L-AAA administration into the amygdala resulted in a significant increase in immobility time in the FST \( t=3.593; \text{df}=20; p=0.0018 \) [Figure 5.3.1.7].

![Bar chart showing immobility time comparison between Saline and L-AAA](image)

Figure 5.3.1.7: L-AAA administration into the basolateral amygdala induces an increase in immobility in the FST paradigm

Treatment dependent differences in depressive-like behaviour were assessed using the FST paradigm. Data expressed as mean ± SEM (n=10-12). ** p<0.01 vs. saline treated control group
5.3.1.8 L-AAA administration into the basolateral amygdala induces anxiety-related behaviour as assessed by the open field test

Student's t-test revealed that L-AAA administration into the amygdala resulted in a significant decrease in total distance moved in the open field arena \([t=2.935; \text{df}=20; \ p=0.0082]\) [Figure 5.3.1.8 (a)], in distance moved in the inner zone of the open field arena \([t=3.001; \text{df}=19; \ p=0.0073]\) [Figure 5.3.1.8 (b)], in entries into the inner zone of the open field arena \([t=3.299; \text{df}=18; \ p=0.004]\) [Figure 5.3.1.8 (d)], and in total rears \([t=4.59; \text{df}=19; \ p=0.0002]\) [Figure 5.3.1.8 (e)]. Student's t-test revealed that L-AAA administration into the amygdala resulted in a significant increase in latency to enter the inner zone of the open field arena \([t=2.316; \text{df}=19; \ p=0.0319]\) [Figure 5.3.1.8 (c)] and in time spent grooming \([t=2.950; \text{df}=18; \ p=0.0086]\) [Figure 5.3.1.8 (f)].
Figure 5.3.1.8: L-AAA administration into the basolateral amygdala induces anxiety-related behaviour in the open field test

Treatment dependent differences in locomotor activity and anxiety-related behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena, (c) Latency to enter the inner zone, (d) Entries into the inner zone of the OF arena, (e) Total number of rears in the open field and (f) Total time spent grooming. Data expressed as mean ± SEM (n=10-12). * p<0.05; ** p<0.01; *** p<0.001 vs. saline treated control group.
5.3.1.9 There is no significant difference in GFAP positive cell number in any of the regions analysed following L-AAA administration into the basolateral amygdala. Student's t-test revealed that L-AAA administration into the amygdala did not result in a difference in GFAP positive cell number per mm² in any of the regions analysed relative to saline treated controls.

Table 5.3.3: There is no difference in GFAP positive cell number in any of the regions analysed 5 days following L-AAA administration in the basolateral amygdala

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basolateral amygdala</td>
<td>246.9 ± 17.31</td>
<td>281.2 ± 14.58</td>
</tr>
<tr>
<td>CA1</td>
<td>284.5 ± 12.22</td>
<td>263.2 ± 12.87</td>
</tr>
<tr>
<td>CA2</td>
<td>313.1 ± 18.60</td>
<td>300.5 ± 9.84</td>
</tr>
<tr>
<td>CA3</td>
<td>328.6 ± 17.23</td>
<td>341.3 ± 11.57</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>292.8 ± 17.78</td>
<td>300.8 ± 20.22</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm² ± SEM (n=10-11).
5.3.1.10 L-AAA administration into the CA3 subfield of the hippocampus does not alter behaviour in the FST paradigm

Student’s t-test revealed that L-AAA administration into the hippocampus resulted in no change in immobility time in the FST \[t=0.8938; \text{df}=10; \text{p}=0.3924\] [Figure 5.3.1.10].

Figure 5.3.1.10: L-AAA administration into the CA3 subfield of the hippocampus does not alter behaviour in the FST paradigm

Treatment dependent differences in depressive-like behaviour were assessed using the FST paradigm. Data expressed as mean ± SEM (n=6).
5.3.1.11: L-AAA administration into the CA3 subfield of the hippocampus does not alter behaviour in the open field test

Student's *t*-test revealed that L-AAA administration into the CA3 subfield resulted in no change in total distance moved in the open field arena \( t=0.2412; \text{df}=10; p=0.8142 \) [Figure 5.3.1.11 (a)], in distance moved in the inner zone of the open field arena \( t=0.4556; \text{df}=10; p=0.6584 \) [Figure 5.3.1.11 (b)], in latency to enter the inner zone of the open field arena \( t=0.4037; \text{df}=10; p=0.6950 \) [Figure 5.3.1.11 (c)], in entries into the inner zone of the open field arena \( t=0.1557; \text{df}=10; p=0.8793 \) [Figure 5.3.1.11 (d)], in total rears \( t=0.5933; \text{df}=10; p=0.5662 \) [Figure 5.3.1.11 (e)] and in time spent grooming \( t=1.688; \text{df}=10; p=0.1223 \) [Figure 5.3.1.11 (f)].
Figure 5.3.1.11: The L-AAA treated group does not exhibit anxiety-related behaviour in the open field test

Treatment dependent differences in locomotor activity and anxiety-like behaviour were assessed via (a) Total distance moved in the OF arena, (b) Total distance moved in the inner zone of the OF arena, (c) Latency to enter the inner zone, (d) Entries into the inner zone of the OF arena, (e) Total number of rears in the open field and (f) Total time spent grooming. Data expressed as mean ± SEM (n=6).
5.3.1.12 There is no difference in GFAP positive cell number in any of the regions analysed 5 days following L-AAA administration into the CA3 subfield of the hippocampus.

Student's t-test revealed that L-AAA administration into the CA3 subfield of the hippocampus did not result in a difference in GFAP positive cell number per mm$^2$ in any of the regions analysed relative to saline treated controls.

Table 5.3.4: There is no difference in GFAP positive cell number in any of the regions analysed following L-AAA administration into the CA3 subfield of the hippocampus

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basolateral amygdala</td>
<td>251.1 ± 24.07</td>
<td>312 ± 23.99</td>
</tr>
<tr>
<td>CA1</td>
<td>347.6 ± 15.85</td>
<td>303.1 ± 25.47</td>
</tr>
<tr>
<td>CA2</td>
<td>353.3 ± 12.10</td>
<td>329.8 ± 15.38</td>
</tr>
<tr>
<td>CA3</td>
<td>376.9 ± 18.98</td>
<td>374.4 ± 23.71</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>352 ± 16.70</td>
<td>370.2 ± 14.53</td>
</tr>
</tbody>
</table>

Data expressed as mean number of GFAP positive cells per mm$^2$ ± SEM (n=6).
5.3.2 Characterisation of MR imaging markers associated with pre-limbic cortical L-AAA administration

In order to assess if astroglial ablation was associated with changes in MR imaging markers, animals underwent MR imaging at two time points, 24 and 96 hours following L-AAA administration into the pre-limbic cortex.

5.3.2.1 The L-AAA treated group does not exhibit a change in resting state cerebral perfusion in the frontal cortex section 24 hours following L-AAA administration into the pre-limbic cortex

There were no differences in mean transit time (MTT), capillary transit time (CTT) or signal amplitude ASL perfusion parameters detected in the frontal cortical section 24 hours following L-AAA administration [Table 5.3.5].

Table 5.3.5: The L-AAA treated group does not exhibit any change in resting state cerebral perfusion in the frontal cortex section 24 hours following L-AAA administration into the pre-limbic cortex

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>MTT (s) ± SEM</th>
<th>CTT (s) ± SEM</th>
<th>Amplitude (a.u.) ± SEM</th>
<th>MTT (s) ± SEM</th>
<th>CTT (s) ± SEM</th>
<th>Amplitude (a.u.) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-limbic cortex</td>
<td>1.856 ± 0.100</td>
<td>1.654 ± 0.092</td>
<td>0.1176 ± 0.010</td>
<td>1.928 ± 0.063</td>
<td>1.687 ± 0.069</td>
<td>0.1143 ± 0.007</td>
</tr>
<tr>
<td>Primary motor cortex</td>
<td>2.079 ± 0.066</td>
<td>1.706 ± 0.049</td>
<td>0.9013 ± 0.003</td>
<td>2.122 ± 0.041</td>
<td>1.706 ± 0.038</td>
<td>0.09139 ± 0.005</td>
</tr>
<tr>
<td>Secondary motor cortex</td>
<td>2.117 ± 0.078</td>
<td>1.856 ± 0.059</td>
<td>1.013 ± 0.007</td>
<td>2.087 ± 0.067</td>
<td>1.758 ± 0.065</td>
<td>0.1018 ± 0.008</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>1.858 ± 0.471</td>
<td>1.690 ± 0.060</td>
<td>0.1112 ± 0.004</td>
<td>1.856 ± 0.066</td>
<td>1.674 ± 0.074</td>
<td>0.1204 ± 0.007</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.786 ± 0.043</td>
<td>0.115 ± 0.03</td>
<td>0.1112 ± 0.004</td>
<td>1.87 ± 0.067</td>
<td>0.1179 ± 0.008</td>
<td>0.1204 ± 0.007</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=7)
5.3.2.2 The L-AAA treated group exhibits an increase in T2 relaxation times in the pre-limbic cortex 24 hours following L-AAA administration into the pre-limbic cortex

Student's t-test revealed that L-AAA administration into the pre-limbic cortex results in an increase \([t=2.322; \text{df}=11; \text{p}=0.0405]\) [Table: 5.3.6] in T2 relaxation time in the pre-limbic cortex. There were no other differences in T1 or T2 relaxation parameters detected in the frontal cortex section 24 hours following L-AAA administration [Table 5.3.6].

Table 5.3.6: The L-AAA treated group exhibits an increase in T2 relaxation times in the pre-limbic cortex 24 hours following L-AAA administration into the pre-limbic cortex

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(T1) (ms)</td>
<td>(T2) (ms)</td>
</tr>
<tr>
<td><strong>Pre-limbic cortex</strong></td>
<td>(2208 \pm 25.76)</td>
<td>(53.49 \pm 0.23)</td>
</tr>
<tr>
<td><strong>Primary motor cortex</strong></td>
<td>(2138 \pm 13.07)</td>
<td>(52.73 \pm 0.31)</td>
</tr>
<tr>
<td><strong>Secondary motor cortex</strong></td>
<td>(2332 \pm 36.08)</td>
<td>(57.13 \pm 1.08)</td>
</tr>
<tr>
<td><strong>Somatosensory cortex</strong></td>
<td>(2064 \pm 17.59)</td>
<td>(51.43 \pm 0.38)</td>
</tr>
<tr>
<td><strong>Striatum</strong></td>
<td>(1899 \pm 11.15)</td>
<td>(49.64 \pm 0.37)</td>
</tr>
</tbody>
</table>

Data expressed as mean \(\pm\) SEM (\(n=6-7\))
5.3.2.3 The L-AAA treated group does not exhibit a change in resting state cerebral perfusion in the hippocampal section 24 hours following L-AAA administration into the pre-limbic cortex.

There were no differences in ASL perfusion parameters detected in the hippocampal section 24 hours following the second L-AAA administration [Table 5.3.7].

Table 5.3.7: The L-AAA treated group does not exhibit a change in resting state cerebral perfusion in the hippocampal section 24 hours following L-AAA administration into the pre-limbic cortex

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT (s)</td>
<td>CTT (s)</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td>2.022 ± 1.699 ± 0.09884 ± 0.005</td>
<td>2.046 ± 1.708 ± 0.1032 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>0.048</td>
<td>0.041</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.717 ± 1.579 ± 0.1221 ± 0.005</td>
<td>1.786 ± 1.648 ± 0.1234 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>0.036</td>
<td>0.034</td>
</tr>
<tr>
<td>Parietal association</td>
<td>2.018 ± 1.688 ± 0.1005 ± 0.007</td>
<td>1.973 ± 1.707 ± 0.1069 ± 0.007</td>
</tr>
<tr>
<td>cortex</td>
<td>0.086</td>
<td>0.072</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
<td>1.991 ± 1.722 ± 0.1048 ± 0.007</td>
<td>1.947 ± 1.644 ± 0.1137 ± 0.101</td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>0.045</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.609 ± 1.456 ± 0.1332 ± 0.009</td>
<td>1.662 ± 1.430 ± 0.1379 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>0.034</td>
<td>0.034</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>2.027 ± 1.755 ± 0.09742 ± 0.006</td>
<td>2.023 ± 1.769 ± 0.1043 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>0.023</td>
<td>0.072</td>
</tr>
<tr>
<td>Third ventricle</td>
<td>2.014 ± 1.382 ± 0.1662 ± 0.150</td>
<td>2.151 ± 1.606 ± 0.1618 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>0.136</td>
<td>0.122</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=7)
5.3.2.4 The L-AAA treated group does not exhibit a change in resting state cerebral perfusion in the frontal cortex section 4 days following L-AAA administration into the pre-limbic cortex

There were no differences in ASL perfusion parameters detected in the frontal cortex section 4 days following the second L-AAA administration [Table 5.3.8].

Table 5.3.8: The L-AAA treated group does not exhibit a change in resting state cerebral perfusion in the frontal cortex section 4 days following L-AAA administration into the pre-limbic cortex

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT (s)</td>
<td>CTT (s)</td>
</tr>
<tr>
<td>Pre-limbic cortex</td>
<td>1.677 ± 1.398 ± 0.1064 ± 0.003</td>
<td>1.573 ± 1.347 ± 0.1121 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>0.042</td>
<td>0.050</td>
</tr>
<tr>
<td>Primary motor cortex</td>
<td>1.717 ± 1.406 ± 0.1073 ± 0.004</td>
<td>1.648 ± 1.333 ± 0.1075 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>0.073</td>
<td>0.062</td>
</tr>
<tr>
<td>Secondary Motor cortex</td>
<td>1.73 ± 1.416 ± 0.1065 ± 0.004</td>
<td>1.741 ± 1.411 ± 0.1042 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>0.071</td>
<td>0.064</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>1.485 ± 1.334 ± 0.1217 ± 0.003</td>
<td>1.459 ± 1.275 ± 0.1174 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>0.049</td>
<td>0.083</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.710 ± 1.520 ± 0.1058 ± 0.003</td>
<td>1.751 ± 1.631 ± 0.1070 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>0.073</td>
<td>0.130</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=9-10)
5.3.2.5 The L-AAA treated group exhibits a decrease in T1 relaxation time in the striatum 4 days following L-AAA administration into the pre-limbic cortex.

Student’s t-test revealed that L-AAA administration into the pre-limbic cortex results in a significant decrease \( t=2.57; \text{df}=16; p=0.0205 \) [Table: 5.3.9] in T1 relaxation time in the striatum.

Table 5.3.9: The L-AAA treated group exhibits a decrease in T1 relaxation time in the striatum 4 days following L-AAA administration into the pre-limbic cortex

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1 (ms)</td>
<td>T2 (ms)</td>
</tr>
<tr>
<td>Pre-limbic cortex</td>
<td>1911 ± 23.18</td>
<td>52.79 ± 0.25</td>
</tr>
<tr>
<td>Primary motor cortex</td>
<td>1950 ± 32.84</td>
<td>52.18 ± 0.13</td>
</tr>
<tr>
<td>Secondary motor cortex</td>
<td>2015 ± 44.77</td>
<td>53.34 ± 0.26</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>1816 ± 20.18</td>
<td>51.06 ± 0.44</td>
</tr>
<tr>
<td>Striatum</td>
<td>1792 ± 27.42</td>
<td>50.71 ± 0.35</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=9-10). * \( p<0.05 \) vs. saline treated control group.
5.3.2.6 The L-AAA treated group exhibits an increase in resting state cerebral perfusion in the hippocampal section 4 days following L-AAA administration into the pre-limbic cortex.

Student's t-test revealed that the L-AAA treated group exhibited a significant decrease in MTT in the parietal association cortical \[t=2.219; df=18; p=0.0395\] [Table 5.3.10], the retrosplenial cortical \[t=2.283; df=18; p=0.0348\] [Table 5.3.10], the thalamic \[t=2.477; df=17; p=0.024\] [Table 5.3.10], and the visual cortical \[t=3.115; df=18; p=0.006\] [Table 5.3.10] regions analysed. Student's t-test revealed that the L-AAA treated group exhibited a significant decrease in CTT in the retrosplenial cortical \[t= 2.128; df=17; p=0.0483\] [Table 5.3.10] and the visual cortical \[t=2.889; df=18; p=0.0098\] [Table 5.3.10] regions analysed. Student's t-test revealed the L-AAA treated group exhibited a significant increase in signal amplitude in the auditory cortical \[t=2.338; df=17; p=0.0318\] [Table 5.3.10], the parietal association cortical \[t=2.244; df=18; p=0.0377\] [Table 5.3.10], and the visual cortical \[t=2.294; df=18; p=0.034\] [Table 5.3.10] regions analysed.
Table 5.3.10: The L-AAA treated group exhibits an increase in resting state cerebral perfusion in the hippocampal section 4 days following L-AAA administration into the pre-limbic cortex.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>L-AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT (s)</td>
<td>CTT (s)</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td>1.699 ± 1.349 ± 0.1118 ± 0.005</td>
<td>1.559 ± 1.269 ± 0.1236 ± 0.006 *</td>
</tr>
<tr>
<td></td>
<td>0.039</td>
<td>0.018</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>1.417 ± 1.233 ± 0.1271 ± 0.004</td>
<td>1.330 ± 1.185 ± 0.1366 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>0.036</td>
<td>0.022</td>
</tr>
<tr>
<td>Parietal association</td>
<td>1.56 ± 1.309 ± 0.114 ± 0.004</td>
<td>1.437 ± 1.232 ± 0.1271 ± 0.005 *</td>
</tr>
<tr>
<td>cortex</td>
<td>0.048</td>
<td>0.035</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
<td>1.634 ± 1.312 ± 0.1244 ± 0.005</td>
<td>1.462 ± 1.239 ± 0.1244 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>0.066</td>
<td>0.032</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.369 ± 1.266 ± 0.1537 ± 0.005</td>
<td>1.266 ± 1.164 ± 0.1633 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>0.035</td>
<td>0.020</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>1.772 ± 1.42 ± 0.1042 ± 0.114</td>
<td>1.529 ± 1.27 ± 0.1184 ± 0.005 *</td>
</tr>
<tr>
<td></td>
<td>0.062</td>
<td>0.047</td>
</tr>
<tr>
<td>Third ventricle</td>
<td>1.418 ± 1.276 ± 0.1621 ± 0.004</td>
<td>1.389 ± 1.23 ± 0.1560 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>0.079</td>
<td>0.079</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n=9-10). * p<0.05; ** p<0.01 vs. saline treated control group
Figure 5.3.2.6: Representative cerebral blood volume maps comparing hippocampal blood volume 4 days following L-AAA administration into the pre-limbic cortex

Representative cerebral blood volume (CBV) maps depicting increased CBV in the hippocampal coronal section analysed 4 days following L-AAA administration into the pre-limbic cortex. Brighter colours indicate increased signal amplitude which is proportional to CBV.
5.4 Discussion

5.4.1 Characterisation of the behavioural change induced following regional L-AAA administration

The first aim of the present experiments was to further investigate the effects of central regional administration of the astroglial toxin L-AAA on anxiety and depressive-related behaviours in a rodent model. A number of behavioural tests were undertaken following L-AAA administration to specific brain regions to assess if astrocytic ablation may be associated with the development of anxiety or depressive-related behaviours.

5.4.1.1 L-AAA administration into the pre-limbic cortex produces transient depressive and anxiety-related behaviours in Wistar rats

Banasr and Duman (2008) have previously shown that L-AAA infusion into the pre-limbic cortex produces a depressive-like phenotype in Sprague-Dawley rats (Banasr and Duman, 2008). Here we report that L-AAA microinjection into the pre-limbic cortex of Wistar rats also produces depressive-like behaviour as evidenced through increased immobility in the FST. These data also suggest that pre-limbic cortical astrocytic dysfunction is associated with mild anxiety-like behaviour expressed as decreased exploration of the inner zone of the open field arena. Impaired neuronal function in this region has previously been shown to increase anxiety-like behaviour in rodent models. Induction of a neuronal lesion in the pre-limbic cortex via localised administration of a 1 mA current for 10 seconds, increases anxiety-like behaviour in the open field and in the elevated plus maze tests in rodents when compared to sham lesioned controls (Jinks and McGregor, 1997). Hence anxiety-like behaviour demonstrated here may be related to impaired neuronal function resulting from dysfunctional astroglial cells in this region. L-AAA administration into the pre-limbic cortex has previously been reported to decrease dendritic length of pyramidal neurons at the L-AAA lesion site, thus confirming that L-AAA induced astrocytic dysfunction is associated with changes in neuronal function in this region (Lima et al., 2014). One limitation of the present studies is that we did not confirm that there was no neuronal loss induced by toxin administration, although several previous studies have reported that L-AAA administration is not associated with neuronal loss (Banasr and Duman, 2008; Khurgel et al., 1996). Furthermore it has previously been shown that neurons exhibit negligible uptake of L-AAA (Pow, 2001). L-AAA administration was not associated with a different microglial response to the surgical procedure as assessed by staining for ionized calcium-binding...
adapter molecule 1 (Iba-1). Iba-1 stains both activated and resting microglial cells. Under resting conditions microglia exhibit ramified processes, however once activated following an inflammatory insult microglia alter their cellular morphology and appear as amoeboid cells (Graeber, 2010). Activated microglial cells can be seen around the site of injection following pre-limbic cortical drug administration, but this microglial response does not appear to differ between L-AAA and saline treated groups and is not evident at the 5 day time point in either group.

A role for dysfunctional astrocytes within the pre-limbic cortical region in the development of depressive-like behaviours in animal models is supported by the literature. Indeed several studies have shown that modulating astrocyte function in the pre-limbic cortex is sufficient to produce depressive-like behaviours in rodent models. Astrocytic gap junctions mediate direct communication between large groups of astrocytes (Pannasch et al., 2011). Blockade of these astrocytic GAP junctions via infusion of gap junction blocker carbenoxolone into the pre-limbic cortex produces decreased hedonic behaviour as assessed via sucrose consumption in the SPT and increased latency to feed in the novelty suppressed feeding test (NSFT) (Sun et al., 2011). Intracerebroventricular administration of dihydrokainic acid (DHK), a blocker of astrocytic glutamate transporter type 1 (GLT-1), has been shown to reduce hedonic behaviour as assessed by intracranial self stimulation (ICSS) and was coupled with increased neuronal activation as indicated by increased immunoreactivity for the immediate early gene marker c-fos in the infra-limbic cortex, dentate gyrus and the basolateral amygdala (Bechtholt-Gompf et al., 2010). Furthermore direct administration of DHK into the infra-limbic cortex also results in an increase in ICSS thresholds when compared to saline treated control groups (John et al., 2012). These studies suggest that increased extracellular glutamate concentrations resulting from impaired astrocytic uptake are causal in the induction of decreased hedonic behaviour in this model. These studies did not however assess the effects of DHK on immobility time in the FST.

In the case of L-AAA administration induced increases in immobility in the FST, there is evidence to suggest that rather than being a consequence of increased extracellular glutamate concentrations, it appears to be related to decreased glutamine concentrations in the pre-limbic cortical region. Administration of glutamine into the pre-limbic cortex attenuates L-AAA induced increases in immobility time in the FST. Furthermore post-mortem analysis of glutamate and glutamine levels following L-AAA
administration via high performance liquid chromatography (HPLC) reveal a decrease in both glutamate and glutamine in the pre-limbic cortex of L-AAA treated rats relative to saline treated control groups. While administration of glutamine synthetase inhibitor methionine sulfoximine (MSO) or glutamine transporter (SAT2) blocker α-methyl-aminoisobutyric acid (MeAIB) into the pre-limbic cortex also results in increased immobility in the FST in a murine model (Lee et al., 2013). These studies targeted the infra-limbic and pre-limbic cortices respectively, it should be noted however that preliminary work conducted in our lab shows that administration of 2μl of evans blue dye into the pre-limbic cortex produces an approximately 1mm² dyed zone (data not shown). Hence administration of agents to either the infra-limbic or the pre-limbic cortices is unlikely to be regionally specific due to their close anatomical proximity. The results of these studies are not necessarily contradictory as DHK induced decrease in the astrocytic uptake of glutamate, while initially resulting in an increase in synaptic glutamate concentrations, would eventually result in decreased astrocytic glutamate to glutamine cycling and hence reduced concentrations of glutamine and glutamate.

The saline treated control group shows an increase in GFAP positive cell number relative to the L-AAA treated group at the 48 hour time point, this difference is not seen at the later 5 day time point. Khurgel and colleagues (1996) have reported that when L-AAA is administered via stereotactic injection into the rodent brain, the maximum decrease in GFAP immunoreactivity is observed 48 hours following surgery. The level of GFAP immunoreactivity then returns towards saline treated control levels within 7 days of surgery (Khurgel et al., 1996). This recovery in astrocytic function is the probable cause of the transient nature of the behavioural phenotype induced following L-AAA administration into the pre-limbic cortex. In a separate study we report that when behavioural testing began 8 days following surgery, the L-AAA treated animals showed no differences in immobility time in the FST or anxiety-like behaviour as assessed in the open field when compared to saline treated control animals. This is in agreement with a previous study which reported that 10 days following L-AAA infusion into the pre-limbic cortex in mice, immobility times in the FST had returned to saline treated control levels (Lee et al., 2013).

It should be noted that several other studies have reported a decrease in GFAP positive cell numbers following L-AAA administration at a 6 day time point (Banasr and Duman, 2008; Domin et al., 2014; Lee et al., 2013). This contrast with the data reported in the
present experiments can most likely be attributed to methodological differences between the studies. All other studies in the literature [with the exception of Khurgel et al. (1996)] involved the implantation of in lying guide cannulae into the pre-limbic cortical region of interest one week prior to toxin administration. Astrocytes proliferate through the process of reactive astrogliosis and form glial scars in response to brain trauma (Sofroniew and Vinters, 2010). The prolonged nature of cannulation is likely to induce more robust astrogliosis. Polarisation of astrocytes towards the site of injury coupled with increased GFAP expression has been shown to take 3 to 5 days following injury and may be maintained for weeks afterwards (Bardehle et al., 2013). Hence the reductions in GFAP positive cell number reported in these studies at the later time point are relative to saline treated control levels which may be higher than those seen with the method of toxin delivery employed in the present experiments. As appears to be the case here, L-AAA has previously been shown to inhibit astrogliosis induced by acute trauma (Cho et al., 2005; McGraw et al., 2001). The decrease in GFAP positive cell number in the saline treated control group following recovery from surgery is in keeping with reports that GFAP immunoreactivity returns to baseline levels 7 days following a ballistic injury in the rat cortex (Williams et al., 2007). Future studies may include sham operated control groups which receive anaesthesia only but no microinjection to further elaborate on the GFAP response to this surgical procedure.

5.4.1.2 L-AAA administration into the basolateral amygdala produces a depressive and high anxiety-like phenotype in Wistar rats

We report that L-AAA administration into the basolateral amygdala produces a depressive-like phenotype as evidenced by an increase in immobility time in the FST. Basolateral amygdala L-AAA administration is also associated with a highly anxious phenotype as assessed in the open field. The lack of any significant change in GFAP positive cell number in any of the regions analysed is most likely due to the recovery of astrocytic function as discussed above. As a result of this recovery, it is likely that behavioural change induced following L-AAA administration into the basolateral amygdala is also transient. These findings are in agreement with data which show that blocking astrocyte glutamate uptake through administration of DHK into the basolateral amygdala of Wistar rats produces a dose dependent deficit in a social interaction test, considered an anxiogenic effect, which is attenuated by co-administration with NMDA receptor antagonist amino-5-phosphonopentanoate (AP5) (Lee et al., 2007). Administration of DHK into the central amygdala has also been shown to be anxiogenic,
resulting in decreased entries into the open arms of an elevated plus maze and increased freezing behaviour in a fear conditioning task, it also resulted in an anhedonic response as evidenced by higher minimum stimulation frequencies in ICSS (John et al., 2015).

Bowley and colleagues (2002) have reported that there is decreased glial cell density and decreased glia to neuron ratio in the amygdala of depressed patients relative to non-psychiatric controls, and that lithium and valproate treatment attenuated this reduction in glia cell numbers in the region. This study did not however differentiate between which class of glial cells were reduced (Bowley et al., 2002). It has been reported that this reduction in glial cell number is primarily as a result of decreased numbers on oligodendrocytes in the region (Hamidi et al., 2004). It should be noted here that astrocytes were quantified through staining for calcium binding protein S100B. Decreases in GFAP positive cells in the absence of any change in the number of cells positive for S100B have previously been reported in the amygdala of the WKY rat animal model of depressive-like behaviour (Gosselin et al., 2009). Hence the lack of change in S100B immunoreactivity does not preclude a decrease in GFAP positive cells in the region. Indeed Altshuler and colleagues (2010) have reported a decrease in GFAP positive astrocyte density in the amygdala of depressed patients relative to non-depressed controls (Altshuler et al., 2010). Decreases in astroglial enzyme glutamine synthetase mRNA levels previously reported in the amygdala of depressed patients further implicate altered astroglial function in this region in depressive illness (Sequeira et al., 2009). The data presented here supports the hypothesis that astrocytic dysfunction in the amygdala plays a role in depressive symptomatology.

Basolateral amygdala L-AAA administration induced more pronounced anxiety-like behaviour than pre-limbic cortical administration. This is perhaps unsurprising as hyperactivity in the amygdala in response to negative stimuli in patients with anxiety disorders is a common finding in the clinical neuroimaging literature (Etkin and Wager, 2007; Forster et al., 2012), although it should be noted that the prefrontal cortex is important in inhibiting the activity of the amygdala as part of normal fear extinction processes (Davidson, 2002). It is possible that the increased anxiety-like behaviour in response to amygdala L-AAA administration is associated with altered glutamatergic function in the region. Future studies may employ magnetic resonance spectroscopy to
investigate if L-AAA administration is associated with changes in glutamate or glutamine concentrations in the region and the temporal nature of such changes.

5.4.1.3 L-AAA administration into the CA3 subfield of the hippocampus does not result in depressive-like behaviour in Wistar rats

We report that L-AAA administration into the CA3 subfield of the hippocampus of Wistar rats does not result in a change in behaviour as assessed in the FST or the open field test. The data suggest that decreased levels of GFAP positive cells in this region of the hippocampus are not related to performance in these tasks. Thus we can surmise that the previously reported decrease in GFAP positive cell number in this region of the hippocampus of the WKY rat relative to the Wistar (As reported in chapter 4) and Sprague-Dawley (Gosselin et al., 2009) rat strain is not directly related to the depressive and anxiety-like behaviours exhibited in that strain. Post-mortem tissue analysis from clinically depressed patients suggests a decrease in GFAP immunoreactivity in the CA1 and CA2 subfields of the hippocampus relative to non psychiatric controls (Müller et al., 2001). Accordingly it may be of interest in future studies to examine the behavioural effects of astroglial dysfunction in these regions. It has previously been reported that injection of L-AAA into the rodent dorsal hippocampus does not produce a significant reduction in GFAP immunoreactivity 48 hours after injection (Saffran and Crutcher, 1987). Consistent with previous findings discussed above we did not observe any significant difference in GFAP positive cell number in any of the regions analysed in the hippocampus when tissue was acquired 5 days post surgery. Future studies will assess if differences in GFAP positive cell number are evident in tissue acquired at earlier time points following surgery.

Although hippocampal L-AAA administration produced no depressive or anxiety-related behaviour, it may be of interest in the future to investigate if L-AAA treated animals exhibit cognitive deficits in hippocampal dependent memory tasks. Future studies will investigate the cognitive effects of regional L-AAA administration. Lima and colleagues (2014) have previously reported that cortical astrocytic ablation is associated with cognitive deficits. Administration of L-AAA into the pre-limbic cortex produces a deficit in a task of non spatial working memory, the attentional set shifting task, as well as in a task of spatial working memory, the Morris water maze. This deficit in cognitive function was associated with a decrease in neuronal branching within the lesion site (Lima et al., 2014). Furthermore astrocytes have previously been shown to play a role in
hippocampal dependent memory via an interleukin (IL-1) dependent mechanism (Ben Menachem-Zidon et al., 2011).

5.4.2 Characterisation of MR imaging markers associated with L-AAA induced astrocytic dysfunction

The second aim of the present studies was to investigate if L-AAA administration into the pre-limbic cortex resulted in a detectable change in resting state blood perfusion markers or in local T1 or T2 relaxometry measurements. As we reported a positive correlation between regional GFAP positive cell numbers and blood perfusion in the pre-limbic cortical region in the WKY rat strain in the previous chapter, we hypothesised that L-AAA induced astroglial ablation may cause reduced regional cerebral blood perfusion.

5.4.2.1 L-AAA administration into the pre-limbic cortex was not associated with robust changes in MR imaging markers in the frontal cortex

Blood perfusion and relaxometry data were acquired 24 and 96 hours following L-AAA administration. There were no significant changes in blood perfusion parameters in the frontal cortex section at either time point. This is not the result which was expected, we anticipated based on our previous work that localised reductions in astrocyte cell number may lead to changes in resting state blood perfusion in that region. There are several possible explanations as to why this was not observed in the present studies. It is probable that any change in resting state regional cerebral blood flow caused by astrocytic dysfunction would be relatively small. Hence it is possible that any such change was masked by the effects of the surgical procedure employed to administer L-AAA into the pre-limbic cortex. Brain trauma activates astrocytes via a process of reactive gliosis, causing an up-regulation in the expression of GFAP (Burda and Sofroniew, 2014). Activated astrocytes are potent producers of IL-6, a cytokine with broad effects involved in the regulation of the acute inflammatory response (Dong and Benveniste, 2001). It is possible that the acute trauma caused by the microinjection procedure, led to an inflammatory response and consequential production of proinflammatory cytokines. In vitro work has previously been shown that activation of astrocytes by inflammatory cytokines IL-1β, tumour necrosis factor alpha (TNFα) and Interferon gamma (IFNy) results in increased expression of phospholipase A2 and cyclooxygenase 2 as well as an increase in the production of vasodilatory prostaglandin E2 (Xu et al., 2003). Increased expression of astrocytic inducible nitric oxide synthase and production of vasodilatory nitric oxide also forms part of the normal inflammatory response.
response (Chen and Swanson, 2003). Such inflammatory mediators may lead to an increase in blood flow in perilesion areas, counteracting any astrocytic loss induced reduction in cerebral blood flow. Activated microglial cells were visible at the injection site in both the saline and L-AAA treated groups at the 48 hour time point indicative of a normal localised inflammatory response to the surgical procedure. A possible technical factor is the spatial resolution and imaging section thickness used in the ASL data acquisition in the present studies, thus the region may include tissue which was not lesioned, again possibly masking any effect of astroglial dysfunction on localised blood flow within and around the lesion site.

A decrease in T1 relaxation time in the striatal region 4 days post L-AAA administration and an increase in T2 relaxation time into the pre-limbic cortex 24 hours post L-AAA administration into the pre-limbic cortex were the only changes observed in localised relaxation times in the present studies. Again this is not what might have been expected. We expected to see alterations in T1 relaxation time at the point of injection into the pre-limbic cortex. Although there was no change in T1 relaxation times in the region 24 hours post toxin administration, there is a statistical trend suggesting a positive correlation between increased GFAP positive cell number and increased T1 relaxation time as has been previously reported ($r=0.5162$, $p=0.0588$, $n=20$) (Cowley et al., 2012). It is possible that L-AAA may have diffused to the striatal region and affected astrocytic function with a knock on effect on T1 relaxation time but we have no post-mortem data to confirm this. Astrocyte cell number was not quantified in the striatum as there was little GFAP immunoreactivity in the region making consistent between subject quantification difficult. Future studies may use alternative astrocytic staining markers to further elucidate if the delayed change in striatal T1 relaxation time following L-AAA administration into the pre-limbic cortex reported here is related to alterations in astrocyte function.

Increases in T2 relaxation time have previously been associated with oedema and a breakdown in the blood brain barrier (BBB) (Qiao et al., 2001). Therefore the increase in T2 relaxation time in the pre-limbic cortex 24 hours following the second toxin dose may suggest that astroglial dysfunction is associated with localised oedema and BBB damage in the vicinity of the injection site. This is perhaps to be expected as astrocytes play a key role in the normal response to brain trauma and maintenance of the BBB (Abbott et al., 2006; Burda and Sofroniew, 2014). The finding that T2 relaxation time 4 days following
toxin administration returns to saline treated control levels may suggest a recovery in astrocyte function. In the future it may be of interest to investigate if L-AAA administration is associated with prolonged damage to the BBB. It should be noted that previous results relating decreased T2 relaxation time with microglial activation have been in aged animals (Blau et al., 2012) and following several weeks recovery from an ischaemic insult (Justicia et al., 2008). Such changes are thought to be associated with a build up in ferromagnetic iron as a result of microglial heme metabolism (Justicia et al., 2008). Consequently any acute inflammation on foot of cortical L-AAA administration may not be detectable via changes in T2 relaxation times at the MR imaging data acquisition time points in the current studies. Future studies may investigate if L-AAA administration is associated with any delayed decrease in T2 relaxation time.

5.4.2.2 L-AAA administration into the pre-limbic cortex was associated with increased blood perfusion in the hippocampal section 4 days post L-AAA administration

The increase in blood perfusion reported 96 hours following L-AAA administration into the pre-limbic cortex in the visual and retrosplenial cortical regions occurs in the absence of any detectable difference in GFAP positive cell number in either region or in the pre-limbic cortex at the 5 day time point. It is possible that the general trend towards an increase in blood flow in the hippocampal section is a delayed neuroinflammatory response to the surgical procedure. Such a delay in inflammatory response in regions distal to the site of injury has previously been reported in thalamic regions 7 days following a ballistic injury in the rat frontal cortex, and is thought to be associated with increased retrograde nerve fibre degeneration (Williams et al., 2007). However as neither the retrosplenial nor visual cortices in either the L-AAA or saline treated groups exhibited any qualitative difference in microglial activation at the 5 day time point, a prolonged inflammatory response is unlikely to be the cause of these observed changes in blood perfusion parameters.

Another possibility is that aberrant astrocyte function in the pre-limbic cortex is resulting in altered activity of pre-limbic cortical efferents and thus causing a change in resting state activity in distal brain regions. There are indirect neuronal connections between the pre-limbic, visual and retrosplenial cortical regions. In humans, the dorsal prefrontal cortex is connected to the retrosplenial cortex, while the frontal cortex is indirectly linked to the visual cortex through the amygdala (Price and Drevets, 2010). The retrosplenial cortex also receives input from the visual cortex in rodents (Wyss and
Van Groen, 1992). Both the visual and retrosplenial cortices have been reported to be involved in the rodent default mode network, thought to be analogous to the default mode network in humans (Lu et al., 2012). The default mode network in humans is a series of brain regions, identified in neuroimaging studies that are functionally and structurally connected (Greicius et al., 2009). Increased resting state functional connectivity between the dorsomedial prefrontal cortex and the default mode network has been reported in depressed patients relative to non psychiatric controls (Sheline et al., 2010). The retrosplenial cortical region in humans has also been suggested to be part of a group of cortical midline structures involved in the processing of self-referential tasks (Northoff and Bermpohl, 2004). These cortical midline structures exhibit resting state hyperactivity in depressive patients (Northoff and Wiebking, 2011). Furthermore 10 days of immobilisation stress has been show to increase resting state connectivity in the rodent visual and default mode network (Henckens et al., 2015). Due to the region of interest approach presented here it is difficult to establish from the current data if such findings have relevance to the delayed increase in central cortical blood perfusion following L-AAA induced astrocytic dysfunction in the pre-limbic cortex. Future studies may employ resting state blood-oxygen-level dependent (BOLD) contrast functional MR imaging, to further map alterations in resting state functional connectivity induced following pre-limbic cortical L-AAA administration. Such information may provide us with additional information on the validity of the L-AAA treated Wistar rat as a model of depressive-like behaviour.
5.5 Conclusion

In these studies we report for the first time that administration of astroglial specific toxin L-AAA produces differential behavioural phenotypes dependent on the site of administration. Administration of L-AAA into the pre-limbic cortex of Wistar rats results in increased immobility in the forced swim test as well as mild anxiety-like behaviours in the open field test. We report that administration of L-AAA into the basolateral amygdala also produces increased immobility in the forced swim test with more pronounced anxiety-related behaviour in the open field, while administration of L-AAA into the CA3 subfield of the hippocampus results in no detectable change in behaviour as assessed in the forced swim and open field test. These data add to the growing body of literature implicating astrocytic dysfunction in the development of depressive symptomatology. Specifically they suggest that altered glial function in the amygdala may be involved in anxiety which exhibits high co-morbidity with depression.

Furthermore we report for the first time a series of temporally dependent neuroimaging markers associated with astroglial dysfunction. These data suggest that astroglial dysfunction in one region can, over time, lead to changes in resting state blood flow in distal brain regions. As there were no localised changes in GFAP positive cell numbers at these distal sites, it is likely that changes in blood flow in these regions are related to changes in resting state neuronal activity. Future studies will investigate whether astroglial dysfunction in other brain regions leads to similar changes in resting state brain activity. Such studies may employ whole brain BOLD contrast resting state functional MR imaging to allow us to assess the effect of discrete regional alterations in astrocytic activity on regional functional connectivity in the rodent brain. These experiments could potentially help elucidate if astroglial dysfunction forms part of the physiological processes underlying changes in resting state functional connectivity reported in depressed patients in the literature.
Chapter 6 – General Discussion and future directions
6.1 General discussion

Depression is a debilitating and potentially fatal disorder for which the pathophysiology underlying the illness is still unknown. The search for peripheral biomarkers in depression is ongoing, markers such as increased peripheral levels of inflammatory cytokines and changes in circulating levels of the astrocytic marker S100B as well as changes in circulating glutamate concentrations have been reported in depressed cohorts (Dowlati et al., 2010; Mitani et al., 2006; Schroeter et al., 2013). However these peripheral markers currently lack specificity for depressive illness and thus are of limited diagnostic or prognostic value at present. Neuroimaging studies have identified a series of detectable changes in brain structure and function in depressed patients; however the physiological processes underlying these markers are poorly understood. Animal models provide us with the opportunity to examine these processes in a way that is not possible in the human population. Due to the heterogeneity of the disorder there is unlikely to be a single pathophysiological process related to depressive illness. It is more likely that several different physiological mechanisms may result in the development of depressive symptoms or subtypes of depression in patients. There is evidence from in vivo magnetic resonance spectroscopy data and post-mortem brain tissue analysis that changes in glutamate metabolism and astrocytic function in discrete brain regions may be pathological processes related to, or predisposing factors in depressive illness. The primary aim of this thesis was to establish if three animal models of depression which are known to exhibit altered glutamatergic neurotransmission and/or astrocyte function also exhibit brain structural and functional abnormalities similar to those reported in human depressed patients. Furthermore we sought to investigate if astroglial dysfunction was associated with discrete magnetic resonance (MR) neuroimaging markers which may be of translational significance.

The olfactory bulbectomised (OB) rat, a lesion model with known predictive validity exhibited an increase in lateral and third ventricular volume and a decrease in cortical T2 relaxation time relative to sham-operated controls. The OB rat has been proposed as a potential animal model for neurodegenerative diseases like Alzheimer’s disease due to reported deficits in cognitive tasks and alterations in cholinergic neuronal function in the model (Hozumi et al., 2003; Yehuda and Rabinovitz, 2014a, 2014b). Animal models of Alzheimer’s disease have been reported to exhibit decreased cortical T2 relaxation time related to iron containing plaques (Jack et al., 2005), while decreases in cortical T2 relaxation time have been seen in aged relative to young animals (Blau et al., 2012).
These results support the suggestion that the OB rat may be a suitable animal model of Alzheimer's disease. Chronic dosing with nitric oxide synthase (NOS) inhibitor L-NA and tri-cyclic antidepressant imipramine were shown to attenuate OB-induced hyperactivity in the open field as well as partially attenuate the OB-related decrease in cortical T2 relaxation time, while increasing total ventricular volume. In contrast sub-acute dosing with NMDA receptor antagonist ketamine was not sufficient to produce an antidepressant response in the model and did not alter OB-related neuroimaging markers. As chronic dosing with the NMDA receptor antagonist MK-801 has previously been shown to attenuate OB-related hyperactivity in the open field, it is probable that chronic ketamine administration would do likewise. This re-affirms the need for chronic dosing to produce an antidepressant response in the OB rat model, suggesting that the “OB syndrome” can only be attenuated following prolonged treatment-induced changes in the OB brain. These data also suggest that cortical T2 relaxation time may be a state dependent marker for depressive-like behaviour in this model. Although there was a small increase in glial fibrillary acidic protein (GFAP) mRNA expression reported in the frontal cortex of the OB rat relative to sham-operated controls, the OB rat does not appear to be a suitable animal model to investigate the role of astrocytes in depressive-like behaviours.

The Wistar-Kyoto (WKY) rat, a genetic model predisposed to depression-related behaviours exhibited an increase in lateral and third ventricular volume and associated decrease in hippocampal volume relative to the Wistar rat strain. A small but significant increase in midbrain volume was also recorded. The WKY strain also exhibits resting state functional hypoactivity in frontal cortical regions relative to the Wistar strain which was correlated with decreased GFAP positive cell number. As the WKY rat has previously been proposed as an animal model of treatment resistant depression (Lahmame et al., 1997), the effects of electroconvulsive stimulation (ECS), the rodent equivalent of electroconvulsive therapy (ECT) were assessed in the strain. The effects of two different kinds of ECS treatment on behavioural and brain protein markers in the WKY and Wistar strain were assessed. Both brief pulse (BP) and ultrabrief pulse (UBP) ECS treatment resulted in a decrease in immobility time in the forced swim test (FST) in the WKY but not in the Wistar strain. This is in contrast with previous reports which show that BP but not UBP ECS treatment results in a decrease in immobility in the FST in naive Sprague-Dawley rats (O'Donovan et al., 2012). Differences in baseline FST immobility and response to antidepressant treatment have previously been reported between Sprague-
Dawley and Wistar rats which may partially explain this discrepancy (Crespi, 2010). Both BP and UBP treatment resulted in strain dependent cognitive deficits. Thus contrary to reports from the clinical literature, UBP ECS treatment was not associated with fewer cognitive side effects than BP ECS treatment (Verwijk et al., 2012). Future studies may further explore the effects of BP and UBP ECS treatment on cognition in rodents using alternative testing paradigms. BP ECS treatment was associated with an increase in frontal cortical actin protein levels in the WKY rat strain while having no effect on actin levels in the frontal cortex or hippocampus of the Wistar rat. As both BP and UBP ECS treatment resulted in an antidepressant response in the FST in the WKY rat, these data suggest that frontal cortical actin protein levels are not a marker of antidepressant response in the model. The WKY rat has previously been reported to be a highly stress sensitive strain, however the effect of stress on astrocytic function in the strain has not been well characterised. Hence we conducted an experiment to assess the effect of a ten day chronic restraint stress paradigm on behaviour, mRNA expression of markers of astrocyte cell function and GFAP protein expression in the WKY rat. Chronic stress produced an increase in immobility time in the WKY rat in the FST which was associated with regional dependent changes in mRNA expression for astrocyte related markers. Restraint stress was not associated with changes in GFAP protein expression in the frontal cortex or the hippocampus of the WKY rat. Unfortunately MR imaging was not possible in these later experiments due to technical problems. It would be of interest to investigate if chronic stress and antidepressant treatment have divergent effects on MR imaging markers in the WKY rat strain which would allow us to establish if the functional and structural imaging markers in the strain are state or trait dependent markers, and potentially further elucidate if frontal cortical resting state blood perfusion is related to GFAP positive cell number.

To further elucidate the effect of astrocytic dysfunction in sub-regions of the rodent brain, experiments were carried out to assess behavioural and neuroimaging markers associated with administration of astrocyte specific toxin L-alpha aminoacidic acid (L-AAA) into the rodent brain. Administration of L-AAA into the rodent pre-limbic cortex has previously been shown to produce depression related behaviours. Here we report that L-AAA administration results in administration site dependent changes in behaviour in the Wistar rat. Both pre-limbic cortex and basolateral amygdala administration result in an increase in immobility in the FST, basolateral amygdala administration was associated with increased anxiety-like behaviour as assessed in the open field.
Hippocampal L-AAA administration was not associated with any difference in behaviour as assessed in either the FST or in the open field. It is of interest that a similar behavioural phenotype as exhibited in the WKY rat can be induced in the Wistar rat by selectively altering astrocytic function in the basolateral amygdala. Changes in GFAP positive cell count in the basolateral amygdala have previously been reported in the WKY rat (Gosselin et al., 2009). Changes in cortical cerebral blood perfusion unrelated to local changes in GFAP positive cell number were recorded 4 days following L-AAA administration. Qualitative staining with the microglial activation marker Iba-1 suggests that these changes are not as a result of a generalised inflammatory response to toxin administration. The cause of the observed changes in localised perfusion parameters is still unknown but may be related to astrocytic dysfunction induced changes in resting state neuronal function.

These experiments provide further evidence in support of a role for aberrant astrocytic dysfunction in depression and anxiety related behaviours. The data suggest that changes in astrocyte function may lead to changes in cerebral blood flow as assessed by MR ASL. Future studies in animal models are required to further develop potential neuroimaging markers associated with astrocyte dysfunction. Figure 6.1 presents a brief visual summary of the results outlined in this thesis. Figure 6.2 presents a summary of potential alterations in neuroimaging markers which may be related to astrocyte cell function in the future. Future studies combining multimodal in vivo MR imaging in animal models of depression with post-mortem immunohistochemical analysis should provide us with further insights into the cellular and molecular mechanisms which impact on the structural and functional imaging markers reported in the depressed clinical population.
Figure 6.1: Summary of results to date

**Behaviour**
- Hyperactive in the novel open field
- Hyperactivity attenuated following chronic treatment with L-NA or Imipramine
- Sub-acute treatment with Ketamine not sufficient to attenuate hyperactivity
- WKY rats exhibit depressive and anxiety-related behaviours in the FST and OF test
- WKY rats exhibit deficits in spatial memory as assessed in the object displacement task
- L-AAA administration is associated with administration region specific alterations in rodent behaviour
- L-AAA administration to PLC and BLA increases immobility in the FST and anxiety-related behaviour in the OF

**MRI**
- OB is not associated with robust changes in mRNA expression of markers of neuroinflammation or astrocytic activity in either the frontal cortex or the hippocampus
- OB rats exhibit depressive and anxiety-related behaviours in the FST and OF test
- WKY rats exhibit deficits in spatial memory as assessed in the object displacement task

**Molecular**
- L-AAA administration induces transient astrocytic dysfunction at the site of injection
- No change in frontal cortical GFAP protein expression following stress

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**OB**
- Sham OB
- Saline OB
- L-AAA OB

**WKY**
- WIS WKY
- WIS WKY
- Saline WKY
- L-AAA WKY

**L-AAA**
- Saline L-AAA
- L-AAA L-AAA
Figure 6.2: Potential MR neuroimaging markers related to astrocyte cell function

**MRS**
- Changes in regional Glx signal reported in depression are likely to be associated with changes in astrocytic glutamate metabolism
- At higher field strengths detectable alterations in regional glutamine concentrations may represent a marker for astrocytic glutamate metabolism.

**Structure**
- Decreased trophic support for neurons may lead to dendritic remodelling and decreased neuronal branching leading to a change in volume in brain regions seen in depressive illness
- Decreased astrocyte cell number and altered astrocyte morphology may play a role in changes in regional brain volume
- Changes in astrocyte cell number may impact of parenchymal cell packing density and result in detectable changes in T1 and T2 relaxation times

**Functional changes**
- Changes in resting state fMRI markers are likely to be related to aberrant glutamatergic signalling, potentially due to altered astrocytic glutamate metabolism
- Changes in astrocytic regulation of cerebrovascular tone may be detectable as changes in resting state blood perfusion

**Astrocyte**

- Astrocytes are connected to oligodendrocytes via gap junctions, changes in astrocyte function may impact of oligodendrocyte cell function and hence impact on CNS white matter integrity. This may be detectable as changes in T1 and T2 relaxation times and changes in white matter fractional anisotropy values
6.2 Limitations

In this thesis astrocyte cell number was quantified via GFAP positive cell number using a method adapted from Gosselin and colleagues (2009). This technique was adopted because robust and reproducible quantification was possible. It should be noted that astrocyte cell quantification via GFAP positive cell number has limits as a technique; under resting conditions the majority of astrocytes in grey matter will express relatively small amounts GFAP protein. Furthermore the increase in GFAP positive cell number in response to acute trauma relates primarily to an increase in the expression of GFAP by astrocytes in situ at the site of trauma, rather than recruitment of astrocytes to the site or large scale proliferation. Only a small subpopulation of astrocytes have been shown to proliferate in response to an acute stab wound in the cerebral cortex (Bardehle et al., 2013). Thus changes in GFAP positive cell number reported in this thesis are unlikely to represent a change in astrocyte cell number. GFAP positive cell number reported in these experiments is instead best interpreted as a marker of astrocyte cell function. Future studies may use multiple markers for astrocytes to gather more information on regional astrocyte function in the animal models discussed in this thesis. Glutamine synthetase and/or S100B immunostaining combined with GFAP staining may provide a more information on astrocyte function in these models.

A second limitation of the present studies is that it was not possible to measure rodent central blood pressure during MRI scans, hence we cannot exclude the possibility that strain and treatment related differences in peripheral blood pressure may account for some of the central changes in blood perfusion parameters reported in these studies. Respiration rate was monitored and maintained at a rate of between 60-75 per minute for all strains and treatments during scanning. Physiological parameters in the OB rat are relatively well characterised, no baseline differences in heart rate or body temperature, between OB rats and sham-operated Sprague-Dawley controls have been reported previously (Roche et al., 2007; Vinkers et al., 2009). Blood pressure in the WKY rat strain has been well characterised, WKY rats have been reported as showing no basal difference in blood pressure relative to Sprague-Dawley rats (Markgraf et al., 1993) and Wistar rats (Preuss et al., 1998). As changes in cerebral blood flow parameters only reached significance in a sub-regional dependent manner it suggests that localised changes in vascular dynamics rather than altered peripheral blood pressure are the cause of the changes reported in these parameters here.
Lastly the role of anaesthesia in detected changes in MR imaging parameters cannot be discounted. Anaesthesia is required to provide immobility and minimise stress on the animal during MR scanning, inhalant anaesthetics such as isoflurane are the preferred method of anaesthesia when available (Hanusch et al., 2007). Isoflurane is a vasodilator which produces an increase in resting state blood perfusion in the rodent brain (Hendrich et al., 2001; Sicard et al., 2003). However it is possible to detect changes in cerebral blood flow in response to peripheral stimulation in isoflurane anaesthetised rodents (Masamoto et al., 2007). There have been several studies which have conducted MR scans in conscious animals (Duong, 2007; Sicard et al., 2003), however this is unsuitable for resting state measures as trained habituation of the animal to the scanning procedure may represent the conductance of a “task” which may influence results. As such MRI under anaesthesia, though presenting potential confounds, is still the preferable method for acquisition of resting state perfusion data. Future studies may use alternative anaesthesia to assess the effect of isoflurane on cerebral blood flow parameters reported in this thesis.
6.3 Future directions

There are several further studies which could be carried out to further elaborate upon and aid in our interpretation of the results presented in this thesis.

Future studies in the OB rat model should conduct post-mortem immunohistochemical analysis to investigate the cellular and molecular correlates of the imaging markers reported in chapter 3 of this thesis. Perfused post-mortem tissue was not available for analysis in the OB studies presented here. Prussian blue staining could further elaborate if the changes in cortical T2 relaxation time observed in the OB rat are associated with changes in cortical iron content, while Iba-1 staining to assess cortical microglial activation may also be of interest to confirm that OB is not associated with a robust inflammatory response. Such analysis could elaborate on the mechanisms through which antidepressant action in the model appears to attenuate OB-induced changes in T2 relaxation times. It would be of interest to assess whether chronic dosing with NMDA receptor antagonist ketamine is sufficient to attenuate OB-induced hyperactivity in the open field and if such attenuation was associated with similar changes in neuroimaging markers reported for L-NA and imipramine.

Future studies in the WKY rat model should conduct MR imaging following ECS treatment to assess if an antidepressant response in the WKY rat is associated with changes in resting state blood perfusion markers, and if any such changes are associated with regional changes in astrocytic function assessed post-mortem. Furthermore the effect of chronic ECS treatment on WKY brain structure should also be investigated. Future studies may employ voxel based morphometry to allow for whole brain analysis of changes in grey and white matter density (Ashburner and Friston, 2000). This would allow enable detection of differences in brain structure in regions which are not anatomically well defined such as frontal cortical sub-regions. Assessment of WKY white matter structure via diffusion tensor imaging may also be of interest to establish if the WKY strain exhibits alterations in white matter integrity and brain structural connectivity similar to those reported in depressed patients in the clinical literature (see section 1.5.1), further improving the validity and utility of the model for use in future studies. It may be of interest to assess if altered astrocyte function reported here is associated with alterations in regional Glx signal as assessed by in vivo magnetic resonance spectroscopy (MRS). Assessment of whether the cerebral blood flow response in the WKY rat strain following a hypercapnic challenge differs from that of comparator strains like the Wistar and Sprague-Dawley rat may provide information on whether alterations in astrocyte function reported here have functional consequences. Changes in cortical
functional MRI activity in response to CO₂ challenge have previously been reported in animal models following chronic stress and may be of interest in the WKY rat, given its known anxious like phenotype (Rahman et al., 2014).

In the L-AAA model we have shown that pre-limbic cortical administration is associated with a transient increase in immobility time in the FST and a transient change in the number of GFAP positive cells in the pre-limbic cortex. We have not confirmed that this is the case in the basolateral amygdala and hippocampus. Tissue should be collected at an acute time point following basolateral L-AAA administration to confirm that behavioural change is associated with a change in astrocyte function as assessed via altered GFAP positive cell number. It would be of interest to investigate if there are differences in the duration of anxiety and depression-related behaviour following basolateral amygdala L-AAA administration by conducting further behavioural experiments at later time points. Increased information on the effect of the surgical procedure on GFAP positive cell number at both the acute and later points may be of interest to aid in the interpretation of results gathered to date, such information could be acquired by adding a sham-surgery group to future experiments. We have also inferred that behavioural change following toxin administration is resultant from a change in astroglial cell function, however we have not confirmed that toxin administration did not affect neuronal viability. It should be noted that several previous studies have reported that toxin administration into the rodent brain is not associated with changes in neuronal cell number (Banasr and Duman, 2008; Khurgel et al., 1996), and unpublished in vitro work from our own lab suggests that L-AAA is only toxic to neurons at high concentration. Future work should confirm that toxin administration related behavioural change presented here is not associated with a change in neuronal cell number as assessed through staining for neuronal marker neuN at the site of injection. Although hippocampal L-AAA administration was not associated with any change in depression or anxiety-related behaviour, it may be of interest to investigate if hippocampal astrocytic ablation is associated with deficits in cognitive tasks, pre-limbic cortical L-AAA administration has previously been shown to be associated with cognitive deficits in rats (Lima et al., 2014).

Acute L-AAA administration was not associated with changes in cerebral blood flow parameters. This may have been related to methodological issues such as the method of administration used in the present studies as well as the voxel size used in the ASL sequence. Improved receiver coils may allow for better signal to noise ratio, allowing for a smaller voxel size to be used and thus more localised changes in cerebral blood flow to
be assessed. The toxin was administered via microcapillary in the present studies due to concerns about minimising reactive gliosis and the inability to acquire MR compatible plastic guide cannula in the dimensions required for pre-limbic cortical administration. It may be of interest to compare changes in blood perfusion related parameters and to assess the level of reactive astrogliosis following insertion of a cannula into another region such as the basolateral amygdala. Finally in vivo MRS could be used to investigate the temporal change in Glx signal following toxin administration to further elaborate on the relationship between changes in behaviour in response to toxin administration and altered glutamate metabolism.
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Publications


Published abstracts


Oral presentations

“Animal models of depression: Preclinical approaches to develop new and improved antidepressant treatments”. Presented to “Assessment of rodents’ behaviour: methods and rationale”, 27-30/8/2012, Culture Center Sofia, Helsinki, An international course by FPDP Pharmacy and Biocenter Finland.
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