Investigating the neurochemistry of depression and the novel mood stabilising agent, ebselen, using magnetic resonance spectroscopy

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Abstract

RATIONALE: Magnetic resonance spectroscopy (MRS) offers a tool for investigating in vivo neurochemical changes in depression, as well as for guiding targeted drug development. Using MRS, changes to the glutamatergic system have been demonstrated in depressed patients, although results from different studies have often been inconsistent. Elucidating the role of glutamate in depression can be helped by carrying out assessment with ultra-high field MRS and in addition to this, identifying patient characteristics that may contribute to variability in neurochemical findings. MRS can also be used to demonstrate in vivo effects of pharmacological manipulation, and by so doing, confirm mechanisms of drug action. Recently, a potential novel mood-stabilising and antidepressant agent, ebselen, has been identified based on preclinical evidence of lithium-mimetic effects. This drug offers the possibility of an alternative new treatment for mood disorders, without the side effects of lithium.

OBJECTIVES: This thesis aimed to investigate glutamatergic neurochemistry in depression, as well as identify its clinical correlates. Further to this, the effects of ebselen treatment on neurochemistry and performance on neuropsychological tasks relevant to psychiatric treatment were investigated.

METHODS: The first study involved 40 patients with major depressive disorder and 32 healthy controls. Subjects were scanned with 7-tesla Siemens scanner equipped with a 32 channel receive array head coil. High-resolution MP-RAGE structural images were acquired and used to guide the placement of MRS voxels. Proton-MRS was carried out sequentially on three voxels placed on the anterior cingulate cortex (ACC), occipital cortex and putamen, using a semi-LASER sequence (TE=36ms, TR=7s, NT=64). Metabolites were quantified with LCModel, using an unsuppressed water signal as a reference. The primary metabolites of interest were glutamate, glutamine and Glx. The second study featured exploratory analysis of associations between the concentrations of ACC glutamate, ACC Glx, and putamen glutamine with patient clinical parameters. In the third and fourth studies, 20 healthy volunteers were tested on two occasions following treatment with either ebselen (3600 mg in three doses over 2 days) or identically matching placebo, in a within-subject, double-blind, crossover design. Participants underwent 7-tesla MRS scanning 2 hours after the final dose of ebselen, with voxels placed over the ACC and occipital cortex. Scanning was performed with a STEAM sequence (TE = 11ms, TR=5s, NT = 64). Three hours after the final dose (immediately following the scan), participants completed the Cambridge Gambling Task (CGT) and the Facial Emotion Recognition Task (FERT).

STATISTICS: Separate MANOVA for each voxel region were used to assess metabolite concentration differences between depressed patients and controls, with significant main effects followed up by comparisons with independent t-tests. Associations between neurochemistry and clinical variables were tested using Pearson's correlation tests for continuous variables and independent samples t-tests for categorical outcomes. Separate repeated measures MANOVA for each region were used to test for the effects of ebselen treatment on neurochemistry, with significant main effects followed up by comparisons with paired t-tests. Correlations were also determined between neurochemistry and neuropsychological task performance using Spearman's-rho test.

RESULTS: There were main effects of gender on ACC metabolite levels, with depressed female patients having lower levels of glutamate ($p = 0.005$) and Glx ($p = 0.010$) relative to female controls, while depressed male patients had elevated levels of Glx ($p = 0.046$) relative to male controls. In the putamen, depressed patients had elevated levels of glutamine ($p = 0.014$) without gender effects. There were no group effects on occipital cortex metabolite levels. No significant associations were found between several patient variables and neurochemistry. Ebselen treatment lowered concentrations of inositol ($p = 0.028$), glutamate ($p = 0.010$), glutamine ($p = 0.024$), Glx ($p = 0.001$) and glutathione ($p = 0.033$) in the ACC. There were no effects of ebselen treatment on occipital cortex metabolite levels. On the CGT, ebselen treatment lowered the delay aversion score ($p = 0.010$), and increased reward seeking ($p = 0.046$). On the FERT, ebselen treatment increased the accuracy of recognition of positive facial expressions ($p = 0.035$).

CONCLUSIONS: Glutamatergic changes are present in depression, although these may show gender specificity in the ACC. Ebselen treatment decreases brain inositol levels, suggestive of a lithium-mimetic effect. Ebselen treatment also decreases impulsivity, increases reward seeking and produces a positive bias in emotional processing, suggesting potential uses as an antidepressant and anti-impulsivity agent.

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Declaration

Some parts of this thesis are available in publication

Chapter 5
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Abbreviations:

3-D	3-Dimensional	MANOVA	Multivariate Analysis of Variance
5-HIAA	5-Hydroxyindoleacetic acid	MAO	Mono-amine Oxidase
5HT	Serotonin	MDD	Major Depressive Disorder
ACC	Anterior cingulate cortex	mGluR	Metabotropic Glutamate Receptor
AFP	Adiabatic full passage	NAA	N-Acetyl Aspartate
B_0	Main magnetic field	NICE	National Institute for Health and Care Excellence
BDI	Beck depression inventory	NIH	National Institute of Health
CFS	Chalder Fatigue Scale	NIMH	National Institute of Mental Health
CRLB	Cramer Rao Lower Bound	NMDA	N-Methyl-D-Aspartate
CSDE	Chemical shift displacement error	NMR	Nuclear Magnetic Resonance
CTQ	Childhood Trauma Questionnaire	ppm	Parts per million (MRS scale)
DSM	Diagnostic and statistical manual	PRESS	Point-RESolved Spectroscopy
DSM IV TR	Diagnostic and statistical manual-Text Revision	RF pulse	Radiofrequency pulse
EAATs	Excitatory Amino Acid Transporters	rTMS	Repetitive-Transcranial Magnetic Stimulation
FAST	FMRIB Automated Segmentation Tool	SAR	Specific Absorption Rate
FWHM	Full width at half maximum	SEM	Standard Error of Mean
GABA	γ-Aminobutyric acid	semi-LASER	semi-Localized by Adiabatic Selective Refocusing
Glx	Glutamate + Glutamine	SHPS	Snaith-Hamilton Pleasure Scale
GWAS	Genome-wide association studies	SNR	Signal-to-Noise
HAM-D	Hamilton Depression Rating Scale	STAI	State-Trait Anxiety Inventory
LASER	Localized by Adiabatic Selective Refocusing	STEAM	Stimulated Echo Acquisition Mode
LCModel	Linear Combination of Model Spectra	TCAs	Tricyclic Antidepressants

Chapter 1: An Overview of Major Depressive Disorder

1.1 Introduction

Major Depressive Disorder (MDD) is a debilitating neuropsychiatric illness that is estimated to affect 350 million people globally. At population level, the 12-month prevalence is estimated to be about 5-6% and lifetime prevalence 13-16% (Hasin *et al.*, 2005, Kessler *et al.*, 2007). The peak time of diagnosis is in early adulthood, although most patients report having experienced symptoms before the time of diagnosis (Kessler *et al.*, 2010). Wide regional variations have been reported in the prevalence of depressive disorders, although these may be related to case identification (Kessler *et al.*, 2007). In particular, mental health stigma and cross-cultural differences in health seeking behaviour may have a significant influence on the regional variations (Probst *et al.*, 2006). The prevalence of MDD shows gender variation, being almost twice as common in women (Kuehner, 2003). Biological factors are thought to explain some of the gender variability, though social-cultural issues, such as the greater risk of women to experience sexual abuse and partner violence, are also likely to be important (Cutler *et al.,* 1991).

MDD is among the leading contributors to the global burden of disease, ranking first among the mental, neurological and substance use disorders and third across all categories (Collins *et al.*, 2011). Its prevalence is on the rise, and is projected to rank second in global disease burden by the year 2020 (Mathers and Loncar, 2006). Depressive episodes are associated with severe disability and loss of work productivity. In addition, suicide rates are substantially increased among patients with MDD, further contributing to the disease burden (Harris and Barraclough,

1997, Hawton *et al.*, 2013). Patients with MDD are also very likely to have a co-morbid psychiatric illness. In addition to psychiatric morbidity, depressed patients have an increased risk for general medical conditions, particularly cardiovascular disease and metabolic syndrome (Luppino *et al.*, 2010, Moussavi *et al.*, 2007).

Advances in techniques for neuroscience and an ever-increasing wealth of knowledge about the structure and function of the brain have helped to elucidate possible mechanisms in the pathophysiology of MDD. Several theories have been put forward to explain the neurocognitive features of depression; of which the most intensely researched on is the monoamine hypothesis (discussed in section 1.4 below). These theories have also served to guide research on new therapies.

Currently available pharmacotherapies for MDD are largely based on targets realised through serendipitous discoveries and not through rational drug development. As a consequence, response to the available treatments has remained modest, with more than a third of patients failing to achieve remission in spite of four different treatment iterations (Nierenberg *et al.*, 2006, Rush *et al.*, 2006, Trivedi *et al.*, 2006).

Recent evidence suggests that the pathophysiology of MDD involves cytoarchitectural and molecular changes involving the predominant excitatory neurotransmitter system, glutamate (Sanacora *et al.*, 2012). This has formed the basis for intense research with the aim of developing rapidly acting and better efficacious antidepressant agents (Krystal *et al.*, 2013).

Several questions remain unanswered in the research on the neurobiology of MDD. While there is evidence that MDD results, at least in part, from chemical alterations in areas of the brain involved in mood regulation, the precise nature and direction of these alterations remain equivocal. It is also not clear what the specific mechanisms of action of the currently available antidepressant medications are, especially given the shortcomings of the most widely postulated monoamine depletion hypothesis (Healy, 2015).

Advances in the techniques for brain imaging have allowed us to interrogate some aspects of neurochemistry in vivo. Using the tool of magnetic resonance spectroscopy (MRS, discussed in chapter 2), we can assess neurochemical levels within defined brain regions and in so doing establish patterns that may underlie neuropsychiatric diseases. Evidence from MRS studies supports the involvement of the amino-acid neurotransmitter systems, and particularly the glutamatergic system, in MDD. Availability of higher field imaging scanners (4 and 7-tesla MRI) has allowed us to investigate such chemical changes with more precision. In addition to determining underlying neurochemical changes, there is the potential for applying the technique of MRS for targeted drug discovery in neuropsychiatry, including for the treatment of MDD.

This Chapter will carry out an overview of MDD. Diagnostic criteria and treatment options will be reviewed. In addition, the neurobiological theories on the pathophysiology of MDD, including evidence supporting a glutamatergic hypothesis of depression (a key aspect of this thesis), will be discussed.

1.2 Diagnosis of Major Depressive Disorder

Major Depressive Disorder is a heterogeneous syndrome whose core symptoms are presence of a low mood and diminished interest in pleasurable activities. Other symptoms include sleep disturbances, excessive and abnormal guilt, loss of energy, decreased ability to concentrate, decreased appetite, psychomotor agitation and retardation and suicidality (Diagnostic and Statistical Manual of Mental Health Disorders, DSM-5, American Psychological Association 2013; Table 1.1). While decreased appetite and weight loss are common, some patients experience an abnormal increase in appetite (comfort eating) and weight gain. Psychomotor changes are particularly common in severe depression and manifest in the form of slowed movement and cognitive processing (retardation), and distressed and restless behaviour (agitation). MDD is related to normal experiences of sadness and bereavement. Unlike these however, symptoms of MDD are usually out of proportion to that expected of normal sadness or bereavement, and do not remit once the identified external precipitating factor is removed (Belmaker and Agam, 2008).

The diagnosis of depression is currently made through the use of specified criteria, of which the Diagnostic and Statistical Manual (used for the studies presented in this thesis) and the International Classification of Diseases criteria are most widely used. These criteria use clustering of clinical symptomatology as well as course and duration of symptoms to define psychiatric diagnostic categories. In the DSM-5 criteria (Table 1.1), depression is diagnosed on the basis of presence of at least five symptoms, of which one must be either depressed mood or loss of interest/pleasure

(anhedonia). The symptoms must have been present continuously over a period of at least two weeks, must represent a change from previous functioning and be associated with significant psychosocial dysfunction. In the ICD-10 criteria, two of three core symptoms: depressed mood, anhedonia and loss of energy, must be present for a diagnosis of MDD to be made.

Most patients with depression experience an episodic course of illness, with depressive symptoms separated by periods of relatively normal mood. More than half of patients experience a relapse following the initial depressive episode (Eaton *et al.*, 2008). Some patients however do not experience any intervals of normal mood between distinct depressive episodes, and are described as having chronic MDD. Even in such patients, intervals with mild depressive symptoms (dysthymia) separated by overt depressive episodes are more common (Keller *et al.*, 1992, Mueller *et al.*, 1999).

Some patients experience psychotic symptoms during the course of a depressive episode (MDD with psychotic features). These are usually in form of mood-congruent (consistent with the patients low mood) delusions and second person auditory hallucinations. In MDD with melancholic features, patients experience prominence of biological symptoms such as decreased appetite, weight loss, psychomotor agitation/ retardation, excessive guilt and decreased libido. In addition, these patients often experience diurnal variations in the severity of symptoms, typically reporting feeling markedly worse in the early morning and improving gradually as the day progresses (Table 1.2).

Table 1.1: Diagnostic criteria for major depressive disorder (adapted from DSM 5, American Psychiatric Association, 2013). Criteria A-C define a major depressive episode.

Criteria for Major Depressive Disorder – DSM 5

A	Presence of five (or more) of the following symptoms during the same 2-week period, representing a change from previous functioning. At least one of the symptoms must be either depressed mood or loss of interest or pleasure 1. Depressed mood most of the day nearly every day 2. Markedly diminished interest or pleasure in all, or almost all activities 3. Significant weight loss when not dieting, or weight gain 4. Insomnia or hypersomnia 5. Psychomotor agitation or retardation 6. Fatigue or loss of energy 7. Feelings of worthlessness or excessive or inappropriate guilt 8. Diminished ability to think or concentrate, or indecisiveness 9. Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or suicide attempt or a specific plan for committing suicide
B	Symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning
C	Episodes not attributable to the physiological effects of a substance or to another medical condition
D	The occurrence of a major depressive episode (meeting criteria A-C) is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders
E	There has never been a manic episode or hypomanic episode

Patients with melancholic features are more likely to score higher on scales measuring depressive symptoms, such as the Hamilton Rating Scale for Depression (HAM-D). Other features of melancholic depression include a greater likelihood of

familial predisposition and poor response to placebo treatment (Cowen *et al.,* 2013). Other specifying categories of MDD include 'with anxious distress' in which anxiety symptoms are prominent, 'with mixed features' in which manic or hypomanic symptoms not meeting full criteria for hypomania or mania are present, 'with catatonia' in which characteristic abnormalities of movement are present, 'with peripartum onset' when symptoms occur during pregnancy or peripartum period and 'with seasonal pattern' in which symptoms show a cyclical pattern that varies according to seasons. In atypical depression, patients experience some distinct behavioral features that include paradoxical mood reactivity (mood brightens in response to positive events), increased appetite (comfort eating), increased sleep (hypersomnia) and sensation of heaviness in the limbs (leaden paralysis). In addition, patients with MDD with atypical features show a longstanding pattern of rejection sensitivity.

Comorbid neuropsychiatric illnesses are common in MDD, with the most frequent being anxiety disorders, personality disorders and substance use disorders (Alonso *et al.*, 2004). Up to half of patients with MDD have a co-morbid anxiety disorder which may worsen the course of illness, delaying recovery and increasing the rate of relapse (Hirschfeld, 2001).

Table 1.2: Specifying features for the diagnosis of major depressive disorder with melancholic features (adapted from DSM 5, American Psychiatric Association, 2013)

DSM 5 Major Depressive Disorder with Melancholic Features

A	Presence of one of the following during the most severe period of the current episode 1. Lack of pleasure in all, or almost all, activities 2. Lack of reactivity to usually pleasurable stimuli
B	Presence of three or more of the following 1. Profound despondency / despair 2. Symptoms regularly worse in the morning 3. Early morning awakening 4. Marked psychomotor agitation or retardation 5. Significant anorexia or weight loss 6. Excessive or inappropriate guilt

A challenge to the accurate diagnosis of MDD is the overlap with symptoms of bipolar disorder. The most common initial presenting symptoms in bipolar disorder are of depressive polarity (Berk *et al.*, 2007, Duffy *et al.*, 2014, Mesman *et al.*, 2013). As such, the early course of bipolar disorder may be wrongfully diagnosed as MDD. Treatment of such patients, the so called 'hidden bipolars', with antidepressants is associated with adverse outcomes including switching from depressive to manic polarity and increased risk of suicide (Goldberg and Ernst, 2002, Post *et al.*, 2010).

The currently available diagnostic approaches (DSM and ICD) are based on clustering of clinical symptoms into diagnostic categories. This approach results in the grouping of patients into broad heterogeneous categories that are not informed by the underlying pathophysiology. This is particularly concerning for MDD, where emerging evidence suggests that distinct sub-categories may have unique genetic

and molecular characteristics. For instance, patients with specific defining features such as psychosis or melancholia may represent unique populations that at the basic neuroscience level may not be related to other sub-categories. The age of onset has also been suggested as a possible modifier, with early onset and late life depression representing distinct categories separate from the more common adult onset type. For instance, patients with pre-pubertal onset MDD are more likely to have familial risk and a distinct genetic profile (Kendler *et al.*, 2007, Kessler *et al.*, 2001, Shi *et al.*, 2011).

The heterogeneity of the symptom-based classification may account for the poor treatment response and difficulty in identifying disease biomarkers. An emerging initiative proposed by the National Institute of Mental Health (NIMH) suggests categorisation based on biologically defined phenotypes: genetic, neuroimaging, behavioural as well as clinical features (Insel *et al.*, 2010). This approach is more likely to yield homogenous groups with more relevance to research on targeted pharmacotherapy.

Although depression is a frequently diagnosed illness, patients presenting with co-morbid psychiatric or general medical conditions are likely to be undiagnosed (Bair *et al.*, 2003). One approach towards improving the diagnostic yield would be through the use of depression specific biomarkers (Lakhan *et al.*, 2010). In addition, biomarkers would help to further refine the diagnostic sub-categorisation. There have been numerous attempts to identify biomarkers of relevance to the diagnosis of MDD. Several have been proposed including peripheral cortisol measurements

(Carroll *et al.*, 2007), the neurotrophic hormone, brain derived neurotrophic factor (Hashimoto, 2010), genetic markers (Pajer *et al.*, 2012) and neuroimaging biomarkers (Hahn *et al.*, 2011).

1.3 Treatment of major depressive disorder

Several treatment options for MDD are available, each with specific indications and limitations. The National Institute for Health and Care (NICE, 2005) guidelines recommends pharmacotherapy with antidepressants and psychotherapy as the initial treatment options. For mild depression, psychotherapy is recommended before pharmacotherapy. For moderate to severe depression, better response rates are achieved through a combination of pharmacotherapy and psychotherapy (Schramm *et al.*, 2007). In addition to antidepressants, low-dose atypical antipsychotic drugs, lithium and thyroid hormone may be used as add-on treatments for patients who fail to respond to antidepressants monotherapy. In patients with MDD with psychotic features, treatment with antidepressants combined with atypical antipsychotics is usually recommended (Cowen and Anderson, 2015).

Newer agents such as ketamine and other drugs that modulate the activity of the neurotransmitter glutamate are being investigated as potential alternatives for treatment resistant MDD (Krystal *et al.*, 2013). Patients with treatment resistant MDD may also benefit from somatic treatments such as electroconvulsive therapy, repetitive-transcranial magnetic stimulation and deep brain stimulation.

Psychological treatments include cognitive behavior therapy and interpersonal therapy. Although these have been shown to be equally efficacious to pharmacotherapy for the treatment of mild to moderate MDD, they are resource intense and have remained less accessible (Thase *et al.*, 2007). In the United Kingdom, programs such as the 'Improved Access to Psychological Treatment' (IAPT) have been initiated to increase psychological treatment access at primary care level.

Although the use of pharmacotherapy for the treatment of depression has been reported since antiquity, modern pharmacological approaches have only been present for about six decades. Serendipitous discoveries of the mood elevating effects of iproniazid and imipramine, and subsequent discovery of their actions on the monoamine system, laid the foundation for modern psychopharmacology of MDD (discussed in section 1.4 below). Subsequently, several antidepressants were developed targeting the monoamine neurotransmitter systems (serotonin, noradrenaline and dopamine). In particular, the introduction in the 1980's of the selective serotonin reuptake inhibitors (SSRIs), which are better-tolerated and safer than traditional tricyclic antidepressants such as imipramine, stimulated antidepressant use for the treatment of depression. Success with the SSRIs led to the development of additional antidepressants acting on the monoamine system, including the Serotonin and Noradrenaline Reuptake Inhibitors (SNRIs, venlafaxine and duloxetine), Noradrenaline Reuptake Inhibitors (NRIs, reboxetine) and Noradrenergic and Specific Serotonergic Antidepressants (NaSSAs, mirtazapine).

Response rates to the currently available treatment options are modest. Only about a third of patients achieve remission following treatment with the first line antidepressant agents (Trivedi *et al.*, 2006). Second and third line treatments have marginal gains on response rates (Nierenberg *et al.*, 2006, Rush *et al.*, 2006). Identification of new targets beyond the monoamine system would be necessary for reducing the non-responder gap. Additional factors beyond the efficacy of pharmacotherapy contribute to treatment non-response. These include inaccurate diagnosis, comorbid psychiatric and general medical conditions, psychosocial factors and unique individual biological factors (Bennabi *et al.*, 2015).

The lag time between initiation of antidepressant treatment and improvement in mood symptoms represents a challenge to the treatment of MDD. Although antidepressants have been shown to exert subtle positive effects fairly early on during treatment (Harmer *et al.*, 2009a, Pringle *et al.*, 2011a, Warren *et al.*, 2015), and significant improvement in scores on rating scales (Taylor *et al.,* 2006), it usually takes at least 6-8 weeks for their overt mood alleviating effects to be fully appreciated by patients. In addition, side effects remain a considerable concern for antidepressant use, although the newer agents such as SSRIs have better adverse effect profiles. Although many pharmacological agents have been made available since the original antidepressants were introduced, biological targets for these have remained the same. No antidepressants have been developed based on understanding of the biological pathways of importance to mood regulation.

Neuroimaging biomarkers of treatment response have been proposed as useful adjuvants to guide antidepressant drug development. For example, the observation that depressed patients have an exaggerated amygdala response to stimuli with a negative emotional valence, and that treatment with SSRIs reverses this pattern of reactivity, suggests that drugs capable of achieving this effect could be screened for at the neural level using neuroimaging (Murphy *et al.*, 2009, Sheline *et al.*, 2001). Further evidence for potential neuroimaging biomarkers has come from studies investigating brain connectivity patterns during conditions of rest (resting state networks). Observations that depressed patients have increased connectivity within particular brain networks at rest (Greicius *et al.*, 2007, Sheline *et al.*, 2010) and that antidepressant treatments reverse these connectivity patterns (Dichter *et al.*, 2015, McCabe and Mishor, 2011), offer the potential to incorporate this imaging modality during the early phases of drug development.

1.4 Monoamine depletion hypothesis

The monoamine depletion theory posits that depression results from synaptic deficit of the monoamines serotonin and /or, noradrenaline, and that antidepressants act by reversing this deficiency (Bunney and Davis, 1965, Schildkraut, 1965). Dopamine is also a monoamine and plausibly involved in the pathophysiology of depression through its role in reward and incentive behavior. However, the re-uptake blockers used in the pharmacological treatment of depression, with the possible exception of bupropion (Stahl *et al.*, 2004), do not affect dopamine re-uptake directly.

The momoamine hypothesis was formulated some six decades ago following chance discoveries of the antidepressant effects of the monoamine oxidase inhibitor, iproniazid and the tricyclic antidepressant, imipramine (Kuhn, 1957, Loomer *et al.*, 1957). In the 1980s, the SSRIs were introduced after about three decades of research into their possible use for the treatment of obesity and hypertension, indications that were originally thought to be potentially more profitable. The SSRIs have since become the principal pharmacological treatment for MDD. In addition to the SSRIs, all other first line pharmacological treatments act on the monoamine system. These are the Serotonin and Noradrenaline Reuptake Inhibitors (SNRIs), Noradrenaline Reuptake Inhibitors (NRIs) and Noradrenergic and Specific Serotonergic Antidepressants (NaSSAs). Bupropion, licensed for the treatment of depression in the United States, probably inhibits the re-uptake of noradrenaline and dopamine (Stahl *et al.*, 2004).

Serotonin is synthesized from the amino-acid tryptophan through the action of the enzymes tryptophan hydroxylase and aromatic amino acid decarboxylase. It is then stored in vesicles in nerve endings until it is released into the synapse. Secreted serotonin is taken back up by the pre-synaptic neuron, where it is broken down into 5-hydroxyindoleactetic acid (5-HIAA) by the enzyme monoamine oxidase (MAO). Since serotonin does not cross the blood brain barrier, brain levels result from synthesis and breakdown mechanisms occurring in the brain. Serotonergic neurons are located within the raphe nuclei found in the brainstem. From here, these neurons project widely to cortical and subcortical structures. Serotonin exerts its

effects through the serotonin receptors, of which seven main subtypes have been characterized (5-HT_{1-7}, Barnes and Sharp, 1999).

Several lines of evidence support the enhancement of synaptic monoamines as the principal mechanism for pharmacological action of antidepressants. Limitations to this mechanism however suggest the need to look beyond the raphe nuclei and their projections for answers to the biological underpinnings of depression and its treatment. The evidence for and against the monoamine depletion hypothesis will be discussed in the next paragraphs.

1.4.1 Evidence for the monoamine depletion hypothesis

A single nucleotide polymorphism in the human tryptophan hydroxylase-2 gene, the rate limiting step in serotonin synthesis, that results in 80% loss of function in serotonin production, has been found to be more common in depressed patients (Zhang *et al.*, 2005), though this has not been confirmed by GWAS studies (Cohen-Woods *et al.*, 2013). Dysfunction of the synaptic protein P_{11}, which is crucial for the activity of the serotonin receptor (5-HT_{1B}) has also been associated with depressive behavior in animal models (Svenningsson *et al.*, 2006). Decreased responsiveness of the 5-HT_{1A} receptor has been linked to suicidal behavior in major depression (Pitchot *et al.*, 2005). Decreased beta-adrenergic receptor-linked stimulation of adenylate cyclase has been reported in post-mortem samples of patients with MDD (Valdizan *et al.*, 2003).

Further evidence in support of the monoamine depletion hypothesis has come from studies in which the monoamine precursor tryptophan has been experimentally depleted (Ruhe *et al.*, 2007). Conversion of the essential amino-acid tryptophan into 5-hydroxytryptophan by the enzyme tryptophan hydroxylase is the rate-limiting step in the synthesis of serotonin. Acute tryptophan depletion can be achieved through administration of a drink containing all amino acids except tryptophan. This has been demonstrated to induce moderate decreases in mood in medication free recovered MDD patients (Ruhe *et al.*, 2007). In depressed patients currently treated with serotonergic antidepressants, acute tryptophan depletion induces depressive relapse. Similarly, tyrosine hydroxylase inhibition with the false substrate α-methyl-p-tyrosine induces depressive symptoms among patients successfully treated with a noradrenergic potentiating antidepressant as well as in unmedicated recovered depressed patients. Acute tryptophan depletion also lowers mood slightly in healthy controls with a family history of MDD, although it does not generally lower mood in healthy subjects (reviewed in Ruhe *et al.,* 2007).

Positron Emission Tomography (PET) studies have also provided evidence in support of the monoaminergic theory. Decreased ligand binding to serotonin 5-HT_{1A} receptors has been reported in both unmedicated acutely unwell MDD patients and recovered depressed patients (Bhagwagar *et al.*, 2004a, Drevets *et al.*, 2007, Sargent *et al.*, 2000). However, one group has consistently reported increased 5-HT_{1A} receptor binding in depression, and the reason for this discrepancy remains unresolved (Parsey *et al.*, 2006). A meta-analysis has shown that PET ligand binding

to the serotonin transporter is decreased in acute depression (Gryglewski *et al.*, 2014).

1.4.2 Evidence against the monoamine depletion hypothesis

The monoamine hypothesis however has some limitations. For example, the monoamine theory does not provide an explanation for the time lag between the onset of monoamine potentiation at synaptic level and the resolution of depressive symptoms. This means that while antidepressants may act by enhancing synaptic monoamines in the short term, their long-term effects are likely to be more complex (Racagni and Popoli, 2008), perhaps involving changes in synaptic plasticity and neurogenesis (Warner-Schmidt and Duman, 2007). Second, the results from biochemical studies of monoamine activity in depressed patients are inconsistent, perhaps because of clinical heterogeneity. However, it is clear that no particular monoamine change can be used as a diagnostic marker of depression. Indeed the widespread nature of the projections of serotonergic neurons from the raphe nuclei and noradrenergic neurons from the locus coeruleus suggests interactions with other neurotransmitter systems are likely to be of great importance in the pathogenesis of depression. Finally, neither tryptophan depletion nor α-methyl-p-tyrosine induce low mood in healthy subjects (Ruhe *et al.*, 2007) suggesting that monoamine depletion is not sufficient in itself to trigger depressive symptoms. These shortcomings support the need to investigate the role of additional systems that may be involved in the pathophysiology of depression, such as the glutamatergic system (a focus for this thesis).

1.5 Glutamate hypothesis of depression

There is emerging evidence linking dysfunction in the glutamatergic systems to MDD. This has led to the formulation of a glutamate hypothesis of depression (Sanacora *et al.*, 2012). Glutamate's neurotransmitter roles were discovered in the 1950s by Curtis and Watkins (Curtis and Watkins, 1960). Glutamate is the major excitatory neurotransmitter, being present in about 85% of cortical synapses (Douglas and Martin, 2007, Orrego and Villanueva, 1993). Glutamatergic synapses involve interactions between pre and post-synaptic neurons, as well as surrounding glia cells, in an arrangement referred to as the tripartite glutamate synapse (discussed in section 1.5.1 below). In addition to its role in excitatory neurotransmission, glutamate is a key component of the cellular protein synthesis and detoxification mechanisms. Hyperactivity of the glutamatergic system has been linked to neurotoxicity leading to neurodegeneration (Hardingham and Bading, 2010).

The possible mood elevating effects of the anti-infectious agents amantadine and d-cycloserine, which possess antagonistic activity to the glutamate N-methyl-D-aspartate (NMDA) receptor, were suggested in the 1950s (Trullas and Skolnick, 1990). In the 1980s, zinc-like NMDA receptor blocking properties of imipramine were reported, and suggested to be a possible mechanism for the antidepressant effects of the TCAs (Reynolds and Miller, 1988). The most compelling evidence for antidepressant effects of NMDA receptor blockage however has come from clinical studies demonstrating that the anesthetic agent ketamine produces rapid improvement in depressive symptoms in patients who have failed to respond to

conventional monoamine potentiating treatments (Berman *et al.*, 2000, Zarate *et al.*, 2013, Zarate *et al.*, 2006a).

Structural changes observed in MDD affect regions known to be under glutamatergic control (Campbell and MacQueen, 2006). The role of glutamate in the formation of memory through the processes of long-term potentiation and long-term depression has also been established (Citri and Malenka, 2008). Formation and reinforcement of negative emotional memories is thought to be involved in the altered cognitions of MDD. The established MDD treatments, including those targeting the monoaminergic system, are also known to interact with the glutamatergic system. In addition, pharmacological research on a variety of glutamate modulating agents beyond ketamine supports the role of the glutamatergic system in the pathophysiology and treatment of MDD. This section will review the evidence implicating the glutamatergic system to MDD.

1.5.1 The Glutamate Synapse

Glutamatergic neurons interact with post-synaptic neurons and surrounding glia, forming a structural arrangement referred to as the tripartite glutamate synapse (Figure 1.1). Glutamate, being unable to cross the blood brain barrier, must be synthesized within the brain (Hediger, 1999). This is achieved through two principal mechanisms: de novo synthesis from glucose and alpha-ketoglutyrate in Krebs cycle, and from glutamine in the glutamate-glutamine cycle (Erecinska and Silver, 1990). Within glutamatergic neurons, glutamate is packaged into secretory vesicles through uptake by the vesicular glutamate transporters (VGLUT). Following

depolarization, glutamate-laden vesicles are translocated to the presynaptic membrane resulting in synaptic secretion.

At the synaptic cleft, glutamate interacts with a variety of ionotropic and metabotropic receptors (Schoepfer *et al.*, 1994). Ionotropic glutamate receptors are classified based on ligand selectivity into the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), Kainate and N-methyl-D-aspartate (NMDA) subgroups. AMPA and NMDA receptors are present in about 70% of synapses, and are most abundant in the cerebral cortex, hippocampus, amygdala and striatum (Bekkers and Stevens, 1989). AMPA and NMDA receptors mediate fast-acting excitatory neurotransmission. NMDA receptors are tetrameric in structure, being made up of two NR_1 and two NR_2 subunits. Depolarizing currents through the AMPA receptors lead to extrusion of a blocking magnesium ion from the NMDA receptor allowing influx of calcium through the channel. The calcium influx through open NMDA channels activates intracellular pathways involving kinases and phosphatases. NMDA mediated neurotransmission is thought to underlie mechanisms of neuroplasticity, including long-term potentiation and memory formation. Four AMPA receptor subtypes ($GluA_{1-4}$), 5 Kainate ($GluK_{1-5}$) and 7 NMDA subtypes ($GluN_1$, $GluN_{2A-D}$, $GluN_{3A-B}$) have been characterized.

The metabotropic glutamate receptors (mGluR) belong to the seven-transmembrane domain family and exert their effects though intracellular G-proteins coupled systems. Eight sub-classes of the mGluR receptors ($mGluR_{1-8}$) have been described, based on on sequence homology and ligand selectivity. These are

further grouped into three based on the second messenger signaling cascades activated: Group 1 ($mGluR_1$and$_5$) act through the phospholipase C pathway while Group2 ($mGluR_2$ and $_3$) and Group 3 ($mGluR_{4,6,7,8}$) act through adenylate cyclase (Palmada and Centelles, 1998).

Following release into the synapse, glutamate is taken up by the excitatory amino acid transporters (EAATs) located principally within astrocytes ($EAAT_1$ and $EAAT_2$) and to a minor extent within the pre-synaptic neuron (EAAT $_{3-5}$) (Belanger and Magistretti, 2009, O'Shea, 2002). Since there are no glutamate degrading enzymes within the synapse, uptake through EAATs represents the main mechanism for synaptic clearance of glutamate. Within astrocytes, glutamate is combined with ammonia to form glutamine, a reaction carried out by the astrocyte specific enzyme glutamine synthetase.

Glutamine is transported out of the astrocytes and into glutamatergic neurons through the coordinated activity of two transport systems belonging to the sodium-dependent neutral amino acid (SNAT) family of transporters (Mackenzie and Erickson, 2004). Within neurons, glutamine is converted to glutamate through the action of the enzyme glutaminase. This completes the glutamate-glutamine cycle. Astrocyte-derived glutamine is also the precursor for the synthesis of the inhibitory amino-acid neurotransmitter γ- amino butyric acid (GABA) and the antioxidant glutathione. Glutamate release, uptake by EAATs, receptor stability and efficiency are all under tight regulatory control. Alterations in these are thought to underlie mechanisms of disease in some neuropsychiatric disorders including MDD.

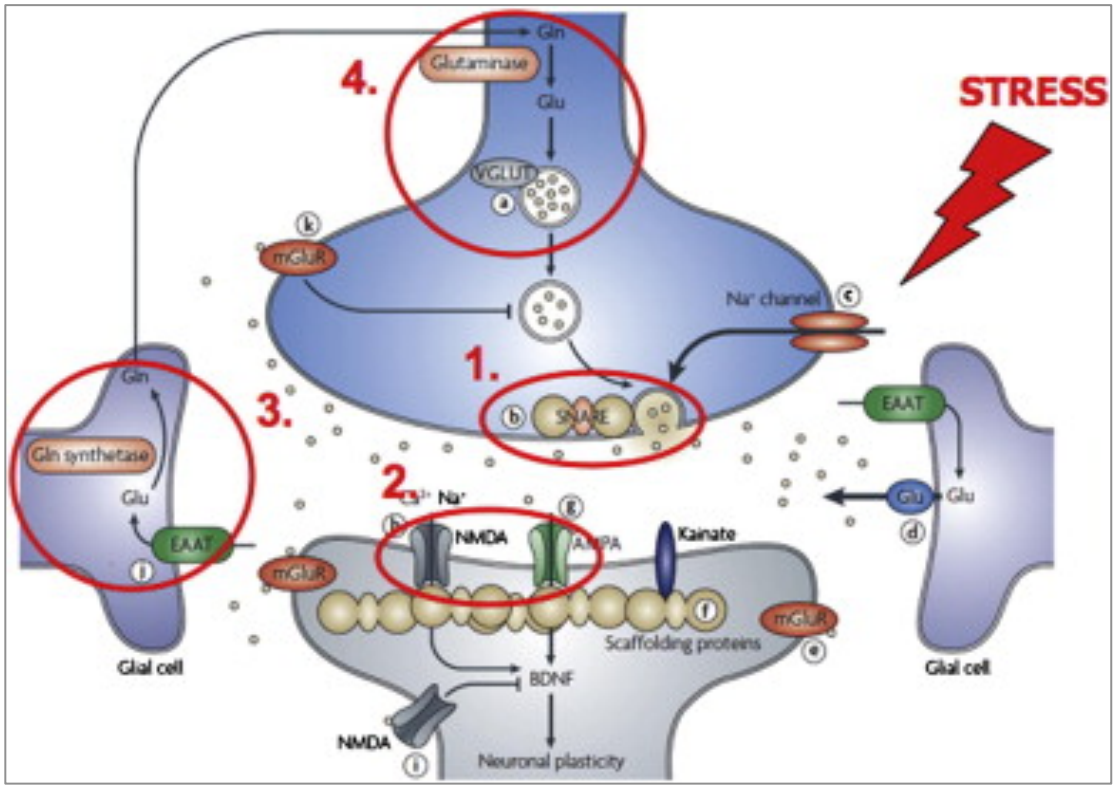

Figure 1.1: The tripartite glutamate synaptic arrangement involving the presynaptic glutamate secreting neuron, the post-synaptic cell and surrounding neuroglia. Synaptic dysfunction can result from impairment in the release of glutamate (1), stimulation of post-synaptic receptors (2), reuptake by glia cells and synthesis of glutamine (3) or synthesis of glutamate (4). Adapted from Sanacora *et al.*, 2012.

1.5.2 Evidence from animal models of depression

Animal models have been used to study structural and functional changes in depression as well as for screening of potential antidepressant drugs. Investigations on a learned helplessness rat model demonstrated reductions in the expression of group 2 and 3 metabotropic glutamate receptors in the hippocampus (Matrisciano *et al.*, 2008). In vivo measurements of glutamate and GABA in a similar rat model demonstrated increased ratio of glutamate to GABA in the hippocampus and prefrontal cortex. These ratios were normalized following treatment with desipramine, as well as following electroconvulsive therapy (Sartorius *et al.*, 2007).

In a study looking at the effects of corticosterone on glutamate receptors, Hunter and colleagues (2009) demonstrated that both chronic unconditioned stress and administration of the rodent stress hormone corticosterone increased the expression of the Kainate KA_1 subunit in the dentate gyrus.

Genetic modifications to the glutamatergic system have also helped to elucidate possible glutamatergic system involvement in depression. Animals with reduced levels of the presynaptic vesicular glutamate transporter VGluT1 demonstrate a depressive phenotype (Tordera *et al.*, 2007). Animals in which the gene for the NR_{2A} subunit of the NMDA receptor has been knocked out demonstrate antidepressant-like profiles when compared to wild type controls (Boyce-Rustay and Holmes, 2006). Exposure of rodents to stress results in a decrease in the levels of the neurotrophic hormone BDNF, a mechanism that is reversed by activation of AMPA receptors (Duman, 2004). These studies provide further evidence linking the glutamatergic system to depression and to the mechanism of action of antidepressants.

1.5.3 Evidence from post-mortem studies

Post-mortem brains have been useful to study neuropathological changes associated with MDD. Use of these samples is however limited by confounds of post-mortem change. Francis and colleagues (1989) provided one of the earliest attempts to use post-mortem samples to investigate possible changes to the glutamatergic system in MDD. In their study looking at the concentrations of glutamate, aspartate and GABA in post-mortem samples of MDD patients, they did not find any evidence for amino-acid alterations. A subsequent study by Hashimoto and colleagues (2007) however

found increased levels of glutamate in the frontal cortex after correcting for post-mortem changes.

Glia play a critical role in regulating synaptic glutamate concentrations. Pathological involvement of glia cells has been reported from post-mortem samples of depressed patients. Loss of glia cells in a wide array of brain regions including ACC and PFC has been reported from post-mortem samples (Rajkowska and Miguel-Hidalgo, 2007). Decreased immunostaining for the glutamate uptake transporters, the EAATs, has also been reported (Miguel-Hidalgo *et al.*, 2010).

1.5.4 Evidence from peripheral measures of glutamate

Elevated levels of plasma glutamate have been reported in depressed patients (Kim *et al.*, 1982, Mitani *et al.*, 2006). These elevations have been correlated to illness severity (Mitani *et al.*, 2006). Although Maes and colleagues (1998) found no differences in baseline plasma glutamate levels between patients with treatment resistant depression and controls, they demonstrated significant decreases among depressed patients following 5-weeks of treatment with antidepressants.

Interpretation of plasma glutamate levels is limited by the fact that glutamate does not cross the blood brain barrier. As such, peripheral measures may not accurately represent changes occurring within the brain. Platelets express glutamate uptake transporters similar to those found in astrocytes (Zoia *et al.*, 2004). It is therefore possible that changes in glutamate uptake occurring within the brain could be mirrored by changes occurring peripherally within platelets. In a study measuring glutamate levels in platelets, numerically but not statistically significant increases

were found in patients with MDD (Mauri *et al.*, 1998). The clinical and pathophysiological significance of the changes to peripheral glutamate levels remain to be elucidated.

1.5.5 Evidence from measurement of CSF glutamate

Measurements of CSF glutamate and glutamine could provide more accurate assessment of changes occurring within the brain. Levine and colleagues (2000) reported increased levels of CSF glutamine in MDD patients. In a study measuring levels of CSF glutamate in a mixed group of unipolar depressed and bipolar subjects, Frye and colleagues (2007) reported reductions in glutamate levels. These studies suggest possible reductions in glutamate and increases in glutamine in MDD. CSF glutamate measures are however a broad representation of overall glutamate metabolism and may not reflect localized changes occurring at synaptic level.

1.5.6 Evidence from magnetic resonance spectroscopy studies in human

Converging evidence from studies measuring in-vivo concentrations of glutamate and glutamine using MRS suggest that changes in the concentrations of these metabolites may be linked to the pathophysiology of MDD (reviewed in Arnone et al., 2015, Luykx et al., 2012, Yildiz-Yesiloglu and Ankerst, 2006, Yuksel and Ongur, 2010). These studies suggest that levels of glutamate and the combined signal from glutamate and glutamine, Glx, are reduced in prefrontal cortical areas of patients with MDD. In the occipital cortex however, glutamate and Glx levels have been reported to be elevated in depressed patients (Sanacora *et al.*, 2004), recovered depressed patients (Palmada and Centelles, 1998) and in healthy subjects with familial risk of depression (Taylor *et al.*, 2011). Although subcortical areas such as the striatum have been implicated in MDD and particularly in explaining anhedonia

symptoms, no consistent neurochemical patterns involving glutamatergic metabolites have been established in these regions (Luykx *et al.*, 2012).

Proton-MRS provides information on overall metabolite pool and thus cannot determine more precise changes occurring at synaptic or neuronal level. In attempt to investigate whether changes in the energy coupled mechanisms involved in the neurotransmission of glutamate are altered in MDD, Abdallah and colleagues (2014) used a combination of ^{1}H-MRS and ^{13}C-MRS to investigate glutamate-glutamine cycling in medication free subjects with MDD. In this study, they found evidence of decreased mitochondrial energy production in glutamate neurons in the occipital cortex of patients with MDD, although rather surprisingly, no differences in the rate of glutamate/glutamine cycling were present. In addition, there were no differences in basal levels of glutamate between depressed patients and controls.

Overall, the data suggest that MRS glutamate and Glx levels are lower in anterior cortical regions in depressed patients, though the data are inconsistent. This might be due to a number of factors including effects of concomitant antidepressant drug treatment, patient heterogeneity, in conjunction with small sample sizes, and the technical difficulty of resolving MRS glutamate accurately at magnetic field strengths of 3-tesla or lower. Resolving these confounds, as well as further discussion on MRS findings in depression, is the basis of chapter 3 in this thesis.

1.5.7 Evidence from pharmacotherapy with glutamate modulators

The most compelling evidence implicating the glutamatergic system in MDD has come from observations that glutamate receptor modulators have antidepressant

effects. Evidence for this has been obtained from research with a number of glutamate modulating agents including ketamine, lamotrigine, amantadine, riluzole, memantine, traxoprodil, d-cycloserine, as well as with other unlicensed investigational compounds (Caddy *et al.*, 2015, McCloud *et al.*, 2015, Sanacora *et al.*, 2012).

The best evidence has been obtained from studies on ketamine (reviewed in Caddy et al., 2015). Ketamine is a non-competitive NMDA receptor antagonists primarily used as a dissociative general anaesthetic (Mealing *et al.*, 1999). Sub-anesthetic doses produce rapid antidepressant effects, including in patients with treatment resistant depression (Berman *et al.*, 2000, Zarate *et al.*, 2006a). Ketamine's antidepressant effects are evident within 24 hours of a single intravenous infusion and can last up to 14 days. Repeated infusions can delay relapse for up to 19 days (aan het Rot *et al.*, 2010, Murrough *et al.*, 2013). Case studies suggest that prolonged IV infusion of ketamine can prolong the duration of remission for a period of up to 12 months (Correll and Futter, 2006). Active metabolites of ketamine, such as dehydronorketamine and hydroxynorketamine, are thought to be responsible for its prolonged antidepressant effects (Zhao *et al.*, 2012). Indeed a recent study in animals suggested that the antidepressant effect of ketamine might be due solely to the action of hydroxynorketamine and its ability to activate AMPA receptors (Zanos *et al.*, 2016).

There have however been some limitations in the clinical studies looking at the antidepressant effects of ketamine. Most have used small sample sizes, or included

patients with severe or treatment resistant depression. These samples are not representative of community psychiatric patients. It is also difficult to carry out blinded studies on ketamine due to its psychotomimetic side effects. In-vivo measurements of ketamine's effect on glutamate release using ^{1}H-MRS have been equivocal. While ketamine administration has been reported to increase glutamate release in both healthy subjects (Rowland *et al.,* 2005, Stone *et al.,* 2012) and in depressed patients (Milak *et al.,* 2016), this effect has not been replicated in other studies (Taylor *et al.,* 2012, Valentine *et al.,* 2011).

Whether ketamine's antidepressant effects are due to blockade of NMDA receptors or to mechanism involving non-NMDA glutamate receptors such as AMPA receptors remains to be determined. Quite apart from the possible direct action of norketamine on AMPA receptors, it has been previously hypothesized that NMDA receptor blockade might result in a 'feedback' increase in glutamate release, which then acts via AMPA and metabotropic glutamate receptors to produce ketamine's antidepressant effects. The excess glutamate released following blockade of NMDA receptors may also act indirectly through non-glutamatergic pathways, such as through serotonin and dopamine receptors, or through activation of the mammalian target of rapamycin (mTOR) pathway (Dutta *et al.*, 2015, Moghaddam *et al.*, 1997).

Antidepressant effects have also been observed with other glutamate modulating agents. Memantine is an NMDA antagonist approved for clinical use in Alzheimer's disease. High doses have been shown to result in reductions in depressive symptoms in MDD (Ferguson and Shingleton, 2007); however, more standard doses

have shown no antidepressant effect (Zarate *et al.*, 2006b). Riluzole, a drug used in the treatment of amyotrophic lateral sclerosis, has also been shown to be effective in preliminary studies of the treatment of MDD (Zarate *et al.*, 2004). Further evidence has come from the anti-tuberculosis drug d-cycloserine, a partial agonist at the glycine-B site of the NMDA receptor (Heresco-Levy *et al.*, 2013, Heresco-Levy *et al.*, 2006). Similarly, antidepressant effects have been reported with the antiviral drug amantadine that is used as a neuroprotective agent in the treatment of Parkinson's disease (Huber *et al.*, 1999). Traxoprodil, an antagonist at $GluN_{2B}$ subunit of the NMDA receptor, also demonstrated efficacy in treatment resistant depression in early trials (Preskorn *et al.*, 2008) but disappointing results in phase 2 trials led to its discontinuation (Newport *et al.*, 2015).

1.5.8 Evidence from non-pharmacologic treatments of depression

Changes to the glutamatergic system have been reported following electroconvulsive therapy (Pfleiderer *et al.*, 2003) and repetitive-transcranial magnetic stimulation (Zangen and Hyodo, 2002). Electroconvulsive therapy has been shown to increase Glx concentration within the anterior cingulate cortex (Pfleiderer et al., 2003), reversing decreases present in MDD patients. In a study looking at the effects of repetitive-transcranial magnetic stimulation on the neurochemistry of the nucleus accumbens in the rat, glutamate levels were increased following stimulation (Zangen & Hyodo, 2002). These studies provide further evidence linking the glutamatergic system to depression and its treatment.

1.5.9 GABA-Glutamate imbalance

GABA and glutamate are intricately linked through the glutamate-glutamine cycle. Astrocyte derived glutamine is the source for the synthesis of both glutamate and

GABA. GABA is the predominant inhibitory neurotransmitter and counterbalances the excitatory effects of glutamate. One possible mechanism through which dysfunction within the glutamatergic system may result in depressive phenotype is through facilitation of unopposed inhibitory GABAergic signaling. However, proton-MRS studies have reported reductions in GABA concentrations within the ACC that normalize following treatment with antidepressants (Hasler *et al.*, 2007, Price *et al.*, 2009, Sanacora *et al.*, 1999, Sanacora and Saricicek, 2007).

1.5.10 Interaction between glutamatergic and monoaminergic systems

Several lines of evidence suggest that there are interactions between glutamatergic and monoaminegic systems that may be of relevance to mood regulation. SSRI treatment has effects on the expression and function of glutamate receptors, which may contribute towards their antidepressant effects. Administration of the SSRI paroxetine results in an increase in AMPA receptor subunit trafficking to the synaptic membranes (Martinez-Turrillas *et al.*, 2005). Modulatory effects on glutamate receptors have also been demonstrated with citalopram, fluoxetine and desipramine (Nowak *et al.*, 1996, Szasz *et al.*, 2007). Genetic variation in the GRIK4 gene, which codes for the Kainate receptor KA_1, has been linked to response to citalopram treatment (Paddock *et al.*, 2007). The effects of SSRI treatment on MRS glutamate levels in depressed patients have not been much studied and thus far there is no evidence of a change (Godlewska *et al.*, 2015, Taylor *et al.*, 2012a). In healthy participants the effects of short-term SSRI treatment shows regional variation with an increase in occipital cortex but no change in ACC (Taylor *et al.*, 2008, Taylor *et al.*, 2010).

1.5.11 The Stress-Glutamate Model

Mechanisms linking stress, depression and glutamatergic signaling provide further evidence for a glutamatergic hypothesis of depression (Musazzi *et al.*, 2011, Popoli *et al.*, 2012). At the center of this axis is the hypothalamus-pituitary-adrenal (HPA) axis. Many aspects of stress, including enhancement of HPA axis activity, are thought to be mediated by glutamatergic neurotransmission in the prefrontal cortex (Moghaddam, 2002). Excessive secretion of glucocorticoids has been linked to mechanisms of neurotoxicity and is thought to be the basis for the atrophic brain changes in MDD (Hardingham and Bading, 2010). Acute stress has been shown to enhance frontal cortical glutamatergic neurotransmission in rats through mechanisms involving glucocorticoid receptors (Yuen *et al.*, 2009). The metabotropic glutamate receptor mGluR5 in the nucleus accumbens has been shown to be critical for promoting resilience to chronic stress (Shin *et al.*, 2015). The detrimental effects of stress on memory are thought to be mediated by AMPA and NMDA receptors (Lisman *et al.*, 1998). These studies provide further evidence for the role of the glutamatergic system in MDD pathophysiology.

1.6 Lithium in the treatment of mood disorders

Lithium is a naturally occurring element used extensively for the treatment of mood disorders. Its principal use is in the maintenance treatment of bipolar disorder, where it has the ability to decrease the risk for both manic and depressive episodes (Geddes *et al.*, 2010). Lithium is also effective in the treatment of acute manic episodes (Cipriani *et al.*, 2011). It has some possible efficacy as monotherapy in the treatment of MDD (Guzzetta *et al.*, 2007, Lewitzka *et al.*, 2015). There is also some

evidence for lithium's role in relapse prevention following recovery from a depressive episode in MDD (Cipriani *et al.*, 2006). Its main use in major depression is to 'augment' the therapeutic effects of antidepressant treatment in non-responsive patients (Nelson *et al.,* 2014). Lithium use is however associated with many limiting side effects and the need for frequent monitoring of drug levels. In addition, it has a low therapeutic to toxicity ratio and interacts with multiple drugs, with potential for renal and neurological side effects (Price and Heninger, 1994). This section will discuss lithium's role in the treatment of MDD.

Some of the earliest evidence for lithium's role in the treatment of mood disorders came from reports of the Danish psychiatrist Carl Lange in 1886 (Shorter, 2009). It was however not until the re-discovery of its therapeutic effects in 1949 by the Australian psychiatrist John Cade that lithium gained widespread use for the treatment of mood disorders (Cade, 1949). Cade's experiments focused on investigating the role of uric acid in neuropsychiatric disorders. Serendipitous observation that lithium urate salts had a calming effect in patients with varied psychiatric illnesses led to the discovery of lithium's role in the treatment of manic episodes. Subsequent methodical experiments carried out by two Danish psychiatrists, Mogens Schou and Poul Baastrup, confirmed lithium to be a mood stabilizer (Baastrup and Schou, 1967) as well as being effective in the prophylaxis of mood episodes during the inter-episode (euthymic) period (Baastrup *et al.*, 1970).

The molecular and cellular mechanisms through which lithium acts remain debatable. At least 10 different biological targets have been identified (Lenox and

Wang, 2003, Quiroz *et al.*, 2004). At therapeutic concentrations, the most likely targets are the inositol monophosphatase (IMPase) and the glycogen synthase kinase-3B pathways (Berridge *et al.*, 1982, Lenox and Wang, 2003, Williams *et al.*, 2002). Both of these are involved in second-messenger intracellular signaling cascades.

Inhibition of cellular IMPases has been of particular interest, especially from observations that it may be a shared mechanism of action for the commonly prescribed mood stabilizing agents lithium, valproate acid and carbamazepine (Harwood and Agam, 2003, Lee *et al.*, 2015, Williams *et al.*, 2002). Some researchers have however disputed the importance of the inositol-signaling pathway to lithium's cellular actions (Berry *et al.*, 2004). The observed lag in onset of lithium's beneficial effects suggest that its mechanisms of action are most likely mediated though modification of gene expression (Lenox and Wang, 2003). One of the proposed downstream genetic actions of lithium is enhancement of BDNF synthesis (Hashimoto *et al.*, 2002).

As noted above, lithium has two broad applications in the treatment of MDD. It is used as an add on treatment for the augmentation antidepressants in patients who fail to respond to initial treatment. In addition, it has a role in the long-term prophylaxis of mood episodes.

1.6.1 Lithium use in the augmentation of antidepressant actions

The role of lithium in the augmentation of antidepressant action has long since been known, since De Montigny and colleagues (1981) reported on its beneficial effects

as an add on treatment to tricyclic antidepressants for treatment resistant depression. A recent meta-analysis of randomised studies confirmed the efficacy of lithium as an augmentation treatment but overall the total number of patients studied is relatively small (Nelson *et al.,* 2014). In addition many lithium augmentation studies involved a primary TCA treatment and the efficacy of lithium in augmentation of newer antidepressants is less firmly established. Particular patient characteristics have been suggested as being predictive of likelihood of response to lithium augmentation. Patients with a positive family history of depression, significant weight loss and psychomotor retardation may be more likely to benefit from lithium augmentation therapy (Alvarez *et al.*, 1997, Bschor and Bauer, 2006, Sugawara *et al.*, 2010).

1.6.2 Lithium use in the prophylaxis of mood episodes in MDD

Lithium's role in the prophylaxis of mood episodes in bipolar disorder has been well established (Baastrup *et al.*, 1970, Baastrup and Schou, 1967, Geddes *et al.*, 2010). There is also some evidence that lithium prophylaxis is helpful in averting depressive relapses in MDD. Burgess and colleagues (2001) determined that lithium was significantly more effective than placebo in preventing new depressive episodes, with odds ratio of 0.46. Subsequently, Cipriani and colleagues (2006) determined that both lithium and antidepressants were efficacious for prophylaxis in unipolar affective disorder.

A limitation to the use of lithium as a prophylactic agent is its long-term toxicity (McKnight *et al.*, 2012, Shine *et al.*, 2015) and the need for regular blood monitoring of drug levels. However, in a naturalistic study with average follow-up periods of 7

years, Baethge and colleagues (2003) determined that lithium was efficacious in the long-term prophylaxis of mood episodes in MDD, with minimal need for treatment change due to its side effects.

Death due to suicide is common in MDD, with depression being the most common psychiatric disorder among people who die by suicide (Harris and Barraclough, 1997, Hawton *et al.*, 2013). Lithium has been shown to be useful in the prevention of suicide among patients with mood disorders including those with MDD (Cipriani *et al.*, 2013, Lewitzka *et al.*, 2015). Augmentation of antidepressant treatment with lithium has been shown to have the added benefit of reducing suicide risk (Cipriani *et al.*, 2006).

An intriguing observation is that lithium's anti-suicide properties are not solely accounted for by its mood-stabilising effects. Other effective treatments for mood symptoms have not demonstrated similar efficacy in lowering suicide risk (Cipriani *et al.*, 2013). In addition, lithium lowers suicidal behaviour even in patients who fail to respond to its mood stabilising effects (Ahrens and Muller-Oerlinghausen, 2001). Lithium's ability to decrease impulsive aggression has been demonstrated in both animal work (Ohmura *et al.*, 2012) and human studies (Jones *et al.,* 2011) and has been suggested to contribute to its anti-suicide effects.

1.7 Cognitive Neuropsychological Model of Depression

One of the proposed models for the neurobiology of depression, that offers a compelling explanation for the lag in time between the neurochemical effects of antidepressants and their clinical benefits, is the cognitive neuropsychological

model (Harmer *et al.*, 2009a). This model also offers a plausible explanation for the mechanisms through which antidepressants act at the molecular level to alter cognitive processing perceived at the behavioural level.

The importance of pathological cognitions in MDD has long been established. In the 1960s, the psychologist Aaron Beck proposed the cognitive model of depression that postulates that depressive disorders result from three negative affective schema: negative thoughts about one's self, the world and one's future (Beck, 1967). In depression, these negative cognitions render more salience to negative emotional information.

The way in which cognitive processing interacts with biological theories has been the subject of intense research. Earlier dualistic thinking suggested that biological and psychological mechanisms acted in separate and independent ways. In the cognitive neuropsychological model, interaction between a receptive biological substrate and positive cues in the environment are needed to alter the pathological cognitions present in depression. Antidepressants act almost immediately at the neural level to implicitly alter the processing of emotional information. These subtle effects have been demonstrated using experimental medicine models that assess biases in the processing of emotional information (Harmer *et al.*, 2009a, Pringle *et al.*, 2011a, Warren *et al.*, 2015). These effects of antidepressants are however not sufficient in themselves to fully alter the pathological cognitions of depression. To achieve this, an enabling environment must be present to facilitate the re-learning of positive emotional associations. The requirement for re-learning as a substrate for

developing positive emotions is supported by neuroanatomical studies reporting neurotrophic changes during recovery from depressive episodes (Duman and Monteggia, 2006). The cognitive neuropsychological model, and its possible interrogation using experimental medicine models, will be tested in this thesis to investigate a potential mood stabilising and antidepressant agent called ebselen (chapter 6).

1.8: Conclusion

Major depressive disorder is a complex neuropsychiatric illness with pathophysiological involvement of biological, psychological and social factors. For an illness with such a great social-economic burden, understanding its biological mechanisms holds key to the development of effective therapies. Multiple lines of evidence from both clinical and pre-clinical studies support a glutamate- based hypothesis of depression. Understanding the glutamatergic system in depression therefore offers an avenue for targeted pharmacology of MDD. There is some evidence from clinical trials that drugs modulating the glutamatergic system could have clinically useful antidepressant properties. These effects need to be explored further, alongside the development of newer agents with better safety and efficacy profiles.

In this thesis, the technique of ^{1}H-MRS (discussed in chapter 2) has been used to investigate changes to the glutamatergic system in MDD (chapter 3). As noted above, MRS is a feasible, non-invasive means of detecting levels of glutamate in the living human brain. However results to date in depressed patients have been somewhat inconsistent. The development of ultra-high field imaging allows a clearer

resolution of the glutamatergic metabolites glutamate and glutamine in the brain, and a main aim of this thesis was to take advantage of the availability of a 7-tesla MRI to study levels of these metabolites in depressed patients compared to healthy controls (Chapter 3). Recruited patients were free of current psychotropic medication and clinically characterized to allow correlation between glutamate levels and clinical features (Chapter 4).

The Introduction has also described the important therapeutic effects and drawbacks of lithium treatment. A second aim of this thesis was to use both MRS and experimental medicine models to characterize a potential lithium-mimetic agent called ebselen. One of the important therapeutic targets of lithium is thought to be an inhibitory effect on the enzyme IMPase. Ebselen was identified as an IMPase inhibitor by Churchill and colleagues in the University Department of Pharmacology in Oxford (Singh *et al.*, 2013). As well as inhibiting IMPase, ebselen is also an inhibitor of glutaminase (Thomas *et al.*, 2013), which, as noted above, converts glutamine to glutamate in glutamatergic neurons. Inhibition of IMPase should lower brain levels of inositol which can also be detected by proton MRS, and inhibition of glutaminase could modify brain glutamate and glutamine which makes MRS potentially useful in confirming target engagement of ebselen in the human brain (Chapter 5). At the same time it is possible to test ebselen in models of emotional processing and decision making, to see if it produces evidence of potentially beneficial effects on mood and impulsive responding (Chapter 6).

Chapter 2: General Methods

2.1 Study Approvals

The studies presented in this thesis received ethical approval from the National Research Ethics Service Committee (NRES), South-Central Oxford. Each participant gave full-informed written consent after presentation of a Patient Information Leaflet describing the study.

2.2 Magnetic Resonance Spectroscopy

The technique of Magnetic Resonance Spectroscopy (MRS) offers a non-invasive way to assess neurochemical profiles within defined areas. In neuroscience, MRS has allowed us to study aspects of brain neurochemistry in both physiological and pathological conditions. MRS is particularly useful because it allows such changes to be assessed in-vivo, and by so doing, assist in narrowing the knowledge gap between anatomical, physiological and pathological processes. The quantitative nature of the information derived from MRS enables comparisons to be made between different brain regions, as well as allowing for changes to be monitored over time. MRS however has some limitations that have hindered its broad application, particularly in clinical psychiatry. This chapter will give an overview of the technique of MRS with a focus on proton-MRS (^{1}H-MRS) which is the methodology used in this thesis. A brief historical background will be given, followed by discussion on the physical properties of the MRS signal and techniques for signal acquisition and interpretation. Finally, challenges specific to MRS imaging at ultra-high field, that are relevant to the work carried out in this thesis (7-tesla MRS) will be discussed.

2.2.1 Historical overview of MRS

Identification of the phenomenon of nuclear magnetic resonance (NMR) led to the development of magnetic resonance imaging and subsequently MRS. NMR refers to the property that describes the angular momentum and associated magnetic moment of atomic nuclei within a magnetic field. NMR was established in 1946 by the independent but simultaneous work of two groups: Felix Bloch and colleagues (1946) at Harvard University and Edward Purcell and colleagues (1946) at Stanford University.

Subsequently, NMR was widely applied in analytical chemistry to investigate molecular structure and kinematics. Spatial localization using NMR became possible after the seminal work carried out in by Lauterbur and colleagues (1973) and Mansfield and Grannell (1977). The decades that followed saw the application of NMR in medical imaging in the form of magnetic resonance imaging (MRI). MRI proved particularly useful in providing better soft tissue differentiation and avoiding radiation safety concerns that characterised the more widely available medical imaging techniques: CT-scan and X-ray.

Further to the characterisation of spatial encoding of the NMR signal, Proctor and Yu (1950) and Dickinson (1950) observed that even within the same molecule, identical nuclei may emit electromagnetic radiation at different resonant frequencies. This observation was subsequently attributed to the variations in the local magnetic environment acting on the individual nuclei, forming the basis for MRS.

Earlier studies utilizing MRS were carried out on cell suspensions and isolated perfused organ systems (Damadian 1971; Shulman *et al.,* 1979). These studies demonstrated the feasibility of carrying out non-invasive biochemical investigations on biological systems. The study by Damadian and colleagues (1971), who used MRS to differentiate between malignant and healthy tissue, also demonstrated the feasibility of using MRS in clinical pathological states.

The first in-vivo MRS study of the brain was carried out by Ackerman and colleagues (1980). In this study, ^{31}P-MRS was used to monitor metabolism in rats, with the MRS signal being collected using surface radiofrequency coils. By being placed right next to the anatomical area within which the signal of interest is localised, surface coils improved signal acquisition and marked an important technical advance. In addition to ^{31}P-based MRS, ^{13}C-MRS was also developed and used by Alger and colleagues (1981) to investigate in-vivo metabolic changes in a rat liver.

Human in-vivo MRS was first carried out using ^{31}P spectroscopy to investigate biochemical changes in the forearm muscles of a patient with McArdle's syndrome (Ross *et al.*, 1981). The first application of MRS of the brain for human in-vivo studies was carried out by Cady and colleagues (1983) who used ^{31}P spectroscopy to investigate biochemical changes among neonates with birth asphyxia.

Investigations using ^{31}P were favoured in the initial MRS investigations because they offered the ability to study the core products of cellular bioenergetics (ATP, ADP, phosphocreatine and inorganic pyrophosphate) at a large chemical shift range

and without the need for water suppression (a requirement for ^{1}H-MRS). Subsequently however, ^{1}H-MRS superseded ^{31}P-MRS, as it offered the ability to carry out spectroscopy using clinical scanners without the need for additional hardware. The relatively high magnetic sensitivity (gyromagnetic ratio) of the ^{1}H nucleus coupled with its high natural abundance in biological tissues also favoured development of ^{1}H-MRS over other MRS modalities. In Psychiatry, ^{1}H-MRS offers the additional advantage of measuring neurometabolites of great significance to psychiatry disease and treatment, for instance N-acetyl- aspartate (NAA), inositol, glutamate, glutamine and GABA.

The first in-vivo ^{1}H-MRS imaging of the brain was carried out in rats by Behar and colleagues (1983). In this, the researchers demonstrated feasibility of measuring key MRS metabolites such as NAA, glutamate, glutamine, choline, creatine and lactate in anesthetized rats (at 360.13 MHz). They also demonstrated MRS-detectable variations in lactate concentrations with changing oxygen content in administered gas mixtures. Further to this, the same researchers successfully carried out MRS of the rat brain at scanner field strength of 1.9-tesla (Damadian, 1971) thus demonstrating the feasibility of employing ^{1}H-MRS of the brain at more clinically relevant scanner field strengths.

The first application of localized ^{1}H-MRS in human brain scanning was carried out by Bottomley and colleagues (1985) using a 1.5-tesla scanner. Since then, MRS has found broad applications in the study of neurological and psychiatric illnesses, including investigating neurochemistry of mood disorders. Despite a substantial

research effort, thus far no clinically useful psychiatric application has been identified for MRS. However, the increasing availability of higher field scanners coupled with improved techniques for generating homogenous field gradients and quantification of MRS spectra will hopefully lead to a better understanding of the neurochemical basis of psychiatric illness and thereby perhaps to practical applications.

2.2.2 Basic principles of magnetic resonance spectroscopy

The principles that govern the formation of the signal in MRS are similar to those for conventional MRI imaging of protons in water. The main magnetic field (abbreviated as B_0) is generated by superconducting electromagnets. To achieve spatial selection, which is important for localization of the generated MRI signal, additional magnets are used to generate gradients in three orthogonal directions (x, y and z). These introduce spatial variations in the magnetization that forms the basis for frequency selective excitation. In addition to the superconducting magnets and gradient magnets, radiofrequency coils are used to generate the excitation energy (the radiofrequency pulse, RF) that tips the nuclei of interest off the longitudinal alignment.

When atomic nuclei with a magnetic moment are placed in a magnetic field, they will exhibit directional orientation. The resultant orientation of the nuclei within a tissue will be such that alignment will be either parallel or antiparallel to the magnetic field. In additional to directional orientation, nuclei within a magnetic field will demonstrate characteristic-spinning behaviour referred to as magnetic resonance. Mathematically, this can be represented by the Larmor equation as

follows:

$$\omega_o = \Upsilon B_0$$

where ω_o is the angular frequency of precession of protons in an external magnetic field, Υ the scaling constant (gyromagnetic ratio) that is specific to the nucleus of interest and B_0 the main magnetic field.

Precession in the longitudinal direction does not generate detectable magnetic resonance signal. As such, it is necessary to tip the spins off along the transverse direction, where signal can be detected and quantified. This is achieved by application of a second external magnetic field, the RF pulse, which is applied perpendicular to the B_0. Only the nuclei precessing at a similar frequency to the RF pulse experience its excitation. Nuclei tipped by the RF pulse undergo relaxation along the longitudinal and transverse planes, and in so doing emit electromagnetic radiation. The resultant energy emitted along the transverse plane can be detected with a receiver coil, constituting the MRS signal.

2.2.2.1 Chemical Shift

The most important determinant of the precession frequency is the main magnetic main genetic field, B_0. However, within molecular configuration, precessing nuclei experience magnetic effects from shielding electrons, which modulate their precession frequencies. In particular, electrons surrounding a nucleus create a shielding effect that diminishes the effective magnetic field perceived by the nucleus. This effect, referred to as chemical shielding, forms the basis for discriminating nuclear spins based on molecular configuration in MRS. The effective

magnetic field (Beff) can be mathematically represented as

$$Beff = (1- \sigma) B_0$$

where σ is the chemical shielding constant, which is unique to each nucleus. Accounting for the chemical shielding effects, the Larmor equation can be represented as follows

$$\omega o = \Upsilon (1- \sigma) B_0$$

2.2.2.2 J-coupling

An additional effect influencing the appearance of spectra in MRS results from interactions between adjacent nuclei that are electrochemically coupled. This is referred to as J-coupling or scalar coupling. This effect may result in splitting or dispersal of peaks, even from otherwise identical nuclei within the same molecule. J-coupling is field independent but varies with echo time (a phenomenon referred to as J-evolution). The characteristic multiplets seen in an MRS spectrum are as a result of these J-coupling effects. While J-coupling may prove to be a challenge during quantification of metabolites, it can be useful in identification of molecules based of their unique J-coupling forces. This forms the basis of editing techniques such as the MEshcher-GArwood Point RESolved Spectroscopy (MEGA-PRESS) used for GABA quantification (Mescher, 1998).

2.2.3 Choice of MRS Isotope

Theoretically, MRS can be acquired from any nuclei that posses a magnetic moment. Additional factors however favour particular nuclei as sources of MRS signal. Among these is the gyromagnetic ratio of the nuclei that determines isotope sensitivity to excitation. Others are the natural abundance of the isotope, and the chemical shift

range. Proton (^{1}H) has emerged as a popular target for MRS because of its relative high gyromagnetic ratio and high natural abundance in biological tissues. In addition, proton-MRS can be carried out with clinical scanners without the need for additional hardware, making the technique more accessible.

2.2.4 The MRS Spectrum

When the signal from all the nuclei of interest within a tissue is collected and Fourier transformed, the resultant plot comprises peaks distributed according to chemical shift effects. Mathematically, the dispersal of the metabolite peaks along the x-axis (the chemical shift axis) is represented with reference to a chemical that experiences very minimal chemical shift effects, as follows

$$\delta = \frac{\omega - \omega\, ref}{\omega\, ref}\ 10^{6}$$

The chemical shift is thus quantified in parts per million (ppm). The most commonly used reference chemicals are tetramethylsilane (TMS) and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). In proton MRS, metabolites of interest typical lie between 0 and 5ppm. Metabolites at larger chemical shift ranges have been characterised with non-proton MRS methods (such as ^{13}C and ^{31}P).

2.2.5 Metabolites Quantified with MRS

Signal detection in MRS is limited to mobile low molecular weight metabolites. Molecules within structural macromolecular complexes are generally considered to be outside the MRS visible range, although they may contribute to aspects of the spectrum, such as the baseline. Since the concentrations of most low molecular weight metabolites within the brain are thought to be under tight control, MRS has been used to detect subtle changes that may reflect underlying physiological or

pathological processes. Due to low sensitivity of MRS, a critical concentration of a metabolite, estimated to be about 0.5 mol/g tissue, is necessary to achieve a reasonable signal to noise ratio (Duarte *et al.*, 2012). This means that unlike MRI, MRS generally uses large voxels, typically in the range of 1-10mm^3. Larger voxels achieve better Signal to Noise Ratio (SNR), but at a cost to region and tissue selectivity. The critical concentrations required for MRS visibility excludes some molecules that are of interest particularly in psychiatric research, such as the monoamines: dopamine, noradrenaline and serotonin. The MRS visible range however includes metabolites such as N-acetyl- aspartate (NAA), inositol, glutamate, glutamine and GABA, which have been implicated in many neuropsychiatric illnesses including MDD. An additional 15-20 metabolites of varied significance can also be quantified, including creatine, which is often used as an internal reference compound (Emir *et al.*, 2012, Govindaraju *et al.*, 2000).

2.2.5.1 N-Acetyl-aspartate

N-Acetyl-aspartate (NAA) is present in relatively large quantities within the brain (Rigotti *et al.*, 2007). It forms the most prominent peak, a singlet at 2.01ppm, in a water suppressed ^{1}H-MRS spectrum. Additional less prominent coupled resonances from NAA are also present at 2.49, 2.67 and 4.38ppm. NAA is predominantly localised within neuronal soma, where it is synthesised by the mitochondria and transported into the cytoplasm. The exact function of NAA remains uncertain. One possible role is that it functions as osmolyte (Baslow, 2002, 2003). NAA loss has been reported in white matter diseases such as multiple sclerosis and hypoxic encephalopathy (Rosen and Lenkinski, 2007), suggesting a possible role as a marker for neuronal viability. Increased levels of NAA however characterise the autosomal

recessive neurodegenerative disease Canavan's disease (Castillo *et al.*, 1998). NAA and its derivative N-acetylglutamate are closely correlated in MRS analysis and are thus often quantified together as total-NAA.

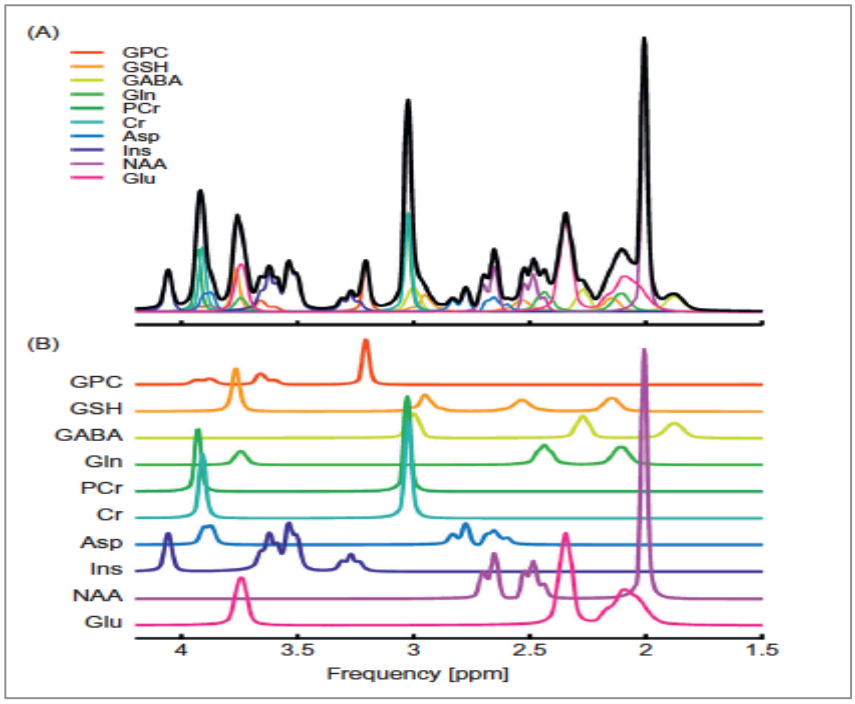

Figure 2.1: Model ^{1}H-MRS Spectra. A combination of linear model spectra with resonant peaks displayed along the chemical shift (x) axis. Prominent peaks are visible at 2.02 for NAA, 3.22 for choline, and 3.0, 3.99 for creatine. (Adapted from Stagg and Rothman, 2014)

2.2.5.2 Inositol

Inositol is a cyclic sugar containing a six-carbon ring with a hydroxyl attached to each of the six carbon atoms. Six inositol isomers have been described, of which the most abundant (~90%) in human tissues is *myo*-inositol (Stagg and Rothman, 2014). *Myo*-inositol does not cross the blood brain barrier and thus changes in concentrations detected with ^{1}H-MRS reflect those occurring centrally. In ^{1}H-HMS, *myo*-inositol forms a coupled multiplet visible at 3.52 and 3.61ppm. *Myo*-inositol has a short T_2, and is thus best imaged at ultra-short echo time, which can be achieved at

7-tesla using the STEAM sequence (discussed in section 2.2.7, below). This approach was used in the present thesis (chapter 5). Glia are the predominant source of cerebral *myo*-inositol and elevated levels have been reported in astrogliosis (Flogel *et al.*, 1994). Most of the inositol in the brain is found intracellularly, being taken up by cells via two sodium-*myo*-inositol co-transporters, SMT1 and SMT2, and a hydrogen-*myo*-inositol symporter, HMIT (Stagg and Rothman, 2014). Some of this intracellular pool is thought to function as an osmolyte. Another pool participates in the G-protein coupled, inositol triphosphate (IP_3) second messenger signalling.

2.2.5.3 Glutamate and Glutamine

The amino acids glutamate and glutamine are components of the tripartite glutamate synapse, with important roles in neurotransmission and detoxification mechanisms (discussed in Chapter 1). In ^{1}H-MRS, glutamate and glutamine form complex overlapping peaks at 3.7ppm (resulting from the ^{2}CH protons) and between 2.1 and 2.4ppm (from the $^3CH_2$ and $^4Ch_2$ protons). At field strengths ≤ 3T, glutamate and glutamine peaks are difficult to resolve, and are thus quantified together as the composite metabolite, Glx. Several approaches however have been suggested for resolving the glutamate and glutamine peaks at 3-tesla (Hurd *et al.*, 2004, Provencher, 1993). At higher scanner strengths (≥ 4T), there is better resolution of their $^4CH_2$ resonances, allowing for separate quantification (Tkac *et al.*, 2001). The role of glutamate in the pathophysiology of mood disorders has been the subject of intense research (discussed in Chapter 3). Investigations in this field have been advanced by the availability of ultra high field imaging that has enabled separate quantification of glutamate and glutamine concentrations.

2.2.5.4 γ -Aminobutyric acid

γ -aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the brain, being present in about 20% of synapses (Douglas and Martin, 2007). It has additional important roles in cerebral metabolism. The CH_2 groups in GABA form coupled resonances at 1.89, 2.28 and 3.01ppm. Due to its low concentration and the fact that its peaks overlap with those of strongly resonant molecules, accurate GABA quantification at field strengths ≤ 3T is challenging (Mullins *et al.*, 2014). Spectral editing techniques based on GABA's J-coupling effects have been developed, and these used to measure GABA with in-vivo ^{1}H-MRS. The MEGA-PRESS sequence is designed specifically to achieve this (Mescher *et al.*, 1998). In MEGA-PRESS, two spectral acquisitions are obtained. In one, a frequency selective pulse is applied at 1.9ppm to target GABA. In the second acquisition, frequency selective pulses are applied outside the metabolite range and hence have little effect on the GABA spectrum. Through subtraction, a residual resonance attributed to GABA is obtained. Subtraction removes all peaks that did not perceive the 1.9ppm selective pulse. GABA is thought to play various roles in the brain, including being involved in cortical plasticity (Jones, 1993). Low GABA levels have been reported in some studies in depressed patients.

2.2.5.5 Creatine

Creatine (Cr) forms two prominent singlets at 3.03 and 3.93ppm. These, together with the NAA and choline singlets, dominate the skyline of in-vivo water suppressed ^{1}H-MRS spectrum. Creatine is an integral constituent of many metabolic processes and is thus used as a surrogate marker for cellular energy metabolism. Since de-novo synthesis of creatine does not occur within the brain, alterations in its

concentration may reflect systemic changes. In the absence of disease, creatine concentrations are thought to remain fairly stable. As such, creatine is often used as an internal reference metabolite, from which other metabolite concentrations can be derived. While such an approach is useful particularly for making cross-centre comparisons, it may also be the source of bias in conditions where the concentrations of creatine (or any other reference metabolite) are altered.

2.2.6 Nuisance Metabolites

The aim of ^{1}H-MRS is to collect and quantify signal from low molecular weight metabolites that are significant in understanding brain health and disease. Accurate quantification of these metabolites however requires knowledge on undesired signal from nuisance metabolites. When unaccounted for, these may decrease the quality of the MRS spectra and hence introduce errors in the estimation of metabolite concentrations. While some of the nuisance signals can be accounted for during data processing, others require tweaking of acquisition protocols such that the nuisance signal is minimised. The most crucial nuisance signals in ^{1}H-MRS come from macromolecules and lipids.

2.2.6.1 Macromolecules

Macromolecules form broad complexes between 0.9 and 2.4 ppm, overlapping with resonances of most metabolites (Cudalbu et al., 2012). The predominant contribution is thought to arise from methyl and methylene groups of amino acids in cytosolic proteins (Behar *et al.*, 1994). Because of their short T_2, macromolecules cause more spectral interference with ultra-short, and short echo imaging. If unaccounted for, they may lead to overestimation of metabolite concentrations (Považan, 2015). Several approaches have been proposed for handling the

macromolecule signal. These include mathematical estimations of the signal (Cudalbu *et al.*, 2012), direct acquisition of a macromolecule free spectrum (Knight-Scott, 1999) or incorporation of a macromolecule spectrum into the basis set during metabolite fitting (Emir *et al.*, 2012), which is the approach used in the data presented in this thesis (chapters 3 and 5). Because macromolecules have T_1 times that are different from those of metabolites, a macromolecule only spectrum can be obtained using an inversion recovery sequence that nulls metabolite resonances. The macromolecule signal can then be incorporated into the basis set used for fitting analysis.

2.2.6.2 Lipids

Intracranial lipids are mainly localised within fixed structural components. Since MRS targets mobile low molecular weight metabolites, lipids are generally outside the MRS visible range. Prominent lipid resonances may however arise from extra-cranial lipids, masking the metabolite peaks. These are usually spread between 0.9-2.8 ppm. Like macromolecules, lipids have short T_2 signals and hence are more problematic with short-echo time imaging. Robust in-vivo MRS must therefore include techniques for lipid signal suppression. Inadequate lipid suppression may lead to an irregular metabolite baseline and poor spectral fit. Lipids are however not invariably nuisance signals. Levels may be elevated in pathological processes such as in neoplastic conditions. In such cases, suppression of lipid resonances may mask an important underlying biochemical process. One of the techniques for lipid suppression with in-vivo H-MRS is outer volume saturation, used in the experiments presented in this thesis. In this approach, slabs covering regions containing the unwanted extra cranial lipids are excited using frequency selective pulses. The

selectively excited lipid signal is then dephased using spoiler gradients before acquisition of a lipid-suppressed metabolite spectrum from the voxel of interest.

2.2.7 Single-Voxel In Vivo Magnetic Resonance Spectroscopy

Key to single voxel spectroscopy is the ability to selectively excite and detect signal from a pre-defined voxel-of-interest (VOI). Current approaches aimed at achieving such localisation involve the use of serial pulses in the form of pulse sequences. Theoretically, simple pulse sequences such as the spin-echo could be utilised for in-vivo MRS. This would however not achieve sharp 3-D volume selection, thus limiting the accuracy of the MRS acquisition. Currently used pulse sequences aim at achieving frequency selective excitation along three orthogonal planes, thus defining a cuboidal voxel. Excitation on each plane is achieved through a combination of a gradient fields and selective RF pulses. When the two are applied, the RF pulses are capable of exciting only a limited range of frequencies defined by the gradients, thus enabling selection of excitable bands along three planes. To ensure robustness in signal localisation, dephasing (crusher or spoiler) gradients are applied to remove unwanted signals (see figure 2.2 below).

Of the available pulse sequences, the best characterized are the Point RESolved Spectroscopy (PRESS, Bottomley et al., 1985) and the STimulated Echo Acquisition Mode (STEAM, Frahm et al., 1987). These have the advantage of achieving 3-D volume localization with a single excitation. They are however susceptible to movement artefacts, some of which may be corrected for with post-processing techniques. Attempts at improving on the PRESS sequence, and particularly for application at ultra-high field MRS, led to the development of the Localization by

Adiabatic SElective refocusing (LASER; Garwood & DelaBarre, 2001) and semi-LASER (Scheenen *et al.,* 2008). In this thesis, the semi-LASER sequence was used to carry out spectroscopic imaging of the glutamatergic system (Chapter 3) while the STEAM sequence was used to measure inositol concentrations (Chapter 5). These two sequences will be briefly outlined in this section.

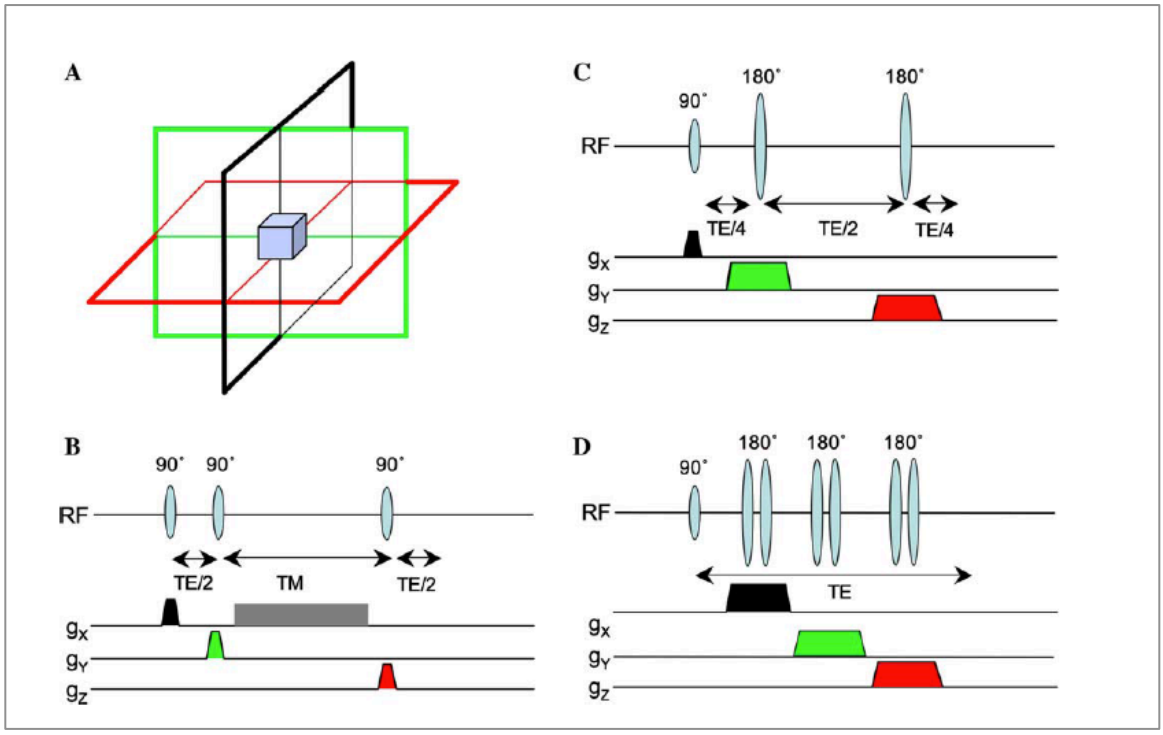

Figure 2.2: 3-Dimensional volume selection with MRS sequences. Single voxel excitation can be achieved by using pulse sequences in which selective excitations are carried out along three orthogonal planes. A) The three orthogonal excitations define a cuboidal voxel of interest located at their intersection. B) The STEAM sequence consisting of three 90° excitations. C) The PRESS sequence comprising a 90° excitation pulse followed by two 180° refocusing pulses. D) The LASER sequence, in which a non-slice selective adiabatic half-passage excitation pulse is applied, followed by three pairs 180° refocusing pulses. In the semi-LASER sequence, a slice-selective excitation pulse replaces the non-selective 90° excitation, and is then followed by two pairs of 180° refocusing pulses. (Adapted from Baker and Lin, 2006)

2.2.7.1 Semi-LASER Sequence

The semi-Localization by Adiabatic SElective Refocusing (semi-Laser) sequence (Scheenen *et al.,* 2008) was developed as a modification of the PRESS sequence (Bottomley, 1984) and with particular utility for MRS imaging at ultra-high field. In PRESS, a slice selective 90°excitation is used followed by two slice selective 180° refocusing pulses. It thus constitutes a double spin-echo arrangement. A magnetic gradient is applied during each excitation to ensure band selective frequency excitations. The three pulses are applied in orthogonal planes thus achieving 3-D volume selection. In addition to selective excitation, unwanted spin-echoes are removed through the use of spoiler gradients applied on either side of the refocusing pulses. The spoiler gradients remove any excitations that did not experience the initial 90° excitation but were then refocused with the subsequent 180° pulses.

In semi-LASER, the refocusing pulses used in PRESS are replaced with pairs of adiabatic full passage (AFP) pulses. Adiabatic pulses have the advantage of generating homogenous excitations. This is because adiabatic pulses generate uniform flip angles independent of the radio frequency field amplitude, in addition to achieving larger bandwidths in comparison to PRESS. Magnetic field inhomogeneity is an inherent feature of in-vivo MRS, and is more amplified at higher magnetic fields (such as 7-tesla MRS, used in the present thesis). Use of adiabatic pulses thus allows for minimisation of errors arising from magnetic field inhomogeneities (Baker and Lin, 2006).

2.2.7.2 STEAM Sequence

Selection of an ideal pulse sequence should also take into account the minimum possible echo time that can be achieved with the sequence. This consideration favours the choice of STEAM over semi-LASER when the primary metabolite of interest has a very-short T_2 signal. In this thesis, the STEAM sequence was used to measure *myo*-Inositol levels (chapter 5). In the STEAM sequence (Frahm *et al.,* 1987), three 90° selective pulses are used to achieve 3D volume localisation (see figure 2.2 above). The use of 90° refocusing pulses, in place of the 180° pulses used in PRESS, confers some advantages in that a sharper 3-D volume can be defined. In addition, very short echo times are possible (such as 11ms used in chapter 5). Use of 90° pulses however rotates only half of the transverse magnetisation back to the longitudinal axis, with the other half being crushed by spoiler gradients. As such, the signal collected using STEAM is only about half that collected with PRESS.

2.2.8 Technical Considerations

In addition to the right choice of pulse sequence for an MRS experiment, some technical issues need particular consideration, especially with in-vivo imaging at ultra-high field. These include the requirements for shimming to improve magnetic field homogeneity, achieving water suppression, and minimisation of errors resulting from chemical shift displacement.

2.2.8.1 Shimming

Proton-MRS is dependent on detecting differences between spins based their chemical shift and J-coupling effects. To achieve this, it is assumed that the magnetic field within the voxel of interest remains constant and that any observed differences

are as a result of electrochemical shielding effects. This is however not completely the case, particularly with in-vivo MRS, where biological tissues inherently introduce local variations in the magnetic field. In addition, placement of a subject into the MRI scanner results in the formation of an air-tissue boundary that distorts the magnetic field. To facilitate spectroscopic imaging, methods for mapping and correcting for the inhomogeneous fields, referred to as shimming techniques, have been developed. Shimming is particularly important since errors resulting from magnetic field inhomogeneity cannot be corrected for during post-processing.

MRI scanners are usually fitted with two types of shims; primary shim coils (that correct inhomogeneities along the x, y and z planes) and quadratic shim coils. While higher order shims may be desirable for robust MRS imaging, this cannot be achieved with clinical MRI scanners without additional hardware. Failure to carry out proper shimming results in broadening of spectral linewidths and hence poor spectral resolution (Blamire *et al.*, 1996).

2.2.8.2 Water Suppression

The water content in brain tissue is estimated at 80%, with some variation specific to region and tissue composition (Emir *et al.*, 2012). In contrast, the concentration of metabolites is about 10^3 to 10^4 times less. While the ubiquitous and abundant distribution of water within biological tissues is an advantage in conventional MRI, in ^{1}H-MRS, it is the spins from non-water ^{1}H atoms that are desired. In the absence of suppression, water forms a prominent resonance at about 4.65ppm, overlapping with that of adjacent metabolites and masking signal from the rest of the spectrum. In addition, the prominent water resonance distorts the spectral baseline thus

interfering with metabolite quantification.

In-vivo ^{1}H-MRS must therefore include techniques for water suppression. The most commonly used methods are chemical shift selective (CHESS) suppression (Haase *et al.*, 1985) and Variable Power and Optimised Relaxation Delays (VAPOR) suppression (Tkac *et al.*, 1999). CHESS involves selective excitation of resonances about the water selective frequency of 4.65 ppm; followed by application of crusher gradients to dephase the excited water spins. In VAPOR, the excitation-dephasing protocol used in CHESS is repeated several times, with the delay between the excitations carefully selected so as to take advantage of the T_1 differences between water and metabolites. Undesired residual water following VAPOR suppression can be removed by post-processing subtraction methods, although this may interfere with the quantification of metabolites adjacent to the water peak.

2.2.8.3 Chemical Shift Displacement Errors

While differences in resonance frequencies determine the MRS spectrum, in conventional MRI, such differences are used to map spatial distribution. In frequency selective slice excitation, nuclei precessing at different frequencies will experience different slice selections. It is thus possible that nuclei from outside a voxel may be included in the spectra because they experienced the selective pulse of the MRS sequence. This is referred to as the chemical shift displacement error (CSDE). Chemical shift differences are however generally small compared with spatial frequency variations generated by magnetic gradients. As such, using an RF pulse with a sufficiently large bandwidth would minimise CSDE. Increasing the RF bandwidth would however require an increase in the RF power, which may result in

an increase in the specific absorption rate (SAR - the power absorbed per unit mass). Allowable SAR for clinical scans is strictly defined, making it impossible to increase the bandwidth beyond certain limits.

2.2.9 Ultra-high Field Magnetic Resonance Spectroscopy

Technical advances in MRS have been facilitated by the advent of ultra-high-field imaging (≥ 4T). The low field strengths of most research and clinical scans is limiting because of spectral overlaps and low SNR. While MRS signal increases linearly with the magnetic field, the noise increases as a function of the square root of the field. Thus increasing the field strength results in an overall SNR gain. In addition, chemical shift dispersion between resonances increases linearly with the main magnetic field resulting in better metabolite resolution. Ultra high field imaging is particularly useful for quantifying metabolites such as glutamate and glutamine, whose quantification at field strengths <4T would require use of specialised metabolite-selective pulses. Such pulses could introduce errors in estimation, as well as limiting the range of metabolites quantified.

Spectroscopy at ultra high field however requires that some limitations specific to this modality be considered. These include the need for advanced shimming techniques (to correct for the enhanced field inhomogeneities) and use of pulse sequences that minimise errors due to chemical shift dispersal. To overcome inhomogeneities in the transmit field, a higher RF power is required, which results in increased SAR (Vaughan *et al.*, 2001). Even with advanced shimming approaches, metabolite linewidths broaden at ultra high field, resulting in shorter T2 relaxation times and increased tissue dependent susceptibility effects (Tkac *et al.*, 2009).

2.2.10 Analysis of MRS Spectrum

MRS spectra analysis involves deriving metabolite concentrations from the peaks within the spectrum. This can be done in the time domain (Vanhamme *et al.*, 1997) or, more frequently, in the chemical shift domain (Mierisova and Ala-Korpela, 2001). In the chemical shift domain of an ^{1}H-MRS spectrum, the amplitude of the protons on the y-axis corresponds to their relative abundance. Estimation of the area under peak would therefore provide information on the relative concentrations. Computation of peak areas with in-vivo spectroscopy is however complicated by the overlapping resonances, distortion of the baseline and lineshapes that are difficult to model. Several software tools for carrying out such computations have been developed, an example of which is the LCModel (Linear Combination of Model spectra; Provencher, 1993, 2001). The user-friendly interface of LCModel allows for standardised non-expert use, giving it an advantage for clinical MRS.

LCModel fits the in vivo spectrum as a combination of pure, model spectra from each of the expected compounds in the brain. Analysis with LCModel requires fitting metabolite spectra alongside a prediction of the expected spectra. This predicted spectrum is referred to as the basis set, and is specific to the experimental conditions (scanner strength, pulse sequence and timing). Estimates of the chemical shifts and coupling constants for most metabolites observed in-vivo is available in literature (Govindaraju *et al.*, 2000) and these can be used to generate the basis spectra. Software packages can be used to generate basis sets, an example of which is the GAMMA/PyGAMMA simulation library available through VESPA (Versatile Simulation, Pulses and Analysis, http://scion.duhs.duke.edu/vespa/ (Soher *et al.,*

2011), which was used in the experiments presented in this thesis. Better accuracy of fit can be obtained by inclusion of the predicted macromolecule spectrum (obtained experimentally) into the basis spectrum.

Following basis-set fitting, metabolite concentrations can be derived by scaling based on the concentrations of a known metabolite, such as water, creatine or NAA. Post-processing techniques are used to correct for scanner artefacts such as eddy currents and phase errors. Because of spectral overlap, a correlation matrix is used to determine the confidence with which the concentrations of individual metabolites can be independently reported. Metabolites with correlation values less than - 0.5 are considered to be strongly coupled, and are reported together (for example creatine and phosphocreatine as total-Creatine (Emir *et al.,* 2012, Oz and Tkac, 2011).

Several steps are utilised to assess the quality of the MRS spectra during analysis. First is the visualisation of the spectra and LCModel fit. A good quality spectrum should have a smooth baseline and small random residue (see figure 2.3 below). Quantification errors are estimated with Cramer Rao Lower Bounds (CRLBs), which estimate the lowest possible standard deviations of the measured parameters. Generally metabolites with CRLBs < 20% are considered for inclusion. Strict filtering of metabolites based on CRLB can however introduce a bias in the quantification (Kreis, 2015). In addition to the inspection of fit and CRLBs, linewidths and SNR are also used to assess for quality.

2.2.11 This Thesis

In this thesis, ^{1}H-MRS was used as a tool to investigate glutamatergic changes in major depressive disorder, taking advantage of ultra-high field (7-tesla) imaging to accurately quantify glutamate and glutamine separately (chapter 3). Further to this, MRS was used as a tool to guide drug development (chapter 5), through the utilisation of the accuracy of 7-tesla MRS to quantify metabolites targeted by an investigational antidepressant agent, ebselen.

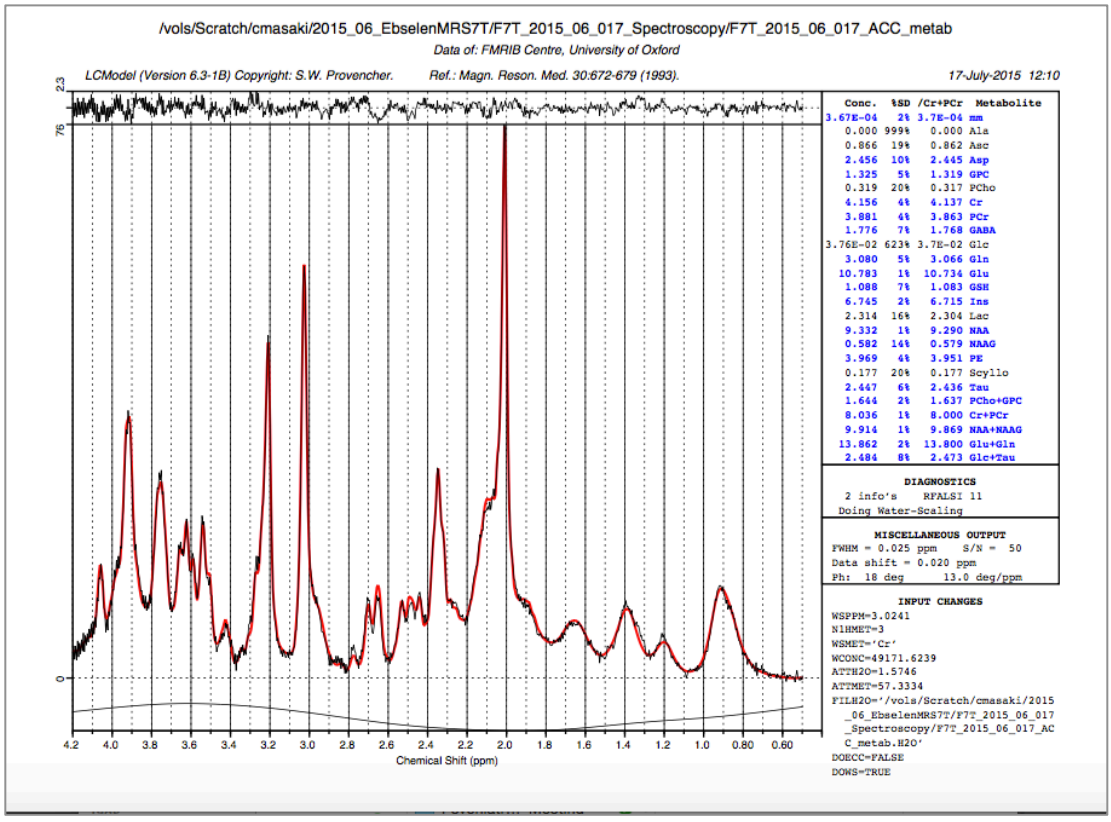

Figure 2.3: LCModel output. Water suppressed ^{1}H-MRS spectrum from the Anterior Cingulate Cortex (ACC). An example of an LCModel output from a subject following MRS acquisition on an 8mm^{3} voxel with STEAM sequence at TE=11ms, NT = 64 averages. The MRS spectrum was fitted as a linear combination of model spectra of pure compounds defined in the basis set. Post-processing correction of frequency and phase errors was also carried out. Absolute metabolite concentrations are presented based on scaling to an unsuppressed water spectrum collected during the same acquisition.

2.3 Neuropsychological tasks

Neuropsychological tasks are increasingly used to guide the development of novel psychotropic drugs and are a useful bridge between animal behavioural experimental investigations and clinical studies in patients. The aim is to devise tasks that tap neuropsychological processes thought to be impacted by the drug and the disease upon which its effects are to be investigated. The drug assessed in this thesis, ebselen, is being developed as a lithium-mimetic. As noted in chapter 1, lithium is effective in the treatment of mood disorders such as bipolar disorder and resistant depression. In addition, lithium is known to decrease both suicidal behaviours and impulsive aggressive behaviour. Therefore in the thesis, the effects of ebselen treatment on decision-making were investigated using the Cambridge Gambling Task (Clark *et al.*, 2008, Rogers *et al.*, 1999) and on the processing of emotional information using the Facial Expression Recognition Task (Harmer *et al.*, 2003).

2.3.1 Cambridge Gambling Task

The Cambridge Gambling Task (CGT) was developed to assess decision-making and risk-taking behaviour outside a learning context (Clark *et al.*, 2008, Rogers *et al.*, 1999). Unlike most of the commonly used tasks to assess reward seeking, in the CGT, all the relevant information is presented to the participant 'up-front', thus eliminating the need to retrieve previous information in subsequent trials. The CGT therefore minimises confounding effects due to demands from learning, executive function and working memory.

2.3.1.1 Design of the CGT

In each trial, participants are shown a row of ten boxes at the top of a black screen, with some boxes being red and some blue. The ratio of red to blue boxes varies from 9:1; 8:2; 7:3; 6:4; 5:5 and vice versa in pseudo-random order. At the bottom of the screen, a response panel containing two rectangles with the words 'red' and 'blue' is also shown.

Participants are informed that a yellow token is hidden inside one of the red or blue boxes and asked to select the box in which the token is most likely hidden, by pressing either the red or blue box in the response panel. After a response is selected, participants are shown a bet box from which they can gamble on their confidence in the selection. In this, bets are presented in either ascending (rising) order or descending (falling) order. Bets are presented in proportions of 5%, 25%, 50%, 75% and 95% of the total points at the start of each trial, with bets changing every 2.5 seconds.

100 points are given to the participants at the start of each block, displayed on the middle left of the screen. When a bet is selected, the points are either added to the total points if a correct response was made (win trials) or subtracted from the total if an incorrect response was made (loose trials). If no bet is chosen, then the last bet presented (95% of total points in ascend trials, or 5% in descend trials) is automatically selected. Participants are asked to accumulate as many points as possible.

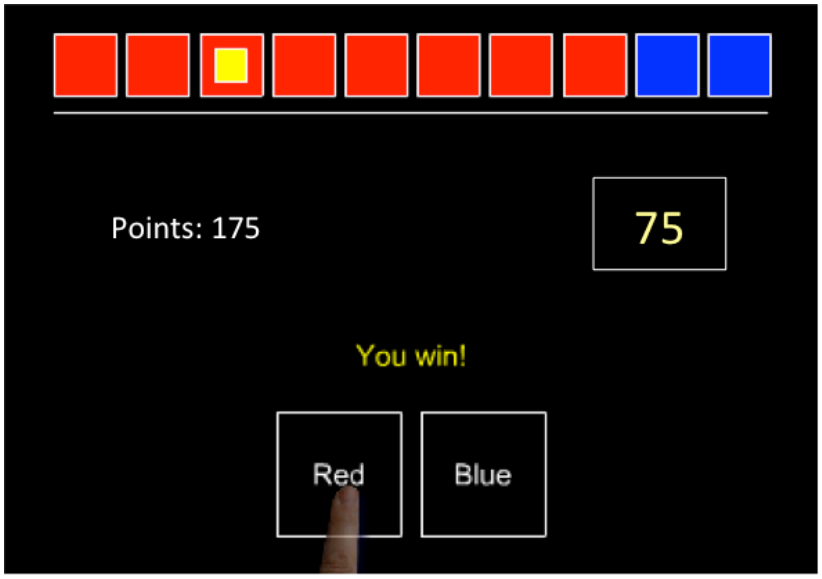

Figure 2.4: The CGT display screen showing a gambling trial in which the participant has selected the red box as the most likely location where the yellow token is hidden and staked 75 points in the bet. The participant in this case has made the right selection (win trial) and the total points staked have been added to the initial 100 points presented at the start of the trial. If the participant had selected the blue box, this would have been a lose trial and 75 points would have been taken out of the 100 points.

2.3.1.2 CGT Administration

The CGT is administered in 5 stages, with instructions given at the start of each stage. In the first stage, participants are asked to decide on the location in which the yellow token is likely to be hidden without any bets being presented. This is a practice decision-making stage. In the second stage, in addition to selecting the token location, participants are given a few trials (not assessed) with the bets presented in either ascending or descending order. The third stage is an assessed gambling stage, given in four blocks, with bets presented in the same order as in the second (training) stage. The fourth stage is a training stage similar to the second stage, but with the bets presented in an order in the opposite direction to that presented in the 2nd and 3rd stages. The fifth stage is a gambling stage, given in four blocks as well, with bets presented in the same order as in the fourth stage. Two

possible modes of the CGT can be administered. In the ascending first mode, the first bets presented in stages 2 and 3 are in ascending order. In the descending first mode, the first bets are presented in descending order. The task lasts about 30 min.

2.3.1.3 CGT Outcome Measures

There are six possible outcome measures from the CGT. In the decision making stage (selection of the location of the yellow token), outcome measures are the quality of decision making and the deliberation time. Outcome measures from the gambling stages are the reward seeking, risk adjustment and delay aversion. Some of these outcome measures can be further categorised based on the gamble type (ascending or descending) or bet ratio (ratio of red: blue boxes).

In most trials, there is asymmetry in the ratio of red: blue boxes presented. Participants may chose to gamble on the more likely outcome (more favourable ratio) or on the less likely outcome. The *quality of decision-making* measures the proportion of trials on which the more likely ratio is selected.

The *deliberation time* is a measure of the mean latency time from the presentation of coloured boxes to when the participant makes a selection.

Risk taking (also used as a measure of reward seeking) is a measure of the proportion of current points (between 5% - 95%) that the participant is willing to stake on trials in which the more likely ratio of red: blue boxes is selected. Trials in which less favourable ratios are selected are not included in calculating the risk taking.

Risk adjustment is designed to measure the extent to which participants bet a higher proportion of points when the chosen ratio of red to blue boxes (or vice versa) is higher (such as 9:1 and 8:2) compared with when the proportion bet when a smaller majority of boxes are of the chosen colour (such as 6:4). Mathematically it is calculated using the formula:

$$\frac{(2v + w - x - 2y)}{z}$$

Where v, w, x and y represent the proportion risked in trials in which the ratio of red to blue boxes (or vice versa) is 9:1, 8:2, 7:3 and 6:4 respectively, and z the mean proportion risked across all trials.

Delay aversion assesses a subject's unwillingness to wait for later bets to be presented. It is calculated by subtracting the risk taking measure in trials in which the bets are presented in descending order from the risk taking measure in trials in which the bets are presented in ascending order. Low bets in the ascend blocks and high bets in the descend blocks reveal an impulsive betting strategy, while high bets in both conditions reveal a risk taking or reward seeking strategy.

Overall proportion bet is a measure of the average proportion of current total points that a participant staked on the gambles, irrespective of whether the more likely ratio of red: blue boxes was selected or not.

2.4. Facial emotion recognition task

Depression is associated with negative biases in the processing of emotional information. Psychological and pharmacologic therapies are hypothesised to act by

reorienting these negative affective schema (Harmer *et al.*, 2009a). Cognitive biases in the processing of emotional information relevant to clinical depressive symptoms can be assessed by using a battery of tasks, including the Facial Emotion Recognition Task (FERT). This task has proved to be useful not only in detecting negative biases among depressed patients but also in guiding antidepressant drug development where drug-induced positive biases can be detected in both healthy participants and depressed patients (Harmer *et al.*, 2009b, Pringle *et al.*, 2011a, Warren *et al.*, 2015).

The FERT features six basic facial emotional expressions - happiness, surprise, sadness, fear, anger and disgust. These expressions have been taken from Ekman's pictures of affect series (Ekman and Friesen, 1976). In this task, faces from 10 individuals expressing the six basic emotions are used. In order to achieve varied intensities of each of the emotions, the facial expressions are morphed between each prototype and neutral in 10% incremental steps, using a technique described by Young and colleagues (1997). As such, 10 possible expressions of varying intensities are available for each of the emotions. Forty expressions are presented for each emotion, 4 at each intensity (between 10% - 100%). This leads to a total of 240 faces expressing the six basic emotions.

Before the start of the task, instructions are given to the participants asking them to identify each of the expressions which will be presented as quickly and accurately as possible. Responses are indicated by pressing labelled keys on a laptop keyboard. Facial stimuli are then presented in a random order on a laptop screen for 500ms then replaced by a blank screen. During this time participants make a selection. As

soon as a selection has been made, the next face is presented. The task is broken down into two parts with an untimed rest period between the two. The number of stimuli accurately classified as each emotion as well as the number incorrectly assigned to each emotion, and reaction times are recorded. In this thesis, the FERT was used to assess the cognitive effects of the investigational drug ebselen (chapter 6).

2.5. Clinical Rating Scales

Current approaches to psychiatric diagnoses rely on specified criteria based on patient report and behavioural observations. However, diagnoses based on the most widely used tools such as the Diagnostic and Statistical Manual for Mental Disorders (DSM, American Psychiatric Association) yield broad heterogeneous patient groups, making reliable assessment of psychological domains difficult. Questionnaire based psychological screening tools have developed to provide assessment of psychiatric dimensions further to the broad categorization of diagnostic approaches. These tools have proved useful in both clinical and research settings in not only identifying more homogenous patient populations, but also in the assessment of symptom severity and change. Some of these tools have also proved useful for healthy volunteers, for example in the assessment of personality characteristics known to constitute risk for future psychopathology. The use of questionnaire-based screening tools has also allowed for cross-institutional comparisons and collaborations, as well as making it easier to interpret research findings.

Psychological questionnaires have also allowed correlations to be drawn with other

behavioural measures such as those obtained from neuroimaging studies. The scales used in this thesis, for the assessment of depressed patients (chapters 3 and 4) and healthy volunteers following pharmacological intervention (chapters 5 and 6) will be presented in this section. For each, a brief background and scope will be covered as well as description of questionnaire administration.

2.5.1. Beck Depression Inventory

The Beck Depression Inventory (BDI, Beck *et al.*, 1961), is a 21-item questionnaire scale developed for screening depressive symptoms. It is a self-report scale developed around the common symptoms reported by depressed patients. Its development was based on Aaron Beck's cognitive theory of depression that attributes depressive symptoms to negative thoughts about the world, future and self. The BDI measures such symptoms such as severity of depressed mood, feelings of hopelessness, irritability and various cognitive domains affected in depression. It also assesses vegetative symptoms such as fatigue, weight loss and changes in sleep.

The original scale was published in 1961 consisting of 21 items each scored in single best answer multiple-choice format (Beck *et al.*, 1961). Each item is scored between 0-3, with higher numbers representing increased symptom severity. Some items have two alternative statements each scored with the same weight. In the original scale, scores of 0-9 indicate minimal depression, 10-18 mild depression, 19-29 moderate depression, and 30-63 severe depression.

The BDI however has some limitations, including those generalizable to other self-administered questionnaires for depression. The setting in which the questionnaire is administered could introduce a bias in the way the items are rated. In addition, concomitant physical illness could exaggerate scores derived from items assessing somatic symptoms. The BDI has also been shown to be less useful in differentiating moderate from severe depression (Kearns *et al.*, 1982).

The BDI has however proved to be a useful tool for the assessment of depressive symptoms, with particular utility for screening. It has been showed to correlate well with global severity scores determined by psychiatrists (Bech *et al.*, 1975; Crawford, 1973) and with the Hamilton Depression Rating Scale, an interviewer administered questionnaire frequently used in clinical trials, discussed in 2.5.5 (Davies *et al.,* 1975). It has also been shown to be sensitive to clinical improvement and hence useful for follow-up assessments (Metcalfe and Goldman, 1965).

2.5.2. Chalder Fatigue Questionnaire

The Chalder fatigue scale (CFQ 11, Chalder *et al.,* 1993) is a self-rating scale developed to measure severity of fatigue. Originally developed to assess chronic fatigue symptoms in a clinical setting (Butler *et al.*, 1991), it has since been modified and is now applicable for both clinical and non-clinical settings. It consists of 11-items designed to assess physical fatigue (items 1-7) and mental fatigue (items 8-11). Each item is scored on a 4 point scale as; 0 ‘better than usual’, 1 ’no more than usual’, 2 ‘worse than usual’ and 3 'much worse than usual’.

Two scoring systems are used. The first is a simple summation of the scores, with overall scores ranging from 0 to 33. The second is a bimodal scoring system designed to eliminate errors due to end-users and middle-users. In this, responses in the two left hand columns are scored 0 while those in the two right hand columns are scored 1. Thus the maximum score with the bimodal system is 11, with respondents being categorised as 'not fatigued' (scores of ≤ 3) and fatigued (scores of ≥ 4). The CFQ-11 has been shown to be highly reliable in studies with patients with chronic fatigue syndrome (Morriss *et al.*, 1998) as well as in assessment of occupational fatigue (Loge *et al.*, 1998).

2.5.3. Childhood Trauma Questionnaire

The Childhood Trauma Questionnaire (CTQ, Bernstein *et al.,* 1994) is a tool developed for the assessment of histories of abuse and neglect. It is a self-report that includes 28-items and assesses 5 types of maltreatment: emotional abuse, physical abuse, sexual abuse, emotional neglect and physical neglect. It uses a 5 point Likert scale, with items scores as 1 'Never True', 2 'Rarely True', 3 'Sometimes True' and 4 'Very Often True'. The questionnaire has been shown to be reliable with high internal consistency scores (Bernstein *et al.*, 1997, Bernstein *et al.*, 1994, Fink *et al.*, 1995). High scores on the CTQ have been associated with structural brain changes in adults (Dannlowski *et al.*, 2012).

2.5.4. Eysenck Personality Questionnaire

The Eysenck Personality Questionnaire (EPQ, Eysenck and Eysenck, 1975), also referred to as the Eysenck Personality Inventory, was developed to assess

personality traits. The questionnaire assesses four trait dimensions of personality: Extraversion (E), Neuroticism (N), Psychoticism (P) and Social desirability (L). The original form consists of 90 questions relating to personality characteristics, each scored as 'Yes' or 'No'. The questionnaire has been validated for cross-cultural appropriateness (Barrett et al., 1998, Eysenck and Eysenck, 1983) and is available in multiple translations. The most widely employed measure is that of neuroticism which has emerged as an important risk factor for depression (Kendler *et al.*, 2004).

2.5.5. Hamilton Depression Rating Scale

The Hamilton Depression Rating Scale (HAM-D, Hamilton 1960) is a clinician-administered scale used to assess severity of depression among adults. It is the most widely used tool for assessment of depressive severity (Hedlund and Viewig, 1979). The original form was a 17-item multiple-choice questionnaire developed by Max Hamilton in 1960. It has since undergone several revisions (Hamilton, 1966, 1967, 1969). The HAM-D is intended to be administered to patients with a confirmed diagnosis of depression. Since the questionnaire itself does not come with an accompanying structured interview, it is intended to be administered by a psychiatrist. Semi-structured interview guides have since been developed (Williams, 1988), but these remain to be validated. Information obtained during the questionnaire interview, as well as from any other sources available to the psychiatrist, is factored in during the scoring.

The 17-items of the HAM-D assess depressive mood (maximum of 4 points), psychic anxiety (4), depressive thought such as guilt, suicidality, hypochondriasis (12),

vegetative symptoms (18), psychomotor symptoms (6), occupational impairment (4) and lack of insight (2). Four additional unscored items were included in the 1969 revision, to assess diurnal variation (important in melancholic depression), depersonalisation and derealisation, as well as presence of paranoid and obsessive compulsive symptoms. Scores of 0-7 indicate absence of depression, 8-19: mild depression and ≥ 20: moderate, severe or very severe depression. The HAM-D has demonstrated good internal, inter-rater and retest reliability (Bagby *et al.*, 2004).

The HAM-D is however restrictive since it is designed to be administered by a clinical expert. It has also been shown that the 17-items measure multiple dimensions and as such make it difficult to interpret overall summated scores. In addition, emphasis on negative symptoms of depression (such as weight loss, or insomnia) and not positive symptoms (weight gain and excessive sleep) also limits the scope to which it captures depressive symptomatology. Some modifications have been developed to overcome these shortcomings, including the revision by the Depression Rating Scale Standardization Team, GRID-HAMD (Kalai *et al.,* 2002).

2.5.6. Leeds Sleep Evaluation Questionnaire

The Leeds Sleep Evaluation Questionnaire (Parrot and Hindmarch, 1980) is a 10-item questionnaire that assesses self-reported measures of sleep. The questionnaire was developed to assess changes in sleep quality during the course of pharmacological intervention. The 10-items are grouped to assess four sleep domains namely ease of initiating sleep, quality of sleep, ease of waking and behaviour immediately following wakefulness. The questions are presented in the form of a visual analog scale.

2.5.7. Positive and Negative Affective Schedule

The Positive and Negative Affective Schedule is a 20-item self-report questionnaire developed by Watson, Clark and Tellegen (1988) that assesses positive and negative affect. Participants are given 20 mood descriptors (10 positive and 10 negative). On each, they are asked to rate on a 5-point scale (with 1 being very slightly or not at all and 5 extremely) as to how they feel at the time of filling in the questionnaire. It has been shown to have good reliability and validity (Crawford and Henry, 2004).

2.5.8. Snaith-Hamilton Pleasure Scale

The Snaith-Hamilton Pleasure Scale (Snaith *et al.*, 1995) is a 14-item self-report questionnaire that assesses the inability to experience pleasure (anhedonia), a core feature of depression. Questions are presented in the form of a table with four possible responses for each potentially pleasurable experience listed- for example, 'I would be able to enjoy my favourite meal': 'Definitely Agree', 'Agree', 'Disagree', 'Strongly Disagree'. The two 'Disagree' responses are scored 1 and the 'Agree' responses 0. Thus possible scores range from 0 to 14, with higher scores indicating increased anhedonia.

2.5.9. State-Trait Anxiety Inventory

The State-Trait Anxiety Inventory is a scale that measures self reported symptoms of anxiety. It was developed by Spielberger and colleagues in the 1960s as a tool to measure enduring personality characteristics of anxiety (trait symptoms) and present anxiety symptoms (state symptoms). The 'State' form asks questions such as, 'I feel calm', 'I feel secure', 'I am tense'. The trait form asks questions such as, 'I

am a steady person', 'I am content', 'I worry too much over something that doesn't really matter'. The currently used version is a 1983 revision, labelled form Y (Spielberger *et al.,* 1983). It consists of two similar but not identical forms, the state measure (Y1) and the trait measure (Y2). Each form (Y1 and Y2) comprise 20 questions about half of which ask for presence of anxiety symptoms, and the other half absence of such symptoms. Each question is rated on a 4-point scale. The scale is useful for differentiating between symptoms of anxiety and depression, conditions that are frequently comorbid. It has been shown to be applicable across cultures and multiple translations are available. It has an advantage in that it can be applied even at low literacy levels.

2.5.10. Structured Clinical Interview for DSM-IV

The Structured Clinical Interview for DSM-IV (First *et al.,* 1996) is a semi-structured interview guide used for screening of major (axis 1) psychiatric disorders (SCID-I) or personality (Axis-II) disorders according to the DSM-IV. The SCID was originally developed based on the DSM-III-TR (American Psychiatric Association, 1987) to assist clinicians in making diagnosis based on the new proposed criteria (Spitzer *et al.*, 1992). Its purpose was to assist a trained clinician familiar with the psychiatric interview in carrying out a standardised interview to screen for DSM-III-TR diagnoses. The most widely used form of the SCID is based on the DSM-IV, although a DSM 5 version is also currently available.

Although originally intended to be administered by clinicians, the SCID can also be used by mental health professionals familiar with the DSM diagnostic criteria

following training. The SCID-I contains subsections assessing different diagnostic domains such as mood, anxiety, psychoactive substance use and eating disorders. Each sub-section begins with a broad entry question. If the response to this question is negative, the interviewer can skip the particular sub-section. If however the response is positive or doubtful, the interviewer can then proceed with asking more detailed questions present in the manual to explore the specifics of the symptom. The SCID is however designed to be a screening tool and does not provide detailed information about any psychopathology. As such, it is not a replacement for a detailed psychiatric interview.

2.5.10. This Thesis

The questionnaires discussed above were used to assess patients and healthy volunteers in the studies presented in this thesis. Screening for major psychopathology was carried out using the SCID-I, with additional psychiatrist administered interviews for patients (chapters 3 and 4). Mood, anxiety and personality assessment were carried out suing the HAM-D, BDI, state measure of the STAI and the Eysenck Personality Questionnaire. The Snaith Hamilton Pleasure Scale, Chalder Fatigue Scales and Childhood Trauma Questionnaire were used in the assessment of depressed patients and control participants in the study looking at neurochemistry of depression (Chapters 3 and 4). In Chapters 5 and 6, the PANAS and the Leeds Sleep Evaluation Questionnaire were used to assess mood and sleep measures following treatment with the pharmacologic agent/placebo.

Chapter 3: Investigating the neurochemistry of major depressive disorder using 7-tesla magnetic resonance spectroscopy

3.1 Introduction

There is a wealth of evidence implicating the amino-acid neurotransmitter systems in the pathophysiology of major depressive disorder (MDD). In particular, changes within the glutamatergic system have been demonstrated across multiple studies using pre-clinical models, post-mortem pathological samples and in-vivo MRI imaging (Machado-Vieira *et al.*, 2009, Sanacora *et al.*, 2012). These findings, coupled with the evidence that glutamate modulating agents have antidepressant effects, have led to the formulation of a glutamate based hypothesis of depression (Sanacora *et al.*, 2012). They have also nurtured an interest in elucidating specific cellular and molecular mechanisms that link the glutamatergic system to depressive phenotype. Early evidence suggests that a glutamate based mechanism could provide a common pathway linking other proposed pathophysiological mechanisms of depression, such as those focusing on the monoamine and the corticosteroid systems.

Synaptic signaling within the glutamatergic system involves complex interactions between the glutamate secreting neuron, the post-synaptic cell expressing glutamate receptors and surrounding neuroglia. This arrangement is referred to as the tripartite glutamate synapse (discussed in chapter 1, section 1.5.5.1). Signal transmission within this synapse is an energy intense process that consumes as much as 80% of the energy derived from cerebral oxidative metabolism (Shulman *et al.*, 2002). Theoretically, perturbations to any of the components of this synaptic arrangement could contribute to the pathogenesis of MDD.

In order to understand the neurochemical underpinnings of the glutamatergic system in MDD, it is necessary to carry out assessments in vivo. This has been facilitated by the availability of the technique of Magnetic Resonance Spectroscopy (MRS, discussed in chapter 2). Since its first application for brain research by Cady and colleagues (1983), who used it to investigate biochemical changes in neonatal birth asphyxia, MRS has undergone many technical advances (Oz *et al.*, 2014, Soares and Law, 2009). In particular, improvements in methods for acquisition and analysis of spectra, together with availability of ultra-high-field (≥ 4 tesla) MRI scanners, have enabled more accurate quantification of neurometabolites in both physiological and pathological states.

Earlier studies investigating ^{1}H-MRS quantifiable neurometabolites in MDD focused on imaging the occipital cortex (Sanacora *et al.*, 1999, Sanacora *et al.*, 2003, Sanacora *et al.*, 2002). Although this region is not considered to be a key component of the emotional regulatory network (Drevets *et al.*, 2008, Phillips *et al.*, 2003), it was favored by these studies due to the relative ease of signal acquisition from it. Increasingly however, ^{1}H-MRS has been applied to investigate neurochemical changes in a wide variety of regions including those within established emotional regulatory networks such as the prefrontal cortex and the striatum.

Most of the clinical and research scanners capable of carrying out ^{1}H-MRS are available at field strengths 1.5 tesla to 3-tesla. While such scanners are useful in quantifying metabolites with single, non-overlapping resonances on the MRS spectrum, for example N-acetyl-aspartate (NAA) and creatinine, they are less

reliable for quantify the glutamatergic metabolites glutamate and glutamine. At field strength of 3-tesla or lower, glutamate and glutamine form complex overlapping resonances distributed between 2.1 and 2.4 ppm on the MRS chemical shift axis. This is further complicated by the fact that with short-echo time imaging, a technique that provides increased metabolite coverage, this region (2.1-2.4ppm) also contains nuisance signals from macromolecular complexes and unsuppressed lipids. As such, most of the investigations carried at 3-tesla or lower have mainly reported a composite resonance composed mainly of glutamate and glutamine, and referred to as Glx.

Accurate quantification of glutamate and glutamine using short-echo time MRS has been demonstrated at scanner field strengths of 4-tesla and higher (Emir *et al.,* 2012, Gruetter *et al.*, 1998, Mekle *et al.*, 2009, Tkac *et al.*, 2009). Separate quantification of these metabolites is particularly important for localizing pathology to either neuronal cells, which predominantly contain glutamate, or to neuroglia, which contain glutamine (Erecinska and Silver, 1990, Lehmann *et al.*, 1983). In addition, there is evidence that glutamine (and perhaps glutamate) concentrations may vary in opposite directions in the two primary mood disorders, MDD and bipolar disorder (Taylor, 2014). Therefore, separate quantification of glutamate and glutamine could potentially serve as a biomarker that could assist in the diagnosis of the two disorders, which often present with similar symptoms at the outset (Phillips and Kupfer, 2013).

Studies investigating glutamatergic changes in MDD have yielded a plethora of findings, at times contradictory. These have ranged from isolated changes to glutamate, glutamine or Glx concentrations, to a lack of demonstrable metabolite concentration differences (reviewed in Arnone et al., 2015; Yildiz-Yesiloglu & Ankerst, 2006; Yuksel & Ongur, 2010). These findings have been further complicated by observations that the glutamatergic changes in MDD may show regional and sub-regional variations. The direction of change has however been more consistently reported for the prefrontal cortex, a region with established roles in emotional regulation (Drevets *et al.*, 2008, Phillips *et al.*, 2003). Review studies have reported decreases in either glutamate or Glx concentrations within this region (Arnone *et al.*, 2015, Yildiz-Yesiloglu and Ankerst, 2006, Yuksel and Ongur, 2010). This is in contrast to the reported findings from the occipital cortex, where glutamate and Glx levels are suggested to be elevated (Yildiz-Yesiloglu and Ankerst, 2006).

In order to determine more accurately the specific changes to the glutamatergic system in MDD, it is necessary to accurately quantify the metabolites at ultra-high field strength. The gains in spectral dispersal and signal-to noise ratio (SNR) with this approach allow for disambiguation of the underlying neurometabolite concentration changes, thus providing a clearer picture of the pathophysiological mechanisms (Emir *et al.*, 2012, Tkac *et al.*, 2009).

Beyond investigating the prefrontal and occipital cortices, there is increasing interest into the role of subcortical areas in the regulation of mood. Preclinical work

and functional neuroimaging studies have provided evidence for the involvement of the ventral striatum (a region that includes the putamen) in the pathophysiology of MDD (Phillips *et al.*, 2003). This region is involved in the processing of aversive and rewarding emotional information, and could therefore mediate some of the symptoms of depression, such as anhedonia and decreased motivation (Berton and Nestler, 2006).

Understanding glutamatergic neurochemistry in MDD would be useful not only in elucidating disease mechanisms, but also in guiding targeted drug development based on known effects of pharmacological manipulation on the system. It could also serve as a diagnostic adjuvant with utility where the presenting mood symptoms are ambiguous. In addition, glutamatergic MRS could assist in sub-categorisation of patients into more homogenous research and treatment groups, and by so doing, assist in the selection of patients more likely to benefit from treatment with glutamate modulating drugs. This study therefore aimed to investigate glutamatergic neurochemistry in MDD using 7-tesla ^{1}H-MRS, focusing on the anterior cingulate cortex (ACC), occipital cortex and putamen.

3.2: Methods:

3.2.1 Study Eligibility

Participants aged between 18 to 70 years were recruited through Oxford Health NHS Foundation Trust mental health clinics following referral by consultants and research assistants. Additional recruitment was carried out through advertisement in local newspapers. The study group comprised medication free patients with MDD and healthy control subjects. These subjects were recruited as part of an ongoing study investigating the link between depression, neurochemistry and inflammation.

Healthy control subjects were selected to match for age and gender to the patients already enrolled in the study. Due to the approach of recruiting control subjects only after recruitment of patients, the final sample included in the analysis for this thesis was not balanced in numbers within the specific gender groups (details in the results section). As this is an ongoing study, this limitation will be overcome at completion of subject recruitment.

Before enrolment, patients were screened for current MDD according to the DSM-IV TR criteria (American Psychiatric Association, 2000), and for other Axis 1 Disorders using the Structured Clinical Interview for DSM-IV Axis I Disorders Schedule (SCID-I, First *et al.,* 1996). Medication free patients (not on antidepressant medication for a period of at least two weeks) with confirmed MDD were included in the study. Patients at clinically significant risk of suicidal behavior were excluded from participation. Healthy controls were excluded if they had a present or past Axis I psychiatric disorder.

Exclusion criteria for all participants included current history of DSM-IV TR substance dependence or psychosis, contraindications to MRI scanning (including claustrophobia and metallic implants), concurrent medications that could interfere with MRS scanning, pregnancy or lactation and participation in any research study within a month prior to inclusion. All participants gave written informed consent to participate in the study.

3.2.2 Participants

The final participant sample included 40 patients with MDD (22 Female) and 32 healthy control subjects (14 Female). All patients had a psychiatric interview at the screening visit. In addition to the psychiatric interview, demographic data (age, gender, and body mass index), smoking status and details on alcohol consumption were also collected. Results of the various clinical parameters are presented in chapter 4.

On the scan day, the Hamilton Depression Rating Scale (HAM-D, Hamilton, 1960), was administered. Participants also completed the Beck Depression Inventory (BDI, Beck *et al.*, 1961), Snaith-Hamilton Pleasure Scale (SHPS, Snaith *et al.*, 1995), state measure of the State-Trait Anxiety Inventory, (STAI, Spielberger *et al.,* 1983), the Chalder Fatigue Scale (CFS, Chalder *et al.*, 1993) and the Childhood Trauma Questionnaire (CTQ, Bernstein *et al.*, 1994).

3.2.3 Magnetic Resonance Spectroscopy

Proton-MRS scanning took place at the Functional Magnetic Resonance Imaging of the Brain (FMRIB) Centre. Scanning was performed on a 7-tesla Siemens MAGNETOM scanner (Siemens, Erlangen, Germany) equipped with a Nova Medical 32 channel receive array head coil.

To assist with MRS voxel placement, high resolution 3-dimensional T1-weighted structural images were acquired with a Magnetisation Prepared RApid Gradient-Echo (MP-RAGE) sequence (Brant-Zawadzki *et al.*, 1992), with FOV=192×192 mm^2, TR = 2.2 s, TE = 2.82 ms, slice thickness = 1 mm, non- selective inversion, scan time 3 min.

Spectra were measured from three voxels manually placed in the pre-genu ACC (20x20x20mm^3), the occipital cortex, centered on the calcarine sulcus (20x20x20mm^3), and the right putamen (oblique voxel, 10x16x20mm^3, Figure 3.1). First- and second-order shims were first adjusted by gradient-echo shimming (Shah *et al.,* 2009) followed by fine adjustment of first order shims using FASTMAP (Gruetter and Tkac, 2000).

Spectra were acquired using a semi-LASER sequence (Scheenen *et al.,* 2008), TE=36 ms, TR = 7 s, NT = 64, with variable power radiofrequency pulses with optimized relaxation delays (VAPOR) water suppression and outer volume saturation (Emir *et al.*, 2012). Unsuppressed water spectra acquired from the same voxel were used to remove residual eddy current effects and to reconstruct the phased array spectra.

Metabolites were quantified using LCModel (Provencher, 2001). The model spectra of aspartate (Asp), ascorbate/vitamin C (Asc), glycerophosphocholine (GPC), phosphocholine (PC), creatine (Cr), phosphocreatine (PCr), γ-amino-butyric acid (GABA), glucose (Glc), glutamine (Gln), Glutamate (Glu), glutathione (GSH), myo-inositol (myo-Ins), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphoethanolamine (PE), scyllo-inositol (scyllo-Ins) and taurine (Tau) were generated based on previously reported chemical shifts and coupling constants (Govindaraju *et al.*, 2000) by using GAMMA/PyGAMMA simulation library of VESPA (VErsatile Simulation Pulses and Analysis, Soher *et al.,* 2011) for carrying out the density matrix formalism. Simulations were performed with the same RF pulses and sequence timings as that on the 7-tesla system. A macromolecule spectrum acquired from the occipital cortex using an inversion recovery sequence was included in the model spectra. Metabolite concentrations were obtained relative to an unsuppressed water spectrum acquired from the same voxel of interest, assuming water content of 82% for the ACC and OCC, and 78% for the putamen (Emir *et al.*, 2012).

The MPRAGE images were segmented using FAST (FMRIB 's Automated Segmentation Tool, part of the FSL toolbox (Zhang *et al.*, 2001), to determine CSF fraction, gray matter and white matter content. Metabolite concentrations were then corrected for CSF fraction with the following formula:

$$[\,M_{corr}\,] = [M].\left(\frac{1}{1-\mathrm{fCSF}}\right)$$

where $[M_{corr}]$ = corrected concentration and [M] = metabolite concentration from LCModel output.

Metabolites quantified with Cramér-Rao lower bounds (CRLB, estimated error of the metabolite quantification) >50% were classified as not detected. As a secondary filter to select reliable metabolite concentrations, only metabolites quantified with CRLB ≤50% in at least half of the spectra from a brain region were included. The primary metabolites of interest for this study were glutamate and glutamine, and their combined signal, Glx.

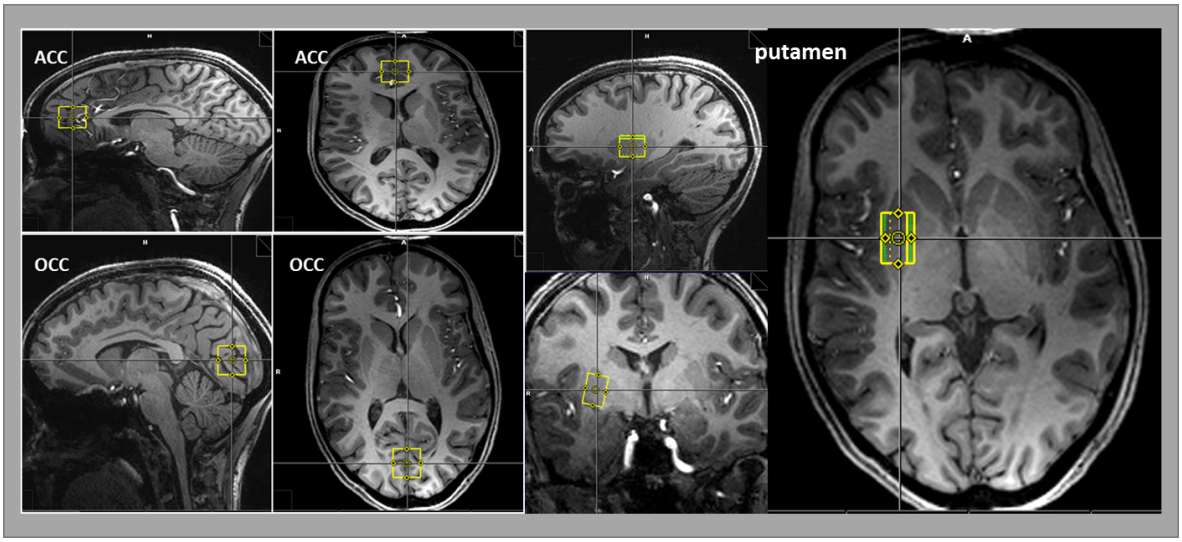

Figure 3.1: MRS Voxel placement in the anterior cingulate cortex (ACC), occipital cortex (OCC) and putamen. Voxels were manually placed with reference to high resolution (1mm isotropic) T1 weighted images.

3.2.4 Statistical Analysis

Statistical analyses were performed in SPSS version 22. Independent samples t-tests were used to compare differences between patients and controls on the demographic data and questionnaire scores. A chi-square test was performed to investigate differences in the proportions of male and female subjects between the groups (depressed patients and controls). MRS data (glutamate, glutamine and Glx) were analysed using separate multivariate analysis of variance (MANOVA) for each

region (ACC, OCC and putamen). A 3x2 MANOVA was used for each region, with the metabolites glutamate, glutamine and Glx (glutamate+glutamine) as dependent variables, and group (depressed patients vs. controls) and gender (male vs. female) as fixed factors. Significant main effects on the MANOVA were followed up with pairwise comparisons using t-tests. Post-hoc comparisons of group differences in the glutamine to glutamate ratio were also carried out using t-tests.

3.3: RESULTS

3.3.1 Participants

Depressed patients and controls did not differ significantly with respect to age and gender. There was however proportionately more women in the depressed group (22 females patients and 14 female controls, compared with 18 male patients and 18 male controls), a weakness for this study. This difference was however not statistically significant {$X2$ (2, N = 72) = 0.900, p = 0.343}. The mean HAM-D score in the patient group was 21.1 ± 5.2 (±SD; range 11–32). Patients and controls differed in their ratings of current symptoms of depression, anxiety, anhedonia and fatigue (Table 3:1). Depressed patients and controls also differed on the measures of childhood trauma (Table 3.2).

3.3.2 Magnetic Resonance Spectroscopy

MRS spectra were obtained from all 40 patients and 32 control subjects included in the study. Four spectra from the ACC, 6 from the OCC and 2 from the putamen were excluded following analysis due to poor quality. The final sample analyzed consisted of 68 spectra from the ACC (39 patients), 66 from the occipital cortex (36 patients) and 70 from the putamen (39 patients). There were no significant differences between patients and controls in the voxel water-linewidths, SNR, gray matter, white matter or CSF content (Table 3.3). Metabolite CRLB values have been reported in table 3.4. Spectral averages and distribution have been plotted in figure 3.2.

3.3.3 Anterior Cingulate Cortex

MANOVA for the ACC revealed no main effect of group (controls vs. patients) on the absolute metabolite concentrations (Wilks' Lambda $F_{2,63} = 1.109$, $p = 0.336$). There was however a main effect of gender on metabolite concentrations ($F_{2,63} = 3.944$, $p = 0.024$), and significant group x gender x metabolite interactions ($F_{2,63} = 5.757$, $p = 0.005$). Post-hoc analysis revealed that the gender effect was driven by differences in the concentrations of glutamate ($F_{1,64} = 7.735$, $p = 0.007$) and Glx ($F_{1,64} = 4.450$, $p = 0.039$). Follow-up pairwise comparisons of metabolite concentrations revealed significant decreases in the concentrations of glutamate [$t_{(33)} = 3.044$, $p = 0.005$] and Glx [$t_{(33)} = 2.719$, $p = 0.010$] in female patients relative to female controls (Table 3.5, Figure 3.3). Male patients had a significantly elevated Glx concentration relative to male controls [$t_{(31)} = -2.080$, $p = 0.046$, Figure 3.3]. There were no significant group differences in the glutamine to glutamate ratio. Analysis was repeated using relative (/Cr) metabolite concentrations with similar trends being observed (Table 3.6).

3.3.4 Occipital cortex

MANOVA for the occipital cortex revealed neither a main effect of group ($F_{2,61} = 2.161$, $p = 0.124$) nor gender ($F_{2,61} = 1.973$, $p = 0.148$) on absolute metabolite concentrations. There were no significant group x gender x metabolite interactions ($F_{2,61} = 1.625$, $p = 0.205$).

3.3.5 Putamen

There was no main effect of gender on the putamen metabolite concentrations.

There was a main effect of group (control vs. patients) on absolute metabolite concentrations ($F_{2,65} = 3.372$, $p = 0.040$). There were no significant group x gender x metabolite interactions ($F_{2,65} = 0.230$, $p = 0.795$).

Follow up pairwise comparisons revealed that the significant group effect was driven by differences in putamen glutamine concentrations ($F_{1,66} = 6.374$, $p = 0.014$), with depressed patients having a higher glutamine concentration relative to controls (Table 3.7, Figure 3.4). There was a significantly higher glutamine to glutamate ratio in depressed patients relative to controls ($p = 0.036$). A similar pattern was obtained with analysis using relative (/Cr) metabolite concentrations (Table 3.7).

Table 3.1: Participant characteristics. BDI, Beck Depression Inventory; HAM-D, 17-item Hamilton Depression Rating Scale; STAI, state measure of the State-Trait Anxiety Inventory; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale - total sum of scores; CFS-11, Chalder Fatigue Scale - 11-item scoring. Values represent mean ± SEM.

		Controls (N=32)	Patients (N=40)	Statistic: p value
Age, mean ± SEM (range)	**All**	32.3 ± 2.0 (19-62)	32. 7 ± 1.9 (18-63)	0.570
	M	34.6 ± 3.0 (19-62)	35.4 ± 3.3 (20-63)	0.862
	F	29.3 ± 2.4 (19-51)	30.5 ± 2.2 (18-59)	0.722
Gender	**M**	18	18	
	F	14	22	
BDI (Mean ± SEM)	**All**	1 ± 0	30 ± 1	<0.001
	M	1 ± 0	27 ± 2	<0.001
	F	1 ± 1	33 ± 2	<0.001
HAM-D (Mean ± SEM)	**All**	0 ± 0	21 ± 1	<0.001
	M	0 ± 0	19 ± 1	<0.001
	F	0 ± 0	23 ± 1	<0.001
STAI (Mean ± SEM)	**All**	25 ± 1	51 ± 2	<0.001
	M	26 ± 2	50 ± 3	<0.001
	F	24 ± 1	51 ± 3	<0.001
SHPS	**All**	18 ± 1	34 ± 1	<0.001
	M	20 ± 1	33 ± 1	<0.001
	F	17 ± 1	34 ± 2	<0.001
CFS	**All**	10 ± 1	22 ± 1	<0.001
	M	9 ± 1	21 ± 1	<0.001
	F	10 ± 1	23 ± 1	<0.001
CFS-11	**All**	0 ± 0	8 ± 0	<0.001
	M	1 ± 0	7 ± 1	<0.001
	F	0 ± 0	9 ±1	<0.001

Table 3.2: Participant scores on the childhood trauma questionnaire (CTQ, mean ± SEM)

		Controls	Patients	Statistic: p value
Emotional abuse	**All**	6.4 ± 0.4	12.8 ± 1.1	<0.001
	M	6.1 ± 0.4	9.8 ± 1.5	0.013
	F	6.8 ± 0.8	14.6 ± 1.4	<0.001
Physical abuse	**All**	5.1 ± 0.1	7.8 ± 0.6	<0.001
	M	5.0 ± 0.1	7.1 ± 0.9	0.009
	F	5.2 ± 0.2	8.3 ± 0.8	0.011
Sexual abuse	**All**	5.1 ± 0.1	6.4 ± 0.6	0.054
	M	5.1 ± 0.1	5.4 ± 0.3	0.267
	F	5.0 ± 0.0	7.0 ± 1.0	0.155
Emotional neglect	**All**	8.0 ± 0.6	13.2 ± 0.9	<0.001
	M	8.2 ± 0.9	11.8 ± 1.6	0.040
	F	7.7 ± 0.9	14.1 ± 1.0	<0.001
Physical neglect	**All**	6.0 ± 0.4	8.5 ± 0.7	0.005
	M	6.1 ± 0.6	6.8 ± 1.0	0.549
	F	5.8 ± 0.5	9.5 ± 0.9	0.009
CTQ _ Total score	**All**	30.6 ± 1.3	48.6 ± 3.0	<0.001
	M	30.6 ± 1.7	40.8 ± 4.5	0.023
	F	30.5 ± 2.0	53.4 ± 3.7	<0.001

Table 3.3: Voxel tissue concentrations, signal-to-noise ratio (SNR) and water linewidths for the ACC, occipital cortex and putamen.

Parameter: (mean ± SEM)	Controls	Patients	p value
ACC grey matter	0.65 ± 0.01	0.64 ± 0.01	0.187
ACC white matter	0.20 ± 0.01	0.20 ± 0.01	0.538
ACC CSF	0.15 ± 0.01	0.16 ± 0.01	0.575
ACC SNR	47.36 ± 2.10	43.22 ± 1.76	0.134
ACC water linewidth	15.42 ± 0.52	15.51 ± 0.45	0.896
OCC grey matter	0.54 ± 0.01	0.52 ± 0.01	0.138
OCC white matter	0.36 ± 0.01	0.37 ± 0.01	0.477
OCC CSF	0.10 ± 0.01	0.11 ± 0.01	0.234
OCC SNR	43.54 ± 1.83	44.36 ± 1.83	0.741
OCC water linewidth	13.72 ± 0.29	13.54 ± 0.42	0.748
Putamen grey matter	0.29 ± 0.02	0.27 ± 0.01	0.231
Putamen white matter	0.71 ± 0.02	0.73 ± 0.01	0.216
Putamen CSF	0.00 ± 0.00	0.00 ± 0.00	0.154
Putamen SNR	18.14 ± 1.02	18.39 ± 0.86	0.853
Putamen water linewidth	19.01 ± 0.60	18.11 ± 0.41	0.207

Table 3.4: Metabolite Cramér–Rao lower bound (CRLB, mean ± SEM) values

	Glutamate	Glutamine	Glx
ACC	2.8 ± 0.8	10.7 ± 0.5	3.4 ± 0.1
OCC	5.4 ± 0.1	9.3 ± 0.3	4.9 ± 0.1
Putamen	8.8 ± 0.5	19.0 ± 0.9	6.1 ± 0.2

Table 3.5: Absolute metabolite concentrations of glutamate, glutamine and Glx, as well as the glutamine: glutamate (Gln:Glu) ratio in the ACC. Significant differences in glutamate and Glx concentrations were present between patients and controls among female subjects. There were significant differences in the Glx concentrations among male subjects. * $p < 0.05$

Metabolite		Control (mean ± SEM)	Patients (mean ± SEM)	Statistic: p value
ACC glutamate	All	10.54 ± 0.28	10.21 ± 0.15	0.269
	M	9.83 ± 0.22	10.27 ± 0.27	0.207
	F	11.43 ± 0.46	10.17 ± 0.17	**0.005***
ACC Glutamine	All	4.75 ± 0.17	4.68 ± 0.14	0.730
	M	4.50 ± 0.13	4.91 ± 0.21	0.119
	F	5.06 ± 0.32	4.50 ± 0.19	0.115
ACC Glx	All	15.30 ± 0.40	14.90 ± 0.22	0.347
	M	14.33 ± 0.22	15.18 ± 0.34	**0.046***
	F	16.50 ± 0.73	14.68 ± 0.28	**0.010***
ACC Gln:Glu	All	0.45 ± 0.01	0.46 ± 0.02	0.737
	M	0.46 ± 0.02	0.48 ± 0.02	0.531
	F	0.44 ± 0.02	0.44 ± 0.02	0.981

Table 3.6: Relative (/Cr) metabolite concentrations of glutamate, glutamine and Glx, as well as the glutamine: glutamate (Gln:Glu) ratio in the ACC. Significant differences in glutamate and Glx concentrations were present between patients and controls among female subjects. There were significant differences in the Glx concentrations among male subjects. * $p < 0.05$

Metabolite		Control (mean ± SEM)	Patients (mean ± SEM)	Statistic: p value
ACC glutamate	All	11.05 ± 0.34	10.68 ± 0.15	0.279
	M	10.15 ± 0.16	10.67 ± 0.27	0.109
	F	12.15 ± 0.60	10.68 ± 0.18	**0.008***
ACC Glutamine	All	4.99 ± 0.21	4.91 ± 0.17	0.745
	M	4.67 ± 0.15	5.13 ± 0.28	0.159
	F	5.40 ± 0.41	4.73 ± 0.20	0.114
ACC Glx	All	16.04 ± 0.51	15.58 ± 0.26	0.391
	M	14.82 ± 0.18	15.81 ± 0.43	**0.048***
	F	17.54 ± 0.97	15.41 ± 0.31	**0.017***
ACC Gln:Glu	All	0.45 ± 0.01	0.46 ± 0.02	0.737
	M	0.46 ± 0.02	0.48 ± 0.02	0.530
	F	0.44 ± 0.02	0.44 ± 0.02	0.981

Table 3.7: Concentrations of glutamate, glutamine and Glx, as well as the glutamine: glutamate (Gln:Glu) ratio, in the putamen. Significant differences in the glutamine concentrations, and in the Gln:Glu ratio, were present between patients and controls. Relative concentrations have been scaled with reference to total-creatine. * $p < 0.05$

		Control (mean ± SEM)	Patients (mean ± SEM)	Statistic: p value
Putamen (Absolute)	Glutamate	7.44 ± 0.20	7.06 ± 0.21	0.207
	Glutamine	4.36 ± 0.20	4.95 ± 0.17	**0.027***
	Glx	11.79 ± 0.25	12.01 ± 0.24	0.533
	Glu:Gln	0.60 ± 0.03	0.76 ± 0.06	**0.036***
Putamen (Relative)	Glutamate	5.83 ± 0.18	5.58 ± 0.16	0.310
	Glutamine	3.41 ± 0.16	3.97 ± 0.16	**0.019***
	Glx	9.23 ± 0.23	9.55 ± 0.21	0.319
	Gln:Glu	0.60 ± 0.03	0.76 ± 0.06	**0.036***

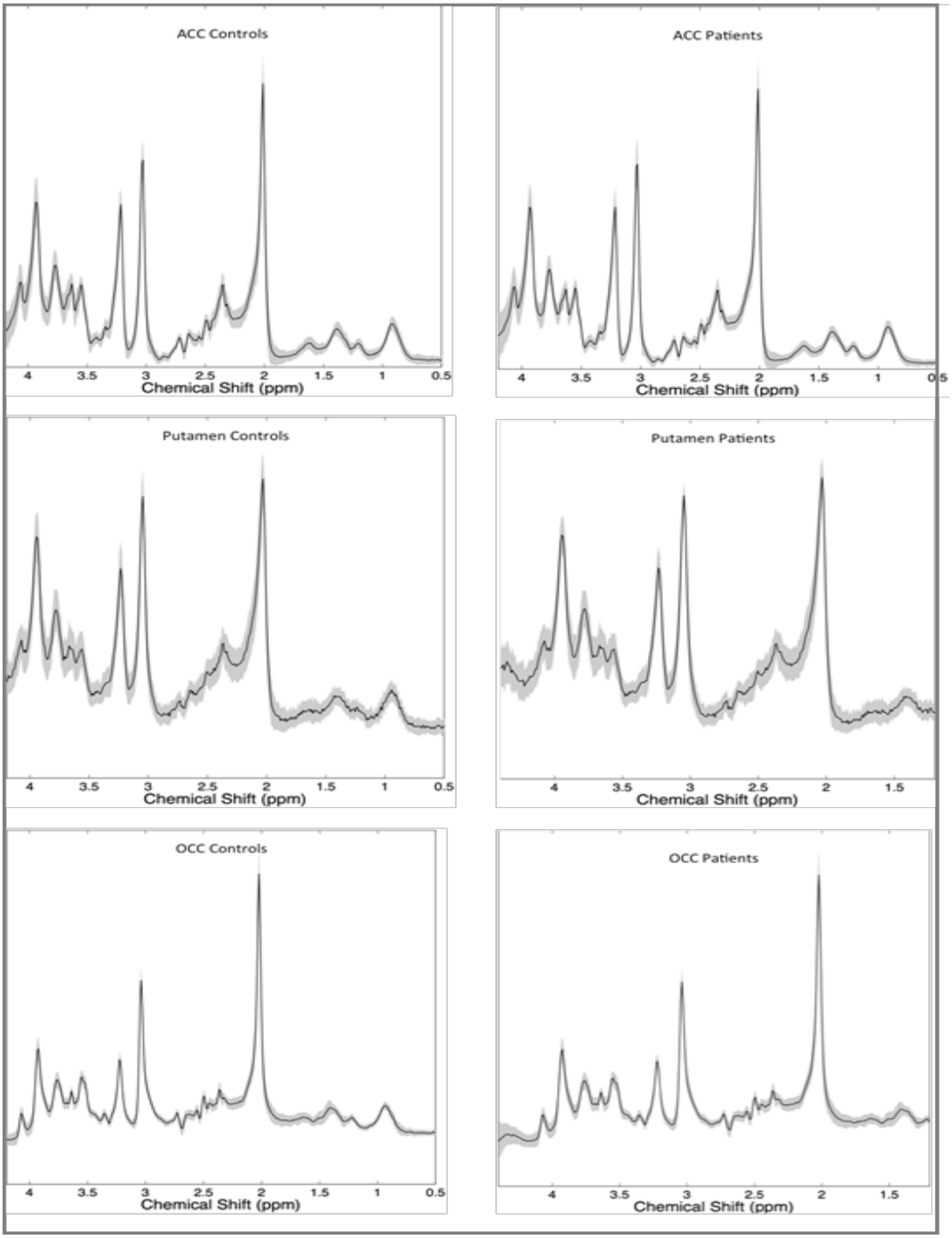

Figure 3.2: Average plots for ^{1}H-MRS spectra acquired from the Anterior Cingulate Cortex (ACC), Occipital Cortex (OCC) and Putamen. Spectra were acquired with a semi-LASER sequence (TR = 7s; TE = 36 ms; NT = 64). Shaded regions correspond to the standard deviations.

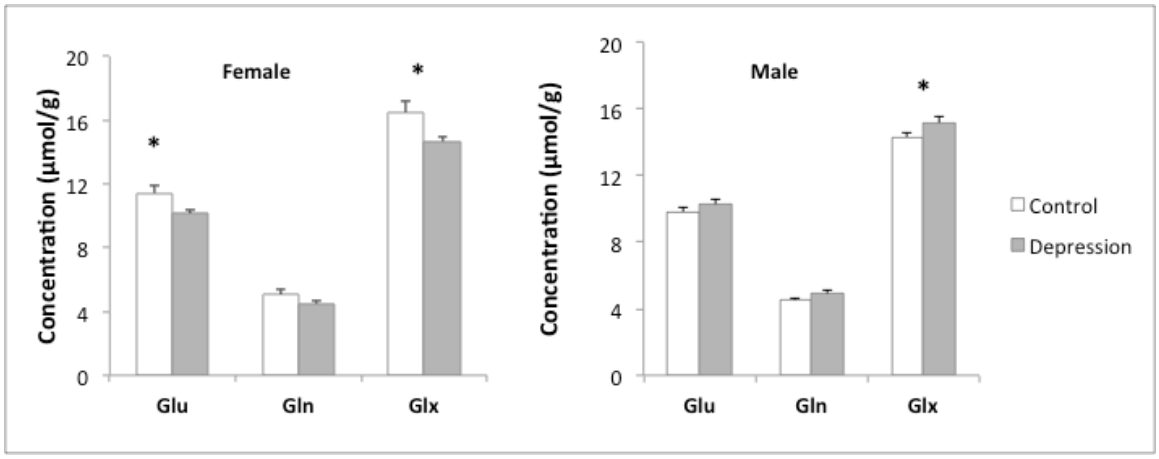

Figure 3.3: Comparison of the absolute concentrations of glutamate, glutamine and Glx measurements from the ACC, between depressed subjects (dark bars) and controls. Significant metabolite concentration differences between patients and controls were present in the glutamate and Glx concentrations for female subjects, and in the Glx concentrations for the male subjects.

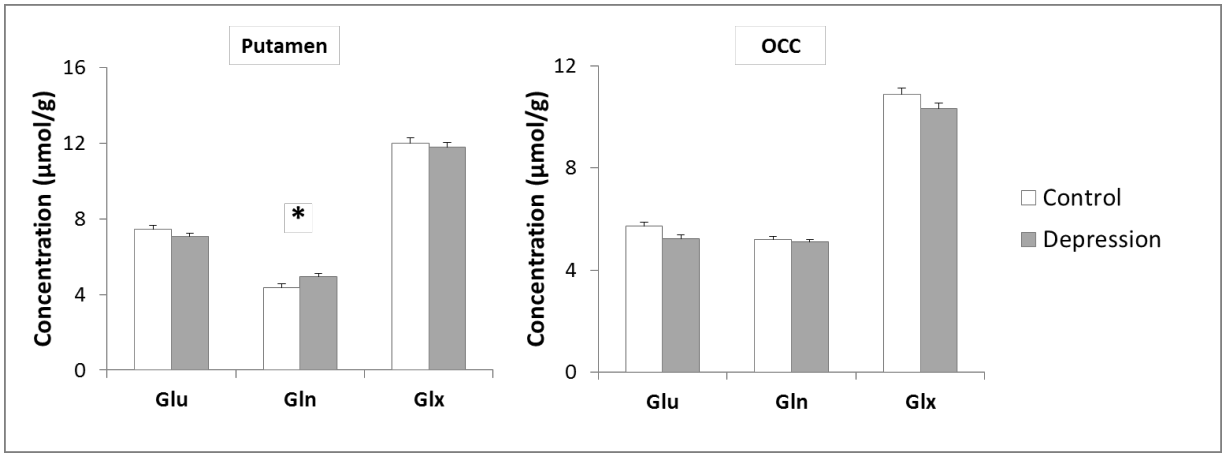

Figure 3.4: Comparison of the absolute concentrations of glutamate, glutamine and Glx measurements from the putamen and occipital cortex, between depressed subjects (dark bars) and controls. Significant group differences were present in the putamen glutamine concentrations (*).

3.4: Discussion

The main findings of this study were a decrease in the ACC glutamate and Glx concentrations in female depressed patients relative to controls, without significant changes to the glutamine concentrations. There was also a significant increase in the putamen glutamine concentrations in patients (both male and female) relative to controls.

3.4.1 Anterior Cingulate Cortex

The ACC subserves emotional and cognitive roles (Kennerley *et al.*, 2006) and has been the subject of intense research into the neurobiological mechanisms of MDD (Ebert and Ebmeier, 1996). At the neuropathological level, changes within this region that are relevant to depression pathophysiology have been demonstrated. These include decreased number and density of glia cells (Rajkowska and Miguel-Hidalgo, 2007), decreased neuronal soma size (Chana *et al.*, 2003, Nestler *et al.*, 2002), increased white matter intensities (Coffey *et al.*, 1993), and enzymatic alterations involving astrocytic uptake transporters and the glutamine synthetase enzyme (Choudary *et al.*, 2005, Ongur *et al.*, 1998). Neuroimaging studies have demonstrated decreased metabolic activity and blood flow in the prefrontal cortex that normalizes following antidepressant treatment or with symptom resolution (Bench *et al.*, 1995, Kennedy *et al.*, 2001).

Although MRS studies have identified a potential neurochemical basis for MDD involving the ACC and other prefrontal cortical areas, the nature and direction of these changes has largely remained equivocal. A recent meta-analysis that specifically focused on the glutamatergic system within the prefrontal cortex

reported a decrease in Glx, without significant change to the glutamate signal (Arnone *et al.*, 2015). Based on this observation, the authors postulated that the decrease in Glx levels was most likely being driven by a decrease in glutamine levels. Taking into consideration that glutamine is predominantly synthesized and localized within astrocytes, a selective decrease in glutamine concentrations points towards a glia-based neuropathological mechanism. The results from the individual studies included in the meta-analysis were however quite varied, including reports of no changes to glutamate, glutamine or Glx concentrations (Brambilla *et al.*, 2005, Hasler *et al.*, 2005, Nery *et al.*, 2009, Price *et al.*, 2009). In addition, the reported decrease in prefrontal cortical concentrations of glutamate and glutamine contradict neurosurgical and post-mortem findings reporting no changes (Francis *et al.*, 1989) or increased glutamate levels (Hashimoto *et al.*, 2007).

Several methodological limitations could have contributed to the variability in the reported neurochemical findings in the prefrontal cortex in MDD. First of these is the use of low scanner field strengths resulting in inaccurate quantification of neurometabolite levels. Secondly, the use of modest sample sizes (typically less than 30 patients, see Table 3.8 below) that decreases the ability to identify consistent change patterns. The biochemical changes may also show sub-regional specificity (Grimm *et al.*, 2012, Hasler *et al.*, 2007, Palomero-Gallagher *et al.*, 2009), such that while decreased glutamate, or Glx levels may be present in one sub-region, others may demonstrate no change, or change in the opposite direction. Two out of three published studies that used an ACC voxel (similar to the present study) reported a

decrease in glutamate concentrations within this region in depressed patients (Table 3.8).

Additional confounding factors include patient selection (medicated vs. non-medicated, treatment resistant vs. treatment responders, severely depressed vs. moderately depressed) and variability in MRS techniques. There have not been any publications to date using ultra-high field imaging to investigate neurometabolite concentrations in depression, an approach that would be necessary to overcome some of the technical limitations of 1.5 and 3-tesla imaging.

In the present study, a decrease in ACC glutamate levels was present only in female patients (when compared to female controls). In fact, male patients had significantly elevated ACC Glx concentration. This gender specific finding was unexpected and would need to be investigated further. It is possible that the finding represents a type 1 error, arising from the non-balanced gender proportions in the depressed and control groups (a limitation for the present study). The decrease in glutamate and Glx concentrations among female patients was however strongly significant, suggesting that this effect may persist even after the proportions are balanced at the completion of the study. Male and female patients differed on some clinical parameters such as measures of treatment severity and childhood trauma (Tables 3.1 and 3.2, also discussed in chapter 4), which could have accounted for the gender effects. A review of the studies included in the most recent published meta-analysis of prefrontal ^{1}H-MRS metabolites in depression (Arnone *et al.*, 2015) revealed that in some of the studies the gender proportions were also not balanced (Table 3.8,

modification reflecting gender proportions). Indeed the majority of the studies included more female depressed participants than male- unsurprisingly because, as noted in chapter 1, depression is more common in women. This observation highlights the importance of considering gender effects in MRS metabolite quantification. Although some studies in the meta-analysis carried out analysis for gender effects, no significant effects were reported for the glutamate metabolites. Nery and colleagues (2009) however reported significant gender effects on creatinine concentrations, which might influence studies where glutamate metabolites are referenced to creatine.

Gender differences in neurometabolite concentrations are an important finding and if indeed true, would suggest gender specific pathophysiological mechanisms in depression. Effects of sex steroids on the developing brain, and particularly on the GABA system, have been described (McCarthy *et al.*, 2002). Phase of the menstrual cycle has also been reported to affect brain neurochemistry (Batra *et al.*, 2008, Epperson *et al.*, 2002, Silveri *et al.*, 2013). Previous MRS studies on healthy subjects have found an effect of gender on MRS measurements of glutamate (O'Gorman *et al.*, 2011, Sailasuta *et al.*, 2008). Results from these studies suggest that concentrations of glutamate are higher in men relative to women. These differences may however change with age, as men but not women show significant age related decreases in glutamate concentrations (Sailasuta *et al.*, 2008). These observations would however not explain the significant elevated concentrations of Glx in depressed men, but decreased concentrations in depressed women, that were noted in the present study. In fact, women (both depressed and controls) had numerically higher concentrations of both glutamate and Glx relative to men. Hasler and colleagues

(2007) however observed that Glx reductions within two prefrontal cortical voxels were more prominent in depressed women than in men, when compared with control subjects, although the gender differences did not attain statistical significance.

Table 3.8: Studies investigating changes to glutamate or Glx levels within the prefrontal cortex in patients with major depressive disorder. The table shows the study sample distribution by gender (with columns representing the percentage of female subjects in the patient and control groups), as well as voxel of interest region. WM, white matter; PFC, prefrontal cortex; dl, dorsolateral; A, anterior, DA, dorsal-anterior; VM, ventromedial; M, medial; MCC, mid-cingulate cortex. Studies were identified from Arnone et al. 2015.

↓ Decreased concentration ↑ Increased concentration ↔ No change

Region	Study	Patients (% Female)	Controls (% Female)	Glutamate or Glx changes
ACC	Jarnum et al. 2011	70%	50%	↓
ACC	Walter et al. 2009	58%	75%	↔
ACC	Auer et al. 2000	68%	56%	↓
Left and right frontal	Gruber et al. 2003	53%	53%	↔
Frontal WM	Frey et al. 2003	58%	58%	↔
Left DLPFC	Michael et al. 2003	50%	50%	↓
Left APFC	Pfleiderer et al. 2003	71%	71%	↓
VM-PFC & DM-DA-PFC	Hasler et al. 2005	75%	75%	↔
DLPFC	Ajilore et al. 2007	71%	71%	↓
VM-PFC & DM-DA-PFC	Hasler et al. 2007	65%	65%	↓
Left DPFC	Bernier et al. 2009	100%*	100%*	↔
Left DLPFC	Nery et al. 2009	65%	65%	↔
VM-PFC	Portella et al. 2011	78%	67%	↓
MPFC	McEwen et al. 2012	100%*	100%*	↑
Left DLPFC & MCC	Grimm et al. 2013	50%	50%	↑
PFC	Sozeri-Varma et al. 2013	8%	25%	↔

* Only female subjects included in study

The phase of menstrual cycle was not determined in the present study (a limitation). This information would have enabled determination of any menstrual phase effects on metabolite concentration differences among female subjects. Although age has also been identified as a potential modifier of neurometabolite levels (Chang *et al.*, 2009, Kaiser *et al.*, 2005, Sailasuta *et al.*, 2008), age effects have been shown to be minimal when only adult subjects are recruited. In the present study, addition of age as a covariate on the MANOVA (data not presented) did not change the overall findings.

3.4.2 Occipital cortex

Although the occipital cortex is not considered a core component of the mood regulatory network, PET studies investigating serotonin 1A receptor binding (Drevets *et al.*, 1999) and functional neuroimaging studies on emotional processing (Li *et al.*, 2013) have provided some evidence for its involvement in mood regulation. The lack of significant changes to glutamatergic metabolite concentrations within the occipital cortex observed in the present study was unexpected, as elevation in Glx concentrations have been previously reported (Sanacora *et al.*, 2004). Elevated occipital glutamate levels have also been reported in unmediated MDD patients in remission (Bhagwagar *et al.*, 2007) as well as in young unaffected individuals (ages 16-21 years) at increased risk of depression based of familial predisposition (Taylor *et al.*, 2011). These findings suggest that elevations in occipital glutamate levels could be a trait marker representing vulnerability to depression. Other studies have however not found any significant changes to glutamatergic metabolites within the occipital cortex in MDD (Godlewska *et al.*, 2015, Price *et al.*, 2009). Occipital cortex glutamate and glutamine

concentrations have also been reported to remain unchanged even after 6 weeks of antidepressant treatment, in spite of mood improvement (Godlewska *et al.*, 2015).

3.4.3 Putamen

There is increasing evidence linking the ventral-striatum (putamen and caudate nucleus) to mood regulation. Functional brain imaging studies have associated anhedonia symptoms to altered BOLD activity within the putamen (Connolly *et al.*, 2015, O'Doherty *et al.*, 2002). At the genetic level, polymorphisms in the serotonin transporter gene (5-HTTLPR) have been linked to morphometric changes in the putamen (Jaworska *et al.*, 2016). At the neurochemical level, processing of information relating to reward and motivation has been linked to dopaminergic activity within this region (Gerfen and Surmeier, 2011). The putamen receives glutamatergic input from corticofugal projections. Convergent synaptic and extra synaptic glutamate-dopamine interactions have been proposed as a possible contributor to the pathophysiological mechanisms of neuropsychiatric diseases involving this region and other basal ganglia areas (Greenamyre, 2001). Recently, increased inflammation in major depression has been proposed to result in increased glutamate levels in the basal ganglia and manifestation of symptoms of MDD such as anhedonia and psychomotor slowing (Haroon *et al.*, 2016).

The depressed patients in this study showed a significant increase in the putamen glutamine concentrations, as well as the glutamine to glutamate ratio, in the absence of changes to the glutamate concentrations. The significance of this observation, not previously reported for the putamen, and how it relates to pathophysiological mechanisms of depression, needs to be evaluated further. Intriguingly, increased

glutamine levels, and elevated glutamine to glutamate ratios, have been reported in the prefrontal cortex of patients with hepatitis C during treatment with the depressogenic immune stimulant interferon-alpha (Taylor *et al.,* 2014). Elevated levels of glutamine in the CSF have also been reported in depressed patients (Levine *et al.*, 2000). The present study findings, although limited to the putamen, suggest that a similar neurochemical mechanism may be present in MDD. This observation needs to be investigated further.

Few studies have investigated neurochemistry of the putamen in MDD with ^{1}H-MRS. One possible reason for this could be that the low signal-to-noise ratio in this region makes spectroscopic acquisitions particularly challenging at low field strengths. There is however some evidence for increased choline concentrations in the putamen in MDD, an observation that suggests increased membrane turnover (Yildiz-Yesiloglu and Ankerst, 2006).

In the present study, the MRS voxel was placed on the right putamen. Although a previous functional neuroimaging study reported decreased activation in the right putamen in an affect-reactivity task (Connolly *et al.*, 2015), it is not clear whether neurochemical changes within this region would demonstrate lateralization.

3.4.4 Conclusions

Proton-MRS at 7-tesla offers a tool to investigate neurochemistry in vivo at precision levels that allow accurate quantifications of glutamate and glutamine concentrations. In MDD, such information would be useful in understanding the pathophysiological mechanisms that could assist with patient sub-categorisation

based on neurochemical profile as well as the development of targeted pharmacotherapies. Although ^{1}H-MRS is not currently used as a diagnostic tool in psychiatry, there is a potential for its application alongside other clinical parameters especially where diagnosis is equivocal. For instance, early evidence suggests that the direction of change for glutamine and glutamate in the two primary mood disorders (bipolar disorder and MDD) could be in opposite directions (Taylor, 2014). MRS measurements of glutamate and glutamine could therefore serve as a diagnostic aid that could assist with the correct identification of patients during the early phase of bipolar disorder when the presenting symptoms mimic those of MDD (the so called 'hidden bipolars').

Although convergent evidence from previous studies points towards decreased prefrontal glutamate concentrations in MDD, the present study suggests that this effect could be gender specific. Several limitations to the present study, such as imbalanced gender proportions, highlight the need for cautious interpretation of this finding. Specific patient characteristics, some with gender effects, could have contributed to this observation. If however the gender specific effects are indeed true, they would suggest that gender is an important consideration when determining the use of a glutamate modulator for the treatment of depression. Pre-treatment levels of glutamate and Glx have been reported to predict treatment response to ketamine (Salvadore *et al.*, 2012). Given that female patients had more evidence of glutamate dysfunction relative to male patients, it is possible that some female patients may be better suited for treatment with glutamate modulators.

A gender effect on neurochemistry of MDD however raises additional questions on the neurobiological mechanisms of the disease. Although epidemiological studies have unequivocally shown that depression is more common in women than in men, studies looking at possible biological and non-biological underpinnings of this have rarely revealed plausible explanations (Heim *et al.*, 2008, Piccinelli and Wilkinson, 2000). In particular, it has been observed that the gender differences in prevalence are unlikely to be due to differential exposure to stressful life events or to differential stress sensitivity (Kendler *et al.*, 2001), though the latter study did not consider the issue of childhood maltreatment, including childhood sexual abuse. Nonetheless, possible gender specific mechanisms in the neurobiology of depression are an important consideration, and warrant further investigation.

Only medication free subjects were included in the present study, thus eliminating confounding effects of current treatment. Some patients however (9 patients) had received antidepressant treatment with previous depressive episodes. The effect of previous treatment exposure on neurochemistry is explored in chapter 4.

The mechanisms through which changes at the glutamatergic synapse lead to behavioral manifestations of depression have not been well defined. One hypothesis that is based on a synaptic excitotoxicity model, suggests that the antecedent event is excess in glutamate secretion at the synapse (Rothstein *et al.*, 1993). This excess glutamate has been mainly attributed to dysfunctional uptake transporters (the astrocytic excitatory amino acid uptake transporters), which serve as the main clearance mechanism for synaptic glutamate. In the excitotoxicity model, spillover of

the excess glutamate activates extra-synaptic receptors, resulting in aberrant neuronal activations and triggering compensatory mechanisms. The overall effect of this is a decrease in glutamatergic synaptic activity that manifests at the behavioral level as depressive illness. Yuksel and Ongur (2010) have thus proposed that ^{1}H-MRS measurements of Glx are a reflection of the overall glutamatergic pool, and that this pool is constricted in MDD.

Several limitations in ^{1}H-MRS are however of important consideration when interpreting findings on glutamatergic neurochemistry. Proton-MRS measurements, even at the accuracy of 7-tesla imaging, do not provide information on neurometabolite compartmentalisation. It is thus difficult to attribute any changes to fluxes occurring specifically within the synaptic or metabolic pools, intracellular or extracellular processes, or to neuronal or glia pathology. Some inferences can be made where separate quantifications of glutamate and glutamine have been obtained. For instance, the two metabolites are known to be overwhelmingly localised intracellularly, within neuronal cells and glia respectively (Erecinska and Silver, 1990, Lehmann *et al.*, 1983).

Proton-MRS does not provide information on dynamic metabolite changes. This can however be investigated using specialized techniques, such as ^{13}C-MRS. Using this technique (combined with ^{1}H-MRS), Abdallah and colleagues (2014) found no changes to the rate of glutamate/glutamine cycling in depressed patients when compared to controls. They however observed that mitochondrial energy

production of glutamatergic neurons was significantly reduced in depressed patients relative to control subjects.

Structural neuropathological changes have been extensively investigated in MDD (Harrison, 2002), findings of which have been corroborated by anatomic neuroimaging studies (Arnone *et al.*, 2012). Whether ^{1}H-MRS neurochemical changes represent unique cellular and molecular processes of relevance to disease pathophysiology, or are simply reflections of the anatomic pathological changes, remains to be elucidated. In addition, changes to frontal lobe perfusion in MDD have been reported using single-photon emission computed tomography (SPECT) imaging (Kohn *et al.*, 2007) as well as with arterial spin labeling (Jarnum *et al.*, 2011). These changes have been shown to be reversed following antidepressant treatment. As such, neurochemical measurements obtained with ^{1}H-MRS could be a representation of the altered metabolic processes and not independent pathological processes involving cellular neurochemistry. Anatomic, metabolic, cellular and molecular processes are however intricately linked, and observations from any of these processes are more likely representative of the same disease process at different levels of analysis.

The focus of the present study was to investigate neurochemistry specific to the glutamatergic system. This system is however linked to the inhibitory amino-acid neurotransmitter system that utilizes GABA. GABAergic changes in MDD have been described, including with ^{1}H-MRS (Luscher and Fuchs, 2015, Sanacora, 2010). These studies have mainly reported decreased GABA levels, an observation that is

intriguing given that glial derived glutamine serves as the precursor for the synthesis of GABA. It is therefore possible that the neurochemical changes present in the two systems in MDD are in the same direction, and representative of a constricted pool of glutamatergic and GABAergic metabolites.

Changes to the glutamatergic system have been reported in a wide array of psychiatric and general medical conditions. Whether the changes present in MDD are suggestive of a unique pathophysiological process, or general manifestations of brain adaptation to injury, remains to be determined. For instance, decreased glutamate to glutamine ratio has been reported in breast cancer patients with chemotherapy induced depression (Cousins and Harper, 1996). An increase in the glutamine to glutamate ratio has been reported in patients with hepatitis C during treatment with the depression-inducing agent interferon-alpha (Taylor *et al.,* 2014). These studies suggest that glutamatergic changes could be representative of depressive phenotype across multiple psychiatric and general medical conditions, and not a unique feature of the primary mood symptoms of MDD. Another possibility is that glutamatergic changes could delineate a specific subgroup of patients among the heterogeneous MDD category. There is evidence that some patient clinical characteristics, for example frequency of depressive episodes, age at onset and treatment response, correlate with glutamatergic changes (Price *et al.*, 2009). These associations are the basis for the analysis carried out in chapter 4.

Another consideration when interpreting glutamatergic neurochemistry in depression is whether any observable changes are mood state specific, or mood-

independent. Current evidence suggests that decreased prefrontal cortical glutamate levels are more specific to periods of depressive episodes, with some studies reporting correlations between decreased Glx concentrations and severity of symptoms (Hasler *et al.*, 2007, Portella *et al.*, 2011). This has been supported by observations that Glx and glutamate levels are not altered during periods of euthymia in recovered depressed patients (Hasler *et al.*, 2005, Taylor *et al.*, 2009). Glx concentrations have also been reported to normalize with symptom resolution in MDD patients treated with electroconvulsive therapy (Michael *et al.*, 2003) and following repetitive-transcranial magnetic stimulation (Luborzewski *et al.*, 2007), further supporting a mood-state dependent change in glutamatergic metabolites.

Metabolites were primarily quantified in absolute values scaled to a water peak obtained from a non- suppressed spectrum. This approach improves the robustness of the analysis, as it is not confounded by disease related changes to a reference metabolite. In particular, creatine concentrations have been reported to vary in depressed patients relative to controls (Michael *et al.*, 2003, Portella *et al.*, 2011) making it less ideal as an internal reference. Analysis was however repeated using relative metabolite concentrations scaled to the total creatine concentration, assumed to be 8.0 mol/g tissue (Du et al., 2012; Faro and Mohamed, 2011), with similar trends being observed.

Metabolites were corrected for the concentration of CSF within each individual voxel, an approach that is recommended where metabolites are scaled to tissue water content (Emir *et al.*, 2012). No corrections were made for gray matter volume

as these were determined not to be statistically different between the patients and controls. Gray matter content has however been reported to influence ^{1}H- MRS glutamate concentrations (Auer *et al.*, 2000, Pouwels and Frahm, 1998) and this effect could have had some influence, albeit minimal, on the quantified concentrations. Addition of gray matter as a covariate on the MANOVA (data not presented) did not change the overall findings.

This study was carried out at 7-tesla thus utilising the advantages of ultra-high field imaging. In addition, the use of the semi-LASER sequence at an echo time of 36ms, together with incorporation of a separately acquired macromolecular spectrum on the basis set, helped to improve the accuracy of quantification of glutamate and glutamine. Evidence for this was from the low error estimates (CRLBs) for the individual concentrations measured (mostly <10%). The sample size of 40 patients and 30 controls is also relatively large when compared to those reported from previous studies (see Table 3.8 above). The use of a cross-sectional design is however limiting, as it does not allow for temporal changes to metabolites levels to be assessed. Such information would be useful, particularly when investigating the link between neurochemistry, mood state, symptom severity and recovery.

Voxels were manually placed with reference to high-resolution structural images. A standard template was used as a reference for the voxel placement to ensure consistency. A limitation to the study was that no calculations were made to determine the extent of overlap of the MRS voxels across participants. There were

however no significant group differences in the tissue contents, water linewidths and SNR, supporting reliability of the voxel placement.

In summary, this study has demonstrated decreased ACC glutamate and Glx among female patients, an increase in ACC Glx among male patients, and an increase in putamen glutamine concentrations in both male and female patients with MDD. These findings, not previously reported with ^{1}H-MRS at 7-tesla, support a glutamatergic basis for MDD. They also raise questions on the specific disease mechanisms that need to be investigated further.

CHAPTER 4: Correlation between neurochemistry and clinical variables

4.1: Introduction

Converging evidence from neuropathological and functional neuroimaging studies implicates the anterior cingulate cortex (ACC) in the pathophysiology of MDD (Drevets *et al.*, 2008). At the neurochemical level, changes to the glutamatergic neurotransmitter system have been characterised in this region, although the nature and direction of these changes remains equivocal (Arnone *et al.*, 2015). There is also increasing evidence for the role of subcortical regions such as the putamen in emotional regulation (Fitzgerald *et al.*, 2008, Kupfer *et al.*, 2012).

In Chapter 3, changes to the concentrations of glutamate and glutamine were observed in both the ACC and putamen in patients with MDD using ^{1}H-MRS. These findings, together with those from previous studies (reviewed in Arnone et al., 2015; Yildiz-Yesiloglu & Ankerst, 2006; Yuksel & Ongur, 2010), support a glutamate-based mechanism for the pathophysiology of depression. Unlike previous ^{1}H-MRS studies, the results presented in chapter 3 were carried out at 7-tesla, thus utilizing the precision of ultra-high field imaging to accurately quantify glutamate and glutamine separately. Glutamate (and Glx) levels were observed to be decreased in the ACC in depressed patients relative to controls, although this was only present in female subjects. This finding is similar to that observed in some previous studies on the neurochemistry of the ACC in depression, although the gender specific changes have not been previously reported. There was also an increase in glutamine concentrations within the putamen, a finding not previously reported.

A challenge to the determination of brain neurochemistry in depression is the variability in findings across studies, even where similar methodological approaches have been used. One factor that could contribute to this variability is the heterogeneity of the patient population investigated. As discussed in chapter 1, MDD is a broad diagnostic category that includes patients presenting with some core behavioral symptoms without consideration for specific clinical characteristics or neurobiological factors. As such, the diagnosis includes patients with varying clinical and biological phenotype, though all meeting formal diagnostic criteria based on clustering of some common symptoms of depression.

Previous attempts to identify clinical characteristics that may be relevant to the neurochemistry of depression have been limited by the inaccuracies in the quantification of MRS metabolites at low field strengths. At the typical MRI scanner field strengths utilized (1.5 and 3-tesla), overlap between metabolite peaks leads to inaccuracies in measurements, a shortcoming that is particularly limiting when quantifying glutamatergic metabolites. This can however be overcome through the use of ultra-high field (≥ 4 tesla) imaging, as demonstrated in chapter 3.

Identifying patient characteristics that correlate with neurochemical measurements would be useful in delineating patient subgroups within the heterogeneous category of MDD. Such groups could be amenable to targeted pharmacotherapy based on known neurochemical alterations and their clinical correlates. Selection of patients into more homogenous clinical and neurobiological groups could also assist with research into the mechanisms of disease that are informed by neurochemical

phenotype. The aim of this chapter was to carry out exploratory analysis investigating relationships between clinical parameters in MDD patients and concentrations of the glutamatergic metabolites measured using ^{1}H-MRS at 7-tesla.

4.2: Methods

This was an exploratory analysis to look for relationships between patient variables and neurochemistry measured using 7-tesla ^{1}H-MRS. The participant sample for this analysis was the same as that reported in Chapter 3. Only data from depressed patients were included.

The clinical variables were derived from information obtained during the psychiatric interview as well as from the questionnaires completed during the MRS scan visits. The variables of interest were the severity of illness scored using the Hamilton Depression Rating Scale (HAM-D) and the Beck Depression Inventory (BDI), the participant age, age during the first depressive episode, anhedonia levels scored with the Snaith-Hamilton Pleasure Scale (SHPS), fatigue levels scored with the Chalder Fatigue Scale (CFS), anxiety levels scored with the state-measure of the State-Trait Anxiety Inventory (STAI), scores of childhood trauma obtained from the Childhood Trauma Questionnaire (CTQ), previous antidepressant use, history of depression in a first degree family member and meeting criteria for melancholic depression.

Proton-MRS was carried out as described in chapter 3. Gray matter volumes were obtained from segmentation of high-resolution structural images (1mm isotropic) using FMRIB's Automated Segmentation Tool, FAST (Zhang *et al.,* 2001). Although ^{1}H-MRS was carried out on three brain regions (ACC, occipital cortex and putamen), only data from the ACC and putamen were used for this chapter. This was because analysis carried out in chapter 3 did not reveal any significant neurometabolite changes within the occipital cortex in depressed patients.

Thirty-nine complete datasets for each region (ACC and putamen) were available for analysis. ACC glutamate and Glx, and putamen glutamine were selected as the neurometabolites of interest, to explore the findings revealed in chapter 3. Absolute metabolite concentrations (scaled relative to the water peak) were used in the analysis. Statistical analyses were performed on SPSS version 22.

Continuous variables

Normality of distribution of the MRS measures (ACC glutamate, Glx and putamen glutamine) was determined based on inspection of Q-Q plots. Continuous variables were the HAM-D score, BDI score, participant age, age at first depressive episode, duration of illness, voxel gray matter volume, anhedonia score, fatigue score and childhood trauma scores. Bivariate correlations were carried out using Pearson's product-moment to determine the relationships between the patient variables and the MRS measures. In addition, correlations between patient variables and ACC glutamate and Glx were also performed separately for each gender, since significant effects of gender on metabolite concentrations had been revealed for this region.

Categorical variables

Patients were categorized based on depression severity using the HAM-D and BDI scores, with separate categorization and analysis being done for each scale. Moderate depression was taken as a HAM-D score of ≤ 22 or BDI score of ≤ 29, and severe depression as a HAM-D score of ≥ 23 or BDI score of ≥ 30 (Beck *et al.,* 1961, Hamilton, 1969). Patients were also grouped based on previous antidepressant use (into antidepressant naïve or antidepressant exposed groups), based on family

history of depression (positive vs. negative family history), on whether they met the criteria for melancholic depression (melancholic vs. non-melancholic) and on age at onset of depression, with early onset taken as onset before age 25 years (Kessler *et al.,* 2001). Independent samples t-tests were used to compare means for the neurometabolite concentrations between the groups. As the analyses were considered exploratory, no corrections were made for multiple comparisons.

4.3: Results

The patient scores on the various clinical measures have been presented in Table 4.1. There were significant gender differences in the BDI scores and scores of childhood trauma, with a trend towards significant gender differences in the HAM-D scores (Table 4.2). ACC glutamate and Glx, and the putamen glutamine concentrations were determined to be normally distributed.

Anterior cingulate cortex

There were no significant correlations between ACC glutamate concentrations and any of the patient variables. Similarly, there were no significant correlations between ACC Glx concentrations and any of the patient variables (Table 4.3). Separate determinations of correlations for each gender did not reveal any significant correlations between ACC glutamate or Glx with clinical parameters (Tables 4.4 and 4.5).

Severely depressed patients had lower ACC glutamate and Glx concentrations, although these differences were not statistically significant (Figure 4.1, Table 4.6). There were no significant group differences in ACC glutamate or Glx concentrations, based on previous antidepressant use, family history of depression, melancholic status or age at onset of depression (Table 4.7).

Putamen

There were no significant correlations between putamen glutamine and any of the patient variables (Table 4.8). There were no significant group differences in the putamen glutamine concentrations based on the depression severity, previous

antidepressant use, family history of depression, melancholic status or age at onset of depression (Table 4.9).

Table 4.1: Patient clinical and imaging variables. HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory, GM, gray matter; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale; STAI, state-measure of the State-Trait Anxiety Inventory; CTQ, Childhood Trauma Questionnaire; SEM, standard error of mean; CI, Confidence intervals. Values represent mean ± standard error of mean.

		Mean	SEM	95% CI
HAM-D		21.1	0.8	19.4 – 22.7
BDI		30.0	1.4	22.3 – 32.8
Age (years)		32.7	1.9	28.8 – 36.6
Age at first episode (years)		23.6	2.0	19.7 – 27.6
Duration of illness (years)		9.5	2.0	5.5 – 13.6
GM volume	**ACC**	0.64	0.00	0.63 – 0.65
	Putamen	0.28	0.01	0.26 – 0.29
Anhedonia (SHPS)		33.5	1.1	31.3 – 35.7
Fatigue levels (CFS, total score)		21.8	0.7	20.3 – 23.3
Anxiety levels (STAI)		50.6	2.0	46.6 – 54.6
Childhood trauma (CTQ)	**Emotional abuse**	12.8	1.1	10.5 – 15.0
	Physical abuse	7.8	0.6	6.6 – 9.1
	Sexual abuse	6.4	0.6	5.1 – 7.6
	Emotional neglect	13.2	0.9	11.4 – 15.0
	Physical neglect	8.5	0.7	7.0 – 9.9
	CTQ Total Score	48.6	3.0	42.5 – 54.7

Table 4.2: Gender differences in the patient clinical and imaging variables. HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory, GM, gray matter; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale; STAI, state-measure of the State-Trait Anxiety Inventory; CTQ, Childhood Trauma Questionnaire; SEM, standard error of mean; CI, Confidence intervals. Values represent mean ± standard error of mean.
* significance at $p < 0.05$.

		Male N = 18	Female N = 22	Significance – p value
HAM-D		19.3 ± 1.0	22.5 ± 1.2	0.051
BDI		26.7 ± 1.6	32.8 ± 2.0	**0.023***
Age (years)		35.4 ± 3.3	30.5 ± 2.2	0.209
Age at first episode (years)		27.0 ± 3.4	20.6 ± 2.0	0.104
Duration of illness (years)		8.5 ± 3.1	10.5 ± 2.5	0.611
GM volume	**ACC**	0.64 ± 0.01	0.64 ± 0.01	0.912
	Putamen	0.29 ± 0.01	0.25 ± 0.01	0.067
Anhedonia (SHPS)		32.6 ± 1.2	34.3 ± 1.7	0.425
Fatigue levels (CFS, total score)		20.6 ± 1.0	22.8 ± 1.1	0.135
Anxiety levels (STAI)		50.4 ± 2.6	50.8 ± 2.9	0.924
Childhood trauma (CTQ)	**Emotional abuse**	9.8 ± 1.5	14.6 ± 1.4	**0.032***
	Physical abuse	7.1 ± 0.9	8.3 ± 0.8	0.338
	Sexual abuse	5.5 ± 0.3	7.0 ± 0.1	0.243
	Emotional neglect	11.8 ± 1.6	14.1 ± 1.0	0.214
	Physical neglect	6.8 ± 1.0	9.5 ± 0.9	0.056
	CTQ Total Score	40.8 ± 4.5	53.4 ± 3.7	**0.040***

Table 4.3: Bivariate correlations between ACC glutamate and Glx concentrations and patient variables. First episode refers to the age at first depressive episode; Duration, to the duration of illness; HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory, GM, gray matter; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale; STAI, state-measure of the State-Trait Anxiety Inventory; CTQ, Childhood Trauma Questionnaire; sig, significance (p value). N = 39.

	ACC Glutamate		ACC Glx	
	Pearson's r	**sig**	**Pearson's r**	**sig**
HAM-D	- 0.182	0.267	- 0.062	0.707
BDI	- 0.209	0.202	- 0.084	0.611
Age (years)	- 0.172	0.269	- 0.092	0.577
First episode	- 0.119	0.481	- 0.199	0.239
Duration	- 0.025	0.883	0.103	0.545
GM ACC	0.091	0.581	0.088	0.595
Anhedonia (SHPS)	0.031	0.852	- 0.037	0.822
Fatigue levels (CFS)	0.063	0.701	- 0.011	0.945
Anxiety levels (STAI)	- 0.138	0.404	0.029	0.862
CTQ Total Score	- 0.015	0.932	0.049	0.782

Table 4.4: Bivariate correlations (Pearson's product moment) between ACC Glx and glutamate concentrations with female patient variables. First episode refers to the age at first depressive episode; Duration, to the duration of illness; HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory, GM, gray matter; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale; STAI, state-measure of the State-Trait Anxiety Inventory; CTQ, Childhood Trauma Questionnaire; sig, significance (p value). N = 22.

	ACC Glx		ACC Glutamate	
	Pearson's r	**sig**	**Pearson's r**	**sig**
HAM-D	0.080	0.723	0.016	0.942
BDI	- 0.038	0.868	- 0.131	0.560
Age (years)	0.119	0.599	- 0.020	0.930
First episode	- 0.156	0.512	- 0.047	0.843
Duration	0.301	0.198	0.113	0.634
GM ACC	0.189	0.400	0.165	0.464
Anhedonia (SHPS)	- 0.127	0.573	- 0.074	0.744
Fatigue levels (CFS)	- 0.027	0.907	0.023	0.919
Anxiety levels (STAI)	0.143	0.525	0.086	0.703
CTQ Total Score	0.174	0.450	0.078	0.736

Table 4.5: Bivariate correlations (Pearson's product moment) between ACC Glx and glutamate concentrations with male patient variables. First episode refers to the age at first depressive episode; Duration, to the duration of illness; HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory, GM, gray matter; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale; STAI, state-measure of the State-Trait Anxiety Inventory; CTQ, Childhood Trauma Questionnaire; sig, significance (p value). N = 17.

	ACC Glx		ACC Glutamate	
	Pearson's r	**sig**	**Pearson's r**	**sig**
HAM-D	- 0.147	0.573	- 0.409	0.103
BDI	0.000	1.000	- 0.320	0.211
Age (years)	- 0.365	0.149	- 0.303	0.237
First episode	- 0.342	0.179	- 0.205	0.429
Duration	- 0.041	0.877	- 0.102	0.697
GM ACC	- 0.053	0.840	0.020	0.939
Anhedonia (SHPS)	0.194	0.455	0.209	0.421
Fatigue levels (CFS)	0.119	0.648	0.144	0.582
Anxiety levels (STAI)	- 0.151	0.564	- 0.405	0.107
CTQ Total Score	0.023	0.940	- 0.098	0.751

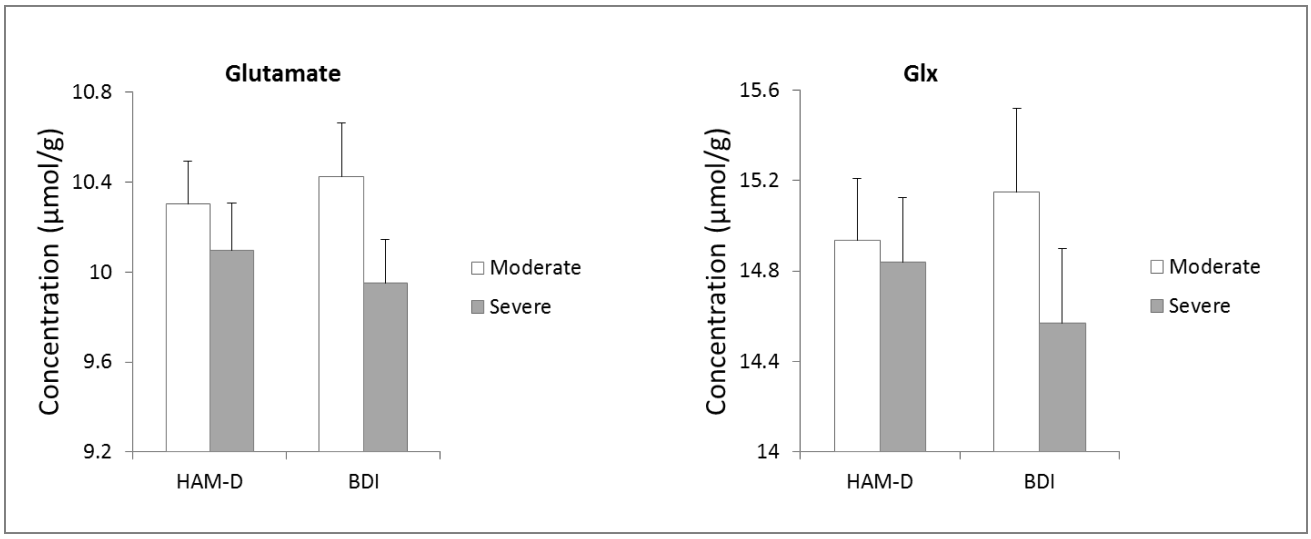

Figure 4.1: Absolute metabolite concentrations of glutamate and Glx in the anterior cingulate cortex, between moderately depressed patients (HAM-D score ≤ 22 or BDI score ≤ 29) and severe depressed patients (HAM-D score ≥ 23 or BDI score ≥ 30). There were no statistically significant group differences. Bars represent mean ± standard error of mean.

Table 4.6: Depression severity group differences in neurometabolite concentrations

		Moderate depression (HAM-D ≤ 22) N = 23	Severe depression (HAM-D ≥ 23) N = 16	Significance (Independent samples t-test)
HAM-D	ACC Glutamate	10.30 ± 0.19	10.10 ± 0.24	p = 0.506
	ACC Glx	14.94 ± 0.27	14.84 ± 0.37	p = 0.827
		Moderate depression (BDI ≤ 29) N = 22	Severe depression (HAM-D ≥ 30) N = 17	Significance (Independent samples t-test)
BDI	ACC Glutamate	10.42 ± 0.21	9.95 ± 0.20	p = 0.115
	ACC Glx	15.15 ± 0.29	14.57 ± 0.33	p = 0.194

Table 4.7: ACC glutamate and Glx concentrations in patients grouped according to previous antidepressant use, family history of depression, melancholic depression status and age at onset of depressive symptoms. Values represent mean ± standard error of mean.

ACC glutamate and glutamine concentrations				Significance (Independent samples t-test)
Antidepressant status		Antidepressant naïve N = 29	Antidepressant exposed N = 9	
	ACC glutamate	10.25 ± 0.18	10.18 ± 0.31	0.847
	ACC Glx	14.88 ± 0.24	14.91 ± 0.55	0.955
Family history of depression		Negative N = 10	Positive N = 21	
	ACC glutamate	10.21 ± 0.23	10.25 ± 0.23	0.917
	ACC Glx	14.73 ± 0.28	15.05 ± 0.36	0.576
Melancholic depression		Non-melancholic N = 26	Melancholic N = 13	
	ACC glutamate	10.26 ± 0.18	10.14 ± 0.27	0.713
	ACC Glx	14.92 ± 0.28	14.85 ± 0.36	0.881
Age at onset		Early onset (< 25 years) N = 23	Adult onset (≥25 years) N = 14	
	ACC glutamate	10.28 ± 0.19	9.99 ± 0.25	0.355
	ACC Glx	15.07 ± 0.27	14.51 ± 0.38	0.227

Table 4.8: Bivariate correlations between putamen glutamine concentrations and patient variables. First episode refers to the age at first depressive episode; Duration, to the duration of illness; HAM-D, Hamilton Depression Rating Scale; BDI, Beck Depression Inventory, GM, gray matter; SHPS, Snaith-Hamilton Pleasure Scale; CFS, Chalder Fatigue Scale; STAI, state-measure of the State-Trait Anxiety Inventory; CTQ, Childhood Trauma Questionnaire; sig, significance (p value). (N = 39).

	Bivariate correlations	
	Pearson's r	**sig**
HAM-D	- 0.046	0.783
BDI	0.002	0.988
Age (years)	- 0.073	0.658
First episode	- 0.127	0.453
Duration	0.033	0.845
GM putamen	- 0.007	0.966
Anhedonia (SHAS)	0.150	0.363
Fatigue levels (CFS)	- 0.101	0.542
Anxiety levels (STAI)	- 0.016	0.923
CTQ Total Score	- 0.083	0.641

Table 4.9: Putamen glutamine concentrations in patients grouped according to previous antidepressant use, family history of depression and melancholic depression status

Putamen glutamine concentrations			Significance (Independent samples t-test)
Depression severity (HAM-D score)	Moderate (HAM-D ≤ 22) N = 23	Severe (HAM-D ≥ 23) N = 16	
	5.01 ± 0.21	4.87 ± 0.30	0.689
Depression severity (BDI score)	Moderate (BDI ≤ 29) N = 22	Severe (HAM-D ≥ 30) N = 17	
	5.08 ± 0.22	4.78 ± 0.28	0.394
Antidepressant status	Antidepressant naïve N = 29	Antidepressant exposed N = 9	
	4.98 ± 0.21	4.90 ± 0.33	0.866
Family history of depression	Negative N = 10	Positive N = 20	
	5.31 ± 0.22	4.95 ± 0.29	0.427
Melancholic depression	Non-melancholic N = 26	Melancholic N = 13	
	4.88 ± 0.20	5.10 ± 0.34	0.552
Age at onset	Early onset (< 25 years) N = 23	Adult onset (≥25 years) N = 14	
	5.17 ± 0.24	4.63 ± 0.27	0.148

4.4: Discussion

This chapter carried out an exploratory analysis to determine whether clinical variables in MDD have an association with glutamatergic neurochemistry. The main findings were lack of significant correlations between ACC glutamate, ACC Glx, or putamen glutamine concentrations, and clinical parameters in unmedicated patients with MDD.

Response rates to pharmacotherapy for depression are at best modest, even where several treatment iterations are trialed (Trivedi *et al.*, 2006). Identification of clinical and neurobiological markers of significance to the course of illness and response to treatment is crucial to narrowing this non-response gap. Several modifiers of disease expression in MDD have been identified through epidemiological surveys. These include chronological age, age at illness onset, employment status, marital status, years in education and socio-economic factors (Alonso *et al.*, 2004, Kessler *et al.*, 2007, Kessler *et al.*, 2010). The specific mechanisms through which these factors act at the biological level to influence phenotypic behavior have not been clearly established. One possible mechanism could be that these social factors lead to differential exposure to and perception of stress, and in the background of genetic vulnerability, increase predisposition to MDD.

At the neurochemical level, there is evidence for changes to the glutamatergic system within brain regions involved in the processing of emotional information (discussed in chapter 3). Studies investigating the neurochemistry of depression have however often yielded variable findings, suggesting that additional factors may

act at the biological level to contribute to this variability. Identification of such factors could assist in selecting patients with more coherent patterns of neurochemical changes, who may be amenable to targeted pharmacotherapy. It could also assist in research towards identifying pathophysiological mechanisms specific to subtypes of depression by eliminating confounds of group heterogeneity. Previous studies have attempted to identify such associations, with factors such as age at illness onset, number of depressive episodes, first episode vs. recurrent episode, episodic vs. chronic course, and capacity to achieve remission without medication, being proposed as possible correlates of neurochemistry (Hasler *et al.*, 2005, Portella *et al.*, 2011).

Some studies have linked glutamatergic changes to severity of depressive symptoms (Michael *et al.*, 2003, Portella *et al.*, 2011). These studies have mainly demonstrated a correlation between high scores on depression scales with lower concentrations of Glx or glutamate. This association was confirmed by a recent meta-analysis on the neurochemistry of the prefrontal cortex in MDD (Arnone *et al.*, 2015). ACC glutamate levels have also been reported to increase following ECT treatment, in a magnitude reflective of treatment response (Zhang *et al.*, 2013), further supporting the link between glutamate levels and severity of mood symptoms. Some studies have however not identified any associations between glutamatergic metabolites and illness severity, an observation similar to the findings in the present study (Grimm *et al.*, 2012, Hasler *et al.*, 2007, Price *et al.*, 2009, Sozeri-Varma *et al.*, 2013).

A possible explanation for the lack of correlation between symptom severity and glutamate levels in the present study could be the recruitment of patients with predominantly moderate symptom severity (average HAM-D score of 21). The study participants, while meeting criteria for major depression, were mainly recruited through outpatient clinics and the community, and were thus of moderate to moderately severe depressive subtype. It would therefore be useful to extend these investigations into a more severely depressed group, as well as into patients with treatment resistance, so as to establish whether glutamate neurochemistry might indeed correlate with illness severity.

Melancholic depression represents a specific sub-category of MDD in which patients experience prominence of biological symptoms such as decreased appetite, weight loss, psychomotor agitation/ retardation, excessive guilt, decreased libido, and marked diurnal variations in symptoms (Cowen *et al.,* 2013). In a previous study investigating occipital cortex metabolites in MDD, patients with melancholic features were identified to be more likely to have decreased GABA and increased glutamate concentrations (Sanacora *et al.*, 2004). In the present study, there were no associations between presence of melancholic features and neurochemistry. The small number of patients meeting the criteria for melancholic depression (13 out of 40) could however have contributed to this negative finding.

Major depressive disorder is heritable, with about a third of the risk attributed to genetic factors (Kendler *et al.*, 2006, Sullivan *et al.*, 2000). Several clinical correlates of familial MDD have been described, among them an early age at onset of illness, a

longer duration of depressive episodes, frequent recurrences, high levels of impairment and increased suicidality (Kendler *et al.*, 1999, Kendler *et al.*, 2007, Shi *et al.*, 2011). These associations suggest that familial MDD may have unique neurobiological mechanisms separate from non-familial MDD, and that these mechanisms may be expressed at the neurochemical level. Investigations for associations between familial MDD and neurochemistry in the present study did not establish any significant effects. The groups (familial vs. non-familial) were however not balanced, with most patients (21 out of 31) being classified as having familial depression (familial status could not be established for some patients). The determination of familial nature of illness was based upon patient self-reports (of having a first-degree relative with the disorder), with no further confirmation being sought. This approach could have introduced some inaccuracies in the categorisation.

Another factor that may influence neurochemical measurements is the chronological age. Glutamate concentrations have been reported to decrease with age (Chang *et al.*, 2009, Kaiser *et al.*, 2005, Sailasuta *et al.*, 2008). There were no significant correlations between the patient age and glutamatergic neurochemistry in the present study. The study group consisted of adult patients with minimal variations in ages across subjects, a factor which could account for the lack of age effects.

Neuroplasticity takes place in the brain during the course of depressive illness. At the neuroanatomical level, these changes manifest as progressive volumetric loss in

diverse areas of the brain (Frodl *et al.*, 2008). It is therefore possible that the length of period during which the brain is exposed to the pathological processes of depression could have an effect on neurochemistry. The limitation to this hypothesis however is that an assumption is made on the timing of onset of disease mechanisms, being assumed to be at about the time of onset of depressive symptoms. There were no significant correlations between duration of illness (estimated from the patients' age and age at onset of symptoms) and metabolite concentrations. Determination of duration of illness in MDD is however difficult as most patients report having had sub-threshold symptoms before the time of overt symptomatology or first clinical diagnosis. In addition, this approach does not separate patients with typical episodic illness from those with a chronic course.

MDD usually starts in late teen and early adult years (Kessler *et al.*, 2010). There is evidence that age at onset may define distinct sub-categories of MDD, with early onset depression (before age 25 years) representing an entity separate from typical adult onset depression (Jaffee *et al.*, 2002, Pajer *et al.*, 2012, Truong *et al.*, 2013, Weissman *et al.*, 1999). Early onset MDD has been shown to have poorer prognosis, including poorer response to treatment (Harrington *et al.*, 1990). In addition, patients with early onset MDD are more likely to have comorbid substance use disorder and general medical conditions (Pajer *et al.*, 2012). Several studies have investigated associations between age at onset of MDD and neurochemistry. Hasler and colleagues (2005) reported negative relationships between prefrontal cortex Glx: GABA ratio and age at onset of depression. Nery and colleagues (2009) found a negative correlation between the age at MDD onset and glutamate levels. There

were however no significant correlations between age at onset and neurometabolite concentrations in the present study, an observation that needs to be investigated further.

The prefrontal cortex and striatum have been linked to the manifestation of anhedonic behaviour in depression (Keedwell *et al.*, 2005). Depressed patients with high anhedonia scores have been shown to have low levels of glutamine within the ACC (Walter *et al.*, 2009). Although glutamine changes have not been previously characterised within the putamen in MDD, this region is known to be important in the regulation of motivation and reward seeking behavior (Connolly *et al.*, 2015, O'Doherty *et al.*, 2002). In the present study, there were however no significant correlations between anhedonia scores and neurochemistry in either the ACC or putamen.

Traumatic events in early childhood have been shown to result in structural changes to the brain in adulthood (Dannlowski *et al.*, 2012), which may be linked to the development of depressive illness (Heim *et al.*, 2008). Childhood trauma is thought to exert an effect on the brain through the hypothalamic-pituitary-adrenal axis, by increasing corticotropin secretion (Lee *et al.*, 2005). Associations were determined between measures of childhood trauma and glutamate neurochemistry, but none were identified.

Antidepressants have neurotrophic effects, which may influence neurochemistry (Bessa *et al.*, 2009, Pittenger and Duman, 2008, Schmidt and Duman, 2007).

Preclinical studies have demonstrated changes to glutamatergic neurotransmission following chronic antidepressant treatment (Bonanno *et al.*, 2005, Michael-Titus *et al.*, 2000). Clinical studies have also provided evidence linking antidepressant treatment to change in neurochemistry (Bhagwagar *et al.*, 2004b, Block *et al.*, 2009, Price *et al.*, 2009, Sanacora *et al.*, 2002), although this effect may not be present in healthy controls (Taylor *et al.*, 2010, Taylor *et al.*, 2012b). Although all the patients included in the present study were antidepressant-free, 9 patients had been previously treated with antidepressants (29 were antidepressant naïve). There were no significant group differences between medication naïve and antidepressant exposed patients on the neurochemistry of the ACC or putamen.

Glutamate concentrations have been reported to be higher in gray matter tissue compared with white matter (Sailasuta *et al.*, 2008). Since gray matter loss has been reported in depressed patients (Soares and Mann, 1997), correlations were determined between voxel gray matter volume and glutamate levels. There were no significant correlations between gray matter content and glutamate or glutamine concentrations. There were also no significant correlations between gray matter content and illness severity (data not presented). Gray matter content therefore is unlikely to have had a significant influence on the neurochemical measurements.

Limitations

This chapter involved carrying out analysis on data primarily collected to investigate differences in glutamatergic neurochemistry between MDD patients and control subjects. As such, the study was not adequately powered to carry out all the

correlations and comparisons that have been reported. The investigations were therefore treated as exploratory, with the purpose of determining associations that may inform the design of future studies primarily investigating clinical correlates of neurochemistry in MDD.

4.5 Conclusion

In this study, glutamatergic neurochemistry in the ACC and putamen was not significantly associated with the severity of illness, patient age, age at onset of depressive illness, duration of symptoms, gray matter content, antidepressant use, having a family history of depression, melancholic status or scores of anhedonia, fatigue, anxiety and childhood trauma. Determination of the clinical characteristics that are associated with the neurochemistry of depression would be useful in informing possible disease mechanism as well as in selection of appropriate pharmacotherapy informed by the underlying neurobiological mechanisms. These associations therefore need to be investigated further.

CHAPTER 5: Effects of the potential lithium-mimetic, ebselen, on brain neurochemistry

5.1: Introduction

5.1.1: Lithium

Lithium has important roles in the treatment of mood disorders (discussed in chapter 1). Lithium's primary role is in the maintenance treatment of bipolar disorder, where it has been shown to be effective in decreasing the risk for both manic and depressive episodes (Geddes *et al.*, 2010). Lithium also has utility in the treatment of MDD where it is mainly used to augment treatment in patients who fail to respond to antidepressant monotherapy (Cowen and Anderson 2015). There is also evidence in support of lithium's use for prophylaxis against depressive episodes in MDD (Cipriani *et al.*, 2006). In addition, lithium is the only pharmacological agent that has demonstrated efficacy in decreasing suicide risk in patients with mood disorders (Cipriani *et al.*, 2013).

Lithium use is however limited by its acute and long-term side effects (McKnight *et al.*, 2012, Shine *et al.*, 2015). It has a narrow therapeutic index and is toxic at only twice its therapeutic concentration. It has many side effects that include hypothyroidism, hyperparathyroidism, hypercalcemia, tremor and weight gain. Lithium use is also associated with renal toxicity that may progress to end-stage renal failure (McKnight *et al.*, 2012, Shine *et al.*, 2015).

More than six decades since the rediscovery of lithium's role in the treatment of mood disorders, the molecular and cellular mechanisms through which it exerts its

actions remain equivocal (Quiroz *et al.*, 2004). The observation that there is a lag in onset of its therapeutic effect and that these effects persist after discontinuation of treatment suggests that lithium's actions involve gene reprogramming (Lenox and Hahn, 2000, Tondo *et al.*, 1998).

Lithium inhibits at least 10 targets within intracellular signaling cascades (Quiroz *et al.*, 2004). At therapeutic concentrations (0.6 - 1.2mM), the effects of lithium are thought to result from inhibition of two signal transduction pathways: the inositol monophosphatase (IMPase) pathway (Belmaker *et al.*, 1995, 1996, Berridge *et al.*, 1982, 1989) and the glycogen synthase kinase-3beta (GSK-3B) pathway (O'Brien and Klein, 2009). Inhibition of inositol-linked signaling may in fact be a shared mechanism between lithium and the other most commonly prescribed mood stabilisers, valproic acid and carbamazepine (Williams *et al.*, 2002).

IMPase is part of an intracellular second messenger-signaling cascade triggered by the activation of G-protein coupled receptors. Activation of this pathway leads to intracellular hydrolysis of phosphoinositol 4,5-bisphosphate (PIP_2) with subsequent formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 is a soluble molecule that diffuses to the endoplasmic reticulum where it binds to the IP_3 receptor, a ligand gated calcium channel. Diacylglycerol activates protein-kinase C, which then acts downstream to regulate gene expression (Figure 5.1).

Lithium is a non-competitive inhibitor of IMPase (ki = 0.8 mM, Hallcher and Sherman, 1980). IMPase inhibition results in depletion of inositol and accumulation

of inositol 1-phosphate. It also results in decreased formation of diacylglycerol. Since regeneration of PIP_2 is dependent on recycling of inositol from inositol 1-phosphate, lithium administration results in dampening of signal transduction through this pathway. The noncompetitive nature of inhibition (binding to the enzyme-substrate complex) also suggests that hyperactive pathways would be more vulnerable to lithium's inhibition. This is the basis for the inositol depletion hypothesis of lithium's action in the treatment of mood disorders (Berridge *et al.*, 1982).

Inositol penetrates the blood-brain barrier poorly, making brain cells particularly vulnerable to perturbations in IMPase signaling (Berridge *et al.*, 1982). Lithium's inositol lowering effects have been shown to demonstrate regional selectivity, being more pronounced in the frontal cortex (Moore *et al.*, 1999) than temporal and occipital cortices. Given that the frontal cortex is involved in the top-down control of emotional processing (Drevets *et al.*, 2008), the selectivity of lithium's IMPase inhibition to this region provides further evidence supporting the role of IMPase inhibition in the treatment of mood disorders.

Decrease in brain *myo*-inostiol levels following acute lithium treatment has been demonstrated using ^{1}H-MRS (Davanzo *et al.*, 2001, Moore *et al.*, 1999). In addition, lithium responders have been reported to be more likely to have reductions in myo-inositol concentrations (Davanzo *et al.*, 2001). Proton-MRS detectable decrease in *myo*-inositol concentrations following lithium treatment has however not been consistently demonstrated, with Silverstone and colleagues (1996) reporting no

significant change and Machado-Vieira and colleagues (2015) reporting an increase in *myo*-inositol concentrations. Given the evidence from preclinical studies demonstrating lithium's inhibition of IMPase, it is more likely that *myo*-inositol concentrations are decreased following lithium treatment.

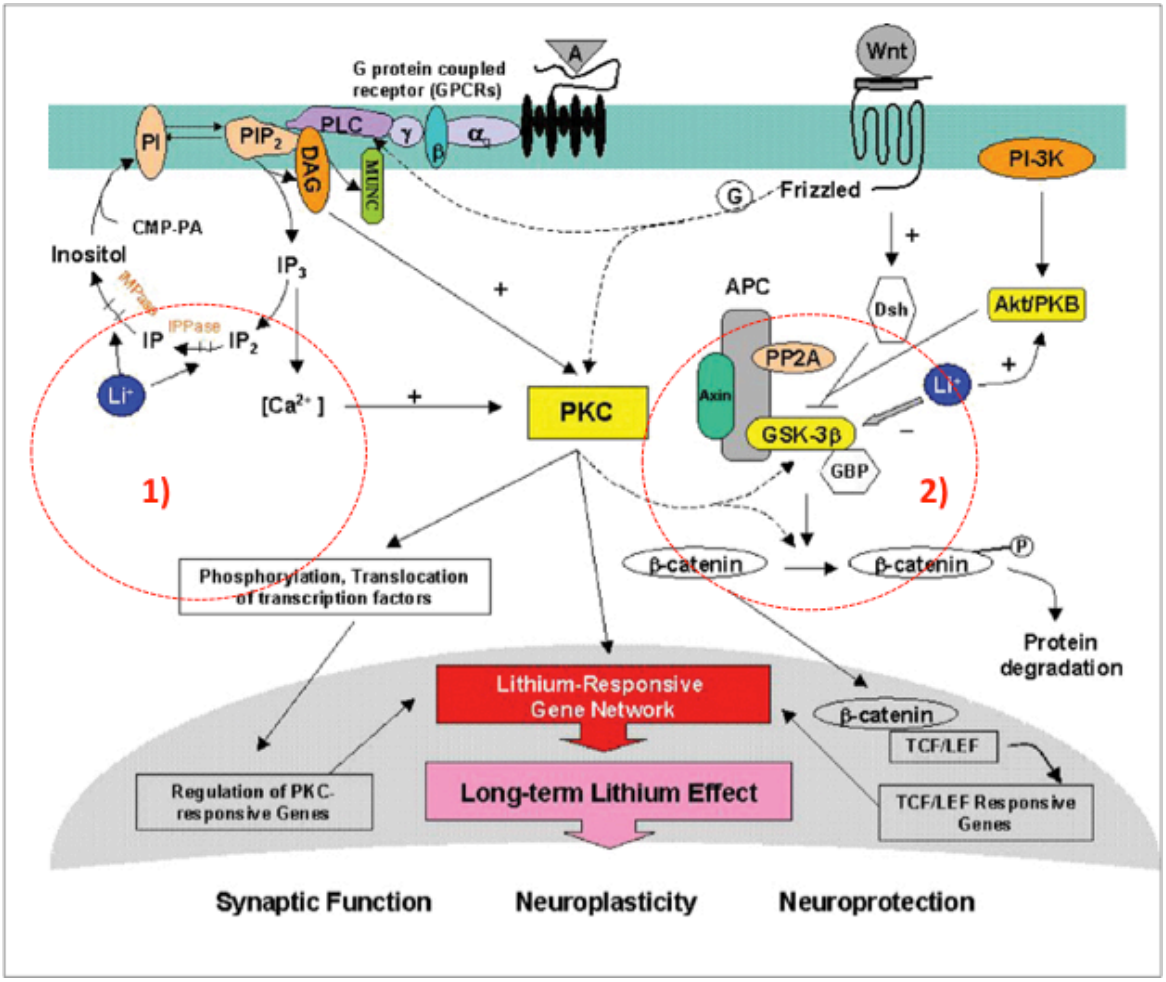

Figure 5.1: Signal transduction pathways perturbed by lithium at its therapeutic concentrations (0.6 - 1.2mM). **1)**. Lithium noncompetitively inhibits inositol monophosphatase (IMPase), thus dampening signaling through the phosphoinositol-bisphosphate pathway. This results in depletion of cellular inositols including inositol-1,4,5-triphosphate (IP3). It also results in decreased concentrations of diacylglyecerol (DAG) with subsequent downregulation of the activity of protein kinase C. **2)** Lithium binds to and inhibits glycogen synthase kinase-3Beta (GSK-3B) resulting in stabilisation of B-catenin, a regulator of gene expression. Adapted from Lenox and Wang, 2003.

Treatment response to currently available antidepressant and mood stabilising agents is at best modest, even where several iterations are trialed (discussed in chapter 1). Currently prescribed antidepressants were developed through serendipitous discoveries of their mood elevating effects, and spin-offs based on chemical modifications of original discoveries. None were developed through drug discovery based on known biological targets. Target based drug discovery would be necessary for the development of novel pharmacotherapies with better efficacies in the treatment of mood disorders. A key question therefore is whether an antidepressant or mood-stabilising agent could be developed based on lithium's biological targets, and whether such an agent would demonstrate clinical efficacy in the treatment of mood symptoms, but without lithium's side effects. This question forms the basis for the experiment carried out in this chapter.

5.1.2 Ebselen

Ebselen is a bioavailable, synthetic organoselenium compound, with anti-inflammatory and antioxidant properties (Muller *et al.*, 1984, Parnham and Kindt, 1984, Schewe, 1995). The molecular structure of ebselen and the proposed mechanism for its antioxidant effects have been presented in Figure 5.2. Ebselen has molecular mimicry activity to the enzyme glutathione peroxidase, and has demonstrable effects in the regulation of cellular redox reactions (Azad and Tomar, 2014). It is a member of the library of molecules in the National Institute of Health (NIH) Molecular Libraries Roadmap Initiative (Austin *et al.*, 2004). This repository contains a collection of small molecular entities that are thought to be safe for human use based on clinical trials, but without definite proof of clinical efficacy.

Identifying potential uses for such molecules through the process of drug repurposing, or the so called 'drug rescue of orphaned drugs', would be very beneficial since these molecules may be more readily available for human use (Cavalla, 2009).

The antioxidant properties of ebselen have been known for over 30 years, since the publication by Müller and colleagues (1984) describing its glutathione peroxidase-like activity. Since then, investigations have been carried out to determine whether these antioxidant properties could be exploited for clinical use (reviewd in Parnham and Sies, 2013). Although there were initial concerns that long term use of ebselen would lead to selenium deposition and subsequent toxicity, these concerns were allayed following discoveries that the selenium moiety is not released during redox reactions (Nogueira *et al.*, 2004).

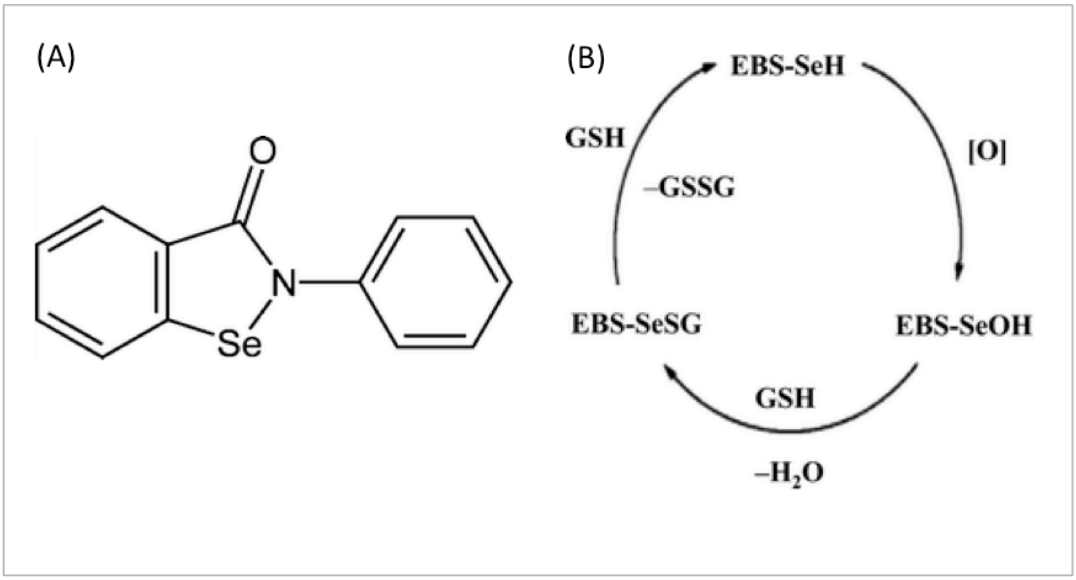

Figure 5.2: (A) Molecular structure of ebselen, chemical formula 2-phenyl-1,2-benzisoselenazol-3(2H)-one. (B) Ebselen's catalytic mechanisms in oxidative reactions. Reactive oxygen species oxidise the resting state selenol (SeH) in ebselen (EBS), forming selenic acid (EBS-SeOH). Selenic acid is then reduced to the active selenol (EBS-SeH) by glutathione (GSH) through a selenyl sulfide intermediate (EBS-SeSG). Adapted from Azad and Tomar, 2014.

The first in man study with ebselen was carried out by Fischer and colleagues (1988) who investigated its pharmacokinetic effects. Subsequently ebselen was investigated for potential use in the treatment of inflammatory conditions including rheumatic diseases and hepatitis (Parnham and Sies, 2013). Concerns for potential toxicity with long-term use (later disproved), however led to search for alternative indications for which non-chronic use of ebselen would be indicated. This formed the basis for investigations into ebselen's use for the treatment of stroke (Ogawa *et al.*, 1999, Saito *et al.*, 1998, Yamaguchi *et al.*, 1998). These initial studies investigating ebselen's role for the treatment of subarachnoid hemorrhage (Saito *et al.*, 1998) and ischaemic stroke (Ogawa *et al.*, 1999, Yamaguchi *et al.*, 1998) demonstrated some benefit over placebo, but these therapeutic effects were not considered sufficient by the regulatory authorities to warrant licensing. This led to abandonment of further development of ebselen for these indications (Parnham and Sies, 2013). Nearly a decade since the abandonment of the stroke studies, there was an interest in the potential clinical use of ebselen for the prevention of sound induced hearing loss (Kil *et al.,* 2007, Lynch, 2009). There are currently ongoing clinical trials investigating ebselen's use for Meniere's disease, prevention of aminoglycoside induced ototoxicity, chemotherapy induced ototoxicity and for mitigating inflammation in type 1 diabetes mellitus (www.clinicaltrials.gov).

5.1.3 Ebselen inhibits Inositol Monophosphatase

Ebselen has recently been identified as a molecular inhibitor of IMPase using cell cultures and animal models (Singh *et al.*, 2013). This mechanism was identified through a screening of the compounds within the NIH molecular libraries initiative

with the aim of finding those with activity against IMPase. The binding of ebselen to IMPase was determined to be covalent and irreversible. In the same study, ebselen produced inhibition of 5-HT_2 agonist induced head twitches in mice, a mechanism that has also been reported with lithium administration (Goodwin *et al.,* 1986). $5HT_2$ receptor actions are mediated through PIP_2 signaling, inhibition of which is thought to contribute to lithium's antidepressant effects (Antoniadou, 2015, Friston *et al.*, 1989). Ebselen also reversed mania-type behavior in mouse models, producing a phenotypic effect that has also been demonstrated with lithium. Administration of inositol reversed the effects of ebselen, providing evidence for an IMPase dependent mechanism of action.

In a recent 3-tesla ^{1}H-MRS study, acute administration of ebselen was shown to decrease *myo*-inositol levels within the anterior cingulate cortex (ACC) of healthy volunteers (Singh *et al.*, 2015). In the same study, Ebselen was also found to have effects on sleep architecture (diminishing slow wave sleep), an effect, however, opposite to that seen with lithium administration (Friston *et al.*, 1989).

5.1.4 Ebselen: Additional targets

Additional enzymatic targets for ebselen have been demonstrated in animal studies and cell culture work, raising the potential for its application in a wide range of diseases including Alzheimer's disease (Luo *et al.*, 2013, Xie *et al.*, 2012), spinal cord injury (Kalayci *et al.*, 2005) and skin depigmentation syndromes (Kasraee *et al.*, 2012). There is also pre-clinical evidence that ebselen lowers brain glutamate levels, through mechanisms involving inhibition of the enzyme glutaminase (Thomas *et al.*,

2013) and inhibition of synaptic release of glutamate (Nogueira *et al.*, 2002). Such an effect would have potential application for the treatment of diseases characterized by excess glutamate release.

5.1.5 Aim

Basic science work has confirmed ebselen as an inhibitor of IMPase, with enzymatic and behavioral effects suggestive of a lithium-mimetic agent. Additional targets of ebselen, such as inhibition of glutamate release, have also been reported. A recent ^{1}H-MRS study at 3-tesla has demonstrated that ebselen decreases brain *myo*-inositol levels in human. Imaging neurochemistry at 3-tesla is however hindered by poor resolution of spectra, thus limiting the accuracy and range of metabolite quantification. The aim of the present study was to investigate effects of acute treatment with ebselen on brain neurochemistry in healthy volunteers, using ^{1}H-MRS at 7-tesla. Twice the dose used in the 3-tesla ^{1}H-MRS study was selected, so as to investigate whether ebselen's effects demonstrate a dose response.

5.2 Methods – Ebselen MRS

5.2.1 Participants and study design

The study sample consisted of twenty healthy volunteers (7 females, 13 males, mean age 25.1 years, range 20 – 38 years; mean BMI 22.7 kg/$m^{2,}$ range 18.7 - 30.0 kg/m^2) who were included after giving full written informed consent.

Exclusion criteria included a history of any DSM-IV Axis I psychiatric disorder, determined using the Structured Clinical Interview for DSM-IV (First *et al.,* 2002), significant current medical condition, current regular medication (apart from the contraceptive pill), pregnancy or lactation, heavy smoking (defined as more than 5 cigarettes per day), having taken part in another study involving an investigational drug within the last 3 months and contraindications to MRI scanning. Participants were asked to maintain stable exercise and diet as well as refrain from alcohol during study participation.

Ebselen capsules and identical matching placebo (containing microcrystalline cellulose) were purchased from Shasun pharmaceuticals Ltd. Participants were tested twice (seven days apart) receiving on one occasion ebselen and on the other, placebo in a random-order, double-blind, cross-over design. Ebselen was administered in 6 x 200mg capsules in three doses given over two days. On the day before the scan visit, participants were asked to take the first dose at 1pm and the second dose at 10pm. The final dose was taken 2 hours prior to the MRI scan session, at a time when peak plasma levels of ebselen would expected (Lynch and

Kil, 2009). Participants were sent text message reminders a few minutes before they were due to take medication, and were asked to confirm receiving the messages. Participants completed various questionnaire assessments for mood, personality and sleep, and these are presented in chapter 6. Immediately before the MRI scan, participants completed a side effect profile questionnaire. Participants also completed neuropsychological tasks immediately following the MRI scan, results for which are also presented in chapter 6.

5.2.2 Proton Magnetic Resonance Spectroscopy

Proton-MRS scanning took place at the Functional Magnetic Resonance Imaging of the Brain (FMRIB) Centre. Scanning was performed on a 7-tesla Siemens MAGNETOM scanner (Siemens, Erlangen, Germany) equipped with a Nova Medical 32 channel receive array head coil. Spectra were measured from two 8ml voxels, one in the anterior cingulate cortex (ACC) and the other in the occipital cortex (OCC, Fig. 5.3). Voxels were positioned manually by reference to 1-mm isotropic T1-MPRAGE images. To ensure reproducibility of voxel placement during both ^{1}H-MRS scan visits, screenshots of each anatomical region showing voxel placement in 3 planes were taken from each subject during the first visit. These were used to guide voxel placement during the second visit.

First- and second-order shims were first adjusted by gradient-echo shimming (Shah *et al.* 2009). The second step involved only fine adjustment of first order shims using FASTMAP (Gruetter and Tkac, 2000). Spectra were acquired using a Stimulated Echo Acquisition Mode (STEAM) pulse sequence (TE = 11ms, TR = 5s, number of transients = 64) with variable power radiofrequency pulses with optimized

relaxation delays (VAPOR) water suppression and outer volume saturation (Emir *et al.*, 2012). Unsuppressed water spectra acquired from the same voxel were used to remove residual eddy current effects and to reconstruct the phased array spectra.

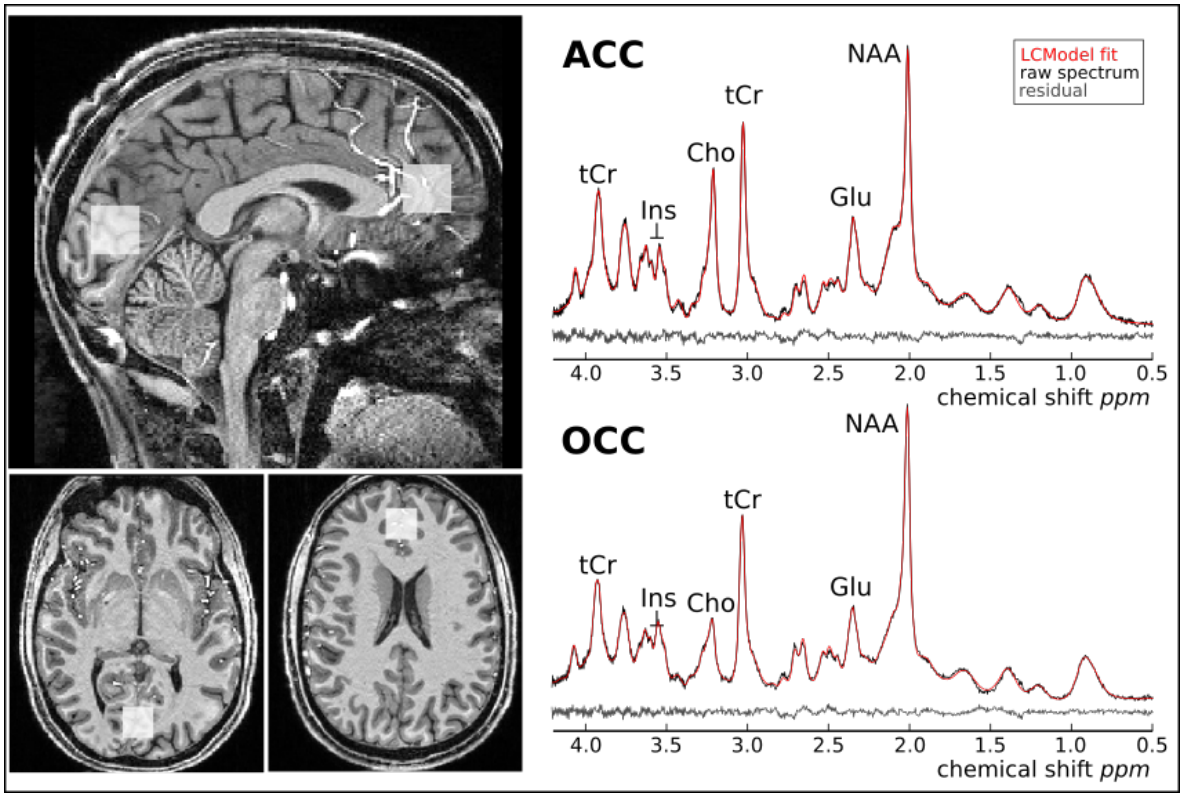

Figure 5.3: Voxel placement and representative spectra from the anterior cingulate cortex (ACC) and occipital cortex (OCC). Each acquired spectrum (64 averages) is overlaid with the metabolite fit from LCModel (red line) with major peaks labeled. The difference between the metabolite fit and underlying spectrum is shown below as a residual, which remains small and uniform indicating a high quality spectral fit. tCR, total creatine; Ins, *myo*-Inositol; Cho, choline; Glu, glutamate; NAA, N-acetylaspartate.

Metabolites were quantified using LCModel (Provencher, 2001). Model spectra were generated based on previously reported chemical shifts and coupling constants as described in Chapter 3 (section 3.2.3). A macromolecule spectrum acquired from the occipital cortex, using an inversion recovery sequence (TR = 3 s, TE = 11 ms, inversion time TI = 0.685 s), was included in the model spectra. Metabolite

concentrations were obtained relative to an unsuppressed water spectrum acquired from the same VOI assuming a water content of 82% for the ACC and occipital cortex, which primarily contain gray matter (Emir *et al.*, 2012).

The MPRAGE images were segmented using FAST (FMRIB 's Automated Segmentation Tool) to determine CSF fraction (fCSF) in the voxels (Zhang *et al.*, 2001). Concentrations were then corrected for CSF fraction with the following formula:

$$[\,M_{corr}\,] = [M].\left(\frac{1}{1-fCSF}\right)$$

where $[M_{corr}]$ = corrected concentration and [M] = metabolite concentration from LCModel output.

Pairs of MRS spectra with a difference in full width at half-maximum (FWHM) greater than 0.01ppm were excluded (three for the anterior cingulate cortex, none for the occipital cortex).

Metabolites quantified with Cramér-Rao lower bounds (CRLB, estimated error of the metabolite quantification) >50% were classified as not detected. As a secondary filter to select reliable metabolite concentrations, only metabolites quantified with CRLB ≤50% in at least half of the spectra from a brain region were reported. This led to the selection of neurochemicals with average CRLB ≤∼15%.

5.2.3 Statistics

Statistical analyses were performed in SPSS version 22. Differences in metabolite concentrations between placebo and ebselen administration were determined using

separate repeated measures multivariate analysis of variance (MANOVA) for the anterior cingulate cortex and occipital cortex. For each region, separate 7x2 MANOVAs were used, with metabolites (inositol, N-acetyl aspartate, glutathione, GABA, glutamate, glutamine and Glx) and treatment group (placebo or ebselen) as the within subject factors. Significant effects on the MANOVA were followed up with post-hoc paired samples t-test. The change in *myo*-inositol concentration was taken as the primary end point.

5.3 Results

Ebselen was well tolerated and no participant dropped out of the study. Five participants reported feeling drowsy while on ebselen treatment compared with none reporting the same while on placebo. Otherwise, there was a low and comparable frequency of side effects reported during ebselen and placebo treatment (the side effect profile is presented in chapter 6).

MRS voxel placement and representative spectra from the ACC and occipital cortex are shown in Figure 5.3. Nineteen pairs of spectra were obtained for the ACC, with one pair being discarded due to poor quality. All 20 pairs of spectra were obtained for the occipital cortex. To ensure technical comparability in the spectra acquired between the first and second scan visits, spectral pairs with a FWHM (measure of linewidth) difference > 0.01ppm were excluded (3 pairs for the ACC and none for the occipital cortex). The final samples included in the analysis were 16 pairs for the ACC and 20 for the occipital cortex.

All the spectra analysed were of high quality, with average signal to noise ratio (SNR) of 39.97 ± 1.04 (mean ± SEM), linewidth of 9.60 ± 0.34 Hz for the ACC, and SNR of 45.65 ± 0.92, linewidth 9.62 ± 0.12 Hz for the occipital cortex. All the metabolites of interest had an average CRLB below 15%, in keeping with high quality MRS spectra at 7-tesla. As there were significant correlations between inositol and total-creatine concentrations following ebselen treatment (Pearson's correlation coefficient 0.576, p=0.020), absolute metabolite concentrations were used for analysis.

The MANOVA for the anterior cingulate cortex (Wilks Lamda) showed a main effect of ebselen treatment ($F_{1,15} = 12.48$; p= 0.003) and a significant interaction between treatment and neurometabolite concentrations ($F_{6,10} = 3.38$; p= 0.044). Follow-up pairwise comparisons revealed that ebselen decreased inositol concentrations [$t_{(15)} = 2.437$, p = 0.028] within this region (Table 5.1, Figure 5.4). There were also significant reductions in the glutathione [$t_{(15)} = 2.342$, p = 0.033], glutamine [$t_{(15)} = 2.508$, p = 0.024], glutamate [$t_{(15)} = 2.954$, p = 0.010] and Glx [$t_{(15)} = 4.047$, p = 0.001] levels (Table 5.1, Figures 5.5 and 5.6). There was no change in concentrations of GABA or total NAA. The MANOVA for occipital cortex (Wilks' Lamda) showed neither a main effect of ebselen treatment ($F_{1,19} = 0.01$; p= 0.93) nor significant interaction between treatment and neurometabolite levels ($F_{6,14} = 0.99$; p= 0.47, Table 5.2).

Table 5.1: Absolute metabolite concentrations (μmol/g) given as mean ± SEM, in the anterior cingulate cortex following treatment with ebselen (3600mg over 24 hours) or placebo. The averages of the linewidth (Hz) and signal to noise ratio (SNR) have also been reported. NAA, n-acetyl-aspartate (NAA); GSH, glutathione, GABA, γ-aminobutyric acid (GABA); Glx, glutamate + glutamine.

	Placebo	Ebselen	Significance - paired t-test
Inositol	7.82 ± 0.15	7.53 ± 0.14	**0.028**
NAA	10.53 ± 0.23	10.49 ± 0.26	0.789
GSH	1.31 ± 0.043	1.17 ± 0.07	**0.033**
GABA	2.04 ± 0.08	2.04 ± 0.07	0.984
Glutamate	11.66 ± 0.17	11.34 ± 0.15	**0.010**
Glutamine	3.60 ± 0.10	3.37 ± 0.10	**0.024**
Glx	15.26 ± 0.19	14.71 ± 0.18	**0.001**
Linewidth	9.66 ± 0.53	9.55 ± 0.43	0.743
SNR	39.5 ± 1.4	40.4 ± 1.6	0.264

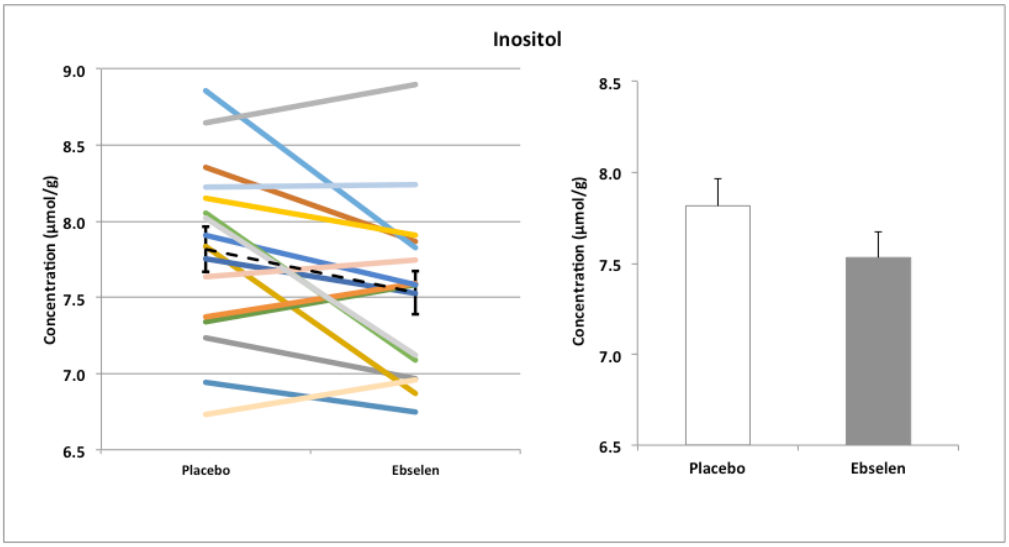

Figure 5.4: Anterior cingulate cortex concentrations of inositol (µmol/g) following treatment with ebselen (3600mg over 24 hours, dark bar) or placebo (light bar) in 16 individual subjects. Ebselen treatment resulted in a significant decrease in inositol (p=0.028, paired t-test). Black dotted line and error bars represents mean (and standard error) for group at each visit.

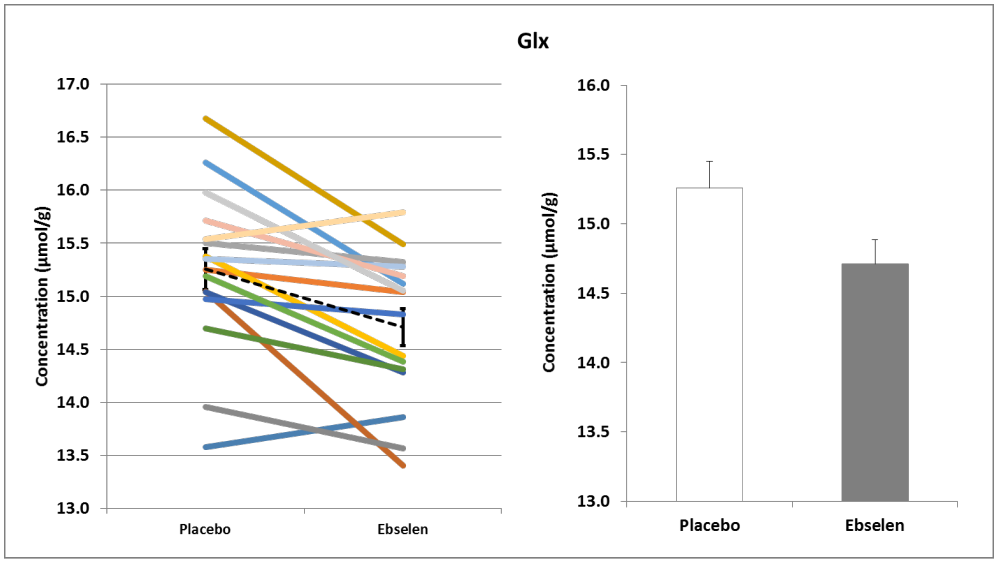

Figure 5.5: Anterior cingulate cortex concentrations of Glx (µmol/g) following treatment with ebselen (3600mg over 24 hours, dark bar) or placebo (light bar) in 16 individual subjects. Ebselen treatment resulted in a significant decrease in Glx (p=0.001, paired t-test). Black dotted line and error bars represents mean (and standard error) for group at each visit.

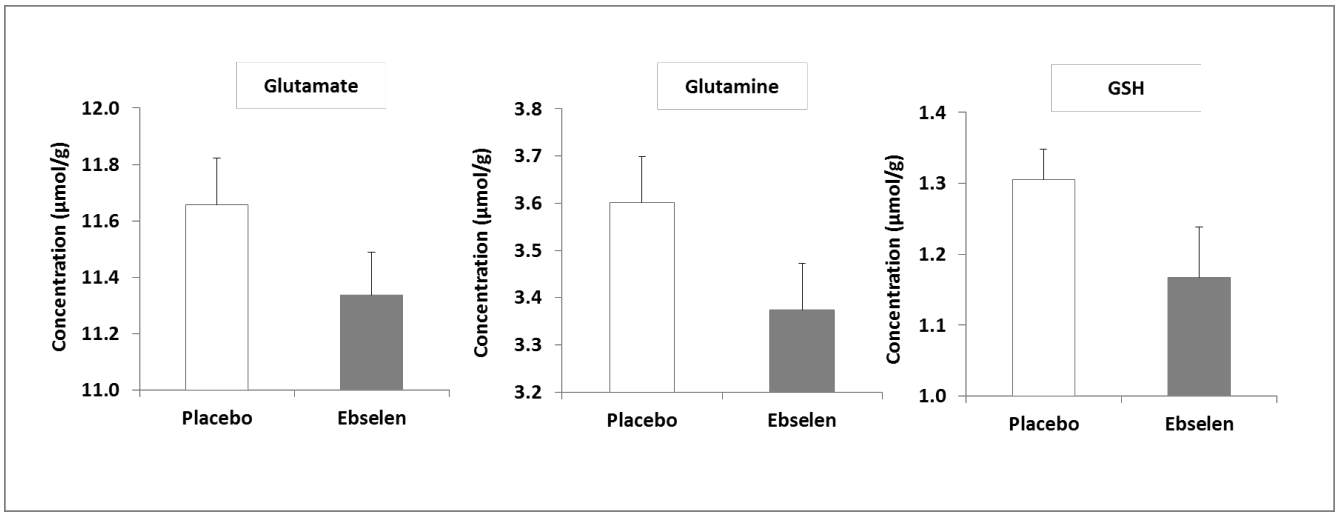

Figure 5.6: Anterior cingulate concentrations of glutamate, glutamine and glutathione (GSH) in µmol/g, following treatment with ebselen (3600mg over 24 hours, dark bars) or placebo (light bars) in 16 individual subjects. Ebselen treatment resulted in a significant decrease in the glutamate ($p = 0.010$), glutamine ($p = 0.024$), and GSH ($p = 0.033$) concentrations. Error bars represent SEM.

Table 5.2: Absolute metabolite concentrations (µmol/g) given as mean ± SEM, in the occipital cortex following treatment with ebselen (3600mg over 24 hours) or placebo. The averages of the linewidth (Hz) and signal to noise ratio (SNR) have also been reported. NAA, n-acetyl-aspartate (NAA); GSH, glutathione, GABA, γ-aminobutyric acid (GABA); Glx, glutamate + glutamine.

	Placebo	Ebselen	Significance - paired t-test
Inositol	6.66 ± 0.14	6.69 ± 0.15	0.651
NAA	11.95 ± 0.17	11.88 ± 0.15	0.567
GSH	0.95 ± 0.03	0.93 ± 0.03	0.570
GABA	1.79 ± 0.07	1.85 ± 0.06	0.314
Glutamate	9.32 ± 0.14	9.27 ± 0.15	0.610
Glutamine	2.80 ± 0.08	2.83 ± 0.08	0.770
Glx	12.13 ± 0.17	12.10 ± 0.15	0.843
Linewidth	9.66 ± 0.17	9.58 ± 0.17	0.748
SNR	46.1 ± 1.3	45.3 ± 1.4	0.484

5.4: Discussion

The main finding in this study was that short-term treatment with ebselen decreased *myo*-inositol concentrations within the ACC. In addition, ebselen treatment reduced the concentrations of glutamate and glutamine, metabolites important in the pathophysiology of mood disorders.

5.4.1 Ebselen decreases brain *myo*-Inostiol concentrations

The brain is uniquely sensitive to IMPase inhibition as it is relatively impervious to inositol within the circulation (Berridge *et al.*, 1982). As such, brain inositols are largely generated from de-novo synthesis from glucose-6-phosphate via *myo*-inositol-1-phosphate. Animal work has demonstrated that ebselen decreases brain *myo*-inositol levels through the inhibition of IMPase (Singh *et al.*, 2013). The results of the present study confirm mechanistic target engagement of IMPase by ebselen in a human model. It also replicates recent findings reported with ^{1}H-MRS at 3-tesla (Singh *et al.*, 2015). Inhibition of IMPase has been shown to deplete cellular inositols, thus dampening signal transduction through the PIP_2 second messenger system. Hyperactivity in signal transduction pathways within brain regions involved in emotional regulation has been linked to the pathophysiology of mood disorders (Berridge *et al.*, 1982, Mertens *et al.*, 2015). Lithium, a pharmacological agent with antidepressant and mood-stabilizing properties, may exert its effects through dampening signaling through these pathways.

The reduction in *myo*-inositol concentrations observed in the present study was modest (≈ 4%). These measurements however, obtained through ^{1}H-MRS, are more

likely representative of steady state concentrations, and the actual fluxes may be more in terms of absolute quantities. In addition, ^{1}H-MRS measures overall *myo*-inositol concentrations without being specific to the pool that participates in phosphoinositol signaling. Glia cells contain large quantities of inositol, which is reported to function as a cerebral osmolyte (Brand *et al.*, 1993). Given that this glia pool of inositol is not sensitive to IMPase inhibition, it may explain the modest decrease in concentrations following ebselen treatment.

The reductions in *myo*-inositol levels were in keeping with the hypothesis that ebselen depletes cellular inositols based on preclinical investigations. In addition, the finding confirmed a similar observation reported with in-vivo 3-tesla ^{1}H-MRS (Singh *et al.*, 2015). On the basis of these observations, it is more likely that the *myo*-inositol reductions are as a result of perturbations in the IMPase signaling and that the modest changes recorded could be the result of a masking effect from the larger inositol pool that is not involved in the IMPase signaling.

Twice the dose of ebselen was used in the present study (1200mg x 3, over 24 hrs.) compared with the study by Singh et al. (2015, 600mg x 3, over 24 hrs.). Reductions in *myo*-inositol concentrations were however similar in both studies. This observation suggests that there is no dose-response effect at doses above that used in the Singh et al. study, and that ebselen's effects on IMPase could be achieved with the lower dose.

Whether lithium treatment lowers ^{1}H-MRS detectable *myo*-inositol in the brain remains equivocal. Previous studies have reported decreased levels (Davanzo *et al.*, 2001, Moore *et al.*, 1999), no change (Silverstone *et al.*, 1996) or increased levels (Machado-Vieira *et al.*, 2015) of *myo*-inositol following lithium treatment. One possible explanation for these discrepant findings could be the timing of measurement of *myo*-inositol concentrations. Large enough reductions in *myo*-inositol levels that could be detected by ^{1}H-MRS could be a feature of early lithium treatment, with long-term use activating compensatory mechanisms that obfuscate this effect. This could explain the decreased concentrations reported with short-term (up to 1 week) lithium use (Davanzo *et al.*, 2001, Moore *et al.*, 1999), but not long-term (6-week) use (Machado-Vieira *et al.*, 2015). Alternatively, it is possible that the overall changes in *myo*-inositol levels are very modest, beyond the accuracy of 1.5 and 3-tesla^{1}H-MRS. Ebselen, being a more potent inhibitor of IMPase than lithium (Singh *et al.*, 2013), would be more likely to result in significant reductions in myo-inositol concentrations.

5.4.2 Ebselen decreases concentrations of glutamatergic metabolites

The finding that ebselen lowered glutamate and glutamine levels is in keeping with pre-clinical studies demonstrating that ebselen inhibits the enzyme glutaminase (Azad and Tomar, 2014). This neuronal specific enzyme converts glutamine to glutamate and is critical for the recycling of glutamatergic metabolites. Ebselen is also reported to inhibit synaptic uptake and release of glutamate (Nogueira *et al.*, 2002). These effects would be expected to lower the overall glutamate pool.

Glutamatergic excitotoxicity has been implicated in the pathophysiology of mood disorders (Gigante *et al.*, 2012, Taylor, 2014). Spectroscopic studies have demonstrated elevated levels of Glx or glutamate in patients with bipolar disorder (Taylor, 2014). Given that ebselen treatment decreases glutamate concentrations, it could have an additional benefit in the treatment of bipolar disorder through a glutamate-dependent mechanism. Elevations in brain glutamate levels have also been reported in neurodegenerative diseases (Hardingham and Bading, 2010). The proposed mechanism of glutamate toxicity in these disorders is through activation of extra-synaptic metabotropic glutamate receptors resulting in initiation of mechanisms for cellular apoptosis. Decreasing glutamate levels pharmacologically with ebselen treatment could therefore counteract this mechanism, resulting in a neuroprotective effect. Although further development of ebselen for the treatment of stroke was abandoned partly due to inadequate efficacy, the reported studies demonstrated some beneficial effects suggestive of a neuroprotective mechanism (Parnham and Sies, 2013). Ebselen's effects on glutamate metabolism could have contributed to this neuroprotective effect.

A recent ^{1}H-MRS study investigating the effects of lithium treatment on brain neurochemistry suggested that 6-weeks treatment resulted in an increase in glutamate levels within the ACC (Machado-Vieira *et al.*, 2015). This effect is opposite that observed with ebselen treatment, although the two studies differ in the duration of pharmacological treatment (6 weeks vs. 2 days in the present study). If indeed lithium treatment increases glutamate levels, while ebselen treatment

achieves an opposite effect, ebselen might have an additional advantage over lithium of achieving a glutamate-dependent neuroprotective effect.

5.4.3 Ebselen decreases glutathione concentrations

Ebselen treatment lowered the levels of glutathione within the ACC. This effect is significant, given the link between glutathione levels and mood disorders. Ebselen was originally developed in a program designed to identify organoselenium compounds with molecular mimicry to the enzyme glutathione peroxidase (Muller *et al.*, 1984, Sies, 1993). These compounds were expected to have anti-inflammatory properties, achieved through increased glutathione peroxidase dependent clearance of reactive oxygen radicals. Antioxidant effects of ebselen have been demonstrated in preclinical studies, and these are thought to be glutathione peroxidase dependent (Cabungcal *et al.*, 2014, Parnham and Sies, 2013).

While the decrease in glutathione levels following ebselen treatment is in keeping with a glutathione-peroxidase-like activity, this effect warrants careful consideration. Glutathione, by acting as a free radical scavenger, helps to protect cells from oxidative stress (Bains and Shaw, 1997), and reduced levels have been linked to pathophysiology of mood disorders (Berk *et al.*, 2008, Gawryluk *et al.*, 2011, Rosa *et al.*, 2014). In addition, ebselen has been reported to increase glutathione levels in rat cortical neurons (Pawlas and Malecki, 2007), although this effect may be dose dependent (Pawlas *et al.*, 2009). It is however not clearly established whether ^{1}H-MRS detectable changes to glutathione levels could be significant to the pathogenesis of mood disorders. For instance, several studies have

failed to demonstrate ^{1}H-MRS differences in glutathione levels in patients with bipolar disorder (Fusar-Poli *et al.*, 2012, Lagopoulos *et al.*, 2013), although this has been suggested in preclinical investigations (Dean *et al.*, 2009).

Several explanations could be given for the decrease in glutathione levels observed in the present study. Glutathione exists in two species: reduced form (GSH) and oxidised form (GSSG). Activation of glutathione peroxidase leads to increased generation of the oxidised form. Decreased activity of this enzyme has been reported in MDD (Gawryluk *et al.*, 2011, Maes *et al.*, 2011, Shungu *et al.*, 2012). While ^{1}H-MRS predominantly measures reduced (GSH) levels, it is difficult to tell apart the two species even at ultra-high field (Satoh and Yoshioka, 2006). It is therefore possible that although overall levels of glutathione were decreased with ebselen treatment, the levels of the reduced form may have actually been increased. A decrease in GSH levels would however not in itself suggest deleterious effects on the brain, as it may be a reflection of increased consumption of harmful oxidative radicals through activation of glutathione peroxidase-dependent redox reactions.

Another possible explanation for the decreased glutathione levels could through ebselen's ability to decrease brain glutamate levels. Glutathione metabolism is linked to that of glutamate, and thus the glutamatergic changes observed in the present study could have contributed to the observed decrease in glutathione levels (Berk *et al.*, 2011). Alternatively, the decrease in GSH levels, which though significant was very modest, could have been a chance finding.

5.4.4 Ebselen has no effects on neurometabolite concentrations in the occipital cortex

Ebselen treatment did not affect occipital cortex metabolite concentrations. This observation was somewhat unexpected, given that ebselen's actions on the brain were expected to be ubiquitous. This regional selective effect however supports a lithium-like mechanism of action, since lithium's inhibition of IMPase has been determined to be preferentially localised to the frontal cortex (Moore *et al.*, 1999). The inositol depletion hypothesis works on the premise that hyperactive signaling pathways within emotional processing networks, such as in the ACC, are preferentially targeted by pharmacological IMPase inhibition (Berridge *et al.*, 1982).

In the present study, spectroscopy was acquired while the subjects were resting, lying in the scanner and not performing any tasks. Under such conditions, a network of brain regions within the so-called default mode network (Greicius *et al.*, 2003), would be expected to have increased activation relative to regions outside the network. The default mode network includes the dorsal medial prefrontal cortex, a region that overlaps with the ACC voxel used in the present study. A possible explanation for the regional selective effects of ebselen treatment therefore could be that under the scanning conditions used, the ACC voxel had relatively increased activity compared to the occipital voxel, and was thus more vulnerable to IMPase inhibition.

5.4.5 Technical advantages

Proton-MRS was carried out at 7-tesla, thus utilizing the advantages of ultra-high field imaging. The use of the STEAM sequence allowed for accurate quantification of

myo-Inositol, an ultra-short T_2 metabolite. Seven-tesla imaging also allowed for accurate measurements of glutamate and glutamine, with most of the individual concentrations being quantified at CRLB < 10%. Metabolites were quantified in absolute concentrations scaled to water, thus avoiding confounding effects due to changes in the concentration of a reference metabolite.

5.4.6 Repurposing ebselen

Drug development is a lengthy process that is fraught with costly failures. Most of the failures occur at the later stages of development during evaluation of efficacy (Scannell 2011; Kola and Landis 2004). The failure costs are not only in form of direct investment into the specific drug development, but also in opportunity costs for drugs not developed. In particular, the development of pharmacological treatments for mood disorders is hindered by the lack of plausible preclinical models (Nestler, 2002). Animal models have generally proved to be poor predictors of drug efficacy, particularly in the evaluation of non-monoamine based treatments (Conn and Roth, 2008; Harmer 2011). Using in vivo MRS, this study has confirmed the hypothesis that ebselen treatment decreases inositol levels in the brain, suggestive of a lithium-mimetic effect. The study therefore demonstrates the application of MRS in guiding targeted drug discovery.

Ebselen's potential role for psychiatric treatment was based on screening of small chemical entities from a library of molecules with likely clinical safety but no proven efficacy. These drugs have the great advantage in that they could be available for human use in the event that clinical efficacy is established. This process, referred to

as drug repurposing, offers a path for efficient and less costly drug development. Expending investigations on ebselen into patient groups would therefore be suggested.

Although the primary aim of the study was to determine whether ebselen decreased brain inositol levels, additional effects on the glutamatergic system were also established. This suggests that ebselen could have broader applications for the treatment of neurological and psychiatric diseases in which regulation of glutamatergic neurotransmission is warranted.

In addition to repurposing ebselen for treatment purposes, the drug could also function as a tool for interrogating the link between IMPase signaling and mood disorders. Apart from lithium, ebselen is the only known bioavailable and brain penetrant IMPase inhibitor. Ebselen's inhibition of IMPase is also more potent than that of lithium (Singh *et al.*, 2013). Although ebselen also inhibits the enzyme GSK3Beta, another putative target for lithium's actions, this inhibition is of much lower magnitude (about 20x less) compared to IMPase inhibition (Singh *et al.*, 2013). Ebselen could therefore function as a compound that could be used to study the effects of IMPase inhibition on mood, as well as in the development of other IMPase inhibitors that could have utility for pharmacotherapy of mood disorders.

5.4.7 Shortcomings

The extent to which the voxel placement between the first and second scan visits overlapped was not determined. However, voxel placement during the second scan

visit was guided by screenshots taken during the first visit, and intra-scan water linewidth measurements were used to ensure coherence in voxel placement. In addition, the voxel content and quality parameters did not differ between the two visits, suggesting that reliability in voxel placement was achieved.

5.5 Conclusion

This study has demonstrated that short-term treatment with the potential lithium mimetic agent ebselen decreases *myo*-inositol levels in the brain. Since IMPase is a putative target for the mechanism of action of lithium and other mood-stabilizing agents, it suggests a potential role for ebselen in the treatment of mood disorders. Ebselen treatment also decreases brain glutamate levels, in a manner suggestive of a neuroprotective agent. This could have broad applications in the treatment of neuropsychiatric illnesses characterized by glutamate excitotoxicity. As a repurposed drug, ebselen could be directly available for human use once efficacy has been demonstrated with patient groups. In addition, ebselen, as a safe, bioavailable and brain penetrant IMPase inhibitor, could be used as a pharmacological tool for further investigations into the role of IMPase signaling in the pathophysiology and treatment of mood disorders.

Chapter 6: Effects of the potential lithium-mimetic, ebselen, on impulsivity and emotional processing

6.1: Introduction

6.1.1 Lithium

Lithium has important roles in the treatment and prophylaxis of mood disorders (discussed in chapter 1). In addition to this, lithium is the only psychotropic agent shown to reliably reduce suicidal behaviour (Cipriani *et al.*, 2013, Geddes *et al.*, 2010, Miura *et al.*, 2014). Lithium's anti-suicide effects do not appear to be accounted for solely by its effects on mood (Cipriani *et al.*, 2013). One of the mechanisms through which lithium may exert anti-suicide properties is through diminishing impulsive aggression. This effect has been demonstrated across several groups, including in patients without mood symptoms (Craft *et al.*, 1987, Jones *et al.*, 2011, Sheard *et al.*, 1976).

Six decades since the re-discovery of lithium's role in the treatment of dysregulated mood, its mechanisms of action remain equivocal (discussed in chapter 5). Inhibition of two putative targets within intracellular second messenger signaling pathways has been postulated to contribute to a large part of its mechanistic actions. These are the inositol monophosphatase (IMPase) pathway and the glycogen synthase kinase 3B (GSK3B) pathway.

Lithium use is however associated with many limiting side effects and safety issues (McKnight *et al.*, 2012, Shine *et al.*, 2015). Its poor tolerance, narrow therapeutic index, and long-term toxicities suggest the need to identify alternative treatments.

One approach towards the development of lithium surrogates would be through repurposed drug discovery based on lithium's biological targets (IMPase and GSK3B inhibition).

6.1.2 Ebselen

As discussed in chapter 5, ebselen is an organoselenium antioxidant that has been shown to inhibit IMPase as well as induce lithium-like effects in pre-clinical models. Recently, ebselen has also been shown to decrease brain *myo*-inositol concentrations in healthy human subjects, providing evidence for IMPase inhibition in a human model (Singh *et al.*, 2015). This effect was confirmed by the results reported in chapter 5. In addition to lowering brain *myo*-inositol concentrations, ebselen has additional effects on the glutamatergic system that suggest its potential use as a neuroprotective agent.

Ebselen therefore offers a potential replacement for lithium in the treatment of mood disorders. Data available from Phase I-III clinical trials investigating ebselen's use for non-psychiatric indications have confirmed its safety for human use (Lynch E, 2009, Ogawa *et al.*, 1999, Saito *et al.*, 1998, Yamaguchi *et al.*, 1998). Therefore, determining whether ebselen induces behavioral effects relevant to the treatment of psychiatric illnesses would be useful in guiding its further development for this purpose.

6.1.3 Investigating ebselen with experimental medicine models

A challenge to the development of new pharmacotherapies for neuropsychiatric diseases is the high failure rate during the evaluation of efficacy (Scannell *et al.*, 2012). Most of the targets identified in animal models fail to prove efficacy in

clinical trials (Conn and Roth, 2008). Human experimental medicine models such as the Cambridge Gambling Task (CGT) and Facial Emotion Recognition Task (FERT) offer tools to assess clinical efficacy during the initial stages of drug development (Dawson and Goodwin, 2005, Harmer, 2013). Use of these models in healthy volunteers has allowed behavioral effects to be investigated outside confounds of clinical mood disturbances.

Drugs with antidepressant properties have been shown to induce positive cognitive biases in the processing of emotional information. On the FERT, this manifests as increased identification of ambiguous faces as expressing positive emotions or a decreased ability to detect negative expressions. These effects are present early on in treatment before overt perception of mood change (Harmer, 2010, 2013, Harmer and Cowen, 2013). Performance on the FERT has also been shown to be predictive of future treatment response (Tranter *et al.*, 2009). The FERT has also demonstrated potential in identifying agents that are unlikely to be efficacious as antidepressant in human (Chandra *et al.*, 2010, Pringle *et al.*, 2011b), thus offering a mechanism for weeding out ineffective compounds early on during drug development.

The CGT was developed as a task to assess decision-making, which would be independently sensitive to risky choice and impulsivity. Interestingly, people at risk of bipolar disorder show an impulsive betting strategy on the CGT (Wessa et al, 2015). In contrast, both depressed patients and those at risk of depression demonstrate conservative betting strategies suggestive of a risk averse / low reward seeking phenotype (Mannie *et al.*, 2014, Murphy *et al.*, 2001, Rawal *et al.*,

2013). The CGT has not been used previously as a tool for drug discovery but clearly has potential as a mean of detecting agents that might lower impulsivity.

6.1.4 Correlations between neurochemistry and behaviour

The anterior cingulate cortex (ACC) plays important roles in both affective and cognitive processing (Kennerley *et al.*, 2006). Neurochemistry within the ACC has been linked to performance in cognitive decision-making tasks, including those assessing impulsive behaviour and risky choice decision-making (Fujihara *et al.*, 2015). Assessment of pharmacologic manipulations of ACC neurochemistry and its link to cognition would therefore provide useful information that could guide the development of treatments for cognitive and emotional disorders.

6.1.5 Aims

The aims of this chapter were to use human experimental medicine models to determine whether short-term treatment with ebselen would

1. Decrease a laboratory measure of impulsive behaviour
2. Produce a positive bias in the processing of emotional information
3. Produce changes in cognitive processing that correlate with changes to neurochemistry

6.2 Methods

6.2.1 Participants and study design

The study sample comprised the same participants who took part in the neuroimaging study presented in chapter 5. The inclusion and exclusion criteria for the cognitive and emotional processing assessment were similar to those reported in chapter 5. All 20 healthy participants (7 females, 13 males) completed two validated neuropsychological tasks, the Cambridge Gambling Task (CGT) and the Facial Emotion Recognition Task (FERT). This was after taking 3600mg of ebselen (or identical matching placebo) in three separate doses (1200mg per dose) administered over two days. The first two doses were taken at 1pm and 10pm on the day preceding cognitive testing. The final dose was taken 3 hours prior to cognitive testing. Participants were tested twice (7-days apart) in a within-subject, placebo controlled, double-blind, cross-over design (Figure 6.1).

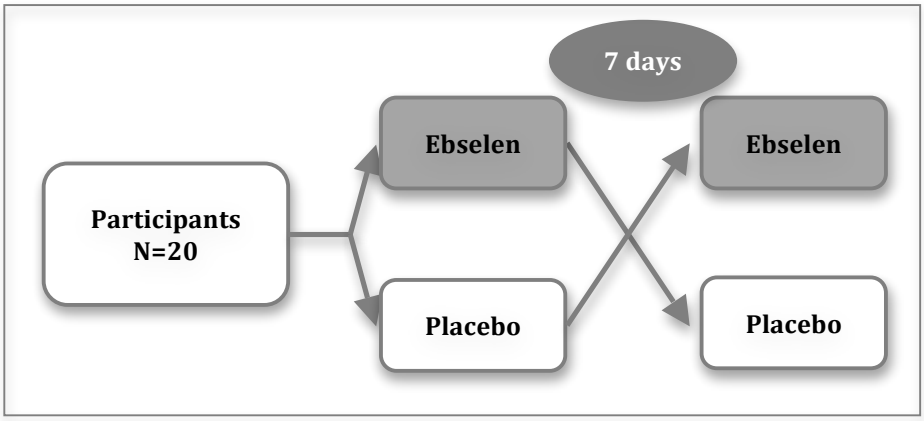

Figure 6.1: Study experimental design. 20 healthy participants were included following screening. Ebselen (1200mg per dose) was administered at 1pm and 10pm on the day preceding cognitive testing, as well as 3 hours prior to cognitive testing (Total dose 3600mg).

6.2.2 Mood, personality and sleep assessment

During the screening visit, participants were assessed for baseline mood symptoms with the Beck Depression Inventory (Beck *et al.*, 1961), for anxiety symptoms with the state measure of the State-Trait Anxiety Inventory (Spielberger et al. 1983); and for personality with the Eysenck Personality Questionnaire (Eysenck and Eysenck, 1983). On the morning preceding psychological testing, participants completed the Leeds Sleep Evaluation Questionnaire (Parrot and Hindmarch, 1980) within 30 minutes of waking. Before cognitive testing, participants were asked to rate their mood using the Positive and Negative Affective Schedule (Watson *et al.*, 1988).

6.2.3 Cambridge Gambling Task

The Cambridge Gambling Task has been described in detail in chapter 2 (section 2.3). In summary, participants were shown a row of 10 boxes, some red, others blue. The ratios of red to blue boxes varied in pseudo-stochastic order. Participants were asked to guess whether a token was hidden under a red or blue box, and to stake a proportion of their points (from an initial allocation of 100) on the bet. Bets were offered in either ascending order (5% - 95% of total points at start of trial) or descending order (95% - 5%). The order of presentation (ascending or descending first) was balanced across participants. When a correct bet was selected, the staked points were added to the total in the trial. Conversely, when an incorrect response was made, the staked points were subtracted from the total. Participants were asked to maximize their points while minimizing losses. Measures assessed on the CGT were the deliberation time, reward seeking, risk adjustment and delay aversion.

6.2.4 Facial expression recognition

A detailed description of the FERT has been given in Chapter 2 (section 2.4). In summary, the task featured six basic expressions of emotion (happiness, surprise, sadness, fear, anger and disgust) taken from the Pictures of Affect Series (Ekman and Friesen, 1976). These had been morphed between each prototype and neutral (Young *et al.,* 1997). Four examples of each emotion, and at each intensity, were given (total of 10 individuals). The facial stimuli were presented on a computer screen (in random order) for 500 ms, and then replaced by a blank screen. Participants made their responses by pressing a labeled key on the keyboard. Participants were asked to respond as quickly and accurately as possible. Accuracy, misclassifications (false alarms) and reaction times were measured.

6.2.5 Correlation with MRS measures

Metabolite concentrations were measured using proton-MRS at 7-tesla as described in chapter 5. Absolute concentrations of glutamate, glutamine, GABA, inositol and glutathione were used for the correlation analysis. Correlation was done between the change in MRS signal (ebselen visit minus placebo visit) and change in the neuropsychological measures (delay aversion, risk adjustment and reward seeking on the CGT, and accuracy of recognition of positive and negative expressions on the FERT).

6.2.6 Statistics

Statistical analyses were performed on SPSS 22, with two-sided significance level set at 5%. Demographic data and subjective scores of mood and anxiety were analyzed with one-way analysis of variance (ANOVA), with group (placebo or

ebselen) as the factor. Accuracy of detecting the randomization arm during each visit was assessed using a chi-squared test.

For the CGT, analyses were conducted with repeated measures ANOVA with 'treatment' (placebo vs. ebselen) as the within subject factor and 'order' (placebo first vs. ebselen first) as the between subject factor. A similar analysis was carried out for the FERT but 'emotion' (specific facial expression) was added as a further within-subject factor. Significant differences on the ANOVA were followed up with pairwise comparisons using the paired samples t-tests.

Correlations between change in ^{1}H-MRS metabolite concentrations and performance on the neuropsychological tasks were done using Spearman's rho test. This was to avoid errors out of assumptions of normality since some of the MRS metabolite concentrations were determined not to be normally distributed based on inspection of Q-Q plots.

6.3: Results

6.3.1 Subjective state, energy and side effects

At baseline, all participants had low scores on self-ratings scales of mood and anxiety (Table 6.1). There were no main or interactive effects of treatment on mood, assessed using the positive and negative affective schedule (PANAS) questionnaire (Table 6.2). Ebselen was well tolerated and no participants dropped out of study. Five participants reported feeling drowsy while on ebselen treatment, compared with none reporting the same while on placebo treatment. Otherwise, there was a low and comparable frequency of side effects reported during ebselen and placebo treatment (Figure 6.2). There were no significant differences in the subjective measures of sleep (Figure 6.3). Participants were 35% accurate on guessing whether they had received ebselen during visits in which ebselen (and not placebo) was administered.

Table 6.1: Baseline personality and mood scores (mean ± SEM). EPQ: Eysenck Personality Questionnaire; BDI: Beck's Depression Inventory, STAI: state measure of the State-Trait Anxiety Inventory, N = neuroticism, P = psychoticism, L = lie scale, E = extraversion

EPQ: N	4.3 ± 0.7
EPQ: P	3.4 ± 0.5
EPQ: L	6.2 ± 0.7
EPQ: E	15.3 ± 0.8
BDI	2.0 ± 0.7
STAI	27.2 ± 1.2

Table 6.2: Subjective mood ratings scored with the positive and negative affective schedule (PANAS) questionnaire, during placebo and ebselen study visits. The number of participants who correctly guessed the randomization arm at each visit has also been presented.

	Placebo Mean ± SEM	Ebselen Mean ± SEM	Statistical significance
PANAS - Positive	28.50 ± 1.62	29.75 ± 1.20	$F_{1,19} = 1.7$, $p = 0.204$ [a]
PANAS - Negative	11.75 ± 0.73	11.90 ± 0.68	$F_{1,19} = 0.1$, $p = 0.818$ [a]
Correct guesses for randomization	14/20 (70%)	7/20 (35%)	$p = 0.056$ ($\chi 2$) [b]

a. Repeated measures ANOVA
b. Chi-squared statistic

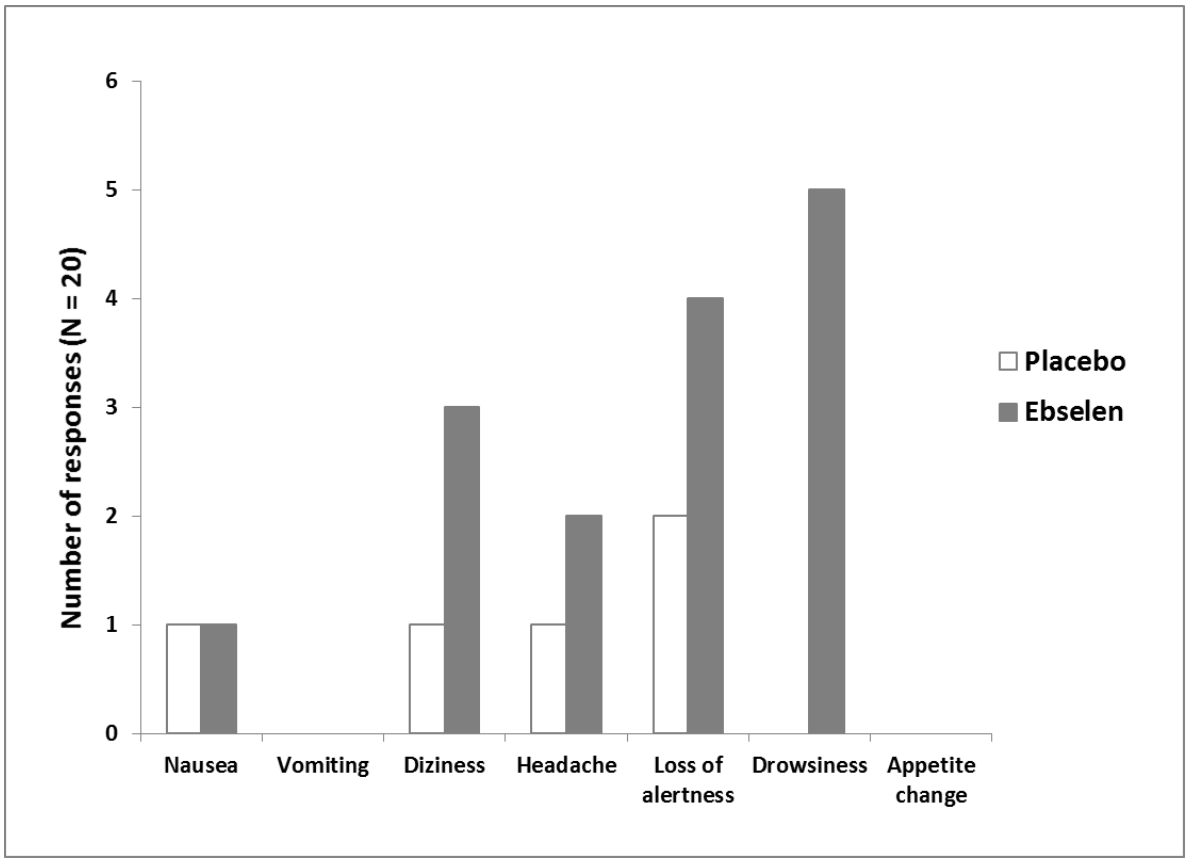

Figure 6.2: Comparison of the frequency of side effects reported by participants following placebo (light bars) or ebselen (dark bars) treatment.

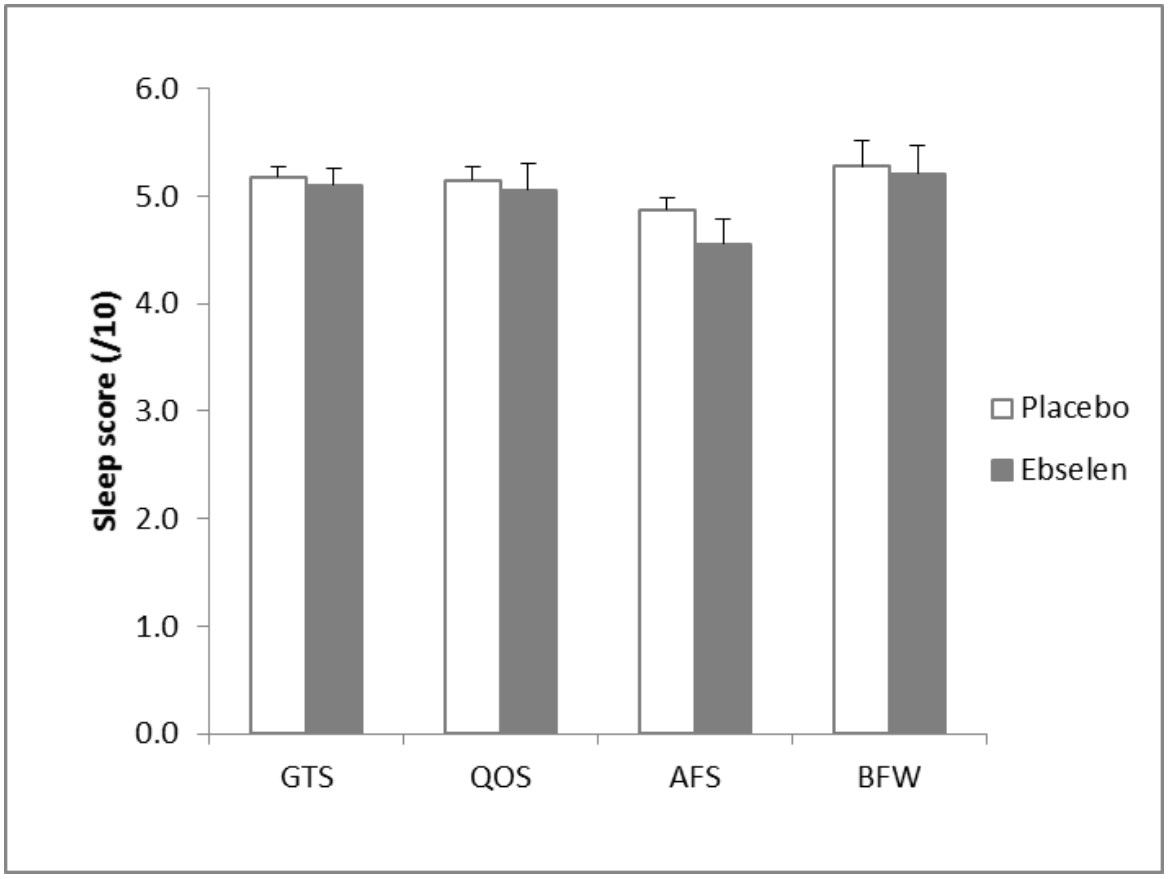

Figure 6.3: Sleep assessment following placebo or ebselen treatment. Subjective measures of sleep were taken using the Leeds Sleep Evaluation Questionnaire (LSEQ) on the morning following the second ebselen dose (morning preceding psychological testing). The following LSEQ measures were assessed using a 10 point visual analogue scale: GTS – getting to sleep, QOS – quality of sleep, AFS – awake following sleep, BFW – behaviour following wakening. There were no main effects of treatment ($F_{1, 19} = 0.575$, $p = 0.457$) or significant treatment x sleep-measures interactions ($F_{3, 57} = 3.900$, $p = 0.758$) on the ANOVA. Values represent mean (out of 10) ± standard error of mean.

6.3.2 Cambridge Gambling Task

There were no significant effects of treatment on the quality of decision making, deliberation time or risk adjustment (Table 6.3). Ebselen treatment was associated with a significant decrease in delay aversion ($F_{1, 18} = 8.205$, $p = 0.010$). This effect was present irrespective of the probability of a favourable outcome (ratio of the bets presented, Figure 6.4). Ebselen treatment was also associated with an increase in reward seeking ($F_{1, 18} = 4.605$ $p = 0.046$).

There were significant main effects of order on the deliberation time ($F_{1, 18} = 38.405$, $p < 0.001$) and a trend towards significant main effects of order on reward seeking ($F_{1, 18} = 3.391$, $p = 0.082$). There were no significant main effects of order on delay aversion, quality of decision-making or risk adjustment. There were no significant interactions involving order and treatment (all p values >0.1).

Table 6.3: Results of the Cambridge Gambling Task. Ebselen treatment decreased delay aversion, and increased reward seeking

	Placebo Mean ± SEM	Ebselen Mean ± SEM	Statistical significance (Repeated measures ANOVA)
Delay aversion (%)	13.65 ± 2.19	9.04 ± 2.14	**$F_{1,18} = 8.205$, $p = 0.010$**
Reward seeking (%)	60.32 ± 1.82	63.85 ± 1.45	**$F_{1,18} = 4.605$, $p = 0.046$**
Deliberation time (ms)	1475 ± 99	1408 ± 78	$F_{1,18} = 2.500$, $p = 0.131$
Quality of decision-making (%)	98.3 ± 0.8	99.3 ± 0.3	$F_{1,18} = 0.204$, $p = 0.155$
Risk adjustment	2.25 ± 0.15	2.06 ± 0.18	$F_{1,18} = 2.884$, $p = 0.107$

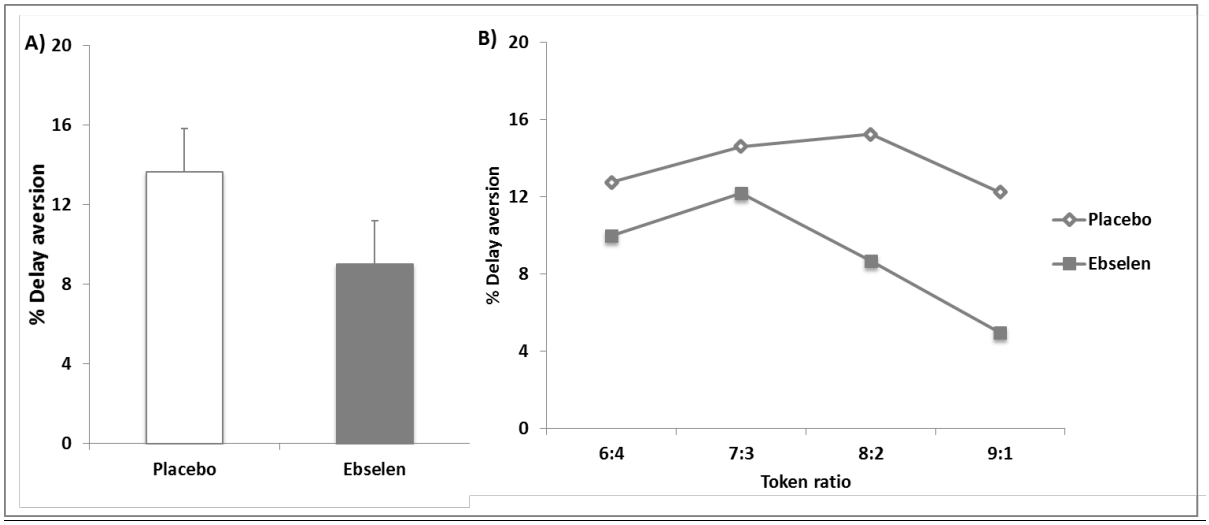

Figure 6.4: Results of the Cambridge Gambling Task. A) Ebselen treatment was associated with a significant decrease in the mean delay aversion (main effect of treatment on ANOVA, $F_{1, 18 =}$ 8.208, p = 0.010). B) The decrease in delay aversion following ebselen treatment was present irrespective of the token ratio presented.

6.3.3 Facial emotion recognition task

There was a trend towards significant interactions between treatment group (ebselen vs. placebo) and emotion on the accuracy of recognition of the six individual emotional expressions ($F_{5, 90}$ = 2.147 p = 0.067). Follow-up pairwise comparisons revealed that ebselen treatment improved accuracy of recognition of happy facial expressions [$t_{(19)}$ = 2.298, p = 0.033] without significant effects on the accuracy of recognition of other emotional expressions (Figure 6.5). There were no significant interactions between treatment group and emotion for the misclassifications or reaction times to the six emotional expressions.

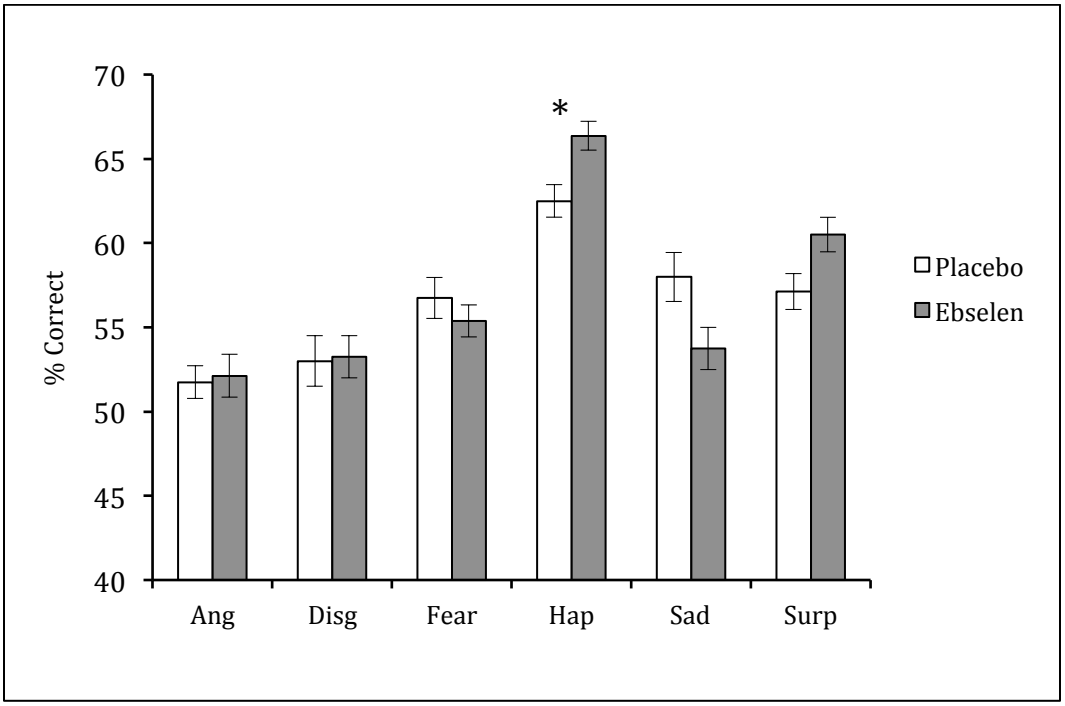

Figure 6.5: Effect of ebselen on accuracy of facial expression recognition. Values represent the mean percentage correct for each of the six basic emotions summed over different intensity levels. There was a tendency towards significant interactions between treatment and emotion ($F_{5, 90} = 2.147$, $p = 0.067$). Follow-up pairwise comparisons revealed that ebselen treatment was associated with significantly improved accuracy in the recognition of happy facial expressions (* $p = 0.033$), without significant effects in the recognition of other expressions. All data are plotted as mean ± standard error of mean, N=20. ang, angry; disg, disgust; hap, happy; surp, surprised.

There was a significant interaction between treatment and emotion for the accuracy of recognition of positive (happy + surprise; averaged) and negative (angry + disgust + sad + fear; averaged) facial expressions ($F_{1, 18} = 8.267$, $p = 0.010$). Follow up pairwise comparisons revealed that ebselen treatment significantly increased the accuracy of recognition of positive expressions [$t_{(19)} = 2.267$, $p = 0.035$], without significant effects on negative expressions [$t_{(19)} = - 0.899$, $p = 0.380$, Figure 6.6]. There was a significant main effect of order of treatment on the accuracy of recognition of facial emotion ($F_{1, 18} = 6.338$, $p = 0.022$). However, there were no

significant three-way interaction between order, treatment and emotion ($F_{1, 18}$ = 0.012, p = 0.913). There were no significant main effects of order, or significant order x treatment x emotion interactions when considering misclassifications or reaction times to emotional expressions. Absences of such suggest that neither misclassifications nor reaction times confounded the observed differences in accuracy of recognition of emotional expressions.

6.3.4 Correlation between MRS and Cognition

There were no significant correlations between the changes in ACC absolute ^{1}H-MRS metabolite concentrations with change in any of the CGT measures (Table 6.4). For the FERT, there was a trend towards significant negative correlation between change in ACC absolute glutamine concentration with change in accuracy of recognition of positive emotions [$r_{(14)}$ = - 0.459, p = 0.074; Table 6.6).

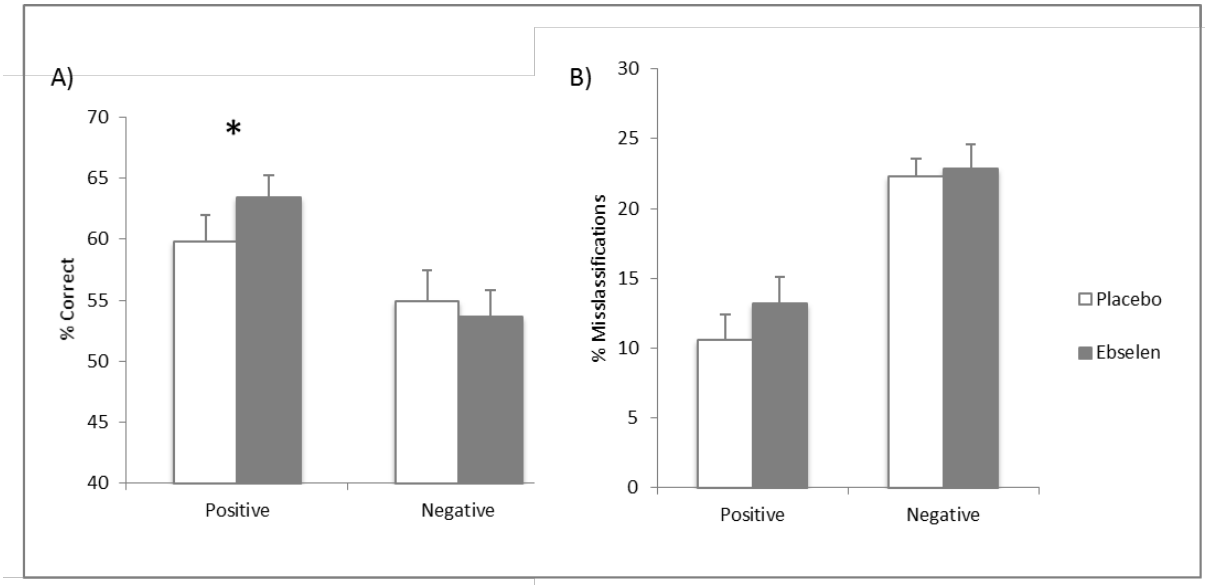

Figure 6.6: Results of the Facial Emotion Recognition Task. A) There were significant interactions between treatment and emotion, for accuracy of recognition of positive and negative facial expressions ($F_{1, 18} = 8.267$, $p = 0.010$). Ebselen treatment was associated with a significant increase in the accuracy of recognition of positive expressions without significant effects in recognition of negative expressions. B) Ebselen treatment was not associated with any significant differences in the misclassifications of emotional expressions. All data are plotted as mean ± standard error of mean, N=20. Values represent the percentage of average total responses for positive (happy + surprise) and negative (angry + disgust + fear +sad) expressions. * $p = 0.035$.

Table 6.4: Correlations between neurometabolite concentrations and performance on the CGT. There were no significant correlations between changes in absolute neurometabolite concentrations with changes in CGT measures.
r = spearman rho correlation coefficient; d = change (value under ebselen treatment - value under placebo treatment); Gln, glutamine; Glu, glutamate; Glx, glutamate+glutamine; Ins, inositol; GSH, glutathione.

		d_Delay aversion	**d_Risk adjustment**	**d_Reward seeking**
d_GABA	r	.032	-.226	-.324
	p value	.905	.399	.222
d_Gln	r	-.006	.053	-.326
	p value	.983	.846	.217
d_Glu	r	.244	.132	-.015
	p value	.362	.625	.957
d_Glx	r	.079	.059	-.259
	p value	.770	.829	.333
d_Ins	r	-.132	.082	-.368
	p value	.625	.762	.161
d_GSH	r	.138	.156	-.229
	p value	.610	.564	.393

Table 6.5: Correlations between changes in absolute neurometabolite concentrations with change in the accuracy of recognition emotional expressions on the FERT. There was a trend towards significant correlation between change in glutamine concentrations with change in accuracy of recognition of positive expressions (p = 0.074).
r = spearman rho correlation coefficient; d = change (value under ebselen treatment - value under placebo treatment); Gln, glutamine; Glu, glutamate; Glx, glutamate+glutamine; Ins, inositol; GSH, glutathione; acc, average accuracy.

		d_Positive_acc	**d_Negative_acc**
d_GABA	r	-.015	-.287
	p value	.957	.282
d_Gln	r	**-.459**	-.284
	p value	**.074**	.287
d_Glu	r	.361	.095
	p value	.169	.726
d_Glx	r	-.083	-.083
	p value	.760	.759
d_Ins	r	-.190	-.091
	p value	.482	.739
d_GSH	r	.078	.001

6.4: Discussion

Preclinical work has demonstrated that ebselen inhibits a target linked to lithium's antidepressant and anti-manic effects (Singh *et al.*, 2013). This mechanism has also been confirmed with in vivo brain imaging, in the results reported in chapter 5. The main findings in the present study were that short-term treatment with ebselen decreased a laboratory measure of impulsivity and produced a positive bias in the processing of emotional information. These observations demonstrate the potential use of ebselen for the treatment of impulsive disorders and dysphoric mood.

6.4.1 Ebselen's effects on mood and sleep measures

As in previous clinical studies with ebselen, the drug was well tolerated with the only distinguishing side effect being increased reports of drowsiness with ebselen treatment compared with placebo. Participants were 35% accurate at guessing whether they had received ebselen treatment during the drug visit, confirming success of blinding. There were no reported differences in mood ratings or sleep measures between ebselen and placebo treatment, suggesting that the observed neuropsychological effects were unlikely to be the result of medication side effects. The lack of noticeable improvement in subjective measures of mood following acute treatment with ebselen is also in keeping with effects of established antidepressant agents, which are known to exert neural and cognitive effects long before overt perception of mood improvement (Harmer *et al.*, 2009a).

6.4.2 Cambridge Gambling Task

Ebselen decreased the delay aversion measure of the CGT. This measure is calculated by subtracting bets made during the ascending trials from those made during the descending trials. In the descending trials, higher bets are presented first followed by increasingly smaller bets. In the ascending trials, smaller bets are presented first. Therefore, when impulsive tendencies are present, subjects are more likely to select higher bets in the descending trials and lower bets in the ascending trials ('inability to wait for later presented bets'), resulting in a higher delay aversion score. The delay aversion score has therefore been used as a surrogate measure of impulsive behavior (Deakin *et al.*, 2004, Newcombe *et al.*, 2011). High delay aversion on the CGT has been reported in participants at increased risk for bipolar disorder based on familial predisposition, as well as in healthy subjects scoring high on a hypomanic personality scale (Wessa *et al.*, 2015). Ebselen's ability to decrease delay aversion therefore demonstrates its potential in the treatment of disorders characterized by impaired impulse control.

Lithium's anti-suicide effects are thought to result partly from its ability to inhibit impulsive aggressive tendencies. This is because the effect of lithium in decreasing suicidal behavior is present even in patients who do not demonstrate mood improvement (Ahrens and Muller-Oerlinghausen, 2001). Ebselen's ability to decrease a laboratory measure of impulsivity therefore supports a lithium-like mechanism of action. Whether this effect is due to IMPase inhibition or some other mechanism shared between lithium and ebselen is unknown. Other mood stabilizers, such as valproic acid and carbamazepine, which also exert effects on

inositol cycling (Williams *et al.*, 2002), have not demonstrated anti-suicide properties of the same order as lithium. This suggests that some additional mechanism of action may be involved in mediating lithium's anti-suicide effects. There is preclinical evidence that non-IMPase dependent mechanisms may be involved in lithium's anti-impulsive actions (O'Donnell and Gould, 2007). Whether ebselen also targets these additional pathways remains to be established.

The reward seeking (risk taking) measure of the CGT is calculated based on the proportion of bets that subjects gamble on trials in which the more likely ratio of bets (red: blue box ratio) is selected. This measure is used as a surrogate marker for reward seeking or loss aversion. Low reward seeking on the CGT has been demonstrated in depressed patients (Murphy *et al.,* 2001), as well as in unaffected subjects at high familial risk for depression (Mannie *et al.*, 2015, Rawal *et al.*, 2013). In the present study, ebselen treatment increased reward seeking, in a manner suggestive of an antidepressant effect. Although there are no published studies looking at the effects of lithium on the CGT, lithium has some antidepressant effects particularly when used to augment first line agents and perhaps, though less certainly, for the treatment of bipolar depression (Nelson *et al.*, 2014; Young *et al.,* 2010).

A recent study looking at the effects of acute ebselen treatment on learning reported that ebselen decreased reward reinforcement (Singh *et al.*, 2015). The CGT measures reward seeking outside a learning context and thus no effects of reinforcement through learning could be determined. These observations however

suggest that ebselen may enhance pleasure seeking (antidepressant effect) without pathological reinforcement of reward-seeking mechanisms.

6.4.3 Facial Emotion Recognition Task

Ebselen treatment produced a positive bias in the processing of emotional information. Subjects had significantly better accuracies at recognizing positive facial expressions relative to negative expressions while on ebselen treatment. In addition, there were no differences in the misclassifications of emotional expressions between ebselen and placebo treatment, suggesting that the enhanced recognition of positive expressions was not driven by selective misclassification of expressions. When considering individual emotional expressions, there was a tendency towards more accurate identification of happy expressions, an observation similar to that reported by Singh and colleagues (2015), who used the FERT in a parallel arm design. The study by Singh et al. also reported enhanced recognition of expressions of disgust with ebselen treatment, a finding that was not present in this study. Differences in the ebselen dose used (twice as much in the present study) and study design (within subject vs. parallel group) could explain this difference.

Depressed patients have impaired recognition of facial expressions of emotion, being less likely to identify positive expressions and more likely to mislabel ambiguous expressions as sad (Gur *et al.*, 1992, Surguladze *et al.*, 2004). Enhancement of processing of positive relative to negative affect has been demonstrated in both healthy subjects and patients following treatment with antidepressants from across different pharmacological categories (Murphy *et al.*, 2008, Warren *et al.*, 2015). That ebselen enhanced accuracy of recognition of

positive expressions therefore points towards an antidepressant effect. This supports evidence from pre-clinical models reporting that ebselen reverses depressive symptoms by targeting biological pathways relevant to antidepressant action (Posser *et al.*, 2009, Singh *et al.*, 2013). For instance, Sing et al (2013) demonstrated that ebselen treatment attenuated $5HT_2$ receptor mediated responses. Intriguingly, this receptor pathway, that utilizes IMPase signaling, has been suggested as a mechanism for lithium's antidepressant effects (Friston *et al.*, 1989, Goodwin *et al.*, 1986).

In this study, the CGT and FERT were used in a within-subject design, with participants taking the same task on two separate occasions a week apart. This is unlike most previous publications with these tasks, where they have been used in parallel group design so as to avoid learning effects. Studies looking at the neural effects of repeated exposure to emotional faces have reported response habituation (Ishai *et al.*, 2004) or selective sensitization to specific emotional expressions (Strauss *et al.*, 2005).

The effects of order of treatment (ebselen first or placebo first) were assessed in both the CGT and FERT. On the CGT, there were no effects of order of treatment on the delay aversion, suggesting that learning did not play a significant role on this measure. There were however significant effects of order on the deliberation time and a trend towards a significant effect on reward seeking. On the FERT, there were no significant three-way interactions between order, treatment group and emotion, although 'order of treatment' has a significant effect on the accuracy of recognition

of emotional expressions. In a recent study looking at the reliability of repeat testing with the FERT (with a week interval between testing), Adams and colleagues (2015) reported increased accuracy in the recognition of emotional expressions on repeat testing, without selective effects to any individual emotional expressions. Repeat testing therefore may improve accuracy of emotional recognition across all expressions, as was the case in the present study, but without altering the selective effects on individual expressions (the main outcome confirming antidepressant action). In addition, learning effects in a within subject design would be expected to be counterbalanced across participants, thus minimizing their salience. Order of treatment is therefore unlikely to have had a significant effect on the CGT and FERT outcomes, and the results obtained can therefore probably be attributed to pharmacologic manipulation.

6.4.4 Correlations between neurochemistry and decision-making

The ACC has established roles in control of impulsive behavior, risk taking and reward seeking behaviour (Clark *et al.*, 2008, Rogers *et al.*, 2004). Optimal choice decision-making is regulated by fine balance between excitatory (glutamatergic) and inhibitory (GABAergic) signaling in this region (Jocham *et al.*, 2012). Low ACC GABA has been linked to impaired response inhibition (Silveri *et al.*, 2013). On the CGT, high ACC Glx has been associated with decreased ability to make adjustments to risk taking, while low GABA levels have been linked to increased delay aversion (Fujihara *et al.*, 2015).

In the present study, there were no significant correlations between changes in ACC glutamate, Glx or GABA following ebselen treatment with any of the measures of the CGT. A possible explanation for this would be that the small sample size used was not sufficient to detect neurochemical changes that correlate with neuropsychological task performance. Since the correlations between neurochemistry and cognition were not primary outcome measures, this study was not adequately powered to investigate them.

6.4.5 Limitation

The phase of the menstrual cycle has been shown to influence ^{1}H-MRS measures (Batra *et al.*, 2008, Epperson *et al.*, 2002). This was not taken into consideration during scheduling of study participation, an effect that may have confounded some of the measurements obtained. Female participants were however relatively fewer (7/20) and any menstrual cycle phase effects, if present, would not be expected to be a major confound.

6.5 Conclusion

This study has demonstrated that short-term treatment with the potential lithium-mimetic ebselen results in significant effects on behavioral measures of relevance to psychiatric treatment. Ebselen treatment decreased a laboratory measure of impulsivity, increased reward seeking and enhanced recognition of positive relative to negative emotional expressions. These effects are suggestive of a pharmacological agent with anti-impulsive and antidepressant effects. As a repurposed drug, ebselen is available for use in humans, and research on these indications using patient groups would be suggested.

Chapter 7: General Discussion

7.1 Overview

Major depressive disorder remains a significant global burden in spite of some advances in the understanding of its pathophysiological mechanisms and developments of new pharmacological and psychological treatments. More than six decades since the discovery of the original antidepressant drugs, the currently available agents largely work on the same pharmacologic principle - facilitation of monoaminergic neurotransmission. On the other hand, therapeutic outcomes have remained modest with many patients failing to achieve remission even with adequate trials of pharmacotherapy and psychotherapy.

Current algorithms recommend trialing different antidepressants in case of non-response, although this approach is limited by the similarities in mechanistic targets for the available agents. Medication side effects have also remained a significant concern. Several approaches have been proposed for overcoming these challenges. Among them is the utilization of neurobiological understandings into disease mechanisms towards targeted drug development. In particular, knowledge of the neurochemistry of depression and effects of pharmacological manipulations on it has the potential for guiding research into the pathophysiological mechanisms of depressive disorders, as well as the development of treatments for targets identified within the neurochemical pathways.

7.2 Summary of findings

Chapter 1 reviewed the neurobiology of depression with an emphasis on the role of pharmacotherapy and its limitations. In addition, the role of lithium in the treatment and prophylaxis of mood symptoms, as well as current understanding of its mechanisms of action were discussed.

Chapter 2 looked at the various tools that have been used to answer the research questions in this thesis. In particular, the technique of MRS and its application in psychiatric research was discussed. Two experimental medicine neuropsychological models used in this thesis, the Cambridge Gambling Task and the Facial Emotion Recognition Task, were also described.

Chapter 3 and 4 focused on the neurochemistry of depression and its link with clinical variables. There is increasing evidence for the involvement of the glutamatergic system in depression, although results from MRS studies have often been inconsistent. Several limitations could explain the variability in the reported findings, among them technical challenges with spectroscopic imaging at low field strengths and heterogeneity of the patient population. Chapters 3 and 4 attempted to overcome these challenges.

In Chapter 3, measurements of glutamatergic metabolites were carried out with 7-tesla MRS, an approach that enabled accurate and separate quantification of glutamate and glutamine. Levels of these metabolites were compared between patients and controls. Previous studies had suggested that levels of glutamate, or Glx (the combined signal of glutamate and glutamine) are decreased in the prefrontal

cortex in depressed patients. In the present study, this was observed only in female patients. These results were unexpected given that previous studies had not reported significant effects of gender on glutamatergic metabolites in depression. Although the gender effects were strongly significant, they could have arisen from a type 1 error due to the unbalanced proportions of participants within the gender groups. This was a limitation for the analysis carried out for this thesis, but these proportions are expected to be matched at completion of the study. Male and female patients differed on a number of clinical parameters and these could have accounted for a part of the gender effects. Nonetheless, the results suggest the need to factor in gender when assessing glutamatergic metabolites in depression. A review of previous publications revealed that most had used modest sample sizes, which could explain why the gender effects had not been reported, or why the findings on glutamatergic changes were fairly inconsistent. As expected most of the studies had a preponderance of females with small numbers of males. One study had noted that glutamate changes in depression were more prominent in female patients, although these changes did not attain statistical significance (Hasler *et al.,* 2005).

Glutamatergic metabolites in a subcortical region, the putamen, were also investigated. The gains in signal over noise with 7-tesla MRS allowed measurements to be obtained from this deep gray matter region, thus demonstrating another advantage of ultra-high field imaging. Elevations in the levels of glutamine were observed, a finding not previously reported for this region in MDD. In addition, post-hoc analysis revealed a significant increase in the glutamine to glutamate ratio in depressed patients relative to controls. Intriguingly, this finding (increased

glutamine: glutamate ratio) has been reported in the ACC in patients with hepatitis C while undergoing treatment with the depression-inducing immune cytokine, interferon-alpha (Taylor *et al.,* 2014). Whether this observation stems from similar pathophysiological mechanisms within the two regions, and its relevance to the neurochemistry of MDD, remains to be elucidated.

Chapter 4 explored further the findings from chapter 3, with the aim of identifying associations between patient clinical variables and neurometabolite levels. Although the study was not originally designed to answer this question, exploring such relationships was considered useful for informing the design of future studies specifically aimed at determining these relationships with the objective of understanding more about the clinical and neurochemical heterogeneity of depression. A wide variety of clinical variables were investigated, that ranged from patient demographics and early life experiences to measures of severity of depressive symptoms. None of these variables however associated to a significant level with neurochemistry. This could have been a type II error given that the study was probably not sufficiently powered to investigate these relationships.

Chapters 5 and 6 focused on a novel pharmacological entity called ebselen, which has recently been demonstrated to have antidepressant and lithium-like effects in preclinical models. Ebselen is an organoselenium compound with proven safety based on clinical trials for non-psychiatric indications. It currently remains unlicensed, as it has not demonstrated clinical efficacy for any condition. Several enzymatic and receptor targets for ebselen have been reported, including inhibition

of the inositol monophosphatase (IMPase) enzyme that is believed to be important for the action of mood stabilizing agents such as lithium. In addition, ebselen targets the glutamatergic system and the glutathione peroxidase antioxidant pathways suggesting broad applications for neuropsychiatric treatment.

Lithium-like behavioral effects have also been demonstrated with animal models following ebselen treatment. In Chapter five, 7-tesla ^{1}H-MRS was used to demonstrate that ebselen targets brain IMPase, through measurement of brain *myo*-inositol levels. This effect was region selective, being present in the prefrontal but not the occipital cortex. Interestingly, a similar effect has previously been noted with lithium (Moore *et al.*, 1999). Decrease in inositol levels with ebselen treatment, and the regional nature of this change, both support a lithium-mimetic effect. Ebselen was also found to decrease levels of glutamate in the prefrontal cortex, a mechanism suggestive of a neuroprotective effect and consistent with its ability to inhibit glutaminase.

Chapter 6 investigated the neuropsychological effects of ebselen treatment, using two tasks validated for the assessment of behavior relevant to the treatment of mood and impulsivity disorders. Ebselen was found to decrease a laboratory measure of impulsivity suggesting that it could be used for the treatment of disorders characterised by an impulsive phenotype, including in the prevention of impulse associated suicidal behaviour in mood disorders. Intriguingly, impulse-control has been suggested as contributing to lithium's anti-suicide properties. Ebselen was also found to increase reward-seeking and to produce a positive bias in

the processing of emotional information, mechanisms which have been associated with antidepressant treatments from across different pharmacological categories. These results offer promise for the use of ebselen in the treatment of dysphoric mood.

7.3 Suggestions for Future Experiments

This thesis aimed to use 7-tesla MRS to determine neurochemical alterations in depression, and to further use this technique to determine effects of pharmacological manipulation with a potential lithium-mimetic agent on brain neurochemistry. The results obtained on the neurochemistry of depression did not enable a clear conclusion to be reached. However, they highlighted the importance of considering gender as a modifier in glutamatergic neurochemistry in depression.

It would be useful to carry out methodical assessments on glutamate changes in depression with an emphasis on gender specific effects on neurometabolite levels, so as to confirm or negate the findings reported in this thesis. If indeed there are gender differences in glutamatergic neurochemistry in depression, it would suggest the need to consider gender as an additional factor when prescribing pharmacological treatments for depression and particularly when choosing a glutamate modulating agent. It will also be important to control carefully for phase of the menstrual cycle in future MRS studies.

Depression is partly a genetic disorder and neurochemical measurements with MRS offer a means to assess possible impacts of genetic variation on brain neurochemistry. However, there are limitations to the application of cross-sectional

proton-MRS studies in investigating brain neurochemistry. Even at the precision of 7-tesla imaging, ^{1}H-MRS cannot answer questions on the dynamic changes to the amino-acid neurotransmitter systems. In addition, cross-sectional analyses are not best suited to investigate neurochemical changes during the course of mood disorders.

These limitations can be overcome in two ways. First, through the incorporation of non-proton based spectroscopic methods, such as ^{13}C and ^{31}P MRS, that are capable of measuring dynamic metabolite and energy changes respectively. Second, by applying these techniques in follow-up studies in which neurochemical measurements are correlated with clinical parameters and treatment response over the course of illness. Although glutamatergic neurochemistry did not correlate with any of the assessed patient variables in this thesis, this could have resulted from the weakness of the study design. Identification of clinical correlates of neurochemistry in depression would be useful in guiding treatment selection as well as in building phenotypic patterns that could assist with diagnosis and patient sub-categorisation. These associations therefore need to be investigated further.

The MRS studies on ebselen provide clinical evidence for its mechanistic targets that have hitherto been reported in preclinical models. The results from this thesis support the use of ebselen as a lithium-mimetic agent, with potential antidepressant and mood stabilising properties. Neuropsychological assessments show potential use of ebselen not only for treating mood symptoms, but also for the treatment of disorders of impulse control. It would therefore be important to extend these

investigations into patient groups, so as to establish ebselen's clinical utility. The glutamatergic effects of ebselen also suggest that it could have applications beyond pharmacotherapy for mood disorders, for instance, in the treatment of neurodegenerative diseases and psychosis linked to glutamate excitotoxicity. It would thus be worthwhile exploring its use for these indications as well.

Finally, the imaging results presented in this chapter focused on the technique of MRS. Additional information on biological mechanisms of disease and effects of pharmacological manipulation can be obtained through the use of multimodal imaging. This is particularly with consideration that while spectroscopy measures changes occurring within a small region of the brain defined by the MRS voxel, psychiatric diseases and their treatments are more likely to work at the neural network level. It would therefore be advisable to extend the findings reported in this thesis, both on neurochemical analysis in depression and on the effects of ebselen treatment on neurochemistry and behaviour, to other imaging modalities. Functional MRI, arterial spin labeling and ligand binding studies such as Positron Emission Tomography (PET) are some potential tools that could be used to achieve this.

7.4 Conclusion

In summary, the results in this thesis demonstrate that there are changes to glutamatergic neurochemistry in depression, and that some of these changes may be gender specific. The lithium-mimetic agent ebselen decreases brain *myo*-inositol concentrations, confirming inhibition of IMPase in the human brain and potential use as a mood-stabilising drug. Ebselen treatment decreases brain glutamate levels

suggestive of a neuroprotective effect. Behavioural assessments show that ebselen treatment decreases impulsivity, increases reward-seeking and produces a positive bias in the processing of emotional information. These findings support the use of ebselen as an anti-impulsive and antidepressant agent. As a repurposed drug, ebselen is likely to be safe for human use and extending these investigations into patient groups would be warranted. On a whole, these studies demonstrate the utility of ^{1}H-MRS to investigate neurochemical changes in psychiatric diseases, as well as in guiding targeted drug development.

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