

Molecular Pathways of Depression and Antidepressant Therapies for Treatment Resistant Depression



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Doctor of Philosophy

By
Claire McGrory
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Supervised by: Prof. Declan McLoughlin

Department of Psychiatry (School of Medicine) and
Trinity College Institute of Neuroscience
Trinity College Dublin, Dublin 2

Declaration

I declare that this thesis is submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College, and has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work with the exception of patient and healthy control recruitment for the EFFECT-Dep and KARMA Dep trials, and the electroconvulsive stimulation (ECS) study.

For studies using the EFFECT-Dep trial (Chapters 3 and 4), I carried out all the cDNA synthesis, qRT-PCR and data analyses. I also carried out RNA extractions on ~60% of the healthy controls. A team of 10 researchers was responsible for the trial and Dr Erik Kolshus carried out the majority of patient blood RNA extractions.

For studies using the KARMA-Dep trial (chapter 7), I extracted all RNA samples and carried out the molecular analyses. Dr Bronagh Gallagher and a team of five researchers recruited all patients and ran the clinics.

For the *in vivo* studies (Chapters 3 and 4), I carried out all qRT-PCR runs and gene expression analyses. Dr Sinead O'Donovan and Dr Karen Ryan carried out the animal studies outlined in Section 2.2.2 before my time in the lab.

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Claire McGrory
December 2019

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Summary

According to the World Health Organisation, depression is the most common psychiatric disorder. Despite this, the pathophysiology of depression is unclear. Electroconvulsive therapy (ECT) is the most acutely effective treatment available for severe, sometimes life-threatening, depression. However, its molecular mechanism of action is not understood. Ketamine is a newly emerging rapid antidepressant pharmacotherapy for treatment resistant depression, but its mechanism is also unclear. Advances in scientific techniques are aiding the discovery of novel targets for depression. A deep sequencing study of microRNAs in depression conducted by my lab identified two microRNAs that are altered in patients with depression. Three genes were identified as shared targets of these: *VEGFA*, *E2F1* and *SIRT1*. Peripheral whole blood *VEGFA* mRNA levels were also increased in depression and decreased by ECT. Therefore, *E2F1* and *SIRT1* are genes of interest in depression and the molecular mechanism of ECT. Finally, a proteomics study by my lab identified PEDF as a protein of interest. PEDF was increased in medicated patients with depression and increased further by ECT. Therefore, I hypothesised that antidepressants may target PEDF.

The first aim of this study was to carry out gene expression analysis of *E2F1* and *SIRT1* in patients with depression in comparison to healthy controls. qRT-PCR results showed that levels of both genes were lower in patients with depression, but this was not attenuated by ECT. With *E2F1*, there were no differences in levels among depressive subgroups (psychosis, polarity, response status) and there were no associations with depression severity as measured by the HAM-D-24 rating scale. These results indicate that *E2F1* may be a trait marker of depression but it is not useful as a clinical marker. With *SIRT1*, there were no significant differences among subgroups, but levels were lower in patients with bipolar depression in comparison to unipolar depression. Numbers in the bipolar group were small ($n = 19$); so repetition in a larger cohort may be of use. In patients who responded to ECT and attained remission, there was also a trend for a correlation between an increase in *SIRT1* with ECT and a decrease in HAM-D24 scores. As correlation analysis was exploratory, this would also need repeating in a larger cohort. Finally, gene expression analysis was performed on blood and brains of rats treated with chronic and acute electroconvulsive stimulation (ECS). *E2F1* mRNA was decreased specifically in the dentate gyrus, with no change in peripheral blood. *Sirt1* was increased in the hippocampal formation alone, with no change in peripheral blood. These results indicate that both *E2F1* and *SIRT1* may have a role in the mechanism of ECT in the brain but which may not be translated to the periphery.

The next aim of this study was to evaluate a role for PEDF in the mechanism of antidepressants using an *in vitro* model. Primary rat cortical neurons, astrocytes and microglia were cultured. Astrocytes were found to express the highest levels of *Serpinf1* (PEDF) mRNA. Therefore, astrocytes were treated with fluoxetine, imipramine and noradrenaline for 30 minutes, 1, 3, 6, 12 and 24 hours. Only imipramine (at 24 hours) and noradrenaline (at 3 hours) significantly decreased *Serpinf1* mRNA expression. However, neither antidepressant drug had an effect on PEDF secretion from astrocytes. Therefore, I concluded that PEDF may not have a role to play in the molecular mechanism of antidepressants in so far as could be discerned from studying primary cultures. PEDF, however, may have a role in other brain regions or it may have a peripheral role.

The final aim of this study was to evaluate the effect of a single infusion of ketamine or midazolam on *VEGFA*, *PEDF (SERPINF1)*, *E2F1* and *SIRT1* mRNA expression. Of note, PEDF and VEGF are both neurotrophic and also known to interact, with PEDF blocking VEGF to inhibit angiogenesis. The ratio of *VEGFA* to *SERPINF1* was also evaluated, based on evidence from previous literature, as this may provide a measure of pathway activity. Samples from the KARMA-Dep pilot trial were used and depressed patients' peripheral whole blood samples were collected 60 minutes before and 4 hours after an infusion of ketamine or midazolam. Ketamine alone significantly increased *VEGFA* levels. *SERPINF1* levels were lower following ketamine treatment but this was not significant. The *VEGFA/SERPINF1* mRNA ratio was also significantly increased following ketamine. No change was found with *E2F1* levels. *SIRT1* levels were increased in both groups post infusion, which may be due to diurnal variation. Plasma levels of *VEGFA* and PEDF protein were also analysed but no changes were found.

The findings in this thesis show that *E2F1* and *SIRT1* may not be useful clinical markers in depression but there may be a role for them in the CNS that could be investigated further using animal models. This work points to a possible role for *VEGFA* and *SERPINF1* in the molecular action of ketamine treatment but replication in a larger sample set is required. This is the first report of a relationship between these two genes in treatment for depression, and provides an interesting link to the findings of the deep-sequencing study and proteomic study that this work is based on. The interplay between *VEGFA* and PEDF could be a new pathway of interest in the molecular mechanism of treatments for patients with depression.

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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin/5-hydroxytryptamine
5-HT1A	serotonin receptor 1
5-HTTLPR	serotonin-transporter-linked polymorphic region
8-OHdG	8-hydroxy-2'-deoxyguanosine
AAV	adeno-associated virus
ACC	anterior cingulate cortex
ACTH	adrenocorticotrophic hormone
Akt	protein kinase B
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
APA	American Psychiatric Association
apaf-1	apoptotic protease activating factor 1
Ara-C	cytarabine
ATM	ATM serine/threonine kinase
ATP	adenosine triphosphate
AUC	area under the curve
A β	amyloid- β
BBB	blood brain barrier
bcl-2	B-cell lymphoma 2
BD	bipolar disorder
BDI	Beck Depression Inventory
BDNF	brain-derived growth factor
BK	big potassium channel
BMI	body mass index
BPD	bipolar disorder
BPRS	Brief Psychiatric Rating Scale
BSA	bovine serum albumin
CADSS	Clinician-Administered Dissociative States Scale
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CDK2	cyclin-dependent kinase 2

CDMEM	complete DMEM/F12
cDNA	complementary DNA
CM	conditioned media
cNBM	complete NBM
CNS	central nervous system
CO ₂	carbon dioxide
CREB	cAMP response element binding
CRH	Corticotrophin-releasing hormone
CSF	cerebrospinal fluid
CT	cycle threshold
CV	coefficient of variancy
CVS	chronic variable stress
DA	dopamanergic
DBC1	deleted in breast cancer 1
DG	dentate gyrus
dH ₂ O	distilled water
DHFR	dihydrofolate reductase
DIV	days in vitro
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DP	dimerization partner
DPBS	Dulbecco's phosphate buffered saline
DSM	Diagnostic and Statistical Manual for Mental Disorders
ECS	electroconvulsive shock
ECT	electroconvulsive therapy
EDTA	Ethylenediaminetetraacetic acid
EFFECT-Dep	Enhancing the Effectiveness of Electroconvulsive Therapy in Severe Depression
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial NOS
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FACS	fluorescence-activated cell sorting
FAM	6-carboxyfluorescein

FBS	foetal bovine serum
FC	fold change
FMN	flavin mononucleotide
FOXO1	forkhead box O1
FOXO3	forkhead box O3
FU	follow up
G1 phase	growth phase cell cycle
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCL	granular cell layer
GDNF	glial-derived growth factor
GILZ	glucocorticoid-induced leucine zipper
GLM	general linear model
GM-CSF	granulocyte-macrophage colony-stimulating factor
GR	glucocorticoid receptor
GSH-PX	glutathione peroxidase
GWAS	genome-wide association
H	histone
H ₂ O ₂	hydrogen peroxide
HAM-D24	Hamilton Depression Rating Scale, 24 item version
HDAC	histone deacetylase
H-DHA	dihydroalprenolol
HPA	hypothalamic-pituitary adrenal
hTERT	human telomerase reverse transcriptase
HVA	homovanillic acid
ICD-10	International Classification of Diseases, 10th revision
IDO	indoleamine-pyrrole 2,3-dioxygenase
IFN- γ	interferon gamma
IL	interleukin
IQR	inter quartile range
I κ B α	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JNK	c-Jun N-terminal kinases
KARMA-Dep	The Ketamine as an Adjunctive Therapy for Major Depression trial
KCL	potassium chloride

KH ₂ PO ₄	potassium phosphate monobasic
Kir6.2	potassium voltage gated channel subfamily
LC	locus coeruleus
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LTP	long term potentiation
MAO-1	monoamine oxidase A
MAOI	monoamine oxidase inhibitors
MAPK	mitogen-activated protein kinases
M-CSF	macrophage colony-stimulating factor
MDD	major depressive disorder
miR	micro-RNA
miRNAs	micro-RNAs
MOA-A	monoamine oxidase A
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MTL	medial temporal lobe
Mtor	the mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyoD	myoblast differentiation protein
NA	noradrenaline
NaCl	Sodium Chloride
NAD ⁺	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaHPO ₄	sodium phosphate dibasic
NAM	nicotinamide
NAMPT	nicotinamide phosphoribosyltransferase
NBM	neurobasal media
NET	noradrenaline transporter
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NK	natural Killer
NMDA	N-methyl-D-aspartate

NMN	nicotinamidemononucleotide
NNMAT	nicotinamide N-methyltransferase
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
NRI	norepinephrine reuptake inhibitor
NRT	no reverse transcriptase control
NSAID	nonsteroidal anti-inflammatory drugs
NT-3	neurotrophin-3
NTC	no template control
ORC	origin recognition complex
P/S	penicillin-streptomycin
P14arf	ARF tumor suppressor
p53	tumour protein p53
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEDF	pigment-epithelial derived factor
PEDF-R	pigment-epithelial derived factor receptor
PER2	period circadian regulator 2
PET	positron emission tomography
PFC	pre-frontal cortex
PGC1- α	peroxisome proliferator- activated receptor gamma
PKA	protein kinase A
PPAR γ	peroxisome proliferator-activated receptor
pRb	phosphorylated Rb
PRL	prolactin
qRT-PCR	quantitative real-time polymerase chain reaction
QUIN	quinolinic acid
Rb	retinoblastoma protein
RNA	ribonucleic acid
ROC	receiver operating characteristic
ROS	reactive oxygen species
RPE	retinal pigment epithelium
rPgACC	perigenual anterior cingulate cortex

rpm	revolutions per minute
RQ	relative quantificaion
RT	reverse transcriptase
S phase	DNA synthesis phase cell cycle
SCID-1	Structured Clinical Interview for DSM-IV Axis I Disorders
SD	standard deviation
SEM	standard error of the mean
SERT	serotonin transporter
Sir2	silent information regulator-2
SIRT1	Sirtuin1
SNP	single nucleotide polymorphism
SNRI	serotonin and noradrenaline reuptake inhibitor
SOD-1	superoxide dismutase 1
SOD-2	superoxide dismutase 2
SSRI	selective serotonin reuptake inhibitor
TAC	total antioxidant capacity
TCA	tricyclic antidepressant
TMB	3,3',5,5'-tetramethylbenzidine
TNF- α	tumor necrosis factor alpha
TSH	thyroid stimulating hormone
VEGF	vascular endothelial growth factor
WHO	World Health Organisation
α 1-AR	alpha-1 adrenergic receptor
α 2-AR	alpha-2 adrenergic receptor
Δ	change in

Chapter 1.

Introduction

1.1 Depression

1.1.1 General overview

Depression is recognised as one of the most common psychiatric disorders. Globally, roughly 300 million people of all ages suffer from this condition (WHO, 2017). According to the World Health Organisation (WHO), depression is now the leading cause of disability worldwide, contributing largely to the overall global burden of disease, and is comorbid with other diseases such as cardiovascular disease, diabetes and arthritis (Vos et al., 2012, Katon et al., 2007). These symptoms range from mild, moderate to severe and patients can suffer disruptions to their work, family and social life. The worst outcome for depression sufferers is suicide, with over 800,000 deaths occurring each year (Marcus et al., 2012).

Despite this large individual, economic and social burden, the pathogenesis of depression remains unclear, and treatment for this illness is not ideal, with many patients failing to respond to standard treatments (Rush et al., 2006). Depression is also a heterogeneous disorder and identifying markers that differ between subtypes of depression is be important. Despite the large number of studies on the pathophysiology of depression, there have been no reliable outputs that link any of these data to a specific subtype of depression (National Collaborating Centre for Mental Health (UK), 2010). Therefore, increasing our understanding of the molecular mechanisms underlying depression will help with identifying new treatments, and will allow for biomarker discovery to aid diagnosis, prognosis and treatment choice.

1.1.2 Diagnosis and classification

One of the main issues with depression is the heterogeneous nature of the disorder. Although people present with similar symptoms, the cause of this illness may differ (National Collaborating Centre for Mental Health (UK), 2010). Several depression subtypes have been identified over the past number of years including melancholia, atypical depression, depression with a seasonal pattern/seasonal affective disorder, and psychotic/non-psychotic depression (American Psychiatric Association, 2013). Depressive features can also occur in both unipolar and bipolar depression.

Today, there are two main classification systems in use; the Diagnostic and Statistical Manual for Mental Disorders 5 (DSM-5) (American Psychiatric Association, 2013) and the International Classification of Diseases, 10th revision (ICD-10) (WHO, 1992). For my own studies, the DSM 4th edition (DSM-IV) criteria were used. Apart from some minor changes in classification, DSM-5 and IV are relatively similar with the criteria for a depressive episode remaining the same (Regier et al., 2013). Table 1 outlines the different symptoms present in depression and the DSM-IV criteria for classifying a patient as having a depressive episode.

A. During the same two week period at least five of the following symptoms must be present and must represent a change from previous functioning. They must not be clearly attributable to another medical condition; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.

Depressed mood most of the day, nearly every day, as indicated by either subjective report or observation made by others.

Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).

Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day.

Insomnia or hypersomnia nearly every day.

Psychomotor agitation or retardation nearly every day (observable by others).

Fatigue or loss of energy nearly every day.

Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day.

Diminished ability to think or concentrate, or indecisiveness, nearly every day.

Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

C. The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.

D. The episode is not attributable to the physiological effects of a substance.

E. There has never been a manic episode or a hypomanic episode.

Table 1.1 DSM-IV criteria to be met to be diagnosed with having a depressive episode

1.1.2.1 Psychotic depression and other subtypes

Psychotic depression was recognised as a type of depression by the ICD10 and the DSM IV (Ostergaard et al., 2012). The delay in this classification came from the discussion on whether psychotic depression is a distinct illness or if it just represents a severe form of depression (Rothschild, 2013). The main features of psychotic depression include hallucinations and delusions, as well as suffering from the typical symptoms of depression. An example of a typical case would be anhedonia, loss of interest, inability to concentrate and accompanied by delusions of guilt, worthlessness and thoughts of impending disaster (Rothschild, 2009). Psychotic depression also has a higher relapse rate and more often ends in fatal consequences (Johnson et al., 1991, Vythilingam et al., 2003). In terms of treatment, patients with psychotic depression respond poorly to antidepressant treatment and psychotherapy (Rothschild, 2009). Patients respond more favourably to electroconvulsive therapy (ECT) than non-psychotic depressed patients, with the American Psychiatric Association (APA) and other organisations recommending ECT as a first line treatment (American Psychiatric Association, 2013, Petrides, 2002).

The DSM-IV identifies several other subtypes of depression including melancholic depression, seasonal affective disorder and atypical depression.

1.1.2.2 Bipolar depression

Unipolar and bipolar depression have long been thought to be linked. Studies have indicated that roughly 25% of patients first diagnosed with unipolar depression go on to develop some sort of manic/hypomanic episode, which can then progress to bipolar disorder (McIntyre et al., 2018). Bipolar depression is often difficult to diagnose as in a depressive state the patient may be misdiagnosed as unipolar depressed (Han et al., 2018).

The main issue with this is that treatment differs for the two diagnoses, highlighting the importance of developing biological markers that can differentiate between the two disorders.

1.1.3 Epidemiology of depression

The first study to compare occurrence of depression among different nationalities was published in 1996. This study indicated that the lifetime prevalence of depression ranged from 1.0% (Czech Republic) to 16.9% (United States) (Weissman et al., 1996). Updated reports have indicated that lifetime prevalence rates of unipolar depression are in the region of 10-20% (Weissman et al., 1996, Kessler et al., 2003, Kessler and Bromet, 2013). Women are twice as likely to suffer from unipolar depression, and the typical age of onset for a major depressive episode ranged from mid 20s to late 40s (Kessler and Bromet, 2013).

Lifetime prevalence of bipolar disorder tends to be lower, with studies indicating the overall lifetime prevalence of bipolar spectrum disorders was 2.4% (Merikangas et al., 2011). The influence of sex on bipolar disorder is unclear, with the average age of onset reported as 20-30 years (Rowland and Marwaha, 2018).

1.1.4 Pathophysiology of depression

Despite the large number of people that suffer from depression and its impact on the global burden of disease, the exact pathophysiology of depression remains largely unclear. One of the main reasons for this is probably the heterogeneity of the disorder (Ciobanu et al., 2016). However, research into this area is extensive with a wide range of biological systems being implicated in the development of depression.

1.1.4.1 Monoamines and depression

Despite the lack of knowledge on the precise aetiology of depression, there is still a number of pharmacological treatments available, most of which are based on the accidental discovery of the therapeutic effect of tricyclic antidepressants (TCAs) in the 1950s (Berton and Nestler, 2006). The belief that these drugs target monoamine membrane transporters led to the monoamine theory of depression; the idea that depression is related to decreased levels of centrally available noradrenaline or serotonin (also known as 5-hydroxytryptamine, 5-HT) (Mulinari, 2012, Jesulola et al., 2018, Delgado, 2000). Although it is accepted that elevated levels of monoamines can improve depressive symptoms, these antidepressants take weeks to take effect, and in some cases do not work at all (Marathe et al., 2018, Rush et al., 2006). Monoamine oxidase inhibitors (MAOIs), which inhibit the enzymes that break down serotonin and noradrenaline, are still used to good effect today. However, as with other antidepressants, there is a latent effect and not all patients respond (Berton and Nestler, 2006). Therefore, a better knowledge of how drugs target this system is needed as molecules downstream of monoamine signalling pathways may have a more direct involvement in depression.

Even though targeting the monoamine system is not always effective, there is evidence that monoaminergic systems play a role in the behavioural symptoms of depression (Jesulola et al., 2018). It has been reported that 5-HT binding is reduced in the prefrontal-cortex of post-partum brains in patients who committed suicide (Mann et al., 2000). The 5-HTTLPR (serotonin-transporter-linked polymorphic region) polymorphism is associated with major depression (Mann et al., 2000), and reduced cerebrospinal fluid (CSF) concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of serotonin, has been reported in drug-free depressed patients (Owens and Nemeroff, 1994, Asberg and Bertilsson, 1976, Asberg et al., 1976).

Abnormalities in noradrenergic signalling have also been detected in the brains and CSF of depressed patients. Reduced density of the noradrenaline transporter has been reported in the locus coeruleus (LC) of depressed patients and this was later confirmed by radioligand binding studies (Klimek et al., 1997, Maletic et al., 2017). Concentrations of a major metabolite of noradrenalin, 3-methoxy-4-hydroxyphenylglycol, have been correlated with lifetime mood burden (Maletic et al., 2017), and salivary levels of this metabolite have been correlated with mood scores in men (Watanabe et al., 2012). Changes in the two receptors for noradrenaline, alpha-1 adrenergic receptor (α 1-AR) and alpha-2 adrenergic receptor (α 2-AR), have also been reported; α 1-ARs are desensitised in the post-partum brains of depressed patients, but the density of α 2-ARs has been reported to be increased, possibly as a compensatory mechanism (Ordway et al., 2003, Maletic et al., 2017).

Noradrenaline and serotonin have been implicated in many of the features of depression including low mood, reduced motivation and concentration, fatigue, and psychomotor agitation/retardation (Jesulola et al., 2018). However, for the reasons mentioned above (latent response to drugs that target these systems and no response in some patients) research into depression has started to focus on other areas.

1.1.4.2 The hypothalamic-pituitary-adrenal axis in depression

Over the past number of years dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis has been one of the most consistent findings in depression (Pariante and Lightman, 2008). The HPA axis is a feedback loop that consists of the hypothalamus, pituitary and adrenal glands (Varghese and Brown, 2001, Keller et al., 2017), Figure 1.1 summarises the HPA axis. The axis begins with the secretion of Corticotrophin-releasing hormone

(CRH) and vasopressin from the hypothalamus, to activate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary (Pariante and Lightman, 2008). ACTH in turn stimulates the release of cortisol (a glucocorticoid) from the adrenal glands (Pariante and Lightman, 2008, Keller et al., 2017). Cortisol then binds to glucocorticoid and mineralocorticoid receptors (GRs and MRs) in both peripheral tissue and the brain, including the HPA axis itself, forming a negative feedback loop (Keller et al., 2017, Varghese and Brown, 2001). Cortisol plays a role in various functions of the brain, including regulation of neuronal survival, neurogenesis, memory formation and emotional response (Pariante and Lightman, 2008, Herbert et al., 2006). The HPA axis is activated by physical and emotional stress and is needed in healthy physiological functions. However, over-activation of this axis, possibly due to an impairment of GR-mediated negative feedback (Pariante, 2006), has been reported in depression (Varghese and Brown, 2001, Pariante and Lightman, 2008).

It has been reported that depressed patients have increased levels of cortisol in saliva, plasma and urine, and increased size of the pituitary and adrenal glands (Pariante and Lightman, 2008). One measurement that is frequently reported in studies is a higher cortisol awakening response and a blunted response to the dexamethasone test (Vreeburg et al., 2009, Hinkelmann et al., 2013). GR receptor function has also been reported to be dysfunctional in depressed patients with antidepressants being shown to normalise this (Pariante, 2006). Over-activation of this pathway has been proposed to lead to neuronal death/decreased cognitive function (Herbert et al., 2006) In patients, increased cortisol has been linked to decreased hippocampal volumes (Frodl and O'Keane, 2013) and also could be involved in reduced glia numbers/function in depression (Cotter et al., 2001). However, there is also conflicting reports that cortisol is not associated with depressive symptoms in patients and cannot predict treatment response (Suijk et al., 2018).

Although there is evidence to support the HPA axis in depression, and drugs targeting hypercortisolism have some promise as an adjunctive therapy for depression (Jahn, 2005), however clinical data on other drugs targeting the HPA axis in depression are lacking (Pariante and Lightman, 2008) and it is unclear how effective targeting this system would be.

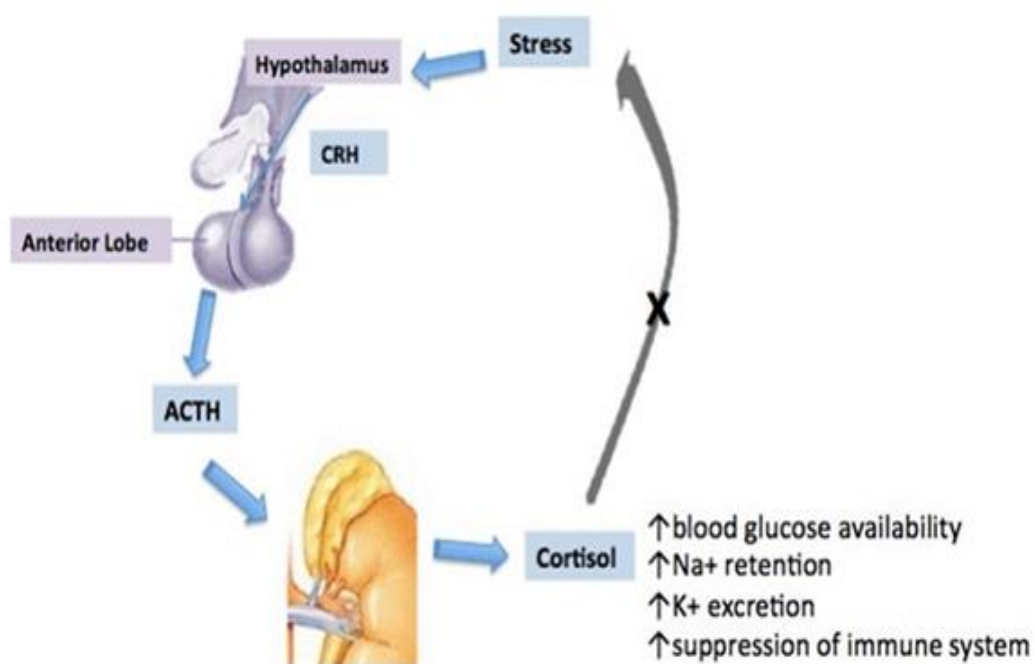


Figure 1.1 The HPA axis

In health the HPA axis functions to release cortisol in response to stress, this can become hyperactive in depression (Hine et al., 2019). Abbreviations: CRH; Corticotropin-releasing hormone, ACTH; adrenocorticotrophic hormone.

1.1.4.3 The inflammatory model of depression

For many years now it has been established that depression can be accompanied by inflammation and changes in immune cell function (Maes, 2011). As well as these biological observations, patients receiving cytokine therapy for cancer treatment reportedly developed mood changes (Capuron et al., 2002). These clinical findings, along with the observations that the features of ‘sickness behaviour’ are quite similar to some

of the features of depression, has led to an inflammatory model of depression (Dantzer et al., 2008). However, whether inflammation is a cause of depression or merely another symptom/consequence remains unclear (Jeon and Kim, 2016).

Historically, the brain was considered an ‘immune privileged’ organ with the blood brain barrier (BBB) preventing entry of pathogens, circulating immune cells, and factors within the blood (Louveau et al., 2015a). The discovery that immune cells are present in high numbers in the meninges of the brain, and participate in immunosurveillance of the central nervous system (CNS) have contradicted this view (Rua and McGavern, 2018). The recent discoveries that the peripheral lymphatic system branches to the CNS (Louveau et al., 2015b) and the observation of perivascular macrophages in the brain parenchyma (Cai et al., 2018) support a role for the immune system in the CNS, and highlight the importance of investigating immune dysfunction in psychiatric illnesses such as depression.

Clinically, patients with depression present with altered peripheral immune cell populations and cytokine levels in comparison to healthy controls. Changes in the number and activity of natural killer cells (NK cells), cytotoxic T cells, and regulatory T cells have been reported (Park et al., 2015, Suzuki et al., 2017, Grosse et al., 2016, Becking et al., 2018). Differences in peripheral cytokines such as tumour necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), Interleukin (IL)-6, IL-10, IL-12, IL-13, IL-18 levels (Kohler et al., 2017) are also detected. Activation of immune cells has also been reported in the CSF of patients with depression, with markers on macrophage/monocyte activation (including neopterin and soluble CD14) correlating with depression severity (Kranaster et al., 2017).

The inflammatory theory of depression is also supported by animal data. Inflammation induced depressive-like behaviour is well characterised in rodent models, where the administration of either cytokines or an inflammatory reagent (e.g. lipopolysaccharide (LPS)) will initially induce sickness behaviour. This is characterised by reduced appetite and locomotor activity, and increased levels of pro-inflammatory cytokines, such as TNF- α and IFN- γ , followed by a period of depression-like behaviours (Remus and Dantzer, 2016, Henry et al., 2008, O'Connor et al., 2009). Chronic stress can also induce changes in cytokine levels in rodents (Glovatchcka et al., 2012).

Cytokines and other inflammatory mediators in depression may interact with the HPA axis, the metabolism of monoamine neurotransmitters, and cellular and synaptic plasticity in the brain (Furtado and Katzman, 2015, Miller et al., 2009, Singhal and Baune, 2017). This is potentially through activation of the kynurenine pathway, which diverts tryptophan, an essential amino acid, away from serotonin synthesis in favour of kynurenic acid and quinolinic acid. Inflammation has been thought to divert the kynurenine pathway down a more neurotoxic pathway, with quinolinic acid in microglia reported to activate the N-methyl-d-aspartate (NMDA) receptor and glutamate release (Schwarcz and Pellicciari, 2002). This can have detrimental effects in the CNS and may lead to the features of depression (Miller et al., 2009, Davis and Liu, 2015). Figure 1.2 gives an overview of how these systems may interact.

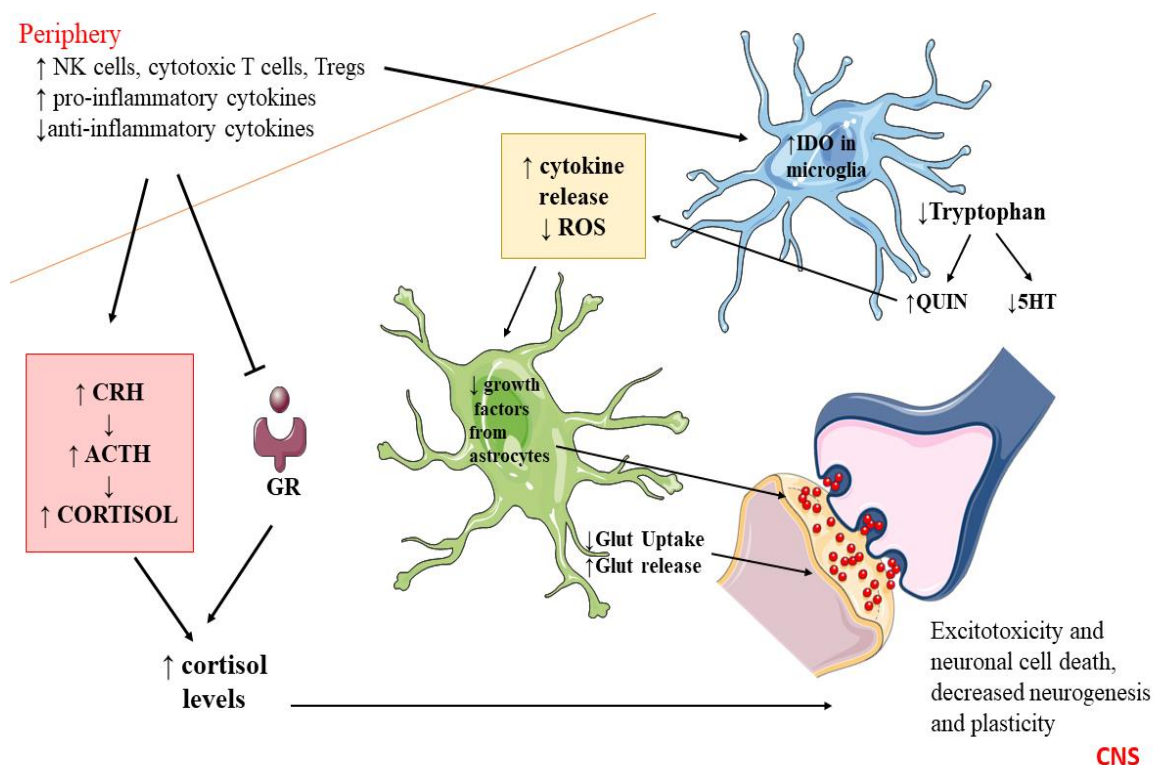


Figure 1.2 Interactions between inflammation and other mediators of depression

Inflammatory signals interact with the kynurenine pathway in the brain resulting in increased glutamate induced excitotoxicity and reduced secretion of protective growth factors. Cytokines also interact with the HPA axis to increase cortisol secretion, which may also cause cell damage. Adapted from Miller *et al.*, 2009. Abbreviations: NK; natural killer, ROS; reactive oxygen species, IDO; indoleamine-pyrrole 2,3-dioxygenase, QUIN; quinolinic acid, CRH; Corticotropin-releasing hormone, ACTH; adrenocorticotrophic hormone, GR; glucocorticoid receptor.

1.1.4.4 The neurotrophic theory of depression

The latent response time to antidepressants suggests that a simple change in neurochemical signalling in the brain is not sufficient for an antidepressant response, and that this delay may be down to the need to induce changes in plasticity (Duman *et al.*, 1999). The neurotrophic hypothesis suggests that depression may be the result of reduced trophic factors in the brain, leading to reduced plasticity of neuronal cells and reduced neurogenesis leading to a reduction in volume in key brain areas associated with depression (Duman and Li, 2012, Duman and Monteggia, 2006). Early studies have

focused on the effect of stress on the hippocampus and much of the evidence for this comes from animal data.

In rodents, chronic corticosterone treatment or daily restraint stress for 21 days in causes selective dendritic atrophy of CA3 pyramidal neurons in the hippocampus (Magarinos and McEwen, 1995). the neurotrophic theory of depression has to date mainly focused on the role of brain-derived growth factor (BDNF), a member of the neurotrophin family that includes nerve growth factor, neurotrphin-3 and neurotrophin-4 (Huang and Reichardt, 2001, Castren and Kojima, 2017). Neurotrophins are vital to the survival of brain cells, playing important roles in brain development and plasticity of neuronal cells (Smith et al., 1995, Huang and Reichardt, 2001). Chronic stress has been repeatedly reported to reduce BDNF protein and mRNA expression in the hippocampus and cortex of rodent brains (Castren and Kojima, 2017, Smith et al., 1995, Duman and Li, 2012).

In patients changes in brain volumes compared to controls have frequently been reported. One of the first studies used magnetic resonance imaging (MRI) data to report depressed patients in remission having smaller left hippocampal volumes than healthy controls, and hippocampal volumes correlating with lifetime duration of depression (Sheline et al., 1999). Since this study, individual patient studies and meta-analysis have reported reduced volumes in the hippocampus and other systems involved in emotion such as the limbic-cortical brain circuit (Santos et al., 2018, Sacher et al., 2012). However, it is unclear in patients what causes this reduction. Reports of peripheral blood BDNF protein levels in patients with depression are conflicting. Reduced serum BDNF in depression is a consistent finding (Kishi et al., 2018, Molendijk et al., 2014, Karege et al., 2002). However, reports on plasma vary with some studies reporting reduced plasma BDNF (Kishi et al., 2018) and others finding no difference between patients with depression and healthy controls (Ryan et al., 2018a, Bocchio-Chiavetto et al., 2010).

Although most research into the neurotrophic theory has focused on BDNF, other trophic factors have been implicated based on peripheral studies in humans. These include glial-derived growth factor (GDNF) (Tsybko Anton et al., 2017, Sharma et al., 2016) vascular endothelial growth factor (VEGF) (Sharma et al., 2016, Kolshus et al., 2017, Berent et al., 2014) and more recently pigment-epithelial derived factor (PEDF) (Ryan et al., 2017, Ditzen et al., 2012). Of these, PEDF has been linked to regulation of VEGF, GDNF and BDNF signalling (Cai et al., 2006, Yabe et al., 2004). These studies highlight that BDNF alterations alone should not be the focus of research in this area, with Figure 1.3 showing a proposed mechanism for this theory. However, more research into the role of neurotrophins and depression is needed as due to the limitations of human studies, no direct link between levels of neurotrophins and plasticity in the brain of depressed patients have been made.

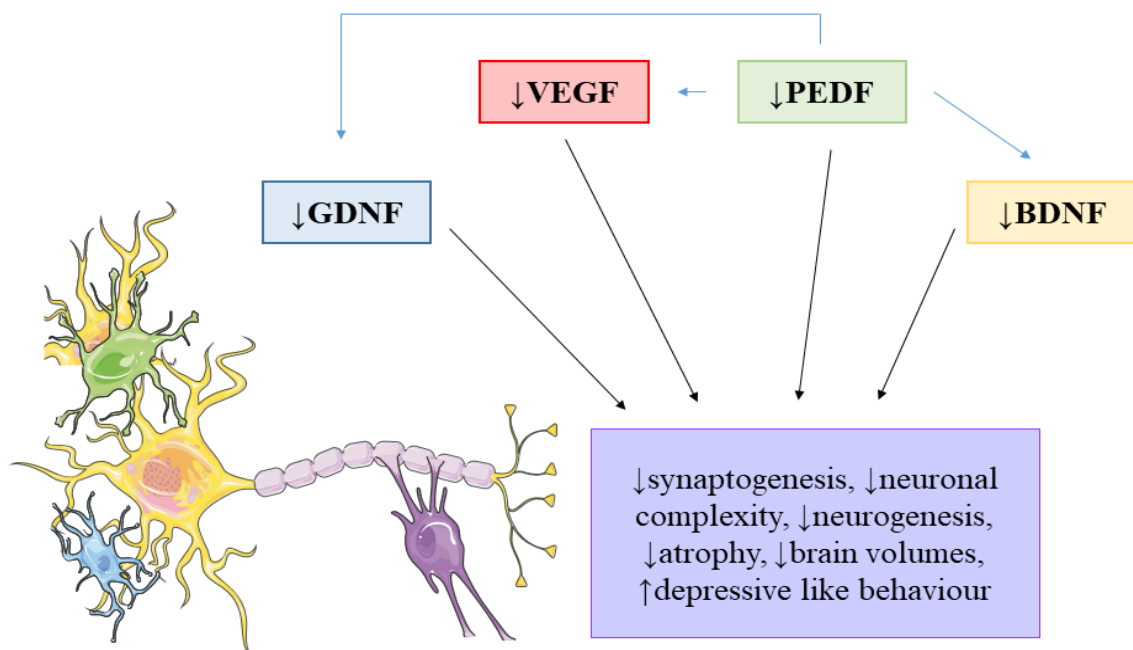


Figure 1.3 The neurotrophic hypothesis of depression

Evidence from animal models indicate that lower levels of BDNF may lead to impaired plasticity, neurogenesis and depression like behaviour. Human studies indicate VEGF, GDNF and PEDF may also be involved in this pathway. Abbreviations: GDNF; glial-derived neurotrophic factor, VEGF; vascular endothelial growth factor, PEDF; pigment epithelial-derived growth factor, BDNF; brain-derived neurotrophic factor.

1.1.4.5 Mitochondrial stress and depression

Mitochondria function to produce adenosine triphosphate (ATP), the main energy source of all eukaryotic cells (Karabatsiakos et al., 2014, Allen et al., 2018b). These vital organelles also function in calcium signalling, apoptosis and cell metabolism, particularly of reactive oxygen species (Mattson et al., 2008). The high energy consumption of the brain and its lack of energy reserves leave it strongly dependent on mitochondrial function (Allen et al., 2018b). The brain uses roughly 20% of the oxygen and glucose taken in by the body, even though it only accounts for 2% of body mass (Magistretti, 2006, Magistretti et al., 1999). It has been reported that a cortical neuron uses roughly 4.7 billion molecules of ATP every second (Allen et al., 2018b). This energy is used for both neurotransmission, neurogenesis and synaptic plasticity (Andreazza and Nierenberg, 2018). Given the changes in neurotrophin levels, neurotransmission, inflammatory profiles and HPA axis that occur in depression, it is no surprise that abnormalities in mitochondrial functions and oxidative stress have also been reported (Rezin et al., 2009).

Investigations into the role of mitochondrial function in depression are at an early stage but there is already evidence to support this. Research has mainly focused on mitochondria genetics in bipolar disorder/schizophrenia (Pei and Wallace, 2018, Kasahara and Kato, 2018), but differences in peripheral oxidative stress markers between depressed patients and controls have also been reported (Liu et al., 2015).

Several studies and meta-analysis that support abnormal mitochondrial function in both peripheral cells and brains of patients with depression have been published (Hirose et al., 2016, Black et al., 2015, Liu et al., 2015, Karabatsiakos et al., 2014). Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels, a marker of oxidative stress, have been reported to be associated with depressive symptoms, with women who have high levels of 8-OHdG scoring higher for both depression and anxiety (Hirose et al.,

2016). 8-OHdG measures oxidative DNA damage and, along with F2-isoprostane (which measures lipid damage), is reported to be the most commonly reported marker of oxidative stress (Black et al., 2015, Liu et al., 2015). A recent meta-analysis has indicated increased levels of both of these markers in depression (Black et al., 2015). Patients with depression have been shown to have impaired mitochondrial respiration, which correlates negatively with mood scores (Karabatsiakis et al., 2014). Plasma activities of mitochondrial enzymes, such as superoxide dismutase 1 (SOD-1), have also been reported to be decreased in patients with depression while levels of hydrogen peroxide (H₂O₂) have been shown to be increased (Rybka et al., 2013). A meta-analysis has also indicated lower total antioxidant capacity (TAC) in depressed patients during an acute episode of depression, lower levels of antioxidants in patients with depression and increased oxidative damage products in patients with depression in comparison to controls (Liu et al., 2015).

Studies have also looked at mitochondrial DNA (mtDNA) copy number but the data for this are mixed. One study reported lower mtDNA copy number whereas other studies show no differences between patients with depression and controls (Chang et al., 2015, He et al., 2014, de Sousa et al., 2014). Another study showed that childhood adversity and a lifetime of depression result in significantly higher mtDNA copy numbers (Tyrka et al., 2016). Finally, peripheral whole blood mRNA levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), a master regulator of mitochondrial biogenesis, have been reported to be reduced in patients with depression in comparison to healthy controls (Ryan et al., 2018b).

Interest in altered mitochondrial activity as a possible regulator of depression pathophysiology is fairly recent. Nonetheless, there is already a substantial amount of evidence to support a role for this in depression. Some clinical trials have already tested

the therapeutic effects of antioxidant supplements such as omega-3 fatty acids but with mixed results (Robinson et al., 2018, Jahangard et al., 2018, Bai et al., 2018). Therefore, more work is needed to assess the therapeutic value of targeting mitochondrial function for treating depression.

1.1.4.6 Conclusion

Depression is a complex and heterogeneous disorder. Clinical and pre-clinical studies support a role for a range of biological systems in its pathophysiology. Therefore, depression is a difficult disorder to treat. Available antidepressant therapies that reportedly interact with these systems are not ideal.

1.1.5 Treatment of depression

1.1.5.1 Psychotherapy

Psychotherapy is often recommended for treating mild-moderate depression as a standalone option or as an adjunctive therapy to pharmacological treatments (Kolshus et al., 2014b). Several studies have shown that both cognitive behavioural therapy and interpersonal therapy are beneficial to the patient when combined with pharmacotherapy, overall reducing symptoms of depression (Cuijpers et al., 2009, Cuijpers et al., 2011). Of these, interpersonal therapy is reported to be a slightly superior option (de Mello et al., 2005).

1.1.5.2 Antidepressants and other pharmacological treatments

Since the original antidepressant discoveries that were made in the 1950s, little progress has been made and most available antidepressant drugs still act on the noradrenaline or

serotonin receptors, increasing availability of these monoamines at the synapse (Berton and Nestler, 2006). Newer classes of antidepressants such as the selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (NRIs), and serotonin/norepinephrine reuptake inhibitors (SNRIs) are based on the original TCAs and MAOIs, but with a more selective mechanism of action (Rang et al., 2012). These drugs have not shown increased efficacy in comparison to earlier drugs but they have shown increased tolerability in patients (Morilak and Frazer, 2004, Ferguson, 2001, Wang et al., 2018a).

Results from randomised control trials have established that these traditional antidepressants have efficacy but it is far from ideal; approximately 50% of patients will respond to treatment with reduced depression scores being recorded. However, of these only 50-70% will attain remission and the majority of patients will relapse after this (Rush et al., 2006, Clevenger et al., 2018). Therefore, researchers and clinicians are still searching for new drugs for depression that may target some other aspect of the pathophysiology. Despite this, pharmacotherapy is the preferred/first-line treatment for moderate-severe depression, with treatment augmentation drugs such as lithium or Triiodothyronine being given in severe/resistant cases (Kolshus et al., 2014b).

1.1.5.3 Rapid-acting antidepressants

A breakthrough in recent years was the discovery of the rapid-acting anti-depressant properties of ketamine, an NMDA glutamate receptor antagonist that is a dissociative anaesthetic with abuse potential (Duman, 2018, Harkin and McLoughlin, 2019). A growing body of evidence for the involvement of the NMDA glutamate receptors in depression from the 1990s led to the first clinical trial with Ketamine, which reported a

significant decrease in depression scores in patients who received 2 x 40min 0.5mg/kg infusion doses of ketamine hydrochloride in comparison to patients who received placebo (saline) (Berman et al., 2000). Since this first trial several papers have reported the rapid antidepressant effects of ketamine (i.e. depressive symptoms improve within hours), as well as showing it is safe and tolerable at low doses (Zarate et al., 2006, Singh et al., 2016, Murrough et al., 2013, Perry et al., 2007, Kim and Mierzwinski-Urban, 2017). Although these trials initially led to excitement over a new possible understanding of depression and a possible change to a more rapid, robust treatment than slow acting antidepressants, the effects of ketamine only last a short period of time ($\leq$ 1week) (Kim and Mierzwinski-Urban, 2017, Han et al., 2016).

Although originally reported to act on the NMDA glutamate receptor, there have been reports that ketamine may mediate its anti-depressive action through the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) or opioid receptors (Aleksandrova et al., 2017, Williams et al., 2018). Furthermore, ketamine has been reported to be a ligand for the alpha estrogen receptor (Ho et al., 2018). How it mediates its effects from here are unknown but it seems to be linked to synaptic plasticity mechanisms (Aleksandrova et al., 2017). Animal data reports ketamine activates the mammalian target of rapamycin (mTOR signalling) pathway, resulting in increased BDNF translation and secretion (Zhou et al., 2014, Zanos et al., 2018). More recently, the antidepressant actions of ketamine have been linked to VEGF signalling in neurons (Deyama et al., 2019).

Current studies are aiming to establish if ketamine could be used as an adjunctive therapy to enhance the effects of other treatments. These studies have mainly focused on electroconvulsive therapy, and report that ketamine does not improve depressive symptoms over ECT alone (Ren et al., 2018, McGirr et al., 2017). Nonetheless, molecular

studies using patient plasma or serum are being conducted to further knowledge of ketamine's mechanism of action and to identify a possible clinical marker to monitor or predict response. These studies have focused on neurotrophic factors, cytokines and D-serine, a co-agonist of the NMDA receptor (Moaddel et al., 2015, Yang et al., 2015, Park et al., 2017, Allen et al., 2015, Rybakowski et al., 2013), with IL-6 and D-serine identified as a possible predictor of ketamine response (Moaddel et al., 2015, Yang et al., 2015). However, these studies have small sample numbers and larger ketamine trials would be needed to further research on the biological effects of ketamine.

1.1.5.4 Electroconvulsive therapy

Electroconvulsive therapy (ECT), the application of an electrical current to the brain in order to induce a generalized seizure for therapeutic effects, was first demonstrated in the 1930s. (Leiknes et al., 2012, Cerletti and Bini, 1938). Today, it is the most acutely effective treatment available for severe, sometimes life-threatening, depression with approximately one million patients worldwide receiving ECT annually (UK ECT Group, 2003, Leiknes et al., 2012, Kellner et al., 2012). ECT is most often used as a third or fourth line treatment, where one or more pharmacological treatments have failed, and in these treatment resistant patients can offer up to 60% remission rate (Greenberg and Kellner, 2005, Kellner et al., 2012). Despite its clinical effectiveness, the use of ECT is limited due to its side effects, mainly on cognition but for the most part these impairments are temporary (Kellner et al., 2010, Sackeim et al., 2007, Semkovska and McLoughlin, 2010, Rose et al., 2003). However, like other depression treatments the clinical efficacy of ECT is temporary, with a 37% relapse rate at 6 months post-treatment being recorded across studies (Jelovac et al., 2013).

Despite being one of the most effective treatments for depression, its exact mechanism of action is still unknown (Sienaert, 2014). Studies have demonstrated that ECT regulates many of the systemic pathways indicated in depression, with a summary of these effects shown in Figure 1.4. Small patient studies have indicated that ECT can alter concentrations of monoamine metabolites in the CSF and result in a global decrease of serotonin-1A receptor binding in the brain (Lanzenberger et al., 2013, Nikisch and Mathe, 2008). Electroconvulsive stimulation (ECS), the animal model equivalent of ECT, has also been reported to alter monoamine signalling (Deakin et al., 1981, Tsen et al., 2013, Lillethorup et al., 2015).

ECT has also been implicated in regulation of the HPA axis, with ECT increasing serum levels of ACTH, cortisol, and prolactin in patients with depression (Apéria et al., 1985). ECT also decreased whole blood mRNA levels of glucocorticoid-induced leucine zipper in patients with depression (Ryan and McLoughlin, 2019). In a separate study, ECT has also been shown to reduce the cortisol awakening response in patients who respond to ECT (Allen et al., 2018a).

ECT and the immune system has a complex relationship, with studies reporting an initial transient increase in pro-inflammatory markers in response to ECT. However, after repeated treatments with ECT this seems to be reversed and cytokine levels decrease (van Buel et al., 2015) (Lehtimäki et al., 2008), with one study showing that one hour after a single ECT TNF- α levels are raised, but after repeated stimulation these return to healthy control levels (Hestad et al., 2003). Similarly, it has been shown that ECT increased baseline levels of IL-6 in patients 4 hours after treatment, and in patients who remit these levels are shown to be reduced towards the end of the course of ECT treatment, indicating a long term anti-inflammatory effect (Jarventausta et al., 2017). The reasons for an initial pro-inflammatory response to ECT are unclear with further studies

needed to clarify how this relates to depression treatment. ECT has been reported to acutely increase the number of white blood cells in whole blood, which could explain the initial increase in pro-inflammatory cytokines (Fluitman et al., 2011).

Changes in neurotrophic factors have also been linked to the mechanism of action of ECT, with changes in peripheral BDNF, VEGF and PEDF being reported (Rocha et al., 2016, Ryan et al., 2017, Kolshus et al., 2017, Minelli et al., 2011). ECT has also been reported to increase the volume of brain areas linked to depression, which may be associated with treatment outcomes in depression (Cao et al., 2018, Cano et al., 2017, Olteidal et al., 2018, Gbyl and Videbech, 2018). Results from rodent studies using ECS, may be more informative of the role of neurotrophic factors in the response to ECT. ECS has been reported to increase levels of BDNF and BDNF related miRNAs in several ECS studies, indicating a role for growth factors in the molecular mechanism of ECT (Meyers et al., 2018, Maynard et al., 2018, Ryan et al., 2013).

Data on ECT and oxidative stress are limited as it is a recent area of interest. However, one study showed no change in total antioxidant capacity with ECT (contradicting a theory that ECT might cause oxidative damage), while another reported ECT increased mRNA levels of *PGC-1 α* in patients with psychotic depression (Ryan et al., 2018b, Senyurt et al., 2017). ECS has also been shown to increase the activity of glutathione peroxidase (GSH-PX) and SOD, which are protective against oxidative stress (Župan et al., 2008)

ECT interacts with a wide range of systems/pathways that are implicated in depression, making it difficult to determine an exact mechanism(s) of action. Numerous studies have also tried to determine biomarkers that might predict response to ECT, and although studies have identified different factors that may influence response (age,

presence of psychotic features) there is currently no clear biomarker available (Pinna et al., 2018).

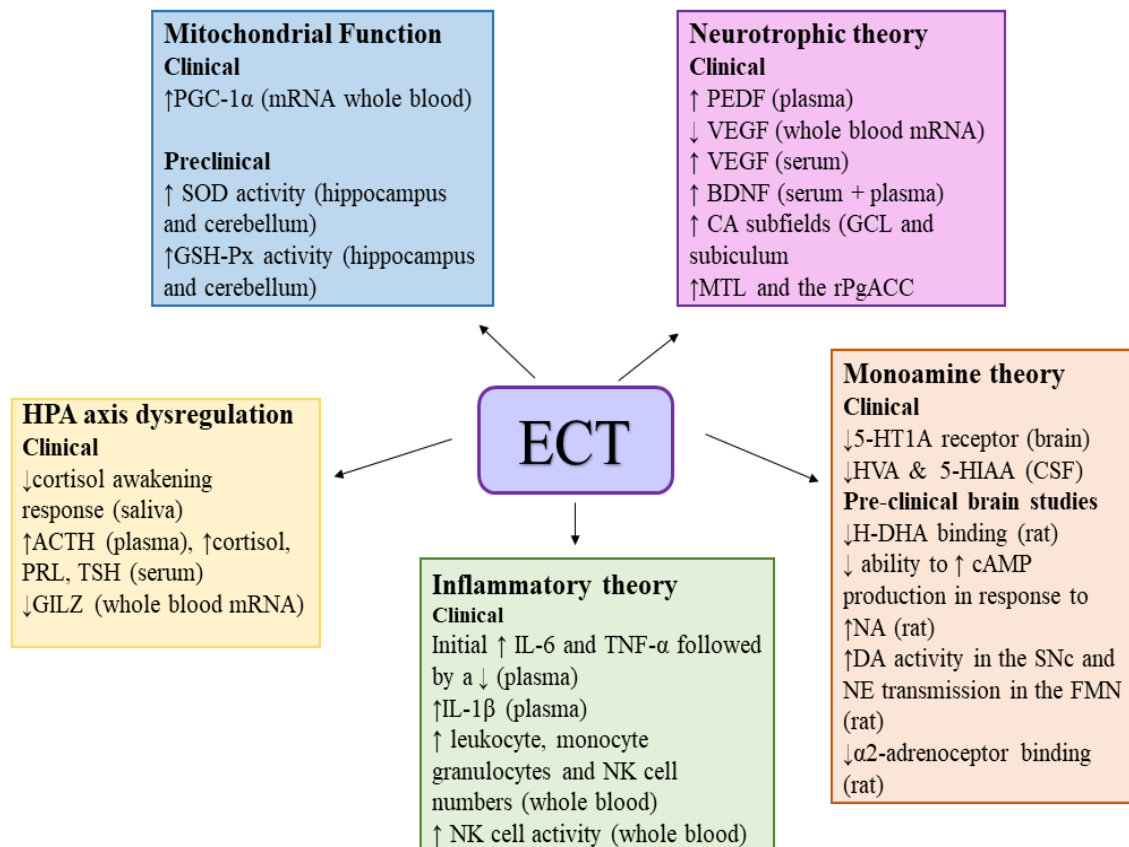


Figure 1.4 Molecular and biological changes in response to ECT

ECT acts on proteins/genes related to the different theories of depression. Abbreviations: PGC-1α; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, SOD; superoxide dismutase, GSH-Px; glutathione peroxidase, GDNF; glial-derived neurotrophic factor, VEGF; vascular endothelial growth factor, PEDF; pigment epithelial-derived growth factor, BDNF; brain-derived neurotrophic factor GCL; granular cell layer, MTL; medial temporal lobe, rPgACC; perigenual anterior cingulate cortex, ACTH; adrenocorticotrophic hormone, PRL; prolactin, TSH; thyroid stimulating hormone, GILZ; glucocorticoid-induced leucine zipper, NK; natural killer, 5-HT1A; serotonin receptor 1 HVA; homovanillic acid, 5-H1AA; 5-Hydroxyindoleacetic acid, H-DHA; dihydroalprenolol NA; noradrenaline, DA; dopaminergic, FMN; flavin mononucleotide.

1.1.5.5 Other treatments

Other alternative therapies for patients who do not respond to antidepressants include transcranial magnetic stimulation, deep brain stimulation, transcranial direct

current stimulation and vagus nerve stimulation. All of these therapeutic neuromodulation methods have shown moderate efficacy when combined with antidepressants based on meta-analysis (McGirr and Berlim, 2018, Garnaat et al., 2018, Wei et al., 2017). However, it is an area that still needs further study with larger sample sizes to fully optimise treatment parameters and evaluate clinical usefulness (Wei et al., 2017, Croarkin and MacMaster, 2019).

1.1.5.6 Conclusion

Treatment of depression is still not ideal. The molecular mechanisms of the most effective treatments are still unknown. Greater understanding of these mechanisms is needed to aid in the development of better treatments. Clinical studies may also help predict which patients will respond to each treatment, cancelling out the need for a patient to try several course of drugs.

1.1.6 Changes in peripheral blood markers in depression

The current diagnosis of depression relies on clinical examination of depressive symptoms, with no simple measurement from peripheral blood available to diagnose depression (Hacimusalar and Esel, 2018, Perlman et al., 2019). One of the main issues with studying biological mechanisms is the choice of tissue. Studies investigating molecular markers in the brain, while informative and the optimal choice for depression research, are impractical for diagnostic purposes and may also be influenced by other factors in death (Mehta et al., 2010). Studies using cerebral spinal fluid (CSF) may also reflect changes in the brain, but it is difficult to recruit patients to CSF studies. Therefore,

peripheral blood (which shares roughly 82% of its gene expression with brain tissue), is usually the tissue of choice (Liew et al., 2006).

As well as having the potential to be used as biomarkers and predict treatment response, changes in peripheral blood markers might also help us understand the biological mechanisms of depression by identifying new targets beyond the scope of traditional theories.

1.1.6.1 MicroRNAs

MicroRNAs (miRNAs) have emerged as key regulators of post-translational gene expression (Bartel, 2009, Pasquinelli, 2012). They consist of small non-protein coding genes of approximately 21-23 bases that bind to target messenger RNA (mRNA) to regulate gene expression, by both inhibition of translation and RNA degradation (Kolshus et al., 2014a, Lopez et al., 2017). Depression heritability is estimated to be 37% (Bienvenu et al., 2011, Sullivan et al., 2000); therefore, regulation of gene expression could play an important role in depression. miRNAs have been implicated in many neuronal functions, with high turnover of the miRNAs shown to be activity dependent, supporting an important role in adaptive features of the CNS such as plasticity (Krol et al., 2010).

More recently, studies have started to investigate the role of miRNAs in antidepressant response, stress resilience and depression characterisation. The first study to link a possible depression target to a miRNA was in 2010, when miR-16 was found to target the serotonin transporter (SERT) *in vitro* (Baudry et al., 2010). Since this study, clinical miRNA research in depression has grown. For example, miRNAs 146a/b-5 and 425-3p and 24-3p have been implicated in treatment response in patients (Lopez et al.,

2017). Previous research from my lab reported miR-126-3p and miR-106a-5p to be differentially expressed in patients with depression with psychotic features in comparison to patients without psychotic features and return to control levels following ECT, implicating miRNAs in the mechanism of action of ECT (Kolshus et al., 2017).

Despite these interesting findings, data regarding miRNAs vary among studies and no miRNA has been clearly linked to depression (Kolshus et al., 2014a). There are miRNAs that are reported frequently in studies, usually with roles in neuroplasticity, but no miRNA has been identified as a biomarker of depression (Gruzdev et al., 2019). However, this is still a new field and further studies on miRNAs, using modern techniques such as deep-sequencing may broaden our insights into the pathology of depression and its therapeutic response.

1.1.6.2 Gene expression studies

Advances in technology have changed the way genetic markers can be studied, with next generation sequencing and microarrays allowing us to profile thousands of genes simultaneously. These technologies have been applied successfully to many diseases, including cancer, aiding diagnosis, prognosis and in some cases predicting outcome to therapy (Ciobanu et al., 2016). Over the past number of years numerous large genome-wide association (GWAS) studies and candidate gene studies have attempted to find a risk gene for depression, however no reproducible results have been reported (Menke, 2013).

Despite this, research into genetic markers of depression has continued, because discovering a robust genetic marker of depression would provide tremendous insight into its pathophysiology. However, the heterogeneity of depression makes this difficult

(Ciobanu et al., 2016). Studies have also tried to find genetic markers of antidepressant response, but no reliable predictors of antidepressant response have been identified. (Uher et al., 2013).

Investigating gene targets regulated by miRNAs previously linked to depression is also an interesting way to explore novel targets. For example, a deep-sequencing study conducted by my lab identified two microRNAs that were altered in depression that dually regulate three gene targets: *VEGF*, *E2F1* and *SIRTUIN1* (*SIRT1*). Of these, *VEGF* was found to be significantly altered in psychotic depression (compared to healthy controls and non-psychotic depression) and normalised following a course of ECT, indicating VEGF as a potential marker to distinguish between psychotic and non-psychotic depression (Kolshus et al., 2017).

Genes emerging from these studies are from various biological pathways, indicating that there is possibly some master regulator upstream of these that could potentially link all these together (Belzeaux et al., 2012). Therefore, more genetic screens, with ideally larger sample sizes, are needed to identify a gene/miRNA that converges on these pathways.

1.1.6.3 Protein studies

As the evidence for an effect of depression in the periphery has grown, so has the use of molecular profiling for depression from plasma or serum (Guest et al., 2016). Hypothesis-driven protein studies have been popular to try and identify trait/state features of depression, but have also been unsuccessful at identifying a reliable marker for depression (Comes et al., 2018). However, these studies can provide evidence to support

the more modern theories of depression such as inflammation and neurotrophin dysregulation (Levy et al., 2018, Kohler et al., 2017, Kohler et al., 2018).

More recently, proteomic approaches have been utilised to identify targets in depression (Guest et al., 2016). Proteomics refers to the large scale analysis of the proteome, the entire set of proteins that are produced by a cell, to obtain a more integrated view of the biology of a disorder by studying all the proteins in the cell/tissue of interest (Comes et al., 2018). These studies allow unbiased discovery of new targets for depression as well as supporting existing theories (Carboni, 2015). Two-Dimensional Gel Electrophoresis, mass spectrometry and multiplex immunoassays are popular methods for proteomic studies, with enzyme-linked immunosorbent assay (ELISA) and western blots typically being used to validate results (Guest et al., 2016).

To date a small number of proteomic studies comparing patients with depression and healthy controls have been conducted, with all studies identifying neurotrophic factors and proteins involved in inflammatory responses to be differentially expressed in patients. However, these studies have also identified markers from other systems, particularly metabolism. Differences in insulin expression has been detected in several studies (Domenici et al., 2010, Arnold et al., 2012, Lamers et al., 2016), as well as several proteins involved in lipid metabolism (Xu et al., 2012), and energy balance (Lamers et al., 2016). Oxidative stress markers have also been identified in proteomic studies (Stelzhammer et al., 2014). Extracellular matrix proteins (such as matrix metalloproteinase 9), proteins involved in wound healing (tenascin-C, von Willebrand factor) and signalling proteins (extracellular newly identified receptor for advanced glycation end-products binding protein) have also been identified (Domenici et al., 2010, Bot et al., 2015).

A recent study from my lab took plasma samples from patients with depression ($n = 30$) pre/post ECT and carried out protein analysis using 2-dimensional difference in gel electrophoresis and mass spectrometry. Of the protein spots identified and proteins chosen for confirmation analysis, PEDF was confirmed to be increased in patients and increased further following ECT, implicating PEDF in its mechanism of action (Ryan et al., 2017). However, the initial PEDF increase merits further investigation.

Although these new studies identify new targets of potential interest in depression (particularly metabolism), there have only been a few studies, with not much overlap among them. However, many of these studies were performed in medication free patients indicating that the results are due to the disorder, highlighting the ability of proteomic studies to identify new directions for depression research.

1.1.6.4 Conclusion

Gene expression/proteomic studies are useful for providing supporting evidence to existing theories of depression as well as identifying new pathways of interest. However, the lack of continuity between studies indicates a lot more research is needed, ideally with increased numbers and shared methodology between studies. Some studies also provide opportunities for future exploration, as with our lab's previous studies indicating that further research into E2F1, SIRT1 and PEDF is needed (Kolshus et al., 2017, Ryan et al., 2017).

1.2 E2F1

The E2F transcription factors were discovered as a family of proteins that bind to the promotor region of the adenovirus E2 gene (Kovesdi et al., 1986). To date, eight family

members (E2F1–8) have been identified. The E2F family play an important role in cell cycle regulation (DeGregori and Johnson, 2006). E2F1, the first family member to be cloned, is typically known as an activator E2F because it binds and activates the transcription of proteins involved in cell cycle progression (Ertosun et al., 2016). However, it has also been known to activate apoptosis in certain conditions (Ginsberg, 2002). Other functions, independent of the cell cycle, have also been reported for E2F1 including the detection and repair of damaged DNA, induction of cell senescence and, more recently, regulation of metabolism (Stevens and La Thangue, 2004, Dimri et al., 2000, Denechaud et al., 2017). These different functions depend on the various pathways that E2F1 has been reported to signal through.

1.2.1 E2F1 signalling

E2F1 was found to be a cellular target of the retinoblastoma (Rb) protein, which in its hypophosphorylated form binds and inhibits E2F1 function via a pocket domain on the Rb protein (Bernards, 1997, Helin et al., 1993, Chellappan et al., 1991). Phosphorylation of Rb by cyclin dependent kinases results in the release of E2F1, which then forms a heterodimer complex with dimerization partner (DP) 1 or DP2 to become transcriptionally active (Ertosun et al., 2016, Magae et al., 1996, Bernards, 1997). Once active, E2F1 can regulate both proliferative and apoptotic pathways, as well as cell cycle independent genes (see Figure 1.5).

In pro-proliferative pathways, activation of E2F1 progresses the cell cycle from the late G1 (growth) phase to the S phase (DNA synthesis) through transcriptional activation of targets related to DNA replication and cell cycle progression (Johnson et al., 1993, Stanelle et al., 2002). E2F1 binding sites have been found in genes promoting the

transition from G1-S phase, including the origin recognition complex, thymidine kinase, dehydrofolate reductase, DNA polymerase- α , cdc2, c-myc, N-myc, c-myb, E-myb, cyclin D1, and cyclin E (Sala et al., 1994, Muller and Helin, 2000, DeGregori et al., 1995, Ohtani et al., 1996).

However, overexpression of E2F1 was found to induce apoptosis in cells via premature S phase entry, indicating that E2F1 could have dual roles. It was also reported that tumour protein 53 (p53) was involved in E2F1 mediated cell death (Qin et al., 1994, Shan and Lee, 1994). Since these two original publications, it has been established that E2F1 can induce apoptosis through both p53 dependent and p53 independent pathways (Ginsberg, 2002). Abnormal expression of E2F1 induces p53 accumulation in cells, which results in apoptosis via the p14ARF tumour suppressor gene and consequent p53 stabilisation and activation (Nip et al., 1997, Bates et al., 1998, Kowalik et al., 1995, Ginsberg, 2002). Independent of p14ARF, E2F1 activates the ATM serine/threonine kinase signalling pathway in cell culture, leading to phosphorylation of p53 and apoptosis/DNA damage (Pusapati et al., 2006, Lindstrom and Wiman, 2003). E2F1 increases p53 expression through activation of p73 (Irwin et al., 2000). E2F1 can also activate apoptotic protease activating factor 1 (Apaf-1), a key molecule in mitochondrial induced apoptosis through caspase activation, independent of p53 (Shakeri et al., 2017, Moroni et al., 2001). Finally, E2F1 can inhibit the function of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), preventing the transcription of anti-apoptotic genes (Tanaka et al., 2002, Ginsberg, 2002). Activation of these pathways result in the activation of pro-apoptotic proteins including caspases and members of the B-cell lymphoma 2 (bcl-2) family (Ertosun et al., 2016, Ginsberg, 2002).

E2F1 activates cell cycle arrest through regulation of p18, p19, p21 and p27, indicating a possible role in cell senescence (Shats et al., 2017, Radhakrishnan et al.,

2004). It has been proposed that levels of E2F1 in the cell determine cell fate with low levels driving proliferation, moderate levels driving cell arrest and high levels inducing apoptosis (Shats et al., 2017).

Separate to cell proliferation and death, E2F1 regulates genes/proteins that are not directly linked to the cell cycle. For example, E2F1 has been shown to decrease Kir6.2 expression (a key component of the channel involved in insulin secretion), increase peroxisome proliferator-activated receptor gamma (PPAR γ) expression (regulating adipocyte differentiation), and repress myogenic differentiation protein (MyoD) and myogenin, suggesting a role in myogenic regulation (Fajas et al., 2002, Annicotte et al., 2009, Wang et al., 1995). Overall, these studies suggest an important role in metabolism (Denechaud et al., 2017). Research into E2F1 signalling has proposed diverse roles for this protein, in both normal physiological and pathological conditions.

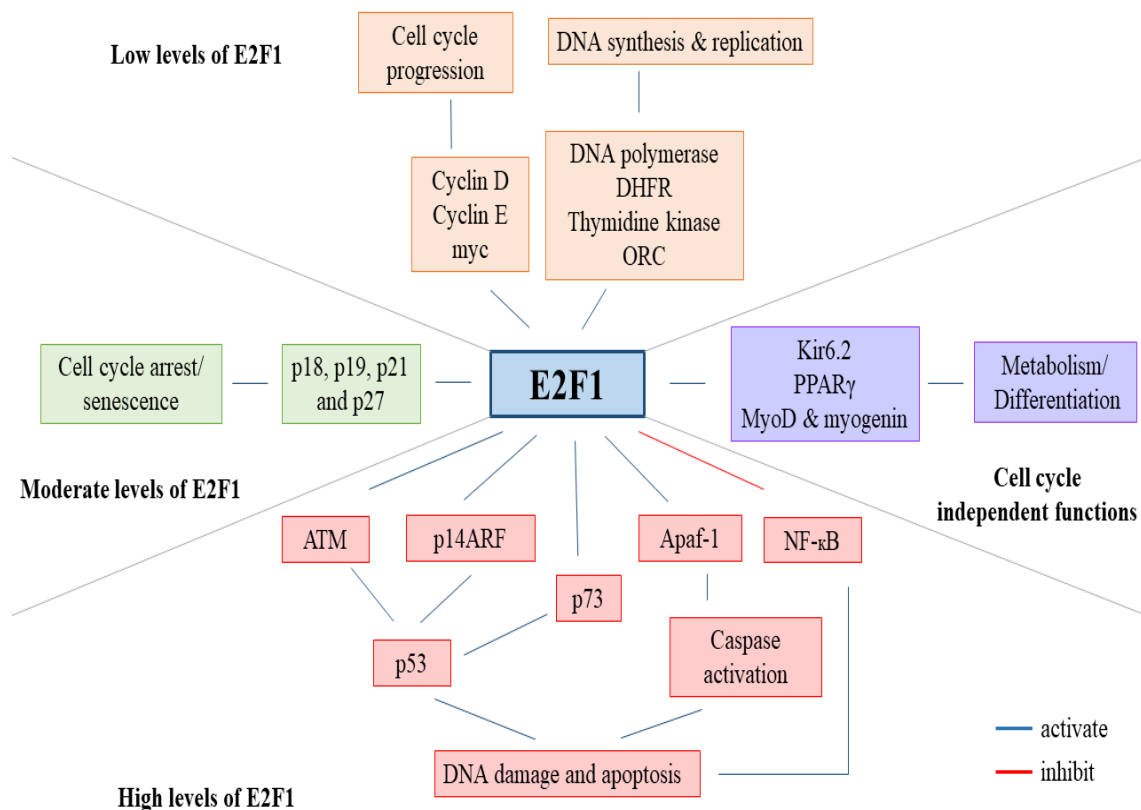


Figure 1.5 E2F1 cell signalling

E2F1 signals through various pathways to promote cell cycle proliferation, arrest or apoptosis depending on cell conditions and pathway activated. Independent of this E2F1 functions to regulate aspects of metabolism including cell differentiation. Abbreviations: DHFR; dihydrofolate reductase, ORC; origin recognition complex, Kir6.2; potassium voltage-gated channel subfamily, PPAR γ ; peroxisome proliferator-activated receptor gamma, MyoD; myogenic differentiation protein, ATM; ATM serine/threonine kinase, p14ARF; ARF tumour suppressor, apaf-1; Apoptotic protease activating factor 1; NF- κ B; nuclear factor kappa-light-chain-enhancer of activated B cells.

1.2.2 E2F1 and depression

No studies to date have directly linked E2F1 to depression. However, there has been some interest in the effect of antidepressant treatment on cell cycle proteins, given the links between depression and neurogenesis. Chronic treatment with antidepressants (imipramine, fluoxetine and desipramine) in mice was shown to inhibit P21 expression in the hippocampus (Pechnick et al., 2011). E2F1 and P21 have been reported to negatively regulate each other by several studies (Gartel et al., 2000, Dimri et al., 1996, Sharma et al., 2006). One study has also reported that ECS activates E2F1 protein, along

with other cell cycle proteins such as cyclin-dependent kinase 2 (CDK2) (Kim et al., 2005). However, whether E2F1 is dysregulated in depression or involved in antidepressant treatment response is currently unknown.

1.3 Sirtuin1

The sirtuins are a group of seven (SIRT1-7) nicotinamide (NAD⁺)-dependent deacetylases, classed as type III histone deacetylases (HDACs) (Michan and Sinclair, 2007, Whittle et al., 2007). Sirtuins are orthologous to the silent information regulator-2 (Sir2) protein which was first discovered in *Saccharomyces cerevisiae* and found to extend their lifespan (Buler et al., 2016, Kaeberlein et al., 1999). Sir2 and mammalian sirtuins function as enzymes that catalyse post-translational modifications of both histone and non-histone proteins through direct deacetylation (Michan and Sinclair, 2007, Imai et al., 2000).

Sirtuins have been reported to be expressed in all tissues, including the CNS (Jayasena et al., 2016, Buler et al., 2016). SIRT1-7 have a highly conserved central NAD⁺-binding and catalytic domain which is referred to as the core domain; however, they can differ in their N and C termini, with SIRT1 being the closest orthologue to Sir2 (Haigis and Guarente, 2006, Haigis and Sinclair, 2010). SIRT1 is defined as a nuclear protein, although there are reports that it can be released to the cytosol (Michishita et al., 2005, Jin et al., 2007). SIRT1 has multiple substrates, including p53, Ku70, NF- κ B, forkhead proteins, and PGC-1 α (Haigis and Guarente, 2006). Through deacetylation of these targets, SIRT1 has peripheral functions in glucose homeostasis, mitochondrial biogenesis, DNA repair, inflammation and apoptosis, as well as aging (Inoue et al., 2017, Oberdoerffer et al., 2008, Rodgers et al., 2005, Tissenbaum and Guarente, 2001, Vaziri

et al., 2001). SIRT1 also functions in the central nervous system (CNS) and regulates circadian rhythms, synaptic plasticity, and memory in animal studies (Asher et al., 2008, Gao et al., 2010, Michan et al., 2010).

1.3.1 Sirtuin1 signalling

SIRT1 interacts with both histone and non-histone proteins (Stunkel and Campbell, 2011). *In vitro*, the yeast protein Sir2 has been reported to deacetylate specific lysine residues on various histones including histone H3 (K9,K14) and H4 (K16) (Imai et al., 2000). Deacetylation of H4-K16Ac and H3-K9 by human SIRT1 has also been demonstrated *in vitro*, a function which has been reported to be essential for the formation of heterochromatin and gene silencing (Vaquero et al., 2004, Vaquero et al., 2006). SIRT1 also regulates a large number of non-histone protein targets, suggesting a wider function than epigenetic silencing (Stunkel and Campbell, 2011, Huber and Superti-Furga, 2011).

In the periphery and CNS, SIRT1 interacts with targets that are linked to cell cycle regulation, metabolism, immune function and neuroprotection. One of the first non-histone targets identified for SIRT1 was p53. SIRT1 was reported to bind to and reduce its transcription and p53 dependent apoptosis in response to stress and DNA damage (Luo et al., 2001, Vaziri et al., 2001). SIRT1 has been linked to deacetylation of Rb, leading to activation of E2F1 and increased proliferation of both macrophages and glia (Jablonska et al., 2016, Imperatore et al., 2017). SIRT1 can also deacetylate E2F1 and forkhead box O3 (FOXO3; a tumour suppressing protein) directly, which, along with its effect on p53, strengthens its role as a cell cycle regulator and highlights its possible importance in cancer biology (Imperatore et al., 2017, Jablonska et al., 2016, Brooks and Gu, 2008).

SIRT1 signalling plays a role in immune function and has been demonstrated to deacetylate the RelA/p65 subunit of NF- κ B and forkheadbox O1 (FOXO1), inhibiting the transcription of pro-inflammatory cytokines (Yeung et al., 2004, Zhu et al., 2019, Schug et al., 2010, Zhao et al., 2015). The interaction between SIRT1 and NF- κ B has been reported to be protective in response to pro-inflammatory stimuli (e.g. TNF- α), amyloid-beta induced cytotoxicity and brain injury (Chen et al., 2005, Liu et al., 2018, Zhu et al., 2019). In response to these stimuli, SIRT1 reduces oxidative stress, through both the NF- κ B and FOXO pathways, as well as through regulation of SOD, NADPH oxidase (NOX) and endothelial NOS (eNOS) pathways (Zhu et al., 2019, Zhang et al., 2017). SIRT1 can also be protective against oxidative stress through deacetylation of PGC-1 α , the master regulator of mitochondrial function (Guo et al., 2014). Through deacetylation of PGC-1 α and FOXO1, SIRT1 signalling has been linked to cell metabolism processes, including lipogenesis and gluconeogenesis (Dominy et al., 2010, Rodgers et al., 2005, Rodgers and Puigserver, 2007, Frescas et al., 2005).

Other signalling pathways for SIRT1 include regulation of CLOCK genes through deacetylation of Period Circadian Regulator 2 (PER2), possibly linking circadian rhythms and cell metabolism (Asher et al., 2008). SIRT1 signals through ERK/Mitogen-activated protein kinases (MAPK) signalling, which could be linked to a role in synaptic plasticity (Abe-Higuchi et al., 2016, Gao et al., 2010, Michan et al., 2010).

Less is known about methods that regulate SIRT1, apart from levels of NAD⁺. Figure 1.6 give an overview of how SIRT1 might be regulated. It has recently been shown that deleted in breast cancer 1 (DBC1) protein forms a complex with SIRT1 to suppress its function (Nin et al., 2012), and phosphorylation by unknown kinases can release SIRT1 from this complex, a process requiring AMP-activated protein kinase (AMPK) (Nin et al., 2012, Lau et al., 2014). SIRT1 has been reported to have

phosphorylation sites at sites that bind cell cycle proteins, indicating a potential role for regulation of SIRT1 by the cell cycle (Sasaki et al., 2008). Finally, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway has also been reported to phosphorylate SIRT1 (independent of NAD^+), increasing its intrinsic deacetylase activity and could be linked to its metabolic functions (Gerhart-Hines et al., 2011).

SIRT1 is a complex protein that, through its many targets, participates in numerous cell functions that are vital to cell biology.

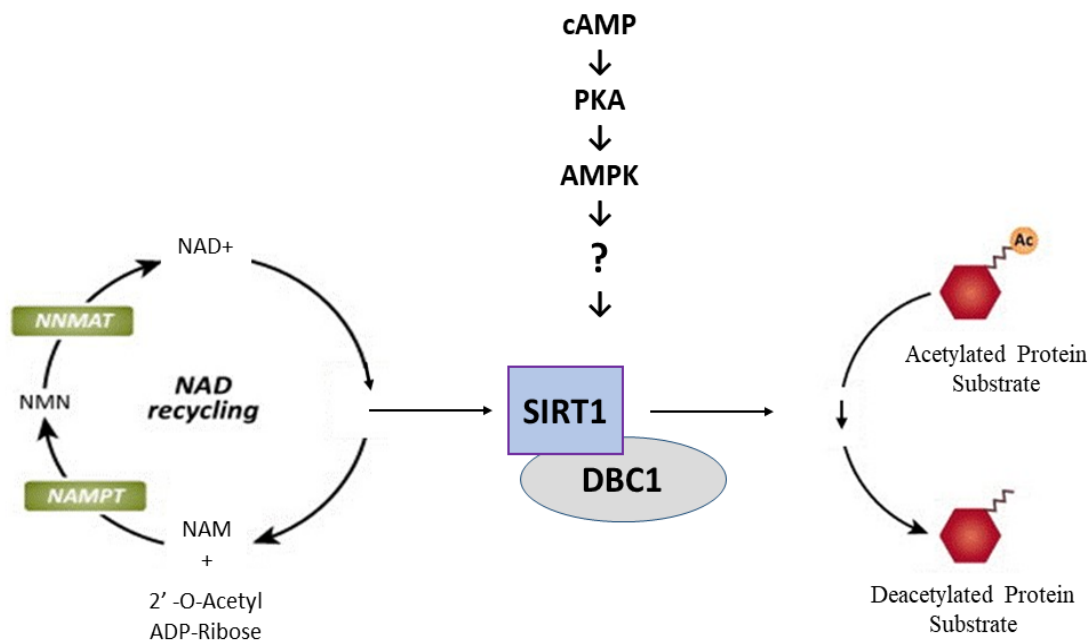


Figure 1.6 SIRT1 regulation

SIRT1 is primarily thought to be regulated by NAD^+ but recently adrenergic signalling through cAMP and AMPK has been thought to activate SIRT1. Adapted from 'The ways and means that fine tune Sirt1 activity', Figure 1 (Revollo and Li, 2013). Abbreviations: NAD^+ ; nicotinamide adenine dinucleotide, NNMT; nicotinamidemononucleotidoadenyltransferase, NAMPT; nicotinamidephosphorybosyltransferase, NAM; nicotinamide, NMN; nicotinamidemononucleotide.

1.3.2 Sirtuin1 and depression

To date, a substantial amount of evidence for a role of SIRT1 in depression has been reported. SIRT1 was first investigated in depression because of its links to circadian rhythm, with an association between a SNP in the *SIRT1* gene and depression in Japanese patients with major depressive disorder (MDD; $n = 450$) reported (Kishi et al., 2010). A subsequent GWAS linked *SIRT1* to MDD, identifying a locus in the *SIRT1* gene in Han Chinese women with recurrent depression (5,303 cases; 5,337 controls (Cai et al., 2015).

Three studies have demonstrated reduced peripheral blood *SIRT1* mRNA levels in depression. The first study reported reduced *SIRT1*, *SIRT2* and *SIRT6* mRNA in a small group of MDD ($n = 20$) and bipolar depressed ($n = 12$) patients compared to controls ($n = 28$). Similar *SIRT1* mRNA levels to controls were found in a group of MDD patients ($n = 39$) who attained remission (Abe et al., 2011). A second study demonstrated reduced blood *SIRT1* mRNA levels in a larger group of MDD patients ($n = 50$) from Shanghai compared to controls ($n=50$), and this was confirmed in a separate group of MDD patients ($n = 635$) (Luo and Zhang, 2016). Finally, a recent study reported reduced *SIRT1* mRNA levels in a group of drug naïve depressed patients in comparison to controls, and reported 4 weeks treatment with citalopram attenuated this (Wang et al., 2018b). Table 1.2 summarises the available clinical studies on SIRT1 and depression.

Animal studies have linked SIRT1 to depression but with conflicting results. Table 1.3 summarises this data. One of the first studies showed that mice lacking functional SIRT1 are less anxious than wild-type littermates and less susceptible to depressive-like behaviours (Libert et al., 2011). In contrast, chronic stress reduces *Sirt1* mRNA, SIRT1 activity, and protein levels in a mouse model of depression (Abe-Higuchi et al., 2016). They also showed that SIRT1 inhibition in the dentate gyrus leads to a depressive phenotype, whereas SIRT1 activation inhibited a chronic stress-induced

depressive phenotype (Abe-Higuchi et al., 2016). In contrast, chronic variable stress has also shown to increase SIRT1 activity in the dentate gyrus in male rats and inhibition of SIRT1 has been reported to be protective in this model (Ferland et al., 2013). Therefore, further animal studies would be needed to clarify the role of SIRT1 in depression as these studies vary in both their species and depression model.

To date, as far as I am aware, no studies have assessed if there is any association between *SIRT1* mRNA levels and clinical outcomes, or if there are any differences between different depression subtypes. To my knowledge, no studies have investigated the effects of ECT on *SIRT1* mRNA levels in human patients. Therefore, more work is needed to evaluate the clinical usefulness of SIRT1 and to confirm if SIRT1 is involved in the mechanistic action of ECT.

Study Type	Disorder	Controls	Summary	Citation
GWAS	MDD (n = 5303)	Yes (n=5337)	Identified, and replicated in a second group, two loci contributing to risk of MDD on chromosome 10: one near the SIRT1 gene	Cai et al., 2015
Gene association study	MDD (n =4 50)	Yes (n=766)	Found an association between rs10997875 in SIRT1 gene and MDD but no association with therapeutic response to SSRIs	Kishi et al., 2010
Gene association study	BPD (n = 180)	No	Found an association between rs10997870 SIRT1 gene variant and suicidal behaviour in patients with bipolar depression	Nivoli et al., 2016
Gene expression study	MDD (n = 20) BPD (n = 12)	Yes (n = 28)	<i>SIRT1</i> mRNA levels were significantly lower in patients (in a current depressed state) in comparison to controls, no differences observed with patients in a remissive state	Abe et al., 2011
Gene expression study	MDD (n = 50)	Yes (n = 50)	<i>SIRT1</i> mRNA levels were significantly lower in unmedicated patients with depression in comparison to controls	Luo and Zhang, 2016
Gene expression study	MDD (n = 68)	Yes (n = 42)	<i>SIRT1</i> mRNA levels were significantly lower in unmedicated patients with depression in comparison to controls, this was attenuated after 4weeks treatment with citalopram (20 mg/day)	Wang et al., 2018

Table 1.2 Summary of the clinical data to support a role for SIRT1 in depression

Abbreviations: GWAS; genome wide association study, MDD; major depressive disorder, SSRIs; selective serotonin reuptake inhibitors, BPD; bipolar depression.

Species	Animal model & techniques used	Summary	Citation
mice	Brain specific SIRT1 knock-out mice, social defeat model	SIRT1 knock-out mice show a less anxious phenotype and have lower levels of MAO-A and less susceptible to depression and anhedonia following social defeat. Mice overexpressing SIRT1 are more susceptible to depression	Libert et al., 2011
rat	Chronic variable stress	Sirtinol (a sirtuin inhibitor) infusion into the DG prevented the CVS-mediated decrease in performance on the novel object location memory task and increase in anhedonic behaviour and rescued CVS induced molecular changes	Ferland et al., 2013
mouse	n/a (naïve)	Electroconvulsive shock resulted in a gradual increase of SIRT1 immunoreactivity in the mouse hippocampus and hypothalamus	Chung et al., 2013
mice	Chronic Ultra-Mild Stress	Chronic stress reduced SIRT1 in the dentate gyrus, inhibition SIRT1 resulted in a depressive phenotype, SIRT1 activation blocked the development of depression-related phenotypes and aberrant dendritic structures elicited by chronic stress	Abe-Higuchi et al., 2016
mice and rats	Chronic variable stress, electrophysiology	SIRT1 activity rapidly modulates intrinsic and synaptic properties of glutamate signalling in the dentate gyrus granule cells. These effect were mediated through BK channels, and the primary α subunit of BK channels was shown to be a direct target of SIRT1 activity. SIRT1 activity was shown to modulate anxiety behaviour with a SIRT1 activator increasing time spend in the middle of an open field (blocking BK channels inhibited this).	Yu et al., 2018

Table 1.3 Summary of the animal literature to support a role for SIRT1 in depression

Abbreviations: MAO-A; monoamine oxidase A, DG; dentate gyrus, CVS; chronic variable stress, BK; big potassium channel

1.4 Pigment epithelial-derived factor

Pigment epithelium-derived factor (PEDF) is a 50 kDA glycoprotein of the serine protease inhibitor family that is widely expressed in both the CNS and periphery (Tombran-Tink and Barnstable, 2003). It was first discovered in the medium of human retinal pigment epithelium (RPE) cell cultures and was shown to induce a neuronal phenotype of Y29 retinoblastoma cells, indicating it could be an important neurotrophic factor (Tombrantink et al., 1991). Studies have subsequently shown that PEDF is neuroprotective, against both oxidative stress from high glucose (DeCoster et al., 1999), and neurotoxicity from high glutamate conditions (Zheng et al., 2010). PEDF has also been shown to increase expression of other growth factors (e.g. BDNF) important for neurogenesis (Yabe et al., 2004), and to alter the inflammatory profile of microglia (Sanagi et al., 2005).

PEDF has been implicated in neurodegenerative diseases. For example, PEDF has been implicated as a potential predictive biomarker of Alzheimer's disease progression (Hye et al., 2014). Memantine, an NMDA non-competitive antagonist used in the treatment of Alzheimer's, increases expression of PEDF and is involved in its enhancing effect on hippocampal progenitor cells and neurogenesis (Namba et al., 2010). Therefore, PEDF could have an important role to play in brain disorders.

1.4.1 Pigment epithelial-derived factor signalling

PEDF binds to a newly-identified membrane-linked receptor with lipase activity. This membrane receptor has been shown to contain a functional phospholipase domain and a transmembrane topology with extracellular loops that are able to interact with the PEDF ligand (Subramanian et al., 2010). From here PEDF seems to be involved in a number of

signalling pathways: both apoptotic pathways and pathways that lead to its antiangiogenic and neurotrophic effects. Figure 1.7 summarises the protective effects of PEDF.

Studies have shown that PEDF mediates its antiangiogenic and apoptotic effects through MAPK signalling – by increasing p38 and JNK1/2 (c-Jun N-terminal kinases) phosphorylation and also through increasing levels of caspase 3 (Konson et al., 2018). It has also been shown to inhibit the pro-angiogenic VEGF through activation of γ -secretase, which then cleaves the VEGF1 receptor (Cai et al., 2006). This increased γ -secretase activity, coupled with reduced VEGF1 receptor surface expression, has also been reported to be anti-inflammatory and protective in oxidative stress through reduction of superoxides (Qi et al., 2012). Three main pathways have been linked to the neuroprotective effects of PEDF: Protein kinase B (Akt); extracellular signal-regulated kinase (ERK); and peroxisome proliferator-activated receptor (PPAR γ) signalling (Yabe et al., 2001, Yabe et al., 2005, Zhuang et al., 2016).

PEDF has been reported to increased phosphorylation of Akt, which in turn leads to increased phosphorylation of cAMP response element binding protein (CREB) and I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha). I κ B α then activates NF- κ B (). This leads to increased expression of pro-survival genes such as superoxide dismutase 2 (SOD2) and Bcl-2, and neurotrophins such as nerve growth factor, BDNF and GDNF (Yabe et al., 2001, Yabe et al., 2005).

However, it has also been reported that ERK signalling is necessary for the neuroprotective effects of PEDF (Tsao et al., 2006). ERK has also been shown to activate CREB and NF- κ B. This could, therefore, be a common mechanism on which the two pathways converge (Tsao et al., 2006).

Finally, PEDF has been reported to activate PPAR γ resulting in decreased reactive oxygen species (ROS) production and caspase activation and blocking PPAR γ inhibits the protective effects of PEDF (Zhuang et al., 2016). Therefore, PPAR γ and Akt/ERK signalling may be required for the neuroprotective effects of PEDF in the CNS.

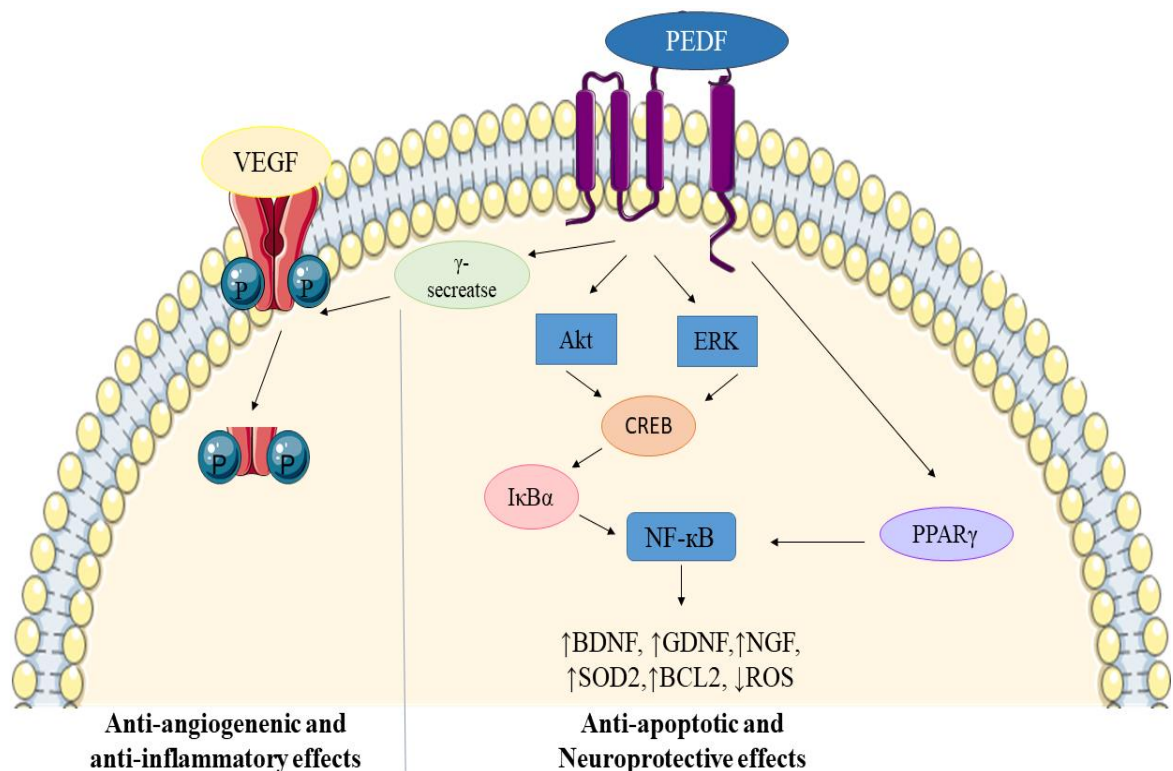


Figure 1.7 Pigment epithelial-derived factor protective pathways

PEDF signals through Akt, ERK and PPAR γ to activate NF- κ B to produce neurotrophic and anti-apoptotic effects. PEDF also activates γ -secretase, cleaving the VEGF-1 receptor to produce anti-angiogenic and possible anti-inflammatory effects. Abbreviations: VEGF; vascular endothelial growth factor, Akt; protein kinase B, ERK; extracellular-signal-regulated kinase, CREB; cAMP response element binding protein, I κ B α ; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha, NF- κ B; nuclear factor kappa-light-chain-enhancer of activated B cells; PPAR γ ; Peroxisome proliferator-activated receptor gamma, BDNF; brain-derived growth factor, GDNF; glial-derived growth factor, NGF; nerve growth factor, SOD; superoxide dismutase, BCL2; B-cell lymphoma 2, ROS; reactive oxygen species.

1.4.2 Pigment epithelial-derived factor and depression

Research on PEDF in depression is limited, with a previous study from my lab being one of two clinical reports from the literature. This study reported that PEDF protein was

increased in patients with depression, which was increased further by ECT. ECS was also reported to increase PEDF expression in the rat hippocampus and blood (Ryan et al., 2017). Previous to this there was just one clinical study that reported altered PEDF in patients ($n = 12$) with depression (Ditzen et al., 2012). This study analysed the proteome of the CSF from patients with depression and healthy controls using two-dimensional polyacrylamide gel electrophoresis and time-of-flight mass spectrometry peptide profiling. They reported an increase in PEDF using 2D-gel electrophoresis. In contrast, the results of mass spectrometry showed a decrease in PEDF. The only other link between PEDF and depression is an animal study that shows chronic fluoxetine treatment increased *SERPINF1* (PEDF encoding gene) mRNA expression in the hippocampus of male mice (Miller et al., 2007). These studies support a role for PEDF, possibly in its pathogenesis but also in the therapeutic action of antidepressants. However, much more research is needed to assess what role PEDF may have in response to antidepressant treatment.

1.5 Objectives

1.5.1 Aims

The exact molecular mechanisms behind the pathophysiology of depression and the therapeutic action of ECT and ketamine still remain unclear. Evidence from my lab has indicated four potential targets that merit further investigation. These include VEGFA, E2F1 and SIRT1 which were indicated in a deep sequencing and gene target analysis study (Kolshus et al., 2017), and PEDF which was identified in a proteomics study (Ryan et al., 2017). The overall aim of the study is to investigate the role these genes may play in depression and the antidepressant action of two treatments for treatment resistant depression; ECT and ketamine.

To my knowledge, no study has yet investigated if *E2F1* or *SIRT1* are involved in treatment response or other clinical aspects of depression. The proteomics study from my lab, which identified PEDF as a growth factor induced by ECT in the plasma of depressed patients, also reported that PEDF levels are increased in medicated patients at baseline. Therefore, this thesis aimed to investigate the effects of antidepressants on PEDF levels *in vitro* to study how PEDF might be involved in their mechanisms of action.

Given the limited knowledge on the peripheral biological effects of ketamine in patients, another aim of this thesis was to investigate if the genes highlighted from the deep sequencing and proteomics studies conducted by my lab also change with a single infusion of ketamine. As VEGF has been reported to play a role in the mechanism of action of ketamine (Deyama et al., 2019), and is linked to PEDF signalling (Falk et al., 2010), the role of these two genes in the molecular action of ketamine is of particular interest.

Based on previous studies from my lab, and evidence from the literature my thesis is based on the following hypotheses:

1. mRNA levels of *E2F1* and *SIRT1* will be altered in patients with depression in comparison to healthy controls.
2. Levels of *E2F1* and *SIRT1* will be normalised following ECT.
3. There will be differences between patients with and without the presence of psychosis.
4. As astrocytes play an important role in neuroprotection and production of growth factors (Karki et al., 2014), it was hypothesised that astrocytes will be the primary source of PEDF and that treatment with antidepressants will increase *PEDF* mRNA and protein expression.

5. Ketamine will have a similar effect to ECT on these genes (*VEGFA*, *SERPINF1* (*PEDF*), *E2F1* and *SIRT1*).

1.5.2 Objectives

1. To analyse the levels of *E2F1* mRNA in whole blood collected from patients with depression and controls using RT-qPCR and to assess differences in the levels of *E2F1* mRNA in patients pre-/post-ECT.
2. To assess differences in *E2F1* mRNA levels pre-/post-ECT in depression subtypes (psychotic/non-psychotic, unipolar/bipolar).
3. To determine if *E2F1* can predict clinical outcomes or if levels are associated with mood scores based on the HAM-D24.
4. To analyse the levels of *SIRT1* mRNA in whole blood collected from patients with depression and controls using RT-qPCR and to assess differences in the levels of *SIRT1* mRNA in patients pre-/post-ECT.
5. To assess differences in *SIRT1* mRNA levels pre-/post-ECT in depression subtypes (psychotic/non-psychotic, unipolar/bipolar).
6. To determine if *SIRT1* can predict clinical outcomes or if levels are associated with HAM-D24 scores.
7. To analyse the effects of antidepressants on PEDF signalling in primary neuronal and glial cell cultures.
8. To determine the effect of a single infusion of ketamine on *VEGFA*, *E2F1*, *SIRT1* and *SERPINF1* (*PEDF*) mRNA expression.

Chapter 2.

Materials and Methods

Materials

General consumables and equipment

Olympus DP72 Microscope	Olympus Life Science
Heraeus Multifuge 3SR Plus Centrifuge	Thermo Scientific
Pipette tips (non-sterile)	Starstedt
TipOne [®] sterile pipette tips	StarLabs
10ml serological pipettes	VWR
25ml serological pipettes	Fisher
RNase/DNase free PCR tubes	Starstedt
RNase/DNase free 1.5ml microtubes	Starstedt
Plastic syringes (5ml, 10ml, 15ml)	BD Plastics
Sterile 15ml tubes	Starstedt
Sterile 50ml tubes	Starstedt
Sterile 1.5ml microtubes	Starstedt

General chemicals and reagents

Absolute Ethanol	Hazmat, TCD
Molecular Grade Ethanol	Honeywell
Propanol	Fisher Scientific
β-mercaptoethanol	Sigma-Aldrich (Merck)
Tween-20	Sigma-Aldrich (Merck)

Bovine Serum Albumin	Sigma-Aldrich (Merck)
Sodium Chloride (NaCL)	Sigma-Aldrich (Merck)
Potassium Chloride (KCL)	Sigma-Aldrich (Merck)
Sodium Phosphate Dibasic (NaHPO ₄)	Sigma-Aldrich (Merck)
Potassium Phosphate Monobasic (KH ₂ PO ₄)	Sigma-Aldrich (Merck)
Urea	Sigma-Aldrich (Merck)

RNA extraction

PAXgene Blood RNA tubes	Qiagen
PAXgene Blood mRNA Kit	Qiagen
RNase Zap Wipes	Ambion
NanoDrop [®] 1000 UV-Vis Spectrophotometer	Fisher Scientific

cDNA synthesis and polymerase chain reaction (PCR)

Eppendorf [®] Master Gradient Cycler	Eppendorf
Eppendorf [®] PCR Cooler	Sigma-Aldrich (Merck)
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
RNase/DNase-free water	Sigma-Aldrich (Merck)
MicroAmp Fast 96 well optical reaction plates	Applied Biosystems

MicroAmp Fast 96 well support base	Applied Biosystems
Optical adhesive covers	Applied Biosystems
TaqMan [®] gene expression assays	Applied Biosystems
TaqMan [®] Fast Advanced Master Mix	Applied Biosystems
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
StepOne Plus [™] Real-Time PCR system	Applied Biosystems

Animals

Wistar rats (1-3 days old)	Bioresources, TCD
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Cell culture plastics and equipment

Microflow horizontal laminar-flow hood	Astec Microflow
Olympus CKX41 inverted microscope	Olympus Life Science
Steri cycle CO ₂ incubator	Fisher Scientific
Stuart si500 shaking incubator	Stuart Equipment
Cell strainers (40µM)	Corning
Disposable sterile scalpels	Swann-Morton
Filter Sterilisers (0.2µm)	Millipore (Merck)
Sterile 24 well plates	Starstedt
Sterile 96 well plates	Starstedt

Sterile pasteur Pipettes	Starstedt
Sterile petri dishes	VWR
Sterile T25 flasks	Starstedt
Sterile T75 flask	Starstedt
Haemocytometer	Sigma-Aldrich (Merck)

Cell culture reagents

Aquasist	VWR
B-27 growth factor	Gibco
Biocidal ZF™	VWR
Dimethyl Sulfoxide	Sigma-Aldrich (Merck)
Dulbecco's Modified Eagle's Medium/	
Nutrient F-12 Ham (DMEM/F12)	Sigma-Aldrich (Merck)
Dulbecco's phosphate buffered saline (DPBS) (1X)	Gibco
Fluoxetine Hydrochloride	Sigma-Aldrich (Merck)
Foetal Bovine Serum	Gibco
Glutamax	Gibco
GM-CSF	Biolegend
Imipramine Hydrochloride	Sigma-Aldrich (Merck)

LDH assay kit	Fisher Scientific
M-CSF	Biolegend
MTT Salts	Sigma-Aldrich (Merck)
Neurobasal-A media	Gibco
Norepinephrine bitartrate salt	Sigma-Aldrich (Merck)
NucleoSpin RNA kit	Macherey-Nagel
Penicillin-streptomycin	Sigma-Aldrich (Merck)
Poly-D-Lysine	Sigma-Aldrich (Merck)
Resazurin sodium salt	Sigma-Aldrich (Merck)
Trypan Blue	Sigma-Aldrich (Merck)
Trypsin-EDTA	Sigma-Aldrich (Merck)
Virkon	Fisher Scientific

Protein analysis reagents and equipment

MSD [®] V-PLEX VEGFA Human kit	Mesoscale
ChemiKine [™] PEDF human ELISA	Millipore (Merck)
PEDF ELISA kit (rat)	ELISAGenie
Synergy [™] HT plate reader	Biotek (Agilent)
MESO QuickPlex SQ 120 plate reader	Mesoscale
PMS 1000i microplate shaker	Grant Instruments

2.2 Methods

2.2.1 Human studies

The studies in this thesis included participants recruited as part of the EFFECT-Dep (Enhancing the Effectiveness of ECT in Severe Depression, ISRCTN23577151) and the KARMA-Dep (The Ketamine as an Adjunctive Therapy for Major Depression NCT03256162) trials.

2.2.1.1 The EFFECT-Dep Trial

The EFFECT-Dep Trial (Enhancing the Effectiveness of ECT in Severe Depression, ISRCTN23577151) (Semkovska et al., 2016) took place in St Patrick's Mental Health Services, Dublin (www.stpatricks.ie) and recruitment was carried out between May 2008 and October 2012. The study was approved by the St. Patrick's University Hospital research ethics committee and written informed consent was obtained from all participants. This was a pragmatic, randomised, patient- and rater-blinded, non-inferiority trial. To be eligible, participants were over 18 years old and referred for ECT for a major depressive episode as diagnosed by the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I) (First et al., 1995). Patients were also assessed using the Hamilton Depression Rating Scale 24-item version (HAM-D24) (Beckham and Leber, 1985) and had a score ≥ 21 . Exclusion criteria included a history of schizophrenia, schizoaffective disorder, dementia; alcohol or substance abuse in the previous six months; involuntary status; inability or refusal to provide consent. Patients remained on their regular medication throughout the trial. Unilateral or bitemporal ECT was administered twice-weekly (Semkovska et al., 2016).

Patients who gave consent to participate in the trial were administered electroconvulsive therapy (ECT) twice weekly using a Mecta 5000M device, (Mecta Corp., Portland, Ore.; maximum 1200mC) according to Royal College of Psychiatrists' guidelines. Methohexital (0.75 mg/kg – 1.0 mg/kg) was used for anaesthesia, and succinylcholine (0.5 mg/kg – 1.0 mg/kg) for muscle relaxation (Benbow, 2002). Seizure threshold was established at the first session. Subsequent treatments were administered at $1.5 \times$ threshold for bitemporal and $6 \times$ threshold for unilateral ECT. Up to 12 ECT sessions were administered, the number for each patient was determined by referring clinicians in consultation with patients.

Response to ECT was defined as a $\geq 60\%$ reduction in baseline HAM-D24 and a score ≤ 16 . For remission status a patient had to have a $\geq 60\%$ reduction in baseline HAM-D24 and a score ≤ 10 for two consecutive weeks.

Patient fasting blood samples were collected 07.30 – 09.30 on the morning of the first ECT and 1 – 3 days after the final treatment. Their medical/clinical history was collected from patient interviews and their patient files.

2.2.1.2 The KARMA-Dep Trial

The Ketamine as an Adjunctive Therapy for Major Depression (KARMA-Dep) trial (NCT03256162) was carried out in St Patrick's Mental Health Services, Dublin (www.stpatricks.ie). St. Patrick's University Hospital research ethics committee approved this study and recruitment took place from September 2017 to September 2018. This study was a pragmatic, randomised, controlled, parallel-group, double-blinded pilot trial. As a pilot trial, the main aim of the study was to assess trial procedures (such as recruitment and safety) to inform a larger definitive trial (Arnold et al., 2009, Thabane et

al., 2010). Trial participants were patients admitted to St Patrick's University Hospital for treatment of a depressive episode. Inclusion and exclusion criteria are listed in Table 2.1 below.

Inclusion criteria
≥18 years old
Hamilton Rating Scale for Depression-24 item version (HAM-D24) score of ≥ 21
Voluntary admission for treatment of an acute depressive episode
Meet Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V), criteria for a major depressive disorder (MDD) or bipolar affective disorder (current episode depression)
Exclusion Criteria
Current involuntary admission
Medical condition rendering unfit for ketamine/midazolam
Active suicidal intention
Dementia
History of Axis 1 diagnosis other than major depression
Electroconvulsive Therapy (ECT) administered within the last two months
Alcohol/substance dependence in previous six-months
Pregnancy or inability to confirm use of adequate contraception during the trial
Breastfeeding women

Table 2.1 Criteria list for the KARMA-Dep Trial

Abbreviations: HAM-D24, Hamilton Rating Scale for Depression-24 item version; DSM-V, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; MDD, major depressive disorder; ECT, electroconvulsive therapy.

To be considered for the trial a patient had to be referred for treatment of a major depressive episode as diagnosed by the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I), 5th edition (First et al., 2002), and score ≥ 21 on the Hamilton Depression Rating Scale 24-item version (HAM-D24) (Beckham and Leber, 1985). If written consent was obtained and a patient met all other criteria, they were then block randomised to receive either ketamine or midazolam infusions. Midazolam was used as an active placebo to minimize the unblinding risk as it has pharmacokinetic

characteristics similar to those of ketamine (including fast onset of action and short elimination half-life) (Fava et al., 2018, Murrough et al., 2013). Participants received either four once-weekly slow (40 minutes) infusions of ketamine at 0.5 mg/kg or midazolam at 0.045 mg/kg. PAXgene blood samples were taken one hour before and four hours after the first infusion. Both groups continued their usual inpatient care as prescribed by their treating team. The primary outcome measurement for this trial was the Hamilton Rating Scale for Depression-24 item version (HAM-D24). Secondary outcome measures included the Clinician-Administered Dissociative States Scale (CADSS) to measure dissociative symptoms (Bremner et al., 1998).

2.2.1.3 Healthy controls

Additionally, healthy controls were recruited through St. Patrick's Mental Health Services via newspapers and social media. To be included, a healthy control had to score ≤ 10 on the HAM-D24 and could not have a history of a psychiatric disorder or any other severe medical issues. Fasting blood samples and medical/clinical history were obtained from healthy controls on the morning of their assessment.

2.2.1.4 Clinical variables

2.2.1.4.1 Diagnostic assessment

The SCID-I was used to confirm the presence or absence of a major depressive disorder in all patients and controls (First et al., 2002). This is a semi-structured clinical interview designed to diagnose mental illness based on DSM-IV criteria. The research version differs slightly from the clinical version. It is slightly longer and contains more subtypes (including psychotic depression, bipolar episodes) and a severity rating.

2.2.1.4.2 Depression ratings

Depression severity at baseline and at end of treatment was measured using the Hamilton Depression Rating Scale (HAM-D) (Beckham and Leber, 1985). This is one of the most commonly used rating scales in major depression and has been widely used in the ECT literature. The Hamilton Depression Rating Scale originally had 17 items and has been updated several times to include more items linked to depression. For the studies used in this thesis the 24 item version was used. The HAM-D24 measures depressive symptom severity and can track changes over time. The HAM-D24 takes approximately 20 minutes to complete and the total score ranges from 0 to 77 with a high score reflecting a higher depression severity. The HAM-D24 includes questions on mood, appetite, sleep, anxiety as well as thoughts of guilt, suicide and worthlessness. The HAM-D has been shown to have good validity as well as inter-rater reliability (Miller et al., 1985, Maier et al., 1988).

The presence of psychosis and its severity was measured using the Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962). The BPRS assesses the level of 18 psychotic symptom constructs including hostility, suspiciousness, hallucination, and grandiosity.

2.2.1.4.3 The Clinician-Administered Dissociative States Scale (CADSS)

Dissociative symptoms include: gaps in memory not due to ordinary forgetting; out of body experiences; changes in visual perception; and identity disturbances (Bremner et al., 1998). These symptoms can occur with ketamine administration and have been linked to its clinical efficacy (Luckenbaugh et al., 2014). The Clinician-Administered Dissociative States Scale (CADSS) is a 27-item scale with 19 subject-rated items and 8 items scored by an observer. The subjective component consists of 19 items administered

by a clinician. The subject can respond with five answers: 0 (not at all), 1 (slightly), 2 (moderately), 3 (considerably), 4 (extremely). The observer component consists of eight behavioural items that are consistent with the presence of a dissociative state. The CADSS has shown high inner-rater reliability and consistency. It has also been shown to be a valid instrument for measuring dissociative state (Bremner et al., 1998).

2.2.1.5 Blood collection

As samples were taken in a clinical setting whole blood samples for mRNA analysis were unable to be processed on collection. Therefore, the PAXgene system was used for collection to prevent degradation of RNA by RNases (enzymes) ex-vivo (Qiagen Inc., USA) and for subsequent molecular analysis. These tubes contain a reagent that will disrupt blood cells and stabilise the released RNA so that molecular results will reflect the intracellular state (Rainen et al., 2002). Samples were left at room temperature for 2 hours, transferred to -20°C for 24 hours and then stored at -80°C until use. A technical note from Qiagen recommends that samples stored at -80°C can be used for up to 96 months without any change in RNA stability. Blood samples for plasma will be collected using K₂EDTA vacutainer tubes (BD, Oxford, UK). Tubes were centrifuged at 2000rpm for 10 minutes. Plasma was then aliquoted and stored at -80°C until use.

2.2.1.6 RNA extraction

mRNA was extracted from whole blood using the PAXgene Blood RNA kit according to the manufacturer's instructions (Qiagen Inc., USA). Before RNA extraction was carried out all work surfaces and equipment were cleaned using RNase Zap wipes to remove any degrading RNases. Figure 2.1 gives an overview of the extraction steps.

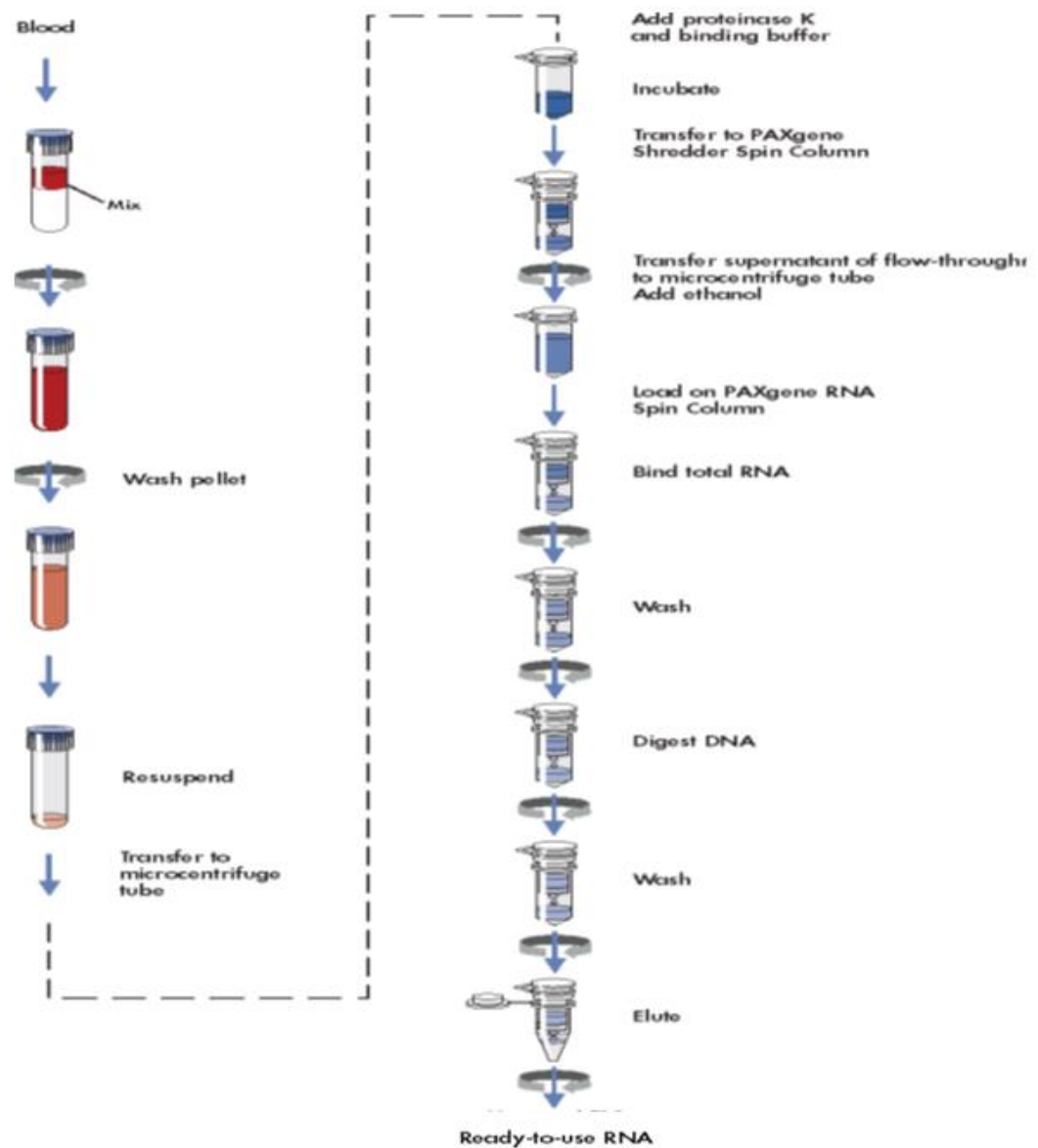


Figure 2.1 The Manual PAXgene Blood RNA Procedure

Adapted from the PAXgene Blood Protocol booklet, available at www.qiagen.com.

Frozen PAXgene tubes were thawed at room temperature for a minimum of 2 hours, and then centrifuged for 10 minutes at $3000 \times g$. The supernatant was then discarded and 4 mL RNase-free water was added to the pellet. The pellet was vortexed until dissolved and then centrifuged for a further 10 minutes at $3000 \times g$. The supernatant was discarded

and 350µL of resuspension buffer 1 was added to the pellet. The pellet was vortexed until dissolved and added to a 1.5mL microcentrifuge tube together with 300µL of binding buffer. 40µL of proteinase K was then added to remove any endonucleases that might contaminate the RNA.

The sample was incubated for 10 minutes at 55°C in a shaker-incubator at 300rpm. The samples were then passed through PAXgene shredder spin columns at $18,000 \times g$ for three minutes. The flow-through supernatant was added to a new 1.5mL microcentrifuge tube and 350µL of molecular grade ethanol was added. The sample was then vortexed.

Samples were passed through PAXgene spin columns, which allows the RNA to bind to silica membranes. 350µL of wash buffer 3 was added to the spin columns, which were then centrifuged for 1 minute at $18,000 \times g$. 80µL of DNase incubation mix was then pipetted directly onto the spin column membrane to remove any DNA contamination. This was left to incubate at room temperature for 15 minutes.

A series of washes were then carried out: 350µL wash buffer 3 for 1 minute at $18,000 \times g$; 500µL of wash buffer 4 for 1 minute at $18,000 \times g$; 500µL of wash buffer 4 for 3 minutes; followed by a final spin for 1 minute at $18,000 \times g$ to dry the column. The column was then placed in a fresh 1.5ml microcentrifuge tube and 40µL of elution buffer 5 was then added directly to the spin columns. The samples were centrifuged for one minute at $18,000 \times g$. This step was repeated with another 40µL of elution buffer 5 to release the RNA from the silica membranes. The RNA was then divided into 10µL aliquots and stored at -80°C.

2.2.1.7 RNA quality analysis

RNA quantification and quality analysis were performed using the NanoDrop[®] 1000 UV-Vis Spectrophotometer. Before quantification, the spectrophotometer was blanked using 1 µL of the elution buffer used on the sample. The spectrophotometer was cleaned using a tissue (between each sample analysis) and 1 µL of the RNA sample was then placed on the NanoDrop and the absorbance at 260 nm (A260) and 280 nm (A280) was measured. The A260 measures the RNA concentration of the sample, and the A260/A280 ratio gives an indication of the purity of the sample. A A260/A280 ratio >1.8 is largely considered suitable for gene expression analysis (Becker et al., 2010). Samples were then equalized to 10 ng/µL using RNase-free water.

2.2.1.8 cDNA synthesis

cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) on mRNA samples equalised to 10ng/µL. The components of this kit were thawed on ice. A master mix was made up as shown in Table 2.2, with the enzyme added just before the master mix was added to the samples.

Component	Volume (per reaction)
10xRT Buffer	2.0 µL
25c dNTP Mix (100mM)	0.8 µL
10x RT Random Primers	2.0 µL
Multiscribe [™] Reverse Transcriptase	1.0 µL
Nuclease-free water	4.2 µL
Total	10.0 µL

Table 2.2 cDNA synthesis master mix reagents

Abbreviations: RT; reverse transcriptase, dNTPS; deoxynucleotide triphosphates

The master mix was added to the mRNA samples at a 1:1 ratio. Samples were then mixed and spun down before being placed in the thermal cycler. A no template control (NTC) and a no reverse transcriptase control (NRT) were also made up. The NTC was made by adding the master mix to an equal volume of RNase free water to test for contamination of the reagents. For the NRT a separate master mix was made up that replaced the enzyme with water. This was then added to a selected mRNA sample to test for genomic DNA contamination. The thermal cycler was then run at the following conditions: 25°C for 10 minutes; 37°C for 120 minutes; 85°C for 5 minutes; followed by a hold at 4°C until samples were taken from the cycler. Samples were then diluted to a 1:3.5 ratio as recommended by Applied Biosystems.

2.2.1.9 Multiplex quantitative real-time polymerase chain reaction (qRT-PCR)

For mRNA gene expression analysis a two-step multiplex quantitative real-time polymerase chain reaction (qRT-PCR) was carried using a StepOne Plus™ Real-Time PCR system (Applied Biosystems). Multiplex qRT-PCR is advantageous in that the analysis of both the target of interest and the endogenous control occur on the same plate. TaqMan® gene expression assays and TaqMan Fast Advanced Master Mix® (Applied Biosystems) were used for this. These assays contain a primer for the target of interest containing a 6-carboxyfluorescein (FAM) labelled probe as well as a VIC labelled probe for the endogenous control, which in these studies was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH was chosen as an endogenous control as it had been previously tested against five controls in my lab and shown to have the highest stability in response to ECT treatment. Labelling primers with different fluorescent probes allows the detection of multiple targets in the same PCR run (Blacket et al., 2012). The assay IDs for the primer used in this thesis are presented in Table 2.3.

Gene Target	Assay ID
<i>GAPDH</i>	Hs02758991_g1
<i>SIRT1</i>	Hs01009006_m1
<i>E2F1</i>	Hs00153451_m1
<i>VEGFA</i>	Hs00900055_m1
<i>SERPINF1</i> (PEDF)	Hs01106934_m1

Table 2.3 Assay IDs for primer used in human gene expression analysis

The qRT-PCR was then carried out according to the manufacturer's instructions. All necessary components were first thawed on ice. A master mix of 5µL of TaqMan Fast Advanced Master Mix and 0.5µL of the target primer and 0.5µL of the endogenous control primer was then made for every sample. The master mix (6µL) was added to each well of the PCR plate along with 4µL of cDNA. Samples were plated in duplicate. Inner plate calibrators were used to control for variation between plates. cDNA from four healthy participants were used as inner plate calibrators. The plates were then sealed using an optical film (Applied Biosystems) and scored between wells to prevent mixing of samples. The plate was then spun down at 800rpm for 1 minute.

The PCR plates were run using the following thermal cycling conditions: Uracil-N-glycosylase incubation at 50°C for 2 minutes; a polymerase activation step of 95 °C for 20 seconds followed by 40 cycles of two-step PCR consisting of a denaturation step at 95°C for 1 second and an annealing/extension step at 60°C for 20 seconds. At the end of RT-PCR the StepOne Plus™ Software generated cycle threshold (CT) values for the genes of interest.

2.2.1.10 Gene expression analysis

The comparative CT (or $2^{-\Delta\Delta CT}$) method was used to assess gene expression (Livak and Schmittgen, 2001). This method compares relative gene expression between two different samples, such as treated vs. controls or before and after treatment rather than absolute quantification. A reference sample is needed for this and one of the inner plate calibrators was used. For all plates the same threshold and baseline cycle was selected for each gene. Once analysis on the StepOne Plus™ Software was complete, qRT-PCR files were analysed using qBase+ to correct for variability between qRT-PCR runs (Hellemans et al., 2007).

2.2.1.11 Protein analysis

Plasma samples for protein analysis were thawed on ice. Samples were then centrifuged at $2000 \times g$ for 3 minutes to pellet any debris.

2.2.1.11.1 Vascular endothelial growth factor A protein analysis

VEGFA protein analysis was carried out using the MSD® V-PLEX Cytokine Panel 1 Human Kit (Mesoscale, USA) according to the manufacturer's instructions. Plasma samples were thawed on ice, and then diluted 1:2 with the sample diluent 43 provided with the kit. 9 calibrators for generating a standard curve were then prepared by serial dilution using diluent 43 and a lyophilised calibrator. The plate was then washed 3 times with 250µL of wash buffer (1X PBS pH 7.3 with 0.05% Tween 20). 1X PBS was made up as follows; 137mM NaCl (MW = 58.44g/mol, 8.01g/L), 2.7mM KCL (MW = 74.55g/mol, 0.20g/L), 8.1mM NaHPO₄ (MW = 141.96g/mol, 1.15g/L) and 1.5mM KH₂PO₄ (MW = 136.09g/mol, 0.20g/L). 50µL of samples and the calibrators were then

added to the wells. The plate was sealed, and incubated overnight at 4°C on a plate shaker at 1000rpm. The next day the plate was washed 3 times with 150µL of wash buffer. 25µL of detection antibody was then added to each well. The plate was sealed and incubated for 2 hours at room temperature on a plate shaker at 1000rpm. Following this incubation the plate was washed 3 times with 150µL of wash buffer. 150µL of read buffer was then added to the wells. The plate was then read using the Mesoscale QuickPlex SQ 120 machine. Data was analysed using the mesoscale discovery workbench version 1300.

2.2.1.11.2 Pigment epithelial-derived factor protein analysis

PEDF protein was analysed using the ChemiKine™ Pigment Epithelium Derived Factor, Sandwich ELISA (Merck, UK) according to the manufacturer's instructions. For total PEDF protein analysis samples were incubated with 8M Urea (MW = 60.06g/mol). A 16M urea solution was made up and diluted 1:2 with the samples (9.9096g in 10ml dH₂O) for 1 hour on ice. Samples were then diluted 1:400 with the reagent diluent supplied by the kit. The PEDF standard vial was then reconstituted in 500µL of H₂O to give a concentration of 500ng/ml. This was diluted to 62.5ng/ml in reagent diluent for the top standard, and a 1:2 dilution was then carried out to yield 7 standards (62.5ng/ml – 0.98ng/ml) and a 0 dose/blank. 100µL of standards and samples were then plated in duplicate and incubated at 37°C for 1 hour. The plate was then washed four times with 250µL of wash buffer. 100µL of Biotinylated Mouse Anti-Human PEDF monoclonal antibody (diluted 1:250) was added to each well and incubated at 37°C for 1 hour. The wash step was repeated. 100µL of streptavidin peroxidase conjugate (diluted 1:10000) was added and incubated at 37°C for 1 hour. The wash step was repeated. Warm 3,3',5,5'-Tetramethylbenzidine (TMB) solution was then added (100µL/well). After 5 minutes at room temperature the reaction was stopped using 50µL of stop solution. The

absorbance was then read at 450nm. A standard curve was plotted and used to determine the concentration of each sample.

2.2.1.12 Statistical analysis

Data were analysed using SPSS version 21.0 (IBM Corporation, NY, USA) and graphs generated using GraphPad Prism 5 (GraphPad Software, California, USA). Data were tested for normality using a Shapiro–Wilk test and log-transformed where necessary. Baseline clinical and demographic factors are presented as means with standard deviation (SD), or number (%) per group. I adjusted for potential variance covariates that were selected from the literature for each gene of interest, as well as demographic and clinical characteristics that differed significantly between patient and control groups.

Data were analysed using independent t-tests, paired t-tests, Mann-Whitney U tests for non-parametric data, or general linear models. Categorical data were tested using a chi-square test (χ^2) or Fisher's exact test when group numbers were small. For correlational analyses, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) for parametric data and Spearman's rank correlation coefficient ρ for non-parametric data.

Differences with $p < 0.05$ were deemed statistically significant. For exploratory correlation analyses, a p -value < 0.01 was used to correct for testing on multiple subgroups.

2.2.2 Animal studies

2.2.2.1 Animals and electroconvulsive stimulation procedures

Experimental procedures were carried out in compliance with the European Council Directive (86/609/EEC). Male Sprague-Dawley rats, weighing 150 - 200g on arrival, were housed four per cage. Animals were maintained at an ambient temperature of 20 ± 1 °C with lighting on a 12 hour light-dark cycle. Animals were given free access to food and water. Prior to experimentation animals were handled for one week to habituate them to the ear-clips used for ECS and to alleviate any stress that may be caused by animal handling.

Animals were randomised to receive either acute (single) or chronic electroconvulsive stimulation (ECS) or sham treatment as a control ($n = 8$ per group). ECS or sham treatment was given three times per week (Monday, Wednesday and Friday) to mimic clinical settings. For chronic ECS 10 treatments were given. ECS was administered via ear-clip electrodes using the ECT Unit 57 800 device (Ugo Basile, Italy). Brief-pulse ECS treatment parameters were as follows: 0.5 millisecond pulse width, 100 pulses per second, 75 milliAmps, 0.7 second duration. For sham treatment no charge was administered. Animals were only included in the study if a tonic-clonic seizure was successfully induced on each treatment day.

Four hours following a single (acute) treatment or after the tenth (chronic) treatment animals were sacrificed under isoflurane anaesthesia. Whole blood was harvested by cardiac puncture into K2EDTA coated syringes. Blood was then transferred to RNAprotect Animal Blood Tubes (Qiagen). Rats were then immediately decapitated and the brains removed from the skull. Any large blood vessels were removed and then the dentate gyrus, hippocampal formation, dentate gyrus, frontal cortex and the cerebellum were dissected out by hand and snap-frozen on dry-ice.

2.2.2.2 RNA extraction

Total RNA was extracted from the brain tissue using a mir-Vana miRNA Isolation kit according to the manufacturer's instructions. For whole blood RNA extraction the RNeasy Protect Animal Blood Kit was used.

2.2.2.2.1 Brain tissue extraction

The tissue sample was first weighed. 10 volumes of Lysis/Binding Buffer per tissue mass was placed on ice. The tissue was grinded into a powder and placed into the Lysis/Binding Buffer and mixed rapidly. The mixture was homogenised until all visible clumps were removed.

1/10 volume of miRNA Homogenate Additive was added to the tissue homogenate, and mixed by vortexing. A volume of acid-Phenol:Chloroform that is equal to the lysate

volume before addition of the miRNA Homogenate Additive was added. The sample was then mixed by vortexing for 30 - 60 seconds. The sample was centrifuged for five minutes at maximum $10,000 \times g$ at room temperature to separate the aqueous and organic phases. The aqueous (upper) phase was removed and transferred to a fresh tube.

1.25 volumes of room temperature 100% ethanol was added to the aqueous phase. The sample was pipetted onto a Filter Cartridge and centrifuged for 15 seconds to pass the mixture through the filter. The flow through was discarded. A series of wash steps was then performed. The sample was washed twice with 500 μL of Wash Solution 2/3. The filter cartridge was then placed in a collection tube and 100 μL of Nuclease-free Water was added to the sample to elute the RNA. The tube was spun at maximum speed for 1 minute to collect the RNA which was then aliquoted and stored at -80°C

2.2.2.2.2 Whole blood RNA extraction

The RNAprotect Animal Blood Tube was placed in a centrifuge for 3 minutes at 5000 x g and the supernatant removed. 1ml of RNase-free water was added to the pellet which was then mixed by vortexing. The sample was spun again for 3 minutes. The entire supernatant was then removed by pipetting. 240µl of a resuspension Buffer was added and then mixed by vortexing until the pellet is visibly dissolved. The sample was placed in a 1.5 ml collection tube. 200µl Buffer RBT and 20µl proteinase K was added. The sample was mixed by vortexing for five seconds, and incubated for 10 minutes at 55°C in a shaker-incubator at 300rpm.

The sample was pipetted into a QIAshredder spin column centrifuged for 3 minutes at 17000 × g. The entire supernatant was placed in a 1.5 ml collection tube and 240µl ethanol was added. The sample was then pipetted into an RNeasy MinElute spin column and placed in the centrifuge for 1 minute at at 17000 × g. 350µl Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 seconds. 10µl DNase I stock solution was added to 70µl Buffer RDD in a 1.5 ml microcentrifuge tube and mixed. The DNase I incubation mix was added directly to the spin column membrane and incubated for 15 minutes at room temperature.

350µl Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged for 15 seconds. 500µl Buffer RPE was then added to the RNeasy MinElute spin column and the sample was spun at 17,000 × g for 15 seconds. The column was then washed with 500 µl of 80% ethanol at 17,000 × g for 15 seconds. The tube was then spun for 5 minutes to dry the column. 30µl Buffer REB was added directly onto the spin column membrane to elute the RNA. This was spun for 1 minute at 17,000 × g to collect the RNA. Samples were then stored at -80°C until use.

2.2.2.3 RNA quality analysis

RNA quality analysis was carried out as described in section 2.2.1.7 with one minor change. Samples were normalised to 30ng/μL using RNase-free water.

2.2.2.4 cDNA synthesis and multiplex quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA synthesis, qRT-PCR and gene expression analysis were carried out as described in section 2.2.1.8 – 2.2.1.10 with the primers used outlines in Table 2.4 below.

Gene Target	Assay ID
<i>Gapdh</i>	Rn99999916_s1
<i>Sirt1</i>	Rn01428096_m1
<i>E2f1</i>	Rn00153451_m1

Table 2.4 Assay IDs for primer used in rat gene expression analysis

2.2.2.5 Statistical analysis

All data are presented as mean ± SD and were analysed using GraphPad Prism, version 5.0 (GraphPad Software, USA). A Shapiro-Wilk normality test was first carried out. When data was found to be normal a student t-test was carried out. When data was not normal non parametric tests were used (Mann-Whitney U test). Statistical significance was set at $p < 0.05$.

2.2.3 *In vitro* studies

2.2.3.1 Aseptic technique

During all cell culture work and preparation of reagents, aseptic techniques were implemented. This was to maintain a sterile environment that was free from fungal, bacterial and viral infections that could affect cellular function and growth.

All pipette tips, tubes and distilled water (dH₂O) were autoclaved at 121°C for 20 minutes prior to use. Dissection equipment was soaked in Virkon after use and cleaned before being autoclaved for use. All culture work was carried out in a Microflow laminar flow station that only allows filtered air to pass through, preventing contamination from airborne contaminants.

Prior to any cell culture work, the laminar flow hood was sprayed with 70% ethanol (EtOH; 30% Dh₂O, 70% EtOH v/v) and wiped down. Any equipment taken into the hood was also sprayed with 70% EtOH. Disposable latex gloves were worn as well as clean lab coats designated for culture work; these were both sprayed with 70% EtOH before use and gloves were changed regularly. At the end of each day the hood surface was also exposed to ultraviolet (UV) light.

Cells were maintained in a sterile incubator (humidified 5% CO₂: 95% air environment at 37°C) and any items being placed in the incubator were sprayed with 70% EtOH to prevent contamination. Aquarest was added to the dH₂O a working concentration of 0.1% (1ml solution/1ml dH₂O as per manufacturer's instructions) in the incubator to prevent the growth of algae and bacteria.

The incubator and laminar flow were regularly cleaned with Biocidal ZF™, Virkon and EtOH to prevent contamination.

2.2.3.2 Preparation of tissue culture media and test compounds

Glial Culture Media: Primary astrocytes and microglia were cultured in Dulbecco's modified Eagle's medium (DMEM:F12) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) and 1% (v/v) Penicillin-Streptomycin (P/S). This was prepared by filter-sterilising 5ml P/S and 50ml FBS through a 0.2µm syringe filter into a 500ml bottle of DMEM:F12. This complete DMEM (cDMEM) was then used in all glial cell preparations.

Neurobasal media: Primary neurons were cultured in neurobasal media (NBM) supplemented with 1% (v/v) P/S and 1% (v/v) Glutamax. This was prepared by filter sterilising 5ml P/S and 5ml glutamax through a 0.2µm syringe filter into a 500ml of NBM. This was then divided up into 50ml aliquots. As required, 500µL 100X B-27 growth supplement was then added to a 50ml aliquot resulting in 50ml completer-NBM (cNBM).

Poly-D-Lysine: a 5mg/ml vial of Poly-D-Lysine was dissolved in 50ml dH₂O to make a 0.1mg/ml solution. This was divided into 5ml aliquots and frozen at -20°C until future use.

Ara-C (Cytarabine): A 25mM stock solution was prepared by adding 16.44ml dH₂O to 100mg vial of Ara-C (MW = 243.22). Once dissolved, this was filter-sterilised using a 0.2µm syringe, aliquoted and frozen at -20°C until future use.

Macrophage colony-stimulating factor (M-CSF): a 50µg/ml solution M-CSF was prepared by adding 50µL 200µg/ml M-CSF to 150µL DPBS/1% bovine serum albumin (BSA). This was aliquoted and frozen at -20°C until future use.

Granulocyte-macrophage colony-stimulating factor (GM-CSF): A 10µg/ml solution GM-CSF was prepared by adding 50µL 200µg/ml M-CSF to 950µL DPBS/1% bovine serum albumin (BSA). This was aliquoted and frozen at -20°C until future use.

Fluoxetine: A 10mM stock solution was prepared by adding 2.89ml of dH₂O to 10mg fluoxetine (MW = 345.79) in the vial. Once dissolved this was filter-sterilised using a 0.2µm Syringe, diluted 1:10 to a 1mM stock, aliquoted and frozen at -20°C until future use.

Imipramine: A 10mM stock solution was prepared by dissolving 0.031g Imipramine (MW = 345.79g/mol) in 10ml dH₂O. This was then filter-sterilised using a 0.2µm syringe filter, aliquoted and frozen at -20°C until future use.

Noradrenaline (NA): A 100mM stock solution was prepared by dissolving 0.034g NA (MW = 337g/mol) in 1ml dH₂O. This was then filter-sterilised using a 0.2µm syringe filter. As NA is light sensitive light exposure was kept minimal. This was then diluted 1:10 to 10mM stock, aliquoted and frozen at -20°C until future use.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): A 5mg/ml solution of MTT formazan was made up in dH₂O. Once dissolved this was filter-sterilised

through a 0.2µm syringe filter. MTT formazan is light sensitive so this solution was kept away from light. MTT salts were made up fresh for every experiment.

Resazurin (AlamarBlue): A 440µM stock solution of alamar blue was prepared by dissolving 2.76mg resazurin (C₁₂H₆NNaO₄, MW = 251.17g/mol) in 25ml dH₂O. This stock solution was filter-sterilised through a 0.2µm syringe filter and stored away from light.

2.2.3.3 Coating coverslips/plates with poly-D-lysine

For primary neuronal cultures, coverslips and 24 plate wells were covered with Poly-D-Lysine. Prior to use, Poly-D-Lysine was defrosted, diluted 1:2 with dH₂O to make a final concentration of 50µg/ml, and filter-sterilised through a 0.2µm syringe filter. For plates, 100µL of poly-D-lysine was added to the centre of each well. The plate was then incubated at 37°C for 1hour and the poly-d-lysine solution removed. Plates were left to dry for 2 hours. They were then ready to use, or frozen at -20°C until future use.

2.2.3.4 Primary cell preparation and culture

Primary cultures for enriched astrocytes, enriched microglia and primary cortical neurons were prepared following protocols published in studies by colleagues in Trinity College Institute of Neuroscience (McNamee et al., 2010, Day et al., 2014). All procedures were performed in the laminar flow hood.

2.2.3.4.1 Preparation of mixed glial cultures for enriched astrocyte and microglial cultures

Mixed glial cultures were prepared from the brains of newborn Wistar rat pups (postnatal day 2-3) under sterile conditions. The pups were decapitated using a large sharp/blunt scissors. A small sharp scissors was then used to cut the skin down the midline to reveal the skull. The skull was then carefully cut on each side at ear level using a sharp scissors. The skull was then pulled back using a fine forceps to expose the brain. The meninges were removed using a fine forceps. The cortical tissue on both hemispheres was then removed using a curved forceps and any meninges under the cortical tissue were also removed. The tissue was then placed in a drop of pre-warmed cDMEM and cross-chopped using a scalpel. Any extra blood vessels were removed and the tissue was placed in a 15ml Falcon tube with 2ml of pre-warmed cDMEM and incubated at 37°C for 20 minutes.

After this, the tissue was triturated until all visible clumps were removed and passed through a sterile mesh filter (40µm). The cells were then centrifuged at 2000rpm for 3 minutes at 20°C. The supernatant was discarded and the pellet was resuspended in 1ml pre-warmed cDMEM. The resuspended cells were added to a T75 flask along with 3mls of pre-warmed cDMEM. The cells were left to adhere for 2 hours and 10ml of cDMEM was then added to the flask. Media (cDMEM) was replaced every 4-5 days.

2.2.3.4.2 Isolation of enriched primary astrocyte cell cultures

When the T75 flasks of primary mixed glial cells were confluent (days *in vitro*: DIV 10-14) plates were sealed with parafilm and placed on a shaker/incubator at 37°C at 200rpm for 2 hours. Microglia sit on a layer above the astrocytes and break away from the astrocytes when agitated. Plates were banged up to 10 times to remove all microglia. The

media was then discarded and the remaining astrocytes were washed with 10ml pre-warmed 1X DPBS. 1ml of trypsin-EDTA was then added and the plate was incubated at 37°C for 5 minutes. A microscope was used to check that the astrocytes had dissociated from the flask before the trypsin-EDTA was neutralised with 7ml cDMEM (FBS in cDMEM contains inhibitors for trypsin). This media/trypsin-EDTA was then added to a 15ml tube and placed in a centrifuge for 2000rpm for 3 minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in 1ml cDMEM.

Live cells were then counted using the tryphan blue dye exclusion/haemocytometer method. For this method 0.4% tryphan blue are added to the cells before counting. Intact cells (live viable cells) will exclude tryphan blue due to their intact membrane. The cells/tryphan blue solution is added to a haemocytometer. Viable cells are counted in the four large squares highlighted in Figure 2.3 and an average cell number taken.

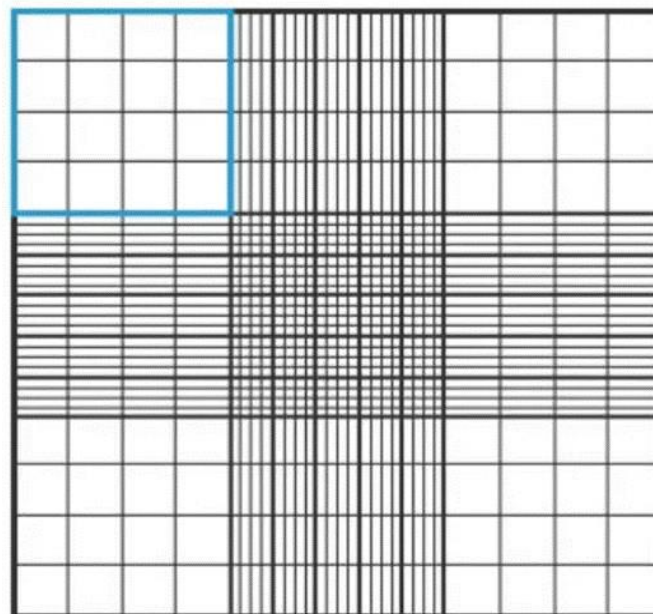


Figure 2.2 Hemocytometer gridlines.

Highlighted in blue is one of the four large squares that should be counted and an average count taken to get your cell number/ml. Image taken from www.abcam.com.

Approximately 1×10^5 cells were plated in each well. These cells were left in the incubator to adhere before being gently flooded with 300ml cDMEM. Primary enriched astrocytes were used for experiments up to 5 days after plating. Figure 2.3 outlines the steps needed to obtain an enriched astrocyte culture.

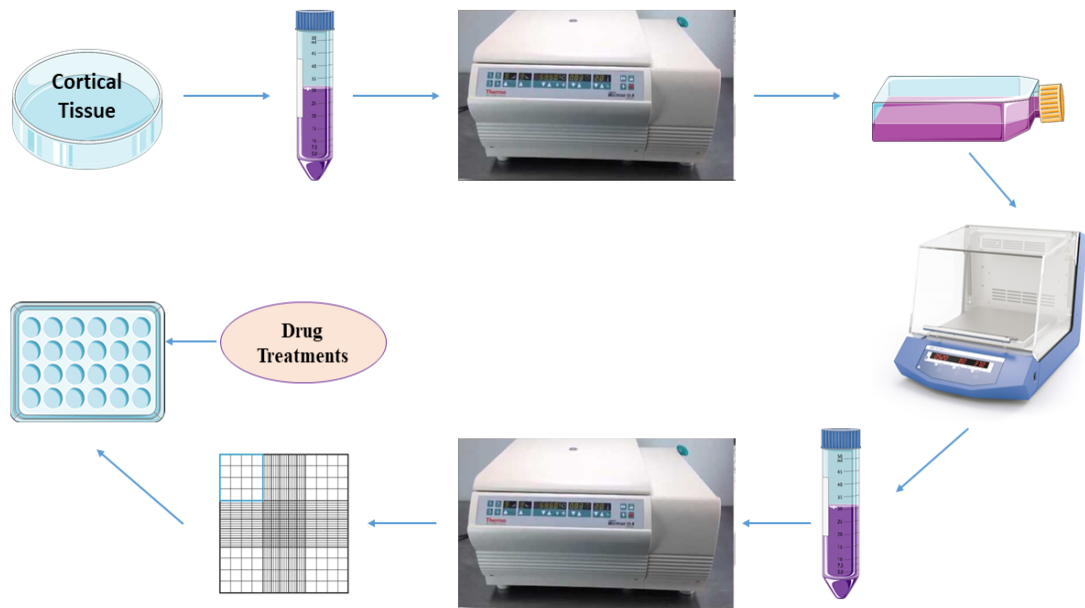


Figure 2.3 Isolation of enriched primary astrocyte cell cultures

Cortical tissue is harvested from 1-3 day old neonatal pups and grown in a flask to confluence before microglia are shaken off. Enriched astrocytes are then grown and treated in well plates.

2.2.3.4.3 Isolation of enriched primary microglia cell cultures

As with enriched primary astrocyte cultures, T75 flasks of primary mixed glial cells were grown until confluent, approximately after DIV 10-14 with one additional step. Microglia require growth factors to proliferate and reach the number of cells required for experiments. Therefore, all cDMEM used for the culture of microglia was supplemented with 10ng of M-CSF and 10ng of GM-CSF from DIV1.

When the flasks were confluent, they were sealed with parafilm and placed on a shaker/incubator at 37°C at 200rpm for 2 hours. The media containing the microglia was

then transferred to a 15ml tube. This was then placed in a centrifuge for 2000rpm for 3 minutes at 20°C. The supernatant was discarded and the cell pellet was resuspended in 1ml cDMEM.

Live cells were then counted using the trypan blue dye exclusion/haemocytometer method. Approximately 50×10^4 cells were plated in each well. These cells were left in the incubator to adhere before being gently flooded with 300ml cDMEM supplemented with M-CSF and GM-CSF. Primary enriched microglia were used for experiments up to 5 days after plating.

2.2.3.4.4 Primary neuronal cell preparation and culture

The cortex was dissected from 1 day old Wistar rat pups as described above in section 2.2.3.4.1. Once the cortical tissue was removed it was finely cross-chopped in pre-warmed NBM and then transferred into 5ml of trypsin-EDTA. This was incubated for 2 minutes at 37°C. 5ml of cDMEM was then added to the tissue. This was then resuspended and centrifuged at 2000rpm for 3 minutes at 20°C. The supernatant was then discarded and the pellet resuspended in 1ml cDMEM. The cell suspension was triturated until no visible clumps remained, passed through a sterile mesh filter (40µm) and centrifuged at 2000rpm for 3 minutes at 20 °C. The supernatant was discarded and the pellet was resuspended in 1ml pre-warmed cNBM. This cell suspension was then counted using the trypan blue/haemocytometer method to obtain the number of live cells. 1×10^5 cells were plated in each well. 100µL of the appropriate cell suspension pipetted onto the centre of a poly-D-coated coverslip/well in a 24-well plate. This was placed into an incubator (5% CO₂, 95% air at 37°C) for 2 hours to allow the cells to adhere. They were then gently flooded with 200µl cNBM. After DIV1 the cells were treated with Ara-C to

prevent proliferation of glial cells and blood vessels. A 25mM stock was diluted to 25 μ M in dH₂O and 30 μ L of this was added to the 300 μ L of media in the well for a final working concentration of 2.5 μ M. After 24 hours (DIV2) the media was removed and replaced with fresh cNBM. Immature neurons were then treated at DIV3 or DIV4.

2.2.3.5 Cell culture treatments

For each experiment, primary cortical enriched astrocytes, microglia or neurons were plated in 24-well plates. Each plate was considered an $n = 1$. Cell cultures were incubated at 37°C with 5% CO₂ with the various drug treatments for a time course of 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours or 24 hours for gene expression changes. For conditioned media experiments and changes in protein production or viability assays, cells were incubated with the various drug treatments for 24 hours. For conditioned media experiments, cell supernatants were removed after 24 hours drug treatments and transferred onto primary neuronal cultures. Cell numbers were kept at approximately the same numbers for each experiment type.

2.2.3.6 Viability assays

To ensure any of my treatments were not affecting the viability of the primary cells three viability assays were used.

2.2.3.6.1 Pierce™ Lactate Dehydrogenase (LDH) cytotoxicity assay

The Thermo Scientific Pierce LDH Cytotoxicity Assay Kit is a reliable colorimetric assay that quantitatively measures lactate dehydrogenase (LDH) released into the media from damaged cells as a marker for cellular cytotoxicity and cytolysis. LDH is an enzyme

found in the cytoplasm; therefore its presence in the media indicates cell death. The assay measures extracellular LDH in culture media using an enzymatic reaction that results in a red formazan product, which can be spectrophotometrically measured. The assay was carried out according to the manufacturer's instructions. Prior to carrying out the assay, a vial of assay buffer was added to 11.4mls of the provided substrate mix. This was protected from light and stored at -20°C until use. For experiments, additional wells were used to account for a triplicate of spontaneous LDH activity control and a maximum LDH control. Cells were treated as normal for 24 hours. For the spontaneous LDH control, 30µL of ultrapure H₂O was added. Nothing was added to the maximum LDH control wells. Following the required incubation, 10µL of lysis buffer was added to the maximum LDH control well. Following a 45 minute incubation, 50µL of medium from the control and treatment wells was transferred to 96-well plates in duplicate. Using a multi-channel pipette, 50µL of reaction mixture was added to the wells, the plate lightly tapped and left at room temperature for 30 minutes protected from light. 50µL of stop solution was then added and the plate lightly tapped again. The absorbance was read at 490nm and 680nm. The 680nm value was subtracted from the 490nm value and the % cytotoxicity was calculated using the formula provided by the manufacturer. This was as follows; % cytotoxicity = (Compound-treated LDH activity – Spontaneous LDH activity)/(Maximum LDH activity - Spontaneous LDH activity)*100.

2.2.3.6.2 AlamarBlue Assay

Resazurin sodium salt (C₁₂H₆NNaO₄), or the AlamarBlue assay, is used to measure the metabolic activity and proliferation of living cells. The reduction of the dye by mitochondrial enzyme reduces the amount of the oxidized form (blue) and increases the fluorescent intermediate (red). Figure 2.3 gives an overview of this reaction.

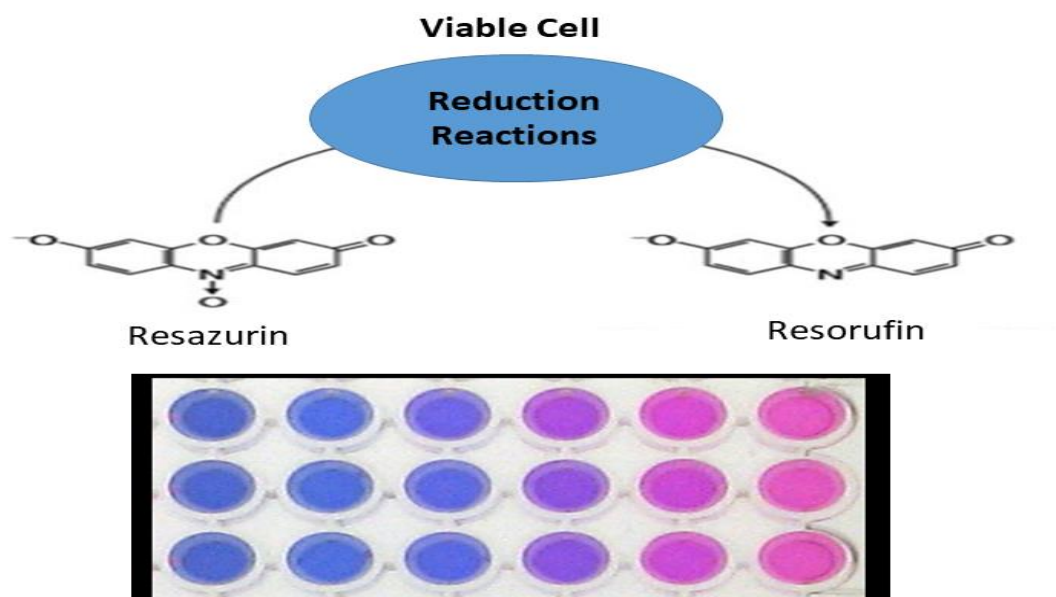


Figure 2.4 The AlamarBlue Assay

Mitochondrial activity converts resazurin (coloured blue on the left wells) to reserufin (pink on the right wells). Adapted from (Shenoy et al., 2017).

Therefore, the more viable or mitochondrial active the cells, the greater the colour change. For the assay, resazurin was prepared as outlined in section 2.2.2.2. After the cells were treated for the appropriate time, this solution was diluted 1:10 in the wells as described in the Abcam protocol. The plate was then protected from light and left to incubate (5% CO₂, 95% air at 37°C) for 1-3 hours. Once a colour change was observed 100µL of sample was transferred to a 96well plate in duplicate. The absorbance was then read at 600nm and 570nm. The 600nm value was subtracted from the 570nm value and the % viability was calculated as follows: % viability = (average absorbance of a treated sample)/(average absorbance of a control sample)*100.

2.2.3.6.3 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) produces a yellowish solution that is converted to dark blue, water-insoluble, MTT formazan by mitochondrial dehydrogenases of living cells. Therefore, like the AlamarBlue assay, it is used to measure the metabolic activity and proliferation of living cells. A 5mg/ml solution of MTT was made up as described in section 2.2.2.2. After 24 hour treatment, this was then diluted to 1mg/ml in the wells/media. The plate was then protected from light and left to incubate (5% CO₂, 95% air at 37°C) for 1-3 hours. Once the MTT formazan was produced, the media was removed and the crystals dissolved in isopropanol (300ml) as per the manufacturer's instructions. 100µL of sample was transferred to a 96well plate in duplicate and the absorbance was 570nm. The % viability was calculated with the same formula used for the AlamarBlue assay (section 2.2.2.6.2).

2.2.3.7 Tissue preparation for quantitative real-time polymerase chain reaction (qRT-PCR)

For RNA experiments, cells were plated at 1×10^5 cells/well and two wells were pooled for each sample to ensure enough RNA. RNA was then extracted using the NucleoSpin RNA kit from Macherey-Nagel according to the manufacturer's instructions with one minor change. Samples were centrifuged at a higher speed than in the protocol as columns were not drying out fully and there was minor sample contamination. Figure 2.4 gives an overview of the steps carried out.

1. Lyse Cells	 350µL RA1 + 3.5µL β-mercaptoethanol per sample
2. Filter Lysate	  16,000 × g 1 minute
3. Adjust RNA binding conditions	 350 µL 70 % ethanol Vortex 5 seconds
4. Bind RNA	  Load sample 16,000 × g 30 seconds
5. Desalt silica membrane	  350 µL MDB 16,000 × g 1 minute
6. Digest DNA	 95µL DNase reaction mixture Room temperature, 15 minutes
7. Wash and dry silica membrane	 1st wash 200 µL RAW2 2nd wash 600 µL RA3 3rd wash 250 µL RA3  1st and 2nd 16,000 × g, 30 seconds 3rd wash 16,000 × g, 2 minutes
8. Elute RNA	  40µL RNasefree H ₂ O 16,000 × g, 1 minute

Figure 2.5 mRNA extraction protocol

Adapted from Macherey-Nagel NucleoSpin RNA extraction kit protocol available at <https://www.mn-net.com>.

350µl lysis buffer RA1 (supplemented with 10% β-mercaptoethanol) was divided across the wells being pooled for each sample. The cell lysate was then either immediately frozen at -80°C for future extraction or was extracted straight after lysing. If frozen, the lysate was left to defrost slowly on ice. The cell lysate was then filtered through a NucleoSpin® Filter to reduce viscosity and clear the lysate. The RNA binding conditions were then adjusted by adding 350µL 70% ethanol. The lysate was then centrifuged through a NucleoSpin® RNA column for 30 seconds at 16,000 × g. 350µL of Membrane Desalting Buffer was then added to the RNA column and this was centrifuged at 16,000

× g for 1 minute. The flow through was discarded and the column centrifuged again for 30 seconds to ensure the RNA membrane was dry. 95µL of DNase reaction mixture was applied directly to the centre of the silica membrane of the column to digest any DNA. This was left to incubate at room temperature for 15 minutes.

A series of wash steps was then carried out: the silica membrane was washed with 200µL wash buffer RAW2 at 16,000 × g for 30 seconds; followed by 600µL Buffer RA3 at 16,000 × g for 30 seconds; and a final wash with 250µL Buffer RA3 at 16,000 × g for 2 minutes. The RNA was then eluted with 40µL RNase-free H₂O and centrifuged at 16,000 × g for 1 minute. The RNA was then aliquoted and stored at -80°C for future use.

2.2.3.8 RNA quantification and qualification

RNA was quantified and quality analysed as in section 2.2.1.4 with one minor change. For cell culture experiments, RNA was equalised to 20ng/µL with RNase free water unless otherwise stated. In other experiments with a low yield of RNA, the samples were equalised to the lowest concentrated sample, which will be stated in the particular experiment.

2.2.3.9 cDNA synthesis and qRT-PCR

cDNA synthesis and qRT-PCR were carried out as in section 2.2.1.5 and 2.2.1.6. A list of rat primers used can be found in Table 2.5.

Gene target	Assay ID
<i>Gapdh</i>	Rn99999916_s1
<i>Gfap</i>	Rn00566603_m1
<i>Cd11b</i>	Rn00709342_m1
<i>Tubb3</i>	Rn01431594_m1
<i>Serpinf1</i> (Pedf)	Rn00709999_m1
<i>Pnpla2</i> (Pedf-R)	Rn01479968_g1

Table 2.5 Assay IDs for cell culture gene targets

2.2.3.10 Harvesting supernatants from in vitro cell cultures

For secreted protein analysis supernatants were harvested from cells after appropriate treatment. Media was removed from the wells and the cells were washed with PBS. Serum free media was then added to the wells for 24 hours before collecting the supernatant. Treatments were added to this serum free media as needed. Samples were collected on ice and placed in the centrifuge at $1000 \times g$ for 20 minutes to remove insoluble impurities and debris. The supernatant was then aliquoted and stored at -80°C for future use.

2.2.3.11 Pigment epithelial derived-factor protein analysis

Pigment Epithelial Derived-Factor (PEDF) secreted protein analysis was carried out using the ELISAGENIE rat sandwich PEDF ELISA kit. This kit arrived pre-coated with an anti-PEDF antibody. Samples were prepared as described in section 2.2.3.10 above. The assay was carried out according to the manufacturer's instructions.

A standard curve was prepared using a lyophilized standard diluted to 10 different concentrations ranging from 10ng/ml to 0 ng/ml. Samples were then diluted to a suitable concentration (a 1:10 dilution was found to be suitable for supernatant taken from astrocyte cultures). 100 μL of the standard curve and test samples were plated in duplicate

and left to incubate at 37°C for 90 minutes. The ELISA plate was then washed twice with wash buffer. Next, 100µL of Biotin-labelled antibody was added to each well. The plate was then incubated at 37°C for 1 hour. After the incubation period the wells were washed 3 times with wash buffer and 100µL of Horseradish Peroxidase (HRP) Streptavidin Conjugate (SABC) was added to the wells for 30 minutes at 37°C. Following SABC incubation the wells were washed 5 times with wash buffer, which was left in the wells for 2 minutes each time to remove all traces of the solutions. 90µL of pre-warmed TMB was added to each well and this was left to incubate at 37°C until the optimal colour change occurred (15 minutes). 50µL of stop solution was then added and the absorbance was read at 450nm.

A standard curve was then plotted using the relative absorbance results (the absorbance of each well – the absorbance of the zero standard well), and the PEDF concentration of the samples was determined using this curve. The intra-assay coefficient of variability (CV) was then calculated from the results for each ELISA to test variation within the plate run. This was calculated by dividing the mean of each sample duplicate by its standard deviation and then multiplied by 100. The CV for all samples were then averaged. As each experiment was conducted on one plate no inter-assay CV was calculated. Assay CVs <20% are deemed acceptable (USDHHS and Research., 2001, Kifude et al., 2008)

2.2.3.12 Statistical Analysis

All cell culture experiments were analysed using GraphPad Prism version 5 (GraphPad Prism, California, USA). Graphs were also created using GraphPad. A Kolmogorov–Smirnov normality test was carried out on data. For parametric analyses, student t test or

analysis of variance (ANOVA) was used where appropriate. When significance was detected with the ANOVA a Dunnett's post-hoc test was performed. For non-parametric data a non-parametric Mann-Whitney U test or a Kruskal-Wallis ANOVA was used. When significance was detected with the ANOVA a Dunn's post-hoc test was performed. A p value ≤ 0.05 was deemed statistically significant. All data are expressed as mean $\pm$ standard error of the mean (SEM).

Chapter 3.

Peripheral blood *E2F1* mRNA in depression and following electroconvulsive therapy

3.1 Introduction

Depression is a severe mental illness that affects more than 300 million people globally and is the leading cause of disability worldwide (WHO, 2017). Despite this, the pathogenesis of depression is not clear and 30% of patients do not respond to multiple courses of antidepressants (Rush et al., 2006). Electroconvulsive therapy (ECT) is the most effective treatment for severe, treatment-resistant, and sometimes life-threatening depression and offers approximately 60% of patients remission (UK ECT Group, 2003). Despite its efficacy, the mechanism of action of ECT is still not fully understood (Sienaert, 2014). ECT has been linked to changes in several molecular systems and pathways, one of which is a change in trophic factors, including brain-derived neurotrophic factor (BDNF), pigment-epithelial derived factor (PEDF) and vascular endothelial growth factor (VEGF) (Minelli et al., 2011, Rocha et al., 2016, Ryan et al., 2017).

MicroRNAs (miRNAs) have recently emerged as a potential regulator of such changes (Kolshus et al., 2014a). MiRNAs are short (21–23 bases), non-coding RNA molecules that regulate the expression of multiple genes and may be useful for identifying potential targets for the treatment of psychiatric disorders (Maffioletti et al., 2014). My lab recently performed a deep sequencing study of miRNA changes in peripheral whole blood following ECT and identified two miRNAs of interest(miR-126-3p and miR-106a-5p) (Kolshus et al., 2017). Both miRNAs were elevated in patients with psychotic depression at baseline and returned to healthy control levels following ECT. Gene target analysis using miRTarBase 6.0 (Chou et al., 2016) and TarBase 7.0 (Vlachos et al., 2015) was carried out to identify gene targets that were regulated by both of these miRNAs. To be included, a gene had to be validated using strong evidence (such as reporter assay or blotting) or validated with more than one form of weaker evidence (microarray, next

generation sequencing) in both databases. There was overlap with three genes when these criteria were applied: *VEGFA*, *E2F1* and *SIRT1*. *VEGFA* mRNA expression analysis has previously been carried out by my lab, with mRNA levels reported to be significantly higher in patients with psychotic depression in comparison to patients without psychotic depression and healthy controls. This increase was attenuated following a course of ECT (Kolshus et al., 2017). Therefore, studies on the role of E2F1 and SIRT1 in depression are of interest.

The E2F transcription factors were identified as proteins that bind to the promotor region of the adenovirus E2 gene (Kovesdi et al., 1986). Eight family members (E2F1–8) have been identified to date and play an important role in cell cycle regulation (DeGregori and Johnson, 2006). In the central nervous system (CNS), E2F1 has been implicated in both neurogenesis and apoptosis of neuronal cells. Staurosporine treatment, a model of cell apoptosis activation (Bertrand et al., 1994), has been reported to increase *E2F1* mRNA and E2F1 protein in a time-dependent manner in cortical neurons (Hou et al., 2000). In contrast, E2F1 has been linked to VEGF-induced neurogenesis in mouse cortical cultures (Zhu et al., 2003), and electroconvulsive shock (ECS) has been reported to activate the cyclin-dependent kinase 2-phosphorylated retinoblastoma protein (pRb)-E2F1 pathway in the rat frontal cortex, resulting in cell cycle progression (Kim et al., 2005). As ECS/ECT is associated with neurogenesis (in animal studies) and increases in human brain volumes (Wilkinson et al., 2017), dysfunctional E2F1 signalling could be linked to the pathogenesis of depression and mechanism of action of ECT. To my knowledge, no studies have examined *E2F1* mRNA levels in depressed patients or in response to ECT. The one study that investigated the E2F1 response to ECS only reported on the frontal cortex. Therefore, the effect of ECS on other brain regions would be of interest.

3.2 Aims and objectives

The aim of this study was to examine *E2F1* mRNA levels in whole blood samples collected from patients with depression pre- and post-treatment with ECT and age- and sex-matched healthy controls in order to gain an understanding of the effect of ECT. To my knowledge, no previous data is published on *E2F1* mRNA and depression. This work is a follow on of a previous published study, where two miRNAs that target *E2F1* were increased in patients with depression, and returned to control levels following a course of ECT (Kolshus et al., 2017). Therefore, it was hypothesised that *E2F1* mRNA levels would be decreased in patients with depression and return to control levels following a course of ECT. This is because of the report that miRNAs typically downregulate their target gene (Orang et al., 2014). The objectives of the study were to:

1. To analyse the levels of *E2F1* mRNA in whole blood collected from patients with depression and controls using RT-qPCR.
2. To assess differences in the levels of *E2F1* mRNA in patients pre-/post-ECT.
3. To assess differences in *E2F1* mRNA levels pre-/post-ECT in depression subtypes (psychotic/non-psychotic, unipolar/bipolar).
4. To determine if there are differences in the levels of *E2F1* mRNA in responders/non-responders and remitters/non-remitters.
5. To perform correlation analyses to determine if levels of *E2F1* are associated with mood scores.
6. To investigate the effects of acute and chronic ECS on *E2f1* mRNA levels in different brain regions of naïve rats.

3.3 Methods

3.3.1 Human participants

This study included participants recruited as part of the EFFECT-Dep Trial (Enhancing the Effectiveness of ECT in Severe Depression, ISRCTN23577151) that took place in St Patrick's Mental Health Services, Dublin (www.stpatricks.ie) (Semkovska et al., 2016). Full details of this trial, clinical assessments and healthy control recruitment can be found in sections 2.2.1.1 – 2.2.1.4.

3.3.2 Blood sampling and mRNA extraction

Full details for blood sampling and mRNA extraction are given in section 2.2.1.3 – 2.2.1.5. Fasting whole blood samples for mRNA analysis were taken using the PaxGene© system (Qiagen Inc., USA). Samples were left at room temperature for 2 hours, transferred to -20°C for 24 hours and then stored at -80°C until use. mRNA was extracted using the PaxGene© Blood RNA Kit according to the manufacturer's instructions (Qiagen Inc., USA). Quality analysis (to check RNA concentration and integrity) was carried out on the NanoDrop© 1000 UV-Vis Spectrophotometer (Fisher Scientific, UK). RNA samples with a 280/260 ratio of 1.8-2.2 were considered acceptable for analysis and RNA was equalised to 10ng/µl.

3.3.3 Multiplex quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). qRT-PCR was carried out on the StepOne Plus™ Real-Time PCR system (Applied Biosystems, UK) using TaqMan® (Applied Biosystems, UK) gene expression assays (*E2F1*; assay ID:Hs00153451_m1) with

glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; assay ID:Hs03929097_g1) as the endogenous control. Full details of cDNA synthesis, qRT-PCR and gene expression analysis can be found in sections 2.2.1.8 – 2.2.1.10.

3.3.4 Electroconvulsive stimulation study

Methods for the acute/chronic ECS/sham treatment study can be found in section 2.2.2.

3.3.5 Statistical analysis

Data were analysed using SPSS version 21.0 (IBM Corporation, NY, USA) and GraphPad Prism 5 (GraphPad Software, California, USA). Receiver Operating Characteristic curve analysis was carried out using MedCalc© (MedCalc Software, Belgium). Data were tested for normality using a Shapiro–Wilk test and log-transformed when necessary.

Baseline clinical and demographic factors are presented as means with standard deviation (SD), or number (%) per group. I adjusted for potential variance with possible confounding factors from the literature. These included the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and diabetes (Prabhanjan et al., 2016, Valle et al., 2013). Variables that were significantly different between patients and controls (current smoking status, BMI and education) were also included as covariates. When analysing the effect of ECT, I also corrected for baseline HAM-D24 scores, presence of psychosis, depression polarity and electrode placement as they may influence how a patients responds to ECT.

Data were analysed using independent t-tests, paired t-tests, Mann-Whitney U tests for non-parametric data, or a general linear model (GLM). Categorical data were tested using a chi-square test (χ^2) or Fisher's exact test (when sample size > 5). For

correlational analyses, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) for parametric data and Spearman's rank correlation coefficient ρ for non-parametric data.

Animal data were tested for normality using a Kolmogorov-Smirnov normality test. Parametric data were tested with a student t test and non-parametric data were tested using a Mann-Whitney U test. Values are represented as a fold-change (a ratio of how much the treatment group changed in comparison to the control group).

Statistical significance was set at $p < 0.05$. For exploratory correlation analyses a p value of <0.01 was used to control for multiple testing.

3.4 Results

3.4.1 Sample characteristics

For this study 88 out of 138 patient participants from the EFFECT-Dep trial had both pre- and post-ECT blood samples available for analysis. The demographic and clinical features of the patients were compared to the entire trial group to ensure my sample was comparable to the original complete real-world group of patients with depression. As shown in Table 3.1 there were no significant differences between groups.

	Patients included (n = 88)	EFFECT group (n = 140)	Statistical Test
Age (SD)	56.97 (13.6)	56.70 (14.8)	$t(224)=-1.35, p=0.32$
Sex, female n (%)	57 (64.8)	87 (62.1)	$\chi^2=0.07, p=0.45$
Smoker, yes n (%)	38 (43.2)	61 (43.6)	$\chi^2=0.23, p=0.50$
BMI (SD)	26.91 (5.2)	26.62 (4.8)	$U(228)=5995.00, p=0.73$
Education Completed, n (%)			
Primary	18 (20.5)	23 (16.5)	
Secondary	49 (55.7)	80 (57.6)	
Tertiary	21 (23.9)	36 (25.9)	$\chi^2=0.58, p=0.51$
Bipolar Depression, yes n (%)	19 (21.6)	32 (22.9)	$\chi^2=0.08, p=0.46$
Psychotic Subtype, yes n (%)	22 (25.0)	29 (20.7)	$\chi^2=0.48, p=0.30$
Treatment Resistant, yes n (%)	64 (72.7)	98 (70.0)	$\chi^2=0.78, p=0.45$
Previous ECT, yes n (%)	28 (31.8)	52 (37.1)	$\chi^2= 0.77, p=.23$
No. of Previous Episodes (SD)	5.29 (4.8)	5.33 (4.5)	$t(224)=0.06, p=0.70$
Pre-ECT HAM-D24 (SD)	30.26 (6.5)	29.91 (6.2)	$U(226)=5908.00, p=0.73$
Post-ECT HAM-D24 (SD)	10.57 (7.5)	11.79 (8.4)	$U(223)=5499.50, p=0.36$
No. ECT sessions (SD)	8.27 (2.3)	8.14 (2.4)	$t(224)=-0.81, p=0.42$
Electrode Placement, n (%)			
Unilateral	42 (47.7)	69 (50.0)	
Bilateral	46 (52.3)	69 (50.0)	$\chi^2=0.11, p=0.42$
Responder, yes n (%)	55 (62.5)	77 (55.0)	$\chi^2= 0.99, p=0.20$
Remitter, yes n (%)	46 (52.3)	61 (43.6)	$\chi^2=1.40 p=0.15$
Relapser (of those that remitted), yes n (%)	15 (32.6)	17 (27.9)	$\chi^2=1.48 p=0.30$
Medications, yes n (%)			
SSRI	23 (26.1)	28 (20.0)	$\chi^2=1.15, p=0.18$
SNRI	41 (46.6)	65 (46.4)	$\chi^2<0.00, p=0.55$
TCA	22 (25.0)	43 (30.7)	$\chi^2=0.90, p=0.21$
Mirtazapine	32 (36.4)	45 (32.1)	$\chi^2=0.41, p=0.31$
Bupropion	3 (3.4)	5 (3.6)	$\chi^2=0.01, p=0.63$
MAOI	10 (11.4)	15 (10.7)	$\chi^2=0.02, p=0.52$
Lithium	32 (36.4)	55 (39.3)	$\chi^2=0.10, p=0.43$
Antipsychotic	65 (73.9)	96 (68.6)	$\chi^2=0.70, p=0.25$
Valproate	5 (5.7)	9 (6.4)	$\chi^2=0.06, p=0.53$
Pregabalin	5 (5.7)	8 (5.7)	$\chi^2<0.00, p=0.62$
Benzodiazepine	47 (53.3)	76 (54.3)	$\chi^2=0.02, p=0.50$
Non-benzodiazepine Hypnotic	52 (59.1)	87 (62.1)	$\chi^2=0.24, p=0.36$

Table 3.1 Demographic and clinical details of patients used for this study compared to the whole EFFECT-Dep patient sample

Abbreviations: BMI, body mass index; NSAID, Nonsteroidal anti-inflammatory drugs; HAM-D24, Hamilton Depression Rating Scale, 24-item; ECT, Electroconvulsive therapy; SSRI, Selective serotonin reuptake inhibitor; SNRI, Serotonin and noradrenaline reuptake inhibitor; TCA, Tricyclic antidepressants; MAOI, Monoamine Oxidase Inhibitor; U, standardised Mann-Whitney test. Data are presented in means (SD) or n (%).

Samples were also available for 79 healthy controls that were balanced with the patient group for age and sex. The patient group had more smokers ($p < 0.01$), a larger body mass index (BMI) ($p < 0.01$), lower educational attainment ($p < 0.001$) and higher NSAID use ($p < 0.001$) than the control group. Therefore, these measures were included as covariates in all analyses for this group. Demographic and clinical characteristics are presented in Table 3.2.

	Patient (n = 88)	Control (n = 79)	Statistical Test
Age	56.97 (13.6)	54.42 (10.9)	$t(165)=-1.33, p=0.19$
Sex, female n (%)	57 (64.8)	51 (64.6)	$\chi^2<0.01, p=0.53$
Smoker, yes n (%)	38 (43.2)	16 (20.3)	$\chi^2=10.00, p<0.01$
BMI (SD)	26.91 (5.2)	24.91 (4.0)	$U=2592.00, p=0.01$
Education Completed, n (%)			
Primary	18 (20.5)	4 (5.1)	
Secondary	49 (55.7)	14 (17.7)	
Tertiary	21 (23.9)	61 (77.2)	$\chi^2=51.96, p<0.000$
Diabetes, n (%)	6 (6.8)	3 (3.8)	$p=0.51$
NSAID use	22 (25)	2 (2.5)	$p<0.001$
HAM-D24 Pre-ECT	30.26 (6.5)	3.34 (2.4)	$U<0.001, p<0.001$

Table 3.2 Demographic and clinical details of patients and controls for *E2F1* mRNA analysis.

Abbreviations: BMI, body mass index; NSAID, Nonsteroidal anti-inflammatory drugs; HAM-D24, Hamilton Depression Rating Scale, 24-item; ECT, Electroconvulsive therapy; U, standardised Mann-Whitney test. Data are presented in means (SD) or n (%).

3.4.2 *E2F1* mRNA levels in depression and following electroconvulsive therapy

RQ values for *E2F1* mRNA were not normally distributed. Following log₁₀ transformation these values were normal and log values for *E2F1* were used for analysis. Before log transformation, healthy controls had a mean RQ value of 1.32 (SD: 0.63). Patients had a mean RQ value of 1.01 (SD: 0.41) prior to ECT and this changed to 1.10 (SD: 0.49) following a course of ECT.

Baseline (pre-ECT) peripheral *E2F1* mRNA levels were significantly lower in patients ($n = 88$) with depression compared to healthy controls ($n = 79$) ($p < 0.001$; Figure 3.1). Adjusting for relevant covariates (NSAID use, diabetes, smoking, BMI, and education) using a GLM altered this slightly ($F(1,157) = 6.94$, $p = 0.009$), with levels still lower in patients and no covariates having a significant effect on *E2F1* levels. Table 3.3 lists the effects of the covariates on *E2F1* mRNA levels.

A paired t-test revealed that *E2F1* mRNA levels were not altered by ECT ($p = 0.42$; Figure 3.1). Correcting for covariates (NSAID use, diabetes, smoking, BMI, education, baseline HAM-D24 score, electrode placement, presence of psychosis, and polarity of depression) did not change this ($F(1,81) = 0.02$, $p = 0.89$).

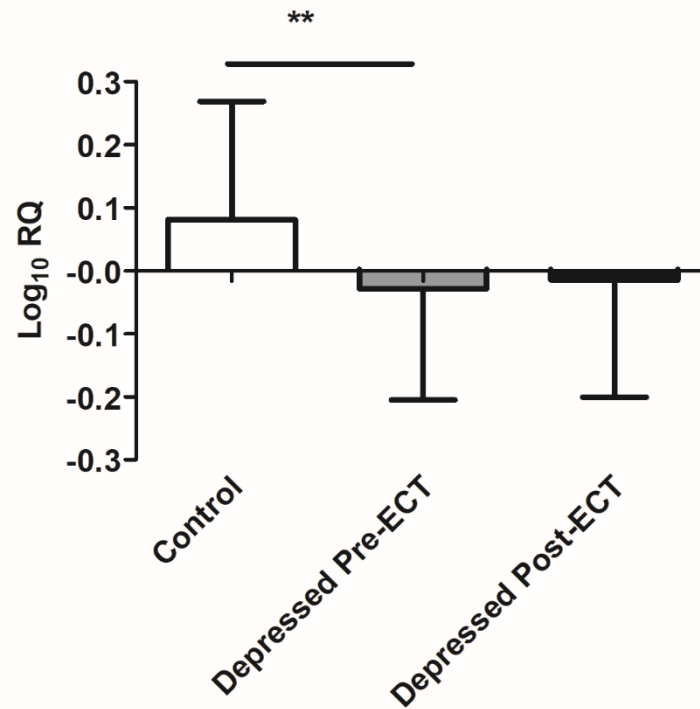


Figure 3.1 *E2F1* mRNA levels in controls and patients pre-/post-ECT

Log transformed RQ values of *E2F1* mRNA in peripheral whole blood as measured by quantitative real-time chain reaction and a general linear model. Data are presented as unadjusted means $\pm$ SD. ** $p = 0.009$, healthy controls ($n = 79$) versus depressed pre-ECT patients ($n = 88$) as reported by a general linear model. Abbreviations: RQ, relative quantification; ECT, electroconvulsive therapy.

Covariate	df	F	p-value
Patient Group	1,164	6.94	<0.01
NSAID use	1,164	1.08	0.30
Smoking	1,164	1.20	0.28
Diabetes	1, 164	1.11	0.29
BMI	1,164	0.33	0.57
Level of Education	1,164	0.98	0.38

Table 3.3 Effects of relevant covariates on *E2F1* mRNA expression.

Only the patient group (control vs. depressed patient) had a significant effect on *E2F1* mRNA levels as measured by a general linear model. Abbreviations: df, degrees of freedom; F, F value (variance group means); NSAID, Nonsteroidal anti-inflammatory drugs; BMI, body mass index.

3.4.3 Receiver operating characteristic curve analysis

Next, a receiver operating characteristic (ROC) curve was generated to determine if *E2F1* mRNA levels at baseline could be used to distinguish patients with depression from controls. A ROC curve plots the true positive results against the true negative results and the area under the curve (AUC) is used to determine the accuracy of a diagnostic marker (Zweig and Campbell, 1993). An AUC value of 1 indicates an excellent diagnostic test where as a value of 0.5 indicates a poor test. The AUC value of *E2F1* was 0.663, with a sensitivity of 69.3% and specificity of 58.2%; see Figure 3.2.

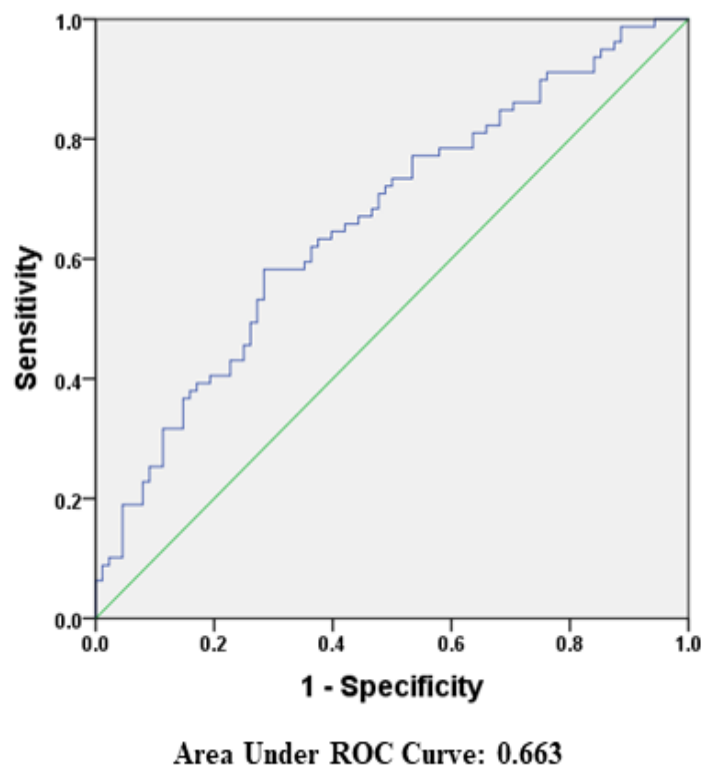


Figure 3.2 ROC of *E2F1* mRNA levels and patient/control classification

ROC analysis of depressed patients ($n = 88$) and healthy controls ($n = 79$).

3.4.4 The effects of different depression subtypes on *E2F1* mRNA levels

Subgroup analysis was carried out to determine if *E2F1* mRNA levels differed between different subsets of depression, of if levels differed between ECT responders/remitters and non-responders/remitters. These analyses were carried out to investigate if *E2F1* mRNA levels could be used in a clinical setting to distinguish between subtypes of depression or predict response to ECT. Results for the individual subtypes will be reported in the following sections with a summary of all the results found in Table 3.5 at the end of section 3.4.4.

3.4.4.1 *E2F1* mRNA levels and the presence of psychosis

Since the initial miRNA findings from my lab group were in the psychotic depression subtype (Kolshus et al., 2017), the effect of psychosis on *E2F1* levels was assessed. Table 3.4 summaries the demographic and clinical features for the patients with/without the presence of psychotic features.

	Non-Psychotic Depressed (n = 66)	Psychotic Depressed (n = 22)	Statistical Test
Age	55.41 (14.0)	61.64 (11.5)	$t(86)=-1.89, p=0.06$
Sex, female n (%)	44 (66.7)	13 (59.1)	$\chi^2=0.42, p=0.35$
Smoker, yes n (%)	30 (45.5)	8 (36.4)	$\chi^2=0.556, p=0.31$
BMI (SD)	27.21 (5.3)	26.00 (4.8)	$U=645.00, p=0.44$
Education Completed, n (%)			
Primary	11 (16.7)	7 (31.8)	
Secondary	38 (57.6)	11 (50.0)	
Tertiary	17 (25.8)	4 (18.2)	$p=0.34$
Diabetes, n (%)	5 (7.6)	1 (4.5)	$p=0.53$
NSAID use	17 (25.3)	5 (22.7)	$\chi^2=0.08, p=0.51$
Bipolar Depression, n (%)	14 (21.2)	5 (22.7)	$\chi^2=0.02, p=0.55$
Treatment Resistant, yes n (%)	57 (86.4)	7 (31.8)	$\chi^2=24.76, p<0.000$
Previous ECT, yes n (%)	20 (30.3)	8 (36.4)	$\chi^2=0.24, p=0.41$
No. of Previous Episodes (SD)	5.67 (5.2)	4.18 (3.0)	$t(86)=1.26, p=0.21$
HAM-D24 Pre-ECT	28.94 (5.1)	34.32 (8.4)	$U=453.00, p<0.01$
HAM-D24 Post-ECT	10.9 (7.3)	9.6 (8.2)	$U=615.50, p=0.33$
No. ECT sessions	8.35 (2.4)	8.05 (2.2)	$t(86)=0.53, p=0.60$
Electrode Placement			
Unilateral	30 (45.5)	12 (54.5)	
Bilateral	36 (54.5)	10 (45.5)	$\chi^2=0.55, p=0.31$
Responder, n (%)	39 (59.1)	16 (72.7)	$\chi^2=1.31, p=0.18$
Remitter, n (%)	34 (51.1)	12 (54.5)	$\chi^2=0.61, p=0.50$
Medications, n (%)			
SSRI	15 (22.7)	8 (36.4)	$\chi^2=1.49, p=0.17$
SNRI	30 (45.5)	11 (50.0)	$\chi^2=0.20, p=0.47$
TCA	20 (30.3)	2 (9.1)	$p=0.04$
Mirtazapine	25 (37.9)	7 (31.8)	$\chi^2=0.31, p=0.39$
Bupropion	3 (4.5)	0 (0.0)	$p=0.41$
MAOI	5 (7.6)	5 (22.7)	$\chi^2=3.62, p=0.07$
Lithium	22 (33.3)	10 (45.5)	$\chi^2=0.95, p=0.23$
Antipsychotic	49 (74.2)	16 (72.7)	$\chi^2=0.06, p=0.51$
Valproate	3 (4.5)	2 (9.1)	$p=0.37$
Pregabalin	5 (7.6)	0 (0.0)	$p=0.22$
Benzodiazepin	36 (54.5)	11 (50.0)	$\chi^2=0.19, p=0.42$
Non-benzodiazepine	39 (59.1)	13 (59.1)	$\chi^2=0.01, p=0.57$
Hypnotic			

Table 3.4 Demographic and clinical details of patients with/without the presence psychotic features

Abbreviations: BMI, body mass index; NSAID, Nonsteroidal anti-inflammatory drugs; HAM-D24, Hamilton Depression Rating Scale, 24-item; ECT, Electroconvulsive therapy; SSRI, Selective serotonin reuptake inhibitor; SNRI, Serotonin and noradrenaline reuptake inhibitor; TCA, Tricyclic antidepressants; MAOI, Monoamine Oxidase Inhibitor; U, standardised Mann-Whitney test. Data are presented in means (SD) or n (%).

Patients with depression in the absence psychotic features had a significantly higher percentage of patients with treatment resistance in comparison to patients with psychotic features. There were no other significant differences between the groups. Treatment resistance had no effect on *E2F1* mRNA levels ($F(1,86) = 0.56, p = 0.46$). There was no difference between non-psychotic ($n = 66$) and psychotic ($n = 22$) groups at baseline (as measured by a student t test; see Figure 3.3). A general linear model was then used to adjust for covariates (NSAID use, diabetes, smoking, BMI, education, baseline HAM-D24 score, and polarity of depression) but this did not alter the result, see Figure 3.3. There were no differences between psychotic and non-psychotic groups for *E2F1* levels pre-/post ECT.

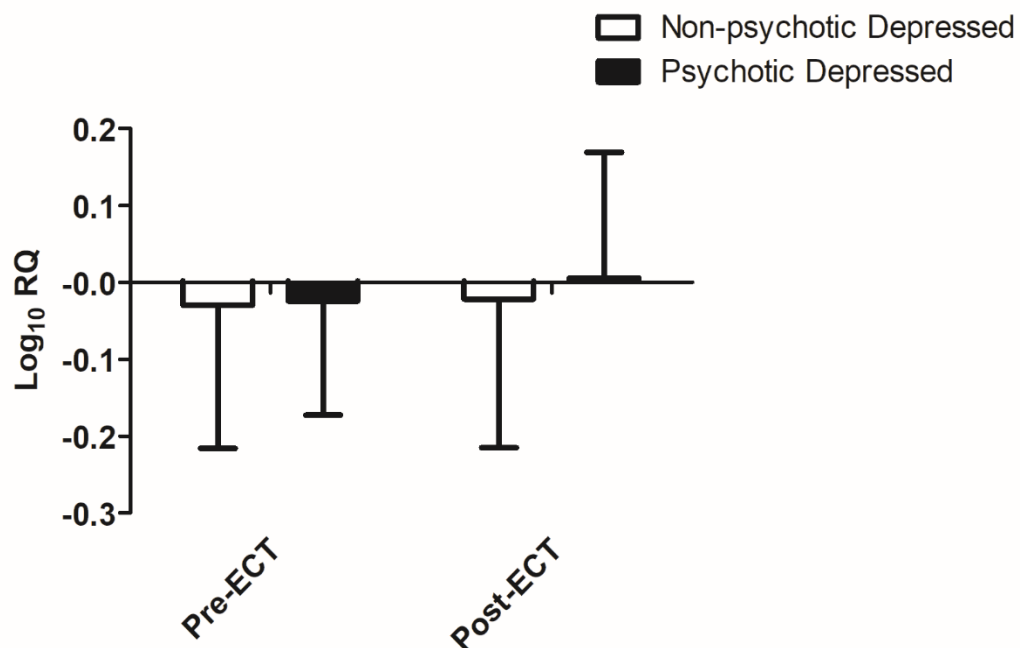


Figure 3.3 The effect of psychosis on *E2F1* mRNA levels

There were no differences in *E2F1* mRNA levels between patients with non-psychotic depression ($n = 66$) and patients with psychotic depression ($n = 22$). This did not change following ECT. Data are presented as unadjusted means $\pm$ SD.

3.4.4.2 *E2F1* mRNA levels and depression polarity

As whole blood peripheral gene levels might vary between bipolar and unipolar depression, the effect of polarity on *E2F1* mRNA levels was analysed (Figure 3.4). There were no differences between patients with unipolar ($n = 68$) or bipolar ($n = 19$) depression at baseline and there was no time $\times$ polarity interaction as measured by a GLM and repeated measures AVOVA respectively.

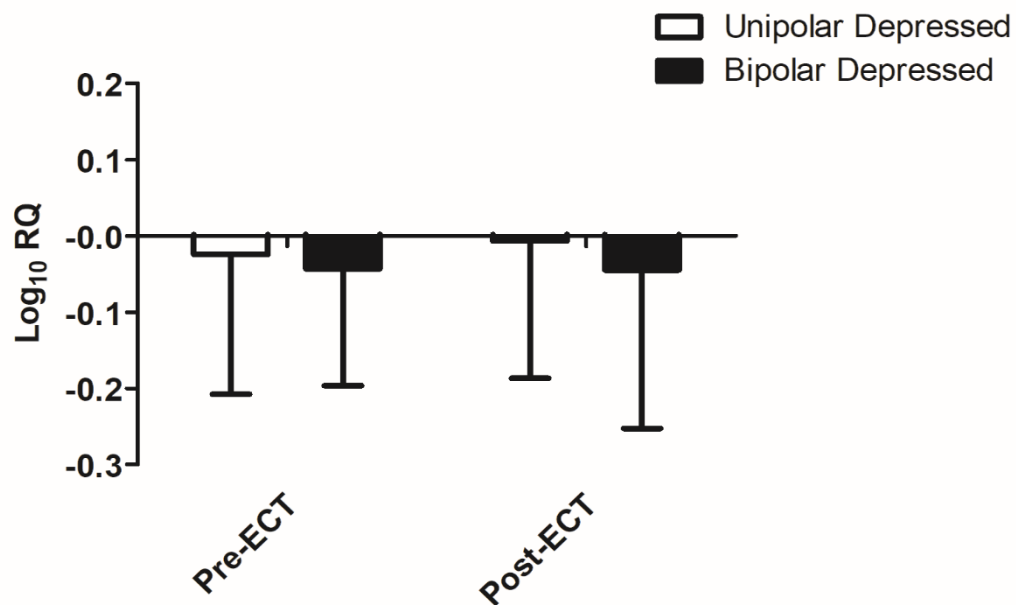


Figure 3.4 The effect of polarity on *E2F1* mRNA levels

No differences in *E2F1* mRNA levels between patients with unipolar ($n = 68$) and patients with bipolar depression ($n = 19$) were observed. This did not change with ECT. Data are presented as unadjusted means $\pm$ SD.

3.4.4.3 *E2F1* mRNA levels and response or remission

Next, to determine if *E2F1* mRNA levels could help predict if a patient would respond to ECT or enter remission, exploratory analysis was performed to determine whether *E2F1* mRNA levels were different at baseline between ECT responders ($n = 55$) and non-responders ($n = 33$), and also explored if levels changed after completing a course of

ECT. Differences in *E2F1* levels in ECT remitters ($n = 46$) and non-remitters ($n = 42$; Figure 3.5) were also assessed.

As shown in Figure 3.5, there were no differences in *E2F1* mRNA levels before or after ECT between responders and non-responders, or between remitters and non-remitters (as measured respectively by a student t-test and a repeated measured ANOVA). Adjusting for covariates (NSAID use, diabetes, smoking, BMI, education, baseline HAM-D24 score, electrode placement, presence of psychosis, and polarity of depression) using a GLM did not change these findings.

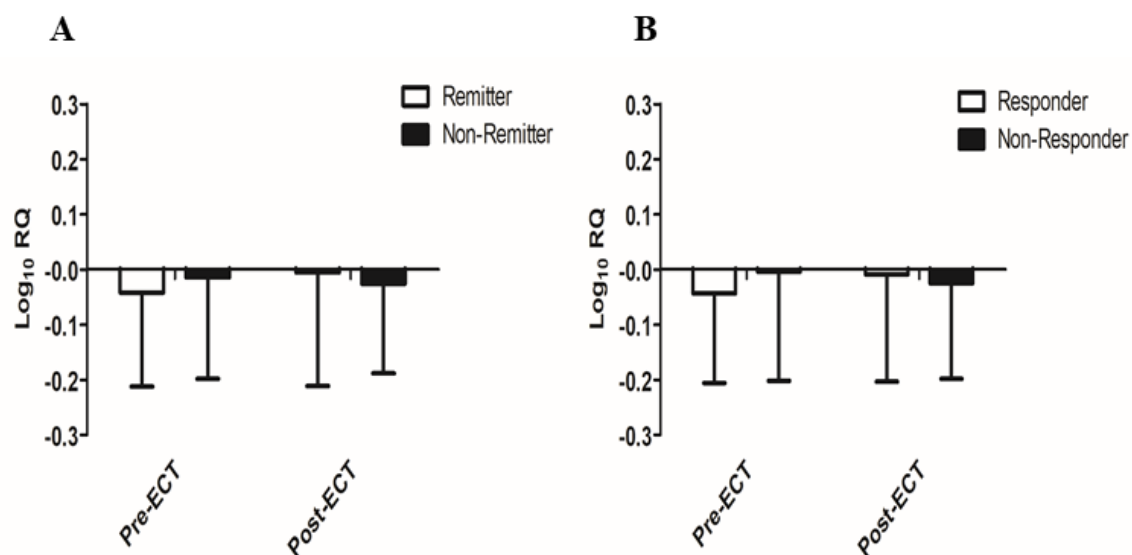


Figure 3.5 *E2F1* mRNA levels and response/remission status

There were no differences in *E2F1* mRNA levels between (A) ECT responders ($n = 55$) and non responders ($n = 33$), or (B) between patients who attained remission ($n = 46$) and those who did not ($n = 42$). This did not change with ECT. Data are presented as unadjusted means $\pm$ SD.

Subgroup	Depressed Pre-ECT	Depressed Post-ECT	Test	Statistics	Adjusted Statistics
Psychosis			Group Baseline	F(1,86) = 0.01, $p=0.91$	F(1,78)=0.10, $p=0.78$
Yes ($n = 22$)	-0.0253 (0.15)	0.0052 (0.16)	Time	F(1,86)=0.92, $p=0.34$	F(1,77)=0.26, $p=0.61$
No ($n = 6$)	-0.0303 (0.19)	-0.0210 (0.20)	Group x Time	F(1,86)=0.16, $p=0.70$	F(1,77)=0.16, $p=0.69$
Polarity			Group Baseline	F(1,86)=0.15, $p=0.70$	F(1,78)=0.22, $p=0.64$
Bipolar ($n = 19$)	-0.0430 (0.15)	-0.0433 (0.21)	Time	F(1,86)=0.18, $p=0.68$	F(1,77)=0.26, $p=0.61$
Unipolar ($n = 68$)	-0.0252 (0.18)	-0.0065 (0.18)	Group x Time	F(1,86)=0.43, $p=0.52$	F(1,77)=0.03, $p=0.85$
Responder			Group Baseline	F(1,86)=1.10, $p=0.32$	F(1,77)=1.73, $p=0.19$
Yes ($n = 55$)	-0.0437 (0.16)	-0.0082 (0.20)	Time	F(1,86)=0.17, $p=0.68$	F(1,76)=0.29, $p=0.59$
No ($n = 33$)	-0.0046 (0.20)	-0.0249 (0.17)	Group x Time	F(1,86)=0.10, $p=0.75$	F(1,76)=0.55, $p=0.46$
Remission			Group Baseline	F(1,86)=0.56, $p=0.46$	F(1,77)=0.88, $p=0.36$
Yes ($n = 46$)	-0.0425 (0.17)	-0.0047 (0.21)	Time	F(1,86)=0.57, $p=0.45$	F(1,76)=0.43, $p=0.51$
No ($n = 41$)	-0.0143 (0.18)	-0.0251 (0.16)	Group x Time	F(1,86)=0.01, $p=0.91$	F(1,76)=0.13, $p=0.72$

Table 3.5 Subgroup analysis of *E2F1* mRNA levels at baseline and after a course of ECT

Abbreviations: ECT, electroconvulsive therapy. Data are represented as unadjusted mean log₁₀ E2F1 relative quantification levels (SD).

3.4.5 Correlation analysis

Next, *E2F1* mRNA levels were correlated with mood scores (using the HAM-D24) to analysed if they could predict depression severity at baseline, or the response to ECT. This was carried out in the group as a whole as well in the different subgroups. The following sections (3.4.5.1 – 3.4.5.4) give the full result for each group with table 3.6 at the end of this section summarising the findings in the different subgroups.

3.4.5.1 Associations between mood ratings and *E2F1* mRNA levels in the patient group as a whole

First, the relationship between *E2F1* mRNA levels and HAM-D24 mood ratings was assessed (Figure 3.6). There was no statistically significant correlation between baseline *E2F1* levels and baseline HAM-D24 scores ($\rho = -0.07, p = 0.52$), between baseline *E2F1* levels and the change in HAM-D24 score pre-/post-ECT ($r = 0.17, p = 0.12$), or between changes in *E2F1* levels and HAM-D24 scores pre-/post-ECT ($\rho = -0.22, p = 0.04$).

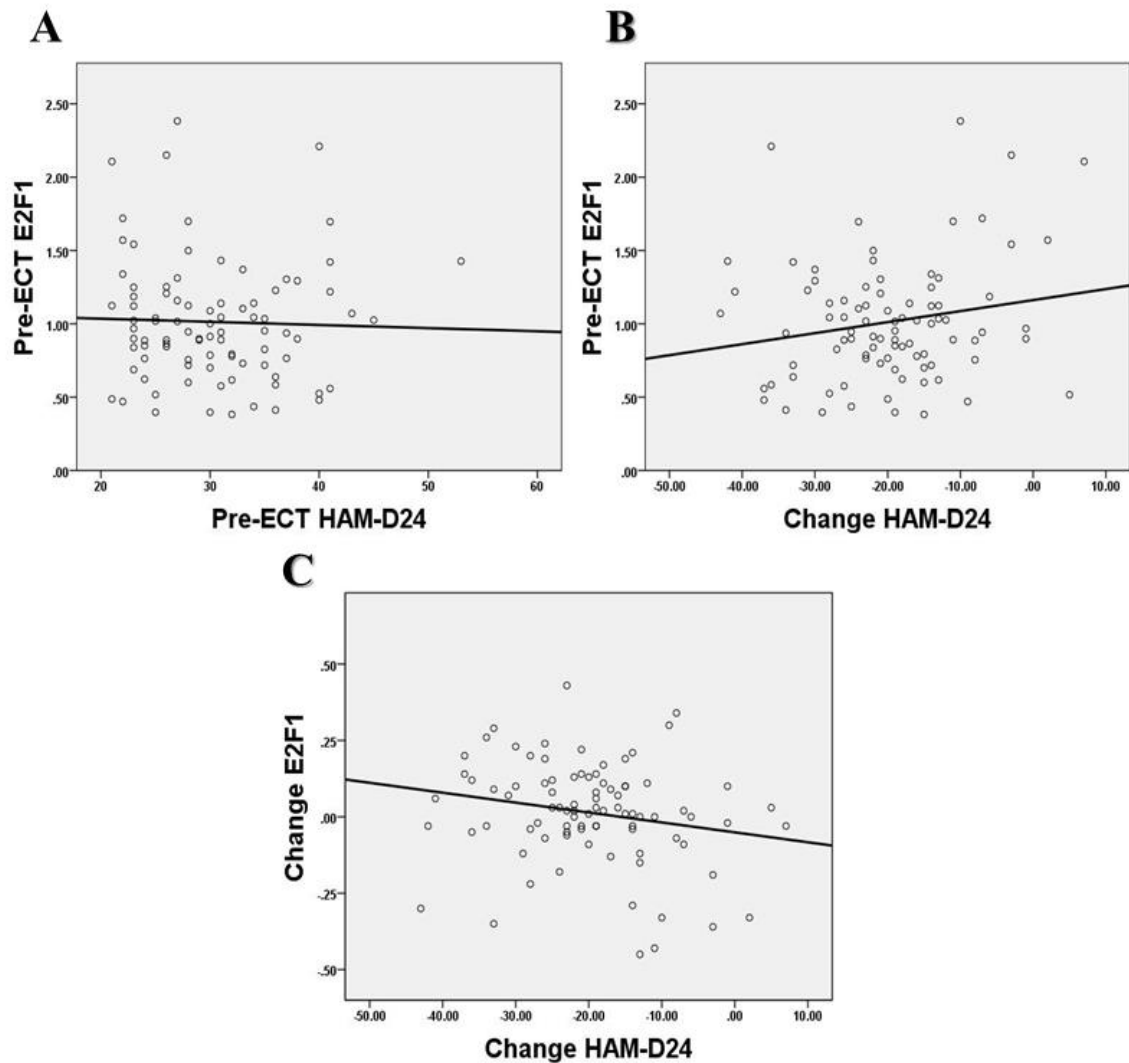


Figure 3.6 Relationship between *E2F1* mRNA levels and mood ratings

There was no statistically significant correlation between baseline (A) *E2F1* mRNA levels and baseline HAM-D24 scores ($\rho = -0.07$, $p = 0.52$), between (B) baseline *E2F1* levels and the change in HAM-D24 score pre-/post-ECT ($r = 0.17$, $p = 0.12$), or (C) between changes in *E2F1* levels and HAM-D24 scores pre-/post-ECT ($\rho = -0.22$, $p = 0.04$).

3.4.5.2 Associations between mood ratings, *E2F1* mRNA levels and the presence of psychosis

The presence of psychosis had no effect on the association between mood scores and *E2F1* mRNA levels. This indicates baseline *E2F1* mRNA levels cannot predict depression severity before ECT ($r = 0.27$, $p = 0.27$; figure 3.7), cannot predict symptom

improvement with ECT ($r = 0.27$, $p = 0.27$), and a change in *E2F1* mRNA pre-/post-ECT cannot predict symptom improvement ($\rho = -0.05$, $p = 0.83$).

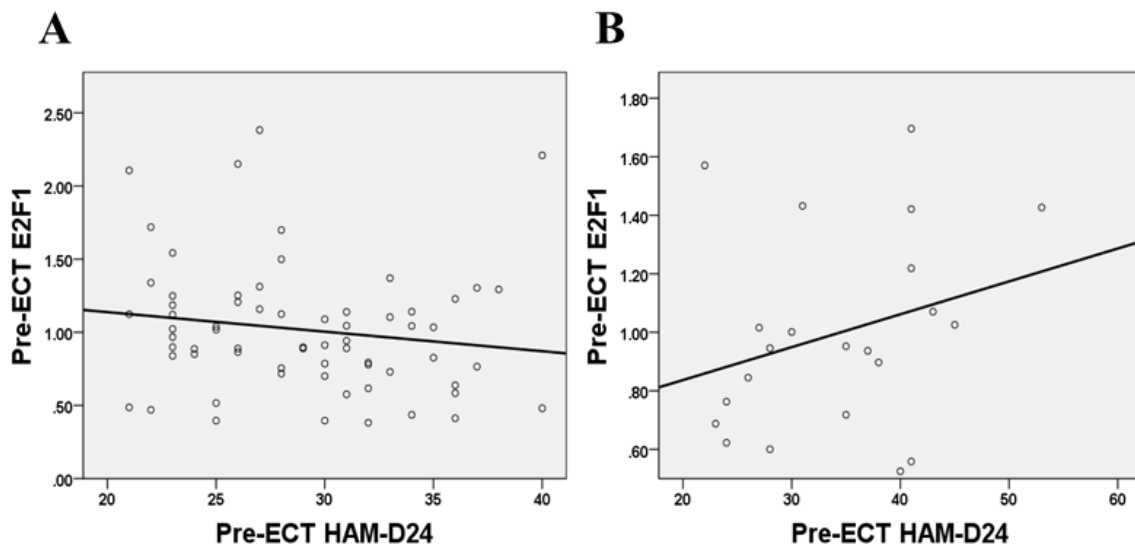


Figure 3.7 Relationship between *E2F1* mRNA levels and mood ratings and psychosis pre-ECT

There was no statistically significant correlation between baseline *E2F1* mRNA levels and baseline HAM-D24 scores in patients with (A) non-psychotic depression ($n = 66$, $\rho = -0.19$, $p = 0.12$) or (B) with the presence of psychosis ($n = 22$).

3.4.5.3 Associations between mood ratings, *E2F1* mRNA levels and polarity of depression

Correlation analysis indicated there was a negative relationship between a change in *E2F1* mRNA levels and a change in mood scores ($r = -0.24$, $p = 0.05$) in patients with unipolar depression as measured by the HAM-D24 (Figure 3.8). However, this significance did not survive correcting for multiple comparisons as the p value was > 0.01 . There were no other associations between mood scores and *E2F1* mRNA in either group.

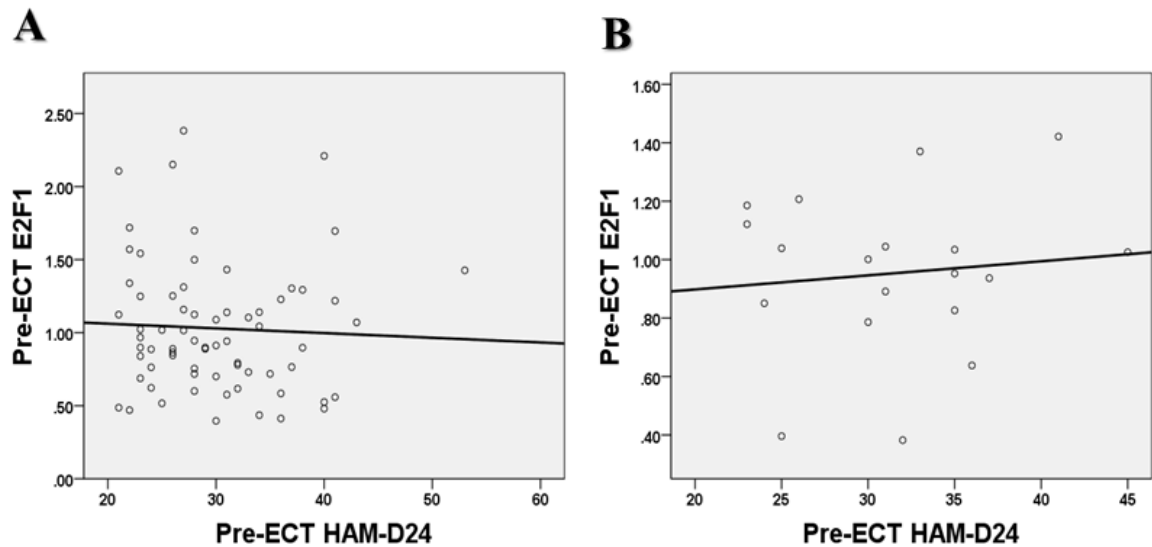


Figure 3.8 Relationship between *E2F1* mRNA levels and mood ratings and polarity of depression

There was a trend for a correlation between a change in *E2F1* mRNA levels with ECT and a change in HAM-D24 scores in (A) unipolar depressed patients ($n = 68$, $r = -0.24$, $p = 0.05$), there were no correlations in patients with (B) bipolar depression ($n = 19$).

3.4.5.4 Associations between mood ratings, *E2F1* mRNA levels and response/remission status

Associations between mood scores and *E2F1* mRNA levels were also assessed with response/remission status (Figure 3.9). There was a trend for a negative correlation between a change in HAM-D24 scores and a change in *E2F1* levels over time in patients who did not respond to ECT ($\rho = -0.40$, $p = 0.02$) and those who did not obtain remission ($n = 41$; $\rho = -0.31$, $p = 0.05$). Neither of these findings survived correction for multiple comparisons, indicating no relationship between mood scores, *E2F1* mRNA levels and response/remission status.

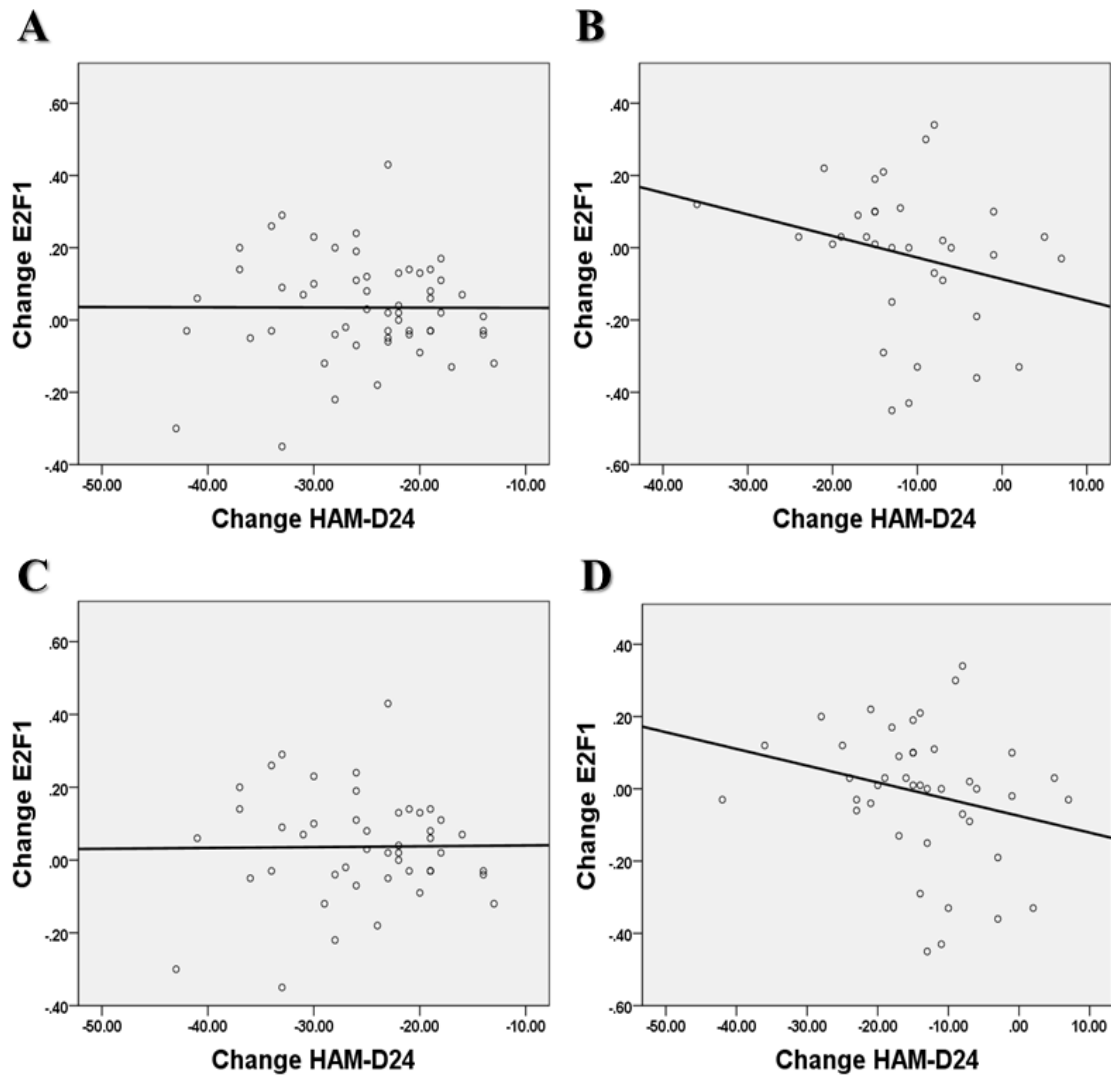


Figure 3.9 Relationship between *E2F1* mRNA levels and mood ratings with response/remission status

There was no correlation between a change in *E2F1* levels and change in HAM-D24 score in patients who (A) responded to ECT ($n = 55$), however there was a trend for a negative correlation in those who (C) did not respond to ECT ($n = 33$; $\rho = -0.40$, $p = 0.02$). Similar to this there were no correlations between *E2F1* levels in patients who (C) did attain remission ($n = 46$) but there was a trend for a negative correlation in those who (D) did not attain remission ($n = 41$; $\rho = -0.31$, $p = 0.05$).

Subgroup	Pre-ECT <i>E2F1</i> and HAM-D24	Pre-ECT <i>E2F1</i> and Δ HAM-D24	Δ <i>E2F1</i> and Δ HAM-D24
Psychosis			
Yes (<i>n</i> = 22)	$r = 0.27, p=0.27$	$r = 0.01, p=0.96$	$\rho = -0.05, p=0.83$
No (<i>n</i> = 66)	$\rho = -0.19, p=0.12$	$r = 0.23, p=0.06$	$r = -0.20, p=0.12$
Polarity			
Bipolar (<i>n</i> = 19)	$\rho = -0.06, p=0.80$	$\rho = 0.03, p=0.90$	$r = 0.02, p=0.93$
Unipolar (<i>n</i> = 68)	$\rho = -0.10, p=0.40$	$r = 0.21, p=0.09$	$r = -0.24, p=0.05$
Responder			
Yes (<i>n</i> = 55)	$\rho = 0.06, p=0.66$	$\rho = 0.01, p=0.96$	$\rho = -0.10, p=0.45$
No (<i>n</i> = 33)	$r = -0.17, p=0.35$	$r = 0.29, p=0.10$	$\rho = -0.40, p=0.02$
Remission			
Yes (<i>n</i> = 46)	$\rho = -0.09, p=0.57$	$\rho = 0.02, p=0.91$	$r = 0.01, p=0.94$
No (<i>n</i> = 41)	$\rho = -0.23, p=0.14$	$r = 0.23, p=0.15$	$\rho = -0.31, p=0.05$

Table 3.6 Clinical subgroup correlations between *E2F1* levels and HAM-D-24 scores
Abbreviations: HAM-D24, Hamilton Depression Rating Scale, 24-item; Δ *E2F1*, change in *E2F1* pre/post ECT; Δ HAM-D24, change in HAM-D24 score pre/post ECT

3.4.6 The effect of ECS on *E2f1* mRNA levels in the rat brain and peripheral whole blood

To determine if an effect of ECS in the brain might translate to the periphery, quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate the effect of acute (single) and chronic (10 sessions) ECS on rat *E2f1* mRNA levels in the hippocampal formation, dentate gyrus, frontal cortex, cerebellum and peripheral whole blood. Neither acute nor chronic ECS had an effect on *E2f1* mRNA in the hippocampal formation, frontal cortex, cerebellum and peripheral whole blood. In the dentate gyrus, chronic ECS had no effect on *E2f1* mRNA levels. However, acute ECS significantly reduced *E2f1* mRNA levels in the dentate gyrus. Full data can be found in Table 3.7 and Figure 3.10 below.

		Acute		Chronic	
		Control	ECS	Control	ECS
Peripheral whole blood	<i>n</i>	8	8	5	5
	mean	1.00	0.93	1.00	1.24
	SD	0.20	0.11	0.27	0.55
	SEM	0.07	0.04	0.12	0.23
	<i>p</i> value		0.40		0.93
Hippocampal Formation	<i>n</i>	8	8	8	7
	mean	1.00	0.95	1.00	0.94
	SD	0.11	0.09	0.13	0.10
	SEM	0.04	0.03	0.04	0.04
	<i>p</i> value		0.31		0.69
Dentate Gyrus	<i>n</i>	8	8	8	7
	mean	1.00	0.72	1.00	0.82
	SD	0.11	0.18	0.25	0.15
	SEM	0.04	0.06	0.09	0.06
	<i>p</i> value		<0.01**		0.13
Frontal Cortex	<i>n</i>	8	8	8	7
	mean	1.00	1.00	1.00	0.97
	SD	0.16	0.12	0.16	0.14
	SEM	0.06	0.05	0.05	0.05
	<i>p</i> value		0.97		0.74
Cerebellum	<i>n</i>	8	8	8	7
	mean	1.00	0.93	1.00	0.95
	SD	0.29	0.27	0.29	0.37
	SEM	0.10	0.10	0.10	0.14
	<i>p</i> value		0.72		0.78

Table 3.7 Data for the effect of ECS on *E2f1* mRNA levels in rat brain and peripheral blood

Abbreviations: ECS, electroconvulsive stimulation; SD, standard deviation; SEM, standard error of the mean.

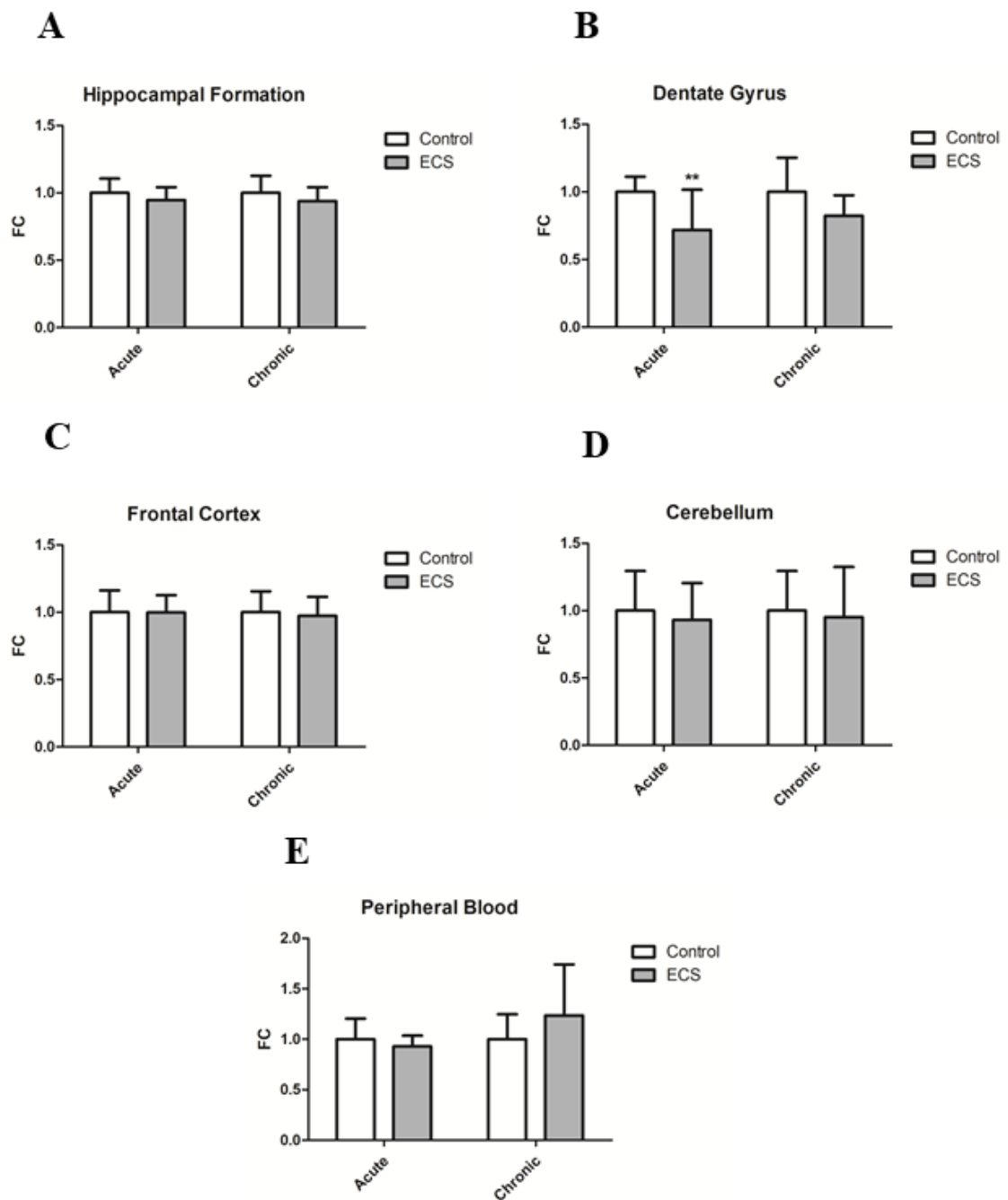


Figure 3.10 The effect of ECS on *E2f1* mRNA animals in the rat brain and peripheral whole blood

All data is presented as mean $\pm$ SD, $n = 5-8$. ** represents a $p < 0.01$ as measured by a student t-test. Abbreviations: ECS, electroconvulsive stimulation; FC, fold change.

3.5 Discussion

3.5.1 Results summary

To my knowledge, this is the first study to examine peripheral blood *E2F1* mRNA levels in a relatively large cohort of a well characterised group of patients with depression. *E2F1* mRNA levels were found to be significantly lower in patients with depression in comparison to healthy controls. There was no change in *E2F1* mRNA levels in patients following ECT. There were no correlations between *E2F1* levels and mood ratings using the HAM-D24. Based on previous findings (Kolshus et al., 2017), the effect of psychosis on *E2F1* levels was also investigated. However, there were no differences between patients with and without psychosis. Polarity of depression also had no effect on *E2F1* levels. There was a trend for a negative correlation between a change in HAM-D24 scores and a change in *E2F1* levels over time in patients with unipolar depression. There was also a trend for a negative correlation between a change in HAM-D24 scores and a change in *E2F1* levels over time in patients who did not respond to ECT. In the rat brain, acute ECS significantly reduced *E2f1* mRNA levels in the dentate gyrus.

3.5.2 Lower *E2F1* mRNA and depression

Patients with depression had significantly lower levels of peripheral whole blood *E2F1* mRNA in comparison to controls. This is what was expected from this study as miRNAs typically downregulate their target genes (Orang et al., 2014) and miR-106a has been reported to be inversely correlated with levels of *E2F1* mRNA (Diaz et al., 2008). However, ROC analysis reported an AUC of 0.667. This score indicates that *E2F1* is not a very good marker for depression, at least when it is used alone. Combining *E2F1* with related markers (for example other cell cycle proteins could be dysregulated in depression

also) may give a stronger diagnostic result. This result still gives some evidence for a role of E2F1 in depression.

It is unclear how lower *E2F1* mRNA levels are linked to the pathophysiology of depression as this has not been investigated before. However, E2F1 has been linked to peripheral systems and proteins that have been shown to be dysregulated in depression. For example, E2F1 has been shown to interact with several cytokines, e.g. interferon- γ has been reported to reduce E2F1 levels (Reimer et al., 2006). Therefore, the inflamed status that has been reported in patients with depression could be affecting *E2F1* mRNA levels (Kohler et al., 2017). Growth factors, including VEGF, BDNF and PEDF, have also been linked to E2F1 (Ertosun et al., 2016). VEGF is an angiogenic mitogen with roles in vasculogenesis stimulation and neurogenesis that has been implicated in depression (Sharma et al., 2016). Patients with depression have higher levels of peripheral blood *VEGF* mRNA in comparison to healthy controls (Kolshus et al., 2017, Berent et al., 2014, Shibata et al., 2013). E2F1 knock-out mice have increased VEGF levels in peripheral cells (Wu et al., 2014, Wang et al., 2016). Although it is unclear what role increased peripheral VEGF has for depression, increased brain angiogenesis has been linked to recovery from depressive episodes (Yamada, 2016). Reduced E2F1 could therefore have a role in this via upregulation of VEGF. As mentioned in the introduction, E2F1 is increased in response to VEGF treatment to induce proliferation of rat cortical neuronal progenitor cells (Zhu et al., 2003).

In contrast, other growth factors have been reported to reduce E2F1 protein levels to either induce apoptosis or inhibited cell cycle re-entry. Under hypoxic conditions, BDNF reduces excitotoxicity and cell death by preventing cell cycle re-entry via E2F1 activation (Boutahar et al., 2010). PEDF induces apoptosis of chondrocytes by downregulating E2F1 but its effect in the CNS is unknown (Tan et al., 2010). Through

its interactions with growth factors E2F1 may play an important role in the CNS that could depend on cell conditions.

E2F1 has also been linked to telomere maintenance and, as reduced telomere length has been reported in depression (Ridout et al., 2015), lower *E2F1* mRNA could be linked to reduced telomere length in depression. In normal human cells, E2F1 has been shown to activate human telomerase reverse transcriptase (hTERT) (Won et al., 2002). Activated hTERT, the catalytic subunit of telomerase, has been reported to maintain telomere length, through an assembly of E2F1 and a histone deacetylase (HDAC) complex (Won et al., 2004). Therefore, reduced E2F1 as demonstrated in this study and reduced HDAC expression, already reported in depression (Hobara et al., 2010), could result in reduced telomerase activity and telomere length. It has also been suggested that telomerase activity can mediate the effects of trophic factors in the brain (Mattson et al., 2001) and peripheral blood mononuclear cell telomerase activity correlates with hippocampal volumes in depression (Wolkowitz et al., 2015). Therefore, this E2F1/hTERT interaction could be linked to changes in brain volumes in depression.

Overall, there are a number of pathways implicated/reported in depression that have links to E2F1.

3.5.3 The effect of ECT on *E2F1* mRNA levels

ECT had no effect on peripheral blood *E2F1* mRNA levels. To date only one study has examined at effect of ECS on E2F1 protein levels (Kim et al., 2005). They focused on rat frontal cortex samples and did not examine peripheral blood levels. Rats were treated with a single ECS or either five or ten days of chronic ECS, with E2F1 protein levels significantly increasing with the number of ECS sessions given. In that study animals

were sacrificed 24 hours after their last ECS. For our study, patient bloods were taken 1–3 days after their last ECT session, and it is possible that the effect of ECT on *E2F1* mRNA was missed if it was transient.

The exact source of *E2F1* mRNA in peripheral whole blood is not clear but peripheral blood mononuclear cells (PBMCs) express *E2F1* and are a potential source of the gene expression detected in this study (Sarhan et al., 2015). I am unaware of any studies that show *E2F1* mRNA can cross the blood brain barrier. One way for mRNA to cross the BBB is by exosomes, specialized membranous vesicles produced in the endosomal compartment that have a role in cell-to-cell communication (Morales-Prieto et al., 2018). However, to my knowledge no studies have studied if exosomes release *E2F1*. Therefore, ECT may increase *E2F1* protein and mRNA levels in the brain and this may not be detectable in peripheral blood.

3.5.4 The effect of psychosis and other depression subgroups on *E2F1* mRNA levels

The main reason for exploring *E2F1* levels in depression was the result of a deep sequencing study and gene target analysis that showed altered levels of two miRNAs in patients from the EFECT-Dep Trial (miR-126-3p and miR-106-5p). Gene target analysis identified *E2F1* as a target of both these miRNAs. These miRNAs were elevated at baseline in psychotic depression and returned to control levels following ECT (Kolshus et al., 2017). However, no statistically significant difference in *E2F1* mRNA levels between the non-psychotic and psychotic groups at baseline or after ECT were found. This indicates that *E2F1* might not be directly regulated by these miRNAs in depression. Another possibility is that *E2F1* is linked to other miRNAs and may be suppressed by other pathways. For example, activation of *E2F1* protein has been shown to directly

regulate miR-16 (Ofir et al., 2011), possibly through tumour protein 53 (p53), which has been linked to the therapeutic response to antidepressants and has been reported to be downregulated in depression (Song et al., 2006). It has been suggested that *E2F1*-regulated miRNAs function as part of a feedback loop, activating E2F1 function (Ofir et al., 2011). It is possible that miR-16 plays a close regulatory role in E2F1 function. Therefore, changes in *E2F1* could be independent of our previously reported miRNA changes.

3.5.5 Correlation studies

This study reported a few weak correlations between a change in *E2F1* levels and a change in HAM-D24 scores after ECT. These associations were found between unipolar patients, and patients who did not respond to ECT or attain remission. However, after correcting for multiple testing these correlations were not significant. It is unlikely that *E2F1* mRNA could be used clinically to monitor response to ECT. The only result that still had a trend for significance after correcting was the relationship between a change in *E2F1* levels and a change in HAM-D24 scores after ECT in non-responders. This indicates that increasing *E2F1* mRNA levels results in decreasing HAM-D24 scores in patients who do not respond to ECT, a result that is difficult to interpret and may have been a Type I error as a result of multiple testing.

3.5.6 The effect of ECS on *E2f1* mRNA levels in the rat brain

As previous animal data had shown an effect of ECS on E2F1 protein levels in the rat frontal cortex (Kim et al., 2005), I wanted to investigate if the effects of ECS in the brain would translate to the periphery by analysing *E2f1* mRNA expression in response to acute

and chronic ECS in naïve rats. I found decreased *E2f1* mRNA in the dentate gyrus following acute ECS but no changes in any other region after both acute and chronic ECS. To my knowledge, this is the first study to examine the effects of ECS or any antidepressant therapy on *E2f1* mRNA in the brain and periphery. The study by Kim *et al.* (2005) examined E2F1 protein and samples were collected after 24 hours. In their study, E2F1 protein gradually increased with the number of ECS sessions, possibly indicating sustained E2F1 protein activation. However, *E2f1* mRNA was only decreased in the dentate gyrus after an acute ECS session.

It is not clear why *E2f1* mRNA decreased in the dentate gyrus. Based on previous work by Kim *et al.* (2005), I would have expected ECS to activate cell cycle progression and E2F1. However, mRNA levels do not always reflect protein levels. Studies have shown that steady-state transcript abundances only partially predict protein concentrations in most organisms, with protein localisation, degradation and post-translational modifications affecting protein concentrations (Vogel and Marcotte, 2012). Therefore, I cannot say for certain how reduced mRNA in the dentate gyrus could translate to protein levels. It does, however, give some support to the point that changes in *E2F1* mRNA post ECT might be transient and not translate to the periphery. To fully understand the role of E2F1 in depression an animal model investigating changes in E2F1 and other cell cycle regulatory proteins may be needed.

3.5.7 Study limitations and future studies

The main limitation of this study is that the EFFECT-Dep study was not originally designed for molecular analysis. Therefore, recruitment did not take into consideration the power needed to statistically detect changes in gene levels between patients and

controls. However, this study has a relatively large sample size, that is in line or higher than other studies that have detected molecular changes in whole blood between patients and controls, or in patients before and after treatment (Jarventausta et al., 2017, Allen et al., 2018a, Abe et al., 2011).

One limitation of this study is that all the patients received pharmacological treatment as normal throughout their course of ECT. It therefore cannot be ruled out that reduced peripheral *E2F1* is an antidepressant effect in the depressed patients in comparison to controls. For example, one study has reported that amitriptyline, a tricyclic antidepressant, can reduce cyclin D transactivation and protein expression (Mao et al., 2011). Reduced cyclin D expression can result in suppressed E2F1 protein levels through reduced Rb phosphorylation (Jan et al., 2011). Therefore, antidepressants could alter *E2F1* mRNA levels. It would be interesting to repeat this study in an antidepressant naïve cohort to clarify if this is a marker of depression or an antidepressant response.

3.6 Conclusion

In conclusion, *E2F1* mRNA levels were significantly lower in patients with depression. *E2F1* was not associated with any mood ratings in depression and did not change among subgroups of depression. Further studies would be useful to clarify if reduced *E2F1* is an antidepressant effect or a marker of depression and to examine the effects of treatment in an antidepressant naïve cohort. As ECS has previously been shown to increase E2F1 in the rat frontal cortex (Kim et al., 2005), and I reported reduced mRNA expression in the dentate gyrus in response to ECS, it would be interesting to investigate the role of E2F1 in an animal model of depression. Overall, these results indicate that *E2F1* mRNA could be a peripheral trait feature of depression.

Chapter 4.

Peripheral blood *SIRT1* mRNA in depression and following electroconvulsive therapy

4.1 Introduction

The sirtuins are a family of seven (SIRT1-7) nicotinamide (NAD⁺)-dependent deacetylases, that have been classed as type III histone deacetylases (HDACs) (Michan and Sinclair, 2007, Whittle et al., 2007). Through deacetylating both histone and non-histone protein targets, they have been reported to regulate glucose homeostasis, mitochondrial biogenesis, DNA repair, inflammation, apoptosis, and aging in the periphery (Inoue et al., 2017, Oberdoerffer et al., 2008, Rodgers et al., 2005, Tissenbaum and Guarente, 2001, Vaziri et al., 2001). SIRT1 also has roles in the central nervous system (CNS), including improvement of cognition, regulation of circadian rhythms, synaptic plasticity, and memory in animal studies (Asher et al., 2008, Gao et al., 2010, Michan et al., 2010, Cao et al., 2018).

There is strong evidence linking SIRT1 to depression that comes from human genetic studies (Kishi et al., 2010, Cai et al., 2015), animal studies (Libert et al., 2011, Abe-Higuchi et al., 2016), and human gene expression studies (Abe et al., 2011, Luo and Zhang, 2016, Wang et al., 2018b). One of these studies (Wang et al., 2018b), also reported that four weeks of treatment with citalopram attenuated reduced *SIRT1* mRNA levels in drug naïve patients, indicating SIRT1 is involved in the therapeutic response to antidepressants. However, as far as I am aware, no studies have assessed if ECT has an effect on *SIRT1* mRNA levels, or if levels are associated with clinical outcomes.

As mentioned in Chapters 1 and 3, a recent study from my lab identified two microRNAs (miR-126-3p and miR-106a-5p), for which SIRT1 is a shared target, that were elevated in psychotic depressed patients and returned to control levels following electroconvulsive therapy (ECT) (Kolshus et al., 2017). There is further evidence to link SIRT1 to the antidepressant response of ECT. A recent study showed that acute electroconvulsive stimulation (ECS) increases SIRT1 immunoreactivity levels in mouse

hippocampus and hypothalamus (Chung et al., 2013), and ECS has been reported to induce histone modification of hippocampal genes (Tsankova et al., 2004). Together, the above findings suggest a role for SIRT1 in depression and ECT treatment response.

4.2 Aims and objectives

The aim of this study was to examine *SIRT1* mRNA levels in peripheral whole blood samples collected from patients with depression pre- and post-treatment with ECT and age- and sex-matched healthy controls in order to gain an understanding of the effect of ECT on these markers. Based on the studies on *SIRT1* mRNA published at the time of this study (Abe et al., 2011, Luo and Zhang, 2016), I hypothesised that *SIRT1* mRNA levels would be lower in patients with depression than in healthy controls. As this study was based on the previous miRNA data where the miRNA levels that target SIRT1 returned to control levels (Kolshus et al., 2017), I hypothesised *SIRT1* mRNA would also return to control levels following a course of ECT. The objectives of the study were to:

1. To analyse the levels of *SIRT1* mRNA in whole blood collected from patients with depression and controls using real-time quantitative polymerase chain reaction (RT-qPCR).
2. To assess differences in the levels of *SIRT1* mRNA in patients pre-/post-ECT.
3. To assess differences in *SIRT1* mRNA levels pre-/post-ECT in depression subtypes (psychotic/non-psychotic, unipolar/bipolar).
4. To determine if there are differences in the levels of *SIRT1* mRNA in responders/non-responders and remitters/non-remitters.
5. To perform correlation analyses to determine if levels of *SIRT1* are associated with mood scores.

6. To investigate the effects of acute and chronic ECS on *Sirt1* mRNA levels in peripheral whole blood and brain regions of naïve rats.

4.3 Materials and methods

4.3.1 Human participants

This study included participants recruited as part of the EFFECT-Dep Trial (Enhancing the Effectiveness of ECT in Severe Depression, ISRCTN23577151) that took place in St Patrick's Mental Health Services, Dublin (www.stpatricks.ie). Full details of this trial, clinical assessments and healthy control recruitment can be found in sections 2.2.1.1 – 2.2.1.4.

4.3.2 Blood sampling and mRNA extraction

Full details for blood sampling and mRNA extraction are given in section 2.2.1.3 – 2.2.1.5. Fasting whole blood samples for mRNA analysis were taken using the PaxGene[®] system (Qiagen Inc., USA). Samples were left to incubate at room temperature for two hours, transferred to -20°C for 24 hours, and then stored at -80°C until use. mRNA was extracted using the PaxGene[®] Blood RNA Kit according to the manufacturer's instructions (Qiagen Inc., USA). Quality analysis (to check RNA concentration and integrity) was carried out on the NanoDrop[®] 1000 UV-Vis Spectrophotometer (Fisher Scientific, UK). RNA samples with a 280/260 ratio of 1.8-2.2 were considered acceptable for analysis and RNA was equalised to 10ng/µl.

4.3.3 Multiplex quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). qRT-PCR was carried out on the StepOne Plus™ Real-Time PCR system (Applied Biosystems, UK) using TaqMan® (Applied Biosystems, UK) gene expression assays (*SIRT1*; assay ID:Hs01009006_m1) with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; assay ID:Hs03929097_g1) as the endogenous control. Full details of cDNA synthesis, qRT-PCR and gene expression analysis can be found in sections 2.2.1.8 – 2.2.1.10.

4.3.4 Electroconvulsive stimulation study

Methods for the acute/chronic electroconvulsive stimulations (ECS)/sham treatment study can be found in section 2.2.2.

4.3.5 Statistical analysis

Data were analysed using SPSS version 21.0 (IBM Corporation, NY, USA) and GraphPad Prism 5 (GraphPad Software, California, USA). Receiver Operating Characteristic curve analysis was carried out using MedCalc® (MedCalc Software, Belgium). Data were tested for normality using a Shapiro–Wilk test and log-transformed when necessary.

Baseline clinical and demographic factors are presented as means with standard deviation (SD), or number (%) per group. I adjusted for potential variance with confounders from the literature as well as variables that were significantly different between groups (current smoking status, BMI and education). For *SIRT1* mRNA analysis, potential covariates from the literature included age, body mass index (BMI), diabetes,

current smoking status and ischemic heart disease (Breitenstein et al., 2013, Owczarz et al., 2017, Li et al., 2017, Santos-Bezerra et al., 2017, Zillikens et al., 2009). When analysing the effect of ECT, I also corrected for baseline HAM-D24 scores, presence of psychosis, depression polarity and electrode placement as these may affect how a patient responds to treatment.

Data were analysed using independent t-tests, paired t-tests, Mann-Whitney U tests for non-parametric data, or a general linear model (GLM). Categorical data were tested using a chi-square test (χ^2) or Fisher's exact test. For correlational analyses, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) for parametric data and Spearman's rank correlation coefficient rho (ρ) for non-parametric data.

Animal data were tested for normality using a Kolmogorov-Smirnov normality test. Parametric data were tested with a student t test and non-parametric data were tested using a Mann-Whitney U test. Values are represented as a fold-change (a ratio of how much the treatment group changed in comparison to the control group). Animal data are presented as means $\pm$ standard error of the mean (SEM).

Statistical significance was set at $p < 0.05$. For correlation analysis $p < 0.01$ was used to account for multiple testing.

4.4 Results

4.4.1 Sample characteristics

For this study, 91 out of 138 participants from the EFFECT-Dep trial had both pre- and post-ECT bloods available for analysis. Similar to chapter 3.4.1, these samples were representative of the whole EFFECT-Dep patient group (see Table 4.1).

	Patients included (n = 91)	EFFECT group (n = 140)	Statistical Test
Age (SD)	56.78 (13.5)	56.70 (14.8)	$t(227)=-0.04, p=0.97$
Sex, female n (%)	59 (64.8)	87 (62.1)	$\chi^2=0.08, p=0.45$
Smoker, yes n (%)	38 (41.8)	61 (43.6)	$\chi^2=0.13, p=0.41$
BMI (SD)	27.02 (5.1)	26.62 (4.8)	$U(231)=6101.00, p=0.58$
Education Completed, n (%)			
Primary	18 (19.8)	23 (16.5)	
Secondary	50 (54.9)	80 (57.6)	
Tertiary	23 (25.3)	36 (25.9)	$\chi^2=0.40, p=0.66$
Bipolar Depression, yes n (%)	19 (20.9)	32 (22.9)	$\chi^2=0.17, p=0.40$
Psychotic Subtype, yes n (%)	22 (24.2)	29 (20.7)	$\chi^2=0.32, p=0.34$
Treatment Resistant, yes n (%)	67 (73.6)	98 (70.0)	$\chi^2=0.19, p=0.39$
Previous ECT, yes n (%)	29 (31.9)	52 (37.1)	$\chi^2=0.78, p=.23$
No. of Previous Depression Episodes (SD)	5.33 (4.8)	5.33 (4.5)	$t(227)=0.01, p=0.65$
Pre-ECT HAM-D24 (SD)	30.27 (6.4)	29.91 (6.2)	$U(229)=6803.00, p=0.70$
Post-ECT HAM-D24 (SD)	10.94 (8.2)	11.79 (8.4)	$U(227)=5768.50, p=0.39$
No. of ECT sessions (SD)	8.24 (2.3)	8.01 (2.4)	$t(227)=-0.73, p=0.47$
Electrode Placement, n (%)			
Unilateral	42 (46.2)	69 (50.0)	
Bilateral	49 (53.8)	69 (50.0)	$\chi^2=0.33, p=0.32$
Responder, yes n (%)	56 (61.5)	77 (55.0)	$\chi^2=0.74, p=0.24$
Remitter, yes n (%)	47 (51.6)	61 (43.6)	$\chi^2=1.22, p=0.17$
Relapse (of those that remitted), yes n (%)	15 (31.9)	17 (27.9)	$\chi^2=1.27, p=0.31$
Medications, yes n (%)			
SSRI	24 (26.4)	28 (20.0)	$\chi^2=1.26, p=0.17$
SNRI	43 (47.3)	65 (46.4)	$\chi^2=0.01, p=0.51$
TCA	23 (25.3)	43 (30.7)	$\chi^2=0.83, p=0.22$
Mirtazapine	33 (36.3)	45 (32.1)	$\chi^2=0.40, p=0.31$
Bupropion	4 (4.4)	5 (3.6)	$p=0.51$
MAOI	11 (12.1)	15 (10.7)	$\chi^2=0.10, p=0.46$
Lithium	34 (37.4)	55 (39.3)	$\chi^2=0.10, p=0.43$
Antipsychotic	66 (72.5)	96 (68.6)	$\chi^2=0.38, p=0.32$
Valproate	5 (5.5)	9 (6.4)	$\chi^2=0.01, p=0.50$
Pregabalin	5 (5.5)	8 (5.7)	$\chi^2=0.01, p=0.59$
Benzodiazepine	49 (53.8)	76 (54.3)	$\chi^2=0.01, p=0.52$
Non-benzodiazepine Hypnotic	54 (59.3)	87 (62.1)	$\chi^2=0.21, p=0.37$

Table 4.1 Comparison of the patients used for the *SIRT1* mRNA study to the entire EFFECT-Dep patient sample

Abbreviations: BMI, body mass index; HAM-D24, Hamilton Depression Rating Scale, 24-item version; ECT, electroconvulsive therapy; SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin and noradrenaline reuptake inhibitor; TCA, tricyclic antidepressants; MAOI, monoamine oxidase inhibitor; U, standardized Mann-Whitney test. Data are presented as means (SD) or n (%).

Healthy controls ($n = 85$) were included who were balanced with the patients for age and sex. The patient group had a higher BMI ($p < 0.01$), were more likely to smoke ($p < 0.01$) and had a lower educational attainment ($p < 0.001$) than the control group. These variables were therefore included as covariates for analysis. Demographic and clinical characteristics are presented in Table 4.

	Patient ($n = 91$)	Control ($n = 85$)	Statistical Test
Age	56.78 (13.5)	54.74 (10.7)	$t(174)=-1.10, p=0.27$
Sex, female n (%)	59 (64.8)	53 (62.4)	$\chi^2 = 0.12, p=0.76$
Smoker, yes n (%)	38 (41.8)	19 (22.4)	$\chi^2=7.56, p<0.01$
BMI	27.02 (5.1)	25.14 (4.0)	$U=2922.00, p<0.01$
Education Completed, n (%)			
Primary	18 (19.8)	4 (4.7)	
Secondary	50 (54.9)	16 (18.8)	
Tertiary	23 (25.3)	65 (76.5)	$\chi^2=52.10, p<0.001$
Diabetes, yes n (%)	7 (7.7)	3 (3.5)	$p=0.34$
Ischemic Heart Disease, yes n (%)	11 (12.1)	4 (4.7)	$p=0.11$
HAM-D24 Pre-ECT	30.27 (6.4)	3.29 (2.5)	$U<0.001, p<0.001$

Table 4.2 Demographic and clinical features of patients and controls for *SIRT1* mRNA analysis

Abbreviations: BMI, body mass index; HAM-D24, Hamilton Depression Rating Scale, 24-item version; ECT, electroconvulsive therapy; U, standardized Mann-Whitney test. Data are presented as means (SD) or n (%). *SIRT1* mRNA levels in depression and following ECT.

4.4.2 Sirtuin1 levels in depression and following electroconvulsive therapy

RQ values for *SIRT1* mRNA were not normally distributed. Values were normal following log transformation. Therefore, log values for *SIRT1* were used for analysis. Healthy controls had a mean *SIRT1* mRNA RQ value of 1.31 (SD: 0.44), whereas depressed patients had a mean value of 1.06 (SD: 0.34). Patient *SIRT1* levels increased to 1.10 (SD: 0.34) post-ECT.

Patient peripheral blood *SIRT1* mRNA levels were initially compared to controls using an unadjusted student's t-test. Baseline peripheral *SIRT1* mRNA levels were significantly lower in patients with depression ($n = 91$) compared to healthy controls ($n = 85$) ($p < 0.001$; Figure 4.1). Adjusting for potential covariates (age, current smoking status, BMI, education, diabetes, ischemic heart disease) using a GLM altered the result slightly, ($F(1,165) = 8.20, p = 0.005$), but there was still a significant difference between groups with lower *SIRT1* levels in patients. Only age was found to have an effect on *SIRT1* levels ($F(1,165) = 10.13, p = 0.002$), with a lower age being correlated with higher *SIRT1* mRNA levels. The effects of the other covariates are reported in Table 4.3.

A paired t-test revealed that *SIRT1* mRNA levels were not significantly altered by ECT treatment ($p = 0.23$; Figure 4.1). Correcting for covariates did not change this ($F(1,79) = 0.06, p = 0.80$).

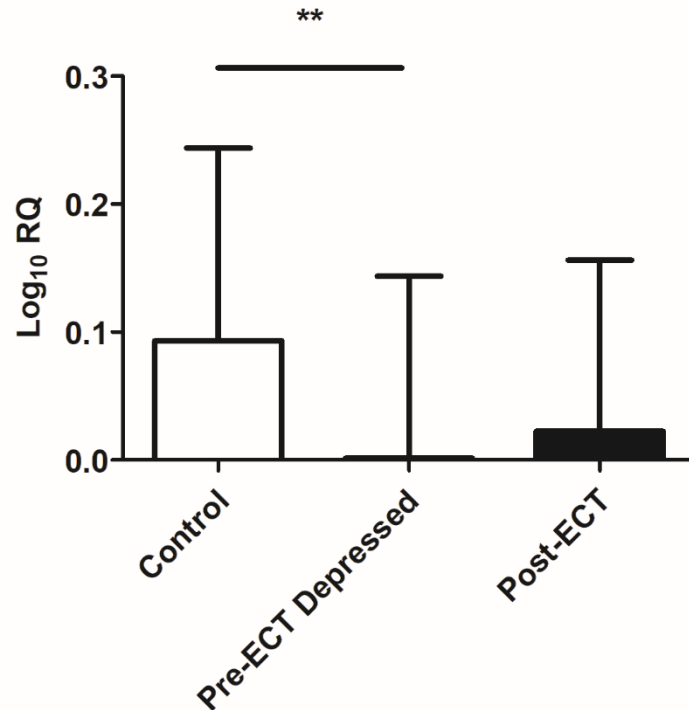


Figure 4.1 *SIRT1* mRNA levels in controls and patients pre-/post-ECT

Log transformed RQ values of *SIRT1* mRNA in controls and pre-ECT in depressed patients, and after a course of ECT in patients. *SIRT1* levels were significantly decreased in patients ($n = 91$) in comparison to healthy controls ($n = 85$). Data are presented as mean $\pm$ SD. ** $p = 0.005$ healthy controls versus depressed pre-ECT.

Covariate	df	F	P Value
Patient Group	1, 173	8.20	<0.01
Age	1, 173	12.15	<0.01
Smoking	1, 173	2.91	0.09
BMI	1, 173	1.84	0.18
Level of Education	2, 173	0.01	0.25
Diabtetes	1, 173	1.93	0.17
Ischemic Heart Disease	1, 173	0.16	0.69

Table 4.3 The effect of relevant covariates on *SIRT1* mRNA expression.

Age had a significant effect on *SIRT1* mRNA levels. However, the patient group (control vs. depressed patient) still had a significant effect on *SIRT1* mRNA levels. Abbreviations: df, degrees of freedom; F, F value (variance group means); BMI, body mass index.

4.4.3 Receiver operating characteristic curve analysis

I next performed a receiver operating characteristic (ROC) curve to determine if *SIRT1* mRNA levels at baseline could be used to distinguish patients with depression from controls. This is done by plotting the true positive results against the true negative results and the area under the curve (AUC) is used to determine the accuracy of a diagnostic marker (Zweig and Campbell, 1993). An AUC value of 1 indicates a good diagnostic test where as a value of 0.5 indicates a poor test. The AUC value of *SIRT1* was 0.677, with a sensitivity of 70.3% and a specificity of 58.8%; see Figure 4.2.

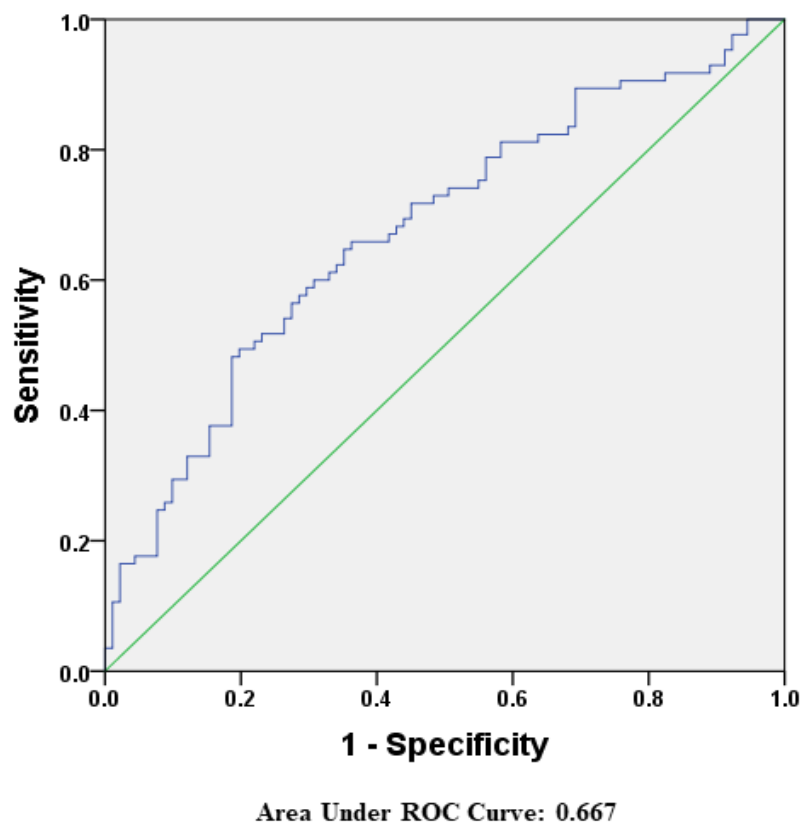


Figure 4.2 ROC of *SIRT1* mRNA levels and patient/control classification.
ROC analysis of depressed patients ($n = 91$) and healthy controls ($n = 85$).

4.4.4 The effects of different depression subtypes on *Sirtuin1* mRNA levels

Subgroup analyses were performed to determine if *SIRT1* mRNA differed between different subsets of depression (depression with/without the presence of psychosis and unipolar/bipolar depression), or if levels differed between ECT responders/remitters. These analyses were performed to investigate if *SIRT1* mRNA levels could be used in a clinical setting to distinguish between subtypes of depression or predict response to ECT. The sections below detail the effect of the different clinical groups on *SIRT1* mRNA levels with a summary table (Table 4.6) provided at the end of section 4.4.4.

4.4.4.1 The presence of psychosis and *Sirtuin1* mRNA levels

As the initial miRNA findings were found in patients with psychotic depression (Kolshus et al., 2017), the effect of psychosis on *SIRT1* levels was assessed.

There was no difference in *SIRT1* mRNA levels between non-psychotic ($n = 69$) and psychotic ($n = 22$) groups at baseline ($p = 0.63$; Figure 4.3) and adjusting for covariates (age, current smoking status, BMI, education, diabetes, ischemic heart disease, baseline HAM-D24 score, and polarity of depression) did not change this. There were no differences between psychotic and non-psychotic groups for *SIRT1* levels over time ($p = 0.16$).

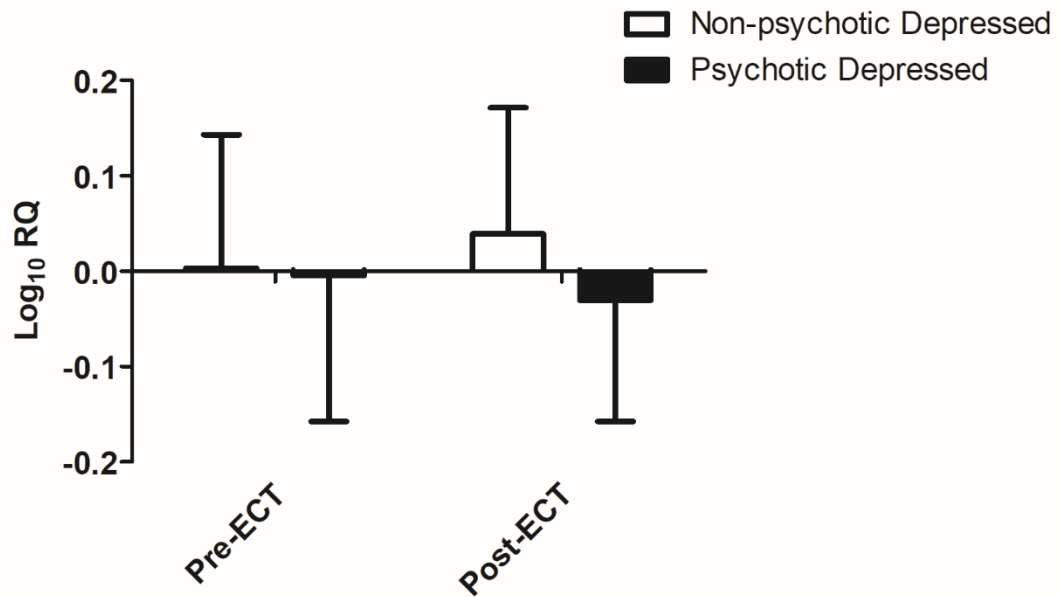


Figure 4.3 *SIRT1* mRNA levels and the presence of psychosis pre-/post-ECT

There were no differences in *SIRT1* mRNA levels in patients with ($n = 22$) or without ($n = 69$) the presence of psychosis pre-ECT and this did not change over time. Data are presented as unadjusted mean $\pm$ SD.

4.4.4.2 Depression polarity and *Sirtuin1* mRNA levels

As *SIRT1* mRNA levels had previously been shown to be reduced in both MDD and bipolar depressed patients (Abe et al., 2011), I also investigated the effects of depression polarity on *SIRT1* mRNA levels. Table 4.4 compares the clinical and demographic features of the patients with unipolar depression and patients with bipolar depression. Patients with bipolar depression were more likely to be smokers ($p = 0.002$) and there was a difference in education completed with less bipolar depressed patients attaining third level education ($p = 0.05$). Both of these measures were included as covariates. Patients with bipolar depression also had a greater number of previous depressive episodes ($p = 0.003$), this was therefore included as a covariate for this analysis. Patients with bipolar depression were also less likely to be on SNRI medication ($p = 0.003$). However, due to the heterogeneity of the medications taken by our patients there was no clear way to adjust for this.

	Unipolar Depression (<i>n</i> = 72)	Bipolar Depression (<i>n</i> = 19)	Statistical Test
Age	57.26 (13.1)	54.95 (15.1)	$t(89)=0.66, p=0.51$
Sex, female <i>n</i> (%)	49 (68.1)	10 (47.6)	$\chi^2=1.57, p=0.16$
Smoker, yes <i>n</i> (%)	24 (33.3)	14 (66.6)	$\chi^2=10.06, p<0.01$
BMI (SD)	27.10 (4.9)	26.71 (6.2)	$U=584.50, p=0.33$
Education Completed, <i>n</i> (%)			
Primary	12 (16.7)	6 (31.5)	
Secondary	40 (55.6)	10 (52.6)	
Tertiary	20 (27.8)	3 (15.9)	$p=0.30$
Diabetes, yes <i>n</i> (%)	6 (8.3)	1 (5.3)	$p=0.55$
Ischemic Heart Disease, yes <i>n</i> (%)	8 (11.1)	3 (15.8)	$p=0.41$
Psychosis Subtype, <i>n</i> (%)	17 (23.6)	5 (26.3)	$\chi^2=0.06, p=0.51$
Treatment Resistant, yes <i>n</i> (%)	56 (77.8)	11 (57.9)	$\chi^2=3.06, p=0.08$
Previous ECT, yes <i>n</i> (%)	22 (30.6)	7 (36.8)	$\chi^2=0.24, p=0.41$
No. of Previous Depression Episodes (SD)	4.58 (4.1)	8.20 (5.6)	$t(89)=-3.04, p<0.01$
HAM-D24 Pre-ECT	29.97 (6.5)	31.42 (6.2)	$U=575.50, p=0.29$
HAM-D24 Post-ECT	10.92 (8.2)	11.10 (8.1)	$U=667.00, p=0.94$
No. ECT sessions	8.19 (2.3)	8.42 (2.6)	$t(89)=-0.37, p=0.71$
Electrode Placement			
Unilateral	32 (44.4)	10 (52.6)	
Bilateral	40 (55.6)	9 (47.4)	$\chi^2=0.41, p=0.35$
Responder, <i>n</i> (%)	44 (61.1)	12 (63.2)	$\chi^2=0.03, p=0.55$
Remitter, <i>n</i> (%)	35 (48.6)	10 (52.6)	$\chi^2=0.09, p=0.57$
Medications, <i>n</i> (%)			
SSRI	18 (25.0)	6 (31.6)	$\chi^2=0.30, p=0.39$
SNRI	40 (55.6)	3 (15.8)	$P<0.01$
TCA	19 (26.4)	4 (21.1)	$p=0.43$
Mirtazapine	25 (34.7)	8 (4.1)	$\chi^2=0.31, p=0.38$
Bupropion	4 (5.6)	0 (0.0)	$p=0.38$
MAOI	8 (11.1)	3 (15.8)	$p=0.42$
Lithium	28 (38.9)	6 (31.6)	$\chi^2=0.39, p=0.36$
Antipsychotic	52 (72.2)	14 (73.7)	$\chi^2<0.01, p=0.61$
Valproate	2 (2.8)	3 (15.8)	$p=0.06$
Pregabalin	4 (5.6)	1 (5.3)	$p=0.72$
Benzodiazepine	40 (55.6)	9 (47.4)	$\chi^2=0.49, p=0.33$
Non-benzodiazepine Hypnotic	44 (61.1)	10 (52.6)	$\chi^2=0.55, p=0.32$

Table 4.4 Demographic and clinical details of patients with unipolar depression and bipolar depression

Abbreviations: BMI, body mass index; NSAID, Nonsteroidal anti-inflammatory drugs; HAM-D24, Hamilton Depression Rating Scale, 24-item; ECT, Electroconvulsive therapy; SSRI, Selective serotonin reuptake inhibitor; SNRI, Serotonin and noradrenaline reuptake inhibitor; TCA, Tricyclic antidepressants; MAOI, Monoamine Oxidase Inhibitor; U, standardised Mann-Whitney test. Data are presented in means (SD) or *n* (%).

An unadjusted GLM found a significant difference between the two groups ($p = 0.049$), with lower levels of *SIRT1* mRNA in the bipolar depressed group ($n = 19$) compared to the unipolar depressed group ($n = 72$). However, after adjusting for the relevant covariates (age, current smoking status, BMI, education, diabetes, ischemic heart disease, baseline HAM-D24 score, the presence of psychosis and the number of previous depressive episodes) this significance was no longer seen ($p = 0.13$; Figure 4.4). There was also a trend for a polarity $\times$ time interaction ($p = 0.08$) but this effect was also lost after correction ($p = 0.23$). Of the relevant covariates mentioned above, only age had a significant effect on the effect of polarity and *SIRT1* mRNA levels, see Table 4.5.

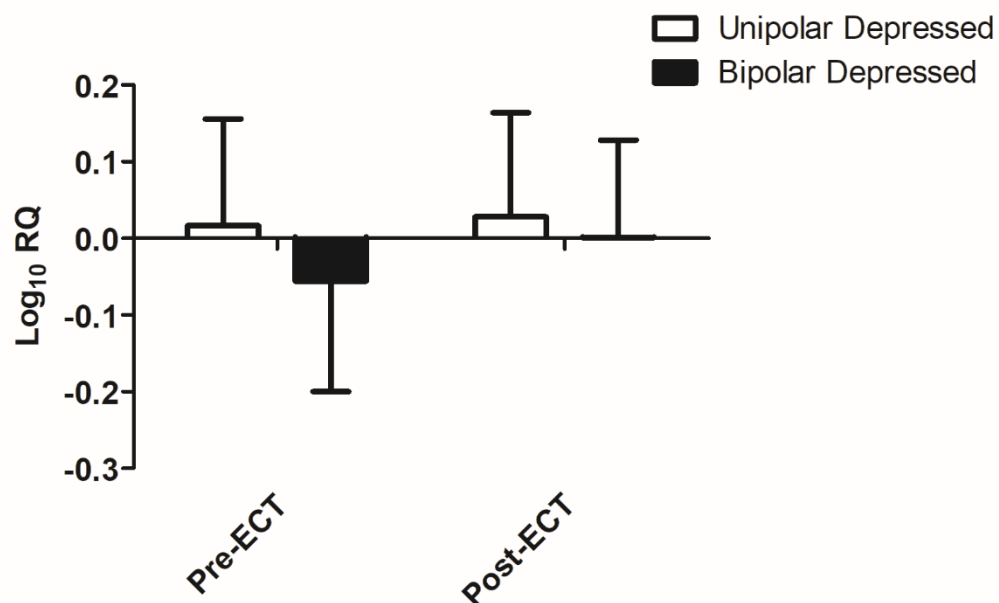


Figure 4.4 *SIRT1* mRNA levels and the polarity of depression pre-/post-ECT

An unadjusted GLM found a significant difference in *SIRT1* mRNA levels between the two groups ($p = 0.049$). However, after adjusting for covariates this significance was no longer seen ($p = 0.13$). (b) There was also a trend for a polarity $\times$ time interaction ($p = 0.08$) but this effect was lost after correction ($p = 0.23$). Data are presented as unadjusted mean $\pm$ SD.

Source	df	F	P value
Patient Group	1, 79	2.09	0.15
Age	1, 79	8.63	<0.01
Smoking Status	1, 79	2.04	0.16
BMI	1, 79	1.17	0.28
Level of Education	2, 89	.31	0.73
Diabetes	1, 79	3.08	0.08
Ischemic_Heart_disease	1, 79	0.09	0.77
HAM-D24 baseline	1, 79	<0.01	0.98
Presence of Psychosis	1, 79	0.19	0.66
No. of previous depressive episodes	1, 79	0.94	0.34

Table 4.5 The effects of relative covariates on *SIRT1* mRNA levels and polarity of depression

After adjusting for covariates polarity of depression had no effect on *SIRT1* mRNA levels, with age having a significant effect on this interaction (patient group = unipolar vs bipolar depression). Abbreviations: df, degrees of freedom; F, F value (variance group means); BMI, body mass index; HAM-D24, Hamilton Depression Rating Scale, 24-item.

4.4.4.3 *Sirtuin1* mRNA levels and response or remission

Next, I wanted to assess if *SIRT1* mRNA levels could be used to predict clinical response to ECT. Analyses was performed to explore if *SIRT1* mRNA levels were different at baseline between patients who responded to ECT ($n = 56$) and those who did not ($n = 35$), and if these levels changed over time (pre-/post-ECT). Differences in *SIRT1* levels in patients who did ($n = 47$) and did not ($n = 44$) attain remission were also assessed.

As shown in Figure 4.5 there were no differences in *SIRT1* mRNA levels between (a) responders and non-responders at baseline ($p = 0.70$) or pre-/post-ECT ($p = 0.40$), or between (b) remitters and non-remitters at baseline ($p = 0.89$) or over time ($p = 0.77$). Adjusting for covariates (age, current smoking status, BMI, education, diabetes, ischemic heart disease, baseline HAM-D24 score, electrode placement, presence of psychosis, and polarity of depression) did not change these findings.

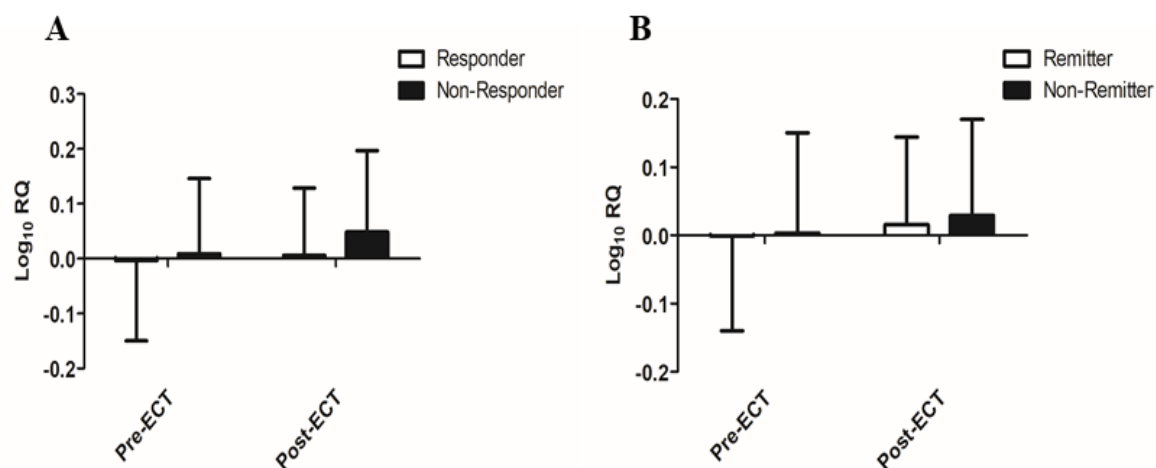


Figure 4.5 *SIRT1* mRNA levels and response/remission status

A general linear model found no differences in *SIRT1* mRNA levels between (A) ECT responders ($n = 56$) and non responders ($n = 35$), or (B) between patients who attained remission ($n = 47$) and those who did not ($n = 44$). This did not change with ECT. Data are presented as unadjusted means $\pm$ SD.

Subgroup	Depressed Pre-ECT	Depressed Post-ECT	Test	Statistics	Adjusted Statistics
Psychosis			Group Pre-ECT	F(1,89)=0.06, $p=0.80$	F(1,80)=0.19, $p=0.66$
Yes ($n = 22$)	0.0033 (0.14)	-0.00301 (0.13)	Time	F(1,89)=0.08, $p=0.78$	F(1,79)=0.06, $p=0.80$
No ($n = 69$)	-0.0055 (0.15)	0.0394 (0.13)	Group $\times$ Time	F(1,89)=2.12, $p=0.15$	F(1,79)=2.00, $p=0.16$
Polarity			Group Pre-ECT	F(1,89)=4.00, $p=0.05$	F(1,80)=2.09, $p=0.15$
Bipolar ($n = 19$)	-0.0562 (0.14)	0.0019 (0.13)	Time	F(1,89)=2.62, $p=0.12$	F(1,79)=0.06, $p=0.80$
Unipolar ($n = 72$)	0.0163 (0.14)	0.0281 (0.14)	Group $\times$ Time	F(1,89)=3.07, $p=0.08$	F(1,79)=1.61, $p=0.21$
Responder			Group Pre-ECT	F(1,89)=0.16, $p=0.70$	F(1,79)=0.01, $p=0.92$
Yes ($n = 56$)	-0.0035 (0.15)	0.0062 (0.12)	Time	F(1,89)=1.91, $p=0.17$	F(1,78)=0.10, $p=0.75$
No ($n = 35$)	0.0086 (0.14)	0.0488 (0.15)	Group $\times$ Time	F(1,89)=1.33, $p=0.25$	F(1,78)=1.38, $p=0.24$
Remission			Group Pre-ECT	F(1,89)=0.02, $p=0.89$	F(1,79)=0.03, $p=0.87$
Yes ($n = 47$)	-0.0008 (0.14)	0.0158 (0.13)	Time	F(1,89)=1.50, $p=0.22$	F(1,78)=0.06, $p=0.82$
No ($n = 44$)	0.0032 (0.15)	0.0299 (0.14)	Group $\times$ Time	F(1,89)=0.15, $p=0.70$	F(1,78)=0.28, $p=0.60$

Table 4.6 Clinical subgroup analysis of *SIRT1* mRNA levels before and after a course of ECT

Abbreviations: ECT, electroconvulsive therapy. Data are presented as unadjusted mean log₁₀ *SIRT1* relative quantification levels (SD).

4.4.5 Correlation analyses

Next, I carried out exploratory correlation analyses to assess if *SIRT1* mRNA levels could predict depression severity at baseline, or the response to ECT by correlating mood scores (using the HAM-D24) with *SIRT1* mRNA levels. These analyses were performed in the patient group as a whole as well in the different subgroups. The following sections give the data for each individual group with a summary table (Table 4.7) provided at the end of the section.

4.4.5.1 Associations between mood ratings and *Sirtuin1* mRNA levels in the patient group as a whole

First, the relationship between *SIRT1* mRNA levels and HAM-D24 mood scores in the patient group were assessed, see Figure 4.6. There were no correlations between baseline *SIRT1* mRNA and mood ratings at baseline ($\rho = -0.02$, $p = 0.85$) or a change in HAM-D24 score pre-/post-ECT ($r = -0.25$, $p = 0.81$), or between changes in *SIRT1* levels and HAM-D24 scores pre-/post-ECT ($r = 0.03$, $p = 0.81$).

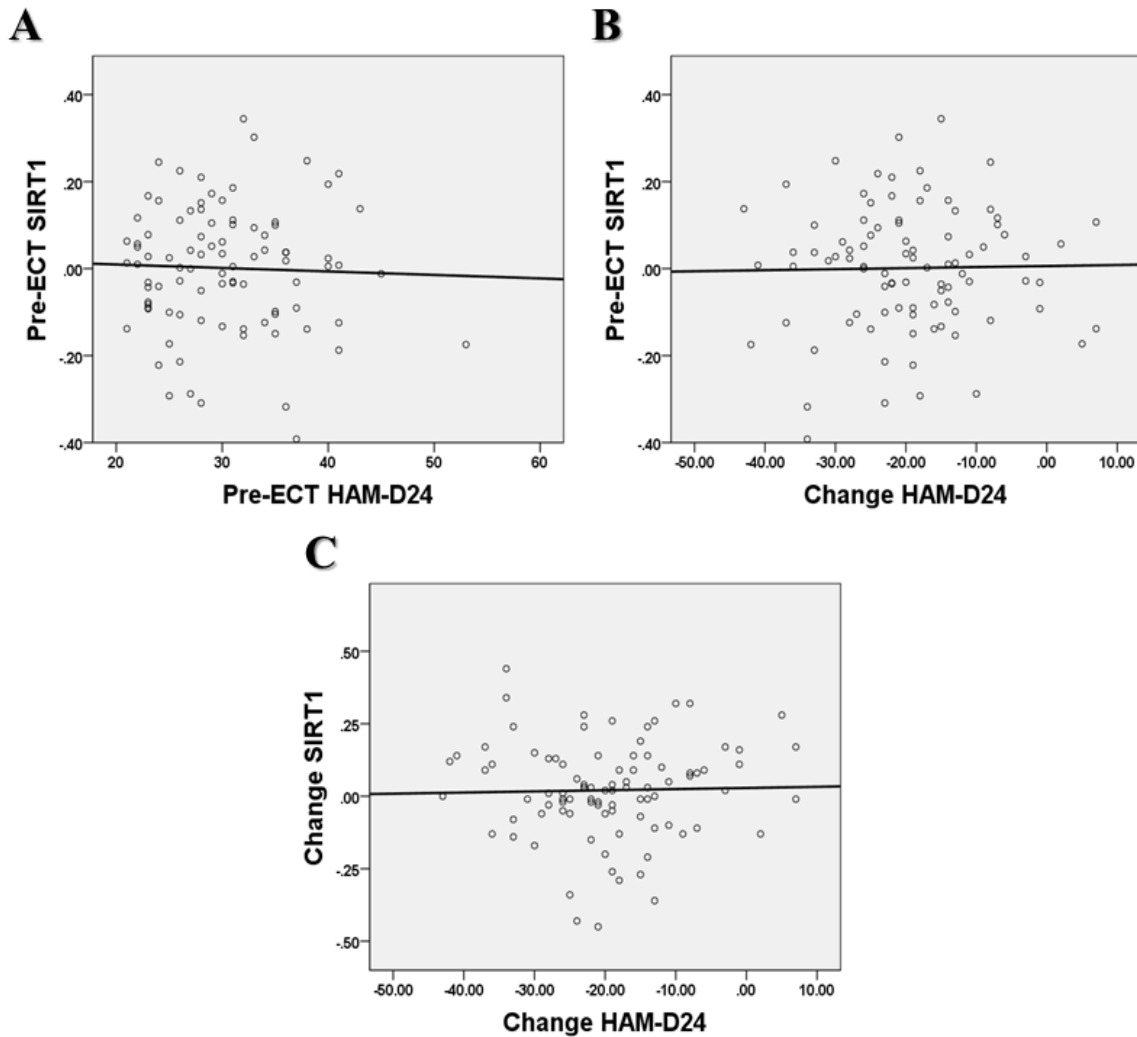


Figure 4.6 Relationship between *SIRT1* mRNA levels and mood ratings

There was no correlation between baseline *SIRT1* mRNA levels and mood ratings at (A) baseline as measured by Spearman's rank correlation coefficient rho (B) a change in HAM-D24 score pre-/post-ECT, or (C) between changes in *SIRT1* levels and HAM-D24 scores pre-/post-ECT, both measured by Pearson's *r*.

4.4.5.2 Associations between mood ratings and *Sirtuin1* mRNA levels in patients with the presence of psychosis

The presence of psychosis had no effect on the association between mood scores and *SIRT1* mRNA levels. This indicates that in the patients with psychosis ($n = 22$), baseline *SIRT1* mRNA levels cannot predict depression severity before ECT ($r = -0.14$, $p = 0.53$), cannot predict symptom improvement with ECT ($r = 0.14$, $p = 0.53$), and a change in *SIRT1* mRNA pre-/post-ECT cannot predict symptom improvement ($\rho = -0.24$, $p = 0.30$).

4.4.5.3 Associations between mood ratings and *Sirtuin1* mRNA levels in with bipolar depression

As with the presence of psychosis, polarity of depression had no effect on the association between mood scores and *SIRT1* mRNA levels. In patients with bipolar depression ($n = 19$),

baseline *SIRT1* mRNA levels cannot predict depression severity before ECT ($r = -0.20$, $p = 0.49$), cannot predict symptom improvement with ECT ($r = 0.29$, $p = 0.23$), and a change in *SIRT1* mRNA pre-/post-ECT cannot predict symptom improvement ($r = -0.32$, $p = 0.19$).

4.4.5.4 Associations between mood ratings, *Sirtuin1* mRNA levels and response or remission status

Associations between mood scores and *SIRT1* mRNA levels were also assessed with response/remission status. There was a trend for a negative correlation between a change in HAM-D24 scores and a change in *SIRT1* levels over time in patients who responded to ECT ($n = 56$; $r = -0.34$, $p = 0.01$; Figure 4.7) and those who attained remission ($n = 47$; $r = -0.34$, $p = 0.02$). No other associations were statistically significant.

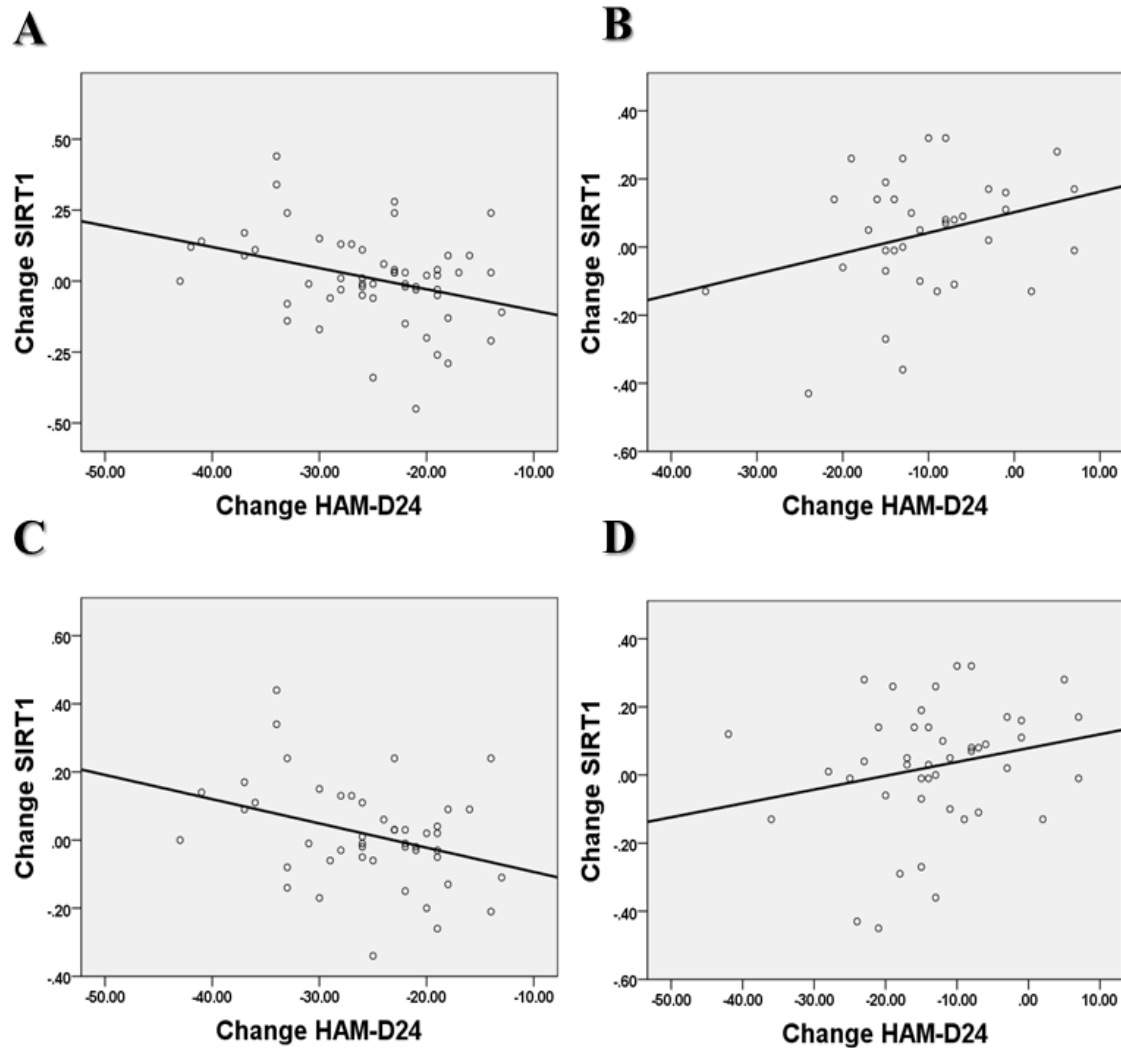


Figure 4.7 Relationship between *SIRT1* mRNA levels and mood scores with response/remission

There was a trend for a negative correlation between the change in *SIRT1* levels and change in HAM-D24 score in patients who (A) responded to ECT ($n = 56$), with (B) no relationship with patients who did not respond to ECT ($n = 35$). Similarly, there was a trend for a negative correlation between the change in *SIRT1* levels and change in HAM-D24 score in patients who (C) attained remission ($n = 47$). There were no relationships in the patients who (D) did not attain remission ($n = 44$). Data for (A-C) were obtained using Pearson's r correlation analysis and (D) was measured using Spearman's rank correlation coefficient ρ .

Subgroup	Pre-ECT and HAM-D24	<i>SIRT1</i> Pre-ECT <i>SIRT1</i> and Δ HAM-D24	Δ <i>SIRT1</i> and Δ HAM-D24
Psychosis			
Yes (<i>n</i> =22)	$r = -0.14, p=0.53$	$r = 0.14, p=0.53$	$\rho = -0.24, p=0.30$
No (<i>n</i> =69)	$\rho = -0.11, p=0.36$	$\rho = -0.12, p=0.38$	$r = 0.14, p=0.25$
Polarity			
Bipolar (<i>n</i> =19)	$r = -0.20, p=0.49$	$r = 0.29, p=0.23$	$r = -0.32, p=0.19$
Unipolar (<i>n</i> =72)	$\rho = -0.04, p=0.75$	$r = -0.05, p=0.71$	$r = -0.08, p=0.50$
Responder			
Yes (<i>n</i> =56)	$\rho < 0.01, p=0.99$	$r = 0.06, p=0.66$	$r = -0.34, p=0.01$
No (<i>n</i> =35)	$r = -0.03, p=0.70$	$r = -0.10, p=0.57$	$r = 0.30, p=0.08$
Remission			
Yes (<i>n</i> =47)	$\rho = 0.04, p=0.79$	$r = -0.02, p=0.92$	$r = -0.34, p=0.02$
No (<i>n</i> =44)	$\rho = -0.09, p=0.56$	$r = 0.03, p=0.84$	$\rho = 0.23, p=0.13$

Table 4.7 Clinical subgroup correlations between *SIRT1* mRNA levels and HAM-D-24 scores

Abbreviations: HAM-D24, Hamilton Depression Rating Scale, 24-item; Δ *SIRT1*, change in *SIRT1* pre/post ECT; Δ HAM-D24, change in HAM-D24 score pre/post ECT.

4.4.6 The effect of ECS on *Sirtuin1* mRNA levels in the rat brain and peripheral whole blood

To determine if an effect of ECS in the brain might translate to the periphery, quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate the effect of acute (single) and chronic (10 sessions) ECS on rat *Sirt1* mRNA levels in the hippocampal formation, dentate gyrus, frontal cortex, cerebellum, and peripheral whole blood. Neither acute nor chronic ECS had an effect on *Sirt1* mRNA in the dentate gyrus, frontal cortex, cerebellum and peripheral whole blood. In the hippocampal formation, both acute and chronic ECS significantly increased *Sirt1* mRNA levels in the dentate gyrus. Full data can be found in Table 4.8 and Figure 4.8 below.

		Control	Acute ECS	Control	Chronic ECS
Hippocampal Formation	<i>n</i>	8	8	8	7
	mean	1.00	1.24	1.00	1.25
	SD	0.07	0.12	0.06	0.10
	SEM	0.02	0.04	0.02	0.04
	<i>p</i> value		<0.001		<0.0001
Dentate Gyrus	<i>n</i>	8	8	8	7
	mean	1.00	1.02	1.00	0.92
	SD	0.11	0.11	0.16	0.12
	SEM	0.04	0.04	0.06	0.04
	<i>p</i> value		0.88		0.28
Frontal Cortex	<i>n</i>	8	8	8	7
	mean	1.00	1.01	1.000	1.07
	SD	0.12	0.21	0.08337	0.10
	SEM	0.04	0.07	0.03	0.04
	<i>p</i> value		0.88		0.19
Cerebellum	<i>n</i>	8	8	8	7
	mean	1.00	1.03	1.00	0.94
	SD	0.10	0.08	0.12	0.08
	SEM	0.04	0.03	0.04	0.03
	<i>p</i> value		0.58		0.26
Peripheral whole blood	<i>n</i>	8	8	5	5
	mean	1.00	0.91	1.00	0.98
	SD	0.31	0.21	0.24	0.44
	SEM	0.11	0.08	0.11	0.20
	<i>p</i> value		0.28		0.94

Table 4.8 Data for the effect of ECS on *Sirt1* mRNA levels in rat brain and peripheral blood

Abbreviations: ECS, electroconvulsive stimulation; SD, standard deviation; SEM, standard error of the mean.

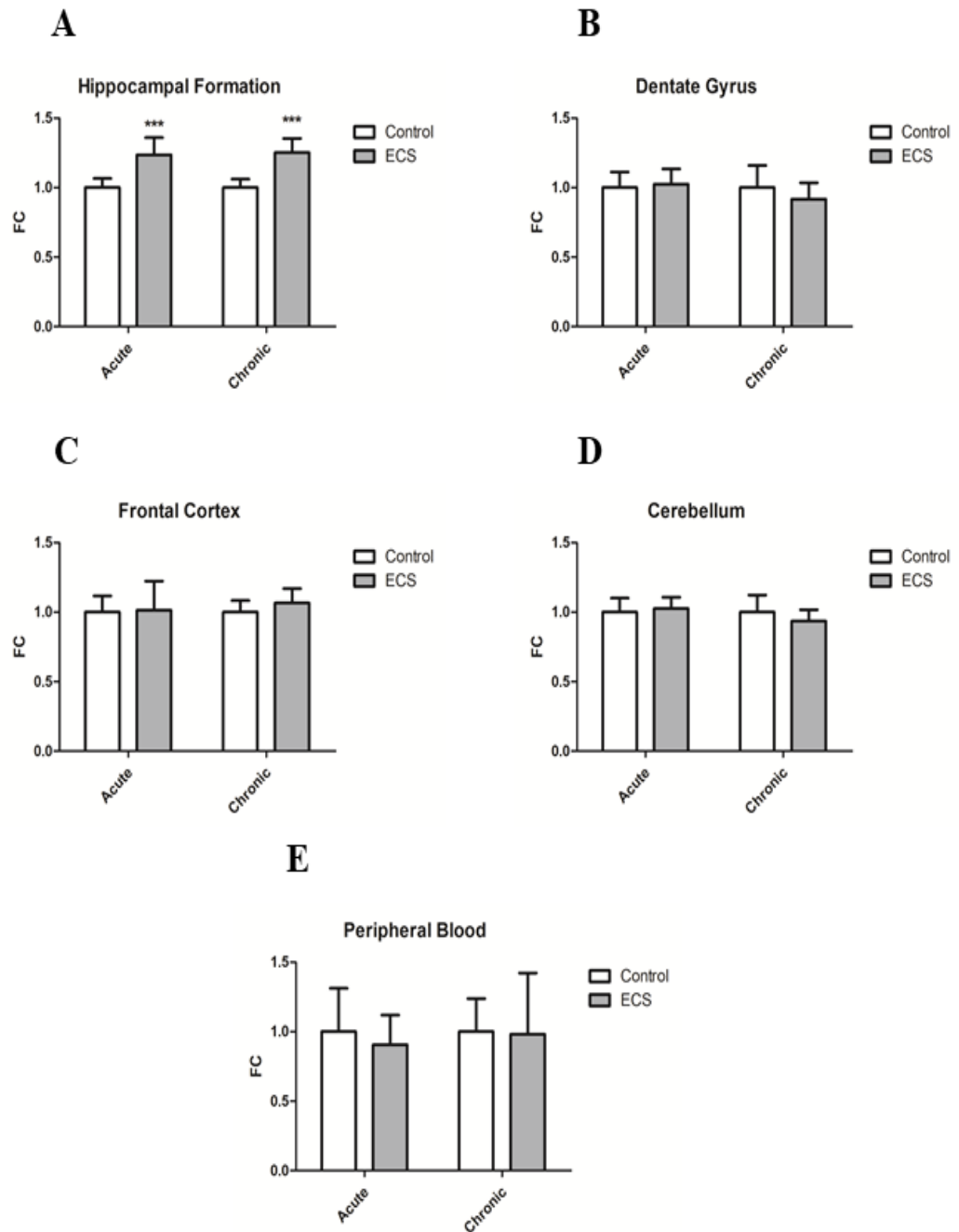


Figure 4.8 The effect of ECS on *Sirt1* mRNA animals in the rat brain and peripheral whole blood

All data is presented as mean $\pm$ SD, $n = 5-8$. *** represents a $p < 0.001$ as measured by a student t-test. Abbreviations: ECS, electroconvulsive stimulation; FC, fold change.

4.5 Discussion

4.5.1 Results summary

As far as I am aware, this study is the first to examine the effect of a course of ECT on *SIRT1* mRNA levels in a relatively large cohort of well characterised depressed patients. *SIRT1* mRNA levels were found to be lower in depressed patients in comparison to healthy controls. However, ECT had no effect on these levels. There were no correlations between *SIRT1* levels at baseline and mood ratings, assessed using the HAM-D24, in the group as a whole. There was a trend for negative correlations between the change in *SIRT1* levels and change in HAM-D24 scores in both ECT responders and remitters. In addition, I investigated the effect of the presence of psychosis on *SIRT1* mRNA levels, owing to my lab's previous finding (Kolshus et al., 2017); however, there were no differences in levels in patients with and without psychosis. The effect of depression polarity on *SIRT1* levels was also assessed, *SIRT1* was lower in patients with bipolar depression pre-ECT in comparison to patients with unipolar depression, but this was not statistically significant after correcting for covariates. ECS significantly increased *Sirt1* mRNA in the hippocampal formation but not in the peripheral blood or any other brain region.

4.5.2 *Sirtuin1* mRNA levels are significantly lower in patients with depression

This finding of lower levels of *SIRT1* mRNA in depressed patients in comparison to healthy controls is in line with previous studies (Abe et al., 2011, Luo and Zhang, 2016, Wang et al., 2018b). This also corresponds with animal studies showing that inhibition of SIRT1 leads to a depressive-like phenotype (Abe-Higuchi et al., 2016). However, AUC curve analysis indicated that *SIRT1* mRNA level would not be a good clinical biomarker when tested on its own. As depression is quite a heterogeneous disorder, it is

unlikely one biological marker alone would be sufficient to test for depression. Nonetheless, my finding contributes to the research that indicates dysregulated SIRT1 in depression. Of the covariates included in our analysis only age was found to have a significant effect on *SIRT* mRNA levels. Correlation analysis revealed a significant negative association between age and *SIRT1* mRNA levels, indicating increased age was associated with lower *SIRT1* mRNA levels ($r=-0.25$, $p=0.001$). This is in line with previous literature that reported SIRT genes were downregulated in peripheral blood mononuclear cells of aging humans (Owczarz et al., 2017).

It is unclear how decreased *SIRT1* levels could lead to depression but there is a lot of evidence linking SIRT1 to other systems that have been shown to be altered in depression.

In animal studies, inhibition of SIRT1 protein and the *Sirt1* gene, both pharmacologically and by the adeno-associated virus (AAV) mediated gene transfer system, results in a depressive phenotype that is associated with hippocampal atrophy (Abe et al., 2011). The author's also reported that chronic stress can reduce SIRT1 protein levels (Abe-Higuchi et al., 2016). Sensitivity to stress has been clinically linked to depression, with stress being linked to structural changes in the hippocampus (MacQueen and Frodl, 2011). A meta-analysis has also shown reduced hippocampal volumes in depressed patients (Campbell et al., 2004). Animal data showing the role of SIRT1 in protecting against neuronal atrophy, and clinical data of reduced *SIRT1* mRNA in depression, could therefore implicate this mechanism in depression.

In the periphery, SIRT1 has been shown to be protective in oxidative stress and has roles in mitochondrial function (Brenmoehl and Hoeflich, 2013, Tang, 2016). In recent years there have been implications that oxidative stress and mitochondrial

dysfunction have a role in mood disorders (Bansal and Kuhad, 2016). For example, lower levels of endogenous anti-oxidants and their enzymes have been found in depressed patients, with another meta-analysis showing altered oxidative stress markers in depression, indicating a lower anti-oxidant capacity (Liu et al., 2015). As well as increased oxidative and nitrosative stress, reduced mitochondrial function (reduced mitochondrial DNA) has been reported in depressed patients (Chang et al., 2015). This increased stress can reduce the availability of NAD⁺, which is necessary for SIRT1 function (Anderson, 2018). SIRT1 has been shown to regulate peroxisome proliferator-activated receptor gamma (PGC1- α), a master regulator of mitochondrial function, this could therefore lead to the reduced mitochondrial function seen in depression and could explain the reduced *SIRT1* mRNA levels in patients (Guo et al., 2014). Of note, *PGC1- α* mRNA has also been reported to be lower in patients with depression (Ryan et al., 2018b). Increased oxidative and nitrosative stress is also associated with decreased telomere length (Anderson, 2018) and SIRT1 has been shown to be a positive regulator of telomere length (Palacios et al., 2010). Decreased telomere length has been reported in depressed patients (Ridout et al., 2015). Therefore, reduced SIRT1 levels could play many roles in the pathways linked to depression.

4.5.3 The effect of ECT on *Sirtuin1* mRNA levels

Contrary to what I expected, ECT had no effect on *SIRT1* mRNA levels in the peripheral blood of patients with depression. There was a negative association between a change in *SIRT1* levels and a change in HAM-D24 scores in ECT responders and remitters, indicating that in patients who respond to ECT and attain remission, as your *SIRT1* mRNA levels increase, your HAM-D24 score decrease. This could implicate *SIRT1* in ECT response and may provide a marker for monitoring response to ECT. However, these

correlations were exploratory and replication of this study in a larger group of responders/remitters would be required for a more accurate interpretation of these findings.

There are several possible reasons why *SIRT1* mRNA levels did not change with treatment as expected. The source of *SIRT1* mRNA in peripheral whole blood samples is unknown. To my knowledge, there have been no studies to demonstrate that SIRT1 protein or mRNA can cross the blood brain barrier (BBB). In the CNS, SIRT1 protein and *SIRT1* mRNA expression has been shown in most areas of the human brain. SIRT1 protein has also been detected in cerebral spinal fluid (CSF) (Jayasena et al., 2016). However, to my knowledge, no studies have examined SIRT1 levels in the CSF. SIRT1 is located in the nucleus of most peripheral cells (Michan et al., 2010). Therefore, ECT may not affect peripheral cells but could be having a response in the CNS that may not translate to the periphery.

Only one animal study, using mice, to date has examined the effect of ECS on *Sirt1* mRNA levels (Chung et al., 2013). The authors reported that a single ECS increased SIRT1 immunoreactivity in the hippocampus and hypothalamus, with levels peaking at 2–8 hours and returning to normal after 24 hours. However, a limitation of my study is that end of treatment bloods were taken 1–3 days after the last ECT. Thus, if there is only a very brief transient increase in *SIRT1* mRNA following ECT a response may have been missed. There may also be a change in SIRT1 protein or activity levels in response to ECT and future studies are needed to investigate this.

4.5.4 *SIRT1* mRNA levels and psychosis

As mentioned, the main reason for exploring *SIRT1* levels in depression was the result of a deep sequencing study and gene target analysis that showed altered levels of two micro-RNAs (miRNAs) in patients with psychotic depression (miR-126-3p and miR-106-5p). These miRNAs were elevated at baseline in psychotic depression and returned to control levels following ECT (Kolshus et al., 2017). However, as with *E2F1* in Chapter 3, no statistically significant difference in *SIRT1* levels between the non-psychotic and psychotic groups at baseline or after ECT was found. Reduced *SIRT1* at baseline is what might be expected from miRNA regulation. Typically, miRNAs reduce levels of mRNA and protein (Orang et al., 2014), and these miRNAs were increased at baseline in our patients. The miRNA levels returned to control level following ECT but the *SIRT1* levels did not. One possible reason for this is that *SIRT1* is also associated with other miRNAs that have been linked to depression, miR-134 and miR-155 for example (He et al., 2014, Wang et al., 2018b) (Dwivedi, 2014, Gao et al., 2010); therefore, it may not be specific to psychotic depression, and may not be directly regulated by miR-126-3p and miR-106-5p in depression.

4.5.5 *Sirtuin1* mRNA levels and polarity of depression

Baseline *SIRT1* levels were lower in bipolar depressed patients compared to unipolar depressed patients, but this was no longer significant after adjusting for relevant covariates. To my knowledge, this is the first report of reduced *SIRT* mRNA levels in bipolar patients. A previous study analysed *SIRT1* mRNA in both major depressive disorder (MDD) and bipolar disorder (BD) patients in comparison to controls and reported them both to be lower but they did not directly compare the MDD vs BD groups although based on their reported graphs/results the BD group had slightly lower levels

than the MDD group (Abe et al., 2011). For my group of patients with bipolar depression the sample size was small ($n = 19$). Therefore, the study may not have had the power to detect statistically significant differences after adjusting for all potential covariates. Therefore, it would be informative to replicate this study with a larger number of bipolar depressed patients, as it could potentially represent a peripheral blood difference in patients with bipolar or unipolar depression.

3.1.1 The effect of ECS on *Sirtuin1* mRNA levels in the rat brain

As I wanted to investigate if ECS has an effect on *Sirt1* mRNA levels in different brain regions, and if changes might also be detectable at a peripheral level, I measured *Sirt1* mRNA levels in the hippocampal formation, dentate gyrus, frontal cortex, cerebellum, and peripheral whole blood of naïve rats treated with acute (single) or chronic (10 sessions) ECS. ECS (both chronic and acute) significantly increased *Sirt1* mRNA levels in the hippocampal formation in comparison to rats who received sham ECS, but no other changes were detected including in peripheral whole blood. This finding may support my theory that ECT may act specifically on *Sirt1* in the brain and may not have a peripheral impact.

I found that the effect of ECS on *Sirt1* mRNA was specific to the hippocampal formation. As far as I am aware, only one study to date has examined the effect of ECS on SIRT1 protein expression in rodents (Chung et al., 2013). This study reported increased *Sirt1* immunoreactivity in the mouse hippocampal CA1, CA2, CA3 and dentate gyrus at 2 hour and 8 hour post ECS (acute). Increased immunoreactivity in the hypothalamus was also reported. Based on the results of this study (Chung et al., 2013), I would have expected the increase I detected in the hippocampal formation. However, I

also would have expected a change in *Sirt1* mRNA levels in the dentate gyrus. One potential explanation for this is that Chung *et al.* (2013) reported a change in protein where as I analysed mRNA and mRNA changes may not reflect protein changes. Differences may also occur in the mouse and rat response to ECS. No differences were detected in any other brain region. As far as I am aware, no other study has looked at the effect of any treatment on SIRT1 protein or mRNA in these regions. Therefore, there are no results to compare my finding to and it is possible that changes in SIRT1 are very specific to the hippocampus, and as reported by Chung *et al.*, (2013) the hypothalamus. Overall, these results indicate a role for SIRT1 within the hippocampus in the molecular response of ECS.

4.5.6 Study limitations and future studies

The main limitation of this study is that the EFFECT-Dep study was not originally designed for molecular analysis. Therefore, recruitment did not take into consideration the power needed to statistically detect changes in gene levels between patients and controls. However, the numbers for the whole group (patient and control) are relatively large, and are bigger than previous studies that detected statistically significant differences in *SIRT1* mRNA levels (Abe *et al.*, 2011, Luo and Zhang, 2016). However, our subgroups have low numbers, particularly the bipolar group ($n = 19$), and are likely not powered to detect differences, especially when controlling for covariates.

Another limitation of this study is that the patients are already on stable doses of pharmacological treatment prior to starting ECT and this could be affecting *SIRT1* levels. However, previous studies have showed that unmedicated patients had lower *SIRT1* mRNA levels compared to non-depressed controls (Luo and Zhang, 2016, Wang *et al.*,

2018b). Therefore, lower *SIRT1* levels in patients may more likely be a feature of depression and not a result of antidepressant treatment. One of these studies also reported that *SIRT1* mRNA levels increased with citalopram treatment (Wang et al., 2018b). Therefore, other pharmacological treatments could still influence *SIRT1* expression and affect response to ECT. It would therefore be instructive to examine the effect of ECT or other treatments on *SIRT1* levels in patients who are antidepressant drug naive.

4.6 Conclusion

In conclusion, I found that peripheral *SIRT1* mRNA levels were significantly lower in depressed patients in comparison to healthy controls, and ECT did not affect *SIRT1* mRNA levels. There was a trend for a correlation between a change in *SIRT1* levels and a change in HAM-D24 scores in patients who responded to ECT, and in those who attained remission. Future studies are required to investigate changes in SIRT1 protein or activity in depression as these changes might be less transient than mRNA and might tell us more about the role of SIRT1 in ECT response. There was also a trend towards lower levels of *SIRT1* in bipolar depressed patients in comparison to unipolar depression that merits further investigation. In the rat brain ECS significantly increased *Sirt1* mRNA in the hippocampal formation, indicating a role for SIRT1 in the mechanism of ECS in the brain. Overall, clinically in patients, the results of this study indicate that reduced peripheral blood *SIRT1* mRNA could be a trait marker of depression.

Chapter 5.

Characterisation of PEDF in primary cells from rat brain and the effects of antidepressants on PEDF

5.1 Introduction

Pigment Epithelium-Derived Factor (PEDF) is a 50 kDA glycoprotein of the serine protease inhibitor family that is widely expressed in both the CNS and periphery (Tombran-Tink and Barnstable, 2003). It was first discovered in the medium of human retinal pigment epithelium (RPE) cell cultures (Tombrantink et al., 1991), with subsequent studies reporting its neuroprotective effects and regulation of other neuroprotective factors such as brain-derived neurotrophic factor (BDNF) (DeCoster et al., 1999, Zheng et al., 2010, Yabe et al., 2001).

PEDF has been recently implicated in depression by a previous proteomics study from my lab (Ryan et al., 2017). Before this, two other studies had linked PEDF to depression. The first, a proteomic study of cerebrospinal fluid (CSF) from depressed patients and controls identified altered PEDF, with results from two dimensional polyacrylamide gel electrophoresis showing reduced PEDF protein in patients, and results from mass spectrometry showing increased PEDF protein in patients with depression (Ditzen et al., 2012). The second study reported that chronic fluoxetine treatment increased *Serpinf1* (PEDF) mRNA in the hippocampus of male mice (Miller et al., 2007). In the study reported by my lab patients had higher plasma levels of PEDF protein, which was further increased by ECT (Ryan et al., 2017). They also found that electroconvulsive stimulation (ECS) increased *Serpinf1* mRNA in the rat hippocampus. Investigating the effects of antidepressants on PEDF would therefore be of interest.

The impact of stress on synaptic plasticity in the brain has been studied as a model of depression for the past number of years, with the majority of evidence reported from animal models. Two rodent models in particular, chronic unpredictable stress and chronic restraint stress, have been reported to impair plasticity and long term potentiation (Joels et al., 2004, Alfarez et al., 2003, Pavlides et al., 2002). Although particularly well studied

in the rodent hippocampus, with long term potentiation (LTP) data focusing on the CA1, CA2 and CA3 subfield regions of the hippocampus, synaptic impairments are also reported in the pre-frontal cortex (PFC), thalamus and the amygdala with stress (Quan et al., 2011, Fogaca and Duman, 2019, Danielewicz et al., 2017). Additionally, stress induced alterations include changes in proteins and receptors, such as lowered gamma-aminobutyric acid (GABA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartic acid (NMDA) receptor subunit expression (Marsden, 2013, Fogaca and Duman, 2019).

In rodents antidepressant administration can reverse these impairments in plasticity and attenuate depressive-like behaviour (Levy et al., 2018). For example, imipramine, a tricyclic antidepressant (TCA), ameliorates the negative effect of maternal separation on LTP in rats (Danielewicz et al., 2017); furthermore, imipramine attenuates the deficits in LTP caused by chronic corticosterone injections in rats (Bobula et al., 2011). The selective serotonin reuptake inhibitor (SSRI) fluoxetine inhibits dendritic atrophy in the chronic mild stress model of depression in rats (Luo and Tan, 2001). Additionally, fluoxetine promotes neurogenesis to restore hippocampal regulation of the HPA-axis (Surget et al., 2011). Novel compounds used for antidepressant therapy, such as the rapidly acting antidepressant ketamine, also enhance neurogenesis and synaptogenesis through modulation of growth factors and possibly the reduction of pro-inflammatory cytokines (Deyama et al., 2018, Deyama et al., 2019, Clarke et al., 2017).

Therefore, when investigating a protein/signalling pathway in relation to an antidepressant response, neuronal plasticity in primary cultures is often used as a measurement of the protein's role in antidepressant efficacy and their neuroprotective effects (Deyama et al., 2018, Deyama et al., 2019, O'Neill et al., 2016). Astrocytes also express transporters for both noradrenaline (NA) and serotonin (5-HT), as well as NA

and 5-HT receptors (Inazu et al., 2003, Hirst et al., 1998, Marathe et al., 2018). Astrocytes provide trophic support to neurons through the release of several growth factors (Marathe et al., 2018). Antidepressants can therefore have direct effects on astrocyte function, possibly by increasing the levels of growth factors secreted by astrocytes (Marathe et al., 2018, Martin et al., 2013). For example: imipramine increased *Bdnf* mRNA in cultured rat astrocytes (Takano et al., 2012); fluoxetine increases *Bdnf* and *Vegf* mRNA expression in astrocytes (Allaman et al., 2011); and NA increases the secretion of BDNF and other growth factors from astrocytes (Day et al., 2014, Takano et al., 2012). Furthermore, the conditioned media (CM) from NA-treated astrocytes increased the complexity of primary immature rat cortical neurons (Day et al., 2014). Therefore, the effects of antidepressants on astrocyte function may lead to synaptic plasticity changes in neurons.

5.2 Aims and objectives

There is little known about the role of PEDF in the brain but, given its role in the protection of neurons (DeCoster et al., 1999), it is possible that PEDF is secreted by astrocytes to provide further trophic support to neurons. Therefore, the aim of this study was to characterise the mRNA expression of PEDF (*Serpinf1*) and the PEDF receptor (*Pnpla2*) in primary rat cortical neurons, microglia and astrocytes. Next, I aimed to determine the effects of antidepressant treatment on *Serpinf1* mRNA expression in the cell type that primarily expresses *SERPINF1*. Finally, I wanted to analyse the effect of antidepressants on PEDF protein secretion.

I hypothesised that *Serpinf1* would be expressed in astrocytes as with other neurotrophic factors (Marathe et al., 2018). *In vitro*, incubation with recombinant PEDF results in physiological effects in neurons, astrocytes and microglia (Yabe et al., 2005,

Sanagi et al., 2005, Yabe et al., 2001), indicating the PEDF-R might be expressed by all three cell types. Receptors for other neurotrophic factors are also expressed on neurons, astrocytes and microglia (Gupta et al., 2013, Honda et al., 1999, Rudge et al., 1994, Sarabi et al., 2003). Therefore, I hypothesised all three cell types would express *Pnpla2*.

Based on the results of the proteomic study from my lab and the data reporting increased *SERPINF1* mRNA in response to fluoxetine treatment (Ryan et al., 2017, Miller et al., 2007), I hypothesised that antidepressant treatment would increase *Serpinf1* mRNA expression and PEDF secretion from astrocytes.

5.3 Methods

5.3.1 Primary cell culture

Primary cultures for enriched astrocytes, enriched microglia and primary cortical neurons were prepared following protocols published in studies by colleagues in Trinity College Institute of Neuroscience (Day et al., 2014, McNamee et al., 2010) and are described in detail in Chapter 2, section 2.2.3.4.

In brief, primary glial cultures were prepared from 2-3 day-old male Wistar rats. Cortical tissue was isolated in Dulbecco's Modified Eagle Medium (F12) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin for 20 minutes at 37°C (cDMEM). The tissue was then triturated and filtered. This cell suspension was centrifuged and the pellet was re-suspended in cDMEM. Cells from one neonate were seeded in T75 flasks. Following 10-14 days in vitro (DIV), astrocytes or microglia were separated and seeded in 24 well plates at 1×10^5 or 5×10^4 cell/ml respectively.

Primary neuronal cultures were prepared from 0-1 day old male Wistar rats. Cortical tissue was dissociated and incubated in trypsin-EDTA for 2 min at 37°C.

cDMEM was added and tissue was triturated, followed by centrifugation. The pellet re-suspended in cDMEM filtered and centrifuged. The supernatant was discarded and the pellet re-suspended in neurobasal media complete with 1% Glutamax, 1% penicillin/streptomycin and 1% B-27. Neurons were seeded at 1×10^5 cells/ml on poly-D-lysine-coated coverslips in 24-well plates for RNA analysis. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C. This protocol has been reported to yield 95% pure astrocytes and microglia, with 97% pure neurons (McNamee et al., 2010).

5.3.2 Cell treatments

Stock drugs were dissolved to their desired concentrations using DMEM:F12, and control wells received DMEM:F12 alone. For the effects of the different drugs on *Serpinf1* mRNA five independent experiments were carried out ($n = 5$). For protein analysis, samples from 6-7 independent experiments were carried out due to variability with the assay ($n = 6-7$). The dose of noradrenaline was chosen based on previous work by another lab in TCIN (Day et al., 2014). Imipramine doses were based on previous published work (Hwang et al., 2008, Obuchowicz et al., 2014), and the fluoxetine doses were chosen based on previous culture studies, as well as data from a human study that reported the concentrations of fluoxetine that reach the brain after treatment (Vasu et al., 2016, Miner et al., 1995). To my knowledge there were no comparison studies on a time point for PEDF analysis. I therefore conducted a time course based on treatment times for other neurotrophic factors factors (Allaman et al., 2011, Kajitani et al., 2012). All cells were treated when confluent and healthy in the 24 well plates (2-3 days after plating).

5.3.3 Viability assays

Viability assays were performed to determine non-toxic doses of each treatment. The alamarBlue assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the Pierce™ Lactate Dehydrogenase (LDH) cytotoxicity assay were performed as described in section 2.2.3.6. data were then presented as a percentage of live cells in comparison to the control treatment group.

5.3.4 Gene expression analysis

After treatment with the different drugs for various time points, RNA was collected and gene expression analysis was carried out as described in Chapter 2. RNA isolation is described in section 2.2.3.7, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR) are described in section 2.2.1.5 and 2.2.1.6. A full list of primers used is given in Table 2.5. For all experiments RNA was equalised to 20ng/μl where possible; if not, RNA was equalised to the same concentration as the sample with lowest RNA content. All data in gene expression graphs are expressed as a fold change (FC) relative to the control group.

5.3.5 Enzyme-linked immunosorbent assay

For secreted protein analysis in cell supernatants, cDMEM was removed from wells, cells were washed in PBS, and then fresh medium without fetal bovine serum was added to the wells to prevent background signal in the ELISA. Cells were then treated and supernatant collected. The ELISA was carried out as described in chapter 2, section 2.2.3.11. For imipramine, treatment time was based on the gene analysis experiments. For noradrenaline treatment, a time course was conducted based on a previous study on

the effect of noradrenaline on PEDF secretion from retinal pigment epithelium cells (Lashbrook and Steinle, 2005), my own gene expression results and also previous work on noradrenaline and growth factors (Day et al., 2014).

5.3.6 Statistical analysis

All cell culture experiments were analysed using GraphPad Prism version 5 (GraphPad Prism, California, USA). Graphs were also created using GraphPad. A Kolmogorov–Smirnov normality test was performed on data. For experiments with less than 5 independent samples non-parametric statistics were used as a normality test was not possible. For parametric analyses, student t-tests or one-way analysis of variance (ANOVA) was used where appropriate. When significance was detected with the ANOVA, a Dunnett’s post-hoc test was performed. For non-parametric data a Mann-Whitney U test or a Kruskal-Wallis ANOVA was used; when significance was detected with the ANOVA a Dunn’s post-hoc test was performed. A p value of less than 0.05 was deemed statistically significant. All data are expressed as mean $\pm$ standard deviation (SD).

5.4 Results

5.4.1 Confirmation of primary neuron, microglia and astrocyte purity

The purity of neurons, microglia and astrocytes was assessed using qRT-PCR. Gene expression analysis was carried out for cell specific markers in all cell types. Beta-III tubulin (*Tubb3*) for neurons, glial fibrillary acidic protein (*Gfap*) for astrocytes and cluster of differentiation molecule 11b (CD11B, *Itgam*) for microglia (Katsetos et al., 1998, Cahoy et al., 2008, Martin et al., 2017), see Figure 5.1. *Tubb3* mRNA was highly

expressed in neurons (RQ: 0.74 ± 0.12), with very low expression detectable in microglia (RQ: $0.0003991 \pm <0.001$) and astrocytes (RQ: $0.004439 \pm <0.001$). Astrocytes had high expression of *Gfap* mRNA (RQ: 1.062 ± 0.26), with low expression levels in neurons (0.2339 ± 0.07) and microglia (RQ: 0.003610 ± 0.002). Lastly, the highest expression of *Itgam* was found in microglia (0.9546 ± 0.04) with low expression in both neurons (0.01173 ± 0.007) and astrocytes (0.02125 ± 0.003). This shows that the cell isolation protocol worked as previously described and these cultures were reasonably enriched.

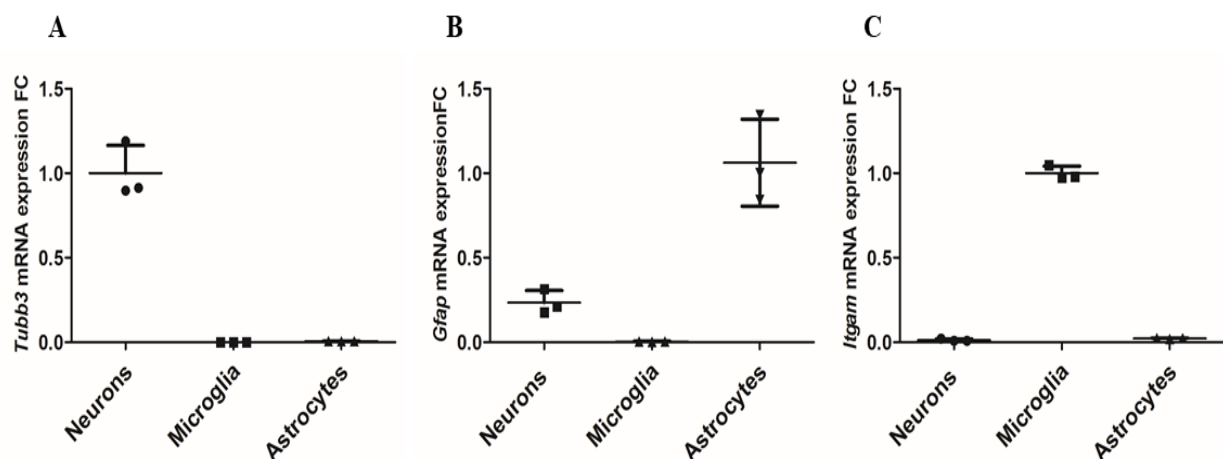


Figure 5.1 Confirmation of primary cell purity

Gene expression analysis with qRT-PCR of (A) beta-tubulin III (*TUBB3*) with neurons as the control group, (B) *Gfap* with astrocytes as the control group and (C) CD11B (*Itgam*) with microglia as the control group. All data are expressed as mean $\pm$ SD, $n = 3$. Abbreviations: FC; fold change relative to the average of the control group.

5.4.2 Characterisation of PEDF (*Serpinf1*) and PEDF-R (*Pnpla2*) mRNA expression in the different primary cell types

As far as I am aware, there are no reports to date of the cell source of PEDF or its receptor in the brain. qRT-PCR was carried out to determine the expression *Serpinf1* and *Pnpla2* mRNA in rat cortical primary neurons, microglia and astrocytes (Figure 5.2). Astrocytes had the highest expression of *Serpinf1* mRNA (RQ: 11.12 ± 3.7), followed by neurons

(0.9508 ± 0.11) with very low expression in microglia (RQ: 0.02398 ± 0.02). Both astrocytes and microglia had high expression of (*Pnpla2*) mRNA (RQ: 1.703 ± 0.31 and 1.568 ± 0.16 respectively), with a relatively high expression in neurons also detected (RQ: 0.8865 ± 0.11).

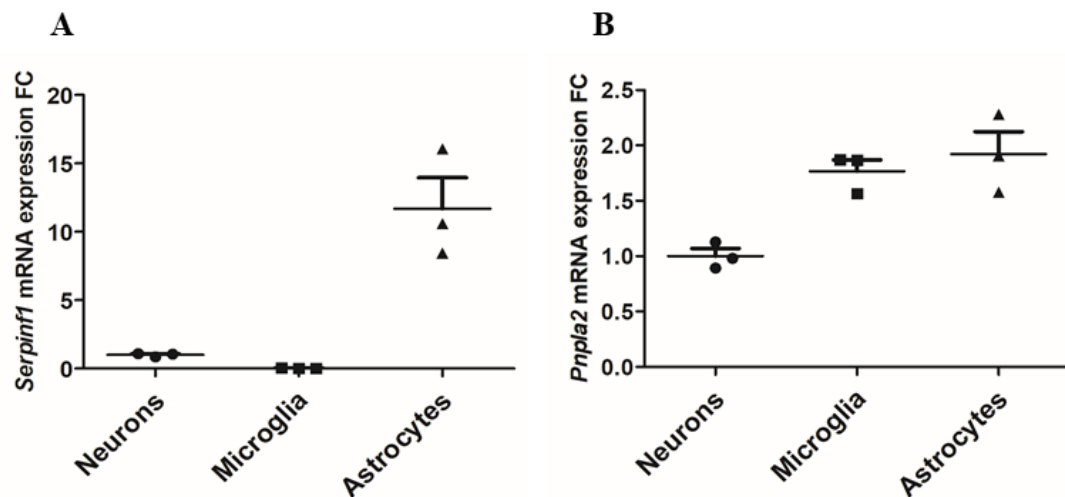


Figure 5.2 Characterisation of PEDF and PEDF-R expression in the different primary cell types.

(A) *Serpinf1* and (B) *Pnpla2* gene expression was measured by qRT-PCR in primary neurons, microglia and astrocytes. All data is expressed as mean $\pm$ SD, $n = 3$. Abbreviations: FC; fold change, with neurons used as the control group.

5.4.3 The effect of treatment on astrocyte cell viability

To ensure my treatments were not causing a reduction in cell viability or cell death three viability assays were used.

5.4.3.1 The effect of fluoxetine on astrocyte cell viability

An AlamarBlue assay confirmed that 24 hours of $45\mu\text{M}$ fluoxetine treatment significantly reduced astrocyte cell viability (with $85 \pm 5.0\%$ cell viability detected in comparison to control; Figure 5.3A), with no significant effect of $5\mu\text{M}$ and $15\mu\text{M}$ Fluoxetine treatment ($91 \pm 3.6\%$ and $89 \pm 2.1\%$ cell viability in comparison to control respectively). A similar

result was found with the MTT assay: $93.7 \pm 9.3\%$ viability was detected with $5\mu\text{M}$ Fluoxetine and $86.7 \pm 3.8\%$ with $15\mu\text{M}$ Fluoxetine treatment (Figure 5.3B). $45\mu\text{M}$ fluoxetine treatment significantly decreased cell viability to $54.0 \pm 5.3\%$ in comparison to control treatment. However, the LDH assay, which is a direct measurement of cell death, reported no decrease in viability with any treatment dose of fluoxetine ($5\mu\text{M}$: $91.1 \pm 14.0\%$, $15\mu\text{M}$: $91.5 \pm 5.3\%$, $45\mu\text{M}$: $78.0 \pm 7.5\%$. Figure 5.3C).

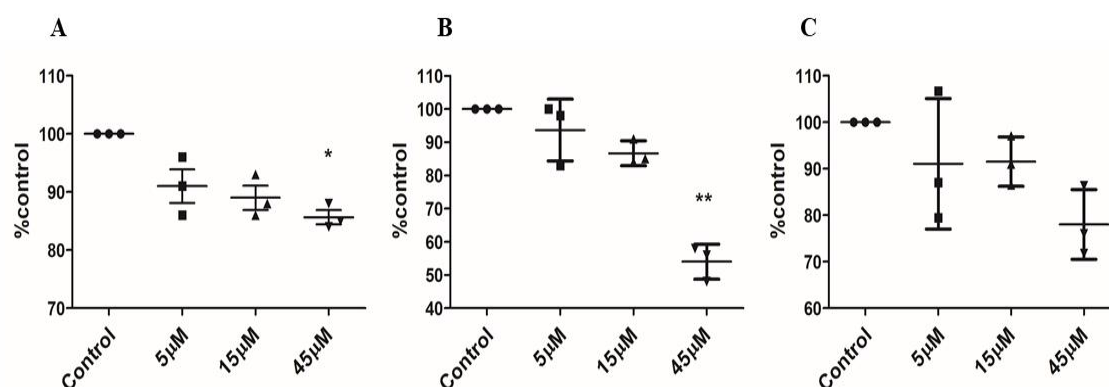


Figure 5.3 The effect of fluoxetine treatment on astrocyte cell viability

Astrocyte cell viability was measured after treatment with three doses of fluoxetine for 24 hours using (A) AlamarBlue assay, (B) MTT assay and (C) the LDH assay. All data are represented as mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$ vs. control group, as analysed by a Kruskal-Wallis test and Dunn's post-hoc test.

5.4.3.2 The effect of imipramine on astrocyte cell viability

An alamarBlue, MTT and LDH assay confirmed that 24 hours of $100\mu\text{M}$ imipramine treatment significantly reduced cell viability, with no significant changes at any other time point, (Figure 5.4). AlamarBlue data showed that: $10\mu\text{M}$ imipramine reduced viability to $82.0 \pm 4.0\%$; $30\mu\text{M}$ imipramine reduced viability to $81.3 \pm 4.9\%$; and $100\mu\text{M}$ imipramine significantly decreased viability to $73.3 \pm 4.9\%$ in comparison to control treatment. MTT data showed that: $10\mu\text{M}$ imipramine reduced viability to $65.5 \pm 4.5\%$;

30 μ M imipramine reduced viability to $66.0 \pm 4.6\%$; and 100 μ M imipramine significantly decreased viability to $45.7 \pm 3.2\%$ in comparison to control treatment. The LDH assay showed a similar result with: 10 μ M imipramine decreasing viability to $91.7 \pm 14.8\%$; 30 μ M imipramine decreasing viability to $90.9 \pm 5.1\%$; and 100 μ M imipramine significantly decreased viability to $25.8 \pm 2.0\%$ in comparison to control treatment.

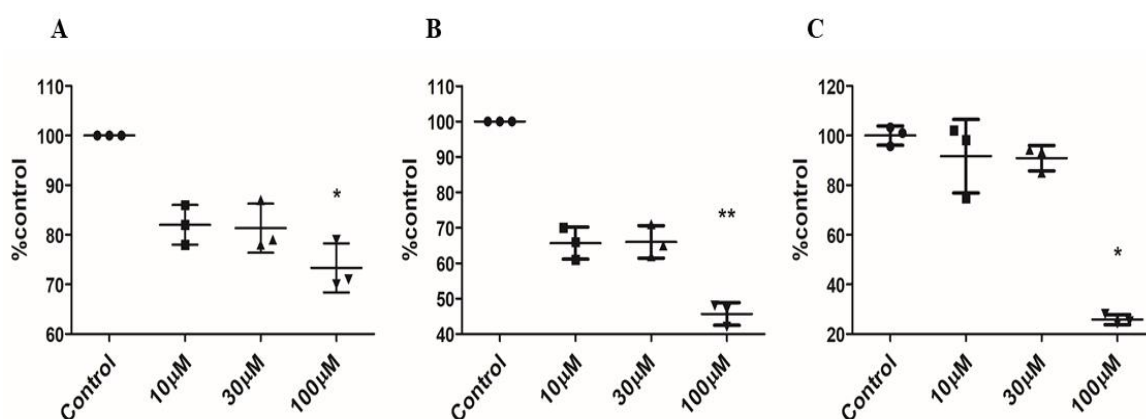


Figure 5.4 The effect of imipramine treatment on astrocyte cell viability.

Astrocyte cell viability was measured after treatment with three doses of imipramine for 24 hours using (A) alamarBlue assay, (B) MTT assay and (C) the LDH assay. All data are represented as mean $\pm$ SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$ vs. control group, as analysed by a Kruskal-Wallis test and Dunn's post-hoc test.

5.4.3.3 The effect of noradrenaline on astrocyte cell viability

An alamarBlue assay confirmed no dose of noradrenaline significantly reduced astrocyte cell viability after 24 hours of treatment and a similar result was found with an MTT assay (Figure 5.5). AlamarBlue data showed that: 1 μ M noradrenaline increased viability to $105.9 \pm 9.9\%$; 10 μ M noradrenaline reduced viability to $92.9 \pm 9.0\%$; and 100 μ M noradrenaline reduced viability to $89.1 \pm 5.7\%$ in comparison to control treatment. Similarly, MTT data showed that: 1 μ M noradrenaline increased viability to $107.7 \pm 8.3\%$; 10 μ M noradrenaline reduced viability to $84.9 \pm 4.7\%$; and 100 μ M noradrenaline

significantly decreased viability to $78.2 \pm 11.2\%$ in comparison to control treatment. The LDH assay showed: an increase in viability with $1\mu\text{M}$ noradrenaline to $102.1 \pm 26.7\%$; $10\mu\text{M}$ slightly decreased viability to $82.3 \pm 15.4\%$; and $100\mu\text{M}$ decreased viability to $93.9 \pm 11.9\%$.

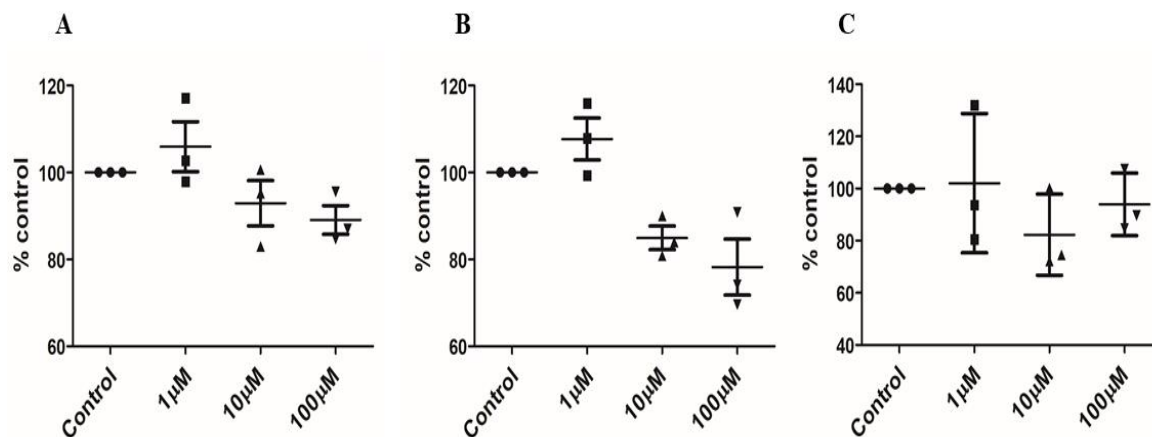


Figure 5.5 The effect of noradrenaline on astrocyte viability

Astrocyte cell viability was measured after treatment with three doses of imipramine for 24 hours using (A) alamarBlue assay, (B) MTT assay and (C) the LDH assay. All data are represented as mean $\pm$ SD, $n = 3$.

5.4.4 Fluoxetine treatment has no effect on PEDF (*Serpinf1*) mRNA levels in astrocytes

Cortical primary rat astrocytes were cultured in 24 well plates until ready to treat. Cells were then treated with DMEM:F12 (control), $5\mu\text{M}$ fluoxetine or $15\mu\text{M}$ fluoxetine for 30 minutes, 3 hours, 6 hours, 12 hours or 24 hours and RNA was collected for mRNA analysis. Fluoxetine had no statistically significant effect on *Serpinf1* mRNA at any dose or time point. A summary of the raw data is given in Table 5.1, with data given as a fold change of the average control from the 30 minute treatment group, $n = 5$. Data are also shown in Figure 5.6.

	Control	5μM fluoxetine	15μM fluoxetine	n	p value
30 minutes	1.00 ± 0.1	1.03 ± 0.2	1.04 ± 0.2	5	F(2) = 0.22, <i>p</i> = 0.81
1 hour	1.09 ± 0.2	1.06 ± 0.1	1.00 ± 0.1	5	H(2) = 0.78, <i>p</i> = 0.68
3 hour	1.02 ± 0.1	1.03 ± 0.1	0.98 ± 0.1	5	F(2) = 0.21, <i>p</i> = 0.81
6 hour	1.13 ± 0.2	1.09 ± 0.1	1.04 ± 0.09	5	F(2) = 0.73, <i>p</i> = 0.50
12 hour	1.06 ± 0.1	1.03 ± 0.1	1.03 ± 0.1	5	F(2) = 0.01, <i>p</i> = 0.90
24 hour	0.99 ± 0.2	1.03 ± 0.1	0.92 ± 0.1	5	F(2) = 1.31, <i>p</i> = 0.31

Table 5.1 Raw data of the effect of fluoxetine treatment on PEDF (*Serpinf1*) mRNA levels in astrocytes

Data presented as mean fold change ± standard deviation. Abbreviations: F; F ration for one-way analysis of variance, H; Kruskal-Wallis H test.

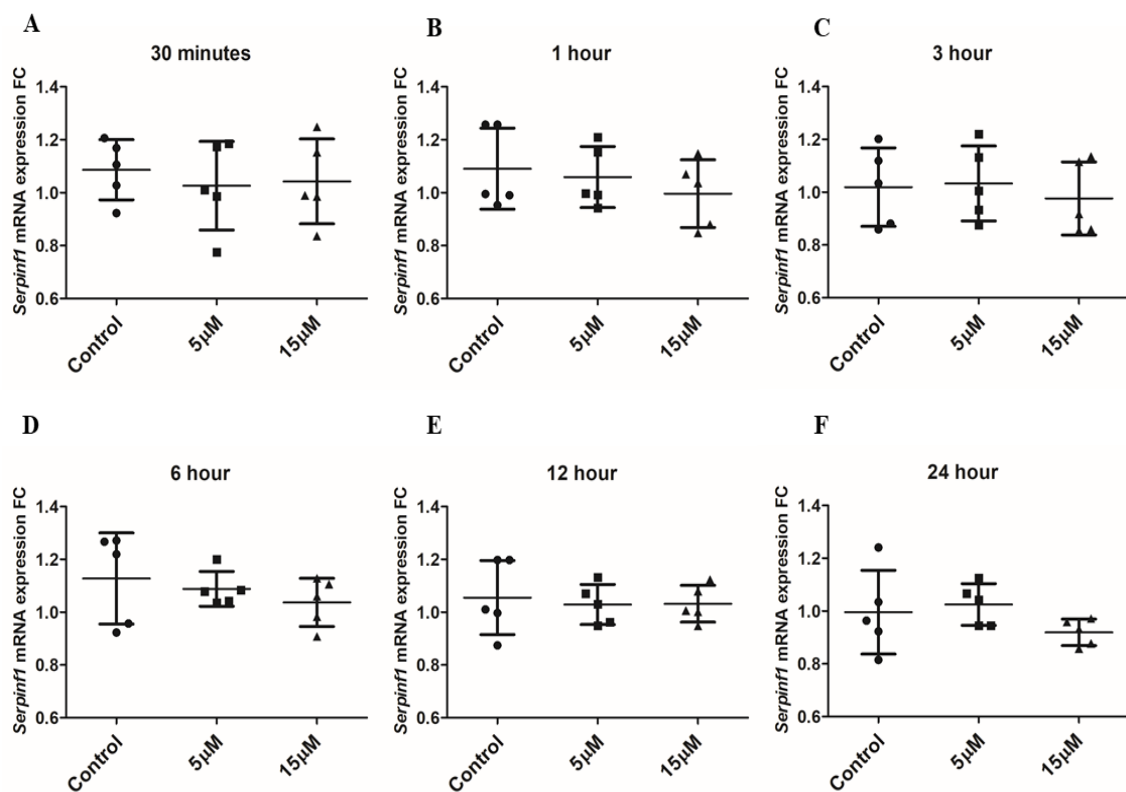


Figure 5.6 The effect of fluoxetine treatment on PEDF (*Serpinf1*) mRNA levels in astrocytes

Astrocytes were treated with 5 μM and 15 μM Fluoxetine for (A) 30 minutes, (B) 1 hour, (C) 3 hours, (D) 6 hours, (E) 12 hours, and (F) 24 hours. All data are expressed as mean fold change ± standard deviation, *n* = 5.

5.4.5 24 hour imipramine treatment decreases PEDF (*Serpinf1*) mRNA levels in astrocytes

Cortical primary rat astrocytes were cultured in 24 well plates until ready to treat. Cells were then treated with DMEM:F12 (control), 10 μ M imipramine or 30 μ M imipramine for 30 minutes, 3 hours, 6 hours, 12 hours or 24 hours and RNA was collected for mRNA analysis. Imipramine had no statistically significant effect on *Serpinf1* mRNA at any dose at any time point from 30 minutes to 12 hours. A statistically significant decrease in *Serpinf1* mRNA was found with both doses of imipramine at 24 hours ($F(2) = 47.43$, $p < 0.001$). A summary of the raw data is given in Table 5.2, with data given as a fold change of the average control from the 30 minute treatment group, $n = 4-5$. Data are also shown in Figure 5.7.

	control	10 μ M imipramine	30 μ M imipramine	n	p value
30 minutes	1.00 $\pm$ 0.1	1.06 $\pm$ 0.04	0.99 $\pm$ 0.03	5	$F(2) = 2.11$, $p = 0.16$
1 hour	0.98 $\pm$ 0.04	1.02 $\pm$ 0.1	0.99 $\pm$ 0.1	5	$F(2) = 0.21$, $p = 0.82$
3 hour	1.00 $\pm$ 0.1	1.08 $\pm$ 0.05	1.03 $\pm$ 0.1	4-5	$H(2) = 3.88$, $p = 0.14$
6 hour	0.90 $\pm$ 0.1	0.90 $\pm$ 0.2	0.86 $\pm$ 0.05	5	$H(2) = 3.42$, $p = 0.18$
12 hour	0.80 $\pm$ 0.03	0.79 $\pm$ 0.05	0.78 $\pm$ 0.04	5	$H(2) = 2.42$, $p = 0.30$
24 hour	0.87 $\pm$ 0.02	0.70 $\pm$ 0.1	0.62 $\pm$ 0.04	5	$F(2) = 47.43$, $p < 0.001$

Table 5.2 Raw data of the effect of imipramine treatment on PEDF (*Serpinf1*) mRNA expression in astrocytes

Data presented as mean fold change $\pm$ standard deviation. Abbreviations: F; F ratio for one-way analysis of variance, H; Kruskal-Wallis H test.

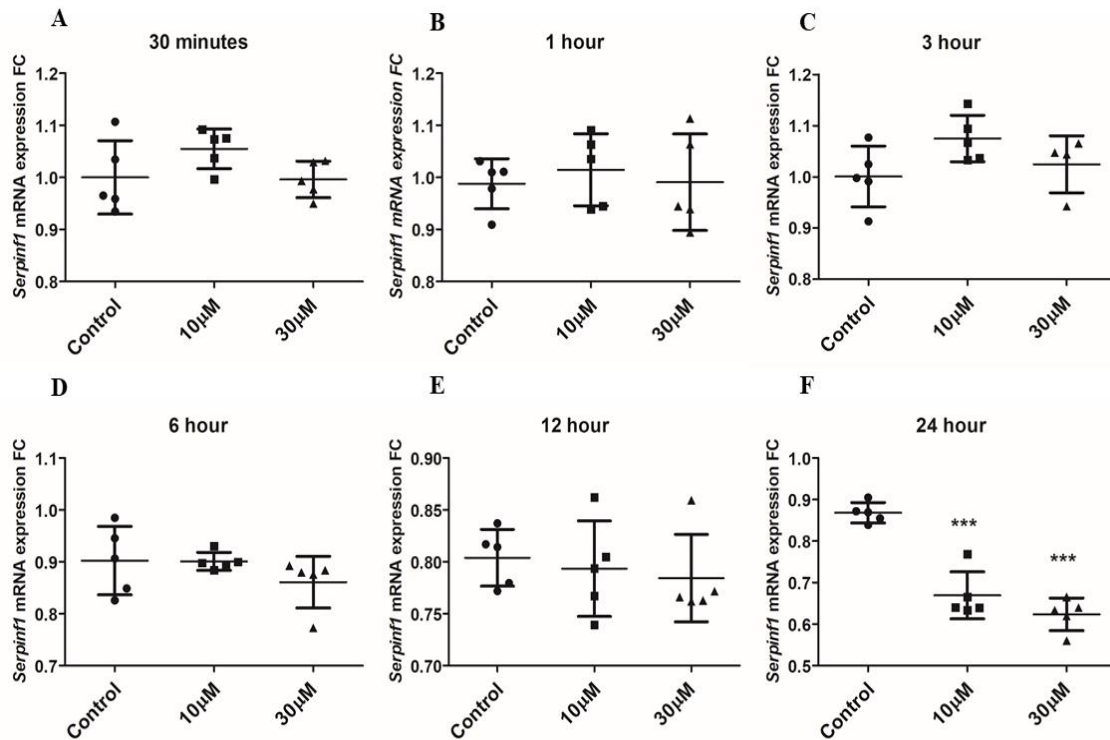


Figure 5.7 The effect of imipramine treatment on PEDF (*Serpinf1*) mRNA expression in astrocytes

Astrocytes were treated with 10 μ M and 30 μ M imipramine for (A) 30 minutes, (B) 1 hour (C) 3 hour, (D) 6 hour, (E) 12 hour, and (F) 24 hour. All data are expressed as mean fold change $\pm$ standard deviation, $n = 4-5$. *** $p < 0.001$, as analysed by a one way ANOVA and Dunnet's post-hoc test analysis.

5.4.6 3 hour and 12 hour noradrenaline treatment alters PEDF (*Serpinf1*) mRNA levels in astrocytes

Cortical primary rat astrocytes were cultured in 24 well plates until ready to treat. Cells were then treated with DMEM:F12 (control) or 10 μ M noradrenaline for 30 minutes, 3 hours, 6 hours, 12 hours or 24 hours and RNA was collected for mRNA analysis. Noradrenaline significantly decreased *Serpinf1* mRNA after 3 hours of treatment ($t(8) = 4.67$, $p = 0.002$) and significantly increased *Serpinf1* mRNA after 12 hours of treatment ($t(8) = 3.28$, $p = 0.01$). No other significant changes were found. A summary of the raw data is given in Table 5.3, with data given as a fold change of the average control from the 30 minute treatment group, $n = 5$. Data are also shown in Figure 5.8.

	control	10µM noradrenaline	n	p value
30 minutes	1.00 ± 0.1	1.06 ± 0.04	5	t(8) = 1.96, p = 0.09
1 hour	1.01 ± 0.03	1.01 ± 0.03	5	t(8) = 0.24, p = 0.81
3 hour	1.01 ± 0.04	0.87 ± 0.05	5	t(8) = 4.67, p = 0.002
6 hour	0.92 ± 0.1	0.93 ± 0.04	5	t(8) = 0.48, p = 0.64
12 hour	0.82 ± 0.03	0.90 ± 0.05	5	t(8) = 3.28, p = 0.01
24 hour	0.88 ± 0.02	0.94 ± 0.1	5	t(8) = 1.75, p = 0.12

Table 5.3 Raw data of the effect of noradrenaline treatment on PEDF (*Serpinf1*) mRNA expression in astrocytes

Data presented as mean fold change ± standard deviation. Abbreviations: t; t test statistic of the student t test.

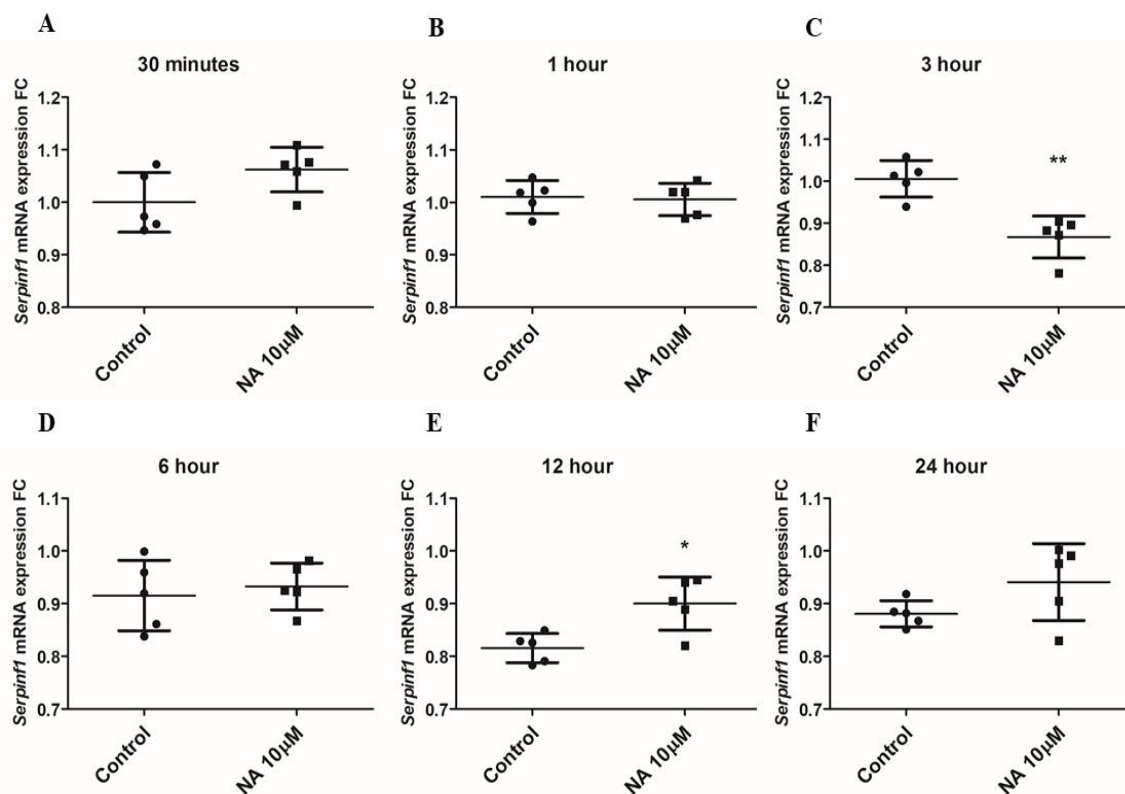


Figure 5.8 The effect of noradrenaline treatment on PEDF (*Serpinf1*) mRNA expression in astrocytes

Astrocytes were treated with 10 µM noradrenaline for (A) 30 minutes, (B) 1 hour (cC) 3 hours, (D) 6 hours, (E) 12 hours, and (F) 24 hours. All data are expressed as mean fold change ± standard deviation, n = 5. * p < 0.05, ** p < 0.01, as analysed by a student t test.

5.4.7 Imipramine treatment does not affect PEDF protein secretion from astrocytes

Cortical primary rat astrocytes were cultured in 24 well plates until ready to treat. Cells were then treated with DMEM:F12 (control) or 30 μ M imipramine for 24 hours and the supernatant was collected and analysed for PEDF protein secretion by ELISA. There was no difference between control (6.71 ± 6.3 ng/ml) or imipramine (6.93 ± 7.4 ng/ml) treated samples ($U = 22.0$, $p = 0.80$, $n = 7$; Figure 5.9). The intra-assay coefficient of variance (CV) for this ELISA was 7.6%.

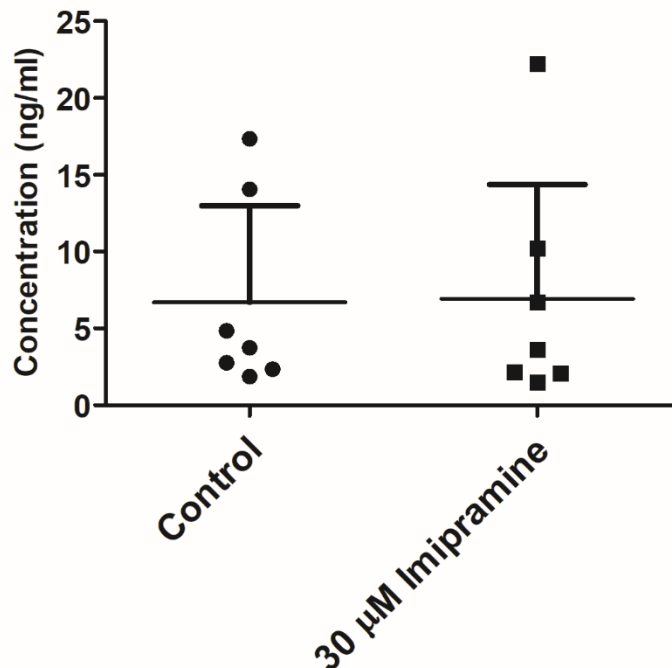


Figure 5.9 The effect of imipramine treatment on PEDF secretion from astrocytes

24 hour treatment with 30 μ M imipramine had no effect on PEDF protein secretion from astrocytes as analysed by a Mann-Whitney U test. All data expressed as mean $\pm$ standard deviation, $n = 7$.

5.4.8 Noradrenaline treatment has no effect on PEDF protein secretion from astrocytes

Cortical primary rat astrocytes were cultured in 24 well plates until ready to treat. Cells were then treated with DMEM:F12 (control) or 10 μ M imipramine for 5 minutes, 3 hours and 24 hours. The supernatant was then collected and analysed for PEDF protein secretion by ELISA. There was no difference between control (3.69 ± 2.5 ng/ml) or noradrenaline (5 minutes; 5.11 ± 3.1 ng/ml, 3 hours; 4.86 ± 4.9 , 24 hours; 1.26 ± 0.5) treated samples, [$H(3) = 7.3$, $p = 0.06$, Figure 5.10]. The intra-assay CV for this ELISA was 2.9%.

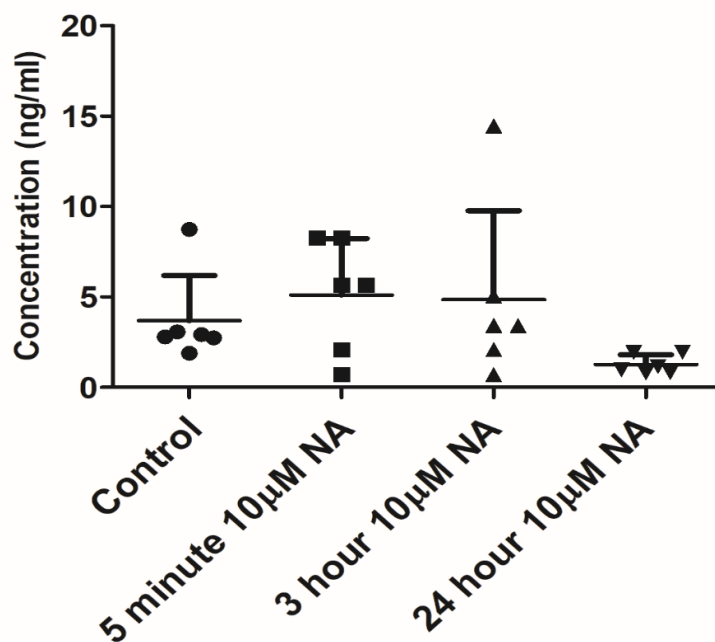


Figure 5.10 The effect of noradrenaline treatment on PEDF secretion from astrocytes
5 minute, 3 hours or 24 hours treatment with 10 μ M noradrenaline had no effect on PEDF protein secretion from astrocytes as analysed by a Kruskal-Wallis test. All data are expressed as mean $\pm$ standard deviation, $n = 6$.

5.5 Discussion

5.5.1 Results summary

My results demonstrate that *Serpinf1* (PEDF) mRNA is highly expressed in rat primary enriched astrocyte cultures, with low expression in both microglia and neuronal cultures. The PEDF-R receptor (*PNPLA2*) was expressed in all cell types at an mRNA level. Fluoxetine treatment had no significant effects on *Serpinf1* mRNA at any time point. Imipramine treatment significantly decreased *Serpinf1* mRNA at 24 hours. Noradrenaline significantly decreased *Serpinf1* mRNA after 3 hours of treatment and increased *Serpinf1* mRNA after 12 hours of treatment. However, neither imipramine nor noradrenaline affected the secretion of PEDF protein from astrocytes.

5.5.2 *Serpinf1* (PEDF) is mostly expressed in primary rat astrocytes

qRT-PCR was used to validate the primary cell isolation and separation protocols and to characterise the mRNA expression of PEDF and its receptor PEDFR in all three cell types. Results showed that *Tubb3* was mostly expressed by neurons, *Cd11b* by microglia and *Gfap* by astrocytes, with very little expression in the other two cells types (close to 0). The protocol followed for this study had previously been reported to yield 95% pure astrocytes and microglia, with 97% pure neurons (McNamee et al., 2010). Given that PCR can detect very small quantities of nucleic acid sequences (Sanders et al., 2014), a slight amount of cell specific marker gene contamination would be expected in cell cultures that are not 100% pure.

Serpinf1 mRNA was mainly detectable in astrocytes with low expression in both neurons and microglia. This result was as hypothesised as growth factors are highly expressed in astrocytes (Marathe et al., 2018). To my knowledge, this is the first study

that has determined the main source of *Serpinf1* (PEDF) mRNA in the brain. As hypothesised, *Pnpla2* expression was detectable at high levels in all cell types. This was as expected as incubation with recombinant PEDF has been reported to alter cell signalling and function of microglia, astrocytes and neurons (Sanagi et al., 2005, Yabe et al., 2005, Eichler et al., 2017). Based on these results, primary rat astrocytes were treated with antidepressants to study their effect on *Serpinf1* expression.

5.5.3 Fluoxetine does not affect *Serpinf1* mRNA expression in cultured rat astrocytes

As no previous *in vitro* data had been reported on the effect on fluoxetine on *Serpinf1* mRNA (to my knowledge), a time course was conducted. Fluoxetine had no significant effect on *Serpinf1* mRNA at any time point. The only previous report on fluoxetine and PEDF was an *in vivo* report where chronic fluoxetine treatment (21 days) increased *Serpinf1* mRNA expression in the mouse hippocampus, as measured by microarray (Miller et al., 2007). This result was not as hypothesised.

There are several reasons why fluoxetine may not have affected *Serpinf1* mRNA *in vitro*. My cells were only treated acutely and not chronically. It is possible that sustained treatment with fluoxetine is needed to induce changes in *Serpinf1* mRNA. I also used cortical rat cultures for my work whereas the previous report on fluoxetine and PEDF was in mouse hippocampi (Miller et al., 2007). Changes in PEDF expression may be specific to the hippocampus or may differ between species. Finally, the previous report was also in the whole hippocampus and not specific to a certain cell type (Miller et al., 2007); it is therefore possible that fluoxetine may be influencing protein secretion from neurons or microglia that may be responsible for an upregulation of PEDF.

5.5.4 Imipramine and noradrenaline have a significant effect on *Serpinf1* mRNA expression in astrocytes

qRT-PCR gene expression analysis showed that 24 hour treatment with both 10 μ M and 30 μ M imipramine significantly downregulated *Serpinf1* mRNA expression in primary rat astrocytes. As far as I am aware, there are no previous data that describe an effect of imipramine on PEDF. However, I hypothesised imipramine would have a similar effect on *Serpinf1* mRNA as it does on other neurotrophic factors, as imipramine increases their expression (Takano et al., 2012). Similarly, 10 μ M noradrenaline downregulated *Serpinf1* mRNA expression after 3 hours of treatment, but also increased it after 12 hours of treatment. It is unclear why imipramine would downregulate *Serpinf1* mRNA expression as literature supports a role for imipramine and other antidepressants in promoting neurogenesis and the production of growth factors (Alves et al., 2017, Levy et al., 2018). Similarly, noradrenaline has been reported to promote growth factor mRNA and protein expression, as well as synaptic plasticity in cortical rat astrocytes (Day et al., 2014).

Research on the role of PEDF in the cortex is limited, with a few studies reporting the increase of its gene expression by hypoxia (Julian et al., 2015). The PEDF gene has binding sites for both retinoic acid and oestrogen but there are few reports of its regulation (He et al., 2015). Hence, it is unclear what mechanism of noradrenaline or imipramine could downregulate PEDF. Additionally, noradrenaline significantly increased *Serpinf1* mRNA at 12 hours of treatment. It is possible that PEDF gene expression could be regulated by a feedback loop when stimulated, but further experiments would be required to fully understand the mechanism of this. mRNA expression is also not a clear indicator of what is happening at a protein level, as protein is constantly being produced and degraded (Wang, 2008). Therefore, analysing protein secretion in response to treatment would give a better indication the role of antidepressants on PEDF expression.

5.5.5 Imipramine and noradrenaline have no effects on PEDF protein secretion from astrocytes

Protein analysis of secreted PEDF in conditioned medium showed no significant effect of either imipramine or noradrenaline treatment. Based on the gene expression results in section 5.5.4 and the previous report on an increase in PEDF secretion with noradrenaline treatment (Lashbrook and Steinle, 2005), I would have expected a change in protein secretion. It is unclear why there is not. One previous study reported hypoxia increasing PEDF at a gene expression level, but with no change in protein (Julian et al., 2015). This could indicate different molecular profiles for PEDF gene and protein expression in response to antidepressants. As far as I am aware, previous reports on the effect of treatment on PEDF in the brain only analysed gene expression (Ryan et al., 2017, Miller et al., 2007). Furthermore, although the intra plate CVs were low, there was a lot of variability between samples, with some biological replicates decreasing secretion with treatment and others increasing. One possible reason for this could be the presence of astrocyte subtypes. At least two subsets of astrocytes have been confirmed in rodents; protoplasmic astrocytes, which are found in grey matter and whose functions have been well characterised, and fibrous astrocytes whose functions are not well known (Tabata, 2015, Verkhratsky and Nedergaard, 2018). Therefore primary astrocytes cultures show cellular heterogeneity (Schildge et al., 2013), and it is possible that different subtypes show different PEDF responses after antidepressant treatment. Looking at a more specific subset may give less heterogeneous data.

5.5.6 Limitations and future directions

The main limitation of this work is that the different cell types are being studied in isolation and not as a whole, which would mimic conditions in the intact central nervous system (CNS). Neurons and glia do not function independently in the brain; signals from the other cell types could therefore influence how a cell responds to treatment (Perea et al., 2014). *In vivo* studies or co-cultures of both glia and neurons could be a way to improve this study in the future (Carter and Shieh, 2015). This study was designed to determine if antidepressants had an effect on PEDF as my group had previously reported that protein levels were increased in medicated patients and increased further following ECT (Ryan et al., 2017). Although I did not find any data to support this, *in vivo* studies to investigate a role for PEDF in animal models of depression may be more useful.

Had antidepressants affected PEDF secretion from astrocytes the next step in the study would have been to examine the effect of antidepressant treated conditioned media (CM) from astrocytes on neuronal complexity *in vitro* and the role of PEDF in this. Primary cortical rat neurons would have been treated with antidepressant treated CM with or without the PEDF-R inhibitor (Taxiarchis et al., 2019). Sholl analysis would then have been used to examine neuronal complexity. This would have determined if blocking PEDF signalling blocks the effect of antidepressants on neuronal complexity. Unfortunately, the results of this study did not support following on with these experiments.

A second limitation is that this study only examined levels of mRNA and protein, which does not give a clear insight into functionality. Mature PEDF undergoes post-translational modification, possibly extracellularly by casein kinase 2 (CK2) and intracellularly by protein kinase A (PKA) (He et al., 2015). Phosphorylation by CK2 is reported to inhibit the neurotrophic effects of PEDF, and phosphorylation by PKA is

reported to abolish the anti-angiogenic effects of PEDF (Maik-Rachline et al., 2005). Monoamines, the target of traditional antidepressants such as imipramine (Berton and Nestler, 2006), signal through different protein kinases (O'Neill and Harkin, 2018, Wirth et al., 2017). Therefore, investigating the post-translational modification of PEDF in response to antidepressants (for example by comparing the level of PEDF phosphorylation in cell lysates to the amount of PEDF phosphorylation in the supernatant) may give more insight into its potential role in depression.

Finally, this study only used cortical cells. Previous studies on the effect of fluoxetine and electroconvulsive simulation on PEDF report changes in the hippocampus (Miller et al., 2007, Ryan et al., 2017). I chose to study cortical cells because of established protocols and reports on plasticity and growth factor changes in cortical cells (Day et al., 2014, McNamee et al., 2010, Deyama et al., 2018, Deyama et al., 2019). Future studies repeating the above experiments in hippocampal cultures may be of benefit as the role of PEDF in depression, and possibly the molecular mechanism of antidepressants, may be specific to the hippocampus.

5.6 Conclusion

As far as I am aware this is the first study to characterise the expression of *Serpinf1* (PEDF) mRNA and *Pnpla2* (PEDF-R) in rodent brain cells *in vitro*. The principal findings of this study were that *Serpinf1* mRNA is mainly expressed in astrocytes, and that imipramine and noradrenaline decrease *Serpinf1* mRNA but do not affect PEDF protein secretion. Although I did not find evidence to support my hypothesis, my lab's previous clinical study still supports a role for PEDF in depression (Ryan et al., 2017), and possibly a role for PEDF in response to treatments for treatment resistant depression.

Of note, PEDF is reported to have a close relationship with VEGFA (Falk et al., 2010). VEGF, has recently been implicated in the antidepressant actions of ketamine *in vivo* (Deyama et al., 2019). Therefore, investigating peripheral levels of these two genes/proteins in response to ketamine would also be of interest and is the focus of the work presented in the next chapter.

Chapter 6.

Gene expression and protein analysis: a comparison of a single ketamine or midazolam infusion

6.1 Introduction

In the past number of years the use of ketamine as a potential treatment for depression has been investigated. Ketamine is an antagonist of the NMDA glutamate receptor and is widely used as a dissociative anaesthetic (Duman, 2018, Harkin and McLoughlin, 2019). Ketamine is reported in a number of studies to be a rapid-acting anti-depressant (Berman et al., 2000, Zarate et al., 2006, Singh et al., 2016, Murrough et al., 2013, Kim and Mierzwinski-Urban, 2017). How it mediates this antidepressant effect is unclear, with studies also reporting ketamine acts through the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) or opioid receptors, and more recently ketamine has been reported to act as a ligand for the estrogen receptor (Aleksandrova et al., 2017, Williams et al., 2018, Ho et al., 2018). A few studies have reported on clinical measures that might predict or affect response to treatment. These include anxiety during infusions, which is associated with a negative effect on treatment (Aust et al., 2019), and increased dissociative scores during infusions which may predict a positive response (Niciu et al., 2018). For example, a higher dissociative score on the Clinician-Administered Dissociative States Scale (CADSS) has been reported to be associated with an improvement in depressive symptoms as measured by the Hamilton depression rating scale 17 item version (HAM-D17) (Luckenbaugh et al., 2014).

Studies on the effect of ketamine on molecular changes in humans are limited, with only a handful of papers to date reporting on protein changes in plasma or serum. These studies have focused on cytokines (Park et al., 2017, Yang et al., 2015, Kiraly et al., 2017, Chen et al., 2018), growth factors (Allen et al., 2015, Haile et al., 2014, Duncan et al., 2013, Rybakowski et al., 2013), metabolomics (Moaddel et al., 2018, Villasenor et al., 2014) and the kynurenine pathway (Allen et al., 2018a). Increases in brain derived

neurotrophic factor and IL-6 are the most reported findings (Haile et al., 2014, Duncan et al., 2013, Yang et al., 2015, Park et al., 2017).

The molecular pathways that underlie ketamine's antidepressant effects have not been identified, with the above findings not consistent among studies. In addition, no peripheral markers identify ketamine responders from non-responders. As with other antidepressant treatments, a peripheral marker that could predict the patient's response to ketamine would be of great clinical use. Therefore, more biological studies are needed to identify new potential peripheral gene or protein markers.

Recently, a study was published suggesting that VEGF signalling is necessary for the antidepressant action of ketamine in rodents (Deyama et al., 2019). VEGF signalling is also closely linked to pigment epithelial-derived factor (PEDF) (Falk et al., 2010), and a ratio of the two genes has been analysed as a potential peripheral biomarker, with one study reporting the ratio of the two genes can predict preeclampsia (Zhang et al., 2018). Therefore, analysing the effect of ketamine on these two genes/proteins would be of interest.

6.2 Aims and objectives

Given the small amount of human data on biological changes with ketamine, and the animal study implicating VEGF in the antidepressant action of ketamine, the aim of this study was to examine the effects of ketamine on targets from the previously reported deep sequencing and proteomic studies: VEGF-A and PEDF (Kolshus et al., 2017, Ryan et al., 2017), especially as these are already known to interact (Falk et al., 2010). I also wanted to explore the effect of ketamine on my other genes of interest related to depression: *Sirtuin1* and *E2F1*. For this, samples from the KARMA-Dep pilot trial (*clinical trials.gov*

ID: NCT03256162), which took place in St Patrick's Mental Health Services in Dublin (www.stpatricks.ie), from September 2017 – September 2018, were used. The effect of a single infusion of ketamine or midazolam on depression severity was also assessed, with a reduction in HAM-D24 scores following ketamine, but not midazolam, expected as reported in previous studies (Fava et al., 2018, Phillips et al., 2019, Murrough et al., 2013).

Based on the previous studies reporting changes in *VEGFA* mRNA and PEDF protein with ECT (Kolshus et al., 2017, Ryan et al., 2017), and recent studies that show a similar biological effect of ECT and ketamine on BDNF and kynurenine metabolites (Allen et al., 2015, Allen et al., 2018a), I hypothesised that both *VEGF* and *SERPINF1* (PEDF) mRNA will be increased following a single ketamine infusion. I also hypothesised that ketamine would affect the ratio of *VEGFA* and *SERPINF1* mRNA, based on reports of their close signalling relationship in the periphery (Falk et al., 2010). As no data are yet published on ketamine, *E2F1* and *SIRT1* this work is more exploratory. However, these genes have previously been implicated in depression by my lab and other work (Abe-Higuchi et al., 2016, Abe et al., 2011, Kim et al., 2005, McGrory et al., 2018, McGrory et al., 2019). The effect of ketamine on their expression is therefore of interest. The objectives of this study therefore were:

1. To analyse differences in peripheral whole blood levels of *VEGFA*, *E2F1*, *SIRT1*, *SERPINF1* mRNA and the ratio of *VEGFA/SERPINF1* mRNA in patients pre-/post- infusions of either ketamine or midazolam.
2. To perform correlation analyses to determine if mRNA levels are associated with mood scores and measured by Hamilton depression rating scale, 24-item version (HAM-D24).

3. To perform correlation analyses to determine if a change in mRNA levels with ketamine are associated with dissociative scores as measured by the Clinician-Administered Dissociative States Scale (CADSS).
4. To analyse differences in plasma protein concentrations of VEGFA and PEDF in patients pre-/post- infusions of either ketamine or midazolam.

6.3 Methods

6.3.1 Participant recruitment

This study included patients recruited from the KARMA-Dep pilot trial (clinicaltrials.gov ID: NCT03256162) who received four once-weekly 40 minute infusions of either 0.5mg/kg ketamine or 0.045mg/kg midazolam. Full details of the trial procedure and eligibility criteria can be found in section 2.2.1.2. For the purpose of the present study, I focused on peripheral blood analyses before and after the very first infusion.

6.3.2 Blood sampling and mRNA extraction

Full details for blood sampling and mRNA extraction are provided in section 2.2.1.3 – 2.2.1.5. Fasting whole blood samples for mRNA analysis were taken using the PaxGene[®] system (Qiagen Inc., USA). For the KARMA-Dep pilot trial, blood for RNA was taken 60 minutes before (-60 minutes) and 4 hours after the start of the first infusion (+240 minutes).

6.3.3 Multiplex quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK). qRT-PCR was carried out on the StepOne

PlusTM Real-Time PCR system (Applied Biosystems, UK), using TaqMan® (Applied Biosystems, UK) gene expression assays (VEGFA; assay ID:Hs00900055_m1, *E2F1*; assay ID:Hs00153451_m1, *SIRT1*; assay ID:Hs01009006_m1, *SERPINF1*; assay ID:Hs01106934_m1) with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; assay ID:Hs03929097_g1) as the endogenous control. Full details of cDNA synthesis, qRT-PCR and gene expression analysis can be found in sections 2.2.1.8 – 2.2.1.10.

6.3.4 Plasma protein vascular endothelial growth factor A and pigment epithelial-derived factor measurement

Plasma collection is detailed in section 2.2.1.5. Plasma samples were collected -60 minutes pre-infusion, +40 minutes post-infusion and +240 minutes post-infusion. For VEGFA plasma protein analysis the MSD® V-PLEX Cytokine Panel 1 Human Kit (Mesoscale, USA) was used according to the manufacturer's instructions. The ChemoKine™ Pigment Epithelium Derived Factor Sandwich ELISA (Merck, UK) kit was used for PEDF protein analysis. Full protocols are described in section 2.2.1.11. The intra assay coefficient of variation (CV) for the VEGFA and PEDF protein assays were < 4% and < 5% respectively. As all samples were run on a single plate no inter-assay CV was calculated.

6.3.5 Statistical analysis

Data were analysed using SPSS version 21.0 (IBM Corporation, NY, USA) and GraphPad Prism 5 (GraphPad Software, California, USA). Data were tested for normality using a Shapiro–Wilk normality test and log-transformed when necessary.

Baseline clinical and demographic factors are presented as means with standard deviation (SD), or number (%) per group. Adjusting for potential covariance was not

included in this analysis due to the small sample sizes and the exploratory nature of the study.

Data were analysed using independent t-tests, paired t-tests, Mann-Whitney U tests for non-parametric data, or a general linear model (GLM). Categorical data were tested using a chi-square test (χ^2) or Fisher's exact test when numbers were small (<5). As sample numbers were small (especially for protein), changes pre/post-infusions were first assessed using paired t test or one-way ANOVAs. A repeated measure GLM was then used as this adjusts for multiple testing using Bonferroni correction. Where significant interactions were found, main effects were determined. For non-parametric groups Friedman's test, the non-parametric equivalent to the repeated measures was used. For correlational analyses, data were analysed with Pearson's product-moment correlation coefficient (Pearson's r) for parametric data and Spearman's rank correlation coefficient rho (ρ) for non-parametric data. As numbers were small, correlation analysis was only carried out when a gene changed with ketamine treatment. Statistical significance was set at $p < 0.05$. For correlations analysis $p < 0.01$ was deemed significant to control for multiple testing.

6.4 Results

6.4.1 Patient characteristics

Of the 25 patients randomized to either ketamine or midazolam infusions, 23 had both pre- and post-infusion RNA samples available for analysis. Of these, 12 patients received ketamine infusions. Clinical and demographic data for both groups are shown in Table 6.1. There were no statistically significant differences between groups in any demographic characteristic. A statistically significant higher number of patients who

received ketamine were on antipsychotics in comparison to patients in the midazolam group ($p = 0.01$), as measured by a Fisher's exact test. Patients receiving ketamine also had a higher dissociative score 30 minutes after the beginning of their infusion ($U = 17.000$, $p = 0.002$). There were no other clinical differences between the two groups.

	Ketamine (<i>n</i> = 12)	Midazolam (<i>n</i> = 11)	Statistical Test
Age	48.17 (13.3)	51.64 (12.9)	$t(21)=-0.66$, $p=0.53$
Sex, female <i>n</i> (%)	8 (66.7)	4 (36.4)	$p=0.15$
Smoker, yes <i>n</i> (%)	3 (25.0)	3 (27.3)	$p=0.64$
BMI	27.58 (7.1)	29.27 (3.2)	$t(21)=-0.73$, $p=0.48$
Education Completed, yes <i>n</i> (%)			
Secondary	2 (16.7)	6 (54.5)	
Tertiary	10 (83.3)	5 (45.5)	$p=0.10$
Bipolar Depression, yes <i>n</i> (%)	3 (21.6)	2 (18.2)	$p=0.55$
HAM-D24 Pre-Infusion 1 (-60 mins)	28.50 (4.4)	28.00 (4.0)	$t(21)=0.28$, $p=0.78$
HAM-D24 Post-Infusion 1 (+240 mins)	20.67 (4.1)	17.72 (4.7)	$t(21)=1.60$, $p=0.12$
CADSS 0 minutes	1.83 (2.0)	1.91 (2.4)	$U=64.50$, $p=0.93$
CADSS +30 minutes	15.00 (11.6)	3.73 (4.2)	$U=17.00$, $p=0.002$
CADSS +60 minutes	3.17 (3.6)	3.00 (2.9)	$U=65.10$, $p=0.79$
Medications, <i>n</i> (%)			
SSRI	4 (33.3)	4 (36.4)	$p=0.61$
SNRI	7 (83.3)	3 (72.7)	$p=0.14$
TCA	2 (16.7)	3 (27.3)	$p=0.46$
Mirtazapine	3 (25.0)	2 (18.2)	$p=0.55$
Bupropion	0 (0.0)	1 (9.1)	$p=0.48$
Lithium	3 (25.5)	2 (18.2)	$p=0.55$
Antipsychotic	10 (83.3)	3 (27.3)	$p=0.01$
Pregabalin	4 (33.3)	1 (9.1)	$p=0.19$
Benzodiazepines	4 (33.3)	6 (54.5)	$p=0.27$

Table 6.1 Demographic and clinical details of patients receiving ketamine or midazolam infusions

Abbreviations: BMI, body mass index; HAM-D24, Hamilton Depression Rating Scale, 24-item version; CADSS, Clinician-Administered Dissociative States Scale; SSRI, Selective serotonin reuptake inhibitor; SNRI, Serotonin and noradrenaline reuptake inhibitor; TCA, Tricyclic antidepressants; U, standardised Mann-Whitney test. A p value and no other test statistic indicates Fisher's exact test. Data are presented as means (SD) or n (%).

6.4.2 Response to ketamine or midazolam infusions

Both groups had a similar HAM-D24 score prior to an infusion of either ketamine or midazolam ($t(21) = 0.284, p = 0.78$). A single infusion of both ketamine and midazolam significantly decreased HAM-D24 scores ($F(1,21) = 102.40, p < 0.000$; Figure 6.1), with no interaction between time (pre-/post-infusion) and treatment group ($F(1,21) = 1.129, p < 0.282$). Following the first infusion no patient met response criteria.

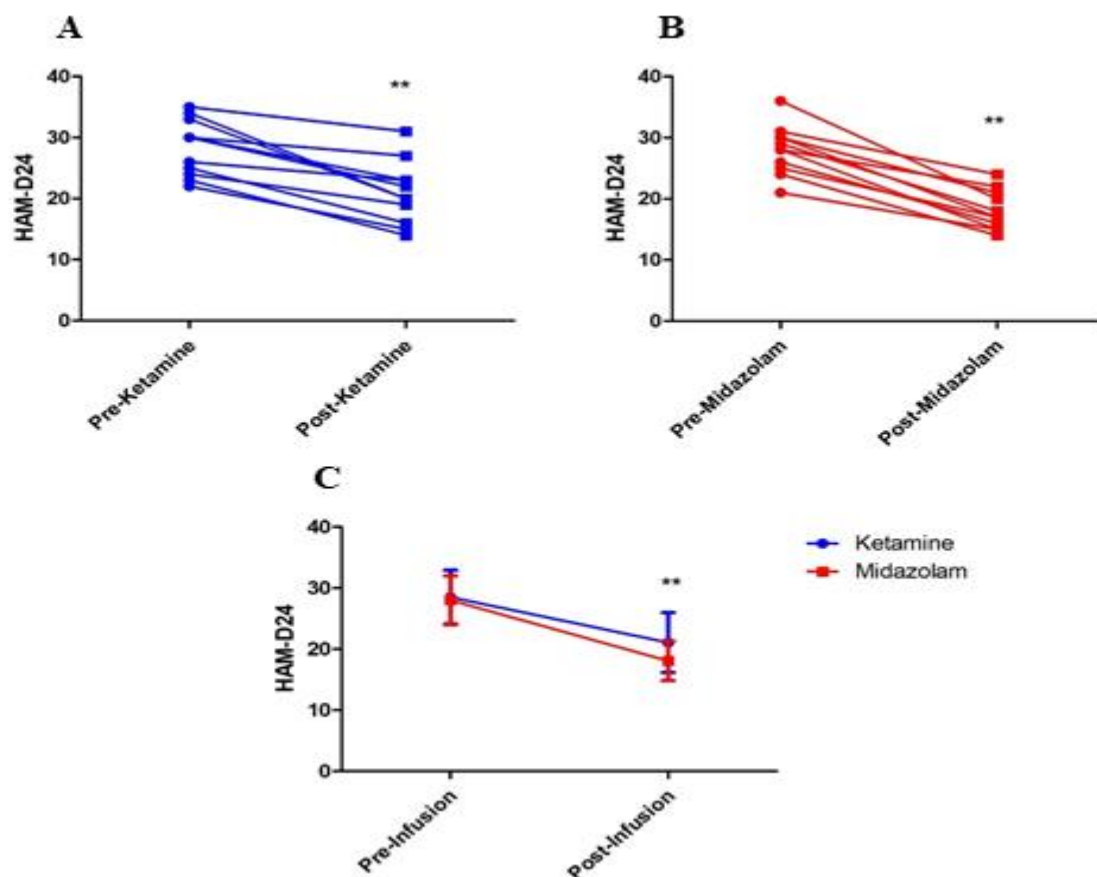


Figure 6.1 The effect of a single ketamine or midazolam infusion on depression severity

A single infusion of both (A) ketamine ($n = 12$) and (B) midazolam ($n = 11$) significantly reduced depression severity as measured by the HAM-D24. (C) A repeated measures ANOVA showed a significant overall reduction in HAM-D24 scores with no time $\times$ treatment effect. $**p < 0.01$ for both treatment groups. Data represented as mean $\pm$ SD. Abbreviations: HAM-D24, Hamilton Depression Rating Scale, 24-item.

There was also no significant difference in the CADSS scores between each group prior to infusion ($t(21) = -0.082, p = 0.94$). There was a significant effect of time on CADSS scores (as measured at 0 minutes, +30 minutes and +60 minutes; $F(1,21) = 14.030, p < 0.001$) with a statistically significant time $\times$ group interaction ($F(1,21) = 1.578, p = 0.03$), with the CADSS score for the ketamine group increasing 30 minutes after the start of the infusion before returning towards baseline. Figure 6.2 shows an interaction plot for the CADSS scores.

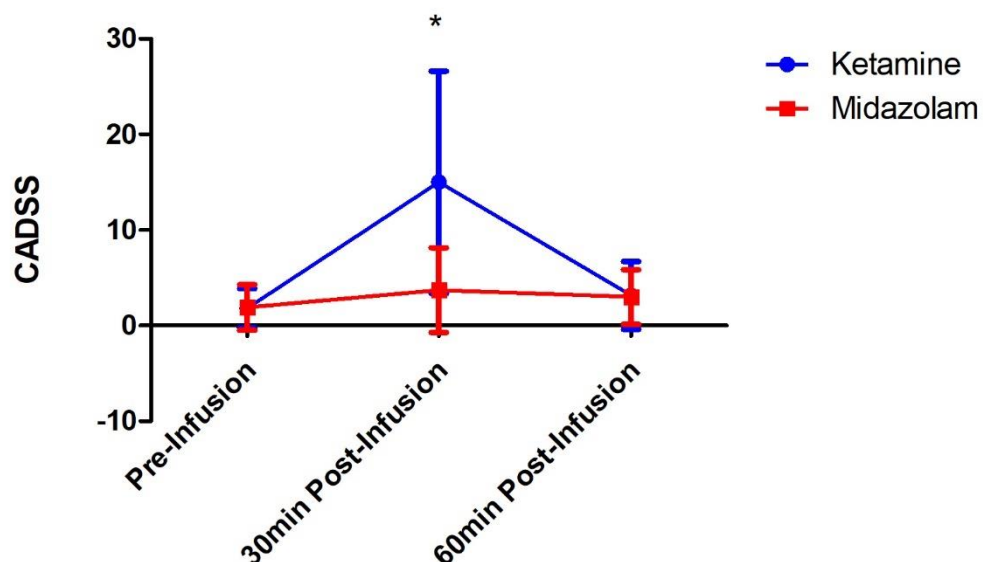


Figure 6.2 Interaction plot of the change in CADSS scores with treatment

There was a significant time $\times$ group interaction for a change in CADSS scores with treatment. CADSS scores significantly increased with a single ketamine infusion until 30 minutes after the infusion before returning to baseline. * $p < 0.05$, ketamine group, as analysed by a repeated measures ANOVA. Abbreviations: CADSS; Clinician-Administered Dissociative States Scale.

6.4.3 Vascular endothelial growth factor peripheral gene expression analysis

Data were tested for normality using a Shapiro-Wilk normality test. Pre-infusion, *VEGFA* mRNA RQ values were not normal for the ketamine group ($p = 0.001$), but were for the midazolam group ($p = 0.482$). Post-infusion RQ values were also not normal for the ketamine group ($p = 0.002$) and normal for the midazolam group ($p = 0.574$). Log transformation did not render the RQ values for ketamine treatment normal pre-treatment ($p = 0.015$). However, as the post-infusion RQ values were normal for the ketamine group following log transformation ($p = 0.060$), log values were used as the data were less skewed.

6.4.3.1 *VEGFA* mRNA levels following ketamine and midazolam infusions

VEGFA mRNA levels were not significantly different between the ketamine and midazolam groups pre-infusion ($U = 58.000$, $p = 0.65$). A Wilcoxon signed rank test showed a significant effect of ketamine treatment on *VEGFA* mRNA levels ($p = 0.01$; Figure 6.3). Midazolam had no effect on *VEGFA* mRNA levels as measured by a paired t test ($t(10) = 0.4312$, $p = 0.68$). A repeated measure ANOVA reported no overall main effect of time on *VEGFA* mRNA levels ($F(1, 21) = 1.542$, $p = 0.23$; within-subject model) or overall main effect of treatment group ($F(1, 21) = 1.632$, $p = 0.15$; between-subject model). However, there was a significant time $\times$ treatment group interaction ($F(1, 21) = 5.207$, $p = 0.03$). Therefore, simple main effect analysis was carried out to determine the nature of this interaction. A Friedman's test reported a significant effect of time on *VEGFA* mRNA levels in the ketamine group ($\chi^2(1) = 5.333$, $p = 0.021$; Figure 6.3). A repeated measures ANOVA reported no change with the midazolam group ($F(1, 10) = 0.349$, $p = 0.57$). A summary of the data can be seen in Table 6.2.

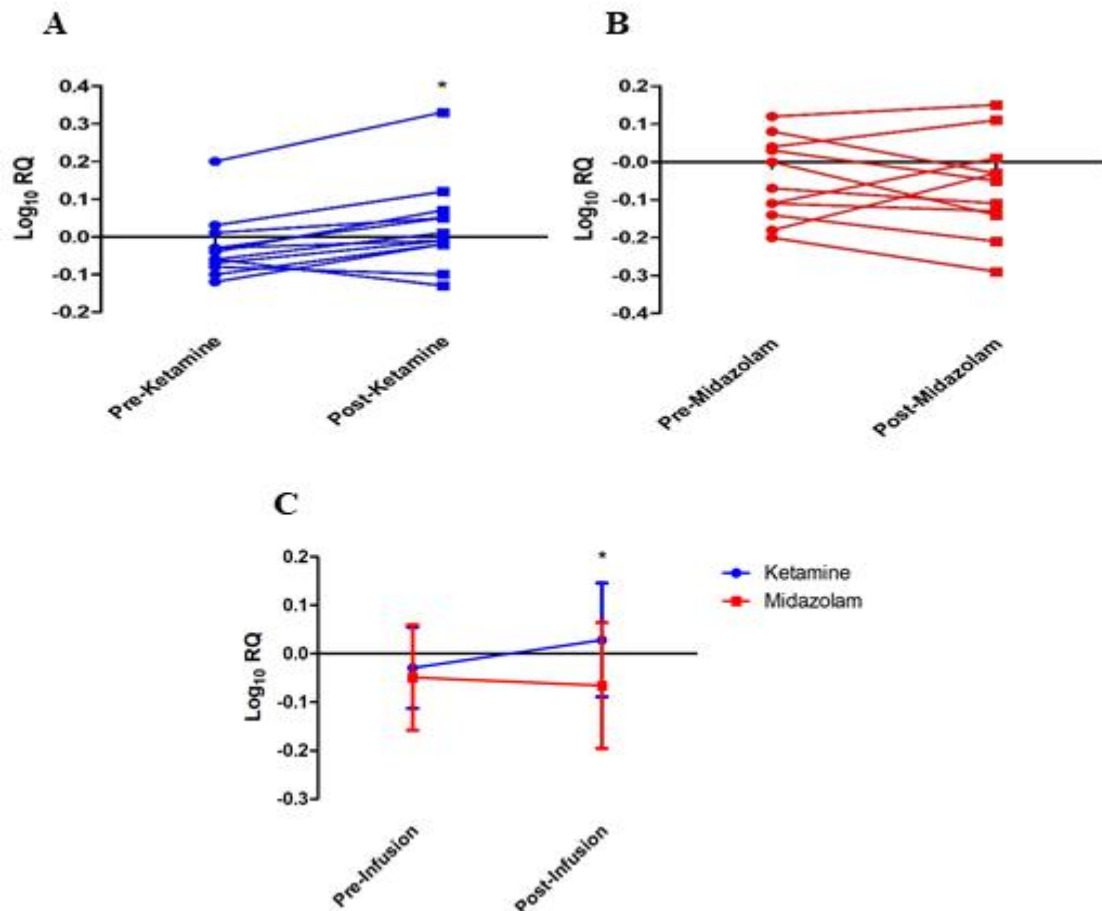


Figure 6.3 The effect of a single ketamine or midazolam infusion on peripheral blood VEGFA mRNA levels

(A) Ketamine ($n = 12$) significantly increased VEGFA mRNA levels while (B) midazolam ($n = 11$) had no effect. (C) There was a significant time $\times$ treatment interaction with ketamine, specifically increasing VEGFA gene expression; $*p < 0.05$, $*p < 0.05$ (pre-/post-ketamine infusion only). Data presented as mean $\pm$ SD.

	Ketamine ($n = 12$)		Midazolam ($n = 11$)	
	Pre-Infusion	Post-Infusion	Pre-Infusion	Post-Infusion
Mean RQ	0.95	1.11	0.92	0.90
SD	0.22	0.36	0.23	0.27
SEM	0.06	0.11	0.07	0.08
Median RQ	0.89	1.01	0.86	0.90
IQR	0.84 - 1.01	0.96 - 1.16	0.72 - 1.09	0.73 - 1.02

Table 6.2 VEGFA mRNA data pre- and post-infusions

Abbreviations: RQ; relative quantification value, SD; standard deviation, SEM; standard error of the mean, IQR; inter-quartile range.

6.4.3.2 Correlation analysis of *VEGFA* mRNA with mood ratings

Exploratory correlation analysis was performed to assess if *VEGFA* mRNA levels could predict depression severity as measured by the HAM-D24 at baseline or the response to a single infusion of ketamine. For baseline severity correlations the whole group was used in the analysis, with all further analyses breaking the groups down into ketamine or midazolam to assess specific treatment associations. As reported in Table 6.3 no significant associations were found.

	Baseline <i>VEGFA</i> & Baseline HAMD-24	Baseline <i>VEGFA</i> & Δ HAM-D24	Δ <i>VEGFA</i> & Δ HAM-D24
Whole Group (n = 23)	$\rho = -0.241, p = 0.326$	Not analysed	Not analysed
Ketamine (n = 12)	$r = 0.303, p = 0.338$	$r = 0.199, p = 0.534$	$r = 0.257, p = 0.421$
Midazolam (n = 11)	$r = 0.128, p = 0.708$	$r = 0.193, p = 0.579$	$r = -0.015, p = 0.965$

Table 6.3 Associations between *VEGFA* mRNA levels and depression severity before and after a single infusion of either ketamine or midazolam

Abbreviations: Δ ; a change in, HAMD-24; Hamilton Depression Rating Scale, 24-item.

6.4.3.3 Correlation analysis of peripheral blood *VEGFA* mRNA with dissociative scores

As a higher dissociative score has been reported to be associated with better treatment response to ketamine (Niciu et al., 2017), exploratory analysis was carried out to determine any relationship between a change in *VEGFA* mRNA levels with ketamine and dissociative scores (as measured by the Clinician-Administered Dissociative States Scale – CADSS). As the CADSS scores peaked at 30 minutes post infusion, a change in gene expression was correlated with a change in CADSS score (from 0 to +30 minutes) and the change in *VEGFA* mRNA levels from -60 to +240 minutes. No relationship was found, see Figure 6.4.

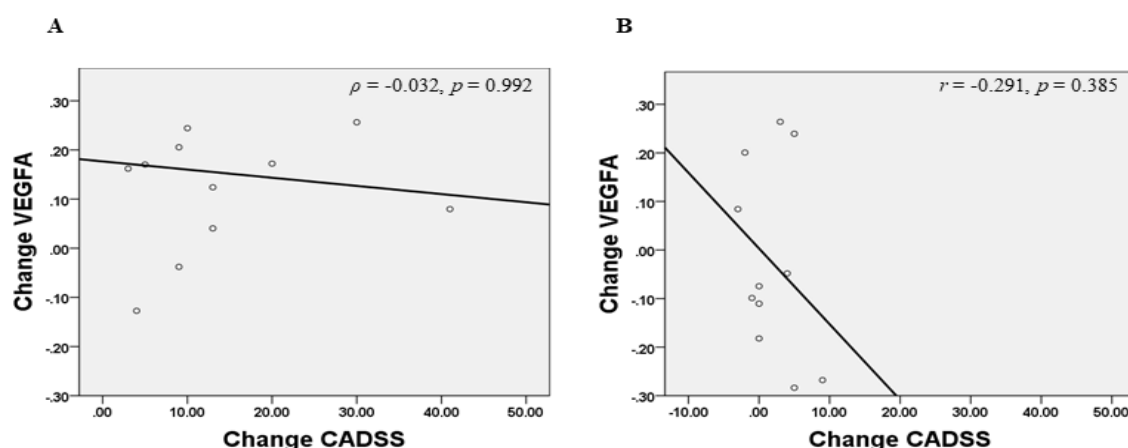


Figure 6.4 Associations between VEGFA mRNA levels and change in CADSS scores

No associations between a change in VEGFA mRNA levels and a change in CADSS scores were found with (A) ketamine treatment ($n = 12$) or (B) midazolam treatment ($n = 11$). Data measured with Pearson's r or Spearman's rho (ρ) correlation analysis.

6.4.4 Pigment epithelial derived-factor peripheral gene expression analysis

SERPINF1 (PEDF) mRNA RQ values were normal for the ketamine group (pre-infusion; $p = 0.786$, post-infusion; $p = 0.134$), but not for the midazolam group (pre-infusion; $p = 0.030$, post infusion; $p = 0.029$). Log transformation rendered the RQ values normal for all groups (pre-ketamine; $p = 0.946$, post-ketamine; $p = 0.999$, pre-midazolam; $p = 0.100$, post-midazolam; $p = 0.426$). Log transformed data were therefore used for all analyses.

6.4.4.1 *SERPINF1* (PEDF) mRNA levels following ketamine and midazolam infusions

There was no significant difference in *SERPINF1* mRNA levels prior to infusions ($t(21) = -0.832$, $p = 0.415$). A paired t test reported a significant effect of ketamine treatment on *SERPINF1* mRNA levels ($t(11) = 2.371$, $p = 0.03$; Figure 6.5), but no effect of Midazolam ($t(10) = 0.1863$, $p = 0.86$). A repeated measures ANOVA reported no overall main effect of time on *SERPINF1* mRNA levels ($F(1, 21) = 2.228$, $p = 0.150$), no main effect of treatment group ($F(1, 21) = 1.777$, $p = 0.197$), and no significant time $\times$ treatment group

interaction ($F(1, 21) = 3.287, p = 0.084$; Figure 6.5). A summary of the data can be seen in Table 6.4.

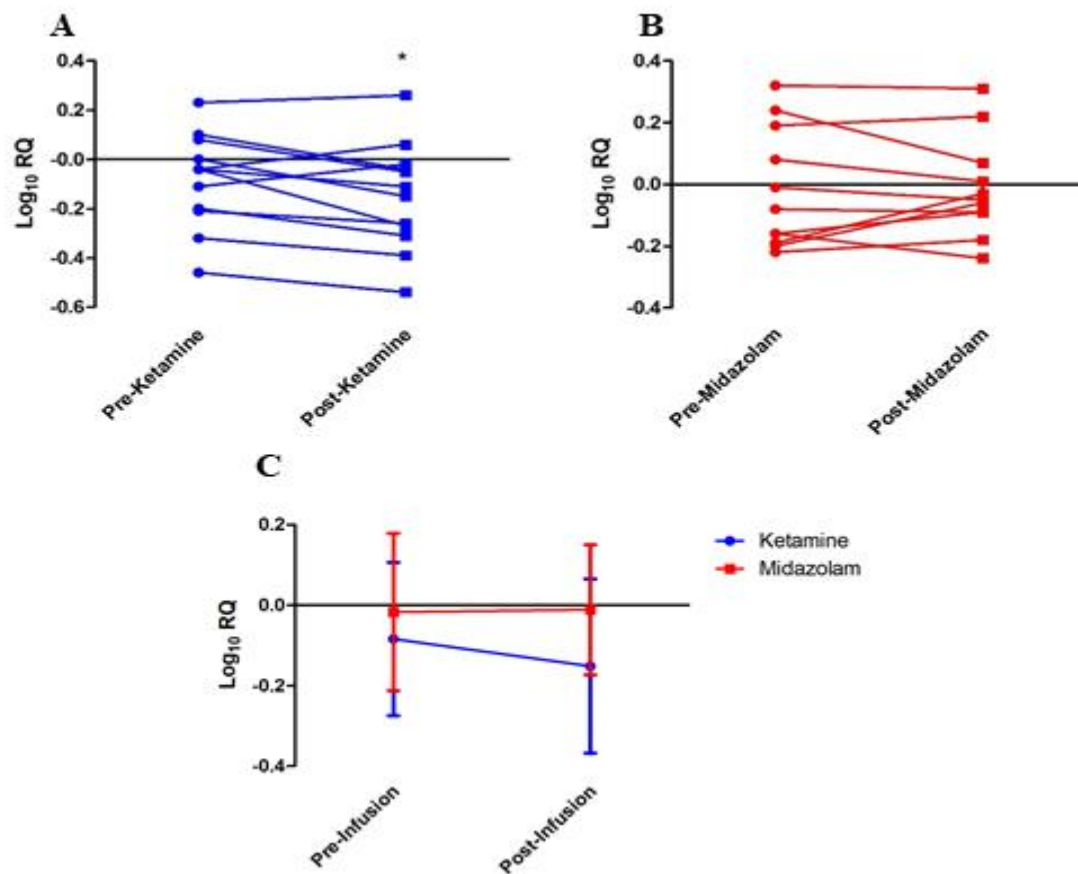


Figure 6.5 The effect of a single ketamine or midazolam infusion on *SERPINF1* mRNA levels

A paired t test reported (A) ketamine ($n = 12$) significantly increased *SERPINF1* mRNA levels. (B) Midazolam ($n = 11$) had no effect. (C) a repeated measures ANOVA indicated no significant time $\times$ treatment interaction. $*p < 0.05$, data presented as mean $\pm$ SD.

	Ketamine ($n = 12$)		Midazolam ($n = 11$)	
	Pre-Infusion	Post-Infusion	Pre-Infusion	Post-Infusion
Mean RQ	0.90	0.79	1.06	1.04
SD	0.37	0.41	0.5	0.43
SEM	0.11	0.12	0.15	0.13
Median RQ	0.91	0.74	0.84	0.90
IQR	0.62 - 1.16	0.50 - 0.94	0.65 - 1.53	0.81 - 1.18

Table 6.4 *SERPINF1* mRNA data pre- and post-infusions

Abbreviations: RQ; relative quantification value, SD; standard deviation, SEM; standard error of the mean, IQR; inter-quartile range.

6.4.4.2 Correlation analysis of *SERPINF1* (PEDF) mRNA with mood ratings

Exploratory correlation analysis was then performed to assess if *SERPINF1* mRNA levels was associated with depression severity at baseline or could predict the response to a single infusion of ketamine. The Pearson correlation coefficient r reported no significant correlations. Table 6.5 summarises the statistics.

	Baseline <i>SERPINF1</i> & Baseline HAMD-24	Baseline <i>SERPINF1</i> & Δ HAM-D24	Δ <i>SERPINF1</i> & Δ HAM-D24
Whole Group ($n = 23$)	$r = 0.153, p = 0.468$	Not analysed	Not analysed
Ketamine ($n = 12$)	$r = 0.420, p = 0.174$	$r = 0.004, p = 0.994$	$r = 0.245, p = 0.442$
Midazolam ($n = 12$)	$r = -0.135, p = 0.693$	$r = -0.007, p = 0.983$	$r = 0.167, p = 0.624$

Table 6.5 Associations between *SERPINF1* mRNA levels and depression severity at baseline and following treatment

Abbreviations: Δ ; a change in, FU; follow up, HAMD-24; Hamilton Depression Rating Scale, 24-item.

6.4.4.3 Correlation analysis of *SERPINF1* (PEDF) mRNA with dissociative scores

Correlation analyses were carried out to determine if a change in *SERPINF1* mRNA levels with treatment was associated with a change in dissociative scores. There was a relationship reported with midazolam treatment ($r = -0.731, p = 0.011$; Figure 6.6). However, this was not significant as the significance level for correlation analysis was set at 0.01 to account for multiple testing.

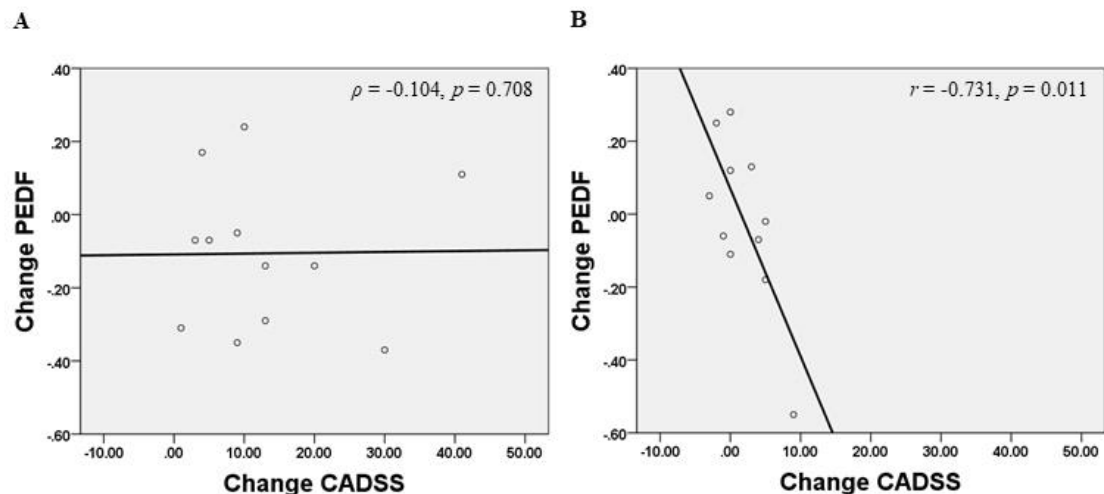


Figure 6.6 Associations between *SERPINF1* mRNA levels and CADSS scores

No associations between a change in *SERPINF1* mRNA levels and a change in CADSS scores was found with (a) ketamine ($n = 12$) treatment or (b) midazolam ($n = 11$) treatment. Data measured with Pearson's r or Spearman's rho (ρ) correlation analysis.

6.4.5 Ratio analysis of vascular endothelial derived-factor and pigment epithelial derived-factor gene expression

Studies report a strong relationship between VEGF and PEDF signalling (Falk et al., 2010), and the use of a ratio of the two genes can be used for predicting preeclampsia (Zhang et al., 2018) and may also play a role in infant haemangioma and nicotine induced neovascularisation (Zhang et al., 2016, Zhu et al., 2018). Therefore, the role for the ratio of the two genes in the response to ketamine was assessed. RQ values were normal for all groups (pre-ketamine; $p = 0.110$, post-ketamine; $p = 0.488$, pre-midazolam; $p = 0.516$, post-midazolam; $p = 0.453$). Parametric statistics were used throughout this section.

6.4.5.1 *VEGFA/SERPINF1* mRNA ratio following ketamine and midazolam infusions

The *VEGFA/SERPINF1* mRNA ratio levels were not significantly different between the ketamine and midazolam groups pre-infusion ($t(21) = 0.669$, $p = 0.45$). A paired t test reported a significant effect of ketamine treatment on the *VEGFA/SERPINF1* mRNA

ratio ($t(11) = 3.47$, $p = 0.005$; Figure 6.7) but no effect of midazolam ($t(10) = 0.95$, $p = 0.36$). A repeated measure ANOVA reported a significant main effect of time on the *VEGFA/SERPINF1* mRNA ratio levels ($F(1, 21) = 7.005$, $p = 0.015$), but no significant main effect of treatment group ($F(1, 21) = 2.949$, $p = 0.101$). There was a significant time $\times$ treatment group interaction ($F(1, 21) = 11.836$, $p = 0.002$). A simple main effects model revealed a significant effect of time in the ketamine group ($F(1, 11) = 12.085$, $p = 0.005$) but not in the midazolam group ($F(1, 10) = 0.837$, $p = 0.382$). A summary of the data can be seen in Table 6.6.

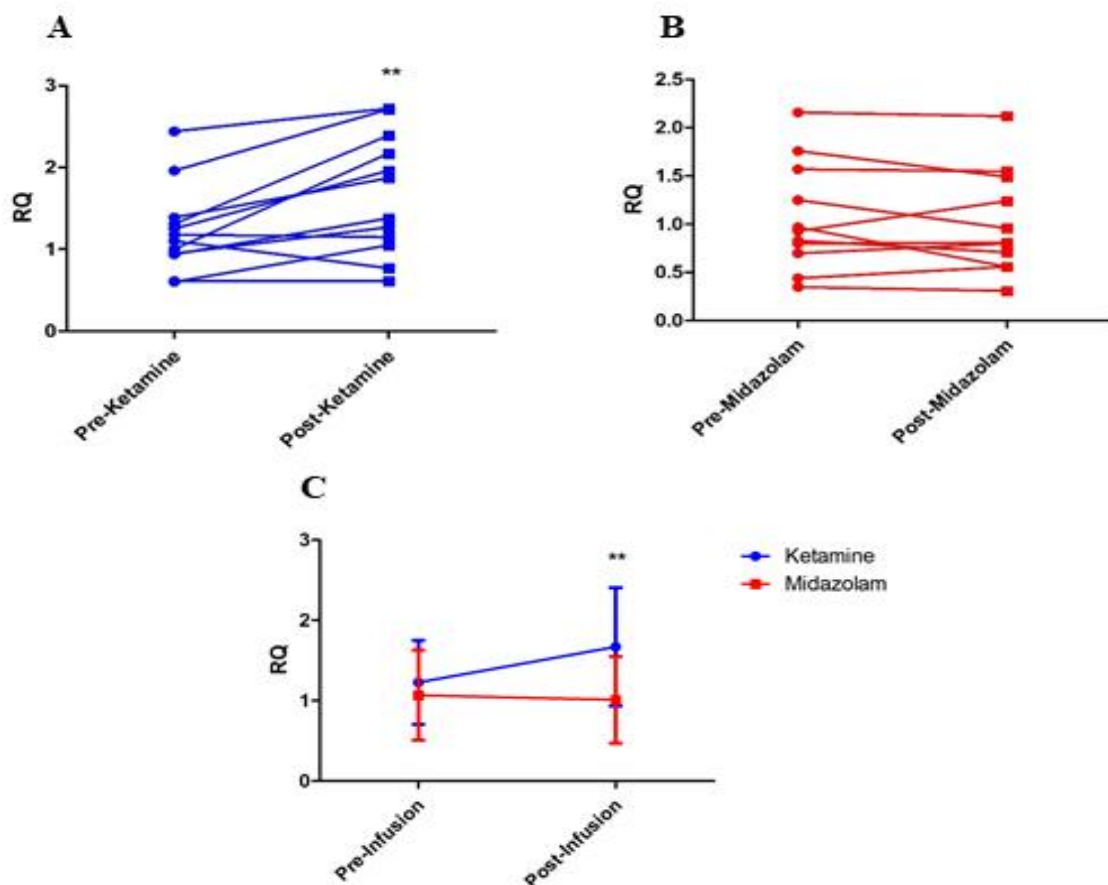


Figure 6.7 The effect of a single ketamine or midazolam infusion on *VEGFA/SERPINF1* mRNA level ratio

(A) Ketamine ($n = 12$) significantly increased the *VEGFA/SERPINF1* mRNA ratio. (B) Midazolam ($n = 11$) had no effect. (C) A repeated measures ANOVA indicated a significant time $\times$ treatment interaction with ketamine specifically increasing the *VEGFA/SERPINF1* ratio. $**p < 0.01$, data presented as mean $\pm$ SD.

	Ketamine (n = 12)		Midazolam (n = 11)	
	Pre-Infusion	Post-Infusion	Pre-Infusion	Post-Infusion
Mean ratio	1.23	1.67	1.07	1.01
SD	0.53	0.73	0.56	0.54
SEM	0.15	0.21	0.17	0.16

Table 6.6 VEGFA/SERPINF1 mRNA ratio data pre- and post-infusions

Abbreviations: SD; standard deviation, SEM; standard error of the mean.

6.4.5.2 Correlation analysis of VEGFA/SERPINF1 mRNA ratio with mood ratings

Pearson's correlation coefficient (r) reported no significant correlations between the VEGFA/SERPINF1 RQ ratio and HAM-D24 scores, see Table 6.7.

	Baseline VEGFA/SERPINF1 & Baseline HAMD- 24	Baseline VEGFA/SERPINF1 & Δ HAM-D24	Δ VEGFA/SERPINF1 & Δ HAM-D24
Whole Group (n = 23)	$r = -0.142, p = 0.517$	Not analysed	Not analysed
Ketamine (n = 12)	$r = -0.433, p = 0.16$	$r = 0.162, p = 0.614$	$r = 0.011, p = 0.973$
Midazolam (n = 11)	$r = -0.162, p = 0.635$	$r = 0.112, p = 0.743$	$r = -0.239, p = 0.480$

Table 6.7 Associations between VEGFA/SERPINF1 mRNA ratio and depression severity at baseline and following treatment

Abbreviations: Δ ; a change in, FU; follow up, HAMD-24; Hamilton Depression Rating Scale, 24-item.

6.4.5.3 Correlation analysis of VEGFA/SERPINF1 mRNA ratio with dissociative scores

Next, correlation analysis was carried out to determine any relationship between a change in VEGFA/SERPINF1 mRNA ratio with treatment and a change in dissociative scores. No significant associations were reported, Figure 6.8.

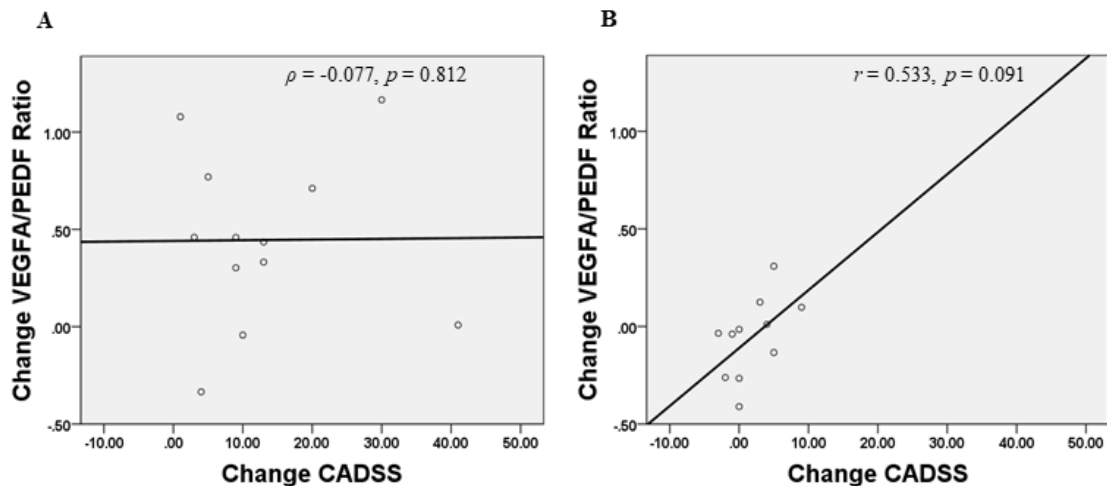


Figure 6.8 Associations between VEGFA/SERPINF1 mRNA ratio and CADSS scores
No associations between a change in the VEGFA/SERPINF1 mRNA ratio and a change in CADSS scores were found with (a) ketamine (n = 12) treatment or (b) midazolam (n = 11) treatment. Data measured with Pearson's r or Spearman's rho (ρ) correlation analysis.

6.4.6 *E2F1* peripheral blood gene expression analysis

E2F1 mRNA RQ values were normal for both groups before (ketamine; $p = 0.08$, midazolam; $p = 0.55$) and after (ketamine; $p = 0.92$, midazolam; $p = 0.08$) infusions. Data were therefore analysed with parametric statistics.

6.4.6.1 *E2F1* mRNA levels following ketamine and midazolam infusions

E2F1 mRNA levels were not significantly different between the ketamine and midazolam groups pre-infusion ($t(21) = -0.778$, $p = 0.45$). There was no significant effect of ketamine ($t(11) = 0.073$, $p = 0.94$; Figure 6.9) or midazolam ($t(10) = 0.8829$, $p = 0.40$) on *E2F1* mRNA levels. There was no main effect of time on *E2F1* mRNA levels ($F(1, 21) = 0.731$, $p = 0.402$), no main effect of treatment group ($F(1, 21) = 0.594$, $p = 0.449$) and no time $\times$ treatment group interaction ($F(1, 21) = 0.236$, $p = 0.632$). A summary of the data can be seen in Table 6.8.

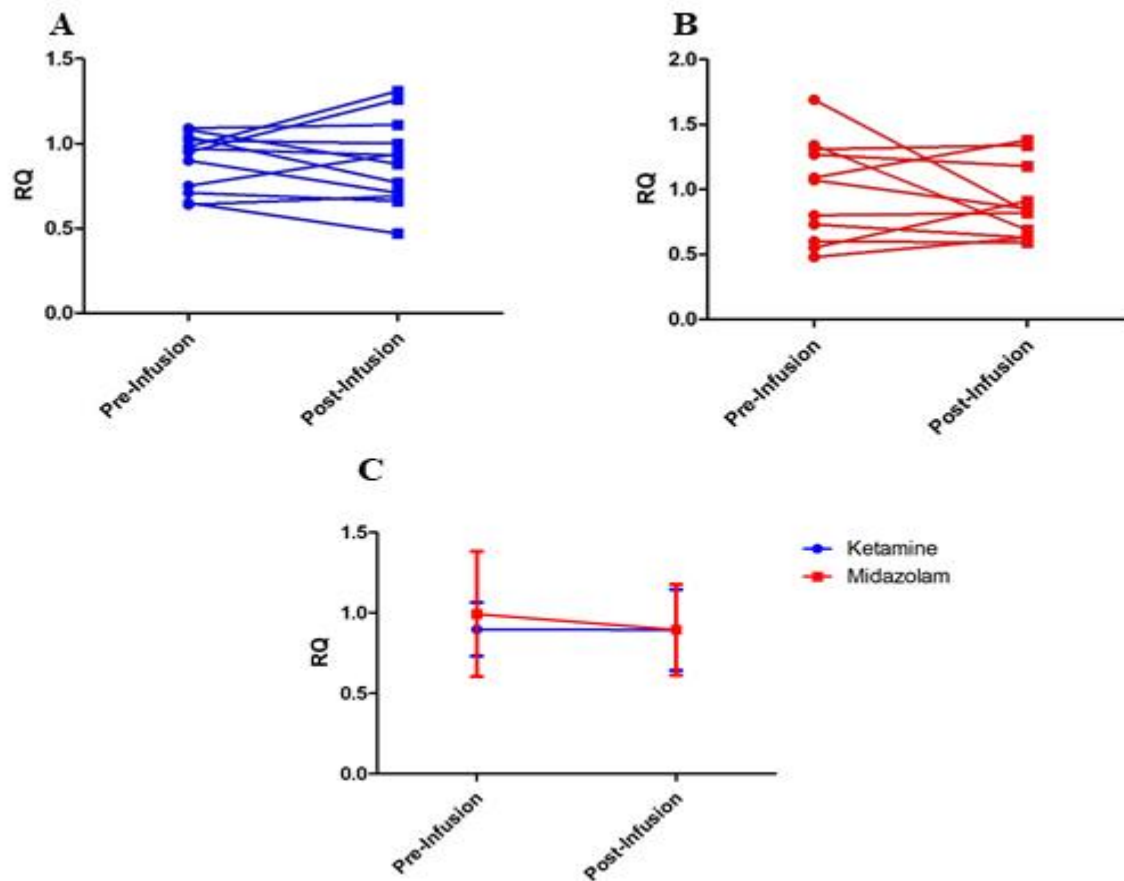


Figure 6.9 The effect of a single ketamine or midazolam infusion on *E2F1* mRNA levels

There were no significant effects of (A) ketamine ($n = 12$) or (B) midazolam ($n = 11$) on *E2F1* mRNA levels as measured by a paired t test. (C) There was no time $\times$ treatment interaction effect. Data presented as mean $\pm$ SD.

	Ketamine ($n = 12$)		Midazolam ($n = 11$)	
	Pre-Infusion	Post-Infusion	Pre-Infusion	Post-Infusion
Mean RQ	0.90	0.89	0.99	0.90
SD	0.17	0.25	0.39	0.28
SEM	0.05	0.07	0.12	0.09

Table 6.8 *E2F1* mRNA data pre- and post-infusions

Abbreviations: RQ; relative quantification value, SD; standard deviation, SEM; standard error of the mean.

6.4.7 *Sirtuin 1* peripheral gene expression analysis

SIRT1 mRNA RQ values were normal for both groups before (ketamine; $p = 0.11$, midazolam; $p = 0.75$) and after (ketamine; $p = 0.11$, midazolam; $p = 0.64$) infusions. Data were therefore analysed with parametric statistics.

6.4.7.1 *SIRT1* mRNA levels following ketamine and midazolam infusions

There was no significant difference in *SIRT1* mRNA levels between the ketamine and midazolam groups pre-infusion ($t(21) = -1.145$, $p = 0.27$). A paired t test found a significant effect of ketamine treatment on *SIRT1* mRNA levels ($t(11) = 2.535$, $p = 0.03$; Figure 6.10) but not midazolam ($t(10) = 0.9683$, $p = 0.40$). However, a repeated measure ANOVA found no overall significant main effect of time on *SIRT1* mRNA levels ($F(1, 21) = 3.508$, $p = 0.079$) and there was no main effect of treatment group ($F(1, 21) = 0.00$, $p = 0.100$) or time $\times$ treatment group interaction ($F(1, 21) = 1.637$, $p = 0.215$). A summary of the data can be seen in Table 6.9.

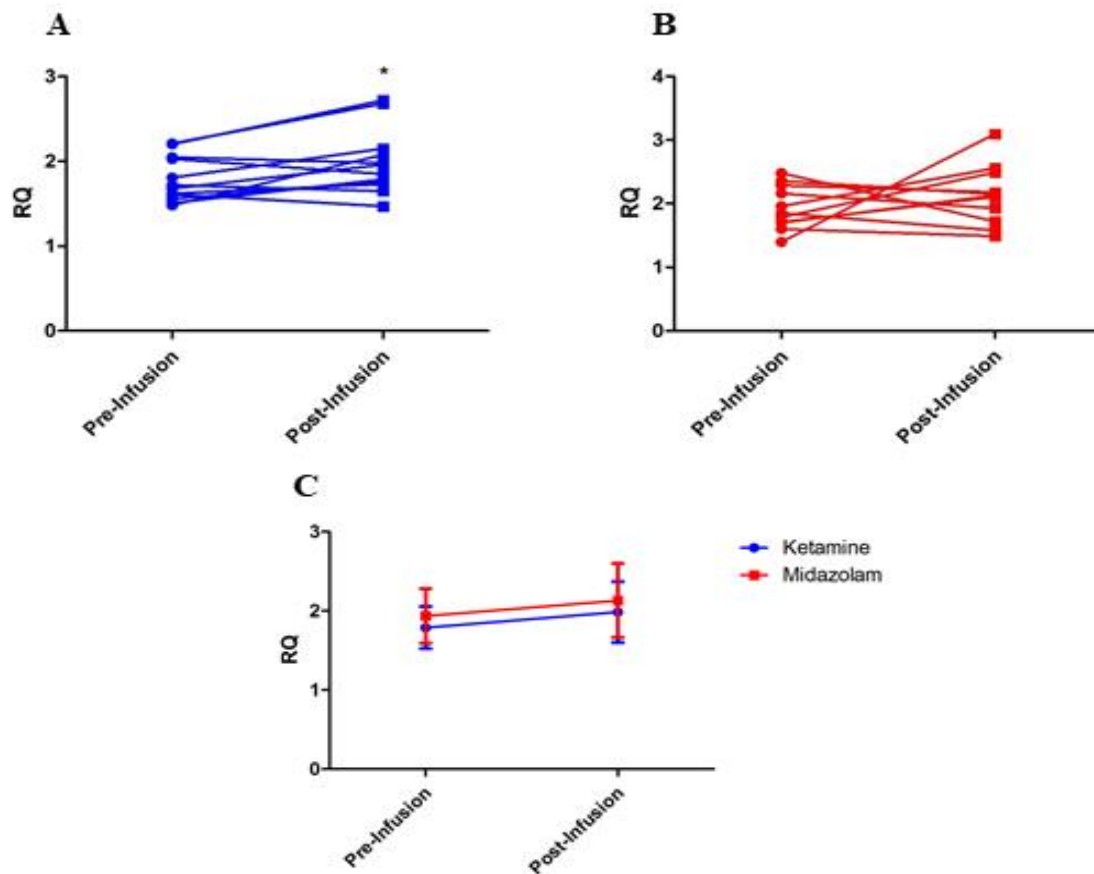


Figure 6.10 The effect of a single ketamine or midazolam infusion on *SIRT1* mRNA levels

There was a significant effect of (A) ketamine ($n = 12$) on *SIRT1* mRNA levels but no significant effect of (B) midazolam ($n = 11$) on *SIRT1* mRNA levels as measured by a paired t test. (C) There was no time $\times$ treatment interaction effect. * $p < 0.05$, data presented as mean $\pm$ SD.

	Ketamine ($n = 12$)		Midazolam ($n = 11$)	
	Pre-Infusion	Post-Infusion	Pre-Infusion	Post-Infusion
Mean RQ	1.79	1.99	1.94	2.13
SD	0.27	0.38	0.35	0.47
SEM	0.08	0.11	0.10	0.14

Table 6.9 *SIRT1* mRNA data pre- and post-infusions

Abbreviations: RQ; relative quantification value, SD; standard deviation, SEM; standard error of the mean.

6.4.8 Vascular endothelial growth factor and pigment epithelial-derived factor protein analysis

I found a significant change in both *VEGFA* and *SERPINF1* mRNA expression with ketamine treatment, and a change in the ratio of the two genes. Therefore, Peripheral plasma protein analysis was carried out to assess the effect of ketamine on VEGFA and PEDF protein levels. Plasma samples were available for six patients who received ketamine and six patients who received midazolam. Table 6.10 provides the clinical and demographic data for these groups. Patients who received a ketamine infusion had a higher CADSS score at 30 minutes after the infusion ($p = 0.01$). There were no other significant differences between groups.

	Ketamine (n = 6)	Midazolam (n = 7)	Statistical Test
Age	49.00 (13.2)	50.86 (10.8)	$t(11)=-0.28, p=0.79$
Sex, female n (%)	4 (66.7)	2 (28.6)	$p=0.21$
Smoker, yes n (%)	2 (33.3)	3 (42.9)	$p=0.59$
BMI	25.50 (4.5)	29.42 (3.8)	$t(11)=-1.71, p=0.12$
Education Completed, yes n (%)			
Secondary	2 (33.3)	4 (57.1)	
Tertiary	4 (66.7)	3 (42.9)	$p=0.48$
Bipolar Depression, yes n (%)	2 (33.3)	1 (14.3)	$p=0.44$
HAM-D24 Pre-Infusion (-60)	29.50 (4.8)	27.43 (4.8)	$t(11)=0.78, p=0.45$
HAM-D24 Post-Infusion 1 (+240)	20.83 (5.2)	16.43 (3.5)	$t(11)=1.82, p=0.10$
CADSS 0 minutes	1.33 (2.4)	1.14 (1.3)	$U=18.00, p=0.73$
CADSS +30 minutes	17.67 (15.7)	3.29 (3.9)	$U=4.00, p=0.01$
CADSS +60 minutes	3.17 (4.0)	2.43 (1.8)	$U=20.00, p=0.95$
Medications, n (%)			
SSRI	2 (33.3)	1 (14.3)	$p=0.44$
SNRI	2 (33.3)	2 (28.6)	$p=0.66$
TCA	1 (16.7)	2 (28.6)	$p=0.56$
Mirtazapine	3 (50.0)	1 (14.3)	$p=0.22$
Bupropion	0 (0.0)	1 (14.3)	$p=0.54$
Lithium	1 (16.7)	2 (28.6)	$p=0.56$
Antipsychotic	5 (83.3)	2 (28.6)	$p=0.08$
Pregabalin	3 (50.0)	1 (14.3)	$p=0.22$
Benzodiazepines	1 (16.7)	3 (42.9)	$p=0.34$

Table 6.10 Demographic and clinical details of ketamine/midazolam patients with plasma samples available

Abbreviations: BMI, body mass index; HAM-D24, Hamilton Depression Rating Scale, 24-item; CADSS, Clinician-Administered Dissociative States Scale; SSRI, Selective serotonin reuptake inhibitor; SNRI, Serotonin and noradrenaline reuptake inhibitor; TCA, Tricyclic antidepressants; U, standardised Mann-Whitney test. A p value and no other test statistic indicates Fisher's exact test. Data are presented in means (SD) or n (%). Tests with only a p value represent Fisher's exact test.

6.4.8.1 Vascular endothelial growth factor plasma protein analysis

Pre-infusion, VEGFA protein values were normal for the ketamine group ($p = 0.54$) and the midazolam group ($p = 0.21$). Protein values of plasma samples taken 40 minutes after the infusion were not normal for the ketamine group ($p = 0.03$) but were normal for the midazolam group ($p = 0.73$). At 4 hours after the infusion values for the ketamine group were normal ($p = 0.15$) but were not for the midazolam group ($p < 0.001$). Following log transformation ketamine values 40 minutes after the infusion were normal ($p = 0.14$) but midazolam values at 4 hours after the infusion were not ($p < 0.01$). Log10 transformed values were used as more data groups were normal and non-parametric statistics were used when necessary.

6.4.8.1.1 VEGFA protein levels following a single ketamine or midazolam infusion

A student t test showed no significant difference between groups at baseline ($t(11) = -2.216$, $p = 0.05$). A one way-ANOVA reported no change in VEGFA protein levels with ketamine ($F(2, 15) = 0.22$), $p = 0.80$). A Kruskal-Wallis test also reported no effect of midazolam on VEGFA protein levels ($H = 0.30$, $p = 0.86$). A repeated measured GLM reported no main effect of time ($F(1,11) = 0.94$, $p = 0.41$) or treatment group ($F(1,11) = 0.76$), $p = 0.48$), with no significant time $\times$ group interaction effect ($F(1,11) = 4.10$, $p = 0.07$; Figure 6.11). A summary of raw data can be seen in Table 6.11.

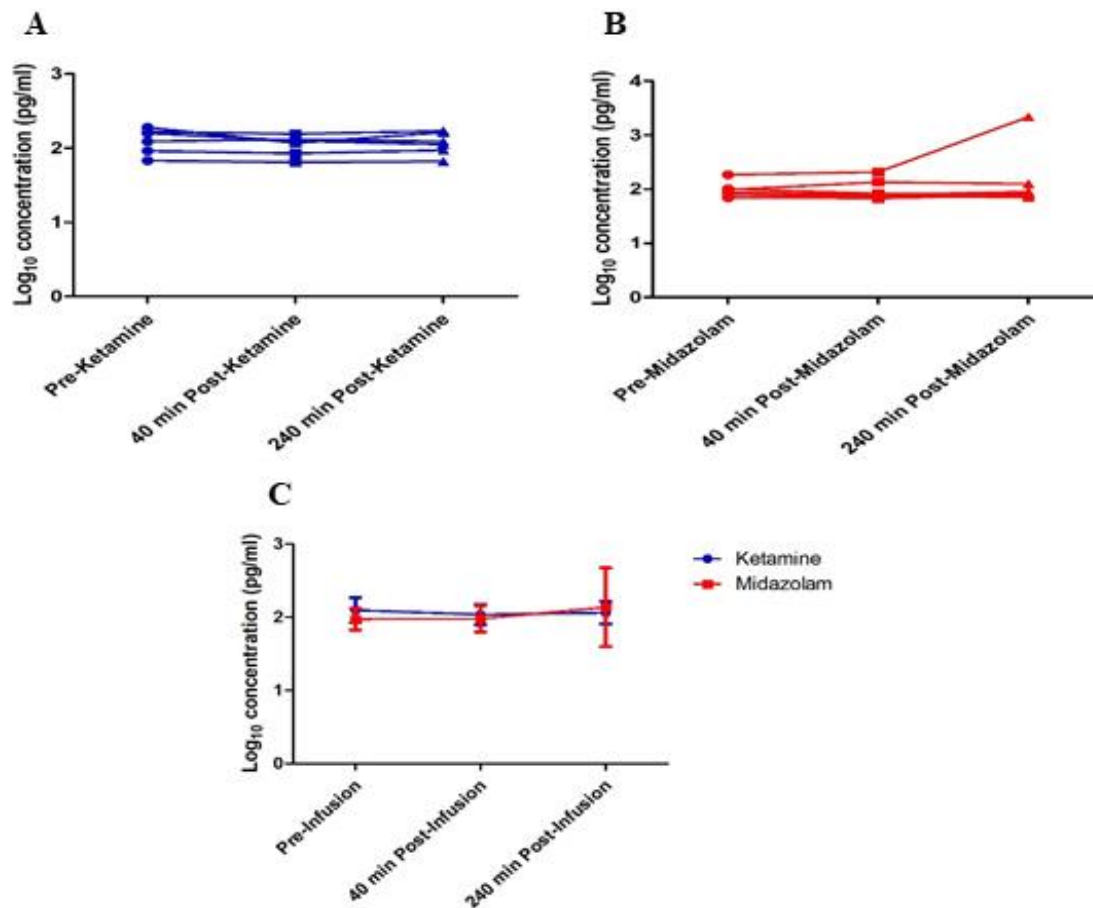


Figure 6.11 The effect of a single ketamine or midazolam infusion on plasma VEGFA protein levels

There was no significant effect of (A) ketamine ($n = 6$) on VEGFA protein levels and no significant effect of (B) midazolam ($n = 7$) (C) There was no time $\times$ treatment interaction effect. Data presented as mean $\pm$ SD.

	Ketamine ($n = 6$)			Midazolam ($n = 7$)		
	Pre-Infusion	40 min Post-Infusion	240 min Post-Infusion	Pre-Infusion	40 min Post-Infusion	240 min Post-Infusion
Mean	2.10	2.04	2.06	1.97	1.98	2.14
SD	0.17	0.14	0.15	0.15	0.18	0.54
SEM	0.07	0.06	0.06	0.06	0.07	0.20
Median	2.15	2.09	2.07	1.94	1.90	1.92
IQR	1.93 - 2.24	1.90 - 2.14	1.93 - 2.22	1.85 - 2.27	1.87 - 2.13	1.89 - 2.10

Table 6.11 VEGFA protein data pre- and post-infusions

Values represent Log₁₀ protein concentrations (pg/ml)

Abbreviations: SD; standard deviation, SEM; standard error of the mean, IQR; inter-quartile range.

6.4.8.2 Pigment epithelial-derived factor protein analyses

Pre-infusion PEDF protein values were normal for both groups (ketamine group; $p = 0.65$, midazolam group; $p = 0.21$). At 40 minutes after the infusion data were normal for the ketamine group ($p = 0.46$) but were not normal for the midazolam group ($p = 0.04$). At 4 hours after the infusion values for the ketamine group were normal ($p = 0.29$) but were not for the midazolam group ($p = 0.04$). Following log transformation all values were normal. Therefore, log10 transformed values were used.

6.4.8.2.1 PEDF protein levels following a single ketamine or midazolam infusion

There was a significant difference between the two groups at baseline as reported by a student t test ($t(11) = -2.755$, $p = 0.02$). Patients receiving midazolam had higher PEDF protein levels at baseline. A one way-ANOVA reported no change in PEDF protein levels with ketamine ($F(2, 15) = 0.12$, $p = 0.89$) or midazolam ($F(2, 15) = 1.30$, $p = 0.29$). A repeated measured GLM reported no main effect of time ($F(1,11) = 1.56$, $p = 0.21$) or treatment group ($F(1,11) = 1.30$, $p = 0.28$). There was a significant time $\times$ group interaction effect ($F(1,11) = 4.04$, $p = 0.03$) because of the significant baseline difference. See Figure 6.12. A summary of raw data can be seen in Table 6.12.

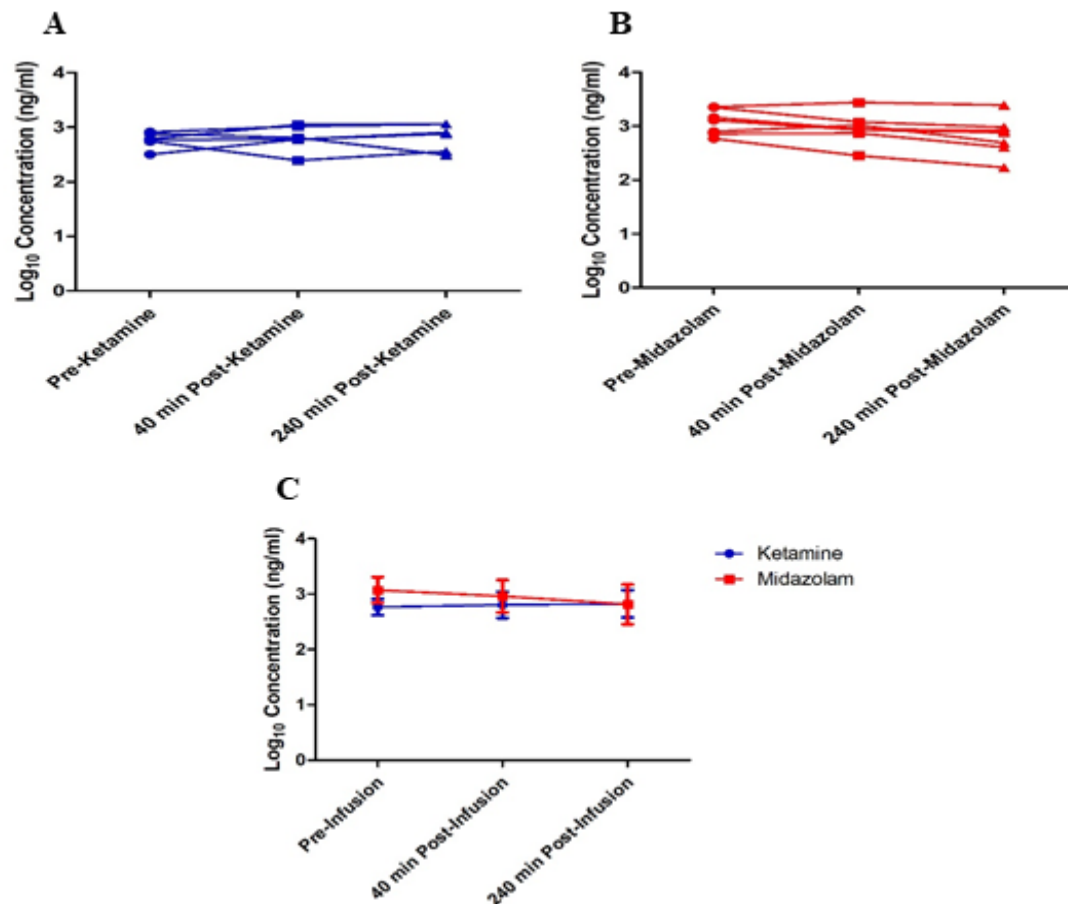


Figure 6.12 The effect of a single ketamine or midazolam infusion on PEDF protein levels

There was no significant effect of (A) ketamine ($n = 6$) on PEDF protein levels and no effect of (B) midazolam ($n = 7$) (C) There was no time $\times$ treatment effect. Data presented as mean $\pm$ SD.

	Ketamine ($n = 6$)			Midazolam ($n = 7$)		
	Pre-Infusion	40 min Post-Infusion	240 min Post-Infusion	Pre-Infusion	40 min Post-Infusion	240 min Post-Infusion
Mean	2.77	2.81	2.83	3.07	2.96	2.81
SD	0.15	0.24	0.25	0.24	0.29	0.36
SEM	0.06	0.10	0.10	0.09	0.11	0.14

Table 6.12 Plasma protein data pre- and post-infusions

Values represent Log₁₀ protein concentrations (ng/ml).

Abbreviations: SD; standard deviation, SEM; standard error of the mean.

6.4.8.3 VEGFA/PEDF protein ratio analysis

As with the mRNA data, a role for the ratio of VEGFA to PEDF protein ratio was investigated. Pre-infusion, VEGFA/PEDF ratio values were not normal for the ketamine group ($p = 0.02$) but were for the midazolam group ($p = 0.16$). Ratio values of plasma samples taken 40 minutes after the infusion were normal for both groups (ketamine; $p = 0.06$, midazolam; $p = 0.67$). At 4 hours post-infusion values for the ketamine group were normal ($p = 0.07$) but were not for the midazolam group ($p < 0.001$). Following log transformation, all data were normal. Therefore, log10 transformed values were used.

6.4.8.3.1 VEGFA/PEDF ratio levels following a single ketamine or midazolam infusion

A student t test showed no significant difference between groups at baseline ($t(11) = 1.480$, $p = 0.17$). A one way-ANOVA reported no change in ratio levels with ketamine ($F(2, 15) = 0.26$, $p = 0.78$) or midazolam ($F(2, 15) = 2.48$, $p = 0.11$). A repeated measured GLM reported no main effect of time ($F(1,11) = 2.50$, $p = 0.11$) or treatment group ($F(1,11) = 0.32$, $p = 0.59$), with no significant time $\times$ group interaction effect ($F(1,11) = 2.19$, $p = 0.16$; Figure 6.13). A summary of raw data can be seen in Table 6.13.

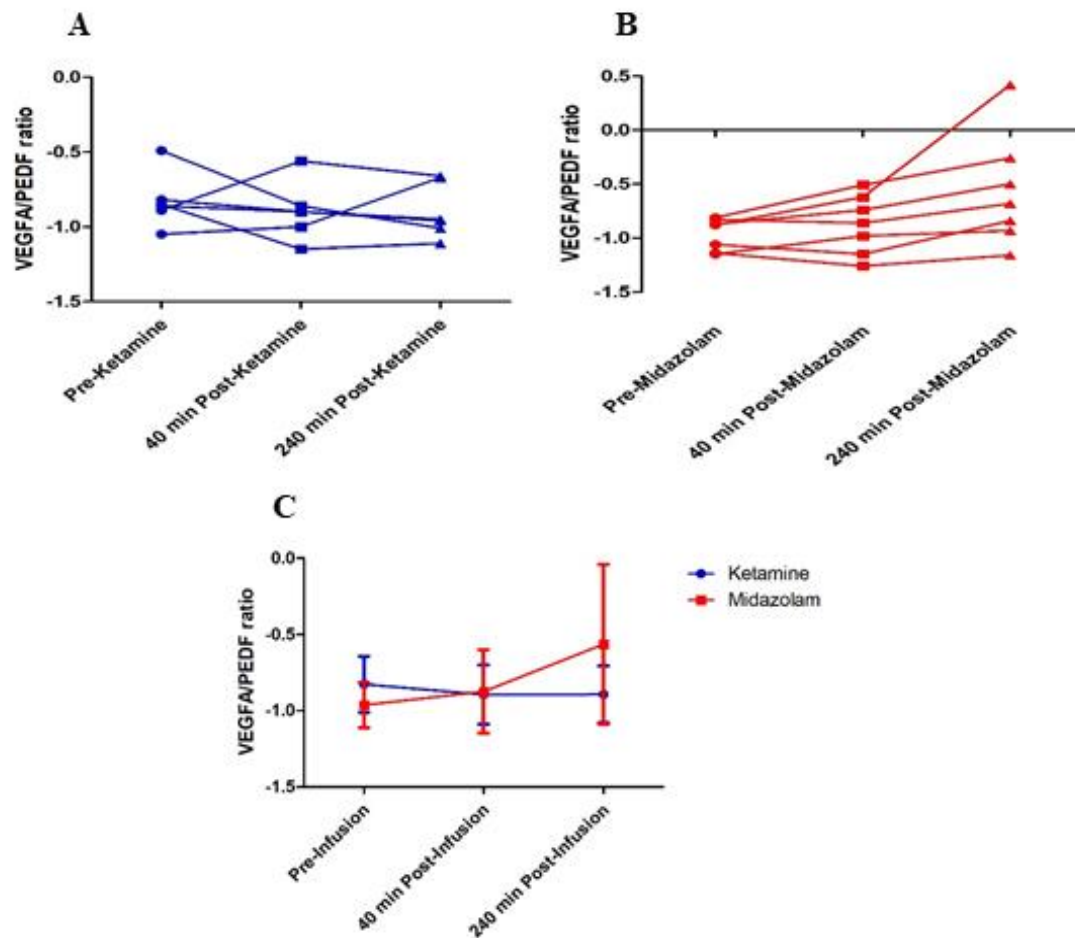


Figure 6.13 The effect of a single ketamine or midazolam infusion on the VEGFA/PEDF protein ratio

There was no significant effect of (A) ketamine ($n = 6$) on ratio levels and no significant effect of (B) midazolam ($n = 7$) (C) There was no time $\times$ treatment effect. Data presented as Log₁₀ mean $\pm$ SD.

	Pre-Infusion	40 min Post-Infusion	240 min Post-Infusion	Pre-Infusion	40 min Post-Infusion	240 min Post-Infusion
Mean	-0.96	-0.87	-0.56	-0.83	-0.90	-0.90
SD	0.15	0.27	0.52	0.18	0.19	0.19
SEM	0.06	0.10	0.20	0.08	0.08	0.08

Table 6.13 VEGFA/PEDF ratio data pre- and post-infusions

Values represent a Log₁₀ ratio of VEGFA/PEDF values

Abbreviations: SD; standard deviation, SEM; standard error of the mean.

6.5 Discussion

6.5.1 Results summary

This study reported that both ketamine and midazolam significantly decreased depression severity as measured by the HAM-D24 4 hours after a single infusion of ketamine. Although a significant antidepressant effect of ketamine over midazolam was not reported, molecular changes specific to ketamine were reported. *VEGFA* mRNA was significantly increased following ketamine treatment only. Ketamine alone reduced *SERPINF1* mRNA levels but this was not statistically significant. Ketamine also significantly increased the *VEGFA/SERPINF1* mRNA ratio, suggesting ketamine may have an effect on the interaction of the two genes. Neither ketamine nor midazolam had an effect on *E2F1* or *SIRT1* mRNA. There were no associations between mRNA levels of any gene and improvement in depression scores or changes in the CADSS. No significant changes were reported in VEGFA, PEDF or the VEGFA/PEDF protein ratio levels with ketamine or midazolam treatment. There were no statistically significant associations between mRNA or protein levels and clinical measures (HAM-D24 and CADSS scores).

6.5.2 A single infusion of ketamine, but also midazolam, improves depression scores in patients

Both ketamine and midazolam significantly decreased depression scores as measured by the HAM-D24 as measured 4 hours after a single infusion. A significant reduction in depression severity following ketamine treatment is in line with other studies and supports its efficacy as an antidepressant treatment (Berman et al., 2000, Zarate et al., 2006, Singh et al., 2016). However, a similar improvement with midazolam treatment was not expected as previous trials have reported a greater efficacy of ketamine treatment

over midazolam (Murrough et al., 2013, Fava et al., 2018, Phillips et al., 2019, Haile et al., 2014). It is unclear why midazolam treatment had the same effect on HAMD-24 scores as ketamine. Previous reports differed in their patients and the use of medications. In two of these previous trials, patients were taken off any psychotropic medications except for non-benzodiazepine hypnotics (Murrough et al., 2013, Haile et al., 2014). In a third trial medications were maintained as normal once the dose had been stable for the previous 4 weeks (Fava et al., 2018). In the fourth trial patients were medicated but avoided benzodiazepines 24 hours before the clinics as they may attenuate ketamine response (Phillips et al., 2019). As all these trials reported a greater efficacy of ketamine over midazolam, it is difficult to say if any medications influence a patient's response to ketamine, but it is unlikely as both medicated and unmedicated patients responded favourably to ketamine over midazolam. The dose of ketamine (0.5mg/kg) and midazolam 0.045mg/kg used in the KARMA-Dep pilot trial was also in line with previous studies, as well as the method of slow infusion (Murrough et al., 2013, Fava et al., 2018). As the present study had a small sample size, and as a pilot trial was not designed to measure efficacy, a clear conclusion on the efficacy of ketamine versus midazolam cannot be made.

6.5.3 A single infusion of ketamine, but not midazolam, increases peripheral blood VEGFA mRNA levels but not plasma protein levels

Peripheral whole blood VEGFA mRNA levels were increased following a single infusion of ketamine, but were not altered by midazolam treatment. This was as I had hypothesised based on studies that show an effect of antidepressant treatments on VEGFA (both mRNA and protein levels) (Kolshus et al., 2017, Minelli et al., 2011) and the report that increased VEGF protein signalling is necessary for the antidepressant actions of ketamine

(Deyama et al., 2019). One of these two clinical studies reported ECT decreased *VEGFA* mRNA levels (Kolshus et al., 2017), whereas the second reported increased *VEGFA* levels in serum one month post ECT (Minelli et al., 2011). No changes have been reported with other antidepressant treatments, but chronic ketamine abuse has been reported to reduce *VEGFA* mRNA levels (Fan et al., 2015). The effect of a single infusion of ketamine on *VEGFA* mRNA levels cannot be directly compared to these studies as they measure VEGF after a chronic antidepressant treatment or after ketamine abuse. Animal studies investigating the role of VEGF in antidepressant response are more consistent. Fluoxetine, desipramine, and ECS increase rodent VEGF gene expression in the brain (Warner-Schmidt and Duman, 2007, Newton et al., 2003). In animal models, VEGF release is also necessary for the antidepressant effects of ketamine (Deyama et al., 2019), and VEGF administration mimics the effects of ketamine and desipramine (Deyama et al., 2019, Warner-Schmidt and Duman, 2007). Therefore, an increase in *VEGFA* mRNA or protein following an antidepressant treatment is in line with what would be expected based on evidence from animal model studies.

One limitation of the *VEGFA* gene expression study carried out for this thesis is that data was still non-parametric after log transformation. Although data was only mildly skewed (with one data point identified by SPSS as an outlier), and non-parametric analysis was carried out were possible with the GLM, a larger replication study of this data would hopefully allow for fully parametric analysis and strengthen this finding.

In contrast to the mRNA data, neither ketamine nor midazolam altered *VEGFA* protein levels. Although my hypothesis was that ketamine would increase *VEGFA* protein levels, this result is not completely unexpected. My lab have previously reported a change in *VEGFA* mRNA levels but not plasma protein levels in patients from the EFFECT-Dep study (Kolshus et al., 2017, Ryan and McLoughlin, 2018). Studies on the

effect of antidepressant treatment on VEGFA protein levels in the periphery and cerebral spinal fluid are conflicting. Some studies report an increase (Minelli et al., 2011), others report no change (Ryan and McLoughlin, 2018, Kranaster et al., 2019, Ventriglia et al., 2009) or report opposite changes in patients who respond or do not respond to treatment (Fornaro et al., 2013). In a study that did report a change in protein levels in response to treatment, this change was one month following treatment and not immediately after (Minelli et al., 2011). Furthermore, one study on the effect of ketamine on BDNF, another neurotrophin, reported an increase only 1 week after ketamine infusion (Allen et al., 2015). Therefore, the time the bloods in this KARMA-Dep pilot trial were taken may not be suitable for detecting a change in protein. mRNA levels were increased at 4 hours post-infusion but protein levels may take longer to reflect this change. The sample numbers for the protein analysis were also small. It may still be important to investigate VEGFA protein in a larger sample size, and at different time points post-infusion (possibly 1-week) or after a course of infusions.

6.5.4 Ketamine, but not midazolam, treatment reduces peripheral *SERPINF1* mRNA levels but neither affect plasma PEDF protein

Ketamine significantly decreased *SERPINF1* mRNA (as measured by a paired t-test), and there was a trend towards a time $\times$ treatment group interaction, indicating ketamine, but not midazolam, decreased peripheral whole blood *SERPINF1* mRNA levels. As ECT treatment had previously shown to increase plasma protein PEDF concentration (Ryan et al., 2017) an effect of ketamine was hypothesised. However, an increase was hypothesised. This is the first study to investigate the effect of ketamine on PEDF, and no animal data have been published. Therefore, there is no study to directly compare my results to. In chapter five of this thesis, antidepressants significantly decreased *Serpinf1*

mRNA in cortical astrocytes. It may be possible that gene and protein levels of PEDF are affected differently in response to treatment. Therefore, measuring PEDF protein would give a better insight into the functional role of PEDF in response to ketamine.

Based on the mRNA results and the previous work by my lab, an effect on PEDF protein would be expected. Neither ketamine nor midazolam significantly altered plasma PEDF protein levels. PEDF protein values increased slightly from 607.3ng/ml to 760ng/ml with ketamine treatment and decreased from 1130.0ng/ml to 865.3ng/ml with midazolam treatment. There was a high degree of variability with the PEDF protein data, with a significant difference between the groups at baseline. A reason for this is unclear, and is possible down the variation between the groups and the small sample number. Therefore, this study would have to be repeated with a larger sample size. A change in timing for collection of plasma could also be considered, similar to VEGFA protein a change in PEDF protein may take longer. For ECT, bloods were taken 1-3 days after a course of treatment, so a 1 day follow up blood after a single or course of ketamine infusions could be considered.

6.5.5 Ketamine increases the ratio of *VEGFA/SERPINF1* mRNA levels but not the protein ratio

A close relationship between VEGF and PEDF signalling has been reported in literature (Falk et al., 2010). The VEGF/PEDF ratio has also been studied as a potential diagnostic marker, with one study reporting the ratio as a possible diagnostic factor for pre-eclampsia (Zhang et al., 2018). Therefore, as ketamine increased *VEGFA* mRNA levels and decreased *SERPINF1* mRNA levels in the KARMA-Dep patients, a relationship between the two is of interest. As hypothesised, ketamine alone increased the ratio of the two genes indicating that as *VEGFA* mRNA levels increase, *SERPINF1* mRNA levels

decrease. Neither ketamine nor midazolam affected this ratio at a protein level. However, as the protein levels of VEGFA and PEDF were not changed by ketamine a change in the ratio of the two would not be expected. Reasons for the protein not changing have already been discussed in sections 6.5.3 and 6.5.6 above. As significant changes were found at an mRNA level, follow-up studies with the protein as mentioned in these sections may be important.

To my knowledge, this is the first study to look at the relationship of these two genes in response to ketamine treatment. The increasing ratio indicated a higher expression of *VEGFA* mRNA and lower expression of *SERPINF1* mRNA with ketamine treatment. However, it is difficult to interpret what this means in relation to ketamine response. In the periphery and retinal cells, VEGF and PEDF signalling has been studied in relation to angiogenesis and psoriasis (Ohno-Matsui et al., 2001, Yan et al., 2018). In these published findings, higher levels of VEGF are associated with lower levels of PEDF, and a balance between the two is reported to be important for normal physiological function (Yan et al., 2018, Wang et al., 2017, Deeg et al., 2007). In the brain, there is little reported on the relationship between the two genes. One study does report a positive relationship between the two proteins, with a positive correlation of the two proteins reported in the striatum of patients with Parkinson's disease (Yasuda et al., 2007). This suggests the proteins may react differently in different cell types. As both proteins are reported to be neurotrophic, exploring this relationship in rodent models of depression, and investigating the effects of ketamine on these proteins in the CNS, would be of interest and would give more clarity to the roles of these two genes/proteins and their signalling in depression.

Further studies with a larger sample size would also be of interest for both protein and gene expression analysis to determine if there is a change in the protein ratio and if

this has a role in depression, or a change in mRNA levels could be a more stable marker with clinical use.

6.5.6 Neither ketamine nor midazolam influence *E2F1* mRNA expression

Peripheral whole blood *E2F1* mRNA levels were unaffected by ketamine or midazolam treatment. To date, the study that formed chapter three of this study provides the only available clinical data on *E2F1* and depression (McGrory et al., 2019). *E2F1* mRNA was decreased in patients with depression but remained unchanged by ECT. This may have been due to the time frame in which post-ECT bloods were taken. For the KARMA-Dep trial, post-infusion bloods were taken at this same time - 4 hours post the commencement of infusions. Only a single study has analysed the effects of ketamine on *E2F1* signalling in the mouse brain (Soriano et al., 2010). Ketamine increased the protein expression of *E2F1* and other cell cycle related proteins 6 hours after infusion. This indicates that the time frame in which bloods were taken in the KARMA-Dep trial could have been appropriate to detect a change in *E2F1* mRNA levels. Therefore, as hypothesised in section 3.5.6, *E2F1* may be involved in the antidepressant response of both ECT and ketamine in the brain, but this may not translate to a peripheral level. *E2F1* mRNA may not be useful as a clinical marker of depression, or as a marker of treatment response.

6.5.7 *Sirtuin1* mRNA levels are elevated 4 hours post ketamine and midazolam infusions

SIRT1 mRNA levels were increased, but not significantly, following treatment of both ketamine and midazolam. Although a paired t test reported a significant increase with ketamine infusion, a repeated measures ANOVA uses bonferroni correction, therefore which could account for the loss in significance. No study has examined the effect of

ketamine or midazolam on SIRT1 in patients or in rodent studies. Sub-anesthetic doses of ketamine have been reported to lower *Sirt1* mRNA in zebrafish (Zakhary et al., 2011). It is difficult to interpret in this study if *SIRT1* mRNA is increased non-specifically by both treatments, or if the change is because of diurnal variation. SIRT1 is reported to follow a diurnal pattern as it is closely linked to circadian genes such as CLOCK (Wallace et al., 2018, Logan et al., 2018). A second control group, such as a non-active control such as saline infusion, or a healthy control with bloods taken at the same time points as the patients receiving infusions, would be needed to establish if this change is due to diurnal variation or if both drug treatments influence SIRT1. Nonetheless, SIRT1 may not be a suitable specific marker for response to ketamine treatment.

6.5.8 Limitations of this study

The main limitation of this study is that KARMA-Dep was a pilot trial and the main purpose was to evaluate feasibility and safety of the trial. It was not intended to test the efficacy of ketamine over midazolam, or to test for molecular changes in response to treatments (Arnold et al., 2009, Thabane et al., 2010). Therefore, it was not designed with the statistical power to detect differences or correlations and all work was exploratory. Nevertheless, molecular differences were detected. It would be of interest to follow these up with a bigger cohort, with more power to detect associations with clinical response.

A second limitation is the use of antidepressants throughout the trial. Publications on molecular changes with ketamine are varied. Some trials take patients off all medications (Murrough et al., 2013), others do not allow the use of benzodiazepines because of a possible interaction with ketamine (Phillips et al., 2019) and some allow medication as usual (Fava et al., 2018). These trials all reported a similar efficacy of

ketamine. As our patients do not alter their medication throughout the trial any change in gene expression is likely because of the use of ketamine.

Finally, molecular analysis was only performed pre/post the first infusion with no follow up data available. Therefore it is unknown if the changes detected are transient or if they are sustained for a longer period of time. Follow up data would be of interest to determine if a sustained effect on gene expression may be linked to response or remission in patients. For correlation analysis, the time point at which the bloods were taken do not match the time the CADSS scores were taken. Therefore there may still be some relationship between *VEGFA* and *SERPINF1* mRNA levels and CADSS scores, future studies would need to match time points to clarify this.

6.6 Conclusions

Overall, this study indicates that changes in *VEGFA* and *SERPINF1* mRNA levels may be involved in the molecular response of ketamine. To my knowledge, this is the first study that reports ketamine alters *VEGFA* mRNA levels, and is the first to indicate *SERPINF1* mRNA in the antidepressant actions of ketamine. Although this result did not translate to protein, numbers were low and this study should be repeated in a larger sample size, and maybe with plasma samples taken at a later time point. Finally, this study supports a novel role for the relationship between *VEGFA* and PEDF in ketamine's molecular action. Future studies will be needed to clarify how this novel finding is involved in the molecular effect of ketamine.

Chapter 7.

Conclusions and Future Directions

7.1 Introduction

Depression is the most common psychiatric disorder and is now the leading cause of disability worldwide, contributing substantially to the overall global burden of disease (WHO, 2017, Vos et al., 2012). Electroconvulsive therapy (ECT) is the most effective treatment for depression, but due to the limited knowledge of its molecular mechanism of action and the concern over the cognitive side effects its use is limited (UK ECT Group, 2003). Ketamine has emerged as a rapid antidepressant therapy for treatment resistant depression. However, its effects are short lived and its antidepressant molecular mechanism not fully understood (Duman, 2018). Therefore, the aim of this thesis was to further the knowledge of the molecular mechanisms of these two treatments. Previous studies from my lab, a deep-sequencing study and a proteomic study, have implicated vascular endothelial growth factor-A (VEGFA), E2F1, Sirtuin1 (SIRT1) and pigment epithelial-derived factor (PEDF) in depression (Kolshus et al., 2017, Ryan et al., 2017). This work presented in this thesis aimed to answer the following questions:

1. Are E2F1 and SIRT1 downregulated in depression at a gene expression level and does ECT normalise any such changes? As with VEGFA, are these changes more specific to patients with a psychotic subtype of depression?
2. What cells in the brain express PEDF and do antidepressants alter mRNA levels and secretion of PEDF *in vitro*?
3. Does a single ketamine infusion have a similar or different effect on VEGFA, E2F1, SIRT1 and PEDF as previously reported for ECT?

7.2 *E2F1*, depression and ECT

This was the first study that evaluated a role for *E2F1* in depression at a clinical level. *E2F1* mRNA levels were downregulated in patients with depression in comparison to healthy controls. Levels were unchanged by ECT and there were no differences among the different depressive subgroups (McGrory et al., 2019). As a change in *E2F1* mRNA levels with ECT was expected, as were differences between patients with or without psychotic features based on my lab's previous deep sequencing study (Kolshus et al., 2017), gene expression analysis was conducted on brain tissue and peripheral whole blood of male rats who had received acute or chronic electroconvulsive stimulation (ECS). ECS downregulated *E2f1* mRNA in the dentate gyrus and there was no peripheral effect. This indicates that *E2F1* may have a role in the molecular mechanism of ECT in the brain, but it may not be useful as a clinical marker for response to ECT. Clinically, as a peripheral measurement, this study does not point to any immediate future direction for *E2F1* research. However, cell cycle related proteins are of interest in pre-clinical depression research given the extensive evidence on reduced brain volumes in depression and a role for synaptic plasticity in animal models of depression (Duman, 2014, Duman, 2002, Santos et al., 2018, Holmes et al., 2019).

7.3 *Sirtuin1*, depression and ECT

Before my study, lower levels of peripheral *SIRT1* mRNA had been reported in depression by two studies (Abe et al., 2011, Luo and Zhang, 2016) but neither had looked at the effect of treatment on these levels. Similar to the *E2F1* study, *SIRT1* mRNA levels were downregulated in medicated patients with depression in comparison to healthy controls. Levels were unchanged by ECT and there were no differences among the patients with or without psychotic depression. There were differences among the bipolar

and unipolar subgroups, but these were not statistically significant after controlling for covariates. There was also a trend for an association between a change in *SIRT1* mRNA levels with treatment and a change in HAM-D24 scores; as *SIRT1* levels increased, HAM-D24 scores decreased (McGrory et al., 2018). Although this study did not find any statistically significant evidence to support the use of *SIRT1* as a clinical marker, my results support replication of the analysis in a larger group of patients.

Prior to correction for covariates, *SIRT1* mRNA levels were lower in patients with bipolar depression in comparison to unipolar depression. Following ECT, these levels returned to a similar level as the unipolar patients but not to control levels. A previous study that reported on *SIRT1* mRNA in major depressive disorder ($n = 20$) and bipolar disorder ($n = 12$) did not analyse differences between the two subgroups, but in Figure 1 of Abe et al's (2011) paper *SIRT1* mRNA levels in the bipolar group appeared lower (Abe et al., 2011). As with the study in the present thesis, the number of patients in the bipolar group was small. This would be interesting to follow up in a larger sample size as it might represent a biological difference between the two groups.

In patients who responded to ECT and those who attained remission, there was a trend towards a negative correlation between a change in *SIRT1* mRNA levels with ECT and a change in HAM-D24 scores. As these correlations were exploratory, this would merit further investigation in a larger cohort. It would also be interesting to measure *SIRT1* mRNA after each ECT session, to determine if a change early on in treatment might predict if a patient responds to ECT as this would be more clinically useful than looking at the change between the first and last session.

An important future direction for Sirtuin research in depression would also be to examine *SIRT1* activity in patients with depression. To date, only mRNA levels of *SIRT1*

have been reported. SIRT1 activity, would give a better functional insight into its role in depression. Animal studies have also reported a change in SIRT1 activity with antidepressants when there is no change at a gene or protein level (Abe-Higuchi et al., 2016). Therefore, this might be an important area of research given the role of SIRT1 in regulating many peripheral systems linked to depression.

As with E2F1, as there was no overall peripheral change, *Sirt1* mRNA levels in brain tissue and peripheral whole blood of male rats who had received acute or chronic ECS were analysed. *Sirt1* mRNA levels were increased specifically in the hippocampal formation but no change was observed in the periphery. This may indicate that future work on SIRT1 in the molecular mechanisms of ECT should focus on the CNS rather than the periphery.

In conclusion, although I have found some preliminary evidence that SIRT1 might be useful at a clinical level, research focusing on the CNS may give more insight into its role in depression and any potential link to the molecular mechanism of ECT.

7.4 The effect of antidepressants on pigment epithelial-derived factor in vitro

A previously reported proteomics study by my lab identified PEDF as a protein of interest in depression. In a separate validation study PEDF protein levels were found to be increased in patients with depression, but then increased further with ECT (Ryan et al., 2017). As these patients were on medication this thesis hypothesised that antidepressants might regulate PEDF expression and, as PEDF is neuroprotective, this may be linked to neuronal plasticity. A series of experiments showed that astrocytes were the main source of PEDF from neuronal and glial cells. A time and dose response analysis was carried out with two antidepressants. Fluoxetine did not regulate PEDF expression at any time

point but imipramine significantly decreased its expression at 24 hours. However, imipramine did not affect protein levels. Cells were also treated with noradrenaline as these antidepressants target the monoamine system, and noradrenaline previously increased PEDF secretion in epithelial cells (Lashbrook and Steinle, 2005). Based on this study I hypothesised noradrenaline would increase PEDF secretion from astrocytes. Noradrenaline did not affect PEDF secretion from astrocytes. These results went against the hypothesis and point to the conclusion that PEDF does not have a role in antidepressant induced plasticity in the cortex or at least in cultured primary neurons. However, imipramine and noradrenaline did reduced *Serpinf1* mRNA. Further studies to investigate the role of PEDF in animal models of depression, or looking at post-translational modifications and function of PEDF in response to treatment, would be useful to explore if PEDF is involved in the molecular pathophysiology of depression. It is also possible that PEDF is of more interest at a peripheral level.

7.5 The effect of ketamine on VEGFA, PEDF, E2F1 and Sirtuin1

Ketamine is an emerging effective treatment for treatment resistant depression but its antidepressant effect is short lived (Duman, 2018). Identifying ketamine's molecular targets may help understand its mechanism of action, or identify markers that may predict response to ketamine. Therefore, my work for this thesis investigated the effects of ketamine on the peripheral blood levels of genes of interest that were previously identified in the deep sequencing and proteomics studies by my lab in depressed patient treated with ECT (Kolshus et al., 2017, Ryan et al., 2017). Of note, increased VEGF secretion has very recently been implicated in the antidepressant actions of ketamine (Deyama et al., 2019). VEGF and PEDF signalling are also linked and the two proteins are reported to regulate each other (Falk et al., 2010). Therefore, the ratio of the two genes

was also of interest. When changes were identified at a gene level I also performed analysis of relevant proteins in peripheral blood plasma. As my study used samples from a small trial, the KARMA-Dep study, all analysis is exploratory and these are preliminary results.

7.5.1 Ketamine and vascular endothelial growth factor A

I found that a single dose of ketamine (0.5 mg/kg) increased whole blood peripheral *VEGFA* mRNA levels four hours after beginning of a slow 40 min infusion. In contrast midazolam had no effect. Neither ketamine nor midazolam affected plasma VEGF protein levels 40 minutes or 4 hours after a single infusion. Animal models have implicated an increased release of neurotrophins, both brain-derived neurotrophic factor (BDNF) and VEGF, in the antidepressant mechanism of action of ketamine (Deyama et al., 2018, Deyama et al., 2019). As BDNF protein levels increase with a single infusion ketamine (Haile et al., 2014), an increase in *VEGFA* would be expected. Therefore, the significant increase in *VEGFA* mRNA levels were as I had hypothesised. Reports on plasma/serum neurotrophins in response to ketamine are varied: some report no change (Rybakowski et al., 2013), others a rapid change (Haile et al., 2014), and others a delayed change (Allen et al., 2015). Therefore, as plasma sample numbers in my study were small and only taken 40 minutes and 240 minutes post-infusion, an effect of ketamine on *VEGFA* protein cannot be ruled out. Further studies with increased sample numbers and bloods taken one week post infusion may be of interest. An increase in the mRNA levels may still be of interest at a clinical level. As the KARMA-Dep trial was a pilot study with a small sample size there was only a small number of ketamine responders. Repeating this study in a larger sample size would be of use to assess the utility of *VEGFA* mRNA levels as a clinical marker of ketamine response.

7.5.2 Ketamine and pigment epithelial-derived factor

A single infusion of ketamine moderately decreased whole blood peripheral *SERPINF1* (*PEDF*) mRNA expression 4 hours after the infusion, but this was not significant. Midazolam had no effect. Neither ketamine nor midazolam significantly affected plasma PEDF protein levels 40 minutes or 4 hours after a single infusion. Based on previous work, ECT increases plasma PEDF protein levels (Ryan et al., 2017). Therefore, as ECT and ketamine have been reported to produce similar effects (Allen et al., 2018a, Allen et al., 2015), an increase in *SERPINF1* mRNA and PEDF protein was hypothesised. *SERPINF1* mRNA levels were decreased (though not statistically significantly so) but a change in protein levels was not seen. Reasons for no effect of ketamine on PEDF protein might be similar to those for VEGF protein levels discussed in section 7.5.1 above. Also, plasma PEDF protein levels were quite variable, ranging from 318 to 2774ng/ml. With a high level of variability, bigger numbers may be needed to see a statistically significant effect. As there are no studies to which to compare an effect of antidepressant treatment on *SERPINF1* mRNA levels, it is possible that gene expression and protein levels are affected differently. Repeating this study with a larger cohort may therefore be of interest.

7.5.3 Ketamine and the ratio of VEGFA and PEDF

The relationship between VEGF and PEDF is widely reported in the literature, most often their roles in regulating angiogenesis (Falk et al., 2010, Zhang et al., 2018, Cai et al., 2011, Li et al., 2019, Long et al., 2016). A ratio of the two have been reported to play a role in preeclampsia and infant haemangioma (Zhang et al., 2018, Zhu et al., 2018). As both genes/proteins have been implicated in depression (Kolshus et al., 2017, Ryan et al., 2017), investigating their relationship in the response to ketamine was of interest. A

single infusion of ketamine significantly increased the ratio of *VEGFA/SERPINF1* mRNA, with an increase indicating higher *VEGFA* levels and lower *SERPINF1* levels. No change in the ratio of the protein was reported but as there were no individual changes this was to be expected. This is the first report of a change in the *VEGFA/SERPINF1* ratio following an antidepressant treatment. A significant change in the gene levels indicates that this would be worth following up in a larger number of patients. Both PEDF and VEGFA have been linked to biological pathways that are implicated in depression. PEDF regulates oxidative stress, inflammation and is neuroprotective (Wang et al., 2019, Yabe et al., 2005, Tombran-Tink and Barnstable, 2003, Farnoodian et al., 2018). VEGFA has been linked to inflammation, although the literature is not strong, but has also been reported to play a role in neurogenesis and neuroprotection (Shim and Madsen, 2018, Martin et al., 2009, Zhu et al., 2003). Therefore, using rodent models of depression, investigating how these two proteins interact in depression could lead to a better understanding of a new interesting pathway relevant to depression.

7.5.4 Ketamine, *E2F1* and *Sirtuin1*

Similar to ECT, there were no statistically significant effects of ketamine or midazolam on *E2F1* or *SIRT1* peripheral whole blood mRNA levels. *SIRT1* mRNA levels increased slightly with both ketamine and midazolam treatment. This is possibly due to diurnal variation. As in the ECT (EFFECT-Dep) study with these genes, the results indicate that *E2F1* and *SIRT1* may not be useful as clinical markers and are not involved in the peripheral blood molecular response to ketamine.

7.6 Future directions

The results of this thesis point towards further work needed in the role of VEGFA and PEDF in depression and its treatment. Although the other two genes of interest, E2F1 and SIRT1, were downregulated in depression, the results of this work indicate that from a peripheral blood perspective they may not be involved in treatment response. As ECS increased levels of *Sirt1* in the hippocampal formation, and decreased *E2f1* levels in the dentate gyrus, investigating their signalling in rodent brains in response to ECS may be of use, but clinically they may be trait markers of depression. *VEGFA* mRNA levels, and the ratio of *VEGFA/SERPINF1* mRNA levels, did increase with a single infusion of ketamine. Therefore, these genes would be of interest in a follow-up, larger trial with ketamine that is designed for molecular analysis. Comparison of levels with a group of healthy controls would also be of interest as this has not yet been reported. It would also be of interest to explore the relationship of VEGFA and PEDF in the brain using rodent models as current research on their relationship focuses on the peripheral and the retina. Based on the findings of this thesis the following follow up studies would be of interest:

1. Compare the peripheral blood *VEGFA/PEDF* mRNA ratio in depressed patients to healthy controls.
2. Analyse gene expression levels in a larger group of patients pre-/post-infusion and determine any relationship with response to ketamine.
3. Analyse protein levels in a larger group of patients and over a longer post-infusion time (1 week).
4. Investigate in a rodent models if VEGFA/PEDF signalling in the brain has a role in the pathophysiology of depression (using gene knock-out animal models).
5. Investigate the effect of ketamine on VEGFA/PEDF signalling in the rodent brain.

7.7 Conclusions

Deep-sequencing and proteomic studies, such as the ones on which my thesis work is based can lead to interesting new discoveries for depression. Overall, the findings in my work link two proteins of interest from these separate studies, VEGFA and PEDF, as a potential new signalling pathway in the molecular response to ketamine. Future studies are needed to investigate if this signalling pathway is also involved in the pathophysiology of depression.

Appendix:

Publications and Presentations

Publications

McGrory CL, Ryan KM, Gallagher B, McLoughlin DM. 2020. Vascular endothelial growth factor and pigment epithelial-derived factor in the peripheral response to ketamine. *J Affect Disord* 273: 380-383.

McGrory CL, Ryan KM, Kolshus E, McLoughlin DM. 2019. Peripheral blood *E2F1* mRNA levels in depression following electroconvulsive therapy. *Prog Neuropsychopharmacol Biol Psychiatry* 89: 380-385.

McGrory CL, Ryan KM, Kolshus E, Finnegan M, McLoughlin DM. 2018. Peripheral blood *SIRT1* mRNA levels in depression and treatment with electroconvulsive therapy. *Eur Neuropsychopharmacol* 28: 1015-1023.

Conference Proceedings

McGrory CL, Ryan KM, McLoughlin DM. Effects of Antidepressants on PEDF Secretion from Astrocytes & Neuronal Complexity – BNA Festival of Neuroscience, Dublin, Ireland 2019.

McGrory CL, Ryan KM, McLoughlin DM. Peripheral blood *SIRT1* mRNA levels in depression and treatment with electroconvulsive therapy - 31st ECNP Congress, Barcelona, Spain 2018.

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