Investigation of α-tubulins as translational biomarkers of major depressive disorder: indications for disease severity and treatment efficacy

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i. Declaration

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ii. Summary

Introduction: Major Depressive Disorder is a significant cause of disability worldwide. In a 2017 report, the World Health Organisation estimated that 320 million people suffered from depression globally. Although several antidepressant drugs have been delivered to the market over recent years, many patients do not respond adequately, or at all, to treatment. This has created an urgent need for safer and more effective treatments, which requires a better understanding of the underlying pathophysiology of depression. Disruption in neuronal plasticity; the ability of neurons to physically and functionally change over time, has been implicated in the pathophysiology of depression in both preclinical and clinical investigations. Microtubule dynamics, the rapid assembly and disassembly of microtubules and its constituents is a cellular mechanism allowing for neuronal plasticity. Microtubules contribute to this process primarily through axonal and dendritic remodelling that is required for the maintenance of synaptic connections. Microtubule stability and dynamic states affecting neuronal plasticity can be measured by assessing one of the microtubule heterodimers, α-tubulin and its post-translational modifications. While α-tubulins have been studied in the brain, they can also be detected in blood plasma, providing the possibility of peripheral detection of neuronal states. In order to evaluate if α-tubulins indicate a depressed state and subsequently a treatment response, analysis of tissue from a preclinical model of depression and blood plasma from depressed individuals was conducted.

Hypothesis: That α-tubulins are biomarkers for depression severity and treatment efficacy.

Methods: The Forced Swim Test was used to measure “depressive-like” behaviour of Wistar Kyoto and Sprague Dawley rats following chronic or acute antidepressant treatment. Due to the reported spontaneous immobility of the Wistar Kyoto rat, a pre-test session was omitted. The total time animals spent immobile during the test session was assessed in addition to individual behaviours of swimming, climbing, diving, and immobility. Following the Forced Swim Test, animals were immediately sacrificed for tissue collection. The hippocampi and prefrontal cortex were dissected out and blood plasma was isolated from trunk blood. For the human samples, blood plasma and corresponding medical histories from depressed individuals and healthy controls were obtained through academic collaborators at Trinity College Dublin and the University of Regensburg. Individuals with depression were selected that reported high Hamilton Depression Rating Scale Scores at time point one and returned to a “normal” score at a second time point following a course of treatment. Blood plasma samples from healthy

control participants were available for one of the two cohorts. Western blotting with the Li-Cor Odyssey imaging system was used to assess the levels of α-tubulin in all tissues. Acetylated-α-tubulin (Acet-Tub), Tyrosinated-α-tubulin (Tyr-Tub), Detyrosinated-α-tubulin (Glu-Tub), Deglutamylated-α-tubulin (Δ2-Tub), and Total¬-α-tubulin (Total-α-Tub) were measured with commercially available antibodies. Two additional analyses were carried out to assess brain-derived neurotrophic factor (BDNF) and corticosterone levels in rodent hippocampus and blood plasma, respectively. These analyses were conducted using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Statistical analyses were carried out using both Microsoft Excel and InVivoStat software.

Results: Wistar Kyoto rats displayed higher spontaneous immobility in the FST compared to Sprague Dawley controls. Consistent with previous reports, Wistar Kyoto rats did not show reduced immobility following acute or chronic selective serotonin reuptake inhibitor (escitalopram) treatment but did show reduced immobility to acute and chronic desipramine and acute ketamine and pregnenolone-methyl-ether treatment. Sprague Dawley rats showed a significant decrease in immobility following chronic desipramine treatment, highlighting the adrenergic effects of desipramine. No significant changes in BDNF or corticosterone were found. No robust changes in α-tubulin were found in brain tissue between Sprague Dawley and Wistar Kyoto rats nor was there a robust response to treatment, although a number of markers showed nominally significant differences. Wistar Kyoto rats overexpressed all α-tubulins in blood plasma compared to Sprague Dawley rats but no treatment effects were found. In the first human cohort, no significant changes in α-tubulins were found between the healthy controls and depressed individuals at either time point, nor were any significant changes found in α-tubulin for depressed individuals between the two time points where their symptoms improved following electroconvulsive therapy. Likewise, no significant changes in α-tubulin were found in the second cohort where their symptoms subsided following six weeks of pharmacological antidepressant treatment.

Conclusions: From the studies carried out here it is reasonably clear that α-tubulins are not effective markers for either a depressed state or treatment efficacy and provide no intuitive translational value. Acknowledging that neuronal plasticity plays a meaningful role in the pathophysiology of depression, further investigation into related markers is warranted.

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vi. List of Abbreviations

α-Tub	α-Tubulin
Δ2-Tub	Deglutamylated-α-Tubulin
5-HT	Serotonin
Acet-Tub	Acetylated-α-Tubulin
ACTH	Adrenocorticotropic Hormone
Akt	Protein kinase B
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMPAR	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor
ANOVA	Analysis of Variance
AREC	Animal Research Ethics Committee (Trinity College Dublin)
aTAT1	Tubulin acetyltransferase
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CMS	Chronic Mild Stress
CORT	Corticosterone
CRF	Corticotrophin Releasing Factor
DMI	Desipramine
DNA	Deoxy Ribonucleic Acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5th Edition
ELISA	Enzyme-Linked Immunosorbent Assay
EEAT2	Excitatory Amino Acid Transporter 2
eEF2	Eukaryotic Elongation Factor 2
eEF2K	Eukaryotic Elongation Factor 2 Kinase
EGTA	Ethylene Glycol Tetraacetic Acid
ESC	Escitalopram
FST	Forced Swim Test
GABA	γ-Aminobutyric Acid
GABAR	γ-Aminobutyric Acid Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glu-Tub	Detyrosinated-α-Tubulin
HAM-D	Hamilton Depression Rating Scale
HDAC-6	Histone Deactylase 6
HPA	Hypothalamic Pituitary Adrenal
IFWB	Infrared Western Blot
IgG	Immunoglobulin G

MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase Inhibitor
MAP	Microtubule Associated Protein
MAP-2	Microtubule Associated Protein 2
MDD	Major Depressive Disorder
MRI	Magnetic Resonance Imaging
MT	Microtubule
MTOC	Microtubule-Organising Centre
mTOR	Mammalian Target of Rapamycin
MWM	Molecular Weight Marker
NA	Noradrenaline
NAT	Noradrenaline Transporter
NMDA	N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartate Receptor
OBB	Odyssey Blocking Buffer
O/N	Overnight
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCPA	Para-Chlorophenylalanine
peEF2	Phosphorylated Eukaryotic Elongation Factor 2
PFC	Prefrontal Cortex
PME	Pregnenolone-Methyl-Ether
PSD-95	Postsynaptic Density Protein 95
PTM	Posttranslational Modification
PVDF	Polyvinylidene Fluoride
RCF	Relative Centrifugal Force
R^2	Coefficient of Determination
REM	Rapid Eye Movement
RIMA	Reversible Inhibitor of MAO_A
RT	Room Temperature
s.c.	*Sub Cutaneous*
SD	Sprague Dawley
SDS	Sodium Dodecyl Sulfate
SERT	Serotonin Transporter
SHR	Spontaneously Hypertensive Rat
SIRT-2	Sirtuin Type 2

SOMREC	School of Medicine Research Ethics Committee (Trinity College Dublin)
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressant
TCP	Tubulin Carboxypeptidase
TRD	Treatment Resistant Depression
TrkB	Tropomyosin Receptor Kinase B
Tris-HCL	Tris(hydroxymethyl)aminomethane Hydrochloride
TTL	Tubulin Tyrosine Ligase
Tyr-Tub	Tyrosinated-α-Tubulin
VEH	Vehicle
WB	Western Blotting
WHO	World Health Organisation
WKY	Wistar Kyoto

Chapter 1: General Introduction and Background

1. Major Depressive Disorder

Depression is a significant cause of disability worldwide that was estimated in 2010 to have a yearly economic burden of $210 billion in the United States alone[1]. In a 2017 report, the World Health Organisation (WHO) estimated that 320 million people suffered from depression globally with numbers expected to rise with growing populations[2]. A recent report showed that depression is also a growing problem among adolescents throughout Ireland and the European Union. It is estimated that 12% of Irish young people (aged 15-24) suffer from chronic depression, the highest rate in Europe[3].

Generally characterised as persistent low mood and loss of interest in things normally thought of as pleasurable, depression is actually a very heterogeneous disorder with considerable differences in clinical presentation. While reported prevalence rates differ greatly by region, depression is estimated to effect at least one in ten people with a high rate of relapse leading to the most common diagnosis of Major Depressive Disorder (MDD)[4]. Although several antidepressant drugs have been delivered to the market over the years, many patients do not respond adequately or at all to treatment. One major contributing factor is that it is estimated only 30% of patients adhere to their medication plan due to unwanted side effects even when their depressive symptoms are relieved[5]. MDD patients that fail to respond entirely to treatment can be characterised in a distinct sub-type called treatment resistant depression (TRD)[6]. These issues highlight the need for safer and more effective treatments, while also suggesting aetiological heterogeneity of MDD and the imperative for a better understanding of its underlying pathology.

1.1. Diagnosis

A clinical diagnosis of MDD by the Diagnostic and Statistical Manual of the American Psychiatric Association 5th Edition (DSM-V) requires that five out of nine symptom criteria are met (Figure 1.1). While seemingly straight forward, diagnosing MDD can be counterintuitive in some cases due to several of the symptom criteria describing opposite states. For example, criteria item 4: insomnia *or* hypersomnia. The DSM-V criteria highlight the heterogeneity of MDD as it presents itself clinically, which further suggests the underlying pathology may be equally heterogeneous and help explain why antidepressants do not always work as expected. It is clear that a diagnosis of MDD does not by itself offer enough information to the clinician to prescribe adequate treatment. This discrepancy between diagnosis and finding adequate treatment is supported by estimates that 50-60% of patients will not respond to antidepressant treatment[6] and when antidepressant treatment is effective, it normally takes several

weeks for the medication to have its desired effect[7]. This delayed onset coupled with unwanted side effects is what often leads patients to give up on their treatment.

- Depressed mood or a loss of interest in daily activities for more than two weeks.
- Mood represents a change from the person's baseline.
- Impaired function: social, occupational, educational.
- Specific symptoms, **at least 5 of these 9**, present nearly every day:

1. **Depressed mood or irritable** most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful)
2. **Decreased interest or pleasure** in most activities, most of each day
3. **Significant weight change (5%) or change in appetite**
4. **Change in sleep:** Insomnia or hypersomnia
5. **Change in activity:** Psychomotor agitation or retardation
6. **Fatigue or loss of energy**
7. **Guilt/worthlessness:** Feelings of worthlessness or excessive or inappropriate guilt
8. **Concentration:** Diminished ability to think or concentrate, or more indecisiveness
9. **Suicidality:** Thoughts of death or suicide, or has suicide plan

Figure 1.1. Diagnostic and Statistical Manual of Mental Disorders 5th Edition Criteria for Major Depressive Disorder[8].

1.2. Aetiology

Several hypotheses have been proposed over the years to explain how MDD occurs mechanistically as well as psychosocially. Historically, new insights into the aetiology of MDD often occurred serendipitously with the discovery that certain types of compounds could either produce or relieve depressive symptoms. More often than not these compounds were intended to target different diseases and in some cases not even neurological ones. However, through these chance discoveries several neurological systems were identified and implicated as being mechanistic causes of MDD.

1.2.1. Monoamine Hypothesis

The monoamine hypothesis of depression proposes that depleted circulating concentrations of monoamines (dopamine, noradrenaline, and serotonin (5-HT)) in the synaptic cleft result in a "chemical imbalance" that leads to depression. Antidepressants act by correcting this imbalance through inhibiting of the reuptake of monoamines or by promoting their synthesis and release into the cleft (Figure 1.2).

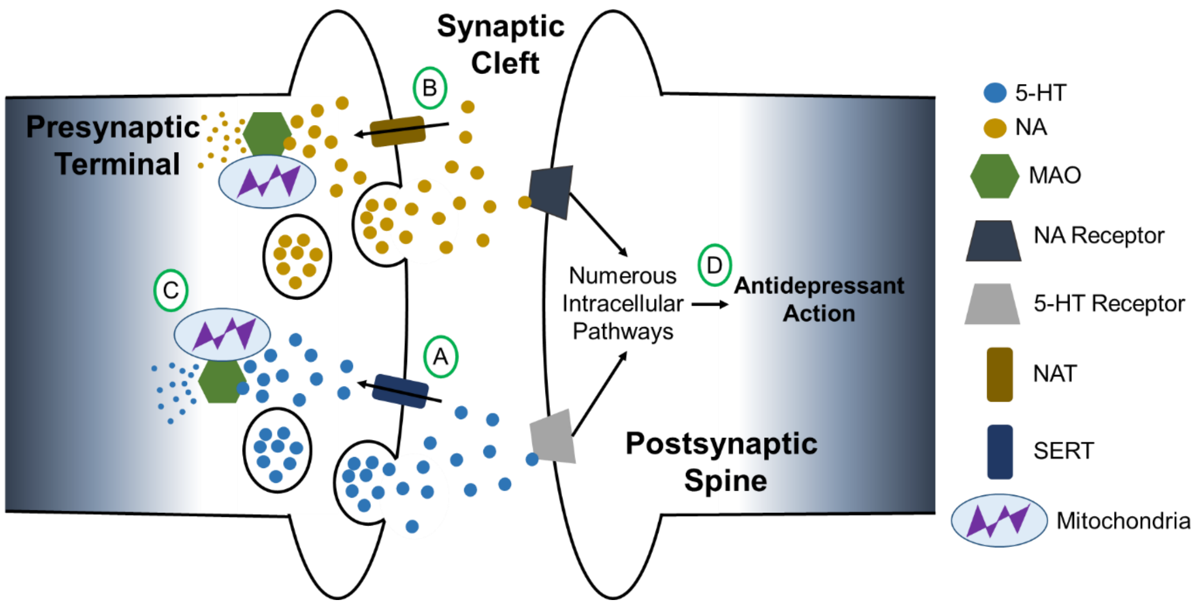

Figure 1.2. Overview of antidepressant action on the monoaminergic system. [A] SSRIs block the reuptake of 5-HT by preventing SERTs from channelling 5-HT back into the presynaptic neuron. By blocking reuptake of 5-HT, its concentration in the synaptic cleft rises, increasing availability for postsynaptic binding. **[B]** SNRIs selectively block the reuptake of NA by preventing NATs from channelling back into the presynaptic neuron. Blocking NA reuptake increases its availability for post synaptic binding. Some TCAs, such as DMI, act by blocking both NATs and SERTs. **[C]** MAOIs increase the availability of the monoamines (*e.g.* 5-HT and NA) for recirculation into the synaptic cleft by preventing MAOs from breaking them down. This occurs primarily inside the presynaptic neuron but it is not limited to that site. **[D]** The antidepressant action resulting from A, B, and C is driven by intracellular pathways that restore neuroplasticity through increasing dendritic arborisation and increasing synaptogenesis.

The monoamine hypothesis originated in the 1950s where several studies discovered by chance that certain pharmacological agents acting on monoamines could either produce or relieve depressive symptoms. One notable example is reserpine, which was administered to patients to treat hypertensive vascular disease but instead induced a depressed state in them by depleting 5-HT concentrations[9].

The connection between circulating levels of monoamines and depressive symptoms wouldn't be made for several years but in that time several compounds were discovered that acted on them. Other notable compounds included imipramine, the first tricyclic antidepressant originally developed as a chlorpromazine analogue, and iproniazid, a monoamine oxidase inhibitor originally developed as an antitubercular drug[9].

The monoamine hypothesis remains the prevailing theory of MDD and has driven the development of several blockbuster drugs, most notably the selective 5-HT reuptake

inhibitors (SSRI) fluoxetine and citalopram. However, recent reviews of the monoamine hypothesis have demonstrated several lingering issues, including but not limited to the altered expression of GABA in MDD[10], the efficacy of these compounds in other mood and psychiatric disorders, and the exclusion of the inflammatory hypothesis[11].

1.2.2. Inflammatory Hypothesis

Emphasising the parallels between sickness behaviour and depressive symptoms, the inflammatory hypothesis proposes that inflammatory signalling molecules can act on the brain to produce MDD[12]. Clinical trials using anti-inflammatory compounds were able to relieve depressive symptoms for some participants, suggesting that they might only work in a subtype of depressed individuals where neuroinflammation was the driving cause[13]. Traditionally, this hypothesis has been used to explain how external stimuli such as physical stress can cause depressive symptoms through peripheral and central nueroinflammation, however, it is well documented that psychosocial stress also causes inflammation and depressive phenotypes[14] as well as autoimmune disorders[15].

Rather than being a completely unique explanation for the underlying pathophysiology of MDD, this hypothesis somewhat augments the monoamine hypothesis by arguing that inflammation leads to a depressed state by disrupting monoamine regulation. Several studies have demonstrated that inflammatory proteins both increase the reuptake of monoamines and disrupt their synthesis, reducing their availability in the synaptic cleft[16–18]. Additionally, neuroinflammation can lead to the synthesis of quinolinic acid, a neurotoxic metabolite, which through a series of mechanisms increases glutamate concentrations[19]. Excess glutamate then binds NMDA receptors and consequently decreases brain derived neurotrophic factor (BDNF) production, reducing neurogenesis and neuroplasticity[20].

1.2.3. Neuroplasticity and Neurogenesis

Neuroplasticity, or neuronal plasticity, is the ability of neurons (and by extension the brain) to physically and functionally change over time and it is strongly connected to MDD pathophysiology[21]. The link between MDD, neuronal plasticity, and antidepressant action has been investigated extensively in the context of how stress affects different regions of the brain[22]. Several animal studies have demonstrated that stress can reduce hippocampal volume by structurally affecting neuronal plasticity[23,24]. Interestingly, these stress induced changes can then be rescued by antidepressant treatment[25–29]. Hippocampal volume is also reduced in depressed individuals, as shown in post-mortem analysis[30]. Additionally, several MRI studies have found decreased hippocampal volume

in unipolar depressed patients[31] suggesting that structural neuronal plasticity and neurogenesis is translationally relevant.

A clear distinction must be made between neuronal plasticity, the ability of neurons to physically and functionally change, and neurogenesis, the brain's ability to form new neurons. While there is an inherent connection between the two, the difference between forming new neurons and changing existing neurons, in both pathology and treatment, cannot be understated. As mentioned above, impaired neurogenesis also contributes to MDD[32] and several studies have shown a reduction of hippocampal volume in response to stress can be recovered by antidepressant treatment. It is important to note that the cause of this reduction is due to both reduced structural neuronal plasticity and neurogenesis, as is evident in immunohistochemistry studies showing reduced dendritic arborisation[33] (neuronal plasticity) and number of neurons[24] (neurogenesis).

Brain derived neurotrophic factor (BDNF) is a crucial protein for neurogenesis and neuronal plasticity[34]. BDNF dysregulation, specifically a decrease in BDNF expression, has been implicated in several neurodegenerative disorders, not only MDD. Work in rodents has shown that stress and the release of corticosterone also decreases the expression of BDNF, establishing a link between stress, BDNF, corticosterone, and anxiety and mood disorders[35]. However, several studies using BDNF as a treatment to correct depleted BDNF levels have failed, suggesting that BDNF may only play a supporting role in these conditions. Consequently, a large but conflicting literature[36] exists on BDNF as a marker for these disorders and target for novel treatment, however it has been consistently shown to be a sensitive marker of stress and has been repeatedly reported as a marker for antidepressant efficacy[37–39].

1.2.4. Glutamate Theory of Depression

More recently glutamatergic transmission has become a prominent avenue for MDD research. Glutamatergic synapses consist of three actors: a pre-synaptic neuron, post-synaptic neuron, and astrocytes. Once released by the presynaptic neuron, glutamate binds to various receptors on the pre- and post-synaptic neurons, as well on astrocytes. Glutamate reuptake is primarily performed by the surrounding astrocytes, via the excitatory amino acid transporter 2 (EEAT2), where it is converted into glutamine and resupplied to the pre-synaptic neurons. Several studies have found an overabundance of glutamate in individuals with MDD compared to healthy controls[40,41] and imaging studies have found that the glutamate/glutamine exchange is reduced in the hippocampus and anterior cingulate cortex of MDD patients[42–44]. This increase in extracellular glutamate may be primarily driven by the reduction in EEAT2 expression,

as supported by post-mortem studies[45,46], however the reason for the reduction in EEAT2 is unclear. Interestingly, antidepressant treatment lowers plasma glutamate levels in depressed patients[41].

The primary glutamatergic receptor of interest in MDD is the n-methyl-D-aspartate (NMDA) receptor. Made up of seven subunits, the NMDA receptor is a ligand-gated ion channel, requiring the binding of two agonists to open, glutamate and glycine. NMDA receptor pores are blocked by magnesium ions that require α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation to depolarise the membrane and expel the ion. Once activated, NMDA receptors will allow calcium and sodium ions to enter the neuron, where they are each responsible for activating numerous signalling cascades that drive neuronal plasticity. Post-mortem studies have found region specific changes in the expression of NMDA receptor subunits in MDD that impede its function in conjunction with elevated extracellular glutamate[47,48].

1.2.5. Hypothalamic-pituitary-adrenal Axis

Another bridge linking stress and MDD is the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis leads to the release of corticotrophin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus. CRF then triggers the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which in turn stimulates the adrenal cortex and the release of cortisol in humans or corticosterone in rodents (Figure 1.3). Once released, cortisol and corticosterone act on glucocorticoid receptors in the hippocampus, hypothalamus, and pituitary gland inducing inhibition feedback. Both hyperactivity of the HPA axis and impairment of glucocorticoid receptors have been repeatedly shown in depressed patients[49]. Hypercortisolemia, overexpression of cortisol, is often seen in depressed patients[50] and has negative effects on neuronal plasticity and induces morphological changes in the hippocampus and prefrontal cortex[51]. Several other region specific changes in the HPA axis have been connected to specific MDD symptoms. For example, low mood has been connected to changes in the prefrontal cortex-amygdala/hippocampus circuit, anhedonia may result from changes in the prefrontal cortex-nucleus accumbens circuit[52], and cognitive dysfunction has been tied to changes in the medial-dorsolateral prefrontal cortex circuit[53].

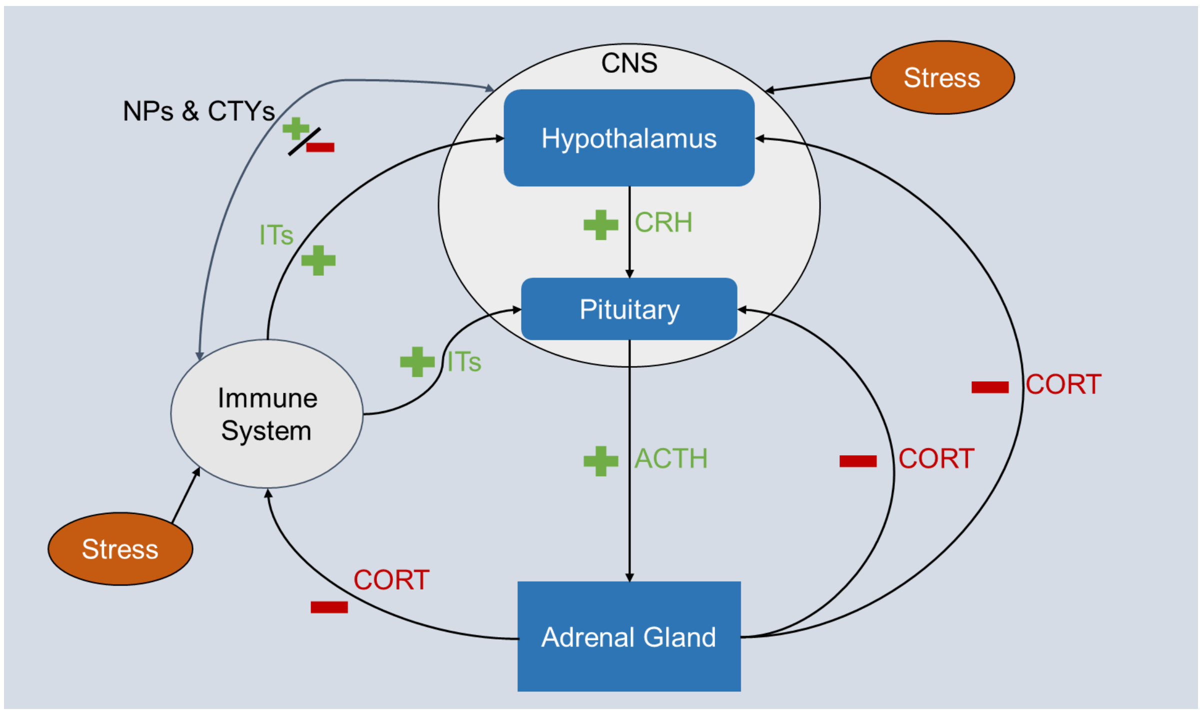

Figure 1.3. Overview of Hypothalamic-Pituitary-Adrenal (HPA) Axis. Neuropeptide (NP), Cytokines (CTY), Immunotransmitter (IT), Corticotrophin-Releasing Hormone (CRH), Adrenocorticotropic hormone (ACTH), Cortisol/Corticosterone (CORT).

HPA axis dysregulation has also been directly tied to impaired neurogenesis and synaptic plasticity. Chronic activation of glucocorticoid receptors in the hippocampus brought on by stress and persistent elevated levels of cortisol causes downstream effects that result in decreased neurogenesis, decreased synaptogenesis, and even apoptosis[54–56]. The loss of hippocampal neurons due to over-activation of the HPA axis is consistent with imaging and post-mortem studies showing reduced hippocampal volume in depressed patients as previously discussed.

1.3. Treatment Options

Numerous treatment options exist for MDD, both pharmacological and non-pharmacological. In mild cases, Cognitive Behavioural Therapy is often the first option before medication is prescribed and there exists a wealth of research conducted on the benefits of exercise, diet, and meditation. The focus of this research is on pharmacological treatments.

1.3.1. Traditional Antidepressants

For the purposes of this project, traditional antidepressants are those medications that have been developed to target the monoaminergic system and have been studied

extensively preclinically and clinically. These are the most commonly prescribed medications for treating MDD and other psychiatric disorders.

1.3.1.1. Monoamine Oxidase Inhibitors

Monoamine oxidase inhibitors (MAOI) were discovered by chance to have antidepressant properties in tuberculosis trials. Iproniazid and isoniazid were introduced in 1952 as potential treatments for tuberculosis when they were found to cause euphoria in patients. At the time, the mechanisms by which these compounds exerted their antidepressant effect was not known but it became exigently clear that they caused a number of life-threatening side effects. The most alarming of which was liver necrosis, which quickly led to their discontinuation as antitubercular and antidepressant treatments in favour of safer and more effective options[57].

We now know that MAOIs act by blocking the activity of monoamine oxidases (MAO), of which there are two isoenzymes: MAO_A and MAO_B. Located in the presynaptic terminal, MAOs are responsible for breaking down biogenic amines (*e.g.* dopamine, 5-HT, noradrenaline). Inhibiting MAOs therefore increase concentration of amines in the presynaptic terminal and subsequently their availability when released into the synaptic cleft. The health risk posed by early MAOIs, such as iproniazid, was due to irreversible binding to both MAOs that caused surges in monoamine circulation. Consequently, safer MAOIs were developed that reversibly and selectively bound to MAO_A, the isoenzyme responsible for breaking down 5-HT[58]. One example of a reversible inhibitor of MAO_A (RIMA) is moclobemide, which is arguably as safe and effective as other available antidepressants[59].

1.3.1.2. Tricyclic Antidepressants

Tricyclic antidepressants (TCA), so named for their molecular structure of three rings rather than by their mechanism, were the first widely used antidepressant drugs. The first TCA, imipramine, was originally synthesised as an antipsychotic rather than a mood stabilizer and was approved by the U.S. Food and Drug Administration (FDA) for the treatment of MDD in 1959. The first reported use of imipramine as an antidepressant stated that the compound was preferable to the available MAOIs as it produced fewer serious side effects[60], although the mechanism of action for either class was not known. Desipramine (DMI), an active metabolite of imipramine, was found to have the same therapeutic benefit but with even fewer unwanted side effects[61,62]. Consequently, DMI is now the preferred TCA.

It is now understood that TCAs act through a number of receptors and reuptake proteins, making their pharmacological profile more complicated than newer antidepressants. The main therapeutic action of TCAs in MDD is thought to be mediated through the inhibition of noradrenaline and 5-HT reuptake transporters, while common side effects such as drowsiness and memory impairment are caused due to their high affinity for adrenergic, muscarinic, and histaminergic receptors[63,64]. TCAs are rarely given as first option antidepressants today due to these side effects.

1.3.1.3. Selective Serotonin Reuptake Inhibitors

The development of selective serotonin reuptake inhibitors (SSRIs) began independently of the knowledge that TCAs exerted their therapeutic benefit through the inhibition of monoamine reuptake. By their own account, the researchers that developed fluoxetine, the first marketed SSRI in the United States, began their efforts from the discovery that serotonergic neurons were distinct from dopaminergic and noradrenergic neurons[65] and simply decided to target them. Further evidence to target 5-HT came later from post-mortem studies showing that neuronal 5-HT was depleted in depressive suicides[66] and the eventual discovery that certain TCAs also acted through the inhibition of 5-HT reuptake[67].

SSRIs, such as fluoxetine and escitalopram, act by preventing the reuptake of 5-HT from the synaptic cleft by blocking 5-HT transporters thereby increasing 5-HT availability. SSRIs are highly selective for their respective transporters and therefore have fewer side effects than the TCAs. It was originally though that SSRIs do not bind to postsynaptic 5-HT receptors, meaning that their pharmacological action was purely from increasing the availability of 5-HT in the synaptic cleft[63,68], however more recent research as shown that SSRIs can bind 5-HT_2 receptors[69,70].

1.3.2. Novel Treatments

For the purposes of this project, novel antidepressant treatments are those that do not target the monoaminergic system.

1.3.2.1. Ketamine

N-methyl-D-aspartate (NMDA) antagonist ketamine is not the first antidepressant drug to target the glutamatergic system. In 1959, D-cycloserine was given to tuberculosis patients who also suffered from depression and was found to have antidepressant efficacy[71]. Amantadine and memantine followed in the 1960s. It wasn't until the 1980s that it became clear that these compounds were NMDA receptor antagonists and that

they also had antidepressant efficacy in preclinical models of depression[72]. Collectively, these studies suggest that NMDA receptors may be dysfunctional in depression.

Clinical trials demonstrated the rapid relief of depressive symptoms with low dose ketamine infusion[73] and signalling studies have shown that ketamine administration leads to an increase in synaptic glutamate and AMPA activation that in turns leads to activation of the Akt/mTOR pathway[74,75]. Activation of the Akt/mTOR pathway leads to in an increase in synaptic plasticity and the formation of dendritic spines[76]. The formation of dendritic spines requires BDNF, which is promoted by the inhibition of the NMDA receptors and reduction in eukaryotic elongation factor 2 (EEF2) signalling (Figure 1.4). Even with this knowledge and while it is now generally accepted that ketamine is a viable option for antidepressant treatment[77] many questions remain about its exact mechanism of action and safety[78].

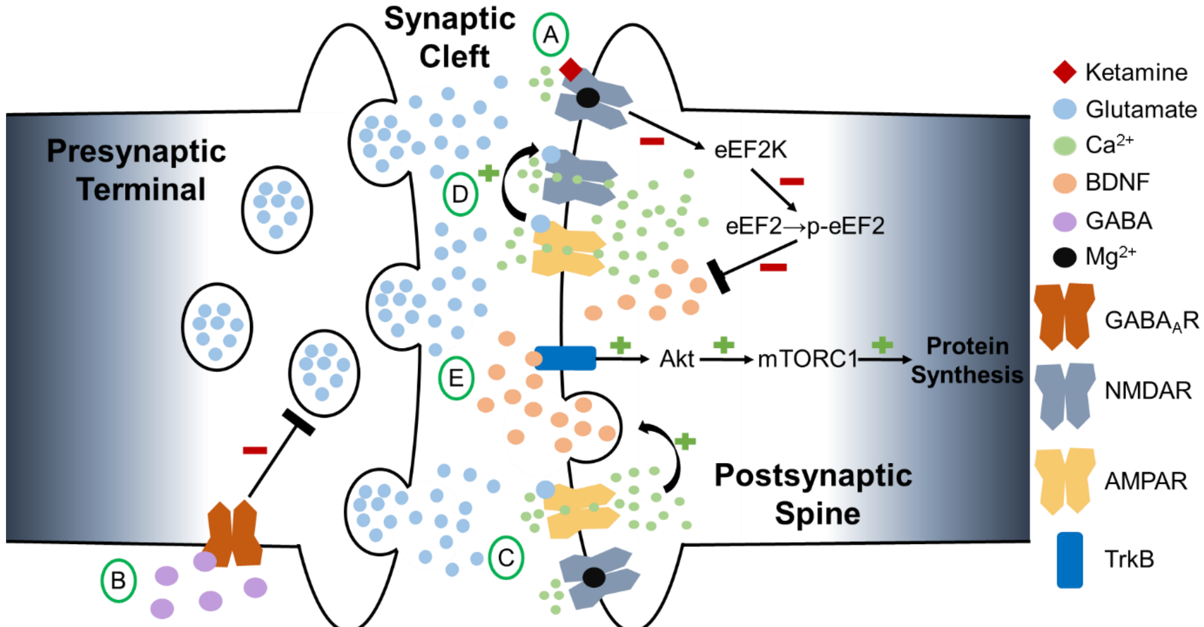

Figure 1.4. Overview of ketamine mechanism of action. [A] Ketamine blocks NMDAR activity consequently inhibiting activation of eEF2K and the phosphorylation of eEF2, which in turn increases BDNF translation. **[B]** Ketamine also drives the release of the GABA by interneurons that cause the release of Glutamate by the presynaptic neuron by inhibiting $GABA_AR$ activity. **[C]** Glutamate activates AMPAR which allows an influx of calcium ions into the postsynaptic neuron stimulating the release of BDNF. At rest, magnesium ions block the NMDAR channel preventing other ions from passing through. **[D]** AMPAR activation leads to NMDAR activation and the magnesium ion is removed allowing calcium ions to enter the postsynaptic neuron. The influx of calcium ions activates BDNF translation. **[E]** Increased availability of BDNF activates TrkB signalling pathways that drives protein synthesis that is thought to restore synaptic plasticity.

1.3.2.2. Pregnenolone-Methyl-Ether

Like the first generation of antidepressant drugs developed in the 1950s that were discovered serendipitously, Pregnenolone-Methyl-Ether (PME) was first synthesised as a treatment for rheumatoid arthritis and hypersensitivity diseases. While it failed to treat those two conditions, patients reported a greater feeling of wellbeing during these trials, which led to the investigators administering PME to psychiatric patients. Notably, the group that most benefited from PME treatment were those suffering from depression and anxiety with the investigators reporting improved mood, reduced anxiety, and restored sleep cycle in those individuals[79]. Interestingly, no serious side effects were reported with only mild nausea occurring with the highest doses. In a follow-up study, PME was administered to healthy US Air Force pilots to test if the compound might aid in recovery from long flight missions by reducing stress and mental fatigue. The investigators found that PME did not aid in recovery in these healthy individuals compared to their control group and while they expressed disappointment in this result, they noted the difference may be due to the pilots being in good health rather than suffering from depression and anxiety[80].

Many years later, the discovery that pregnenolone and PME selectively bind microtubule associated protein 2 (MAP-2) rejuvenated interest in neurosteroids as mood modulators[81]. Activation of MAP-2 by PME was found to increase neural plasticity and microtubule dynamics while recovering depressive phenotypes in rodent models[26].

1.4. Why Do Antidepressant Treatments Fail?

To briefly summarise this chapter so far, several cellular systems and markers have been implicated in the pathophysiology of MDD that may account for its heterogeneity in clinical presentations. It is also clear that antidepressant treatments targeting the monoaminergic system leave a lot to be desired both in efficacy and in onset of efficacy. SSRIs have been shown to rapidly increase the availability of 5-HT in the synaptic cleft although the therapeutic benefit is not seen until weeks later. More recent studies depleting 5-HT have failed to induce depressive symptoms as expected[82]. In a clear dissenting opinion, Stephen Stahl writes in his pharmacology textbook, "…there is no clear and convincing evidence that monoamine deficiency accounts for depression[83]." Novel treatments targeting the glutamatergic system and synaptic plasticity have shown promise while also expanding our understanding of MDD's underlying pathology. It has also been demonstrated that an increase in inflammatory markers can lead to a decrease in monoamine availability[16–18], connecting inflammation to the monoamine hypothesis of depression and that glutamate also plays a role. What all this evidence

suggests is that while MDD is clearly heterogeneous in its presentation, it may be even more heterogeneous in its underlying pathology. What is classed as a single disorder may in fact be several closely linked but distinct disorders (in terms of cause and pathology) resulting in a common, albeit at times conflicting, set of clinical symptoms. This heterogeneity makes prescribing medications a game of chance more than an exact science.

2. Finding a Translational Biomarker for Depression

2.1. What is a Biomarker?

As defined by the United States Food and Drug Administration and National Institute of Health, a biological marker or biomarker is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention[84]." Generally, this definition applies to any measurable molecular, histologic, radiographic, or physiologic marker of health or biological function.

More specific definitions of biomarkers exist depending on the area of application. These include: diagnostic biomarkers, monitoring biomarkers, pharmacodynamic/response biomarkers, predictive biomarkers, prognostic biomarkers, safety biomarkers, and susceptibility/risk biomarkers[84].

The title of this thesis names α-tubulins (α-Tubs) as a "translational" biomarker, referring to its potential use in both preclinical and clinical applications. This translational relevance of α-Tub assumes that it is a functional diagnostic and monitoring biomarker. A diagnostic biomarker "…detects or confirms the presence of a disease or condition of interest[85]" where a monitoring biomarker "…detects an effect of a medical product or biological agent[85]". α-Tub is considered and investigated as both in this thesis.

It is important to note the difference between biomarkers and preclinical or clinical endpoints in this thesis. Biomarkers are objective quantifiable measures of biological processes, where clinical endpoints, such as the Hamilton Depression Rating Scale (HAM-D), are used to assess an individual's wellbeing and experience. (For context, the preclinical endpoints investigated here are the behavioural measures in the rodent Forced Swim Test.) Clinical endpoints are generally considered the gold standard for any investigation of disease progression or intervention study; however, it is important to note that they may not coincide with changes in the biomarker of interest[86].

Indeed, the goal of biomarker research and development is to identify "surrogate" markers that not only inform the outcome of a disease or intervention but also explain what is happening biologically. Understandably, the bar for a biomarker to be considered

a surrogate is quite high. Not only does the biomarker have to consistently match the clinical endpoint, in terms of outcome, but it must also be thoroughly validated to explain what is happening biologically[85].

2.2. Biomarkers for Depression

For MDD, finding robust biomarkers has been a challenge for the field. While many potential biomarkers have been identified and our understanding of MDD's underlying pathophysiology has expanded greatly, none so far have met the rigorous standard of a surrogate for a diagnostic or monitoring biomarker[87,88].

Numerous inflammatory markers, proteins, and electrophysiological markers have been explored in MDD[89]. Some examples are: BDNF, cortisol, tumour necrosis factor α, interleukin 6, and theta waves[89]. A recent meta-analysis of papers published on the leading theories of predictive MDD biomarkers found that only cortisol was a potential target, however the authors note that this result may be confounded by the quality of the studies analysed[90]. In another review of the state of MDD biomarker research, the authors note that MDD biomarker research has still to overcome the heterogeneity of the disease and suggests that single markers may not be as beneficial as using several markers in a panel, making use of "big data" algorithm techniques[91].

There remains a considerable amount of work to do in identifying and validating biomarkers for MDD. One possible target that may act as biomarker is α-Tub, a microtubular protein.

2.3. Background on Microtubules and Microtubule Dynamics

Microtubule (MT) dynamics, the rapid assembly and disassembly of MTs and their constituent proteins is an integral cellular mechanism allowing for neuronal plasticity to work properly. As part of the cytoskeleton, MTs form long stable tubes from two tubulin isotypes (α-Tub and β-tubulin) that give cells shape.

In neurons this is particularly important as they play a significant role in axonal and dendritic formation and remodelling[92], intracellular transport[93], and may even act as signalling proteins[94]. These functions of MTs in regulating morphological changes has been implicated in the underlying pathology of MDD as seen in dendritic spine loss[33] and reduction in hippocampal volume[31]. Interestingly, MTs will self-repair in response to mechanical stress[95], implying that the microtubular system is disrupted in MDD rather than structurally damaged. As one of the fundamental building blocks of neuronal architecture that have been shown to be dysregulated in depression and respond to antidepressant treatment, MTs are a key target for further investigation.

2.4. Neuronal Microtubules

MT proteins and their structure are highly conserved across species. The α- and β-tubulin heterodimers align into 13 protofilaments that wrap around to form a hollow tube with a diameter of 25 nm. Because the heterodimers align in the same direction uniformly, MTs are asymmetrical and can be described as having "plus" and "minus" ends[96,97]. β-tubulin is exposed at the plus end and is considered the growth end for assembly and disassembly, while the minus end with an exposed α-Tub is more stable. In non-neuronal cells, it is the minus end of the MT that is bound at the site of nucleation, named the microtubule-organising centre (MTOC), "anchoring" the MT and maintaining the structural polarity.

In neurons, axonal MTs are not bound to MTOCs and instead organise more freely to accommodate the structure of the neuron, although whether the axonal MTs are generated at MTOCs or by other γ-tubulin ring complexes is unclear[98–100]. Likewise, dendritic MTs are regulated locally to produce dendritic spines[101–103]. Axonal MTs are relatively long and have uniform polarity that provide structural rigidity compared to dendritic MTs that are shorter and have mixed polarity allowing for more dynamic states[92,104]. From this obvious difference in axonal and dendritic MTs, a much more complicated story emerges. The structural organisation of axonal and dendritic MTs informs widely different functions, relying on many different tubulin isotypes and posttranslational modifications[105]. Additionally, a variety of microtubule associated proteins (MAPs) and enzymes regulate MT activity and differ greatly in activity depending on where in the neuron they are located.

2.4.1. Microtubules and Synaptic Regulation

Synaptic regulation is conducted through complex interactions of signalling pathways from both intra- and extracellular mechanisms, such as NMDA receptor pathways mediated by BDNF and post-synaptic density protein 95 (PSD-95). MTs not only have a central role in the growth and maintenance of axons and dendritic shafts but also play an important role in the growth and regulation of dendritic spines[106]. F-actin and G-actin are the primary cytoskeletal protein responsible for maintaining dendritic spine shape and growth but it was recently discovered that MTs also enter dendritic spines, contributing to their growth and regulation[101]. Furthermore, it has been demonstrated that MT dynamicity is directly involved with the growth of dendritic spines, where stable MTs are located primarily in the dendritic shaft and dynamic MTs are located in the spines[107]. These changes in microtubule functionality depend on distinct tubulin

isotypes[108]. Post-translational modifications (PTMs) to α-Tub have been identified and connected to MT stability and dynamic states affecting neuronal plasticity[109,110].

2.5. α-Tubulin Posttranslational Modifications in Neurons

There are four α-Tubs PTMs that are the focus of this research: Acetylated-α-tubulin (Acet-Tub), Tyrosinated-α-tubulin (Tyr-Tub), Detyrosinated-α-tubulin (Glu-Tub), and Deglutamylated-α-tubulin (Δ2-Tub). Total-α-tubulin (Tot-α-Tub) will also be investigated. Tyr-Tub, Glu-Tub, and Δ2-Tub are C terminus modifications of α-Tub that can be measured to assess dynamic states while Acet-Tub occurs in the lumen of the MT at the lysine-40 site and indicates stable long lived MTs (Figure 1.5).

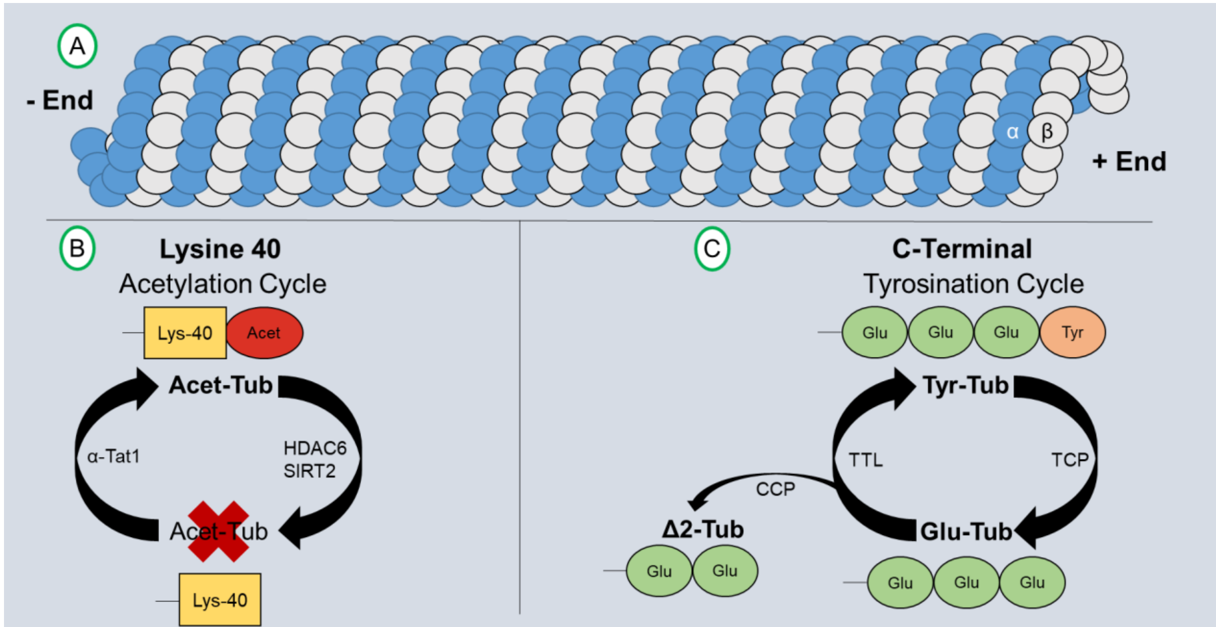

Figure 1.5. α-Tubulin posttranslational modification sites and cycles. **[A]** The microtubule forms a tube structure from α- and β-Tubulin dimers. This creates an asymmetrical structure with plus and minus ends. The rapid assembly and disassembly of microtubules takes place at the plus end. **[B]** Acetylation of α-Tubulin occurs in the lumen of the microtubule at the Lysine 40 site by the enzyme α-Tat1. The acetyl group (Acet) can be removed by enzymes, such as histone deacetylase 6 (HDAC6) and NAD-dependent deacetylase sirtuin 2 (SIRT2). **[C]** The α-Tubulin tyrosination cycle takes place at the C-terminal of the of the α-Tubulin protein. The removal and addition of the tyrosine residue (Tyr) by tubulin carboxypeptidase (TCP) and tubulin tyrosine ligase (TTL) can be measured to assess microtubule dynamic states. The removal of the penultimate glutamate group (Glu) by a cytosolic carboxypeptidase (CCP) forms Δ2-Tub, which is thought to be irreversible and indicate very stable microtubules.

2.5.1. Acetylation of α-Tubulin

High levels of Acet-Tub (Lysine-40) have been previously reported to indicate stable long-lived microtubules[111] and have more recently been shown to indicate resilience to mechanical stress[112]. Acet-Tub is highly expressed in the axon of neurons compared to dendrites, in order to provide structural stability. Located in the lumen of the microtubule, the Lys-40 site is thought to be more independent of the microtubule associated proteins and enzymes that regulate modification at the c-terminal[113]. Tubulin acetyltransferase (aTAT1) is the enzyme primarily responsible for the acetylation of α-Tub and can enter the lumen of the microtubule by either the open ends or by cracks that may occur along its surface where it will acetylate locally. This is important as the acetylation of α-Tub is necessary for the repair of damaged microtubules, i.e. where cracks along the surface occur from mechanical stress[114]. Depletion of aTAT1 prevents microtubules repair[115].

Extensive work has been conducted on the role Acet-Tub plays in microtubule dynamics and stability with a large portion focusing on the activity histone deacetylase 6 (HDAC6), which along with sirtuin type 2 (SIRT2) deacetylates tubulin. Studies investigating HDAC6 knockout and inhibition have found that while Acet-Tub increases as a result of these interventions and microtubule stability is increased, overall microtubule function is generally unaffected[113,116], implying that Acet-Tub by itself is not sufficient as a marker of stability or dynamic states.

2.5.2. Tyrosination and Detyrosination of α-Tubulin

Occurring at the c-terminal, the tyrosination and detyrosination of α-tubulin is a reversible cycle that is important to many cellar processes, including: mitosis, muscle contraction, and neuronal differentiation. It is generally accepted that high Tyr-Tub indicates increased microtubule dynamics while high Glu-Tub indicates increased microtubule stability[117]. As part of the same cycle these modifications can be expressed in a direct ratio, which can be used to infer changes in microtubule dynamics and by extension neuronal plasticity. This cycle is especially prevalent in the regulation of dendritic spines that requires rapid MT assembly and disassembly.

Although these post-translational modifications were first described many years ago[118], it wasn't until recently that the tubulin carboxypeptidase responsible for removing the tyrosine residue was identified as vasohibin-1 with the small vasohibin binding protein[119]. The enzyme that adds a tyrosine residue, tubulin tyrosine ligase (TTL), has been extensively studied[120–122]. TTL knockout studies have confirmed that TTL is vital to brain development and neuronal circuitry and is hallmarked by abnormal neurite growth and increased Δ2-Tub formation[123].

2.5.3. Deglutamylation of α-Tubulin

Δ2-Tub is formed by the removal of the penultimate glutamate after detyrosination by cytosolic carboxypeptidases[124]. In the brain, Δ2-Tub is only expressed in neurons and is localised primarily in axons. Although Δ2-Tub's role in microtubule dynamics is not fully understood, it may indicate stable microtubules with changes in its expression reflecting alterations in neuronal Δ2-Tub metabolism[125] and the tyrosination cycle.

2.5.4. Tubulins as Potential Biomarkers for Major Depressive Disorder

PTMs to α-Tub and their interaction with MAPs are well characterised in several models of depression.

It has been reported that Acet-Tub has elevated expression in the hippocampus in animal models of depression and can be decreased by chronic SSRI treatment, while at the same time increasing Δ2-Tub expression[25,126]. This may suggest that Acet-Tub expression is sensitive to depressed states and antidepressant treatment and may be a suitable marker for both, while Δ2-Tub may also be a marker for antidepressant efficacy. In the same study it was found that acute SSRI treatment increased the expression of Glu-Tub, showing that antidepressants may also act on α-Tub PTMs associated with dendritic and synaptic remodelling. In a separate study, it was found that animals administered with para-chlorophenylalanine (PCPA), a compound that depletes 5-HT, had significantly reduced 5-HT levels that coincided with reduced Tyr-Tub expression. Interestingly, acute SSRI treatment rescued and increased 5-HT and Tyr-Tub expression[127]. Taken together, there is sufficient evidence to propose that antidepressants targeting the monoamines affect synaptic and neural plasticity through α-Tub and that α-Tubs are crucial targets for treating MDD.

Likewise, there is data to support the novel antidepressants previously discussed act on α-Tub. Neurosteroid derivative, PME, has been shown to increase Tyr-Tub to Glu-Tub ratio and reduces Acet-Tub expression in the hippocampus of naïve animals and recovers immobility in the Forced Swim Test (FST). Additionally, PME selectively binds MAP-2, providing a novel mechanism to target for α-Tub modulation and potential antidepressant treatment[26]. Healthy female volunteers receiving an intravenous 0.5mg/kg ketamine infusion showed an increase in expression of Acet-Tub in blood plasma in correlation with glutamate change in the pregenual anterior cingulate cortex[128]. This points to the possibility that these α-Tubs may be viable central and peripheral markers for disease severity and treatment efficacy.

2.6. Measuring the Expression of α-Tubulins in Biological Tissue

The primary means of molecular analysis used in this project is Western blotting (WB), or protein immunoblotting, a well-established technique allowing the detection and relative quantification of proteins, in this case α-Tubs.

WB was initially developed in the late 1970s to analyse ribosomal proteins, following the development of the Southern blot, which is used to analyse DNA fragments[129]. Along with the name "Western", an homage to the Southern blot, an improved method using SDS-PAGE was introduced in 1981, which led to wide spread use of the technique[130]. In general, proteins are separated by their molecular weights using gel electrophoresis (*i.e.* electric field current) and are then transferred to a porous membrane, which is incubated with antibodies for the protein(s) of interest. Following primary and secondary antibody incubations, the membranes are imaged and Integrated Intensity (or Pixel Volume) can be measured to assess relative protein concentration.

Modern improvements to the technique have included fluorescently labelled secondary antibodies that enhance sensitivity and reliability compared to older chemiluminescent antibodies that rely on enzymatic activity[131]. Additionally, the transfer of protein from the gel to the membrane can now be completed more quickly and inexpensively using "dry" transfer equipment.

2.6.1. Sampling Tissue from Animals and Humans

WB can be used to relatively quantify target protein expression in numerous tissue sources. To establish if there is translational relevance for the α-Tubs as biomarkers for MDD, tissue will be acquired from both rodents and humans. Rodent tissue will be collected following behavioural studies described below while human tissue will be acquired through academic collaborators who specialise in human MDD research. Both brain tissue and blood plasma will be analysed from rodent samples while only blood plasma will be analysed from human participants.

2.7. Detecting Neuronal Microtubules in Blood Plasma

How neuronal alpha-tubulins may be detectable in the blood stream may be explained by a rapidly growing area in biomarker research; extracellular vesicles or exosomes. Exosomes can contain a variety of cellular material, depending on their cell of origin and size. The contents of exosomes may include: mRNAs, miRNAs, proteins, and lipids[132].

Within the past few years there has been a growing interest in investigating exosomal contents for potential biomarker candidates and neuroscientists have begun considering the role of exosomes in psychiatric and neurological diseases[133].

Exosomal function has been described as an important facilitator for both neurogenesis[134] and neuroplasticity[132], acting as an intercellular signalling mechanism. Exosomes from both neurons[135] and astrocytes[132] have been assessed for their role in these processes. However an intriguing aspect of exosomes in relation to this thesis is their ability to cross the blood brain barrier[136,137], bringing their cellular contents into the blood stream and other organs. This creates the possibility to detect changes in the CNS with peripheral measurements. Indeed, neuronal exosomes have been isolated from blood plasma samples and were shown to have contain important potential biomarkers for neurological disorders, such as Alzheimer's and Parkinson's disease[138]. Among the markers found in these exosomes were MAPs and synaptophysin, a protein involved in synaptic transmission[138]. These markers are not only implicated in neurological disease but also psychiatric. Of particular interest here are cytoskeletal proteins, such as actin and tubulin, that not only have a role in the release of exosomes from a cell[139] but can also be contained within the exosome[140]. Exosomes carrying these cytoskeletal proteins into the blood stream may allow for peripheral detection of the CNS disorders and treatments affecting synaptic plasticity.

3. Assessing Antidepressant Efficacy in Animal Models

An animal model of any disorder must have face, construct, and predictive validity[141]. For MDD this is especially difficult as it is a complex emotional and cognitive disorder, while at times also presenting physical symptoms. If characterising MDD in humans is difficult, finding an adequate animal model is near impossible. One rodent strain that is commonly used in MDD research for its unique behaviour and physiology is the Wistar Kyoto (WKY) rat[142]. The WKY is often described as an endogenously "depressed" rat for its numerous similarities to human MDD, including: altered rapid eye movement (REM) sleep[143], HPA axis dysregulation[144,145], and SSRI resistance[142,146]. Here the WKY is used in a behavioural paradigm commonly used to screen for potential antidepressants, the FST, as well as for tissue analyses of α-Tubs alongside Sprague Dawley (SD) rats, a standard strain in this field selected to represent "healthy controls".

3.1. Forced Swimming Test

The rodent FST was first described by Porsolt in 1977 as a model of behavioural despair[147]. It assumes that when a rodent is placed in a tank of water, from which it cannot escape, it will eventually give up and become immobile in the tank. This

immobility is interpreted as "depressive-like" behaviour and can be modulated with antidepressant treatment. Successful antidepressant treatment will reduce the total time the rat or mouse is immobile in the tank, normally during a five-minute testing period. Consequently, the Porsolt FST and modified versions have become popular screening methods for new antidepressants due to their simplicity and relatively fast scoring method. Since its introduction, many researchers have reported on the effects of different classes of antidepressants (*e.g.* SSRI, TCA, and MAOI), different rodent strains (*e.g.* Han Wistar, SD, and WKY), and scoring methods in the FST[148].

Ketamine has also been studied in FST paradigms with numerous studies finding that acute ketamine administration reduces immobility[149–151]. The "antidepressant" efficacy of ketamine in the FST has also been reported to last up to 2 weeks following a single administration[152,153] and chronic dosing studies have reported even longer effect durations[154].

The FST is not without controversy as a viable model for MDD or even depressive symptoms. Critics have noted several problems with both the design and implementation of the paradigm, demonstrating issues with face, construct, and predictive validity, since it is impossible to say if a rodent is truly depressed and pharmacological treatments other than antidepressants have been found to be efficacious[155]. Additionally, antidepressants that take several weeks to have their effect in humans are often reported to work after an acute treatment in the FST. Many researchers have made the case that the FST is actually a measure of stress coping mechanisms rather than MDD[156]. The FST, or simply water immersion in some cases, has been widely used as a physical stressor in investigations of HPA axis dysregulation. Connectedly, animals that have been subjected to this kind of chronic stress show changes in neural endocrine regulation that leads to disruptions in synaptic plasticity[155].

3.2. Chronic Mild Stress

Many animal models of depression have been developed using stress exposure to trigger certain behavioural phenotypes through activation of the HPA axis. For example, the chronic mild stress (CMS) model of depression has been used extensively to induce behavioural changes in rodents that are considered homologous to human psychiatric conditions. Several different stressors, ranging from mild to severe, and behavioural changes have been characterised with this model, as well as antidepressant response, giving this model high face and construct validity. Predictive validity and reliability remain an issue with CMS studies however, which is most clearly demonstrated by the lack of novel antidepressant treatments succeeding translationally. This may also speak to the

inherent heterogeneity of stress response in animals and humans[157]. The CMS model is mentioned here in order to put the dosing regimen (Chapter 2 Section 1.3) selected for the FST study into context as an additional stressor for the animals and will be discussed further in Chapter 3 Section 1.4.3.

4. Research Aims and Questions

The main hypothesis of this thesis is that α-Tubs are suitable biomarkers for both MDD severity and treatment efficacy. Two main questions will be asked to investigate this: Can α-Tub expression be used to identify a depressed state and does α-tubulin expression change with antidepressant treatment?

This will be accomplished by assessing WKY rats as model of MDD in the FST using different classes of antidepressant treatment and comparing them to healthy SD rats. Tissue will be collected from these animals to investigate if behavioural changes in the FST correspond to α-Tub expression and if α-Tub expression corresponds to a depressed phenotype. This analysis will be conducted using WB techniques and by enzyme-linked immunosorbent assay (ELISA).

To investigate if there is translational value for these markers, the animal study will be followed by the analysis of α-Tub expression in plasma collected from individuals suffering from MDD from two cohorts. In both cohorts, individuals with MDD were selected for high HAM-D scores that indicated severe to very severe depressive illness. The purpose of selecting samples from these individuals with high HAM-D scores was to ensure a significant behavioural contrast existed between time points and in comparison to healthy controls. This selection should yield the greatest chance to detect if any differences in α-Tub exist not only between healthy controls and individuals with MDD but also if treatment affects α-Tub expression.

In the first cohort, individuals with MDD who were unresponsive to pharmacological treatment enrolled in a study investigating if electroconvulsive therapy (ECT) would relieve their symptoms. Healthy controls were also recruited and donated plasma for comparison. Blood plasma was acquired at two time points for each group, pre- and post-ECT treatment.

In the second cohort, individuals donated plasma before starting treatment and once a week for six weeks once treatment began. Comparing α-Tub expression from week 0 and week 6 will answer if there is a correlation with antidepressant treatment and α-Tubs. These individuals also completed HAM-D questionnaires so correlations can also be sought with a clinical endpoint.

Chapter 2: Materials and Methods

1. Sprague Dawley and Wistar Kyoto Rats

The two rat strains used in this project are the Sprague Dawley (SD) and Wistar Kyoto (WKY). Here, SD rats are used as a "healthy control" strain and WKY rats as a "depressed" strain. SD rats were chosen as a control for this study for two reasons: they have been used extensively in Forced Swim Test (FST) studies with their behaviour being well characterised, compared to WKY[146], and as a "healthy control" they have lower baseline and reactivity corticosterone (CORT) levels compared to Han Wistar, providing greater contrast to the WKY "depressed" phenotype[158]. WKY rats have been extensively characterised as a model for posttraumatic stress disorder, anxiety, and depression[144,159,160], displaying both behavioural and physiological differences compared to other strains. For example, much like patients with Major Depressive Disorder (MDD), WKY rats have an altered REM sleep cycle compared to SD rats[143] and exhibit an altered stress response compared to other strains[145,161–163]. Critically for this project, WKY rats have been shown to be resistant to selective serotonin reuptake inhibitor (SSRI) treatment in the FST and display spontaneous immobility in the FST compared to SD[142,146,164].

1.1. Ethical Permission

Ethical approval for this study was obtained from the Animal Research Ethics Committee (AREC) in Trinity College Dublin (Appendix A.1). Animal welfare parameters were recorded daily to ensure animals were in good health throughout the study.

1.2. Treatment Groups and Housing

Animals were allowed to habituate for at least two weeks upon arrival from the supplier (Envigo UK). Male SD and WKY rats (approx. 350-400 g) were randomly assigned to treatment groups (n=10/group/strain, total n=140): Vehicle, Acute Escitalopram (ESC_{Acute}), Acute Desipramine (DMI_{Acute}), Chronic ESC ($ESC_{Chronic}$), Chronic DMI ($DMI_{Chronic}$), Acute Ketamine, and Acute Pregnenolone-Methyl-Ether (PME). Animals were housed 2-3/cage in individually ventilated cages, with a 12 hour day/night cycle, and had food and water *ad libitum*.

Due to the number of animals and other logistical considerations, the FST study was divided into 3 blocks. Group numbers were balanced across the three blocks and animals were randomly assigned to groups and cages using a random number generator in Microsoft Excel. In order to dose the animals, the experimenter was not blind to the group each animal belonged to.

1.3. Dosing and FST Protocol

Animals were treated chronically for 21 days with either ESC (10 mg/kg/ml *s.c.*)[165], DMI (10 mg/kg/ml *s.c.*)[146], or corresponding vehicle solution (injectable water 1 ml/kg *s.c.*). PME (10 mg/kg/ml *s.c.*)[26], Ketamine (5 mg/kg/ml *s.c.*)[166], ESC (10 mg/kg/ml *s.c.*), or DMI (10 mg/kg/ml *s.c.*) were administered acutely on day 21 to rats receiving daily injection of vehicles for 20 days (Figure 2.1). PME is not water soluble and was suspended in sesame oil, therefore all rats also received an additional daily vehicle administration of sesame oil 1 ml/kg *s.c.* On day 21, FST was performed 2h following the last administration of drugs or vehicle. WKY rats display higher spontaneous immobility in the FST compared to SD, therefore no pre-test session was required.

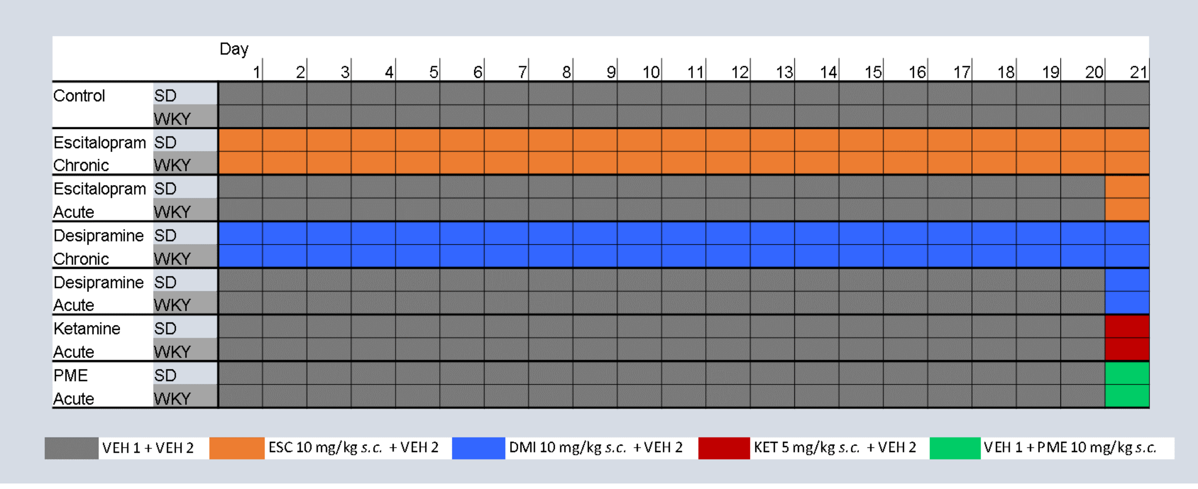

Figure 2.1. Forced Swim Test Study timeline. Animals were dosed for 21 days, receiving two injections per day to accommodate two treatment vehicles.

For the FST procedure, rats were placed in a tank of water 50 cm high and a diameter of 20 cm, which was maintained at a water depth of approximately 30 cm and at 25±1 °C. 5 min test sessions were recorded by a digital video camera placed above the tank. Water was replenished after every test session.

Study environmental conditions have been shown to effect outcomes in the FST, such as lighting, time of the day[167]. To control for these factors, every block was conducted at the same time of day and under a 60 lux light level.

After the 5 min test session, animals were removed from the tank and immediately sacrificed by decapitation and tissue was collected as described below.

1.4. Scoring FST Videos

The experimenter was blind to treatment group when analysing the FST videos as each video was automatically given a generic code generated by the digital camera. Several

behaviours in the FST have previously been characterised in response to different classes of antidepressants, including: swimming, climbing, and immobility (Figure 2.2). For example, TCAs have been found to greatly increase climbing behaviour while SSRIs increase swimming behaviour, reducing immobility in both cases[168,169]. A stopwatch was used to assess the total time animals were completely immobile in the tank during the 5 min test session. In addition to measuring total time spent immobile, individual behaviours of swimming, climbing, diving, and immobility were assessed[170] using a tally counter and stopwatch. Counts were tallied every five seconds, totalling in 60 counts for each five minute test session.

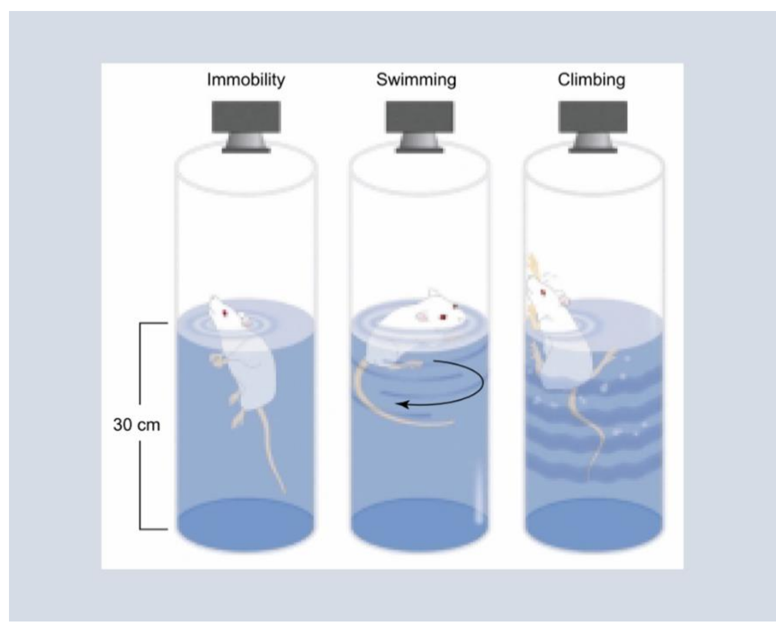

Figure 2.2. Rat behaviour in the Forced Swim Test (Image from Cryan *et al.* 2002[170]).

2. Western Blotting Protocol

2.1. Tissue Acquisition and Preparation

2.1.1. Animal Tissue

Immediately following the FST, all animals were sacrificed by decapitation and trunk blood was collected in lithium-heparin tubes and centrifuged at 200 RCF for 15 min at room temperature to obtain a platelet rich plasma supernatant. The platelet rich plasma supernatant was transferred to eppendorf tubes and spun again at 2100 RCF for 10 min at 4°C to remove platelets from the blood plasma, leaving platelet poor plasma. Hippocampi and prefrontal cortex were dissected out and immediately transferred to dry ice. Both brain regions were homogenised in Lysis Buffer (5mM Tris-HCl, 2mM EGTA, pH 8.0) using a Sonya Soniprep 150 MSE[171]. A protease inhibitor cocktail (Sigma) was

added to each plasma and brain sample, in a concentration of 2% of total sample volume for preservation[172]. All samples were stored at -80°C.

2.1.2. Human Plasma

Human blood plasma samples were acquired through academic collaborators at Trinity College Dublin and the University of Regensburg. In both cases, participant medical histories and approximately 4 ml of venous blood was collected from each donor in lithium-heparin tubes. Blood samples were immediately centrifuged at 2000 RCF for 15 min at room temperature to isolate platelet poor plasma. A protease inhibitor cocktail was added to each sample, in a concentration of 2% of total sample volume to ensure preservation. Samples were stored at -80°C.

2.2. Bradford Protein Assay

Protein content of brain and plasma samples were determined using Bradford Colorimetric Assay[173]. A standard curve was produced from a stock solution of 10 mg/ml bovine serum albumin (BSA) diluted in Milli-Q ultra-pure water. Standards ranging from 25 µg/ml to 3.125 µg/ml BSA were then diluted from the stock solution in saline for plasma samples or Lysis Buffer for brain samples. A blank of saline or Lysis Buffer was also included in the curve. Samples were run in triplicate on a clear 96-well plate (Sigma). In order to read the samples in the BSA standard range, a two-step dilution was required. Plasma samples were first diluted 1:40 in saline and then again 1:250 once the Bradford reagent (Sigma) was added. Brain samples were first diluted 1:10 in Lysis Buffer and then again 1:250 once the Bradford reagent was added. 200 µl of diluted sample, standard, or blank was added to each well of the plate. Once the plates were loaded, they were left to stand for 2 min before absorbance was measured at 595 nm using a SpectreMax M2e plate reader. A linear regression of the standard curve was calculated and protein concentration of the samples was extrapolated in Microsoft Excel.

2.3. Laemmli Buffer

Rodent plasma samples were normalised to have a protein concentration of 1 µg/µl in saline and Laemmli Buffer (Sigma; 4% SDS, 20% Glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blur, 0.125 M Tris-HCl, pH 6.8) and brain samples were normalised to have protein concentration of 0.1 µg/µl in Lysis Buffer and Laemmli Buffer. Human plasma samples were normalised to have a protein concentration of 6 µg/µl in saline and Laemmli Buffer (Table 2.1). Once the Laemmli Buffer was added, samples were heated to 95 °C for 3 min and then stored at -20 °C.

Tissue	Normalised Sample Concentration (µg/µl)	Loading Volume (µl)	Protein Loaded (µg)
Rodent Plasma	1	7	7
Rodent Brain	0.02	10	0.20
Human Plasma	6	10	60

Table 2.1. Tissue protein concentration for gel loading.

2.4. Electrophoresis

Electrophoresis was carried out using 10% Bisacrylamide/Trisacrylamide pre-cast gels (Bio-Science) running at constant 200 V for approximately 1 h using a PowerEase 90W (Life Technologies). 4 gels containing 26 wells each could be run simultaneously using Invitrogen XCell SureLock^{TM} Midi Cell Chambers (Figure 2.3). 2 wells on each gel were reserved for molecular weight marker (MWM), leaving 24 wells per gel for samples to be loaded. The recommended running buffer and antioxidant were used (Invitrogen). For all studies, gels were run in duplicate, resulting in two readings for each sample.

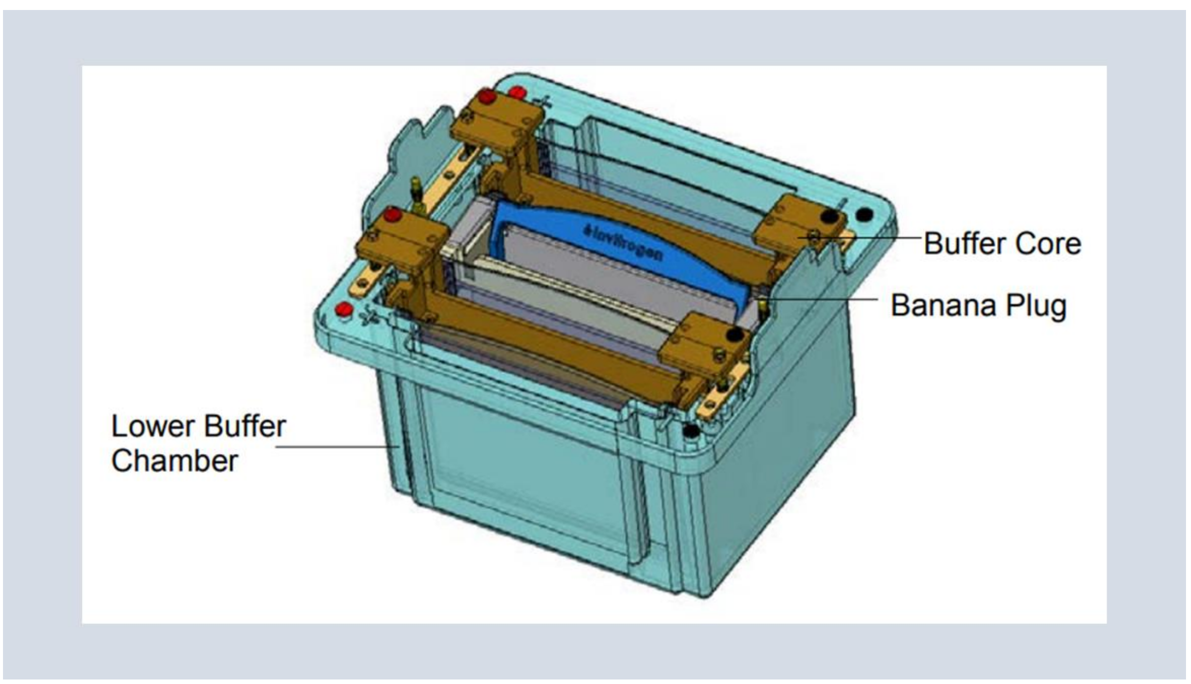

Figure 2.3. Diagram of Electrophoretic XCell SureLock^{TM} Midi Cell Chamber. (Image from thermofisher.com). Each chamber can hold up to 4 gels containing 26 wells each.

2.5. Gel Transfer

Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) with a 0.45 μm pore size using an iBlot2 transfer unit (Invitrogen). iBlot2 transfer stacks (Invitrogen) were used to complete the transfer of protein from the gels to the PVDF membrane running at 20 V for 7 min (Figure 2.4).

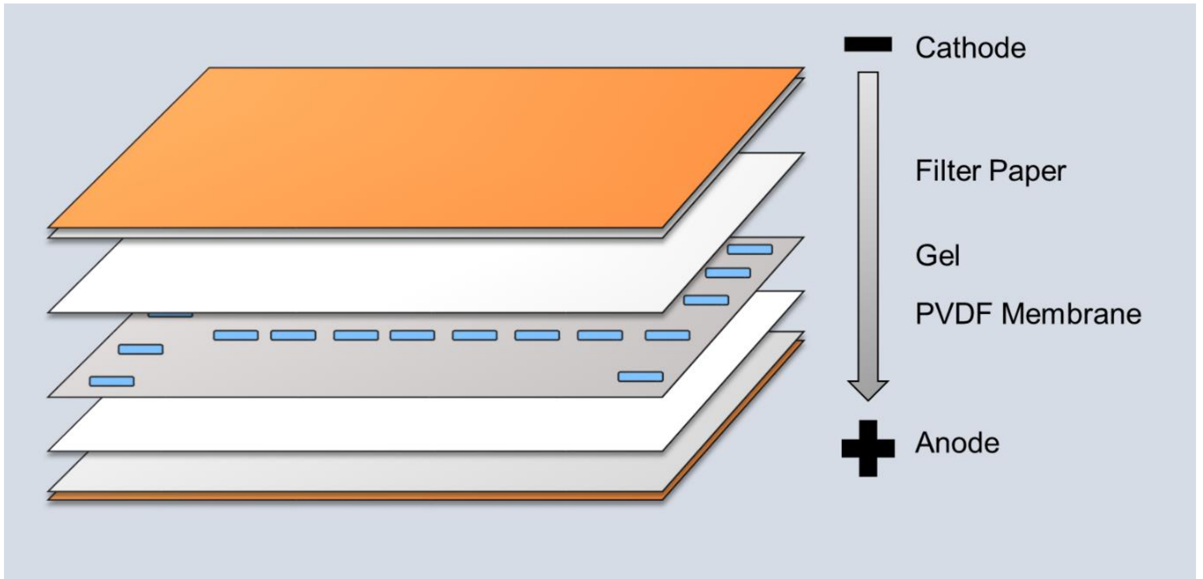

Figure 2.4. Simple diagram of iBlot2 transfer stack. Following electrophoresis, gels are removed from their cassettes and placed in the transfer stack. Similar to electrophoresis, protein is pulled out of the gel and embedded in the PVDF membrane by electrical current flowing through copper electrodes on the top and bottom of the stack.

2.6. Incubations

All membranes were blocked immediately after transfer for 1 hour in a 1:1 Phosphate-buffered saline (PBS) and Odyssey Blocking Buffer (OBB, Li-Cor) solution at room temperature to prevent non-specific binding of antibodies to the membrane.

Following blocking, membranes were incubated in the primary antibody solution (1:1 PBS-OBB, 5% Tween 20) for 1 hour at room temperature for brain samples or overnight at 4°C for plasma samples. After primary incubation, membranes were given six 10 min washes with either a light washing buffer (PBS, 0.01% Tween 20) for brain samples or a strong washing buffer (PBS, 0.05% Tween 20) for plasma samples. Membranes were then incubated for one hour with the secondary antibody solutions (1:1 PBS-OBB, 5% Sodium Dodecyl Sulphate (SDS), 5% Tween 20). Following the secondary antibody incubation, membranes underwent another six 10 min washes with light washing buffer before being stored in PBS buffer at 4°C.

2.6.1. Antibodies and Infrared Detection

Primary antibodies used in this project have been extensively used and validated elsewhere and where chosen for the reported reactivity in both rodent and human tissue[126,174,175]. All primary antibodies used in this study were of IgG isotype, while both monoclonal and polyclonal were used depending on availability and validation (Table 2.2).

Target	Clone	Isotype	Supplier
Total-α-Tub (DM1A)	Monoclonal	Mouse IgG_1	Sigma-Aldrich T9026
Total-α-Tub (11H10)	Monoclonal	Rabbit IgG	Cell Signalling Technologies 2125
Acet-Tub	Monoclonal	Mouse IgG_{2b}	Sigma-Aldrich T6793
Tyr-Tub	Monoclonal	Mouse IgG_3	Sigma-Aldrich T9028
Glu-Tub	Polyclonal	Rabbit IgG	EMD Millipore AB3201
Δ2-Tub	Polyclonal	Rabbit IgG	EMD Millipore AB3203

Table 2.2. Summary of primary α-Tubulin antibodies used in this project with their clone and isotype

Two colour botting required the use of Li-Cor IRDye IgG secondary antibodies: goat anti-mouse (680RD) and goat anti-rabbit (800CW). These secondary antibodies contain fluorophores, which when excited by infrared light emit an altered light wavelength that is read by the Odyssey scanner (Figure 2.5). IRDye 680RD is excited at 676 nm and emits at roughly 700 nm while IRDye 800CW is excited at 778 nm and emits at roughly 800 nm.

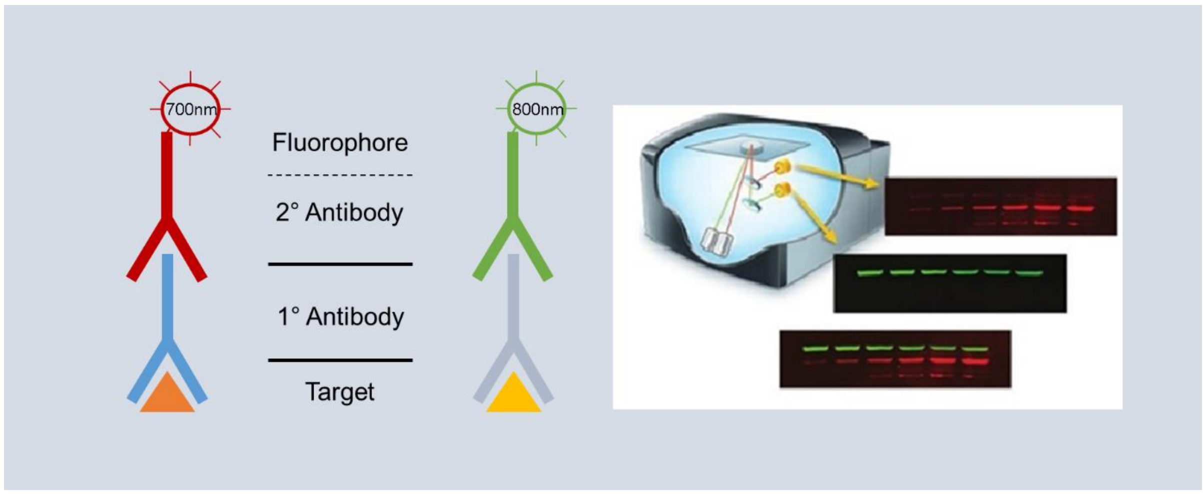

Figure 2.5. Western blotting concept using the Li-Cor Odyssey system. Two different primary antibodies, one anti-mouse and one anti-rabbit, are used to bind to two targets of interest. Secondary IRDye antibodies are added and bind specifically to either the anti-mouse or anti-rabbit primary antibodies. The secondary IRDye antibodies contain fluorophores that when excited by a laser in the scanner emit a 700 nm (anti-mouse targets) and 800 nm (anti-rabbit targets) signal that are then read by the Li-Cor Odyssey Scanner.

2.7. Data and Statistical Analysis

2.7.1. Li-Cor Software

Li-Cor Infrared Imaging System software version 3.0.16 was used to both scan and calculate Integrated Intensity (or Pixel Volume) for each target band. Band Integrated Intensity is automatically calculated by the software by subtracting the background intensity from the target band intensity using a concentric rectangle for each band and immediate background area (Figure 2.6).

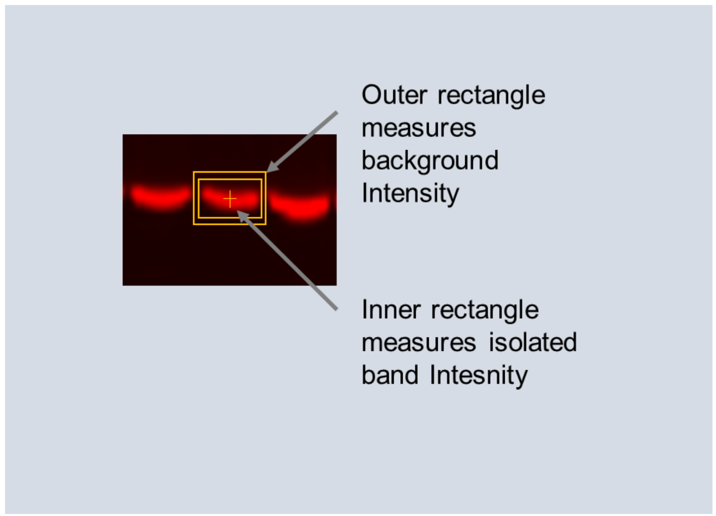

Figure 2.6. Calculation of band integrated intensity. Using the Li-Cor Imaging System Software, Integrated Intensity for each band is calculated by first drawing a band box around it with the software. The reported Integrated Intensity is the intensity of the inner box, containing the band of interest, minus the intensity of the outer box, which measures the background signal or noise. It is crucial to ensure that the boxes around each band are drawn as uniformly as possible to ensure a consistent background calculation.

Once the Intensity was determined for each band, the remaining data organisation and initial analysis was carried out using Microsoft Excel. R^2 for each target was calculated in Graphpad Prism 7.

2.8. Optimisation of α-Tubulin Detection in Animal Fluid and Tissue

2.8.1. Optimisation of α-Tubulin Detection in Rodent Plasma Samples

Optimisation of WB protocols for the analysis of Acet-Tub, Total-α-Tub (DM1A), Tyr-Tub, Glu-Tub, and Transferrin (Abcam, Ab82411) in rat plasma were successful. Δ2-Tub and Tot- α-Tub (11H10) was not reliably detectable in rat plasma. To find the linear range[176] for each target 2, 3, 7, 10, and 15 µg of total protein were loaded in duplicate (Figure 2.7). A dilution of 1:5000 was used for all secondary antibodies, while primary antibody concentration varied by target (Table 2.3). An ideal loading concentration of 7µg total protein was selected for rat plasma samples.

Target	Primary Antibody	Secondary Antibody	Primary Incubation	Secondary Incubation
Total-α-Tub (DM1A)	1:1000	1:5000	O/N 4°C	1 h RT
Acet-Tub	1:1000	1:5000	O/N 4°C	1 h RT
Tyr-Tub	1:1000	1:5000	O/N 4°C	1 h RT
Glu-Tub	1:1000	1:5000	O/N 4°C	1 h RT
Transferrin	1:4000	1:5000	O/N 4°C	1 h RT

Table 2.3. Antibody concentrations for rodent plasma targets and incubation conditions.

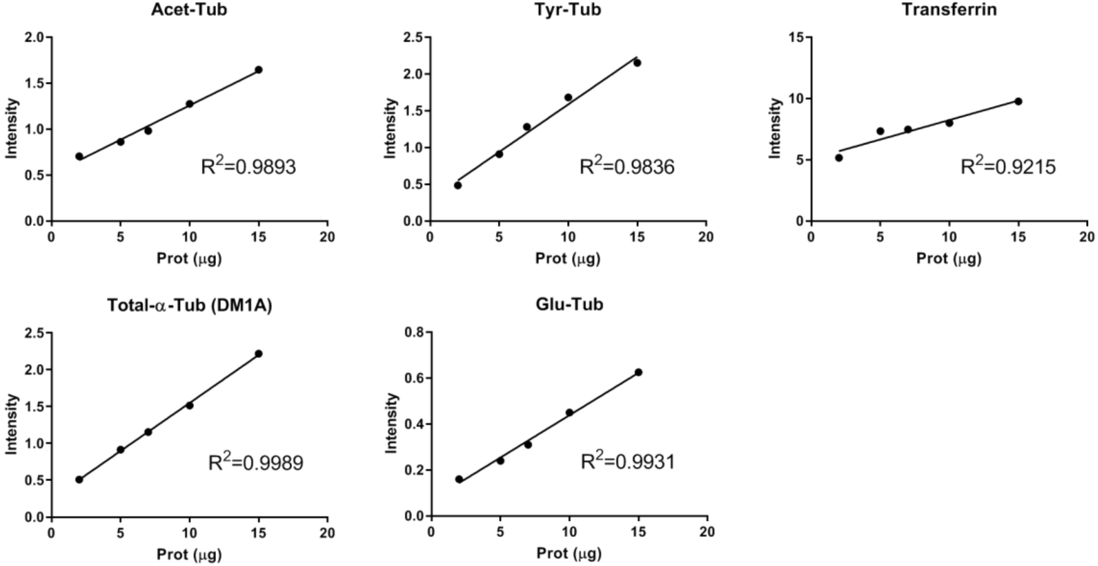

Figure 2.7. Linear ranges for molecular targets in rat plasma. Linear range was confirmed by calculating the correlation coefficient (R^2) between Integrated Intensity and the total protein for each target. Each target was run in duplicate, average Integrated Intensity is shown.

2.9. Optimisation of α-Tubulin Detection Rodent Brain Samples

Optimisation of WB protocols for the analysis of Acet-Tub, Δ2-Tub, Total-α-Tub (DM1A), Tyr-Tub, and Glu-Tub in rat hippocampus and prefrontal cortex were successful. Total-α-Tub (11H10) was not reliably detectable in rat brain. To find the linear range[176] for each target 0.04, 0.10, 0.14, 0.20, and 0.30 µg concentrations were tested in duplicate for each brain region (Figure 2.8 & Figure 2.9). A dilution of 1:5000 was used for all secondary antibodies and 1:4000 for all targets except Tot-α-Tub (DM1A), which had a primary concentration 1:20,000 and secondary concentration 1:10,000 (Table 2.4). Loading concentration of 0.2 µg total protein was selected for loading both hippocampus and PFC samples.

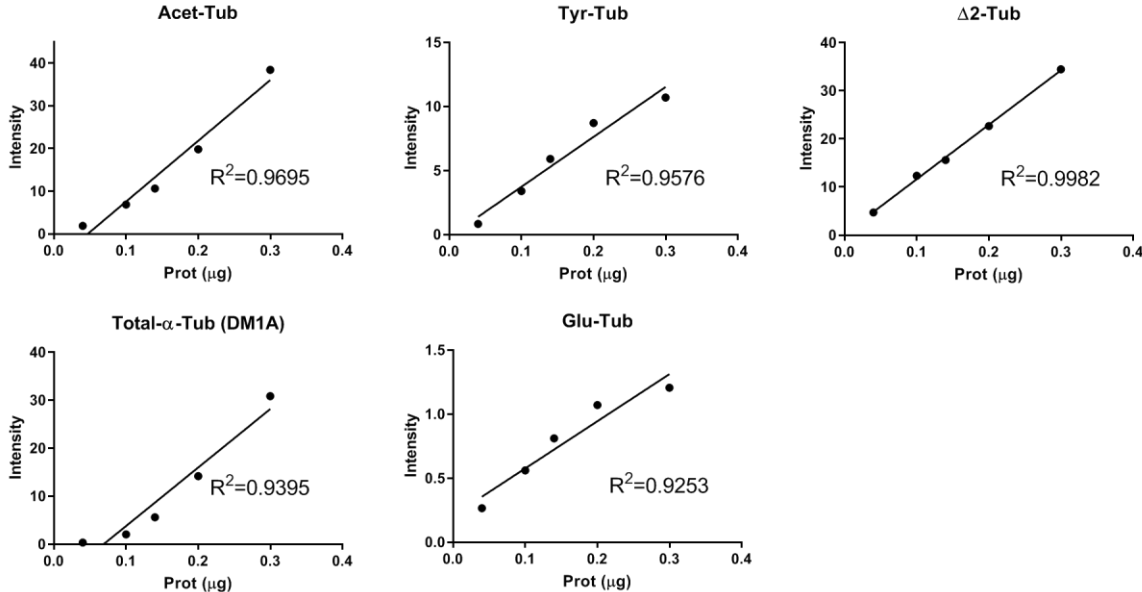

Figure 2.8. Linear ranges for molecular targets in rat hippocampus. Linear range was confirmed by calculating the correlation coefficient (R^2) between Integrated Intensity and the total protein for each target. Each target was run in duplicate, average Integrated Intensity is shown.

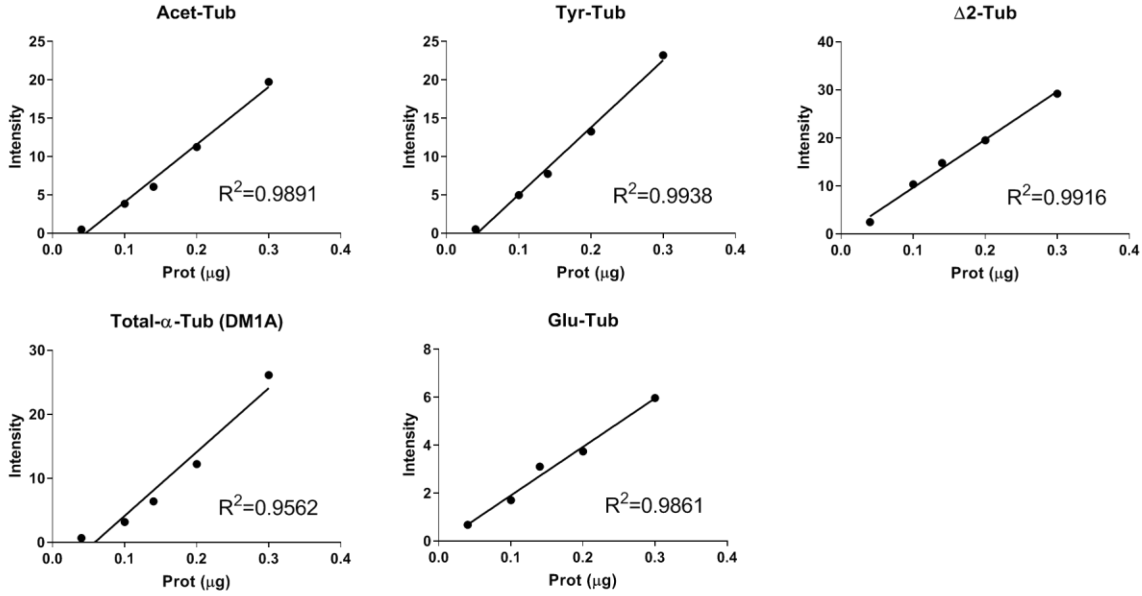

Figure 2.9. Linear ranges for molecular targets in rat prefrontal cortex. Linear range was confirmed by calculating the correlation coefficient (R^2) Integrated Intensity and the total protein for each target. Each target was run in duplicate, average Integrated Intensity is shown.

Target	Primary Antibody	Secondary Antibody	Primary Incubation	Secondary Incubation
Total-α-Tub (DM1A)	1:20,000	1:10,000	1h RT	1h RT
Acet-Tub	1:4000	1:5000	1h RT	1h RT
Tyr-Tub	1:4000	1:5000	1h RT	1h RT
Glu-Tub	1:4000	1:5000	1h RT	1h RT
Δ2-Tub	1:4000	1:5000	1h RT	1h RT

Table 2.4. Antibody Concentrations for Rodent Brain Tissue Targets and Incubation Conditions.

2.10. Optimisation of α-Tubulin detection in human blood plasma

Optimisation of WB protocols for the analysis of Acet-Tub, Δ2-Tub, Total-α-Tub (DM1A), Total-α-Tub (11H10), Tyr-Tub, and Glu-Tub human plasma were successful. To find the linear range[176] for each target 20, 30, 40, 60, and 90 µg concentrations were tested in duplicate (Figure 2.10). A dilution of 1:1000 was used for all secondary antibodies, while primary antibody concentration varied by target (Table 2.5). Loading concentration of 60 µg total protein was selected.

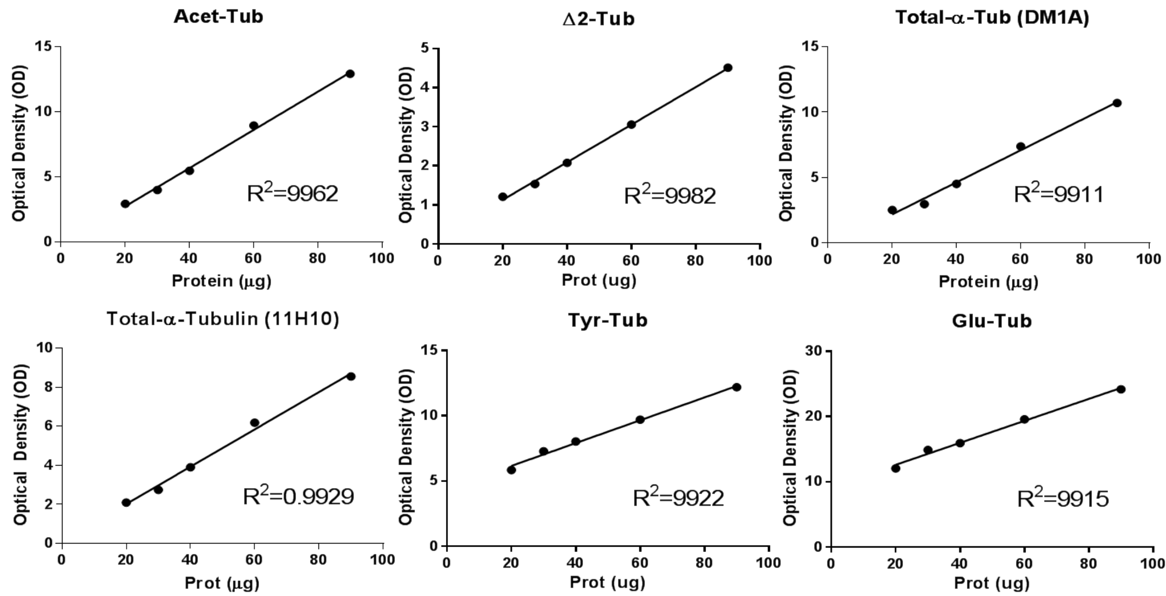

Figure 2.10. Linear ranges for molecular targets in human plasma. Linear range was confirmed by calculating the correlation coefficient (R^2) between Integrated Intensity and the total protein for each target. Each target was run in duplicate, average Integrated Intensity is shown.

Target	Primary Antibody	Secondary Antibody	Primary Incubation	Secondary Incubation
Total-α-Tub (DM1A)	1:500	1:1000	O/N 4°C	1h RT
Total-α-Tub (11H10)	1:500	1:1000	O/N 4°C	1h RT
Acet-Tub	1:500	1:1000	O/N 4°C	1h RT
Tyr-Tub	1:1000	1:1000	O/N 4°C	1h RT
Glu-Tub	1:1000	1:1000	O/N 4°C	1h RT
Δ2-Tub	1:1000	1:1000	O/N 4°C	1h RT

Table 2.5. Antibody concentrations for human plasma targets and incubation conditions.

3. Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was primarily pioneered by Engvall and Perlmann as an alternative to the radioimmunoassay[177]. It has since been greatly expanded for utilisation by several scientific fields and industries and is a preferred method of quantitative analysis due to its simplicity and high throughput capabilities. This project utilises conventional “sandwich” ELISAs with pre-coated wells with the capture antibody (Figure 2.11).

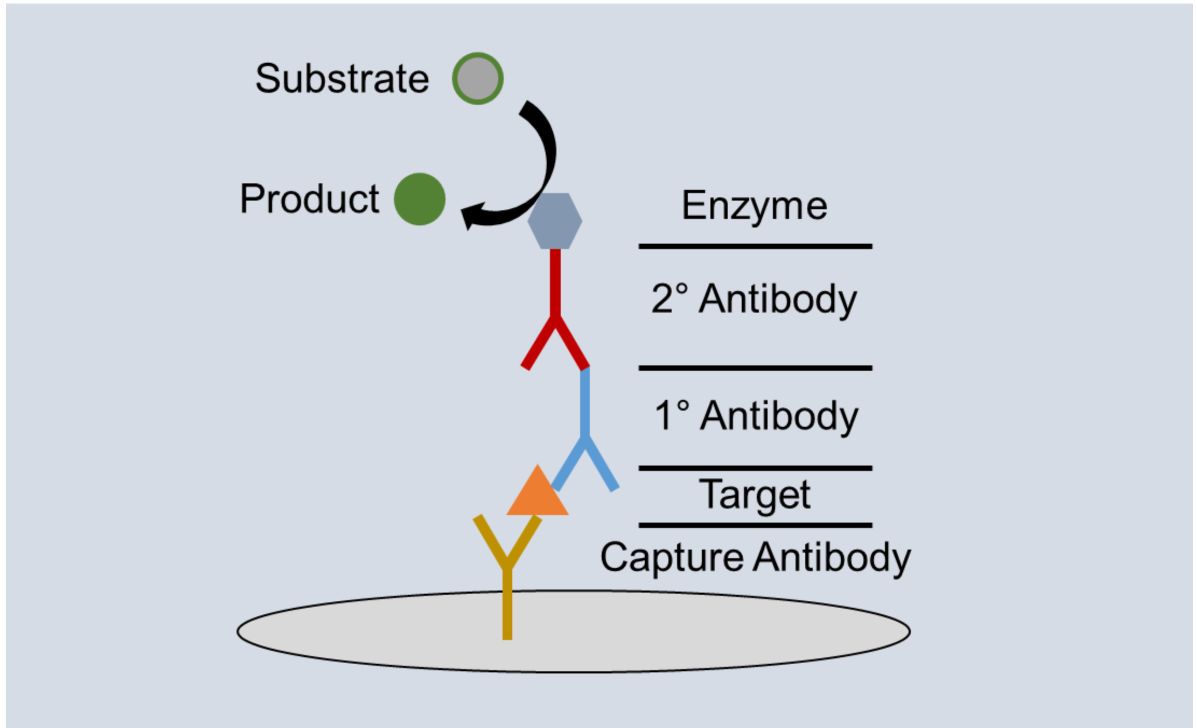

Figure 2.11. Sandwich Enzyme-Linked Immunosorbent Assay diagram.

3.1. Corticosterone Enzyme-Linked Immunosorbent Assay

Corticosterone (CORT) ELISA kits were purchased from Enzo Life Sciences (ADI-900-097[178]) to measure CORT expression in rodent plasma. Plasma samples were diluted 1:40 with steroid displacement reagent. No other deviations were made from the manufacturer's suggested protocol. Samples and standards were randomly assigned to wells and run in duplicate. Plates were read using a SpectreMax M2e plate reader.

3.1.1. Naïve Animals for Corticosterone analysis

To confirm that CORT was elevated in the test animals, naïve animals (n=4/strain) were included in a sub-study for comparison. Naïve animals were sacrificed with blood plasma collected as described above.

3.2. Brain Derived Neurotrophic Factor Enzyme-Linked Immunosorbent Assay

Brain Derived Neurotrophic Factor (BDNF) ELISA kits were purchased from R&D Systems (DBNT00[179]) to measure BDNF expression in rodent hippocampi. Minor deviations were made to the manufacturer's recommended protocol. Lysates were slowly allowed to thaw on ice. Once thawed, 300 µl of lysate was transferred to a new eppendorf and centrifuged at 10,000 RCF for 20 min at 4 °C. Lysate supernatants were collected for use in the ELISA and stored at -80 °C. On the day of the assay, lysate supernatants were diluted 1:150 to ensure that all samples could be read within the

standard curve range. Samples were randomly assigned to wells and run in duplicate. Plates were read using a SpectreMax M2e plate reader. A Bradford protein assay was conducted for all the samples on the same day of the ELISA assay so that data could be represented as a ratio of BDNF to total protein.

4. Data Analysis and Statistical Methods

4.1. Power Calculations for Rodent Studies

Power calculations were made in InVivoStat 3.7.0.0 using historical Transpharmation Ireland Ltd. data for SD and WKY immobility in the FST. The power calculations assume the statistical analysis will be performed using the two sample t-tests. This calculation may lead to slightly conservative estimates of sample sizes and statistical power[180,181].

Assuming p<0.05 significance, the appropriate sample size for SD rats was determined to be 10 per group to detect a biologically relevant 25% change from control with 88% power (Figure 2.12).

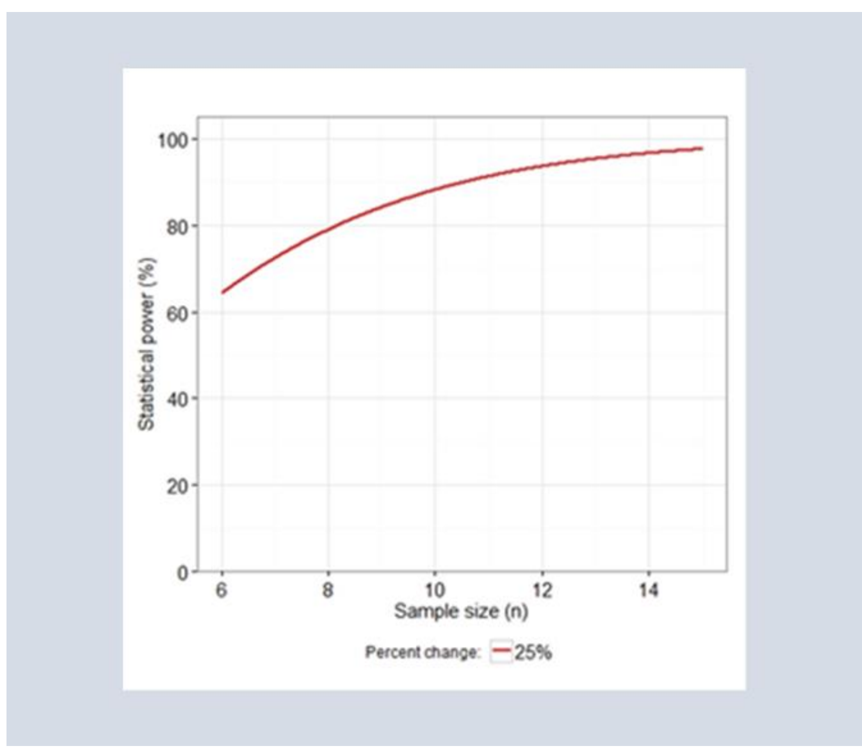

Figure 2.12. Power curve plot for Sprague Dawley rats in the Forced Swim Test.

Again using an assumption of $p<0.05$ significance, the appropriate sample size for WKY rats was determined to be 10 per group to detect a biologically relevant 25% change from control with 86% power (Figure 2.13).

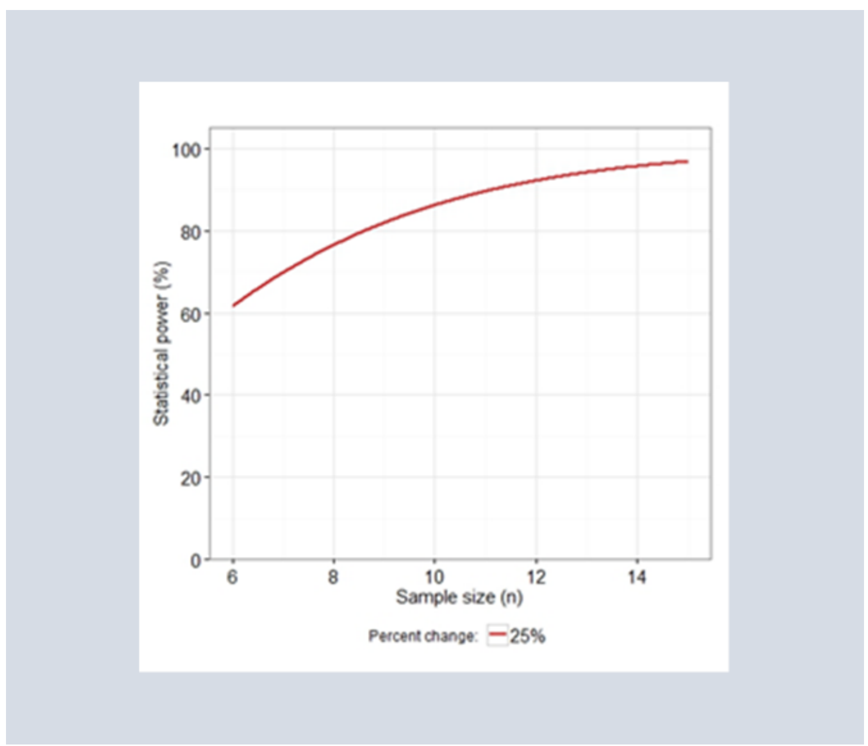

Figure 2.13. Power curve plot for Wistar Kyoto rats in the Forced Swim Test.

Using an assumption of $p<0.05$ significance, the appropriate sample size for the detection of differences in Tot-α-Tub (DM1A) was determined to be 10 per group to detect a biologically relevant 25% change from control with 89% power (Figure 2.14).

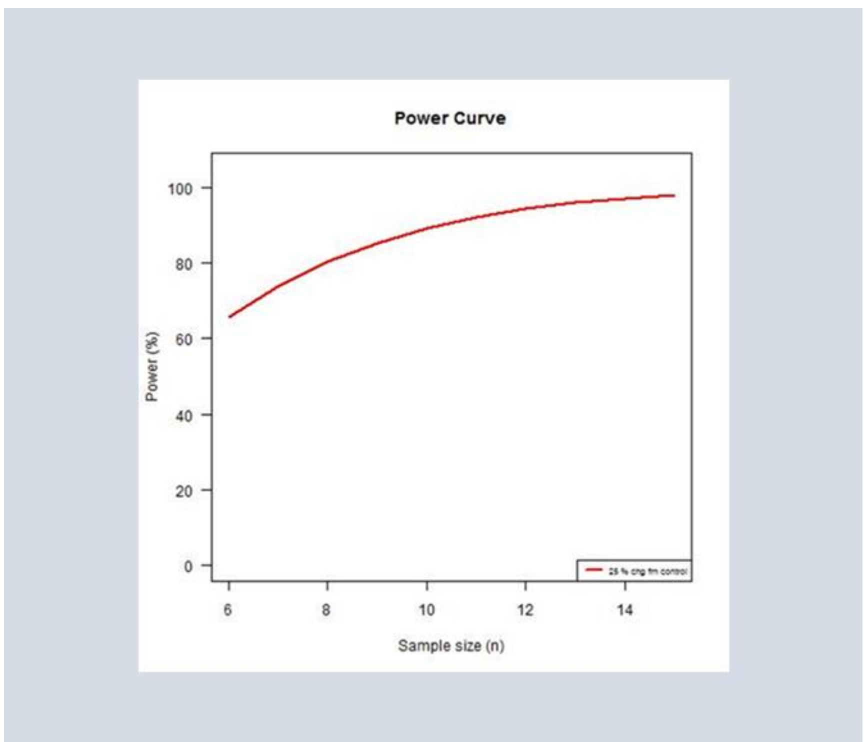

Figure 2.14. Power curve plot for Total-α-Tubulin (DM1A) in Sprague Dawley Hippocampal Tissue.

4.2. Analysis of Rodent Behavioural and Molecular Data

Study design and statistical analysis was aided by Dr. Simon Bate (GSK Stevenage, UK). Analysis was carried out in InVivo Stat 3.7.0.0. Graphpad Prism 8 was used to make all graphs.

Rodent molecular data was Log_{10} transformed for statistical analysis but observed means and standard error are represented in all plots. This layout was chosen to best avoid confusion from the effects of blocking factors and non-intuitive Log transformed data.

Single measure two-way analysis of variance (ANOVA) with blocking factor (as appropriate) followed by unadjusted planned comparisons[180] was employed for each behavioural and molecular endpoint. The risk of false positives is higher when using unadjusted planned comparisons, however this was determined to be the best option for analysis to detect if any relevant biological changes may be occurring. This is especially important to note when considering the molecular data as observed means are not

adjusted for unequal replicates across blocks and the statistical analysis may contradict the observed means plots. The block design for WB analysis of rodent samples is explained further in Chapter 4 Section 4.2.

Because planned comparisons were carried out, there was no formal *post hoc* analysis; however, the mathematics behind the unadjusted planned comparisons and typical *post hoc* test in this case, the Fisher's least significant difference test, would be the same. This is noted as a more theoretical consideration for the analysis than a practical one[180].

4.2.1. Data and Statistical Analysis for Enzyme-Linked Immunosorbent Assays

Initial analysis of CORT ELISAs was carried out using Microsoft Excel and Graphpad Prism 8 for the Log_X extrapolation. Formal statistical analysis was carried out in InVivo Stat 3.7.0.0 using single measure two-way ANOVA with blocking factor followed by unadjusted planned comparisons[180]. The comparison of naïve animals to SD and WKY vehicle groups was carried out in a separate analysis using the same method as above with the exception that no blocking factor was necessary.

Initial analysis of BDNF ELISAs was carried out using Microsoft Excel for wavelength correction. Formal statistical analysis was carried out in InVivo Stat 3.7.0.0 using single measure two-way ANOVA with blocking factor followed by unadjusted planned comparisons.

4.3. Power Calculations for Human Cohorts

Formal power calculations were not conducted for human cohorts due to the limited number of samples available. Instead, samples were selected on a hypothesis driven basis outlined in Chapter 5 Sections 4.1.1 and 4.1.2.

4.4. Data and Statistical Analysis of Human Cohort 1

Analysis was carried out in InVivo Stat 3.7.0.0. Graphpad Prism 8 was used to make all graphs.

A repeated measure mixed model analysis[182] with planned comparisons was employed for all endpoints in cohort 1. HAM-D and individual α-Tub targets were analysed using observed values. Ratios of α-Tub expression required log_{10} transformation prior to analysis however observed means and standard error are represented in plots.

4.5. Data and Statistical Analysis of Human Cohort 2

Analysis was carried out in InVivo Stat 3.7.0.0. Graphpad Prism 8 was used to make all graphs.

A repeated measure mixed model analysis[182] was employed for all endpoints in cohort 2. HAM-D and individual α-Tub targets were analysed using observed values. Ratios of α-Tub expression required log_{10} transformation prior to analysis however observed means and standard error are represented in plots.

Chapter 3: Effects of Acute and Chronic Antidepressant Treatment on an Animal Model of Depression in the Forced Swim Test

1. Introduction

The Forced Swim Test (FST) is a rapid behavioural paradigm commonly used in screening antidepressant compounds. Since its introduction in the 1970s, the FST has proved to be remarkably resilient, even under constant scrutiny as an appropriate and accurate paradigm for assessing depressive-like behaviour.

The FST was chosen as a behavioural measure in the following study over other paradigms due the unique behavioural characteristics of the Wistar Kyoto (WKY) rat in the assay in addition to the wealth of historical data available using Sprague Dawley (SD) rats.

1.1. The Porsolt Forced Swim Test

The original FST paradigm described by Porsolt was widely applied for testing a variety of antidepressant compounds and treatments, such as Monoamine Oxidase Inhibitors (MAOIs), Tricyclic Antidepressants (TCAs), and electroconvulsive therapy[183]. The experimental design consisted of two sessions. The first session, or pre-test session, involves placing a rat in a plexiglass cylinder 40 cm high with a diameter of 18 cm and a water depth of 15 cm at 25 °C for 15 min. This 15 min pre-test session was intended to cause a state of "behavioural despair". Following the 15 min pre-test session, the animals are returned to their home cages. 24 hours later the animals are again placed in the tank of water for a 5 min test session, where their total immobility time is scored. Porsolt found that a 15 min pre-test session resulted in animals being immobile for roughly 75% of the 5 min test session creating a suitable baseline to test pharmacological agents[147].

In his description of the paradigm, Porsolt writes:

> The test is based on the observation that rats when forced to swim in a restricted space from which they cannot escape will eventually cease apparent attempts to escape and become immobile apart from the small movements necessary to keep their heads above water. We suggested that this characteristic and readily identifiable behavioural immobility reflects a state of despair in the rat and showed that immobility was reduced by a variety of agents which are therapeutically effective in depression[184].

Porsolt's anthropomorphic description of "state of despair" has been a constant source of controversy for the FST. We simply cannot know what a rat is thinking let alone in reference to complex human emotion and psychiatric condition such as Major Depressive Disorder (MDD). This disconnect is summed up perfectly in the cartoon pictured below (Figure 3.1).

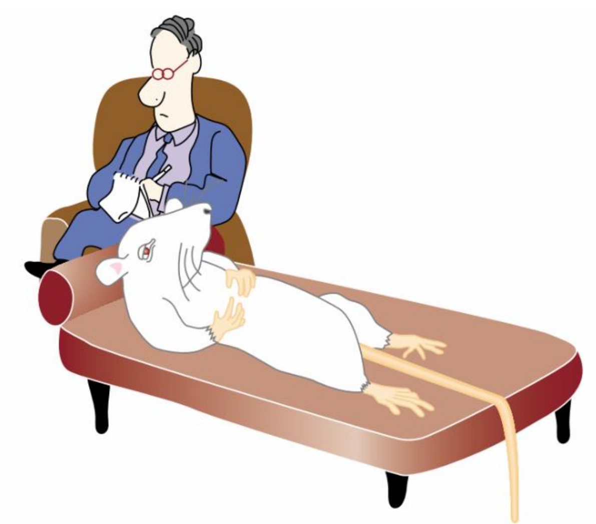

Figure 3.1. A "depressed" rat. Image from Cryan *et al.* 2002[170].

An opposing view to immobility as a state of despair says that the lack of movement seen in the test session is actually a learned coping strategy or habituation[183]. During the 15 min pre-test session, animals learn that they cannot escape from the tank of water so when they are exposed to this situation a second time, they are less likely to try to escape. It has been argued that not only is this behaviour not "depressive" but even a positive adaptation to an adverse event[185].

Regardless of the animals' "state of mind" there is no doubt that pharmacological treatments modulate behaviour in the FST.

1.1.1. Rat Strains in the Porsolt Forced Swim Test

Porsolt's first publication describing the FST used only Sprague Dawley (SD) rats but reported a wide variety of treatment and dose combinations administered by *intra peritoneal* injection[147]. In his follow up study, two rat strains from two suppliers were used: the SD rat and Wistar rat each from Charles River and Iffa-Credo[184]. In this article, Porsolt showed that there were no strain or supplier differences using his paradigm, although this would not bear out in future studies. Once other experimenters adopted the protocol, it was clear that both strain and supplier could impact the results of the Porsolt FST[167].

1.2. Treatments and Dosing in the Porsolt Forced Swim Test

The Porsolt design used a sub-chronic dosing regimen where animals were dosed three times: 24 hours before the test session (immediately following the pre-test session), 5 hours before the test session, and 1 hour before the test session. In addition to optimising the pharmacological effect of the test compounds, the intended purpose of the sub-chronic dosing regimen was to more closely mimic clinical usage of antidepressants[147].

Prescribed antidepressants are not taken once but daily for many weeks before they can be seen to have their desired effect. However, fully replicating this exactly in an animal model is often unnecessary and impractical.

The validity of the FST as a strict depressive phenotypic measure has been called into question due to other psychoactive compounds having an effect on immobility. Psychostimulants, such as caffeine and amphetamine, can produce false positives in the FST as well as numerous GABAergic and anticholinergic compounds acting through various neural and endocrine pathways[167]. Likewise, it is possible to generate a false negative with the most common example being Selective Serotonin Reuptake Inhibitors (SSRIs) in the Porsolt FST (this problem is addressed by the Modified FST described below).

Another question concerning validity is that: if the FST is inducing a state of despair through a stressful act, would anxiolytics not also work in reducing immobility? At best it appears that anxiolytics (specifically benzodiazepines) have conflicting results in the FST[186,187]. Although anxiety and depression are closely linked by dysregulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis and have high comorbidity, the pharmacological profile of benzodiazepines is considerably different to that of antidepressants[188]. Even though SSRIs are often prescribed for anxiety, benzodiazepines are never a first choice for treating depression because they can worsen depressive symptoms and increase suicidality[189,190]. This risk of benzodiazepines worsening depressive symptoms may be reflected in the FST where some studies have shown that benzodiazepines not only increase immobility but can also counteract the antidepressant effects of other treatments[191]. Another major confounding factor of benzodiazepines in the FST is their sedative effects that can cause locomotor deficits thereby increasing immobility.

Locomotor deficits must be accounted for with any antidepressant treatment investigated using the FST and most early investigations included other behavioural paradigms to confirm that changes in the FST were truly “antidepressant” in nature. The Open Field Test is the most common paradigm used in conjunction with the FST for this purpose. Like the FST, the Open Field Test is a fast and easily scored assay with several variants for behavioural scoring[192,193]. Traditionally, the test consists of placing the rat in a large circular arena where it can move freely. The researcher then simply calculates the distance the rat travels in the arena during the test session. In some cases, the Open Field Test can serve a dual purpose as a test for anxiety-like behaviour by calculating

the amount of time the rat spends exploring the centre of the arena (interpreted as the rat being less anxious) versus exploring along the walls[194].

A locomotor test was omitted in this study as the compounds and doses being tested have been extensively investigated in the past and no locomotor effects have been reported[26,152,168,195].

1.3. Modified Forced Swim Test

A major limitation of the Porsolt FST design is its unreliable measurement of SSRI efficacy[196]. The Modified FST, first published by Detke *et al in* 1995[168], refined several design parameters that improved the sensitivity of the FST to SSRIs. The water level was increased from 15-18 cm to 30 cm and clear tanks roughly 20 cm in diameter were used[168]. This revision allowed more thorough characterisation of swimming and escape behaviour by using an interval scoring method where predominant behaviours are tallied at 5 sec intervals[168,197]. This scoring method is useful in reducing intra-rater variability as well as assessing antidepressant effects on behaviours other than immobility[198]. But more importantly than measuring these behaviours in isolation, they could now be tied to specific neurochemical changes, greatly enhancing the investigative power of the FST. An extraordinary amount of research has subsequently been conducted by numerous groups characterising these active and passive behaviours in response to antidepressant compounds acting on specific systems and receptors[148]. For example, three 5-HT receptor subtypes have been identified as playing key roles in SSRI efficacy in the FST: 5-HT_{1A},[199,200] 5-$HT_{1B/1D}$[146,201], and 5-HT_{2C}[202].

(For an extensive review of pharmacological agents used in the FST and the role of specific transporters and receptors see Cryan *et al.* 2005[148])

1.3.1. Antidepressants in the Modified Forced Swim Test

The improvements made by the Modified FST revealed that SSRIs reduced immobility by causing an increase in swimming behaviour[168,197]. This is notably different than drugs acting on catecholaminergic transmission, such as desipramine (DMI), which reduces immobility with an accompanying increase in climbing behavior[168,197].

Novel antidepressant treatments have also been screened in the FST. Though ketamine's antidepressant properties were discovered in clinical trials, it is being used more and more in preclinical models to help identify similar drugs that may be better suited for treating depression. It has been validated in the FST, where it significantly reduces immobility without affecting locomotor activity at sub-anaesthetic doses[203]. Interestingly, ketamine is also used in an FST model of schizophrenia, where repeated

sub-anaesthetic dosing (although at a higher concentration than its antidepressant efficacy window) reportedly causes dissociative symptoms in rats and increases immobility[204]. This reinforces that like the clinical administration of ketamine, the dose range is critical to eliciting an antidepressant effect versus a dissociative or anaesthetic one.

Pregnenolone-Methyl-Ether's (PME) antidepressant efficacy has been explored in the modified FST where it significantly reduces immobility by increasing swimming behaviour similar to SSRI treatments[26].

1.4. Dosing Regimen and Administration Route

While the dose concentration is of obvious experimental importance[148], so too is the number of doses the animal receives. Much of the reported work in the FST has used single or sub-chronic dosing regimens, as in the Porsolt FST. It has been demonstrated sub-chronic dosing is more efficacious in reducing immobility than a signal dose of the same cumulative concentration[183]. Likewise, chronic dosing is more efficacious than single and sub-chronic dosing, particularly for fluoxetine[205,206] and DMI[205], which may have no effect at a single dose. Due to the unique design and aims of this project, the dose selection was necessarily informed from several studies and is summarised below.

Pharmacological agents can be administered by several routes in this paradigm. Most common are *sub cutaneous (s.c.)* and *intra peritoneal*, although *per os*, through drinking water, and even direct administration to the central nervous system by cannula can be used[167]. Choosing an appropriate administration route depends on several factors, including: solubility, pH, ability to cross the blood brain barrier, and pharmacokinetic profile of the compound.

In this study, the *s.c.* route was chosen to be in line with previous reports using the same compounds[26,146,166].

1.4.1. Escitalopram and Desipramine Doses

SD rats administered acutely with fluoxetine or DMI, both at 10 mg/kg *s.c.*, have shown significant reduction in immobility and an increase in swimming and climbing behaviour, respectively[146]. As will be discussed further below, WKYs are resistant to sub-chronic fluoxetine (5, 10, and 20mg/kg *s.c.*) but not DMI, where a sub-chronic administration of 5mg/kg *s.c.* treatment showed significantly lower immobility than control[146]. In the same study, it was shown that sub-chronic DMI significantly reduced immobility in SD with an increase in climbing behaviour at 10mkg *s.c.* but not 5mg/kg *s.c.*

Sub-chronic ESC (10mg/kg *s.c.*) has been shown to reduce immobility in SD with an increase in swimming behaviour matching the effects seen with fluoxetine[207]. While there is a wealth of data for fluoxetine administration to WKYs, to the best of my knowledge the effects of acute and chronic ESC treatment in the FST has not been published.

10mg/kg/ml *s.c.* was selected for acute and chronic ESC and DMI treatment based on these studies.

1.4.2. Pregnenolone-Methyl-Ether and Ketamine Doses

One of the hopes for new antidepressant treatments is that they work faster and more effectively than what is currently available. PME and ketamine were selected for this project as they have been reported to have those two important characteristics.

A single acute administration of PME (10mg/kg *s.c.*)[26] in SD was shown to increase swimming behaviour and reduce immobility. Likewise, a single administration of ketamine (5mg/kg *s.c.*)[166] has been shown to significantly reduce immobility in WKY rats.

As in the previous section, 10mg/kg/ml *s.c.* PME and 5mg/kg/ml *s.c.* ketamine were selected based on the available information on their use in the FST with the relevant strains.

1.4.3. Effects of Chronic Mild Stress

The dosing regimen for this study requires each animal to receive two daily *s.c.* injections due to two vehicle solutions. Thorough steps were taken and records kept throughout the study to assure good animal welfare was maintained. However, the nature of the dosing was understandably a possible mild stressor to the animals and multiple injections were foreseen to result in some discomfort. Therefore it was anticipated that undergoing this regimen may have some impact on the animal's behaviour in the FST, in particular, the WKYs, which have abnormal HPA axis regulation[208] and are hyper-reactive to stress[209,210].

Similar to the pre-test exposure of the Porsolt FST, numerous other stress-inducing paradigms have been run in conjunction with the FST that lead to higher immobility. Most common of these are: pre-natal stress[211], social defeat[212], and restraint stress[213]. The rationale behind these paradigms is that chronic stress is commonly a precursor to depressive symptoms in humans.

Despite the well validated use of stressors prior to the FST test session, there exists some disagreement about the role of corticosterone (CORT) on behaviour in the FST. Some have argued that increased CORT levels brought on by stress are not linked to

behaviour in the FST[214], although there is considerably more evidence to the contrary. Several groups have found that CORT administration increases immobility[215] and modulates antidepressant efficacy[216]. Furthermore, as an acute stressor, the FST itself significantly increases the expression of CORT[155].

To investigate if a heightened state of stress occurred in the test animals that may have impacted behaviour, plasma CORT analysis was conducted.

1.5. Rat Strains in the Modified Forced Swim Test

Sprague Dawley (SD) rats are the most common rat used in the modified FST[148], however other strains are commonly used and in some cases for specific behaviours. For example, Wistar Kyoto (WKY) rats (discussed more below) are known for their spontaneous immobility in the FST[217], likewise Flinders Sensitive Line rats display higher immobility than other strains[218], and Long Evans rats have been shown to have hyper reactivity to stress thereby increasing immobility[219].

1.6. Animals

1.6.1. Sprague Dawley Rats

SD rats, among the most commonly used outbred strains in animal research, were raised during the 1920s as a hybrid between Wistar rats and wild rats. In the following decades Charles River Laboratories and Harlan Laboratories would each maintain SD stocks leading to a slight genetic drift between the two suppliers[220]. As to be expected, this genetic drift resulted in both physical and behavioural differences in SDs between the two suppliers. I note this since SD rats used in this study were sourced from Envigo UK (formerly Harlan Laboratories), however the supplier selection is not anticipated to negatively impact the project given their intended purpose. In this context the SDs are a “healthy control” or reference strain for the WKY as has been reported previously[146].

1.6.2. Wistar Kyoto Rats

The WKY rat was bred in Japan in 1963 from Wistar rats as a normotensive control strain for spontaneously hypertensive (SHR) rats[221]. While WKY rats were a success for their intended use, it was quickly noticed that the new inbred strain displayed many unintended behavioural and physiological differences that collectively resembled symptoms of MDD. For example, WKYs exhibit altered REM sleep[143] and altered HPA axis regulation[208–210]. Specifically, WKYs have higher basal plasma adrenocorticotropic hormone compared to Wistar rats and maintain higher levels of CORT following diurnal peak[208]. WKYS also have a higher response in plasma adrenocorticotropic hormone following acute and chronic stress compared to other strains[209].

Like the SDs, genetic variability and behavioural differences in sub-strains of WKYs from different suppliers has been noted as a confounding factor in these studies[222]. This is most problematic when they are used as a control for SHR rats but should be noted for any future investigations using this strain as behavioural and molecular phenotypes may differ between suppliers. WKYs used in this project were also sourced from Envigo UK.

Behaviourally, WKYs have been thoroughly characterised in a number of paradigms. Most importantly for this project is their spontaneous immobility in the FST[162,217,223] but WKYs also display an increased anxiety phenotype in the Open Field Test[224] and altered passive coping behaviour in the defensive burying test[161].

Perhaps most intriguing is how WKYs respond to pharmacological treatment compared to other strains. WKYs are reportedly resistant to SSRI treatment in the FST[146,225], however to the best of my knowledge the activity of escitalopram (ESC) has not been reported in a WKY FST study. This resistance is thought to be due to combination of dysregulation in the serotonergic system[159,226] and decrease in density of monoaminergic transporters[227]. This is further supported by WKYs unusual response to TCAs compared to other strains, although results are conflicting with evidence for both reduced[228] and increased[146] immobility in the FST. This particular inconsistency may be due to different dosing regimens and type of TCA (imipramine[228] versus DMI[146]).

WKYs respond to both acute and chronic treatment with ketamine in the FST without any impairment to locomotor function[154]. The unique behavioural and pharmacokinetic profile of WKYs has subsequently been identified as a model for MDD and even Treatment Resistant Depression.

2. Aims

This study's main aim is to compare the effects of different classes of antidepressant drugs on immobility in a FST paradigm, using both "healthy" SD and "depressed" WKY rats. The classes of antidepressants that were included in this study were: a SSRI (ESC), a TCA (DMI), N-methyl-D-aspartate (NDMA) antagonist (ketamine), and a neurosteroid derivative (PME). All four of these drug classes have been reported to have antidepressant properties in both human and animal investigations. Additionally, individual behaviours of swimming, diving, climbing, and immobility will be scored using the interval scoring method described in Chapter 2. And lastly, plasma CORT will be assessed.

Tissue samples will be collected for α-Tubulin analysis (Chapter 4).

3. Ethical Permissions

Ethical approval for this study was obtained from the Animal Research Ethics Committee (AREC) of Trinity College Dublin. For ethical permissions see Appendix A.1.

4. Methods

4.1. Animals

As described in Chapter 2 and above, the two strains used in this project are the SD and WKY rat.

4.2. Forced Swim Test Procedure

FST was performed as previously described in Chapter 2 Sections 1.3 and 1.4. To reiterate, due to the WKY's spontaneous immobility in the FST, no pre-test session was needed to induce a depressed state. As the healthy control strain, it was not desirable to induce a state of despair in the SD rats. This point is critical for interpreting their behaviour as they would normally undergo a pre-test session to induce a state of despair in studies where they were the only strain used.

Treatments and doses are summarised in Table 3.1. Last dosing for all groups was 2h pre-FST.

	Vehicle	ESC Acute	DMI Acute	ESC Chronic	DMI Chronic	PME	Ketamine
Dose	VEH 1 + VEH 2	10mg/kg/ml *s.c.* + VEH 2	10mg/kg/ml *s.c.* + VEH 2	10mg/kg/ml *s.c.* + VEH 2	10mg/kg/ml *s.c.* + VEH 2	10mg/kg/ml *s.c.* + VEH 1	5mg/kg/ml *s.c.* + VEH 2
Class	N/A	SSRI	TCA	SSRI	TCA	Neurosteroid derivative	Anaesthetic
Days	1-21	21 (VEHs 1-20)	21 (VEHs 1-20)	1-21	1-21	21 (VEHs 1-20)	21 (VEHs 1-20)
Primary Action	N/A	Reuptake inhibitor (SERT)	Reuptake inhibitor (NET)	Reuptake inhibitor (SERT)	Reuptake inhibitor (NET)	Microtubule dynamics modulator	NMDAR Antagonist

Table 3.1. Summary of treatment groups and dosing regimen prior to the Forced Swim Test

4.3. Corticosterone Analysis

Immediately following the FST, animals were sacrificed and tissue was collected for further analysis. Plasma CORT analysis was performed by enzyme-linked immunosorbent assay (ELISA) as previously described in Chapter 2 Section 3.1.

4.4. Statistics

Statistical analysis was completed using InVivoStat 3.2.0.0 software and plots were prepared in Graphpad Prism 8.

Single measure two-way analysis of variance (ANOVA) with blocking factor followed by unadjusted planned comparisons[180] was employed for each behavioural endpoint. The risk of false positives is higher when using unadjusted planned comparisons, however this was determined to be the best option for analysis to detect if any relevant biological changes may be occurring.

All rats tolerated the dosing regimen and were included in the behavioural and *ex vivo* analyses (Chapters 3 and 4). Predicted values with a residual more or less than three standard deviations of the mean are considered outliers.

4.4.1. Data and Statistical Analysis for Enzyme-Linked Immunosorbent Assay

Initial analysis of CORT ELISAs was carried out using Microsoft Excel and Graphpad Prism 8 for the Log_X extrapolation. Formal statistical analysis was carried out in InVivo Stat 3.7.0.0 using single measure two-way ANOVA with blocking factor followed by unadjusted planned comparisons[180]. The comparison of naïve animals to SD and WKY

vehicle groups was carried out in a separate analysis using the same method as above with the exception that no blocking factor was necessary.

Predicted values with a residual more or less than three standard deviations of the mean are considered outliers.

5. Results

The FST study design required three separate blocks and animals were randomly assigned to treatment groups within each block. A two-way ANOVA was performed with a complete data set from all three blocks. Only planned *a priori* comparisons are represented below.

Two-way ANOVA for total immobility found a main effect of block ($F_{(2,\ 121)}$ =23.39, p<0.001), strain ($F_{(1,\ 121)}$ =72.93, p<0.001), treatment ($F_{(6,\ 121)}$ =14.14, p<0.001), and significant interaction between strain and treatment ($F_{(6,\ 121)}$ =2.86, p=0.0121). Full ANOVA table results can be found in Appendix B.1.

5.1. Behavioural Endpoints in the Forced Swim Test

5.1.1. Total Immobility for Sprague Dawley and Wistar Kyoto Vehicle Groups in the Forced Swim Test

Total immobility was calculated for each five-minute session and is represented here in total seconds (sec) spent immobile for SD and WKY vehicle groups.

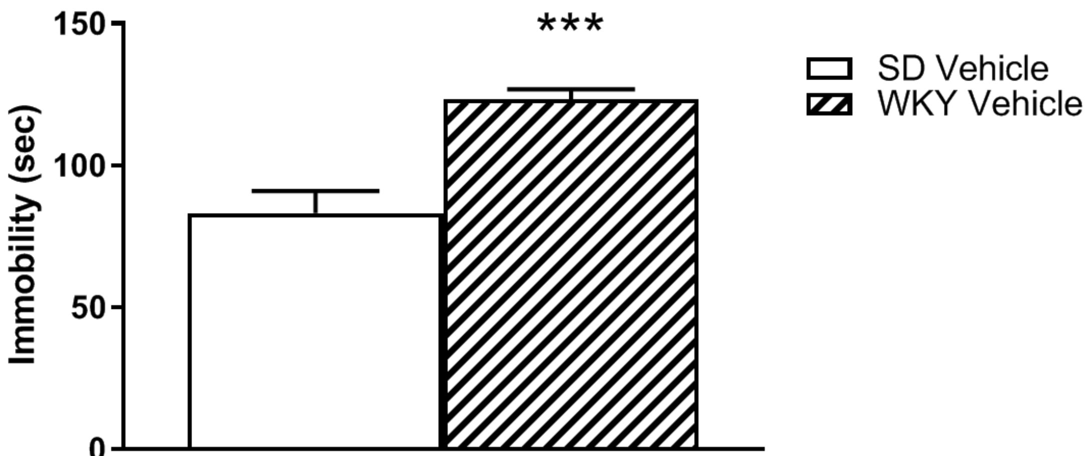

Figure 3.2. Immobility of Sprague Dawley and Wistar Kyoto vehicle groups in Forced Swim Test. n=10/group. ***p<0.001 vs SD vehicle. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

WKY vehicle exhibit significantly higher immobility (p<0.001) compared to SD vehicle (Figure 3.2). Omitting the pre-test session, ensured that the SD vehicle group would have relatively low immobility compared to WKY vehicle and represent a “healthy control” group.

5.1.2. Individual Behaviours for Sprague Dawley and Wistar Kyoto Vehicle Groups in the Forced Swim Test

Individual behaviours (swimming, climbing, immobile, and diving) are represented as counts taken at 5 second intervals over the five-minute test session. There is a total of 60 counts for each test session. The following compares the SD and WKY vehicle groups:

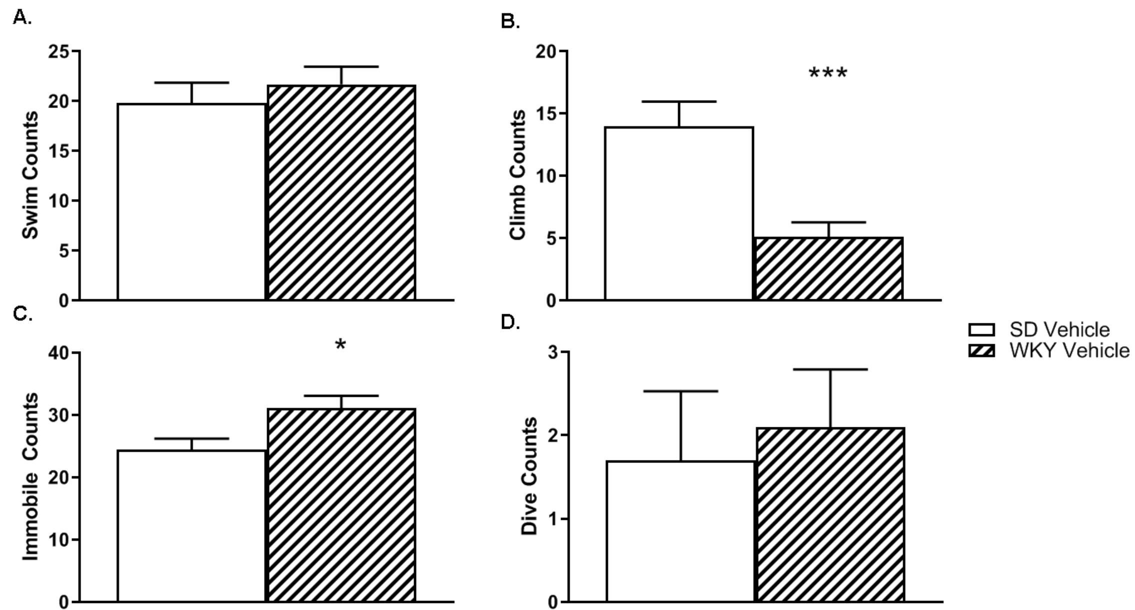

Figure 3.3. Interval behavioural counts for Sprague Dawley and Wistar Kyoto vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Swim counts for SD and WKY vehicle groups. **[B]** Climb counts for SD and WKY vehicles groups. ***p<0.001 vs SD Vehicle **[C]** Immobile counts for SD and WKY vehicle groups. *p<0.05 vs SD Vehicle **[D]** Dive counts for SD and WKY vehicle groups.

Tallying behaviours at 5 sec intervals confirmed that WKY vehicle display significantly higher immobility than SD vehicle (p<0.05, Figure 3.3C). The WKY vehicle group also climbed significantly less than SD vehicle (p<0.001, Figure 3.3B). No differences were found in the number of swim counts or in dive counts between SD and WKY vehicle groups. The WKY's increase in immobility compared to SD appears to be primarily due to a difference in climbing behaviour.

5.1.3. Effects of Acute Escitalopram and Desipramine Treatment on Total Immobility in Sprague Dawley and Wistar Kyoto Rats in the Forced Swim Test

Total immobility was calculated for each five-minute session and is represented here in total seconds (sec) spent immobile for SD and WKY undergoing acute DMI and ESC treatment compared to corresponding vehicle groups.

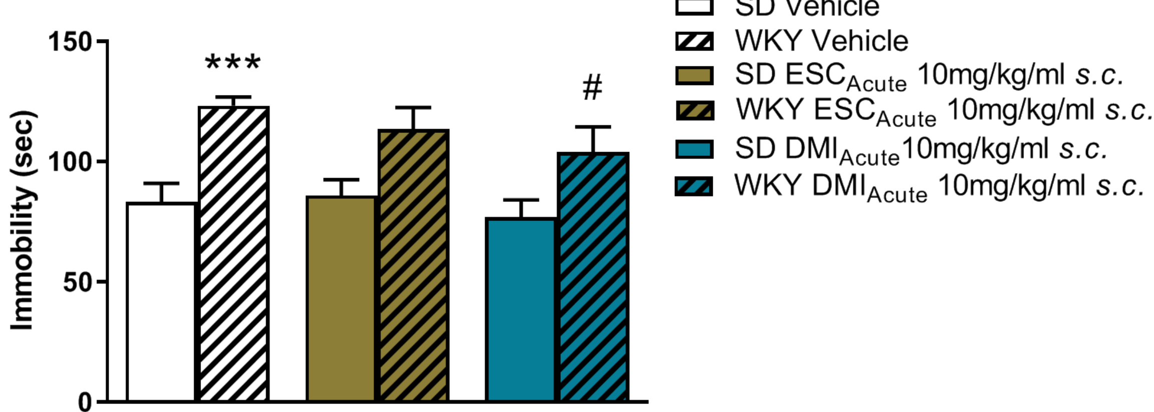

Figure 3.4. Comparison of acute treatment of escitalopram and desipramine to vehicle groups. n=8-10/group. ***p<0.001 vs SD vehicle, #p<0.05 vs WKY vehicle. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

Acute treatments of ESC showed no effect on immobility in either SD or WKY compared to their respective vehicle groups. Acute DMI treated WKYs showed significantly reduced immobility ($p<0.05$) compared to WKY vehicle (Figure 3.4). Acute DMI treatment showed no effect in SD compared to SD vehicle.

5.1.4. Effects of Acute Escitalopram and Desipramine Treatment on Individual Behaviours in Sprague Dawley and Wistar Kyoto Rats in the Forced Swim Test

Individual behaviours (swimming, climbing, immobile, and diving) are represented as counts taken at 5 second intervals over the five-minute test session. There is a total of 60 counts for each test session. The following shows SD and WKY undergoing acute DMI and ESC treatment compared to corresponding vehicle groups:

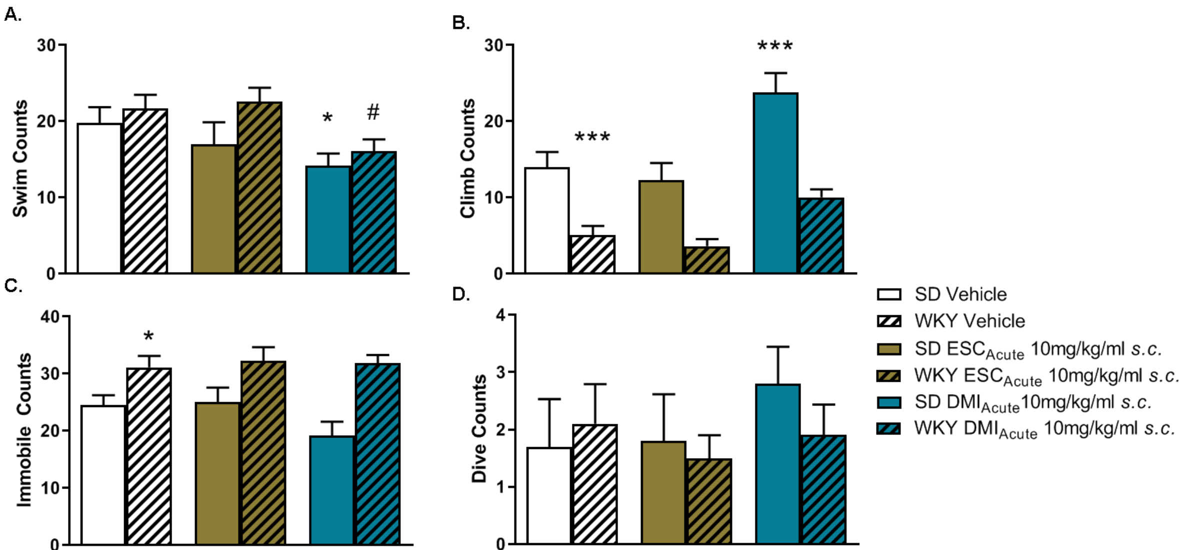

Figure 3.5. Interval behavioural counts of acute treatments of escitalopram and desipramine compared to vehicle groups. n=8-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Swim counts for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD vehicle, #p<0.05 vs WKY vehicle **[B]** Climb counts for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle **[C]** Immobile counts for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD Vehicle **[D]** Dive counts for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group.

Acute DMI treated SDs showed significantly increased climbing behaviour compared to SD vehicle (p<0.05, Figure 3.5B) although there was no accompanying significant decrease in immobility, rather a significant decrease can be observed in swimming behaviour (p<0.05) (Figure 3.5A). This is consistent with acute DMI's lack of effect on total immobility in the prior section (Figure 3.4). Acute DMI treated WKYs showed significantly reduced swimming counts (p<0.05, Figure 3.5A) compared to WKY vehicle while a trend to increase in climbing behaviour compared to WKY vehicle can be seen

(p=0.0561, Figure 3.5B). Acute ESC treated animals showed no effect for any individual behaviour in either SD or WKY compared to their respective vehicle.

5.1.5. Effects of Chronic Escitalopram and Desipramine Treatment on Total Immobility in Sprague Dawley and Wistar Kyoto Rats in the Forced Swim Test

Total immobility was calculated for each five-minute session and is represented here in total seconds (sec) spent immobile for SD and WKY undergoing chronic DMI and ESC treatment compared to corresponding vehicle groups.

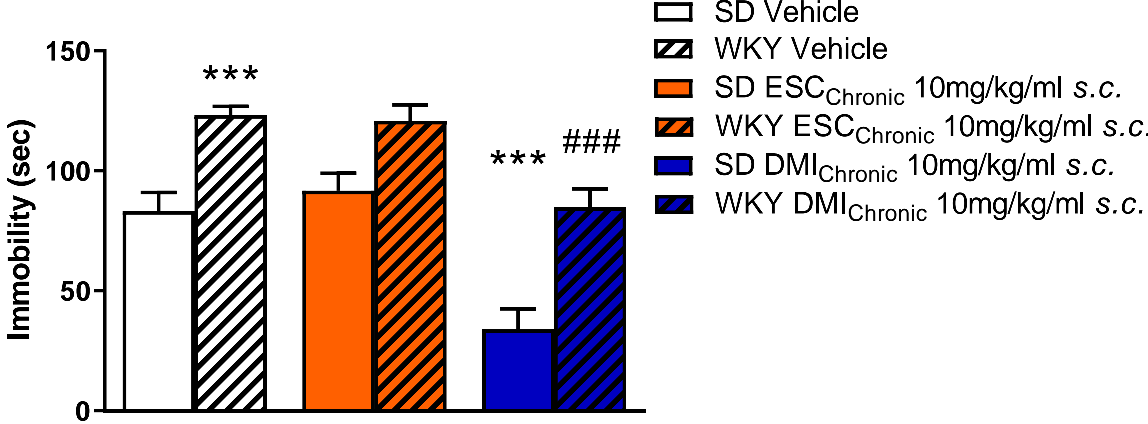

Figure 3.6. Comparison of chronic treatment of escitalopram or desipramine to vehicle groups. n=10/group. ***p<0.001 vs SD vehicle, ###p<0.001 vs WKY vehicle. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

Chronic ESC treatment showed no effect on WKYs while chronic DMI treated WKYs showed reduced total immobility (p<0.001) compared to WKY vehicle. Chronic DMI treated SDs also showed significantly reduced immobility compared to SD vehicle (p<0.001) while chronic ESC treatment did not (Figure 3.6). Chronic DMI treated animals showed the greatest treatment response immobility in both SD and WKY compared to their respective vehicle groups. Interestingly, chronic ESC showed no effect in reducing immobility in SD as might be expected. This is most likely due to the lack of a pre-test session inducing a state of despair to be recovered by antidepressant treatment.

5.1.6. Effects of Chronic Escitalopram and Desipramine Treatment on Individuals Behaviours in Sprague Dawley and Wistar Kyoto Rats in the Forced Swim Test

Individual behaviours (swimming, climbing, immobile, and diving) are represented as counts taken at 5 second intervals over the five-minute test session. There is a total of 60 counts for each test session. The following shows SD and WKY undergoing chronic DMI and ESC treatment compared to corresponding vehicle groups:

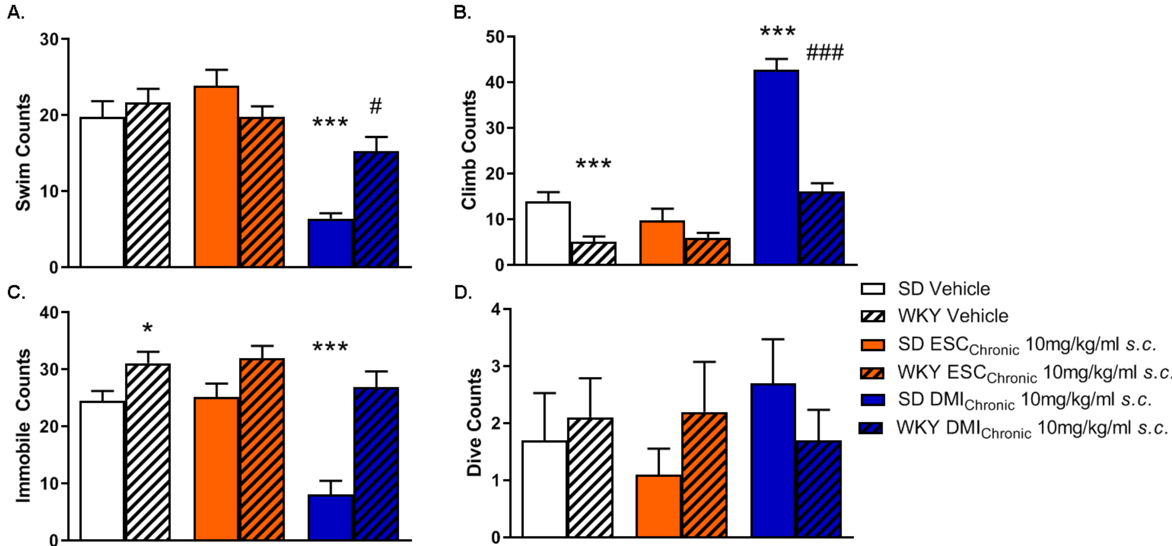

Figure 3.7. Interval behavioural counts of chronic treatment of escitalopram and desipramine compared to vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Swim counts for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle, #p<0.05 vs WKY vehicle **[B]** Climb counts for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD Vehicle, ###p<0.001 vs WKY vehicle **[C]** Immobile counts for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD vehicle, ***p<0.001 vs SD vehicle **[D]** Dive counts for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group.

Chronic DMI treated animals showed significantly increased climbing behaviour in both SD and WKY compared to their respective vehicle groups (p<0.001, Figure 3.7B). The increase in climbing behaviour in chronic DMI treated animals was accompanied by a significant decrease in swimming behaviour in both SD (p<0.001) and WKY (p<0.05, Figure 3.7A) and significant reduction in immobility in SD compared to SD vehicle (p<0.001, Figure 3.7C). Interestingly, a significant reduction in immobility counts was not found in WKY receiving chronic DMI even though a significant reduction was found in total immobility for the same group (Figure 3.6). There were no significant effects of chronic ESC in either SD or WKY for any behaviour compared to vehicle groups. No

significance difference was found in diving behaviour for chronic ESC or DMI in ether SD or WKY compared to vehicle groups.

5.1.7. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on Total Immobility in Sprague Dawley and Wistar Kyoto Rats in the Forced Swim Test

Total immobility was calculated for each five-minute session and is represented here in total seconds (sec) spent immobile for SD and WKY undergoing acute PME and Ketamine treatment compared to corresponding vehicle groups.

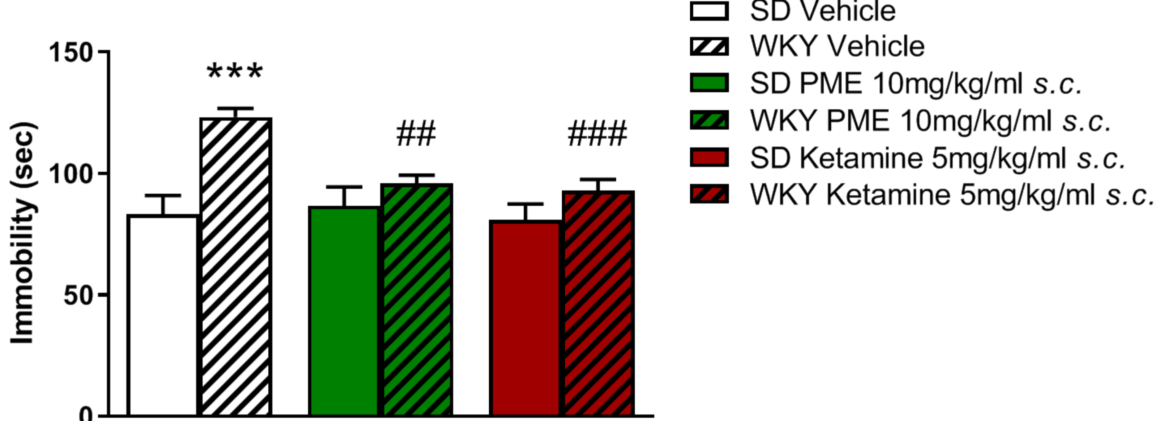

Figure 3.8. Comparison of acute treatment of novel antidepressants pregnenolone-methyl-ether and ketamine to vehicle groups. n=10/group. ***$p<0.001$ vs SD vehicle, ##$p<0.01$ vs WKY vehicle, ###$p<0.001$ vs WKY vehicle. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

Both PME ($p<0.01$) and ketamine ($p<0.001$) treated WKYs showed a significant decrease in total immobility compared to WKY vehicle (Figure 3.8). No effect for ketamine or PME treatment was found in the SDs.

5.1.8. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on Individual Behaviours in Sprague Dawley and Wistar Kyoto Rats in the Forced Swim Test

Individual behaviours (swimming, climbing, immobile, and diving) are represented as counts taken at 5 second intervals over the five-minute test session. There is a total of 60 counts for each test session. The following shows SD and WKY undergoing acute PME and Ketamine treatment compared to corresponding vehicle groups:

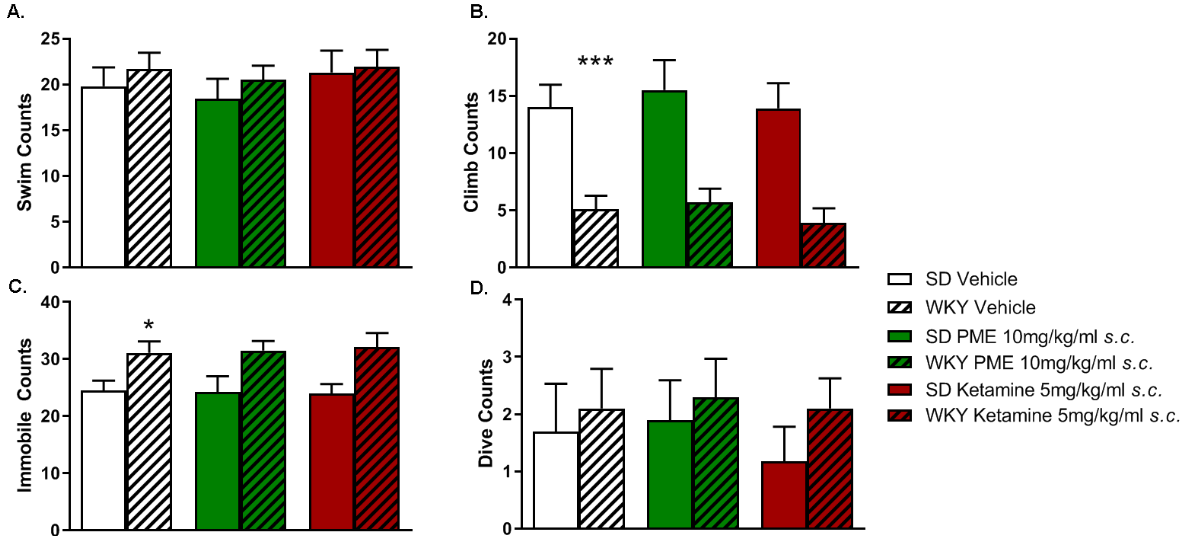

Figure 3.9. Interval behavioural counts of novel treatments of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=10/group. *p<0.05 vs SD vehicle, ***p<0.001 vs SD vehicle. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Swim counts for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[B]** Climb counts for SD and WKY groups treated a cutely with PME or ketamine compared to corresponding vehicle group. ***p<0.001 vs SD vehicle **[C]** Immobile counts for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group.*p<0.05 vs SD vehicle **[D]** Dive counts for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group.

Novel treatments did not show any significant effect on individual behaviours for either strain (Figure 3.9). This was unexpected as both PME and ketamine reduced total immobility in WKY as shown in the previous section and highlights the benefits and disadvantages of both scoring methods.

5.2. Plasma Corticosterone Concentration

Plasma CORT concentration determined by ELISA is represented here in ng/ml. Plasma was isolated from trunk blood immediately following the FST.

Two-way ANOVA for CORT in the main analysis found no main effect of strain ($F_{(1, 119)}$=3.5, p=0.0640), treatment ($F_{(6, 119)}$ =1.55, p=0.1686), or interaction between strain and treatment ($F_{(6, 119)}$ =1.33, p=0.2498). Only planned *a priori* comparisons are represented below.

Two-way ANOVA for CORT in naïve animals compared to vehicle animals found no main effect of strain ($F_{(1, 24)}$=1.10, p=0.3050). However, a main effect of treatment (naïve vs treated) ($F_{(1, 24)}$ =168.78, p<0.001) and interaction between strain and treatment ($F_{(1, 24)}$ =4.35, p=0.0477) was found. Only planned *a priori* comparisons are represented below.

5.2.1. Plasma Corticosterone Concentrations in Sprague Dawley and Wistar Kyoto Vehicle Groups Following Forced Swim Test Exposure and Naïve Animals

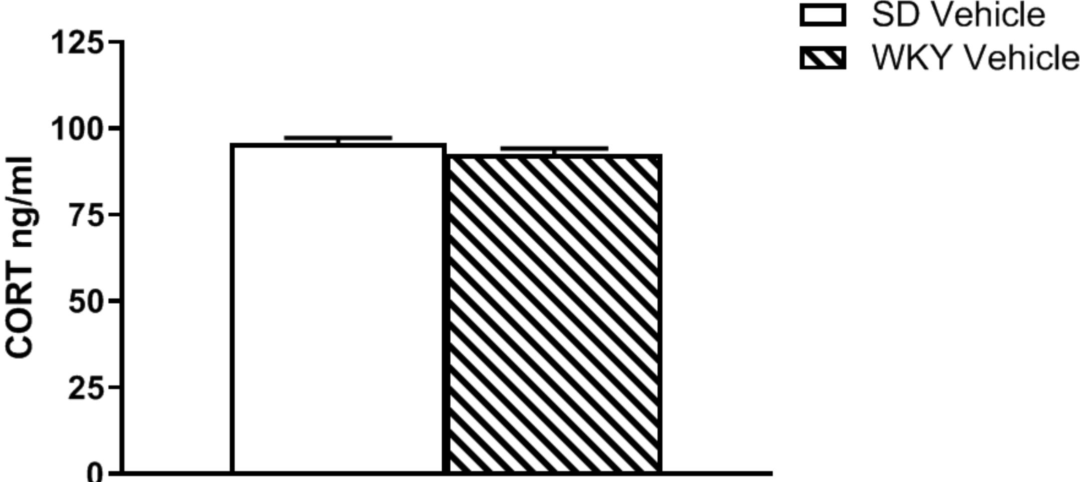

Figure 3.10. Comparison of corticosterone concentrations in Sprague Dawley and Wistar Kyoto vehicle groups. n=10/group. Two-way ANOVA with blocking factor and planned comparisons.

SD and WKY vehicle groups had the same level of plasma CORT (Figure 3.10).

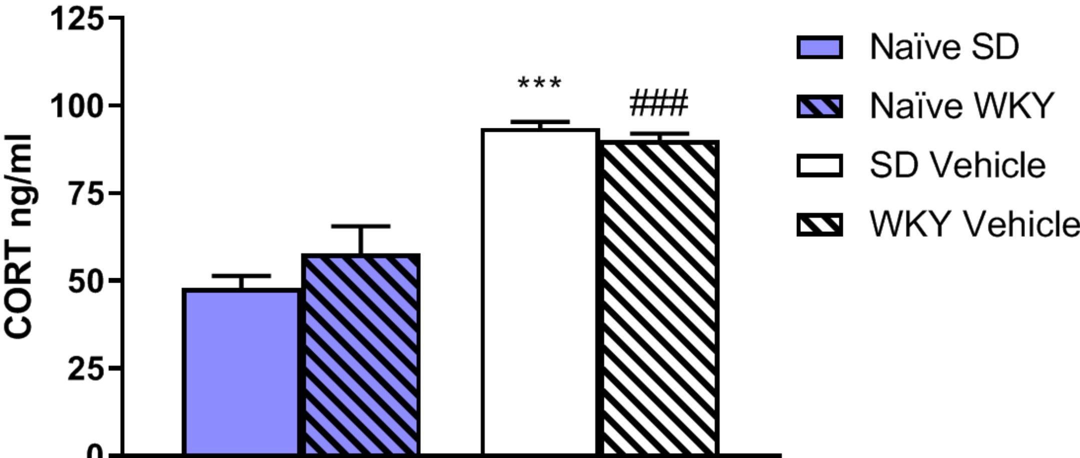

Figure 3.11. Comparison of corticosterone concentrations in study Sprague Dawley and Wistar Kyoto vehicle groups animals and Naïve Sprague Dawley and Wistar Kyoto animals. Naïve animals n=4/group, study vehicle animals n=10/group. ***p<0.001 vs Naïve SD, ###p<0.001 vs Naïve WKY. Two-way ANOVA with planned comparisons. Data: Observed Mean ± SEM.

Plasma CORT was significantly higher in SD and WKY vehicle groups compared to naïve animals of the same strain (p<0.001, Figure 3.11). SD and WKY vehicle groups were exposed to FST and injection stress while the naïve animals were not.

5.2.2. Effects of Acute Escitalopram and Desipramine Treatment on Plasma Corticosterone from Sprague Dawley and Wistar Kyoto Rats Following Forced Swim Test

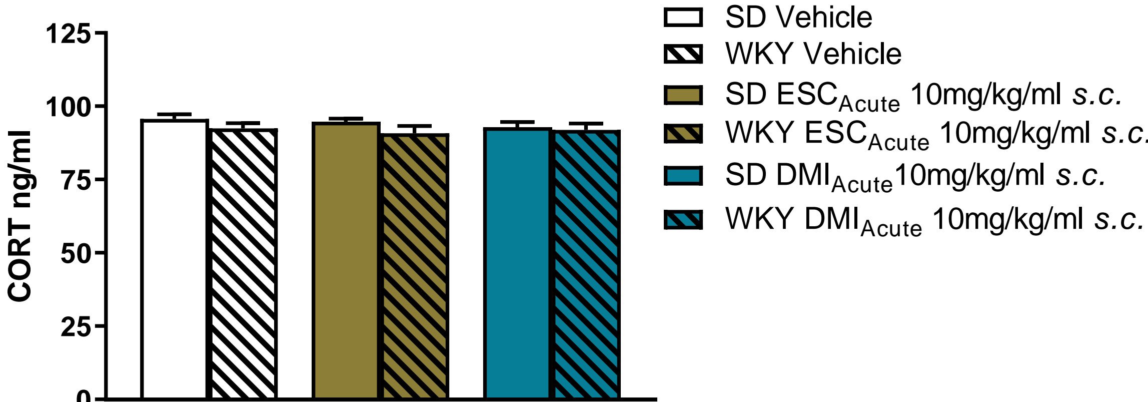

Figure 3.12. Comparison of acute treatment of escitalopram or desipramine on plasma corticosterone concentrations compared to vehicle groups. n=9-10/group. Two-way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

No significant effects of acute DMI or ESC treatment were found on plasma CORT concentrations in SD or WKY (Figure 3.12).

5.2.3. Effects of Chronic Escitalopram and Desipramine Treatment on Plasma Corticosterone from Sprague Dawley and Wistar Kyoto Rats Following Forced Swim Test

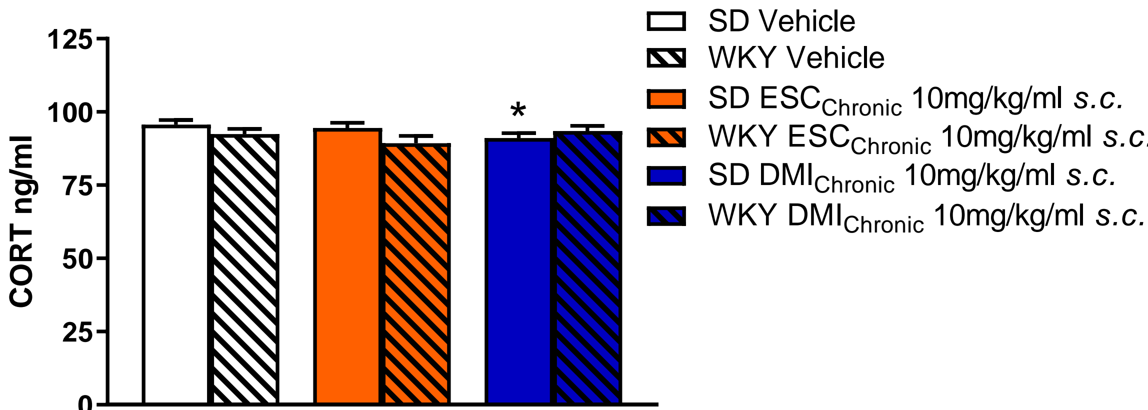

Figure 3.13. Comparison of chronic treatment of escitalopram or desipramine on plasma corticosterone concentrations compared to vehicle groups. n=8-10/group. *p<0.05 vs SD vehicle. Two-way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

Chronic DMI treated SDs showed significantly reduced plasma CORT concentrations compared to SD vehicle ($p<0.05$, Figure 3.13) but no change was found in Chronic DMI treated WKYs. The significant reduction plasma CORT seen in chronic DMI SD group is in line with that group having the lowest immobility time in the FST (Figure 3.6). Chronic ESC treatment showed no effect on plasma CORT in either SD or WKY.

5.2.4. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on Plasma Corticosterone from Sprague Dawley and Wistar Kyoto Rats Following Forced Swim Test

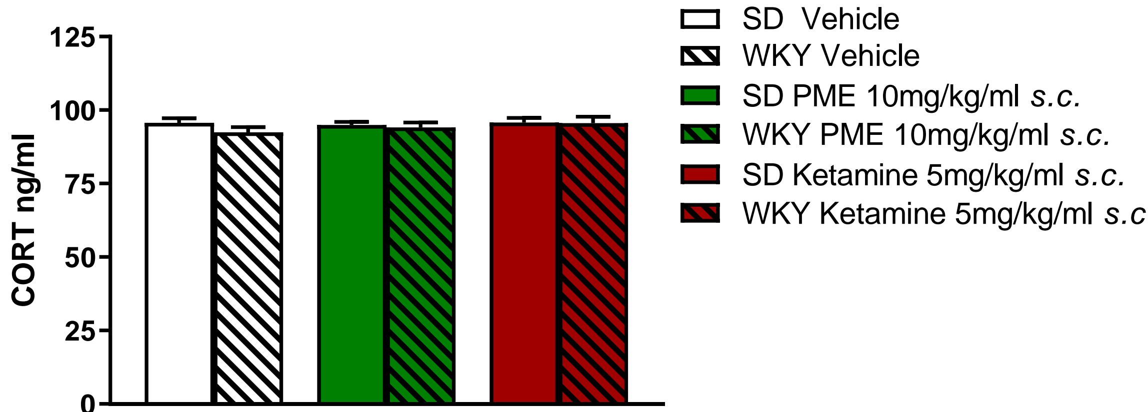

Figure 3.14. Comparison of acute treatment of novel antidepressants pregnenolone-methyl-ether and ketamine on plasma corticosterone concentrations compared to vehicle groups. n=10/group. Two-way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

No significant effect of PME or ketamine was found on plasma CORT concentrations in SD or WKY (Figure 3.14).

6. Discussion

6.1. Modified Forced Swim Test and Scoring Methods

Consistent with the literature, the WKY vehicle group was significantly more immobile than SD[162,217,223] (Figure 3.2). This is a critical point for this study and confirms that a pre-test is not necessary to induce a state of "despair" when using WKYs in reference to a control strain. The description of "endogenously depressed" appears to be valid descriptor for the WKYs in this case[142]. The WKY vehicle group also climbed significantly less than SD vehicle (Figure 3.3B), which has been reported previously[146].

Although the modified FST was intended to increase the sensitivity of the assay to SSRIs[168,197], this did not happen here, probably for two reasons. In the first case, WKYs have been repeatedly shown to be resistant to SSRI treatment[217,225,228]. This is thought to be the result of a dysregulation of the serotonergic system in WKYs where stress leads to an increase in 5-HT_{1A} receptor binding in the hippocampus and hypothalamus and decreased 5-HT transporter density in the cortex and hippocampus[159]. Other studies have suggested that WKYs also exhibit basal differences in the expression of monoamine transporters[226,227]. Second, the inclusion of SD in this study as a "healthy control" is somewhat unusual for an FST study. Without a pre-test session or repeated stressful event to induce a state of despair, it makes sense that the SDs would not respond to ESC treatment as there was no depressive state to recover.

The notable exception in SDs is the significant effect of chronic DMI treatment (Figure 3.6), which highlights the distinct pharmacology of TCAs compared to SSRIs and may suggest that DMI's effect on behaviour in the FST is not strictly antidepressant in nature. This difference in the pattern of behaviour evoked by SSRI treatment versus TCA treatment in the FST has been described previously with DMI causing an increase in climbing behaviour brought on by increasing the availability of noradrenaline[229].

The lack of response in the SDs to all other treatments suggests that the dosing regimen was not in itself stressful enough to influence the behavioural endpoints of the FST. Had the dosing caused significant stress and induced a depressed state we might have expected the SD vehicle group to be much closer to the WKY vehicle for immobility. The similarity in CORT levels between SD and WKY in both the naïve animals and in the test animals following the FST suggests that there are inherent behavioural differences between the two strains not directly influenced by CORT. López-Rubalcava and Lucki in a similar study showed that a 15 min pre-test session resulted in SD rats spending a little more than half the test session immobile while the WKYs spent nearly 95% of the test session immobile[146]. In this study, SDs were immobile roughly a third of the test session

and WKY rats were immobile a little over half of the time of the test session. The immobility measure in this design can therefore be interpreted as a purely passive coping mechanism that is exacerbated by a "depressed state" in the WKYs.

While the interval scoring method and total immobility measure matched in showing the same general trends, there were some notable differences in outcome, particularly for significant changes in immobility counts versus total immobility for some treatments. For example, Figure 3.6 shows a significant reduction in immobility in WKY's receiving chronic DMI although this reduction is not reflected in immobile counts seen in Figure 3.7C. This discrepancy highlights the limitations of both methods. The interval scoring method lacks the temporal resolution to tease out subtle or quick changes in behaviour and the total immobility time leaves out other crucial behaviour for analysis. Including both can help tease apart subtle differences between the profiles of the agents that may reflect mechanistic differences for future investigations.

6.2. Acute Desipramine and Escitalopram Treatments

Acute ESC treatment showed no efficacy in SD or WKY groups for any behaviour or on CORT expression. This was to be expected as previous studies have demonstrated that single antidepressant doses are less robust than chronic dosing in reducing immobility in the FST[205,206] and again confirms WKY resistance to SSRI retreatment[146,225]. It should be noted that the SSRI used in previous studies was typically fluoxetine or paroxetine and not ESC. To the best of my knowledge, this is the first study to show that WKYs are resistant to acute ESC treatment in the FST. Although this has almost certainly been tested by other research groups.

Acute DMI treatment significantly reduced immobility in WKYs (Figure 3.4) but not in SD, although Acute DMI significantly increased climbing behaviour in SD and not WKY (Figure 3.5B). These results may again reflect limitations in the scoring methods but also confirm that the behavioural effects of DMI are more closely regulated by noradrenergic signalling than serotonergic while also highlighting the difference in neurochemistry between the two strains.

6.3. Chronic Desipramine and Escitalopram Treatments

Consistent with the literature, WKY rats are resistant to chronic ESC treatment but not chronic DMI treatment in reducing immobility in the FST[146] (Figure 3.6). Again, to the best of my knowledge, this is the first study to show that WKYs are resistant to chronic ESC treatment in the FST, although others have certainly run similar investigations.

Chronic DMI showed significantly reduced immobility in SD while chronic ESC did not. As mentioned above, this notable exception of chronic DMI treatment significantly reducing immobility in SD only highlights the underlying pharmacology of DMI acting through inhibition of noradrenaline reuptake, which has been shown to directly impact climbing behavior[230].This is reflected in Figure 3.7B.

Chronic DMI greatly increased climbing behaviour in both SD and WKY but not to the same extent (Figure 3.7B). This is further evidence for an underlying dysregulated monoaminergic system in WKY compared to SD[226,227].

6.4. Novel Treatments

Ketamine and PME both showed significantly reduced immobility in the WKY and had similar behavioural effects (Figure 3.8 and Figure 3.9A-D).

This may be somewhat surprising given their starkly different pharmacology, although they are both thought to have their ultimate antidepressant action through recovering synaptic plasticity, however, how quickly this happens after receiving treatment is unclear[26,231]. Depressed patients receiving ketamine by *intra venous* infusion have reported improved symptoms after 2 hours[232] and ketamine has been shown to be efficacious in the FST following a single dose 30 minutes prior to the test session[166]. Unfortunately, no such data exists for PME. The only preclinical data available for PME in an FST study used a sub-chronic dosing regimen[26] and the clinical trials from the 1950s do not specify time points[79,80]. In any event, there appears to be a discrepancy between behavioural effects and molecular effects of antidepressant treatment that will be discussed further in Chapter 4 with the α-Tubulin data.

Ketamine has been described not only as a rapid but also as a long-lasting antidepressant treatment[73] with its sustained efficacy in the FST being attributed to 5-HT dependent mechanisms[150]. The 5-HT_2 receptor has been suggested as a ketamine target[233]. The contrast in robust behavioural changes seen for ketamine (Figure 3.8) and lack of behavioural effects seen for ESC (Figures 3.6 and 3.7) is most likely explained by ketamine acting rapidly though NMDA receptors and rather than any immediate effect on the serotonergic system. ESC's primary mechanism of action is on the 5-HT transporter, having the highest affinity for the 5-HT transporter of all the SSRIs although it also binds the 5-HT_2 receptor[69,70].

The available literature on PME is quite limited in this regard. All that can be said from this data is that it appears to have a similar effect as ketamine in this FST study although the mechanism is known to be quite different. Neither PME nor ketamine had a

significant effect on behaviour in the SDs. This may again be due to the lack of a pre-test session inducing a state of despair in the SD groups, which lends to the confirmation of the antidepressant action of PME and ketamine in the WKY group. PME has been shown to selectively bind MAP-2[26] while ketamine is a NMDA antagonist[78], creating two possible avenues to develop novel antidepressant treatments.

6.5. Plasma Corticosterone Concentration

Plasma CORT analysis did not find any significance difference between the SD and WKY vehicles, nor between naïve SDs and WKYs, suggesting that the stress of dosing and FST did not adversely affect WKY any more than SD, as previously thought. This finding suggests that CORT did not impact behaviour in one strain more than the other.

The significant reduction in plasma CORT seen in the SD chronic DMI group compared to vehicle may reflect this group also showing the lowest total immobility score of all the groups (Figure 3.13). However given the near identical concentrations of CORT across all other groups, CORT does not appear to influence behaviour in the FST as previously argued[214]. Even so, given the HPA axis dysregulation of the WKYs[208–210] it was unexpected that they would have the same CORT concentrations as the SD following the FST. It may have been the case that basal CORT concentrations was indeed different between the two strains following the chronic dosing regimen. Unfortunately, without a sampling immediately prior to the FST, this will have to remain speculative.

7. Conclusion

Overall, the behavioural endpoints in this study reflect what has been reported previously for similarly designed studies and can be taken as a positive result for the robustness of the FST design.

Although the individual elements included in this design are not inherently novel, they have not been employed in this manner previously to the best of my knowledge. For example, the treatments used in this study have been extensively explored elsewhere in the FST and other paradigms but not simultaneously in SDs and WKYs as in this case. And as noted above, including SDs and WKYs without a pre-test is not typical for this type of study.

The experimental design employed here was successful in comparing the effects of acute and chronic antidepressant treatments in two rat strains and was crucial for setting up the following chapter where the tissue collected from these animals is analysed for the expression of α-Tubs.

Chapter 4: The effects of antidepressant treatment on α-tubulins in blood plasma and brain tissue and effects on hippocampal Brain Derived Neurotrophic Factor

1. Introduction

The study design implemented here is initially the same as in Chapter 3 with respect to the test animals and treatment groups, however the transition to proteomic analysis within the tissue samples collected from those animals introduces additional design considerations that will be discussed more below.

All four of the treatment compounds used here have been characterised for their structure, reactivity, and binding profiles in relation to Major Depressive Disorder (MDD)[26,234]. The primary focus of the study is answering the question of whether these known antidepressant compounds affect a specific group of microtubule (MT) proteins: α-tubulin (α-Tub) and its posttranslational modifications (PTMs).

1.1. α-Tubulins and Major Depressive Disorder

Proteomic investigations into MDD have found numerous protein families that have significantly different expression in those with MDD compared to healthy controls. Most importantly for this project, tubulins, among other protein families critical to cytoskeletal function, have been identified as having significantly different expression in individuals with MDD compared to healthy controls in post-mortem studies[235]. Likewise, preclinical work has pointed to α-Tub PTMs as having a role in MDD underlying pathology[236,237] and having the potential to be targets for treatment[25,26].

1.2. Brain Derived Neurotrophic Factor and Synaptic Plasticity

Analysis of hippocampal brain-derived neurotrophic factor (BDNF) was included in this study as an additional measure for antidepressant efficacy[37–39]. Pathways mediated by BDNF are crucial for synaptogenesis and neuronal plasticity[34]. For example, hippocampal neurons treated with BDNF have shown increased post synaptic density protein 95 and MT polymerization in dendritic spines[238]. Consequently, BDNF has been called a “key transducer” for its intermediary role in antidepressant action that ultimately restores synaptic plasticity[239]. Selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) both have been shown to increase BNDF and TrkB mRNA expression in the hippocampus after their initial action of increasing extracellular monoamines. The time lag between the rapid increase in monoamine availability and treatment response is thought to be linked to BDNF transcription[240]. Ketamine’s antidepressant action and capability to rapidly increase dendritic spine growth has been strongly linked to BDNF modulation[241]. In theory, the changes in α-Tub expression would be mediated, at least in part, downstream of BDNF pathways.

2. Aims

This study aims to answer two questions: Does pharmacological treatment influence the expression of α-Tub and its PTMs and does α-Tub expression indicate a depressed state in a preclinical model of MDD. Blood plasma, hippocampi, and prefrontal cortex samples were collected from the same animals described in Chapter 3. The expression of Total-α-Tubulin (DM1A) (Tot-α-Tub (DM1A)), Acetylated-α-Tubulin (Acet-Tub), Tyrosinated-α-Tubulin (Tyr-Tub), Detyrosinated-α-Tubulin (Glu-Tub), and Deglutamylated-α-Tubulin (Δ2-Tub) was assessed in these tissue samples using Western blotting. Total BDNF expression was measured in hippocampal tissue by ELISA.

3. Ethical Permissions

Ethical approval for this study was obtained from the Animal Research Ethics Committee (AREC) of Trinity College Dublin. For ethical permissions see Appendix A.1.

4. Methods

WB and BDNF ELISA were performed as previously described in Chapter 2 Sections 2 and 3.2, respectively.

4.1. Housekeeper Proteins

A great number of WB studies utilise "housekeeper" proteins that are used to establish consistent sample loading and normalise target protein data. As a ubiquitous protein that is otherwise considered to have homogenous expression, α-Tub has been widely used as a housekeeper itself. However, there is considerable debate concerning the need or even validity of housekeeper proteins in disease investigations[242–244]. In fact, there is growing literature on the replacement of the traditional housekeeping proteins for total protein stains[245]. This option is still complicated by several factors such as specific WB techniques that may not be compatible with this type of staining and limitations imposed by disease area[246]. For the purposes of this project the use of a housekeeper protein is problematic due to targets of interest, the nature of the disease, and because antidepressants are metabolised by the liver. Consequently, these factors can all affect the expression of several proteins that circulate in the blood that are also commonly used as housekeeper proteins, such as transferrin and albumin. In the brain, changes in neurogenesis and structural plasticity affect other proteins commonly used as housekeepers such as GAPDH, β-tubulin, and β-actin[247]. To accommodate this, studies were planned to analyse individual targets appropriately, normalise on total α-Tub expression to assess changes in posttranslational modification ratios, or where possible

as a ratio of enzymatic modification indicative of disease changes. Transferrin was included in the analysis of rodent blood plasma samples to exemplify this point.

4.2. Statistical Analysis

Due to the limited number of wells in the WB gels, a nested block design was used to run all of the samples (Figure 4.1)[180]. This design resulted in six blocks of samples that were then run in duplicate and recombined into a full data set for each target.

To reiterate Chapter 2, analyses were carried out in InVivo Stat 3.7.0.0. Graphpad Prism 8 was used to make all plots.

Rodent molecular data was Log_{10} transformed for statistical analysis but observed means and standard error are represented in all plots. This layout was chosen to best avoid confusion from the effects of blocking factors and non-intuitive log transformed data.

Single measure two-way analysis of variance (ANOVA) with blocking factor followed by unadjusted planned comparisons[180] was employed for each molecular endpoint. The risk of false positives is higher when using unadjusted planned comparisons, however this was determined to be the best option for analysis to detect if any relevant biological changes may be occurring. This is especially important to note when considering this data set as observed means are not adjusted for unequal replicates across blocks and the statistical analysis may contradict the observed means plots.

All rats tolerated the dosing regimen and were included in the behavioural and *ex vivo* analyses (Chapters 3 and 4). Predicted values with a residual more or less than three standard deviations from the mean were considered statistical outliers and removed from the data set.

4.5.1. Data and Statistical Analysis for Enzyme-Linked Immunosorbent Assay

Initial analysis of BDNF ELISAs was carried out using Microsoft Excel for wavelength correction. Formal statistical analysis was carried out in InVivo Stat 3.7.0.0 using single measure two-way ANOVA with blocking factor followed by unadjusted planned comparisons. Data was Log_{10} transformed prior to analysis, however observed means and standard error are represented.

Predicted values with a residual more or less than three standard deviations from the mean were considered outliers and removed from the data set.

Sample	Block 1	Block 2	Block 3	Block 4	Block 5	Block 6
1	SD Treatment 1 Replicate 1	SD Treatment 1 Replicate 2	SD Treatment 1 Replicate 3	SD Treatment 1 Replicate 4	SD Treatment 1 Replicate 5	SD Treatment 1 Replicate 7
2	SD Treatment 2 Replicate 1	SD Treatment 2 Replicate 2	SD Treatment 2 Replicate 3	SD Treatment 2 Replicate 4	SD Treatment 2 Replicate 5	SD Treatment 1 Replicate 8
3	SD Treatment 3 Replicate 1	SD Treatment 3 Replicate 2	SD Treatment 3 Replicate 3	SD Treatment 3 Replicate 4	SD Treatment 3 Replicate 5	WKY Treatment 1 Replicate 6
4	SD Treatment 4 Replicate 1	SD Treatment 4 Replicate 2	SD Treatment 4 Replicate 3	SD Treatment 4 Replicate 4	SD Treatment 4 Replicate 5	SD Treatment 2 Replicate 8
5	SD Treatment 5 Replicate 1	SD Treatment 5 Replicate 2	SD Treatment 5 Replicate 3	SD Treatment 5 Replicate 4	SD Treatment 5 Replicate 5	SD Treatment 2 Replicate 9
6	SD Treatment 6 Replicate 1	SD Treatment 6 Replicate 2	SD Treatment 6 Replicate 3	SD Treatment 6 Replicate 4	SD Treatment 6 Replicate 5	WKY Treatment 2 Replicate 6
7	SD Treatment 7 Replicate 1	SD Treatment 7 Replicate 2	SD Treatment 7 Replicate 3	SD Treatment 7 Replicate 4	SD Treatment 7 Replicate 5	WKY Treatment 2 Replicate 7
8	WKY Treatment 1 Replicate 1	WKY Treatment 1 Replicate 2	WKY Treatment 1 Replicate 3	WKY Treatment 1 Replicate 4	WKY Treatment 1 Replicate 5	SD Treatment 3 Replicate 10
9	WKY Treatment 2 Replicate 1	WKY Treatment 2 Replicate 2	WKY Treatment 2 Replicate 3	WKY Treatment 2 Replicate 4	WKY Treatment 2 Replicate 5	SD Treatment 3 Replicate 9
10	WKY Treatment 3 Replicate 1	WKY Treatment 3 Replicate 2	WKY Treatment 3 Replicate 3	WKY Treatment 3 Replicate 4	WKY Treatment 3 Replicate 5	WKY Treatment 3 Replicate 7
11	WKY Treatment 4 Replicate 1	WKY Treatment 4 Replicate 2	WKY Treatment 4 Replicate 3	WKY Treatment 4 Replicate 4	WKY Treatment 4 Replicate 5	WKY Treatment 3 Replicate 8
12	WKY Treatment 5 Replicate 1	WKY Treatment 5 Replicate 2	WKY Treatment 5 Replicate 3	WKY Treatment 5 Replicate 4	WKY Treatment 5 Replicate 5	SD Treatment 4 Replicate 10
13	WKY Treatment 6 Replicate 1	WKY Treatment 6 Replicate 2	WKY Treatment 6 Replicate 3	WKY Treatment 6 Replicate 4	WKY Treatment 6 Replicate 5	WKY Treatment 4 Replicate 8
14	WKY Treatment 7 Replicate 1	WKY Treatment 7 Replicate 2	WKY Treatment 7 Replicate 3	WKY Treatment 7 Replicate 4	WKY Treatment 7 Replicate 5	WKY Treatment 4 Replicate 9
15	SD Treatment 1 Replicate 6	SD Treatment 2 Replicate 7	SD Treatment 3 Replicate 8	SD Treatment 1 Replicate 9	SD Treatment 1 Replicate 10	WKY Treatment 5 Replicate 10
16	SD Treatment 2 Replicate 6	SD Treatment 3 Replicate 7	SD Treatment 4 Replicate 8	SD Treatment 4 Replicate 9	SD Treatment 2 Replicate 10	WKY Treatment 5 Replicate 9
17	SD Treatment 3 Replicate 6	SD Treatment 4 Replicate 7	SD Treatment 5 Replicate 8	SD Treatment 5 Replicate 9	SD Treatment 5 Replicate 10	SD Treatment 6 Replicate 6
18	SD Treatment 4 Replicate 6	SD Treatment 5 Replicate 7	SD Treatment 6 Replicate 8	SD Treatment 6 Replicate 9	SD Treatment 6 Replicate 10	WKY Treatment 6 Replicate 10
19	SD Treatment 5 Replicate 6	SD Treatment 6 Replicate 7	SD Treatment 7 Replicate 8	SD Treatment 7 Replicate 9	SD Treatment 7 Replicate 10	SD Treatment 7 Replicate 6
20	WKY Treatment 3 Replicate 6	WKY Treatment 4 Replicate 7	WKY Treatment 5 Replicate 8	WKY Treatment 6 Replicate 9	WKY Treatment 7 Replicate 10	SD Treatment 7 Replicate 7
21	WKY Treatment 4 Replicate 6	WKY Treatment 5 Replicate 7	WKY Treatment 6 Replicate 8	WKY Treatment 7 Replicate 9	WKY Treatment 1 Replicate 10	
22	WKY Treatment 5 Replicate 6	WKY Treatment 6 Replicate 7	WKY Treatment 7 Replicate 8	WKY Treatment 1 Replicate 9	WKY Treatment 2 Replicate 10	
23	WKY Treatment 6 Replicate 6	WKY Treatment 7 Replicate 7	WKY Treatment 8 Replicate 8	WKY Treatment 2 Replicate 9	WKY Treatment 3 Replicate 10	
24	WKY Treatment 7 Replicate 6	WKY Treatment 8 Replicate 7	WKY Treatment 9 Replicate 8	WKY Treatment 3 Replicate 9	WKY Treatment 4 Replicate 10	

Figure 4.1. Nested Block Design for Western Blotting Analysis.

5. Results

5.1. Expression of α-Tubulins, Transferrin, and Brain Derived Neurotrophic Factor in Sprague Dawley and Wistar Kyoto Rats

For clarity in writing and due to the number of analyses conducted, not all ANOVA table information is presented below. Full ANOVA table results for each analysis can be found in Appendix B.2. However, to briefly summarise significant findings:

A significant effect of strain (SD vs WKY) was found for all targets and ratios of expression in blood plasma however no significant effect of treatment or strain*treatment was found (Appendix B.2.1).

In the hippocampus, a significant effect of strain was found for Tyr-Tub to Glu-Tub ratio ($F_{(1, 116)}$=0.33.20, $p<0.001$) and Glu-Tub ($F_{(1, 119)}$=13.75, $p<0.001$). A significant effect of treatment was found for Glu-Tub ($F_{(1, 119)}$=2.28, $p<0.05$) and Acet-Tub ($F_{(1, 119)}$=2.49, $p<0.05$). No significant effects of strain*treatment for found for any target or ratio of expression (Appendix B.2.2).

In the prefrontal cortex, a significant effect of strain was found for the following targets and ratios of expression: Tyr-Tub to Glu-Tub ratio ($F_{(1, 119)}$=10.91, $p=0.0013$), Glu-Tub ($F_{(1, 120)}$=19.08, $p<0.001$), Tot-α-Tub (DM1A) ($F_{(1, 120)}$=5.38, $p<0.05$), and Acet-Tub ($F_{(1, 120)}$=3.34, $p<0.05$). No main effect of treatment or strain*treatment was found (Appendix B.2.3).

5.1.1. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyrosinated-α-Tubulin, and Transferrin in Blood Plasma in Sprague Dawley and Wistar Kyoto Vehicle Groups

Integrated Intensity (Intensity) was calculated for each α-Tub target in blood plasma using the Li-Cor Odyssey System and is represented here for the SD and WKY vehicle groups.

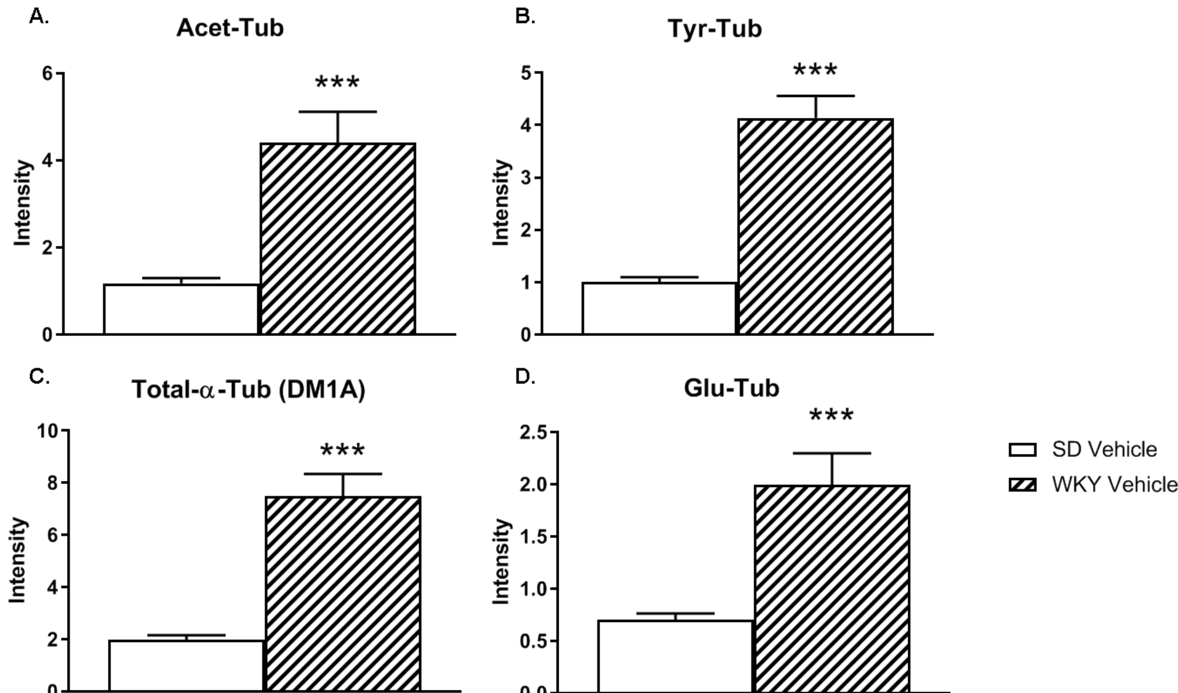

Figure 4.2. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, and Detyrosinated-α-Tubulin in blood plasma for Sprague Dawley and Wistar Kyoto vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in blood plasma for SD and WKY vehicle groups. ***p<0.001 vs SD vehicle. **[B]** Tyr-Tub Intensity in blood plasma for SD and WKY vehicle groups. ***p<0.001 vs SD vehicle. **[C]** Total-α-Tub(DM1A) Intensity in blood plasma for SD and WKY vehicle groups. ***p<0.001 vs SD vehicle. **[D]** Glu-Tub Intensity in blood plasma for SD and WKY vehicle groups. ***p<0.001 vs SD vehicle.

WKY rats significantly overexpress the three α-Tub PTMs that are dateable in blood plasma (Acet-Tub, Tyr-Tub, and Glu-Tub) and Tot-α-Tub (DM1A) compared to SD (p<0.001; Figure 4.2).

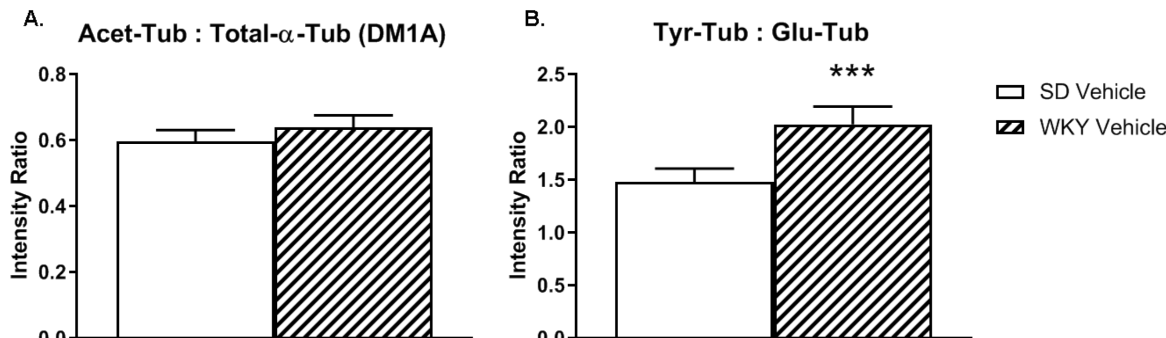

Figure 4.3. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in blood plasma for Sprague Dawley and Wistar Kyoto vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for SD and WKY vehicle groups. **[B]** Tyr-Tub:Glu-Tub Intensity Ratio in blood plasma for SD and WKY vehicle groups. ***p<0.001 vs SD vehicle.

There is no significant difference between WKY and SD for Acet-Tub normalised on Tot-α-Tub (DM1A) (Figure 4.3A. WKY rats have a significantly higher Tyr-Tub to Glu-Tub ratio of expression (p<0.001) compared to SD (Figure 4.3B).

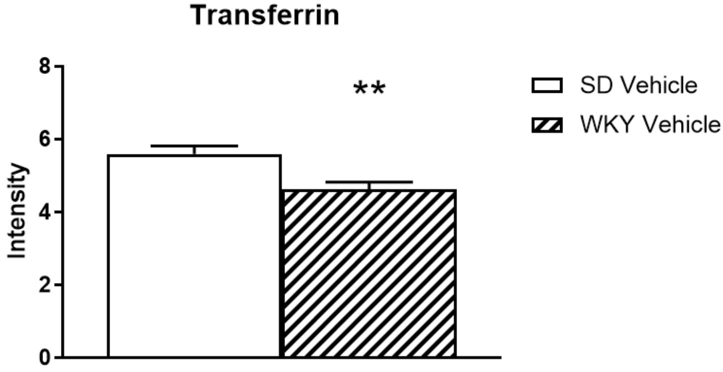

Figure 4.4. Expression of transferrin in blood plasma for Sprague Dawley and Wistar Kyoto vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. Transferrin intensity in blood plasma for SD and WKY vehicle groups. **p<0.01 vs SD vehicle.

WKY rats have significantly lower expression of transferrin (p<0.01) in blood plasma compared to SD (Figure 4.4). Transferrin was measured here as a potential housekeeper protein for normalisation, however due to the significant difference in the vehicle groups it is not included as a housekeeper protein in the following analyses.

5.1.2. Expression of α-Tubulin and its Posttranslational Modifications in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto vehicle Groups

Integrated Intensity (Intensity) was calculated for each α-Tub target in hippocampal tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY vehicle groups.

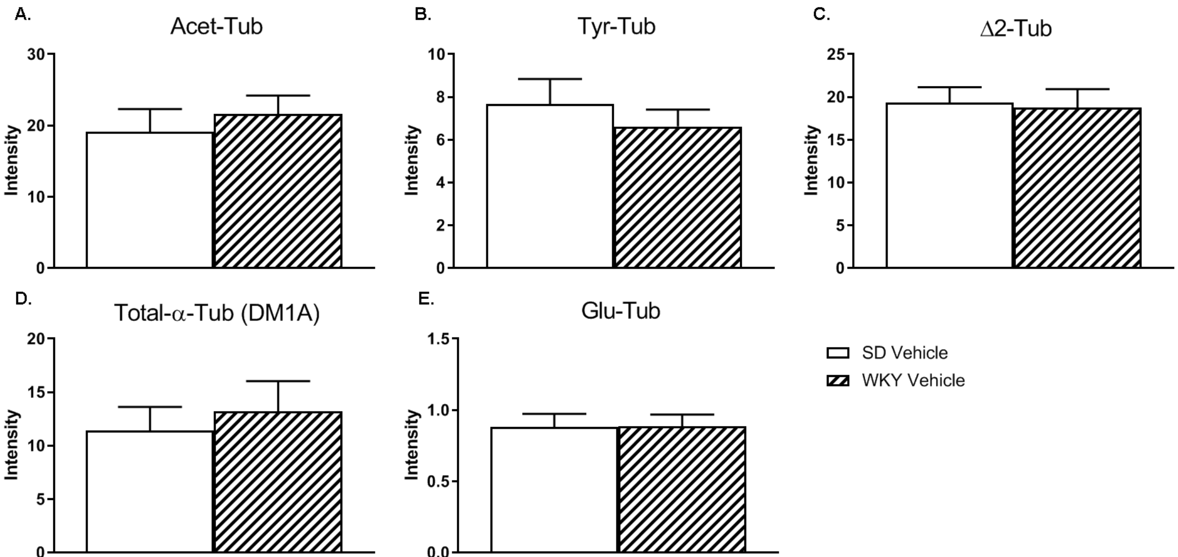

Figure 4.5. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent hippocampus for Sprague Dawley and Wistar Kyoto vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in hippocampus for SD and WKY vehicle groups. **[B]** Tyr-Tub Intensity in hippocampus for SD and WKY vehicle groups. **[C]** Δ2-Tub Intensity in hippocampus for SD and WKY vehicle groups. **[D]** Total-α-Tub(DM1A) Intensity in hippocampus for SD and vehicle groups. **[E]** Glu-Tub Intensity in hippocampus for SD and WKY vehicle groups.

No significant difference was found between SD and WKY for any α-Tub target in the hippocampus (Figure 4.5).

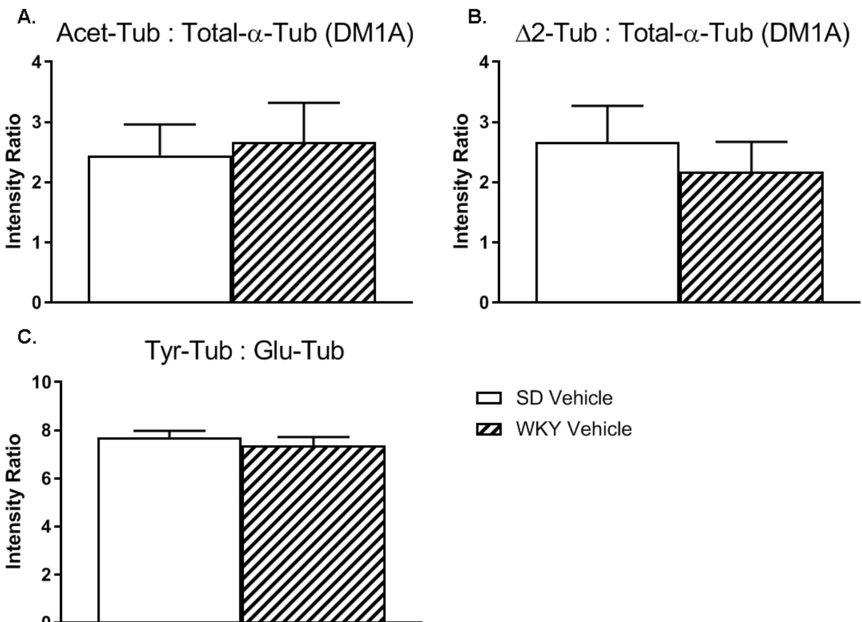

Figure 4.6. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent hippocampus for Sprague Dawley and Wistar Kyoto vehicle groups. n=8-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY vehicle groups. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY vehicle groups. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in hippocampus for SD and WKY vehicle groups.

No significant difference was found between SD and WKY for any α-Tub ratios of expression in the hippocampus (Figure 4.6).

5.1.3. Expression of α-Tubulin and its Posttranslational Modifications in Prefrontal Cortex Tissue in Sprague Dawley and Wistar Kyoto vehicle Groups

Integrated Intensity (Intensity) was calculated for each α-Tub target in hippocampal tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY vehicle groups.

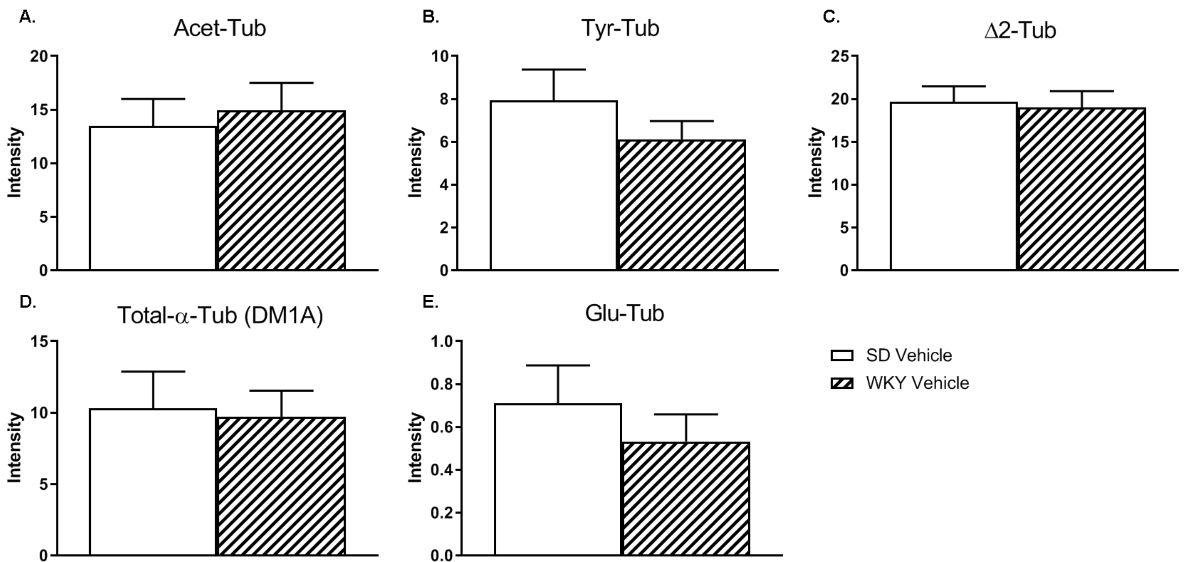

Figure 4.7. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent prefrontal cortex for Sprague Dawley and Wistar Kyoto vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in prefrontal cortex for SD and WKY vehicle groups. **[B]** Tyr-Tub Intensity in prefrontal cortex for SD and WKY vehicle groups. **[C]** Δ2-Tub Intensity in prefrontal cortex for SD and WKY vehicle groups. **[D]** Total-α-Tub(DM1A) Intensity in prefrontal cortex for SD and WKY vehicle groups. **[E]** Glu-Tub Intensity in prefrontal cortex for SD and WKY vehicle groups.

No significant difference was found between SD and WKY for any α-Tub target in the prefrontal cortex (Figure 4.7).

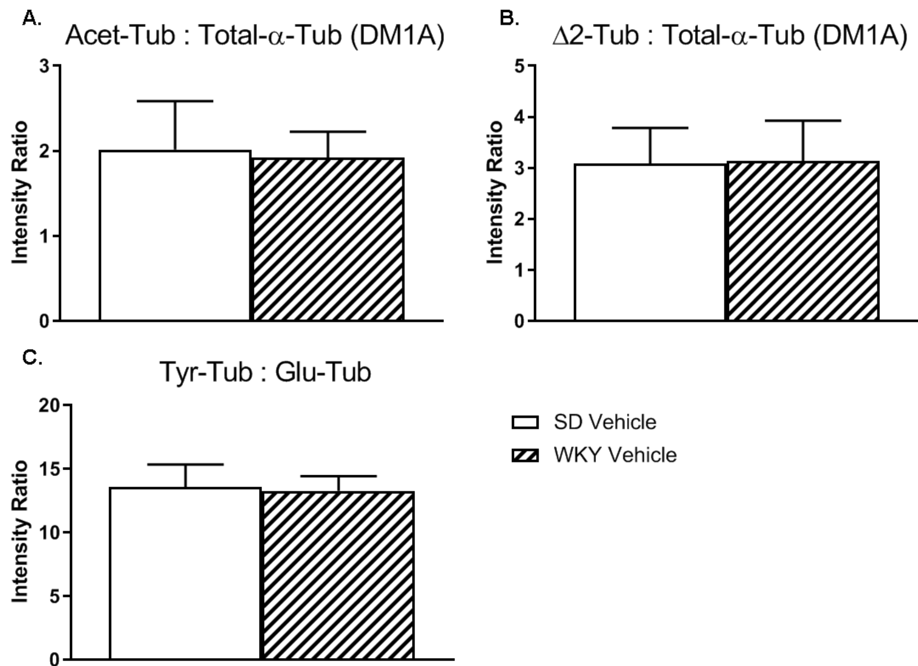

Figure 4.8. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent prefrontal cortex for Sprague Dawley and Wistar Kyoto vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY vehicle groups. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY vehicle groups. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in prefrontal cortex for SD and WKY vehicle groups.

No significant difference was found between SD and WKY for any α-Tub ratio of expression in the prefrontal cortex (Figure 4.8).

5.1.4. Expression of Brain Derived Neurotrophic Factor normalised on Total Protein Content in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto vehicle Groups

Total BDNF expression was determined by ELISA assay and total protein expression was determine by Bradford assay. The ratio of total BDNF Expression to total protein expression is represented here for the SD and WKY vehicle groups. A significant effect of strain on BDNF expression normalised on total protein was found between SD and WKY ($F_{(1,119)}$=5.80, $p<0.05$) but no main effect of treatment was found (Appendix B.2.3.9).

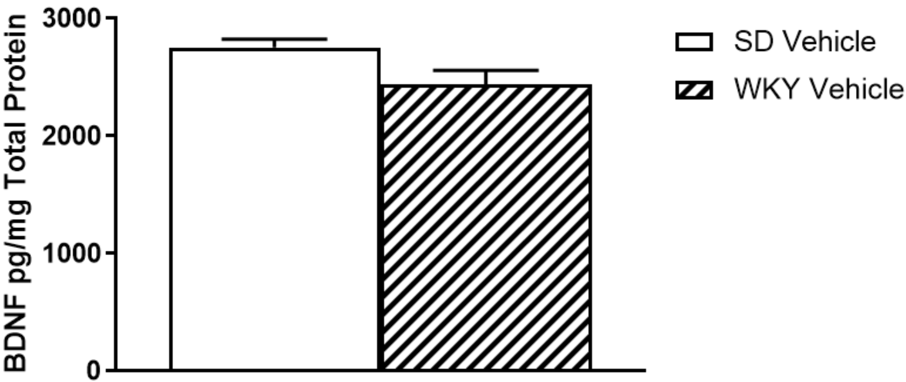

Figure 4.9. Comparison of hippocampal total brain derived neurotrophic factor expression normalised on total protein in Sprague Dawley and Wistar Kyoto vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

There was no significant difference between SD and WKY for total BDNF expression normalised on total protein expression in the hippocampus (Figure 4.9).

5.2. Expression of α-Tubulins and Brain Derived Neurotrophic Factor in Sprague Dawley and Wistar Kyoto Rats Treated Acutely with Escitalopram or Desipramine

5.2.1. Effects of Acute Escitalopram and Desipramine Treatment on α-Tubulin and its Posttranslational Modifications in Blood Plasma in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in blood plasma using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving acute treatment of ESC or DMI.

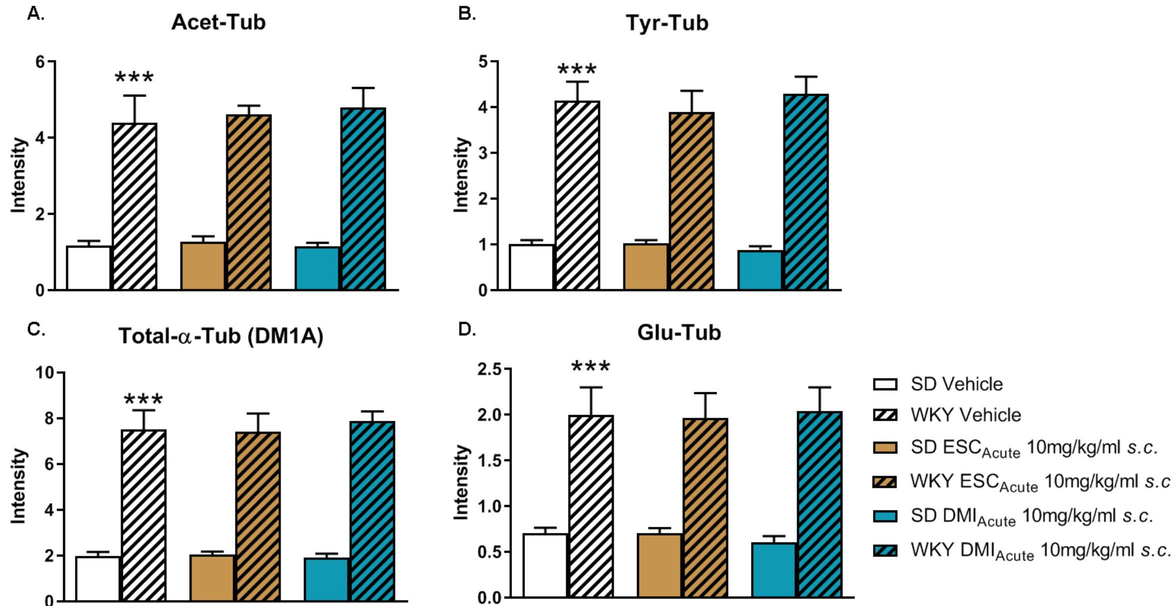

Figure 4.10. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, and Detyronsiated-α-Tubulin in blood plasma with acute treatment of escitalopram or desipramine compared to vehicle groups. n=8-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in blood plasma for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[B]** Tyr-Tub Intensity in blood plasma for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[C]** Total-α-Tub(DM1A) Intensity in blood plasma for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[D]** Glu-Tub Intensity in blood plasma for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle.

Acute treatments of ESC or DMI had no effect on any of the α-Tub targets in blood plasma compared to their respective vehicle groups (Figure 4.10).

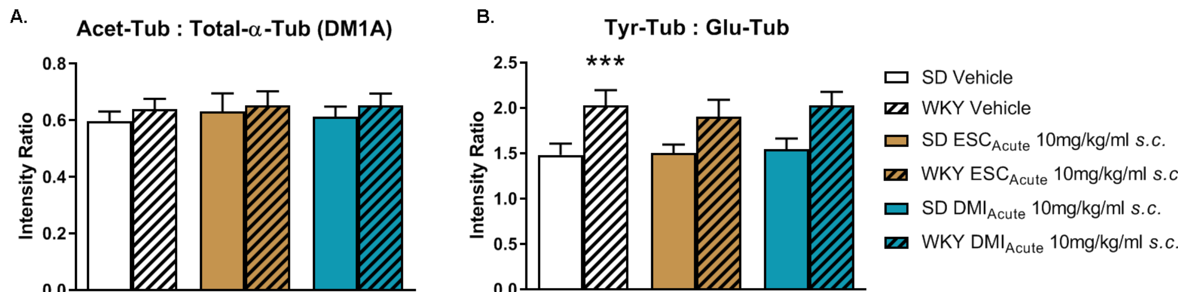

Figure 4.11. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in blood plasma with acute treatment of escitalopram or desipramine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[B]** Tyr-Tub:Glu-Tub Intensity Ratio in blood plasma for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ***$p<0.001$ vs SD vehicle.

Acute treatments of ESC or DMI had no effect on any of the α-Tub ratios of expression in blood plasma compared to their respective vehicle groups (Figure 4.11).

5.2.2. Effects of Acute Escitalopram and Desipramine Treatment on α-Tubulin and its Posttranslational Modifications in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in hippocampal tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving acute treatment of ESC or DMI.

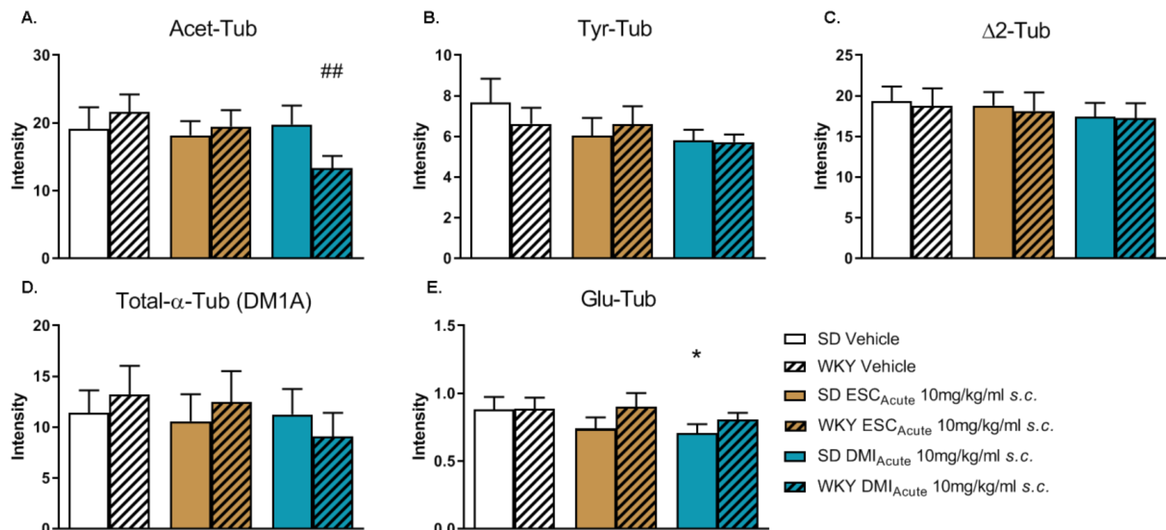

Figure 4.12. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent hippocampus with acute treatment of escitalopram or desipramine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. ##p<0.01 vs WKY vehicle. **[B]** Tyr-Tub Intensity in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[C]** Δ2-Tub Intensity in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[D]** Total-α-Tub(DM1A) Intensity in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[E]** Glu-Tub Intensity in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD vehicle.

Acute DMI treatment significantly decreased Acet-Tub (p<0.01) in WKY compared to vehicle (Figure 4.12A). Acute DMI treatment significantly decreased Glu-Tub (p<0.05) in SD compared to vehicle (Figure 4.12E). No other significant differences were found for acute ESC or DMI treatments in α-Tub targets in the hippocampus.

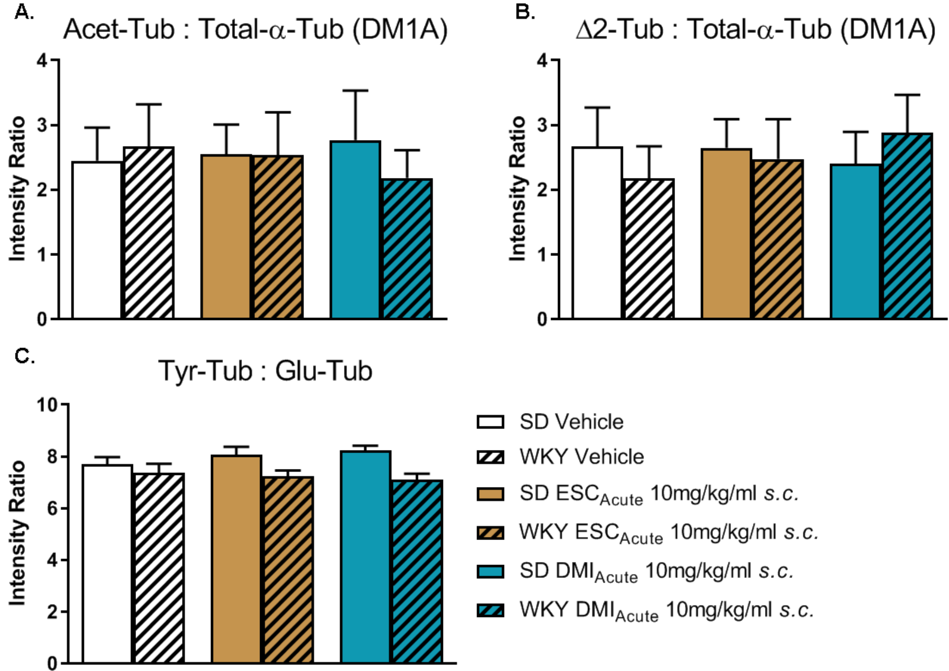

Figure 4.13. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent hippocampus with acute treatment of escitalopram or desipramine compared to vehicle groups. n=8-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in hippocampus for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group.

Acute treatments of ESC or DMI had no effect on α-Tub ratios of expression in hippocampus compared to their respective vehicle groups (Figure 4.13).

5.2.3. Effects of Acute Escitalopram and Desipramine Treatment on α-Tubulin and its Posttranslational Modifications in Prefrontal Cortex Tissue in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in prefrontal cortex tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving acute treatment of ESC or DMI.

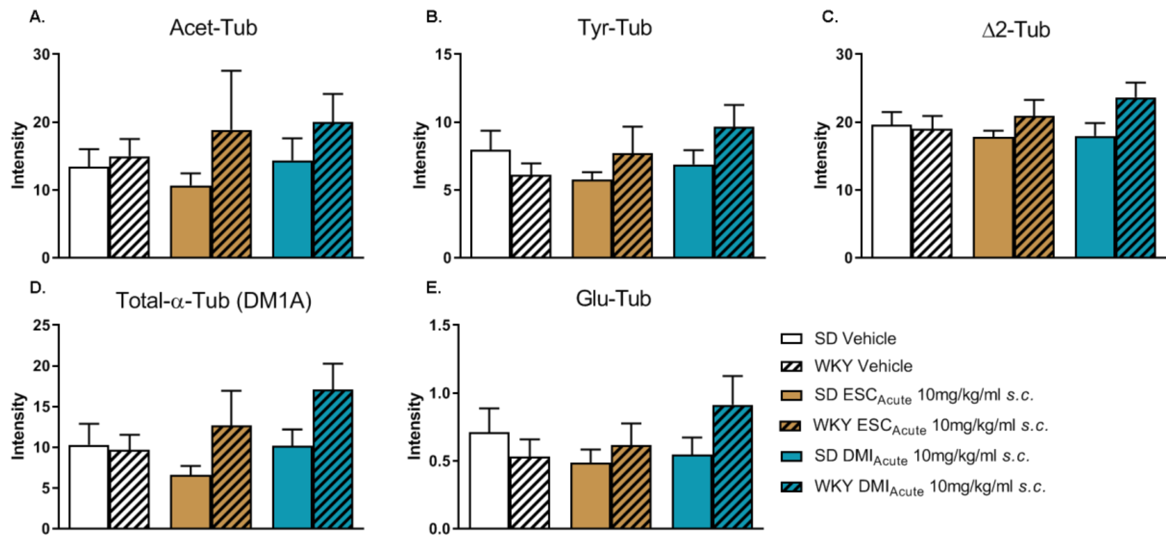

Figure 4.14. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent prefrontal cortex with acute treatment of escitalopram or desipramine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[B]** Tyr-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[C]** Δ2-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[D]** Total-α-Tub(DM1A) Intensity in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[E]** Glu-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group.

Acute treatments of ESC or DMI had no effect on α-Tub targets in the prefrontal cortex compared to their respective vehicle groups (Figure 4.14).

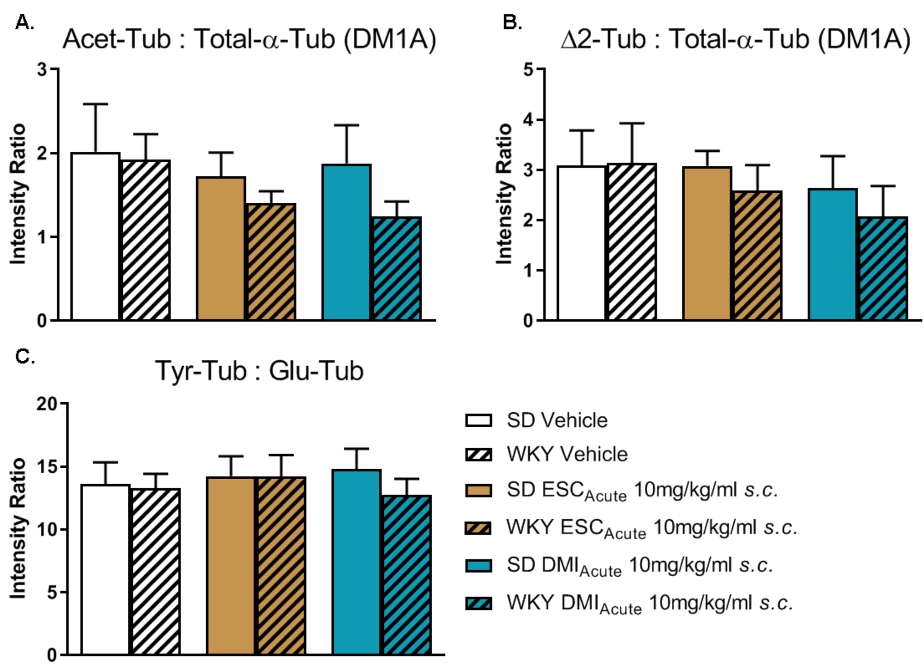

Figure 4.15. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent prefrontal cortex with acute treatment of escitalopram or desipramine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in prefrontal cortex for SD and WKY groups treated acutely with ESC or DMI compared to corresponding vehicle group.

Acute treatments of ESC or DMI had no effect on α-Tub ratios of expression in the prefrontal cortex compared to their respective vehicle groups (Figure 4.15).

5.2.4. Effects of Acute Escitalopram and Desipramine Treatment on Brain Derived Neurotrophic Factor in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto Rats

Total BDNF expression was determined by ELISA assay and total protein expression was determine by Bradford assay. The ratio of total BDNF Expression to total protein expression is represented here for the SD and WKY groups treated acutely with ESC or DMI and compared to vehicle groups.

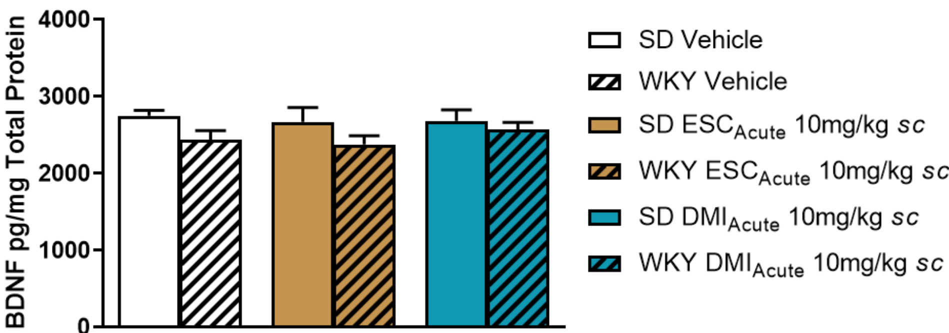

Figure 4.16. Comparison of acute treatment of escitalopram or desipramine on hippocampal total brain derived neurotrophic factor expression normalised on total protein compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

Acute treatments of ESC or DMI had no effect on the ratio of total of BDNF to total protein expression for SD or WKY compared to their respective vehicle groups (Figure 4.16).

5.3. Expression of α-Tubulins and Brain Derived Neurotrophic Factor in Sprague Dawley and Wistar Kyoto Rats Treated Chronically with Escitalopram or Desipramine

5.3.1. Effects of Chronic Escitalopram and Desipramine Treatment on α-Tubulin and its Posttranslational Modifications in Blood Plasma in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in blood plasma using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving chronic treatment of ESC or DMI.

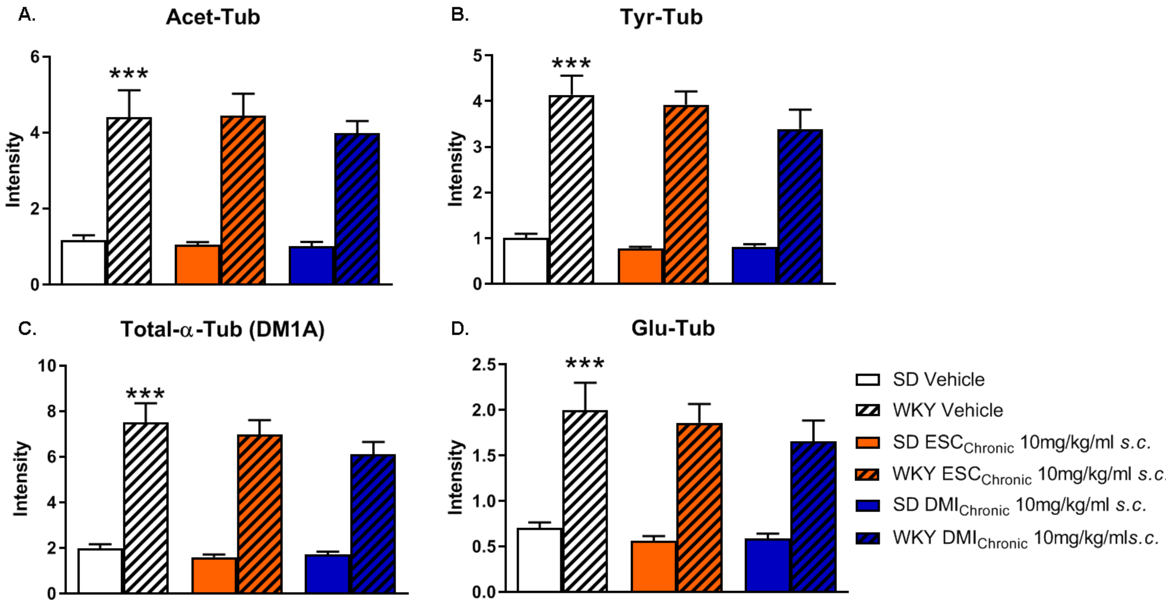

Figure 4.17. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, and Detyronsiated-α-Tubulin in blood plasma with chronic treatment of escitalopram or desipramine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in blood plasma for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[B]** Tyr-Tub Intensity in blood plasma for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[C]** Total-α-Tub(DM1A) Intensity in blood plasma for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[D]** Glu-Tub Intensity in blood plasma for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD vehicle.

Chronic treatments of ESC or DMI had no effect on any of the α-Tub targets in blood plasma compared to their respective vehicle groups (Figure 4.17).

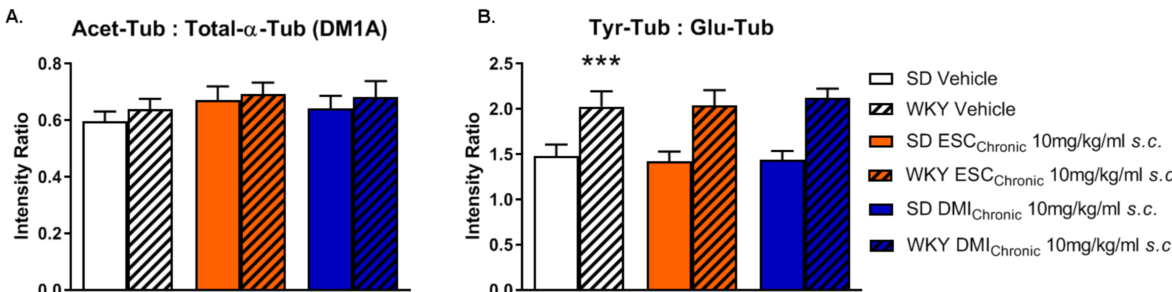

Figure 4.18. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in blood plasma with chronic treatment of escitalopram or desipramine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[B]** Tyr-Tub:Glu-Tub Intensity Ratio in blood plasma for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. ***p<0.001 vs SD Vehicle.

Chronic treatments of ESC or DMI had no effect on any of the α-Tub ratios of expression in blood plasma compared to their respective vehicle groups (Figure 4.18).

5.3.2. Effects of Chronic Escitalopram and Desipramine Treatment on α-Tubulin and its Posttranslational Modifications in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in hippocampal tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving chronic treatment of ESC or DMI.

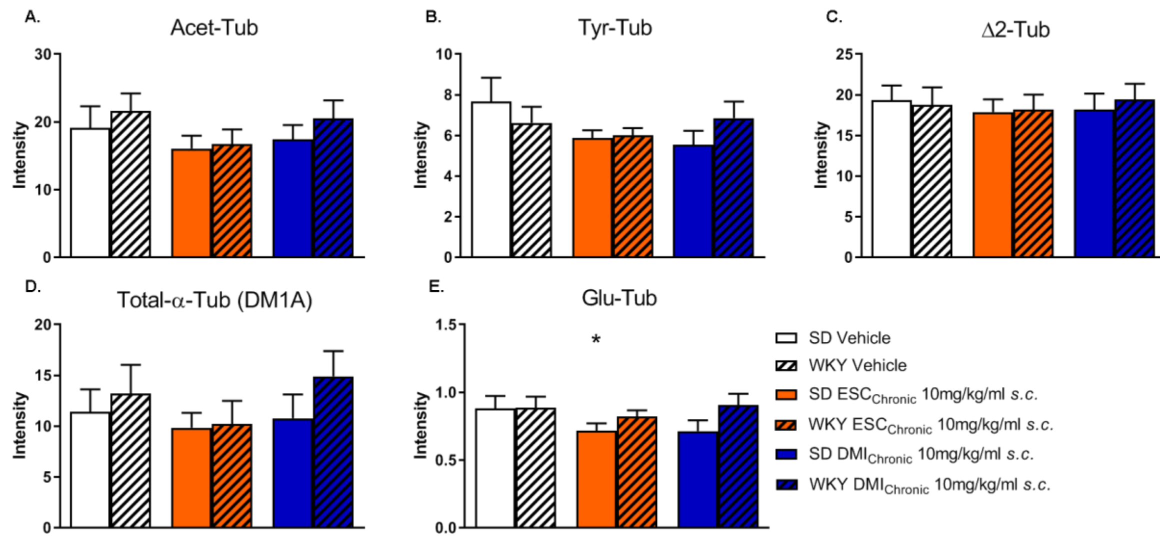

Figure 4.19. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent hippocampus with chronic treatment of escitalopram or desipramine compared to vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[B]** Tyr-Tub Intensity in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[C]** Δ2-Tub Intensity in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[D]** Total-α-Tub(DM1A) Intensity in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[E]** Glu-Tub Intensity in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. *$p<0.05$ vs SD vehicle.

Chronic ESC treatment significantly lowered Glu-Tub in SD ($p<0.05$) compared to vehicle (Figure 4.19E). No other significant differences were found in α-Tub targets with chronic ESC or DMI treatment compared to their respective vehicle group.

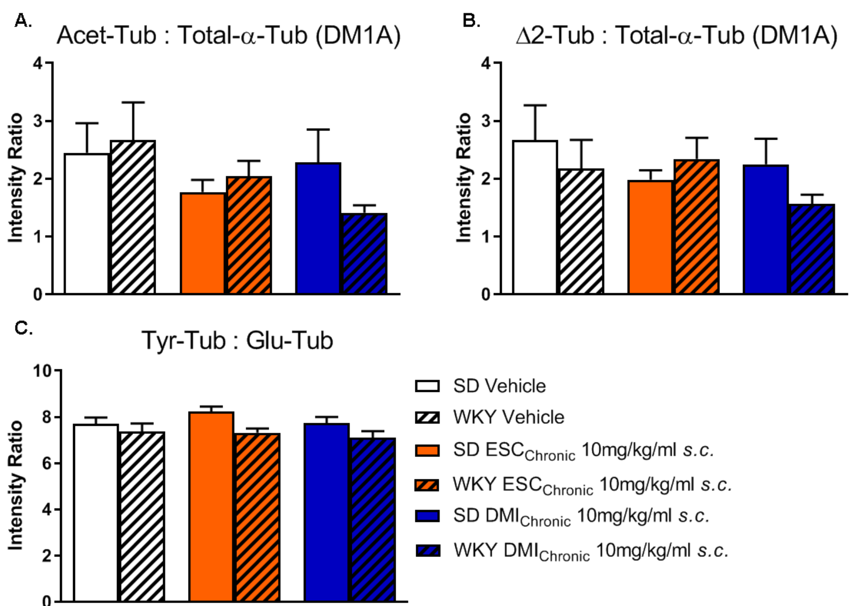

Figure 4.20. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent hippocampus with chronic treatment of escitalopram or desipramine compared to vehicle groups. n=8-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in hippocampus for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group.

Chronic treatments of ESC and DMI had no effect on α-Tub ratios of expression in hippocampus compared to their respective vehicle groups (Figure 4.20).

5.3.3. Effects of Chronic Escitalopram and Desipramine Treatment on α-Tubulin and its Posttranslational Modifications in Prefrontal Cortex in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in prefrontal cortex tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving chronic treatment of ESC or DMI.

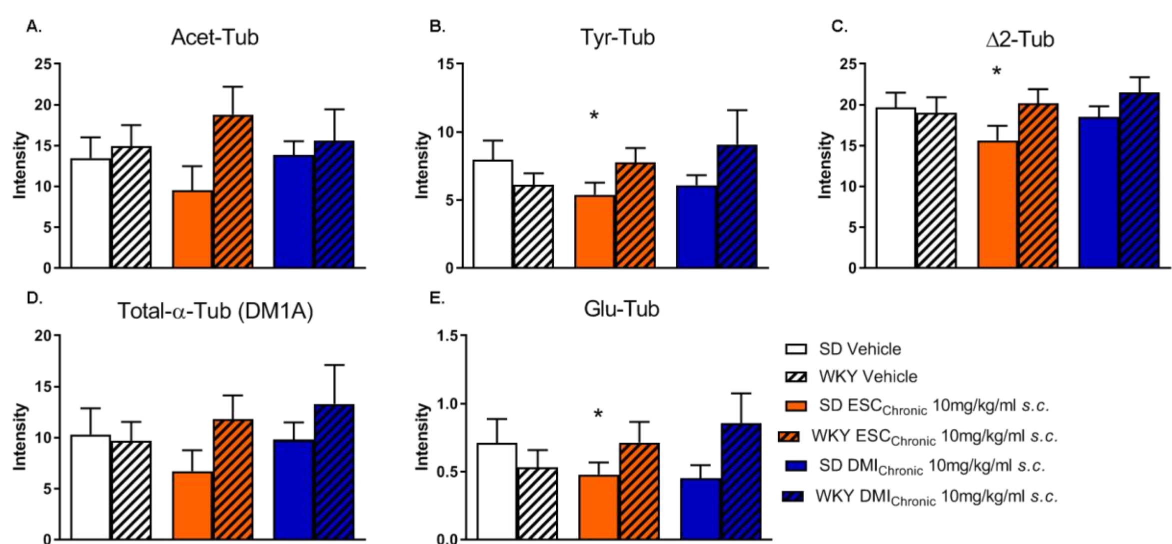

Figure 4.21. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent prefrontal cortex with chronic treatment of escitalopram or desipramine compared to vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[B]** Tyr-Tub Intensity in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD Vehicle. **[C]** Δ2-Tub Intensity in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD vehicle. **[D]** Total-α-Tub(DM1A) Intensity in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[E]** Glu-Tub Intensity in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. *p<0.05 vs SD vehicle.

Chronic ESC treatment significantly decreased the expression of Tyr-Tub, Glu-Tub and Δ2-Tub (p<0.05, Figure 4.21B, 4.21C, and 4.21E) in SD compared to vehicle (Figure 4.21). No other chronic treatments of ESC or DMI had an effect on α-Tub targets in the prefrontal cortex compared to their respective vehicle groups.

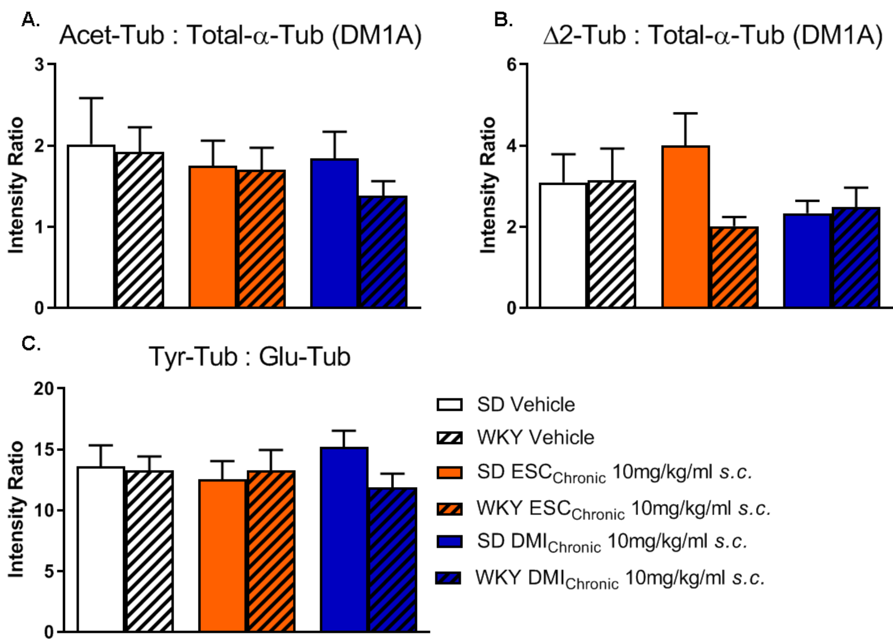

Figure 4.22. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent prefrontal cortex with chronic treatment of escitalopram or desipramine compared to vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in prefrontal cortex for SD and WKY groups treated chronically with ESC or DMI compared to corresponding vehicle group.

Chronic treatments of ESC or DMI had no effect on α-Tub ratios of expression in the prefrontal cortex compared to their respective vehicle groups (Figure 4.22).

5.3.4. Effects of Chronic Escitalopram and Desipramine Treatment on Brain Derived Neurotrophic Factor in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto Rats

Total BDNF expression was determined by ELISA assay and total protein expression was determined by Bradford assay. The ratio of total BDNF Expression to total protein expression is represented here for the SD and WKY groups treated chronically with ESC or DMI and compared to vehicle groups.

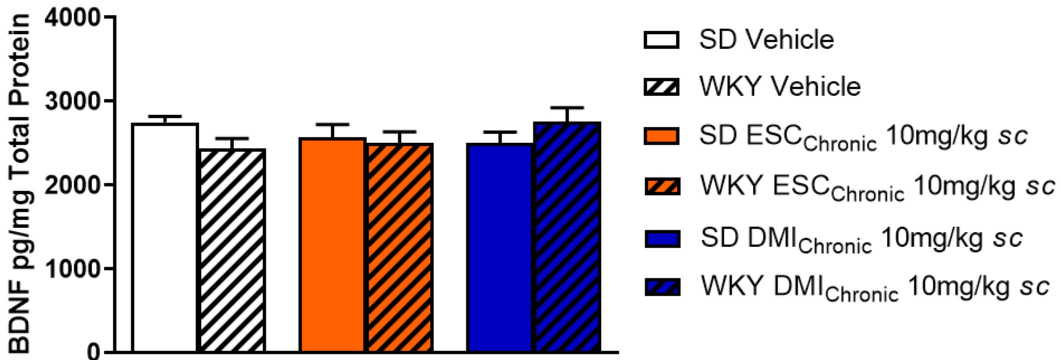

Figure 4.23. Comparison of chronic treatment of escitalopram or desipramine on hippocampal total brain derived neurotrophic factor expression normalised on total protein compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM.

Chronic treatments of ESC or DMI had no effect on the ratio of total BDNF to total protein expression for SD or WKY compared to their respective vehicle groups (Figure 4.23).

5.4. Expression of α-Tubulins and Brain Derived Neurotrophic Factor in Sprague Dawley and Wistar Kyoto Rats Treated Acutely with Pregnenolone-Methyl-Ether or Ketamine

5.4.1. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on α-Tubulin and its Posttranslational Modifications in Blood Plasma in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in blood plasma using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving acute treatment of PME or ketamine.

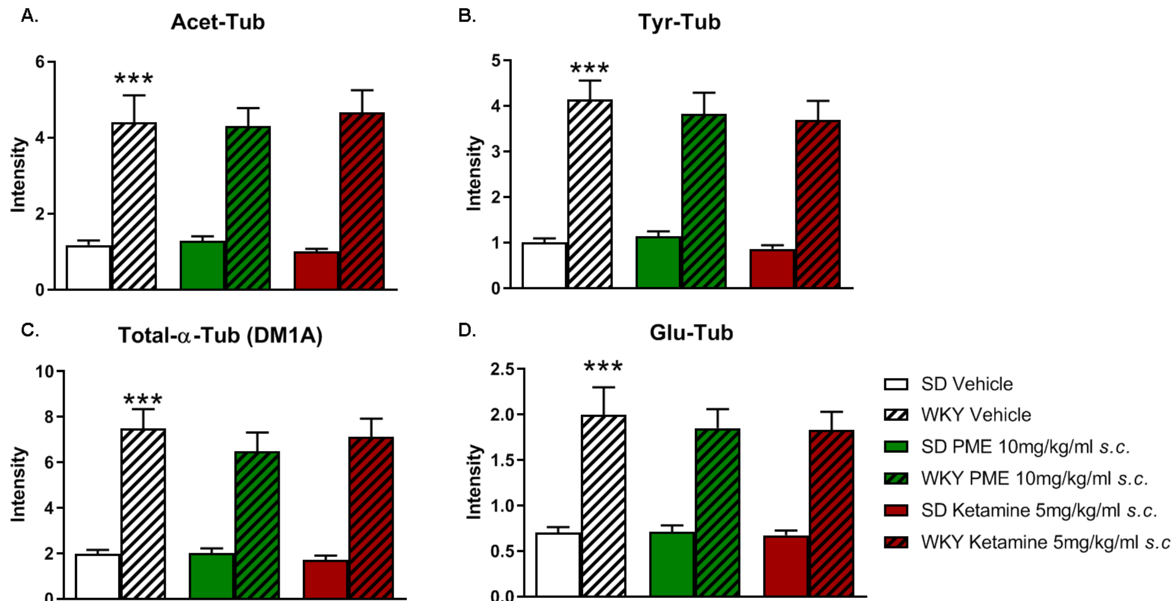

Figure 4.24. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, and Detyronsiated-α-Tubulin in blood plasma with acute treatment of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in blood plasma for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[B]** Tyr-Tub Intensity in blood plasma for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group.***p<0.001 vs SD Vehicle. **[C]** Total-α-Tub(DM1A) Intensity in blood plasma for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. ***p<0.001 vs SD vehicle. **[D]** Glu-Tub Intensity in blood plasma for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. ***p<0.001 vs SD vehicle.

Acute treatments of PME or ketamine had no effect on any of the α-Tub targets in blood plasma compared to their respective vehicle groups (Figure 4.24).

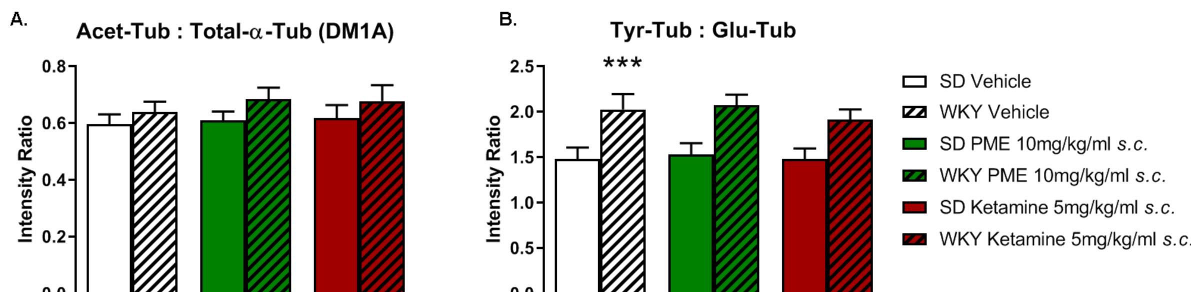

Figure 4.25. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in blood plasma with acute treatment of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[B]** Tyr-Tub:Glu-Tub Intensity Ratio in blood plasma for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. ***$p<0.001$ vs SD vehicle.

Acute treatments of PME or ketamine had no effect on any of the α-Tub ratios of expression in blood plasma compared to their respective vehicle groups (Figure 4.25).

5.4.2. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on α-Tubulin and its Posttranslational Modifications in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in hippocampal tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving acute treatment of PME or ketamine.

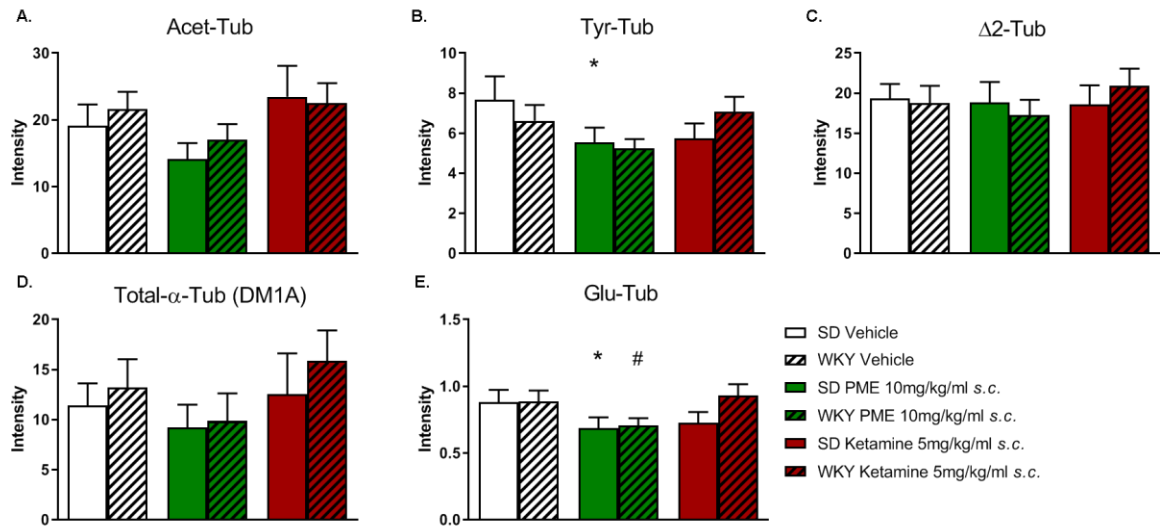

Figure 4.26. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent hippocampus with acute treatment of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[B]** Tyr-Tub Intensity in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. *p<0.05 vs SD vehicle. **[C]** Δ2-Tub Intensity in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[D]** Total-α-Tub(DM1A) Intensity in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[E]** Glu-Tub Intensity in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. *p<0.05 vs SD vehicle, #p<0.05 vs WKY vehicle.

Acute PME treatment significantly decreased both Tyr-Tub and Glu-Tub (p<0.05, Figure 4.26B and 4.26E) in SDs compared to vehicle. Acute PME also decreased Glu-Tub (p<0.05, Figure 4.26E) in WKYs compared to vehicle. No other significant differences were found for acute PME or ketamine treatments compared to their respective vehicle groups.

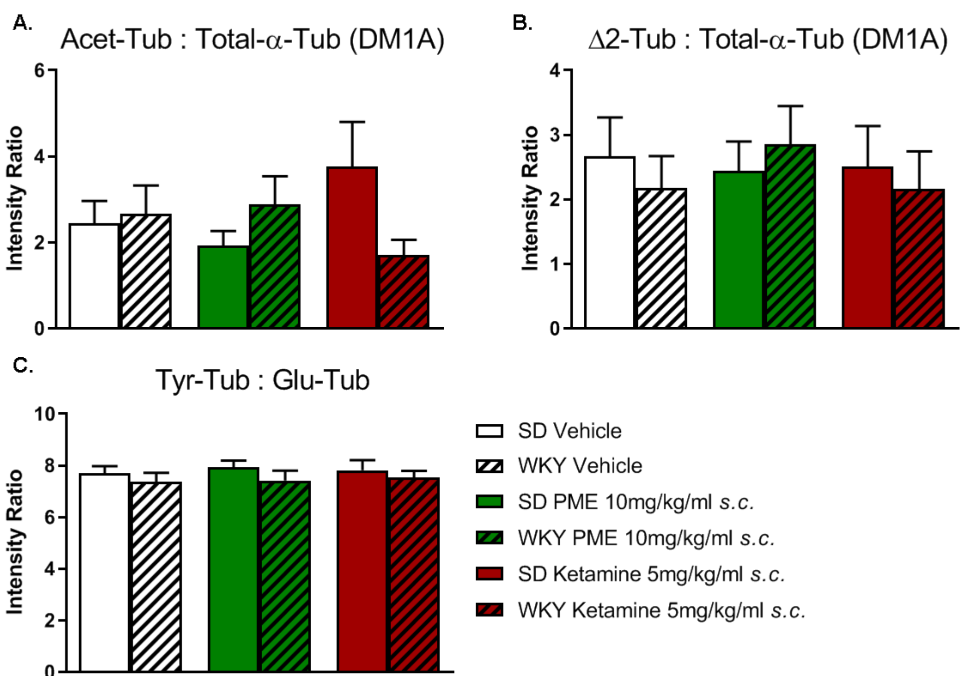

Figure 4.27. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent hippocampus with acute treatment of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=8-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in hippocampus for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group.

Acute treatments of PME and ketamine had no effect on α-Tub ratios of expression in hippocampus compared to their respective vehicle groups (Figure 4.27).

5.4.3. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on α-Tubulin and its Posttranslational Modifications in Prefrontal Cortex Tissue in Sprague Dawley and Wistar Kyoto Rats

Integrated Intensity (Intensity) was calculated for each α-Tub target in prefrontal cortex tissue using the Li-Cor Odyssey System and is represented here for the SD and WKY rats receiving acute treatment of PME or ketamine.

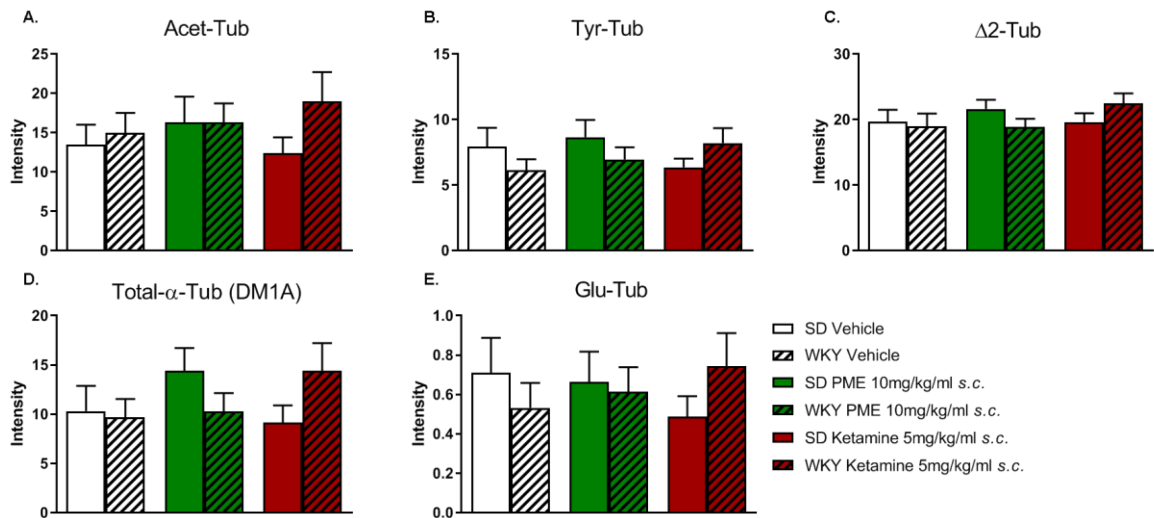

Figure 4.28. Expression of Total-α-Tubulin (DM1A), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyronsiated-α-Tubulin, and Deglutamylated-α-Tubulin in rodent prefrontal cortex with acute treatment of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[B]** Tyr-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[C]** Δ2-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[D]** Total-α-Tub(DM1A) Intensity in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[E]** Glu-Tub Intensity in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group.

Acute treatments of PME or ketamine had no effect on α-Tub targets in the prefrontal cortex compared to their respective vehicle groups (Figure 4.28).

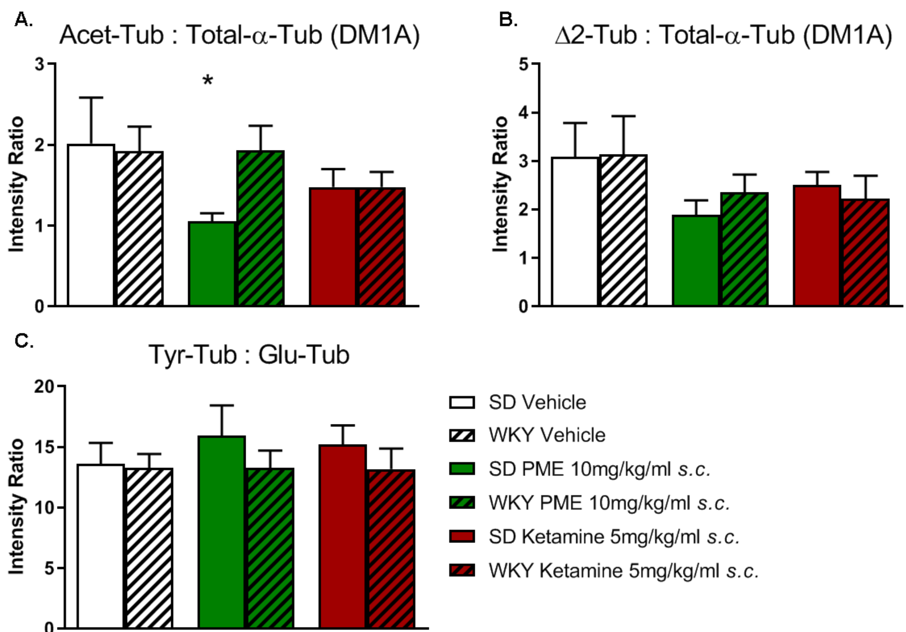

Figure 4.29. Ratios of Acetylated-α-Tubulin to Total-α-Tubulin (DM1A), Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A), and Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin in rodent prefrontal cortex with acute treatment of pregnenolone-methyl-ether or ketamine compared to vehicle groups. n=10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. *p<0.05 vs SD vehicle **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in prefrontal cortex for SD and WKY groups treated acutely with PME or ketamine compared to corresponding vehicle group.

Acute PME treatment significantly reduced the ratio of expression for Acet-Tub to Tot-α-Tub in SD ($p<0.05$) compared to vehicle (4.29A). No other acute treatments of PME or ketamine had an effect on α-Tub ratios of expression in the prefrontal cortex compared to their respective vehicle groups.

5.4.4. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatment on Brain Derived Neurotrophic Factor in Hippocampal Tissue in Sprague Dawley and Wistar Kyoto Rats

Total BDNF expression was determined by ELISA assay and total protein expression was determine by Bradford assay. The ratio of total BDNF expression to total protein expression is represented here for the SD and WKY groups treated acutely with PME or ketamine and compared to vehicle groups.

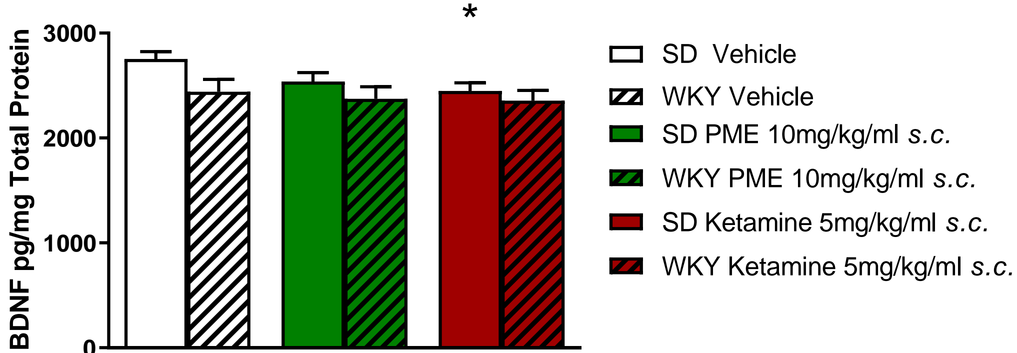

Figure 4.30. Comparison of acute treatment of pregnenolone-methyl-ether or ketamine on hippocampal total brain derived neurotrophic factor expression normalised on total protein compared to vehicle groups. n=9-10/group. Two-Way ANOVA with blocking factor and planned comparisons. Data: Observed Mean ± SEM. *p<0.05 vs SD vehicle.

Acute ketamine treatment significantly lowered total BDNF to total protein expression in SD (p<0.05) compared to vehicle (Figure 4.30). No other effects where observed on the ratio of total BDNF to total protein expression for SD or WKY compared to their respective vehicle groups.

6. Discussion

6.1. Differences in Sprague Dawley and Wistar Kyoto Rats

The clearest finding in this study was that WKY rats significantly overexpress all α-Tubs in blood plasma compared to SD (Figure 4.2). Interestingly, this overexpression is not found in brain tissue (Figure 4.5), suggesting that the cause of the increase in α-Tubs in WKY blood plasma is not connected to CNS dysfunction but rather is a result of unique physiology in other peripheral organs. WKY overexpression of Tyr-Tub to Glu-Tub in blood plasma is particularly striking as the ratio of Tyr-Tub to Glu-Tub remained significantly different in blood plasma (Figure 4.3B) but not in brain tissue (Figures 4.6C and 4.8C).

The discrepancy between α-Tub expression in the brain and blood plasma may reflect the original purpose of WKY rats as a normotensive control strain for the spontaneously hypertensive rat (SHR)[221], where their "depressed" state was an unintended consequence of breeding for a normotensive rat[142]. It is possible that other unintended physiological traits emerged in the WKY, including the overexpression of α-Tubs in blood plasma, however these new traits cannot be assumed to reflect a depressed state. Given the purpose they were bred for, it is more likely that changes in blood-based biomarkers are more directly linked to blood pressure and cardiac health rather than a peripheral marker of neuronal dysfunction. The significantly lower expression of transferrin found in blood plasma also supports this idea (Figure 4.4). Furthermore, genetic variability and behavioural differences in sub-strains of WKYs from different suppliers has been noted as a confounding factor in these studies[222]. Therefore, it cannot be assumed that the differences in α-Tub expression found here will exactly match other WKY sub-strains.

Even though there were significant strain effects for a handful of targets and ratio of expression in the brain, these did not coincide with significant differences in the vehicle groups and there was no consistent change with treatment. Assuming the differences are real for these targets, they would warrant further investigation, perhaps with a higher powered study to clearly demonstrate that they are in fact different. However, the lack of clear differences in α-Tub expression in WKY brain tissue compared to SD is somewhat disappointing considering the robust behavioural differences that were discussed in Chapter 3 (Figures 3.2 and 3.3). It may be concluded then that neuronal α-Tub expression does not have a direct causal link to behavioural endpoints in this model and that any changes in α-Tub are so subtle as to not confer behavioural significance.

Although there was a significant effect of strain on BDNF expression in the hippocampus, the SD and WKY vehicle groups were not significantly different (Figure 4.9). Looking

across all the groups it appears that WKYs tend to have lower BDNF expression in the hippocampus when normalised on total protein expression compared to SD. This is consistent with previous reports that WKYs have reduced BDNF transcription in the hippocampus compared to SD[248].

6.2. Effects of Acute Escitalopram and Desipramine Treatments

Only two significant differences in α-Tub expression were found in groups receiving acute antidepressant treatment: the WKY acute DMI group showed a decrease Acet-Tub in the hippocampus (Figure 4.12A) and the SD acute DMI group showed a decrease in Glu-Tub also in the hippocampus (Figure 4.12E).

In the absence of other effects, these results may be the result of statistical chance rather than a true treatment effect. There were also no changes observed in hippocampal BDNF expression (Figure 4.16). However, assuming that acute DMI was having an effect on these two markers, it would indicate that DMI was increasing MT dynamics in the hippocampus. A decrease in Acet-Tub can be interpreted as a decrease in MT long-term stability[111], suggesting a reorganisation of MTs. Likewise, high Glu-Tub expression is typically associated with stable MTs[117], therefore a decrease may indicate an increase in MT dynamics. Again, it is unlikely a true effect in this case as Tyr-Tub does not increase as would be expected with a decrease in Glu-Tub but appears to be trending downward itself (Figure 4.12B). This is further confirmed in the absence of an effect in both the Tyr-Tub to Glu-Tub ratio (Figure 4.13C) and Acet-Tub normalised on Tot-α-Tub (DM1A) (Figure 4.13A).

It should be noted that rats receiving acute ESC treatment showed no change in behaviour in Chapter 3 (Figures 3.4 and 3.5) while acute DMI treated animals did show some changes. WKYs receiving acute DMI treatment showed a decrease in total immobility (Figure 3.4) and SDs receiving acute DMI treatment showed an increase in climbing behaviour accompanied by a decrease in swimming behaviour (Figures 3.5B and 3.5A). This appears to be in line with acute ESC showing no effect for any α-Tub target and acute DMI having some small effect, however the connection here is inconclusive at best given the variability of the α-Tub data and that the effects are absent when normalised on Tot-α-Tub (DM1A) and Glu-Tub.

6.3. Effects of Chronic Escitalopram and Desipramine Treatments

Unlike the acute DMI treatment described above, chronic DMI treatment did not show any significant effect in any tissue for either SD or WKY. Perhaps the only noteworthy result for chronic DMI here is that WKYs receiving chronic DMI had higher BDNF

expression than the corresponding SDs although not significantly so (Figure 4.23). Considering the main effect of strain for BDNF expression, it is interesting this is the only treatment group where WKY expression appears to be higher than SD. Interestingly, chronic, but not acute, treatment of fluoxetine and DMI at the same doses and durations used in this study have been reported to increase BDNF expression in the frontal cortex but not hippocampuss[249]. This may explain why no significant differences with treatment were found here, as only BDNF in the hippocampus was assessed.

Chronic ESC appears to modulate the tyrosination cycle in the prefrontal cortex with a significant decrease found in Tyr-Tub, Glu-Tub and Δ2-Tub in SDs (Figures 4.21B, 4.21C, 4.21E). While not significant, Acet-Tub and Tot-α-Tub also appear to trend downward, giving the impression that all α-Tubs are knocked down by chronic ESC treatment in the prefrontal cortex. This is also reflected in the expression ratios, where the significant differences disappear. Likewise, SDs treated with chronic ESC showed a decrease in Glu-Tub in the hippocampus (Figure 4.19E) but no other α-Tubs were significantly different, and no differences were found in the ratios of α-Tub expression. No significant differences were found in blood plasma for the chronic treatments (Figures 4.17 and 4.18).

These results are not consistent with the behavioural data in Chapter 3, where both SDs and WKYs treated with chronic DMI showed a robust decrease in total immobility (Figure 3.6) and increase in climbing behaviour (Figure 3.7B), while chronic ESC groups showed no behavioural effects.

6.4. Effects of Acute Pregnenolone-Methyl-Ether and Ketamine Treatments

In line with the previously discussed treatment groups, neither PME nor ketamine treatment groups showed any effect on α-Tubs in blood plasma.

Both SD and WKY PME treated groups showed some differences from vehicle in the hippocampus. Tyr-Tub and Glu-Tub were both significantly decreased in SD receiving PME treatment (Figures 4.26B and 4.26E) while Glu-Tub was significantly decreased in WKYs receiving PME treatment (Figure 4.26E). Like the other treatments, these differences disappear when Tyr-Tub is normalised on Glu-Tub in the hippocampus (Figure 4.27). This is an interesting result as PME's purported mechanisms of action for increasing MT dynamics and restoring neuronal plasticity is through the binding of MAP-2[26,250]. MAP-2 is highly expressed in dendrites[251,252] where the tyrosination cycle also plays a critical role in dendritic formation and maintenance[253].

No individual target differences were found in the prefrontal cortex for either SDs or WKYs but SDs receiving PME treatment showed a significant decrease in Acet to Tot-α-Tub(DM1A) expression ratio (Figure 4.29A).This decrease in Acet-Tub, a marker for MT stability, to Tot-α-Tub(DM1A) may reflect increased neuronal plasticity through MT reorganisation[111].

SDs receiving ketamine treatment showed significantly lower BDNF expression in the hippocampus compared to vehicle (Figure 4.30); unfortunately, no significant changes were found in the α-Tubs to coincide with this result. This finding also contradicts conventional thinking around the mechanism of action for ketamine, which has previously been shown to both decrease total immobility in the FST and increase hippocampal BDNF[152,203,254,255]. It has even been reported that the effects of ketamine on BDNF can be seen in plasma for up to four hours but that BDNF is not a useful central marker of ketamine efficacy[256].

Lastly, the expression of α-Tubs does not appear to match the behavioural data in Chapter 3. While SDs were found to have significant changes in α-Tub expression in the brain, they did not have significant changes in total immobility (Figure 3.8) or in individual behaviours (Figure 3.9) in the FST after receiving the same treatments. Conversely, WKYs receiving PME and ketamine showed a decrease in total immobility in the FST (Figure 3.8) but did not have robust changes in α-Tub expression with the exception of Glu-Tub in the hippocampus.

7. Conclusion

Because all of the α-Tubs are overexpressed in WKY blood plasma, it is difficult to make the case that it is a suitable peripheral biomarker for a “depressed” state in this model. Likewise, the lack of differences in α-Tub expression in the brain of the vehicle groups and inconsistent treatment effects do not support the α-Tubs as a central biomarker for a “depressed” state or treatment efficacy.

The inclusion of both acute and chronic treatments of antidepressants acting by different mechanisms cast a wide net for possible differences to be found in α-Tub expression. It would be understandable to see the acute treatments fail to elicit a response as is often noted in the literature, but it is somewhat surprising to see the chronic treatments also fail. In particular, the chronic desipramine groups, which had the most robust behavioural response in the forced swim test (Chapter 3) did not yield any significant difference for any target compared to vehicle. At the very least this implies that α-Tub expression is not directly linked to the observed behavioural changes in the FST.

Given the near significance of many of the BDNF groups, it would be interesting to investigate the expression levels of the synaptic markers, such as PSD-95, spinophilin, and synaptophysin. As an antidepressant “transducer”, BDNF and other synaptic markers may be more beneficial to look at, especially for acute antidepressant treatments.

Chapter 5: Expression of α-tubulins in Blood Plasma from Individuals with Major Depressive Disorder and Healthy Controls

1. Introduction

The previous chapters have outlined the preclinical elements of this project. This chapter will investigate α-tubulins (α-Tubs) in blood plasma donated by individuals suffering from Major Depressive Disorder (MDD) as well as healthy individuals serving as controls.

As outlined in Chapter 1, the majority of the evidence for synaptic dysfunction in MDD in clinical populations comes from post-mortem[30,257] and neuroimaging[258,259] studies showing reduced hippocampal volume and synapse loss. These changes to both functional and structural neuronal plasticity are also seen in animal models of stress and MDD[24,260,261] and are the evidence supporting the research aims of the earlier chapters.

These post-mortem and neuroimaging investigations are invaluable for expanding our understanding of MDD's underlying pathophysiology in the CNS but similar to the preclinical models of MDD, there is currently no suitable peripheral marker for disease severity or treatment efficacy. To that end α-Tubs were identified as potential peripheral biomarkers for MDD severity and treatment efficacy, which can be easily detected and measured in blood plasma for both humans and animals for translational research.

2. Aims

The main research question addressed in this chapter is: Are α-Tubs peripheral biomarkers for MDD severity and treatment efficacy in clinical populations?

By necessity, this question will be divided into two: do α-Tubs levels relate to MDD severity and do changes in α-Tubs levels reflect treatment efficacy?

The first hypothesis tested is that individuals with MDD will express α-Tubs differently to controls in blood plasma. Cohort 1 will be used to compare depressed individuals and healthy controls.

The second hypothesis tested is that the expression of α-Tubs will change in blood plasma following treatment of MDD. Cohort 2 will be used to compare the same individuals at a baseline measurement and second measurement following six weeks of pharmacological antidepressant treatment.

As in the previous chapter, these questions will be answered by measuring the expression of Tot-α-Tub (DM1A), Tot-α-Tub (11H10), Acet-Tub, Tyr-Tub, Glu-Tub, and Δ2-Tub in blood plasma by Western blot.

3. Ethical Permissions

Both studies described below were carried out in accordance to the Declaration of Helsinki (https://www.wma.net/).

Primary ethical approval for the collection and analysis of samples in cohort 1 was initially applied for and received by collaborators in the Department of Psychiatry, Trinity College Dublin from the St. Patrick's University Hospital Research Ethics Committee.

Primary ethical approval for the collection and analysis of samples in cohort 2 was initially applied for and received by collaborators at the University of Regensburg, Germany from the Intramural Review Panel of the University of Regensburg, Faculty of Medicine. Ethical approval for the analysis of the samples was granted by the School of Medicine Research Ethics Committee (SOMREC) of Trinity College Dublin. For ethical permission see Appendix A.2.

4. Methods

Western blotting analysis and sample collection was carried out as described in Chapter 2 Section 2.

4.1. Sample Acquisition and Time Points

4.1.1. Cohort 1: EFFECT-Dep Study

Cohort 1 (Table 5.1 & 5.2) samples were acquired by researchers in the Department of Psychiatry, Trinity College Dublin, St. Patrick's University Hospital conducting the EFFECT-Dep study[262], which was an investigation into ECT therapies for MDD.

	Healthy Control			**MDD**		
	Male	Female	**Total**	Male	Female	**Total**
n-value	4	5	**9**	5	5	**10**
Age	47.50±15.351	59.60±14.397	**54.60±15.254**	49.00±14.815	60.2±15.959	**54.22±15.671**
T1 HAM-D	3.00±3.559	3.60±2.408	**3.333±2.784**	30.20±7.854	28.6±6.107	**29.40±6.687**
T2 HAM-D	1.75±3.50	3.60±2.302	**2.778±2.863**	4.00±2.645	3.40±1.140	**3.70±1.947**

Table 5.1. General demographic breakdown of cohort 1. Data: Mean ± SD.

	Cohort 1: MDD Participants' Cumulative Medical History		
	Male	Female	**Total**
n-value	5	5	**10**
Previous Major Depressive Episodes	22	25	**47**
Unipolar Depression	3	4	**7**
Bipolar Depression	2	1	**3**
Other Diagnosed Illnesses	6	10	**16**

Table 5.2. Cumulative medical history for MDD participants in cohort 1.

Recruitment was conducted by St. Patrick's Mental Health Services between 2008 and 2012. Inclusion in the ECT trial required participants to be at least 18 years old, be referred for ECT following a major depressive episode as diagnosed by the Structured Clinical Interview for the DSM-IV Axis I Disorders[263], and have a baseline 24-item HAM-D score greater than or equal to 21[264]. Participants were excluded if they were unfit to receive general anaesthesia or ECT, had ECT is the six months leading up to the study, had a history of schizophrenia or schizoaffective disorder, had a neurodegenerative or other neurological disorder, had alcohol or substance abuse in the six months leading up to the study, had involuntary status, or had an inability or refusal to provide consent. Healthy control participants were recruited by public advertisement[262].

MDD participants received ECT twice weekly under anaesthesia (methohexital 0.75-1 mg/kg) and muscle relaxant (succinylcholine 0.5-1 mg/kg)[265,266]. ECT courses varied by participant in consultation with their clinician, however a maximum limit of 12 treatments was enforced in accordance with recommendations made by the Irish Mental Health Commission[267]. MDD participants continued taking their prescribed pharmacological treatments throughout the ECT trial (Table 5.3). The investigators set a remission criteria of a 60% decrease from baseline HAM-D with an end-of-treatment criteria of less than or equal to a HAM-D score of 10 for two consecutive weeks[268]. For the purposes of this study, MDD participants were selected that returned to "normal" HAM-D scores (0-7 points[269]).

	MDD Medications During Trial		
	Male	Female	**Total**
n-value	5	5	**10**
SSRI	2	0	**2**
TCA	2	1	**3**
MAOI	0	1	**1**
SNRI	1	3	**4**
Mirtazapine	2	1	**3**
Lithium	3	3	**6**
Benzodiazepine	2	2	**4**
Antipsychotic	4	2	**6**
Pregabalin	0	1	**1**
Hypnotic	2	1	**3**
Mood Stabilizer	1	0	**1**
Sodium Valproate	1	0	**1**

Table 5.3. Medications taken by cohort 1 MDD group during ECT trial.

All participants were asked to fast before blood samples were collected. Blood was drawn between 07.30 and 09.30 hours on the first day of ECT treatment for a baseline measurement (T1) and at the same time 1-3 days following the last ECT treatment (T2) for MDD participants. The same time points were used for healthy control assessment days. Blood was collected by venipuncture in K_2EDTA tubes (BD) and centrifuged at 2000 rpm for 10 minutes at 4 °C to extract platelet poor plasma. Samples were aliquoted and stored at -80 °C[268].

Of the available samples, participants with MDD were selected for reported moderate to severe HAM-D scores at T1 (17-23 points for moderate; ≥24 points for severe) and a return to normal HAM-D scores at T2 (0-7 points)[269]. Due to the limited number of samples available, it was not possible to select for previous treatments and medical history other than the main criteria described above. Age and gender matched controls were selected for comparison.

4.1.2. Cohort 2: MDD Time Course Study, Regensburg

Cohort 2 (Table 5.4) samples were initially acquired by researchers in the Department of Psychiatry and Psychotherapy, University of Regensburg, Germany, who were investigating plasma chemokines as potential therapy response markers for MDD[270].

Recruitment was conducted between 2012 and 2016 for an open-label six-week trial. All participants were inpatients and had to be free of any pharmacological treatment at

baseline with the exception of zopiclone (up to 7.5 mg/day) for sleep disturbance and lorazepam (up to 2 mg/day) for anxiety[270]. Specific antidepressant medications prescribed to participants for the course of the study were determined at the discretion and clinical judgement of the attending psychiatrist.

Inclusion in the study required participants: to be at least 18 years old, have a major depressive episode as diagnosed by the Structured Clinical Interview for the DSM-IV Axis I Disorders (German version)[271], and have a baseline 21-item HAM-D score greater than or equal to 18[264]. Participants were excluded if: they had a history of any other psychiatric or somatic disorders, had abnormal laboratory test (*e.g.* blood pressure, electrocardiogram, and electroencephalogram), or were pregnant, or were otherwise unable to give consent[270].

HAM-D assessments and blood draws were conducted at baseline (Week 0) and following six weeks of antidepressant treatment (Week 6) by experienced psychiatrists and laboratory staff[270]. Blood was drawn by venipuncture and collected in lithium-heparin tubes (BD). Blood samples were centrifuged at 2000 g for 10 min at 4 °C to extract platelet poor plasma that was then aliquoted and stored at -80 °C[270].

	Male	**Female**	**Total**
n-value	12	12	**24**
Age	37.67±10.585	33.58±13.10	**35.625±11.835**
W0 HAM-D	21.58±3.579	21.17±2.209	**21.38±2.916**
W6 HAM-D	3.167±2.725	4.75±2.896	**3.958±2.866**
Medications	Escitalopram(5), Mirtazapine(3), Agomelatine(2), Duloxetine(1), Venlafaxine(1)	Escitalopram(6), Mirtazapine(2), Sertraline(2), Valdoxan(1), Duloxetine(1)	**Escitalopram(11), Mirtazapine(5), Agomelatine(2), Sertraline(2), Duloxetine(2), Valdoxan(1), Venlafaxine(1)**

Table 5.4. General demographic breakdown of cohort 2. Data: Mean ± SD.

As with cohort 1, plasma samples selected in this study were chosen from participants that reported moderate to severe HAM-D scores at Week 0 (17-23 points for moderate; ≥24 points for severe) and returned to normal HAM-D scores at Week 6 (0-7 points)[269]. Equal number of male and female participants were selected, however due to the limited number of samples, antidepressant class could not be included as a criterion. As with cohort 1, it was determined that choosing samples from participants with a robust change

in HAM-D scores provided the best opportunity to answer if α-Tubs change expression in blood plasma following successful treatment.

4.2. Statistical Analysis

4.2.1. Data and Statistical Analysis of Human Cohort 1

Analysis was carried out in InVivo Stat 3.7.0.0. Graphpad Prism 8 was used to make all graphs.

A repeated measure mixed model analysis[182] with planned comparisons was employed for all endpoints in cohort 1. HAM-D and individual α-Tub targets were analysed using observed values. Ratios of α-Tub expression required $\log_{10}$ transformation prior to analysis however observed means and standard error are represented in plots.

4.2.2. Data and Statistical Analysis of Human Cohort 2

Analysis was carried out in InVivo Stat 3.7.0.0. Graphpad Prism 8 was used to make all graphs.

A repeated measure mixed model analysis[182] was employed for all endpoints in cohort 2. HAM-D and individual α-Tub targets were analysed using observed values. Ratios of α-Tub expression required $\log_{10}$ transformation prior to analysis however observed means and standard error are represented in plots.

5. Results

5.1. Cohort 1: Comparison of Healthy Controls and Individuals Diagnosed with Major Depressive Disorder

5.1.1. Cohort 1: Hamilton Depression Rating Scale Scores

HAM-D scores provided by the collaborator cohort 1 are shown below for Healthy Controls and individuals diagnosed with MDD for T1 and T2.

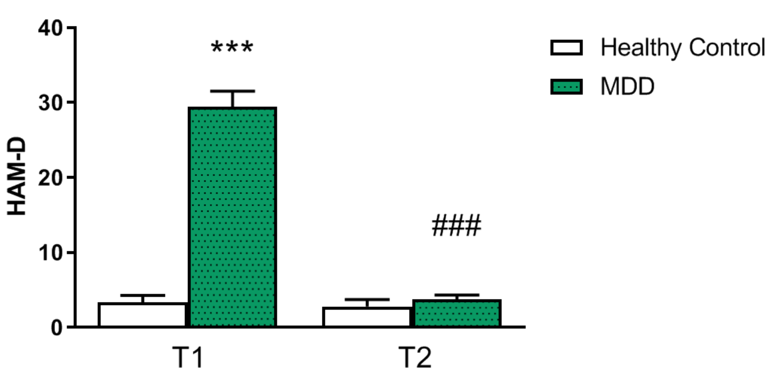

Figure 5.1. Cohort 1 Hamilton Depression Rating Scale (HAM-D) scores. n=9-10/group. Repeated measure mixed model with planned comparisons. Data: Observed Mean ± SEM. ***p<0.001 vs Healthy Control T1. ###p<0.001 vs MDD T1.

At T1 individuals diagnosed with MDD had significantly higher HAM-D scores compared to Healthy Controls ($p<0.001$; Figure 5.1) as would be expected. At T1, the MDD group reported a mean score of 29.40±6.687 standard deviations, which is characterised as severe depression on the scale (≥24 points)[269]. At T2, 1-3 days following ECT treatment, individuals with MDD reported a significantly lower HAM-D score compared to T1 ($p<0.001$; Figure 5.1) with a group mean score of 3.70±1.947 standard deviations, which is characterised as normal on the scale (0-7 points)[269].

5.1.2. Cohort 1: Expression of Total-α-Tubulin (DM1A), Total-α-Tubulin (11H10), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyrosinated-α-Tubulin, and Deglutamylated-α-Tubulin and Ratios in Blood Plasma

Integrated Intensity (Intensity) was calculated for each α-Tub target in blood plasma using the Li-Cor Odyssey System and is represented here for the Healthy Control and MDD groups at T1 and T2. Trends toward a main effect of group was present for Tot-α-Tub (11H10) ($F_{(1,17)}=4.13$, $p=0.0533$) and Δ2-Tub to Tot-α-Tub (11H10) ratio ($F_{(1,17)}=4.34$, $p=0.0527$). Additionally, a trend toward a main effect of time point was present for Δ2-Tub to Tot-α-Tub (DM1A) ratio ($F_{(1,17)}=4.01$, $p=0.0615$).

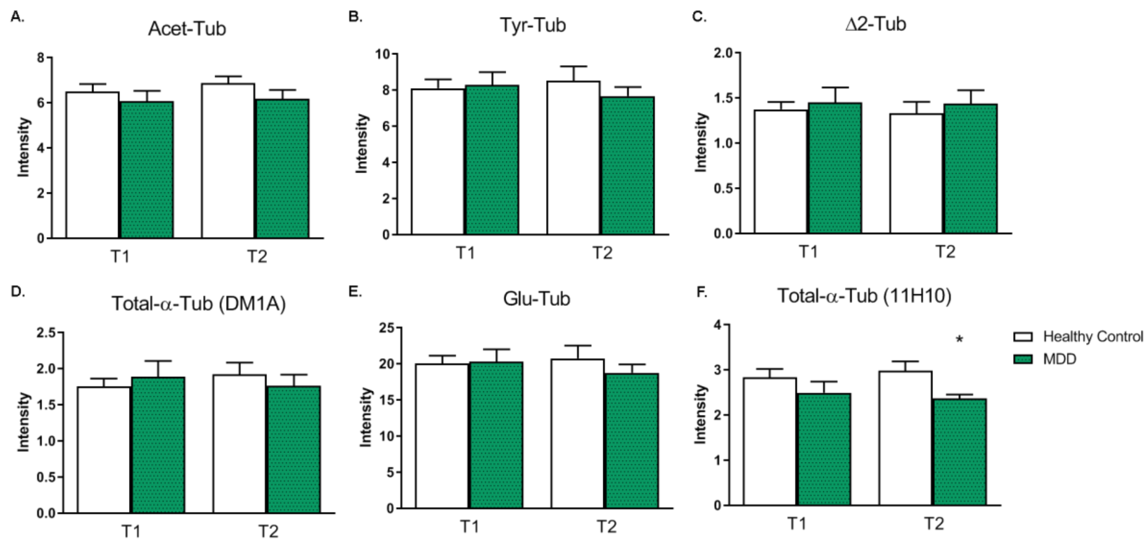

Figure 5.2. Cohort 1 expression of α-Tubulin posttranslational modifications in blood plasma. n=9-10/group. Repeated measure mixed model with planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[B]** Tyr-Tub Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[C]** Δ2-Tub Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[D]** Total-α-Tub(DM1A) Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[E]** Glu-Tub Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[F]** Total-α-Tub(11H10) Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. *p<0.05 vs Healthy Control at T2.

Tot-α-Tub (11H10) was significantly decreased in individuals with MDD compared to Healthy Controls at T2 (p<0.05; Figure 5.2F). A similar trend in Tot-α-Tub (11H10) between Healthy Controls and MDD can be seen at T1 (p=0.0827; Figure 5.2F). No other significant differences between Healthy Controls and MDD were found. Likewise, no significant changes were found within group between T1 and T2 for any target.

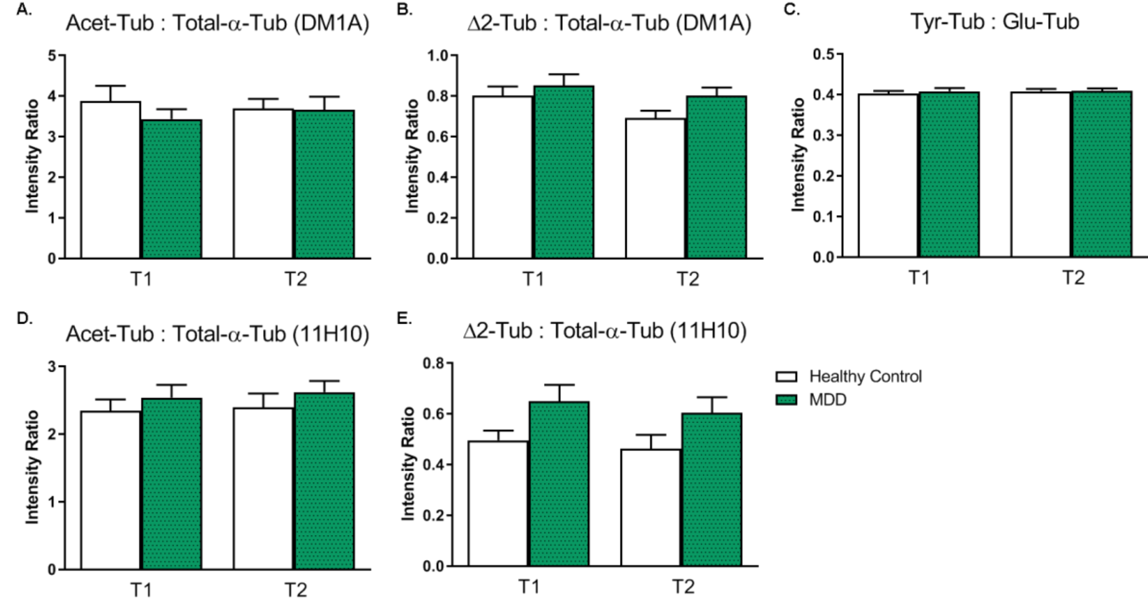

Figure 5.3. Cohort 1 ratios of α-Tubulin posttranslational modifications in blood plasma. n=9-10/group. Repeated measure mixed model with planned comparisons. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[D]** Acet-Tub:Total-α-Tub(11H10) Intensity Ratio in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[E]** Δ2-Tub:Total-α-Tub(11H10) Intensity Ratio in blood plasma for Healthy Controls and MDD groups at T1 and T2.

No significant differences were found between Healthy Controls and MDD at T1 or T2 (Figure 5.3), although several trends were present. Δ2-Tub to Tot-α-Tub (DM1A) ratio showed a trend toward significance (p=0.0824) between Healthy Controls and MDD at T2 (Figure 5.3B) and Δ2-Tub to Total-α-Tub (11H10) ratio neared significance between Healthy Controls and MDD at T2 (Figure 5.3E; p=0.0813).

Likewise, no significant differences were found within groups between time points (Figure 5.3), although a trend in Δ2-Tub to Tot-α-Tub (DM1A) ratio can be seen for Healthy Controls between T1 and T2 (Figure 5.3B; p=0.0569).

5.2. Cohort 2: Comparison of Individuals diagnosed with Major Depressive Disorder Before and After Six Weeks of Treatment

5.2.1. Cohort 2: Hamilton Depression Rating Scale Scores

HAM-D scores provided by the collaborator for cohort 2 are shown below for each individual at baseline and at Week 6.

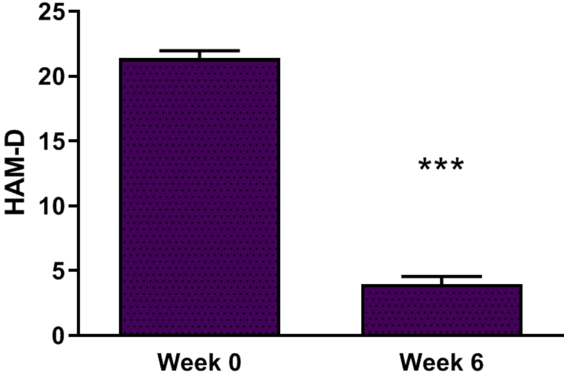

Figure 5.4. Cohort 2 Hamilton Depression Rating Scale (HAM-D) Scores. n=24/group. Repeated measure mixed model. Data: Observed Mean ± SEM. ***p<0.001 vs Week 0.

Individuals diagnosed with MDD reported a mean HAM-D score of 21.38±3.579 standard deviations at baseline (Week 0), which is characterised as moderate depression on the scale (17-23 points for moderate)[269]. Following six weeks of treatment, the group mean HAM-D score decreased to 3.958±2.866 standard deviations, within the normal range on the scale (0-7 points)[269].

5.2.2. Cohort 2: Expression of Total-α-Tubulin (DM1A), Total-α-Tubulin (11H10), Acetylated-α-Tubulin, Tyrosinated-α-Tubulin, Detyrosinated-α-Tubulin, and Deglutamylated-α-Tubulin and Ratios in Blood Plasma

Integrated Intensity (Intensity) was calculated for each α-Tub target in blood plasma using the Li-Cor Odyssey System and is represented here for individuals diagnosed with MDD at baseline (Week 0) and after Week 6 of treatment.

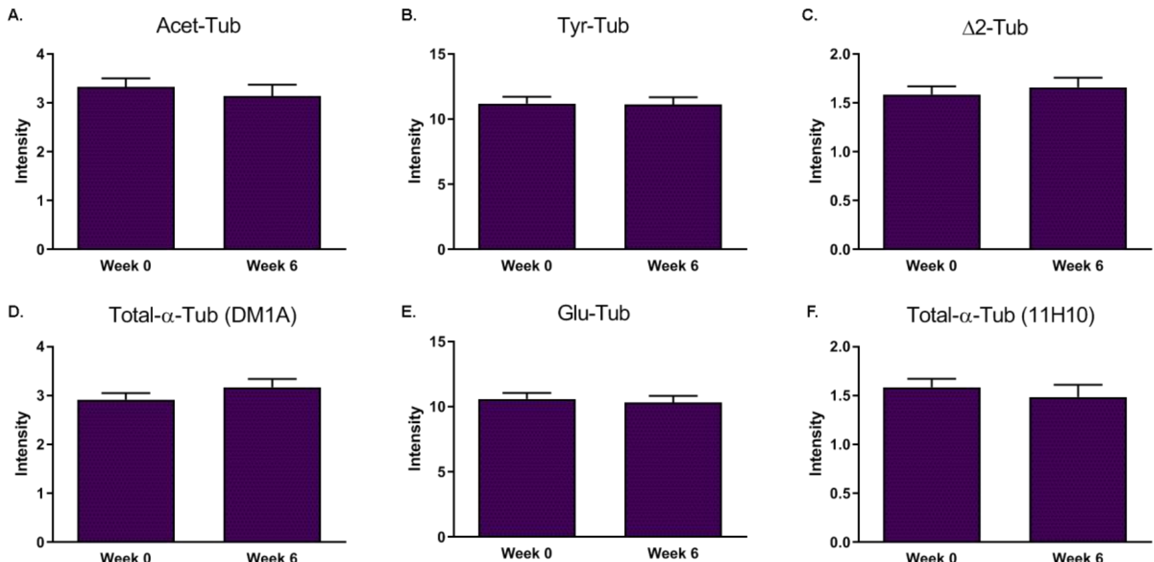

Figure 5.5. Cohort 2 expression of α-Tubulin posttranslational modifications in blood plasma. n=24/group. Repeated measure mixed model. Data: Observed Mean ± SEM. **[A]** Acet-Tub Intensity in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[B]** Tyr-Tub Intensity in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[C]** Δ2-Tub Intensity in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[D]** Total-α-Tub(DM1A) Intensity in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[E]** Glu-Tub Intensity in blood plasma for Healthy Controls and MDD groups at T1 and T2. **[F]** Total-α-Tub(11H10) Intensity in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment.

No significant changes were found between Week 0 and Week 6 for any target in blood plasma from individuals with MDD (Figure 5.5).

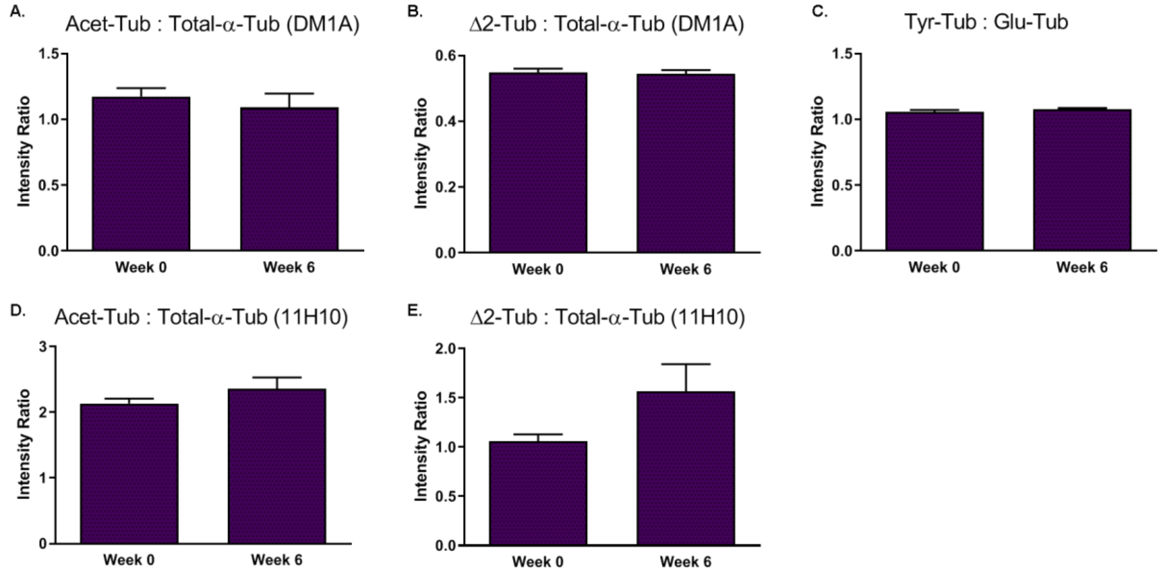

Figure 5.6. Cohort 2 ratios of α-Tubulin posttranslational modifications in blood plasma. n=24/group. Repeated measure mixed model. Data: Observed Mean ± SEM. **[A]** Acet-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[B]** Δ2-Tub:Total-α-Tub(DM1A) Intensity Ratio in blood plasma for individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[C]** Tyr-Tub:Glu-Tub Intensity Ratio in individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[D]** Acet-Tub:Total-α-Tub(11H10) Intensity Ratio in individuals with MDD at baseline (Week 0) and after Week 6 of treatment. **[E]** Δ2-Tub:Total-α-Tub(11H10) Intensity Ratio in individuals with MDD at baseline (Week 0) and after Week 6 of treatment.

No significant changes were found between Week 0 and Week 6 for any α-Tub ratio in blood plasma from individuals with MDD (Figure 5.6).

6. Discussion

6.1. Depressed Individuals Compared to Healthy Controls

Although ECT is thought to recover depressive symptoms by restoring synaptic plasticity mediated by BDNF induction in the hippocampus and PFC[272,273], no significant changes in plasma α-Tubs were identified. For the selected participants in this study, ECT was successful in relieving their depressive symptoms as determined by the HAM-D (Figure 5.1), however there was no difference in α-Tub expression between MDD and controls at baseline (Figure 5.2) or following ECT treatment. Tot-α-Tub (11H10) showed a significant decrease from control post-ECT treatment (Figure 5.2F) but a change in total α-Tub without any accompanying decrease in other PTMs is unconvincing.

A potentially confounding factor in this study is that the participants diagnosed with MDD were already undergoing pharmacological antidepressant treatment, although they were unresponsive. Assuming the near significant group effects for a few of the markers is meaningful, it would suggest that there may be some difference in α-Tub expression between individuals with MDD and healthy controls. However, due to the limited power and MDD participants already receiving pharmacological antidepressant treatment, it is impossible to say if these differences are the result of the disease or treatment. Considering the results of the second cohort and the lack of a change in α-Tub expression from baseline to week six of treatment, discussed below, it would appear that any group effects would more likely be due to the disease itself and that treatment has no impact on α-Tub expression.

The singular significant difference found in Tot-α-Tub (11H10) between MDD and control at T2 (Figure 5.2F) is difficult to interpret, particularly because a similar difference was not found for the other total α-Tub marker (DM1A) (Figure 5.2D) or any other PTMs. This may reflect the different epitopes for the two antibodies. The epitope for Tot-α-Tub (DM1A) is on the C-terminal of α-Tub [274], while the epitope for Tot-α-Tub (11H10) is located at the N-terminus[275]. Both antibodies should have the same relative expression as they are purportedly measuring the same target, so it may be the case that the differences seen here has to do with epitope availability. If there is a difference in epitope availability between the N- and C-terminus of α-Tub, it might suggest a change in function in the whole microtubule; however, what could be leading to this change at this specific epitope in the MDD group is unclear. Further investigation is warranted.

Bearing in mind the limitations and confounding factors in this investigation, it would appear that there is no overall significant difference in the expression of α-Tubs in depressed individual versus healthy controls. Furthermore, given that the MDD

participants were receiving pharmacological treatment prior to and throughout the study and HAM-D scores only changed in response to ECT without a change in α-Tub expression, it is unlikely that α-Tubs indicate a depressed state.

6.2. Effect of Six Weeks of Antidepressant Treatment on α-Tubulins

Pharmacological treatment does not coincide with a significant change in peripheral α-Tub (Figure 5.5 & 5.6) expression although HAM-D scores indicate depressive symptom remission (Figure 5.4). While not of the same specific class, all of the prescribed medications in this cohort act on, at least in part, monoaminergic transmission and in theory should have the same downstream effect of restoring synaptic plasticity as the treatments in cohort 1 and ECT.

It is interesting that Tot-α-Tub (11H10) again had interesting visual results in this cohort (Figure 5.5F & 5.6E). Even with a larger sample size and lack of healthy control group compared to cohort 1, Tot-α-Tub (11H10) expression does not appear to match Tot-α-Tub (DM1A). The different epitopes and implications for MDD versus healthy control are discussed above but it is odd that the same apparent trend exists solely within an MDD cohort. To build from the explanation in the previous section, it may be the case that while antidepressants do not act on individual PTMs of α-Tub, they instead modulate the anchoring of microtubules thereby inflecting neuronal architecture. This explanation infers that measuring total α-Tub levels with the 11H10 antibody is ill-advised as the epitope may be blocked in some circumstances, preventing accurate measurement. Without brain tissue to compare the two total α-Tub antibodies, this potential problem with the 11H10 antibody will be left to speculation. Ultimately, for the purposes of this research the total α-Tub markers are consistent in that neither are significantly different between Week 0 and Week 6 (Figure 5.5D & 5.5F).

Consequently, it appears reasonably clear that the expression of α-Tubs does not indicate treatment efficacy in contrast to significant change in HAM-D from Week 0 to Week 6.

6.3. Peripheral Versus Central Markers

An important caveat to note for the data presented in this chapter and the discussion is that these are peripherally measured markers that are being interpreted as centrally significant. However, the results in Chapter 4 indicated that there was no meaningful connection between α-Tubs expressed centrally and peripherally. How peripheral changes in α-Tub may occur in reference to dysfunction in the CNS is considered more fully in the final chapter.

7. Conclusion

While there are a number of potential confounding factors and limitations in these studies, overall it is clear that a robust change in HAM-D score is not matched by a consistent change in α-Tub expression. In both cohorts, individuals with moderate to severe HAM-D scores (Figures 5.1 and 5.4) returned to normal scores following a course of treatment and at best some potential trends were found. However, potential trends for a treatment effect that can be seen in cohort 1 are not seen in cohort 2.

This is also in line with what was seen in the previous chapter where robust behavioural changes in the Forced Swim Test were not matched by consistent changes in the α-Tub expression in tissue.

Whether individuals with MDD express α-Tubs differently than healthy controls remains inconclusive with this data. Acknowledging the small sample size and confounding factors, this question warrants further investigation.

It is certainly possible that even subtle changes in synaptic plasticity and intracellular mechanisms can result in robust behavioural changes. However, assuming this is the case, using α-Tubs as peripheral marker for disease severity and treatment efficacy seems impractical, at least with the detection methods used here. This will be discussed further in the final chapter.

Chapter 6: General Discussion

1. Aims

The main hypotheses of this thesis is that α-tubulin (α-Tub) and its posttranslational modifications (PTMs) are suitable biomarkers for Major Depressive Disorder (MDD) acting as indicators of depression severity and that they will also indicate treatment efficacy. To investigate these hypotheses, human and animal tissue were assessed for α-Tub expression in conjunction with behavioural endpoints and clinical data.

Post-mortem and magnetic resonance imaging (MRI) analysis of depressed individuals has shown reduced hippocampal volume and decreased neurogenesis and synaptogenesis, implicating changes in synaptic plasticity in the pathophysiology of MDD[21,30,31]. Additionally, preclinical studies have found altered synaptic plasticity in animal models of stress and depression with the microtubule (MT) system being implicated in particular[24,236]. Further investigations found that α-Tub, a main constituent of MTs, has altered expression in brain tissue in these models and could be restored with antidepressant treatment, again indicating a role of synaptic plasticity in the underlying pathophysiology of MDD[26,126]. α-Tubs are readily detectable in blood plasma, creating the opportunity for peripheral detection of antidepressant action and depression severity in patients.

2. Main Findings

In both practical and theoretical terms, it appears reasonably clear from the results of these studies that α-Tubs are not suitable biomarkers for either antidepressant efficacy or MDD disease severity in either preclinical or clinical applications. Much of the research in this area, as within this thesis, has taken a two-pronged approach investigating markers for the disease itself and for treatment efficacy. α-Tubs do not meet the standard of either a diagnostic or monitoring biomarker based on the data presented here and consequently, would not appear to have translational value.

The difficulty in selecting biomarkers for MDD severity is exemplified by the calculation that based on DSM-V criteria there are 227 combinations of MDD symptoms, or to borrow the authors' phrasing, 227 ways to have a depressive episode[276]. This incredible heterogeneity in clinical symptoms makes identifying a common underlying pathology difficult if not impossible and has resulted in the fracturing of MDD into more strictly defined subtypes, such as: treatment resistant depression and immune-metabolic depression[277,278]. That said, the literature on the involvement of synaptic plasticity in MDD appears robust no matter what the underlying cause of the disease may be. However, based on the work presented here, there appears to be a disconnect between dysregulation of α-Tub mediated synaptic plasticity and treatment response as both

clinical and preclinical studies failed to significantly alter α-Tub while promoting recovery of behaviour related to MDD.

While our understanding of psychiatric disease and the brain are growing incrementally, there are limited examples of individual markers being identified for psychiatric diseases and these are primarily genetic markers where the presence of specific alleles can be identified as risk factors[279].

2.1. Animal Behaviour

The results obtained from the forced swim test (FST) matched expectations with the literature considering the combination of experimental factors that were unique to this study. Using Sprague Dawley (SD) rats as a "healthy control" strain proved useful in direct comparison with the "depressed" Wistar Kyoto (WKY) rat. Omitting the pre-test session ensured that the SDs did not experience a state of despair prior to the FST test session and consequently did not respond to antidepressant treatment on the whole. Furthermore, the inclusion of a healthy control strain is also useful in identifying compounds that may create false positives in the FST or at least highlight differences in the therapeutic action of various compounds. For example, although desipramine (DMI) has been shown to be effective as an antidepressant treatment, its activity in the FST does not fully reflect this. The significant reduction in immobility caused by DMI is due to the noradrenergic effects compared to the other treatments as described in the discussion of Chapter 3. The action of the other treatments on individual behaviours is also broken down in the discussion of Chapter 3.

Refining the FST design to accommodate a "healthy control" strain and a "depressed" strain is also useful by eliminating the pre-test session and reducing the time it takes to run a FST study. Eliminating the pre-test also allowed for a more clinically relevant chronic dosing regimen to be employed by not having the dosing regimen bookended by the test sessions, thereby improving the translational relevance of the FST.

Lastly, that no meaningful differences were found in corticosterone levels between SD and WKY from the FST suggests that stress was not impactful on their behaviour or on α-Tub expression.

2.2. α-Tubulins as Biomarkers

Considering the evidence suggesting that antidepressants act by modulating MT dynamics thereby restoring synaptic plasticity[280], it is surprising that these studies did not find α-Tub useful in detecting these changes in either the brain or blood plasma. For example, chronic fluoxetine treatment[281] and DMI[282] treatment have been shown to

increase dendritic spine density, which requires α-Tub as a building block. Furthermore, it has recently been shown that ketamine's rapid antidepressant effect causes a behavioural change in animal models of MDD before restoring dendritic spine formation in the prefrontal cortex, suggesting that restoration in synaptic plasticity is not a prerequisite for successful antidepressant action but may play a role in long-term recovery[283].

One explanation for why no differences were found in α-Tub expression following treatment may be that because of the ubiquity of α-Tub in all cells and there was too much "noise" from numerous sources of α-Tub to detect subtle changes in the target tissue. Even though others have reported being able to detect such changes, they did not use the same Western blotting and statistical techniques used here, which are more accurate than older techniques[131]. Even if the universal expression of α-Tub was unchanged in these tissues, there might still have been changes in the PTMs that would inform the dynamic states of the MTs in these tissues. The lack of PTM differences in brain tissue was particularly surprising here but this may again be due to the PTMs not having different expression on the whole in a depressed state but rather different organisation within the neuron.

Another important theoretical point to consider with these results is the assumption that proteins measured peripherally, in this case blood plasma, directly correlate to what is happening in the brain. There are two ways that neuronal changes might lead to altered peripheral expression in this case. The first is through cellular debris being recycled out of the brain, through the CSF and into the blood stream. This seems unlikely to result in significant changes in protein expression, especially in this case given the ubiquitous expression of α-Tub and although there is evidence for reduced hippocampal volume in depressed individuals, MDD is not generally considered to be neurodegenerative in this way. A similar process is known to happen in true neurodegenerative diseases, such as Parkinson's disease, where α-synuclein in blood plasma has been shown to predict cognitive decline[284].

The second way α-Tub expression might be altered peripherally would be through neuronal signalling pathways regulating peripheral changes. This might also imply that peripheral α-Tub expression had some importance in neuronal regulation of MDD. Although not a direct connection, this peripheral regulation of α-Tubs may be tied to platelet regulation, which has been shown to be important in MDD[285].

2.2.1. α-tubulins in Animal Tissue

The clearest finding here is the consistent higher expression of α-Tub in WKY blood plasma compared to SD. Covered in the discussion of Chapter 4, this is most likely due to the other unique physiological traits bred into the WKY and not necessarily connected to a depressive phenotype.

No consistent differences in α-Tub expression were evident between SD and WKY in brain tissue and no consistent effects of treatment were found. Given the robust behavioural responses seen in the FST this was somewhat surprising but as discussed previously this may in part be due to the ubiquity of α-Tub in the brain. And as noted in the previous section, recovering behaviour in animal models of MDD is not necessarily preceded by recovery in synaptic plasticity or synaptogenesis[283].

2.2.2. α-tubulins in Human Tissue

Based on Hamilton Depressive Rating Scale (HAM-D) scores, participants in these studies reported a remission of depressive symptoms following treatment, although this was not matched by changes in α-Tub in blood plasma. It was in a way beneficial that these participants underwent various treatment plans, or combinations of treatments. If α-Tub was unaffected by these fairly aggressive prescriptions, in particular electroconvulsive therapy, it seems unlikely that it would directly respond to more mild courses of treatment.

However, it should also be stressed that in both cohorts the samples size was relatively small for studies of this kind and was not determined by a power analysis but rather by availability of “responder” participants. Selecting participants with the greatest HAM-D changes was intended to create the highest possibility of seeing any changes or potential trends in α-Tub and its PTMs. It could be said that the one consistent trend was found with the Tot-α-Tub (11H10) marker, however, this requires further validation as it did not match the Tot-α-Tub (DM1A) marker.

Even if the study was underpowered, the inherent variability in the expression of α-Tubs in blood plasma between individuals would make it challenging to use as a marker of disease severity without a robust reference point. There is no “accepted” range for α-Tub expression. As personalised medicine becomes ubiquitous, it may one day be possible to track an individual’s proteomic fingerprint and detect how various treatments effect certain markers. This of course would require data collection at least two time points but also clear expected expression levels for each marker, which are not available for the α-Tubs at present.

With respect to the technique used, it has been argued that only measuring protein levels or expression is not as useful as measuring protein turnover and half-lives[286]. In the case of measuring α-Tub PTMs this may be a better investigative option in identifying subtle changes in expression.

3. Future Directions

It appears clear from these findings that there is no meaningful connection between neuronal α-Tub and α-Tub found in blood plasma. The higher expression of α-Tub seen in WKY blood plasma suggests that other physiological factors are more important in determining α-Tub expression in blood plasma than either a depressed state or antidepressant treatment. Likewise, although no meaningful changes were found in α-Tub expression in blood plasma from depressed patients, participants in these studies reported a remission of symptoms. Any changes that may occur are more likely the result of the liver metabolising the antidepressant rather than the antidepressant changing α-Tub expression in the brain and feeding back into blood plasma.

On a general note for future studies investigating biomarkers for MDD in blood plasma; the potential role of exosomes in delivering these markers into the blood stream should be examined more closely. Techniques now exist that can isolate exosomes from blood samples for a more specific analysis of relevant markers. Although no robust changes were found in α-Tub expression in rodent brain tissue, focusing on exosomal content for either brain or blood plasma may yield interesting results for relevant markers and eliminate the "background noise" from other sources of α-Tub or related markers, such as red blood cells or peripheral blood mononuclear cells. Several of the markers discussed below may be measurable in exosomes in addition to the techniques used in this thesis.

3.1. Tubulins Revisited

Discussed in the last chapter, further investigation of the Tot-α-Tub 11H10 antibody is warranted based on differences found between the MDD and control groups at T2 for both cohorts. The 11H10 antibody has a different epitope than the Tot-α-Tub DM1A antibody and may be sensitive to antidepressant treatment based on these findings.

Additionally, β-Tubulins were not investigated in this thesis but as they also under PTMs and antibodies are commercially available, they may be of interest for comparisons to α-Tubs. It should also be considered that Western blotting is not able to α/β-tubulin polymers and MT polymers. Therefore, other investigative techniques, such as Mass

Spectrometry or High-Performance Liquid Chromatography, should be considered for more detailed analysis of subtle changes to molecular structure.

3.2. Other Targets of Interest

The underlying pathophysiology of MDD involves interplay between α-Tub and other neuronal markers, such as synaptic markers and other cytoskeletal markers. Given the lack of changes in α-Tub expression following treatment, particularly in rodent brain samples, it may be reasonable to investigate other proteins, such as postsynaptic density protein 95 (PSD-95), synaptophysin, and spinophilin. Additionally, proteins that regulate α-Tub expression, particularly in dendrites, should be investigated for their response to antidepressant compounds as they do not necessarily act on α-Tub directly but are mediated through other proteins and signalling pathways, such as BDNF and PI3K/Atk pathway. The recent evidence that antidepressants recover behaviour before restoring synaptic plasticity in the prefrontal cortex at the very least suggests that α-Tub do not mediate a rapid antidepressant response[283].

3.2.1. Actin Filaments

Actin filaments are the primary cytoskeletal protein found in dendritic spines and are responsible for maintaining their structure[287]. PTMs to actin result in the formation of F-actin and G-actin. The ratio of F-actin to G-actin is directly tied to the growth or contraction in dendritic spines. Numerous other modifications to actin have been implicated in depression and have been shown to directly impact dendritic spine morphology[288]. Like the PTMs to α-Tub, these modifications to actin can be assessed by numerous techniques.

3.2.2. Synaptic Markers

Along with α-Tub, spinophilin plays an integral role in dendritic spine morphology although it is more closely linked to the regulation of actin than α-Tub[289–291]. Spinophilin has been demonstrated to closely regulate anxiety phenotypes in animal models[292,293] and is thought to play a role in the underlying pathophysiology of MDD although whether it is up- or down-regulated in unclear[280]. Spinophilin has also been implicated in the therapeutic action of DMI in the FST through pathways activated by the α_2 adrenergic receptor[293]. Likewise, the literature on another synaptic protein, synaptophysin, is unclear. Synaptophysin is nearly ubiquitous in neuronal synapses and consequently can be used as marker for neuroendocrine tumours and quantification of synapses[294]. One post mortem analysis of synaptophysin in brain tissue from depressive suicides did not show significant differences from control tissue however individuals diagnosed with

schizophrenia who committed suicide did, leaving the possibility that altered synaptophysin levels might be tied to suicidality[295].

Lastly, decreased PSD-95 expression in the hippocampus has been tied to depressive phenotypes[296]. NMDA receptor subunits have also been shown to be decreased alongside PSD-95 the frontal cortex of depressed patients, suggesting that PSD-95 expression is tied to glutamatergic signalling[47]. Furthermore, glutamate has been shown to be altered in brain tissue CSF, blood, and brain tissue of depressed patients, which may be useful in assessing treatment efficacy and disease severity[297].

3.2.3. Microtubule Associated Proteins

Microtubule associated proteins (MAPs) are critical for regulating MT structure and function. The primary MAP found in dendrites implicated in MDDs underlying pathophysiology is MAP-2[237,251,252]. MAP-2 was introduced in Chapter 1 as a target of pregnenolone and pregnenolone-methyl-ether, which are purported to be rapid acting antidepressants[26]. MAP-2 can be measured both centrally and peripherally in blood plasma[250]. MAP-1 has also been shown to be upregulated by stress in the hippocampus with a corresponding reduction in synaptophysin[298].

3.3. Multiplexing

One improvement that can be made in future investigations of this kind comes with better technology. While Western blotting is a useful tool in preliminary investigations using a limited number of samples and targets, it is not well suited for high throughput studies with a high number of samples or multiple targets[299]. Depending on the design requirements for a study, there are several options for platforms that allow for customizable multiplexing that would greatly increase reproducibility while reducing the variability introduced by multiple Western blot runs. Two examples of platforms that would improve this area of the study design are Meso Scale Discovery and the Ella ELISA system produced by ProteinSimple.

3.4. Machine Learning and Bioinformatics

Building off the multiplex platforms, the complex nature and interplay of the target proteins in this study would benefit from modern analytical techniques better suited to handling these large data sets. The proteomic studies in this thesis were carefully designed to appropriately analyse specific markers either in isolation or as a direct ratio to another meaningful marker, however new analytic techniques are able to incorporate numerous markers into one statistical framework or model. Indeed, recent reviews of the biomarkers in MDD have called for this kind of analysis.

“Omics integration” in precision medicine is able to account for multiple data sets and create predictive models[300]. To give an example of how it would work within this thesis the human data is best considered. Participants in these studies gave complete medical histories in addition to the HAM-D assessments prior to the analysis of α-Tub in blood plasma. Integrating the “omics” in this case would bring the molecular and medical data together to create unique patient profiles. In theory, this technique would be able to help overcome the limitations of small sample sizes and eliminate the constraint of acceptable α-Tub expression ranges mentioned above by using each individual as their own control. For example, the data collected at two time points from participants in cohort 2 who underwent six weeks of antidepressant treatment would be useful in identifying collective omics change.

To the best of my knowledge there is only one published report successfully using a technique like this to create a “MDDScore”. Researchers combined nine serum based markers with medical histories to create a predictive algorithm for MDD [301]. The markers chosen by these researchers are combination of inflammatory, neurotrophic, metabolic, and HPA- axis markers that were identified from an original 33 marker panel. While psychiatrists may not need help in identifying and diagnosing MDD, there is still room for improvement in identifying efficacious antidepressants for each individual. Interestingly, MDDScore has also been reported to help in clinical situations where the patient is in denial about their condition[302].

4. Final Thoughts

Even though individual α-Tub assessments as biomarkers for MDD did not identify changes in parallel to preclinical behavioural data or human clinical data, α-Tub and MT dynamics remain a focal point of biomarker development for MDD due to their proximity and interplay with other well-established markers and dysregulated systems. Taking a cue from MDDScore, the future of biomarker development for psychiatric diseases most likely lies in new analytical, statistical, and algorithmic techniques that are better able to find subtle changes and trends that can’t be seen with individual marker analysis.

Such improvements would not only help in screening patients for mood disorders and potentially identify subtypes of mood disorders but would also be a valuable tool in preclinical drug screening. Another benefit would be in the identification of which markers are the most translationally relevant and respond to antidepressant treatment thereby increasing the power of preclinical models and increase the chances of success in clinical trials.

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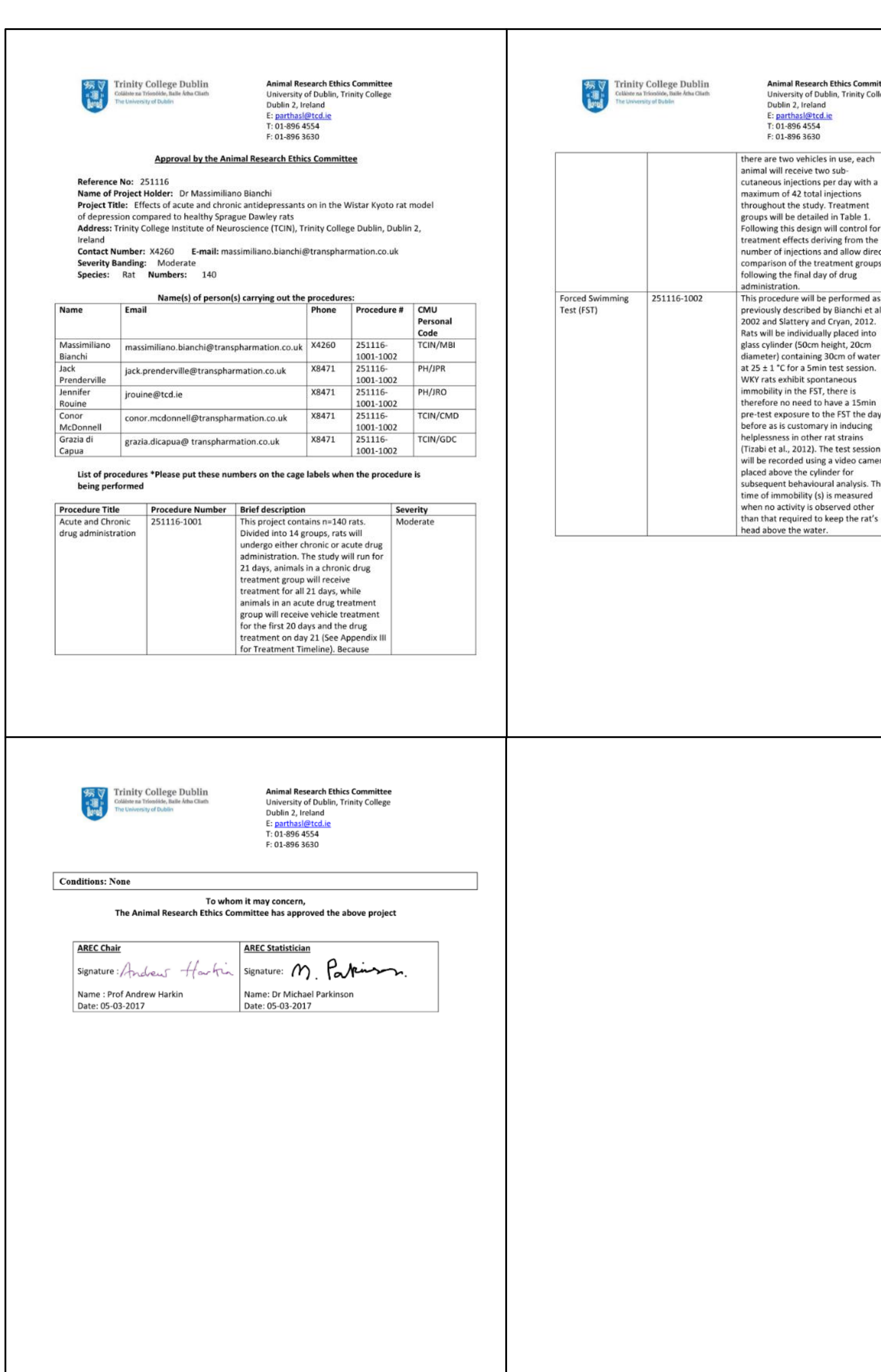
Appendices

A. Ethical Approvals

A.1. Chapters 3 and 4



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Approval by the Animal Research Ethics Committee

Reference No: 251116
Name of Project Holder: Dr Massimiliano Bianchi
Project Title: Effects of acute and chronic antidepressants on in the Wistar Kyoto rat model of depression compared to healthy Sprague Dawley rats
Address: Trinity College Institute of Neuroscience (TCIN), Trinity College Dublin, Dublin 2, Ireland
Contact Number: X4260 **E-mail:** massimiliano.bianchi@transpharmation.co.uk
Severity Banding: Moderate
Species: Rat **Numbers:** 140

Name(s) of person(s) carrying out the procedures:

Name	Email	Phone	Procedure #	CMU Personal Code
Massimiliano Bianchi	massimiliano.bianchi@transpharmation.co.uk	X4260	251116-1001-1002	TCIN/MBI
Jack Prenderville	jack.prenderville@transpharmation.co.uk	X8471	251116-1001-1002	PH/JPR
Jennifer Rouine	jrouine@tcd.ie	X8471	251116-1001-1002	PH/JRO
Conor McDonnell	conor.mcdonnell@transpharmation.co.uk	X8471	251116-1001-1002	TCIN/CMD
Grazia di Capua	grazia.dicapua@ transpharmation.co.uk	X8471	251116-1001-1002	TCIN/GDC

List of procedures *Please put these numbers on the cage labels when the procedure is being performed

Procedure Title	Procedure Number	Brief description	Severity
Acute and Chronic drug administration	251116-1001	This project contains n=140 rats. Divided into 14 groups, rats will undergo either chronic or acute drug administration. The study will run for 21 days, animals in a chronic drug treatment group will receive treatment for all 21 days, while animals in an acute drug treatment group will receive vehicle treatment for the first 20 days and the drug treatment on day 21 (See Appendix III for Treatment Timeline). Because	Moderate

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		there are two vehicles in use, each animal will receive two sub-cutaneous injections per day with a maximum of 42 total injections throughout the study. Treatment groups will be detailed in Table 1. Following this design will control for treatment effects deriving from the number of injections and allow direct comparison of the treatment groups following the final day of drug administration.	
Forced Swimming Test (FST)	251116-1002	This procedure will be performed as previously described by Bianchi et al., 2002 and Slattery and Cryan, 2012. Rats will be individually placed into glass cylinder (50cm height, 20cm diameter) containing 30cm of water at 25 ± 1 °C for a 5min test session. WKY rats exhibit spontaneous immobility in the FST, there is therefore no need to have a 15min pre-test exposure to the FST the day before as is customary in inducing helplessness in other rat strains (Tizabi et al., 2012). The test session will be recorded using a video camera placed above the cylinder for subsequent behavioural analysis. The time of immobility (s) is measured when no activity is observed other than that required to keep the rat's head above the water.	Moderate

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Animal Research Ethics Committee
University of Dublin, Trinity College
Dublin 2, Ireland
E: parthasl@tcd.ie
T: 01-896 4554
F: 01-896 3630

Conditions: None

To whom it may concern,
The Animal Research Ethics Committee has approved the above project

AREC Chair	AREC Statistician
Signature: Andrew Harkin	Signature: M. Parkinson
Name : Prof Andrew Harkin Date: 05-03-2017	Name: Dr Michael Parkinson Date: 05-03-2017

A.2. Chapter 5

Coláiste na Tríonóide, Baile Átha Cliath
Trinity College Dublin
Ollscoil Átha Cliath | The University of Dublin

23rd September 2019

Att: Conor McDonnell

Re: Application 20190219

Title: Time-course study of potential biomarkers of antidepressant efficacy in blood plasma taken from individuals diagnosed with Major Depressive Disorder.

Dear Mr McDonnell,

Your application has been reviewed by the School of Medicine Research Ethics Committee and we are pleased to inform you that the above project has been approved as of 20th March 2019, following the committee meeting.

Applicants must submit an *annual report* for ongoing projects and an *end of project report* upon completion of the study. You will find these forms on the School of Medicine Research Ethics website.

It is the responsibility of the researcher/research team to ensure all aspects of the study are executed in compliance with the General Data Protection Regulation (GDPR) and Data Protection Act 2018.

Yours sincerely,

Dr. Tadhg Stapleton
Chairperson,
School of Medicine Research Ethics Committee.

An tOllamh Michael Gill MD FTCD MRCPsych

Dr. Alex McKee

Scoil an Leighis
Institiúid Eolaíochtaí Bithleighis Choláiste na Tríonóide
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B. Analysis of Variance Tables

B.1. Chapter 3

B.1.1. Total Immobility

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
BLOCK	16622.26	2	8311.132	23.39	< 0.0001
Strain	25916.19	1	25916.189	72.93	< 0.0001
Treatment	30140.31	6	5023.384	14.14	< 0.0001
Strain * Treatment	6104.33	6	1017.388	2.86	0.0121
Residuals	43000.79	121	355.378		

B.1.2. Immobility Counts

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
BLOCK	1595.16	2	797.582	21.12	< 0.0001
Strain	3273.50	1	3273.499	86.70	< 0.0001
Treatment	1915.52	6	319.253	8.46	< 0.0001
Strain * Treatment	615.59	6	102.598	2.72	0.0163
Residuals	4643.94	123	37.756		

B.1.3. Swimming Counts

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
BLOCK	179.76	2	89.881	2.67	0.0733
Strain	191.79	1	191.790	5.70	0.0185
Treatment	1979.34	6	329.891	9.80	< 0.0001
Strain * Treatment	488.10	6	81.350	2.42	0.0306
Residuals	4107.74	122	33.670		

B.1.4. Diving Counts

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
BLOCK	78.22	2	39.111	10.42	< 0.0001
Strain	0.74	1	0.741	0.20	0.6577
Treatment	13.44	6	2.239	0.60	0.7328
Strain * Treatment	22.68	6	3.780	1.01	0.4241
Residuals	465.58	124	3.755		

B.1.5. Climbing Counts

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
BLOCK	332.24	2	166.122	4.96	0.0085
Strain	4796.15	1	4796.150	143.13	< 0.0001
Treatment	7414.00	6	1235.666	36.87	< 0.0001
Strain * Treatment	1568.30	6	261.383	7.80	< 0.0001
Residuals	4155.26	124	33.510		

B.1.6. Plasma Corticosterone

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
BLOCK	935.18	2	467.591	18.32	< 0.0001
Strain	89.22	1	89.215	3.50	0.0640
Treatment	237.00	6	39.500	1.55	0.1686
Strain * Treatment	203.44	6	33.907	1.33	0.2498
Residuals	3037.54	119	25.526		

B.1.7. Plasma Corticosterone: Naïve Animals and Treated Animals

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
Strain	63.33	1	63.335	1.10	0.3050
Treatment (Naïve vs Treated)	9731.04	1	9731.039	168.78	< 0.0001
Strain * Treatment	250.99	1	250.987	4.35	0.0477
Residuals	1383.69	24	57.654		

B.2. Chapter 4

B.2.1. Plasma Markers

B.2.1.1. Acetylated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.27	5	0.054	2.80	0.0199
Strain	11.77	1	11.770	608.16	< 0.0001
Treatment	0.10	6	0.017	0.86	0.5296
Strain * Treatment	0.06	6	0.009	0.48	0.8250
Residuals	2.30	119	0.019		

B.2.1.2. Total-α-Tubulin (DM1A)

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.51	5	0.102	7.31	< 0.0001
Strain	11.04	1	11.039	790.79	< 0.0001
Treatment	0.13	6	0.022	1.56	0.1653
Strain * Treatment	0.06	6	0.010	0.73	0.6264
Residuals	1.62	116	0.014		

B.2.1.3. Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.48	5	0.097	17.00	< 0.0001
Strain	0.03	1	0.034	5.99	0.0159
Treatment	0.03	6	0.005	0.87	0.5228
Strain * Treatment	0.01	6	0.001	0.20	0.9749
Residuals	0.67	118	0.006		

B.2.1.4. Tyrosinated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.60	5	0.119	8.01	< 0.0001
Strain	12.49	1	12.495	841.26	< 0.0001
Treatment	0.12	6	0.021	1.39	0.2255
Strain * Treatment	0.10	6	0.017	1.14	0.3427
Residuals	1.72	116	0.015		

B.2.1.5. Detyrosinated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	1.71	5	0.342	25.22	< 0.0001
Strain	6.82	1	6.823	502.75	< 0.0001
Treatment	0.06	6	0.009	0.69	0.6566
Strain * Treatment	0.04	6	0.006	0.45	0.8456
Residuals	1.61	119	0.014		

B.2.1.6. Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.63	5	0.126	21.77	< 0.0001
Strain	0.59	1	0.592	102.03	< 0.0001
Treatment	0.01	6	0.002	0.34	0.9133
Strain * Treatment	0.03	6	0.006	0.97	0.4491
Residuals	0.68	118	0.006		

B.2.1.7. Transferrin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.30	5	0.061	25.74	< 0.0001
Strain	0.06	1	0.056	23.81	< 0.0001
Treatment	0.03	6	0.006	2.40	0.0319
Strain * Treatment	0.01	6	0.002	0.91	0.4902
Residuals	0.28	120	0.002		

B.2.2. Hippocampus Markers

B.2.2.1. Acetylated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	1.35	5	0.270	9.36	< 0.0001
Strain	0.01	1	0.014	0.50	0.4817
Treatment	0.43	6	0.072	2.49	0.0263
Strain * Treatment	0.21	6	0.035	1.20	0.3087
Residuals	3.43	119	0.029		

B.2.2.2. Total-α-Tubulin (DM1A)

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	12.62	5	2.524	41.39	< 0.0001
Strain	0.10	1	0.100	1.63	0.2036
Treatment	0.35	6	0.058	0.96	0.4563
Strain * Treatment	0.15	6	0.026	0.42	0.8642
Residuals	7.26	119	0.061		

B.2.2.3. Tyrosinated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	1.22	5	0.243	16.57	< 0.0001
Strain	0.02	1	0.018	1.25	0.2657
Treatment	0.16	6	0.027	1.87	0.0920
Strain * Treatment	0.03	6	0.005	0.33	0.9197
Residuals	1.75	119	0.015		

B.2.2.4. Detyrosinated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	1.02	5	0.204	20.14	< 0.0001
Strain	0.14	1	0.140	13.75	0.0003
Treatment	0.14	6	0.023	2.28	0.0407
Strain * Treatment	0.02	6	0.003	0.26	0.9549
Residuals	1.21	119	0.010		

B.2.2.5. Deglutamylated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	1.79	5	0.357	41.99	< 0.0001
Strain	0.00	1	0.002	0.21	0.6461
Treatment	0.08	6	0.014	1.61	0.1499
Strain * Treatment	0.02	6	0.003	0.31	0.9295
Residuals	1.01	119	0.009		

B.2.2.6. Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	5.22	5	1.045	38.82	< 0.0001
Strain	0.05	1	0.047	1.74	0.1894
Treatment	0.06	6	0.010	0.37	0.8942
Strain * Treatment	0.15	6	0.026	0.96	0.4558
Residuals	3.15	117	0.027		

B.2.2.7. Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A) Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	3.70	5	0.741	23.59	< 0.0001
Strain	0.03	1	0.032	1.03	0.3118
Treatment	0.15	6	0.024	0.77	0.5948
Strain * Treatment	0.14	6	0.024	0.75	0.6100
Residuals	3.61	115	0.031		

B.2.2.8. Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.12	5	0.024	16.22	< 0.0001
Strain	0.05	1	0.050	33.20	< 0.0001
Treatment	0.01	6	0.002	1.01	0.4238
Strain * Treatment	0.01	6	0.001	0.60	0.7315
Residuals	0.17	116	0.002		

B.2.3. Prefrontal Cortex Markers

B.2.3.1. Acetylated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.97	5	0.195	2.42	0.0395
Strain	0.51	1	0.511	6.34	0.0131
Treatment	0.25	6	0.042	0.52	0.7905
Strain * Treatment	0.48	6	0.081	1.00	0.4269
Residuals	9.67	120	0.081		

B.2.3.2. Total-α-Tubulin (DM1A)

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	2.97	5	0.595	6.69	< 0.0001
Strain	0.48	1	0.479	5.38	0.0220
Treatment	0.65	6	0.109	1.22	0.3002
Strain * Treatment	0.80	6	0.133	1.49	0.1859
Residuals	10.67	120	0.089		

B.2.3.3. Tyrosinated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	2.26	5	0.452	13.93	< 0.0001
Strain	0.12	1	0.119	3.66	0.0582
Treatment	0.16	6	0.027	0.84	0.5423
Strain * Treatment	0.22	6	0.037	1.15	0.3365
Residuals	3.89	120	0.032		

B.2.3.4. Detyrosinated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	8.79	5	1.759	95.58	< 0.0001
Strain	0.35	1	0.351	19.08	< 0.0001
Treatment	0.13	6	0.021	1.15	0.3367
Strain * Treatment	0.12	6	0.020	1.06	0.3883
Residuals	2.21	120	0.018		

B.2.3.5. Deglutamylated-α-Tubulin

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	0.64	5	0.129	12.69	< 0.0001
Strain	0.08	1	0.082	8.06	0.0053
Treatment	0.10	6	0.017	1.72	0.1222
Strain * Treatment	0.10	6	0.017	1.70	0.1278
Residuals	1.22	120	0.010		

B.2.3.6. Acetylated-α-Tubulin to Total-α-Tubulin (DM1A) Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	2.51	5	0.503	17.02	< 0.0001
Strain	0.00	1	0.001	0.02	0.8939
Treatment	0.13	6	0.022	0.74	0.6190
Strain * Treatment	0.28	6	0.046	1.56	0.1654
Residuals	3.54	120	0.030		

B.2.3.7. Deglutamylated-α-Tubulin to Total-α-Tubulin (DM1A) Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	1.26	5	0.253	5.34	0.0002
Strain	0.16	1	0.165	3.48	0.0646
Treatment	0.33	6	0.055	1.16	0.3333
Strain * Treatment	0.35	6	0.058	1.23	0.2935
Residuals	5.68	120	0.047		

B.2.3.8. Tyrosinated-α-Tubulin to Detyrosinated-α-Tubulin Ratio

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
IFWB SET	2.69	5	0.537	103.91	< 0.0001
Strain	0.06	1	0.056	10.91	0.0013
Treatment	0.02	6	0.004	0.74	0.6166
Strain * Treatment	0.03	6	0.005	0.99	0.4335
Residuals	0.62	119	0.005		

B.2.3.9. Hippocampus BDNF

	Sums of squares	Degrees of freedom	Mean square	F-value	p-value
Plate	0.19	3	0.065	23.49	< 0.0001
Strain	0.02	1	0.016	5.80	0.0176
Treatment	0.03	6	0.005	1.69	0.1291
Strain * Treatment	0.03	6	0.005	1.67	0.1352
Residuals	0.33	119	0.003		

C. **List of Conference Poster Presentations and Publications**

- McDonnell, C.W., Prenderville, J.A., Rouine, J., Burke, T., Dunphy-Doherty, F., Lobato, J.P., Mombereau, C., Gill, M., Bianchi, M. Wistar Kyoto rat in the forced swimming test as a model for treatment-resistant depression: Efficacy of current and novel antidepressants drugs. The International College of Neuropsychopharmacology 2017. Prague, Czech Republic.
- McDonnell, C.W., Prenderville, J.A., Rouine, J., Burke, T., Dunphy-Doherty, F., Lobato, J.P., Gill, M., Bianchi, M. Effects of antidepressant drugs on plasma microtubular proteins in “healthy” Sprague Dawley and “depressed” Wistar Kyoto rats. British Association for Psychopharmacology 2018. London, UK.
- McDonnell, C.W., Gill, M., Upton, N., Bianchi, M. Effect of acute and chronic antidepressant treatment of the Wistar Kyoto rat in Forced Swim test and on corticosterone expression. European College of Neuropsychopharmacology 2019. Copenhagen, Denmark.
- McDonnell, C., Colic, L., Li, M., Woelfer, M., Liebe, T., Kretzschmar, M., Speck, O., Schott, B.H., Bianchi, M., Walter, M. Neuronal glutamatergic changes and peripheral markers of cytoskeleton dynamics change synchronically 24 h after sub-anaesthetic dose of ketamine in healthy subjects. *Behavioural Brain Research* 359: 312-9 (2019).