Time-dependent parallel and synergistic antidepressant-like effects of reelin and ketamine in an animal model of chronic stress

by

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Abstract

Depression is the leading cause of global disability, disproportionately affecting females. There is a pressing need for novel therapeutics as approximately 30% of those diagnosed do not respond adequately to first line treatment. At the forefront of research for novel therapeutics is ketamine, an N-methyl-d-aspartate receptor (NMDAR) antagonist that has rapid antidepressant effects following a single sub-anesthetic dose. Ketamine can ameliorate traditionally hard to treat symptoms (e.g., anhedonia and suicidal ideation), although its use is limited due to psychomimetic side-effects and high abuse potential. Further, ketamine's exact underlying mechanisms remain unknown.

Reelin is an extracellular matrix glycoprotein that appears to be downregulated in the hippocampus of patients with depression. Our lab has shown that exogenous reelin administration can produce rapid-acting antidepressant-like effects. While the molecular signaling pathway of reelin remains elusive, research indicates that reelin and ketamine may share certain mechanisms of action.

The purpose of this thesis is to examine the parallel and synergistic antidepressant-like effects of reelin and ketamine in the pursuit of developing a putative reelin-based therapeutic.

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All figures presented in this thesis were created by the author (or collaboratively when indicated) using a combination of BioRender, Graphpad Prism, and Excel.

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Glossary of abbreviations

(2R,6R)-HNK	(2R,6R)-hydroxynorketamine
5-HT	5-hydroxytryptamine (serotonin)
5-HT2AR	Serotonin 2A receptor
ABC	Avidin-biotin complex
ACTH	Adrenocorticotrophic hormone
AMPA/R	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid / receptor
ANOVA	Analysis of variance
BDI	Beck depression inventory
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CA	Cornu ammonis
cAMP	Cyclic adenosine monophosphate
CK	Corticosterone/ketamine
CKR	Corticosterone/ketamine/reelin
CORT	Corticosterone
CR	Corticosterone/reelin
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CV	Corticosterone/vehicle
DAB	3’-diaminobenzidine
Dab1	Disabled-1
DCX	Doublecortin
DG	Dentate gyrus
DSM-5	Diagnostic and statistical manual of mental disorders (5th Edition)
EC	Entorhinal cortex
ECT	Electroconvulsive therapy
EPM	Elevated plus maze
FDA	Food and drug administration
fEPSP	Field excitatory post-synaptic potential
FST	Forced swim test

GABA	Gamma-aminobutyric acid
$GABA_{A/B}Rs$	$GABA_A$ or $GABA_B$ receptors
GAD65/67	Glutamate acid decarboxylase-65/67
GCL	Granule cell layer
GR	Glucocorticoid receptor
HDRS	Hamilton depression rating scale
HPA	Hypothalamic-pituitary-adrenal
IgG	Immunoglobulin G
ICC	Immunocytochemistry
IHC	Immunohistochemistry
i.p.	Intraperitoneal
iPSC	Induced pluripotent stem cell
i.v.	Intravenous
IR	Immunoreactive
LTD	Long-term depression
LTP	Long-term potentiation
MADRS	Montgomery-Åsberg depression rating scale
MAOI	Monoamine oxidase inhibitor
MCP	Monocyte chemoattractant protein
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
MPC	Membrane protein clustering
MR	Mineralocorticoid receptors
mTORC1	Mechanistic target of rapamycin complex 1
NGS	Normal goat serum
NHS	Normal horse serum
NOS	Nitric oxide synthase
NMDA/R	*N*-methyl-D-aspartate/receptor
p-	phosphorylated-
PBS	Phosphate-buffered saline
PFC	Prefrontal cortex

PKA	Protein kinase A
PKC	Protein kinase C
PL	Polymorphic layer
POR	Patient-oriented research
PSD-95	Postsynaptic density-95
PVN	Paraventricular nucleus
RR	Reelin repeats
s.c.	Subcutaneous
SEM	Standard error of the mean
SERT	Serotonin transporter
SFKs	Src family of non-receptor tyrosine kinases
SGZ	Subgranular zone
SLC6A4	Solute carrier family 6 member 4
SNP	Synaptoneurosome
SNRI	Serotonin–noradrenaline reuptake inhibitor
SPSS	Statistical package for the social sciences
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitor
ST	Sucrose Splash Test
SynI	Synapsin I
TBS	Tris-buffered saline
TCA	Tricyclic antidepressant
TNF-α	Tumor necrosis factor-α
TRD	Treatment-resistant depression
TrkB	Tropomyosin receptor kinase B
UCMS	Unpredictable chronic mild stress
VTA	Ventral tegmental area
VV	Vehicle/vehicle

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Chapter 1: Introduction

1.1 Thesis outline

Major Depressive Disorder (MDD) is the leading cause of disability worldwide and a major contributor to global disease burden (World Health Organization, 2018). Affecting an estimated 350 million people (Gelenberg, 2010; Richards, 2011), MDD is a chronic and debilitating disease characterized by anhedonia, low mood, fatigue, cognitive deficits, and disturbances in sleep and appetite (*Diagnostic and Statistical Manual of Mental Disorders*, 2013). These symptoms cause significant distress accompanied by personal and societal impairment. It is generally accepted that women are twice as likely as men to suffer from MDD (Eid et al., 2019), an effect that emerges in puberty and remains stable throughout adulthood (Salk et al., 2017). Additionally, women have higher symptom severity and more past suicide attempts (Marcus et al., 2008). Depressive disorders are commonly comorbid with other medical and psychiatric disorders (Richards & O'Hara, 2014) and are predictive of poor treatment outcome in medical diagnoses (Oslin et al., 2002; Wang et al., 2020). Despite decades of research the pathophysiology of depression and effective pharmaceuticals remain elusive. This knowledge disparity arises from differences in clinical presentation, treatment-responsiveness, illness course, lack of biomarkers, and genetic polymorphisms (Pryce & Klaus, 2013). Evidently, MDD produces an extraordinary personal, societal, and economic burden. There is a pressing need to understand the biological basis of the disease to develop effective novel antidepressants.

Since its development in the 1950s, the monoamine hypothesis has been used ubiquitously as the basis for pharmacological antidepressant targets (Hirschfeld, 2000). This hypothesis came to fruition when it was observed that inhibiting the breakdown of the monoamine neurotransmitters (serotonin, norepinephrine, and/or dopamine) resulted in affective changes (West & Dally, 1959). Following this discovery, the use of monoamine reuptake inhibitors gained popularity, most notably selective 5-HT reuptake inhibitors (SSRIs) and 5-HT-noradrenaline reuptake inhibitors (SNRIs). Despite their widespread use, these first-line antidepressants have several limitations including adverse side effects, a therapeutic delay of weeks to months, and are ineffective in 30-50% of patients who are considered treatment resistant (Cipriani et al., 2018; Rush et al., 2006; Thase et al., 2001).

Recently it was discovered that ketamine, a non-competitive allosteric N-methyl-D-aspartate receptor (NMDAR) antagonist, can produce rapid acting and transient antidepressant effects. A

single sub-anesthetic dose of ketamine appears to rapidly ameliorate clinical symptoms and biochemical deficits in treatment-naïve and treatment-resistant patients (Browne & Lucki, 2013) without the side-effects from traditional antidepressants (Salvadore & Singh, 2013). This scientific breakthrough led to a surge of research into the cellular and molecular mechanisms involved in ketamine's antidepressant effect, with the goal of developing more effective therapeutics. Preclinical research suggests that synaptic potentiation may underlie ketamine's antidepressant effect (Kim et al., 2021).

Reelin is an extracellular matrix glycoprotein that, in adulthood, regulates many forms of neuroplasticity such as the generation of new-born cells, the formation of dendritic spines, dendritic outgrowth, and increases synaptic connections (Beffert et al., 2006; Bosch et al., 2016; Niu et al., 2004, 2008; Pujadas et al., 2010; Rogers et al., 2013; Teixeira et al., 2012; Ventruti et al., 2011; Weeber et al., 2002). In the 1990s, a reduction in reelin expression in the hippocampus, specifically in the dentate gyrus (DG), was found in post-mortem brain samples from subjects with schizophrenia, bipolar disorder and major depression (Fatemi et al., 2000). This precipitated research into the possible role of hippocampal reelin as a protective factor from depression and related psychiatric illness. Using a chronic stress model of depression, our lab found that repeated corticosterone (CORT) injections decreased reelin expression in the sub-granular zone (SGZ) of the DG and hippocampal neurogenesis, paralleled with depression-like phenotypes (Lebedeva et al., 2017, 2020; Lussier et al., 2009). We then reported that heterozygous reeler mice (*RELN*$^{+/-}$, haplo-insufficient for RELN expressing 40-60% of normal reelin levels), show an increased vulnerability to the depressonergic effects of CORT (Lussier et al., 2011, 2013). Interestingly, both reelin deficiency and behavioural impairments were rescued by conventional and unconventional antidepressants (Brymer et al., 2018; Fenton et al., 2015; Johnston et al., 2020). To explore reelin's potential to produce antidepressant-like effects, we infused reelin first directly into the hippocampus (Brymer et al., 2020) and then peripherally through the tail vein (Allen et al., 2022). We observed that both chronic and acute administration of reelin rescued CORT-induced behavioural and neurochemical deficits at 24 hours post-injection (Allen et al., 2022; Brymer et al., 2020). These findings implicate reelin as a promising candidate for a novel rapid-acting antidepressant. More research into reelin's pharmacokinetics and pharmacodynamics is needed to clarify the time course of drug effect. While the putative molecular mechanisms responsible for reelin's antidepressant effects remain unknown,

significant overlap has been found with that of ketamine (Harraz et al., 2016; Johnston et al., 2020; Koike & Chaki, 2014). Synaptic reelin signaling may even be essential for ketamine's antidepressant effects (Kim et al., 2021).

The purpose of this thesis is to ascertain reelin's time course effect both individually and synergistically with that of ketamine. Using a chronic CORT-model to induce depressive-like phenotypes, I report on the behavioural and neurobiological effects of reelin and ketamine throughout their time-course of action. To evaluate their acute and sustained effects against continued CORT administration, I examined hippocampal reelin levels and serotonergic transporter clusters on peripheral lymphocytes. These experiments were conducted to better understand the pharmacokinetics and pharmacodynamics of reelin, and to lay the groundwork for the development of reelin as a potential fast-acting therapeutic.

1.2 Overview of major depressive disorder

The mental illness crisis is a rising global epidemic. The World Health Organization (2022) reports that about 1 in 4 people suffer from a diagnosable psychiatric disorder at some point in their lives, and that severe mental health conditions cause premature death of 10 to 20 years on average. Global rates of mental illness increased by 25% within the past decade, equivalent to 13% accounting for world population growth, and an astonishing 80% of people do not receive treatment. The two most common mental health conditions in both sexes are depression and anxiety, which are estimated to cost the global economy over US$ 1 trillion each year. In just one year over the COVID-19 pandemic, global disease burden of depressive disorders increased by 28%, and suicide became the second leading cause of death among 15-29-year-olds (World Health Organization, 2022). Depression is pleomorphic in nature, transcending throughout time across all cultures, genders, and ages.

The first documentation of clinical depression can be traced back to 1550 BCE in the Ebers papyrus. Written in hieratic Egyptian writing, this medical papyrus describes a disorder in which the heart is miserable, closed in with darkness, tastes sadness, and is afflicted with moroseness (Bou Khalil & Richa, 2014). Affective disorders were hypothesized to be a disorder of the heart, stemming from an impeded flow of blood and bodily fluids (Bou Khalil & Richa, 2014). This belief was a precursor for Ancient Greek humeral pathology (500 BCE), a system of medicine

that linked mental well-being with harmony in one's physical body. Hippocrates, now known as the Father of Medicine, proposed that imbalances in homeostatic levels of internal fluids led to poor health, attributing melancholy to an excess of black bile from the spleen (Kleisiaris et al., 2014). Despite these medical hypotheses, psychiatric disturbances were most often conceived as a spiritual condition arising from demonic possession and evil spirits. Across ages and cultures, treatment for the mentally ill involved extreme and often harmful practises to exorcise the devils thought to be inhabiting the sufferer's head. The invasive surgery named trepanation, which involved chipping a hole into the skull to provide an opening for the evil spirits to be released, is documented as early as the Neolithic times (Kemp & Williams, 1987). Women often received the worst of these assaults spurred by the theory of the wandering womb, which described female medical pathologies as a result of hysterical suffocation. Female hysteria, which encompassed many symptoms of today's depression and anxiety, was believed to be a source of witchcraft (Hirschfeld, 2000). In extreme cases, these "witches" were burned at the stake, while others were forced to enter an insane asylum or undergo a surgical hysterectomy.

The unequal treatment of women in science and medicine is palpable throughout history. Women were prohibited from participating in all phases of prescription drug clinical trials until the late 1990s (Liu & Mager, 2016). Male bodies provided the basis for scientific advancement while female hormones were seen as insignificant idiosyncrasies. As such, first-generation antidepressants [monoamine oxidase inhibitors (MOAIs) and tricyclic antidepressants (TCAs)] developed in the 1950s and 1960s, followed by antidepressants SSRIs and SNRIs in the 1980s and 1990s, were made publicly available without knowledge on female-mediated pharmacokinetic and pharmacodynamics. This is of particular concern as major depressive disorder (MDD), thought to be the most severe form of depression, is 2- to 3- fold higher in women than men (Labaka et al., 2018), with reports of over twice as many women taking antidepressants than men (Eid et al., 2019). It comes as no surprise then, that several prescription drugs were withdrawn from market in the late 1990s as they posed great health risks for women (Liu & Mager, 2016). Women also tend to experience differences in symptom presentation and severity.

There are several demographic vulnerability factors for depression including sex, age, and culture. Major depressive disorder is the most common psychiatric illness worldwide, with

lifetime prevalence rates of approximately 20% to 25% for women and 7% to 12% for men (Kuo et al., 2015; Richards, 2011). The 12-month prevalence rate ranges from 5-14% in females and 3-9% in males depending on global location (Richards, 2011; Salk et al., 2017). This gender disparity is seen across cultures, suggesting a biological basis separate from race, culture, or socio-economic status (Richards, 2011; Vos et al., 2016). Sex differences in depression can be found as early as ages 8 to 11, with consistent meta-analysis reports of higher symptom severity and more diagnoses in females by age 12 (Salk et al., 2017). Depression is found across all cultures and demographics, with the average age of onset ranging from 18 to 29 (Kessler et al., 2005; Zisook et al., 2004). The onset of depression in youth and adolescence is of particular concern as this often interrupts critical developmental periods. Further, younger development of MDD is associated with greater symptom severity, longer duration of illness, higher suicidality, and more and longer episodes (Hollon et al., 2006; Zisook et al., 2004). Older adults (65+) generally have 2-fold higher rates of depression than in younger cohorts (18-65), presumably due to psychosocial factors and high-stress life experiences such as retirement, loss of loved ones, and physical and cognitive decline (Hollon et al., 2006).

Depression most often presents as a chronic disorder although it is pleomorphic, with considerable variability in disease course. Studies report that the rate of recurrence is over 75%, and over one third of all patients will have episodes that last longer than two years (Hollon et al., 2006; Keller, 2001). Depression is the leading cause of suicide at any age and is the top contributor to suicidal ideations and self-harm behaviours (Cuijpers et al., 2014). Some reports indicate that between 80-98% of those diagnosed with MDD have another comorbid psychiatric illness, most notably 60-70% with anxiety disorders (Richards & O'Hara, 2014). Medical comorbidities are also common including cognitive impairments, and hypertensive, inflammatory, and metabolic disorders (Allen et al., 2018; Dahl et al., 2014; Dowlati et al., 2010; Fancourt & Steptoe, 2020; Ismail et al., 2016; Steffen et al., 2020) which can bidirectionally potentiate severity, slow recovery, and elevate risk of relapse (Oslin et al., 2002; Wang et al., 2020). A diagnosis of depression is a robust predictor of illness outcome, most notably in cancer patients in which there is a 30% increase in mortality risk (Wang et al., 2020). Not only does depression cause significant harm to the sufferer, but it is also associated with considerable social and economic costs. In 2018, the Conference Board of Canada reported $32.3 billion in gross domestic product loss from depression (GBD, 2018). In the United States, one out of every

eight visits to the emergency department relates to psychiatric illness, an equivalent to 12 million visits a year (Owens et al., 2011) and 14% are hospitalized again within 30 days following discharge (Lee et al., 2017). In general hospitals, depression is responsible for 50% of psychiatric consultations and 12% of all hospital admissions (Kuo et al., 2015).

It is imperative that clinical depression be differentiated from the normal human emotion of sadness. While sadness comes and goes often in response to stressful or triggering events, MDD is an overpowering and chronic disorder that drastically impacts daily living. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) has been the standard for psychiatric diagnoses in North America since its publication in 2013. MDD is diagnosed when five (or more) symptoms have been present for most of the day, everyday for two (or more) consecutive weeks. In addition to a depressed mood (e.g., sad, empty, hopeless) and a loss of interest/pleasure in activities, of which at least one must be present to warrant a diagnosis, symptoms include significant weight loss or weight gain, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or loss of energy, feelings of worthless or excessive/inappropriate guilt, decreased concentration, and recurrent thoughts of death/suicide (*Diagnostic and Statistical Manual of Mental Disorders*, 2013). These symptoms must cause significant distress and/or impairment in social or occupational functioning, must not be attributable to the effects of a substance or another medical condition, and must not be better explained by another mental health disorder. Due to pleomorphic nature of depression, there are several specifiers that can accompany a diagnosis of MDD such as with anxious or psychotic features, or with seasonal patterns.

Diagnostically, MDD is just one of many classifications of depressive disorders. The DSM-V defines eight separate depressive disorders including: disruptive mood dysregulation disorder, major depressive disorder, persistent depressive disorder (dysthymia), premenstrual dysphoric disorder, substance/ medication-induced depressive disorder, depressive disorder due to another medical condition, other specified depressive disorder, and unspecified depressive disorder (*Diagnostic and Statistical Manual of Mental Disorders*, 2013). As depression may present across a multitude of facets, it is important to adequately assess patient needs to achieve the best possible treatment outcome.

Clinician-administered assessment scales are commonly used to stratify depression severity and subtype. Commonly used scales include the Hamilton Depression Rating Scale (HDRS), the Montgomery-Asberg Depression Rating Scale (MADRS), and the Beck Depression Inventory (BDI). The HDRS is a 21-item scale that grades symptom severity and subtype on a five-point Likert scale (0-4). Scores below 7 represent the absence or remission of depression, mild to moderate depression ranges from 7-17 and 18-24 respectively, and scores above 25 represent severe depression (Hamilton, 1960). In response to criticisms of poor reliability and discriminative properties, the improved GRID-Hamilton Depression Rating Scale (GRID-HAMD) was released to standardize the HDRS and distinguish symptom frequency from severity (Williams et al., 2008). The BDI is a 21-item four-point Likert scale (0-3), where a score of 1-10 indicates a normal mood, and 30-63 is severe or extreme depression. Although the BDI generally has good reliability and validity, scores can be influenced by maladaptive personality traits (Beck et al., 1961). Lastly, the 10-item MADRS stratifies the severity of depressive episodes in mood disorders to detect depression change over the course of pharmacological treatment (Quilty et al., 2013).

Clinical assessment of psychiatric disorders is gradually shifting from a categorical to a dimensional approach. Traditional categorical models diagnose psychiatric disorders based on the dichotomous presence or absence of disruptive and abnormal behaviours. Conversely, dimensional models place individual characteristics on a continuum of frequency and severity. Including a dimensional model allows a more flexible threshold between normality and disordered behaviour and can help define severity of a condition. The updated DSM-V, a classically categorical diagnostic tool, now integrates both categorical and dimensional approaches by including spectrum measures that are compatible with categorical definitions (American Psychiatric Association, 2022). Research into psychiatric disorders is also incorporating a dimensional approach as seen with the Research Domain Criteria (RDoC) initiative. Developed by the National Institute of Mental Health (NIMH), the RDoC measures and integrates many classes of variables, including behavioural dimensions and biological indicators, to develop a more comprehensive understanding of the full spectrum of mental health (*The National Institute of Mental Health Strategic Plan*, 2021). The integration of these approaches aims to maintain diagnostic reliability while addressing problems with symptom-based disorder diagnoses and comorbidities.

1.2.1 Etiology of depression

The longstanding nature vs. nurture debate about the causative role of psychiatric disorders continues in the medical and clinical fields. The nature side examines the role of genetic predispositions and biological factors whereas the nurture side evaluates environmental influences on psychiatric and medical conditions. While the truth probably lies somewhere in between as a combination of both, each condition has its own unique set of biological and psychosocial contributing factors. The etiology of MDD is particularly complex to define due to the intertwining of genetic, biological, and psychological factors as well as high comorbidity rates and variances in disease course (Beck, 2009). Further, as not all depressions are alike in etiology nor presentation, it cannot be assumed that there is one underlying pathophysiological mechanism. Biological factors that can influence depression include genetic, neurological, hormonal, immunological, and endocrinological influences, which are all affected by stress, sex, and age (NRC, 2009). Environmental stresses can include childhood trauma, sexual abuse, chronic adversities, lack of social support, and social economic status (NRC, 2009). Finally, individual aspects can mediate depression onset and disease course including cognitive ability, sociability, and personality traits (Klein et al., 2011). For instance, depression is more often observed in those who score high in neuroticism and conscientiousness, with personality traits mediating therapeutic outcomes (Klein et al., 2011). Combined, there is a complex array of risk factors that can heighten susceptibility for depression. The following section will discuss biological, environmental, and personal factors which can influence onset of depression, symptom patterns, and disease course.

MDD is generally considered to be moderately heritable stemming from twin studies which suggest a heritability rate between 40% - 50% (Sullivan et al., 2000). The relative risk ratio for those with a first-degree relative diagnosed with depression is 2-to-3 fold higher than the general population (Weissman et al., 1984), dependent on disease severity and recurrence (Marazita et al., 1997; Weissman et al., 1982). Heritability rates are also affected by sex with twin studies indicating that MDD is significantly more heritable in women than men, though most of the genetic risk factors were shared between sexes (Kendler et al., 1999, 2000, 2001). The genome-wide association studies (GWAS), designed to identify specific genetic loci associated with a particular disease, have provided some robust associations for disease and biological phenotypes

and have thus become the focus of gene-finding research (Uffelmann et al., 2021). Unfortunately, the polygenicity of psychiatric disorders complicates these associations, requiring large sample sizes to detect the modest effect loci. Genomic loci reliably associated with psychiatric illness contribute only a small amount to the overall risk of developing this disease. For example, even in psychiatric disorders with higher heritability such as bipolar disorder and schizophrenia where genetic influences explain up to 85% of risk (Barnett & Smoller, 2009; Dennison et al., 2020; Hilker et al., 2018), estimated heritability based on single nucleotide polymorphisms (SNPs) identified in GWAS is modest, with 45% for schizophrenia and 21% for bipolar disorder (Ward et al., 2019). It comes as no surprise then, that in the moderately heritable MDD meta-analyses have found only an 8% SNP heritability out of 102 independent variants, 269 genes, and 15 gene-pathways associated with depression (Howard et al., 2019; Ward et al., 2019). The most note worthy GWAS identified in MDD include a polymorphism in the promotor region of the carrier family 6 member 4 (SLC6A4) gene which codes for SERT and drives serotonin reuptake from the synaptic cleft. Those containing the short allele are at increased risk to experience depression and suicidal ideation compared to those homozygous for the long variant (Caspi et al., 2003; Collier et al., 1996; Kendler et al., 2005).

Numerous studies have investigated the impact of chronic stress and life adversities on the development of depression. Twin studies suggest that genetic influences may increase individual susceptibility to environmental risk factors (Sullivan et al., 2000), although the extent of the interaction between genotype and environment is unclear (Rice, 2009). Experiencing extremely negative life events increases the chances of developing depression by 5- to 16-fold, and first episodes often directly follow these events (Kendler et al., 2001; Sullivan et al., 2000). Particularly, early life adverse events often related to childhood emotional, physical, or sexual abuse especially in females, are one of the strongest predictors of the development and severity of MDD (Cheasty et al., 1998; Lindert et al., 2014; Shapero et al., 2014). In fact, cortisol levels can be linked back to maternal stress in infancy, suggesting that children exposed to more early life stressors have a sensitized stress response (Essex et al., 2002). In adulthood, depression may be triggered by minor stressors such as harassment, redundancy at work, and financial problems as well as major life events such as death of a loved one or medical diagnoses. The prevalence of depression is increased in groups with lower socioeconomic status and minority groups, potentially mediated by discrimination and decreased access to services (Dunlop et al., 2003).

Other pertinent environmental factors include social disconnectedness and perceived social isolation which are strong mediators of symptom severity of depression and anxiety (Santini et al., 2020). This effect of stress and social isolation is mimicked in certain animal models in a sex-dependent manner (Weintraub et al., 2010).

1.2.2 The monoamine hypothesis of depression

The monoaminergic hypothesis of depression, which was proposed over 70 years ago, posits that a deficiency of monoamines in functionally important neuroreceptor sites is associated with depressive symptoms (Schildkraut, 1995). This hypothesis stemmed from the observation that the antihypertensive agent reserpine depleted presynaptic levels of monoamines available for synaptic release, which was paralleled with depressive symptoms (Hirschfeld, 2000). Interestingly, cessation of reserpine treatment as well as administration of the norepinephrine precursor dihydroxyphenylalanine (DOPA) reversed the depressogenic effects, implying a biochemical basis for depression (Hirschfeld, 2000; Schildkraut, 1995). Further evidence for the monoamine hypothesis of depression arose from the discovery that the antimycobacterial agent iproniazid improved mood in depressed tuberculosis patients through the inhibition of monoamine oxidase (MAO), a mitochondrial enzyme that degrades free monoamines in the presynaptic terminal (Hirschfeld, 2000; West & Dally, 1959). Thus began research into monoamine oxidase inhibitors (MAOI), which precipitated the findings that increased levels of serotonin and norepinephrine in the brain correlated with behavioural excitement and amelioration of depressogenic symptoms (Hirschfeld, 2000).

Monoamines are a class of neurotransmitters and neuromodulators involved in the regulation of cognition, learning, emotion, memory, arousal, mood, sleep, and appetite, all of which can be impaired by depression. Monoamines are derived from aromatic amino acids and can be divided into two subclasses: catecholamines and indolamines. Catecholamines include dopamine, norepinephrine, and epinephrine while indolamines include serotonin [otherwise called 5-hydroxytryptamine (5-HT)] (Rus et al., 2018). Along with MAOIs, tricyclic antidepressants (TCA) are another first-generation antidepressant introduced in the 1950s. TCAs act by blocking serotonin and norepinephrine reuptake in presynaptic terminals while acting as competitive antagonists on post-synaptic alpha cholinergic, muscarinic, and histaminergic receptors (H1)

(Moraczewski & Aedma, 2022). Unfortunately, both MAOIs and TCAs have low efficacy rates and several adverse side effects including blurred vision, constipation, xerostomia, confusion, tachycardia, orthostatic hypotension, dizziness, sedation, weight gain, confusion, and increased suicidality (Moraczewski & Aedma, 2022; Ramachandraih et al., 2011). These first-generation antidepressants have been replaced by the safer and more tolerable second-generation antidepressants including SSRIs and SNRIs that more selectively target specific neurotransmitter receptors. However, the efficacy rates of second-generation antidepressants are not much better than their predecessors, causing the monoamine hypothesis to fall largely out of favour.

Despite these challenges, decades of research provide evidence for a pathophysiological role of monoamine depletion in depression. For example melancholic depression, a particularly severe form of MDD, is associated with marked decrease in 5-HT levels in the central nervous system (Sarrias et al., 1987). Impaired serotonin signaling is also associated with suicidality as lower levels of 5-HT are found in post-mortem samples of suicide victims and in the platelet samples of those who attempted suicide (Alvarez et al., 1999; Kohyama, 2011; Sullivan et al., 2015). In addition, low levels of serotonin's primary CNS metabolite 5-hydroxyindoleacetic acid (5-HIAA) in cerebrospinal fluid (CSF) is associated with prior suicide attempts, particularly those which were highly violent and lethal (Bach-Mizrachi et al., 2006; Sullivan et al., 2015). Paradoxically, in the dorsal raphe nucleus (DRN) there is increased serotonergic neurons and neuronal tryptophan hydroxylase-2 (TPH2) expression in post-mortem analysis of depressed suicide patients (Bach-Mizrachi et al., 2008). Increasing the 5-HT precursor tryptophan through diet (De Vriese et al., 2004), exercise (MacGillivray et al., 2012), and sleep (Kohyama, 2011) can be a protective factor against depression and suicidality (Leyton et al., 1997). Receptor- and region-specific alterations in 5-HT reuptake suggest a much more complex pathomechanism underlying serotonin's effects. Recent focus on the putative protective neuropsychological effects of hallucinogens such as lysergic acid diethylamide (LSD) has uncovered evidence of agonist activity at the serotonin 5-HT_{2A} receptor (López-Giménez & González-Maeso, 2018). Overall, it seems apparent that 5-HT and its receptors play an important role in the pathophysiology of depression, however it is difficult to fully ascertain the exact mechanism due to the complex nature of the disease.

While it is probable that monoamines play a mechanistic role in the etiology of depression, there are several major issues that led this hypothesis to fall largely out of favour. One of the main concerns is that while inhibition of the transporter occurs almost immediately, 2-4 weeks of treatment is typically required before alleviation of depressive symptoms (Cipriani et al., 2018; Rush et al., 2006). This delay suggests that a much more complex mechanism underlies the monoamine-based antidepressant effects. Additionally, limited therapeutic efficacy is seen in a large proportion of depressed patients (Berlim & Turecki, 2007; Kasper & Montgomery, 2013). In fact, placebos are often nearly as effective as antidepressants in randomized clinical trials (Perahia et al., 2006); half of depressed patients in clinical practise do not respond adequately to first-line antidepressants (Rush et al., 2006); and there is a lack of remittance in key symptoms such as anhedonia and suicidality (Kasper & Montgomery, 2013). There is a clear and pressing need for the development of more efficacious and faster-acting antidepressants.

1.2.3 The neuroplasticity hypothesis of depression

The more recently proposed neuroplasticity hypothesis of depression attempts to address some of the points of concern with the monoamine hypothesis. This hypothesis proposes that a dysfunction in neuroplasticity might underlie the biological and clinical characteristics of several neuropsychiatric disorders including depression (Massart et al., 2012; Nissen et al., 2010). Neuroplasticity, or brain plasticity, is the capacity of the nervous system to undergo adaptive structural and functional changes in response to external or internal stimuli (Puderbaugh & Emmady, 2023). Plasticity lies at the core of learning and memory in the developing and adult brain and plays a role in recovery from brain injury. Synaptic plasticity refers specifically to the activity-dependent strengthening or weakening of synaptic transmission, as well as the formation or elimination of synapses (Citri & Malenka, 2008). Long-term potentiation (LTP) is the stimulus-driven increase in synaptic strength that leads to an increase in signal transmission between neurons, whereas long-term depression (LTD) involves a reduction in synaptic strength. The dynamic interactions between LTP and LTD play a crucial role in the development of neural circuitry and adult memory storage (Stanton, 1996). A disruption of neural plasticity is thought to play a significant role in the onset and development of depression. This putative role is evidenced by the molecular changes exerted by antidepressants on the regulatory effects of neural plasticity (Castrén & Hen, 2013; Normann et al., 2018).

MDD is associated with deficits in cognition, attention, concentration, memory, and executive function linked to the prefrontal cortex, limbic system, and hippocampus (Harvey et al., 2005; Kritchevsky et al., 2004). Stress plays a complex role in mediating region-specific plasticity dependent on type of stress and chronicity. While prolonged stress can impair the plasticity underlying hippocampal-dependent learning, mild and transient stress can actually facilitate hippocampal-dependent cognition (Sapolsky, 2003). Interestingly, stress-induced hippocampal LTD that causes dendritic atrophy is often paralleled with the enhancement of amygdaloid plasticity (Sapolsky, 2003; Shors, 2006). This amygdaloid LTP is associated with aspects of emotional responses in fear-conditioning and the stress effects on learning and memory (Shors, 2006).

Glutamatergic signaling lies at the heart of LTP through the binding of excitatory neurotransmitters to ionotropic glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA). The binding of glutamate to postsynaptic AMPA receptors (AMPAR) supports fast excitatory transmission, while NMDA receptors (NMDAR) are coincidence detectors for the induction of long-term synaptic plasticity. Ligand binding opens the AMPAR allowing for the flux of Na^+ into the postsynaptic cell, resulting in a depolarization known as the excitatory postsynaptic potential (EPSP). Conversely, glutamate binding at resting membrane potential does not open the NMDAR channel due to a Mg^{2+} cation bound to the GluN1/GluN2 subunits. The magnesium block is only expelled with significant cell depolarization which allows the entry of Na^+ and Ca^{2+}. Thus, NMDAR channel activation requires both pre- and post-synaptic responses. The influx of Ca^{2+} initiates signaling pathways of secondary messengers which phosphorylate existing AMPARs, increase AMPAR sensitivity to Na^+, and traffic AMPARs onto the postsynaptic surface. This upregulation of AMPAR results in a long-lasting increase in EPSP size which underlies LTP.

Antidepressant treatments are associated with the repairing of neuronal plasticity deficits associated with depression and an increase in neurogenesis (Castrén & Hen, 2013; Normann et al., 2018). The exciting discovery that ketamine, a non-competitive NMDAR antagonist, has rapid antidepressant effects within as little as an hour provides further support for the neuroplasticity hypothesis of depression. Ketamine generates synaptic potentiation of field excitatory postsynaptic potentials (fEPSPs) by trafficking AMPARs to the postsynaptic surface.

Ketamine's antidepressant effects will be covered in more detail later in this chapter. Regardless of pharmaceutical treatment choice, cognitive and behavioural therapies should be used in conjunction for the most evidence-based results.

1.2.4 Treatment resistant depression

It is not uncommon that patients with MDD do not respond adequately to first-line antidepressants developed under the monoamine hypothesis. These individuals are considered to be treatment resistant and consist of a startling 30-60% of patients in clinical practice (Kasper & Montgomery, 2013; Rush et al., 2006). While the definition of treatment-resistant depression (TRD) is variable, TRD is generally defined as an inadequate response to at least two different first-line antidepressants of adequate dose and duration (Berlim & Turecki, 2007; Daly et al., 2019). In clinical settings, TRD can range from the failure to reach remission after one course of antidepressants to multiple failures in response to several more complex treatments, referred to as treatment refractoriness (Kasper & Montgomery, 2013). More treatment steps are associated with lower acute remission rates and higher remission rates (Kasper & Montgomery, 2013; Rush et al., 2006). In fact, one meta-analysis found that with each additional treatment, the relapse rates increase from 40% after the first intervention, to a startling 71% by the fourth (Rush et al., 2006). Further evidence suggests that switching to a different class of antidepressants is not associated with a better clinical response (Souery et al., 2011), although that is most often the prescribed treatment course. In these cases, additional pharmaceutical therapies may even be hindering remission due to an increase in despair and learned helplessness (Forgeard et al., 2011). The inconsistent ways that TRD is defined can bring about difficulties in clinical assessments as well as limitations in scientific research. However regardless of definition, there are several pertinent factors that are consistently reported with TRD including ineffective response to treatment, most notably failure to respond adequately to first-line therapeutics. Identifying these clinical and biological signs early in diagnostic and treatment course could help alleviate individual and societal disease burden.

Clinically, TRD is associated with age of onset, sex, longer depressive episodes, higher suicidality, more hospitalizations, and higher dosage of antidepressants (De Carlo et al., 2016). Predictors of TRD include comorbid panic or anxiety disorders, comorbid personality disorders,

and melancholic features (Kasper & Montgomery, 2013). Genetic determinants of treatment response are also being investigated, most notably the functional polymorphism of the gene encoding for SERT in the promoter region (Kasper & Montgomery, 2013; Serretti et al., 2009). A higher baseline inflammation is also uniquely associated with TRD, suggesting that increased levels of pro-inflammatory cytokines such as C-Reactive Protein and TNF-α could be contributing to treatment non-responsiveness (Strawbridge et al., 2015; Yang et al., 2019). TRD should therefore be viewed as a distinct, more severe subset of MDD. Identifying pertinent biomarkers and developing clinically relevant disease profiles of TRD is essential to streamline treatment course.

1.3 Sex dimorphisms in depression

MDD is sexually dimorphic characterized by gender-differences in clinical presentation, disease course, and response to antidepressant treatment. Depression disproportionately affects women, with reports consistently finding that women are twice as likely to suffer from MDD than men (Eid et al., 2019; McLaughlin & Hatzenbuehler, 2017; Salk et al., 2017). Epidemiological studies show this 2:1 ratio is relatively consistent across cultures, between sociodemographic groups, and remains relatively consistent even as the prevalence of MDD has increased in both sexes (Kessler et al., 2003). Due to the pleomorphic nature of MDD it is hard to pinpoint the exact cause of this variability, however most likely socioenvironmental factors are at interplay with biological roots, genetics, and hormones. The clinical expression of MDD differs between sexes, as women are more likely to present with a seasonal component, atypical symptoms (weight gain, hypersomnia), and have increased somatic complaints (Viveros et al., 2012). Neuroanatomical data shows sexual dimorphisms in many regions of the brain responsible for cognitive processes, including the hippocampus, amygdala and neocortex (Cahill, 2006). Examining these sex differences from a biopsychosocial model can help determine some variables in this web of factors.

Social dynamics pertaining to biological sex and gender roles underpin a complex network of variables that create social inequalities. The historical and still-prevalent oppression of women include myriad of human rights violations against women including sexual violence, unjust work practises, and assaults to human dignity. Women experience higher rates of gender-based

violence across life-course which is associated with a significantly increased risk of developing psychiatric disorders (Astbury, 2010). Epidemiological studies suggest that social conditions and institutional practises can produce a chronic strain related to gender roles (McLaughlin & Hatzenbuehler, 2017). Some studies even suggest that gendered social structures and interactions mediate relationship between sex and depressive symptoms (Bebbington, 1998; McLaughlin & Hatzenbuehler, 2017). Other sex-specific socioeconomic factors found to increase susceptibility to MDD include interpersonal inequalities such as lower social status, unequal access to education, bias in income, unwanted pregnancies or difficulties becoming pregnant, and an increased workload of associated motherhood (Tang & Zhang, 2022). As women are more likely to experience anxiety (Verma et al., 2011), sex-specific stressors can significantly contribute to the development of depression (Chiba et al., 2012). Cognitively, females are more likely to engage in psychological behaviours that are associated with MDD including rumination, rejection sensitivity, and negative attribution styles (McLaughlin & Hatzenbuehler, 2017). Women are also at-risk for sex-specific forms of depression including premenstrual dysphoric disorder, postpartum depression, and postmenopausal depression (Albert, 2015). Life course sex-differences in MDD demonstrate that women are more likely to experience earlier onsets, longer depressive episodes, and a greater risk of recurrence of depression, all of which are predictors of increased chronicity and severity (McLaughlin & Hatzenbuehler, 2017). Women are also 10% more likely to develop a comorbid psychiatric disorder (Bromet et al., 2011).

1.3.1 The role of sex hormones in MDD

Sex differences and hormonal fluctuations contribute largely to the heterogeneity of depression symptomology across lifespan. Sex differences in MDD correlate with sex differences in HPA axis function (Zagni et al., 2016), which is mediated by gonadal hormones (Lund et al., 2004; Sheng et al., 2021). Males have a greater ability to inhibit HPA axis via its feedback loop, as testosterone is a known HPA axis modulator and can suppress the release of corticotrophin releasing hormone (CRH) to depress the stress response (Goel & Bale, 2010; Sheng et al., 2021). In contrast, estrogens enhance HPA axis activity and the release of stress hormones, exhibited by stress hormone levels peaking during periods of high circulating estrogen levels (Atkinson & Waddell, 1997). In women, major hormonal transition periods occur during puberty, pregnancy, and menopause, as well as more subtle hormonal fluctuations during the monthly menstrual

cycle. Shifts in female sex hormone levels (estrogen and progesterone) parallel incidence rates of mood disorders such as unipolar depression (Bebbington, 1998; Musial et al., 2021; Wharton et al., 2012). In women, a high occurrence of lifetime depressive events are thought to be related to the reproductive cycle (Soares & Zitek, 2008). Preclinical studies in animal models demonstrate that progesterone or estrogen withdrawal can induce depressive-like behaviours which can be recovered by hormone administration (Morssinkhof et al., 2020).

Sex differences in MDD prevalence begin to arise during puberty and continue throughout the reproductive life cycle (Soares & Zitek, 2008). Adolescent females have a heightened risk to experience depressive episodes following puberty (Graber, 2013), with an earlier onset of puberty associated with more serious and persistent psychopathology (Angold et al., 1998; Grabe et al., 2007). Following the first menstrual cycle, endogenous ovarian hormone level fluctuations are associated with negative mood symptoms, and allopregnanolone levels have been linked to an increase in amygdala activity paralleled in anxiety reactions (Bäckström et al., 2014; Saunders & Hawton, 2006). During the reproductive years, hypoestrogenism caused by normal monthly hormonal fluctuations could lead to an increased risk of MDD (Arpels, 1996). In fact, meta-analyses have shown a positive correlation between menstrual phases characterized by low estrogen levels (the late luteal and follicular phases) and suicide attempts (Saunders & Hawton, 2006). Despite these findings, little evidence shows a direct-effect relationship between depressed mood and monthly hormonal fluctuations. It is rather the more dramatic and at times unpredictable hormonal fluctuations that are thought to contribute to the increased risk for depression (Soares & Zitek, 2008).

Dramatic increases in estrogen and progesterone levels occur throughout pregnancy paralleled by serotonergic activity (Soares & Zitek, 2008). The postpartum period, which is characterized by lower amounts of estrogen and progesterone, is also a period of increased susceptibility to a particular type of depression known as post-partum depression (PDD) (Barth et al., 2015). However, the contribution of hormonal fluctuations in post-partum depression remains unclear. Some studies indicate that higher plasma estrogen and progesterone levels are linked to depressive symptoms (Sha et al., 2021), while others show a low ratio of follicle stimulating hormone (FSH)/luteinizing hormone (LH) in the development of PDD (Raji et al., 2017). There appears to be an inverse relationship between depression and perimenopause, a stage

characterized by unstable levels of FSH and estrogen (Barth et al., 2015). Findings suggest that a lifetime history of depression predisposes the risk of early perimenopause by nearly 3-fold, while early entry to perimenopause in non-depressed women increases the risk of severe mood disturbances and first onset of depression (Cohen et al., 2006). No significant increase in risk of depression is reported in postmenopausal women, reinforcing the putative causal role of ovarian sex-hormone fluctuations in MDD (Soares & Zitek, 2008).

Ovarian hormones exhibit modulatory effects on the synaptic transmission of 5-HT, dopamine, GABA, and glutamate (Barth et al., 2015), hormones which have been implicated in MDD (Hirschfeld, 2000). Both estrogen and progesterone have been shown to modify the functionality and responsiveness of SERT in SSRI treatment, suggesting a role of female sex hormones in therapeutic response (Benmansour et al., 2012). Some studies suggest that males respond better to TCAs while females have an enhanced response to SSRIs (Alshammari, 2021), although these findings are not consistently reported. Estrogen is thought to influence serotonin synthesis as well as serotonin receptor binding and activity (Keers & Aitchison, 2010). Animal models suggest that lower baseline levels of serotonergic activity increase depressive phenotypic susceptibility during periods of neuroendocrinal stress (Soares & Zitek, 2008). Clinical reports indicate that the interaction between ovarian hormonal levels with age and genotype modulate serotonergic reactivity (Barth et al., 2015).

Neurologically, it has been proposed that ovarian hormones and testosterone influence cortical functional connectivity in different manners (Barth et al., 2015). Lowered testosterone levels are also associated with symptoms of depression, and testosterone administration can reduce these symptoms in both human and animal models (Sha et al., 2021). It has been proposed that the consistent levels of testosterone may be a protective factor against MDD (Albert, 2015). Testosterone can be metabolized into metabolites that affect progesterone and estrogen transmission and reception, suggesting an overlapping effect between testosterone, progesterone, and estrogen (Morssinkhof et al., 2020). Overall, it is clear that sex hormone level fluctuations may be a vulnerability factor to the development and course of MDD. In female studies of depression, it is particularly important to track cycle phases to evaluate hormonal contributions.

In preclinical models, the female rat’s estrous cycle lasts around 4-5 days and consists of four stages: proestrus, estrus, metestrus, and diestrus. Proestrus is characterized by peak levels of sex

hormones estrogen, progesterone, luteinizing hormone, and follicle stimulating hormone, and lasts around 14 hours consisting of rapid hormone changes (Andrade et al., 2010; Lovick & Zangrossi, 2021). Proestrus females are typically less anxious than females in other estrous phases (ter Horst et al., 2012), and often have less immobility in the FST (a measure of behavioural despair) than other non-proestrus females and males, thought to be associated with elevated levels of progesterone (Andrade et al., 2010). Females in proestrus have also been shown to have greater resting and CORT-induced HPA axis responses to stress than other stages of the estrous cycle (Babb et al., 2013). Estrus, which lasts 24-48h, is characterized by falling levels of estrogen, moderate levels of progesterone, and high levels of LH and FSH levels (Lovick & Zangrossi, 2021). Metestrus (sometimes diestrus I or early diestrus) is characterized by low levels of estrogen and LH, whereas in diestrus (or diestrus II/-late diestrus) estrogen levels begin to rise (Jenkins et al., 2001; Shors, 1998). In general, higher levels of anxiety-like behaviour and acute stress responsiveness are seen in the metestrus and diestrus phases compared to the proestrus and estrus phases (Lovick & Zangrossi, 2021; ter Horst et al., 2012), possibly from decreased levels of estrogen and progesterone (Jenkins et al., 2001; Lovick, 2012; Shors, 1998). In fact, diestrus rats exhibit increased immobility in the FST that is reversible through low dose progesterone administration, suggesting that lower serum progesterone levels may be associated with depressive-like behaviours (Andrade et al., 2010).

Across the estrous cycle, oscillations in sex hormone levels may have a modulatory effect on stress-induced cognitive deficits relevant to depression (do Nascimento et al., 2019). Female rats in metestrus/diestrus are more susceptible to chronic stress induced memory deficits despite similar CORT levels across estrous phases (do Nascimento et al., 2019). Combined, these results suggest that periods of peak sex hormones may be a protective factor against anxiety and stress-induced memory impairment in females (do Nascimento et al., 2019; Shors, 1998; ter Horst et al., 2012), a result that is paralleled throughout the human female cycle (Lovick & Zangrossi, 2021). Although relevant, these hormonal fluctuations may have a limited impact, as some studies found no differences across antidepressant behavioural response throughout the estrous cycle (Kokras et al., 2012).

1.4 Hippocampus: Anatomy, structure, and function

The hippocampus, named after its resemblance to a seahorse (in Greek, "*hippos*" for horse and "*kampos*" for sea monster), is a grey matter structure involved in mood regulation and consolidation of short- and long-term memory. Residing in the medial temporal lobe of both hemispheres, the hippocampus is a major structure in the limbic system which is involved in emotional and behavioural response. The hippocampus provides a spatiotemporal framework for experience and receives input from most cortical and subcortical regions (Knierim, 2015). The involvement of the hippocampus in memory has been well-established since 1953 when patient H.M. developed antegrade amnesia following bilateral hippocampal resection in an attempt to reduce epileptic seizures (Knierim, 2015; Lisman et al., 2017). Despite retaining full cognitive function, H.M. lost the ability to develop new semantic or episodic memories (antegrade amnesia) or remember events that occurred close to the surgery (partial retrograde amnesia). This scientific breakthrough showed strong evidence for the role of the hippocampus in declarative but not procedural memory. Severe episodic and spatial memory impairments are also found in those with hippocampal lesions, however normal levels of intelligence are generally retained (Hainmueller & Bartos, 2020; Knierim, 2015). Since H.M., the hippocampus is one of the most studied structures of the brain.

The hippocampal formation is a neural network of millions of neurons organized into four main regions: the Cornu Ammonis (CA), the DG, the entorhinal cortex (EC), and the subicular complex (found in Figure 1.1). Each region is highly connected and essential for the proper relay of information between the hippocampus, the PFC, the hypothalamus, and other limbic areas (David & Pierre, 2006). The intrinsic flow of information through intrahippocampal circuits is largely unidirectional and follows a serial progression through either the tri-synaptic circuit (EC → DG → CA3 → CA1) or via the perforant pathway (Knierim, 2015). Highly distributed three-dimensional connections allow integration of multimodal sensory information. The neuroanatomy of the hippocampal formation differs between rodent and primate brains, expressed as C-shaped and vertically oriented in rodents, whereas it is more linear and horizontally oriented in primates (David & Pierre, 2006).

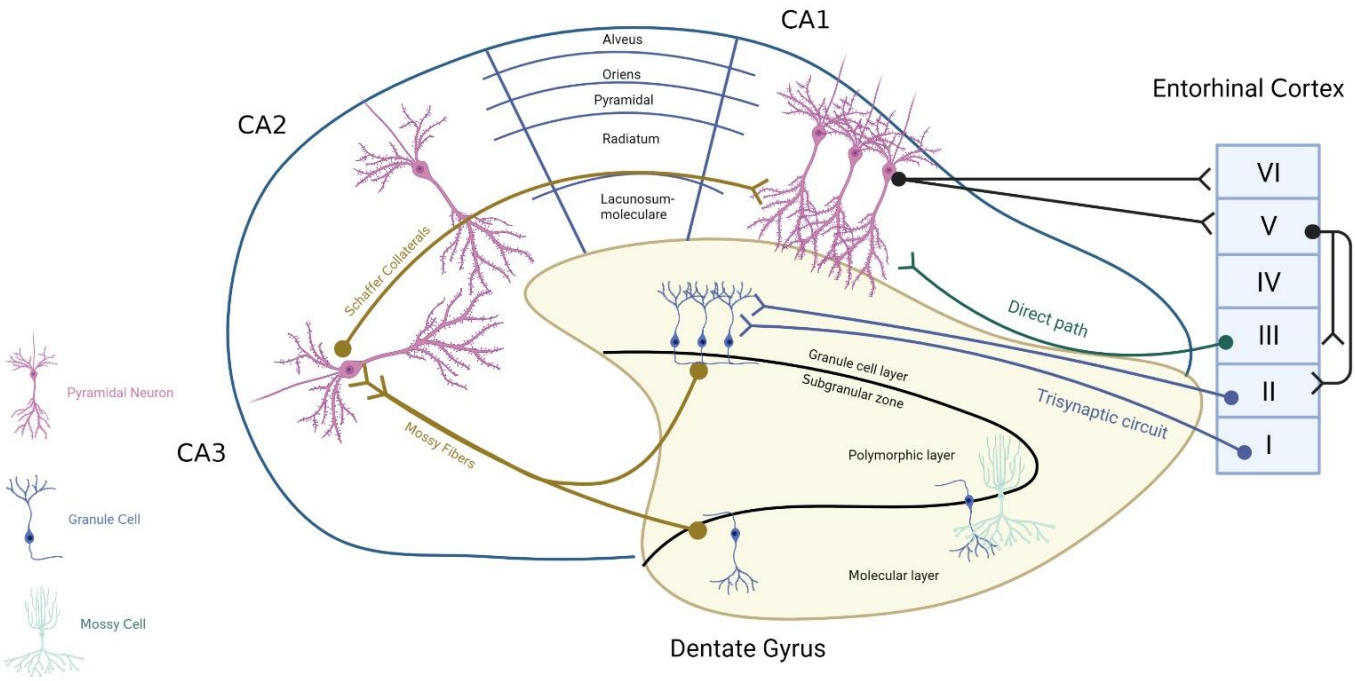


Figure 1.1 General anatomy, structure, and circuitry of the hippocampus. The hippocampus proper consists of 3 subdivisions (CA1, CA2, CA3), which are further divided into 5 strata (stratum oriens, stratum pyramidal, stratum radiatum, stratum lucidum, and stratum lacunosum-moleculare). The dentate gyrus (DG) is made up of 3 layers: the molecular layer, the granule cell layer, and the polymorphic layer. The trisynaptic circuit of the hippocampus is a unidirectional circuit that transfers information from layers I and II of the entorhinal cortex to the granule cells of the DG, and then to pyramidal cells in the CA3 through mossy fibers. Shaffer collaterals connect the CA3 to pyramidal cells in the CA1, which outputs to layers V and VI of the entorhinal cortex. The direct path connects layer III of the entorhinal cortex to the pyramidal cells of CA1. Figure created by author in BioRender adapted from Dr. Jenessa Johnston.

The CA, or hippocampus proper, is made up of 3 subdivisions (CA1 – 3) which have five strata or layers: the stratum oriens, stratum pyramidal, stratum radiatum, stratum lucidum, and stratum lacunosum-moleculare, in order of superficial to deep. Excitatory pyramidal neurons are distributed across the stratum oriens, which houses the dendrites, and the stratum pyramidal, where the soma are tightly packed together. The activity of these pyramidale neurons is fine-tuned by GABAergic interneurons that project to different CA subdivisions. The stratum radiatum contains Schaffer collateral fibers (axonal projections of CA3 pyramidal cells to

CA1/2) which activate GABAergic interneurons to provide feed-forward inhibition on pyramidal cells. The stratum lucidum, the thinnest strata found only in the CA3, contains mossy fibers from the DG granule cells which project to the CA3 and the stratum pyramidal. The stratum lacunosum-moleculare contains the perforant path fibers from the EC which form synapses onto the apical dendrites of pyramidal cells (Basu & Siegelbaum, 2015).

The DG plays a critical role in memory consolidation and spatial navigation. The DG is a trilaminate structure, composed of an outer molecular layer, a middle granule layer, and an inner polymorphic layer (occasionally referred to as CA4 or the hilus). Between the granule cell layer and the hilus is the subgranular zone (SGZ) which is the site of adult neurogenesis (Basu & Siegelbaum, 2015; David & Pierre, 2006; Knierim, 2015; Lisman et al., 2017; Stuchlik, 2018). Adult neurogenesis is the process in which neural stem cells, which are self-renewing multipotent cells, differentiate into functionally integrated and other cell types (Zhang et al., 2018). Neural stem cells in the SGZ develop into granular cells which sprout axons that target neurons in the CA3, establishing synaptic connections with nearby cells (Zhang et al., 2018). These connections are important for controlling the formation of memories and learned behaviours. The EC is involved in memory processing and displays subregional specialization to mediate the cortical information input and output from the hippocampus proper (Garcia & Buffalo, 2020). The subiculum, which lies between the hippocampus proper and the EC, is involved in spatial navigation, mnemonic processing, and mediation of hippocampal-cortical interactions (O'Mara et al., 2001). Long-term synaptic plasticity in the corticohippocampal circuit underlies memory storage and spatial representation (Basu & Siegelbaum, 2015). Interestingly, diseases of the hippocampus including hippocampal sclerosis, medial temporal lobe seizures, and Alzheimer's disease often have affective neuropsychiatric morbidities, most prominently depression (Matthew et al., 2006).

1.4.1 Hippocampal dysfunction in depression

The hippocampus is heavily implicated in the pathophysiology of MDD. Consistent reports find reduced hippocampal volume in depressed patients of up to 18-20% compared to healthy controls (Campbell et al., 2004; Stockmeier et al., 2004; Videbech & Ravnkilde, 2004). High-resolution magnetic resonance imaging (MRI) studies suggest that hippocampal volumetric

reductions may occur in a sex-dependent left-right asymmetrical manner (Kronmüller et al., 2009; Sawyer et al., 2012). Post-mortem hippocampal analysis of patients with MDD suggest this volumetric reduction could arise from a decrease in neurophils and pyramidal neuron soma size, and an increase in packing density of glia, pyramidal neurons, and granule cells neurons (Stockmeier et al., 2004). Additionally, reductions in neuronal density, particularly the CA2 interneurons, is observed in depression (Matthew et al., 2006). Larger volumetric reductions correlate with earlier onset of depression, more recurrent episodes, and longer illness duration (MacQueen & Frodl, 2011; Sheline, 2011). However, the directionality of this relationship remains unclear as smaller hippocampal volumes predict worse clinical outcomes (Sheline, 2011).

The bidirectional relationship between the HPA-axis and the hippocampus is not fully understood, but it is known that cortisol has important regulatory effects on the hippocampus and vice versa. This is evidenced by high expression of hippocampal glucocorticoid receptors in rodents and the finding that activity in the hippocampus can trigger the release or metabolization of CORT (Richard, 2006). While chronic stress is associated with structural changes in the CA3, neurotoxic lesions of CA3 also cause stress-induced hypersecretion of CORT (Charney, 2004; Richard, 2006). One prominent hypothesis, known as the neurotoxicity hypothesis, proposes that hippocampal volumetric reductions in patients with MDD occur from the deleterious effects of chronic stress. The neurotoxicity hypothesis posits that prolonged exposure to glucocorticoids (e.g., cortisol) increases neuronal vulnerability thereby increasing cell death (Duman & Li, 2012). This glucocorticosteroid cascade participates in feed-forward progressive hippocampal damage and promotes dysregulation of the HPA axis (Richard, 2006). Contrarily, the vulnerability hypothesis posits that neurobiological risk factors predispose psychopathology related to allostatic overload (Charney, 2004). Some monozygotic twin studies have found evidence that smaller hippocampal volume may constitute a risk factor for the development of MDD and stress-related psychopathology (Charney, 2004). In rodents, hippocampal volume decreases paralleled with depressive-like phenotypes were reversed by increased hippocampal neurogenesis (Zhang et al., 2018), suggesting an underlying neurobiochemical deficit.

Stress-hormones modulate hippocampal excitability, learning, memory processing, and activity-dependent synaptic plasticity (Richard, 2006). Stress-induced reductions in neurotrophic factors,

particularly brain-derived neurotrophic factor (BDNF), contribute to hippocampal volume loss, dysregulation of dendrite length and spine density, and decreases in neurogenesis (Duman & Li, 2012; Richard, 2006; Sheline, 2011). Stressful experiences elevate circulating levels of glucocorticoids and stimulate the release of hippocampal glutamate, which inhibits granule cell proliferation in the DG through an NMDAR-dependent pathway (Richard, 2006). Chronic-stress induced persistent inhibition of granule cell production could explain the structural changes in the DG and decreases in neurogenesis seen in depression (Richard, 2006). Research shows that stress can impair neurogenesis in the DG demonstrated by an inverted U-shape between level of acute stress and memory (Gould & Tanapat, 1999; Richard, 2006).

Hippocampal neurogenesis appears to play a role in mood regulation and antidepressant efficacy (Eisch & Petrik, 2012). Spurred from the hypothesis that most antidepressants stimulate adult hippocampal neurogenesis, research has found that adult-generated hippocampal neurons may mediate the deleterious effects of chronic stress (e.g., atrophy) and the behavioural effects of antidepressants (DeCarolis & Eisch, 2010; Sahay & Hen, 2007), and that neuron generation in the DG follows the time course seen in the delayed onset of monoaminergic-based antidepressants (Duman et al., 2001). In animals, a lack of new hippocampal neurons is associated with deficits and delays in contextual memory such as fear conditioning, working memory, and long-term spatial memory (Becker & Wojtowicz, 2007). Although the role of neurogenesis in the etiology of MDD is heavily debated, evidence implies its involvement in stress, emotion regulation, memory, and cognition (Bartsch & Bartsch, 2012).

Data suggests that the dorsal hippocampus is involved in cognitive processes such as learning and memory, whereas the ventral hippocampus may modulate the regulation of emotional and motivational behaviour (Fanselow & Dong, 2010). Interestingly, acute swim stress in the forced swim test (FST) is associated with region-specific changes of LTP in the hippocampus; specifically, a decrease in dorsal hippocampus and an increase in the ventral hippocampus associated with fear-conditioning (Stuchlik, 2018). Patients with MDD often exhibit memory deficits in hippocampal-encoded memories (Burt et al., 1995), which can be predictive of a more severe disease course (Sumner et al., 2010). Cognitive deficits also occur in around 2/3 of depressed patients and include impaired decision-making, executive-functioning, attention,

concentration, and deficits in hippocampal-based episodic and semantic memory (Rock et al., 2014; Szabo et al., 2014), which are often predictive of poor remission rates (Austin et al., 2001).

1.5 The HPA axis and MDD

Stress is the physical, emotional, or psychological strain that arises from real or perceived threats. Evolutionarily, the body's ability to adjust physiology and behaviour in response to immediate threats or changing circumstances has a short-term selective advantage. This type of acute stress response, often referred to as fight-or-flight, allows energy relocation away from functions not immediately needed for survival and towards facing the immediate threat. Acute stress activates the sympathetic nervous system which signals a downstream chain of events causing a short-term increase in heart rate, blood pressure, and breathing rate. While the fight-or-flight response has evolutionary advantages for survival by enabling quick reactions, a malfunctioning stress response can be extremely detrimental. In today's modern Western society, humans are faced less often with life-threatening situations and more so with daily hassles. Chronic stress occurs when the body overreacts to non-life-threatening stressors eliciting a similar, but inappropriate stress response. Extensive research has shown that chronic stress significantly increases the risk of many physical and mental health challenges, notably including depression (Kokras et al., 2012). The stress response occurs when a stressor stimulates a neuroendocrinological cascade called the HPA-axis, which regulates brain and body homeostasis (Herman & Cullinan, 1997). Schematic representation of the HPA-axis under stress can be found in Figure 1.2.

The stress response begins when a sensory stimulus from a perceived threat evokes a downstream signalling cascade. Sensory information is processed through the thalamus and analyzed by cortical structures such as the amygdala, responsible for processing fearful stimuli, and the hippocampus. These cortico-limbic structures send a distress signal to the hypothalamus, a structure that sits just above the brainstem and is the command center for hormonal release. The axonal terminals of the hypothalamic paraventricular nucleus (PVN) secrete corticotropin-releasing factor (CRF; used interchangeably with corticotropin-releasing hormone) which triggers the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into blood circulation. ACTH travels to the adrenal glands on top of the kidneys, which triggers

the release of the glucocorticoid hormone cortisol from the zona fasciculata layer of the adrenal cortex. Cortisol is the primary stress hormone in humans, analogous to CORT in rodents. Free fraction cortisol (< 5% of body cortisol) is physiologically active and stimulates glucose metabolism, creating the energy surge associated with the fight-or-flight response within minutes (Bianchi & Esposito, 2012). The free fraction cortisol remains unbound to proteins and its free circulation regulates the production of CRF and ACTH through a negative feedback mechanism. In a functional stress response, an increase of free fraction plasma cortisol inhibits the release of CRH and ACTH which in turn decreases cortisol levels. Endogenous glucocorticoids, primarily glucose, contribute to the suppression of the HPA-axis and the stabilizing neural excitability (Bianchi & Esposito, 2012).

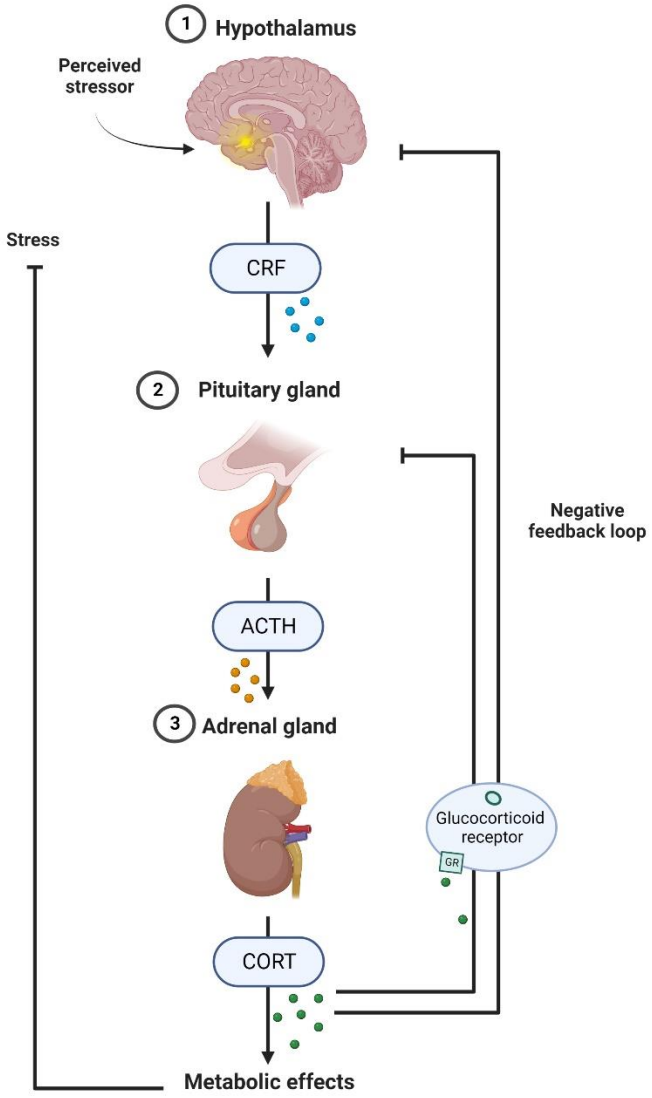


Figure 1.2 The HPA axis under conditions of stress. In response to a perceived stressor, the paraventricular nucleus in the hypothalamus secretes CRF which targets the anterior pituitary gland to in turn release ACTH. ACTH stimulates the release of CORT from the adrenal glands. CORT diffuses through the blood-brain barrier to bind to glucocorticoid receptors (GRs) in the cytosol of target cells. This activates a negative feedback system to shut off the stress response, however chronic stress can lead to maladaptive downregulation of GRs, and dysregulation of the negative feedback loop. Hypercortisolemia is observed in ≈ 60% of patients with diagnosed with depression and is heavily implicated in the associated stress response. Figure created by author in BioRender.

Normal cortisol release is cyclical, with individual variation in diurnal rhythms and magnitude of release. In humans, cortisol levels peak after waking and are lowest before bed (Adam et al.,

2010) while in nocturnal rodents CORT levels are highest at night (Watts et al., 2004). Cortisol levels are highly variable across individuals and can fluctuate up to 60% throughout a given day (Watts et al., 2004). However, hypercortisolemia is associated with brain atrophy, decreases in neurogenesis, and slowed cell maturation (Brymer et al., 2018). Overactivation of the HPA-axis can cause dendritic atrophy in the amygdala and neuronal death in the PFC and hippocampus (Bianchi & Esposito, 2012; Tafet, 2022). It is therefore likely that dysregulation of the HPA-axis may contribute to the reductions in hippocampus volume observed in patients with MDD (Adam et al., 2010; Bianchi & Esposito, 2012; Brymer et al., 2018).

The relationship between chronic stress and depression is well-established. Periods of high stress such as grief or divorce are pertinent risk factors for depression onset (Kessing, 2004). Biologically, high levels of cortisol are consistently reported in the plasma of patients with MDD (Heim et al., 2008; Pariante & Lightman, 2008), paralleled with increased ACTH and CRF in the brain, cerebrospinal fluid, and blood (Carroll et al., 2007; Nestler et al., 2002). In fact, hypercortisolemia is seen in ~60% of depressed patients (Carroll et al., 2007). Higher levels of CRF and cortisol are also recorded in the PFC, blood, hair, and saliva of suicide victims those experiencing suicidal ideation (Johnston et al., 2022; Merali et al., 2004). The relationship between HPA functioning and responsiveness to antidepressant treatment is controversial. Some studies suggest that the HPA-axis functioning can be a predictor of antidepressant response (Fischer et al., 2017), others found no relationship between cortisol level and therapeutic response (Nandam et al., 2020), and yet other studies argue for a sexually dimorphic approach (Holsen et al., 2013; Kokras et al., 2012). However, antidepressants and electroconvulsive therapy (ECT) do appear to normalize HPA-axis function (Du & Pang, 2015; Pariante, 2003; Pariante & Lightman, 2008). Discussed in further detail later in this thesis, exposure of chronic mild stress increases plasma concentrations of CORT in rodents (Li et al., 2008), and chronic CORT administration produces depressive-like phenotypes (Allen et al., 2022; Brymer et al., 2020; Johnston et al., 2020; Romay-Tallon et al., 2018). The consistent association of chronic stress with depression provides evidence that the HPA-axis plays an important role in depression which needs to be studied further.

1.6 Translational neuroscience: Animal models of MDD

Animal models are used to better understand human pathologies and to develop effective treatment approaches, however there are challenges in the translatability of such models. For ethical reasons, limitations on studying human pathologies are abundant including restrictions on human genetic modification. Additionally, while studying post-mortem tissue can provide valuable neurobiological information in psychiatric illness genomics and proteomics, it is not without limitations. Researchers must disentangle the nature of the primary disease from the consequences of illness lifecourse, the potential influence of other comorbid disorders, the impact of, if any, pharmacological agents, and the elapsed time between death and fixation of brain tissue. Further, the morphological and biochemical integrity of the brain structures may be affected during the agonal state such as alterations in acid-base balance, blood oxygenation, and structural alterations (Lewis, 2002). Ultimately, post-mortem brain samples are difficult to obtain and often tell an incomplete story of illness course, riddled with confounding issues. This is particularly true in depression where death by suicide, prolonged post-mortem interval, and influences of psychoactive medications can cause significant changes in the brain. Animal models of depression provide a crucial framework to examine neurochemical circuits in a controlled environment, allowing for methods impossible to employ in human patient studies such as gene editing and pharmacological manipulations (Wang et al., 2017).

Although humans and rodents diverged from a common ancestor approximately 75-125 million years ago, human disease genes are highly represented (~99.5%) in rodent orthologs (Center, 2002; Huang et al., 2004). Rodents are used to model human conditions due to their low cost, size, replicability, and fast reproductive rates. Across pathophysiology systems, rodent neurological genomes exhibit the greatest evolutionary conservation suggesting that rodent models of neuropsychiatric disease can provide an accurate representation of human pathologies (Huang et al., 2004). That said, modeling human neuropsychiatric disorders in animals is not without challenge due to the subjective nature of many key symptoms and the lack of biomarkers and objective diagnostic tests. In depression, the highly heterogeneous symptom characteristics and elusive disease etiology provide challenges in translational validity. For example, hallmark symptoms such as depressed mood, suicidal ideation, feelings of worthlessness, low self-esteem, and excessive guilt are difficult to model due a presumed lack of rodent self-reflection. Further,

the interplay of human genetic predispositions, environmental stressors, and socioeconomic factors create a complex pathophysiology causing researchers to often focus on a particular facet of the disorder. For this reason, there are many different models to study depressive-like behaviour, although the "best model" depends on the specific research question at hand. These include but are not limited to models of chronic or acute exposure to environmental stressors (during development and/or adulthood), targeted genetic manipulations (transgenic gene deletion or overexpression), and biological manipulations (surgical procedures, targeted lesions, and optogenetic control).

Experimental animal models of human disorders must meet standards to ensure reliability and translatability of preclinical research. Models are established based on three basic constructs: face validity (similarity in illness phenotype and clinical symptoms), predictive validity (effectiveness of disease-specific interventions in humans), and construct validity (human pathology recapitulation within the model) (Belzung & Lemoine, 2011; McKinney & Bunney, 1969; Wang et al., 2017; Willner, 1997). Face validity ensures analogy of the model's behavioural phenotype with clinical symptoms, predictive validity refers to the amelioration with clinically effective treatments (but not ineffective ones), and construct validity examines the similarity of neurobiological mechanisms with the etiology of the disorder.

1.6.1 Behavioural tests to evaluate depressive-like phenotypes

There are many behavioural changes that can be observed in animals to evaluate depressive-like phenotypes such as changes in exploratory behaviour, fear-conditioning, escape responses, memory performance, and anhedonia. Although there are many common paradigms to evaluate depressive-like behaviours, this thesis will focus on the most widely used behavioural test for antidepressant efficacy, the forced swim test. The FST was first described by Porsolt and colleagues (1978) as a measure of behavioural despair through learned helplessness. Rodents were subjected to 15 minutes of forced swimming in a deep, inescapable, water-filled cylinder. The following day, animals were returned to the tank for 5 minutes and researchers scored the amount of time spent swimming, climbing (used interchangeably with struggling), immobile, and latency to immobility. Porsolt argued that time spent immobile was indicative of despair-like behaviour brought on by learned helplessness from the 15-minute pre-swim, and that immobility

could be rescued through antidepressant treatment (Porsolt et al., 1978). However, criticisms of the 2-day procedure argued that immobility on the second day of testing could be an adaptive response as rodents learned to conserve energy remembering that they will be removed from the tank. Consequently, a one-day 10-minute protocol was established to remove the confounding effects of memory. In the updated protocol, stressed animals are consistently more immobile than controls, a phenomenon that is reversible with antidepressants (Allen et al., 2022; Brymer et al., 2018, 2020; Fenton et al., 2015; Lebedeva et al., 2020).

However, the updated FST protocol is not without critique, as the complexity of human depression cannot be captured by a simple rodent behavioural test. Critics focus on the translatability to human symptomology, the subjectivity of immobility analysis, and the failure to account for the treatment-resistant population. Some researchers argue that the FST measures coping behaviour rather than despair (Commons et al., 2017), while others see the FST more as a tool for screening antidepressant efficacy (Allen, 2022).

Despite these criticisms, the updated FST remains the most ubiquitous behavioural test for antidepressant efficacy largely due to its predictive validity (defined as similarity in drug response used to treat human condition). Therapeutics that decrease depressive symptoms in humans are often paralleled by decreases in rodent FST-immobility (Nani et al., 2019). For example, chronic (but not acute) treatment with traditional antidepressants decreases FST immobility in a time course similar to humans (Fenton et al., 2015; Holick et al., 2008), a single dose of ketamine rescues this behaviour rapidly (Browne & Lucki, 2013), and psychedelic drugs do so persistently (Hibicke et al., 2020), effects that are also seen in human patients (Kadriu et al., 2021). Genetic animal models of depression were also found to influence the immobility behaviour (Yankelevitch-Yahav et al., 2015). While this demonstrates some form of predictive validity, differences in therapeutic response do exist between human participants and rodents. For instance, while antidepressants rescue depressive-like phenotypes in most rodents, 30-50% of human patients do not respond to initial treatment (Planchez et al., 2019), largely due to the heterogeneity of the human-condition. Nonetheless, the therapeutic parallels found in a broad range of antidepressant drugs provides context for the ubiquitous use of this test.

1.6.2 Chronic stress models of depression

To tackle the heterogeneity of depression, a multitude of animal models have been developed based on stress exposure, brain lesions, and genetic manipulations. As the link between stress and MDD is well-defined, it is not surprising that chronic stress models of depression often show the greatest validity and translational potential (DeCarolis & Eisch, 2010; Willner, 1997, 2016). Commonly used models of chronic stress include exogenous glucocorticoid administration (Sterner & Kalynchuk, 2010), chronic unpredictable mild stress (Willner, 2016), learned helplessness (Vollmayr & Gass, 2013), chronic restraint (Wang et al., 2017), and social isolation or instability (Lopez & Bagot, 2021). However, a lack of experimental control over individual differences in responsivity to physiological and psychological stressors combined with a large variability in animal response makes it difficult to evaluate the direct effect of stress in many of these models. High variability in corticosterone levels is found between different rats exposed to the same experimenter-applied stressor, and many animals habituate to the aversive effects of repeated exposure (Sterner & Kalynchuk, 2010).

To circumvent these differences our laboratory uses a chronic corticosterone administration paradigm to model depressive-like behaviour whereby rats are subjected to 3 weeks of daily subcutaneous injections of 40mg/kg of CORT. This allows us to control the amount of CORT given to each rat dependent on bodyweight, and more directly examine the detrimental effects of stress (Sterner & Kalynchuk, 2010). Rodents are typically subjected to 21 days of CORT injections although shorter and longer exposure periods have been used (Lussier et al., 2013). This paradigm is shown to consistently induce depressive-phenotypes comparable to humans, and antidepressants can reliably rescue CORT-induced behavioural and neurochemical abnormalities (Allen et al., 2022; Brymer et al., 2020; Fenton et al., 2015; Johnston et al., 2020; Lebedeva et al., 2020). While CORT can be administered in a variety of ways including through food, water, pellet implantation, or osmotic pump infusion, injections allow for the most control over quantity administered.

As previously discussed, the best animal models address face, predictive, and constructive validity, as well as external validity (reproducibility) in a sex-standardized manner. Research indicates that face validity of the CORT model is met as it produces robust and reliable behavioural phenotypes that approximates clinical depression symptoms (DeCarolis & Eisch,

2010). Chronic CORT administration increases despair-like behaviour in the FST (Ali et al., 2015; Gourley & Taylor, 2009; Wróbel et al., 2017) in a dose- (5mg/kg, 10mg/kg, 20mg/kg, or 40mg/kg) and time-dependent manner (Johnson et al., 2006; Lussier et al., 2013; Marks et al., 2015). Depressive-like behaviour worsens with each additional 21-day CORT cycle (Lebedeva et al., 2017, 2020), can be reversed by treatment with antidepressants (David et al., 2009; Fenton et al., 2015; Rainer et al., 2012), and is not mediated by weight or muscle strength of the animals. In addition, CORT-treated rats display anhedonic behaviours (Gourley & Taylor, 2009; Kvarta et al., 2015; Ma et al., 2018) and deficits in social behaviours, food-seeking, and sexual behaviours that are normally rewarding (Berger et al., 2019; Chan et al., 2017; Peng et al., 2021). Anxiety-like behaviours, often comorbid with depression in humans, are also increased in the CORT model as demonstrated with the open-field test (Li et al., 2017), the elevated-plus maze (Luo et al., 2017), and the light-dark box test (Murray et al., 2008). Cognitive deficits comparable to those observed in depressed humans are also observed in the CORT model, evidenced by impairments in the rodent's ability to recognize novel objects (Darcet et al., 2014) or familiar objects in a new location (Brymer et al., 2018, 2020), and deficits in spatial memory (Coburn-Litvak et al., 2003).

Biological changes in the chronic CORT model also parallel those found in depressed humans. Body-weight decreases are observed in a dose- and time-dependent manner (Johnson et al., 2006; Lebedeva et al., 2020). Alterations in circadian rhythm, often expressed as sleep deficits in humans, are expressed in CORT-treated rodents (Ma et al., 2018). Moreover, CORT-induced inflammatory responses are thought to contribute to premature aging (Ma et al., 2018; Xie et al., 2018). Of note, sex-differences in low-doses of shorter-length chronic CORT administration exist, showing a paradoxical behavioural effect in females (Brotto et al., 2001).

Neurochemical alterations are also found in CORT-treated rodents that parallel human depressed patients. Our laboratory has found CORT-induced reductions in neurogenesis paralleled depressive-like behaviour (Lussier et al., 2013) and can be rescued by antidepressants (Brymer et al., 2018; Fenton et al., 2015). Depressive-like states induced by CORT were also associated with decreases in excitability of dopaminergic VTA neurons (Peng et al., 2021), reductions in AMPAR-mediated neurotransmission (Kvarta et al., 2015), decreased GluA1 expression in the SGZ (Brymer et al., 2020), dendritic retraction in hippocampal neurons (Sousa et al., 2000),

dendritic hypertrophy in the amygdala (Mitra & Sapolsky, 2008), and mitochondrial damage resulting in cellular excitotoxicity, oxidative stress, and apoptosis (Allen et al., 2021; Ma et al., 2018). CORT-treated rats also show gradual decreases in reelin expression in the SGZ and CA1, and a lower number of dentate granule cells, paralleled in a time-dependent manner with depressive behaviours (Lussier et al., 2009, 2013). Interestingly, chronic restraint stress was ineffective at inducing depressive- and anxiety-like behaviours, did not reduce reelin expression, and failed at inducing GABAergic and glutamatergic neurotransmission generally associated with depression (Gregus et al., 2005; Lussier et al., 2013). Combined, these findings provide rationale for using chronic CORT-injections to model depressive-like phenotypes in rodents.

1.7 Ketamine

Ketamine is a dissociative anesthetic that can be used as an analgesic and has recently been implicated in the treatment of psychiatric disorders, particularly depression. As a non-competitive NDMAR antagonist, ketamine produces its effects in dose-dependent manner. First synthesized in 1962 as a derivative of phencyclidine (PCP), ketamine was observed to produce cataleptic, analgesic, and anaesthetic action without the hypnotic and hallucinogenic effects associated with PCP (Domino & Warner, 2010). Combined with its shorter acting properties, ketamine quickly grew in favour over PCP with the first human patient trials beginning in 1964. The term “dissociative anesthetic” was coined by the patients to describe a floating sensation of environmental disconnection, most likely due to a functional dissociation between the limbic and thalamocortical systems (Mion, 2017). Due largely in part to its large safety margins, ketamine was approved in 1970 by the USA Food and Drug Administration (FDA) as anesthesia, used primarily for field soldiers in the Vietnam war. However, ketamine use was largely discontinued soon after due to lowered patient tolerability from its psychotomimetic actions, seizures, and increases in intracranial pressure (Hirota & Lambert, 2022). Societal concerns surrounding ketamine’s abuse potential led to the US Controlled Substances Act designation of ketamine as a class III substance in 1999.

As an anesthetic, ketamine provides a unique spectrum of pharmacological effects including sedation, analgesia, catalepsy, bronchodilation, and sympathomimetic properties (Kurdi et al., 2014). Although it is most commonly used in veterinary medicine, ketamine is still used as an

intravenous induction agent in shocked or hypotensive patients, for children with congenital heart disease, burn patients, bronchospasms, battlefield analgesia, and in conjunction with benzodiazepines (Kurdi et al., 2014). At high doses, ketamine's antagonism on NMDA receptors produces amnesia and loss of consciousness due to NMDARs role in excitatory neurotransmission, LTP, and memory formation (Kohtala, 2021). Anesthetic doses of ketamine also antagonise μ and κ-opioid receptors (Hirota & Lambert, 2011). Non-analgesic doses can potentiate opioid analgesia in rodents (Campos et al., 2006) and perioperatively in human patients (Bell et al., 2006). Ketamine also appears to reduce the production of excess proinflammatory cytokines and inflammation-induced nitric oxide in a dose-dependent manner (Zanos et al., 2018). NMDAR inhibition also underlies ketamine's antinociceptive actions along with activation of descending inhibitory monoaminergic pain pathways (Hirota & Lambert, 2011). While NMDAR blockade is responsible for ketamine's most pronounced effects, other putative lower-affinity pharmacological targets have been identified including GABAergic, dopaminergic, serotonergic, adrenergic, opioidergic, cholinergic, and sigma receptors (Frohlich & Van Horn, 2014; Sinner & Graf, 2008), serotonin, noradrenaline, and dopamine reuptake transporters (Kohtala, 2021), and various ion channels such as voltage-gated sodium and hyperpolarization-activated cyclic nucleotide-gated channels (Zanos et al., 2018).

As ketamine is water- and lipid-soluble, it can be administered through multiple routes (intravenous or intramuscular injection, intranasally, rectally, and orally) while still rapidly crossing the blood-brain barrier (Sinner & Graf, 2008). Ketamine is characterized by a chiral carbon at the optically active centre (C2 position) of the molecule creating two enantiomers: S(+) isomer and R(−) isomer. Pharmaceutically, ketamine is produced in both racemic and enantiopure forms as its stereoselective binding causes differences in clinical potencies and receptor-affinities (Sinner & Graf, 2008). (S)-ketamine is generally preferred in clinical anesthesia due a higher binding affinity to NMDARs 4x that of (R)-ketamine and 2x that of the racemic mixture (Kohtala, 2021; Zhang et al., 2021). Both stereoisomers exhibit voltage- and use-dependent blockade of NMDAR currents by acting as an open channel block (Zanos, et al., 2018). By binding to the allosteric PCP site localized deep within the NMDAR channel pore, ketamine occludes ions from moving through the channel; although the PCP site is only accessible when the channel is in open confirmation with the Mg 2+ block already removed (MacDonald et al., 1987). Interestingly, women express a 20% greater elimination clearance rate

of (S)-ketamine, (R)-ketamine, and its metabolites, resulting in lower drug plasma concentrations in women (Saland et al., 2017; Sigtermans et al., 2009).

1.7.1 Ketamine as an antidepressant

The discovery of ketamine's rapid-acting antidepressant effects has resulted in a surge of clinical and pre-clinical research to establish ketamine as a novel therapeutic in the treatment psychiatric disorders. Berman and colleagues first demonstrated that subanesthetic doses (0.5mg/kg) of ketamine could elicit rapid-acting (within hours) and sustained (up to 3 days) antidepressant effects in patients previously diagnosed with MDD (Berman et al., 2000). Since this breakthrough, a multitude of randomized placebo-controlled trials have extended upon this findings to validate ketamine's robust antidepressant effects in patients with MDD (Alnefeesi et al., 2022; McIntyre et al., 2020), treatment-resistant depression (Dai et al., 2022; Zarate et al., 2006), treatment-resistant bipolar disorder (Diazgranados et al., 2010; Zarate et al., 2012), and post-traumatic stress disorder (Kim, 2017; Stein & Simon, 2021). Compared to traditional antidepressants, ketamine showed significantly higher response and remission rates and was able to improve traditionally hard to treat symptoms such as suicidality, anhedonia, and amotivation (Monteggia & Zarate, 2015). Meta-analysis shows that reduction in depressive symptoms start as early as within 40 minutes and last up to 8 days, with response peaking at 24 hours post-infusion (Kishimoto et al., 2016). This is remarkable because ketamine's antidepressant effects are sustained well beyond its relatively short half-life (2-4 h) and peak pharmacokinetic exposure (Kohtala, 2021; Monteggia & Zarate, 2015). Considering this, ketamine's antidepressant response is most likely mediated by acute changes in synaptic plasticity that lead to sustained strengthening of excitatory synapses.

These exciting findings led to the approval of intranasal esketamine (the S-enantiomer of ketamine; Spravato) for adults with TRD (defined as non-responsiveness to at least two antidepressant treatments of adequate dose and duration) by the U.S. FDA and the European Union. However, concerns around ketamine's abuse potential persist largely due to its transient efficacy and overlapping neural circuitry between depression and addiction (Kokane et al., 2020; Turner, 2019). Additionally, ketamine must be administered under medical supervision as acute neurocognitive and psychotomimetic side-effects are common at around 40 minutes post-

infusion (Acevedo-Diaz et al., 2020; Sassano-Higgins et al., 2016), although they are generally transient and resolve spontaneously (Short et al., 2016). More recently, a flood of research has focused on (R)-ketamine in attempt to minimize the adverse effects while maintaining the fast-acting antidepressant properties of (S)-ketamine. (R)-ketamine appears to exhibit greater potency and longer-lasting antidepressant effects in rodent models of depression (Fukumoto et al., 2017; Yang et al., 2018; Zhang et al., 2014), with a higher safety profile and lower side effects (Bonaventura et al., 2021; Chang et al., 2019; Tian et al., 2018), as well as in human patients with TRD (He et al., 2022; Leal et al., 2021). Despite intense research efforts, the neurobiological mechanisms underlying ketamine's antidepressant effects remain elusive largely due to its dose-specific effects on molecular mechanisms, multiple pharmacologically active metabolites, and complex mechanism of action.

There are currently two main hypotheses to explain the neural mechanisms of ketamine's antidepressant effects: the disinhibition hypothesis and the direct inhibition hypothesis. At the core of both hypotheses, low dose ketamine's NMDAR antagonism promotes LTP-like synaptic plasticity and increases excitatory synapses in corticolimbic brain regions (Autry et al., 2011; Miller et al., 2016; Monteggia & Zarate, 2015), a dysregulation of which is a biological hallmark of depression (Castrén & Hen, 2013; Massart et al., 2012; Normann et al., 2018). The disinhibition hypothesis proposes that ketamine selectively antagonizes tonically active NMDARs expressed on GABAergic interneurons, which leads to the disinhibition (the loss of tonic inhibition) and indirect excitation of pyramidal neurons (Zanos & Gould, 2018). Enhanced glutamatergic firing increases postsynaptic AMPAR expression, which activates downstream neurotrophic signaling pathways to promote protein synthesis (Li et al., 2010; Miller et al., 2016). Under the second hypothesis, ketamine's direct inhibition on extra-synaptic GluN2B-containing NMDARs lessens their tonic activation resulting in rapid compensatory glutamatergic increases, which then deactivates eukaryotic elongation factor 2 (eEF2) kinase (CaMKIII) (Autry et al., 2011; Nosyreva et al., 2013). From there, common downstream effector pathways increase protein synthesis which ameliorates the chronic-stress synaptic induced deficits associated with depression (Zanos et al., 2018). It should be noted that these two hypotheses are not mutually exclusive and may act complementarily with hydroxynorketamine (HNK) metabolites (2R,6R)-HNK and (2S,6S)-HNK and other mechanisms of action for antidepressant response (Zanos & Gould, 2018).

AMPAR-mediated mechanisms may underlie the downstream signaling pathways to produce the above-described plasticity and neurogenesis (Kohtala, 2021). Briefly, the evoked glutamine release activates to post-synaptic AMPARs which enhances BDNF release (Abdallah et al., 2018). BDNF then binds to the tropomyosin receptor kinase B (TrkB) receptor, which activates mechanistic target of rapamycin complex 1 (mTORC1) through downstream signaling molecules (Zanos et al., 2018). Transient mTORC1 activation upregulates proteins involved in increasing excitatory transmission such as post-synaptic density-95 (PSD-95) and Synapsin I, and increases membrane insertion of GluA1 subunit which mediates AMPAR trafficking (Li et al., 2010). Of note, while the induction of antidepressant response is associated with changes in AMPAR activation, the persistent antidepressant response is associated with changes in AMPAR number and function (Zanos et al., 2018). This promotion of protein synthesis, synaptogenesis, and activity-dependent synaptic plasticity allows for a continued antidepressant response, even when the drug has been metabolized and is no longer in the system (Wohleb et al., 2017).

1.8 Reelin

Reelin is an endogenous glycoprotein that plays an important role in both the developing and adult brain in which our laboratory has been studying for the past decade (Caruncho et al., 2016). The "reeler" mouse was first described in 1951 following a spontaneous autosomal recessive mutation in a colony of mildly inbred mice (Falconer, 1951). These mutant homologous reeler mice (*RELN*$^{-/-}$) exhibited severe neuronal abnormalities accompanied by a "reeling" gait (Caviness, 1976), which was later discovered to be from the complete loss of *RELN* gene transcription (D'Arcangelo et al., 1995). The loss of *RELN*, a protein coding gene that normally produces the extracellular glycoprotein reelin, was accompanied by neuronal ectopia in laminated brain structures including the hippocampus, cerebellum, and cortex (D'Arcangelo et al., 1999). Aberrant cortical lamination was accompanied by other neurological abnormalities including severe cerebellar hypoplasia and size decreases, as well as behavioural deficits of impaired motor coordination, tremors, ataxia, and imbalance (Cooper, 2008; D'Arcangelo et al., 1999). More recently, studies using molecular marker-based phenotyping and fine-scale electron microscopy suggest a more widespread disorganization in the *reeler* neocortex which contain neuronal clusters instead the classic six-layered organization of the neocortex (Prume et al., 2018; Ranaivoson et al., 2016). In humans, *RELN* mutations can cause major brain

malformations such as lissencephaly (smooth brain) due to improper migration of post-mitotic neurons from the ventricular zone (Kato & Dobyns, 2003). Since its discovery, research has implicated reelin in the facilitation of proper neurodevelopment, the maintenance of brain functioning in adulthood, and a dysfunctional role in neuropsychiatric and neurodegenerative disorders.

The reelin sequence is composed of 3461 amino acids that are coded by the *RELN* gene located on chromosome 5 in mouse and 7 (7q22) in humans, with a molecular mass of 388 kDa (DeSilva et al., 1997; Ranaivoson et al., 2016). Mouse and human reelin have a sequence identity of 94.2% for amino acids and 87.2% at DNA level (DeSilva et al., 1997), suggesting some inter-species functional conservation. Structurally, reelin is homomeric protein complex composed of an N-terminal (required for multimerization), 8 consecutive reelin repeats (RR), and a highly basic C-terminal which participates in downstream signaling (de Bergeyck et al., 1998; Koie et al., 2014). Each RR (350-390 amino acids each) is divided into two homologous sub-domains (A and B) separated by an epidermal growth factor (EGF)-like cysteine motif (DeSilva et al., 1997; Ranaivoson et al., 2016). *In vivo,* secreted reelin undergoes proteolysis by which extracellular matrix metalloproteinases degrade reelin into three major fragments (Jossin, 2020). As the central region (RR3-RR6) is necessary for reelin functioning and receptor-binding, central reelin is specifically cleaved at the N-t (between Pro-1244 and Ala-1245 within RR3) and C-t (between Arg3455 and Ser3456 within RR6-RR7) sites (D'Arcangelo et al., 1999; Ranaivoson et al., 2016). Proteolysis occurs by members of the Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMT) family of proteins (ADAMT2/3 cleaves N-t; ADAMT4/5 cleaves C-t) and can occur in endosomes or extracellular space (Jossin, 2020; Koie et al., 2014; Sato et al., 2016). The central fragment of reelin binds to apolipoprotein E receptor 2 (ApoER2) and the very-low-density lipoprotein receptor (VLDLR), members of the lipoprotein superfamily (Ranaivoson et al., 2016; Yasui et al., 2007). It is thought that each fragment may regulate different processes of radially migrating neurons (Jossin et al., 2004, 2007; Kubo et al., 2002), however the functional significance of degradation remains elusive.

1.8.1 Reelin throughout the lifecourse

From early preplate embryonic stages of cerebral cortex development (day 10-12), reelin is secreted from Cajal-Retzius cells in the marginal zone of the cortex and hippocampus, and by glutamatergic cerebellar cells (Curran & D'Arcangelo, 1998; D'Arcangelo et al., 1999; Vílchez-Acosta et al., 2022). Once released into the extracellular space, reelin binds to VLDLR and ApoER2, which induces tyrosine phosphorylation to activate disabled-1 (Dab1) by Src family kinases (SFKs) Fryn and Src (Fuchigami et al., 2013). Dab1 is an adaptor protein that initiates a tyrosine kinase signal transduction cascade to regulate cell positioning in the developing brain (Luque et al., 2003).

During early phase of cortical neurogenesis, reelin (and its receptors VLDLR and ApoER2) is critical in orchestrating the typical inside-out arrangement of cortical neurons by controlling cell-to-cell interactions to appropriately position migrating neurons (Chameau et al., 2009). To do so, reelin induces a specific radial glia phenotype from progenitor cells born in the ventricular zone which have projection fibers that act as ascent scaffolds (Chai et al., 2009). In normal development of the six-layered cortex, early-born neurons occupy the deeper layers and late-born neurons bypass their predecessors to reach more superficial layers (Fuchigami et al., 2013). Several theories suggest that reelin may act as a detachment/positional signal or chemoattractant migrating cortical neurons, while others suggest it to be a repellent to subplate neurons (Gilmore & Herrup, 2000). It is clear however, that reeler mutations affect the ability of postmitotic neurons to correctly position themselves in the developing brain. The cortical plate develops ectopically; young neurons fail to migrate past their predecessors and pile underneath layers of pre-existing cells resulting in an apparent inversion of cortical layers (Chai et al., 2009; Curran & D'Arcangelo, 1998; Vílchez-Acosta et al., 2022). Interestingly, double-knockout of VLDLR and ApoER2, or loss of Dab1 mutants, express a reeler-like phenotype although the receptors appear to have divergent roles in cell migration (Wasser & Herz, 2017). VLDLR mutants (but not in ApoER2$^{-/-}$) express marginal zone neuronal invasion, implying that VLDLR may act as a 'stop signal', while ApoER2 mutants (but not Vldlr$^{-/-}$) show late-generated neuronal migration defects (Hack et al., 2007). Similarly, Dab1 gene mutations produce similar phenotypes as reeler- and VLDLR/ApoER2 knockout mice, and disrupts proper neuronal layering (Howell et al., 1997; Sweet et al., 1996). This illuminates the interconnectedness of reelin, its receptors, and Dab1 in

brain development, and why the central fragment of reelin (RR3-RR6) is sufficient to govern proper cortical layer formation (Jossin et al., 2004; Wasser & Herz, 2017).

Postnatally, reelin is repurposed as a neuromodulator for NMDAR-mediated neurotransmission, which is essential for synaptic plasticity, learning, and memory (Knuesel, 2010). As Cajal-Retzius cell density decreases (in rodents: the first 2 postnatal weeks) due to differentiation and apoptosis, reelin production is shifted to cortical and hippocampal GABAergic and glutamatergic cells in the olfactory bulb, cerebellum, and layer II pyramidal cells in the piriform and EC (Chameau et al., 2009; Jossin, 2020). In these areas, reelin regulates cell signaling by modulating neuronal connections through synaptogenesis, dendritogenesis, and dendritic spinogensis (Beffert et al., 2006; Niu et al., 2004; Rogers et al., 2013; Ventruti et al., 2011; Weeber et al., 2002). Indeed, reelin overexpression accelerates dendritic growth and maturation, while the reelin/Dab1 pathway disruption produces neurons with aberrant dendritic development and orientation (Jossin, 2020; Lossi et al., 2019; Wasser & Herz, 2017). Dysregulation of this pathway can also affect the morphology of dendritic spines by changing the configuration of presynaptic boutons which, in turn, can affect the function of synapses (Bosch et al., 2016).

In adulthood, reelin regulates dendrite development, spine formation, glutamatergic neurotransmission, and neural plasticity. Reelin also modulates the molecular composition of hippocampal synapses, as reelin expression changes alter NMDAR subunit composition (Wasser & Herz, 2017). Reelin signaling is a critical regulator of both number and strength of synaptic connections, which is important because the number and molecular composition of synaptic connections determines the efficacy of neuronal networks (Wasser & Herz, 2017). Interestingly, one intraventricular injection of reelin can enhance LTP and cognitive function in wild-type mice (Rogers et al., 2011) and heterozygous reeler mice (Rogers et al., 2013). Reelin is also thought to have an effect on the immune system, liver fibrosis, multiple cancers, and is essential for the structural and functional organization of the blood-brain barrier (D'Arcangelo et al., 1999; Vílchez-Acosta et al., 2022). Schematic representation of reelin's signaling pathway can be found at Figure 1.3.

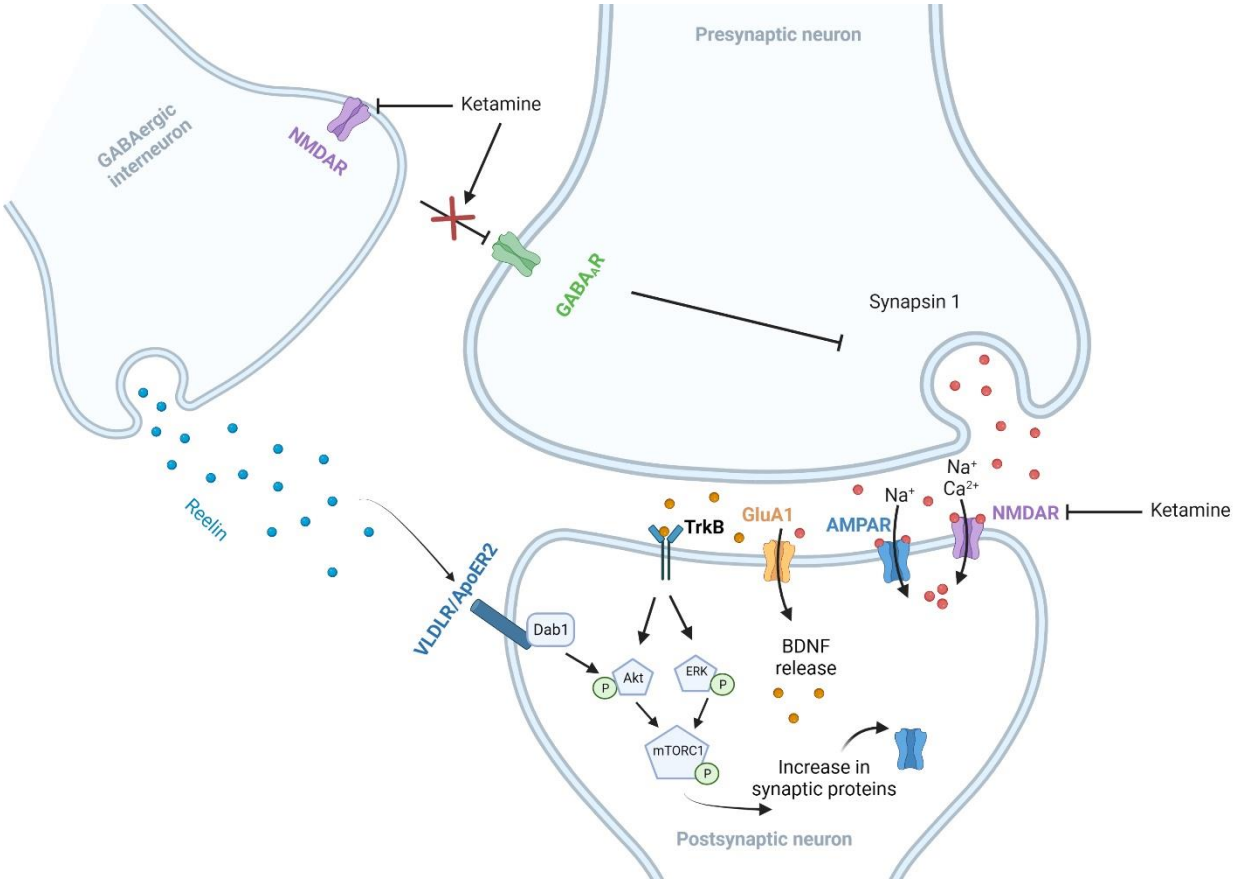


Figure 1.3 Hypothesized overlapping signaling pathways of reelin and ketamine. Reelin is released from GABAergic interneurons and then binds to its receptors VLDLR (very low-density lipoprotein receptor) and ApoER2 (Apolipoprotein E receptor 2). Upon binding, cytoplasmic adaptor protein Dab1 is phosphorylated by SFKs Fyn and Src, which activates PI3K (phosphoinositide-3'kinase) to initiate downstream activation of mTORC1 (mammalian target of rapamycin complex 1). mTORC1 phosphorylation increases expression of synaptic proteins (e.g., GluA1 and PSD-95) which feeds forward for excitatory signalling. Ketamine may antagonize NMDARs on GABAergic interneurons which disinhibits excitatory cells, leading to an influx of synaptic glutamate. Glutamate release triggers Ca^{2+} influx, which increased BDNF release, which in turn binds to TrkB receptors to activate parallel pathways as reelin to upregulate mTORC1 activity. Figure was created in BioRender by author.

1.8.2 Reelin and its involvement in depression

Since its discovery, reelin has been implicated in a myriad of neurodevelopmental and neuropsychiatric disorders including autism, Alzheimer's disease, schizophrenia, bipolar disorder, and depression. Post-mortem brain analyses show a significant decrease of *RELN* mRNA throughout the brain in schizophrenia (Impagnatiello et al., 1998), in the cerebral cortex of those with bipolar disorder (Guidotti et al., 2000), and in the hippocampus (most notably in

the DG) from patients with schizophrenia, bipolar, and MDD (Fatemi et al., 2000; Knable et al., 2004). Abnormal serum reelin levels paralleled this central downregulation in those with mood and psychotic disorders (Fatemi et al., 2000), an effect which may be mediated by epigenetic mechanisms (e.g., psychosocial stress) through the hypermethylation of the *RELN* promoter (Abdolmaleky et al., 2005; Veldic et al., 2004).

The role of reelin in neuropsychiatric disorders is often examined using homozygous ($RELN^{-/-}$) and heterozygous ($RELN^{+/-}$) reeler mice who express null- and 40-60% levels of reelin respectively. The neuroanatomical abnormalities seen in homozygous reeler mice (e.g., improper lamination) are not present in the haplo-insufficient reeler mice, however $RELN^{+/-}$ mice do express some subtle physiological abnormalities that can be linked to psychiatric disorders. For example, reductions in dendritic spine number and density observed in $RELN^{+/-}$ and $RELN^{-/-}$ to varying degrees, is similarly found in patients with schizophrenia (Wasser & Herz, 2017). Along with dendritic spine deficiencies, reductions in PSD-95, parvalbumin, and activity-regulated cytoskeletal protein (Arc) are observed in $RELN^{+/-}$ and $RELN^{-/-}$ mice which combined, weaken synaptic transmission in brain areas pertinent in depression (Dong et al., 2003; Liu et al., 2001). In fact, inactivation of the reelin signaling pathway in adult hippocampal neural progenitor cells impedes proper migration and dendritic maturation, while heightened reelin activity promotes dendritic outgrowth and proper circuit establishment of dentate granule cells (Teixeira et al., 2012). This is important because deficiencies in hippocampal neurogenesis and dendritic atrophy are pertinent characteristics of depression (Nissen et al., 2010; Paizanis et al., 2007; Wainwright & Galea, 2013). Additionally, reelin downregulation alters its co-expression with neuronal nitric oxide synthase (nNOS) which can create imbalances in glutamatergic and GABAergic circuits (Caruncho et al., 2016), and disturbances in the dopaminergic and 5-Htergic systems (Ballmaier et al., 2002; Varela et al., 2015). These systems influence mood and motivational behaviour and are thought to play crucial roles in the pathophysiology of depression (Di Giovanni et al., 2008; Duman et al., 2019; Li et al., 2015; Sanacora et al., 2012; Venzala et al., 2013).

Behaviourally, certain tests such as Open Field Test (OFT), black-white box, FST, novelty-suppressed feeding tests, and cocaine sensitization are unable to phenotypically distinguish between $RELN^{+/-}$ and wild-type mice (Fatemi, 2011; Teixeira et al., 2011). However, compared to wild-type mice, $RELN^{+/-}$ demonstrate working-memory impairments and emotional deficits

particularly in males (Iemolo et al., 2021), and VLDLR/ApoER2 knockout mice display deficits in contextual fear conditioning (Weeber et al., 2002). Further, *RELN*$^{+/-}$ mice display social deficits, poor inhibitory control, and altered stress responses (Iemolo et al., 2021), phenotypes that characterize an array of psychiatric disorders. It is possible that the downregulation of reelin potentiates depressive-like behaviour by delaying new-born cell maturation and interfering with their proper neuronal placement and integration (Caruncho et al., 2016). Haplo-insufficient reeler mice are particularly vulnerable to the depressive-like phenotype; chronic CORT administration increases immobility on the FST and decreases in neurogenesis in *RELN*$^{+/-}$ but not wild-type mice (Lussier et al., 2011).

Chronic stress also consistently decreases reelin expression in rodents that do not express genetic vulnerabilities (Caruncho et al., 2016). Our laboratory has shown that chronic CORT exposure decreased the number of reelin-IR cells by 21% in the CA1 stratum-lacunosum-moleculare and by 26% in the dentate gyrus SGZ (Lussier et al., 2009). We then showed that chronic-stress induced decreases in reelin-IR SGZ expression are paralleled with depressive-like phenotypes, demonstrated by an increase in time spent immobile in the FST indicative of despair (Lussier et al., 2011, 2013). Both traditional and non-traditional antidepressants were able to rescue the CORT-induced behavioural and neurochemical deficits. Chronic administration of the TCA imipramine and TNF-α inhibitor etanercept were both able to rescue deficits in hippocampal reelin and neurogenesis (Brymer et al., 2018; Fenton et al., 2015); repeated treatment with the SSRI citalopram counteracted the downregulation of reelin mRNA and protein levels from kainic acid's deleterious effects (Jaako et al., 2011); and ketamine was able to rescue hippocampal reelin (Johnston et al., 2020). In fact, it was recently demonstrated that the genetic abolishment of reelin, its receptor ApoER2, or certain downstream effectors can abolish the behavioural and biochemical antidepressant- like effects of ketamine (Kim et al., 2021). Together, these studies provide strong evidence that reelin signaling may play a role in the antidepressant therapeutic response.

The accumulating evidence towards reelin's role in depression led our lab to evaluate the antidepressant-like effects of exogenous reelin administration. Following the chronic CORT paradigm, 1µg of reelin was infused directly into the hippocampus in a repeated (once per week for 3 weeks) or acute (one time 24 hours before FST) manner. Both repeated and singular

injections of intrahippocampal reelin rescued despair-like behaviour in the FST immobility, hippocampal-dependent cognitive deficits in object location task, and neurochemical deficits in NMDARs, AMPARs, and GABAARs, but only if AMPARs were not blocked by antagonist CNQX (Brymer et al., 2020). Repeated but not singular injections rescued the number and dendritic complexity of newborn granule cell proliferation, suggesting that increases in neurogenesis are not necessary for reelin's fast-acting antidepressant effects. Other labs have also found therapeutic effects of intracranial reelin infusions in mouse models of depression including intrahippocampal (Ibi et al., 2020), intra-amygdalar (Nelson & Pinna, 2010), and intraventricular (Hethorn et al., 2015; Rogers et al., 2011).

As intracranial infusions are not a viable option in humans, our lab established a peripheral administration paradigm using intraventricular injections (i.v.) of reelin into the lateral tail vein of chronic CORT treated rats (Allen et al., 2022). Repeated and acute i.v. reelin injections were able to rescue FST immobility, hippocampal reelin, GABA Aβ2/3, GluA1, and GluN2B receptors, and serotonin transporter (SERT) clustering deficits in peripheral lymphocytes (Allen et al., 2022). Low reelin levels could therefore provide sensitization to the depressogenic effects of stress whereas increasing reelin signaling could improve stress resilience (Fatemi, 2011). We also found sex-specific differences in stress response as basal density of reelin-positive cells in the medial preoptic and paraventricular hypothalamus were lower in females (Sánchez-Lafuente et al., 2022). Most recently, our lab demonstrated that reelin and ketamine similarly rescue reelin expression in the SGZ, and synaptic expression of mTOR and p-mTOR that were decreased by corticosterone (Johnston et al., 2020). Reelin may work through similar synaptic mechanisms as ketamine to rescue the CORT-induced behavioural and biochemical deficits, further implicating reelin as a potential fast-acting antidepressant.

1.8.3 Reelin in the periphery

Peripheral reelin has important pleiotropic roles in the development, repair, and function of biological systems associated with psychiatric disorders. While most research on reelin expression in MDD has focused on the central nervous system, peripheral reelin could also be heavily implicated in the pathophysiology of depression. Reelin is expressed in non-neuronal tissues during development and adulthood including the spleen, liver, kidney, testes, ovaries,

colon, adrenal glands, and lymphatic tissue (Böttner et al., 2014; Lutter et al., 2012; Samama & Boehm, 2005; Smalheiser et al., 2000). Reelin is also expressed in the lymphatic system in bone marrow (Dou et al., 2021; Maurin et al., 2004) and circulated in blood plasma (Smalheiser et al., 2000; Tseng et al., 2010). While the functional role of reelin in the periphery is not well understood, peripheral reelin expression could have important physiological and therapeutic implications in psychiatric disorders. For instance, altered levels of plasma reelin were found in patients with schizophrenia, bipolar, and depression (Fatemi et al., 2001). Further, patients with schizophrenia who have lower levels of lymphocyte VLDLR expression showed a negative correlation with clinical symptom severity (Suzuki et al., 2008).

Reelin regulates membrane protein clustering (MPC) which appears to influence proper functioning of proteins and is involved in responsiveness to antidepressants (Johnston et al., 2020; Rasenick et al., 2007; Strasser et al., 2004; Zhang & Rasenick, 2010). Embedded within the external leaflet of the plasma membrane's lipid bilayer are lipid microdomains involved in the assembly and organization of neurotransmitter signalling components (Rivera-Baltanas et al., 2010). Lipid rafts are highly ordered and tightly packed dynamic microdomains that move and communicate with one another but can also cluster to form large, ordered platforms (Ouweneel et al., 2020). The efficient functioning of SERT, a free-floating membrane protein and the primary target of antidepressants, is maintained by its clustering into specific lipid raft domains (Magnani et al., 2004), and is thought to be critical in the antidepressant response (Allen et al., 2007). In fact, independent of MDD diagnosis, SERT binding is significantly lower in those who died by suicide (Underwood et al., 2018), and could be a substrate in the biology of suicidality (Purselle & Nemeroff, 2003).

Our laboratory has identified SERT clustering on the lipid rafts of peripheral blood mononuclear cells (PBMC) as a promising therapeutic biomarker. We found that SERT cluster sizes are increased in treatment-naïve depression patients compared to non-psychiatric controls, a finding that is paralleled in preclinical animal models (Caruncho et al., 2019). Additionally, SERT distribution pattern (percent clusters within modal peak) in depressed patients could predict treatment response to antidepressants, differentiating a treatment-resistant depressed group (Rivera-Baltanas et al., 2012). Treatment-resistant patients had ~40% of clusters within the modal peak and relatively few clusters of a larger size, while those that responded better to

treatment expressed more clusters of a larger size and ~25% of clusters within the modal peak (Rivera-Baltanas et al., 2014). As reelin is thought to induce membrane protein clustering (Dong et al., 2003), we analyzed SERT cluster size in reeler mice compared to wild-type mice and found 2x and 60% cluster size increases in homozygous and heterozygous reeler mice respectively (Rivera-Baltanas et al., 2010). We later found that chronic-CORT administration induces alterations in SERT clustering patterns and increases protein cluster size (Romay-Tallon et al., 2018), parameters that can be rescued by a singular i.v. injection of reelin (Allen et al., 2022), but not ketamine (Johnston et al., 2020). Overall, it seems that a lack of reelin expression is associated with abnormal protein clustering in immune cells, many of which can cross the blood-brain barrier (BBB) and are implicated in depression.

Drug transport across the BBB is important in neurotherapeutics, however it is currently unknown if full-length reelin or fragments of reelin can cross the BBB. Formed by the brain capillary endothelium, epithelial-like tight junctions in the BBB prevent the passage of large molecules and ~98% of all small-molecule drugs (Pardridge, 2005). While most antidepressants are able to passively diffuse across the BBB, recombinant proteins such as reelin are often thought to be too large to be delivered across the BBB. Interestingly, reelin-immunoreactivity has been observed in endothelial cells, particularly in caveolae (vesicles of transcytosis), that line the BBB which may represent a mechanism whereby reelin can access the brain (Perez-Costas et al., 2015). Indeed, ApoER2 are localized in the same caveolae, suggesting that reelin may cross in a receptor-mediated mechanism (Riddell et al., 2001). New theories on the putative mechanisms of antidepressants suggest that stimulating peripheral mediators such as insulin-like growth factor 1 (IFG-1) could underlie the antidepressant action of 5-HT (Manev & Manev, 2002). Understanding the relationship between peripheral reelin and the BBB could have important physiological, pathological, and therapeutic implications.

1.9 Specific Research Aims

The research presented in this thesis aims to ascertain reelin's time course effect both individually and synergistically with that of ketamine. Our lab has already shown that individually, one dose of either reelin or ketamine can rescue CORT-induced behavioural and

neurochemical alterations after 24h. Based on our previous work and with the suggestions of patient-partners, I developed two main objectives for my research:

Objective 1: assess the time-course effect of reelin and ketamine individually. Before translation to clinical trials, a drug's pharmacokinetics and pharmacodynamics must be evaluated in preclinical research to determine therapeutic potential. Thus, I decided to examine reelin and ketamine's time course response which is based on drug absorption, distribution, metabolism, and elimination, to evaluate onset of action and sustained duration. Using a chronic CORT-administration paradigm, I evaluated their pharmacological effects at 1h, 6h, and 12h after treatment with either ketamine or reelin. An additional treatment group (1w) received five more days of CORT injections after reelin/ketamine treatment to evaluate the protection of a single dose against continued stress. Behaviourally, the time course response was evaluated using the FST, a measure of despair-like behaviour. Reelin-immunoreactive cells in the SGZ and SERT clustering changes on lymphocytes were examined to evaluate the short-term effects of exogenous reelin and ketamine administration on central and peripheral reelin expression respectively.

Objective 2: evaluate the synergistic, time-dependent effect of ketamine and reelin combined. As evidence suggests that ketamine and reelin may be working in parallel pathways, I decided to investigate their combined effect at sub-anesthetic doses at the above-mentioned time-points. Behavioural testing (FST) was followed by post-mortem analyses of reelin in the hippocampus, and I analyzed the effect of reelin on SERT MPC in blood lymphocytes, all of which are associated with depression.

Chapter 2: Methodology

2.1 Animal husbandry

Female Long Evans rats (N = 120) were acquired from Charles River Laboratories (Montreal, Quebec, Canada) and were 6 weeks old upon arrival. The rats weighed between 150 – 250g for the duration of the experiment. Animals were housed individually in clear polypropylene cages that contained a red hut and a wooden chew cube. Social isolation elicits chronic stress and is a widely used as an animal model to potentiate depression-like behaviour in rodents (Murínová et al., 2017). Access to food (Purina rat chow) and water were provided ad libitum except during behavioural testing procedures, and bedding was changed once per week. The colony was thermal-controlled (21°C) and maintained on a 12-h light/dark cycle, with lights turning on at 07:00 a.m. All procedures were approved by the University of Victoria Animal Research Ethics Board and conducted in accordance with the Canadian Council on Animal Care.

2.2 Experimental procedure

Upon arrival, rats were habituated for one week in quarantine followed by another week of daily handling. Rats were weighed daily and randomly assigned to treatment groups receiving either vehicle (Sigma Aldrich; 0.9% [w/v] sodium chloride and 2% [v/v] polysorbate-80 solution) or corticosterone (Steraloids; 40mg/kg suspended in vehicle solution) injections administered at 1ml/kg. The injections were administered subcutaneously between the hours of 08:00 and 11:00 am daily for 21 days (timepoints 1h, 6h, 12h) or 26 days (1w timepoint). On the last day (21 or 26 group-dependent) vehicle (PBS), ketamine hydrochloride (Narketan; Vetoquinol; Lavaltrie, Quebec, Canada; 10mg/kg), recombinant reelin (R&D systems, 3820-MR-025; composed of RR 3-6; predicted molecular weight of 180kDa by SDS-PAGE using reducing conditions), or recombinant reelin and ketamine were administered. Reelin was administered intravenously into the lateral tail vein at 3μg based off a previous effective dose (Allen, 2022) and suspended in 0.5ml of 0.1M phosphate buffered saline (PBS, pH = 7.4). Ketamine hydrochloride was suspended in saline and injected intraperitoneally at 10 mg/kg in a volume of 1 ml/kg, based off previous pre-clinical research, which found that a dose of 10mg/kg – 15mg/kg is the most effective at rescuing depressive-like behaviour (Ardalan et al., 2016; Zhu et al., 2017). Animals in the 1w treatment group underwent five more days of CORT/vehicle injections following

vehicle/reelin/ketamine/reelin-ketamine treatment. Schematic representation of experimental groups can be found in Figure 2.1 and experimental timeline can be found in Figure 2.2.

Treatment		21 days			26 days
Chronic	Acute	1h	6h	12h	1w
Vehicle	Vehicle	n = 6	n = 6	n = 6	n = 6
CORT	Vehicle	n = 6	n = 6	n = 6	n = 6
CORT	Reelin	n = 6	n = 6	n = 6	n = 6
CORT	Ketamine	n = 6	n = 6	n = 6	n = 6
CORT	Reelin & Ketamine	n = 6	n = 6	n = 6	n = 6

Figure 2.1 Experimental groups. CORT (40mg/kg subcutaneously), reelin (3 µg, intravenously), ketamine (10 mg/kg, intraperitoneally). Figure created by author in BioRender.

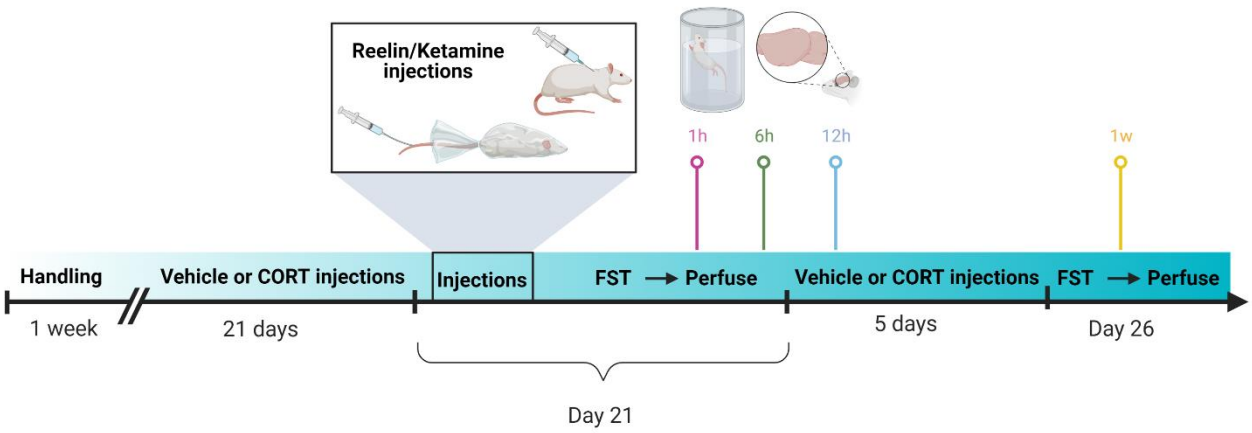


Figure 2.2 Experimental timeline. Female Long Evans rats received daily subcutaneous injections of CORT (40mg/kg) or vehicle (saline) for 21 days. On day 21, rats received injections of reelin (3 µg, intravenously), ketamine (10 mg/kg, intraperitoneally), reelin and ketamine, or vehicle (PBS). Rats underwent the forced-swim test and were perfused immediately following the FST at 1h, 6h, and 12h post-injection. 1w timepoint rats underwent an additional 5 days of either CORT or vehicle injections before the FST and perfusions on day 26. Figure created by author in BioRender.

2.3 Forced Swim Test

A modified one-day version of the Porsolt test was used 1h, 6h, 12h, and 1w following vehicle/reelin/ketamine/reelin-ketamine treatment. The Porsolt test was originally designed to serve as a behavioural assay for the efficacy of antidepressant drugs (Porsolt et al., 1978), with the one-day protocol abolishing potential confounding effects of memory influencing behaviour in a 2-day protocol. Rats were placed into a rectangular Plexiglas swim tank (25cm wide × 25cm long × 60cm high) filled with water (27±2°C) to a depth of approximately 30cm for 10 mins. The amount of time swimming, climbing, immobile, and latency to immobility were manually scored over the 10-minute test time. Immobility was defined as the rat floating or moving just enough to keep afloat as an indicator of despair-like behaviour. As time following treatment was an important factor in this study and behavioural tests should be administered with a minimum of a day apart (Rizzo & Silverman, 2016), only the FST was used as a behavioural measure.

2.4 Perfusions, blood collection, tissue preparation

First, the rats were deeply anaesthetized with 5% isoflurane maintained through an isoflurane machine attached to a nosecone that was placed over the rat's nose. While anaesthetized, 3ml of blood was extracted from the heart with a syringe containing 0.5ml ACD anticoagulant (85mM trisodium citrate, 65mM citric acid, 111mM anhydrous glucose) to make smears on slides. Visual observation and vaginal cytology through the collection of a vaginal lavage smear was used to determine the stage of the estrous cycle by cell type. The rats were then transcardially perfused with 0.1M phosphate buffer (PB, pH 7.4) followed by 500ml of ice-cold 4% (w/v) paraformaldehyde in 0.1M PB (pH 7.4). The brains were then removed and postfixed in 4% paraformaldehyde (w/v) for 48 hours at 4°C. The brains were transferred to 10%, 20%, and then 30% sucrose containing 0.1% sodium azide for 72h before sectioning. Sectioning occurred in the coronal plane at 30 µm on a cryostat (Vibratome ULTRAPRO 5000; CM1850 UV, Leica Biosystems) at -20˚C. Sections were stored in standard cryoprotectant solution [30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4)] at -20°C until use.

2.5 Immunostaining

Standard immunohistochemical and immunocytochemistry techniques were utilized with commercially available antibodies.

2.5.1 Immunohistochemistry protocol

Reelin-immunoreactive (reelin-IR) cells were visualized following immunohistochemical protocols as previously validated by our lab (Lussier et al., 2013). Every 6th section of the coronal brain sections containing the dorsal hippocampus was collected and placed in 6-well tissue culture plates as free-floating sections in tris-buffered saline (TBS) under gentle agitation. All rinses were conducted in TBS (50 mM Tris-Cl, 150 mM NaCl; pH 7.6). No immunoreactive cells were detected when the primary antibody was omitted in an additional well.

Following the initial rinses, the antigen retrieval step took place in which sections were incubated in sodium citrate (pH = 6.0) for 30 minutes at 85 °C. The sections were then preincubated TBS for 30 minutes at room temperature of 15% (v/v) normal horse serum (NHS), 0.5% triton X-100 and bovine serum albumin (1%; w/v) in 0.1 M to block unspecific antibody binding in a blocking solution. Next, mouse anti-reelin primary antibody (1:1000; EMD Millipore, MAB5364) diluted in the previously mentioned blocking solution was applied for 24 h at 4°C. After primary incubation, the endogenous peroxidase activity was blocked by incubating the tissue in 10% (v/v) H_2O_2 in TBS for 30 mins. Sections were subsequently incubated at room temperature for 2 hours with biotinylated goat anti-mouse secondary antibody (IgG, 1:500, Sigma-Aldrich, St. Louis, MO) diluted in above-described blocking buffer. Finally, tissue was incubated for 1h in avidin-biotin complex (1:500, Vecta Stain Elite ABC reagent, Vector Labs) at room temperature. Each step was followed by 3 washes for 5 minutes in TBS. To visualize reelin, sections were stained using 0.002% [w/v] 3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) with 0.0078% [v/v] hydrogen peroxide in TBS (see Figure 2.3 for schematic representation). After approximately 10 minutes, the sections were rinsed to terminate the DAB reaction, and then mounted onto polarized glass slides. After drying overnight, sections were dehydrated using increasing concentrations of ethanol (70, 95 and 100%), cleared in xylene, and then cover slipped using Permount mounting medium (Thermo Fisher Scientific, Waltham, MA).

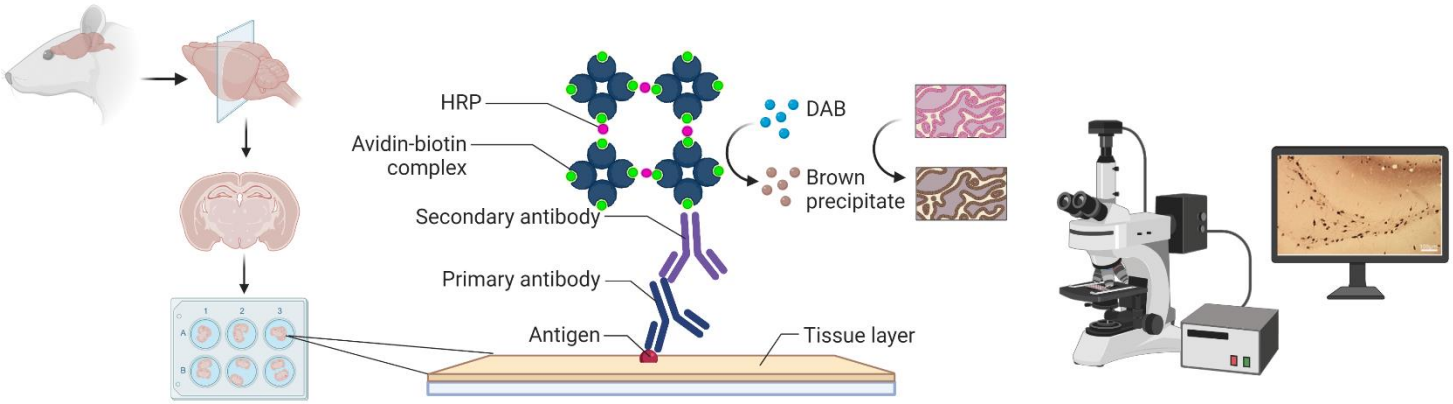


Figure 2.3 Standard immunohistochemistry procedure. Following antigen-retrieval, proteins can be detected using primary and secondary antibodies. The avidin-biotin complex is labelled with horseradish peroxidase (HRP) and can conjugate with the secondary antibody. DAB is added and oxidized by H_2O_2 in a reaction catalyzed by HRP, which produces a brown colorimetric product. Figure created by author in BioRender adapted from Dr. Josh Allen.

2.5.1.1 Reelin imaging and cell counting

Immunohistochemical results were quantified blinded to treatment groups using an unbiased optical fractionator method for stereological estimates as previously described by our laboratory (Botterill et al., 2015; Lussier et al., 2013). Using a Nikon Eclipse E800 microscope with a motorized stage linked to a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc), reelin-IR cells in the SGZ were imaged and counted. The SGZ, defined as a 2-cell width zone in between the inner granule cell layer and the polymorphic layer, was initially traced at 2.5x magnification. Immunopositive cells were then counted in 5 sections in both hemispheres at 40X magnification for stereological analysis. The estimated number of immunoreactive cells was calculated using the formula:

Ntotal: $\Sigma Q- \times 1 / ssf \times A(x,y\ step) / a(frame) \times t/h,$

where ΣQ− is the number of counted cells; ssf is the section sampling fraction (1 in 6); A(x,y step) is the area associated with each x,y movement (10 000μm^2); a(frame) is the area of the

counting frame (3 600μm^2); t is the weighted average section thickness; and h is the height of the dissector plane (12μm). A guard zone of 2μm was used to avoid counting sectioning artifacts.

2.5.2 Immunocytochemistry protocol

Blood smears collected on slides (Fisherbrand, Superfrost Plus) were used an immunocytochemistry protocol for the visualization of SERT clusters as previously described by our lab (Romay-Tallon et al., 2017). First, slides were fixed in 1% paraformaldehyde for 10mins then rinsed with phosphate-buffered saline (PBS). Slides were then incubated in a blocking solution of 3% rat immunoglobulin (Sigma) and Bovine serum albumin (BSA; 1% [w/v]) in PBS for one hour at room temperature to block unspecific antibody binding. Primary antibody (rabbit anti-SERT, 1:100, Millipore Sigma, cat# AB10514P) overnight incubation of slides occurred at 4°C. The following day, slides were rinsed in PBS and then secondary antibody (goat anti-rabbit Alexa Fluor 568, 1:200, Molecular Probes, cat #ab175471 abcam, Cambridge, UK diluted in PBS) diluted in 1% bovine serum albumin (BSA) in PBS was applied for 2 hours at room temperature. Next, Hoescht (1:1000) was applied for 10 minutes at room temperature to ascertain lymphocyte histology. Slides were then cover-slipped with AF1 Citifluor- Mountant Solution (Electron Microscopy Sciences) and stored at −20°C until imaging.

2.5.2.1 SERT imaging and cell counting

SERT clusters on the membrane of 50 individual lymphocytes were imaged per sample at 60x using confocal laser scanning microscopy (Olympus FluoView FV1000). Fiji software (image processing package based on ImageJ2) was used to analyze the lymphocytes in a macro created by PhD candidate Brady Reive and modified by PhD candidate Hannah Reid based on previous principles of cluster analysis (Romay-Tallon et al., 2017). Briefly, lymphocytes are isolated from background and other leukocytes as the region of interest. Threshold nuclei was used for masking and excluded nuclei that did not fit lymphocyte morphology were excluded. The image is transformed into a binary to quantify the number of SERT clusters and their surface area. Quantification of number and size of SERT clusters was used for statistical analyses.

2.6 Statistical analysis

All statistical tests were computed using SPSS (IBM's Statistical Package for Social Sciences version 27). Two-way ANOVAs were run to assess differences between condition (timepoint: 1h, 6h, 12, 1w) and treatment group (vehicle/vehicle or CORT with vehicle, reelin, ketamine, or reelin-ketamine). The assumptions of normality and homogeneity of variance were tested, and Kruskal Wallis tests were used if data was not normally distributed. Tukey post hoc tests were conducted when appropriate. Group means were considered statistically different from one another at $p < 0.05$. Data are expressed as mean ± CI. Pearson's r correlations were also assessed. Analysis of covariance (ANCOVA) was used to examine the influence of an independent variable (treatment group) on a dependent variable (cell count or behavioural data) while removing the effect of the covariate factor (rat estro cycle).

Chapter Three: Results

3.1 Body weight

Body weight analysis indicted that CORT animals (*M* = 25.12, *SD* = 12.14, 95% CI [22.85-27.38]), and vehicle animals (*M* = 50.37, *SD* = 10.24, 95% CI [46.32-54.42]) had significantly different changes in bodyweight over the 21 day injection period in a two-way unpaired t-test, $t(45.2) = 11.09$, $p < 0.0001$, *MD* = 25.26 ± 2.278, 95% CI [20.67 to 29.84]. Although within-group variances between CORT and vehicle animals was non-significant $F(26, 112) = 1.404$, $p = 0.320$, $R^2 = 0.731$.

A one-way ANOVA was run to analyze whether treatment on day 21 had an effect on bodyweight on day 26. The Bartlett's test for homogeneity of variance indicated this assumption had not been violated [$F(4, 15) = 2.211$, $p = 0.116$], and the ANOVA revealed a non-significant relationship between treatment and end weight, $F(4, 32) = 2.293$, $p = 0.081$, $R^2 = 0.223$. *Post hoc* analysis of multiple comparisons can be found in Table 3.1.

Treatment comparisons		Mean diff.	Upper 95% CI	Lower 95% CI	p-value	Significance
CV	VV	-5.125	-15.32	5.065	0.5991	ns
CV	CR	3.125	-7.065	13.32	0.8999	ns
CV	CK	1.286	-9.26	11.83	0.9965	ns
CV	CKR	-5.833	-16.84	5.17	0.5505	ns
VV	CR	8.250	-1.94	18.44	0.1589	ns
VV	CK	6.411	-4.14	16.96	0.4158	ns
VV	CKR	-0.7083	-11.71	10.30	0.9997	ns
CR	CK	-1.839	-12.39	8.71	0.9864	ns
CR	CKR	-8.958	-19.96	2.048	0.1552	ns
CK	CKR	-7.119	-18.46	4.22	0.3834	ns

Table 3.1 Multiple comparisons of body weight changes 5 days post-treatment. Tukey's test for multiple comparisons was used for *post hoc* analysis. No significant differences were found across any group. (Mean diff. = mean difference, 95% CI = upper and lower confidence interval of difference).

3.2 Behavioural results

Several two-way ANOVAs were performed to analyze the effect of drug treatment and time since treatment on the rats' behaviours (Figure 3.2). Mauchly's Test of Sphericity indicated that the assumption of sphericity had not been violated for climbing ($\chi^2(21) = 1.249$, $p = .229$), swimming ($\chi^2(8.9) = 0.756$, $p = .781$), latency to immobility ($\chi^2(17.9) = 1.171$, $p = .294$), of immobility ($\chi^2(8.9) = 0.988$, $p = .493$). For climbing behaviour, a two-way ANOVA revealed no statistically significant interaction between the effects of drug treatment and time since treatment ($F(12, 75) = 1.119$, $p = 0.358$, $\eta^2 = 0.090$), although drug treatment effect was statistically significant ($F(4, 25) = 5.680$, $p = 0.002$, $\eta^2 = 0.210$). For swimming behaviour, there was a statistically significant interaction between the effects of drug treatment and time since treatment ($F(12, 75) = 2.117$, $p = 0.026$, $\eta^2 = 0.120$), and drug treatment effect was statistically significant ($F(4, 25) = 30.04$, $p < 0.0001$, $\eta^2 = 0.428$). Latency to immobility had a non-significant interaction effect between drug treatment and time since treatment ($F(12, 75) = 0.969$, $p = 0.486$, $\eta^2 = 0.071$), and a statistically significant drug treatment effect ($F(4, 25) = 9.168$, $p = 0.0001$, $\eta^2 = 0.263$). There was a statistically significant interaction between the effects of drug treatment and time on immobility ($F(12, 75) = 3.535$, $p = 0.0004$, $\eta^2 = 0.154$), and a significant treatment effect ($F(4, 25) = 32.70$, $p < 0.0001$, $\eta^2 = 0.470$). The effect of time was not statistically significant on climbing ($F(3, 68) = 0.170$, $p = 0.902$, $\eta^2 = 0.003$), swimming ($F(3, 59) = 0.236$, $p = 0.553$, $\eta^2 = 0.009$), latency to immobility ($F(3, 68) = 1.472$, $p = 0.236$, $\eta^2 = 0.027$), or immobility ($F(3, 57) = 1.185$, $p = 0.317$, $\eta^2 = 0.013$) behaviours. Simple main effects were analyzed using Tukey's multiple comparisons test; multiple comparisons with the CV group can be found at Table 3.2.

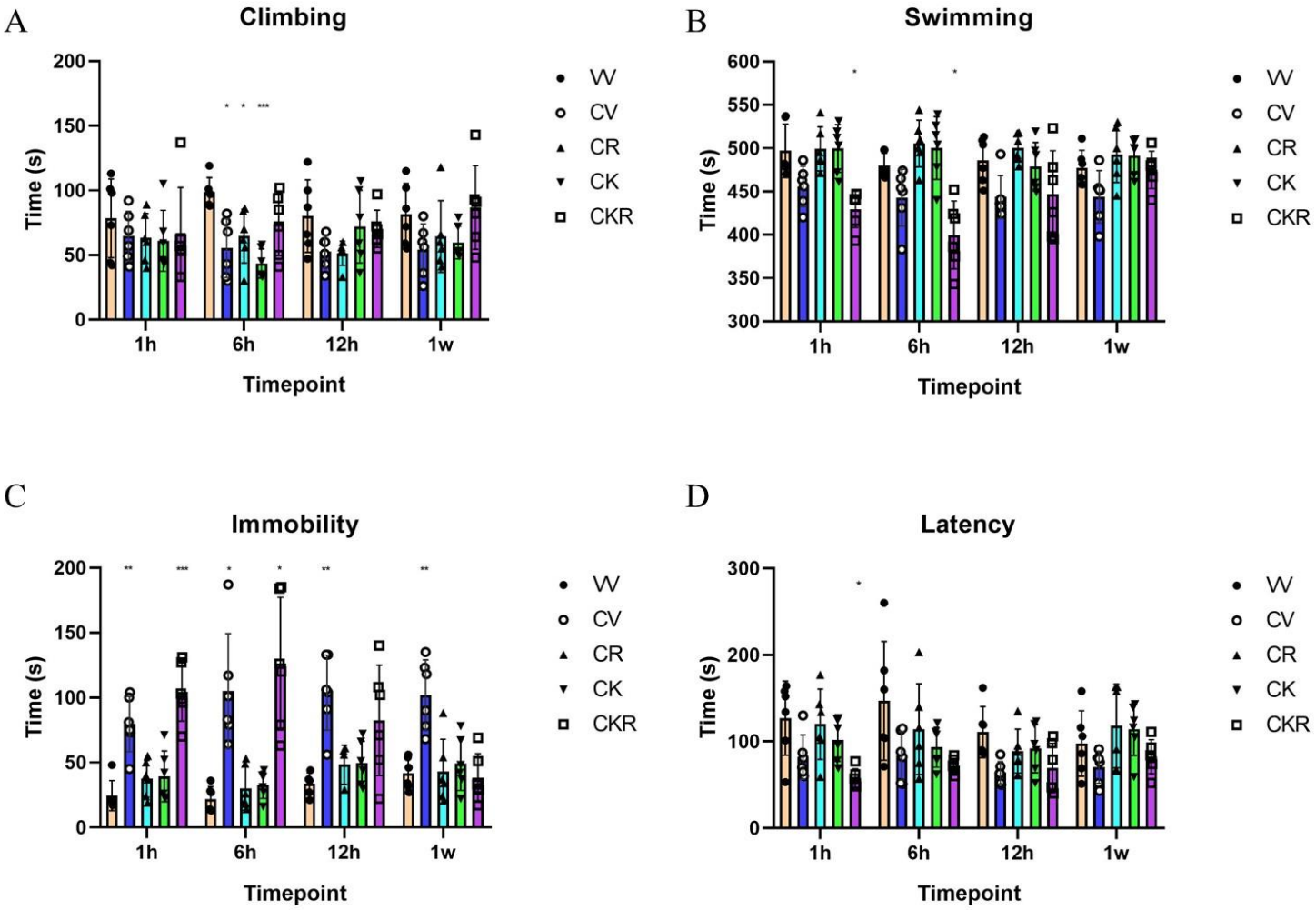


Figure 3.1 Behavioural effects of reelin and ketamine treatment. A) Time spent climbing. B) Time spent swimming. C) Time spent immobile. D) Latency to first immobility. Vehicle rats generally exhibited more active behaviours than CORT treated rats, which were rescued by reelin and ketamine in a time-dependent manner. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Time	Treatment groups		Climbing	Swimming	Latency	Immobility
1h	CV	VV	$p = 0.022$*	ns	ns	$p = 0.004$**
	CV	CR	ns	ns	ns	$p = 0.020$*
	CV	CK	ns	ns	ns	$p = 0.042$*
	CV	CKR	ns	ns	ns	ns
6h	CV	VV	ns	ns	ns	$p = 0.042$*
	CV	CR	ns	$p = 0.032$*	ns	$p = 0.038$*
	CV	CK	ns	$p = 0.036$*	ns	$p = 0.047$*
	CV	CKR	ns	ns	ns	ns
12h	CV	VV	ns	ns	ns	$p = 0.008$**
	CV	CR	ns	$p = 0.009$**	ns	$p = 0.021$*
	CV	CK	ns	ns	ns	$p = 0.024$*
	CV	CKR	ns	ns	ns	ns
1w	CV	VV	ns	ns	ns	$p = 0.009$**
	CV	CR	ns	ns	ns	$p = 0.019$*
	CV	CK	ns	ns	ns	$p = 0.024$*
	CV	CKR	ns	ns	ns	$p = 0.007$**

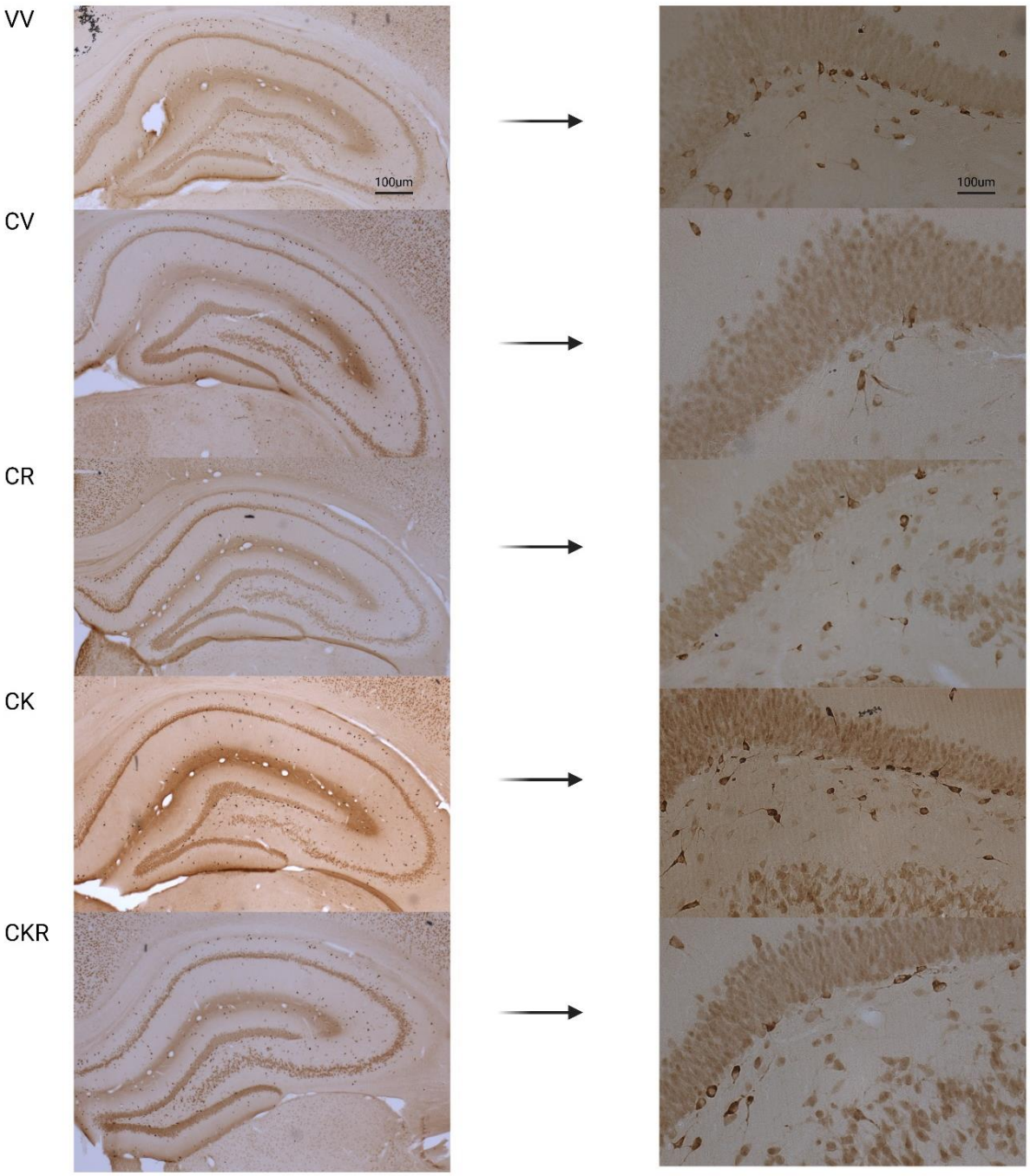
Table 3.2 Simple main effects analysis of behavioural results. Multiple comparisons between the CORT/Vehicle treatment group and all other treatment groups at all time points. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.3 Reelin-IR expression in the SGZ

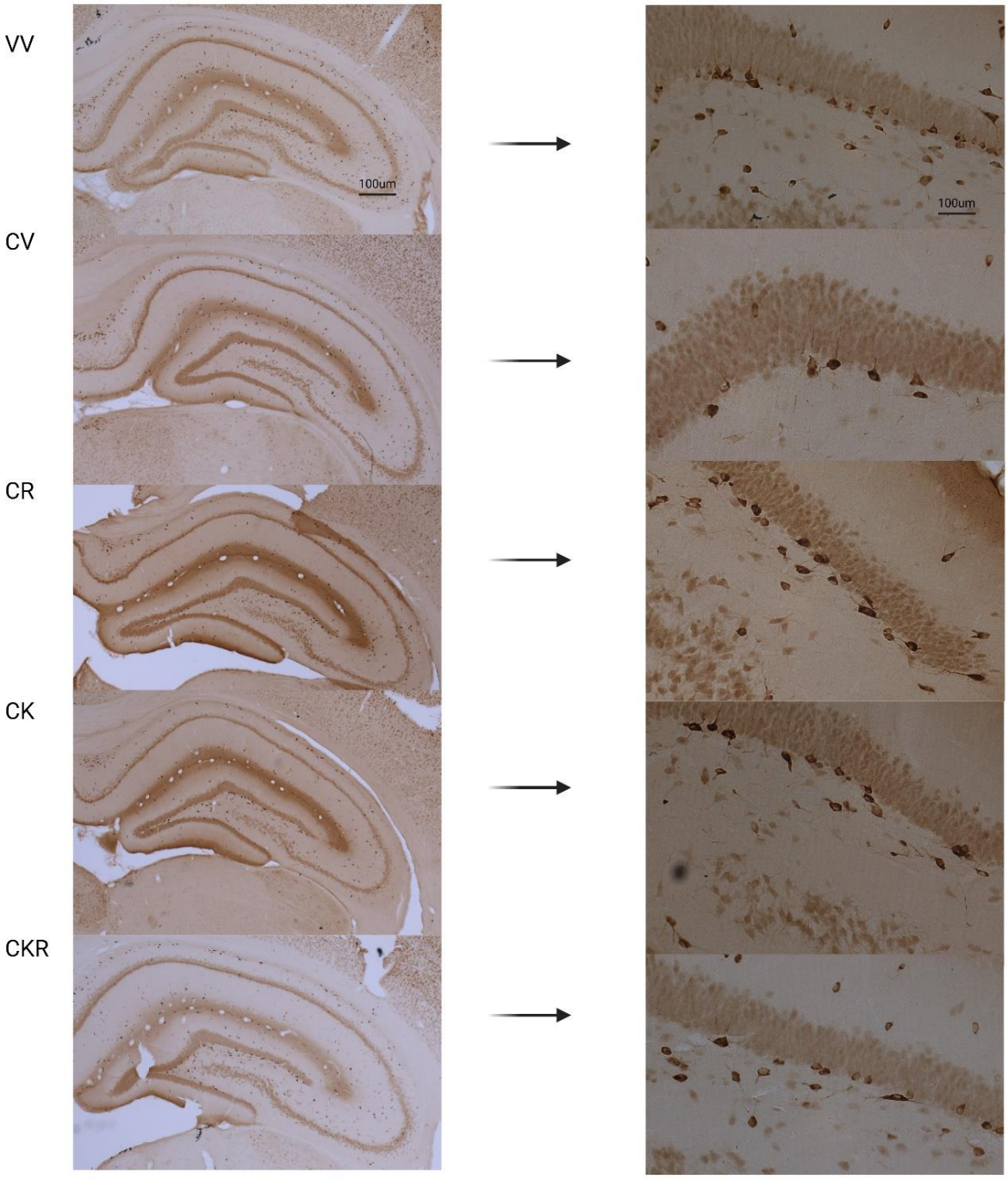
Reelin expression in the subgranular zone of the dorsal hippocampus was affected by condition (CORT/vehicle), treatment (reelin/ketamine), and time (Figure 3.3). Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, $\chi^2(2.25) = 15.6$, $p = .0.004$, therefore the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε) = 0.88. A two-way ANOVA revealed a statistically significant effect of both treatment [$F(4, 25) = 22.130$, $p = 0.003$, $\eta^2 = 0.552$] and time [$F(3, 66) = 5.815$, $p = 0.002$, $\eta^2 = 0.483$] on reelin expression in the SGZ, although there was no statistically significant interaction

effect between treatment group and time point, $F(12, 75) = 1.080$, $p = 0.389$, $\eta^2 = 0.046$. Simple main effects were analyzed using Tukey's multiple comparisons test, found at Table 3.3. Representative images of each treatment/time group are found at Figures 3.3A-D and graphed on Figure 3.3E.

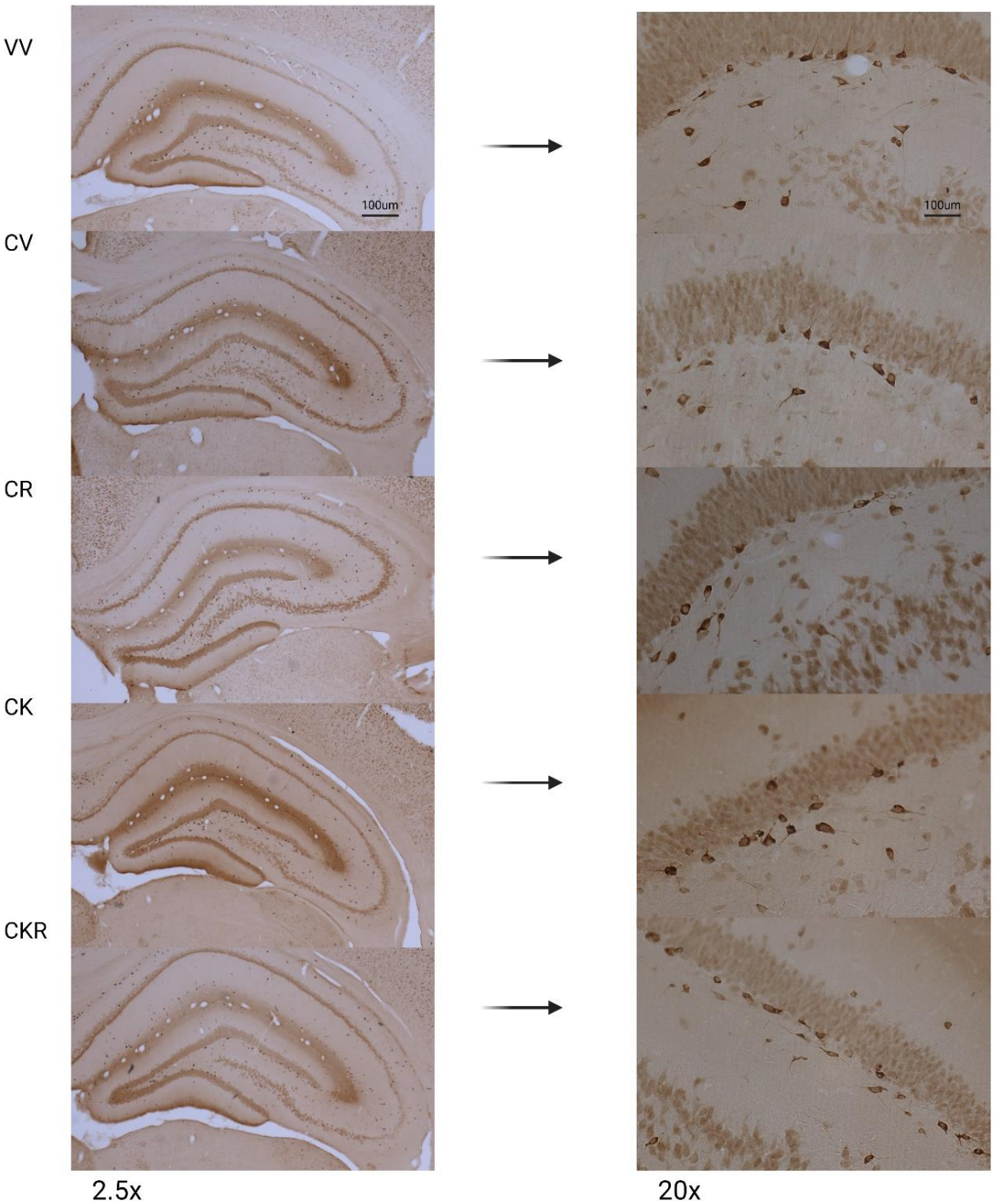
A: 1h



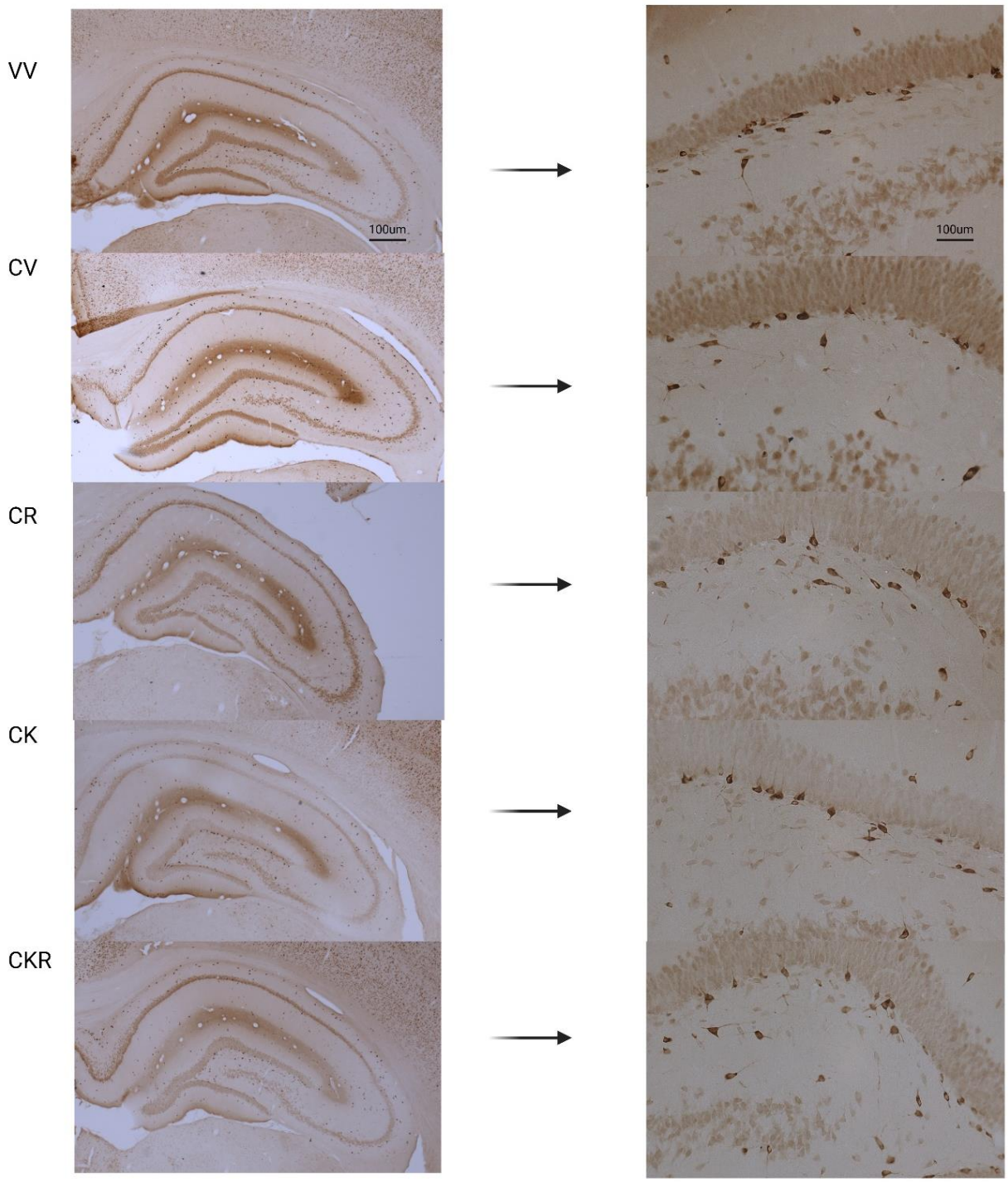
B: 6h



C: 12h



D: 1w



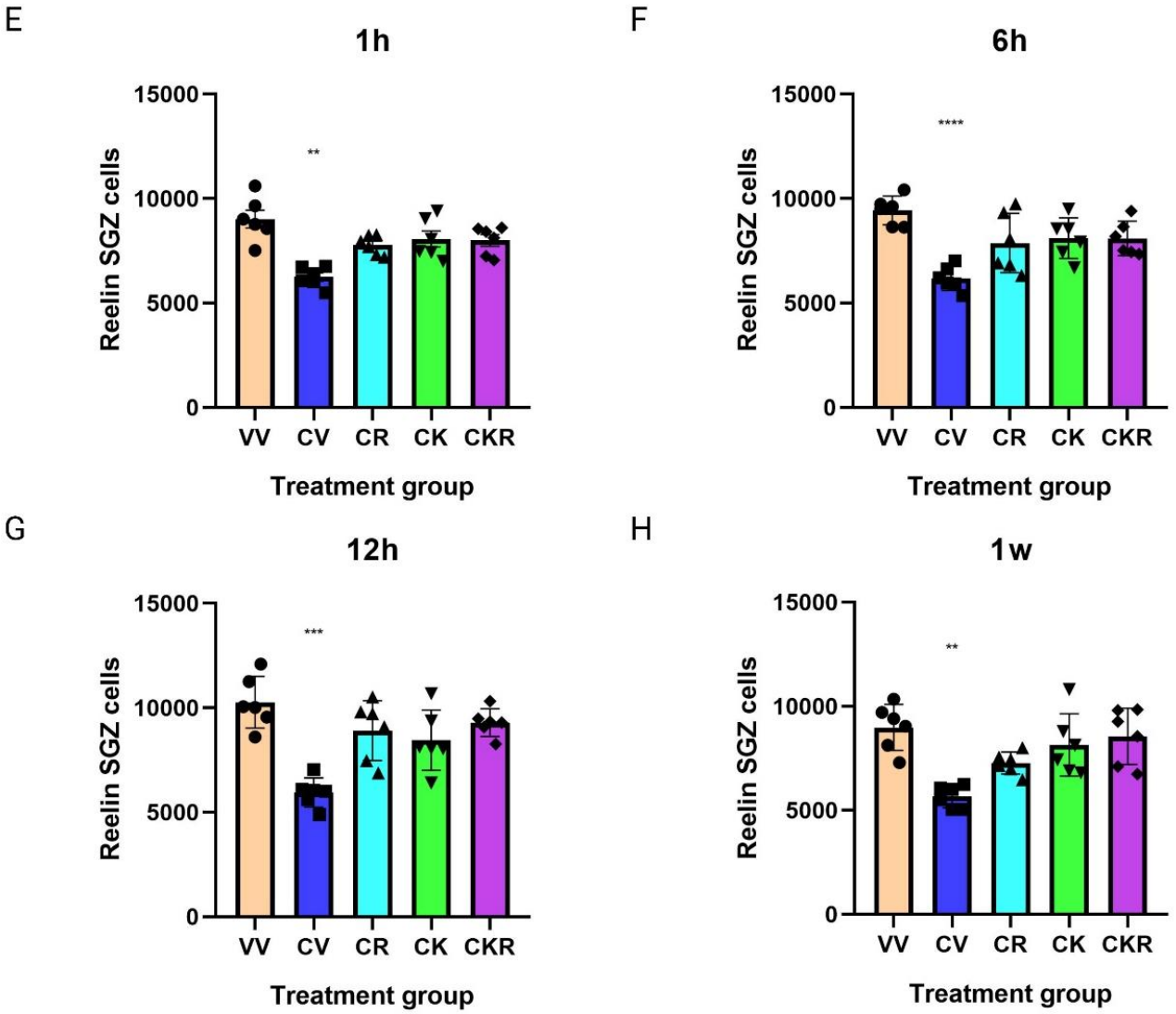


Figure 3.2 Reelin-IR cell counts in the SGZ. A) Representative photomicrographs of reelin expression in the SGZ at 1h post-treatment for all treatment groups (VV, CV, CR, CK, CKR) at 2.5x and 20x. Scale bar = 100 μm. B) Representative photomicrographs of SGZ reelin expression at 6h post-injection. C) Reelin SGZ at 12h imaged at 2.5x and 20x. D) Reelin SGZ at 1w imaged at 2.5xand 20x. E) Reelin-IR expression across groups at 1h post treatment. F) Reelin-IR expression across groups at 6h post treatment. G) Reelin-IR expression across groups at 12h post treatment. H) Reelin-IR expression across groups 5 days (1w) post treatment. Significance denoted between the CORT/Vehicle treatment group and all other treatment groups at all time points. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p > 0.0001$.

Time	Treatment groups		Variability	p value	Significance
1h	CV	VV	$q = 8.392$	$p = 0.004$	**
	CV	CR	$q = 8.022$	$p = 0.015$	*
	CV	CK	$q = 5.898$	$p = 0.022$	*
	CV	CKR	$q = 7.266$	$p = 0.004$	**
6h	CV	VV	$q = 12.450$	$p < 0.0001$	****
	CV	CR	$q = 3.814$	ns	ns
	CV	CK	$q = 5.853$	$p = 0.019$	*
	CV	CKR	$q = 6.581$	$p = 0.008$	**
12h	CV	VV	$q = 10.520$	$p = 0.0005$	***
	CV	CR	$q = 6.432$	$p = 0.015$	*
	CV	CK	$q = 5.404$	$p = 0.035$	*
	CV	CKR	$q = 12.03$	$p < 0.0001$	****
1w	CV	VV	$q = 7.639$	$p = 0.007$	**
	CV	CR	$q = 5.843$	$p = 0.014$	*
	CV	CK	$q = 5.41$	$p = 0.044$	*
	CV	CKR	$q = 9.738$	$p = 0.001$	**

Table 3.3 Simple main effects analysis of reelin-IR cell counts in the SGZ. Multiple comparisons between the CORT/Vehicle treatment group and all other treatment groups at all time points. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p > 0.0001$

3.4 Effect of estrous cycle

A one-way ANCOVA was run to partial out any effect that the covariate, stage of rat estrous cycle, might have on behavioural and neurochemical results. When analyzing time spent immobile in the FST, Levene's assumption of equality of error variances was violated, [$F(4, 120) = 7.464$, $p = 0.04$], however the variance ratio ($\sigma^2 < 2$) indicated that non-parametric tests corrections were not needed. A one-way ANCOVA revealed no statistical significance of estrous cycle stage effect on time spent immobile on the FST, $F(4, 120) = 8.89$, $p = 0.326$, $\eta^2 = 0.229$. For SGZ reelin-IR expression, Levene's assumption of equality of error variances was met, [F(4,

120) = 1.345, $p = 0.257$], and therefore no corrections were needed. A one-way ANCOVA revealed no statistical significance of effect of estrous stage on reelin SGZ cell count, $F(4, 120) = 0.05$, $p = 0.815$, $\eta^2 = 0.424$).

3.5 Correlations with behaviour

Correlations between behaviour (time spent immobile in the FST) and neurochemical alterations were run to examine the extent to which these data were related. Pearson's bivariate correlation coefficient test was used for all comparisons and can be found at Table 3.4. No significance was found across any correlations for immobility-reelin-IR comparisons.

Treatment group		Behavioural correlation with Reelin-IR				
Treatment	Time	r	CI lower limit	CI upper limit	R^2	p-value
VV	1h	-0.646	-0.956	0.3481	0.4173	0.1658
	6h	-0.345	-0.904	0.6480	0.119	0.5031
	12h	-0.527	-0.938	0.4969	0.278	0.2824
	1w	0.794	-0.0482	0.9764	0.631	0.0590
CV	1h	-0.290	-0.8912	0.6819	0.0843	0.5767
	6h	0.268	-0.694	0.8868	0.0721	0.6070
	12h	0.173	-0.7423	0.8633	0.0299	0.7432
	1w	0.010	-0.808	0.8150	0.00010	0.9850
CR	1h	-0.658	-0.958	0.3300	0.433	0.1557
	6h	0.131	-0.762	0.8519	0.0171	0.8049
	12h	0.220	-0.720	0.8754	0.0486	0.6747
	1w	0.349	-0.645	0.9044	0.122	0.4975
CK	1h	-0.445	-0.9231	0.5739	0.198	0.3768
	6h	0.218	-0.721	0.8748	0.0475	0.6784
	12h	0.187	-0.736	0.8670	0.0350	0.7226
	1w	0.010	-0.808	0.8149	0.00010	0.9853
CKR	1h	-0.178	-0.865	0.7404	0.0318	0.7354
	6h	-0.248	-0.882	0.7056	0.0615	0.6356
	12h	0.111	-0.770	0.8462	0.0122	0.8348
	1w	-0.738	-0.969	0.1828	0.545	0.0938

Table 3.4 Correlations between behavioural data, reelin-IR, and SERT MPC. Correlations were run using Pearson's r correlation coefficient. No significance was found across any of the comparisons. r = Pearson's r correlation coefficient, CI LL = 95% confidence interval lower limit, CI UL = 95% confidence interval upper limit.

3.6 Effect of bodyweight

A one-way ANCOVA was run to partial out any effect that the covariate, body weight of rat at time of testing, might have on behavioural and neurochemical results. When analyzing time spent immobile in the FST Levene's assumption of equality of error variances was violated, [$F(4, 120) = 5.615$, $p = 0.04$], however the variance ratio ($\sigma^2 < 2$) indicated that no non-parametric test corrections were needed. A one-way ANCOVA revealed no statistical significance of body weight on time spent immobile on the FST, $F(4, 120) = 2.425$, $p = 0.122$, $\eta^2 = 0.232$). For SGZ reelin-IR expression, Levene's assumption of equality of error variances was met, [$F(4, 120) = 1.213$, $p = 0.309$], and therefore no corrections were needed. A one-way ANCOVA revealed no statistical significance of effect of body weight reelin SGZ cell count, $F(4, 120) = 0.298$, $p = 0.586$, $\eta^2 = 0.529$).

Chapter Four: Discussion

4.1 Summary of main findings

The two main aims of this thesis were to: *(1)* ascertain the time course of action of reelin's antidepressant-like properties and *(2)* evaluate the synergistic effect of sub-anesthetic doses of reelin and ketamine on depressive phenotypes in a paradigm of chronic stress. To the best of our knowledge, no other laboratory has yet measured the reelin's time-course of action either individually or in combination with that of ketamine. This thesis expanded on previous findings from our laboratory on reelin intrahippocampal (Brymer et al., 2020), and i.v. peripheral (Allen et al., 2022) injections to normalize behavioural and neurochemical deficits in a CORT-induced model of chronic stress. As previous findings have focused on males, yet MDD is twice as prevalent in females, I decided to focus on female rats for the purpose of this study.

We first investigated the antidepressant-like properties of reelin on behavioural deficits induced by chronic stress. We used the FST as a screening tool for antidepressant efficacy due to the parallels found between compounds consistently normalize the depressive-like coping strategy in rodents with that MDD patients (Fenton et al., 2015; Hibicke et al., 2020). As expected, 21-days of 40mg/kg subcutaneous CORT injections increased time spent immobile in the FST compared to animals who received saline injections at all time-points (1h, 6h, 12h, and 1w) following PBS treatment. We found that i.v. injections of recombinant reelin rescued the CORT-induced increases in FST immobility at 1 hour, 6 hours, and 12 hours after administration. We also found that one 3µg-dose reelin had a prolonged effect against continued administration of chronic stress 5 days after reelin treatment. Ketamine similarly rescued FST immobility at 1h, 6h, and 12h following treatment. One dose of 10mg/kg of ketamine also had a sustained antidepressant-like effect on behaviour after 5 additional days of CORT administration following ketamine treatment. The combined effect of sub-anesthetic doses of reelin (3µg) and ketamine (10mg/kg) did not rescue FST immobility at the 1h, 6h, or 12h time-period, and in fact increased immobility compared to the CV control group at timepoints 1h and 6h following treatment. However, acute treatment with reelin and ketamine rescued FST immobility after 5 days of continued CORT injections more significantly than that of reelin or ketamine alone. This suggests a lasting synergistic protective effect of reelin and ketamine against continued chronic stress.

Both reelin and ketamine rescued the expression of endogenous reelin in the SGZ thought to be critical for the attenuation of behavioural deficits (Allen et al., 2022; Brymer et al., 2018; Kim,

2017; Lussier et al., 2009). An acute dose of reelin increased SGZ reelin-IR expression at 1h and 12h post-injection and showed a protective effect against 5 days of continued chronic stress administration. Interestingly, recombinant reelin treatment did not rescue reelin-IR expression at 6h post-injection. We also found that acute ketamine injection rescued SGZ reelin-IR expression at all time-points (1h, 6h, and 12h), and had a lasting effect following 5 additional days of CORT-administration. The synergistic effects of sub-anesthetic doses of reelin and ketamine treatment showed an increased effect at all time-points on reelin-IR expression in the SGZ, as well as a lasting protective effect against 5 extra days of continued chronic stress. The stage of the estrous cycle did not have an effect on either behavioural results nor neurochemical deficits rescued by reelin and/or ketamine at any time-point.

4.2 Reelin and ketamine rescue CORT-induced immobility in a time-dependent manner

Independently, both reelin and ketamine rescued CORT-induced immobility in the FST at 1h, 6h, and 12h post-injection. A single dose of either reelin or ketamine had a lasting effect on FST immobility against continued chronic stress. It would be interesting to conduct more research on the time-course effect of reelin to better understand the immediate and lasting behavioural effect against chronic stress.

When administered concordantly, reelin and ketamine rescued CORT-induced FST immobility only at the 1w timepoint. Interestingly, although reelin and ketamine were administered at their respective individual sub-anesthetic doses (3µg reelin; 10mg/kg ketamine), they appeared to produce a combined synergistic psychoactive effect. Shortly following administration of the second pharmaceutical agent [within 60 seconds second injection; CKR rats alternated order of injections (i.v. reelin then i.p. ketamine; or i.p. ketamine followed by i.v. reelin)] the rats displayed alterations in locomotor functions. Lasting approximately 10 mins, rats administered with both reelin and ketamine exhibited balance disturbances including impaired gait and partial loss of righting reflex. This observation was consistent across CKR rats irrespective of weight or time of injection. Plausibly, reelin and ketamine have (a) shared mechanistic target(s) and/or pathway(s), which can produce a synergistic effect. This drug synergism could explain the short-term full-dose-like behavioural response to the administration of sub-anesthetic doses. Interestingly in the 1w cohort, the CKR rats exhibited the greatest resistance to the deleterious

effects of continued CORT administration compared to the CR or CK animals, as exemplified by reductions in FST immobility. This could imply a long-lasting synergistic drug effect which could provide a protective factor against continued stress.

4.3 Reelin and ketamine increase reelin-IR in the SGZ in a time-dependent manner

Reelin and ketamine were both able to individually rescue CORT-induced deficits in SGZ reelin-IR cell expression at 1h and 12h post-injection, although only ketamine rescued reelin-IR expression at the 6h timepoint. A single subanesthetic dose of either reelin or ketamine had a lasting effect on increasing reelin-IR cells after 5 days of continued CORT injections. As mentioned briefly above, decreases in FST immobility were paralleled with increased SGZ reelin-IR cell expression for both recombinant reelin and ketamine treatment individually. Logically this makes sense for ketamine, as research indicates that ketamine can easily cross the BBB (clinical onset within 45-60 seconds) due to its high lipid solubility (Hailu et al., 2021; Herd et al., 2008); rapidly reaching its neurobiological target allows for rapid-acting neurochemical and behavioural changes. Similarly, the sustained behavioural and neurochemical changes observed in the 1w rats can be (at least partially) explained by the changes in synaptic plasticity and upregulation of AMPA receptor trafficking. On the other hand, the time-dependent variability of neurobiological results following recombinant reelin administration demonstrates a more puzzling phenomenon. Particularly of interest is the increased SGZ reelin at the 1h but not 6h timepoint, which is not paralleled by behavioural changes. One theory, which is covered in more depth in section 4.6.1, involves whole or fragments of the injected recombinant reelin being able to cross the BBB which could precipitate the early expression of reelin-IR cells. Alternatively, exogenously increasing peripheral reelin could have downstream implications on neurochemical functions leading to the observed increase in reelin-IR expression. More research with larger sample sizes is needed to better elucidate the mechanisms of action and pharmacokinetics of reelin's neurochemical and behavioural effects.

Increased SGZ reelin-IR expression was observed at all time-points following acute sub-anesthetic doses of both reelin and ketamine, and sustained after continued application of stress. Interestingly, animals treated with both reelin and ketamine showed a more robust effect at each timepoint (1h, 6h, 12h, 1w). In other words, the neurobiological synergistic effect of reelin and

ketamine could be greater than that of reelin or ketamine alone in a fast-acting and persistent manner. This provides evidence for overlapping signaling pathways and/or mechanisms of action which could be promising in the search for novel ketamine-like pharmaceuticals without the psychoactive side-effects.

4.4 Patient-oriented research

The importance of patient-partners in preclinical and clinical research cannot be understated. It is these voices that should ultimately guide the direction of preclinical research and help identify gaps in the health system. Our laboratory has focused on the inclusion of patient partners in our research for the past five years. By increasing patient involvement in clinical and public health research, research can be streamlined to target actual issues that patients experience. However, there is a clear lack of patient voices in the laboratory-based foundational research that provides the framework for larger-scale public health matters. It is for this reason that we formed the patient-oriented committee, whereby patient engagement has enabled us to identify these gaps between patient experience and research focus (Johnston et al., 2021).The primary goals of this thesis were determined with patient involvement in attempt to increase the translatability of our research to a human model disease.

4.5 Limitations

4.5.1 Sex differences

One of the major limitations in this thesis is the exclusion of males from behavioural and neurochemical analysis. Although lifetime depression prevalence rates are double for women than men (Eid et al., 2019), preclinical and clinical pharmaceutical studies are often focused on males, and those that include females often fail to account for the potential effect of estrous hormones. Sex differences are found throughout various chronic stress models and effect drug absorption, distribution, metabolism, and elimination. Estrous hormones easily cross the BBB and alter glucocorticoid receptor activity (Bourke et al., 2012), but may also have a neuroprotective effect, decreasing female sensitivity to the depressogenic effects of chronic unpredictable mild stress (Dalla et al., 2005). While I did not find any mediating effect of estrous

hormones on depressive-like phenotypes, the use of a relatively small sample size (6 rats per group) could mask any significance of effect. Understanding this paradoxical relationship could alleviate the knowledge gap surrounding the increased prevalence rates of depression in women with the potential neuroprotective effects of estrous hormones.

Sex hormones are known to affect reelin expression, exemplified by high levels of testosterone decreasing reelin expression (Absil et al., 2003), and lower baseline levels of reelin in the paraventricular nucleus of the hypothalamus in females (Sánchez-Lafuente et al., 2022). However, our laboratory has not observed any sex differences in response to peripheral reelin administration, nor in hippocampal reelin, GluA1, or GABAAR expression at baseline, after chronic CORT administration, nor with peripheral reelin administration (Allen et al., 2022; Brymer et al., 2020). This is paralleled by a lack of sex differences in behavioural changes following peripheral reelin administration (Allen et al., 2022). Minimal sex differences have been found in the preclinical and clinical studies regarding the antidepressant effects of ketamine and reelin, although many studies fail to differentiate findings between males and females (Ponton et al., 2022; Johnston, 2023). Some preclinical models have found that females are more sensitive to dose of ketamine exhibiting a greater magnitude or initial response, while males tend to have a more prolonged response (Franceschelli et al., 2015; Magnani et al., 2004; Okine et al., 2020). In my own research, the exclusion of males occluded any findings regarding sex differences. Although previous findings suggest that the reported effects would be paralleled in a male population as well, it is possible that the inclusion of males could illuminate sex-differences. Utilizing a different model to study depression could also have an outcome effect, as certain models of chronic stress are known to have sex-specific dependencies.

4.5.2 Necessity of mechanistic studies

In this thesis, I examined behavioural and neurobiological changes in response to reelin and ketamine administration. However, mechanistic studies are required to fully ascertain the proteins and pathways specifically involved in reelin's and ketamine's antidepressant-like effects. While AMPAR signaling inhibition can abolish the antidepressant-like effects of reelin (Brymer et al., 2020) and ketamine (Zhang et al., 2016), there are a multitude of downstream signaling pathways that could mediate reelin's antidepressant effects. Other pathways such as

Rap1 signaling, or Dab1-SFK-induced NMDAR regulation could be responsible for the observed results independent of mTOR. The use of inhibitors such as rapamycin (an mTORC1 inhibitor) and GGTI (a Rap1 inhibitor) could help elucidate reelin's mechanism of action that brings about antidepressant-like results and neurochemical changes. This invaluable information could provide a blueprint for translating preclinical research into a clinical model to develop mechanistically relevant novel antidepressants.

4.5.3 Translatability: extrapolation of preclinical findings

As with all preclinical animal models, the interpretation and extrapolation of experimental findings is a major obstacle in translation to a human model of disease. In fact, preclinical research often fails to leave the bench side due to the complex, intricate differences between basic research and the human condition. Researchers attempt to overcome these barriers by using evidence-based models and examining all aspects of validity, with the ultimate goal of minimizing these differences. At the core of the research presented in this thesis is the foundational objective of developing a fast-acting, safe, and effective antidepressant for human disease, and identification of reliable biomarkers. While all paradigms used in this thesis were chosen with this in mind, preclinical animal research contains inherent limitation in translatability.

The pathophysiology of depression is still poorly understood, paralleled by a significant lack of knowledge surrounding the precise underlying mechanisms of antidepressant action. A significant amount of research is also needed to determine the effect of extraneous factors on depression and pharmacological action including states of chronic stress, comorbid disorders, and age- and sex-related disparities. This is further complicated by the inability to assess an animal's psycho-physical condition, and an uncertainty on whether a rodent can in fact experience states thought to only affect humans such as self-reflection, rumination, suicidal ideation, guilt, and shame. Indeed, it can be argued that the application of chronic stress does not entirely replicate the human expression of any specified or unspecified human depressive disorder. It is important to recognize the disparities between preclinical animal models and the human experience of depression, which are not always directly related, to draw the most accurate conclusions. The preclinical research in this thesis was ultimately designed to lay the foundation

for the potential of developing novel fast-acting antidepressants and identifying biomarkers that could streamline psychiatric illness diagnosis and treatment course.

4.6 Future directions

4.6.1 Reelin and the blood brain barrier

Our laboratory has demonstrated that intrahippocampal and peripherally administered reelin enhances several forms of neuroplasticity and rescue behavioural, cognitive, and neurochemical deficits (Allen et al., 2022; Brymer et al., 2020; Caruncho et al., 2016; Sánchez-Lafuente et al., 2022). Despite the parallels between direct- and peripheral-infusions of reelin, it remains unknown whether full length or fragments of reelin can cross the BBB, information which is invaluable in the development of reelin-based psychotropics. My work in this thesis has provided some evidence that may suggest that reelin crosses the BBB due to the rapid increase in reelin-IR cell counts in the SGZ (1h post-infusion) paralleled with a rescue in behavioural deficits. This is supported by reelin- and ApoER2-immunoreactivity detected in hippocampal stratum lacunosum-moleculare endothelial cells, mainly in putative transcytosis caveolae vesicles, that line the BBB (Perez-Costas et al., 2015; Riddell et al., 2001). This could represent a receptor-mediated mechanism by which reelin could access the brain. However, reelin is a large protein that has limited diffusion properties once released into the extracellular space, meaning a specific transport process would be needed rather than simple diffusion. Accordingly, another putative explanation for the increase in SGZ reelin-IR cells at 1h post-infusion could be peripheral actions with an indirect effect on the CNS that may cause an earlier expression of reelin-expressing GABAergic interneurons migrating from the SGZ.

Evaluating whether peripherally administered reelin can cross the BBB contains an additional challenge in that reelin is an endogenous protein which is already expressed in the brain, therefore simple analysis of analyte concentration will not suffice. Future studies could include PET or single-photon emission-computed tomography (SPECT), which have been employed to study brain uptake kinetics, BBB integrity, cerebral blood flow, and efflux mechanisms. This would involve injecting recombinant reelin labeled with a positron-emitting radionuclide, and

then measuring the emitted γ radiation as a function of tissue depth to produce a three-dimensional image of its distribution.

4.6.2 Reelin signaling and ketamine's antidepressant effects

The research presented in this thesis provides evidence that there is both a fast-acting and long-lasting synergistic effect of reelin and ketamine, suggesting similar pathway effectors. In support of this, recent research from another lab has found that the inhibition of key mediators in reelin signaling (genetic deletion of ApoER2 or inhibition of SFKs or PI3k) abolished ketamine's antidepressant-like behavioural effects and hippocampal NMDAR-mediated synaptic plasticity (Kim et al., 2021). Another study found that GluN2B knockdown in GABAergic interneurons *Gad1*, *Sst*, and *Pvalb* occluded the antidepressant-like effects of ketamine, suggesting an underlying role of GluN2B-containing NMDARs blockade on GABAergic interneurons in the medial prefrontal cortex (Gerhard et al., 2020). Inversely, I have also shown that ketamine administration can increase reelin-IR expression, which should be further examined to determine the exact underlying mechanisms. While evidence suggests some common pathway proteins and receptors, future studies should focus on specifying the ketamine's mechanism of action and the putative reciprocal role of reelin signaling in its antidepressant effects.

4.6.3 Reelin pharmacokinetics and pharmacodynamics

The research in this thesis attempted to uncover aspects of the time course of drug absorption, distribution, metabolism, and excretion, also known as pharmacokinetics. Pharmacokinetic studies focus on enhancing drug efficacy while decreasing toxicity for the entire duration of its exposure with the body. Understanding the body's interaction with reelin throughout the entire time course is fundamental for the development of reelin-based pharmaceuticals and implementing safe and effective therapeutic management. Future studies should focus on elucidating reelin's biochemical interactions during absorption, distribution, metabolism, and excretion, as well as the mechanisms that bring about the time-dependent behaviour and neurochemical alterations described in this thesis. This could be done by examining the time-dependent dose-response relationship by utilizing, for example, the plasma drug concentration-time curve to reflect the rate of drug elimination following exposure. This could help elucidate

not only the time course effect, but also examine different dosages of reelin independently, as well as with mixed dosages of ketamine. Although as reported earlier in this thesis in concordance with findings in Allen et al., 2022, administration of a body-weight independent dose of reelin (3µg) did not have a significant effect on either behavioural or neurochemical outcome, more research on dosages is required.

Equally, preclinical research should focus on uncovering principles of reelin's pharmacodynamics in the search of creating a novel therapeutic. Pharmacodynamics is the study of a drug's molecular, biochemical, and physiologic actions, including the relationship between drug concentration at the action site and the effect. This thesis examined aspects related to pharmacodynamics such as the intensity of therapeutic and adverse effects over time, however more research is needed to understand reelin's site of action and receptor binding. Future studies should focus on understanding reelin's molecular targets and interactions, such as with ketamine, including receptor binding, post-receptor effectors, and chemical interactions. Downstream drug-target effects could include enzyme-binding, cell surface signaling proteins, or binding to molecular targets.

4.6.4 The next steps for reelin-based therapeutics: clinical studies

Ultimately, the goal of preclinical drug developmental research is to test the safety and potential viability of a novel therapeutic for human consumption. The research in this thesis lays the groundwork for developing reelin-based pharmaceuticals for human mental health disorders. While basic scientific research is critical for drug development, human clinical trials are needed to truly evaluate the safety and efficacy of exogenous reelin for the treatment of mental illness. With this in mind, the end goal is to work with patient partners to advance exogenous reelin to the first phase of clinical trial. Before receiving FDA approval for safe consumption, a potential therapeutic will have to pass four phases of clinical trials. The goal of phase I (15-50 patients) is to determine the safety of the treatment and the best route of administration; phase II (<100 patients) focuses on therapeutic efficacy; phase III (100-1000 patients) assesses whether the new treatment is better than standard treatment; and phase IV (1000+ patients) occurs once treatment is approved and available to examine the long-term effects. Transitioning this preclinical research to clinical studies is necessary to truly evaluate the efficacy of reelin-based therapeutics

for major depressive disorder, treatment resistant depression, and/or other related neuropsychiatric disorders.

4.7 Importance of this work and concluding remarks

The prevalence rates of depression have been steadily increasing, with the disease burden of depression at an all-time high. Current first-line therapeutics produce an inadequate response in approximately 1/3 of patients with depression, have a delayed therapeutic onset, and do not target hard-to-treat core symptoms of depression such as suicidality and anhedonia (Cipriani et al., 2018; Rush et al., 2006). At the forefront of research for novel fast-acting antidepressants is ketamine, which is thought to have an effect in the treatment-resistant population while minimizing the traditionally hard-to-treat symptoms. However, ketamine has a relatively high abuse potential due to its ability to induce psychomimetic side effects, and therefore it must be administered under clinician supervision, minimizing its accessibility. Reelin, as demonstrated in this thesis, is a promising putative therapeutic that may act in a parallel manner to ketamine. This thesis provides preliminary, exploratory research into the pharmacokinetics of reelin in the hopes of developing a reelin-based novel fast-acting therapeutic for major depressive disorder or related neuropsychiatric disorders.

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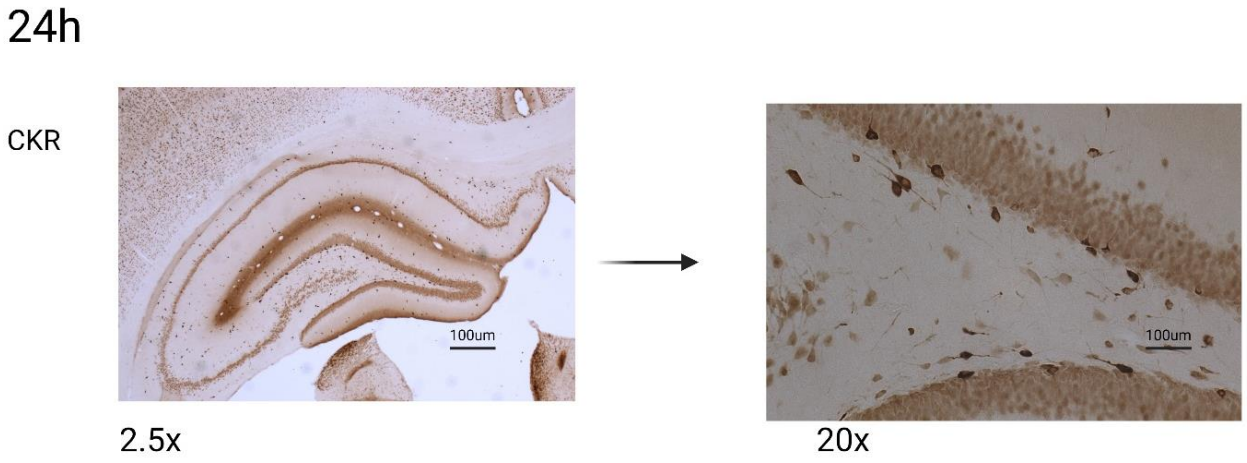
Appendix A (24h CKR cohort)

As described in this thesis, our laboratory has previously focused primarily on behavioural and neurochemical alterations 24h after reelin or ketamine treatment. In this study I included a group of female Long Evans rats (n = 6) who were administered both reelin and ketamine with behavioural testing and perfusions occurring at 24h post-treatment as our lab had not previously collected this data. CKR 24h rats were exposed to the same conditions (animal facilities at University of Victoria), treatment (40mg/kg s.c. CORT; 3µg i.v. reelin; 10mg/kg i.p. ketamine), behavioural testing (10 mins. FST at 27±2°C), and neurochemical analysis (reelin SGZ staining) as the other groups described in this thesis.

Subtle differences in conditions that occur naturally between experiments can significantly impact the outcome of the study. In fact research suggests that seasonal variations (Eccard & Herde, 2013), sex of the researchers (Georgiou et al., 2022), and the neighbouring animals (Saré et al., 2021) can influence behavioural measures in rodents. As such, I did not include a comparative analysis of the CKR 24h cohort with previously run VV/CV/CR/CK 24h groups from our lab. Reelin expression in the SGZ of the CKR 24h cohort is pictured in Figure 1 and correlated with behavioural measures in Table 1.



Appendix A. Figure 1. Representative photomicrographs of reelin expression in the SGZ. Reelin expression at 24h for CKR imaged at 2.5x and 20x.

Treatment group		Behavioural correlation with Reelin-IR				
Treatment	Time	r	LL	UL	R^2	p-value
CKR	24h	-0.254	-0.883	0.7024	0.06446	0.627

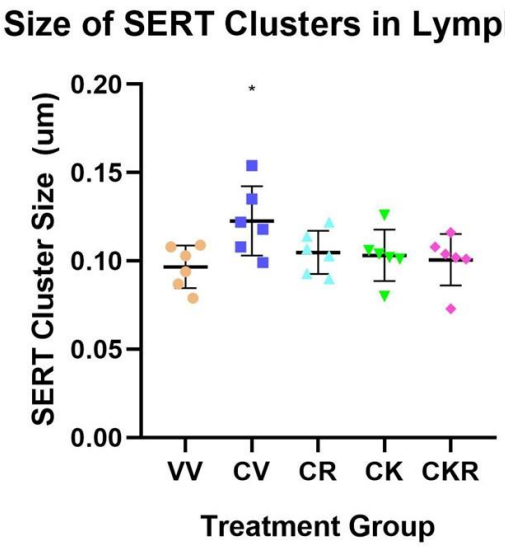
Appendix A. Table 1. Behavioural correlations with Reelin-IR expression for 24h CKR animals. Reelin expression in the SGZ at 24h behavioural correlations. No significance was found.

Appendix B (SERT clustering on MPC of 1w cohort)

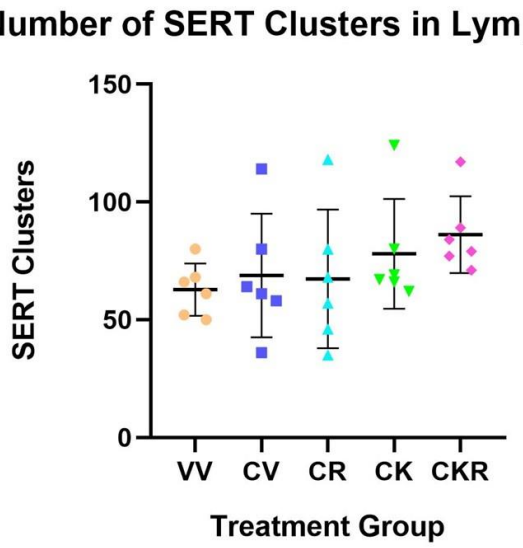
Our laboratory has found extensive evidence for alterations of SERT clustering on MPCs between depressed and control groups in both humans and animals (Caruncho et al., 2019). In this experiment blood smears were taken from all animals for analysis of peripheral SERT clustering. This section will focus on the analysis of the 1w cohort of animals.

When looking at the effect of treatment condition on SERT clustering parameters, I found that size but not number of clusters were significantly related to treatment (Figure 1). To analyze whether treatment condition had an effect on the size of SERT clusters in the 1w cohort a one-way ANOVA was run, which met test assumptions $F(4,25) = 1.536$, $p = 0.820$. The ANOVA revealed a significant relationship between treatment condition and size of SERT clusters 5 days post-treatment, $F(4,25) = 2.715$, $p = 0.048$, $R^2 = 0.303$. Simple main effect analysis can be found at Table 1. A one-way ANOVA was run to analyze whether treatment condition had an effect on the number of SERT clusters in peripheral lymphocytes in the 1w cohort. The Bartlett's test for homogeneity of variance indicated this assumption had not been violated [$F(4,25) = 4.88$, $p = 0.299$], and the ANOVA revealed a non-significant relationship between treatment group and number of SERT clusters, $F(4,25) = 0.578$, $p = 0.399$, $R^2 = 0.145$. *Post hoc* multiple comparisons can be found at Table 2.

A



B



Appendix B. Figure 1. SERT clustering in peripheral lymphocytes post-treatment in the 1w cohort. A) VV and CV had significantly different SERT clusters sizes. B) no significant differences in number of SERT clusters were found between any treatment groups. *$p < 0.05$

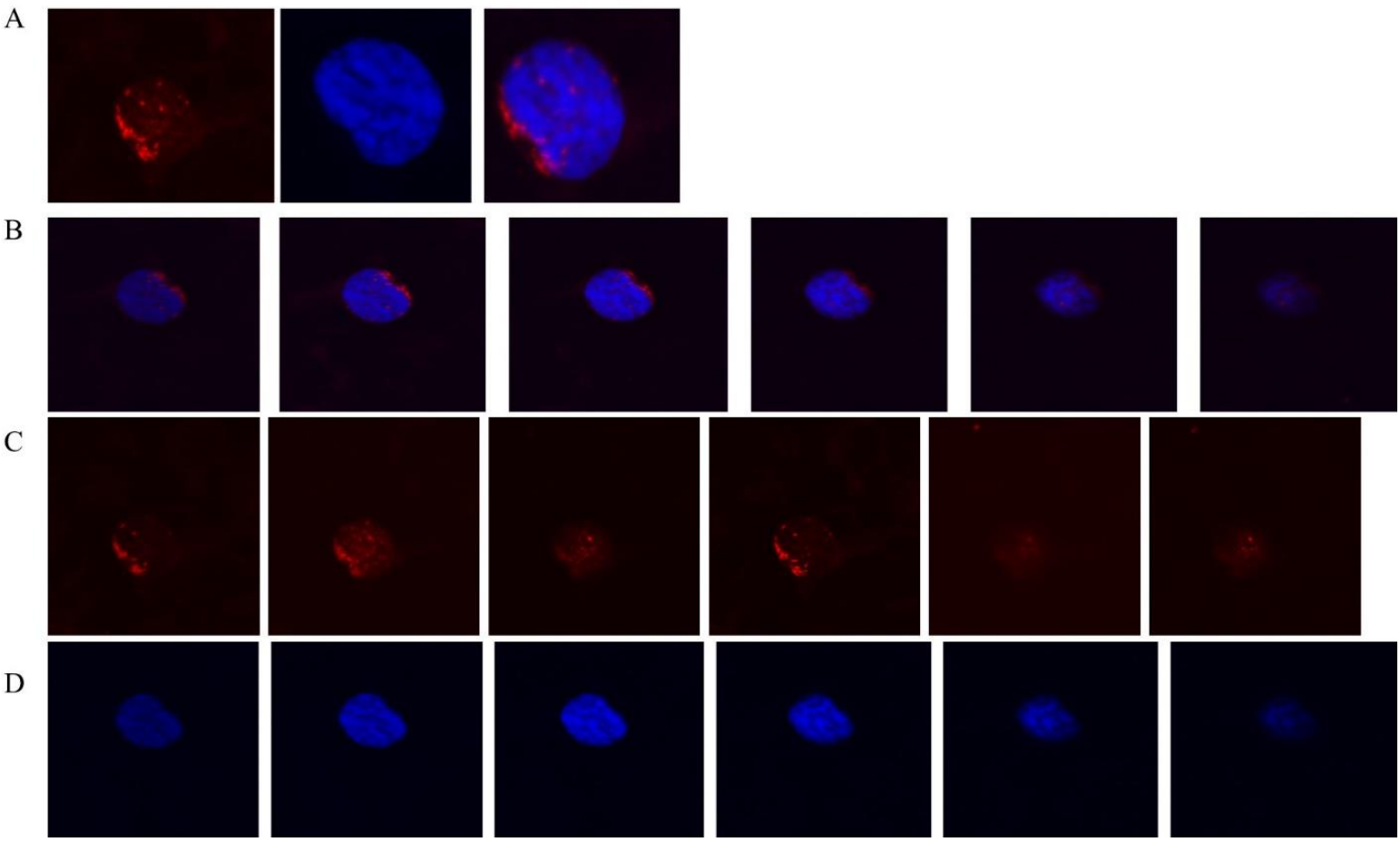
Treatment comparisons		Mean diff.	Lower 95% CI	Upper 95% CI	p-value	Significance
VV	CV	-0.026	-0.0512	-0.00073	0.0415	*
VV	CR	-0.0081	-0.0334	0.01710	0.874	ns
VV	CK	-0.0065	-0.0317	0.01877	0.940	ns
VV	CKR	-0.004	-0.0292	0.02127	0.989	ns
CV	CR	0.0178	-0.0074	0.04310	0.262	ns
CV	CK	0.0195	-0.0057	0.04477	0.189	ns
CV	CKR	0.022	-0.0032	0.04727	0.109	ns
CR	CK	0.0016	-0.0236	0.02693	0.999	ns
CR	CKR	0.0041	-0.0211	0.02943	0.988	ns
CK	CKR	0.0025	-0.0227	0.02777	0.998	ns

Appendix B. Table 1. Simple main effect analysis of SERT cluster size and treatment in 1w cohort. VV and CV had significantly different SERT clusters sizes. (Mean diff. = mean difference; CI = confidence interval) *$p < 0.05$

Treatment comparisons		Mean diff.	Lower 95% CI	Upper 95% CI	p-value	Significance
VV	CV	-6.01	-43.84	31.84	ns	0.989
VV	CR	-4.52	-42.34	33.34	ns	0.996
VV	CK	-15.17	-53.00	22.67	ns	0.763
VV	CKR	-23.33	-61.17	14.50	ns	0.389
CV	CR	1.50	-36.34	39.34	ns	0.998
CV	CK	-9.16	-47.00	28.67	ns	0.951
CV	CKR	-17.33	-55.17	20.50	ns	0.664
CR	CK	-10.67	-48.50	27.17	ns	0.919
CR	CKR	-18.83	-56.67	19.00	ns	0.595
CK	CKR	-8.167	-46.00	29.67	ns	0.968

Appendix B. Table 2. *Post hoc* analysis of SERT cluster number and treatment in 1w cohort. No significance was found across any of the treatment groups. (Mean diff. = mean difference; CI = confidence interval measured at 95%)

SERT data was imaged using confocal laser scanning microscopy (Olympus FluoView FV1000) to obtain a 3-dimensional representation of both lymphocytes and SERT clusters. I coded a macro on Fiji software (image processing package based on ImageJ2) with the help of PhD candidate Hannah Reid adapted from the macro written by PhD candidate Brady Reive for a Nikon Eclipse E800 microscope. Representative images of SERT clustering can be found in Figure 2.



Appendix B. Figure 2. Representative confocal images of SERT clustering on peripheral lymphocytes. A) Images of SERT clusters in red, lymphocytes depicted in blue, and the two channels stacked together. B) Images showing x-y-z stacking on the confocal to obtain a 3-dimensional representation of both SERT clusters and lymphocyte. C) x-y-z stacked images of SERT clustering. D) x-y-z stacked images of a lymphocyte.