# The antidepressant-like effects of intravenous reelin in the repeated-corticosterone paradigm of chronic stress

by

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Bachelor of Arts, National University of Ireland, Galway 2014

Master of Science, National University of Ireland, Galway 2015

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**Dissertation abstract**

Depression is an extremely common, devastating psychiatric syndrome with profound effects on the structure of neurons and the proteins that they express. However, the pathophysiology of depression remains unclear despite decades of extensive research efforts, and this lack of understanding makes it difficult to develop effective treatments. It is extremely problematic that conventional antidepressant drugs do not work for many patients, and those that do respond require weeks to months of continuous treatment before adequate therapeutic improvement is achieved. Therefore, there is a clear unmet need to develop mechanistically novel antidepressant compounds that are well-tolerated, more effective, and faster acting.

Subjecting rats to repeated-corticosterone (CORT; stress hormone analogous to cortisol for humans) injections produces a depressive-like phenotype that can be used to make inferences about the human condition and screen compounds for antidepressant properties. Our laboratory has previously found that stress downregulates hippocampal reelin in a similar manner to that seen in depression patients, and that drugs with antidepressant actions recover this deficit. This provided a rationale to administer reelin directly into the hippocampus, which rescued behavioral and neurochemical deficits, but intrahippocampal infusions are not clinically viable. Reelin is expressed in the periphery and blood as well as the brain, so the aims of the collection of studies described here are to evaluate the antidepressant-like properties of peripheral intravenous (i.v.) reelin. In the first experiment, the antidepressant-like effects of several dosages of reelin (3/5µg given every 5/10 days) were evaluated in rats that were exposed to 3-weeks of daily CORT (40mg/kg) injections. I found that all the dosages of reelin attenuated CORT-induced despair-like behavior in the forced-swim test (FST) and normalized alterations in serotonin (5-HT) transporter (SERT) membrane protein clustering (MPC) in blood lymphocytes. Reelin treatment also increased reelin-immunoreactive (IR) cell counts in the hippocampal dentate gyrus (DG) subgranular zone (SGZ), but it had less of an effect on neurogenesis as measured by the number and maturation rate of doublecortin (DCX)-IR cells. Interestingly, the lowest dosage used also rescued the number of reelin-IR cells in the hypothalamic paraventricular nucleus (PVN). This suggested that the restoration of SGZ-reelin plays a pivotal role in attenuating depressive-like behavior and that 3µg every 10 days was the most effective dosage that was tested.

Using the lowest dosage that showed to be effective in the first experiment, I then evaluated if male and female rats responded similarly to i.v. reelin using a larger battery of behavioral tests. Post-mortem tissue analyses focused on reelin and receptors that bind *gamma*-aminobutyric

acid (GABA) and glutamate in the SGZ, which have been implicated in psychiatric disorders and the mediation of fast-acting antidepressant responses. I found that reelin rescued the FST-behavioral and neurochemical alterations induced by CORT similarly in both sexes, indicating that it may have therapeutic effects by normalizing inhibitory/excitatory transmission. I also evaluated the effect of i.v. reelin on neurogenesis in females and found that, akin to males, the regulation of adult-born cells by peripheral reelin is unlikely to mediate the antidepressant-like effects.

The goal of the third experiment was to examine whether the antidepressant-like effects of peripheral reelin are achieved in a rapid manner. I found that a single 3µg injection after 3 weeks of CORT significantly decreased behavioral deficits in the FST 24 hours later in both sexes. Reelin also partially rescued cognitive deficits and expression levels of reelin, GluN2B, and mitochondrial-related pro-apoptotic factors bcl-2 associated X protein (BAX) and cytochrome C (CytC) in the DG. In addition, a single injection of reelin fully recovered the number of GluA1-expressing cells and partially recovered SERT cluster size in males, whereas reelin partially recovered GluA1-IR cell counts and fully recovered SERT cluster sizes in females. Reelin had modest effects on DCX-IR cells in both sexes.

The final chapter summarizes and discusses my findings, which suggest that the antidepressant-like effects of peripheral reelin are associated with the recovery of neurochemical deficits that strengthen neurotransmission, at least in the hippocampus. Therefore, developing reelin-based therapeutics with antidepressant activity would be a fruitful area of research, although additional mechanistic, pharmacokinetic, and pharmacodynamic studies are essential.

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**Glossary of abbreviations**

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine (serotonin) |
| $5\text{-HT}_{\text{subtype}}\text{R}$ | Serotonin $_{(\text{subtype})}$ receptor |
| ABC | Avidin-biotin complex |
| ACTH | Adrenocorticotrophic hormone |
| AMPA/R | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid / receptor |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| BAX | Bcl-2 associated X protein |
| BDNF | Brain-derived neurotrophic factor |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine serum albumin |
| CA | Cornu ammonis |
| cAMP | Cyclic adenosine monophosphate |
| CUMS | Chronic unpredictable mild stress |
| CORT | Corticosterone |
| CI | Confidence intervals |
| CRF | Corticotropin-releasing factor |
| CytC | Cytochrome C |
| DAB | 30-diaminobenzidine |
| Dab1 | Disabled-1 |
| DCX | Doublecortin |
| DG | Dentate gyrus |

| | |
|---|---|
| DSM(-V) | Diagnostic and statistical manual of mental disorders (5th Edition) |
| DST | Dexamethasone suppression test |
| EC | Entorhinal cortex |
| ECT | Electroconvulsive therapy |
| EPM | Elevated plus maze |
| FDA | Food and drug administration |
| FST | Forced swim test |
| GABA | *Gamma*-aminobutyric acid |
| $GABA_{A/B}Rs$ | $GABA_A$ or $GABA_B$ receptors |
| $GAD_{65/67}$ | Glutamate acid decarboxylase-65/67 |
| GCL | Granule cell layer |
| GR | Glucocorticoid receptors |
| HDRS | Hamilton depression rating scale |
| HIPP | Hilar performant path-associated cells |
| HPA | Hypothalamic-pituitary-adrenal |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| IR | Immunoreactive |
| L-E | Long-Evans |
| LSD | Lysergic acid diethylamide |
| MAO | Monoamine oxidase |

| | |
|---|---|
| MAOA-VNTR | Variable number of tandem repeats polymorphism of MAO-A |
| MAOI | Monoamine oxidase inhibitor |
| MDD | Major depressive disorder |
| MDMA | Methylenedioxymethamphetamine |
| MOPP | Molecular layer performant path-associated cells |
| MPC | Membrane protein clustering |
| MR | Mineralocorticoid receptors |
| mTOR | Mechanistic target of rapamycin |
| MWM | Morris water maze |
| NK | Natural killer |
| NGS | Normal goat serum |
| NHS | Normal horse serum |
| NOS | Nitric oxide synthase |
| NMDAR | N-methl-D-asparate receptor |
| NORT | Novel object recognition test |
| NSF | Novelty suppressed feeding |
| OFT | Open-field test |
| OBIP | Object-in-place test |
| OBL | Object-location test |
| PBS | Phosphate-buffered saline |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PL | Polymorphic layer |

| | |
|---|---|
| PFC | Prefrontal cortex |
| PSD-95 | Postsynaptic density-95 |
| PVN | Paraventricular nucleus |
| REST | Repressor Element 1 Silencing Transcription factor |
| RR | Reelin repeats |
| s.c. | Subcutaneous |
| S-D | Sprague-Dawley |
| SERT | Serotonin transporter |
| SFKs | Src family of non-receptor tyrosine kinases |
| SGZ | Subgranular zone |
| SIT | Social interaction test |
| SLC6A4 | Solute carrier family 6 member 4 |
| SNRI | Serotonin–noradrenaline reuptake inhibitor |
| SPSS | Statistical package for the social sciences |
| SPT | Sucrose preference test |
| SSRI | Selective serotonin reuptake inhibitor |
| TBS | Tris-buffered saline |
| TCA | Tricyclic antidepressant |
| TrkA | Tropomyosin receptor kinase A |
| TrkB | Tropomyosin receptor kinase B |
| TST | Tail suspension test |
| VTA | Ventral tegmental area |
| YmSponAlt | Y-maze percentage of spontaneous alternation |

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# Chapter 1

## An introduction to depression, how we model it, and reelin as a novel candidate antidepressant-like compound

## 1.1 Dissertation outline

Major depressive disorder (MDD) is a chronic, debilitating psychiatric disorder that is characterized by ongoing depressed mood, anhedonia, fatigue, irritability, cognitive deficits, and disturbed appetite and sleep (Nemeroff 1998). MDD affects an estimated 350 million people worldwide (Guilbert 2003), making it the leading cause of disease burden for both sexes (GBD 2018). In fact, the lifetime prevalence rates for depression are about 20% in developed and developing countries (Kessler et al 1994), with about twice as many women suffering than men (Eid et al 2019). Despite decades of extensive research efforts, the pathophysiology of depression and its recurrence remains elusive and our first-line monoaminergic-based medications (including selective 5-HT reuptake inhibitors (SSRIs) and 5-HT–noradrenaline reuptake inhibitors) have several limitations. For example, although antidepressants reach their pharmacological targets within minutes, and activity at the synapse is altered within a few doses, there is a substantial therapeutic delay of weeks to months of continuous treatment and around 33% of the depressed population are considered resistant to conventional treatments (Cipriani et al 2018, Rush et al 2006, Thase et al 2001). This places patients who are experiencing a depressive episode at serious risk considering that MDD is the most notorious predictive factor for suicidal ideation at any age and represents a considerable economic and societal burden (Cuijpers et al 2014a, Roca et al 2019). These are just some reasons why the monoamine hypothesis of depression has largely fallen out of favor and highlights the importance of developing novel compounds with antidepressant properties that are faster acting, longer lasting, and more effective.

In the late 1990s, it was revealed that levels of the extracellular matrix protein reelin, which regulates many forms of neuroplasticity, like the generation of new-born cells, dendritic outgrowth, and the formation of dendritic spines and new synaptic connections (Beffert et al 2006, Bosch et al 2016a, 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), was decreased in post-mortem brain samples from those with psychiatric disorders. Specifically, reelin was substantially decreased in several mood-regulating brain regions from schizophrenic patients (Impagnatiello et al 1998), the cerebral cortex of bipolar patients who have experienced psychotic episodes (Guidotti et al 2000), and in the hippocampus from patients with schizophrenia, bipolar, and MDD, especially in the DG (Fatemi et al 2000, Knable et al 2004). Reelin levels are also abnormal in blood serum from those with mood and psychotic

disorders (Fatemi et al 2001), but it is unknown whether sufferers have altered levels of reelin in peripheral tissues. Nevertheless, these reports suggest that the downregulation of reelin is associated with psychiatric illnesses and that increasing hippocampal reelin could protect one from depression and related issues. To investigate this relationship further, our laboratory has subjected adolescent rats to chronic CORT treatment (their primary stress hormone that is analogous of cortisol for humans) and found that CORT decreased reelin expression in the DG SGZ dose- and time-dependently, and that reductions in reelin parallel the progressive development of depression-like behavior (Lebedeva et al 2020, Lussier et al 2009, 2013a). We went on to show that behavioral impairments and reelin deficits were rescued by drugs that have antidepressant actions such as imipramine, etanercept, and ketamine (Fenton et al 2015, Brymer et al 2018, Johnston et al 2020). Similarly, we reported that animals deficient in reelin, like the heterozygous reeler mouse (*RELN*$^{+/-}$, haplo-insufficient for *RELN* whom express 40-60% of normal reelin levels), are more vulnerable to the depressogenic effects of CORT (Lussier et al 2011). These discoveries provided a rationale to explore whether reelin supplementation has neuroprotective, antidepressant-like effects. Therefore, we infused reelin directly into the hippocampus and found that it rapidly rescued the behavioral and neurochemical deficits induced by repeated CORT and that the putative molecular mechanisms responsible for reelin's antidepressant actions overlapped considerably with those of ketamine (Brymer et al 2020, Johnston et al 2021, Harraz et al 2016a, Koike & Chaki 2014). This is important because ketamine has been shown to significantly improve depression scores within hours for about 2 weeks in some patients that are resistant to conventional treatments (Kadriu et al 2020b, Zarate et al 2006). Another group also recently demonstrated that disrupting synaptic reelin signaling abolishes the antidepressant-like and neuroplasticity-enhancing effects of ketamine in preclinical models (Kim et al 2021). Therefore, the development of reelin-based therapeutics could be a promising avenue of research for the treatment of depression, but intrahippocampal infusions of reelin do not provide a viable treatment approach so other routes of administration should be evaluated.

The focus of this dissertation was to examine the antidepressant-like effects of peripheral reelin in male and female rats and ascertain whether these effects are achieved expeditiously, like those produced by central infusions. Rats were given intravenous (i.v.) injections of reelin via the lateral tail vein, which of course, provides a less invasive and more clinically viable approach than direct brain infusions. In addition to behavioral

outcomes, I report how reelin exerts effects on multiple neurobiological systems that are implicated in depression, such as reelin levels (Fatemi et al 2000, 2001), neurogenesis (Balu & Lucki 2009, Jacobs et al 2000, Malberg et al 2000), mitochondrial apoptotic factors (Allen et al 2018, 2021a), and GABAergic and glutamatergic receptors (Brymer et al 2020, Duman et al 2019, Lussier et al 2013b). I also evaluated how reelin effected SERT MPC in blood lymphocytes which may provide a mechanism whereby peripheral reelin, through the regulation of MPC dynamics, can influence central processes.

These experiments lay the groundwork for subsequent mechanistic studies that can inform which signaling pathways are essential for reelin's antidepressant actions. As well, they suggest that the development of reelin-based therapeutics would make promising, auspicious candidate novel antidepressant compounds that work through the regulation of multiple neuronal and peripheral biological systems. The following sections will discuss the characteristics of MDD in more detail, including biological alterations that are associated with MDD, and how reelin is involved in MDD and how it could be preventative.

## 1.2 Overview of major depressive disorder

Humans are incredibly vulnerable to mental illness, with about 1 in 4 people suffering from a diagnosable psychiatric disorder at some point in their lives, or about 1 in 5 for a given year (Geddes et al 2012). The majority of cases are made up by diagnoses of depression, which has life-time prevalence rates of approximately 20% to 25% for women and 7% to 12% for men (WHO 2017). In a Canadian 2012 study, life-time prevalence rates were 11% and past-year prevalence rates were 5%, which was also found 10 years earlier (Patten et al 2015, Pearson et al 2013). Prevalence estimates often vary substantially between studies which may derive from the fact that depression lacks a clear definition and optimized diagnostic methods. Nevertheless, rates are consistently 2- to 3-fold higher in females than males, a sexual dimorphism that stems from a combination of biopsychosocial factors (Eid et al 2019, Labaka et al 2018). Usually, episodes first occur during adolescence, and they can interrupt critical academic, social, romantic, and occupational developmental periods. The average age for onset of depression is between 18 and 29 in a given year (Kessler et al 2003) and the median age is 32 (Geddes et al 2012). Rates of depression are generally 2-fold higher in adults aged 65 and

over compared to those aged 18-64. One can assume that age differences can be linked to the loss of partners, retirement, and declines in cognitive function and physical health, among other things. That said, depression can afflict someone at any time in life and an earlier age of onset is associated with greater chances of recurrence and chronicity (Hollon et al 2006, Zisook et al 2004). Indeed, it is challenging to accurately estimate the number of people who suffer from depression because not everyone who meets diagnosable criteria pursues treatment and often people are misdiagnosed. Our best estimates are that 300-350 million people suffer from depression, and it is now the leading cause of disease burden for both sexes (Guilbert 2003). It is true that depression has considerable direct costs to healthcare services and indirect economic costs due to an inability to maintain employment; the Conference Board of Canada reported that MDD has an annual cost of $32.3 billion in lost gross domestic product (GBD 2018).

It is normal for people to experience pessimistic thoughts, sadness, and fatigue, especially in response to the slings and arrows of life, but for some, these experiences can be untypically overwhelming, intense, and persistent. The main clinical feature of depression is a low pathological mood which must be discriminated from a normal response to saddening events. Sadness, of course, exists on a large spectrum, and it is often masked by a smile. However, friends, family, and clinicians can often notice a change in character in someone that has lost the zest for life, especially when mood changes are incessant and co-occur with irritability and agitation. The second core symptom of depression is anhedonia, or the loss of interest in once pleasurable activities, which can include hobbies and sexual desire. Persistent sadness and anhedonia are often accompanied by anxiety, fatigue/sluggishness, problems sleeping (insomnia or hypersomnia), irritability, cognitive deficits, changes in weight, inappropriate feelings of guilt or worthlessness, aches and pains, and general malaise. One would typically receive a diagnosis of depression if at least 3 of these symptoms co-occur with low mood and anhedonia most of the day for at least 2 weeks (American Psychiatric Association, 2013). The well-being of patients is typically assessed with a series of standard questions that pertain to things like daily mood and habitual behavior. Clinicians often look out for pessimistic thinking, complaints regarding memory, hypochondriacal preoccupations, loss of libido, lethargy, and changes in task performance, such as house cleaning and grooming behaviors. Secondary issues may arise following things like sleep deprivation, such as increased blood pressure, cognitive impairment, migraines, and over-eating. Symptom profiles can vary considerably but clinically recognizable depression must cause significant distress in educational, occupational, or social settings, and it is often pernicious to family and peer relationships. In

severer cases, more profound functional impairments are inherently observed, and sometimes even strong delusions and hallucinations. At its worst, depression can lead to physical self-harm and it is the number one cause of suicide at any age (Cuijpers et al 2014a); about 4% of patients succumb to suicide, or about 10% of severe cases (Coryell & Young 2005), and 23% of individuals who die by suicide are taking antidepressants at the time (CDC 2009). Biological markers, or biomarkers, can be analyzed in things like blood, saliva, hair, and so on, to help inform the diagnosis and course of treatment, but laboratory tests have generally proven to be unsuccessful at distinguishing different disorders and unpredictive in terms of treatment responsiveness and illness severity. Indeed, atypical, psychotic, bipolar, or major depression, for instance, could all potentially stem from the same phenomena, or perhaps they have unique pathophysiological mechanisms. A physical examination is also usually necessary to rule out that a patient's symptoms are not produced by an underlying medical condition. If one abuses substances, which can often occur as a coping mechanism, then the sequence of the depression and substance abuse must be determined to rule out that the depression is not drug induced. There have also been attempts to map symptoms to specific brain regions using neuroimaging techniques. For instance, anhedonia may be associated with abnormal activity of the nucleus accumbens whereas cognitive impairments and the anterier cingulate cortex may be linked, but most symptoms probably result from the dysfunction of several brain regions simultaneously. It is also true that some regions in the depressed brain may be overactive, like the PVN and amygdala, whereas others may be underactive, like the hippocampus and nucelus accumbens. It seems logical that a loss of function in certain brain regions could cause things like a lack of motivation and low appetite, whereas overactivity of other regions could explain anxiety, insomnia, and intrusive thoughts. Based on a meta-analysis of neuroimaging studies, researchers proposed that depressed patients have more active thalamic pulvinar nuclei, which enhances the response to negative stimuli by activating the amygdala, insula, and anterier cingulate, and less active dopaminergic pathways in the dorsal striatum and dorsolateral prefrtonal cortex (PFC), which causes a failure to feel pleasure and consider or expereince positive reasoning to mitigate negative outlooks (Higgins & George, 2013).

Depression is responsible for 50% of psychiatric evaluations and 12% of hospital admissions (Kuo et al 2015), and patients who tend to meet the diagnostic criteria for depression often meet the criteria for other disorders. For example, common depression comorbidities include anxiety, hypertensive, inflammatory, and metabolic disorders (Fancourt & Steptoe 2020, Groen et al 2020, Steffen et al 2020, Allen et al 2018, Dahl et al 2014, Dowlati et al 2010, Howren et

al 2009), which can potentiate depression severity, slow recovery, and elevate relapse probability and suicidal behavior (Hirschfeld 2001). As well, neurological conditions such as stroke, epilepsy, Parkinson's, and Alzheimer's disease can dramatically increase depression vulnerability (Rickards 2005). Furthermore, a meta-analysis of 57 articles indicated that depression often manifests in 32% of those with mild cognitive impairment (Ismail et al 2017). In fact, 64%, 72%, and 78% of mild, moderately, or severely depressed patients, respectively, experienced a comorbid mental disorder (Steffen et al 2020). Moreover, prevalence rates of depression are high among those with asthma (27%), obstructive pulmonary disease (25%), systemic lupus erythematous (22%), and gout (20%) and rheumatoid arthritis (15%) (Wang et al 2017). One should question, then, whether the importance of studying one disorder independent from another has become overly emphasized. This could even suggest that our research findings are not accurate reflections of the common dimensions that cause psychiatric illness, especially since arbitrary diagnostic and study enrollment criteria exclude the larger spectrum of functioning.

Around 75% of individuals with depression experience multiple recurrent episode relapses as the disease course progresses, making it a chronic, life-long condition (Crown et al 2002, Hollon et al 2006). A longitudinal study found that the median episode length was 12 weeks, and that 15% of individuals did not have an episode-free year for 23 years (Eaton et al 2008). In fact, each episode appears to make one more susceptible to episode relapse, so that episodes come closer together with the passage of time (Judd et al 1998, Kessing et al 2004, Solomon et al 2000). Depressions present differently from patient to patient, but symptoms are dynamic and changeable in single episodes as well, and even after a patient has reached clinical remission, they can continue to experience impaired functioning in family, social, and occupational settings (Judd et al 1998). On the other hand, some people may experience a single episode, perhaps never seek treatment, and go on to fully recover. The age of depression onset also has clinical and etiological implications, because an earlier age of onset positively correlates with symptom severity and greater chances of episode recurrence and chronicity, and this may reflect a genetic etiology (Levinson et al 2003, NRC 2009, Pettit et al 2006). A meta-analysis including 5 family studies concluded that 37% of depression was accounted for by genetic factors and the other 63% was accounted for by individual-specific environmental factors (Sullivan et al 2000).

It is important that depression is diagnosed and treated in a timely fashion to slow its progression and quicken recovery. Patients are typically prescribed drugs that selectively

inhibit SERT or the norepinephrine transporter before tricyclic antidepressants (TCAs), but they all have limitations. For instance, monoaminergic-based medications have poor remission rates (Trivedi et al 2006, Thase et al 2001). To be more detailed, ⅔ of patients fail to respond adequately to the first round of antidepressant treatment, and ⅓ of them will remain unresponsive after multiple trials of combination therapy (Cipriani et al 2018, Rush et al 2006). Occasionally, antidepressants can paradoxically induce suicidal ideation, along with many other side-effects like sexual dysfunction, nausea, weight changes, dry mouth, constipation, and insomnia, which often leads to the discontinuation of treatment (Garland et al 2009, Nischal et al 2012). Another major limitation of antidepressants is that they take around 6 to 10 weeks to reach full effectiveness, which can be especially problematic for patients at high risk of suicide (Stahl 2000). It has been theorized that the therapeutic time-course of antidepressants is related to the time it takes them to increase hippocampal neurogenesis, implying that deficient new-born cell proliferation and maturation is critical to the pathogenesis of depression, although it is probably just one of many contributing factors that is deleterious to overall neuroplasticity (Hanson et al 2011). Despite the inefficacy of antidepressants, there has been little progression in the development of mechanistically novel treatments for over 50 years. Interestingly, the synthetic phencyclidine derivative ketamine, that is widely used as a dissociative anesthetic, was recently shown to have fast-acting antidepressant effects in under an hour that last for 1 to 2 weeks in treatment-resistant populations (Duman 2018, Kim et al 2019). A growing body of evidence suggests that ketamine rescues stress-induced alterations in glutamatergic and GABAergic neurotransmission and suggests that glutamatergic modulators should be considered as a new class of antidepressants (Ghosal et al 2020, Kadriu et al 2021, Koike & Chaki 2014, Pham & Gardier 2019). Ketamine heightens excitatory transmission mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR) that leads to the activation of important plasticity-regulating kinases like tropomyosin receptor kinase B (TrkB) and the mechanistic target of rapamycin (mTOR) (Holubova et al 2016, Kadriu et al 2020b, Li et al 2010). For the past decade, our lab has been studying the reelin protein which we have recently shown can decrease depressive-like behavior in rats that were exposed to stress in 24 hours by working through similar downstream pathways (PI3K/Akt/mTOR and AMPARs) (Brymer et al 2020, Johnston et al 2020, Ly et al 2018). This strongly suggests that the timely promotion of neuroplasticity is a crucial, fundamental event that is necessary for the rapid attenuation of depressive symptoms and can be achieved by targeting more desirable pathways.

### 1.2.1 The evolution of depression over time

Depression manifests as a complex constellation of symptoms that has afflicted mankind across societies since antiquity, well before the formation of our current definitions, and presumably long before we have been documenting. Before we had a better understanding of what caused depression, psychiatric disturbances were assumed to be caused by demonic possession (Asch 1985). In fact, the sufferers often had holes drilled into their skulls (trepanation) through which evil spirits could escape (Foerschner 2010). Over time, our view of depression as a concept has been sculpted and molded by society into what it is today, but it is far from completing its evolution. The oldest document referencing clinical depression that exists is the Ebers papyrus (1550 BCE), which accounts the symptoms, diagnosis, and prognosis of 2 patients with fractured skulls (Kandel 2013). It was the ancient Greeks who first conceptualized that mental well-being was largely influenced by our physical bodies and the need for harmony in one's environment (Kleisiaris et al 2014). Famously, Hippocrates (460-379 BCE), who we know today as the Father of Medicine, postulated that homeostatic levels of internal fluids, called the humors, would bring about good health, and that a substantial deficiency or excess of one would lead to poor health. The humors were blood, phlegm, yellow bile, and black bile, and an excess of the latter was presumed to generate melancholia. In Greek, Malankholia translates to sadness or black bile (Hippocrates 1849). Hippocrates brought forward the idea that depressive behavior, among other ailments, could be explained by physiological phenomena.

The development of the field of psychiatry in the 19$^{th}$ century spawned new ways of thinking about the origin of melancholic behavior as a sickness of the mind. German psychiatrist Emil Kraepelin first described different forms of melancholia as "depressive states", and he categorized manic depression and dementia praecox (precocious madness), which was later replaced by schizophrenia (Del Porto 2004). He largely based distinctions on whether the roots of the depression originated from internal or external sources, and those depressions internal in nature were thought to have "grown" out of one's mind. The notion that material outside of our conscious awareness could influence our mental well-being was famously explored by Sigmund Freud, who referred to melancholia as a sense of loss that co-manifests with an impoverishment of the ego; that it causes delusional thought patterns that interfere with the mental processes of repression (Freud 1917). The acknowledgement that depression could be biological in nature or stem from chronic environmental stress is an important distinction that

developed further with the advent of psychological science; behaviorists arguing that we are social creatures whose behavior is shaped by culture and our interactions in society and determinists favoring the idea that our behavior is dictated by our biological make-up. The modern view is that "nature" predisposes one to psychiatric illness, but "nurture" triggers its manifestation. The importance of nurture was illustrated by Abraham Maslow's well-known "hierarchy of needs", suggesting that depression and mental torment arise when our basic needs are not met or when they are satisfied at the expense of other needs, like those concerning safety and emotional connection (Maslow 1958). Depression is no longer viewed as a mysterious paranormal sickness or emotional weakness, but as a disease of the brain, much like multiple sclerosis is to the peripheral myelin sheaths.

The next breakthrough in our understanding of depression pathology was not until 1952 when the serendipitous discovery that iproniazid, which was being used to treat tuberculosis, induced euphoria by inhibiting the enzyme monoamine oxidase (MAO) (Ramachandraih et al 2011). MAO inhibition raises synaptic levels of indolamine 5-HT and catecholamines noradrenaline and dopamine. Similarly, hypertensive drug reserpine was thought to occasion depression by blocking the vesicular monoamine transporters and depleting norepinephrine, dopamine, 5-HT, and histamine stores in synaptic endings, but evidence of this is lacking considering it may bring about positive mood changes (Baumeister et al 2003, Davies & Shepherd 1955). Nevertheless, more concrete evidence that chemical transmitters regulated mood emerged in the mid-1950s with the detection of 5-HT in the mammalian brain (Twarog & Page 1953). Soon after this came the realization that the chemical structure of 5-HT was very similar to that of lysergic acid diethylamide (LSD), which was synthesized by Albert Hoffman who was first to famously experience its profound psychological effects a decade earlier. This led to the postulation that drugs alter mood by interfering with neurotransmitters, and that targeting a specific deficiency, such as monoamine levels, could be used as a remedy (Woolley & Shaw 1954). The development of MAO inhibitors (MOAIs) and TCAs followed in the 1950s and 60s, and later SSRIs and SNRIs in the 1980s and 1990s which are tolerated better and remain first-line treatments.

With psychologists and psychiatrists analyzing the origin of their patients suffering, there was a need to formulate a standardized mental health classification system to appropriately guide their diagnosis and prognosis which relies on statistical analyses. This prompted the formation of the Diagnostic and Statistical Manual of Mental disorders (DSM, 1952) which is now in its 5$^{th}$ edition (DSM-V) (Kawa & Giordano 2012), as well as questionnaires to standardize

symptom evaluation, such as the Hamilton Depression Rating Scale (HDRS; 1960) and Beck's Depression Inventory (BDI; 1961). The HDRS contains 17 (originally) or 21 (additional questions added to subtype depression but often incorrectly used to interpret severity) items that are graded from 0-2/3/4 and an overall score of 0-7 is considered normal or clinical remission, while 20 and up would indicate moderate and more severe forms of depression, respectively. The HDRS is limited by the fact that atypical depressive symptoms are not assessed (like hypersomnia or hyperphagia) (Hamilton 1960). BDI has 21 items scored 0-3 and an overall score of 1-10 indicates normal mood, 17-20 borderline clinical depression, and 31-40 and over 40 severe and extreme depression, respectively (Beck et al 1961). The Montgomery-Asberg Depression Rating Scale was designed in 1979 to be sensitive to change resulting from pharmacotherapy and comprises 10 items that are scored from 0-6 and focus on core symptoms, and it is influenced less by maladaptive personality traits than the BDI (Svanborg & Asberg 2001).

Over the years we have categorized types of depression to better understand their formation and course of treatment. First, we classify illnesses as a single episode, recurrent, or persistent, and episodes as mild, moderate, or severe. Considering that depression is multifactorial, classifying them as endogenous (stemming from one's biology, like genetic mutations) or reactive (brought on by external stressors) is of little value to clinicians. It is more useful to refer to depressions according to the nature, type of, and intensity of symptoms (Geddes et al 2012). If depressions last for over 2 years, we refer to it as persistent depressive disorder which is typically combatted with monoaminergic pharmacotherapy and often in combination with psychotherapy. It is not uncommon for people to experience intense depressions predominantly in winter months when access to sunlight is limited; once referred to as seasonal affective disorder when occurring over at least 2 consecutive years, but expressed as a course specifier for MDD in the DSM-V. Light therapy has been shown to help, suggesting that depressive symptoms can be traced to disturbances in circadian rhythms (Campbell et al 2017). More severe unipolar depressions co-occur with strong delusions like extreme self-deprecation, nihilism, paranoia, and even hallucinations (more often auditory), which would then be referred to as depression with psychotic features (O'Connor & Agius 2015). It is also common for depression to develop during times of large hormonal fluctuations like puberty and childbirth, so it might be characterized as a premenstrual dysphoric disorder or perinatal depression, for instance (Hofmeister & Bodden 2016, Putnam et al 2017). The DSM-V defines peripartum onset as a specifier that occurs during pregnancy or within a month of giving birth (American

Psychiatric Assocation 2013). In addition, depression can be specified as atypical if the melancholic features of typical depressions are not satisfied and normal functioning is impaired by things like over sleeping and eating, Leadon paralysis (severe bodily exhaustion), and interpersonal rejection sensitivity (Singh & Williams 2006). Adjustment disorder with depressed mood (situational depression) resembles MDD but the symptoms must follow a specific stressor, such as the death of a loved one, divorce, abuse, or unemployment (Bachem & Casey 2018). A particularly common subtype of depression in the elderly is called depressive pseudodementia, which is characterized by severe memory impairments. If at least 3 manic symptoms co-occur within an episode, then it should be characterized by the specifier "with mixed features"; only when the criteria for a manic or hypomanic episode is met does the diagnosis exist in the bipolar spectrum.

A surge of research followed the relatively recent discovery that a single infusion of ketamine can reduce HDRS scores in hours for about 2 weeks (Berman et al 2000, Domino & Warner 2010, Zarate et al 2006), indicating that the modulation of molecular mechanisms other than the inhibition of monoamine reuptake can alleviate depression more effectively. This is arguably one of the most important breakthroughs in the last half-century (Wei et al 2020), and it has raised considerable interest in the direct modulation of glutamatergic transmission as a novel approach to treat depression. Animal models that allow us to make inferences about the human condition are being used to successfully uncover the molecular pathways of ketamine, which rapidly promotes neuroplasticity through an increase in AMPAR and mTOR signaling (Holubova et al 2016, Koike & Chaki 2014, Li et al 2010). Perhaps more excitingly, clinical trials have shown that psilocybin-assisted therapy can produce much longer-lasting antidepressant, anxiolytic, and anti-addictive effects than ketamine (Carhart-Harris et al 2018, Dos Santos et al 2016, Hibicke et al 2020), and studies in mice suggest that this could be true even when the hallucinogenic 5-$HT_{2A}$ receptor (5-$HT_{subtype}$R) is antagonized but more work should be done before this conclusion is made (Hesselgrave et al 2021). It is interesting that more profound "mystical-type" experiences correlate positively with greater mood and habitual improvements following treatment, which could represent a biomarker of therapeutic efficacy (Barrett & Griffiths 2018). Preclinical studies indicate that psychedelics can enhance neuroplasticity like the sustained growth of cortical neurons after only short stimulation periods, and researchers are successfully harnessing their therapeutic potential without inducing hallucinations by manufacturing psychedelic analogues (Hesselgrave et al 2021, Ly et al 2021, Lu et al 2021). It is also believed that the empathogen MDMA

(methylenedioxymethamphetamine) can reopen a novel critical period of neuroplasticity (an epoch during which the brain is sensitive to certain stimuli that are required for experience-dependent learning) which may explain how certain 5-HTergic stimulators persistently reduce negative thinking following drug-assisted therapy (Edsinger & Dölen 2018, Ly et al 2018, Mitchell et al 2021, Nardou et al 2019). In fact, MDMA reliably induces empathy, euphoria, insightfulness, extraversion, gregariousness, and interpersonal trust and it has been boldly described as "penicillin for the soul" (Harris et al 2002, Mitchell et al 2021, Reiff et al 2020). Like ketamine, psilocybin, and MDMA were fast-tracked by the Food and Drug Administration (FDA) following designation as "breakthrough therapies", and while these molecules activate different receptors, their downstream mechanisms converge on glutamatergic and mTOR signaling which strengthens cortico-limbic circuits. It seems that the induction of neuronal growth by such compounds requires an initial epoch of TrkB activity followed by an epoch of sustained mTOR and AMPAR activity (Ly et al 2021).

It is the unfortunate truth that mental disorders are still largely stigmatized by those who are less familiar with psychiatry. This is likely because it is challenging to pinpoint specific pathological characteristics that underlie disorders of the brain; although, one should keep in mind that until recently this has been the basis for most of medicine. Stigmatization is evident in reports that discuss attitudes towards mental illness, for example: 45% of the sample population did not think that individuals with a psychiatric illness should have the same employment rights; 60% thought that mental illnesses arise from a lack of self-discipline or will power; 80% assumed that prevalence rates are lower than they are by a factor of 10; and 66% said they fear those with a mental disorder (Geddes et al 2012). We are left with a picture that describes depression as a vexing syndrome that develops more often in those with genetic vulnerabilities and usually after periods of stress. The amount of stress one experiences seems to correlate with neurochemical dysfunctions, and drugs that enhance neuroplasticity have rapid antidepressant effects.

### 1.2.2 The etiology of depression

Despite the extraordinary prevalence rates of MDD, the pathophysiological mechanisms that trigger its development and recurrence remain elusive. They are difficult to pinpoint considering that depression forms through an interaction of biochemical and psychological

processes that are influenced by the environment. Additionally, not all depressions are alike so we must assume that there can be etiological variances too. Biological factors that influence depression include genetic, neurological, hormonal, immunological, and endocrinological influences, which are affected by stress, sex, and age (NRC 2009). The fact that sex differences in depression emerge after puberty indicates that depression also has a biological basis (Altemus et al 2014, Kang et al 2020). Environmental stressors often precede the development of depressive symptoms, such as sexual abuse or exposure to long term adversities during childhood (NRC 2009, Springer et al 2003). Personal factors that influence depression onset can include things like cognitive ability, sociability, and personality traits. Depression is generally thought to be more common in people who would be characterized as neurotic and conscientious (Klein et al 2011). Personality traits can even influence therapeutic outcomes; for example, patients scoring high in neuroticism preferentially respond to pharmacotherapy rather than psychotherapy (Bagby et al 2008). The following section will discuss findings that pertain to genetic and environmental aspects of the condition. Specific biological systems that are dysregulated by genetic abnormalities and psychosocial stress will be discussed afterwards, such as alterations in systems governing neurotransmission, the stress response, and inflammation.

Several observations, mainly from moderate to severe cases, indicate that MDD is heritable. For instance, studies have found that that those with a first-degree relative that has experienced depression at some point in their lives are more vulnerable to experience depression themselves by up to 3-fold (Lesch 2004, Warner et al 1999, Weissman et al 1984). Similarly, twin studies suggest that genetics account for approximately ⅓ of the risk for depression (Levinson 2006, NRC 2009). Adoption studies support this; children who are raised by mentally healthy parents but were born to a parent with a depressive history are more likely to develop depression, although results may be confounded by obvious methodological limitations (Sullivan et al 2000). The Genome-Wide Association Studies that aimed to identify specific genetic loci involved in depression provided underwhelming results (Power et al 2013), but some specific polymorphisms of the 5-HTergic system have been identified in MDD, which regulate things like emotions, circadian rhythm, body temperature, appetite, and sexual behavior. A polymorphism of the solute carrier family 6 member 4 (*SLC6A4*) gene which codes for SERT has been identified as the most noteworthy, with those possessing copies of the short allele more likely to experience depression and suicidal ideation, especially in response to adverse life events, compared to those homozygous for the long variant (Caspi et al 2003, Collier et al

1996, Kendler et al 2005, Sen et al 2004). In patients with mood disorders and schizophrenia, those carrying 2 short copies of the allele and exposed to high levels of childhood trauma had significantly poorer cognitive ability (Aas et al 2012). Researchers also found a relationship between expression levels of a polymorphism in the promoter region of the MAO-A isozyme (called MAOA-VNTR) with either the short or long allele polymorphism of *SLC6A4* that could explain the adolescent sex differences in depression following stressful situations (Priess-Groben & Hyde 2013). MAOA-VNTR is also associated with reduced cortico-limbic and amygdala-PFC connectivity (Dannlowski et al 2009). These polymorphisms may generate depressive behavior by dysregulating systems that control emotion in response to stress. After reviewing the risks for depression recurrence, it was reported that genetics must account for a large proportion of the underlying vulnerability considering that there were no obvious associations with demographic, clinical, familial, and psychosocial factors in predicting episode relapse (Burcusa & Iacono 2007). However, the polygenic and epistatic nature of depression complexifies our attempts to locate specific genes involved in its manifestation and makes it unlikely that genetic testing will soon be used to identify vulnerable individuals.

The impact that chronic stress and life adversities have on the progressive development of depression has been thoroughly explored. Twin studies suggest that genetic influences make one more susceptible or sensitive to environmental risk factors, but they can also provide evidence of the magnitude of influence that environmental factors account for in causing depression. That said, the extent of the interaction between genotype and the environment is unclear (Rice 2009). Studies generally suggest that experiencing an extremely adverse life event increases the chances that one will develop depression by 5- to 16-fold over the following months (Kendler et al 1998, Sullivan et al 2000). Moreover, first episodes often follow a stressful event and adversities that occur early in life are one of the strongest predictors of depression later in life, such as emotional or sexual abuse, which occur more often in females (Cheasty et al 1998, Lindert et al 2014, Shapero et al 2014). In fact, children that grow up in stressful environments have a sensitized stress response (Essex et al 2002). In adulthood, adversities that trigger depression may include redundancy at work, harassment, financial problems, divorce, and the death of a loved one. Interestingly, the bereavement exclusion criteria published in the DSM-IV was removed in the DSM-V (published in 2013) on the grounds that there are no persuasive studies showing that depressions following the death of a loved one differ in any way from those developed through other means, and depression can be fatal and disqualifying a patient from treatment simply because the symptoms were triggered

by a death is unethical (Pies 2014). Depression is also higher in groups with socio-economic disadvantages, like those living in an urban or rural area with low income (Schiavone et al 2015, Weaver et al 2015). Interestingly, rates of depression with an adolescent-onset have increased over the last few decades, suggesting that psychosocial factors are largely influential (Kessler et al 2003). In a recent longitudinal mediation analysis study, social disconnectedness and perceived social isolation were strong predictors of depression and anxiety symptom severity (Santini et al 2020). Social isolation also has a negative effect on the emotionality of many animals, including rodents in whom it is associated with stress sensitivity (Weintraub et al 2010).

It has been argued that episodes of depression reoccur because each episode lowers the threshold of stress that is needed to cause another (Morris et al 2010, Kendler et al 1999), and therefore that neurobiological alterations caused by stress parallel the progressive disease course (Post 1992). For instance, hippocampal atrophy is associated with depression and correlates with symptom severity over time (Bremner et al 2000, Campbell et al 2004, Sheline et al 2003, Videbech & Ravnkilde 2004). It is interesting that recurring episodes appear to gradually become autonomous of stress, too; that is, stressful events do not always precede recurring episode manifestations. A longitudinal within-person study including over 2000 female twins found a diminishing relationship between depression onset and a preceding adverse life event after the $5^{th}$ or $6^{th}$ episode (Kendler et al 2000, 2001). Exposing rats to cycles of stress sensitizes depression-like behavior and exacerbates neurochemical deficits, such as reductions in reelin expression, and they fail to fully recover after subsequent bouts of stress (Lebedeva et al 2017, 2020).

The question then, is, what are the neuronal mechanisms that gene-environment adversities dysregulate to produce depression? The monoamine hypothesis of depression represented a breakthrough in our pathological understanding, but it has largely fallen out of favor since its conception due to several discrepancies. Research regarding the neurogenic hypothesis of depression provided further insight, but it remains controversial considering that depressive-like behavior can emerge without a reduction in cell proliferation and because antidepressants can influence behavior without potentiating neurogenesis (Bessa et al 2009, David et al 2009, Surget et al 2008). However, antidepressant-induced behavioral changes could be related to their ability to drive dendritogenesis and neuronal remodeling (Bessa et al 2009, Lussier et al 2013a). This notion represents the neuroplasticity hypothesis of depression, which posits that depression arises when neuroplasticity-regulating mechanisms become dysfunctional, such as

after periods of chronic stress. This hypothesis is a broadening of concept that also includes plasticity within cells, like in mitochondria, the organelle that produces chemical energy in the form of adenosine triphosphate (ATP). The dysfunction of mitochondria will lead to a shortage of ATP that is needed to fuel molecular activities like signal transduction and protein synthesis, but it will also exhaust antioxidant defenses, increase inflammation, and possibly induce cell death (Allen et al 2018). The following sections will discuss these theories in more detail.

#### 1.2.2.1 The monoamine hypothesis of depression

The monoamine hypothesis of depression was proposed over half a century ago and posited that MDD was caused by a lack of monoaminergic neurotransmission (Schildkraut 1965). This hypothesis stemmed from the serendipitous clinical observations in the 1950s that iproniazid induced euphoria in patients receiving tuberculosis treatment and reserpine induced depression in those receiving it for hypertension. We now know that iproniazid inhibits the enzyme MAO, which breaks down monoamines, while reserpine depletes monoamine stores by blocking vesicular monoamine transporters (Baumeister et al 2003, Ramachandraih et al 2011). The monoamines are a group of neurotransmitters including 5-HT, dopamine, noradrenaline, and adrenaline, which regulate things like appetite, mood, sleep, reward, the fight-or-flight response, and concentration, which are all impaired by depression to some extent. This led to the development of MAOIs and TCAs, followed by newer classes of antidepressants like SNRIs and SSRIs, which are safer and better tolerated. Therefore, newer SSRIs/SNRIs are prescribed more often but unfortunately their efficacy rates have not improved compared to their predecessors.

There has been some supporting evidence for the monoamine hypothesis of depression since its conception. For instance, melancholia has been associated with reduced plasma 5-HT levels (Sarrias et al 1987), and manipulating one's diet to lower levels of tryptophan, the precursor of 5-HT, can induce a transient decrease in brain 5-HT levels which can produce clinical depressive symptoms in those that have previously experienced an episode (Smith et al 1997). This suggests that deficient 5-HT neurotransmission can lead to depression in some circumstances. There are also reports that plasma tryptophan levels are lower in patients with severe cases of depression compared to healthy controls (Anderson et al 1990), which has been linked to the actions of tryptophan-metabolizing enzymes indoleamine 2,3-dioxygenase and

tryptophan 2, 3-dioxygenase, which are activated by stress and inflammation (Oxenkrug 2010). Researchers have also examined the expression levels of monoamine receptors and transporters and the results generally support the notion that depression is associated with significant alterations in 5-HTergic proteins (Stockmeier & Rajkowska 2004). To give some examples, 5-$HT_{1A}$Rs were increased in the post-mortem midbrain and PFC of depressed suicide victims (Matsubara et al 1991, Stockmeier et al 1998), and the 5-$HT_{2A}$R was increased in the PFC and hippocampus in teenage suicide victims and depressed individuals (Pandey et al 2002, Shelton et al 2009). However, some reports have found no changes in the expression of 5-$HT_{1A}$Rs or 5-$HT_{2A}$Rs (Lowther et al 1997, Stockmeier et al 1997). Region-specific alterations in 5-HT reuptake have also been recorded; SERT availability was decreased in the brainstem of depressed adults but increased in the hypothalamus of younger individuals with depression (Dahlström et al 2000, Malison et al 1998). Many studies have examined the binding of radioligands to SERT in the cerebral cortex of suicide victims and have found mixed results, some groups reporting increases, decreases, or no change in binding potentials (Stockmeier 2003). Decreased densities of SERT have also been noted in the post-mortem dorsolateral prefrontal and ventral/orbitofrontal cortex of depressed suicide victims (Mann et al 2000, Austin et al 2002). Perhaps some of the alterations in protein expression are compensatory, that is, in some cases the brain may increase/decrease the protein translation of SERT and 5-HT receptors to counteract the reductions in 5-HT availability.

Some work from our group has shown that 5-HTergic protein abnormalities also exist in lymphocytes, and that certain alteration patterns can predict therapeutic outcomes in depressed patients. More specifically, we found that the number and size of 5-$HT_{2A}$R membrane protein clusters in blood lymphocytes were increased in naïve depressed patients compared to healthy controls, while only the size of SERT clusters were increased (Rivera-Baltanas et al 2012, 2014). Furthermore, based on the distribution of 5-$HT_{2A}$R and SERT cluster sizes, we differentiated 2 subpopulations of depression patients; both groups had identical HDRS scores prior to 8 weeks of pharmacotherapy, but those with a greater percentage of larger clusters had much lower depression scores afterwards. In fact, the unresponsive group had no improvement in anhedonia scores while the other group showed about a 50% reduction (Rivera-Baltanas et al 2015), indicating that clustering parameters of 5-HTergic proteins could be used as a putative biomarker for antidepressant efficacy (Caruncho et al 2019). We have also shown that similar patterns of alterations are observed in rats that are treated with CORT or mice with genetic vulnerabilities that sensitize them to the depressogenic effects of stress (Rivera-Baltanas et al

2010, Romay-Tallon et al 2018). This suggests that CORT-treated animals can be used to identify potential biomarkers in humans.

Although monoaminergic transmission has a clear role in mood regulation, decades of research efforts convey the unlikeliness that 5-HTergic hypoactivity directly causes depression. This view is oversimplistic and ignores the complexity of MDD. An important discrepancy in the monoamine hypothesis of depression is that antidepressants, which raise monoamine concentrations, have limited therapeutic efficacy in a large proportion of the depressed population. Conventional antidepressants are slow-acting even though they raise synaptic monoamine concentrations in minutes, and they only work in some patients (Cipriani et al 2018, Cuffel et al 2003, Rush et al 2006). Chronically stimulating 5-HT receptors must eventually bring about neurobiological changes that influence mood, but we should assume that there are more direct ways to generate behavioral improvements. Other findings that contradict the monoamine hypothesis include the fact that: placebos are often nearly or just as therapeutic as antidepressants (Evans et al 1997, Goldstein et al 2002, Perahia et al 2006, Tollefson et al 1995); there is no correlation between serum 5-HT levels and depression scores (Saldanha et al 2009); large doses of tryptophan (5-HTs precursor) failed to attenuate depressed mood in a double-blind placebo-controlled study (Mendels et al 1975); reducing synaptic 5-HT through tryptophan depletion did not cause depression in healthy individuals (Ruhé et al 2007), nor did treatment with SERT enhancer tianeptine (Kasper & McEwen 2008); and mice genetically depleted of brain 5-HT did not develop depression-like behavior (Angoa-Pérez et al 2014). However, pharmaceutical companies advertised antidepressants to work by correcting a synaptic 5-HT imbalance based solely on the pharmacological observations that amine-depleting drugs could generate depression and MAOIs ameliorated some symptoms (Coppen 1967, Schildkraut 1965), creating a myth that is echoed throughout society and is not supported by the clinical literature (Lacasse & Leo 2005). In actuality, chronic antidepressant treatment has been shown to decrease the amount of 5-HT in the rodent brain (Bosker et al 2010, Marsteller et al 2007, Siesser et al 2013). Considering the significant lack of therapeutic efficacy and slow onset that are characteristic of conventional antidepressants, it is important that more efficacious, faster-acting, and longer-lasting antidepressant compounds are developed.

#### 1.2.2.2 The neurogenic hypothesis of depression

As we continued to study the depressed brain, we came to notice certain characteristics common among them. One attribute of depression that is observed consistently is a reduction in hippocampal volume, which correlates with illness duration, severity, and executive dysfunction (Bremner et al 2000, Campbell et al 2004, Frodl et al 2006, Kronmüller et al 2009, MacMaster & Kusumakar 2004, MacQueen et al 2003, Videbech & Ravnkilde 2004). Interestingly, the SGZ of the hippocampal DG is one of two neurogenic niches in the adult brain (the other being the subventricular zone of the lateral ventricles), where adult-born cells are produced that appear to play a role in mood, learning, memory, and forgetting (Kang et al 2016, Scott et al 2021, Yau et al 2015b). Although we cannot causally test the involvement of neurogenesis in human patients for obvious reasons, preclinical evidence suggests that stress impairs hippocampal adult-born cell proliferation and the maturation rate of surviving cells, which could contribute to the volumetric reductions in depression (Brummelte & Galea 2010b, Eisch & Petrik 2012, Mayer et al 2006, Murray et al 2008, Santarelli et al 2003). Limbic regions are rich in glucocorticoid receptors (GR) which makes them particularly susceptible to stress-induced impairments (Herman et al 2005). The dampening of cell proliferation and dendritic complexity by stress may invoke a depression-like phenotype by attenuating hippocampal cell connectivity and function (Bessa et al 2009, Lussier et al 2013a). In fact, the hippocampus acts to inhibit the stress response through a negative feedback system, so a weakening of hippocampal circuitry may partially explain why hypercortisolemia is commonly observed in patients (Pariante 2003, Pariante & Miller 2001). Here we discuss the literature surrounding the role of neurogenesis in depression; the stages, regulators, and functions of neurogenesis will be discussed in more detail after a review of hippocampal anatomy and circuitry, in Section 1.8.

Many research groups have aimed to understand the role of adult-born granule cells in the regulation of mood by manipulating neurogenic processes. For instance, hippocampal irradiation or the genetic ablation of glial fibrillary acidic protein-positive neural progenitor cells can generate behavioral deficits and impair synaptic plasticity (Saxe et al 2006), while augmenting neurogenesis can ameliorate anhedonia- and despair-like behaviors induced by exposure to chronic CORT administration or unpredictable mild stress (CUMS) (Eliwa et al 2021, Hill et al 2015). That said, the involvement of neurogenesis in depression and antidepressant responsiveness is inconclusive and there could be methodological limitations in

studies that aim to inhibit neurogenesis, like the possibility of damaging surrounding cells. By subjecting rats to daily CORT injections for 1, 2, or 3 weeks, our laboratory has demonstrated that the progressive development of depressive-like behavior is paralleled by reductions in new-born DG granule cells, slowed neuronal maturation, and decreases in neurogenesis-promoting extracellular protein reelin (Lussier et al 2013a). Furthermore, we found that conventional antidepressants, anti-inflammatory agents, ketamine, and intrahippocampal reelin infusions can protect from this decrease and rescue depressive-like behavior (Brymer et al 2018, 2020, Fenton et al 2015, Johnston et al 2020). Other researchers have found similar results in rodents (Anacker et al 2011, Banasr et al 2006, Dagyte et al 2010, David et al 2009, Nandam et al 2007), bonnet macaques (Perera et al 2011), and human post-mortem tissue (Boldrini et al 2009). Some studies even suggest that hippocampal neurogenesis is essential for antidepressant efficacy (Santarelli et al 2003). In support of the neurogenic hypothesis, electroconvulsive therapy (ECT) stimulates cell proliferation nearly 2-fold that of pharmacological agents in rats and it is often considered the most effective treatment in patients that are unresponsive to pharmacotherapy (Madsen et al 2000, Pagnin et al 2004).

Antidepressants may induce neurogenesis by influencing levels of things like second messenger cyclic adenosine monophosphate (cAMP) and brain-derived neurotrophic factor (BDNF), although there are many important molecules that are involved in signal transduction cascades that regulate neurogenesis and probably emotion in some way (Nakagawa et al 2002, Vithlani et al 2013). cAMP goes on to activate protein kinase A (PKA) which occurs downstream of $G_s$-coupled protein activation. BDNF binds to the tropomyosin receptor kinase B (TrkB) which promotes the survival of cells and synapses, their growth, and the generation of new neurons and their differentiation at neurogenic sites (Huang & Reichardt 2003, Ortiz-López et al 2017). Growth factors like BDNF are best thought of as brain fertilizer. Interestingly, mice who lack TrkB in hippocampal neural progenitor cells were behaviorally insensitive to fluoxetine and imipramine (Li et al 2008). The idea that a disruption in neuronal growth contributes to hippocampal volume loss opens a new way to conceptualize how depression manifests. For these reasons, elevating levels of BDNF is the focus of some therapies and is achieved in the DG by conventional antidepressants, stimulation techniques like ECT, and exercise (Chen et al 2001, Deng et al 2016, Higgins & George, 2013). This is a remarkable discovery considering that it may explain how different treatment approaches bring about similar antidepressant actions.

Findings like these led to the hypothesis that there is a temporal relationship between the therapeutic delay of antidepressants and their ability to increase neurogenesis. This was exemplified in rats that were treated with fluoxetine or placebo; after several days of treatment, there were no differences in new-born cell proliferation or behavior between treatment conditions, but after a month, fluoxetine increased neurogenesis which was paralleled by anxiolytic-like behavioral responses (Santarelli et al 2003). However, although there is enough evidence to confidently state that neurogenesis is involved in at least some depressions, none of it is causative (Schoenfeld & Cameron 2015). A major discrepancy regarding neurogenesis is that the presence of newly generated cells in the human adult is still largely debated, with some researchers arguing that neurogenesis drops sharply after childhood to undetectable levels in adulthood (Sorrells et al 2018), while others are arguing that new-born cells can be detected in the elderly, at least in those that are healthy (Boldrini et al 2018). Another discrepancy that cannot be ignored is that numerous post-mortem studies found no decrease in hippocampal cell proliferation or cell loss in depressed patients compared to controls (Lucassen et al 2010, Muller et al 2001, Reif et al 2006, Stockmeier et al 2004). Preclinical studies have also indicated that the elimination of hippocampal neurogenesis has no effect on the sensitivity to unpredictable stress in mice (Surget et al 2008), nor did it cause depressive- or anxiety-like behavior (Santarelli et al 2003), or abolish the therapeutic efficacy of antidepressants (Bessa et al 2009, David et al 2009). Similarly, exercise increases neurogenesis but refraining from strenuous activity does not cause depression (Trinchero et al 2019, Yau et al 2011). Proper neurogenesis may contribute to normal emotional regulation and hippocampal-dependent cognition rather than protect one from depression, per se (Airan et al 2007, David et al 2009, Eliwa et al 2021, Holick et al 2008). Therefore, it is best to think of reductions in neurogenesis and overall hippocampal volume as a neural scar of depression that serves as an indicator of future episode vulnerability. There is still a tremendous amount of work to be done to better understand the importance of adult-born cells in the regulation of mood, memory, cognitive ability, and executive functions.

#### 1.2.2.3 The neuroplasticity hypothesis of depression

There are an estimated 86 billion neurons in the human brain that communicate with one another using 100 trillion synapses (Herculano-Houzel 2009). Each neuron has an average of around 7000 synaptic connections but some can have up to 20000, and it has been postulated

that any neuron can innervate another neuron by signaling through no more than 6 interneuronal connections (Drachman 2005). The neurons that make up these vast and complex networks are continually forming, eliminating, and modulating their synaptic connections that allow cell-cell communication. This is referred to as neural plasticity, which includes structural and functional neuronal changes that occur in response to intrinsic and extrinsic stimuli such as the chemical perception of reward (via the mesolimbic pathway) or bonding (in response to oxytocin), cellular damage, exercise, playing an instrument, and dangerous encounters. The brain is essentially the most intricate and complicated information processor known to man, that modifies in response to experiences to "store" memories, generate emotions, and choreograph behavior. In other words, neuroplasticity is an umbrella term for the adaptability of the malleable or "plastic" brain to experience, and the neuroplasticity hypothesis of depression posits that symptoms emerge when the neural mechanisms that govern brain plasticity become dysfunctional (Liu et al 2017). There are many studies that support this claim. Rodents that are exposed to chronic stress consistently exhibit dendritic atrophy, a loss of dendritic spines, and slowed cell maturation (Bessa et al 2009, Brymer et al 2020, Fenton et al 2015, Galea et al 1997, Shors et al 2001, Watanabe et al 1992). This is especially true in the hippocampus, where stress reduces dendritic arborization, cell proliferation, and long-term potentiation (LTP) – arguably one of the most studied forms of plasticity (Chen et al 2008, Kim & Diamond 2002, Lussier et al 2013a, McEwen et al 2016, Xu et al 1997).

Glutamatergic transmission is essential for the modulation of synaptic strength. When glutamate binds to its receptors, and the cell depolarizes, a magnesium block is expelled from the channel of NMDARs which allows the influx of calcium ions. Calcium can activate CaMKII which is accepted as a requisite trigger for LTP – albeit like many other biological phenomena – that increases the insertion of excitatory AMPARs in the synapse and their conductance (Coultrap & Bayer 2012, Robison 2014). Receptors that bind the inhibitory neurotransmitter GABA must also play a role in neuroplasticity considering that they ensure neuronal hyperexcitability does not occur. For instance, heightened $GABA_A$ receptor ($GABA_{subtype}R$) activity was associated with a reduction in dendritic spine maturation and levels of postsynaptic density-95 (PSD-95) in rat hippocampal cultures (Jacob et al 2009), but it is difficult to understand the consequences of GABAergic transmission in vivo. This is because $GABA_AR$s (ionotropic) and $GABA_BR$s (coupled to $G_i$-proteins) on GABAergic neurons can dampen inhibitory transmission (disinhibition) and excite principal neuronal populations (Vashchinkina et al 2014). This explains why their antagonism can block the

induction of LTP (Davies et al 1991, Volianskis et al 2015). Interestingly, newly generated adult-born cells are innervated exclusively by GABAergic neurons before other transmitter inputs, and GABA has a depolarizing effect on immature neurons (Song et al 2021). Thus, GABA will inhibit nearby mature granule cells while directing neurotransmission through new-born cells which facilitates their maturation (Markwardt & Overstreet-Wadiche 2008). It is therefore important that the balance in excitatory and inhibitory neurotransmission is efficiently regulated to maintain proper neuroadaptation.

According to the preclinical literature, chronic psychosocial stress reduces levels of both CaMKII and synaptic strengthener protein kinase C (PKC), which is activated downstream of $G_q$-protein coupled receptors (Gerges et al 2003, 2004). Examples of receptors coupled to $G_q$ are the 5-$HT_{2A}$, α1-adrenergic, metabotropic glutamate (group 1), and $M_{1/3/5}$ muscarinic receptors. Other regulators of synaptic plasticity that are dysfunctional in depression are cAMP-dependent PKA which is activated downstream of $G_s$-protein coupled receptors (e.g. 5-$HT_4$ and 5-$HT_7$, β-adrenergic, cannabinoid 2, and dopaminergic D1-like receptors), mitogen-activated protein kinase (MAPK), and tyrosine kinases (Boxall et al 1996, Nguyen & Woo 2003, Pearson et al 2001, Wang & Mao 2019). For example, PKC activates synaptic growth factors like tropomyosin receptor kinase A (TrkA) and TrkB, and potentiates synaptic maturation by accumulating PSD-95 in nerve endings which can be phosphorylated by PKC on serine residues (Sen et al 2016). A lot of attention has focused on the role of neurotrophic signaling (e.g., BDNF-TrkB) in neuroplasticity and depressed patients, who express low circulating levels of BDNF that correlates with HDRS scores and can be reversed by antidepressant treatments (Gonul et al 2005, Lee et al 2007, Shimizu et al 2003, Yoshida et al 2012). Decreased BDNF expression is also observed in the post-mortem hippocampus and PFC from individuals who died by suicide compared to controls (Karege et al 2005). In addition, stressed animals exhibit reductions in neurotrophin expression, like rats who were restrained for a couple of hours, exposed to foot shocks, early maternal separation, social defeat stress, or CORT injections (Pizarro et al 2004, Rasmusson et al 2002, Roceri et al 2002, Schaaf et al 1998). TrkB stimulation leads to increases in synaptic plasticity, neuroprotection, and axonal growth through phospholipase C, PI3K-Akt, and MAPK/ERK pathways, respectively (Deng et al 2016). This explains why TrkB activation leads to proportional changes in cell proliferation in the striatum, septum, thalamus, and hypothalamus after intraventricular infusions of BDNF (Pencea et al 2001). Midbrain infusions of BDNF also rescues FST-immobility and learned helplessness behavior in rat models for depression (Siuciak et al 1997).

These studies provide direct evidence that deficient brain plasticity is related to depressive behavior.

Another regulator of neuroplasticity that has recently received considerable attention is mTOR. This kinase is modulated downstream of glutamatergic neurotransmission, growth factors, hormones, cytokines, and extracellular proteins like reelin (Brymer et al 2020, Perl 2016, Seo et al 2020). Its activation is associated with cell growth, proliferation, motility, survival, and the translation and turnover of synaptic proteins (Zarogoulidis et al 2014). Interestingly, preclinical studies suggest that the rapid antidepressant effects of ketamine that are observed in treatment-resistant patients (Berman et al 2000) rely on the activation of mTOR that occurs downstream of AMPAR-mediated transmission and TrkB activity (Li et al 2010, Maeng et al 2008). Similarly, in vitro studies demonstrate that an hour-long incubation with ketamine can increase dendritic branching and soma size in cultured mouse mesencephalic neurons and human induced pluripotent stem cells derived from dopaminergic neurons, but not when AMPARs, mTOR, TrkB, or $G_i$-coupled dopamine receptor 3 signaling is blocked (Cavalleri et al 2018). A well, ketamine increases the levels of BDNF in the amygdala and pERK in the PFC and hippocampus (Zhang et al 2019a). Other compounds that increase glutamatergic activity like mGlu2/3R antagonists and negative modulators of $GABA_A$Rs – that are enriched in the hippocampus and PFC – also increase neuroplasticity and synaptic strength (Fischell et al 2015, Seo et al 2020). As well as ketamine, the persistent antidepressant effects of "mind manifesting" psilocybin and LSD are associated with structural and functional neuronal plasticity via TrkB and mTOR, corroborating their necessity for the alleviation of psychiatric symptoms (Ly et al 2018). Therefore, it seems likely that the rapid amelioration of synaptic plasticity in brain areas that regulate mood, cognition, and reward by ketamine and other "psychoplastogens" is essential for their timely behavioral changes (Autry et al 2011, Li et al 2010, Ly et al 2021, Olson 2018). However, although there are many studies that correlate depression with deficient neuroplasticity, and suggest that they act on and influence one another, we have yet to determine the initial events that cause depression (Liu et al 2017). This work is interesting to our laboratory because we have shown that the neuroplasticity-enhancing protein reelin requires AMPARs and increases mTOR activity to generate quick behavioral changes, and that reelin deficits may underlie the deleterious effects of stress on behavior, neurogenesis, and synaptic plasticity (Brymer et al 2020, Caruncho et al 2016, Johnston et al 2020). This opens the possibility of developing reelin-based compounds that may potentially provide antidepressant relief without the abuse potential and hallucinogenic properties of these

unconventional but promising treatments. Indeed, one could make a strong argument that the enhancement of neuroplasticity provides an explanatory mechanism as to how disparate modes of treatment (e.g., exercise, SSRIs, ketamine, ECT) produce antidepressant effects.

## 1.3 Hypothalamic-pituitary-adrenal-axis signaling and depression

Stress is considered a significant risk factor for the onset of MDD, so exposing animals to stress forms the basis of many animal models. The perception of physical or psychological stress stimulates a cascade of neuroendocrinological events that are referred to as the hypothalamic-pituitary-adrenal (HPA)-axis. The HPA-axis is necessary for the perception of threatening stimuli and the subsequent behavioral adaptation, and it influences biological processes like inflammation, emotionality, homeostasis, digestion, neuroplasticity, and the storage and expenditure of energy. The stress response begins with the secretion of corticotropin-releasing factor (CRF; used interchangeably with corticotropin-releasing hormone) from axonal terminals in the hypothalamic PVN, which triggers the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into circulation. ACTH acts on steroidogenic cells in the adrenal cortex zona fasciculata to stimulate the synthesis of glucocorticoids like cortisol (this cascade of events is depicted in Figure 1.3). Cortisol is the primary stress hormone in humans (analogous of CORT in rodents) that is released within minutes after synthesis because it is not stored in vesicles (Gjerstad et al 2018). In times of stress, cortisol limits non-essential processes like digestive, reproductive, and inflammatory functions. This quickly mobilizes energy to muscles that are used to escape immediate threats (French et al 2007). In humans, cortisol levels rise as we wake up and decrease in the evening, a cycle that is controlled by the hypothalamic suprachiasmatic nucleus (Adam et al 2010, Kalsbeek et al 2012). For rodents, CORT levels are highest at night when they are more active (Watts et al 2004). The HPA-axis is activated by the fear-registering amygdala and inhibited by the higher-level thinking PFC and memory-consolidating hippocampus (Herman & Cullinan 1997). Pro-inflammatory cytokines can also induce stress hormone release which regulates immune responses such as the inhibition of microglial functions (Silverman et al 2005, Sugama & Kakinuma 2020).

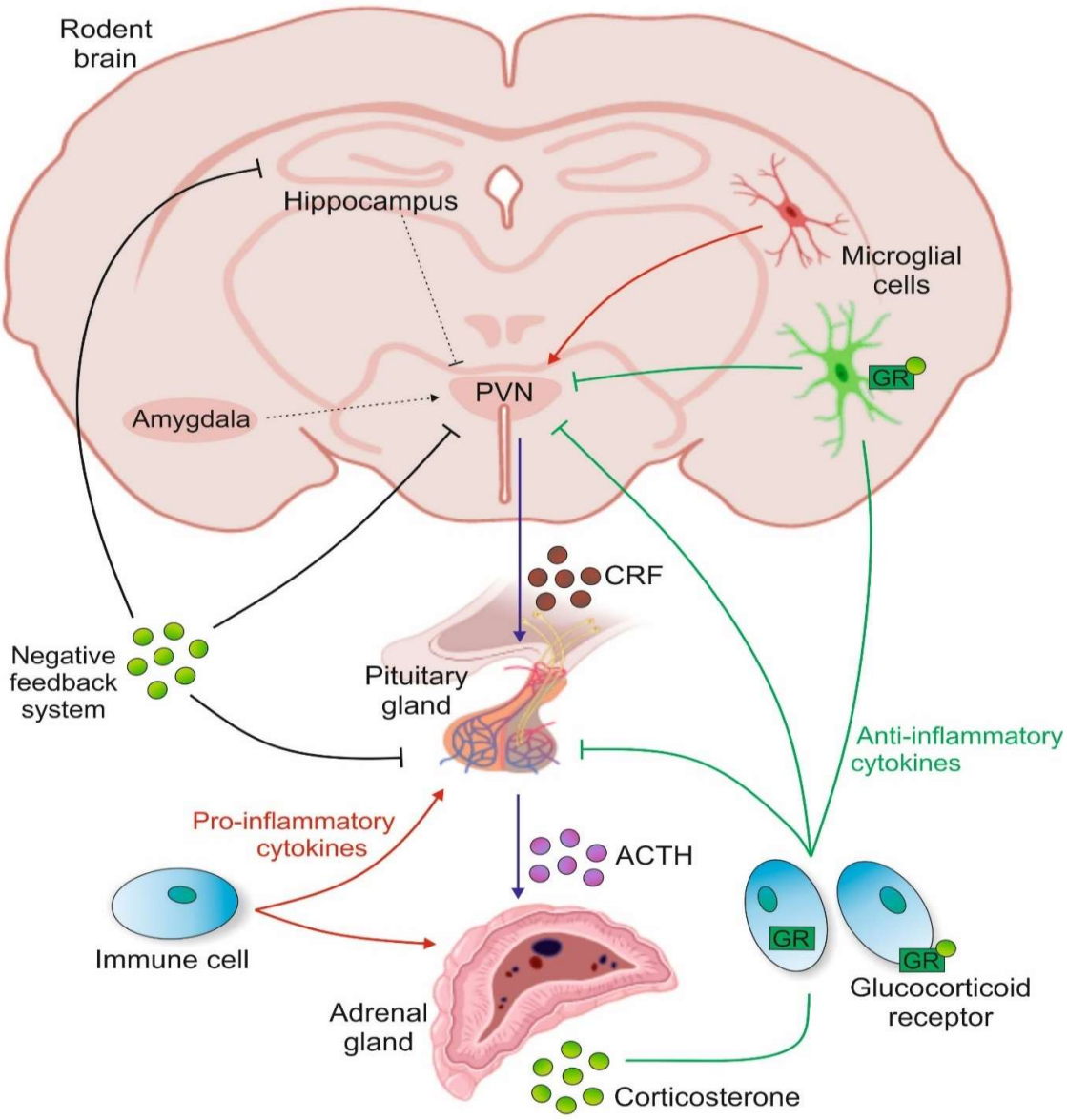


**Figure 1.3. The hypothalamic-pituitary-adrenal axis and its interactions with the immune system.** In response to stress, the PVN secretes CRF which targets the anterior pituitary gland which in turn releases ACTH. This stimulates the release of CORT from the adrenal cortex into circulation. CORT can diffuse through the blood-brain barrier and bind to GRs in the cytosol of target cells that increase the production of anti-inflammatory cytokines and activate a negative feedback system, but chronic stress can desensitize GRs or reduce their number which dampens their inhibition of the stress response and elevates pro-inflammatory events. For instance, hypercortisolemia is observed in approximately 60% of depression patients and is associated with increased TNF-α concentrations which is primarily secreted by microglial cells. Figure created using BioRender by author.

Stress hormones can easily pass through cellular membranes to the cytosol where they bind to mineralocorticoid receptors (MR) with high affinity and GR with 10-times less affinity, which are cytosolic ligand-activated transcription factors (De Kloet et al 1998, Koning et al 2019). This means that MRs are activated at basal hormone levels (resting state) whereas GRs are activated after bouts of stress or when cortisol levels peak over the circadian rhythm (Reul et al 1987). MRs are expressed in many peripheral tissues like the kidneys, heart, sweat glands, and colon, as well as limbic regions of the brain, and GRs are present in nearly all human cells but are particularly abundant in the hippocampus, amygdala, and PFC (de Kloet et al 2005, Reul & de Kloet 1985). When activated, the glucocorticoid-receptor complex translocates to the cell nucleus where they bind to specific DNA response elements, leading to the transcription of anti-inflammatory proteins (Oakley et al 2019). On the other hand, the complex can remain in the cytosol and suppress the translocation of other transcription factors that are considered pro-inflammatory, like nuclear factor-κB (Koo et al 2010, Necela & Cidlowski 2004). Investigations in rodents have also discriminated 2 feedback responses with temporal distinctions. Nongenomic mechanisms that work over seconds are initiated when glucocorticoid concentrations reach a certain level and activate ventral hippocampal projections that synapse with GABAergic interneurons that inhibit PVN-CRF release (De Kloet et al 1998, Gustavo & Charles 2016). Some evidence also suggests that cannabinoid receptors are required for CORT to inhibit glutamatergic inputs to PVN parvocellular neurons (Tasker et al 2006). Genomic mechanisms are delayed by hours to days and involve lowering the transcription of CRF in the PVN and the precursor of ACTH (proopiomelanocortin) in the pituitary (Gjerstad et al 2018, McEwen 2007).

Activation of the HPA-axis under conditions of acute stress is essential to generate appropriate fight-ot-flight responses, but HPA-axis overaction can become problematic. This is because a downregulation and desensitization of GRs occurs, which disrupts many biological processes, including the HPA-axis negative feedback system (De Kloet et al 1998). This leads to an accumulation of glucocorticoids and the jeopardization of their anti-inflammatory signaling potential. In fact, what follows chronic stress is a remodeling of the brain; dendritic hypertrophy is observed in the amygdala whereas retraction of dendritic arbors and neuronal death occurs in the hippocampus and PFC (McEwen 2005, McEwen & Morrison 2013, Mitra & Sapolsky 2008, Sapolsky 2000). As well, post-mortem studies have shown that patients with MDD have a larger number of neurons in the PVN which drives HPA-axis activity, but it is unclear if the density of PVN neurons increases in response to chronic stress or because of

genetic reasons (Higgins & George, 2013). Additionally, rodent studies indicate that hypercortisolemia is associated with reductions in neurogenesis and slowed cell maturation (Brymer et al 2018, Fuchs & Gould 2000, Lussier et al 2013a). Therefore, it is likely that HPA-axis dysfunction contributes to the hippocampal volumetric reductions that are consistently observed in MDD patients (Bremner et al 2000, Kronmüller et al 2009, MacMaster & Kusumakar 2004, Videbech & Ravnkilde 2004).

Chronic stress is a well-established trigger for depression, exemplified by the fact that depressive episodes commonly follow stressful life events, like grief or divorce (Kessing et al 2004). In fact, abnormally high plasma cortisol levels were noticed in those with MDD over half a century ago and has since been replicated numerous times (Gibbons & McHugh 1962, Heim et al 2008, Pariante & Lightman 2008, Varghese & Brown 2001). These findings extend beyond cortisol to ACTH and CRF in the brain, cerebrospinal fluid, and blood (Banki et al 1987, Carroll et al 2007, Hartline et al 1996, Nemeroff et al 1984, Nestler et al 2002). In the PFC of suicide victims, higher levels of CRF and lower levels of mRNA for CRF's receptors were recorded, which may represent a compensatory mechanism (Merali et al 2004). The amount of cortisol that is secreted upon waking is also increased by psychosocial stress, which may represent a premorbid risk factor for depression (Adam et al 2010, Dreger et al 2010). In addition, exposing rodents to stress increases their plasma concentrations of CORT (Li et al 2008, McEwen 2007), and injecting them with CORT for prolonged periods of time produces depressive-like behavior (Brymer et al 2020, David et al 2009, Gregus et al 2005, Lebedeva et al 2020, Murray et al 2008, Romay-Tallon et al 2015, 2018). Another study found a relationship between levels of cortisol in the evening and the severity of depression, but not cortisol levels upon waking or during the day (Van den Bergh & Van Calster 2009). In the preclinical and clinical literature, there are no convincing relationships between cortisol levels and responsiveness to treatment (Nandam et al 2020). That said, antidepressants and ECT normalize HPA-axis dysfunction, supposedly by increasing GRs that generate inhibitory feedback responses (Du & Pang 2015, Holsboer 2001, Mason & Pariante 2006, Pariante 2003, Pariante & Lightman 2008). HPA-axis overactivity can be downregulated by CRF receptor blockers, but the results are inconsistent, and these treatments are associated with hepatoxicity (Higgins & George, 2013). In rodents, chronic but not acute treatment with citalopram protected from increases in CORT and ACTH levels induced by restraint stress (Hesketh et al 2005). Likewise, chronic antidepressant treatment decreased CRF levels and normalized GR density (Barden 2004), suggesting that antidepressants may stimulate GR gene transcription

(Heiske et al 2003). However, it is unlikely that hypercortisolemia (which is seen in only 60% of depressed patients) directly causes depression (Parker et al 2003), but it could disrupt other critical processes, such as neuronal malleability.

The dexamethasone (synthetic glucocorticoid) suppression test (DST) is often used to measure adrenal gland function and is used to aid in the diagnosis of Cushing's syndrome, which is characterized by elevated levels of glucocorticoids and commonly co-occurs with depression (Sonino & Fava 2002). In healthy individuals, dexamethasone will bind to GRs in the pituitary and suppress the release of ACTH, dampening the secretion of cortisol. In patients with depression who exhibit a downregulation of GRs, however, this negative feedback is altered (De Kloet et al 1998, Yokoyama et al 2015). This is important as prolonged stress can suppress neurogenesis, cause dendritic atrophy, compromise cell survival, imbalance neurotransmitter systems, and reduce spine density, neurotrophic signaling, and cell adhesion molecules (Jauregui-Huerta et al 2010). While the DST has generally proven unsuccessful as a predictive biomarker for depression, DST-informed hypercortisolemia has been associated with enhanced suicidal ideation, melancholia, psychotic symptoms, and early episode relapse (Carroll et al 2007, Fountoulakis et al 2008). All in all, depression is consistently paired with chronic stress and altered stress sensitivity, which provides a rationale to use stress-based animal models to further our understanding of human conditions.

### 1.4 Inflammation, apoptosis, and depression

Chronic stress is known to stimulate inflammation (the body's method of fighting against infection, toxins, and injuries) which can influence emotionality and cognition, and inflammation contributes to depression, in at least some patients (Maydych 2019). The relationship between MDD and inflammation is bidirectional; ⅓ of MDD patients have higher levels of pro-inflammatory cytokines in circulation compared to healthy controls (Dahl et al 2014, Dowlati et al 2010, Howren et al 2009), and those who receive cytokine-based therapies or have been diagnosed with an immunological disorder are more likely to suffer from depression (Bachen et al 2009, Capuron et al 2002, Dickens et al 2002). Furthermore, prolonged inflammation can generate a constellation of symptoms referred to as "sickness behavior", characterized by anhedonia, anti-social behavior, a loss of appetite, and insomnia/hypersomnia (Dantzer et al 2008, Krishnadas & Cavanagh 2012). Sickness behavior

closely resembles depression and decreases well-being even in mentally healthy individuals (Fancourt & Steptoe 2020). Interestingly, but perhaps unsurprisingly, longitudinal studies have revealed that heightened plasma pro-inflammatory cytokine concentrations are often noted before one experiences depressive episodes, indicating that immune system dysregulations may represent a vulnerability factor or even trigger symptoms in certain patients (Khandaker et al 2014). In line with this, antidepressants reduce inflammation and anti-inflammatory drugs can improve mood (Brymer et al 2019, Hannestad et al 2011, Köhler et al 2014). That said, we are yet to determine whether causality exists between the correlative nature of inflammation and depression. Therefore, it is unclear how inflammatory processes influence episode onset or relapse, but the relationship presents as a vicious cycle whereby inflammation increases one's susceptibility to mood disturbances and vice versa.

Inflammatory cells include lymphocytes (B-cells, T-cells, and natural killer (NK) cells), monocytes/macrophages, and microglia (that reside in the brain). B-cells develop in bone marrow and then migrate to areas like the spleen and lymph nodes where they await activation by antigens, often with the help of T-cells (Harwood & Batista 2010, Murphy et al 2012). T-cells are born in bone marrow but mature in the thymus, from where their name is derived. There are several types of T-cells: those that are cytotoxic like NK T-cells which destroy infected cells; helper cells which aid B-cell maturation and choreograph the killing of cells by orchestrating other immune functions; regulatory cells that detect cells as foreign or "not self" and provide immune tolerance; and memory cells which augment the immune response after the detection of their cognate antigen which they "remember" (Roberts 2015). NK cells are essential for innate immunity and are so-called because they require no activation to kill foreign invaders (Vivier et al 2011). Macrophages are phagocytic scavengers that protect the body by engulfing and digesting microbes, cancer cells, foreign substances, and other cellular debris. Also in the repertoire of these versatile cells is the production of cytokines (Roberts 2015). Microglia make up 10-15% of the cellular population in the brain and share these qualities (Lawson et al 1992). They scavenge the brain for plaques and neurons that are no longer deemed worthy, among other agents like those that are infectious in nature (Gehrmann et al 1995). Much work is needed to understand how these different cell types communicate with one another to achieve their goal under changing conditions, and how their dysfunction contributes to mood disturbances.

Inflammatory molecules are pleiotropic messengers that can further promote neuroinflammation. For instance, high levels of pro-inflammatory cytokines can disrupt

astrocytic and microglial functions, excitatory transmission, the integrity of the blood-brain barrier, stress reactivity, apoptosis, and neuroplasticity (Lugo-Huitrón et al 2013, Schwarcz et al 2012, Šťastný et al 2000, Yirmiya & Goshen 2011). Cytokines can also induce cortisol release, dose- and time-dependently increase the production of intra- and extra-cellular harmful oxidative molecules (referred to as reactive oxygen species, ROS), and like cortisol and ROS, lower 5-HT levels by activating tryptophan-degrading enzymes that produce neurotoxic metabolites (Anderson & Maes 2017, Badawy 2018, Capuron et al 2002, Chesnokova & Melmed 2002, Mándi & Vécsei 2012, Musso et al 1994, Yang et al 2007, Yirmiya & Goshen 2011). In response to stress and inflammation, microglia release TNF-α which contributes to the development of depressive-like behavior and cell death, and drugs that suppress TNF-α activity can attenuate these phenomena (Belarbi et al 2012, Kaster et al 2012, Liu et al 2004, Souza et al 2013). Our laboratory corroborated this by demonstrating that TNF-α inhibitor etanercept, which cannot cross the blood-brain barrier, rescued neuroplastic insults that follow chronic stress, like alterations in the expression of hippocampal reelin, and GABA and glutamate receptors (Brymer et al 2018, 2019).

Prolonged inflammation can not only potentiate symptom severity but attenuate treatment responsiveness (Haroon et al 2012, Krishnadas & Cavanagh 2012). Inflammatory mediators can access the brain and have the potential to be used as biomarkers, but unfortunately abnormal cytokine levels are not specific to depression, and several antidepressants increase their expression rather than decrease (Lichtblau et al 2013). Extensive research efforts are required to determine if the analysis of cytokine profiles could distinguish MDD from other disorders that present with similar symptoms, like generalized anxiety (Martin et al 2015). Perhaps a combination of pro- and anti-inflammatory cytokine patterns could predict responsiveness to pharmacological agents. While this seems a long way off, our laboratory has found that the size of SERT clusters in lipid raft microdomains on the membrane of peripheral lymphocytes is increased in treatment-naïve depression patients and that the distribution pattern of SERT clusters could differentiate between responders and non-responders (Rivera-Baltanas et al 2012). To be more specific, both groups had similar HDRS and self-assessed anhedonia scores before treatment, but 100% of those who had larger clusters (and therefore a smaller percentage of clusters in the modal peak range of 0.05 to 0.1μm) responded (75% reaching remission) while those with a larger percentage of clusters in the modal range responded much worse (45% responders, 22% reaching remission). The clustering of other neurotransmitter-associated proteins in lymphocytes are also dysregulated, implying that depressed patients have

alterations in these cells that extend beyond the 5-HTergic system. In fact, MDD patients and CORT-treated rats show similar patterns of alterations in SERT and 5-$HT_{2A}$R MPC (Rivera-Baltanas et al 2014), but CORT also altered the clustering of the dopamine transporter, GluN2B subunit-containing NMDARs, Pannexin 1, and prion cellular protein (Romay-Tallon et al 2017, 2018). This suggests that a CORT-administration model can be used to screen biomarkers for depression in a bench-to-bedside-back-to-bench manner and that SERT and 5-$HT_{2A}$R clustering may be putative biomarkers for therapeutic efficacy in MDD (Caruncho et al 2019, Rivera-Baltanas et al 2015). Reelin-deficient mice, which display abnormal cytokine levels and are more susceptible to the depressogenic effect of stress (Green-Johnson et al 1995, Lussier et al 2011), also express altered MPC for SERT (Rivera-Baltanas et al 2010), and recombinant reelin normalized SERT MPC in lymphocytes that were taken from CORT-treated rats, while ketamine had the opposite effect (Johnston et al 2020). Interestingly, the redistribution of proteins in lipid rafts, such as $G_{\alpha}$ protein, is thought to be critical for the responsiveness to antidepressants (Allen et al 2007, Czysz et al 2015, Donati & Rasenick 2005, Erb et al 2016, Zhang & Rasenick 2010). Considering that the inclusion of receptors and transporters into lipid microdomains is essential for neurotransmitter activity and reuptake (Magnani et al 2004), one can assume that the normalization of MPC in lymphocytes would improve their functioning and balance neurotransmission and inflammation overall.

Accumulating evidence suggests that stress and inflammation increase the production of ROS, which damage cellular components, alter gene expression, and induce cytokine release, ultimately potentiating cellular allostatic load and apoptosis (Allen et al 2021a, Guo et al 2019, Sakami et al 2002). Incidentally, reelin may be involved in inflammation and oxidation beyond the regulation of MPC; we found that mice with low levels of reelin had a decreased percentage of cells that co-express reelin and nitric oxide synthase (NOS) in the DG compared to wild-type mice, but that chronic stress decreased and increased the co-expression in wild-type and reelin-deficient mice, respectively (Romay-Tallon et al 2010). This implies that oxidative events are more likely to occur in CORT-treated rats and depressed patients who have lower reelin levels (Fatemi 2011, Lebedeva et al 2020). GR-expressing mitochondria are the primary source of ROS and their membrane polarity can become compromised after periods of oxidative stress (Gao et al 2007). This can occur when high levels of ROS overwhelm antioxidant defenses and eventually lead to the opening of pores on the mitochondrial membranes that results in the release of CytC, which triggers apoptosis (Allen et al 2021b, Brookes et al 2000). Inflammation can be exacerbated if components of the mitochondria leak,

such as mitochondrial DNA, because they are detected by the body as foreign – perhaps because mitochondria originated from bacteria (Allen et al 2021a). Interestingly, those diagnosed with depression or an inflammatory disorder, or who had previously attempted suicide or recently experienced trauma, had higher levels of mitochondrial DNA in circulation and it correlated with HPA-axis hyperactivity (Boyapati et al 2017, Lam et al 2004, Lindqvist et al 2016, 2017, 2018, Nakahira et al 2013). CytC-induced apoptosis can also occur if Bcl-2-associated X protein (BAX) fulfils its goal and punctures the mitochondrial membrane, but not if it is inhibited by anti-apoptotic Bcl-2 which translocates to the mitochondria after forming a complex with activated GRs (Du et al 2009b). Chronic stress could potentiate inflammation and cell death by downregulating GRs and Bcl-2 and upregulating BAX (Du et al 2009b, Juárez-Rojas et al 2015). In fact, deleting *BAX* from neural stem cells in transgenic mice was shown to promote resilience to repeated-CORT treatment and unpredictable stress (Eliwa et al 2021, Hill et al 2015). Similarly, mice that overexpress Bcl-2 had double the number of surviving DCX-expressing cells than wild-type mice (Kuhn et al 2005). BAX is inhibited by the neuroplasticity-promoting PI3K-Akt pathway which can be activated by ketamine and reelin, and this may contribute to their antidepressant properties (Jossin & Goffinet 2007, Kale et al 2018, Li et al 2010, Takino et al 2019). Mitochondrial enzymes are also responsible for the production of steroid hormones (like testosterone, estrogen, progesterone, and cortisol), so their dysfunction would have a wide range of consequences for the organism. Given that cellular processes are very energy-demanding, stress/inflammation-induced mitochondrial dysfunction (such as that driven by BAX and oxidative stress) is receiving considerable experimental attention to explain deficiencies in neuroplasticity (Allen et al 2021a).

Although modern neuroscience conveys the implausibility that 5-HTergic hypoactivity is solely responsible for MDD, there is evidence that high levels of stress and inflammation can excessively break down tryptophan (the precursor for 5-HT) into kynurenine by activating indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase (Wirthgen et al 2018). Kynurenine is not neuroactive by itself, but it can be further processed into neuroprotective kynurenic acid in astrocytes or neurotoxic 3-hydroxykynurenine and quinolinic acid in microglia (Oxenkrug 2010, Savitz et al 2015a), and the degradation of kynurenine along the neurotoxic branch is superior to that of the neuroprotective branch in those with depression (Müller & Schwarz 2008). Indeed, patients with MDD or those at high risk of suicide were shown to have lower tryptophan availability and higher neurotoxic kynurenine metabolites than controls (Bradley et al 2015, Liu et al 2018, Myint et al 2007, Savitz et al 2015b), and

antidepressants can sometimes normalize the concentrations of these metabolites (Ogawa et al 2014, 2018). Quinolinic acid and 3-hydroxykynurenine can increase cellular apoptosis by impairing the synthesis of ATP and the integrity of mitochondrial membranes, and by increasing the production rate of ROS (Behan et al 1999, Guidetti & Schwarcz 1999, Lee et al 2004, Okuda et al 1996, Rodríguez-Martínez et al 2000). This may represent a direct mechanism whereby inflammation can cause cell damage, but inflammation may also bring about excitotoxic events by contributing to the overstimulation of NMDARs (Lugo-Huitrón et al 2013, Ribeiro et al 2006, Schwarcz et al 2012). This is because quinolinic acid competitively agonizes NMDARs that express GluN2A and GluN2B subunits (de Carvalho et al 1996), whereas kynurenic acid has antioxidant properties and protects from excitotoxicity by binding to the glycine B site of NMDARs, where it acts as a non-selective competitive antagonist (Hilmas et al 2001, Majláth et al 2016). Interestingly, fast-acting antidepressant ketamine was shown to protect from increases in quinolinic acid in mice that were subjected to an inflammatory insult, and levels of quinolinic and kynurenic acid may represent promising biomarkers of depression and responsiveness to ketamine (Liu et al 2018, Verdonk et al 2019). This highlights the importance of developing novel compounds to dampen NMDAR-driven excitotoxicity that may improve clinical symptoms more effectively than 5-HTergic medications in a larger percentage of patients. There are still large knowledge gaps shadowing the inner workings of the immune system, how its activation follows cellular processes, and how its prolonged activation creates a vicious, synergistic relationship with stress and depression. The following section will discuss glutamatergic transmission in more detail and how alterations in this system are pertinent to depression beyond the dysregulation of the immune system.

## 1.5 The involvement of glutamatergic transmission in depression

Glutamate is the major excitatory neurotransmitter in vertebrates and glutamatergic neurons make up more than half of excitatory neurons. The modulation of glutamatergic activity has recently received substantial attention as a novel approach to rapidly improve mood. This is compelling to our laboratory because reelin promotes glutamate receptor signaling in several ways (Weeber et al 2002, Beffert et al 2005), which will be discussed in Section 1.11. In neurons, glutamate is synthesized from glutamine by the mitochondrial enzyme glutaminase, and then packed into synaptic vesicles (by vesicular glutamate transporters) that are released

when the cell depolarizes. There are no enzymes that degrade glutamate in the extracellular space once it is released, like how MAO degrades 5-HT, so it is actively taken up by excitatory amino acid transporters that are present mainly on presynaptic neurons and astrocytes. Astrocytes use glutamine synthetase to actively convert glutamate to glutamine, which is trafficked into neurons and the cycle starts again. Therefore, neurons and glial cells must successfully coordinate their actions to maintain an optimal supply of glutamate, which cannot cross the blood-brain barrier.

Upon, release, glutamate targets receptors that are strategically placed on different cell types to generate a variety of responses that are dependent on receptor make-up and localization. Glutamate binds to 3 distinct subtypes of ionotropic receptors (kainite receptors, AMPARs, and NMDARs) that gate a depolarizing current (sodium and calcium ions) that passes through their receptor channels. Additionally, glutamate can activate slower-acting metabotropic receptors (of which there are 8 subtypes that belong to 1 of 3 groups termed mGluR I/II/III), and they are linked to G-proteins and second messenger systems. They are composed of 7 transmembrane domains and activate $G_q$ (mGluR1 and mGluR5 in group I) and $G_i$ (mGluR2 and mGluR3 in group II and mGluR4, mGluR6, mGluR7, and mGluR8 in group III) proteins that modulate neurotransmission (Niswender & Conn 2010). Kainate receptors have 5 subunits (GluK1-5) that are arranged into tetramers and they can increase or decrease excitatory transmission depending on whether they are situated on pre- or post-synaptic neurons, respectively (Contractor et al 2011). More is understood about the roles of AMPARs and NMDARs (which are homo- or hetero-tetrameric) in neurotransmission, synaptic plasticity, cognition, and depression, which will be discussed more thoroughly.

AMPARs are the main drivers of rapid excitatory neurotransmission and an increase in their activity brings about positive mood changes (Thompson et al 2015). They are made up of 4 subunits referred to as GluA1-4, and they are expressed ubiquitously throughout the brain and spinal cord. Each subunit has an extracellular glutamate binding domain that is connected by peptide links to 4 transmembranous domains, and a cytoplasmic C-terminal. In actuality, the second transmembrane domain does not cross the membrane but folds back on itself inside the membrane to the intracellular side (Hollmann et al 1994). The receptor pore or channel is formed by the intracellular transmembrane folds when 4 subunits come together. If 2 or more of the subunits have bound glutamate, the channel will open and allow a depolarizing current to flow into the cell, carried primarily by an influx of sodium ions. The affinity of glutamate to a particular subunit increases as more of the binding sites are occupied (Prieto & Wollmuth

2010). AMPAR-driven cellular depolarization clears a voltage-sensitive magnesium block from the pore of NMDARs and renders them permeable to calcium, which can activate things like CaMKII and increase the delivery of new AMPARs to the synapse and their opening probability (Banke et al 2000, Derkach et al 1999, Hayashi et al 2000). Calcium can also travel through AMPARs if they lack calcium-repellant GluA2 subunits, and the influx of calcium through AMPARs and NMDARs is essential for synaptic strengthening (Liu & Zukin 2007). Around 1ms after opening, AMPAR channels are thought to close by changing the orientation of the binding site (Armstrong et al 2006).

AMPARs are synthesized in the cell body of neurons and they travel along dendrites to their place in the synapse using cytoskeletal motor protein dynein, which transports cellular cargo along microtubules (Krugers et al 2010). The C-terminal sequence dictates how they interact with various cytoskeletal components; for instance, GluA1 interacts with SAP97 and GluA2-4 with PICK1, which traffic and anchor AMPARs, proving their importance for synaptic plasticity (Hirbec et al 2002). At the synapse, AMPARs do not bind with PSD-95 directly but interact via the protein stargazin (Bats et al 2007), and they can be recycled through endo- and exo-cytosic processes (Krugers et al 2010). The delivery of AMPARs to hippocampal synapses follows contextual learning, and studies suggest that they are critical for spatial working memory. This has been demonstrated with GluA1 knockout mice which exhibit significant disruptions in hippocampal LTP and memory tasks that are dependent on the hippocampus (Krugers et al 2010, Sanderson et al 2009, Sanderson et al 2010), where AMPARs are necessary for synapse unsilencing (Selcher et al 2012). GluA1 subunits can be phosphorylated by PKA, PKC, and CaMKII, which enhance AMPAR throughput by modulating channel kinetics, subunit composition, and their localization (Banke et al 2000, Derkach et al 1999, Du et al 2006, Hayashi et al 2000). On the other hand, the internalization of AMPARs can augment long term depression, a form of synaptic plasticity that must go hand-in-hand with LTP to selectively encode new information in response to stimuli (Massey & Bashir 2007). AMPAR dysfunction probably contributes to the learning and memory deficits that are observed in those with depression, who often report cognitive impairment that is difficult to treat (Richardson & Adams 2018).

In post-mortem tissue from depressed patients, mRNA levels for GluA1 and GluA3 were lower in the hippocampus and perirhinal cortex, which interconnects the hippocampus to other limbic regions to aid the formation of memories (Beneyto et al 2007, Duric et al 2013). This is also true in adolescent and aged rats that are subjected to chronic stress, and exposure to inescapable

or unpredictable mild stress lowers the levels of GluA1 in the nucleus accumbens and GluA1 phosphorylation in the dorsal hippocampus and medial PFC, whereas the opposite is observed in the amygdala and ventral hippocampus (Brymer et al 2020, Toth et al 2008, Caudal et al 2010, Kallarackal et al 2013). This is interesting as the dorsal hippocampus is thought to play a role in spatial memory, whereas the ventral hippocampus is more involved in mood regulation (Kheirbek & Hen 2011). Genetic knockout models, like GluA1-lacking mice, highlight the involvement of AMPARs in depression and contextual learning as they exhibit learned helplessness behavior and neurochemical deficits that are associated with mood dysregulation (Chourbaji et al 2008). Acute foot shocks also promptly reduce GluA2 expression at postsynaptic sites (Bonini et al 2016). Similarly, antagonizing AMPARs eliminates the antidepressant actions of fluoxetine, imipramine, and ketamine, which have AMPAR-upregulating effects in the hippocampus and PFC (Aleksandrova et al 2017, Koike & Chaki 2014).

These findings encouraged researchers to evaluate if potentiating AMPAR activity could successfully treat depression. Reports indicated that AMPAR positive allosteric modulators, or ampakines, as well as AMPA itself, dose-dependently rescued depression-relevant phenotypes in rodent paradigms (Akinfiresoye & Tizabi 2013, Du et al 2006, Li et al 2001, Lindholm et al 2012). AMPAR stimulation can elevate neurogenesis, dendritic arborization, and the magnitude of learning and memory; but clinical trials with ampakines have yielded disappointing results, which could be because they have suboptimal pharmacological profiles (Arai & Kessler 2007, Bernard et al 2019, Fumagalli et al 2012, Mendez-David et al 2017, Nations et al 2012, Su et al 2009). Nevertheless, the essentialness of AMPAR activity for various forms of neuroplasticity is well documented and implicates NMDARs, which can strengthen synaptic connections when their agonism coincides with AMPAR-driven cellular depolarization.

NMDARs are also tetrameric and can be composed of various arrangements of 7 subunits, named GluN1/2A/2B/2C/2D/3A/3B. They are usually made up of 2 GluN1 subunits and 2 GluN2 or GluN3 subunits, which have multiple slice variants (Vyklicky et al 2014). Neurons have a larger excess of NR1 subunits as monomers that are waiting to bind GluN2 subunits when they become available, otherwise, they will degrade (Wenthold et al 2003). NMDARs that are made up of GluN1/GluN2 subunits open only when 2 glycine (GluN1/GluN3-targeting and 2 glutamate (GluN2-targeting) molecules are bound to their respective extracellular sites; those made up of GluN1/GluN3 require only glycine to open (Pachernegg et al 2012, Wenthold

et al 2003). However, NMDAR-driven ionic throughput is only occasioned when glutamate and glycine co-agonism co-occurs with cellular depolarization which releases a magnesium block from the inside of the channels pore (Traynelis et al 2010). Because these voltage-sensitive receptors require simultaneous chemical agonism, they are often referred to as coincidence detectors. Once the channel is open and clear, calcium rushes in and triggers a strengthening of the synapse.

NMDARs can produce a variety of effects that are dependent on their location, which is associated with the type of intracellular signaling cascade it activates (Hardingham et al 2002, Kim et al 2005), and subunit composition, because each subunit has distinct pharmacological properties (Liu et al 2007, Massey et al 2004). Receptors that are located at the synapse appear to stimulate pathways that are associated with cell survival, whereas receptors that are extrasynaptically-located promote cell death (Hardingham & Bading 2010, Xu et al 2009). Indeed, synaptic NMDAR stimulation decreases p38MAPK activity and increases BDNF expression and the phosphorylation of ERK1/2, in turn elevating the phosphorylation of Jacob, which binds to calcium sensor Caldendrin, whereas extrasynaptic receptors do the opposite (Hardingham et al 2002, Hardingham & Bading 2010, Li et al 2011b, Parsons & Raymond 2014). Phosphorylated Jacob is associated with heightened CREB phosphorylation whereas ERK1/2 dephosphorylation and unphosphorylated Jacob shuts off the CREB pathway, slowing dendritic maturation and cell-cell connectivity, and compromising cell survival (Dieterich et al 2008, Li et al 2011b, Wang et al 2013b).

In the early postnatal brain, the expression of GluN2B outweighs GluN2A, but the latter predominates as time progresses (Sinagra et al 2005, Wang et al 2009). GluN2A preferentially localize synaptically, whereas GluN2B are often expressed extrasynaptically (Rumbaugh & Vicini 1999, Stocca & Vicini 1998). This may explain why GluN2A-containing receptors promote the insertion of GluA1-AMPARs in mature cultured neurons, whereas GluN2B inhibit their insertion, via the Ras-ERK pathway (Kim et al 2005). GluN2B-containing receptors also stay open longer than receptors containing GluN2A (Cui et al 2013), although they are usually co-expressed (Flint et al 1997, Massey et al 2004). Interestingly, GSK3β is activated by extrasynaptic NMDARs which increases Tau toxicity (a hallmark feature of neurological disorders), and blocking GluN2B-containing receptors inhibits this phenomenon (Tackenberg et al 2013, Toral-Rios et al 2020). However, it is difficult to study the GluN2A:GluNB ratio because differences could be due to age and expression levels of subunits can be altered by experience. In any case, GluN2A- and GluN2B-containing receptors are more responsive to

glutamate and allow increased calcium currents than heteromers containing GluN2C and GluN2D (Yamakura & Shimoji 1999). GluN2A-containing receptors are also more stable at synapses than receptors containing GluN2B, whose stability is heightened by an overexpression of GluN2A (Groc et al 2006).

NMDARs are critically involved in the regulation of learning and the proper formation of memories, especially spatial memories, but not the maintenance or retrieval of memory (Howland & Cazakoff 2010). This is evident by the fact that learning is impaired by NMDAR antagonism before exposure to the task at hand in rodents and humans, but NMDAR blockade after learning a task has no effect on memory retrieval (Danysz et al 1988, Hadj Tahar et al 2004, Hlinák & Krejcí 2002). Spatial orientation memory is impaired when NMDAR activity is disrupted in mice and rats (Heale & Harley 1990, Hlinák & Krejcí 2002), and the consolidation of memory appears to rely on the reactivation of NMDARs at site-specific synapses that somehow code traces of memory (Shimizu et al 2000). NMDAR antagonism and genetic manipulations of GluN2A and GluN2B support this notion, and demonstrate that potentiating NMDAR-mediated currents enhances hippocampal LTP (Cao et al 2007, Cui et al 2011, Heale & Harley 1990, Jacobs & Tsien 2012, Sakimura et al 1995, Wang et al 2009). The disruptions in memory and LTP are more significant in GluN2B knockout mice compared to GluN2A knockouts, which suggests that GluN2B likely preserves a level of synaptic plasticity in mice that are lacking GluN2A (Cui et al 2013). GluN2B-containing receptors do stay open longer than receptors containing GluN2A, and this greater window of stimulation could explain why it is more involved in synaptic plasticity and learning. It is worth noting that synaptic GluN2B-containing receptors are not required for LTD, which is primarily mediated by extrasynaptic NMDARs (Massey et al 2004, Morishita et al 2007). However, NMDARs are a double-edged sword because normal levels of activity promote neuroplasticity whereas their overstimulation leads to excessive calcium influx that can bring about deleterious consequences by activating many signaling cascades – such as endonucleases, phospholipases, proteases, and so on – which increases ROS production, mitochondrial apoptosis, and inflammation. When this occurs, components of the cytoskeleton, membrane, and DNA are subject to damage (Jaiswal et al 2009, Manev et al 1989). The excitotoxic effects of glutamate were first described in 1954 by a Japanese scientist who reported that high levels of glutamate caused seizures (Hayashi 1954). Under normal circumstances, the concentration of glutamate in the synaptic cleft can increase up to 1mM before quickly decreasing in a matter of milliseconds, but neuronal death ensues when high levels of synaptic glutamate are not rapidly

removed (Clements et al 1992). One can assume that depression patients have higher rates of cellular damage and apoptosis given that they have higher concentrations of glutamate in the blood and brain (Ankarcrona et al 1995, Hashimoto et al 2007, Mitani et al 2006).

Indeed, plasma levels of glutamate, glutamine, and glycine were found to be increased in depression patients and glutamate levels correlated positively with HDRS scores (Mitani et al 2006). Those with unipolar or bipolar depression also have elevated levels of glutamate in the frontal cortex (Hashimoto et al 2007), where depressed individuals express half of the levels of GluN2A and GluN2B compared to controls (Feyissa et al 2009). The same group showed that GluN2A was massively increased in the amygdala of those with MDD (Karolewicz et al 2009), and others have found reductions in the expression of GluN2A and GluN2B in the depressed perirhinal cortex, which is highly connected to the hippocampus and involved in memory (Beneyto et al 2007, Lee et al 2006). This is interesting because dendritic atrophy leads to volumetric reductions in the hippocampus and PFC in depression, whereas amygdala volumes are increased due to dendritic hypertrophy (McEwen 2005, McEwen & Morrison 2013, Mitra & Sapolsky 2008, Sapolsky 2000, Sawyer et al 2012, Zhang et al 2018). Therefore, it seems that there is a relationship between stress, the volumetric abnormalities in depression, and deficiencies in synaptic plasticity driven by NMDARs. Alterations in the expression of NMDARs are also seen in animals that express depression-relevant behavior – like olfactory bulbectomized and Flinders Sensitive Line rats – in areas that regulate mood such as the amygdala, hippocampus, and cerebral cortex (Ho et al 2001, Ryan et al 2009). These studies show that disturbances in glutamatergic transmission are region-specific and may be implicated in certain depressive symptoms; for example, altered glutamatergic signaling in the amygdala may be related to anxiety and in the PFC with cognition.

When levels of glutamate in the synapse are excessively high, some molecules will escape into the extracellular space and contact NMDARs that preferentially express GluN2B and are coupled to intracellular signaling pathways that promote cell death (Hardingham & Bading 2010, Rumbaugh & Vicini 1999, Xu et al 2009). In fact, blocking astrocytic glutamate uptake in rats leads to anhedonia-like behavior and cognitive deficits, which are difficult symptoms to treat in depression (Bechtholt-Gompf et al 2010). NMDARs have been described as a double-edged sword considering that their transient activity is critical for synaptic plasticity while their prolonged activation leads to cellular excitotoxicity (Monaco et al 2015). Both in vivo and in vitro work demonstrates that GluN2B is largely responsible for the paradox, regardless of receptor location (Liu et al 2007, von Engelhardt et al 2007). Antagonizing GluN2B, but not

GluN2A, abolishes excitotoxicity in hippocampal slices, corroborating the notion that glutamate and NMDAR-driven processes require tight regulation (Preskorn et al 2008, Zhou & Baudry 2006). In our laboratory, we have shown that chronic exposure to high levels of CORT increases the expression of GluN2B in the dorsal hippocampus (Brymer et al 2020) and others have shown this to be true in the ventral hippocampus as well (Calabrese et al 2012). As a matter of fact, deleting GluN2B appears to mimic the fast-acting antidepressant effects of NMDAR antagonist ketamine; it could be that GluN2B suppresses mTOR-induced protein translation, whereas ketamine does the opposite (Harraz et al 2016, Miller et al 2014, Wang et al 2011).

Ketamine is a phencyclidine derivative that acts as a non-competitive, voltage-dependent NMDAR antagonist by blocking the channel of the receptor, and it has received considerable attention in the last few decades. The first clinical publication of ketamine as a human anesthetic occurred in 1996 and it has since been widely used due to its impressive safety profile (Corssen & Domino 1966). In humans, a single sub-anesthetic dose of ketamine was discovered to combat depression in hours in patients that are resistant to conventional medications, and the antidepressant effects persisted for 1 to 2 weeks (DiazGranados et al 2010b, Harraz et al 2016). Ketamine is made up of a chiral center comprised of 2 enantiomers (S- and R-ketamine), and S-ketamine is more affinitive to NMDARs than R-ketamine by 3- to 4-fold. For this reason, the FDA approved S-ketamine as a nasal spray for treatment-resistant depression in 2019, and for patients displaying suicidal ideation in 2020 (Kadriu et al 2021). That said, R-ketamine may also elicit sustained antidepressant effects with fewer side effects than its counterpart (Yang et al 2015, Zhang et al 2014). There are several hypotheses that have been proposed to explain how ketamine rapidly generates behavioral changes. One is that the direct antagonism of NMDARs on cortical pyramidal neurons somehow triggers the synthesis of proteins in a semblance of homeostatic neuroplasticity that heightens subsequent activity onto these neurons (Miller et al 2016). This is backed up by the fact that ketamine increases AMPAR activity on pyramidal cells even when they are treated with sodium channel blockers that dampen synaptic transmission, and the suppression of eukaryotic elongation factor 2 kinase activity has been implicated in said protein synthesis (Duman & Kystal 2020). Another proposal is that ketamine preferentially inhibits extrasynaptic NMDARs, which are primarily made up of GluN2B and are not activated by the typical release of glutamate but when low levels of ambient glutamate spill into the extracellular space, especially when synaptic levels of glutamate are too high (Miller et al 2014). This may concomitantly induce neuronal

excitation and the disinhibition of protein synthesis (Zanos & Gould 2018). It could also be that ketamine indirectly stimulates excitatory pyramidal neurons by blocking NMDARs on tonically-activated cortical somatostatin- and parvalbumin-expressing GABAergic interneurons that usually dampen pyramidal cell activity (Widman & McMahon 2018). Indeed, interneurons have a higher firing rate than pyramidal neurons which indicates that ketamine may selectively block interneuronal NMDARs that no longer hold magnesium (Seamans 2008). That said, the behavioral improvements that follow ketamine must be more complex than neuronal disinhibition, which does not always reduce depression (Miller et al 2016). Unfortunately, ketamine is limited by its highly addictive, psychotomimetic, and dissociative nature which necessitate the hospitalization of patients during treatment, and its chronic use can lead to brain lesions (Wang et al 2013a). Other compounds that target NMDARs have recently been investigated like glycine binding site and GluN2B-selective allosteric modulators, which seem to produce fewer side-effects but also weaker antidepressant effects than ketamine (Kadriu et al 2020a, Kadriu et al 2019, Park et al 2020, Zanos et al 2015). Curiously, NMDAR antagonists like MK-801, memantine, or AZD6765 do not have antidepressant properties, which suggests that ketamine improves mood by modulating molecular systems other than NMDARs (Gould et al 2019, Hillhouse & Porter 2014). Researchers are trying to identify these mechanisms and develop novel compounds to target them with fewer side-effects, which is difficult considering the pervasive distribution of glutamate and its receptors across the brain.

A shared attribute of drugs that have fast-acting antidepressant effects is that they trigger a "glutamate surge" that dramatically elevates the activity of AMPARs relative to NMDARs (Kadriu et al 2020b, Koike & Chaki 2014, Zhang et al 2016). This flood of AMPAR transmission leads to the release of BDNF, which, as I discussed in section 1.2.2.3, promotes several aspects of neuroplasticity that are impaired in depression (Deng et al 2016, Yoshida et al 2012). BDNF stimulates the activation of mTOR, which strengthens cellular communication by prompting the neurons to synthesize, transport, and insert new AMPARs at the synapse, and blocking the activity of AMPARs and mTOR abolishes the ketamine-driven behavioral and neurochemical antidepressant effects (Koike & Chaki 2014, Li et al 2010, Li et al 2011a, Maeng et al 2008, Zhou et al 2014). Ketamine can quickly rescue deficits in dendritic arborization and the density of dendritic spines induced by chronic stress, albeit differently in males and females (Li et al 2011a, Moda-Sava et al 2019, Sarkar & Kabbaj 2016, Thelen et al 2019). The application of ketamine to hippocampal slices also potentiates transmission driven

by AMPARs in the hippocampal CA1 region (Nosyreva et al 2013). In line with this, the rapid metabolism of ketamine to neuroactive hydroxynorketamine was shown to be essential for its antidepressant actions in mice through the stimulation of AMPARs and independent of NMDARs (Zanos et al 2016); although another group argued that higher doses of hydroxynorketamine block open-channel NMDARs which contributes to the antidepressant-like effects (Suzuki et al 2017). However, hydroxynorketamine did not displace MK-801 from NMDAR-binding or inhibit NMDAR currents that were recorded from GABAergic interneurons in hippocampal slices – contrary to the indirect hypothesis of ketamine – but it did elevate the amplitude and frequency of AMPAR mediated currents in the CA1 following drug washout (Zanos et al 2016). Interestingly, increasing excitatory transmission by antagonizing $G_i$-coupled mGluRs produces rapid and persistent antidepressant-like effects in rats that are exposed to CUMS (Chu & Hablitz 2000, Dwyer et al 2013, Seo et al 2020). These drugs also require AMPARs, but they do not cause adverse reactions that are characteristic of NMDAR antagonists, much like hydroxynorketamine (Aleksandrova et al 2017, Koike & Chaki 2014, Witkin 2020). These reports suggest that positively modulating AMPARs should be considered as a novel approach to rapidly treat depression with fewer and more tolerable adverse side-effects.

### 1.6 The involvement of GABAergic transmission in depression

GABA is the major inhibitory signaling molecule of the central nervous system that around ⅓ of neurons utilize to maintain the temporal and spatial balance of excitatory and inhibitory transsynaptic signaling (Bloom & Iversen 1971, Klausberger & Somogyi 2008). Interestingly, reelin is secreted by GABAergic interneurons in the adult cortex and hippocampus, and stress-sensitive reelin-deficient mice express alterations in GABAergic facilitators, suggesting that the proper regulation of inhibitory transmission is necessary for emotional competency (D'Arcangelo et al 1997, Pesold et al 1998, 1999, Lussier et al 2011, Liu et al 2001). GABA is produced by the enzyme glutamate decarboxylase (GAD), which has two isoforms that convert glutamate into GABA before it is packaged into presynaptic vesicles by the vesicular GABA transporter that are present on glial cells and presynaptic neurons (Fenalti et al 2007, Fogaça & Duman 2019). The $GAD_{65}$ isoform (named for its molecular weight) synthesizes GABA for autocrine and paracrine neurotransmission and is therefore found in nerve terminals whereas $GAD_{67}$ is found evenly distributed throughout cells and generates GABA for purposes other

than neurotransmission – like synaptogenesis and neuroprotection (Kaufman et al 1991, Pinal & Tobin 1998). Evolutionary speaking, distinguishing excitatory and inhibitory neuronal transmission by a single enzyme could represent a sophisticated method to quickly regulate signal transduction. As well, GABAergic neurons of the cortex mainly project axo-axonically, which is strategic considering that the neuronal axon has the lowest threshold for the stimulation of action potentials (Szabadics et al 2006). However, this also suggests that $GAD_{65/67}$ dysregulation would have drastic consequences and lead to a reduction or build-up of glutamate, subsequently attenuating neuronal plasticity or promoting excitotoxicity, respectively.

Like glutamate, GABA is released into the synapse via a calcium-dependent mechanism and is affinitive for ionotropic $GABA_A$Rs and metabotropic $GABA_B$Rs (Hegstad et al 1992). $GABA_A$Rs are hetero-pentameric ligand-gated chloride channels that are made up from α(1-6), β(1-4), γ(1-3), ρ(1-3), and θ, π, ε, or δ subunits (Wongsamitkul et al 2017). They transverse the membrane and form an ionic pore that opens when conformational changes are induced by agonist binding (Horenstein et al 2001). Most $GABA_A$Rs are composed of 2 α subunits, 2 β subunits, and 1 γ subunit and they bind GABA between the α and β subunits and are localized postsynaptically (Chang et al 1996, Sieghart & Sperk 2002). Once the pore opens, the influx of chloride has a hyperpolarizing effect and renders the neuron unable to fire (Mody & Pearce 2004). Depending on the subunit composition and localization of $GABA_A$Rs, they can provide transient phasic inhibition (mediated by receptors present in the synapse) and tonic inhibition (mediated mainly by the persistent firing of extracellular receptors) (Farrant & Nusser 2005). $GABA_A$Rs are the target of many psychotropic drugs, like alcohol, barbiturates, benzodiazepines, and neurosteroids, which have their own distinct binding sites on the receptor. The allosteric binding of benzodiazepines to $GABA_A$Rs occurs between the α and γ subunits and does not open the channel but increases GABA-receptor affinity; some tranquilizing drugs do not recognize receptors that contain α4 or α6 subunits, such as diazepam, which can cause confusion and memory problems (Derry et al 2004). Interestingly, the constitutive intrinsic levels of activity in the absence of ligands suggests that inverse agonists would actually increase neuronal excitability, and such drugs enhance cognition and memory in animal models (Braudeau et al 2011, Kemp et al 1987, Milić et al 2013). Drugs could be designed to target specific GABA subunits that are differentially expressed in different neuronal cell types (McKernan & Whiting 1996). For instance, $GABA_A$-driven activity by receptors that express α1 and α5 subunits is associated with sedation and amnesia, whereas activity from receptors

expressing α2 and α3 are implicated more in anxiety (John 2003). GABAergic drugs are also often used to manage insomnia, mania, and pain, which are associated with central hyperactivity. If glutamate drives activity, then GABA can be thought of as the breaks. Therefore, GABAergic transmission needs to be tightly regulated because deficient signaling could result in a seizure whereas excessive signaling could lead to a loss of consciousness.

$GABA_BRs$ indirectly activate inwardly rectifying potassium channels and outwardly rectifying calcium channels via secondary signaling intermediates in the central nervous system and autonomic division of the peripheral nervous system (Bowery 2010, Hyland & Cryan 2010). $GABA_BRs$ are formed by the heteromeric assembly of 2 $GABA_{B1}$ subunits (which have 2 isoforms: $GABA_{B1a}$ and $GABA_{B1b}$) and 2 $GABA_{B2}$ subunits, which like mGluR subunits, have 7 transmembrane domains (Jones et al 1998). They are situated pre- and post-synaptically to act as autoreceptors and heteroreceptors that dampen inhibitory or excitatory transmission, respectively, by coupling to $G_i$ proteins that reduce the activity of adenyl cyclase (Gassmann & Bettler 2012). After binding to $GABA_{A/B}Rs$, around 80% of synaptic GABA is taken up by 6 different transporters and recycled (Bernstein & Quick 1999). Mitochondrial enzymes like GABA transaminase convert the majority of GABA into succinate, whose metabolites are important for ATP synthesis (Bown & Shelp 1997).

Elevating GABAergic neurotransmission typically induces calming, anxiolytic, and anti-convulsant effects. The majority of GABA-expressing neurons in the brain are interneurons, but there are some extensive thalamic and cortical projections that influence complex cognitive and emotional processes (Nuss 2015). The endings of cortical GABAergic interneurons wrap around many pyramidal cells and some spiny stellate cells and dampen their excitatory projections to important brain regions like limbic structures, the stria terminalis, and brain stem (Fogaça & Duman 2019). These inhibitory interneurons can be divided into groups based on their neurochemical signatures, including those that express parvalbumin, somatostatin, and the ionotropic $5\text{-}HT_3R$, and they each play distinct roles in mediating cellular communication (Rudy et al 2011). For example, parvalbumin-expressing interneurons account for 40% of the GABAergic interneuronal population, they have so-called chandelier and basket morphologies, and they regulate the output of pyramidal cells; somatostatin-expressing interneurons are Marinotti cells that make up 30% of the population – they inhibit parvalbumin-expressing GABAergic cells to regulate the input to pyramidal cells, while also synapsing directly with the dendritic tufts of such pyramidal cells (Fogaça & Duman 2019, Urban-Ciecko & Barth 2016). GABA is also expressed in many peripheral tissues that could influence mood, like in

the gut (Hyland & Cryan 2010). To give another example of peripheral GABAergic activity, lymphocytes dampen their inflammatory response in the presence of GABA (Mendu et al 2012, Tian et al 2011).

Considering the vast number of neurons that are under the governance of GABA, it is unsurprising that GABAergic dysregulations have been implicated in psychiatric disorders, including depression. As expected, several studies have reported that unmedicated depression patients have lower plasma and cerebrospinal fluid levels of GABA (Gerner & Hare 1981, Petty & Sherman 1984), which is also true in the brain where lower levels correlate with higher anhedonia scores and reduced hippocampal volume (Abdallah et al 2015, Gabbay et al 2012, Godfrey et al 2018, Hasler et al 2007, Song et al 2012). Furthermore, they have lower levels of GABA-producing $GAD_{67}$ in the PFC and anterior cingulate cortex (Karolewicz et al 2010, Tripp et al 2012). Interestingly, subjecting patients to a threat-of-shock psychological test that induces acute stress, was shown to reduce GABA levels in the PFC by around 18% when compared to controls who experienced a psychologically safe test (Hasler et al 2010). GABAergic medications are commonly used to treat anxiety, but meta-analyses have reported that the antidepressant effects of alprazolam are comparable to that of first-generation antidepressants (Birkenhäger et al 1995, van Marwijk et al 2012).

Animal models also demonstrate the relationship between stress and GABAergic dysfunction. In our laboratory, we have found that exposing rats to repeated-CORT injections increases depression-like behavior and reduces the expression of $GAD_{67}$ in the amygdala, and $GAD_{65}$ and $GABA_A$Rs in the hippocampus, while restraint stress had no effect on protein expression or behavior (Brymer et al 2018, Lussier et al 2013b). Similarly, CUMS decreased cortical and hippocampal $GAD_{67}$ levels but not $GAD_{65}$ (Banasr et al 2017, Ma et al 2016). Other laboratories have found that environmental stress increases the activity of GABA turnover, and decreases the levels of GABA and $GABA_A$R-mediated signaling in the frontal cortex (Czéh et al 2018, Otero Losada 1988, Shalaby & Kamal 2009). Variations in maternal care also reduces the expression of $GABA_A$Rs in cortical and limbic regions and is associated with increases in depression-like behavior (Caldji et al 2000, 2003). However, there are contradicting reports that show restraint stress increases GABA-$GABA_A$R binding and $GABA_A\alpha1$ mRNA expression in the cortex (Braestrup et al 1979, Gilabert-Juan et al 2013). One way whereby stress-induced disruptions in GABAergic signaling may influence depression is by hindering the inhibition of the stress response. Even acute restraint stress disrupted the GABAergic synaptic inputs that are tasked with inhibiting the HPA-axis (Hewitt et al 2009). The deletion

of $GABA_AR$ α2 and γ2 subunits also impairs the binding of GABA and potentiates HPA-axis activity, and depressive- and anxiety-like phenotypes (Chandra et al 2005, Shen et al 2010, Smith & Rudolph 2012, Vollenweider et al 2011).

Interestingly, GABA has an excitatory, depolarizing effect in the developing brain before a change in the expression of chloride channels causes an influx of chloride instead of an efflux (Li & Xu 2008, Wu & Sun 2015). Indeed, GABAergic signaling arises earlier in the developing hippocampus than glutamatergic signaling because interneurons that expel GABA mature faster, and by doing so aid in the proliferation and differentiation of cells, and their integration into the existing circuitry (Behar et al 1998, Ganguly et al 2001, Salazar et al 2008). This implies that deficient GABAergic inputs to immature neurons, like adult-born dentate granule cells, will slow their maturation rate and impair circuit integration. Unmedicated depression patients present with a reduction in granule cell numbers and lower DG volumes than controls and medicated patients, but it is unclear how insults to these cells directly relate to mood dysfunction (Boldrini et al 2013, Umschweif et al 2021).

Since stress seems to cause a decrease in GABAergic neurotransmission, one wonders whether drugs could be developed with antidepressant activity that selectively target some GABA receptors. Interestingly, the neuroactive steroid allopregnanolone (also known as brexanolone), which is produced naturally from progesterone and cholesterol, was approved as an i.v. medication in 2019 to treat post-partum depression because levels of allopregnanolone drop heavily after childbirth (Schüle et al 2014). Allopregnanolone acts as a $GABA_AR$ positive allosteric modulator, but the implications of this mechanism in behavioral improvements is unclear (Pinna 2020, Walton & Maguire 2019). This is because negative allosteric modulators of α5-containing $GABA_ARs$, which are restricted to the forebrain, also have rapid antidepressant-like effects in mice and rats that is associated with the strengthening of excitatory synapses in the hippocampal CA1 (without the adverse effects of ketamine) (Fischell et al 2015). In addition, ketamine likely initially inhibits NMDARs that are localized to presynaptic tonically-activated GABAergic interneurons which would result in the disinhibition of postsynaptic excitatory pyramidal neurons (Zanos & Gould 2018). Parvalbumin interneurons preferentially contact the somas of pyramidal cells, and they express GluN2C/D subunits, which leave NMDARs more likely to interact with ketamine because of how they associate with magnesium (Wildman & McMahon 2020, Duman & Kystal 2020). Whether caused by direct or indirect mechanisms, pyramidal cell excitation heightens BDNF and mTOR activity (Deutschenbaur et al 2016, Gould et al 2019), which is also achieved by

allopregnanolone administration (Nin et al 2011a). In addition, ketamine increases $GABA_AR$ activity in the cortex and hippocampus (Wang et al 2017a). Interestingly, mouse studies have shown that the antidepressant-like effects of ketamine require $GABA_BRs$ (Rosa et al 2016), but also probably $GABA_ARs$ considering that γ2-$GABA_AR$ deficient mice have less NMDARs and AMPARs, but not when they are treated with ketamine (Ren et al 2016). A GluN2B-selective NMDAR antagonist was also shown to increase protein synthesis by increasing $GABA_BR$ activity (Workman et al 2013, 2015). It is clear that the levels of excitatory and inhibitory neurotransmission are imbalanced in the pathological brain, and that rapid-acting therapeutics correct this imbalance (Milak et al 2016, Silberbauer et al 2020). Unfortunately, these drugs are generally associated with severe side-effects; but targeting specific subtypes of GABAergic cells may provide a method to selectively fine-tune inhibitory/excitatory circuits in certain brain areas.

## 1.7 Hippocampal anatomy, circuitry, and function

The hippocampus is a major structure that resides in the medial temporal lobe of both brain hemispheres that is highly involved in the regulation of mood and the consolidation of short-term memory to long-term memory, but not necessarily its storage. It was named after the Greek word for seahorse ("*hippos*" meaning "horse" and "*kampos*" meaning "sea monster"), which it closely resembles. The hippocampus is accepted as a limbic region that receives inputs from most of the cortex and subcortical regions. In a sense, it provides a spatiotemporal framework for experience and fuses it with the emotional and cognizant components (Knierim 2015). The hippocampus is therefore essential for understanding the background setting of an experience by associating items or events with their context (Lisman et al 2017). The involvement of the hippocampus in memory became very clear in 1957 when an epileptic patient known as H.M. developed anterograde amnesia after his hippocampi were removed to reduce seizures (Squire & Wixted 2011). His cognitive ability was the same after the surgery, but he was no longer able to generate new semantic or episodic memories or remember events that occurred close to the surgery (partial retrograde amnesia). Those with hippocampal lesions who experience severe episodic and spatial memory impairments generally retain normal levels of intelligence (Hainmueller & Bartos 2020). The role of the hippocampus in memory is further exemplified by taxi drivers in London, England who have larger hippocampal volumes because they rely on their spatial memory throughout the workday (Maguire et al 2000). On the other

hand, amnesiac patients have smaller hippocampal volumes and often get lost (Konkel et al 2008, Vijayakumar & Vijayakumar 2012). Animal studies also consistently reveal that damage to the hippocampus impairs memory performance in location-recognition based tests (Broadbent et al 2004, Hampton et al 2004).

The hippocampus is made up of two main regions, namely the Cornu Ammonis (CA; named after the horns of ancient Egyptian god Amun) and the trilaminate DG. The term hippocampus proper is often used to refer to the CA, of which there are 3 divisions (CA1-3), and the term hippocampal formation is used to refer to the CA and DG, along with the subicular and entorhinal cortex (EC), which relay information between the hippocampus and other limbic centers, the PFC, and hypothalamus (Amaral & Witter 1989, Insausti & Amaral 2012). The hippocampus is unique in that the flow of information through each region is unidirectional (Amaral et al 2007); the EC can signal to the CA using the perforant pathway (which perforates through the subiculum) directly or indirectly via a tri-synaptic circuit that utilizes the DG – in other words, information can go the long way around or take the short-cut.

The hippocampus proper has 5 strata, which are the stratum oriens, stratum pyramidal, stratum radiatum, stratum lucidum, and stratum lacunosum-moleculare layers in order of most superficial to deep (see Figure 1.7 below for visual aid). The stratum oriens houses the dendrites of excitatory pyramidal neurons whose soma are tightly packed together in the stratum pyramidal layer below, and their activity is fine-tuned by GABAergic interneurons that can project to other CA subfields. Between the stratum pyramidal and stratum radiatum layers of the CA3, the stratum lucidum can be found. This is the thinnest strata that is made up of axonal projections from granule cells in the DG, referred to as mossy fibers. Deeper is the stratum radiatum, where so-called Schaffer collateral fibers are found, which are the axonal projections of CA3 pyramidal cells to those of the CA1/2. Shaffer collaterals also run through the stratum lacunosum-moleculare where they can excite GABAergic interneurons like basket cells that provide feed-forward inhibition to pyramidal cells. Here the dendrites of CA1 and CA1/2 pyramidal cells receive innervation from excitatory EC layer III cells via the temperoammonic pathway and EC layer II cells via the perforant pathway, respectively (Amaral & Witter 1989, Basu & Siegelbaum 2015). This direct activation of distal apical dendrites of CA pyramidal cells by the EC is referred to as the monosynaptic pathway (Charpak et al 1995).

The DG is comprised of the polymorphic layer (PL; also called the hilus or CA4), the stratum granulosum or granule cell layer (GCL), and the stratum moleculare or molecular layer (ML)

(Blackstad 1956, Insausti & Amaral 2004, Insausti & Amaral 2012). The GCL houses the cell bodies of granule cells which are tightly packed together, and their dendrites are excited by the EC layer II cells of the perforant pathway in the ML. This input to the ML represents the first synapse of the so-called tri-synaptic pathway (depicted by symbol ① in Figure 1.7) and neurotransmission here is modulated by ML performant path-associated interneurons (MOPP) among other cells. In fact, MOPP cells are activated by the perforant pathway before granule cells indicating that they contribute to feed-forward inhibition of granule cells (Li et al 2013). Inhibitory hilar performant path-associated cells (HIPP) and excitatory mossy cells (not to be confused with mossy fibers) are abundant in the PL, and they innervate each other, basket cells, and the output of granule cells (Raza et al 2017, Scharfman & Myers 2013). The SGZ is found between the PL and GCL and is 1 of 2 brain regions where neural stem cells are housed that generate new-born neurons (which will be discussed further in the following section). The axonal mossy fibers of mature dentate granule cells project to the pyramidal cells of CA3 – the second tri-synaptic synapse (②). It is the CA3 Schaffer collateral fibers that relay the information to the adjacent CA2 and CA1 pyramidal cells (accounting for the third synapse ③). Cells in the CA1 project to layers V and VI of the EC, which can project to layers II and III to complete the information loop (Amaral et al 2007, Amaral & Witter 1989, Insausti & Amaral 2004).

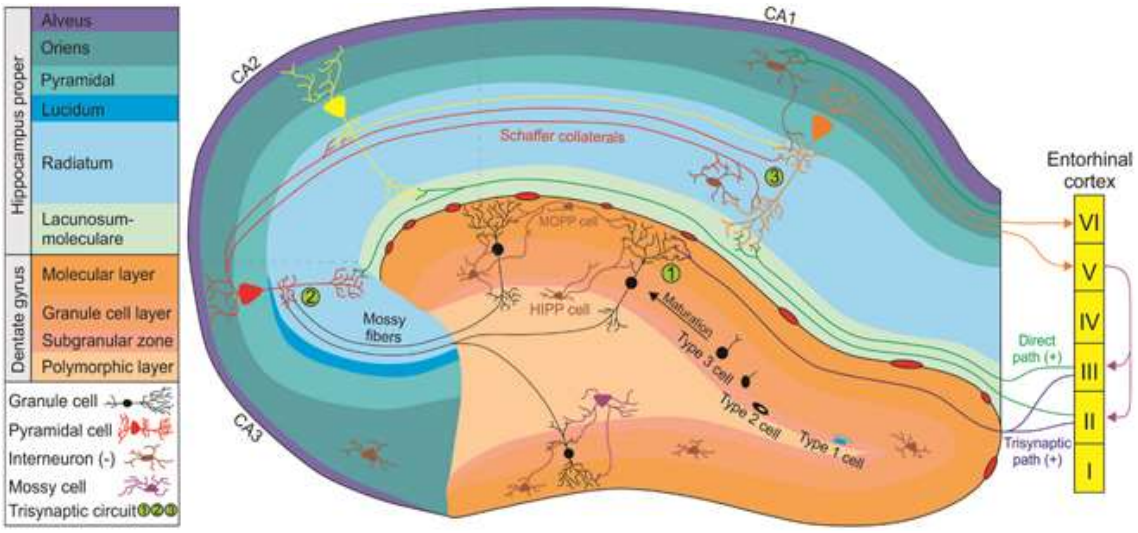


**Figure 1.7. Anatomy and circuitry of the hippocampus.** The hippocampus proper is made up of CA1, CA2 and CA3. The DG is made up of the ML, GCL, PL. The apical dendrites of the granule cells whose soma are tightly packed together in the GCL receive input from layer II/III of the EC via the performant path. The axons of the granule cells form the mossy fibers that project to the CA3 pyramidal cells and these fibers make up the stratum lucidum. The axons of the CA3 pyramidal cells make up what are known as Schaffer collaterals which project to proximal regions (stratum radiatum) of CA1 pyramidal cells and GABAergic interneurons that provide feed-forward inhibition. Thus, this circuit is known as the tri-synaptic circuit and is unique in that it is unidirectional – the 3 synapses are indicated by ①, ② and ③, respectively. The axons of CA1 pyramidal cells project to the subiculum which in turn projects to layers V and VI of the EC which completes the loop. The alveus contains axonal projections from the granule and pyramidal cells. This pathway is modulated by interneurons such as basket cells in the DG, MOPP cells and mossy and HIPP cells, as well as other interneurons throughout the hippocampus proper. In addition to the tri-synaptic pathway, more distal regions (stratum lacunosum) of pyramidal cells of CA1 and CA2 receive a direct excitatory input from EC layer II and CA1 from layer III (and not mossy fibers). CA2 pyramidal cells are excited much more by the EC than other CA subfields and they are the most resistant to injury, and thereafter the CA2 excites CA1 pyramidal cells (neoclassical pathway). The SGZ is a neurogenic site – neural stem cells (known as type 1 cells) give rise to progenitor cells (type 2a/2b cells) which can become neuroblasts (type 3 cells) that migrate to the GCL and mature and integrate into the existing circuitry. Figure created with CorelDraw by author.

The CA2 region is a relatively narrow subfield of the CA interposed between the CA1 and CA3, where pyramidal cells are excited much more by inputs from the EC than the CA3 and can go on to activate CA1 cells (referred to as the neoclassical pathway) (Chevaleyre & Siegelbaum 2010, Hitti & Siegelbaum 2014, Kohara et al 2014). There are also long-range inhibitory neurons in the hippocampus that project to other hippocampal regions, including contralateral areas, the subiculum, lateral hypothalamus, and mammillary nuclei, and the

information runs bi-directionally to the EC (Christenson Wick et al 2019). One can imagine, then, the signals traveling through the tri-synaptic loop can be modified with each iteration by direct monosynaptic and neoclassical circuits, modulatory neurotransmitter projections, and firing patterns of inhibitory and excitatory transmission. It is then consolidated and saved as a rememberable piece of information with links to emotional and environmental for recollection.

The human hippocampus is thought to be 10-fold larger than a monkey and 100-fold larger than a rat, but they share the same basic architectural from and function (Clark & Squire 2013). This, along with the fact that the hippocampus is composed of various neuronal subtypes that are organized into identifiable layers, makes it an ideal model system for investigating neurophysiological phenomena. For example, Bliss and Lomo (1973) first reported the occurrence of LTP in the rabbit hippocampus; which supported Donald Hebb's (1949) earlier prediction that the brain stores memories by altering the strength of connections between neurons that are simultaneously active, which became known as Hebbian learning (Choe 2013). It is estimated that a typical CA1 pyramidal neuron receives an impressive 30000 glutamatergic synaptic inputs and 1700 GABAergic inputs (Megías et al 2001). This emphasizes the magnitude of complexity at which the hippocampus operates, and why atrophy to this region would impair many essential functions.

The hippocampus is critical for spatial and nonspatial forms of declarative memory and it operates with the neocortex to maintain and consolidate memory, but eventually, it becomes independent of long-term memory but remains critical for recall (Squire et al 2004). Different areas of the hippocampus receive modulatory inputs from the 5-HTergic raphe nucleus, noradrenergic locus coeruleus (which co-releases dopamine), and dopaminergic ventral tegmental area (VTA), which generally enhance learning and memory and tie various sensory aspects of an experience together (Bacon et al 2020, Kempadoo et al 2016, Luchetti et al 2020, McNamara & Dupret 2017). Interestingly, when the hippocampus detects novel information (not stored in long-term memory), signals are conveyed to the VTA which in turn releases dopamine in the hippocampus, where dopamine is removed by the noradrenaline transporter due to a lack of dopamine transporter expression (Borgkvist et al 2012, Lisman & Grace 2005). Another important hippocampal input comes from the pleasure-associated medial septal area which sends cholinergic and GABAergic fibers to all areas of the hippocampus and disrupting the septal area severely impairs spatial but not nonspatial working memory in rats (Kelsey & Vargas 1993).

We have lots of evidence that impairing certain regions of the hippocampus disrupts normal memory-related processes. For instance, lesions of the CA3 impairs performance in spatial memory tasks and can also produce hyperactivity (Gilbert & Brushfield 2009, Handelmann & Olton 1981, Jarrard 1983, Sutherland et al 1983). Additionally, CA3 damage was shown to impair object-placed paired associative learning, though lesions to the CA1 or DG did not, which suggests that the CA3 conserves a mechanism to code object-recognition memory (Gilbert & Kesner 2003). The CA2 seems to be more involved in social recognition memory (Lehr et al 2021) which has been demonstrated in mice that display an inability to recognize a conspecific after CA2 inactivation, though sociability and spatial and contextual memory were unchanged (Hitti & Siegelbaum 2014). CA1 pyramidal neuronal loss is also associated with deficient working memory and spatial navigation in a variety of tests (Auer et al 1989, Davis et al 1986, Olsen et al 1994, Whishaw et al 1994). In the Alzheimer's brain, reductions in neuronal density were more prominent of the CA1 than the CA3 (Padurariu et al 2012), and CA1 lesions interrupt normal autobiographical memory, mental time travel, and autonoetic conscious awareness (Bartsch et al 2011). Excitation from the direct pathway reaches the CA1 15-20ms faster than the indirect pathway, as if the CA is involved in recall and compares spatial and nonspatial sensory information from the immediate context to past or memorized mnemonic information (Basu & Siegelbaum 2015). The DG receives the majority of input from the EC and is thought to process this incoming noise into sparse activation of selective groupings of granule cells, which is essential for the discrimination and storage of experiences that are similar (Jung & McNaughton 1993, Kesner 2007). This is known as pattern separation, or the process in which similar or overlapping information/memories is transformed into dissimilar, distinguishable representations (Bakker et al 2008). Indeed, knocking out GluN1 in DG granule cells impairs the ability to distinguish two similar contexts but does not alter contextual fear conditioning (McHugh et al 2007). Other functions of the DG may include novelty detection and linking information to spatial contexts, which aids overall working memory (Hunsaker et al 2008, Lee & Jung 2017, Sasaki et al 2018). Interestingly, slightly changing the shape of an environment can significantly alter the correlative pattern of place-modulated granule cell activity, and more dramatic changes in environment shape recruits additional cell populations in the CA3 (Leutgeb et al 2007). These hippocampal "place cells" fire bursts of potentials when an animal passes through a specific part of an environment (Colgin 2020), and contextual fear conditioning causes a remapping of place cells even if the environment is unchanged, which suggests that a change in place cell firing is associated with learned fear of the environment (Moita et al 2004).

Hippocampal volume is also significantly reduced in depression patients in who longer illness durations correlate with larger volumetric reductions and deficiencies in cognitive ability (Campbell et al 2004, Kronmüller et al 2009, Sawyer et al 2012, Videbech & Ravnkilde 2004). Impairments in cognition are experienced by about ⅔ of depression patients and they predict poor remission rates and often persist after one is considered to have reached clinical remission (Austin et al 2001, Rock et al 2014). That said, it has been argued that depression is associated more with motivational perturbations which can confound cognitive testing and skew findings (Scheurich et al 2007). It is likely that motivation and cognition are altered in depression considering the extensive neuroanatomical abnormalities in MDD patients that consistently report memory problems, even when free from other symptoms (Baumann et al 1999, Marcos et al 1994). Stress also causes significant cellular atrophy in the hippocampus relatively quickly in rodent models, and often selectively (Fenton et al 2015, Goodman & McIntyre 2017, Johnston et al 2020, Kleen et al 2006, Lee et al 2009, Lussier et al 2013a, Park et al 2015a, Rahman et al 2016). These findings corroborate the notion that cognitive deficits are related to the alterations in hippocampal structure and function, but one should keep in mind that alterations in other regions, like the PFC, must also contribute (Levin et al 2007, Meyer et al 2019). As well, we must remember that acute stress brings about a physiological arousal that actually facilitates learning and memory, implying that stress is not always bad, but that biological systems can malfunction progressively (Buchanan & Lovallo 2001, Jelici et al 2004, Payne et al 2007).

Interestingly, the dorsal hippocampus is thought to play a preferential role in spatial, verbal, and conceptual learning and memory (primarily cognitive functions), whereas the ventral hippocampus plays a role in mood and fear conditioning (Cenquizca & Swanson 2007, Zhao et al 2008a). Indeed, lesions to the ventral but not dorsal hippocampus dysregulate stress responses and emotional behavior (Henke 1990), and lesions to the dorsal but not ventral hippocampus dysregulate spatial memory acquisition (Moser et al 1995). As one might expect, the ventral hippocampus projects to the emotional-processing nucleus accumbens, which is critical for decision making about time and activated less by reward in those with depression (Abela et al 2015, Pavuluri et al 2017), but also simultaneously to the medial PFC and amygdala to manage social and emotional memory (Ishikawa & Nakamura 2006, Kim & Cho 2017, Phillips et al 2019). Other ventral projections include the hypothalamus and the bed nucleus of the stria terminalis, while the septum, VTA, and retrosplenial cortex are innervated by the dorsal hippocampus (Tannenholz et al 2014). Dentate granule cells also influence mood

regulation (Eliwa et al 2021, Hill et al 2015, Saxe et al 2006), which I discussed in Section 1.2.2.2, but a thorough review of neurogenesis and its roles in the adult brain is in order.

## 1.8 Adult hippocampal neurogenesis

Neurogenesis is a multistep process of generating functionally integrated neurons from neural stem cells, which can self-replicate and differentiate into multiple cell types. Before the emergence of modern neuroscience, it was assumed that all the neurons we would use throughout life were developed before we are born. In short, that neurogenesis was a developmental process. However, we have come to learn that this is not entirely true; although neurons cannot divide, the adult brain houses at least 2 discrete pools of neural stem cells. One is found in the SGZ of the DG and the other in the subventricular zone of the lateral ventricles. Under normal physiological conditions, these neural stem cells produce progenitor cells, which unlike their parents, can divide a limited number of times (Palmer et al 1997, Seaberg & van der Kooy 2003). Neurons born in the SGZ integrate relatively locally, whereas neurons descendent from the subventricular zone migrate along the so-called rostral migratory stream to the olfactory bulbs, which is more pronounced in animals that rely more heavily on their developed sense of smells than humans (Curtis et al 2007). Here I will review hippocampal neurogenesis only, because optimal cognitive and emotional processing largely relies on this limbic structure (Eliwa et al 2021, Kempermann et al 2015, Ming & Song 2011).

Evidence of hippocampal neurogenesis came in 1965 when a couple of researchers described the presence of adult-born granule cells in rats injected with thymidine-$H^3$ (Altman & Das 1965), but it was not widely accepted as a phenomenon until decades later when more substantial evidence was collected (Gross 2000). Since this pioneering study, we have recorded neurogenic processes in rodents, primates, birds, among other animals, including humans (first study published in 1998) by detecting the proliferative activity of precursor cell populations and the presence of immature neurons with neuron-specific antigens like NeuN, calbindin, and microtubule-associated DCX (Cameron et al 1993, Eriksson et al 1998, Gould et al 1999b, Kaplan & Hinds 1977, Kempermann et al 1997, Kuhn et al 1996, Kukekov et al 1999, Lussier et al 2013a, Nottebohm 2004). Evidence of neurogenesis is also provided by the detection of mature neurons that are labeled with thymidine analog bromodeoxyuridine (BrdU), which has a half-life of 60 minutes and incorporates into the DNA of dividing cells, allowing researchers

to birth-date them (Kuhn et al 2016, Taupin 2007). However, neurogenesis is still a controversial topic because some researchers claim that it continues into old age, at least in healthy individuals (Boldrini et al 2018), while others claim that levels drop after childhood to undetectable levels in the adult – but these post-mortem samples were taken from patients that had epilepsy and were processed after longer post-mortem delays (Sorrells et al 2018). While levels of neurogenesis do decline with age, adult human hippocampal neurogenesis is generally accepted as a notable phenomenon (Encinas et al 2011).

The neurogenic niche comprises the proliferative precursor cells, their immediate progeny, neurons that are still in the immature stages of development, and a vasculature niche (Palmer et al 2000). The whole process of neurogenesis is thought to take about 7 weeks (Kempermann et al 2015), and can be broken down into several stages, which are: proliferation, differentiation, migration, maturation, and integration. Radial glial-like cells are the most common type of neural progenitor cell in the SGZ of the mammalian brain and they are referred to as "type 1 cells". Their cell bodies reside in the SGZ and they extend a radial process through the GCL to the ML of the DG, where their end-feet ensheathe local synapses and vasculature (Filippov et al 2003, Moss et al 2016). Type 1 cells can be detected by the expression of markers like cytoskeletal nestin, glial fibrillary acidic protein, and Sox2, and they can be quiescent or proliferative, mitotically dividing symmetrically and asymmetrically to produce "type 2" intermediate daughter cells which express nestin and nuclear protein ki-67 (which is strictly associated with cellular proliferation) (Bernal & Arranz 2018, Martínez-Cerdeño & Noctor 2018, Scholzen & Gerdes 2000, Seri et al 2001). These intermediate progenitor cells undergo a limited round of highly proliferative divisions and their progeny continue to climb the radial scaffolding fibers of the grandparent type 1 cells (Hunter & Hatten 1995, Martínez-Cerdeño & Noctor 2018). A subset of these cells continue to express markers characteristic of glial cells but are lacking radial glial-like morphologies (type 2a) while others express nestin, prox1, and DCX (type 2b), signifying their commitment to becoming a granule cell (Karalay et al 2011, Klempin et al 2011, Steiner et al 2006, Sun et al 2015). Neuronal precursor cells express DCX as they actively divide and the time-course for hippocampal DCX expression overlaps completely with that of polysialilated neural-cell-adhesion molecule, which was present in BrdU-labeled adult-born dentate granule cells (Seki & Arai 1993). Neuronal daughter cells continue to express DCX for about 2 to 3 weeks (Plümpe et al 2006, Rao & Shetty 2004), but over- or under-expressing DCX does not affect the morphological maturation or migration of adult-born cells (Merz & Lie 2013). Nevertheless, DCX is a valuable marker to identify type

2b and “type 3 cells” (or neuroblasts, which mark the exit of the cell cycle). Neuroblasts migrate to their destination in the GCL, mature, and integrate with the existing granule cell population that receive innervation from the perforant pathway and project to the CA3 (Schloesser et al 2014). GABAergic cells provide ambient GABA and the first synaptic contacts for new-born cells, which depend on sodium-potassium-chloride co-transporters to keep intracellular levels of chloride high to allow GABA to depolarize only immature cells once it opens chloride channels (Suh et al 2009). GABA paces new-born cell activity-dependent regulation and is required for the formation of glutamatergic inputs which triggers a phase of enhanced synaptic plasticity (Chancey et al 2013, Ge et al 2007, Schmidt-Hieber et al 2004, Tozuka et al 2005). As neurons mature, they begin to express post-mitotic markers like NeuN and calcium-binding protein calretinin (Brandt et al 2003). Calretinin sticks around for roughly 3 to 4 weeks as the dendrites mature, before it is replaced with another calcium-binder calbindin (Brandt et al 2003). Adding new cells to the circuit may represent a mechanism to instigate long-term, adaptive changes in the network without catastrophic interference (Wiskott et al 2006).

One should also keep in mind that many of the newly generated cells do not survive long enough to form connections with the CA3 due to apoptotic processes (Biebl et al 2000). Interestingly, mice that overexpress Bcl-2 – which inactivates pro-apoptotic mitochondrial-associated protein BAX (as discussed in Section 1.4) – exhibit an increase in surviving DCX-expressing cells by ~50% (Kuhn et al 2005). The addition of granule cells does not replace older cells but contributes to an expansion of the GCL (Crespo et al 1986), although dentate volumes decrease with age (Dillon et al 2017). Unlike neural progenitor cells, neuroblasts are highly influenced by things like exercise, learning, stress, enrichment, social housing, and drugs (Döbrössy et al 2003, Encinas et al 2006, Gould et al 1999a, Kempermann et al 1997, Kronenberg et al 2003, Li et al 2008, Malberg et al 2000). The survival, adaptability, and overall fine-tuning of adult-born cells in response to environmental triggers is also modulated by projections from the dopaminergic VTA, 5-HTergic raphe nuclei, acetylcholinergic septal nuclei, local GABAergic interneurons, and commissural fibers from the contralateral hippocampus (Kempermann et al 2015). Indeed, learning increases the likelihood that adult-born cells survive which may conversely facilitate further learning (Schmidt-Hieber et al 2004).

Incorporating adult-born cells into the tri-synaptic circuit is an extraordinary example of neuroplasticity and presumably one that has been conserved across mammalian species to serve specific purposes. The importance of continually renewing the granule cell population while neighboring neurons mature, or die, is highly debated, and may differ substantially from animal

to animal. Considering that most of our data come from rodent paradigms, the functional role of adult-born cells in complex human behavior and how disrupting neurogenesis contributes to psychiatric illness remains elusive (Toda et al 2019). An estimated 9000 new-born cells are added to the rat SGZ/GCL each day (Cameron & McKay 2001) or 700 for the adult human, corresponding to an annual cell turnover of 1.75% (Spalding et al 2013). In mice, genetic fate mapping was used to follow adult-born cells over lengthy periods and the data suggested that ⅓ of the granule cell population is generated during adulthood, which is comparable to estimations in the human (Ninkovic et al 2007, Spalding et al 2013). As well as learning and memory, neurogenesis may enhance cognitive flexibility and the ability to distinguish between similar contexts (pattern separation). Separating "patterns" is essential for encoding specific episodic memories so that they can be discriminated among similar experiences and to circumvent interference for their accurate retrieval (Ngo et al 2021). Suppressing neurogenesis in the rodent hippocampus can impair reference memory which can be observed in Morris water and radial arm maze tests (Clelland et al 2009, Garthe et al 2009). This is also true for object-recognition memory (Jessberger et al 2009) which is disrupted in chronically-stressed rats that have fewer DCX-IR cells (Brymer et al 2018, 2020). Some reports have indicated that adult-born cells contribute to the initial acquisition of memory in the Morris water maze (MWM) and contextual fear-conditioning paradigms (Denny et al 2012, Dupret et al 2008, Wojtowicz et al 2008) although some others do not (Deng et al 2009, Garthe et al 2009, Jessberger et al 2009), but these inconsistencies may be due to methodological differences. On the other hand, increasing neurogenesis was demonstrated to improve the ability of mice to differentiate between similar contextual representations while having no effect on normal object recognition, or spatial, contextual, and extinction learning (Sahay et al 2011). Interestingly, it is only the younger granule cells that are required for pattern separation of similar contexts while the mature cells aid memory recall, suggesting that their function switches with age (Nakashiba et al 2012). Another function of adult-generated neurons could be to aid forgetting. For instance, mice that had access to a running wheel for 6 weeks (to stimulate neurogenesis) after learning a hippocampal-dependent memory task showed poorer spatial and contextual memory performance than mice that were kept sedentary when they were re-tested (Akers et al 2014). Similarly, 4 weeks of voluntary exercise caused rats to forget contextual fear memories in a manner dependent on neurogenesis (Scott et al 2021). All in all, one can assume that immature neurons contribute to the encoding of temporal information into long-term episodic memories and may provide a mechanism to increase the efficiency of memory recall (Aimone et al 2006).

The mechanisms underlying cellular self-renewal, fate-choice, migration, and integration are governed by many signaling molecules, including kinases, growth factors, and neurotransmitters. Suppressing extracellular signal-regulated kinase 5 (ERK5) in neural stem cells indicated that the MAPK/ERK pathway regulated SGZ-neuronal differentiation and cognitive flexibility (Pan et al 2012a, 2012b). Interestingly, MAPK/ERK promotes Notch signaling, which is required to keep neural progenitor cells in a state of self-renewal (Yamashita et al 2013). Notch is expressed by type 1 and 2 cells, but neuroblasts stop this expression once they leave the cell cycle and begin to develop neuronal morphological characteristics (Yoon & Gaiano 2005). Like Notch, Disrupted in Schizophrenia 1 (DISC1) promotes basal proliferation but it negatively regulates the maturation of new-born cells; knocking down of DISC1 enhances dendritic growth, spine formation, and synaptic connections (Breunig et al 2007, Duan et al 2007). However, interrupting DISC1 results in the ectopic placement of cells, which could be related to its GSK3β-inhibiting properties (Mao et al 2009). Notch, NGF-TrkA, and BDNF-TrkB have been shown to upregulate the survival and maturation of adult-born cells and they have received attention to address depression and cognitive deficits, much like NMDARs (Bruno et al 2004, Corbin et al 2008, Johnson et al 2009, Nacher & McEwen 2006, Salama-Cohen et al 2005, Vithlani et al 2013, You et al 2021). In the SGZ, many stem cells and a good amount of their progeny express receptors that are targeted by BDNF, which potentiates neurogenesis by activating phospholipase C, PI3K-Akt, and MAPK/ERK pathways (Deng et al 2016, Donovan et al 2008). In fact, BDNF expression is highest in the hippocampus (Katoh-Semba et al 1997) and mice lacking TrkB in dentate neural progenitors had normal levels of basal neurogenesis but had no increases in response to wheel running or chronic antidepressant treatment, to which they were behaviorally insensitive (Li et al 2008). Another regulator of neurogenesis is the Repressor Element 1 Silencing Transcription factor (REST) which prevents precocious neuronal differentiation; mice lacking REST exhibit a transient elevation of new-born cells but eventually a reduction in the capacity of neural progenitor cells (Gao et al 2011). This same pattern of activity is observed in mice after the inactivation of Notch (Ehm et al 2010). Reelin, which is decreased in the hippocampus of depressed patients (Fatemi et al 2000), also governs neurogenesis through a couple of mechanisms. This could be in association with BDNF (which stimulates reelin synthesis) or through the activation of MAPK/ERK and PI3K signaling pathways (Do et al 2013, Simó et al 2006). Indeed, reelin-deficient mice have lower rates of neurogenesis compared to wild-type mice, and the opposite is true for reelin-overexpressing mice (Pujadas et al 2010, Won et al 2006). Reelin signaling is reviewed more broadly in Section 1.11.

## 1.9 Sexual dimorphisms and depression

There are many biological, psychological, and social differences between males and females that largely influence gender identity, mood, and emotional well-being. A combination of these factors contributes to the sexual dimorphisms in depression that span several facets of the disorder, such as epidemiology, symptom and biomarker presentation, treatment responsiveness, and the pathophysiological mechanisms that generate depressive episodes (Labaka et al 2018). It is important to distinguish sex from gender, the latter being a non-biological social construct that is determined by society. Although prevalence rates vary from country to country, meta-analyses indicate that females have a preponderance of most forms of depression by 1.2-2.7 fold, except depression with melancholic features which makes up 1% of depression cases similarly in both sexes (Bogren et al 2018, Luppa et al 2012, Salk et al 2017). This striking disparity represents one of the most robust and consistent findings in psychiatric research that was first discussed in 1977 (Bebbington 1996, Weissman & Klerman 1977). However, medical research has focused predominantly on males because of the female estrous hormones, which fluctuate and cycle variably, which can act as confounding factors that make it difficult to interpret research outcomes.

As well as being more susceptible, females experience depression slightly differently than males. Females are more likely to report hypochondriasis, rumination, suicide attempts, hypersomnia, fatigue/low energy, increased appetite, weight gain, and greater illness severity compared to males, who are more likely to abuse substances, lose weight, develop insomnia, engage in risky behavior, close down, and die by suicide (Marcus et al 2008, Park et al 2015b). Women, then, present with atypical depression more often than men, including somatic- and cognitive-affective symptoms (Penninx et al 2013). However, some have argued that the prevalence rates for both sexes would be equal if alternative features like aggression, risk-taking, and substance abuse behaviors were considered in the DSM criteria for depression, suggesting that men present with other symptoms that are not necessarily being captured (Martin et al 2013). This point has been criticized (Kuehner 2014), but it is important to note that men may present with these secondary symptoms whether they coincide with depression specifically or not. Depressive episodes also last for longer durations of time in females and they are at a higher risk for recurrence (Eaton et al 2008). Additionally, females are 10% more likely to develop a comorbid psychiatric disorder which can slow the diagnostic process and complicate pharmacological regimens, especially since sex influences treatment

responsiveness (Bromet et al 2011, Linzer et al 1996). Females respond better to SNRIs and even more so SSRIs than men (Khan et al 2005), and double-blinded studies indicate that females, predominantly those who are premenopausal, have superior response rates to SSRIs (like fluoxetine and sertraline) than to other classes of antidepressants whereas men respond similarly to both or favor TCAs in some cases (Kornstein et al 2000, Marcus et al 2005). This could suggest that there is a synergistic relationship between sex hormones and 5-HT, which enhances the effects of SSRIs, or it could be an interaction that impedes the effects of older-generation antidepressants. That said, the literature is questionable considering that some meta-analyses have found that there were no sex differences in treatment responsiveness to SSRIs, SNRIs, TCAs, placebo, and cognitive behavioral therapy (Cuijpers et al 2014b, Entsuah et al 2001, Quitkin et al 2002, Wohlfarth et al 2004). Not many studies have evaluated sex as a factor when demonstrating the fast-acting antidepressant effects of ketamine, but those that do show that males and females responded similarly (Freeman et al 2019, Niciu et al 2014).

Analyzing the function and volume of various brain regions can also shed light on some sex differences that likely contribute to depression. The male amygdala, hippocampus, cerebellum, and putamen may be more active than females, who have larger insular and occipital cortex, thalami, and cingulate gyri, but there are conflicting reports (Domes et al 2010, Hofer et al 2006, Madeira & Lieberman 1995, Goldstein et al 2010, Schienle et al 2005). Interestingly, antidepressants increase the volume of the hippocampus more so in responding women than non-responding women but this effect is absent in males (Vakili et al 2000). On a similar note, antidepressants were shown to increase the density of DCX in the hippocampal DG in women but not men, which could represent a mechanism whereby hippocampal circuitry is strengthened (Epp et al 2013). As well as volumetric differences, males and females appear to have impairments in differing brain centers, the former exhibiting prefrontal-striatal abnormalities and the latter prefrontal-limbic abnormalities which supports the notion that males typically show more impulsive and addictive behaviors whereas females present with anxiety (Bahrami & Yousefi 2011, Kong et al 2013, Limbrick-Oldfield et al 2013). There are also sexually dimorphic nuclei, particularly in the hypothalamus which controls hormone release and the stress response (Albert 2015).

Numerous studies conclude that sex differences in the prevalence rates of depression emerge after childhood at around age 13-15, coinciding with puberty, and the disparity widens around ages 15-18 and continues throughout adulthood (Kessler et al 1993, Twenge & Nolen-Hoeksema 2002, Wade et al 2002, Wichstrøm 1999). Not only that, but differences in

prevalence rates between males and postmenopausal women are absent (Cohen et al 2006, Freeman et al 2014), suggesting that sex hormones are largely involved in depression. These dramatic disparities in depression rates are also seen in countries all over the world, which suggests that depression has a biological basis apart from things like race, culture, education, diet, and so on (Labaka et al 2018, Seedat et al 2009). Furthermore, there is little evidence to suggest that sex differences in prevalence rates of depression are greater in countries where women have markedly lower socioeconomic status than men (Rai et al 2013). Sex hormones must be strongly implicated in these relationships. In fact, women are more likely to develop depressions during hormonal fluctuations, such as premenstrual dysphoric disorder, perinatal depression, and menopausal depression (Zagni et al 2016). Women are also more likely to report the onset of depressive episodes after reproductive events like miscarriage, infertility, contraceptive attempts, and hormone replacement therapy (Fernandez-Guasti et al 2012). Therefore, there is a clear relationship between the changing hormonal milieu and the development of depressive episodes in women whether they have a history of depression or not (Freeman et al 2006). Strikingly, women entering menopause are 5 times more likely to suffer from depression if they have a depressive history (Freeman 2010), but as hormonal flux stabilizes, episode susceptibility decreases (Bebbington et al 1998). Indeed, estrogen levels drop 100-1000 fold in the first week after childbirth, indicating that hormonal fluctuations can commonly lead to things like postpartum depression (O'hara & Swain 1996).

There are four kinds of estrogens (estradiol, estrone, estrial, and estetrol which is only produced during puberty) that readily diffuse across cell membranes where they can bind to two types of nuclear estrogen receptors (nERα and nERβ) that regulate gene expression or membrane estrogen receptors (mERs) on the cell surface (Levin 2005). By doing so, estrogens regulate the development of female secondary characteristics, anti-inflammatory events, and mental processes, including learning and memory dose-dependently (Barker & Galea 2009, Korol 2004). Indeed, treatment with estrogen significantly reduces postpartum and perimenopausal depression scores (Ahokas et al 2001, Soares et al 2001) and it has been known to reduce anxiety (Chen et al 2013, Walf & Frye 2007). This could be because estrogens lower MAO activity, increase dopaminergic D2 and 5-$HT_{2A}$ receptor densities in behavioral, emotional, and cognitive brain regions, and promote glutamatergic neurotransmission (Barth et al 2015, Fink et al 1996, Rekkas et al 2014). Glutamate levels are not only higher in females but change over the course of the estrous cycle, with higher levels of estrogen lowering glutamate reuptake

(Frankfurt et al 1984, Sajjad et al). Estrogen is also important for the antidepressant effects of conventional medications like SSRIs (Martínez-Mota et al 2008).

Progesterone binds to the nuclear progesterone receptors that also function as transcription factors by binding to specific response elements on target genes and membrane progesterone receptors that activate G-proteins (Thomas & Pang 2012). This produces anti-inflammatory effects (Hardy et al 2008, Lei et al 2014, Pettus et al 2005) which presumably complement its neuroprotective qualities that have been observed in brain injury models (Cervantes et al 2002, Dang et al 2011, Robertson et al 2006, Roof et al 1996, Roof et al 1997). Progesterone partially agonizes glucocorticoid receptors – 100-fold less than CORT – and antagonizes mineralocorticoid receptors for which it has a higher affinity compared to cortisol (Attardi et al 2007, Lei et al 2012, Rupprecht et al 1993). Progesterone can also be metabolized into allopregnanolone which allosterically increases $GABA_AR$ activity and by doing so, can successfully treat postpartum depression (Frieder et al 2019, Wilkinson & Sanacora 2019). This balance of inhibitory and excitatory signaling by sex hormones (Barth et al 2015) may explain why female rodents exhibit greater behavioral improvements in response to ketamine compared to males, especially considering that ovariectomized rats improve poorly unless they are treated with estradiol and progesterone (Carrier & Kabbaj 2013, Eid et al 2019, Franceschelli et al 2015). Similarly, rats in the proestrous phase of the reproductive estrus cycle are more sensitive to ketamine compared to rodents that are in the diestrus phase or males, who had a decrease in BDNF levels in response to ketamine whereas females had an increase (Zhang et al 2021a).

Interestingly, estrogen and progesterone compliment neurogenesis by boosting cell proliferation and survival (Barker & Galea 2008, Chan et al 2014, Galea 2008, Mazzucco et al 2006, McClure et al 2013). However, it has been shown that cell proliferation is stimulated only with acute treatment and medium doses and not lower or higher doses given chronically (Tanapat et al 2005). As well, rats that are in the pro-estrous phase of the estrous cycle have 50% increases in cell proliferation compared to when they are in phases with lower estrogen levels (Westenbroek et al 2004). Likewise, stunting hormone production by ovariectomizing rodents impairs adult-born cell proliferation (Tanapat et al 1999). Estrogen is known to stimulate the synthesis of BDNF and activate MAPK/ERK signaling pathways which triggers CREB phosphorylation (Galea et al 2017). The question then, is, if estrogen and progesterone can combat depression, then why do men, who lack systemic estrogen have lower rates of depression? The answer seems to lie in the fact that hormone levels fluctuate drastically and during these times one is more susceptible to depression. Moreover, the relatively stable levels

of testosterone in the male brain can be converted into estrogen by the enzyme aromatase (Gillies & McArthur 2010), which may provide more constant protection, especially in hippocampal neurons where hormone receptors are abundant (McEwen & Milner 2007).

Testosterone masculinizes the brain in the early stages of development by activating androgen receptors in the cytoplasm of target cells, which belong to the same nuclear receptor superfamily as progesterone, estrogen, and glucocorticoid receptors (Wu & Shah 2011). Males synthesize around 20 times more testosterone a day than females but it is metabolized faster, leaving circulating levels around 7 times higher (Torjesen & Sandnes 2004). The neuroprotective properties of testosterone are exemplified in hypogonadal males who have much lower levels of testosterone and are more likely to develop depression than males without the disorder (Shores et al 2005, Westley et al 2015, Zarrouf et al 2009). Indeed, testosterone replacement therapies have been found to alleviate depression more effectively than placebo (Kanayama et al 2007, Pope et al 2003, Zarrouf et al 2009). Women who have lower levels of testosterone are also more vulnerable to depression, generalized anxiety disorder, social phobia, and agoraphobia, and antidepressants raise testosterone in circulation (Giltay et al 2012, Kumsar et al 2014). Animal studies have shown that testosterone decreased depression-relevant behavior, normalized hormonal alterations, and enhanced the positive effects of conventional antidepressants in rats (Martínez-Mota & Fernández-Guasti 2004, Wainwright et al 2016). Interestingly, female rats only have antidepressant-like responses to testosterone if they have not been ovariectomized, indicating the importance of normal gonadal tone (Carrier & Kabbaj 2012, Frye & Walf 2009). Similarly, the reduction in antidepressant responsiveness with age is probably related to the paralleled reductions in testosterone with age, which has been shown in male rats that were treated with citalopram (Herrera-Perez et al 2010, Reynolds & Kupfer 1999).

There are several ways by which testosterone may elevate mood, such as promoting the survival of adult-born dentate cells (Spritzer & Galea 2007). This may be related to the fact that testosterone-androgen receptor complexes can non-genomically enhance the action of other androgen receptors by interacting with kinase Src, stimulating the phosphorylation of genomic receptors by activating MAPK and Akt (Leung & Sadar 2017). This may explain how testosterone increases the synthesis of BDNF (Ormerod et al 2004). Testosterone also antagonizes the neurotrophic p75 and TrkA receptors which influence things like cell survival and neuritogenesis (Anagnostopoulou et al 2013, Gravanis et al 2012, Lazaridis et al 2011). Like allopregnanolone, testosterone increases GABAergic transmission by positively

allosterically modulating $GABA_A$Rs which has antidepressant effects (Frieder et al 2019, Kanayama et al 2007, Pope et al 2003, Wang 2011). Additionally, it can also be transformed into dihydrotestosterone which is more potent than testosterone through androgen receptor activation (Askew et al 2007). Evidence suggests that testosterone also has anti-inflammatory effects considering that there is an inverse relationship between plasma testosterone levels and a number of inflammatory markers, including TNF-α and interleukin (IL)-1 and IL-6 (Bianchi 2018).

These huge differences in male and female biology influences one's sensitivity to stress, a strong predictor of depression. In fact, sex differences in depression correlate with sex differences in the HPA-axis (Zagni et al 2016). Males have a greater ability to inhibit the HPA-axis via its feedback loop because estrogen enhances HPA-axis activity, whereas testosterone does the opposite (Bingaman et al 1994, Goel & Bale 2010, Handa et al 1994, Lund et al 2004). Therefore, stress hormone levels peak when females have high circulating levels of estrogen (Atkinson & Waddell 1997). Males may inhibit the HPA-axis more effectively because they have higher levels of GAD and $GABA_AR$ activity (Goel & Bale 2010). Furthermore, stress impacts hippocampal plasticity and dendritic morphology in males and females differentially which may have important implications on depression prevalence rates (McEwen 2000). There are also sex differences in the expression of cytokines, because women have higher levels of IL-6 (Birur et al 2017, O'Connor et al 2007), which correlates with introversion (Chapman et al 2009). Strikingly, women are up to 9 times more likely to get an autoimmune disease compared to men (Whitacre 2001).

There are many neurochemical alterations that are probably related to depression that present differently between the sexes. To name some examples: women have higher levels of MAO in several regions of the brain (Spies et al 2020) and animal studies corroborate that higher levels of estrogen are associated with lower levels of MAO-A activity in the amygdala, locus coeruleus, and hypothalamus (Sacher et al 2010); males produce 52% more 5-HT than females (Nishizawa et al 1997) who have lower $5HT_{2A}R$ activity (Biver et al 1996) and region-specific differences in $5\text{-}HT_{1A}R$ activity (Jovanovic et al 2008, Szewczyk et al 2009); males have lower levels of glutamate in several brain regions (Grachev & Apkarian 2000, Zahr et al 2013) and serum (Stover & Kempski 2005, Teichberg et al 2009); and males have less active dopamine and vesicular monoamine transporters than females (Dluzen & McDermott 2008, Simpson & Kelly 2012). Interestingly, ketamine only increases dopamine in the PFC of male rats (Locklear et al 2016), and female rats express more GluN2B which may suggest that they are more

vulnerable to excitotoxicity (Wang et al 2015). Indeed, women with postpartum depression have higher levels of glutamate than controls which can over-activate NMDARs (McEwen et al 2012). There are also sex differences in molecules that regulate mood like oxytocin. For example, in women, oxytocin facilitates more positive-associated social judgments, but in men, more negative-associated judgments (Ditzen et al 2013, Fischer-Shofty et al 2013, Hoge et al 2014, Preckel et al 2014, Scheele et al 2014). It is also worth noting that oxytocin receptors are differentially expressed and distributed in males and females in areas involved in mood regulation, such as the amygdala (Francis et al 2002). It seems that there are no sex differences regarding the rate of oxytocin synthesis or metabolism, but the density of oxytocin receptors is higher in males (Caldwell 2018, Häussler et al 1990, Rice et al 2017), though there are contradictory findings (Dumais et al 2013).

Social and psychological factors also largely contribute to depression and will be briefly discussed here. Since birth, boys and girls are typically treated somewhat differently, including the types of clothes and toys that we receive. Interestingly, even male monkeys prefer to play with "boyish" toys over "girlish" toys, unlike female monkeys (Hassett et al 2008). There are also different expectations of boys and girls which influences how people treat and interact with one another and assumably the development of personality traits such as bravery, leadership, and aggression. Perhaps these pressures sculpt men into "mentally tough" individuals who internalize negative emotions less often than women, who are thought to be more emotionally intelligent and sensitive to environmental stressors (Chaplin 2015, Cyranowski et al 2000, Hankin & Abramson 2001). It has been suggested that women are exposed to childhood trauma more often than men, but a meta-analysis found that females were actually less likely to experience potentially traumatic events, apart from sexual assault (Tolin & Foa 2006). Victimization is associated with depression in both sexes, but women report more incidences of physical assault by their male partners, sexual harassment in occupational or educational settings, and other horrific acts like rape or kidnappings (Koss et al 1994). On a similar note, higher incidences of depression in women could be related to the fact that they depend more greatly on relationships or affiliative needs (Cyranowski et al 2000, Nolen-Hoeksema 2001). It is also well-accepted that females earn less money than males, increasing financial hardship. Indeed, financial problems or a decrease in socioeconomic status was associated with depression for males and females (Reiss 2013), but poverty is one of the most predictive factors for depression in women, which suggests that the economic inequalities that women often face in society may contribute to the sexual dimorphisms related to depression

(Belle & Doucet 2003). Therefore, women may be more likely to experience learned helplessness, although this is true for males in rodent studies (Dalla et al 2008). Personality traits have also been analyzed in relation to depression and the results indicate that neurotics have higher prevalence rates and more women identify as such (Fernandez-Guasti et al 2012). Introversion/extraversion is also associated with depression and antidepressants increase extraversion scores (Klein et al 2011, Jylhä et al 2009). Personality traits like introversion can also be predicted by high levels of pro-inflammatory cytokine IL-6, which demonstrates the importance of the immune system in mood (Chapman et al 2009). Overall, it seems like gender as a socially constructed status may influence emotion in some individuals more than others (Zagni et al 2016), but that biological femininity is more strongly associated with depression than gender (Sanfilipo 1994, Szpitalak & Prochwicz 2013). However, it is difficult to abolish this disparity considering that we do not fully understand how the environment interacts with genetic and psychological factors to trigger depression. Researchers also argue that advancing experimental efforts in this field are hindered by the lack of consideration of sex as a biological factor and the uniqueness of the female physiology (Eid et al 2019).

### 1.10 Animal models and behavioral tests for the study of depression

It is important that animal models are developed to study the neurochemical alterations that cause human psychiatric disturbances and to develop effective treatment approaches but generalizing experimental findings from laboratory animals to humans can be tricky. For obvious ethical reasons, there is only so much we can learn from human experimentation, which is often hard to interpret considering that confounding variables are abundant in our everyday lives that we cannot control, like genetics, our environment, and social status. Although humans interact with the environment in complex ways, we can hypothesize that the behavior of humans in controlled by similar contingencies as that of animals. In other words, studying an animal cannot tell us why humans act but they can identify factors or neurochemical networks that influence such behavior. Despite rats and humans separating from a common ancestor approximately 80 million years ago, almost all the genes that we have identified to be associated with human disease have highly conserved counterparts in the rat genome (Huang et al 2004). Rodents are used to model human conditions most often due to their low-cost, size, and quick reproductive rates. However, it is difficult to model conditions that are human-specific because we assume that animals do not self-reflect, which means it is

impossible to truly mimic symptoms like suicidal ideation, low self-esteem, excessive guilt, and feelings of worthlessness. Not only that, but the etiology of depression is complex and remains largely elusive, so researchers often focus on a particular facet of a disorder. Behavioral tests have been developed to screen for behavioral alterations that are invoked by experimental manipulation and stress generally increases what is interpreted as depressive-like behavior. But while there are many animal models for depression that researchers use to determine the efficacy of novel compounds and elucidate their mechanisms, there is no "best model" because their value depends on the research question at hand. That said, some common practices have been established that help standardize results across different laboratories and draw conclusions. These include but are not limited to the exposure of animals to environmental stress during development or adulthood, genetic manipulation (gene deletion or overexpression), and biological manipulations (targeted lesions, surgical procedures, and optogenetic control) (Planchez et al 2019).

In 1969, a couple of researchers proposed that valid models should fulfil certain criteria (McKinney & Bunney 1969). They stated that: models should exhibit phenotypes that can be reasonably compared to human symptoms; those behavioral changes can be measured objectively; behavioral abnormalities can be reversed by treatments that are effective in humans; and the results of animal studies should be easily replicable between researchers. These were later molded into similar criteria termed face, predictive and construct validity by Willner (1990). Face validity refers to the degree of similarity between the pathophysiological and phenomenological disturbances in the model and the human condition, including biomarker validity. One must remember that isomorphisms are in regards to the meaning of the behavior and not the material similarity (Belzung & Lemoine 2011). For example, rats often build nests in their home cage which can be thought of as analogous to daily human activities, and they are curious animals so alterations in exploratory behavior in threatening environments may be indicative of anxiety. This is important to keep in mind for molecules as well; CORT is the primary stress hormone in rodents which is analogous to human cortisol. Humans do express CORT, but it has a weaker affinity than cortisol for GRs and it is mainly used as an intermediate in the steroidogenic pathway (Miller & Auchus 2011). Following this line of thought, since many depression patients exhibit cognitive deficits and despair, it is important to evaluate deficits in things like working memory and decision making, and the coping strategies they adopt in aversive situations like inescapable forced-swimming (Commons et al 2017, Porsolt et al 1977). A model will have strong predictive validity if it captures similar

responses to drugs that are used to treat human conditions. For instance, the time course of decreases in despair-like behavior by antidepressants should parallel decreases in the depression scores of patients. The FST somewhat achieves this considering that chronic but not acute treatment with antidepressants decrease FST-immobility (Fenton et al 2015, Holick et al 2008), ketamine does this rapidly (Duman 2018, Murrough et al 2013), and psychedelics but not ketamine do this persistently, which is also true in patients (Hibicke et al 2020, Kadriu et al 2020b). That said, often models have troubles replicating the therapeutic time-delay of antidepressants because acute treatment with drugs like imipramine, for example, has been shown to rescue depressive-like behavior in rodents (Kawai et al 2018), but not always (Detke et al 1997). Of course, there are many variables that would influence both antidepressant responsiveness and stress vulnerability, such as the method of stress, strain, sex, and environmental enrichment like access to a running wheel or paired housing. Something that is also given a lack of consideration for in regard to predictive validity is the percentage of subjects that should be considered non-responders/treatment-resistant (Planchez et al 2019). To have construct validity, the relevant phenotypical alterations must be generated from equivalent etiological factors. An example of a theoretically sound model would be one that exposed animals to stress, considering that stress has deleterious consequences that strongly predict depression. In other words, the model or behavioral test should measure what it is supposed to measure as closely as possible and the more criteria an animal model satisfies the more reliable the model will be (Campos et al 2013). However, the reliability of animal models have been questioned given that it is difficult to distinguish between an animal model for, say, autism, depression, or schizophrenia (Belzung & Lemoine 2011). It is also difficult to feasibly model the cyclicality and chronicity of disorders especially adhering to ethical guidelines, indicating that we know very little about the recurrence of certain conditions. Before we discuss some ways whereby researchers model depression, it is important to describe some behavioral tests that are used to evaluate the product of experimental manipulations.

There are many behavioral changes that can be observed in animals following exposure to chronic stress, such as changes in exploratory behavior, fear-conditioning, escape responses, anhedonia, and changes in memory performance. Although findings are generally replicable, they can sometimes differ drastically between laboratories due to differences in facilities, the experience of researchers, whether the data is manually or automatically collected, the arena or maze, and experimental paradigms. The FST is the most widely used behavioral test to evaluate antidepressant efficacy (see Figure 1.10A). The procedure was demonstrated first by

Porsolt et al (1978) who subjected rodents to a 15-minute forced-swimming session in which the rodents would learn of the inescapable nature of the cylinder that was filled with water to depths that would not allow them to support themselves with their tails. The next day they placed animals in the water for 5 minutes and measured the amount of "despair-like" behavior that they had acquired based on their previous forced-swim. Researchers usually score the amount of time the rodent spends climbing, swimming, and immobile; climbing is used interchangeably with struggling which is given credit when the rodent tries to climb the walls of the tank franticly, swimming is scored when the rodent moves around the tank including diving to the bottom, and immobility is scored when the rodent uses just enough movement to stay afloat. Porsolt argued that immobility is an indicator of behavioral despair brought on by learned helplessness in the 15-minute pre-swim, and that immobility was sensitive to antidepressant treatment in the post-swim (Porsolt et al 1978). In other words, antidepressants increase the amount of time a rat spends trying to escape the tank before giving up or conserving energy. This 2-day procedure attracted criticism, some arguing that immobility could be adaptive rather than depressive-like, such that the rodents learn and remember that they will be removed from the tank and therefore do not need to waste energy. In a 1-day forced-swim session, the confounding effects of memory are removed and rodents that are stressed are consistently more immobile than those that are unstressed, and this phenomenon is reversible with antidepressants (Brymer et al 2020, Fenton et al 2015, Hibicke et al 2020, Lebedeva et al 2020, Marks et al 2009). The TST works on a similar premise; mice who are suspended by their tails struggle for shorter durations if they were previously stressed and more if they are treated with antidepressants. Others have argued that the FST measures coping strategy in a stressful situation rather than despair (Commons et al 2017). Considering the complexity of human depression that is not captured by simple rodent behavioral tests, it is better to think of the FST as a tool to screen for antidepressant efficacy rather than a model of depressive-like behavior. To elaborate, antidepressant drugs reliably decrease immobility, so evaluating how certain compounds influence immobility could shed light on the pharmacological mechanisms that generate antidepressant activity – the issue here is that antidepressants do not work well in humans and so relying on such measures to fulfil predictive validity has been criticized. That said, one could argue that the FST has strong predictive validity considering that SSRIs require chronic treatment to reduce FST-immobility whereas ketamine requires a single dose, and psychedelic drugs normalize immobility behavior persistently (Hibicke et al 2020). Another issue with this test is the subjectivity of behavioral

scoring. For example, the classification/interpretation of immobility by one laboratory is likely to differ from the thresholds set by another laboratory.

The OFT is one of the most common behavioral tests that can shed light on rodent emotionality (see Figure 1.10B). Open-field arenas can be used to assess general locomotor and exploratory (distance traveled, velocity, and rearing) behavior but also anxiety, which commonly co-occurs with depression. Typically, the rat is placed into a well-lit arena that usually has large walls and they are left to explore for 5 minutes. Rodents will usually avoid the center of the arena, especially if they have been subjected to stress, and antidepressants increase the amount of entries and time spent in the center (Ramirez & Sheridan 2016). The shape and diameter of the arena can vary between laboratories, and how/what behavioral parameters are measured. The light/dark test (LDT) is like the OFT, but half of the arena is shaded and the other half well-lit, and reductions in time spent in the lit area would be interpreted as avoidance, i.e., anxiety-like. The EPM is also designed to elicit anxiety and it is so-called because of its "+" shape (see Figure 1.10C). Two of the arms are open and two of the arms are closed-in by large walls (opposite arms), and one would assume that antidepressants would increase exploration into the open arms, which stressed rats are expressly threatened by. It is becoming easier to collect more accurate data with the use of video tracking software that can record the animal's activity live, but there may still be variations in results due to subjectivity. To give an example, rats display peering behavior so one would get vastly different results if they considered a rat to have crossed into an arm when 2 paws have entered the area compared to if all 4 paws have.

The sucrose preference test (SPT) is often used to model anhedonia, or the loss of pleasure in once pleasurable activities (see Figure 1.10D). The rats are habituated to having 2 water bottles of water followed by 2 bottles of a sucrose solution (usually around 1%), before their consumption from both bottles are measured when one is filled with regular water and the other filled with sucrose water. Because rats usually prefer to drink from the sweetened solution, a reduction in sucrose preference fulfils anhedonia face validity. Indeed, stressed rats lose this preference and it can be rescued by drugs that have antidepressant effects in treatment-resistant patients (Hesselgrave et al 2021, Zhang et al 2014). It is important to control for the side preference that animals display. This is because a rat may choose to drink from the right side over the left side of their cage for various reasons, but especially so if before the test, the rat had 1 bottle and it was situated on the right, for instance.

Cognition is modelled mainly be evaluating working and long-term memory performance. Tests like the object-location (OBL) and object-in-place (OBIP) tests are based on the innate preference of rodents to explore a novel object over a familiar one (see Figure 1.10 E/F) and they are hippocampal-dependent. In the former, the rodents are exposed to 2 identical objects in an open-field and then again with one of the objects switched to a different location (Howland & Cazakoff 2010). By doing so, one can measure how long the rat spends exploring the object in the novel location. The OBIP test is similar but 4 different objects are presented and 2 of their positions are switched (novel), and CORT impairs object-recognition in both tasks (Brymer et al 2018, 2020). The preference to explore novelty also extends to environments and spatial recognition memory can be examined using Y-maze arenas. There are different variations of tasks performed using Y-shaped arenas, but usually, the test requires several trials. For instance, rats may be placed into the arena with one of the arms blocked off. In a subsequent trial the rat will have a chance to explore all 3 arms (see Figure 3.10G) and its preference to explore the novel arm is measured to make inferences about memory, which is impaired by stress (Wright & Conrad 2005). This test is heavily influenced by visual cues around the room. The MWM is another good example of a spatial learning and memory task that involves placing a rat into a large pool of water multiple times so that it learns to find a hidden platform that it can stand on. You can measure the latency to find the platform each day to assess learning, and if you remove the platform after they have learned its position, one can measure how long they spend swimming trying to find it in the target area. If one were to place the platform in a novel location, it would allow one to also assess re-learning as a measure of cognitive flexibility (Darcet et al 2014). There is no single test that can capture all depression-like behaviors and so using a battery of tests can provide better insights in the mental state of animals. Other common tests may include social and sexual interaction tests (which are usually rewarding), tail flick tests (pain response), fear conditioning, home-cage monitoring, marble burying, and novelty induced hypophagia (anxiety). Observations of depressive-like behavior in a greater number of tests would increase the face validity of the animal model for the disorder.

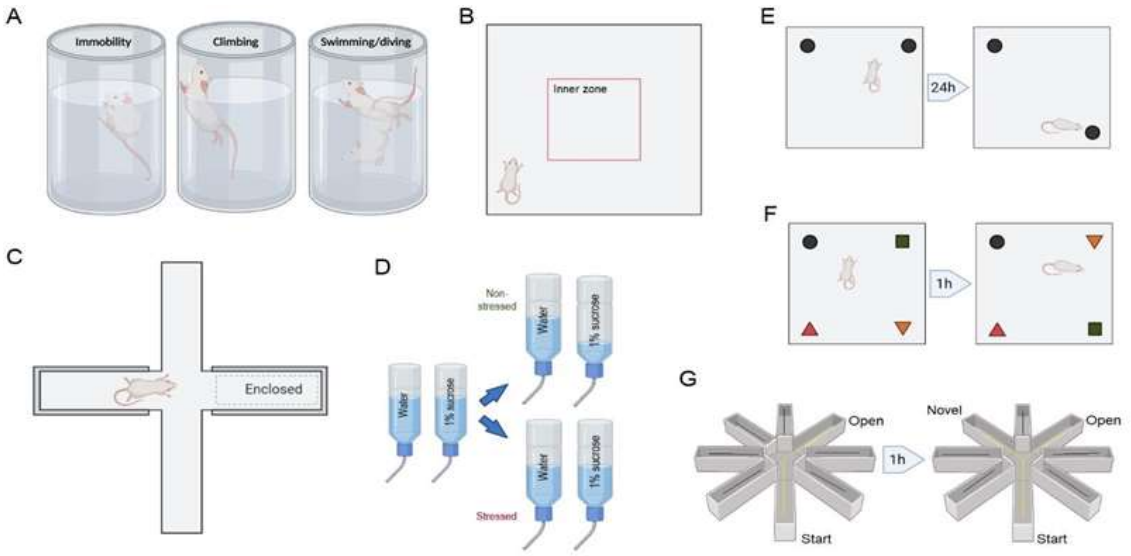


**Figure 1.10. Common rodent behavioral tests used to evaluate depressive- and anxiety-like phenotypes.** The FST is used as a screening tool for antidepressant efficacy and to make inferences about emotionality (A). General exploratory behavior and anxiety can be measured in the OFT (B) and the LDT is similar, but half of the arena is lit up and the other half shadowed. An increased time spent in the closed arms of the EPM is indicative of anxiety (C). Sucrose preference can be measured to evaluate anhedonia-like behavior (D). The OBL test (E) and the OBIP test (F) are used to measure hippocampal-dependent cognition. The Y-maze can be used to assess spatial recognition memory (G). Multiple measures should be used to provide convergent evidence of behavioral abnormalities. Figure created with BioRender and paint by author.

Perhaps the most widely used rodent model is the CUMS model, which involves exposing the animal to a series of mild but unpredictable stressors, such as periods of water and food deprivation, changing the number of animals in the cage or the temperature of the room, exposure to flashing lights and/or mild shocks, and disruption of the 12-hour light-dark cycle for at least 2 weeks. It is important that stressors are not predictable to avoid habituation. After periods of stress, rodents display altered locomotor, sleep, sexual, and anhedonia-relevant behavior (D'Aquila et al 1994, Grønli et al 2004, Schweizer et al 2009). This model has good predictive and construct validity, but the presentation of symptoms (face validity) can vary a lot between laboratories due to things like stressor variations and animal husbandry (Willner et al 1987).

Environmental stress such as exposure to inescapable foot or tail shocks increases learned helplessness-like behavior which is evident by an increase in the latency to escape a threatening environment when given the option, altered sleep patterns, and the development of coat conditions (Bali & Jaggi 2015, Seligman et al 1980). Indeed, depression patients often report

stressful situations to be out of their control so learned helplessness-based models are widely used, and antidepressants and ECT are effective at rescuing helpless behavior (Sartorius et al 2003). Social defeat stress, which consists of placing a rat (usually a male) into a new environment that contains conspecifics, who act defensive and attack the intruder, is another model that instills learned helplessness and other symptoms that are depression-like and rescued by chronic treatment (Blanchard et al 2001, Meerlo et al 1996, Venzala et al 2012).

Some laboratories focus on early life stress considering that childhood adversities are a strong predictor of depression later in life (Lindert et al 2014, Shapero et al 2014, Smith & Pollak 2020). Stressors may include handling the pups at a very early age, separating the pups from dams, or exposing them to prenatal stress which show higher incidences of learned helplessness- and depressive-like behaviors (Andersen 2015). Even providing a limited amount of nesting material causes stress to the dams and results in neglectful behavior by the mother, who spend less time grooming their offspring (Ivy et al 2008). Other laboratories attempt to induce depression-relevant phenotypes by subjecting rodents to chronic restraint stress (Bravo et al 2009, Regenthal et al 2009), but the results with this model are more inconsistent (Gregus et al 2005, Lussier et al 2009). This is likely due to the habituation to the stressor over time and exemplifies why CUMS paradigms are advantageous. Genetic- (e.g., Flinders Sensitive Line and Wistar Kyoto strain rats) and lesion-based (e.g., olfactory bulbectomized rats) models have also been used to screen for antidepressant efficacy, and to help researchers understand the possible underlying mechanisms involved in depression, but a review of these models is beyond the scope of this thesis.

### 1.10.1 Exogenous CORT administration as a model for chronic stress

Our laboratory has been using a chronic CORT-administration paradigm to model the neuroendocrinological abnormalities that are observed in depressed patients. As we have discussed, stress induces depressive-like phenotypes in rodents and depression is associated with an overactive HPA-axis response. It is difficult to evaluate the direct effects of stress on behavior and neurochemistry in many models because there is a lot of variability in the response to psychological and physiological stressors between animals. Our laboratory chooses to model chronic stress (relevant for depression) by subjecting rats to 3 weeks of daily CORT injections, which allows us to provide each rat with the same amount of CORT dependent on

bodyweight and directly examine the deleterious effects of stress on the brain (Sterner & Kalynchuk 2010). CORT can be administered in several ways, such as in food or water, pellet implantation, osmotic pump infusion, or subcutaneously. Rodents are typically exposed to 21 consecutive days of CORT but shorter and longer exposure periods have been used. The CORT model is isomorphic and predictive in that depressive-phenotypes that are comparable to human symptoms are induced reliably and antidepressants can rescue CORT-induced behavioral and neurochemical abnormalities (Brymer et al 2020, Fenton et al 2015, Johnson et al 2006, Lebedeva et al 2020). Construct validity is also met with satisfaction considering that higher cortisol levels are commonly observed in depressed patients (Carroll et al 2007, Holsboer 2001), but it is important to remember that the levels of CORT used are often supraphysiological (exceeding plasma concentrations of 5mg/kg) to induce depression reliably in a given time frame. This physiological difference in concentration levels represents the major limitation of the model. Table 1.10.1a below details experimental findings that show some similarities between CORT-treated animals and depressive symptoms.

The body of literature that demonstrates the face validity of the CORT model is growing. Indeed, plenty of work has demonstrated that CORT increases despair-like behavior in the FST (Ali et al 2015, Brummelte et al 2006, David et al 2009, Gourley & Taylor 2009, Gregus et al 2005, Wróbel et al 2017) in a dose- and time-dependent manner (Lebedeva et al 2017, 2020, Lussier et al 2013a). That is, daily 40mg/kg doses produce greater disturbances in mood than 5mg/kg, 10mg/kg, and 20mg/kg (Johnson et al 2006, Marks et al 2015), as do longer durations of administration compared to shorter durations (Gregus et al 2005, Lussier et al 2013a), and depressive-like behavior worsens with each additional cycle of CORT treatment when interspersed with recovery periods (Lebedeva et al 2017, 2020). Increases in immobility behavior are also observed in the TST as well (Ma et al 2018, Zhao et al 2008b), and like FST-immobility, it can be reversed by treatment with antidepressants (David et al 2009, Fenton et al 2015, Rainer et al 2012). The fact that immobility normalizes after a recovery period in which no CORT is given whereas bodyweight does not, indicates that immobility cannot be explained by the weight of the animals. As well, these behavioral phenotypes occur independent to any differences in muscle strength. CORT-treated rats hold on to a wire suspended in the air above a soft landing just as long as control rats (Marks et al 2009) and travel similar distances in the OFT (Brotto et al 2001, Gregus et al 2005, Peng et al 2021), which suggests that changes in the FST and TST are not accounted for by muscle atrophy.

**Table 1.10.1a. Comparison of human symptoms of depression with CORT alterations.**

| Human symptom | Animal Test | CORT alterations | Reference |
|---|---|---|---|
| Anxiety | OFT | ↓ entries & time in centre | 19, 20 |
| | | ↔ distance travelled | 4, 5, 19, 20, 22, 25 |
| | EPM | ↓ time spent in open arms | 2, 7 |
| | LDT | ↓ time in light section | 2 |
| | POT | ↓ contact with aversive stimuli | 5 |
| Anhedonia | SPT | ↓ sucrose consumption | 11, 13, 14, 33, 34 |
| | NSF | ↑ latency to feed | 6, 19 |
| Despair | Splash test | ↓ grooming | |
| | FST | ↑ immobility | 1, 2, 3, 4, 5, 10, 13, 15, 16, 17, 19, 22, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34 |
| | TST | | 6, 17, 34 |
| Spatial learning & memory deficits | Y-maze | ↔ spatial memory | 21 |
| | | ↓ learning/memory | 25 |
| | NORT | | 23 |
| | Barnes maze | | |
| | MWM | | 12, 23 |
| | OBL | | 24, 28 |
| | OBIP | | 24 |
| Sociability deficits | SIT | ↔ social interaction | 4, 32 |
| Decreased libido | SBT | ↓ sexual behaviour | 8 |
| Weight gain/loss | Weight | ↓ weight | 1, 4, 5, 9, 13, 18, 28, 29, 32 |

**Abbreviations:** ↑ = increase, ↓ = decrease, ↔ = no difference, EPM = Elevated plus maze, FST = Forced Swim Test, LDT = light/dark test, MWM = Morris Water Maze, NORT = Novel Object Recognition Test, NSF = Novelty Suppressed Feeding, OBIP = Object-In-Place Test, OBL = Object Location Test, OFT = Open-field Test, POT = Predator Odor Test, SBT = Sexual Behavior Test, SIT = Social Interaction Test, SPT = Sucrose Preference Test, TST = Tail Suspension Test.

**References**: [1]Lebedeva et al 2017, [2]Luo et al 2017, [3]Wróbel et al 2017, [4]Gregus et al 2005, [5]Kalynchuk et al 2004, [6]David et al 2009, [7]Myers & Greenwood-Van Meerveld 2007, [8]Gorzalka et al 2001, [9]Johnson et al 2006, [10]Brummelte et al 2006, [11]Gorzalka et al 2003, [12]Sousa et al 2000, [13]Gourley & Taylor 2009, [14]Gourley et al 2008, [15]Hill et al 2003, [16]Lussier et al 2013a, [17]Zhao et al 2008b, [18]Lussier et al 2013b, [19]Rainer et al 2012, [20]Li et al 2017, [21]Hill et al 2014, [22]Marks et al 2009, [23]Darcet et al 2014, [24]Brymer et al 2018, [25]Notaras et al 2020, [26]Brummelte et al., 2010a, [27]Romay-Tallon et al 2018, [28]Brymer et al 2020, [29]Lebedeva et al 2020. [30]Yuan et al 2020, [31]Murray et al 2008, [32]Chan et al 2017, [33]Nickle et al 2020, [34]Ali et al 2015, [35]Coburn-Litvak et al 2003.

Behavior that is indicative of anhedonia is also increased in rodents that are treated chronically with CORT. For instance, CORT decreases the amount of sucrose water drank compared to regular water versus controls, who would usually choose to drink from the sweetened solution (Ali et al 2015, Gourley et al 2008, Gourley & Taylor 2009, Kvarta et al 2015, Ma et al 2018). Similarly, normal grooming (Darcet et al 2014, David et al 2009) and social behaviors (Berger et al 2019, Chan et al 2017) are disrupted by CORT which appears to be analogous of the lack of self-care and social isolation that is observed in depression (Little 2004, Taylor et al 2018).

Like humans, rats usually find sweetened solutions and social interactions rewarding, which suggests that CORT interferes with reward-seeking behavior. Indeed, treating male mice and rats with CORT impaired food-seeking (Peng et al 2021) and decreased sexual behavior (Gorzalka et al 2001), respectively, which are also indicative of anhedonia, but CORT may increase sexual behaviors in females (Gorzalka et al 2001, Hanson & Gorzalka 1999).

Anxiety-like behavior has been detected in the CORT model as well using arenas like the open-field, LDT, and elevated plus-maze (EPM). CORT (40mg/kg) given daily for 3 weeks can significantly decrease the time a rat spends in the center of an open-field and increase the latency to enter the inner zone (Li et al 2017); this tendency of rodents to explore the peripheral zone of the open-field is often referred to as thigmotactic behavior (thigmotaxis being an organism's response to touch). The LDT and EPM are also designed to evaluate exploratory behavior in novel, threatening environments, and CORT-treated mice tend to avoid the light or open-arms, respectively (Luo et al 2017, Murray et al 2008, Peng et al 2021). Anxiety can also be measured by evaluating hyponeophagia, or the inhibition to feed induced by novelty which can be used to interpret anxiety, i.e., a care-free rat will approach the food in a novel environment with less hesitation (Berger et al 2019). However, often CORT is less effective at producing anxiety-like behavior in rats than mice (Gregus et al 2005, Hill et al 2014, Kalynchuk et al 2004), and this has been known to extend to FST/TST-immobility (Brotto et al 2001, Notaras et al 2020), social deficits (Gregus et al 2005), and the SPT (Berger et al 2019), which demonstrates that the animal strain, husbandry, and method of drug delivery are important variables when designing experiments. Of course, sex is another factor that largely influences animal behavior and CORT (10 daily 20mg/kg injections) has even been shown to increase immobility in male rats but paradoxically decrease it in females (Brotto et al 2001).

Valuable attempts to model certain aspects of psychiatric disease have focused on fear conditioning and the evaluation of defensive behaviors and learning and memory. Indeed, low, and high doses of CORT heighten the fear response which was demonstrated by an increase in freezing behavior during the retrieval of tone cues that were previously paired with foot shocks (Marks et al 2015). Similarly, CORT potentiated defensive behaviors in male rats who contacted an aversive cat collar less in a predator odor task than CORT-free rats (Kalynchuk et al 2004), which may resemble certain high-anxiety human states, such as walking through dangerous neighborhoods at night, standing up to a bully, or other forms of harassment. Spatial memory and learning deficits are also seen in rodents that are treated with CORT in tests like

the Barnes maze and MWM. Generally, CORT increases the amount of time it takes to learn how to escape aversive environments and impairs cognitive flexibility (Darcet et al 2014, Sousa et al 2000). CORT also impairs the ability of rodents to recognize novel objects (Darcet et al 2014) or familiar objects that have been moved to a novel location (Brymer et al 2018, 2020). Deficits in spatial memory were also evident in a Y-maze task in mice that were treated with CORT (26.8mg/kg) for 56 days but not 21 days (Coburn-Litvak et al 2003). In addition, hippocampal-dependent memory deficits are exacerbated in males that were subject to CORT and maternal separation to form a two-hit model of stress, but females exhibited more anhedonia-like behavior, which was absent in males (Hill et al 2014). This could suggest that females have a protective mechanism in the hippocampus – probably related to sex hormones – but that they are more sensitive to environmental stressors that influence anhedonia-like behavior.

Bodyweight is also altered in those that are depressed and significantly decreased in rodents that receive CORT dose- and time-dependently (Brummelte et al 2006, Gregus et al 2005, Johnson et al 2006, Scherer et al 2011), which may be linked to increases in the levels of the hunger-inhibiting hormone leptin (Perry et al 2019). However, CORT has been shown to increase food intake in mice regardless of leptin levels and on the other hand, leptin reduced food intake regardless of CORT levels, although the animals were only given 3 doses of CORT (Arvaniti et al 1998). Weight loss it most likely related the fact that CORT mobilizes energy stores (glucose) as opposed to reducing food intake during the experimental period, and this process involves the inhibition of insulin which would otherwise store glucose and thereby decrease its availability. That does not mean to say that interactions between things like cortisol, leptin, and insulin are not involved in the regulation of appetite/hunger in humans, or rodents for that matter (Friedman & Ramirez 1994, Rodin 1985).

**Table 1.10.1b. Effects of sex and CORT on behavioral testing.**

| Test | Strain | Sex | Dose | Finding | Reference |
|---|---|---|---|---|---|
| OFT | Wistar | F | 20mg/kg s.c. | No differences | 3 |
| | L-E | M | 40mg/kg s.c. | | 4 |
| | S-D | | 40mg/kg s.c. | ↑thigmotaxic behav. | 10 |
| | C57BL/6Ntac & CD1 mice | | 35 ug/ml/day | | 6, 8 |
| FST | L-E | | 20mg/kg & 40mg/kg s.c. | ↑immobility | 1 |
| | | | 40mg/kg s.c. | | 4, 12, 14, 17, 29 |
| | C57BL/6 mice | | 20mg/kg | | 22 |
| | | | 35 ug/ml/day or 5 mg/kg/day | | 19 |
| | C57BL/6Ntac & CD1 mice | | | | 6, 8, 25 |
| | Swiss Albino mice | | 40mg/kg s.c. | | 2, 20 |
| | Wistar | F | 20mg/kg s.c. | | 3 |
| | S-D | F | 40mg/kg i.p. | | 23 |
| | S-D | M | 40mg/kg s.c. | | 27 |
| | S-D | M | 50µg/ml | | 21 |
| | L-E | M & F | 20mg/kg | No differences but females<males | 26 |
| | L-E | M & F | 40mg/kg s.c. | ↑immobility/females < males | 5 |
| SPT | | M | 50/mg/kg s.c. | ↓sucrose preference | 7 |
| | S-D | F | 40mg/kg i.p. | | 23 |
| | C57BL/6 mice | M | 25-100ug/ml in water | | 11 |
| | C57BL/6 mice | M & F | 45.11mg/L in water | No differences | 24 |
| | Wistar | M & F | 50mg/L in water | No differences | 15 |
| SPT | Swiss Albino mice | M | 40mg/kg s.c. | ↓sucrose preference | 20 |
| TST | | | | ↓mobility | |
| TST | C57BL/6Ntac mice | M | 35 ug/ml dissolved in water | | 6 |
| | | | 20mg/kg s.c. | | 13 |
| POT | L-E | M & F | 40mg/kg s.c. | ↓contact with aversive stimuli | 5 |
| LDT | Swiss Albino mice | M | 40mg/kg s.c. | ↓time spent in light section | 2 |
| EPM | Wistar | M & F | 50mg/L in water | No differences | 15 |
| | Swiss Albino mice | M | 40mg/kg s.c. | ↓time spent in open arms | 2 |
| | C57BL/6Ntac & CD1 mice | | 35 ug/ml/day or 5 mg/kg/day | | 6 |
| NSF | | | 35 ug/ml/day or 5 mg/kg/day | ↑latency to feed | 6, 8 |
| Y-Maze | Wistar | M & F | 50mg/L in water | No differences | 15 |
| | C57BL/6 mice | M | | ↓learning/memory | 18 |
| NORT | | | 35 µg/ml/day in water | ↓time with novel object | 16 |
| Barnes maze | | | | ↓time spent in target section | |
| MWM | | | | | |
| | S-D | | 40mg/kg s.c. | | 9 |
| OBL | L-E | | | ↓learning/memory | 17, 29 |
| OBIP | | | | | |

**Abbreviations**: EPM = Elevated Plus Maze, FST = Forced Swim Test, i.p. = intraperitoneal, L-E = Long-Evans, LDT = Light/Dark Test, MWM = Morris Water Maze, NORT = Novel Object Recognition Test, NSF = Novelty Suppressed Feeding, OBIP = Object-In-Place Test, OBL = Object Location Test, OFT = Open-field Test, POT = Predator Oder Test, S-D = Sprague-Dawley, s.c. = subcutaneous, SPS = Sucrose Preference Test, TST = Tail Suspension Test.

**References**: [1]Lebedeva et al 2017, [2]Luo et al 2017, [3]Wróbel et al 2017, [4]Gregus et al 2005, [5]Kalynchuk et al 2004, [6]David et al 2009, [7]Gorzalka et al 2003, [8]Rainer et al 2012, [9]Yau et al 2011, [10]Li et al 2017, [11]Gourley et al 2008, [12]Lussier et al 2013a, [13]Zhao et al 2008b, [14]Marks et al 2009, [15]Hill et al 2014, [16]Darcet et al 2014, [17]Brymer et al 2018, [18]Notatas et al 2020, [19]Yuan et al 2020, [20]Ali et al 2015, [21]Kvarta et al 2015, [22]Ma et al 2018, [23]Nickle et al 2020, [24]Berger et al 2019, [25]Murray et al 2008, [26]Brotto et al 2001, [27]Chen et al 2017, [28]Sousa et al 2000, [29]Brymer et al 2020.

There are also many neurochemical alterations that are similar in CORT-treated rodents and the depressed patient. Perhaps one of the most studied similarities is the suppression of neurogenesis. Our laboratory found that CORT-induced reductions in neurogenesis, as measured by DCX-IR cell numbers and dendritic complexity, paralleled the progressive development of depression-like behavior (Lussier et al 2013a). Moreover, imipramine and anti-inflammatory agent etanercept can rescue these deficits (Brymer et al 2018, Fenton et al 2015), whereas acute ketamine failed to normalize the expression of DCX (Johnston et al 2020). Interestingly, pre-pubescent female rats that were treated with chronic CORT did not exhibit depressive-like behavior or reductions in neurogenesis, but peri-pubescent rats expressed despair- and anhedonia-like behavioral alterations that were accompanied by reductions in neurogenesis (Nickle et al 2020). As one might expect, the reductions in reward-seeking behavior (and anxiety-like behavior in the EPM) were associated with a decrease in excitability of dopaminergic VTA neurons (Peng et al 2021). Depressive-like states induced by CORT also correlate with impairments in excitatory strength at temperoammonic-CA1 synapses due to reductions in neurotransmission mediated by AMPARs (Kvarta et al 2015). Indeed, our group has shown that CORT dampens the expression of GluA1 in the SGZ, which is essential for neuroplasticity, but increases the expression of hippocampal GluN2B, which is associated with excitotoxicity, as discussed in Section 1.5 (Brymer et al 2020). Similarly, CORT decreased $GAD_{67}$ levels and the number of $GABA_A$Rs in the amygdala, and decreased $GAD_{65}$ and $GABA_A$Rs in the hippocampus, while increasing hippocampal levels of type 2 vesicular glutamate transporters, whereas restraint stress had no effect (Brymer et al 2018, Lussier et al 2013b). This means that less GABA will be produced, and more glutamate will be released from synaptic vesicles. Furthermore, unlike CORT, restraint stress was ineffective at producing depressive-like behavior, emphasizing the involvement of dysfunctional inhibitory/excitatory neurotransmission in the pathogenesis of depression. The hippocampus appears to be expressly sensitive to the deleterious effects of CORT, where it causes dendritic retraction of dentate granule cells, pyramidal cells of the CA, and mossy CA3 fibers (Sousa et al 2000, Woolley et al 1990). Interestingly, acute CORT treatment was sufficient to induce dendritic hypertrophy in the fear-registering amygdala (Mitra & Sapolsky 2008). Stress not only interferes with the growth of neurons but dysregulates their organelles. For instance, 20mg/kg of daily CORT for 3 weeks causes substantial mitochondrial damage which subsequently increases oxidative and inflammatory events, creating a vicious cycle of incidents that lead to cellular excitotoxicity and apoptosis (Allen et al 2021a, Ma et al 2018). As well, CORT increases the expression levels of mitochondrial-associated pro-apoptotic factor BAX, and decreases BAX-inhibitor

Bcl-2, which forms complexes with GRs that also become downregulated following chronic stress (Du et al 2009a, 2009b, Gądek-Michalska et al 2013, Juárez-Rojas et al 2015, Xu et al 2019). Interestingly, chronic CORT treatment did not result in behavioral phenotypes relevant for depression when the BAX gene was deleted from neural stem cells (Eliwa et al 2021, Hill et al 2015). Taken together, the studies outlined above provide concrete evidence that subjecting rodents to repeated CORT can be used as a reliable tool to study the pathological mechanisms that are relevant for depression and how the effects of chronic stress on the brain and behavior can be reversed. Our laboratory has been using this model to evaluate the effects of stress on hippocampal reelin expression, which will be discussed further in the following section.

## 1.11 Reelin has important functions in development and adulthood

Reelin is a protein that has a wide range of functions in the developing and adult brain, and our laboratory has been interested in how reelin activity may combat depression for the last decade (Caruncho et al 2016). Reelin derives its appellation from the "reeler" mouse phenotype that was first described by Falconer in Edinburgh, 1951, when a spontaneous autosomal recessive mutation arose in a colony of mildly inbred mice (Falconer 1951). These mutant homozygous reeler mice (*RELN*$^{-/-}$) exhibited an abnormal "reeling" gait due to some severe neuronal abnormalities which were uncovered in the 1960s and 1970s due to the complete loss of transcription of the *RELN* gene, which was mapped and named in 1995 (Caviness 1976, Hamburgh 1963, Jossin 2020). Another mutation was later identified in Orleans which left mice unable to successfully secrete reelin because it was structurally malformed in that a part of the 8$^{th}$ so-called "reelin repeat" (RR) and the C-terminal failed to transcribe (Ranaivoson et al 2016). Both of these mutations caused neuronal ectopia in laminated brain structures like the hippocampus, cerebellum, and cortex, as well as many other, more subtle abnormalities, that lead to tremors, ataxia, impaired motor coordination, and usually early death around the time of weaning (Cooper 2008, D'Arcangelo et al 1995). Indeed, *RELN* mutations in humans causes lissencephaly, or "smooth brain", because the defective neuronal migration leads to a lack of gyri and sulci formation (Kato & Dobyns 2003). These observations grabbed the attention of scientists who have since gathered large amounts of data that has helped explain how reelin

facilitates proper brain development, the maintenance of brain activity in adulthood, and how disrupting reelin signaling is implicated in psychiatric disorders.

The reelin sequence encompasses 3461 amino acids that are coded by the *RELN* gene (which is localized to chromosome 5 in mice and 7 in man), and it has a molecular mass of 388 kDa (de Bergeyck et al 1998, Ranaivoson et al 2016). Structurally, reelin is composed of an N-terminal, followed by 8 RR (350-390 amino acids each), which connects to a short, basic C-terminal region that is 32 amino acids in length (Ichihara et al 2001). At the amino acid level, human reelin is 94.2% similar to the mouse version which suggests that there is a strong conservation of function across some species (DeSilva et al 1997). Reelin can be cleaved by members of the Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMT) family of proteins between the 2$^{nd}$ and 3$^{rd}$ RR (the N-t site) and between the 6$^{th}$ and 7$^{th}$ RR (the C-t site) (Koie et al 2014, Sato et al 2016). ADAMTS2/3 cleaves reelin at the N-t site and ADAMTS4/5 can cleave reelin at the N-t and C-t site (Jossin 2020, Okugawa et al 2020). Therefore, cleavage separates the N-terminal that is made up of an F-spondin-like amino acid sequence and RR1-2, the central fragment that is made up of RR3-6, and the C-terminal region which includes RR7-8, and certain aspects of reelin signaling are regulated by the individual fragments. Cleavage can occur in the extracellular space and in the endosomes, although the physiological importance of this remains elusive (Koie et al 2014). The C-terminal expresses a Furin recognition site which releases the last 6 amino acids in the chain, and the function of this is also far from being understood (Ranaivoson et al 2016). There is evidence that processing at this site is not required for the proper positioning of neurons during embryonic corticogenesis but that it may be involved in cell placement in the cerebellum and maintenance of the marginal zone in the postnatal cerebral cortex (Kohno et al 2015, Nakamura et al 2016). The necessary binding elements that reelin requires to activate its receptors are located within RR3-6, but possibly just RR5-6 (Knuesel 2010, Nakano et al 2007).

In the developing brain, reelin is secreted by Cajal-Retzius cells in a synthesis-dependent rate in the marginal zone of the cortex and hippocampus, and glutamatergic cerebellar cells (Lacor et al 2000, Schiffmann et al 1997, Tissir & Goffinet 2003). Once released into the extracellular space, reelin binds to the very-low-density-lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), two receptors of the evolutionarily ancient lipoprotein superfamily (Beffert et al 2005, 2006). The volume of the extracellular space comprises ~20% of the brain and is vital for the movement and structural integrity of neurons and their plasticity (Laham & Gould, 2022). Neurons have similar amounts of ApoER2 mRNA than radial glia but they have

10-fold more VLDLR mRNA (Hartfuss et al 2003). Some studies indicate that reelin is more affinitive for ApoER2 in comparison to VLDLR (Andersen 2015, Benhayon et al 2003, Yasui et al 2007), but another found similar affinities (Hiesberger et al 1999).

Binding reelin causes the receptors to cluster together and the signal is relayed by disabled-1 (Dab1) which docks intracellularly to the receptors and gets phosphorylated at specific tyrosine residues by Src family kinases (SFKs) Fyn and Src, inaugurating the downstream effects that regulate neuronal migration (Bock & Herz 2003, D'Arcangelo 2014). Dab1 was brought to our attention when the so-called scrambler and yotari mutant mice, which lack a functional *disabled-1* gene, were identified to have phenotypes that closely resemble reeler and VLDLR/ApoER2 knockout mice (Howell et al 1997, Sweet et al 1996, Yoneshima et al 1997). This illuminates the interconnectedness of reelin, its receptors, and Dab1, and their essentialness in proper brain development (Sheldon et al 1997, Trommsdorff et al 1999). Dab1 goes on to activate important downstream effectors like the PI3K/Akt/mTOR and Crk/CrkL/Rap1 pathways which have multiple roles that are detailed in Figure 1.11 and will be important to keep in mind throughout the following sections.

Reelin could be described as a cyto-architect considering that it orchestrates the typical inside-out birth order of the laminated cortex during embryonic corticogenesis by governing cell-cell interactions that position migrating neurons (Chameau et al 2009). To do so, reelin induces a radial glial phenotype in progenitor cells of the ventricular zone (through interactions with Notch-1 which co-expresses with Dab1 in the DG) that project fibers which are used as scaffolds by new-born neurons to climb to their destinations in the cortex (Chai et al 2009). Cells that develop early are destined to occupy the deep layers of the cortex whereas late-born neurons migrate past these cells using the radial glia to reach more superficial layers (Chai et al 2009). It could be that reelin acts as an attractant for the newly generated neurons that migrate past their predecessors (Gilmore & Herrup 2000). Interestingly, VLDLR knockout mice express neurons that have invaded the marginal zone whereas ApoER2 knockouts express migration defects of late-generated neurons that scaffold past their younger neighbors, indicating that they have divergent roles in cellular migration (Hack et al 2007). Therefore, VLDLR appears to transmit a “stop signal” that terminates the adhesion of the migrating neuron to the radial glial cell (Dulabon et al 2000, Qiu et al 2006a). This is also true for migrating neuroblasts from the subventricular zone along the RMS to the olfactory bulb and from the DG SGZ to the GCL (Hack et al 2002). This illustrates why animals that are deficient in reelin develop an inverted cortex with an outside-in birth order (Cooper 2008), and the

central fragment of reelin alone can sufficiently actuate the necessary intracellular signals that govern proper layer formation in cortical slice cultures (Jossin 2004, Wasser & Herz 2017).

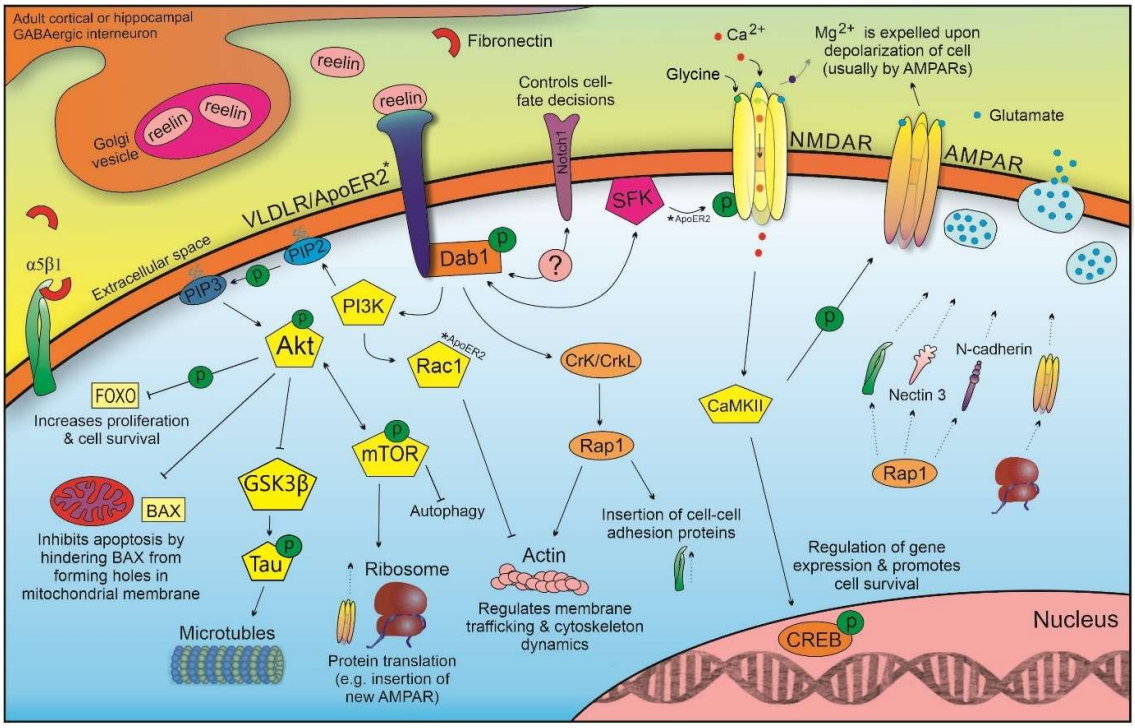


**Figure 1.11. Simplified schematic of reelin signaling pathways.** Upon binding to VLDLR or ApoER2, cytoplasmic adaptor protein Dab1 gets phosphorylated by SFKs Fyn and Src. Phosphorylated Dab1 can also activate SFKs that in turn phosphorylates the NMDAR, which increases the influx of calcium that leads to the activation of CaMKII. CaMKII diffuses into the nucleus and phosphorylates CREB where it regulates gene transcription. CaMKII also phosphorylates AMPARs, increasing signal channel conductance. As well, phosphorylated Dab1 can activate Crk/CrkL. This induces Rap1 activation, which alters the expression of cell-cell adhesion proteins in the membrane. Rap1 also stimulates effector proteins that, when activated, play a role in actin dynamics and membrane trafficking. PI3K is also activated by phosphorylated Dab1, which leads to Rac1 activation (ApoER2-dependent) and the modulation of cytoskeleton dynamics. In addition, PI3K phosphorylates PIP2 into PIP3, which can then activate Akt. Downstream signaling pathways of Akt promote cell survival, protein translation and microtubule dynamics: Akt phosphorylates FOXO which obstructs its ability to inhibit proliferation; it targets BAX to inhibit apoptosis; it inactivates GSK3β to lessen Tau phosphorylation and affects microtubule dynamics; it binds to Rheb which activates mTOR, a regulator of protein translation and autophagy inhibitor. Reelin also promotes Notch1 signaling and vice versa, effecting cell-fate decisions and synaptic plasticity, possibly via Notch1-Dab1 interactions. Figure created with CorelDraw by author.

The expression of reelin ceases by Cajal-Retzius cells in the postnatal brain, but reelin is repurposed as a neuromodulator by cortical and hippocampal GABAergic interneurons and glutamatergic mitral cells in the olfactory bulb, granule cells in the cerebellum, and layer II pyramidal cells in the piriform and EC (D'Arcangelo et al 1997, Knuesel 2010, Pesold et al 1998). In these areas, reelin regulates cell signaling systems that subserve cognitive flexibility by enhancing neurogenesis, the growth of dendrites and formation of dendritic spines, synaptogenesis, and synaptic strength (Beffert et al 2006, Niu et al 2008, Rogers et al 2013, Teixeira et al 2012, Weeber et al 2002). Indeed, transgenic mice that overexpress reelin display increases in adult neurogenesis, cell survival, dendritic hypertrophy, and the number of synaptic contacts (Pujadas et al 2010). Another study found that over- or under-expression of reelin did not affect the number of dendritic spines but led to changes in their morphology, and low levels of reelin compromised the configuration of presynaptic boutons in the DG (Bosch et al 2016a). Dendritic outgrowth seems to depend on the regulation of the actin cytoskeleton and membrane protein trafficking via Crk family proteins, which would explain why low levels of Crk/CrkL are associated with reductions in the dendritic complexity of CA1 neurons (Matsuki et al 2008). Crk is a signaling adapter protein that gets tyrosine phosphorylated following reelin signaling, and it activates Rap1 in a Dab1-dependent manner (Ballif et al 2004, Reedquist et al 2000). Double Crk/CrkL mutant mice exhibit a cortical phenotype much like the reeler mouse (Park & Curran 2008). Interestingly, a single intraventricular injection of reelin was shown to enhance cognitive function, synaptic plasticity, and LTP in a mouse model of Angelman syndrome (Hethorn et al 2015), wild-type mice (Rogers et al 2011), and in heterozygous reeler mice (*RELN*$^{+/-}$) (Rogers et al 2013). Further evidence that reelin modulates neuroplasticity and memory formation was gathered with the use of VLDLR and ApoER2 knockout mice. Mice that lack reelin receptors have reductions in synaptic density and abnormal neuronal migration which leads to memory impairments (Mulder et al 2004, Trommsdorff et al 1999).

One of the ways reelin appears to enhance synaptic plasticity is by regulating glutamate receptor activity via an indirect mechanism. To be more specific, stimulating Dab1 can activate SFKs that are physically associated with NMDARs and phosphorylate GluN2B and GluN2A subunits to enhance their current throughput (Chen & Leonard 1996, Köhr & Seeburg 1996, Yu et al 1997). The application of reelin to hippocampal slices was shown to enhance NMDAR- and AMPAR-dependent synaptic strength in the CA1, but not in slices that were taken from VLDLR- and even more so ApoER2-lacking mice (Weeber et al 2002). This was corroborated

in a later study which found that ApoER2 was essential for reelin to elevate NMDAR-mediated currents at postsynaptic densities (Beffert et al 2005). The increases in AMPAR number and NMDAR-mediated glutamatergic transmission by reelin are also absent when the slices are pretreated with PI3K or Rap1 inhibitors (Qiu et al 2006b). Reelin may even be involved in neurotransmitter release, considering that *RELN*$^{+/-}$ have lower levels of protein SNAP25, which is required for synaptic vesicles to bind to the plasma membrane (Hellwig et al 2011). Another way reelin enhances neuroplasticity is by activation of the PI3K/Akt pathway, which stimulates mTOR and enhances dendritic branching in the hippocampus and insertion of AMPARs (Jossin & Goffinet 2007). This pathway also inhibits GSK3β which protects the microtubule-stabilizing protein Tau from hyperphosphorylation and slows the formation of intracellular neurofibrillary tangles and extracellular amyloid-β fibril accumulation (Beffert et al 2002, Pujadas et al 2014), which are hallmark features of Alzheimer's disease (Hiesberger et al 1999, Tort-Merino et al 2019). This protects from synaptic dysfunction by preserving the trafficking and stability of glutamate receptors, and reelin can prevent amyloid-β-induced LTD (Durakoglugil et al 2009). Interestingly, amyloid-β can activate mGluRs (Renner et al 2010), which prompted researchers to investigate the protective role of reelin in mGluR-agonism-induced LTD. Durakoglugil et al (2021) reported that the application of reelin to hippocampal slices regulated neuronal excitability by preventing the dephosphorylation of GluA2 mediated by mGluRs.

Importantly, reelin is a high-affinity ligand for integrin receptors that co-localize in dendritic spines and postsynaptic densities (Rodriguez et al 2000), and that participate in synaptic plasticity and cognition by modulating the formation, stabilization, and functional maturation of synapses (Qiu et al 2006a). For instance, the N-terminal of reelin binds to the integrin α3β1, which was shown to contribute to the strengthening of synapses by α3β1 function-blocking antibodies (Kramar et al 2002). Another study demonstrated that mice deficient in α3 (as well as α5 and α8) integrins have deficits in spatial memory and LTP in the CA1 (Chan et al 2003). Integrins also contribute to neuronal positioning, as complexes containing reelin, VLDLR/ApoER2, and α3β1 increase the synthesis of activity-regulated cytoskeleton protein in synaptoneurosomes and changes the cellular adhesion of migrating neurons to glia, arresting their journey (Dong et al 2003, Dulabon et al 2000, Förster et al 2002). In response to reelin signaling, other integrins like α5β1 (Dab1/Crk/Rap1-dependent) can promote the adhesion of migrating neurons to extracellular protein fibronectin in a Dab1-dependent manner (Sekine et al 2012). Although integrins contribute to the proper alignments of neurons and influence levels

of Dab1, the inactivation of α3- or β1-class integrins does not induce a phenotype like that of the reeler mouse (Anton et al 1999, Graus-Porta et al 2001), suggesting that, unlike reelin, they are not necessary for cortical development (Dulabon et al 2000, Jossin 2004). That said, integrin receptors are required for reelin to induce the developmental "switch" that occurs in the expression of NMDAR subunits GluN2A and GluN2B. To elaborate, the ratio of NMDARs that express GluN2B compared to GluN2A is higher in the early postnatal brain, but GluN2A begin to predominate as synapses mature over time (Cui et al 2013, Sinagra et al 2005, Wang et al 2009). This switch seems to be dependent on reelin due to its downstream activation of SFKs, which increases the surface mobility of GluN2B and reduces their time at the synapse (Qiu & Weeber 2007, Sinagra et al 2005). This is evident as blocking reelin receptors or β3 integrins interrupts this developmental process (Chavis & Westbrook 2001, Groc et al 2007), and *RELN*$^{+/-}$ have increased GluN2B expression, whereas reelin-overexpressing mice under-express GluN2B (Isosaka et al 2006, Teixeira et al 2011). After full-length or central reelin has bound to its receptor, it is internalized into endocytic vesicles where it can undergo further proteolysis that separates the N-terminal for re-release (Hibi & Hattori 2009). Some reports have suggested that cleavage at the N-t site jeopardizes the phosphorylation of Dab1 (Kohno et al 2009, Kubo et al 2002), but it has also been shown that levels of phosphorylated Dab1 and the downregulation of Dab1 in cultured neurons were similar after several hours of treatment with normal reelin compared to mutated reelin that is uncleavable at this site (Jossin 2004, Koie et al 2014). A more recent study also found that central fragment of reelin was able to phosphorylate Dab1 as efficiently as full-length reelin, which suggests that cleavage of reelin at either site may be necessary to free the central fragment so that it can diffuse and target cells over longer distances (Jossin 2020). However, Koie et al (2014) also found that cleavage here is necessary for the clearance of reelin that has been internalized, and to halt Dab1 phosphorylation machinery; Dab1 was maintained at a low level 24 hours after uncleavable reelin was removed from the cultured neurons, which was not true normal, unmutated reelin. This indicates that cleavage may be necessary to regulate the duration of reelin signaling in the long-term by preventing immoderate Dab1 downregulation, but decreasing N-t cleavage in vivo had milder consequences (Okugawa et al 2020). ApoER2 can also be cleaved by γ-secretase which releases the intracellular domain into the cytoplasm and by proteases like α-secretase which releases a soluble extracellular domain (May et al 2003, Wasser & Herz 2017), and these events can be occasioned by acute reelin activity (Duit et al 2010). The intracellular product can translocate to the nucleus and regulate the transcription of genes, including *RELN* (Balmaceda et al 2014, Telese et al 2015), whereas the extracellular domain can go on to inhibit

reelin activity in primary neurons (Koch et al 2002), providing additional feedback mechanisms to regulate reelin signaling.

### 1.11.1 Reelin and its implications in depression

Reelin has been implicated in multiple psychiatric conditions, including autism, Alzheimer's disease, temporal lobe epilepsy, schizophrenia, bipolar disorder, and depression, which all share common neurobiological risk factors like dysfunctional synaptic plasticity. Two decades ago, the levels of *RELN* mRNA were shown to be decreased substantially in several regions of the schizophrenic post-mortem brain (Impagnatiello et al 1998), the cerebral cortex of bipolar patients with psychotic episodes (Guidotti et al 2000), and in the hippocampus from schizophrenia, bipolar, and MDD patients (Fatemi et al 2000, Knable et al 2004). In addition, Fatemi et al (2001) reported that serum reelin levels were abnormal in those with mood and psychotic disorders, a downregulation that seems to occur through epigenetic mechanisms that effect promotor methylation, like exposure to psychosocial stress (Veldic et al 2004). This has been investigated in the post-mortem brain from schizophrenic patients who were found to have hypermethylated regions of the reelin gene promoter (Abdolmaleky et al 2005). However, these findings were not replicated in a later study, but both reports are limited by a lack of statistical robustness (Tochigi et al 2008). In any case, methylation changes in the brain are paralleled by changes in the blood from schizophrenic patients, suggesting that markers of *RELN* methylation could have value as biomarkers (Auta et al 2013, Nabil Fikri et al 2017) or that patients with depression may present with similar alterations.

$RELN^{-/-}$ and $RELN^{+/-}$ are valuable tools to study the role of reelin in brain pathologies, considering that they express null or 40-60% levels of reelin, respectively. The neuroanatomical abnormalities that are present in $RELN^{-/-}$ are not witnessed in mice that are haplo-insufficient for reelin, but $RELN^{+/-}$ do exhibit subtle physiological abnormalities that have been linked to psychiatric disorders. For example, reductions in the expression PSD-95, activity-regulated cytoskeletal protein, parvalbumin, and the density and number of dendritic spines are observed, which together weaken synaptic transmission in brain areas that are relevant for depression (Dong et al 2003, Liu et al 2001, Nullmeier et al 2011, Pappas et al 2001, Tueting et al 2006, Ventruti et al 2011). Disturbances in dopaminergic and 5-HTergic systems are also evident in mice that are deficient in reelin which assumably influence mood

and motivational behavior (Ballmaier et al 2002, Michetti et al 2014, Nullmeier et al 2014, Varela et al 2015). Additionally, reductions in $GAD_{67}$ are seen in reelin-deficient mice and depression patients, which suggests that a dampening of GABAergic tone occurs when reelin levels are low (Karolewicz et al 2010, Liu et al 2001, Tripp et al 2012). On the other hand, *RELN*$^{+/-}$ express higher levels of GluN2A and GluN2B, but not their mRNA, compared to wild-type mice (Isosaka et al 2006). This dampening and potentiation of inhibitory and excitatory neurotransmission, respectively, leads one to believe that low levels of reelin would advance excitotoxic events. Interestingly, the co-localization of neuronal NOS in reelin-IR cells was reduced in the DG SGZ and ML of *RELN*$^{+/-}$, which suggests that NOS and reelin could synergistically regulate glutamatergic signaling considering that nitric oxide can also enhance dendritic spine formation and LTP (Zhou & Zhu 2009). Additionally, reelin loss-of-function in hippocampal neuroprogenitor cells impedes the proper migration and dendritic maturation of adult-born dentate granule cells, whereas heightened reelin activity promotes dendritic outgrowth and proper circuit establishment (Teixeira et al 2012). This is important because deficient hippocampal neurogenesis, dendritic atrophy, and excitotoxicity are characteristic of depression (Frodl et al 2006, Jacobs et al 2000, Monaco et al 2015).

Despite the neurochemical differences between *RELN*$^{+/-}$ and wild-type mice, they are phenotypically indistinguishable from one another in a variety of behavioral tests. This includes the OFT, black–white box, novelty-suppressed-feeding test, FST, and sensitization to cocaine, which were used to model aspects of mood disorders (Teixeira et al 2011). However, *RELN*$^{+/-}$ mice have a poorer memory span than wild-types, and this is truer for males than females (Iemolo et al 2021). A reelin-deficiency was also shown to contribute to the long-term abnormalities in social behavior and reactivity to aversive situations caused by the heavy consumption of tetrahydrocannabinol, a psychoactive compound found in cannabis (Iemolo et al 2021). Similarly, VLDLR and ApoER2 knockout mice display deficits in contextual fear conditioning (Weeber et al 2002). On the other hand, Teixeira et al (2011) found that reelin over-expressing mice spent less time immobile in the FST and had a reduced behavioral sensitization to cocaine compared to wild-type mice, which was associated with reductions in GluN2B-containing NMDAR-mediated activity. Therefore, low levels of reelin may represent a vulnerability factor for psychiatric illness whereas high levels may be protective. To test the idea, our laboratory subjected *RELN*$^{+/-}$ and wild-type mice to repeated-CORT injections and found that treatment significantly decreased active behaviors in the FST and neurogenesis only in those that were reelin-deficient (Lussier et al 2011). This was corroborated more recently by

another group who revealed that there were no differences in FST-immobility and spatial memory in the Y-maze between *RELN*$^{+/-}$ and wild-type mice in the absence of CORT, but deficits in these tests were potentiated in *RELN*$^{+/-}$ when 50mg/L of CORT was added to their drinking water for 21 days (Notaras et al 2020, Schroeder et al 2015). Furthermore, they found that male *RELN*$^{+/-}$ had poorer social recognition memory in comparison to wild-type mice regardless of CORT treatment, and that CORT increased reelin expression in the PFC whereas we found that s.c. CORT dose-dependently decreased reelin expression in the hippocampus in male rats (Lussier et al 2011, Schroeder et al 2015). Interestingly, chronic CORT exposure decreases the co-expression of neuronal NOS and reelin in the hippocampus of wild-type mice but has the opposite effect in *RELN*$^{+/-}$, which suggests stress exacerbates oxidative events when reelin levels are low and may explain some of the behavioral and cognitive deficits associated with this vulnerability (Romay-Tallon et al 2010, 2015). These experiments indicate that low levels of reelin sensitize one to the depressogenic effects of stress and that increasing reelin signaling could improve stress resilience (Fatemi 2011).

Chronic stress also consistently decreases the expression of reelin in rodents that do not express genetic vulnerabilities. For instance, exposing pregnant rats to restraint stress for 2 hours a day between embryonic days 11 and 20 decreased reelin-IR cell counts in layer I of the cortex in their offspring (Palacios-García et al 2015). Furthermore, they found that there were no differences in the number of NeuN-IR cells (which is widely used to label neurons), indicating that stress-induced reelin deficits are due to differences in gene expression and not neuronal death. Our laboratory has shown that CORT exposure decreased the number of reelin-IR cells in the hippocampal SGZ by 26% and CA1 stratum-lacunosum-moleculare by 21%, but restraint stress had no effect on reelin expression (Lussier et al 2009). This is interesting because restraint stress, unlike chronic CORT treatment, was ineffective at producing depression- and anxiety-like behavior and alterations in GABAergic and glutamatergic neurotransmission (Gregus et al 2005, Lussier et al 2009, 2013b). Considering that reelin expression by GABAergic interneurons is dampened by stress in the SGZ, where adult neurogenesis occurs, we investigated the effects of stress on reelin, neurogenesis, and depression-like behavior over time. Rats were treated with CORT (40mg/kg) for either 7, 14 or 21 days, and we found that the time-dependent increases in FST-immobility were paralleled with reductions in the number of reelin- and in the number and dendritic complexity of DCX-IR cells. Therefore, it is possible that the downregulation of reelin potentiates depression-like behavior by delaying the maturation of new-born cells and interfering with their proper placement and integration into

the existing circuitry (Caruncho et al 2016). We went on to show that acute treatment with ketamine rescued the CORT-induced deficits in hippocampal reelin but not neurogenesis (Johnston et al 2020), and that chronic treatment with imipramine and TNF-α inhibitor etanercept rescued deficits in reelin and neurogenesis (Brymer et al 2018, Fenton et al 2015). Another group also showed that repeated-citalopram treatment counteracted the downregulation of reelin mRNA and protein levels instigated by neurotoxic kainic acid (Jaako et al 2011). Subjecting mice to social isolation can also induce aggression and anxiety-like behavior that is associated with deficits in allopregnanolone and reelin expression in the frontal cortex, hippocampus, and amygdala, and allopregnanolone treatment normalized these deficits (Nin et al 2011b). Interestingly, it was recently demonstrated that the genetic depletion of reelin, ApoER2, or the pharmacological inhibition of their downstream effectors abolishes the neuroplastic and antidepressant-like behavioral effects of ketamine (Kim et al 2021). Together, these studies strongly indicate that antidepressants may require reelin signaling to achieve their therapeutic response.

This motivated our laboratory to evaluate the antidepressant-like and pro-cognitive effects of reelin in the repeated-CORT paradigm of chronic stress. Reelin (1μg) was infused directly into the hippocampus either 3 times over the 3-week CORT-injection period or once at the end, 24 hours before the FST took place. We found that repeated and singular injections of reelin rescued FST-immobility, hippocampal-dependent cognitive deficits in the OBL, and deficits in AMPARs, NMDARs, and $GABA_A$Rs, but not if AMPARs were blocked with antagonist CNQX (Brymer et al 2020). Furthermore, the number and complexity of new-born neurons was recovered by repeated reelin but only the number of cells was increased by a single reelin injection, unless they were sacrificed a week after the infusion as opposed to immediately after forced swimming (Brymer et al 2020). This, and the fact that CNQX did not affect neurogenesis, suggests that the enhancement of new-born granule cell proliferation and their dendritic complexity is not responsible for the fast-acting antidepressant effects of reelin. Indeed, AMPARs are usually co-expressed with NMDARs where they both fundamentally influence the plasticity of cognitive and emotional neurocircuitry and the fast-acting effects of antidepressants (Fraize et al 2017, Kadriu et al 2021, Seo et al 2020, Zhang et al 2016). Additionally, in mice, a micro-infusion of reelin in the hippocampus rescued anxiety-like behavior and cognitive deficits that were induced by antenatal inflammation (Ibi et al 2020), and a micro-infusion into the amygdala rescued aggressive and anxiety-like behavior when examined one month after a period of chronic social isolation (Nin et al 2011b). Other groups

have administered reelin via intraventricular infusions and also recovered deficits in learning and memory, which was associated with increases in GABAergic and glutamatergic tone and a strengthening of synapses in the hippocampus (Hethorn et al 2015, Rogers et al 2011, 2013), which parallel findings from in vitro studies in which hippocampal slices are incubated with reelin (Beffert et al 2005, Qiu et al 2006b, Weeber et al 2002). Table 1.11.1 below details experimental results that relate to reelin.

**Table 1.11.1. Preclinical experiments related to reelin.**

| Animal | Method | Finding | Ref |
|---|---|---|---|
| B6C3Fe WT & RELN+/- mice | Genetic underexpression of reelin | ↓ FC $GAD_{67}$, spine density, neuron & glial cell density | 1 |
| | | ↓ HC GAD67 & parvalbumin, ↔ VTA TH, RN SERT | 2 |
| | | ↓ CA1 spine density, PSD-95*, GluN2A and GluN2B | 3 |
| | | ↑ D2 & $5HT_{2A}R$ expression in frontal cortex | 4 |
| | | ↓ memory span in ♂ but not ♀, ↑ sensitivity to THC-induced abnormalities | 44 |
| | | ↑ HC glutamate & LA after acute stress, ↓cortex dopamine | 5 |
| | | ↓ VTA TH, striatum TH & DAT | 6 |
| B6C3Fe WT, RELN+/- & RELN-/- mice | | ↑ SERT cluster size on MPL (*RELN*$^{-/-}$ > *RELN*$^{+/-}$ > WT) | 7 |
| | | ↑GluN2B (*RELN*-/- > *RELN*+/- > WT) | 8 |
| | | ↓dendritic maturation* & MAP2* | 9 |
| | | ↓HC spine density*, GluN2A, SNS PSD-95 (*RELN*$^{-/-}$ < *RELN*$^{+/-}$ < WT) | 10 |
| C57BL/6J RELN+/- mice | | ↓ antidepressant effects of ketamine, LTP | 45 |
| | | ↑ GSK3β activity & Tau phosphorylation | 11 |
| C57BL/6J mice | Genetic overexpression of reelin | ↑ NMDAR currents, ↓ GluN2B, FST-immobility | 12 |
| | | ↑ dendritic maturation | 13 |
| Tg1/Tg2 mice | | ↑ HC spine density, ↔ GluN1/2A/2B, PSD-95 or CaMKII levels | 14 |
| | | ↔ spine density, ↑mushroom spines, ↓ filopodial spines | 15 |
| | | ↑spine density, neurogenesis & LTP | 16 |
| B6C3Fe mice | Bilateral ventricular injections of reelin | ↑spine density, CA1 LTP, associative & spatial LaM | 17 |
| UBE3A null mutation (AS) mice | | | 18 |
| B6C3Fe WT & RELN+/- mice | | ↑ GAD67, CA1 LTP & associative LaM, ↓spine alterations | 19 |
| ICR mice | Lateral ventricle reelin injection prior to s.c. PCP | ↓ PCP-induced cognitive and sensory-motor gating deficits | 20 |
| Long-Evans rats | s.c. CORT and intrahippocampal reelin infusions | ↑ GluA1, ↓ GluN2B, FST-immobility, cognitive deficits | 21 |
| B16 mice | Apply reelin to CHS | ↑ dendritogenesis (Crk-dependent) | 23 |
| BALB/c mice | | ↑ mTOR phosphorylation (PI3K/Akt-dependent) | 24 |
| C57BL/6J mice | | ↑ TP of GluN2A & GluN2B, NMDAR currents & CA1 LTP | 25 |
| B6C3Fe RELN-/- mice | | ↑SNAP25 & vesicle release, ↔Munc18/synaptobrevin/syntaxin | 26 |
| B6C3Fe mice | | ↑ AMPAR currents & GluN2A, ↓ silent synapses & GluN2B | 27 |
| | | ↑ AMPAR & NMDAR currents, CA1 LTP & Src | 28 |
| Swiss mice | | ↑ GluN2A, ↓ GluN1 & GluN2B | 29 |
| | Blockade of reelin signalling in CHS | ↔ GluN1 & N2B (maturation prevented) | 29, 30 |
| Sprague Dawley rats | Apply reelin to cultured cortical neurons | ↑NMDAR currents, CA2+ influx, GluN2B & CREB phosphorylation | 31 |
| C57BL/6J mice | | ↑ phosphorylation of Dab1, Akt & GSK3β, ↓Tau | 32 |
| C57B1/6J WT & RELN+/- mice | CORT added to drinking water (25-50mg/L) | ↔ in WT, ↓HC GR, GluN2B, GluN2C & PFC GluN1 in ♀ RELN+/- mice | 33 |
| | | ↔ in WT, ↑FST-immobility & ↓spatial memory in RELN+/- mice | 34 |

| | | | |
|---|---|---|---|
| | | CORT ↓ FC & HC reelin in ♂ | 35 |
| | | ↓spatial & social recognition memory in RELN+/- mice | 36 |
| B6C3Fe WT & RELN+/- mice | CORT injections (s.c.) | ↑FST-immobility & deficits in neurogenesis (RELN+/- > WT), ↔OFT | 37 |
| Long-Evans rats | | CORT ↓ SGZ reelin levels | 38, 39, 40, 43 |
| | Etanercept after CORT injections | Etanercept: ↑reelin, ↓FST-immobility, cognitive deficits | 22 |
| | Ketamine after CORT injections | Ketamine: ↑ reelin, ↓ FST-immobility, cognitive deficits<br>Reelin: ↑ mTOR, p-mTOR, ↓ SERT cluster size | 42 |
| Pregnant Sprague Dawley rats | Prenatal stress | Stress ↓ cortical layer I reelin in E20 emybro brains | 41 |

**Abbreviations:** ↑=increase, ↓=decrease, ↔=no difference, *=reversed by applying reelin, AS=Angelman Syndrome, CHS=Cultured Hippocampal Slices, CREB=cAMP-response element binding protein, FC=Frontal Cortex, FST=Forced Swim Test, GR=Glucocorticoid Receptors, HC=Hippocampal, LaM=Learning and Memory, LTP=Long Term Potentiation, MAP2=Microtubule-Associated Protein 2, ML=Molecular Layer, MPL=Membrane of Peripheral Lymphocyte, OFT=Open-field Test, PSD95=Postsynaptic Density 95, RN=Raphe Nucleus, s.c. = subcutaneous, SERT=5-HT Transporter, SNS=Synaptosome, TH=Tyrosine Hydroxylase, THC=Δ9-tetrahydrocannabinol, TP=Tyrosine Phosphorylation, WT=Wild-type, VTA=Ventral Tegmental Area.

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### 1.11.2 The role of reelin in the periphery

The discovery of the reeler mouse phenotype characterized by side-to-side swaying led to intensive experimentation to elucidate the neuronal roles of the reelin signaling pathway. However, reelin is also expressed in non-neuronal tissues during development and adulthood, such as the spleen, liver, kidney, testes, ovaries, adrenal gland, lymphatic tissue, and the colon (Bottner et al 2014, Garcia-Miranda et al 2013, Ikeda & Terashima 1997, Lutter et al 2012, Samama & Boehm 2005, Smalheiser et al 2000). Reelin is also expressed by odontoblasts (Maurin et al 2004), bone marrow, and it circulates in plasma (Chu et al 2014, Dou et al 2021, Smalheiser et al 2000, Tseng et al 2010). That said, the functional roles of reelin in the periphery are much less understood, and we are unsure if full-length reelin or fragments of reelin can cross the blood-brain barrier, which would have important physiological and therapeutic implications. Interestingly, reelin-immunoreactivity has been observed in endothelial cells that line the blood-brain barrier, mainly in putative transcytosis caveolae vesicles, which may represent a mechanism whereby reelin can access the brain (Perez-Costas

et al 2015). Indeed, ApoER2 are localized in the same caveolae, which suggests that reelin may cross via a receptor-mediated mechanism (Riddell et al 2001). VLDLR are highly expressed in the heart, skeletal muscles, and the endothelium of blood vessels, whereas ApoER2 is confined to the central nervous system and testes (Herz & Bock 2002). Evidence suggests that peripheral reelin has important pleiotropic roles in the development, repair, and function of important systems.

In the lymphatic system, reelin is expressed by endothelial cells and is secreted into the extracellular space by more mature collecting lymphatic vessels, which is governed by adjacent smooth muscle cells (Lutter et al 2012). Interestingly, Lutter et al (2012) found that *RELN*$^{-/-}$ had enlarged collecting lymphatic vessels and a decrease in the coverage of smooth muscle cells which contributed to an impairment in lymphatic function. In addition, the lymphatic vessel abnormalities in *RELN*$^{-/-}$ were not present in ApoER2 and VLDLR double knockout mutant mice and Dab1 hypomorphs, suggesting that reelin influences this system by noncanonical mechanisms such as alternative reelin receptors that have yet to be identified. Reelin appears to recruit smooth muscle cells to lymphatic vessels during development by increasing the expression of monocyte chemotactic protein 1, which is involved in smooth muscle cell migration and proliferation and suggests that reelin has autocrine functions (Aplin et al 2010, Lutter et al 2012).

A well as abnormal lymphatic development, low levels of reelin or its downstream effector Dab1 interfere with the proper development of the mammary gland. Reelin signaling appears to be required for the structural organization of the mammary epithelium, considering that reelin and Dab1 knockout mice express structural abnormalities and altered cell migration in this region (Khialeeva et al 2011). This may also be true for the development of the submandibular gland since treatment with reelin stimulated branching morphogenesis and epithelial cell proliferation here (Rebustini et al 2012). Circulating reelin also promotes the vascular inflammatory response by increasing leukocyte adhesion to the vascular epithelium, which ultimately influences atherosclerosis development (Ding et al 2016). Indeed, inactivation of reelin signaling protected the vascular wall from cholesterol-induced atherosclerosis (Baitsch et al 2011). Reelin was first found to access circulation by means of secretion from liver cells (Smalheiser et al 2000), but these cells do not govern liver formation considering *RELN*$^{-/-}$ are free from liver morphological abnormalities, but they may help repair the liver after cellular damage (Ikeda & Terashima 1997). In response to injury, hepatic stellate cells up-regulate reelin expression and become activated, migrating fibrogenic cells, which has

been demonstrated in rodent models of fibrosis (Botella-Lopez et al 2008, Carotti et al 2017). Similarly, human cirrhotic patients have higher levels of reelin in blood plasma (Botella-Lopez et al 2008).

Reelin may improve the integrity of the intestinal barrier that houses stem cells that maintain rapid cellular turnover. Quick cell turnover provides a protective barrier against many lethal microorganisms by decreasing the amount of time that epithelial cells are exposed to deleterious agents (Carvajal et al 2017). *RELN*$^{-/-}$ exhibit a reduction in intestinal cell proliferation, differentiation, and migration, and this was associated with colitis-related tumorigenesis (Carvajal et al 2017). When this protective mechanism is perturbed, such as with low reelin levels, the integrity of the barrier and its functions become compromised, which can lead to intestinal or inflammatory issues (Viggiano et al 2015). A reduction in reelin levels alters the expression of genes in the intestines that are associated with apoptosis, cell proliferation and differentiation, metabolism, membrane transport, tumor development, and the inflammatory response, but no differences in the length or number of villi were reported (García-Miranda et al 2012, 2013). Similar alterations in the dynamics of the intestines are also found in Dab1 hypomorphs (Vázquez-Carretero et al 2014). It could be that low levels of reelin reduces adaptive apoptotic events in the gut (Carvajal et al 2017). This is corroborated by the fact that reductions in reelin are associated with a greater number and size of tumors in the human colon, and reelin levels could potentially be a useful biomarker for colorectal cancer (Serrano-Morales et al 2017). Interestingly, a VLDLR-deficiency partially protects from diet-induced obesity with no clear differences in food intake, with wild-type mice becoming obese and VLDLR knockout mice remaining lean, perhaps due to a lack of fatty acid uptake (Goudriaan et al 2001).

Reelin is also involved in limb development, such as the formation of digits, by utilizing protein kinase B and focal adhesion kinase, at least in mice and chick embryos (Díaz-Mendoza et al 2013). ApoER2 and VLDLR are expressed in developing limb cartilage and tendons where they regulate skeletogenesis by promoting chondrogenesis and inhibiting tendogenic differentiation, respectively (Diaz-Mendoza et al 2014). In limbs, reelin expression is upregulated by cell survival factor FGF2, and downregulated by bone morphogenic protein 7 protein (Khialeeva & Carpenter 2017). However, there must be other signaling pathways that guide proper limb development because reeler mutants do not exhibit limb malformations. Interestingly, reelin is upregulated in cartilage when it is injured or diseased (Magnani et al 2010), which is also true for the regenerative liver and the eye, which suggests that reelin is

required to heal tissue after damage has been caused (Kobold et al 2002, Pulido et al 2007). Reelin may also contribute to sensory perception in limbs because osteocytes (the mechanosensing cells of bone) express much more reelin than osteoblasts (Khialeeva & Carpenter 2017, Paic et al 2009, Rawlinson et al 2009). There is also a relationship with polymorphisms in the *RELN* gene and abnormal bone remodeling that leads to a loss in hearing (Schrauwen et al 2009). Similarly, reelin and Dab1 are expressed by odontoblasts (Heymann et al 2001), whereas VLDLR and Dab1 are found in dental pulp and in the trigeminal ganglion which stimulates tooth development, indicating that reelin could have implications in dentine innervation (Maurin et al 2004).

We know now that circulating blood is supplied with reelin by erythrocytes and platelets and it is thought to play a role in hemostasis (Chu et al 2014, Tseng et al 2010). Reelin-deficient mice have similar prothrombin test clotting times compared to wild-type mice, but they display a prolonged bleeding time and an increased rate of bleeding which is likely due to abnormal clot formation and structure (Tseng et al 2014). Tseng and colleagues (2014) found that this could be due to interactions with liposomes containing phosphatidylserine and phosphatidylcholine and not reelin receptors, and that abnormal clotting resulted from reductions in the synthesis of liver coagulation factor serine protease thrombin and enzyme Factor Xa. As well, $RELN^{-/-}$ have greater counts of red blood cells compared to wild-type mice in plasma and bone marrow which co-occurs with dampened Akt activity (Chu et al 2014).

Of course, the mechanisms that stimulate tumor growth are usually necessary for normal development. Reelin is implicated in the development of several organs by governing cellular migration and proliferation, which explains why it has been implicated in some forms of cancer. Downstream reelin effector Dab1 is also known to activate the PI3K/Akt pathway which has been shown to be hyperactive in some cancers (Larue & Bellacosa 2005). So far we have evidence that reelin is upregulated in retinoblastoma (Seigel et al 2007), esophageal carcinoma (Wang et al 2002), multiple myeloma (Lin et al 2016, Qin et al 2017), and prostate cancer (where it correlates with tumor aggressiveness) (Perrone et al 2007). On the other hand, the downregulation of reelin by transcriptional silencing was associated with hepatocellular carcinoma, breast, pancreatic, and gastric cancer and lower levels of reelin are associated with more advanced stages (Dohi et al 2010, Sato et al 2006). Additionally, the downregulation of reelin was associated with poor prognosis in breast cancer and positive lymph node status (Stein et al 2010). Reelin and Dab1 signaling are also silenced in brain tumors, and decreased reelin mRNA expression correlates with a higher malignancy grade (Schulze et al 2018). Reelin

is necessary for proper cellular migration, proliferation, and housekeeping of different cell types in multiple tissues of the periphery, so maintaining homeostatic levels of reelin is important.

### 1.11.3 The implications of peripheral reelin in depression

A connection between central reelin and depression can be made clearly, but there is a strong chance that peripheral reelin also influences the manifestation of depression. Levels of reelin are not only heavily altered in the hippocampus of depression, bipolar, and schizophrenia patients, but in blood as well, where it regulates cellular homeostasis (Fatemi et al 2001). For example, the activation of VLDLR and ApoER2 on macrophages induces an anti-inflammatory phenotype that is characterized by a reduction in the levels of free radicals and pro-inflammatory cytokines, suppressed antibody-dependent cell cytotoxicity, and upregulated anti-inflammatory cytokine secretion (Baitsch et al 2011). The importance of normal reelin signaling in lymphocytes is demonstrated in patients with schizophrenia who have lower levels of lymphocyte VLDLR expression which negatively correlates with clinical symptom severity (Suzuki et al 2008). Therefore, one can postulate that reelin may protect from depression by shielding from the deleterious effects of inflammation and oxidative stress that are commonly observed in depression patients (Allen et al 2021a, Krishnadas & Cavanagh 2012, Lindqvist et al 2017), especially since lymphocyte and macrophage cytokine and chemokine profiles are dysregulated in *RELN*$^{-/-}$ and *RELN*$^{+/-}$ (Green-Johnson et al 1995).

Reelin regulates MPC, including the clustering of its own receptors, which appears to be important not only for the proper functioning of proteins and, therefore cells, but also for the responsiveness to antidepressants (Allen et al 2007, Czysz et al 2015, Donati & Rasenick 2005, Erb et al 2016, Johnston et al 2020, Strasser et al 2004, Zhang & Rasenick 2010). The plasma membrane is a lipid bilayer that embeds organized proteins that form compartmentalized cellular microdomains that move and communicate with one another in the membrane, and how proteins cluster in these microdomains is known to alter their physiological functions (Rivera-Baltanas et al 2010). An example of a protein that can move freely within the membrane is SERT, the primary target of antidepressants. The efficient functioning of SERT is maintained by its clustering into specific lipid raft membrane domains (Magnani et al 2004), and by its subcellular distribution and oligomerization (Muller et al 2006, Schmid et al 2001). With this in mind, our laboratory hypothesized that reelin-deficient mice would have alterations

in the MPC of SERT in peripheral lymphocytes. We found that the number of SERT clusters per lymphocyte was the same for *RELN*$^{-/-}$ and wild-type mice, but that the size of SERT clusters was increased in *RELN*$^{+/-}$ and even more so in *RELN*$^{-/-}$ compared to wild-types (Rivera-Baltanas et al 2010). We later found that CORT-treated rats and depression patients also have larger SERT and $5HT_{2A}R$ cluster sizes on lymphocyte membranes, which highlights an association between mood dysfunction and decreases in reelin (Rivera-Baltanas et al 2012, 2014, Romay-Tallon et al 2018). CORT also dysregulated the MPC parameters of other proteins like glutamate, noradrenaline, and dopamine transporters/receptors, suggesting that a lack of reelin interrupts the functioning of a wide variety of proteins and that the CORT-model could be used to analyze biomarkers in a bench-to-bedside-back-to-bench manner (Romay-Tallon et al 2018). In patients, increases in SERT cluster size correlated with the poor remittance of anhedonia and the distribution of SERT cluster sizes may represent a putative biomarker to predict therapeutic efficacy (Caruncho et al 2019, Rivera-Baltanas et al 2015). Interestingly, we recently showed that reelin rescued SERT MPC in lymphocytes that were isolated from CORT-treated rats, whereas ketamine had the opposite effect and potentiated the effects of CORT (Johnston et al 2020).

A lack of reelin receptor expression and abnormal protein clustering in immune cells would potentiate inflammatory events, like the enhanced release of pro-inflammatory cytokines, many of which can cross into the brain and are commonly elevated in depression patients (Banks et al 1995, Dowlati et al 2010, Leonard 2010, Young et al 2014). The ability of immune cells to alter central processes and subsequently behavior has been demonstrated by isolating lymphocytes from either stressed or unstressed mice and implanting them into mutant mice that are deficient in lymphocytes (Brachman et al 2015). They found that mice who received lymphocytes from stressed donors were more social and displayed less depressive- and anxiety-like behavior, lower plasma pro-inflammatory cytokine levels, and enhanced neurogenesis, even though pro-inflammatory cytokine levels were increased in the blood of stressed donors compared to unstressed donors (Brachman et al 2015). This exemplifies the adaptive ability of lymphocytes and suggests that chronic stress can make them more resilient and alter their function. It would be interesting to incubate isolated lymphocytes with reelin and evaluate whether their re-administration rescues depressive-like phenotypes, such as after repeated CORT. If successful, this would open many doors for future research and potentially a treatment approach for patients that are resistant to conventional forms of therapy. In any case,

I postulate that peripheral reelin may affect mood by regulating MPC, thereby correcting lymphocyte activity and imbalances in the levels of inflammatory mediators.

We know how stress alters reelin expression in some brain regions, but not whether stress downregulates reelin in peripheral organs, or if reelin administration can protect peripheral organs from stress. This motivated our laboratory to analyze the area of splenic white pulp after treatment with CORT and i.v. reelin, and the preliminary data indicates that multiple doses or a single dose of reelin recovers white pulp area in females whereas only the single dose achieved this in males (unpublished). This protection by reelin will make one more resilient to stress and infection and may increase reelin expression in the brain. Indeed, inhibiting pro-inflammatory cytokine TNF-α with etanercept, which is confined to the periphery, decreases FST-immobility and hippocampal-dependent memory impairments, and increases neurogenesis, which could be secondary to the restoration of reelin in the SGZ (Brymer et al 2018). Thus, there could be a relationship between the quality of lymphocyte populations in tissues like the spleen and thymus and the fact that antidepressants induce anti-inflammatory phenotypes (Slavich & Irwin 2014, Smagula et al 2017) and, along with anti-inflammatory agents, increase hippocampal reelin (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). Interestingly, pro-inflammatory cytokines increase the expression of certain ADAMTS protease enzymes that cleave reelin and may reduce its signaling activity considerably (Hisanaga et al 2012, Koie et al 2014, Kubo et al 2002, Ogino et al 2017, Yasui et al 2007). In this way, controlling inflammation protects from reelin inactivity which may help sustain homeostasis in other tissues, such as the colon, which is receiving copious amounts of experimental attention for its role in depression (Limbana et al 2020, Valles-Colomer et al 2019).

The gut microbiota has a bidirectional communication with the brain and influences neuroimmune, neuroendocrine, and neuronal pathways (Foster & McVey Neufeld 2013). It also influences brain development and how we behave, and mentally healthy individuals have richer and more diverse gut microbiota compared to depressed patients (Kelly et al 2016). A richer microbiota was demonstrated to directly affect the stress response when germ-free mice exhibited higher levels of plasma ACTH and CORT in response to restraint stress compared to specific pathogen free mice (Sudo et al 2004). Indeed, the integrity of the intestinal barrier is compromised in stress-vulnerable reelin-deficient mice (Carvajal et al 2017). A compromised microbiome could lead to a leaky gut that increases cell damage caused by lethal microorganisms (Gareau et al 2008), the risk of developing an inflammatory disease (Viggiano

et al 2015), and disruptions in neuronal communication (Clarke et al 2013). However, we do not understand the interplay of factors that govern intestinal homeostasis or the involvement of reelin in these factors and their relationship with depression, but studies have suggested that reelin protects from colon inflammation and pathology (Serrano-Morales et al 2017).

To summarize, little work has been done to establish the roles of reelin in the periphery and how these factors come to affect emotional behavior. That said, there is mounting evidence to suggest that peripheral reelin would have protective qualities in the periphery and brain, by regulating protein trafficking and organization into lipid rafts, dampening inflammation, and maintaining the integrity of the intestinal barrier.

## 1.12 Specific research aims

There is a pressing necessity to develop novel treatment approaches for depression. The primary goal of the research presented in this dissertation was to determine whether peripheral i.v. reelin has antidepressant-like effects using the repeated-CORT paradigm of chronic stress, relevant for depression. This was investigated by evaluating if reelin could recover CORT-induced behavioral and neurochemical alterations, focusing primarily on the FST, which has been used repeatedly as a preclinical screening tool for antidepressant efficacy. I also assessed whether the antidepressant-like effects of peripheral reelin are brought about in a rapid manner. These questions were addressed through a series of experiments:

**Experiment 1:** CORT-treated male rats were treated with several dosages of reelin because we had no clue what concentrations of peripheral reelin would be effective, but we used higher doses than were shown to be effective when infused into the hippocampus in the same animal model paradigm (Brymer et al 2020). Rats were given 3µg or 5µg of reelin either every 5 or 10 days over a 21-day CORT injection period. Thereafter, they were subjected to the FST to screen for antidepressant-like activity. Behavioral testing was followed by post-mortem analyses of reelin and neurogenesis in the hippocampus, and I analyzed the effect of reelin on SERT MPC in blood lymphocytes, all of which are associated with depression.

**Experiment 2:** I chose the lowest dosage that showed to be effective in the first experiment to evaluate the effect of reelin in males and females using a larger battery of behavioral tests. This is important because depression is twice as prevalent in females, and low mood is associated with deficiencies in excitatory and inhibitory neurotransmission. In fact, the modulation of

glutamatergic and GABAergic signaling is a shared attribute of fast-acting antidepressant drugs. This motivated me to examine whether reelin could rescue CORT-induced alterations in AMPA, NMDA, and $GABA_A$ receptors in the DG, and neurogenesis and SERT MPC in females.

**Experiment 3:** Finally, I used a dose-response experimental design to evaluate if a single injection of reelin given 24 hours before the FST could normalize CORT-induced behavioral deficits. First, I tested 0.5µg, 1µg, 3µg, 5µg, 7µg, and 9µg in males and then tested the most effective dose in females, which was 3µg. I also examined whether a single reelin injection could rescue hippocampal reelin and neurogenesis, AMPARs, SERT MPC, and I explored the effects of reelin on mitochondrial pro-apoptotic factors BAX and CytC.

The work outlined in this dissertation has been published in the journal Neuropharmacology:

Allen J, Romay-Tallon R*, Mitchell MA, Brymer KJ, Johnston J, Pinna G, Kalynchuk LE, Caruncho HJ. 2021. Reelin has antidepressant-like effects after repeated or singular peripheral injections. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2022.109043.

# Chapter 2

## Peripheral intravenous reelin rescues depression-relevant behavior, hippocampal reelin, and SERT MPC in the repeated-CORT paradigm of chronic stress

## 2.1 Abstract

Chronic stress is a significant risk factor for the onset of depression, which is difficult to treat and represents a large economic burden. Depression can be studied preclinically using a CORT-administration paradigm, which results in a behavioral phenotype of depression that is associated with decreased hippocampal neurogenesis and reelin levels, and altered SERT MPC in blood lymphocytes, which parallel changes seen in these parameters in human patients. We have recently shown that intrahippocampal infusions of reelin normalizes CORT-induced behavioral and neurochemical deficits. Here I examined whether peripheral i.v. reelin administration has similar antidepressant-like effects. Rats received daily CORT or vehicle injections for 3 weeks along with either 3μg or 5μg of reelin given every 5 or 10 days. Thereafter, the rats were subjected to the FST which is widely used as a screening tool for antidepressant efficacy. They were then sacrificed to permit immunohistochemical analyses of the number of reelin-IR cells in the SGZ and PVN, as well as the number and dendritic complexity of new-born neurons in the DG GCL. The results show that i.v. reelin rescued the CORT-induced increases in FST-immobility and SERT cluster size and decreases in SGZ-reelin. Interestingly, only the lowest dosage of reelin rescued PVN-reelin, and all dosages had modest effects on the number and maturation rate of new-born cells. These novel findings reveal for the first time that i.v. reelin has antidepressant-like effects that are associated with the restoration of hippocampal reelin and MPC in lymphocytes. Reelin should be evaluated using a larger battery of behavioral tests in both sexes.

## 2.2 Introduction

It has been over half a century since pharmacological observations led to the discovery of antidepressants, which typically target the monoaminergic system and require continuous administration, but we still lack broadly efficacious treatments that can generate fast-acting and sustained effects. It is the unfortunate truth that 66% of patients respond inadequately to their first treatment trial and about 33% of patients remain unresponsive even after multiple trials with different agents (Cipriani et al 2018, Rush et al 2006). Research efforts over the last few decades have uncovered neuronal abnormalities that are associated with depression (Krystal et al 2002, Mayberg 2003, Rajkowska et al 1999), but in this time, the types of drugs that we use to treat to MDD have changed very little. The inefficacy and antidepressant delay of

monoaminergic drugs (Rush et al 2006) place those with MDD at enormous risk, given that depression is the leading global cause of disability (GBD 2018) and the highest predictor of suicide at any age (Cuijpers et al 2014a). The pressing necessity to develop novel, more efficacious, faster acting, and longer lasting antidepressant compounds is of extreme importance now more than ever, considering that rates of MDD are increasing (Hidaka 2012, Klerman & Weissman 1989).

We recently demonstrated that intrahippocampal infusions of the extracellular matrix protein reelin can rescue depressive-like behaviors in rats that were subject to 3 weeks of daily CORT (Brymer et al 2020), and that the putative molecular mechanisms of action underlying the antidepressant-like effect parallel those of ketamine – the recently FDA-approved fast-acting antidepressant for use in treatment-resistant populations (Johnston et al 2020, Kim et al 2019). Reelin is an extracellular matrix protein involved in the regulation of neuronal migration during development, but it is repurposed as a neuromodulator in the adult brain (Pesold et al 1998). Reelin is heavily downregulated (~50%) in multiple regions from schizophrenic post-mortem brain samples (Impagnatiello et al 1998), in the cerebral cortex of bipolar patients with psychotic episodes (Guidotti et al 2000), and in the hippocampus from patients with schizophrenia, bipolar disorder, and MDD (Fatemi et al 2000). Interestingly, we have shown that *RELN*$^{+/-}$ (whom expresses 40-60% of normal reelin levels) have an increased susceptibility to the depressogenic effects of stress (Lussier et al 2011).

Glucocorticoid receptors are abundant in limbic regions which makes the hippocampus particularly susceptible to stress-induced impairments (Herman et al 2005). In fact, patients with MDD have large reductions in hippocampal volume which correlates with the age of depression onset and episode severity (Bremner et al 2000, Frodl et al 2006, Lorenzetti et al 2009, Sheline et al 2003, Videbech & Ravnkilde 2004). The hippocampal volumetric reductions observed in depression may partly be explained by the suppressing effect of stress on new-born cell proliferation which can be rescued by antidepressants in animal models (Brummelte & Galea 2010b, David et al 2009, Mayer et al 2006, Murray et al 2008, Nandam et al 2007). Our laboratory has contributed to this research by demonstrating that CORT downregulates the expression of reelin primarily in the rat hippocampal SGZ which parallels the progressive development of depressive-like behavior and slowed new-born granule cell maturation (Lebedeva et al 2020, Lussier et al 2009, 2013a). We also found that conventional antidepressants like imipramine (Fenton et al 2015), NMDAR antagonist ketamine (Johnston et al 2020), or some anti-inflammatory drugs with antidepressant actions (Brymer et al 2018)

protect against the deleterious effects of CORT on behavior, reelin expression, and neurogenesis.

Reelin is also present in blood plasma where it is believed to be secreted by platelets (Tseng et al 2010) to regulate hemostasis and inflammatory responses (Tseng et al 2014). We have found that *RELN*$^{+/-}$ animals express alterations in MPC in lymphocytes, such as for the SERT (Rivera-Baltanas et al 2010), and that CORT-treated animals show similar alteration patterns (Romay-Tallon et al 2018). Additionally, we also revealed that changes in SERT MPC in lymphocytes from *RELN*$^{+/-}$ and CORT-treated rodents parallel those observed in naïve depression patients, and proposed that these alterations could be used as a putative biomarker for antidepressant efficacy (Caruncho et al 2019, Rivera-Baltanas et al 2015, Rivera-Baltanas et al 2012, Rivera-Baltanas et al 2014).

Considering these effects of peripheral reelin and our previous work on the antidepressant-like effects of intrahippocampal reelin infusions in the repeated-CORT paradigm, I designed an experimental approach to evaluate if peripheral i.v. reelin injections also rescue CORT-induced depressive-like behavior, SERT clustering parameters, hippocampal neurogenesis, and reelin expression in the hippocampal SGZ and stress-regulating hypothalamic PVN.

## 2.3 Materials and methods

### 2.3.1 Animal husbandry

I used 88 adult male Long-Evans rats purchased from Charles River (QC, Canada) that were 6 weeks old on arrival to the facility. They were singly housed in polypropylene cages that contained a red hut and a wooden chew cube, and they had access to food and water ad libitum, except during behavioral testing procedures. Social isolation is often used to potentiate depressive-like behaviors in rodents, but such behavioral changes require longer isolation periods than 3 weeks in adults (Murínová et al 2017). Bedding was changed once a week, Purina rat chow food was topped up at regular intervals, and the room temperature was set to 21°C and maintained on a 12-hour light-dark cycle, lights turning on at 07:00 a.m. All experimental procedures were done in the light phase of the cycle. Protocols were in accordance with the Canadian Council and Animal Care and approved by the University of Victoria animal care committee.

### 2.3.2 Experimental procedures

On arrival to the facility, they were given a week to habituate before another week of handling. During the week of handling, the rats were habituated to a DecapiCone (Braintree Scientific Inc., MA) restraining device that was used throughout the injection period to administer the lateral tail vein injections: on day 3, the DecapiCone was placed into the cage with the rat for 1 minute; on the $4^{th}$, $5^{th}$, $6^{th}$ and $7^{th}$ day, the rat was placed into the DecapiCone for 1 minute.

The animals were weighed and assigned to one of the following treatment groups: 21 days of daily vehicle or CORT (40mg/kg) injections, with the addition of either vehicle or 3µg or 5µg of reelin given every 5 or 10 days (see Figure 2.3.2).

The repeated vehicle or CORT injections were given between the hours of 08:00 and 11:00 a.m. The animals were weighed each day and injections were administered at a volume of 1ml/kg suspended in a 0.9% (w/v) sodium chloride and 2% (v/v) polysorbate-80 (Sigma Aldrich) solution. Vehicle or either 3µg or 5µg of recombinant reelin (R&D systems, 3820-MR-025; composed of RR3-6 and having a predicted molecular weight of 180kDa by SDS-PAGE using reducing conditions) was suspended in 0.5ml of 0.1M phosphate buffered saline (PBS, pH=7.4) and given intravenously into the lateral tail vein every 5 or 10 days over the CORT injection period. We did not have any previous clue about what possible dosages of reelin might be effective and thereby we used higher concentrations than those shown effective (multiple or singular 1µg) at rescuing CORT-induced behavioral and neurochemical deficits when doing intrahippocampal injections in the same animal model paradigm (Brymer et al 2018). Fewer rats were used in the groups that received vehicle in place of reelin to reduce animal suffering, because we could group the vehicle rats that received it every 5 and every 10 days together for the sake of statistical analyses since there were no differences between them (hence they are controls for the study).

| Treatment | Vehicle | | Reelin | | | |
|---|---|---|---|---|---|---|
| | - | | 3µg/0.5ml | | 5µg/0.5ml | |
| | Every 10 days | Every 5 days | Every 10 days | Every 5 days | Every 10 days | Every 5 days |
| Vehicle | n=6 | n=6 | n=8 | n=8 | n=8 | n=8 |
| CORT | n=6 | n=6 | n=8 | n=8 | n=8 | n=8 |

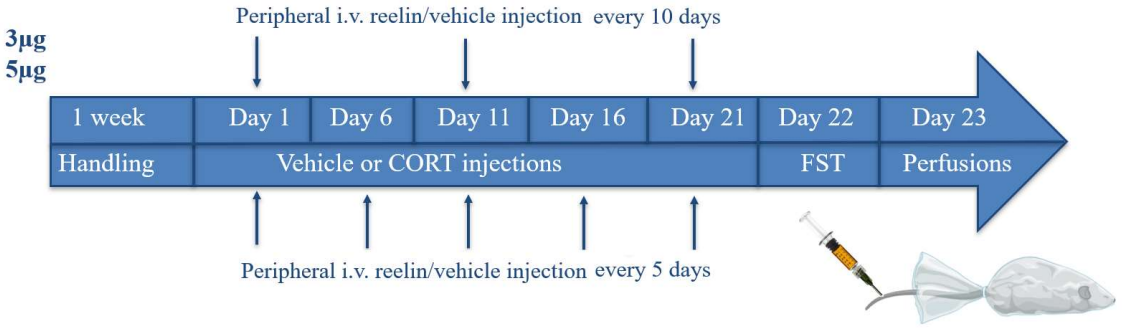


**Figure 2.3.2. Schematic representation of the experimental groups and timeline.** Rats either received vehicle 3 or 5µg of reelin either every 5 or 10 days via the lateral tail vein during a 21-day CORT-injection period, followed by the FST on day 22. Figure created with Microsoft Excel and paint by author.

### 2.3.3 Forced swim test

On day 22 I used a modified one-day version of the Porsolt FST (Porsolt, 1978) that was originally designed to serve as a preclinical behavioral assay for the efficacy of antidepressant drugs, as described previously (Marks et al 2009). A one-day protocol abolishes the potential confounding effects of memory on behavior that would occur if using a two-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 spent immobile was manually scored by timing the duration of time the rat spent floating or moving just enough to keep afloat which can be used to interpret coping strategy in response to inescapable stress and if often indicative of despair-like behavior (Commons et al 2017).

### 2.3.4 Perfusions, blood collection, and tissue preparation

First, the rats were deeply anaesthetized with 5% isoflurane (maintained with an isoflurane machine equipped with a nosecone that was placed over the rat's nose) before 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) and was used to make smears

on slides. The rats were then perfused transcardially using 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). Thereafter, the brains were removed and postfixed in 4% paraformaldehyde (w/v) for 48 hours at 4°C. They were then suspended in a 30% sucrose solution for 72 hours before a cryostat (Vibratome ULTRAPRO 5000) was used to section the tissue at a thickness of 30µm, which was then kept in a standard cryoprotectant solution of 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone and 30% (w/v) sucrose in 0.1M PBS (pH 7.4) at -20°C.

### 2.3.5 Immunostaining

Standard immunohistochemical techniques were utilized with commercially available antibodies on every 6$^{th}$ section of the hippocampus or hypothalamus in 6-well tissue culture plates on free-floating sections under gentle agitation. No IR cells were detected when the primary antibody was omitted from an additional well.

Reelin- and DCX-IR cells were visualized using the following immunohistochemical procedures as previously described (Lussier et al 2013a). First, an antigen-retrieval step took place in which the sections were incubated in sodium citrate (pH 6) for 30 minutes at 85°C. Then, the sections were incubated with either the mouse anti-reelin primary antibody (1:1000; MILLIPORE, MAB5364) in a blocking solution of 15% (v/v) normal horse serum (NHS), 0.5% triton X-100 and bovine serum albumin (BSA, 1%; w/v) in 0.1 M tris-buffered saline (TBS) or with a rabbit anti-DCX primary antibody (1:1000; Cell Signaling, Danvers, MA) in a blocking solution containing normal goat serum (NGS; 5%, v/v), Triton X-100 (0.5%, v/v), and BSA (1%; w/v) in TBS, for 24 hours at room temperature. Next, endogenous peroxidase activity was blocked by incubating the tissue in 10% (v/v) $H_2O_2$ for 30 mins. After this, the tissue was incubated at room temperature for 2 hours with a biotinylated horse anti-mouse or goat anti-rabbit secondary antibody where appropriate (1:500; Vector Laboratories, USA) in block. The tissue was then incubated in an avidin-biotin complex (ABC; 1:500; Vector Laboratories, USA) for 1 hour at room temperature. Avidin can be labeled with an enzyme like horseradish peroxidase (HRP) and it has high affinity for biotin, which can conjugate with the secondary antibody. For reelin, immunolabelling was visualized with 0.02% (w/v) 30-diaminobenzidine (DAB; Sigma Aldrich, St. Louis, MO, PK6100) and 0.0078% $H_2O_2$ diluted in TBS. For DCX, the sections were visualized with 0.025% (w/v) DAB, 4.167% NiSO4 and 0.002% $H_2O_2$ in 0.175 M sodium acetate (pH 6.8). DAB is oxidized by $H_2O_2$ in a reaction

catalyzed by horseradish peroxidase and produces a brown colorimetric product (see Figure 2.3.5 for a schematic representation). After approximately 10 minutes, the sections were rinsed 3 times with TBS to terminate the DAB reaction, before being mounted onto glass slides. They were then air dried overnight, dehydrated using increasing concentrations of ethanol (70, 95 and 100%), cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

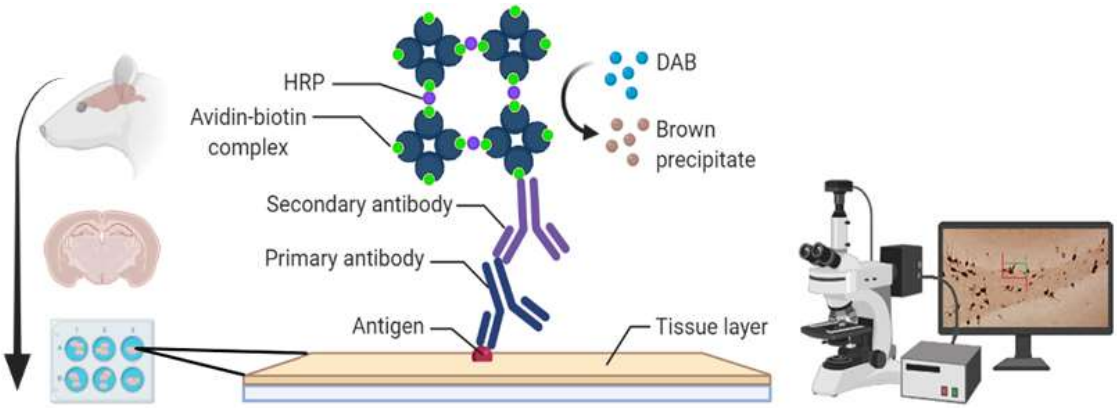


**Figure 2.3.5. Schematic representation of a standard immunohistochemical procedure.** After an antigen-retrieval step, proteins can be detected using primary and secondary antibodies. Avidin-biotin complex that is labelled with horseradish peroxidase (HRP) can conjugate with the secondary antibody. DAB is added and gets oxidized by $H_2O_2$ in a reaction catalyzed by HRP that produces a brown colorimetric product. Figure created with BioRender and Paint by author.

### 2.3.6 Cell counting and SERT analysis

Immunohistochemical results were quantified blind to treatment groups as previously described by our group (Botterill et al 2015a, Lussier et al 2013a). The number of reelin-IR cells in the PVN and the number of reelin-IR cells in the SGZ (defined as a 2-cell width zone in between the inner GCL and the PL) were counted using a Nikon Eclipse E800 microscope with a motorized stage linked to a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc). DCX-IR cells were counted in the SGZ and GCL. Immunopositive cells were counted in 5 sections in both hemispheres at 40X magnification

utilizing unbiased stereology using a modified optical fractionator method to reduce oversampling. The estimated number of IR cells was calculated using the formula:

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

where $\Sigma Q^{-}$ 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 (10000μm$^2$); a(frame) is the area of the counting frame (3600μm$^2$); t is the weighted average section thickness; and h is the height of the dissector (12μm). A guard zone of 4μm was used to avoid counting sectioning artefacts.

SERT MPC parameters were analyzed on 50 individual lymphocytes per sample at 100× magnification. ImageJ software (1.53e, NIH, USA) was used to analyze the images as outlined previously (Romay-Tallon et al 2017). Briefly, this process involves establishing a known distance that the program equivalates to pixels – so that real values can be retrieved – followed by transforming the original image into a binary one and removing background. This binary image can be used to quantify the number of SERT clusters and their surface areas using parameters to define the minimum (0.05μm) and maximum ('infinite') particle size.

### 2.3.7 Categorization of immature DCX-IR cells

The effect of CORT and reelin on the dendritic morphology of immature granule cells was evaluated by characterizing the dendritic branching of 100 randomly selected DCX-IR cells that were evenly distributed across both hemispheres of the 5 sections from each rat. This semi-quantifiable method was performed using a meander scan method blind to treatment groups. Chosen cells were assigned to one of 6 categories (see Figure 2.4.5C): category 1 (no processes) and category 2 (one small process) represented proliferative stages of development; category 3 (medium process reaching the GCL) and category 4 (process reaching the ML) represented an intermediate stage of cell development; and category 5 (one major process extending into the ML) and category 6 (defined dendritic tree with delicate dendritic branching in the GCL) represented more mature stages of development (Lussier et al 2013a, Plümpe et al 2006). Afterwards, the percentage of cells in each category was calculated.

### 2.3.8 Statistical analyses

Statistical analyses were carried out using the SPSS (IBM, USA). The assumptions of normality and homogeneity of variance were tested before two-way ANOVAs were used for data analyses. If a significant main effect of CORT or reelin was found, Tukey *post hoc* tests were conducted. We refer to treatment effects as significant if group means were statistically different from one another at $p<0.05$. We refer to recoveries by reelin as partial if CORT/reelin rats did not significantly differ from CORT/vehicle or vehicle/vehicle rats when CORT/vehicle rats were significantly different from vehicle/vehicle rats. Pearson's bivariate correlation coefficient tests were used for correlational analyses. In addition, I report the percentage of recovery by i.v. reelin as calculated by the following formula:

*Percentage recovered = 100 – (CORT/reelin mean – vehicle/vehicle mean) ÷ (CORT/vehicle mean – vehicle/vehicle mean) × 100*

The data were expressed as mean±confidence intervals (CI).

## 2.4 Results

### 2.4.1 Bodyweight

When looking at bodyweight, sphericity could not be assumed ($p<0.05$) with Mauchly's test of Sphericity and so the Greenhouse-Geisser correction for the two-way ANOVA repeated measures was employed. The results for the within-subjects effects show that there was a significant effect of time on weight gain [$F(1.421, 113.659)=141.857$, $p<0.001$] and an interaction effect of time × CORT treatment [$F(1.241, 113.659)=332.855$, $p<0.001$], but not of time × reelin [$F(1.241, 113.659)=0.129$, $p=0.807$] or time × CORT × reelin [$F(1.241, 113.659)=0.365$, $p=0.622$]; CORT-treated rats lost weight while the vehicle-treated rats gained weight over time (Figure 2.4.1A). The statistical information for the between-subjects effects for Chapter 2 are listed in Table 2.4.5. The percentage of weight change was calculated over the 21-day injection period which clearly shows ~30% reductions in weight gain by CORT ($p<0.001$; Figure 2.4.1B).

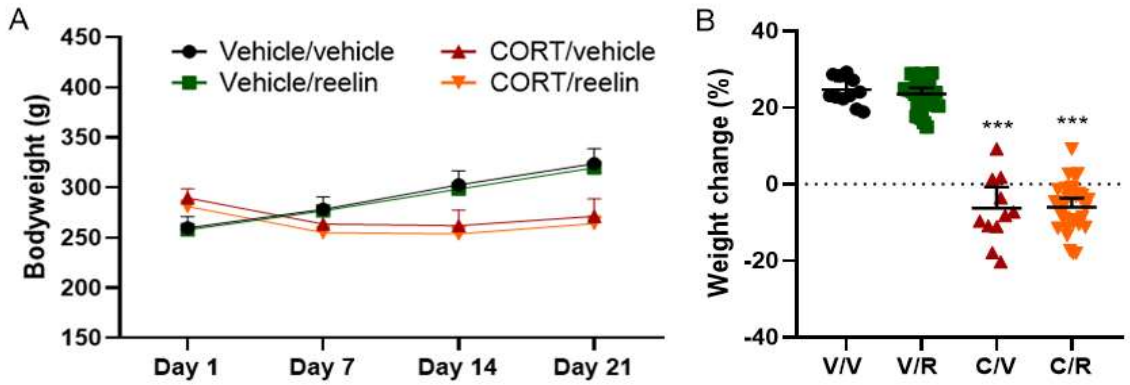


**Figure 2.4.1. CORT decreased bodyweight gain while reelin had no effect.** CORT decreased weight gain over the 21-day injection period (independent of food intake; A) evident by looking at the percentage of weight change (B). Data are expressed as mean±CI. ***$p$<0.001 vs vehicle comparison. Figure created with GraphPad Prism by author.

### 2.4.2 Forced swim test

When looking at time spent immobile in the FST, originally designed by Porsolt (1978), I found that the CORT/vehicle rats (mean±standard error of the mean, SEM: 142±6) spent significantly more time immobile than the vehicle/vehicle ($p$<0.001; mean±SEM: 100±5) and the CORT/reelin rats when treated with 3µg every 10 days (mean±SEM: 112±12), 3µg every 5 days (mean±SEM: 119±7), and 5µg every 5 days (mean±SEM: 115±7; $p$≤0.028). There was a partial (insignificant) recovery of 53% when 5µg was given every 5 days (mean±SEM: 120±8), as shown in Figure 2.4.2 below.

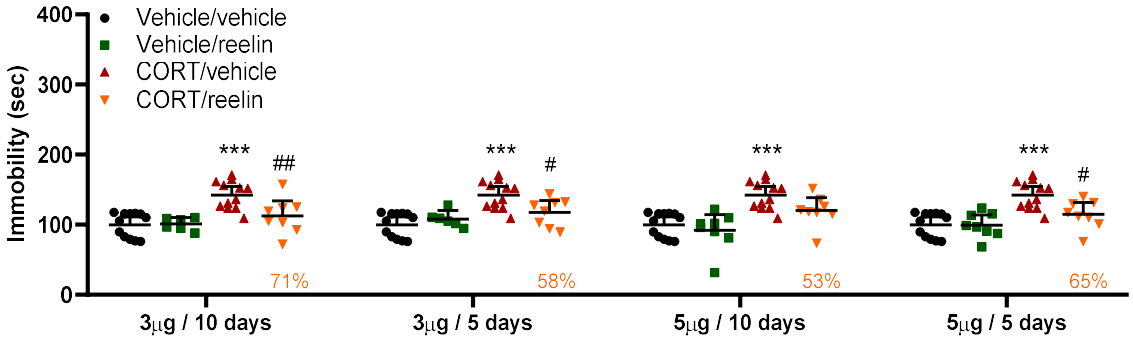


**Figure 2.4.2. The effect of different dosages of reelin on FST-immobility.** CORT increased FST-immobility which was rescued by reelin. Data are expressed as mean±CI and as a percentage change from vehicle/vehicle. Percentage of recovery = %. ***$p$<0.001 vs vehicle/vehicle; #$p$<0.05/##$p$<0.01 vs CORT/vehicle. Figure created with GraphPad Prism by author.

### 2.4.3 SERT clustering parameters

We have observed in the past that CORT alters MPC in lymphocytes and that these changes parallel those that are observed in MDD patients (Caruncho et al 2019). Thereby, I evaluated if i.v. injections of reelin were able to rescue the effects of CORT in the clustering of SERT in lipid raft microdomains (see Figure 2.4.3). I found that the CORT/vehicle rats had significantly larger SERT cluster sizes than the vehicle/vehicle ($p \leq 0.001$) and CORT/Reelin ($p \leq 0.018$) rats, all dosages of reelin successfully normalizing clustering parameters. There was no effect of either treatment on the number of SERT clusters. The functional consequences of SERT MPC and how it relates to depression is unknown.

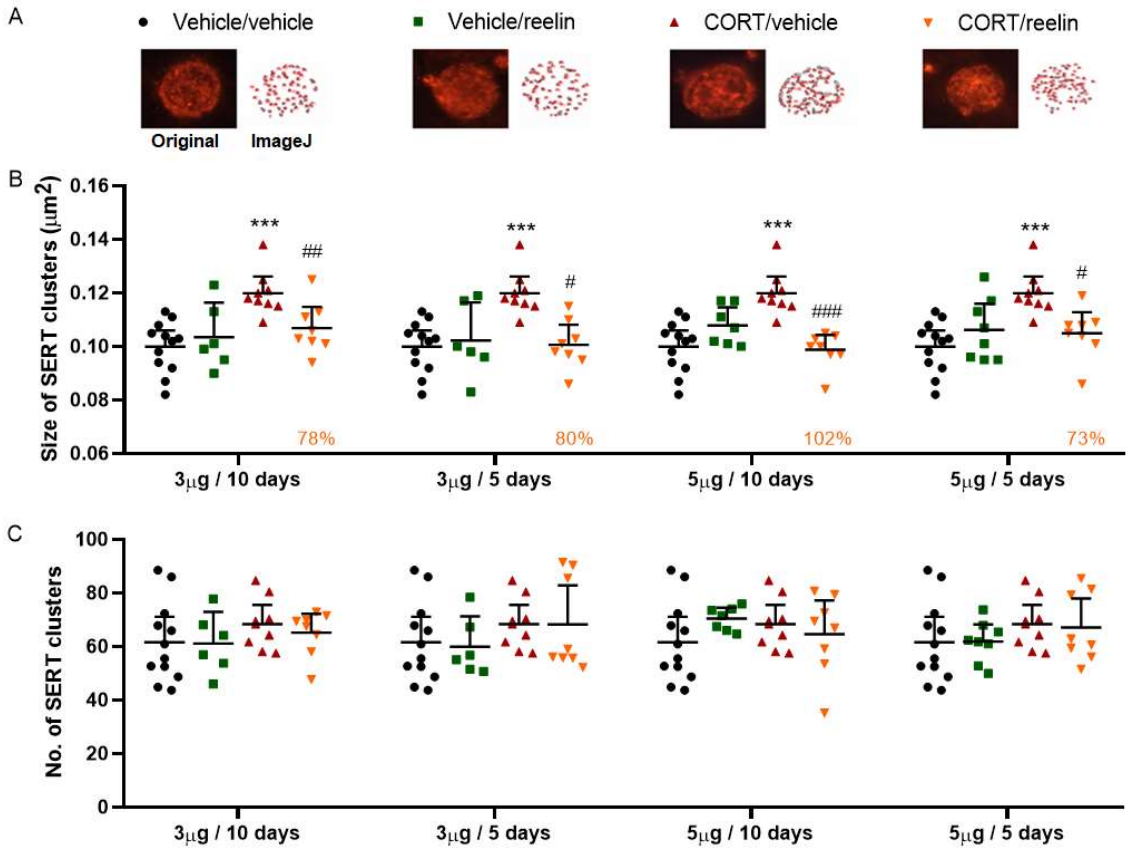


**Figure 2.4.3. The effect of CORT and reelin on SERT clustering parameters.** A) Representative SERT immunostaining in lipid raft microdomains in lymphocytes and the output by ImageJ, which is used to quantify the number and size of each IR product. B) The effect of treatment on SERT cluster size: CORT-treated rats had significantly larger SERT clusters than the vehicle rats, an alteration that was fully recovered by reelin. C) There was no effect of treatment on SERT cluster number. Data are represented as mean±CI. Percentage of partial recovery = %. ***$p<0.001$ vs vehicle/vehicle; #$p<0.05$/##$p<0.01$/###$p<0.001$ vs CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

### 2.4.4 Reelin cell counts

**DG SGZ**

We have previously shown that conventional antidepressants, ketamine, and anti-inflammatory drug etanercept can protect from the CORT-induced downregulation of reelin in the DG SGZ, where it is expressed by GABAergic interneurons (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). Here I assessed if similar effects are achieved by peripheral i.v. reelin injections. Photomicrographs of reelin immunostaining in the SGZ can be found in Figure 2.4.4aA/C. The CORT/vehicle rats had significantly fewer reelin-IR cells than the vehicle/vehicle rats ($p \leq 0.001$) and peripheral reelin significantly recovered this deficit when 3µg were administered every 10 days, 5µg every 10 days, and 5µg every 5 days ($p \leq 0.035$; Figure 2.4.4aB). Reelin expression was partially (insignificantly) recovered (by 48%) when 3µg of reelin was given every 5 days.

**Hypothalamic PVN**

I was also interested to see if CORT would downregulate reelin expression in the stress regulating hypothalamic PVN, which could contribute to HPA-axis dysfunction, and if i.v. reelin could rescue PVN-reelin levels. I found that CORT downregulated ($p \leq 0.003$) PVN-reelin, but only the lowest dosage (3µg every 10 days; $p = 0.021$) significantly recovered reelin expression levels (by 83%, Figure 2.4.4b) while the higher doses had less of an effect (25-34% recoveries).

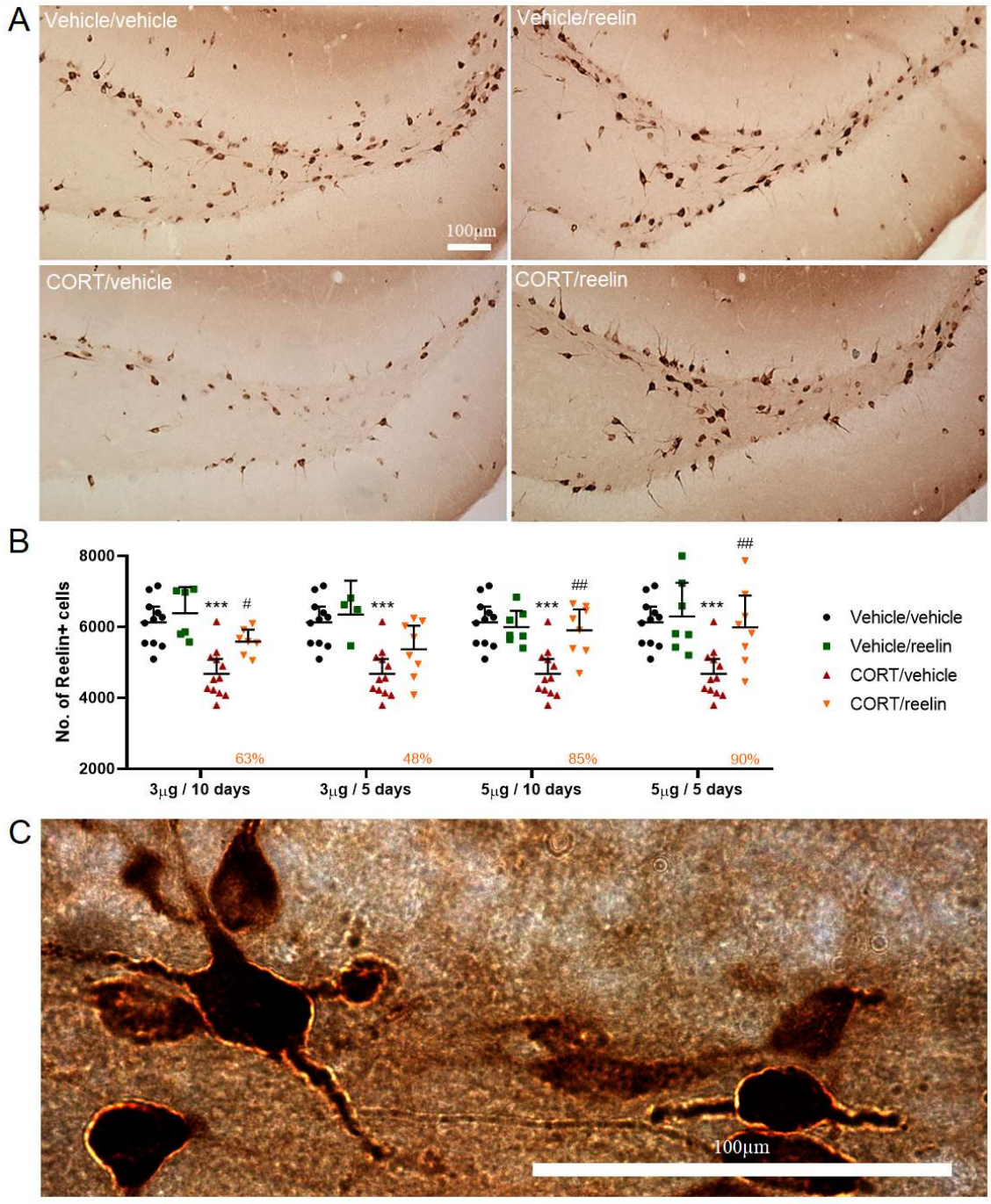


**Figure 2.4.4a. The effect of CORT and i.v. reelin on reelin-IR cell number in the SGZ.** A) Representative photomicrographs of reelin expression in the SGZ. Scale bar = 100µm. B) The effect of treatment on the number of reelin-IR cells: The CORT-induced decreases in SGZ-Reelin expression were significantly rescued by all dosages of reelin except 3µg every 5 days, which showed a partial recovery (48%). C) High magnification image of reelin-IR cells in the SGZ. Data are represented as mean±CI. Percentage of recovery = %. ***$p$<0.001 vs vehicle/vehicle; #$p$<0.05/##$p$<0.01 vs CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

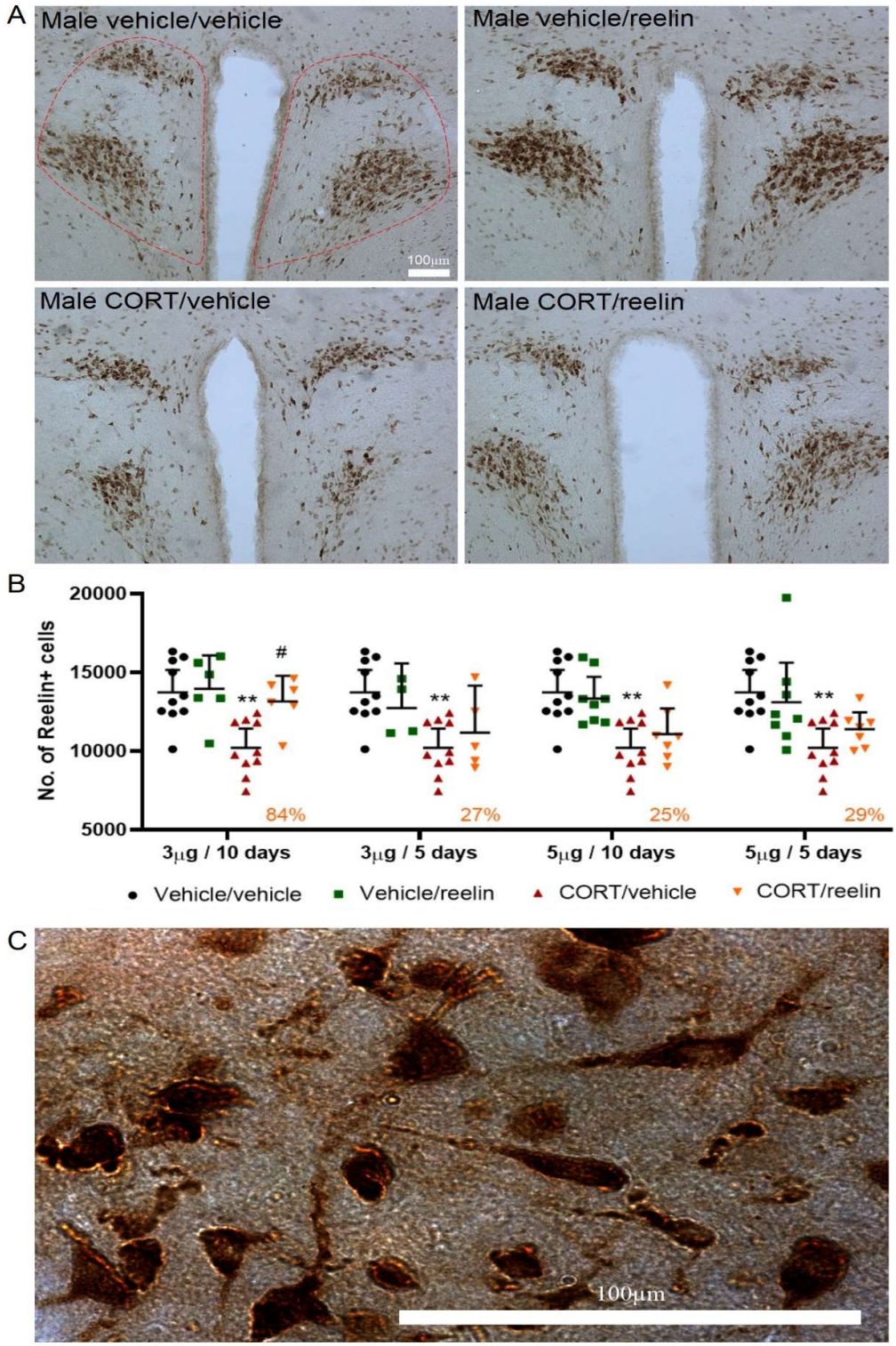


**Figure 2.4.4b. The effect of CORT and reelin on reelin-IR cells in the PVN.** A) Representative photomicrographs of reelin expression in the PVN. B) The effect of treatment on the number of reelin-IR cells for each subgroup. CORT-treated rats had significantly fewer reelin-IR cells than the vehicle rats, which was recovered with 3µg of reelin every 10 days. All data are represented as mean±CI. Percentage of recovery = %. ** $p<0.01$ vs vehicle/vehicle; # $p<0.01$ vs CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

### 2.4.5 DCX cell counts and dendritic categorization

I then used DCX immunostaining (photomicrographs shown in Figure 2.4.5A) to determine if peripheral i.v. reelin injections rescued the deficits in hippocampal neurogenesis induced by CORT. DCX is a microtubule-associated protein that is expressed by adult-born cells that are destined to become neurons. I found that the CORT/vehicle rats had significantly fewer DCX-IR cells than the vehicle/vehicle rats ($p \leq 0.015$, Figure 2.4.5B), while the CORT/reelin rats did not, indicating a slight, partial, insignificant recovery by reelin (ranging from 17% to 37%).

When looking at dendritic complexity, the CORT/vehicle and CORT/reelin rats had a significantly larger percentage of cells in the proliferative (category 1 and 2) and intermediate (category 3) stages of development, and fewer cells in the post-mitotic stage (category 6) than the vehicle/vehicle rats ($p \leq 0.033$, see Figure 2.4.5D). Therefore, peripheral i.v. reelin failed to rescue the slowed dendritic maturation induced by CORT, unlike intrahippocampal infusions of reelin (Brymer et al 2020).

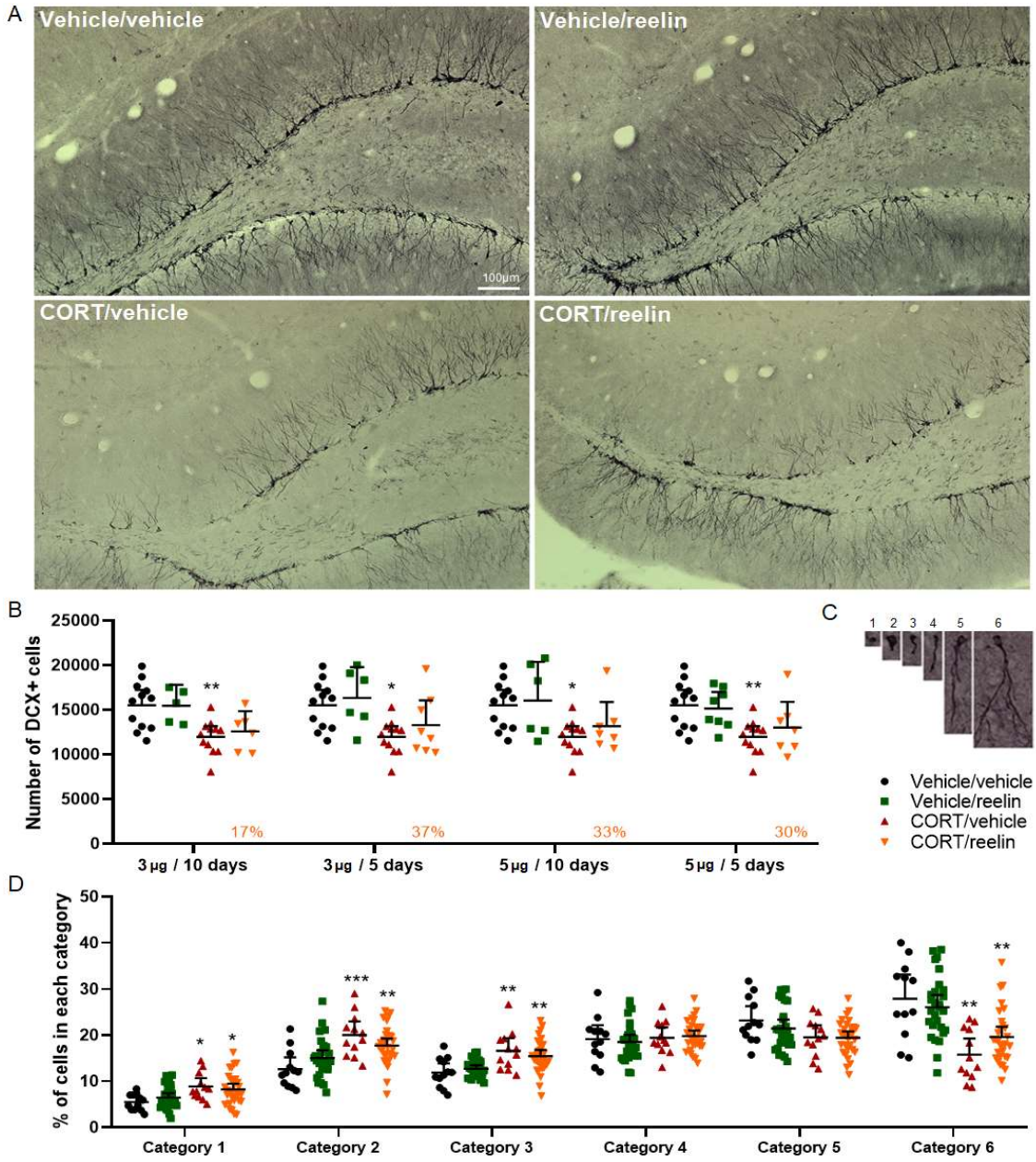


**Figure 2.4.5. The effect of CORT and Reelin on DCX-IR cells in the SGZ and GCL.** A) Representative photomicrographs of DCX expression in the DG. Scale bar = 100µm. B) The effect of treatment on the number of DCX-IR cells: reelin partially recovered the CORT-induced reductions in DCX-IR cell number but not to a significant degree (the percentage of recovery is expressed above the respective error bar). C) Representative photomicrographs of the 6 categories of dendritic complexity. D) The effect of treatment on dendritic complexity after analysis of a random subset of DCX-IR cells: In general, rats treated with CORT had a higher percentage of cells in the proliferative and intermediate (categories 1 to 3) developmental stages and a lower percentage of cells in the post-mitotic stage (category 6), demonstrating that CORT slowed the maturation rate of new-born cells which was not rescued by Reelin. Data are represented as mean±CI. Percentage of recovery = %. *$p$<0.05/**$p$<0.01/***$p$<0.001 vs vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

**Table 2.4.5. Statistical information for the effect of CORT and reelin on behavioral, neurochemical, and MPC parameters.**

| Measure | Dose & frequency | CORT | Reelin | CORT × Reelin |
|---|---|---|---|---|
| Bodyweight (Figure 2.4.1A) | Vehicle/vehicle, Vehicle/Reelin, CORT/vehicle, CORT/Reelin | F(1, 80)=31.556, $p$<0.001 | F(1, 80)=2.083, $p$=0.153 | F(1, 115)=0.66, $p$=0.418 |
| Weight change (Figure 2.4.1B) | | F(1, 80)=465.55, $p$<0.001 | F(1, 80)=0.09, $p$=0.761 | F(1, 80)=0.26, $p$=0.7615 |
| Immobility (Figure 2.4.2) | 3µg / 10 days | F(1, 34)=17.04, $p$<0.001 | F(1, 34)=4.48, $p$=0.034 | F(1, 34)=5.619, $p$=0.024 |
| | 3µg / 5 days | F(1, 34)=18.09, $p$=0.0009 | F(1, 34)=1.78, $p$=0.191 | F(1, 36)=7.22, $p$=0.011 |
| | 5µg / 10 days | F(1, 36)=26.42, $p$=0.0001 | F(1, 36)=4.91, $p$=0.033 | F(1, 36)=1.06, $p$=0.311 |
| | 5µg / 5 days | F(1, 36)=22.77, $p$=0.0003 | F(1, 36)=5.33, $p$=0.027 | F(1, 36)=4.83, $p$=0.034 |
| SERT cluster size (Figure 2.4.3B) | 3µg / 10 days | F(1, 31)=11.969, $p$=0.002 | F(1, 31)=1.953, $p$=0.172 | F(1, 31)=6.049, $p$=0.020 |
| | 3µg / 5 days | F(1, 31)=7.157, $p$=0.012 | F(1, 31)=6.099, $p$=0.019 | F(1, 31)=9.753, $p$=0.004 |
| | 5µg / 10 days | F(1, 32)=3.779, $p$=0.061 | F(1, 32)=5.577, $p$=0.024 | F(1, 32)=27.071, $p$<0.001 |
| | 5µg / 5 days | F(1, 33)=8.463, $p$=0.006 | F(1, 33)=1.767, $p$=0.193 | F(1, 33)=10.874, $p$=0.002 |
| SERT cluster number (Figure 2.4.3C) | 3µg / 10 days | F(1, 31)=1.710, $p$=0.201 | F(1, 31)=0.20, $p$=0.658 | F(1, 31)=0.106, $p$=0.746 |
| | 3µg / 5 days | F(1, 31)=2.444, $p$=0.128 | F(1, 31)=0.033, $p$=0.858 | F(1, 31)=0.028, $p$=0.869 |
| | 5µg / 10 days | F(1, 32)=0.011, $p$=0.916 | F(1, 32)=0.373, $p$=0.546 | F(1, 32)=2.231, $p$=0.145 |
| | 5µg / 5 days | F(1, 33)=2.226, $p$=0.145 | F(1, 33)=0.016, $p$=0.900 | F(1, 33)=0.034, $p$=0.854 |
| SGZ reelin (Figure 2.4.4aB) | 3µg / 10 days | F(1, 32)=26.76, $p$=0.0001 | F(1, 32)=7.11, $p$=0.012 | F(1, 32)=2.25, $p$=0.143 |
| | 3µg / 5 days | F(1, 31)=22.68, $p$=0.0004 | F(1, 31)=3.19, $p$=0.083 | F(1, 31)=0.86, $p$=0.361 |
| | 5µg / 10 days | F(1, 34)=12.87, $p$=0.001 | F(1, 34)=6.45, $p$=0.016 | F(1, 34)=10.08, $p$=0.003 |
| | 5µg / 5 days | F(1, 34)=10.06, $p$=0.003 | F(1, 34)=7.09, $p$=0.012 | F(1, 34)=4.29, $p$=0.046 |
| PVN reelin (Figure 2.4.4bB) | 3µg / 10 days | F(1, 28)=10.329, $p$=0.003 | F(1, 28)=5.612, $p$=0.025 | F(1, 28)=4.149, $p$=0.051 |
| | 3µg / 5 days | F(1, 25)=10.504, $p$=0.003 | F(1, 25)=0.09, $p$=0.984 | F(1, 25)=1.561, $p$=0.223 |
| | 5µg / 10 days | F(1, 31)=22.177, $p$<0.001 | F(1, 31)=1.155, $p$=0.696 | F(1, 31)=1.072, $p$=0.309 |
| | 5µg / 5 days | F(1, 32)=15.387, $p$<0.001 | F(1, 32)=0.078, $p$=0.781 | F(1, 32)=1.408, $p$=0.224 |
| SGZ/GCL DCX (figure 2D) | 3µg / 10 days | F(1, 31)=15.51, $p$=0.0004 | F(1, 31)=0.11, $p$=0.745 | F(1, 31)=0.17, $p$=0.685 |
| | 3µg / 5 days | F(1, 34)=12.97, $p$=0.001 | F(1, 34)=1.33, $p$=0.256 | F(1, 34)=0.08, $p$=0.786 |
| | 5µg / 10 days | F(1, 33)=11.22, $p$=0.002 | F(1, 33)=0.78, $p$=0.348 | F(1, 33)=0.13, $p$=0.725 |
| | 5µg / 5 days | F(1, 35)=12.36, $p$=0.001 | F(1, 35)=0.17, $p$=0.648 | F(1, 35)=0.79, $p$=0.380 |
| DCX cat 1 | Vehicle/vehicle, Vehicle/Reelin, CORT/vehicle, CORT/Reelin (Figure 2.4.5D) | F(1, 78)=13.76, $p$=0.0004 | F(1, 78)=0.06, $p$=0.810 | F(1, 78)=1.35, $p$=0.249 |
| DCX cat 2 | | F(1, 78)=23.68, $p$<0.001 | F(1, 78)=0.002, $p$=0.962 | F(1, 78)=5.13, $p$=0.026 |
| DCX cat 3 | | F(1, 78)=23.19, $p$<0.001 | F(1, 78)=0.04, $p$=0.837 | F(1, 78)=1.72, $p$=0.193 |
| DCX cat 4 | | F(1, 78)=0.72, $p$=0.398 | F(1, 78)=0.03, $p$=0.870 | F(1, 78)=0.31, $p$=0.581 |
| DCX cat 5 | | F(1, 78)=7.09, $p$=0.009 | F(1, 78)=0.72, $p$=0.396 | F(1, 78)=0.60, $p$=0.440 |
| DCX cat 6 | | F(1, 78)=34.16, $p$<0.001 | F(1, 78)=0.39, $p$=0.531 | F(1, 78)=3.18, $p$=0.078 |

### 2.4.6 Correlations with behavior, MPC parameters, and neurochemical alterations

Correlational graphs are depicted in Figure 2.4.6 and were done on separate groups and a combination of groups. A Pearson's bivariate correlation coefficient test including all treatment groups showed that there is a significant negative correlation between immobility and the number of reelin-IR cells in the SGZ (r=-0.365, $p$=0.001, n=78) and PVN (r=-0.372, $p$=0.001, n=72), and the number of DCX-IR new-born neurons (r=-0.312, $p$=0.006, n=77), but no correlation was found between immobility and the size (r=0.085, $p$=0.452, n=80) or number of SERT clusters (r=0.120, $p$=0.290, n=80), or between SERT cluster number and size (r=0.031, $p$=0.778, n=80). There was a significant negative correlation between SERT cluster size and reelin-IR cell counts in the SGZ (r=0.306, $p$=0.008, n=75) but not the PVN (r=-0.109, $p$=0.372, n=69). Finally, there was a positive correlation between the number of reelin-IR cells in the SGZ and PVN (r=0.263, $p$=0.030, n=68).

Additional correlations were computed including values for only CORT/vehicle or CORT/reelin rats. For CORT/vehicle-treated rats, there was no correlation between immobility and SGZ-reelin (r=0.180, $p$=0.576, n=12), PVN-reelin (r=0.018, $p$=0.961, n=10), DCX-IR cell counts (r=-0.458, $p$=0.134, n=12), SERT cluster size (r=-0.350, $p$=0.356, n=9) or SERT cluster number (r=-0.288, $p$=0.452, n=9). SERT cluster size did not correlate with SERT cluster number (r=-0.140, $p$=0.720, n=9), SGZ- (r=-0.411, $p$=0.272, n=9) or PVN-reelin cell counts (r=-0.059, $p$=0.889, n=8). The number of reelin-IR cells did not correlate in the SGZ and PVN (r=0.139, $p$=0.702, n=10).

For CORT/reelin-treated rats, there was no correlation between immobility and SGZ-reelin (r=-0.305, $p$=0.095, n=31), PVN-reelin (r=0.131, $p$=0.525, n=26), DCX-IR cell counts (r=0.114, $p$=0.564, n=28) or SERT cluster number (r=0.114, $p$=0.533, n=32). There was a significant negative correlation between immobility and SERT cluster size (r=-0.355, $p$=0.046, n=31). SERT cluster size did not correlate with SERT cluster number (r=-0.004, $p$=0.984, n=32), SGZ- (r=-0.117, $p$=0.530, n=31) or PVN-reelin cell counts (r=-0.088, $p$=0.669, n=26). The number of reelin-IR cells did not correlate in the SGZ and PVN (r=0.043, $p$=0.833, n=26).

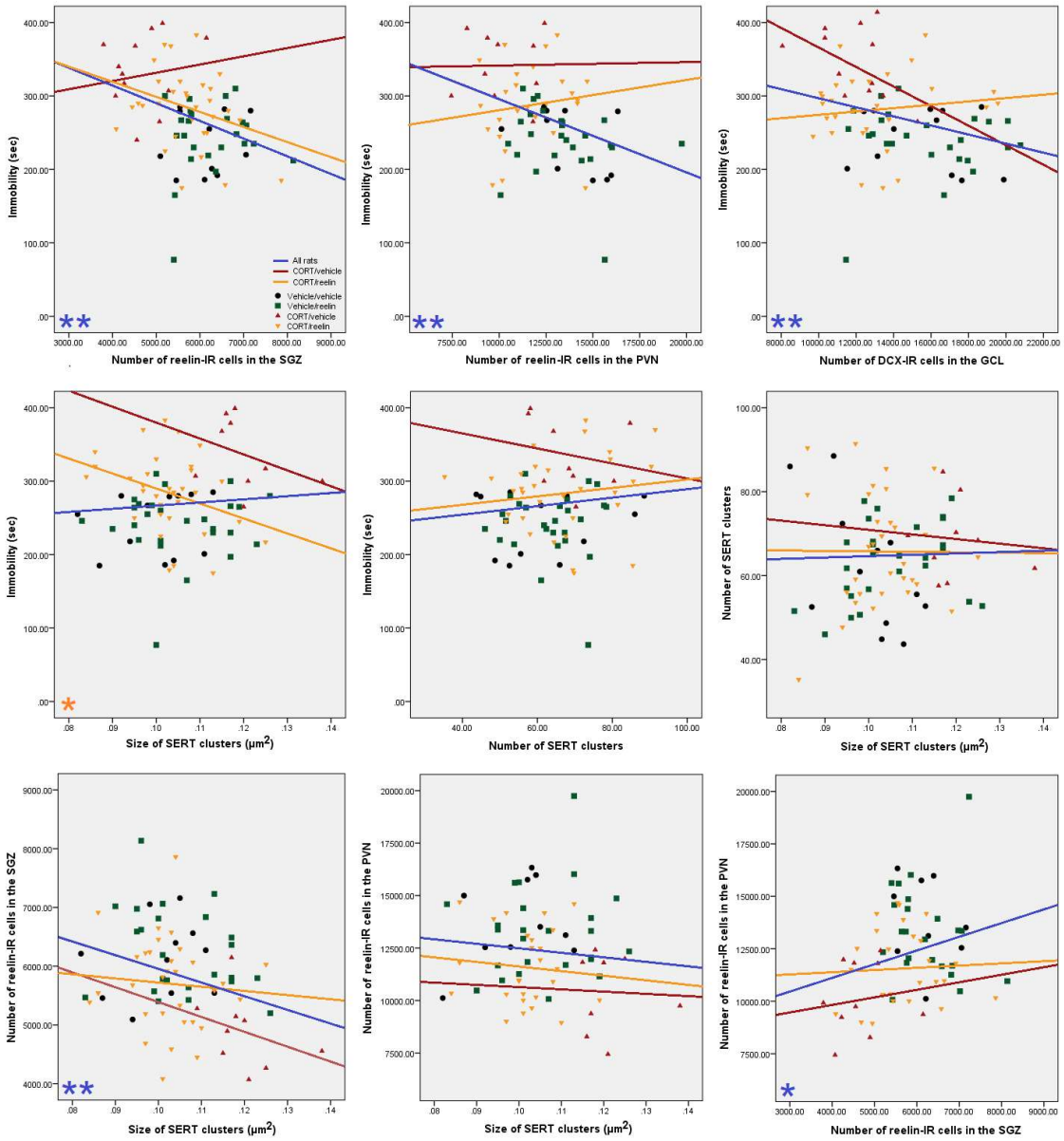


**Figure 2.4.6. Correlations between behavior, SERT MPC parameters and neurochemical alterations.** Significant correlations are illustrated by asterisks (*$p$<0.05/ **$p$<0.01/***$p$<0.001) that are color-coordinated with the line of best fit when all groups were included in the analyses (blue line), or only the CORT/vehicle (red line) or CORT/reelin rats (orange line). Figure created with SPSS and Paint by author.

## 2.5 Discussion

Current antidepressant treatments are laden by their slow therapeutic onset, side effects, and impoverished remission rates. This brings about the need for more efficacious and faster-acting antidepressant compounds. In a recent report, we found that reelin had rapid antidepressant-like and pro-cognitive effects when infused directly into the hippocampus after repeated-CORT (Brymer et al 2020). Here I provided the first evidence, to our knowledge, that peripheral i.v. reelin can also produce similar antidepressant-like effects as ascertained by the FST, and that the restoration of hippocampal reelin and the normalization of MPC in blood lymphocytes may largely contribute to such behavioral recoveries.

Investigators must be aware of the limitations related to the use of animal models in mental health research, and although we focused this study on the FST (which has been repeatedly used to ascertain antidepressant-like effects in preclinical studies and thereby we used it for the evaluation of the putative antidepressant-like effects of i.v. reelin injections), our laboratory understands that additional behavioral testing and the use of other animal models designed to evaluate the putative antidepressant-like effects of i.v. reelin injections is necessary to further verify and extend the data described here. Repeated-CORT produces a behavioral phenotype of depression that is characterized by progressive increases in FST-immobility that are paralleled by decreases in hippocampal reelin and neurogenesis (Lebedeva et al 2020, Lussier et al 2009, 2013a), and conventional antidepressants, ketamine, and anti-inflammatory agents with antidepressant properties can rescue these deficits (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). In the present study I found that FST-immobility can also be recovered by multiple peripheral i.v. reelin injections. While the validity of the FST as a measure of depressive-like behavior – or how rodents cope with inescapable stress – has been questioned (Commons et al 2017), it remains the most commonly used preclinical screening test for antidepressant efficacy (Yuen et al 2017).

There is plenty of evidence that demonstrates the neuroprotective properties of reelin. The genetic overexpression of reelin can protect from behavioral phenotypes related to neuropsychiatric conditions (Teixeira et al 2011) and *RELN*$^{+/-}$ mice have an enhanced vulnerability to stress-induced perturbations (Lussier et al 2011, Notaras et al 2020, Schroeder et al 2015). Findings like these prompted us to evaluate if, like other antidepressant drugs, i.v. reelin could elevate the expression of hippocampal reelin. I found that all dosages significantly recovered the number of reelin-IR cells in the SGZ, except 3µg every 5 days which produced

a partial recovery of 48%. Considering the neuromodulatory roles of reelin that enhance cellular growth and plasticity (Beffert et al 2005, Bosch et al 2016a, Hethorn et al 2015, Niu et al 2004, 2008, Rogers et al 2011, 2013, Weeber et al 2002) – by activating similar molecular pathways as ketamine (Brymer et al 2020, Iafrati et al 2014, Jossin & Goffinet 2007) – one could argue that the behavioral changes observed in this study are secondary to the elevation of hippocampal reelin. Here reelin can promote the strengthening of glutamatergic synapses (Qiu & Weeber 2007, Qiu et al 2006b), much like ketamine and other rapid-acting antidepressants that also require AMPAR-mediated transmission to produce their effects (Brymer et al 2020, Fischell et al 2015, Kadriu et al 2020b, Thompson et al 2015).

Given that the stress response is governed by the hormone-secreting hypothalamic PVN, which is inhibited by the hippocampus, I was also curious to see if i.v. reelin could rescue CORT-induced reelin deficits in this region. One can assume that a strengthening of circuitry in the PVN and hippocampus could protect from the deleterious effects of repeated-CORT, such as HPA-axis feedback dysfunction. Our results were intriguing in that only the lowest dosage, 3µg given every 10 days, significantly recovered PVN-reelin expression, while the higher dosages only heightened expression levels by 30±5%. Therefore, while reductions in hypothalamic reelin may contribute to emotional impairments (and maybe sex differences in prevalence rates which our team is actively investigating), restoring expression levels of reelin in this region appear less critical for behavioral improvements than in the SGZ. For this reason, all future post-mortem analyses outlined in this dissertation will focus on the DG, where the secretion of reelin influences neuro- and synapto-genesis (Bosch et al 2016a, Pujadas et al 2010, Teixeira et al 2012).

Deficient hippocampal neurogenesis is consistently observed in rodents that have been exposed to paradigms of chronic stress, including repeated-CORT (Brummelte & Galea 2010b, Brymer et al 2018, Culig et al 2017, Fenton et al 2015, Lussier et al 2013a, Sliwowska et al 2010), which suggests that there is a link between stress, neurogenesis, and the hippocampal volumetric reductions that are observed in MDD patients (Campbell et al 2004, Frodl et al 2006, Sheline et al 2003, Videbech & Ravnkilde 2004). However, the role of neurogenesis, or lack thereof, in depressive behavior is largely debated. This is because post-mortem studies that show reductions in hippocampal neurogenesis in MDD patients (Berger et al 2020, Boldrini et al 2012) – which can be attenuated by antidepressants (Chen et al 2001) – are not always replicated (Reif et al 2006). Other contradictory findings in the literature are that disrupting hippocampal cell proliferation using genetic and radiological methods in mice has

been shown to abolish the behavioral responses to antidepressants (Santarelli et al 2003), while others have shown that behavioral deficits can be rescued by antidepressants without increasing neurogenesis (Hanson et al 2011), and even when neurogenic processes are blocked (Bessa et al 2009). In any case, reelin is a known regulator and enhancer of neurogenesis by promoting cell migration, dendritic maturation, and cell-cell connectivity (Bosch et al 2016a, Pujadas et al 2010, Teixeira et al 2012, Won et al 2006). This prompted us to use DCX as a marker of neurogenesis to evaluate if i.v. reelin can rescue the CORT-induced reductions in the number of DCX-IR cells and their dendritic complexity, as we demonstrated could be accomplished by etanercept and imipramine (Brymer et al 2018, Fenton et al 2015). I found that reelin produced only slight increases in DCX-IR cell number that ranged from 17% to 37% and failed to rescue the maturation rates of dendritic arbors. This was unlike intrahippocampal reelin infusions, which could rescue new-born cell counts after a single 1µg infusion when sacrificed a week later (Brymer et al 2020). Furthermore, we found that AMPAR antagonist CNQX abolished the antidepressant-like effects of intrahippocampal reelin while having no effect on neurogenesis, indicating that FST-behavioral improvements by reelin are not mediated (at least not completely) by their effects on DCX-expression. That said, one cannot discard the possibility that other behavioral impairments might relate to deficiencies in new-born granule cell proliferation and the arborization of surviving cells. As a whole, deficient neurogenesis probably relates more to antidepressant responsiveness than to episode manifestation (Park 2019).

It is unclear if reelin can cross the blood-brain barrier, therefore, the putative mechanisms whereby i.v. recombinant reelin can increase the expression of reelin in the rodent hippocampus and shield from behavioral deficits as ascertained by the FST are unknown. That said, reelin-immunoreactivity has been observed in some putative transcytosis vesicles in brain capillaries which may represent a mechanism that is mediated by one of the reelin receptors, ApoER2, allowing peripheral reelin to access the brain and vice versa (Perez-Costas et al 2015, Riddell et al 2001). We previously found that reelin and ketamine can both rescue levels of mTOR and p-mTOR in synaptoneurosomes from CORT-treated rats (Johnston et al 2020), which suggests that the antidepressant actions of both compounds share common signaling pathways, like the PI3K/Akt pathway (Jossin & Goffinet 2007, Zunszain et al 2013). Along with possible direct influences on the brain, reelin may trigger certain peripheral activities that indirectly alter central functions. We have shown previously that CORT alters SERT MPC in blood lymphocytes in a similar manner that is observed in depressed patients and *RELN*$^{+/-}$ mice

(Caruncho et al 2016, Rivera-Baltanas et al 2010, 2012, Romay-Tallon et al 2018). Additionally, that antidepressant efficacy correlates with the reversal of these alterations, so we proposed that the clustering of SERT, and perhaps other neurotransmission-influencing proteins, could potentially be used as a putative biomarker of therapeutic responsiveness (Caruncho et al 2019, Rivera-Baltanas et al 2015, Romay-Tallon et al 2018). Here I that show i.v. reelin can rescue the CORT-induced increases in SERT cluster size, which one can assume would influence central functions by modulating lymphocyte activity such as the secretion of blood-brain barrier-crossing cytokines. This is currently under investigation in our laboratory, but may explain why *RELN*$^{+/-}$ mice express altered cytokine levels (Green-Johnson et al 1995). Depressed patients and CORT-treated rats consistently display heightened pro-inflammatory cytokine levels that can be reduced by antidepressants (Dowlati et al 2010, Felger & Lotrich 2013, Kelly et al 2012, Leonard 2010, Miller et al 2009b, Sorrells & Sapolsky 2007). Interestingly, we found that the TNF-α inhibiting drug etanercept – which cannot cross the blood-brain barrier – could rescue CORT-induced FST-immobility and cognitive deficits which was associated with the restoration of hippocampal reelin (Brymer et al 2018).

Here I provided further evidence that raising reelin levels in the SGZ, which can be achieved by multiple i.v. reelin injections, is associated with antidepressant-like behavioral changes. Moreover, that reductions in immobility, which correlated with reelin-IR cell counts in the SGZ and SERT cluster size, are unlikely to be mediated by the enhancement of neurogenesis. I recognize that the effects of reelin should also be evaluated in female rats, considering that prevalence rates for MDD are 2- to 3-fold higher for female patients. As well, a larger battery of behavioral tests should be used to evaluate the effect of CORT and reelin on emotional and cognitive behavior. There is a rationale for future studies to evaluate the effects of peripheral reelin on markers of neuroplasticity that mediate the fast-acting effects of other antidepressant agents, such as excitatory and inhibitory transmission.

# Chapter 3

## Intravenous reelin rescues behavioral and neurochemical alterations relevant for depression in CORT-treated male and female rats

## 3.1 Abstract

Depression is characterized by despair, cognitive deficits, and deficient excitatory and inhibitory neurotransmission. Rats that are treated with CORT express similar alterations, such as reductions in hippocampal reelin and altered levels of $GABA_A$Rs, AMPARs, and NMDARs, an effect that can be recovered by antidepressant treatments. Reelin is a neuromodulator that is expressed by GABAergic interneurons to promote synaptogenesis and dendritogenesis, and we have demonstrated that AMPARs are required for intrahippocampal reelin infusions to generate rapid antidepressant-like effects. In this study, male and female rats received 3μg of reelin in the tail vein every 10 days over the 21-day CORT exposure period. Thereafter, they were subject to a battery of behavioral tests including the forced swim and object-location tests, followed by post-mortem analyses of reelin, $GABA_A$β2/3, GluA1, and GluN2B expression. I also assessed whether reelin could rescue neurogenesis in females. Results show that CORT increased FST-immobility and the number of reelin-, $GABA_A$β2/3- and GluA1-IR cells, and increased GluN2B optical density in the DG. FST-immobility and neurochemical alterations were rescued by i.v. reelin. Reelin partially recovered the number of adult-born granule cells ascertained using DCX as a marker, but it had little effect on their maturation rate. Our findings demonstrate that reelin protects from the deleterious effects of repeated-CORT on glutamatergic and GABAergic transmission, promoting antidepressant-like behavioral changes. Additional mechanistic and pharmacokinetic studies are a necessity.

## 3.2 Introduction

Depression is the most prevalent neuropsychiatric syndrome and the leading cause of disability worldwide with dramatic disparities in lifetime prevalence rates for males (7-12%) and females (20-25%) (Belmaker & Agam 2008, Kessler et al 2005, WHO 2017). Patients with MDD have lower levels of hippocampal reelin (Fatemi et al 2000), the extracellular matrix protein that plays a key role in several forms of plasticity such as the guiding and positioning of migrating neurons, dendritic outgrowth, synaptogenesis, and memory formation (Chameau et al 2009, D'Arcangelo 2014, Niu et al 2008, Rogers et al 2013, Weeber et al 2002). Exposure to repeated-CORT also suppresses reelin expression in the rodent SGZ, but not when CORT is given with a variety of compounds with antidepressant activity like imipramine, ketamine, and anti-inflammatory agent etanercept (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). In

fact, it could be postulated that the rescuing of CORT-induced FST-immobility, memory deficits, and reductions in neurogenesis and $GABA_A\beta2/3$-positive cells by etanercept – the TNF-α inhibitor that cannot cross the blood-brain barrier – are secondary to the restoration of reelin expression in the SGZ. A number of findings support this claim: the progressive development of behavioral phenotypes produced by repeated-CORT is paralleled by reductions in SGZ-reelin (Lebedeva et al 2020, Lussier et al 2013a); *RELN*$^{+/-}$ mice are more susceptible to the depressogenic effects of CORT (Lussier et al 2011, Notaras et al 2020, Schroeder et al 2015); and the genetic overexpression of reelin or intraventricular injections of exogenous reelin recovers behavioral impairments and markers of synaptic plasticity in several animal models (Hethorn et al 2015, Rogers et al 2011, Teixeira et al 2011).

In the experiment that is outlined in the previous chapter, I demonstrated that multiple i.v. reelin injections in male rats can ameliorate despair-like behavior as ascertained by the FST, and that this behavioral recovery is also associated with a restoration of reelin expression in the SGZ. Additionally, we previously reported that a single intrahippocampal infusion of reelin could rescue CORT-induced FST-immobility, cognitive deficits, and dysregulations in neurogenesis and GABAergic and glutamatergic transmission (Brymer et al 2020). Furthermore, we found administration with AMPAR antagonist CNQX precluded the antidepressant-like effects of reelin without interrupting its effect on neurogenesis, which indicates that the antidepressant actions of reelin are independent of its actions on new-born cell proliferation and maturation (Brymer et al 2020). The role of excitatory and inhibitory transmission in reelin's neuroprotectivity was of interest because reelin is expressed by GABAergic interneurons in corticolimbic circuits (Caruncho et al 2016), reelin-deficient mice express lower levels of GluA1 receptors (Qiu & Weeber 2007), and fast-acting antidepressants like ketamine appear to trigger a "glutamate surge" and require AMPAR-mediated activity (Kadriu et al 2020b, Koike & Chaki 2014, Zhang et al 2016). Additionally, reelin regulates learning and memory by governing the activity and subunit composition of NMDARs, downregulating the expression of GluN2B-containing receptors (Chen et al 2005, Groc et al 2007), the genetic depletion of which has been shown to mimic and occlude the effects of ketamine in mice (Miller et al 2014). Therefore, one can expect that raising reelin levels in the SGZ would strengthen excitatory and inhibitory circuits and attenuate the depressogenic effects of CORT. In fact, another group recently demonstrated that the genetic depletion of reelin or its receptor ApoER2, or pharmacological inhibition of their downstream effectors, blocks the neuroplasticity-promoting and antidepressant-like behavioral effects of ketamine, which

suggests that the reelin signaling pathway is required for the positive effects that are produced following ketamine administration (Kim et al 2021).

In the present study, I aimed to replicate and expand on our previous findings regarding the antidepressant-like and pro-cognitive effects of reelin with the addition of female rats using the lowest dose that showed to be effective in Chapter 2 (3µg given i.v. every 10 days over the CORT-injection period). This was done by subjecting the rats to a battery of behavioral tests: the FST, SPT, OFT, and OBL, followed by an examination of the effect of CORT and i.v. reelin on the expression of $GABA_A\beta2/3$, GluA1, and GluN2B in the DG. I also investigated the effects of reelin on neurogenesis in female rats, given that reelin effected neurogenic processes minimally in males (Chapter 2). Research into well-tolerated, novel compounds with antidepressant-like effects is imperative considering that only about 40% of patients respond satisfactorily to first-line monoaminergic-based antidepressants (Trivedi et al 2006), which produce vexing side-effects (Garland et al 2009) and usually require months of daily administration before therapeutic improvement is observed (Huynh & McIntyre 2008).

## 3.3 Methods

### 3.3.1 Experimental procedures

To evaluate the antidepressant-like effects of reelin in both sexes, 40 male and 40 female Long-Evans rats (Charles River Laboratories, Canada) that were 6 weeks old on arrival to the facility were used. They were left for a week to habituate to the facility on arrival, which was followed by another week of handling. The husbandry of the animals was identical to that described previously in Section 2.3.1. After the 7-day habituation period the rats were handled briefly each day for another week. During the handling week, the rats were habituated to a DecapiCone (Braintree Scientific Inc., MA) restraining device that was used throughout the injection period to administer the lateral tail vein injections. On day 3 of handling, the DecapiCone was placed into the cage with the rat for 1 minute. On the $4^{th}$, $5^{th}$, $6^{th}$ and $7^{th}$ day, the rat was placed into the DecapiCone for 1 minute.

Animals of the same sex were then weight-matched and randomly assigned to one of two treatment groups: s.c. vehicle or CORT (Steraloids) injections given at a dose of 40mg/kg. The rats were then subdivided to make a total of 8 subgroups; male or female vehicle or CORT rats

that either received vehicle or reelin (R&D systems, 3820-MR) injections at a dose of 3µg/ml every 10 days (see Figure 3.3.1).

The vehicle or CORT injections were given once per day for 21 consecutive days between the hours of 9:00 and 10:30 a.m. The animals were weighed before each injection which was administered at a volume of 1ml/kg suspended in a 0.9% (w/v) sodium chloride and 2% (v/v) polysorbate-80 (Sigma Aldrich) solution. The reelin that was suspended in 0.5 ml of PBS was injected into the lateral tail vein between the hours of 12:00 and 4:00 p.m. on the first day of the injection period and thereafter every 10 days to receive a total of 3 injections over the course of the experiment.

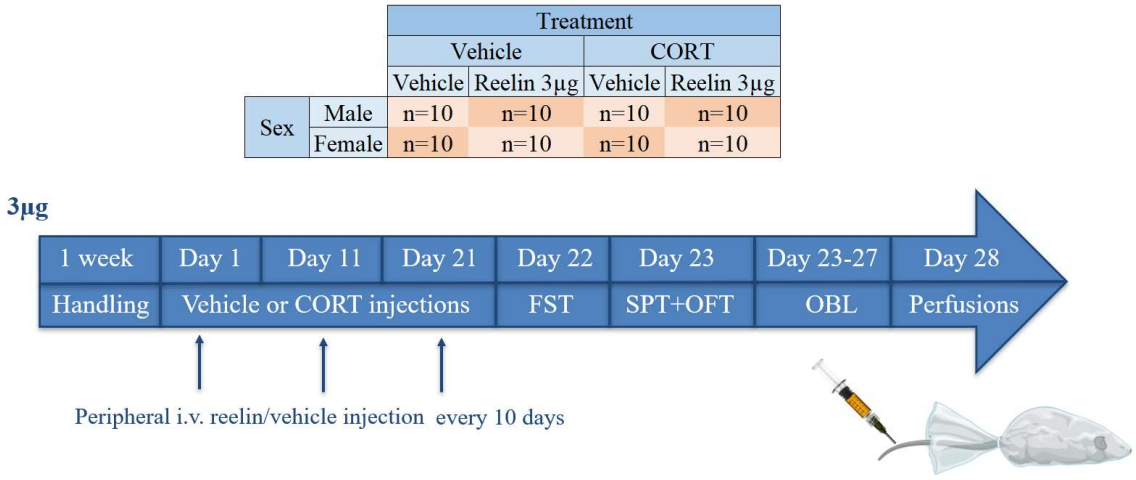


**Figure 3.3.1. Schematic representation of the experimental groups and timeline.** Rats had 7 habituation days followed by 7 handling days on arrival to the facility. They then received s.c. vehicle or CORT injections along with either i.v. vehicle or 3µg of reelin given every 10 days. Rats were subjected to the FST on day 22, the SPT and OFT on day 23, and the OBL on days 23-27, before being perfused on day 28. Figure created with Microsoft Excel and Paint by author.

### 3.3.2 Behavioral tests

Chronic CORT exposure results in behavioral phenotypes relevant for depression that are characterized by increases in immobility in the FST (which has been repeatedly used to ascertain antidepressant-like effects in preclinical studies) and hippocampal-dependent cognitive tests (Brymer et al 2018, 2020). While these alterations are observed somewhat

consistently, behavioral alterations as ascertained by other behavioral tests are not always as reproducible (like tests for anhedonia and anxiety), which represents a limitation of the CORT model and preclinical research for mental health in general. As well as the FST and OBL behavioral assays to evaluate the putative antidepressant-like effects of i.v. reelin injections, we also subjected rats to several other tests like the SPT and OFT to assess anhedonia-like behavior and general locomotor activity/anxiety-like behavior, respectively, which will be discussed in the Appendix. The behavioral tests were conducted in a procedure room that was not used for any other aspect of the study.

#### 3.3.2.1 Forced swim and object-location tests

The FST took place on day 22 in the same manner that was described previously in section 2.3.3. The OBL was conducted in a similar manner to previously established protocols (Howland & Cazakoff 2010). The test took place in a square open-field arena (65cm x 65cm), with a light blue floor and black walls 60cm high. First, the rats received 3 habituation sessions that were 10 minutes in duration each, 24 hours apart, in which they could explore the empty arena before being returned to the colony room. The first habituation session was recorded via video camera and the first 5 minutes scored as the OFT. After the habituation sessions came the sample phase, in which the rats were placed in the center of the arena and allowed to explore for 4 minutes. In this phase, 2 identical objects (Object A and B) were placed in the top 2 corners of the arena, 10cm away from the walls. 24 hours later the test phase took place which also lasted 4 minutes. In this phase, another 2 identical objects were used, but object B was moved to the opposite side of the arena – see Figure 3.5.5D. In between trials, the arena and objects were washed with hot soapy water. The objects were made of porcelain and did not exceed 10cm in height or length. The amount of time the rats spent exploring the objects was recorded. The rats were deemed to be actively exploring an object when its nose was at or under 2cm away, and its head or vibrissae were in motion. Credit was not given for exploration if the rat was standing on top of the object or looking away. The animals explored the objects for more than 15 seconds in each phase and therefore were considered to have reliable recognition memory. A discrimination ratio (DR) was calculated and analyzed by using the following formula from scoring the first 2 minutes of the test phase:

$$DR = (time\ exploring\ B\ -\ time\ exploring\ A)\ /\ (time\ exploring\ B\ +\ time\ exploring\ A)$$

Positive DR scores were indicative of intact object-recognition memory.

### 3.3.3 Perfusions and tissue preparation

On day 28 the rats were sacrificed and perfused. First, the rats were deeply anaesthetized with 5% isoflurane and then perfused transcardially using 0.1M PBS (pH 7.4) followed by 500mls of 4% (w/v) paraformaldehyde in 0.1M PBS (pH7.4). The brains were then removed and post-fixed in 4% paraformaldehyde (w/v) for 48 hours at 4°C. Thereafter, they were suspended in a 30% sucrose solution for 72 hours. Next, the brain tissue was sectioned at a thickness of 30µm using a cryostat (LEICA CM1850 UV) and kept in a cryoprotectant solution of 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone and 30% (w/v) sucrose in 0.1M PBS (pH 7.4) at -20°C.

### 3.3.4 Immunohistochemistry

Standard immunohistochemical techniques were performed with commercially available antibodies on every 6$^{th}$ section of the hippocampus in 6-well tissue culture plates under gentle agitation. An additional well was used to show that no IR cells were detected when the primary antibody was omitted.

Reelin-, DCX-, GluA1-, $GABA_A\beta2/3$-, and GluN2B-IR cells were visualized as follows. Free-floating sections were first incubated in sodium citrate buffer (pH = 6.0) for 30 minutes for epitope retrieval. They were then placed into a blocking solution of 5-15% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 30 minutes, before being exposed to either a mouse anti-reelin (1:1000, MILLIPORE, MAB5364), mouse anti-GluN2B (1:1000, catalog #75-101, RRID: AB-2232584; NeuroMab), mouse anti-$GABA_A\beta2/3$ (clone bd17, 1:1000, catalog #MAB341, RRID: AB_2109419; Millipore), rabbit anti-DCX (1:1000, Cell Signaling, Danvers, MA), or rabbit anti-GluA1 (1:1000, catalog #AB1504, RRID: AB_11212863; Millipore) antibody diluted in blocking solution for either 24 hours (room temperature) or 48 hours (at 4°C). Thereafter, endogenous peroxidase activity was blocked by incubating the sections for 30 minutes with 5-10% (v/v) $H_2O_2$ in 0.1 M TBS. The sections were then incubated in either a biotinylated goat anti-rabbit secondary antibody (1:500, catalog #BA-

1000, RRID: AB_2313606; Vector Laboratories) or a biotinylated goat anti-mouse secondary antibody (1:500, catalog #BA-2000. RRID: AB_2313571; Vector Laboratories) diluted in 5-15% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 1 or 2 hours. Next, they were treated with avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour followed by rinses with TBS or 0.175 M sodium acetate (pH = 6.8). Reelin, GluA1, $GABA_A\beta2/3$, and DCX immunolabelling was visualized with 0.02-0.025% (w/v) DAB and 0.002-0.0078% $H_2O_2$ diluted in TBS. GluN2B immunolabelling was visualized with 4.167% $NiSO_4$ and 0.05% (w/v) glucose oxidase DAB. Finally, the sections were rinsed before being mounted onto glass slides using 0.1 M TBS, air dried overnight, dehydrated using increasing concentrations of ethanol, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

### 3.3.5 Cell counting

The number of reelin-, GluA1-, DCX-, and $GABA_A\beta2/3$-IR cells in the SGZ (defined as a 2-cell width zone in between the inner GCL and the PL) were counted using a Nikon Eclipse E800 microscope with a motorized stage linked to a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc) as previously described in Section 2.3.6.

### 3.3.6 Categorization of immature DCX-IR cells

To determine whether there was an effect of CORT and reelin treatment on the dendritic morphology of immature granule cells in female rats, dendritic branching of 100 randomly selected DCX-IR cells that were evenly distributed across 5 tissue sections for each rat were semi-quantified as previously outlined in Section 2.3.7; see Figure 2.4.5C for representative photomicrographs of the 6 categories of dendritic complexity that was used to classify cells. Categories 1 (no processes) and 2 (one small process) represented the proliferative stage of development, Categories 3 (medium process reaching the GCL) and 4 (process reaching the molecular layer) represented an intermediate stage of cell development, and Categories 5 (one

major process extending into the molecular layer) and 6 (defined dendritic tree with delicate dendritic branching in the GCL) represented more mature stages of cell development.

### 3.3.7 Optical densitometry

In addition to stereological counts of cells, semi-quantitative optical densitometry analyses of GluA1-, $GABA_A\beta2/3$-, and GluN2B-IR cells was performed on 3 sections per brain that were 180µm (6 × 30µm) apart following methods previously described by our laboratory (Botterill et al 2015b). Briefly, grayscale photomicrographs were acquired on our Nikon E800 microscope with exposure and gain settings held constant for every subject. A program called ImageJ (V1.46R, National Institutes of Health, Bethesda, MD, USA) was then used to measure the mean optical density for each region of interest. The program can be used to quantify the intensity of an immunoreaction product within traced regions that were randomly distributed throughout the sections of interest. To control for background staining, the mean optical density of the corpus callosum was subtracted from the immunoreactive measurements. All values were standardized between white (0) and black (255) and expressed as a percentage change from controls (male vehicle/vehicle rats).

## 3.4 Statistical analysis

The results were analyzed using SPSS (IBM, USA). Data were tested for normality and homogeneity of variance before carrying out appropriate statistical analyses. A three-factor repeated measures analysis of variance (ANOVA) was used to analyze data over time, such as for bodyweight, with sex, CORT treatment and reelin treatment as main effects. Sphericity could not be assumed with Mauchly's test of Sphericity, so the Greenhouse-Geisser correction was employed. Three-way ANOVAs were used to analyze the behavioral and neurobiological data, as well as total bodyweight changes. If significant main or interaction effects were found, Tukey *post hoc* tests were conducted. Pearson's bivariate correlation coefficient tests were also used to correlate the behavioral and neurochemical data. Groups were considered to be statistically different from each other at $p<0.05$. The data are expressed as mean±CI.

## 3.5 Results

### 3.5.1 Bodyweight

Sphericity could not be assumed ($p<0.05$) with Mauchly's test of Sphericity for a two-way ANOVA repeated measures when analyzing the bodyweight data over time, so the Greenhouse-Geisser correction was employed. When looking at the within-subjects effects, the results show that there is a significant effect of time on weight gain [$F(2.992, 215.416)=125.402$, $p<0.001$], animals becoming heavier over time. There was also a significant interaction effect of time × sex [$F(2.992, 215.416)=12.712$, $p<0.001$], time × CORT treatment [$F(2.992, 215.416)=160.009$, $p<0.001$], as well as time × sex × CORT treatment [$F(2.992, 215.416)=47.114$, $p<0.001$], but not time × sex × reelin treatment [$F(2.992, 215.416)=1.196$, $p=0.312$]. The male rats weighed more than the female rats, and although CORT treatment decreased the weight of the rats in both sexes, the male rats were more affected (Figure 3.5.1A). The statistical information for the between-subjects effects for bodyweight, and for the behavioral data from each test is outlined in Table 3.5.1.

When looking at the percentage of weight change over the 21-day injection period, *post hoc* analyses revealed that CORT/vehicle- and CORT/reelin-treated rats gained significantly less weight than vehicle/vehicle- and vehicle/reelin-treated rats regardless of sex ($p<0.001$), and that the male CORT/vehicle and CORT/reelin rats gained significantly less weight than the female CORT/vehicle rats and CORT/reelin rats ($p<0.001$). There were on other group differences (see Figure 3.5.1B).

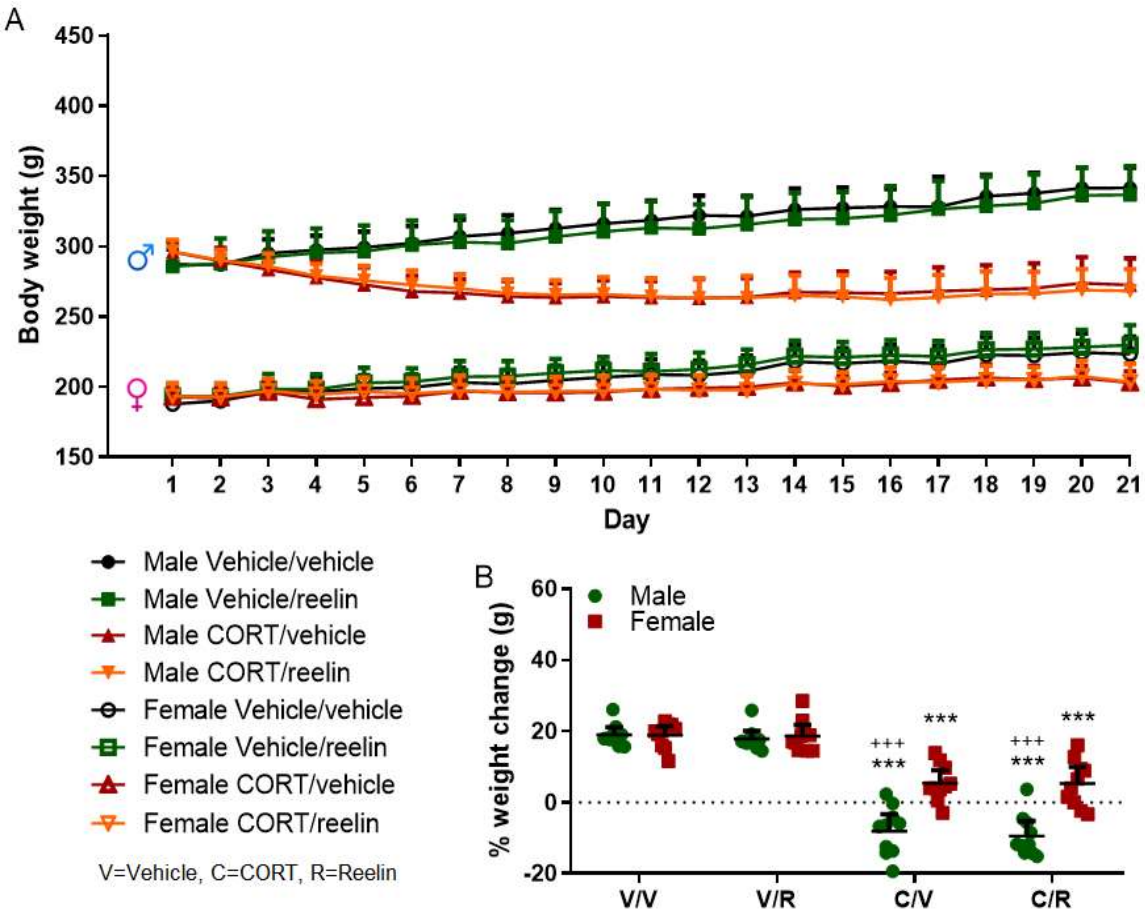


**Figure 3.5.1. The effect of sex, CORT, and reelin treatment on bodyweight.** A) Bodyweight over the 21-day injection period. B) Percentage of weight change over the 21-days. CORT treatment decreased weight gain, and CORT effected the weight of male rats more so than female rats. Data are expressed as mean±CI. ***$p$<0.001 vs vehicle-control; +++$p$<0.001 vs female comparison. Figure created with GraphPad Prism by author.

**Table 3.5.1. Statistical information for the effects of sex, CORT, and reelin on bodyweight and behavioral measures.**

| Measure | Sex | CORT | Reelin | CORT × Reelin | Sex × CORT | Sex × Reelin |
|---|---|---|---|---|---|---|
| Bodyweight | F(1, 72)=530.305, $p$<0.001 | F(1, 72)=51.609, $p$<0.001 | F(1, 72)=0.001, $p$=0.976 | F(1, 72)=0.002, $p$=0.968 | F(1, 72)=17.173, $p$<0.001 | F(1, 72)=0.425, $p$=0.517 |
| % weight change | F(1, 72)=42.265, $p$<0.001 | F(1, 72)=331.884, $p$<0.001 | F(1, 72)=0.427, $p$=0.516 | F(1, 72)=0.427, $p$=0.516 | F(1, 72)=38.617, $p$<0.001 | F(1, 72)=0.221, $p$=0.640 |
| FST-immobility | F(1, 72)=1.990, $p$=0.163 | F(1, 72)=43.062, $p$<0.001 | F(1, 72)=4.131, $p$=0.046 | [F(1, 72)=14.494, $p$<0.001 | F(1, 72)=4.442, $p$=0.030 | F(1, 72)=0.402, $p$=0.528 |
| FST-latency | F(1, 72)=1.691, $p$=0.198 | F(1, 72)=10.273, $p$=0.002 | F(1, 72)=0.240, $p$=0.626 | F(1, 72)=5.941, $p$=0.017 | F(1, 72)=15.00, $p$<0.001 | F(1, 72)=0.007, $p$=0.933 |
| FST-climbing | F(1, 72)=0.11, $p$=0.741 | F(1, 72)=6.897, $p$=0.011 | F(1, 72)=0.0003, $p$=0.985 | F(1, 72)=7.885, $p$=0.006 | F(1, 72)=1.092, $p$=0.299 | F(1, 72)=0.694, $p$=0.408 |
| FST-swimming | F(1, 72)=1.949, $p$=0.167 | F(1, 72)=19.870, $p$<0.001 | F(1, 72)=3.228, $p$=0.077 | F(1, 72)=0.826, $p$=0.366 | F(1, 72)=2.049, $p$=0.157 | F(1, 72)=1.323, $p$=0.254 |
| OBL-DR | F(1, 71)=0.333, $p$=0.566 | F(1, 71)=4.558, $p$=0.036 | F(1, 71)=3.707, $p$=0.058 | F(1, 71)=0.704, $p$=0.404 | F(1, 71)=0.104, $p$=0.748 | F(1, 71)=0.028, $p$=0.867 |
| OBL-distance travelled | F(1, 71)=43.326, $p$<0.001 | F(1, 71)=4.505, $p$=0.037 | F(1, 71)=0.976, $p$=0.327 | F(1, 71)=0.373, $p$=0.544 | F(1, 71)=0.900, $p$=0.346 | F(1, 71)=0.515, p=0.467 |
| OBL-velocity | F(1, 71)=2.363, $p$=0.129 | F(1, 71)=3.184, $p$=0.079 | F(1, 71)=0.019, $p$=0.892 | F(1, 71)=0.470, $p$=0.495 | F(1, 71)=0.245, $p$=0.622 | F(1, 71)=0.058, $p$=0.810 |

### 3.5.2 Forced swim test

Using male rats, we previously found that i.v. reelin can rescue the increases in FST-immobility instigated by chronic CORT exposure. Here I replicated and expanded our findings, revealing that CORT increased time spent immobile in females ($p$=0.013) and even more so in males ($p$<0.001), although there was no statistical difference between the sexes. Reelin reduced FST-immobility by 56% in males ($p$=0.008) and 58% in females ($p$=0.441). Additionally, the male CORT/vehicle rats had a lower latency to immobility time ($p$<0.001) and spent less time climbing ($p$=0.005) and swimming ($p$≤0.043) than the male vehicle/vehicle rats (see Figure 3.5.2).

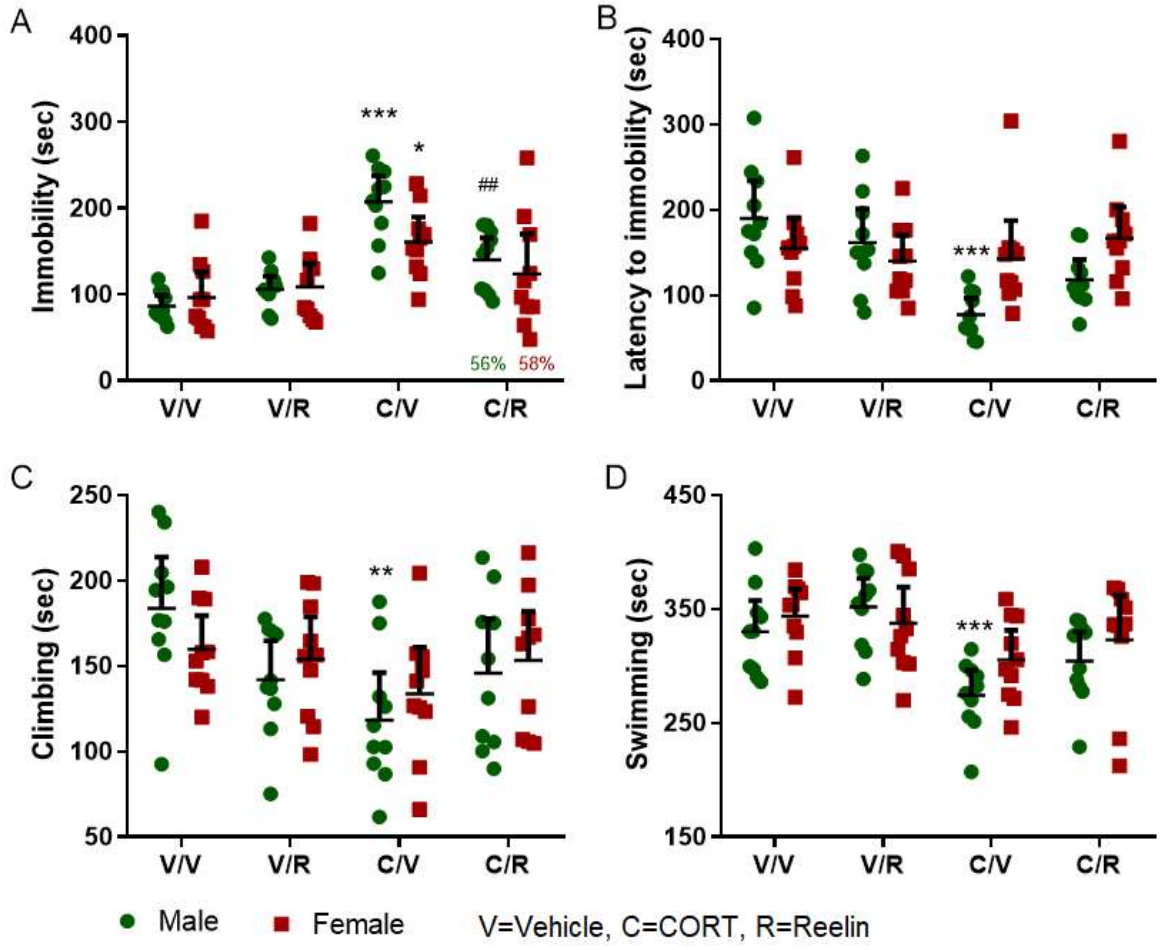


**Figure 3.5.2. The effect of sex, CORT, and reelin treatment on FST-behaviors.** A) Time spent immobility. B) Latency to first immobility. C) Time spent climbing. D) Time spent swimming. Vehicle rats generally exhibited more active behaviors than CORT-treated rats. Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05/**$p$<0.01/***$p$<0.001 vs same-sex vehicle/vehicle; ##$p$<0.01 vs male CORT/vehicle. Figure created with GraphPad Prism by author.

### 3.5.3 Object location recognition-memory test

Cognition was assessed with the hippocampal-dependent OBL. The CORT-induced decreases in DR scores were not significant for either sex ($p \geq 0.380$) but were recovered by reelin in males (by 84%) and females (by 117%) nonetheless (insignificantly). I also found that the females (vehicle/vehicle, vehicle/reelin and CORT/reelin rats) travelled less than the male rats that were treated similarly ($p \leq 0.029$) but there were no group differences regarding velocity.

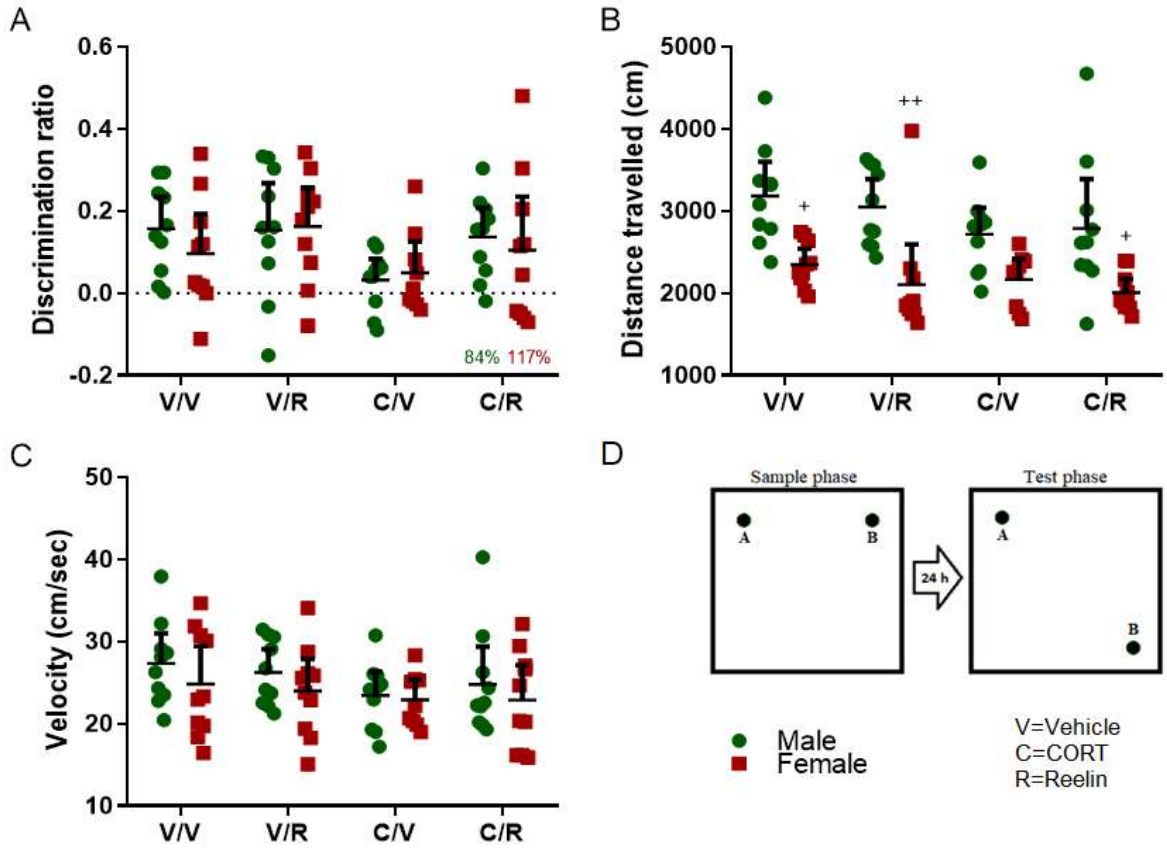


**Figure 3.5.3. The effect of sex, CORT and reelin treatment on OBL-behaviors.** A) Recognition memory as measured by the DR. B) Distance travelled. C) Velocity. D) Schematic representation of the OBL phases. Data are expressed as mean±CI. Percentage of partial recovery = %. +$p$<0.05/++$p$<0.01 vs male comparison. Figure created with GraphPad Prism and Paint by author.

**Table 3.5.5. Time exploring objects in the OBL test phase.**

| Sex | Treatment | Time with A (sec) | Time with B (sec) | Total time with objects (sec) | Discrimination ratio |
|---|---|---|---|---|---|
| Male | Vehicle/vehicle | 21±1.98 | 28.0±0.95 | 49.1±2.76 | 0.157±0.034 |
| | Vehicle/reelin | 18.5±1.18 | 25.8±2.02 | 44.3±2.60 | 0.154±0.050 |
| | CORT/vehicle | 21.3±1.27 | 22.6±1.13 | 43.9±2.20 | 0.033±0.023 |
| | CORT/reelin | 19.8±1.06 | 26.2±1.49 | 46.1±2.09 | 0.137±0.0315 |
| Female | Vehicle/vehicle | 18.9±2.06 | 22.5±1.87 | 41.5±3.63 | 0.097±0.043 |
| | Vehicle/reelin | 16.3+1.35 | 23.2±2.37 | 39.5±3.46 | 0.163±0.042 |
| | CORT/vehicle | 19.1±1.78 | 20.5±1.02 | 39.6±2.69 | 0.049±0.033 |
| | CORT/reelin | 19.1±2.80 | 22.6±2.16 | 41.8±4.44 | 0.104±0.058 |

Time exploring objects was calculated by scoring the first 2 minutes of the test phase. Objects A and B are depicted in Figure 3.5.5D. The DR was calculated using the formula as follows: *DR = (time exploring B – time exploring A) ÷ (time exploring B + time exploring A).*

### 3.5.4 Reelin cell counts

We previously revealed that multiple dosages of i.v. reelin rescued SGZ-reelin-IR cell counts in males. Here I show that the male and female CORT/vehicle rats had fewer immunopositive cells than the vehicle/vehicle ($p<0.001$) and CORT/reelin ($p\leq0.031$) rats (representative photomicrographs are shown in Figure 3.5.4A), i.v. reelin recovering cell counts in males and females by 80% and 72%, respectively (Figure 3.5.4B). The statistical information for the between-subjects effects for neurochemical data is outlined in Table 3.5.8.

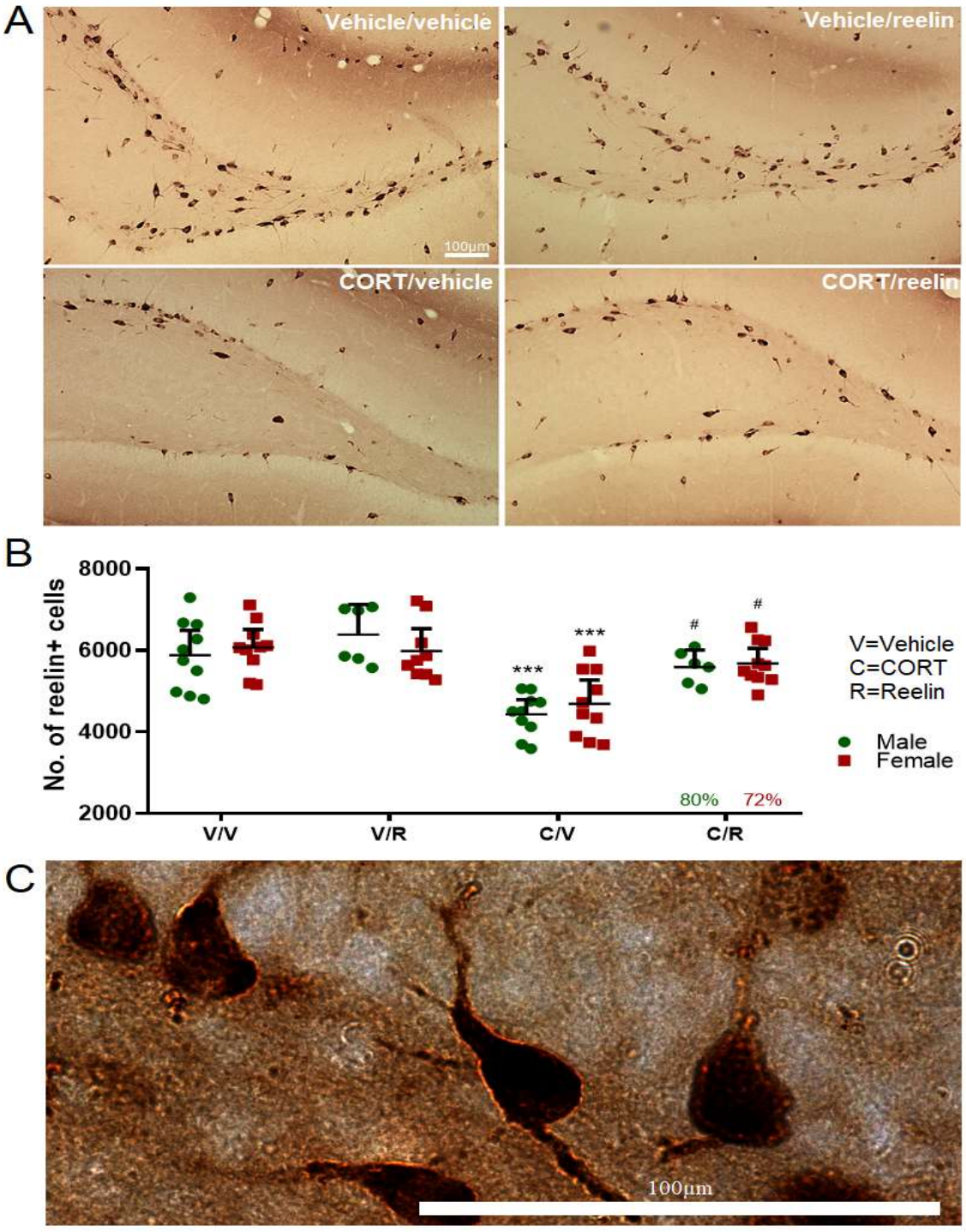


**Figure 3.5.4. The effect of sex, CORT and reelin on the number of reelin-IR cell number in the SGZ.** A) Representative photomicrographs of reelin expression in the SGZ. Scale bar = 100µm. B) The effect of sex and treatment on the number of reelin-IR cells: The CORT-induced decreases in SGZ-reelin expression were significantly rescued by 3µg of reelin given every 10 days by 80% in males and 72% in females. C) High magnification image of reelin-IR GABAergic cells in the SGZ. Data are represented as mean±CI. Percentage of recovery = %. ***$p$<0.001 vs vehicle/vehicle; #$p$<0.05 vs CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

### 3.5.5 DCX-IR cell counts and dendritic categorization

We previously found that reelin had little effect on neurogenesis in males using DCX as a marker of new-born cells. Here I show that female CORT/vehicle rats had fewer DCX-IR cells (photomicrographs shown in Figure 3.5.5A/C) compared to the vehicle/vehicle rats ($p \leq 0.003$; Figure 3.5.5B). When rats were given reelin alongside CORT, DCX-IR cell counts were recovered by 41% and were no longer statistically significantly different from the vehicle/vehicle-treated rats, indicating a partial recovery.

I then categorized 100 randomly selected DCX-IR cells based on their dendritic complexity and found that the CORT/vehicle rats had an increased percentage of cells in the proliferative (category 1 and 2) stages of development compared to the vehicle/vehicle rats ($p \leq 0.021$), but not when treated with reelin, which produced slight recoveries that ranged from 19% to 30% ($p \geq 0.052$). There were no differences in category 3, 4 or 5 cells, but rats that received CORT had a lower percentage of category 6 cells regardless of reelin treatment ($p < 0.001$).

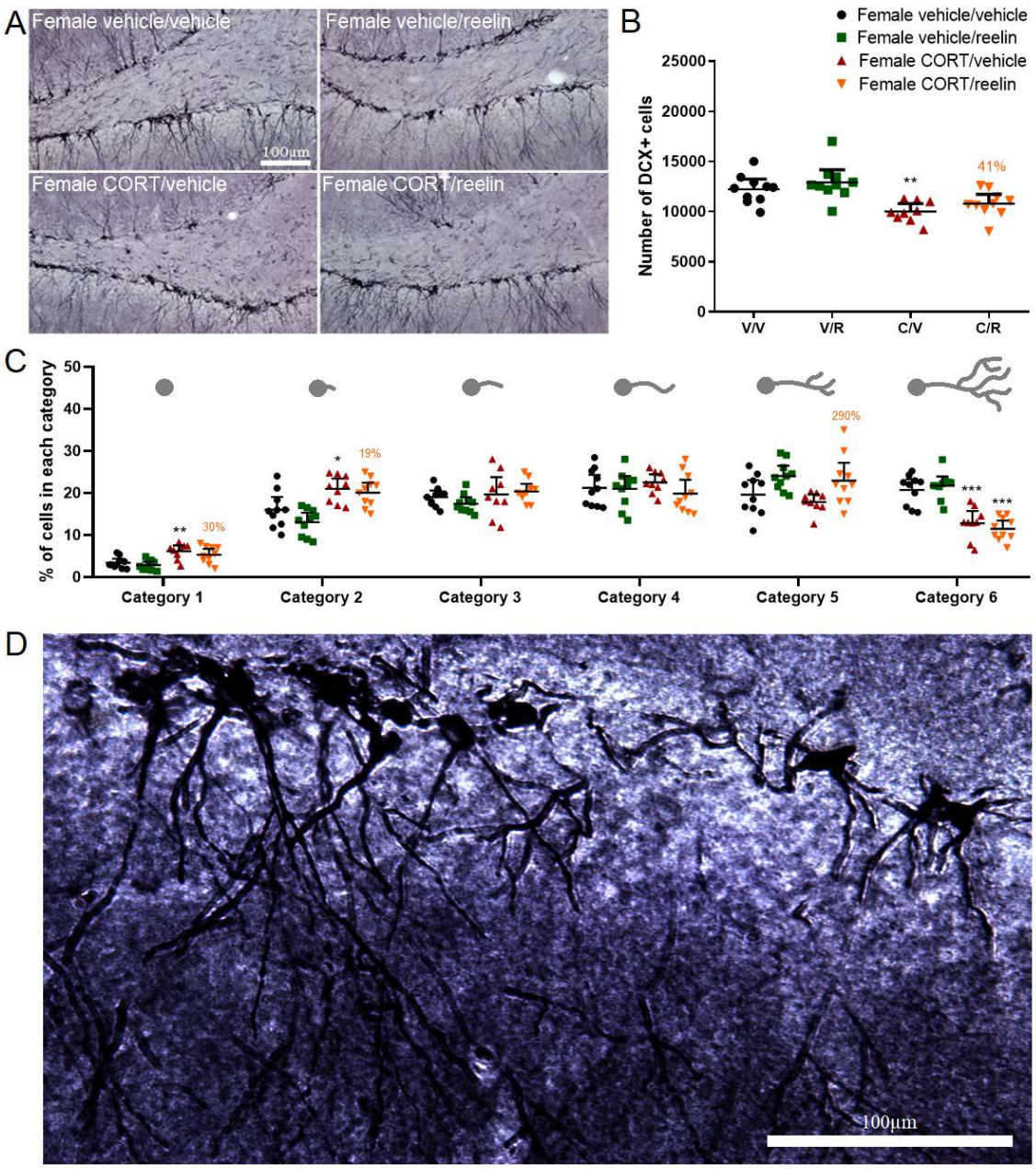


**Figure 3.5.5. The effect of CORT and reelin on DCX-IR cells in females.** A) Representative photomicrographs of DCX expression in the DG. Scale bar = 100µm. B) The effect of treatment on the number of DCX-IR cells: reelin partially recovered the CORT-induced reductions in DCX-IR cell number but not to a significant degree (the percentage of recovery is expressed above the respective error bar). C) The effect of treatment on dendritic complexity after analysis of a random subset of DCX-IR cells: In general, rats treated with CORT had a higher percentage of cells in the proliferative (categories 1 to 2) developmental stages and a lower percentage of cells in the post-mitotic stage (category 6), demonstrating that CORT slowed the maturation rate of new-born cells which was only slightly rescued by reelin. D) High magnification images of DCX-IR cells. Data are represented as mean±CI. Percentage of recovery = %. *$p$<0.05/**$p$<0.01/***$p$<0.001 vs vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

### 3.5.6 GluA1 cell counts and optical density

Given that AMPARs are required for the antidepressant-like effects of intrahippocampally-infused reelin and rapid-acting antidepressant ketamine (Brymer et al 2020, Aleksandrova et al 2017), I investigated whether i.v. reelin could rescue the downregulation of GluA1-IR cells that follows repeated-CORT. Figure 3.5.6A provides photomicrographs of GluA1 immunostaining for each group. Only cells located along the SGZ were included in the analysis. I found that CORT produced a significant decrease in GluA1 expression in females ($p<0.001$), but the decrease in males narrowly missed statistical significance ($p=0.056$). However, the male CORT/vehicle rats did have significantly lower cell counts than the female vehicle/vehicle rats ($p=0.003$). Reelin recovered GluA1 expression in males by 81% ($p=0.215$ vs male CORT/vehicle and $p=0.044$ vs female CORT/vehicle) and in females by 87% ($p=0.003$; see Figure 3.5.6B). I then looked at the optical density of GluA1-immunoractivity in the inner and outer ML, GCL and PL, and found that there were no group differences in any of the layers.

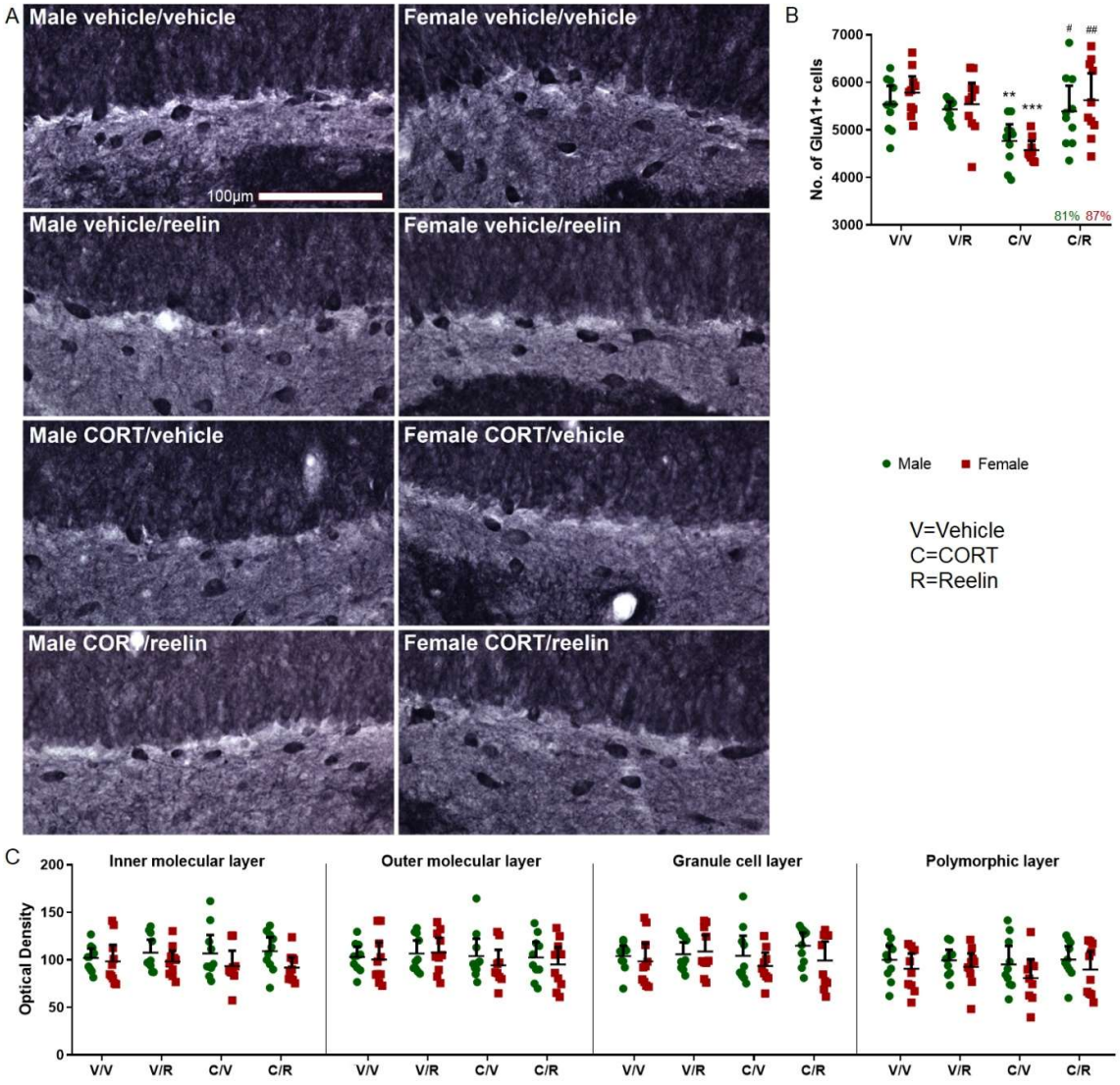


**Figure 3.5.6. The effect of sex, CORT and reelin treatment on GluA1-IR cell counts and optical density in the DG.** A) Photomicrographs of GluA1-IR cells. Scale bar = 100µm. B) Number of GluA1-IR cells in the SGZ. C) Optical density measurements for different areas in the DG, expressed as percentage change from the male vehicle/vehicle rats. In general, CORT-treated rats had significantly fewer GluA1-IR cells than the other groups and CORT and reelin had no effect on GluA1 optical density. Data are represented as mean±CI. Percentage of recovery = %. **$p$<0.01/***$p$<0.001 vs female vehicle/vehicle; #/$p$<0.05/##$p$<0.01 vs female CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

### 3.5.7 GluN2B optical density

I also assessed the optical density of GluN2B across the DG (see Figure 3.5.7A for photomicrographs) and found that the expression of GluN2B was significantly increased by

CORT in the outer ML in male rats ($p$=0.036 vs male vehicle/vehicle), and for both sexes in the GCL (male $p$=0.016 and female $p$=0.044 vs same-sex vehicle/vehicle). Reelin partially (insignificantly) recovered GluN2B expression in males (≥51%) and females (≥46%; see Figure 3.5.7B). Although not shown here, we found that CORT also increased GluN2B expression with western blotting using the same antibody (weighing ~190kDa). It would be valuable to determine where the GluN2B-IR cells are located (i.e., pre/post synaptic and synaptic/extrasynaptic) in follow-up studies.

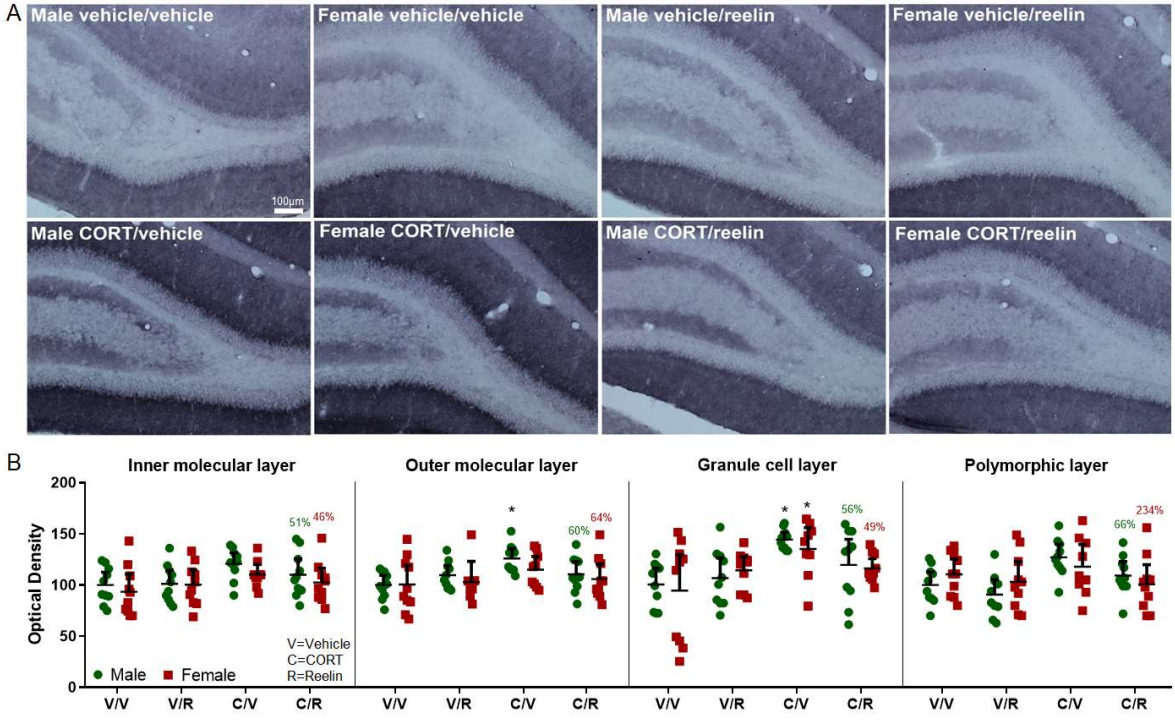


**Figure 3.5.7. The effect of sex, CORT and reelin treatment on GluN2B optical density.** A) Photomicrographs of hippocampal GluN2B. Scale bar = 100µm. B) Optical density measurements for different areas of the DG, expressed as percentage change from the male vehicle/vehicle rats. In general, CORT increased GluN2B immunoreactivity for both sexes but more so in males than females and reelin partially rescued GluN2B immunoreactivity. Data are represented as mean±CI. Percentage of recovery = %. *$p$<0.05 vs same-sex vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

### 3.5.8 GABA$_A$β2/3 cell counts and optical density

Next, I evaluated how i.v. reelin influenced GABA$_A$β2/3-IR cell counts (photomicrographs can be seen in Figure 3.5.10A). I found that CORT decreased the number of GABA$_A$β2/3-IR cells in males and females ($p$<0.001 and $p$=0.009 vs vehicle/vehicle, respectively), and reelin

significantly recovered these deficits by 92% in both sexes (males $p$=0.004, females $p$=0.020; see Figure 3.5.10B). I found no group differences for $GABA_A\beta2/3$ immunoreactive optical density measurements in the inner and outer ML, GCL and PL (Figure 3.5.8C).

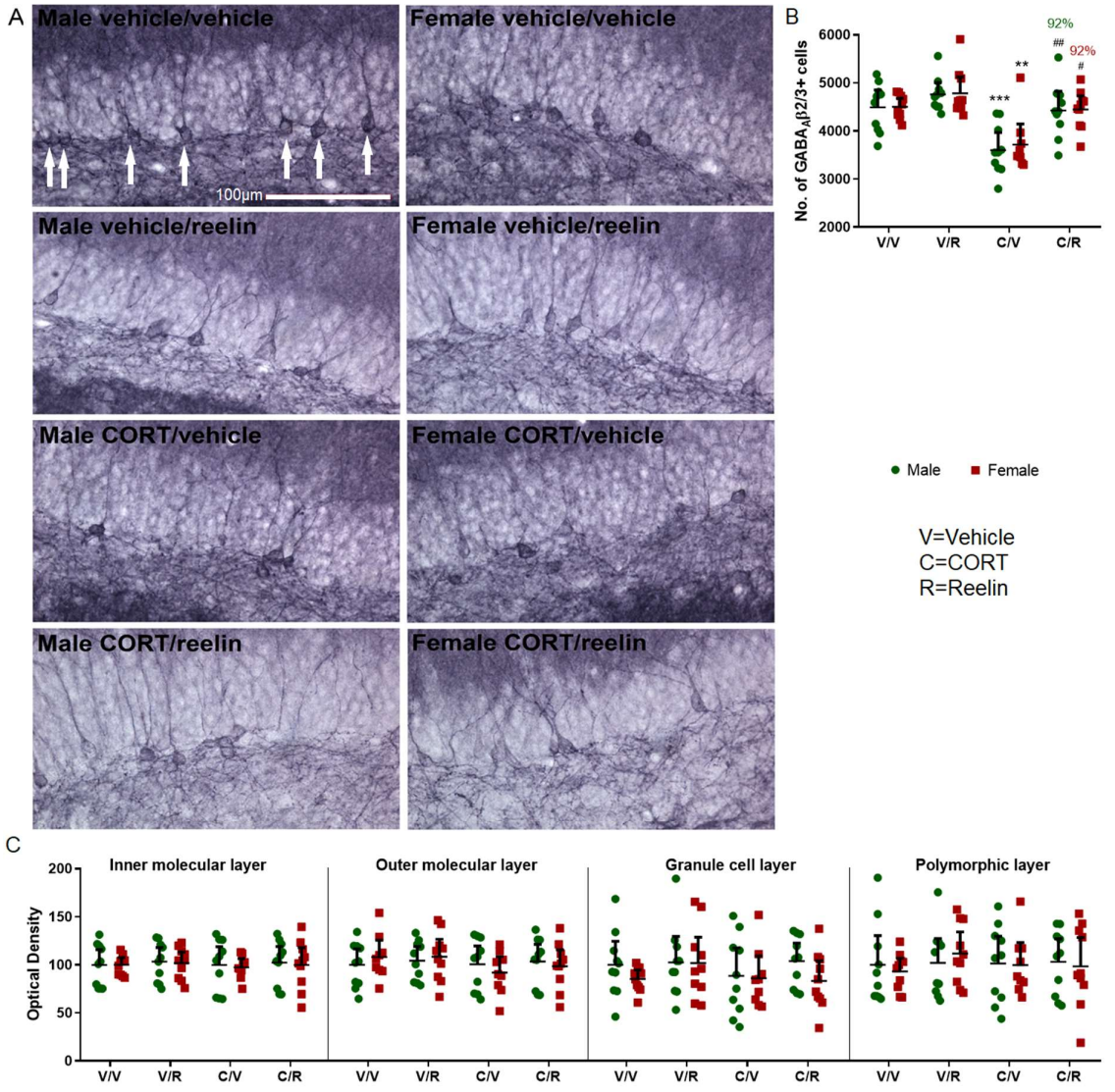


**Figure 3.5.8. The effect of sex, CORT and reelin treatment on GABAAβ2/3-IR cell count and optical density.** A) Photomicrographs of $GABA_A\beta2/3$-IR cells. Scale bar = 100µm. B) Number of $GABA_A\beta2/3$-IR cells in the SGZ: CORT decreased the number of $GABA_A\beta2/3$-expressing cells which was recovered by reelin (92%). C) There was no effect of sex, CORT or reelin on optical density measurements in the DG (expressed as percentage change from the male vehicle/vehicle rats). Data are presented as mean±CI. Percentage of recovery = %. $^{**}p<0.01$/$^{***}p<0.001$ vs same-sex vehicle/vehicle; $\#p<0.05$/$\#\#p<0.01$ vs same-sex CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

**Table 3.5.8. Statistical information for the effects of sex, CORT, and reelin on neurochemical measures.**

| Measure | Sex | CORT | Reelin | CORT × Reelin | Sex × CORT | Sex × Reelin |
|---|---|---|---|---|---|---|
| SGZ-reelin | F(1, 63)=0.047, $p$=0.829 | [F(1, 63)=36.800, $p$<0.001 | F(1, 63)=15.650, $p$=0.00019 | F(1, 63)=7.166, $p$=0.009 | F(1, 63)=0.750, $p$=0.390 | F(1, 63)=1.374, $p$=0.246 |
| DCX cell counts | na | [F(1, 35)=25.829, $p$<0.001 | [F(1, 35)=3.531, $p$=0.069 | [F(1, 35)=0.135, $p$=0.716 | na | |
| DCX cat 1 | | [F(1, 35)=25.977, $p$<0.001 | [F(1, 35)=1.855, $p$=0.182 | [F(1, 35)=0.054, $p$=0.818 | | |
| DCX cat 2 | | [F(1, 35)=27.873, $p$<0.001 | [F(1, 35)=2.982, $p$=0.093 | [F(1, 35)=0.804, $p$=0.376 | | |
| DCX cat 3 | | [F(1, 35)=2.844, $p$=0.101 | [F(1, 35)=0.183, $p$=0.672 | [F(1, 35)=1.196, $p$=0.282 | | |
| DCX cat 4 | | [F(1, 35)=0.005, $p$=0.944 | [F(1, 35)=1.215, $p$=0.278 | [F(1, 35)=0.836, $p$<0.367 | | |
| DCX cat 5 | | [F(1, 35)=1.004, $p$=0.323 | [F(1, 35)=11.425, $p$=0.002 | [F(1, 35)=0.061, $p$=0.806 | | |
| DCX cat 6 | | [F(1, 35)=72.324, $p$<0.001 | [F(1, 35)=0.031, $p$=0.862 | [F(1, 35)=1.207, $p$=0.279 | | |
| GluA1 cell counts | F(1, 71)=0.616, $p$=0.435 | F(1, 71)=14.755, $p$=0.0002 | F(1, 71)=6.953, $p$=0.010 | F(1, 71)=16.057, $p$<0.001 | F(1, 71)=0.368, $p$=0.546 | F(1, 71)=0.312, $p$=0.578 |
| GluA1 OD inner ML | F(1, 71)=2.314, $p$=0.133 | F(1, 71)=0.082, $p$=0.775 | F(1, 71)=1.114, $p$=0.295 | F(1, 71)=0.072, $p$=0.789 | F(1, 71)=0.903, $p$=0.345 | F(1, 71)=0.056, $p$=0.813 |
| GluA1 OD outer ML | F(1, 71)=0.849, $p$=0.361 | F(1, 71)=1.196, $p$=0.278 | F(1, 71)=0.280, $p$=0.598 | F(1, 71)=0.333, $p$=0.565 | F(1, 71)=0.606, $p$=0.439 | F(1, 71)=0.092, $p$=0.762 |
| GluA1 OD GCL | F(1, 71)=1.990, $p$=0.163 | F(1, 71)=0.063, $p$=0.803 | F(1, 71)=1.935, $p$=0.169 | F(1, 71)=0.035, $p$=0.852 | F(1, 71)=1.276, $p$=0.262 | F(1, 71)=0.031, $p$=0.861 |
| GluA1 OD PL | F(1, 71)=4.108, $p$=0.046 | F(1, 71)=0.656, $p$=0.42 | F(1, 71)=0.592, $p$=0.444 | F(1, 71)=0.392, $p$=0.533 | F(1, 71)=0.181, $p$=0.672 | F(1, 71)=0.094, $p$=0.858 |
| GluN2B OD inner ML | F(1, 71)=2.177, $p$=0.145 | F(1, 71)=7.961, $p$=0.006 | F(1, 71)=0.358, $p$=0.551 | F(1, 71)=0.2.391, $p$=0.126 | F(1, 71)=0.358, $p$=0.546 | F(1, 71)=0.234, $p$=0.630 |
| GluN2B OD outer ML | F(1, 71)=2.045, $p$=0.157 | F(1, 71)=7.954, $p$=0.006 | F(1, 71)=0.759, $p$=0.386 | F(1, 71)=4.895, $p$=0.030 | F(1, 71)=0.245, $p$=0.622 | F(1, 71)=0.021, $p$=0.884 |
| GluN2B OD GCL | F(1, 71)=0.186, $p$=0.667 | F(1, 71)=15.660, $p$<0.001 | F(1, 71)=0.500, $p$=0.482 | F(1, 71)=7.718, $p$=0.007 | F(1, 71)=0.331, $p$=0.567 | F(1, 71)=0.584, $p$=0.447 |
| GluN2B OD PL | F(1, 71)=0.069, $p$=0.749 | F(1, 71)=6.090, $p$=0.016 | F(1, 71)=6.353, $p$=0.014 | F(1, 71)=0.821, $p$=0.368 | F(1, 71)=3.988, $p$=0.051 | F(1, 71)=0.019, $p$=0.892 |
| GABAAβ2/3 cell counts | F(1, 71)=0.152, $p$=0.698 | F(1, 71)=32.079, $p$<0.001 | F(1, 71)=25.739, $p$<0.001 | F(1, 71)=5.871, $p$=0.018 | F(1, 71)=0.06, $p$=0.807 | F(1, 71)=0.042, $p$=0.838 |
| GABAAβ2/3 OD inner ML | F(1, 71)=0.119, $p$=0.732 | F(1, 71)=0.096, $p$=0.758 | F(1, 71)=0.319, $p$=0.574 | F(1, 71)=0.00006, $p$=0.994 | F(1, 71)=0.039, $p$=0.844 | F(1, 71)=0.005, $p$=0.943 |
| GABAAβ2/3 OD outer ML | F(1, 71)=0.001, $p$=0.972 | F(1, 71)=1.490, $p$=0.226 | F(1, 71)=0.413, $p$=0.523 | F(1, 71)=0.036, $p$=0.849 | F(1, 71)=1.415, $p$=0.238 | F(1, 71)=0.002, $p$=0.962 |
| GABAAβ2/3 OD GCL | F(1, 71)=2.627, $p$=0.109 | F(1, 71)=0.269, $p$=0.605 | F(1, 71)=0.383, $p$=0.538 | F(1, 71)=0.035, $p$=0.852 | F(1, 71)=0.021, $p$=0.886 | F(1, 71)=0.285, $p$=0.595 |
| GABAAβ2/3 OD PL | F(1, 71)=0.013, $p$=0.910 | F(1, 71)=0.017, $p$=0.898 | F(1, 71)=0.444, $p$=0.508 | F(1, 71)=0.410, $p$=0.524 | F(1, 71)=0.083, $p$=0.774 | F(1, 71)=0.177, $p$=0.675 |

GCL = granule cell layer; ML = molecular layer; OD = optical density; PL = hilus.

### 3.5.9 Correlations

Correlational graphs depicting the relationships between reelin expression in the SGZ and behavioral and other neurochemical alterations that were analyzed are shown in Figure 3.5.9a. When all the groups were included in the analysis, Pearson's bivariate correlation coefficient

tests confirmed that lower reelin levels were associated significantly with higher immobility scores (r=-0.477, $p$=0.001, n=71), a decreased preference for sucrose (r=0.430, $p$=0.001, n=71; see Appendix for data), object-recognition memory scores (r=-0.297, $p$=0.012, n=70), GluA1- (r=-0.392, $p$=0.001, n=70) and $GABA_A\beta2/3$-IR (r=-0.450, $p$<0.001, n=70) cell counts, and higher GluN2B expression in the GCL (r=-0543, $p$<0.001, n=70). There were no significant correlations when the treatment groups were analyzed individually.

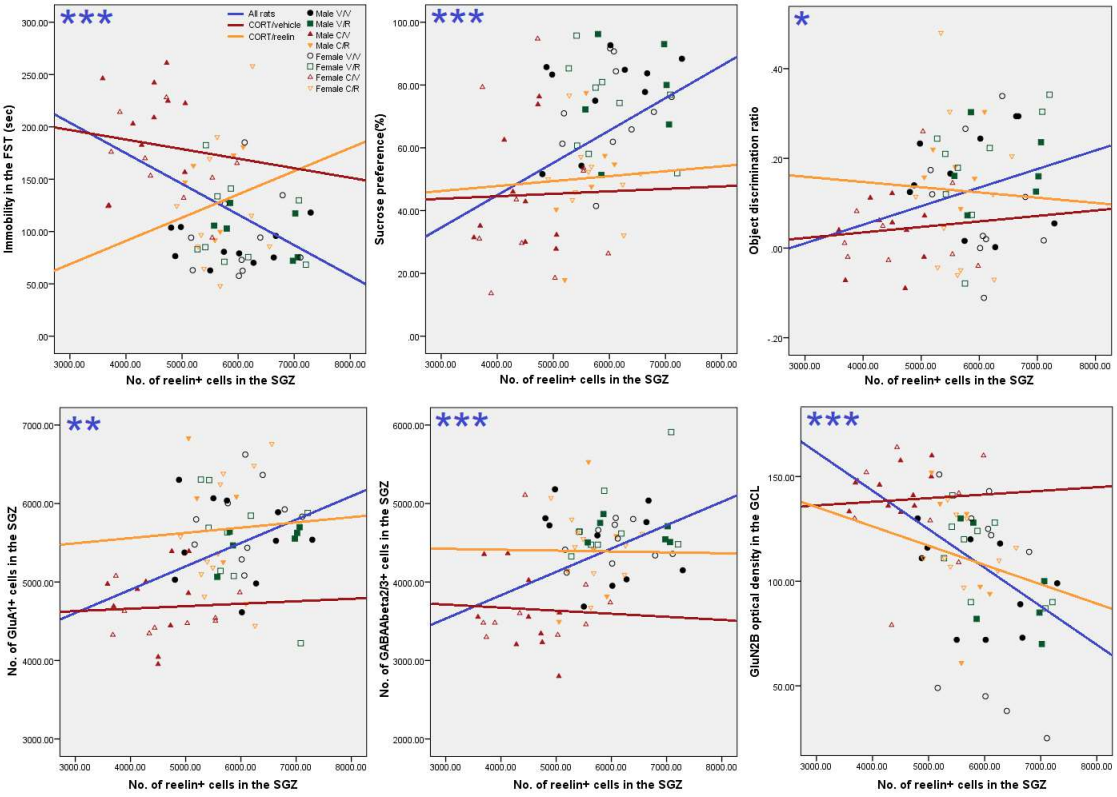


**Figure 3.5.9a. Correlations with reelin in the SGZ.** Significant correlations were observed between reelin-IR cell counts and FST-immobility, sucrose preference, object DR, and the expression of glutamate and GABA receptors when all groups were included in the analyses (*$p$<0.05/**$p$<0.01/***$p$<0.001). Figure created with SPSS and Paint by author.

Correlational graphs depicting the relationships between DCX-IR cell counts and behavioral or other neurochemical alterations that were analyzed are shown in Figure 3.5.9b. When all of the rats were included in the analysis, Pearson's bivariate correlation coefficient tests confirmed that lower DCX-IR cell counts in female rats were associated with higher immobility scores (r=-0.330, $p$=0.040, n=39), a decreased preference for sucrose (r=0.565, $p$<0.001, n=39),

and lower reelin- (r=0.444, $p$=0.005, n=39) and GluA1-IR cells (r=0.351, $p$=0.029, n=39). There were no significant correlations when only the CORT/vehicle or CORT/reelin group were included in the analyses.

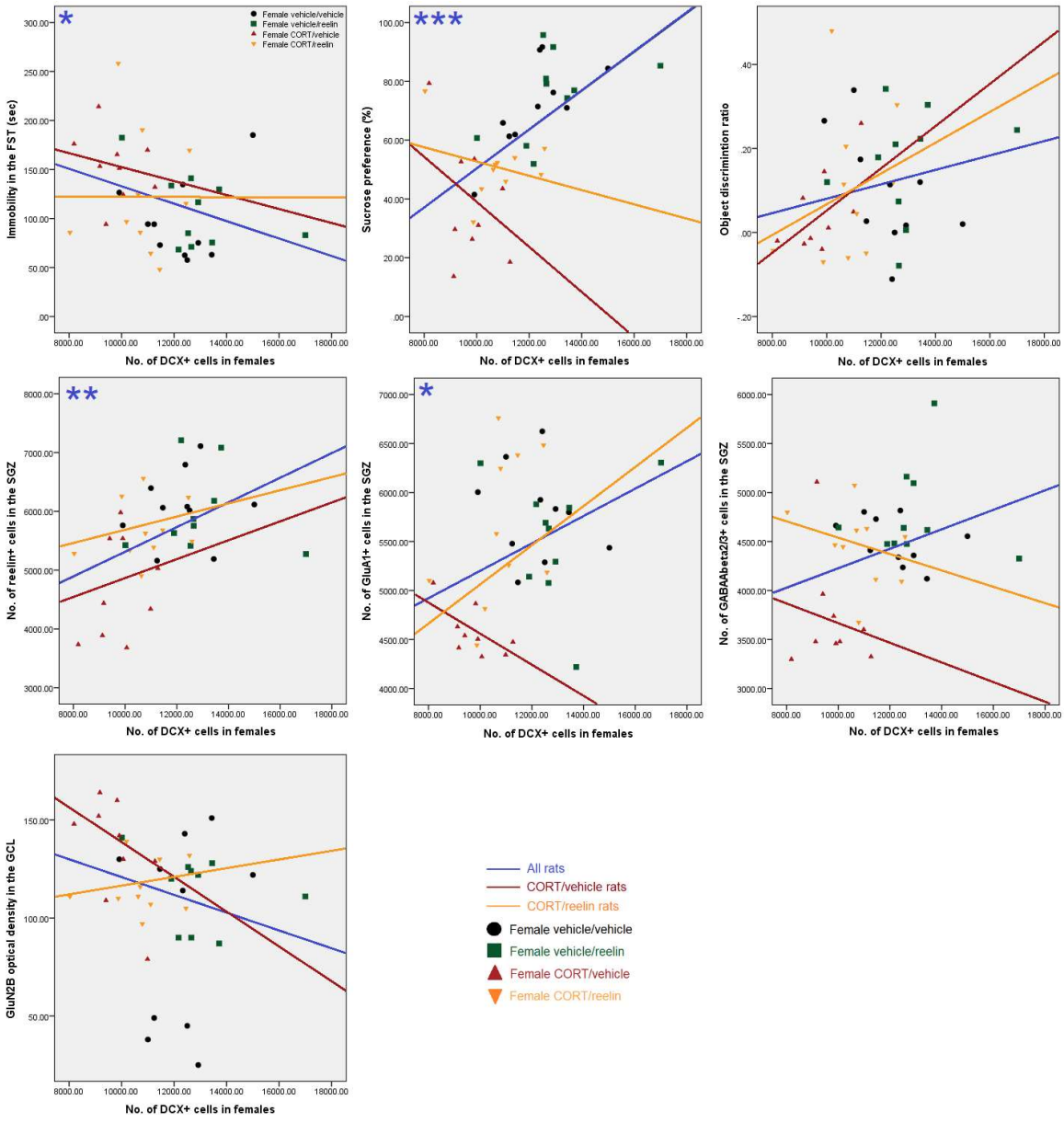


**Figure 3.5.9b. Correlations with DCX-IR cells in the SGZ and GCL.** Significant correlations were observed for DCX-IR cell counts and FST-immobility, sucrose preference, SGZ-reelin and -GluA1 expression when all groups were included in the analyses (*$p$<0.05/**$p$<0.01/***$p$<0.001). Figure created with SPSS and Paint by author.

Correlations with GluA1 and other measures are shown in Figure 3.5.9c. When all the groups were included in the correlation, a lower GluA1-positive cell count significantly correlated with higher FST-immobility (r=-0.417, $p$<0.001, n=79), a decreased preference for sucrose (r=0.417, $p$<0.001, n=79), and increased GluN2B-immunoreactivity (r=-0.417, $p$<0.001, n=79). Interestingly, in the CORT/reelin-treated group, a higher GluA1 cell count was associated with a lower number of $GABA_A\beta2/3$-IR cells. (r=-0.505, $p$=023, n=20).

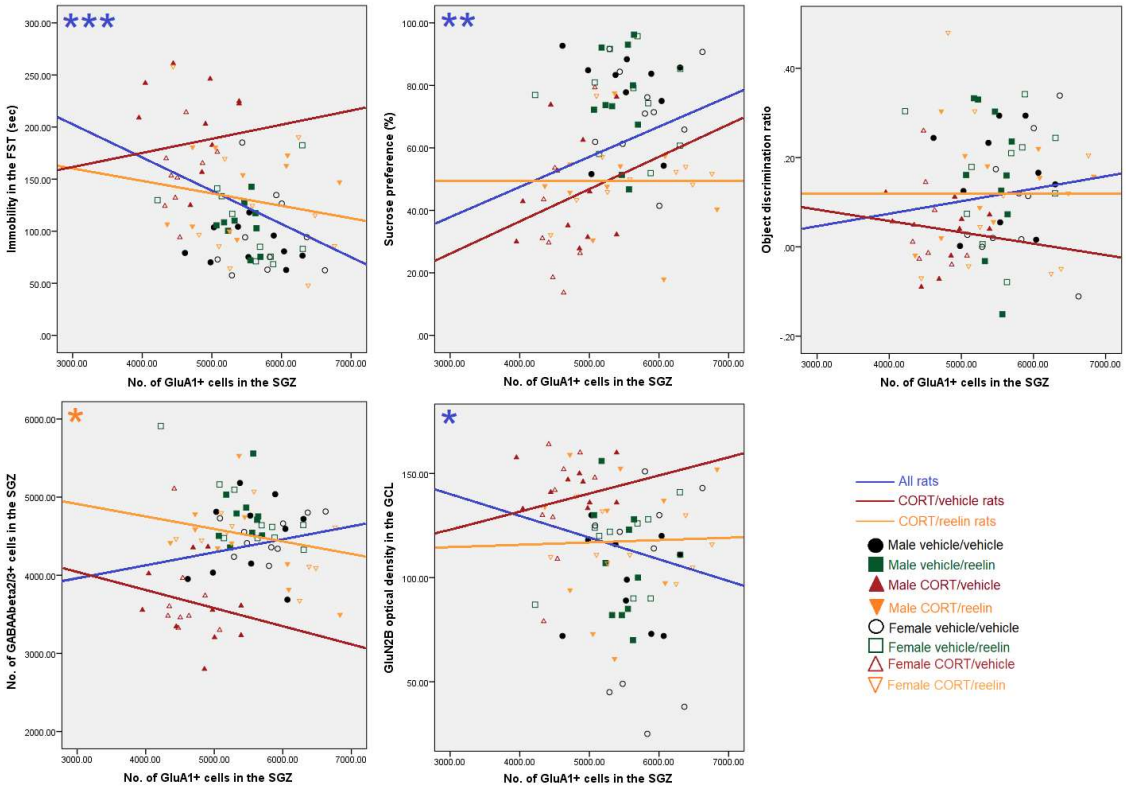


**Figure 3.5.9c. Correlations with GluA1 in the SGZ.** Significant correlations between GluA1 and behavioral and neurochemical alterations are illustrated by asterisks (*$p$<0.05/ **$p$<0.01/***$p$<0.001) that are color-coordinated with the line of best fit when all groups were included in the analyses (blue line), or only the CORT/vehicle (red line) or CORT/reelin rats (orange line). Figure created with SPSS and Paint by author.

When all the rats were included in the analyses, a higher number of $GABA_A\beta2/3$-IR cells were associated significantly with lower immobility (r=-0.417, $p$<0.001, n=79), increased sucrose preference (r=0.406, $p$<0.001, n=79), and lower GluN2B expression (r=-0.261, $p$=0.020, n=79) – see Figure 3.5.9d. No significant correlations were observed for individual groups.

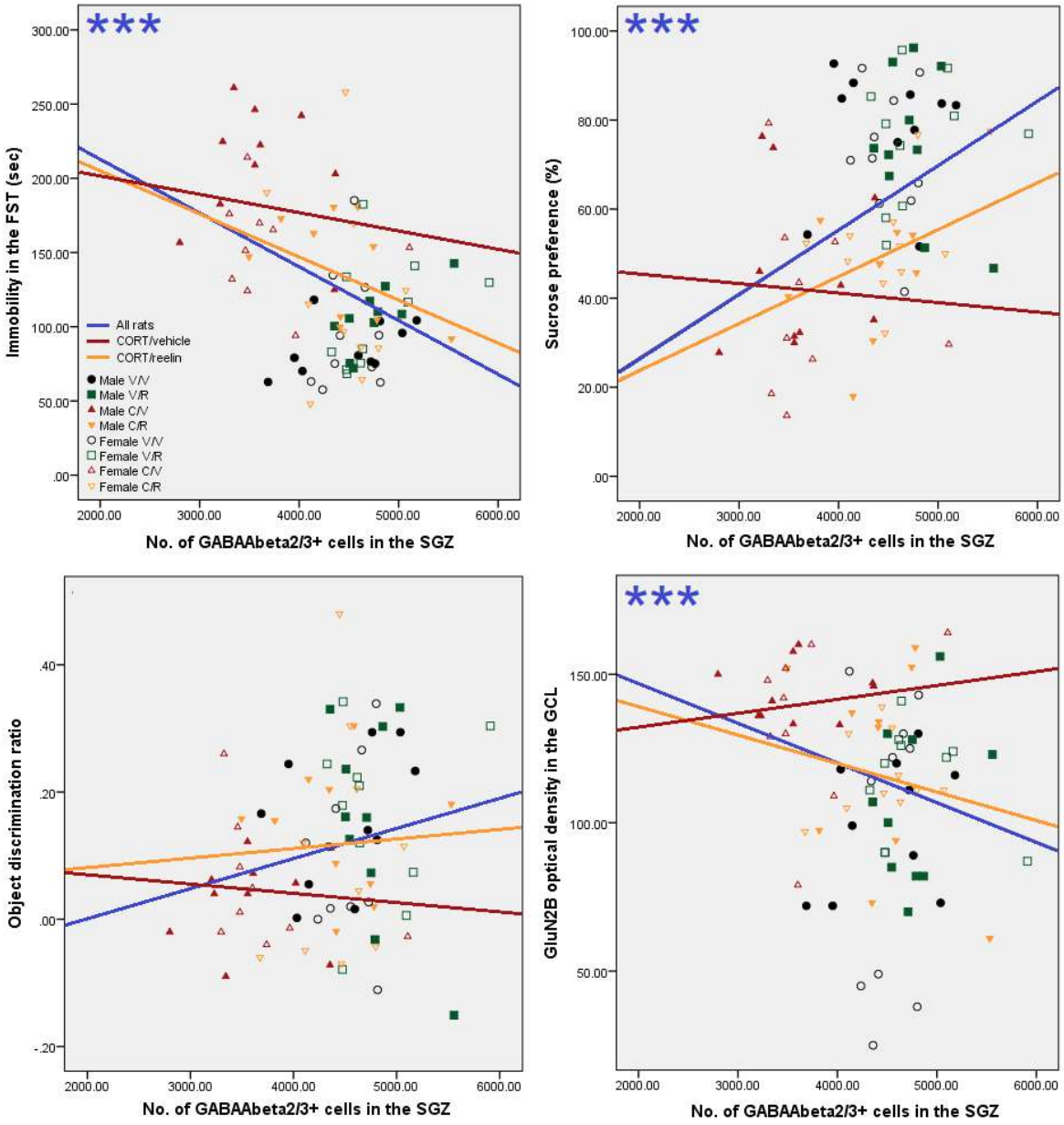


**Figure 3.5.9d. Correlations with GABAAβ2/3 in the SGZ.** Significant correlations were observed for GABAAβ2/3-IR cell counts and FST-immobility, sucrose preference, and GCL-GluN2B expression when all groups were included in the analyses (***$p$<0.001). Figure created with SPSS and Paint by author.

The correlations for GluN2B can be found in Figure 3.5.9e. A higher density of GluN2B in the GCL correlated significantly with a higher time spent immobile in the FST (r=0.380, $p$=0.001, n=79), a decreased preference for sucrose (r=-0.358, $p$=0.001, n=79), and behavior that would indicate hippocampal recognition-memory deficits (r=-0.238, $p$=0.035, n=79). No significant correlations were observed for individual groups.

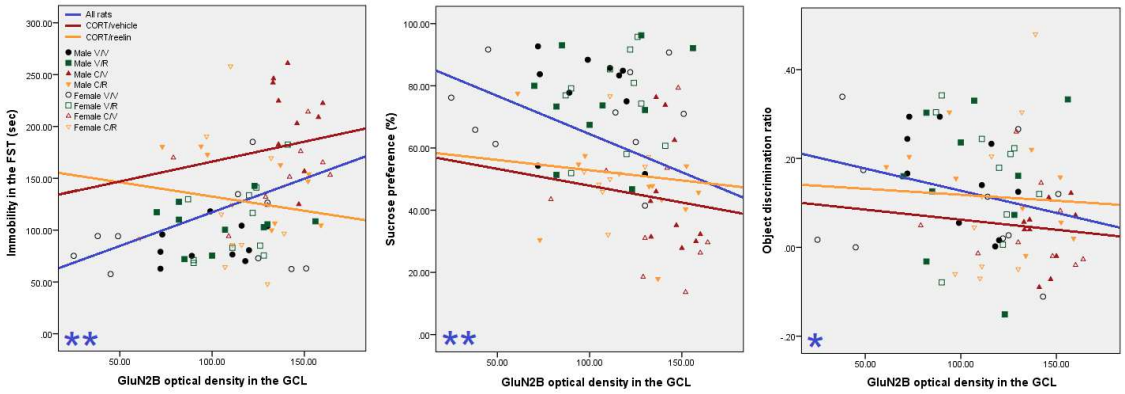


**Figure 3.5.9e. Correlations with GluN2B optical density in the GCL.** Significant correlations were observed for GluN2B immunoreactivity and FST-immobility, sucrose preference, object DR scores when all groups were included in the analyses (*$p$<0.05/**$p$<0.01). Figure created with SPSS and Paint by author.

### 3.6 Discussion

There were 3 main findings in this study: first, I demonstrated that i.v. reelin can recover depression-like behavioral and neurochemical deficits in female rats as well as males; second, that reelin fails to completely solve the deficits in dentate new-born cell maturation as shown by DCX immunostaining; and third, that reelin can protect from the repeated-CORT-induced insults on $GABA_A$Rs, AMPARs, and NMDARs. CORT also decreased the rodent's preference for sucrose, a depression-relevant hedonic behavior, but this was unaffected by reelin. Although depression is uniquely human, experimentation with animal models can be used to make inferences about the signaling pathways that are involved in the human condition. Our findings recapitulate the antidepressant actions of neuroplasticity-promoting compounds that enhance glutamatergic and GABAergic tone and open the possibility of developing reelin-based therapeutics.

In our laboratory, we utilize a well-validated model of chronic stress whereby rats are subjected to 3 weeks of daily CORT (Sterner & Kalynchuk 2010). Repeated-CORT produces behavioral, cognitive, and neurochemical alterations that are rescued by a variety of drugs with antidepressant actions, like imipramine, ketamine, and etanercept (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). The inclusion of females in preclinical experiments with novel compounds is essential considering that women are 2- to 3-fold more likely to develop MDD than males for a combination of biopsychosocial reasons, and there are sex differences in symptom reporting, comorbidity rates, and treatment responsiveness (Linzer et al 1996, Marcus et al 2005, 2008). In the present report, I replicated our finding that i.v. reelin can rescue immobility in the FST in male rats and extended this to females, and reductions in immobility were similar for both sexes (56% for males and 58% for females). The FST is the most commonly used screening tool for antidepressant efficacy in rodent models (Yuen et al 2017), which was our rationale to use it to evaluate the putative antidepressant-like properties of exogenous reelin, but one should be aware that behavioral changes in the FST as a measure of depression-like behavior is questionable and that a battery of behavioral tests should be used to evaluate mood changes (Commons et al 2017).

Reelin-deficient mice have an enhanced vulnerability to the depressogenic effects of CORT as ascertained by the FST (Lussier et al 2011), and this susceptibility to stress also extends to hippocampal-dependent cognition tasks (Notaras et al 2020, Schroeder et al 2015). In fact, the genetic overexpression of reelin or bilateral, intraventricular injections of exogenous reelin was shown to enhance cognitive ability in mouse models of Alzheimer's disease (Pujadas et al 2014) and Angelman syndrome (Hethorn et al 2015), respectively. Therefore, I subjected rats to a hippocampal-dependent object-location recognition task to evaluate if the putative pro-cognitive effects of reelin can be achieved with i.v. administrations. As expected, CORT-treated rats had lower object DR scores in the absence of reelin but not when reelin and CORT were co-administered. Reelin recovered cognitive deficits by 84% in males and 117% in females – however, the effects of CORT and reelin were not statistically significant. Nevertheless, these findings are in line with our previous reports which show that reelin administered multiple times (1µg doses) over the CORT-injection period or once at the end, directly into the hippocampus, significantly augments cognitive ability (Brymer et al 2020), and that increases in SGZ-reelin are associated with cognitive improvements (Brymer et al 2018). This could imply that i.v. reelin has difficulty accessing the brain or perhaps that it

cannot at all but could influence cognition through peripheral mechanisms, like the modulation of inflammatory mediators.

To further evaluate the behavioral effects of exogenous reelin after repeated-CORT, the rats were subjected to additional tests that were designed to elicit responses that can be measured to make inferences about their emotional state, such as the SPT and OFT (see Appendix for data). While these tests are of value, it is important to note that CORT does not reliably alter rodent behavior in these paradigms which also makes it difficult to assess whether reelin can recover relevant behavioral abnormalities. However, our results indicate that i.v. reelin can bring about improve FST-immobility and cognitive ability independent to any apparent exploratory or anxiety-like behavioral abnormalities. Indeed, over the course of the study reelin did not appear to induce any adverse behavioral or physiological reactions in these conditions, which could represent an advantage over ketamine and other drugs with rapid and long-lasting antidepressant effects that also cause clinically-undesirable reactions (Berman et al 2000, Kadriu et al 2020b), which are detectable in rodent models by behaviors like head weaving and stumbling (Hanks & González-Maeso 2013, McDougall et al 2017). I did see, however, that female rats travelled more than males in the arena, probably because they have lighter bodyweights.

Disrupting reelin signaling has been shown to impair dendritogenesis, dendritic spinogenesis, and the glial ensheathment of adult-born granule cells are therefore their integration into hippocampal circuitry (Bosch et al 2016a, Teixeira et al 2012), but reelin failed to significantly rescue the number and maturation rate of new-born granule cells as measured by DCX immunostaining. The involvement of neurogenesis in depression is still debated, but some researchers have found that neurogenic processes are required for the behavioral responses to antidepressants (Santarelli et al 2003), though contradictory reports exist (Bessa et al 2009, Hanson et al 2011). In our laboratory, we found that the fast-acting antidepressant effects of intrahippocampal reelin infusions could be blocked by AMPAR antagonist CNQX without interfering with reelin's effects on neurogenesis (Brymer et al 2020), but one should not rule out the possibility that the proliferation and integration of adult-born cells into hippocampal circuitry influences specific symptoms, such as anhedonia. Thus, it would be interesting to evaluate the effects of reelin administration on sucrose preference, and the behavioral responses in other anhedonia-based tests, in different stress-based models, rat strains, and species.

Over the last decade, our group has provided compelling evidence that a downregulation of reelin-expressing cells in the DG SGZ is associated with behavioral phenotypes relevant for depression. For instance, progressive increases in FST-immobility instigated by CORT is paralleled by decreases in SGZ-reelin and these deficits become sensitized after cyclical CORT administration (Lebedeva et al 2017, 2020, Lussier et al 2013a), and can be rescued by conventional and unconventional antidepressants (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). In Chapter 2, I revealed that multiple i.v. injections of exogenous reelin can also raise the number of reelin expressing cells in the SGZ in male rats; here, I found that 3µg of reelin given every 10 days also rescued this deficit in females in a similar manner. Considering the neuroplasticity-enhancing properties of reelin, one could be persuaded that a strengthening of hippocampal circuitry – which is highly connected to other mood-regulating brain regions – underlies the antidepressant actions of reelin, at least partially. This statement is supported by the fact that the disruption of synaptic reelin signaling eliminates the antidepressant effects of ketamine and its effects on synaptic plasticity in the hippocampal CA1 region (Kim et al 2021). It could be that ketamine requires efficient reelin signaling to maintain baseline NMDAR function, and makes one wonder whether the response rate of ketamine, which helps ½ of those with treatment-resistant MDD, could be improved with the co-administration of reelin (Chen et al 2005, Kim et al 2021, Sinagra et al 2005).

NMDARs are critical for hippocampal neuroplasticity and learning and memory (Barker & Warburton 2008, Monaco et al 2015), and reelin regulates NMDAR activity, trafficking, and subunit composition (Bosch et al 2016a, Chen et al 2005, Groc et al 2007). Therefore, I analyzed if i.v. reelin could rescue CORT-induced increases in GluN2B-immunoreactivity – which is associated with psychiatric disorders (Monaco et al 2015) – and found that reelin produced a partial recovery. Moreover, higher levels of GluN2B expression correlated with lower reelin levels ($p<0.001$, n=70) and DR scores ($p=0.035$, n=79). AMPARs were also of interest because their activity is required for the fast-acting antidepressant effects of ketamine (Zanos et al 2016) and intrahippocampal reelin (Brymer et al 2020). Peripheral reelin normalized the CORT-induced decrease in GluA1-containing AMPARs, and lower numbers of GluA1-IR cells were significantly correlated with depression-like behavior (FST-immobility: $p<0.001$, n=79; sucrose preference: $p<0.001$, n=79). This suggests that i.v. reelin may enhance cognition and mood through glutamatergic mechanisms, but our previous and present data show that repeated-CORT also alters expression levels of GABAergic receptors (Brymer et al 2020, Lussier et al 2013b). Interestingly, reelin is expressed by GABAergic

interneurons in the adult hippocampus (Pesold et al 1998) and I found that i.v. reelin recovered the downregulation of $GABA_A$Rs in the SGZ by a staggering 92% for males and females. Therefore, reelin may produce antidepressant-like effects by correcting the balance in excitatory and inhibitory neurotransmission, promoting a neuroprotective environment with minimal excitotoxicity (Lussier et al 2013b, Teixeira et al 2011).

In conclusion, we provide the first evidence that i.v. can produce antidepressant-like effects in both male and female rats and that glutamatergic and GABAergic receptors are involved. It is unlikely that an enhancement of neurogenesis underlies reelin's effects on FST-immobility but deficient neurogenesis could relate to other behavioral impairments. Additional experiments should aim to replicate these findings in other preclinical models for depression and assess if the antidepressant actions of reelin are fast acting.

# Chapter 4

## Reelin has fast-acting dose-dependent antidepressant-like effects after a single intravenous injection

## 4.1 Abstract

It is extremely necessary that more effective and rapid acting mechanistically novel antidepressants are developed considering that conventional monoaminergic-based treatments have slow acting, are laden by side effects, and have poor remission rates. Inflammation, apoptosis, low reelin levels, and deficient excitatory neurotransmission and neurogenesis are associated with depression and can be investigated using the repeated-CORT paradigm of chronic stress. Our previous work suggests that exogenous reelin, which can promote neuroplasticity, is a promising candidate fast-acting antidepressant compound. Here I show that a single i.v. reelin injection can improve behavioral deficits in just 24 hours and that this behavioral recovery is associated with the normalization of SERT MPC in blood lymphocytes, increasing reelin- and GluA1-positive cell numbers in the SGZ, and dampening the expression of hippocampal CytC and BAX, which works to release CytC from the mitochondria and drive apoptosis. One can assume that the rescuing of these alterations promotes a neuroprotective environment that facilitates neuroplasticity and leads to behavioral and cognitive improvements. Our results suggest that further experimental attention should be paid to the reelin signaling pathway as a target for novel antidepressant treatments.

## 4.2 Introduction

We are far from understanding the complex pathophysiology that underlies depression, which is the most burdensome disease globally that remains difficult to treat. Little progress has been made in generating newer antidepressants that have higher response rates and speedier onsets of action than conventional antidepressants that were discovered from serendipitous pharmacological observations over half a century ago (Ban 2006). Indeed, monoaminergic-based treatments require several weeks to months of continuous administration to achieve therapeutic efficacy, and they only do so in some patients (Cipriani et al 2018, Rush et al 2006, Trivedi et al 2006). The scientific community and patients alike were re-excited by the more recent serendipitous discovery that ketamine, an NMDAR antagonist that was recently approved by the FDA to treat resistant forms of MDD, can decrease depression scores and suicidal ideation in under an hour in unipolar and bipolar patients (Berman et al 2000, Diazgranados et al 2010a, Fava et al 2020, Murrough et al 2013, Zarate et al 2012). That said, the antidepressant effects of ketamine only persist for 2 weeks and its clinical use is limited by

its abuse potential, dissociative nature, and links to neurotoxicity and cognitive impairment after repeated administration (Kishimoto et al 2016, Krystal et al 2005, Neis et al 2020). 5-HTergic psychedelic drugs also produce relatively fast-acting and much longer lasting antidepressant effects than ketamine when paired with integration sessions (Carhart-Harris et al 2018, Davis et al 2020, Hibicke et al 2020, Reiff et al 2020), and compelling evidence suggests that, like ketamine, their mechanisms of action converge on glutamatergic signaling (Hesselgrave et al 2021, Kadriu et al 2020b, Seo et al 2020). However, the enduring psychotomimetic effects of these drugs represent a major complication for their widespread clinical use. Nevertheless, these discoveries have created a blueprint to study novel pharmacological agents and support a neuroplasticity-deficient hypothesis of depression that is associated with weakened excitatory neurotransmission (Holubova et al 2016, Ly et al 2018, Pham & Gardier 2019, Seo et al 2020, Thompson et al 2015, Zhang et al 2016).

Our laboratory recently found that reelin, the extracellular neuromodulator that promotes neurogenesis, cell growth, and synaptogenesis (Bosch et al 2016a, Pujadas et al 2010, Qiu et al 2006b, Rogers et al 2011), has fast-acting antidepressant-like and pro-cognitive effects when infused directly into the hippocampus, and that glutamatergic AMPARs are required for reelin to produce these behavioral changes (Brymer et al 2020). In addition, we have shown that ketamine and conventional antidepressants increase reelin expression in the rat hippocampal SGZ, where reductions in reelin parallel the progressive development of CORT-induced depressive-like behavior (Fenton et al 2015, Johnston et al 2020, Lebedeva et al 2020, Lussier et al 2013a). Another laboratory also revealed that interrupting synaptic reelin signaling abolishes the antidepressant-like and neuroplastic effects of ketamine, suggesting that reelin may underlie its therapeutic responsiveness (Kim et al 2021).

Depressed patients have abnormal levels of reelin in the hippocampus as well as blood plasma (Fatemi et al 2000, Fatemi et al 2001), where it is believed to be secreted by platelets (Tseng et al 2010) to modulate homeostasis and inflammatory responses (Tseng et al 2014). It is true that mice deficient in reelin express abnormal levels of cytokines (Green-Johnson et al 1995), as well as altered MPC in blood lymphocytes, such as for SERT (Rivera-Baltanas et al 2010), the primary target of conventional antidepressants whose clustering into membranous lipid microdomains provides a method to regulate 5-HT uptake activity and subsequently emotion (Magnani et al 2004). Interestingly, we have found that these SERT MPC abnormalities are matched in both CORT-treated animals (Romay-Tallon et al 2018) and depression patients in whom they can predict anhedonia scores and pharmacotherapeutic responsiveness (Caruncho

et al 2019, Rivera-Baltanas et al 2012, 2015). Therefore, the regulation of protein trafficking by peripheral reelin may exert an anti-inflammatory phenotype in immune cells that helps protect one from illness (Baitsch et al 2011, Morimura et al 2005, Santana & Marzolo 2017).

Another connection between inflammation and reelin is apparent by the fact that anti-inflammatory drugs like etanercept can raise hippocampal reelin levels without directly accessing the brain from periphery (Brymer et al 2018). Chronic inflammation and stress increase the expression of pro-apoptotic factors like BAX (Juárez-Rojas et al 2015, Kubera et al 2011, Zhang et al 2019b), which promotes cell death by forming pores in outer mitochondrial membranes and releasing CytC into the cytosol (Allen et al 2018, Kale et al 2018). We recently published a report discussing the possibility that reelin may rescue behavioral phenotypes relevant to depression by stimulating the PI3K/Akt/mTOR pathway, which inactivates BAX (Allen et al 2021a). In fact, activation of the mTOR pathway is a shared attribute among fast-acting antidepressants, like ketamine, psilocybin, and reelin (Brymer et al 2020, Holubova et al 2016, Johnston et al 2020, Li et al 2010, Ly et al 2018). Taking all this into account, I hypothesized that a single injection of reelin could rescue behavioral and cognitive impairments induced by CORT by normalizing SERT MPC in lymphocytes, and expression levels of reelin, AMPARs, BAX, and CytC in the DG.

## 4.3 Methods

### 4.3.1 Experimental procedures

I used 106 male rats that were administered with either a single injection of vehicle or incrementing doses of reelin (0.5µg, 1µg, 3µg, 5µg, 7µg or 9µg) given on day 21 after 3 weeks of daily vehicle or CORT treatment. The FST was performed 24 hours the reelin injection. A subset of rats was then subjected to either the SPT and the LDT or EMP on day 23 followed by a hippocampal-dependent recognition test (OBIP or Y-maze, respectively). I observed 3µg of reelin to be the most effective dose in male rats and so evaluated the effects of this dose in 24 female rats (8 vehicle/vehicle, 8 CORT/vehicle, and 8 CORT/reelin rats) before subjecting them to the FST, LDT and OBIP (Figure 4.3.1).

CORT (Steraloids) was suspended in 0.9% sodium chloride and 2% (v/v) Tween-80 (Sigma Aldrich) and given subcutaneously at a dose of 40mg/kg and at a volume of 1ml/kg. Recombinant reelin (R&D systems, 3820-MR-025; composed of RR3-6 and a molecular

weight of 180kDa by SDS-PAGE using reducing conditions) was suspended in 0.5ml 0.1M PBS (pH=7.4) and given i.v. while rats were restrained by DecapiCones.

| Sex | Treatment | | N |
|---|---|---|---|
| Male | Vehicle | Vehicle | n=16 |
| Male | Vehicle | Reelin 3µg | n=8 |
| Male | Vehicle | Reelin 9µg | n=8 |
| Male | CORT | Vehicle | n=16 |
| Male | CORT | Reelin 0.5µg | n=8 |
| Male | CORT | Reelin 1µg | n=8 |
| Male | CORT | Reelin 3µg | n=18 |
| Male | CORT | Reelin 5µg | n=8 |
| Male | CORT | Reelin 7µg | n=8 |
| Male | CORT | Reelin 9µg | n=8 |
| Female | Vehicle | Vehicle | n=8 |
| Female | CORT | Vehicle | n=8 |
| Female | CORT | Reelin 3µg | n=8 |

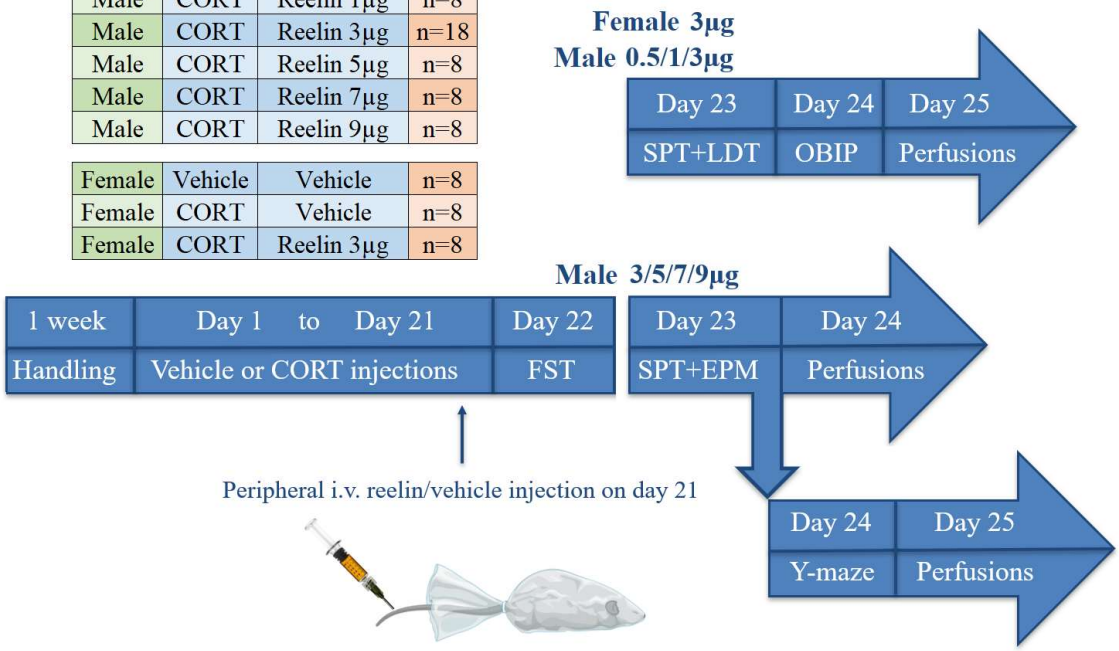


**Figure 4.3.1. Schematic representation of the experimental groups and timeline.** Rats had 7 habituation days followed by 7 handling days on arrival to the facility. They then received s.c. vehicle or CORT injections along with an i.v. injection of either vehicle or 0.5µg, 1µg, 3µg, 5µg, 7µg, or 9µg of reelin administered on day 21, 24 hours of the FST. After the FST came the SPT, and then the rats were subjected to a variety of anxiety- and cognitive-based tasks, such as the LDT and OBIP, or the EPM and Y-maze. Perfusions took place on day 24 or 25, depending on which behavioral tests they were subjected to. I found that the 3µg dose was the most effective in males and so evaluated this dose in females with an additional 24 rats. Figure created with Microsoft Excel, Paint, and BioRender by author.

### 4.3.2 Behavioral tests

All animals were subjected to the FST 24 hours after the i.v. reelin injection to ascertain whether the antidepressant-like effects of reelin have a rapid onset. To get a broader idea of how CORT and reelin would alter anxiety-like behavior and cognition, different groups of rats

were subjected to additional behavioral tests (see Appendix), including the hippocampal-dependent OBIP which is based on a similar premise as the OBL that is described in Chapter 3 – rats have an innate tendency to explore novelty over familiarity (Wright & Conrad 2005).

#### 4.3.2.1 Forced swim and object-in-place behavioral tests

The FST was used as an antidepressant screening tool by measuring the coping strategy of rats in the face of stress (forced swimming); an increased time spent immobile is usually interpreted as despair-like behavior and antidepressants usually decrease immobility time. The FST was conducted as previously described in Section 2.3.3 and was scored manually, blind to experimental conditions.

On day 24, the OBIP test was conducted in a similar manner to previously established protocols (Howland & Cazakoff 2010). The test took place in a square open-field arena (65cm x 65cm), with a light blue floor and black walls 60cm high. First, the rats were given a 10-minute habituation session (the first half of which acted as the LDT on day 23). The next day, the animals were placed back into the arena and could explore 4 different objects that were located about 10cm from the walls of each corner for 5 minutes. They were then returned to the colony room for 1 hour, before re-entering the arena for 4 minutes. This time, they were presented with identical copies of the 4 objects, but 2 of the objects had their positions switched (see Figure 4.4.3). In between trials, the arena and objects were washed with hot soapy water. The objects were made of porcelain and did not exceed 10cm in height or length. The amount of time the rats spent exploring the objects was recorded. The rats were deemed to be actively exploring an object when its nose was at or under 2cm away, and its head or vibrissae were in motion. Credit was not given for exploration if the rat was standing on top of the object or looking away. The animals explored the objects for more than 15 seconds in each phase and therefore were considered to have reliable recognition memory. A DR was calculated and analyzed by using the following formula from scoring the first 2 minutes of the test phase:

$$DR = (time\ exploring\ moved - time\ exploring\ stationary) \div (time\ exploring\ moved + time\ exploring\ stationary)$$

Positive DR scores were indicative of intact object-recognition memory.

### 4.3.3 Perfusions, blood collection, and tissue preparation

Perfusions took place on day 24 or 25. First, the rats were deeply anaesthetized with 5% isoflurane and 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). Several blood smears were made on slides and left to dry before being incubated in 1% paraformaldehyde for 10 minutes and then stored at -20°C until immunocytochemical staining. The rats were then perfused transcardially using 0.1M phosphate buffer (PB, pH 7.4) followed by ice-cold paraformaldehyde (4%, w/v) in PB. The brains were removed and stored in 4% paraformaldehyde for 48 hours, followed by 30% sucrose solution for 72 hours at 4°C. Afterwards, the brains were sectioned at a thickness of 30µm with a cryostat (Vibratome ULTRAPRO 5000) and placed in a cryoprotectant solution and stored at -20°C until immunohistochemical staining was performed.

### 4.3.4 SERT immunocytochemistry and image analyses

SERT was visualized using protocols described elsewhere (Romay-Tallon et al 2017). First, to block unspecific antibody binding, the slides were incubated in a blocking solution of 10% rat IgG (Sigma) in PBS for 1 hour at room temperature. Afterwards, the slides are incubated overnight with the primary antibody (rabbit anti-SERT, 1:250, Millipore cat# AB10514P) in a blocking solution at 4°C. After several rinses, the slides were exposed to a secondary antibody (goat anti-rabbit Alexa Fluor 568, 1:200, Molecular Probes) diluted in 1% bovine serum albumin (BSA) in PBS for 2 hours at room temperature, followed by more rinses to reduce unspecific staining. Finally, the slides were cover-slipped with Citifluor (Electron Microscope Science) and stored at -20°C.

I analyzed the number and size of SERT clusters by obtaining images of 50 individual lymphocytes at 100× magnification using a fluorescent Zeiss Imager M2 microscope. ImageJ software (1.53e, NIH, USA) was used to analyze the images as outlined previously (Romay-Tallon et al 2017).

### 4.3.5 Immunohistochemistry

Reelin-, DCX-, GluA1-, CytC- and BAX-IR cells were visualized as follows. Free-floating sections were first incubated in sodium citrate buffer (pH = 6.0) for 30 minutes for epitope retrieval. They were then placed into a blocking solution of 5-15% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 30 minutes, before being exposed to either a mouse anti-reelin (1:1000, MILLIPORE, MAB5364), rabbit anti-DCX (1:1000, Cell Signaling, Danvers, MA), rabbit anti-GluA1 (1:1000, MILLIPORE, AB1504), rabbit anti-BAX (1:750, ThermoFisher cat# 50599-2-IG), or rabbit anti-CytC (1:750, Novus Biologicals, NBP2-67558) antibody diluted in blocking solution for either 24 hours (room temperature) or 48 hours (at 4°C). Afterwards, endogenous peroxidase activity was blocked by incubating the sections for 30 minutes in 5-10% (v/v) $H_2O_2$ in 0.1 M TBS. The sections were then incubated in either a biotinylated goat anti-rabbit (1:500, abcam, ab6721) or goat anti-mouse secondary antibody (1:500, catalog #BA-2000. RRID: AB_2313571; Vector Laboratories) diluted in 5-15% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 1 or 2 hours. Next, they were treated with avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour, followed by rinses with TBS or 0.175 M sodium acetate (pH = 6.8). Immunolabelling was visualized with 0.02-0.025% (w/v) DAB and 0.002-0.0078% $H_2O_2$ diluted in TBS. Finally, the sections were rinsed before being mounted onto glass slides using 0.1 M TBS, air dried overnight, dehydrated using increasing concentrations of ethanol, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

### 4.3.6 Cell counting and categorization of immature DCX-IR cells

The number of reelin- and GluA1-IR cells were counted in the SGZ (defined as a 2-cell width zone in between the inner GCL and PL), and CytC-IR cells were counted in the SGZ and PL. DCX-IR cells were counted in the SGZ and GCL. Cellular counts were estimated using a Zeiss Imager M2 microscope with a motorized stage linked to a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc) as previously described in Section 2.3.6. Cell counts were taken from 5 sections (both hemispheres) at 40× magnification. Unbiased stereology was utilized using a modified optical fractionator method to reduce oversampling. The following formula was used to estimate the number of positive immunoreactive cells: N total: $\Sigma Q^- \times 1 / ssf \times A(x,y\ step) / a(frame) \times t/h$, where $\Sigma Q^-$ 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 (10000μm$^2$); a(frame) is the area of the counting frame (3600μm$^2$); t is the weighted average section thickness; and h is the height of the dissector (12μm). A guard zone of 4μm was used to avoid counting sectioning artefacts.

The dendritic morphology of adult-born granule cells was categorized after CORT and reelin treatment. Dendritic branching was analyzed in 100 randomly selected DCX-IR cells evenly distributed across the 5 sections for each rat as previously outlined in Section 2.3.7; see Figure 2.4.5C for representative photomicrographs of the 6 categories of dendritic complexity that was used to classify cells.

### 4.3.7 Optical density

Semi-quantitative optical densitometry of BAX and CytC was analyzed in various areas of the DG using 5 sections (30μm) that were 180μm apart, as previously described (Botterill et al 2015b). Briefly, grayscale photomicrographs were captured on the microscope and ImageJ software was used to calculate the intensity of immunoreactive products and the average optical density measurement for each region of interest was subtracted from background staining (mean optical density of the corpus callosum).

### 4.3.8 Statistical analyses

All statistical tests were computed using SPSS. The assumptions of normality and homogeneity of variance were assessed before analyses were computed. Three-way ANOVAs were used to analyze the data with sex, CORT/vehicle, and reelin/vehicle as factors. However, I also ran the male data separately with two-way ANOVAs and the female data with one-way ANOVAs (as a vehicle/reelin control group was not included to reduce animal suffering). Kruskal Wallis tests were used if data were not normally distributed. Tukey *post hoc* tests were conducted when appropriate. Group means were considered statistically different from one another at $p<0.05$. In addition, I report the percentage of recovery by reelin treatment, as calculated by the following formula: *Percentage recovered = 100 – (CORT/reelin mean – vehicle/vehicle mean) ÷ (CORT/vehicle mean – vehicle/vehicle mean) × 100.* Data are expressed as mean±CI. Pearson's r correlations were also assessed.

## 4.4 Results

### 4.4.1 Bodyweight

When looking at bodyweight, sphericity could not be assumed ($p<0.05$) with Mauchly's test of Sphericity for a two-way ANOVA repeated measures so the Greenhouse-Geisser correction was employed. When looking at the within-subjects effects in male rats, the results show that there is a significant effect of time on weight gain [$F(2.335, 224.194)=147.169$, $p<0.001$], animals becoming heavier over time. There was also a significant interaction effect of time × CORT [$F(2.335, 224.194)=280.155$, $p<0.001$], time × reelin [$F(2.335, 224.194)=4.110$, $p<0.001$], and time × CORT × reelin [$F(2.335, 224.194)=2.330$, $p<0.047$]. A one-way ANOVA repeated measures was used to analyze the female data. The within-subjects effects are as follows: there was an effect of time [$F(3.173, 66.636)=76.430$, $p<0.001$] and time × treatment [$F(6.346, 66.636)=13.977$, $p<0.001$]. The male rats weighed more than the female rats, and CORT decreased bodyweight gain in both sexes, but more so in males (Figure 4.4.1A). The statistical information for the between-subjects effects for bodyweight, and for the behavioral data from each test is outlined in Table 4.1.

When looking at the percentage of weight change (Figure 4.4.1B/C), *post hoc* analyses revealed that male and female CORT-treated rats gained significantly less weight than rats that were treated with vehicle ($p<0.001$). The female CORT/vehicle rats gained less weight respective to their bodyweight than the male CORT/vehicle rats ($p<0.041$).

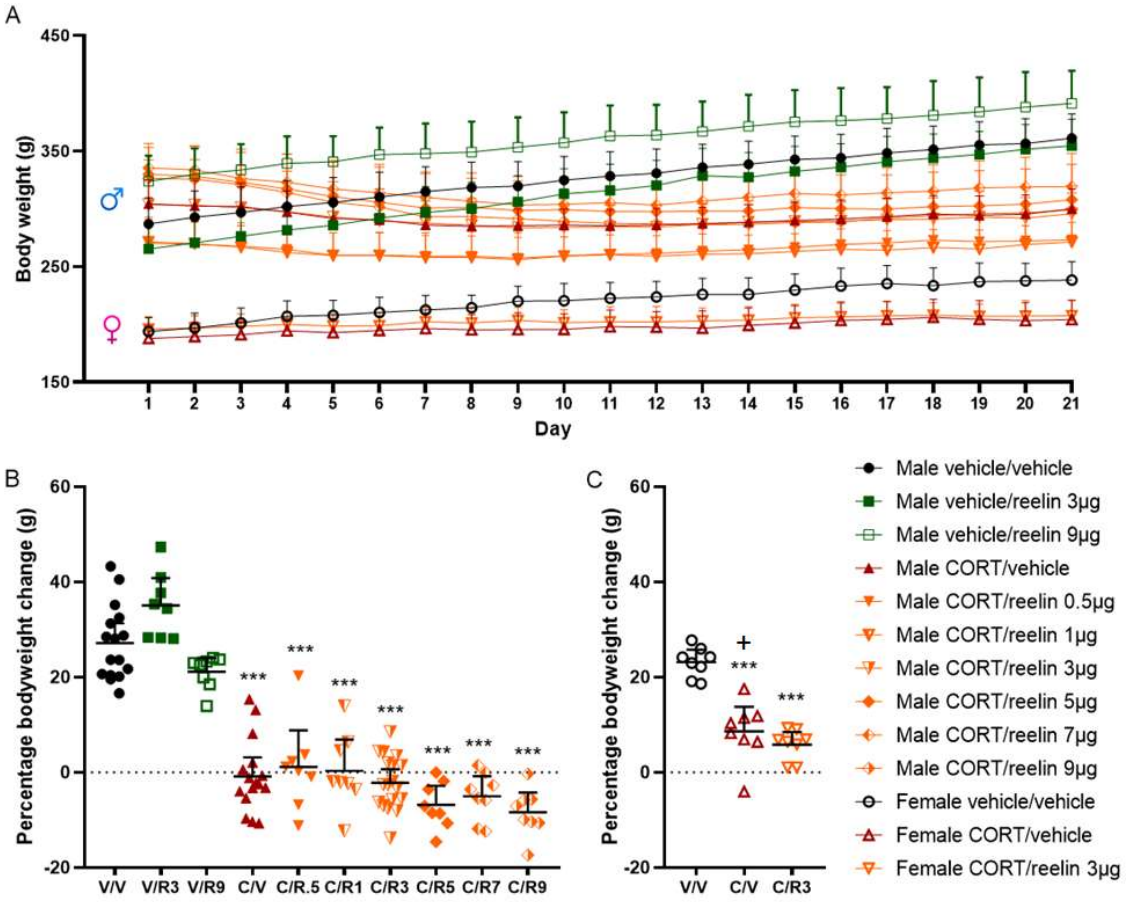


**Figure 4.4.1. The effect of CORT and incrementing doses of reelin on bodyweight.** A) Bodyweight over the 21-day injection period in males and females. Percentage change in weight over the 21-days in males (B) and females (C). CORT treatment decreased weight gain which reelin did not reverse. Data are expressed as mean±CI. ***$p$<0.001 vs vehicle/vehicle or vehicle/reelin; +$p$<0.05 vs male CORT/vehicle. Figure created with GraphPad Prism by author.

### 4.4.2 Forced swim test

A single injection of reelin (0.5µg, 3µg, 5µg, 7µg, or 9µg) was given to the rats on the last day of CORT, 24 hours before the FST. In males, I found that CORT significantly increased FST-immobility ($p$≤0.035) and that 3µg of reelin significantly decreased immobility (by 55%, $p$=0.003; Figure 4.4.2A). This motivated us to evaluate the effects of 3µg of reelin in females and I found that the CORT-induced increases in FST-immobility ($p$<0.001) were normalized by 87% ($p$=0.002; Figure 4.4.2E). I also found that male rats that were treated with CORT along with either vehicle, 0.5µg, 3µg, 5µg, or 7µg of reelin had a significantly lower mean latency to immobility time than the vehicle/vehicle-treated rats ($p$≤0.01), which was also true

for females ($p$=0.048; Figure 4.4.2B/F). There were no differences between groups for time spent climbing at the walls of the tank, but CORT did produce a significant reduction in swimming time ($p$≤0.017) which was rescued by 3µg of reelin in males ($p$=0.020; Figure 4.4.2C/D/G/H).

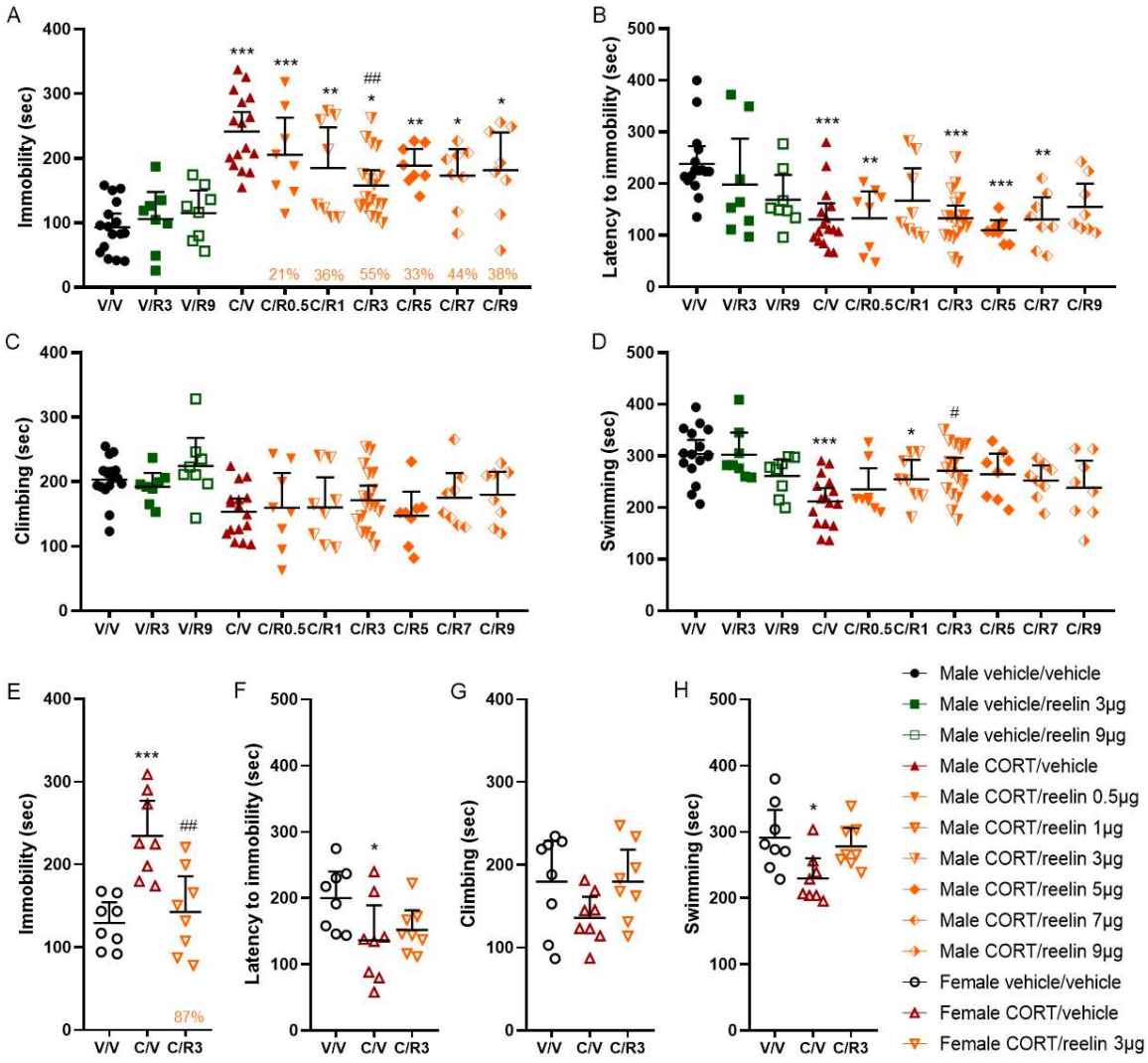


**Figure 4.4.2. The effect of CORT and incrementing doses of reelin on FST-behaviors.** CORT increased immobility and lowered the latency to immobility in males (A/B) and females (E/F), and immobility scores were significantly reduced by 3µg of reelin. Treatment did not affect time spent climbing (C/G) but swimming was increased by CORT in both sexes which was rescued by 3µg in males (D/H). Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05/**$p$<0.01/***$p$<0.001 vs same-sex vehicle/vehicle; #$p$<0.05/##$p$<0.01 vs same-sex CORT/vehicle. Figure created with GraphPad Prism by author.

### 4.4.3 Object-in-place recognition test

We previously demonstrated that intrahippocampal infusions of reelin had pro-cognitive effects (Brymer et al 2020), so I evaluated if this was true for a single i.v. reelin injection. Here I found that CORT-treated rats explored the objects that were moved significantly less than the stationary objects than the vehicle/vehicle group ($p \leq 0.048$; as evidenced by a lower DR score; see Figure 4.4.3), but not when they were given reelin which partially recovered cognitive deficits (albeit insignificantly). The 3µg dose of reelin recovered DR scores by 69% in males ($p=0.061$) and 58% in females ($p=0.321$). There were no differences in general locomotor activity.

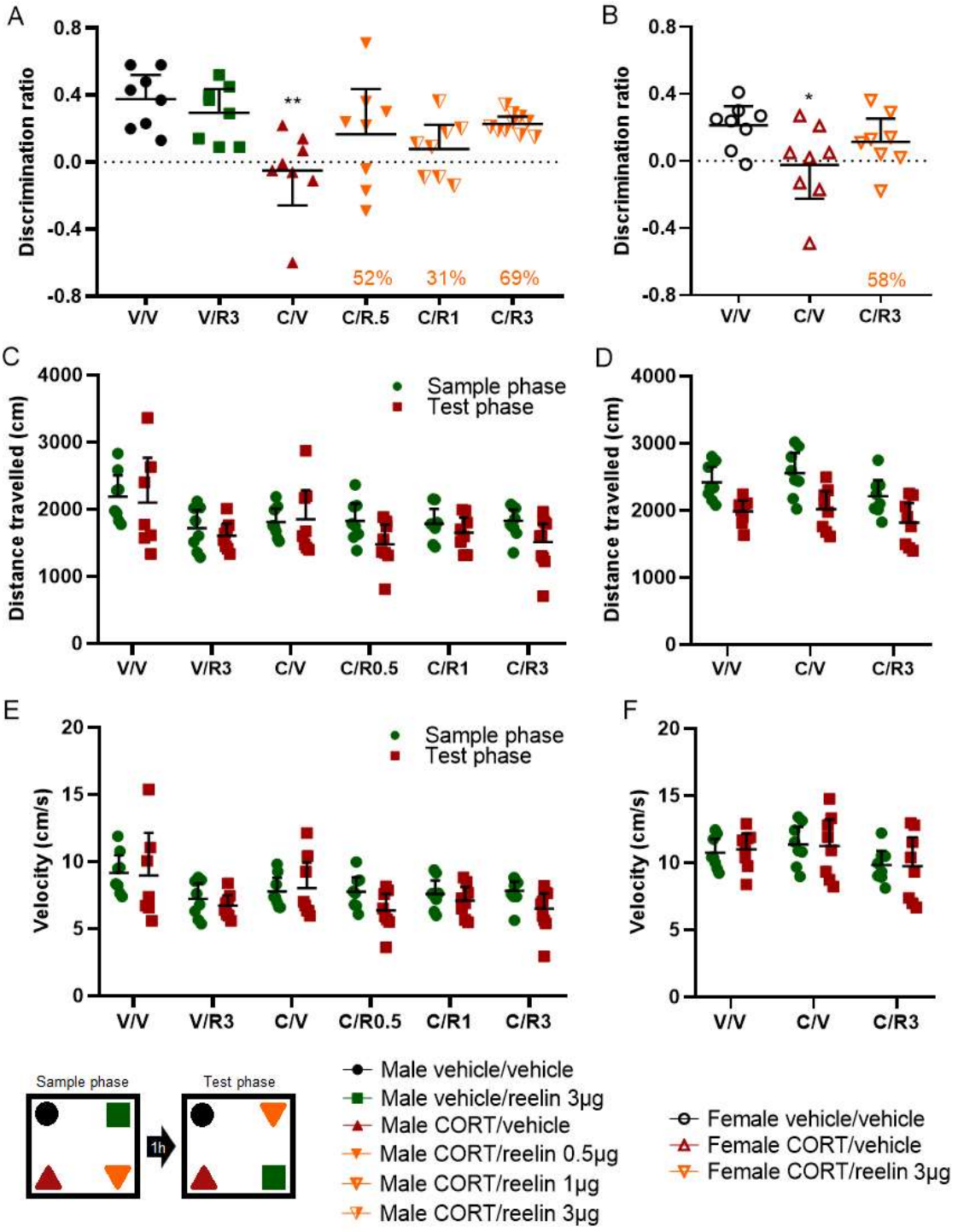


**Figure 4.4.3. The effect of CORT and a single reelin injection on object-recognition memory.** CORT impaired object-recognition memory in males (A) and females (B), which requires proper hippocampal functioning. Reelin partially recovered cognitive deficits. There were no differences in distance travelled or velocity in males (C/E) or females (D/F). Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05/**$p$<0.01 vs same-sex vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

**Table 4.4.3. Statistical information for the effects of CORT and a single injection of reelin on bodyweight and behavioral measures.**

| Measure | Sex | CORT | Reelin | CORT x Reelin | Sex | Sex x CORT | Sex x Reelin |
|---|---|---|---|---|---|---|---|
| Bodyweight | M | F(1, 96)=25.489, *p*<0.001 | F(1, 96)=4.074, *p*=0.001 | F(1, 96)=4.074, *p*=0.001 | na | | |
| | F | One way: F(2, 21)=4.850, p=0.019 | | | | | |
| Weight change | M | F(1, 96)=363.074, *p*<0.001 | F(1, 96)=4.993, *p*<0.001 | F(1, 96)=3.296, *p*=0.041 | | | |
| | F | One way: F(2, 21)=35.606, p<0.001 | | | | | |
| | M & F | F(1, 117)=256.196, p<0.001 | F(1, 117)=5.265, p<0.001 | F(1, 117)=3.667, p=0.029 | F(1, 117)=4.545, p=0.035 | F(1, 117)=12.122, p<0.001 | F(1, 117)=0.149, p=0.700 |
| FST-immobility | M | F(1, 96)=40.695, *p*<0.001 | F(1, 96)=1.017, *p*=0.419 | F(1, 96)=5.157, *p*=0.007 | na | | |
| | F | One way: F(2, 21)=12.753, p<0.001 | | | | | |
| | M & F | F(1, 117)=37.205, p<0.001 | F(1, 117)=2.023, p=0.068 | F(1, 117)=5.468, p=0.005 | F(1, 117)=0.369, p=0.545 | F(1, 117)=1.276, p=0.261 | F(1, 117)=0.196, p=0.659 |
| FST-latency | M | F(1, 96)=16.055, *p*<0.001 | F(1, 96)=1.070, *p*=0.386 | F(1, 96)=3.033, *p*=0.053 | na | | |
| | F | One way: F(2, 21)=3.496, p=0.049 | | | | | |
| | M & F | F(1, 117)=12.299, p=0.001 | F(1, 117)=0.966, p=0.451 | F(1, 117)=3.241, p=0.043 | F(1, 117)=0.166, p=0.684 | F(1, 117)=1.395, p=0.240 | F(1, 117)=0.140, p=0.709 |
| FST-climbing | M | F(1, 96)=12.166, *p*=0.001 | F(1, 96)=0.822, *p*=0.556 | F(1, 96)=0.730, *p*=0.484 | na | | |
| | F | One way: F(2, 21)=2.334, p=0.122 | | | | | |
| | M & F | F(1, 117)=11.035, p=0.001 | F(1, 117)=1.142, p=0.343 | F(1, 117)=0.718, p=0.490 | F(1, 117)=0.676, p=0.413 | F(1, 117)=0.053, p=0.819 | F(1, 117)=0.897, p=0.345 |
| FST-swimming | M | F(1, 96)=16.063, *p*<0.001 | F(1, 96)=1.452, *p*=0.203 | F(1, 96)=3.818, *p*=0.025 | na | | |
| | F | One way: F(2, 21)=5.078, p=0.016 | | | | | |
| | M & F | F(1, 117)=13.875, p<0.001 | F(1, 117)=1.661, p=0.137 | F(1, 117)=4.038, p=0.020 | F(1, 117)=0.009, p=0.924 | F(1, 117)=1.089, p=0.299 | F(1, 117)=0.152, p=0.698 |
| OBIP-DR score | M | F(1, 44)=5.189, *p*=0.028 | F(1, 44)=0.312, *p*=0.817 | F(1, 44)=8.168, *p*=0.006 | na | | |
| | F | F(2, 21)=3.524, *p=0.059* | | | | | |
| | M & F | F(1, 65)=12.639, p=0.001 | F(1, 65)=0.924, p=0.434 | F(1, 65)=6.973, p=0.010 | F(1, 65)=3.318, p=0.073 | F(1, 65)=1.829, p=0.181 | F(1, 65)=1.067, p=0.306 |
| OBIP-distance travelled | M | Sample: F(1, 44)=1.8290, p=0.184; Test: F(1, 43)=1.257, p=0.268 | Sample: F(1, 44)=1.694, p=0.182; Test: F(1, 43)=2.849, p=0.048 | Sample: F(1, 44)=5.879, p=0.019; Test: F(1, 43)=0.243, p=0.625 | na | | |
| | F | Sample: F(2, 21)=2.554, p=0.102; Test: F(2, 21)=1.081, p=0.358 | | | | | |
| | M & F | F(1, 64)=0.401, p=0.529 | F(1, 64)=3.274, p=0.027 | F(1, 64)=0.295, p=0.608 | F(1, 64)=0.899, p=0.347 | F(1, 64)=0.984, p=0.325 | F(1, 64)=0.265, p=0.608 |
| OBIP-velocity | M | Sample: F(1, 44)=0.788, *p*=0.380; Test: F(1, 44)=0.743, p=0.393 | Sample: F(1, 44)=1.613, *p*=0.200; Test: F(1, 44)=2.862, p=0.048 | Sample: F(1, 44)=5.248, *p*=0.027; Test: F(1, 44)=0.267, p=0.608 | na | | |
| | F | Sample: F(2, 21)=2.554, p=0.102; Test: F(2, 21)=1.082, p=0.357 | | | | | |
| | M & F | F(1, 64)=0.141, p=0.709 | F(1, 64)=3.293, p=0.026 | F(1, 64)=0.295, p=0.589 | F(1, 64)=0.050, p=0.824 | F(1, 64)=0.731, p=0.396 | F(1, 64)=0.100, p=0.753 |

### 4.4.4 SERT clustering parameters

We have observed in the past that patients with MDD express alterations in SERT MPC in lymphocytes (Rivera-Baltanas et al 2012, Romay-Tallon et al 2018), and that these changes are paralleled in CORT-treated rats and can be rescued by multiple injections of reelin. Here I show that the CORT-induced increase in SERT cluster size (males: $p$=0.045; females: $p$=0.091) was partially (insignificantly) rescued by a single 3µg dose of reelin in males (by 66%, $p$=0.275; Figure 4.4.8A) and fully in females (by 136%, $p$=0.017; Figure 4.4.4B). There were no effects of treatment on the number of SERT clusters (Figure 4.4.4C/D). While the meaningfulness of a 0.1-0.2µm$^2$ increase in SERT cluster size is unclear, it is possible that this correction is implicated in the responsiveness to antidepressant compounds (Caruncho et al 2019).

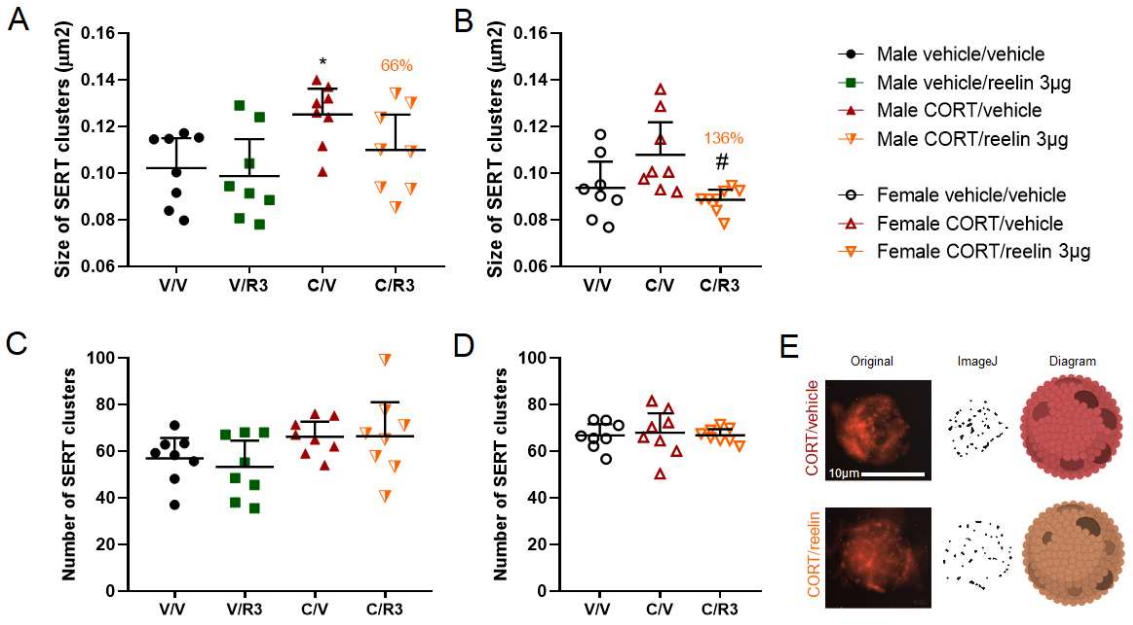


**Figure 4.4.4. The effect of CORT and a single reelin injection on SERT clustering parameters in lymphocytes.** CORT increased the size of SERT clusters which was partially rescued in males (A) and fully in females (B). There was no effect of treatment on the number of SERT clusters in males (C) or females (D). E) Representative images of SERT immunostaining, the binary image produced by ImageJ, and a diagram that shows the effects of reelin on SERT cluster size. Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05 vs vehicle/vehicle; #$p$<0.05 vs CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

### 4.4.5 Reelin-IR cell counts

We previously showed that the number of reelin-expressing cells in the SGZ is normalized in males and females after multiple i.v. reelin injections. Therefore, I evaluated whether a single injection of reelin would rescue the CORT-induced decreases in reelin-IR cell number. The male and female CORT/vehicle rats had significantly fewer reelin-IR cells (photomicrographs shown in Figure 4.4.5A) than the vehicle/vehicle rats ($p \leq 0.019$), but not when given reelin on CORT-administration day 21, indicating that reelin exerted a partial (insignificant) recovery (Figure 4.4.5B/C). In fact, 3µg of reelin rescued the number of reelin-IR cells by a substantial 69% in males ($p=0.161$ vs male CORT/vehicle) and 79% in females ($p=0.068$ vs female CORT/vehicle).

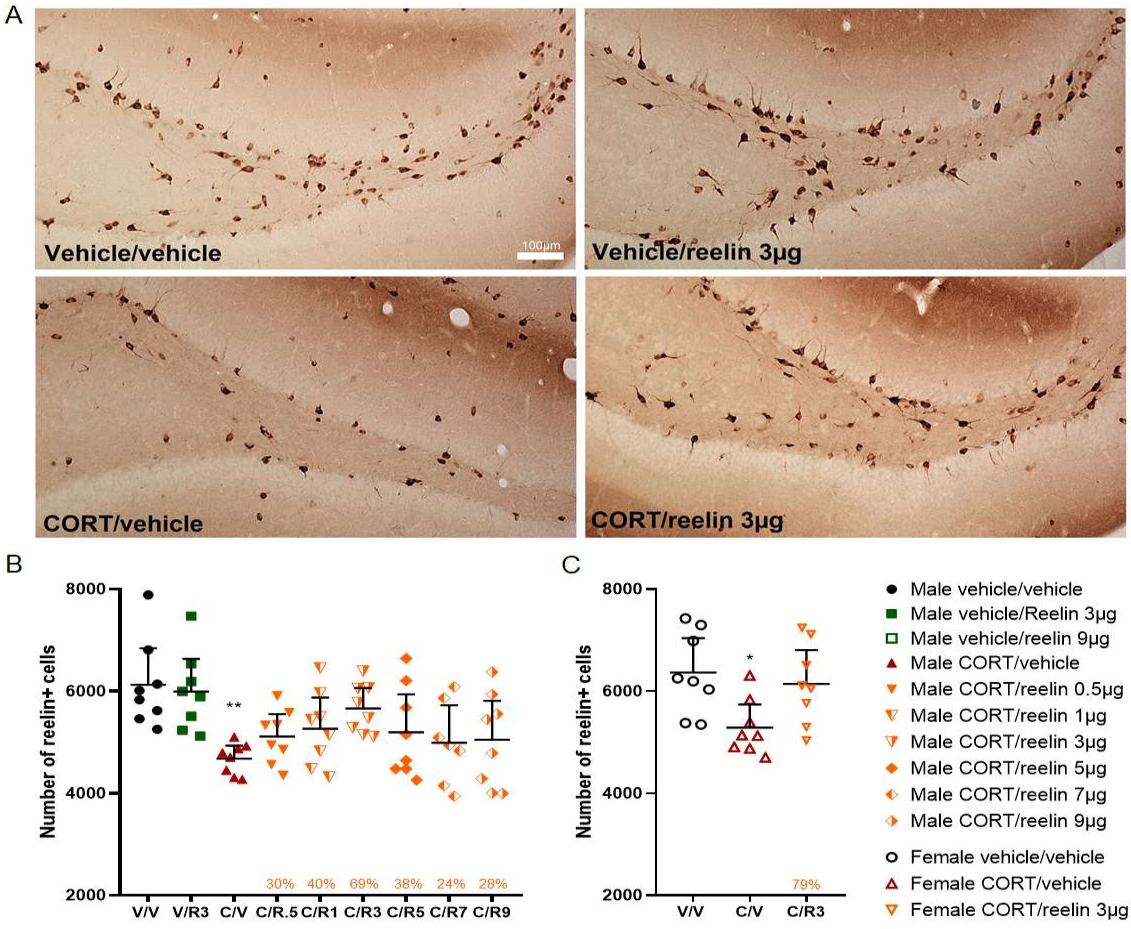


**Figure 4.4.5. The effect of CORT and a single reelin injection on reelin-IR cells counts in the SGZ.** A) Representative photomicrographs of reelin immunoreactivity in hippocampal slices. CORT significantly decreased reelin-IR cell number, but not when reelin was given on CORT-day 21 in both males (B) and females (C). Data are expressed as mean±CI. Percentage of recovery = %. *$p<0.05$/**$p<0.01$ vs vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

### 4.4.6 DCX-IR cell counts and categorization

We previously found that multiple i.v. reelin injections had little effect on neurogenesis as measured by DCX-immunostaining, but I was motivated to see if this was the case after a single 3µg administration. Representative photomicrographs can be seen in Figure 4.4.10A. In males, I found that reelin failed to recover ($p$=0.926, 13%) the CORT-induced decrease ($p$=0.029) in the number of DCX-IR cells (Figure 4.4.6C). When looking at the effect of treatment on the dendritic maturation of new-born DCX-expressing cells, I found that CORT increased the percentage of immature cells in category 2 ($p$=0.017) and intermediate category 4 ($p$=0.015) cells and decreased the percentage of the category 5 cells ($p$=0.004) which have more delicate dendritic branching. Reelin partially recovered these deficits (see Figure 4.4.10C).

In females, reelin partially recovered ($p$=0.476, 45%) the decrease in DCX immunopositive cells that was induced by CORT ($p$=0.040, Figure 4.6D). CORT also increased the number of cells that were assigned to category 1 ($p$=0.016) and 3 ($p\leq$0.042) and decreased the percentage of category 5 cells ($p\leq$0.016). While reelin partially recovered the amount of category 1 cells (by 39%), reelin failed to rescue the dendritic complexity of cells in other categories (Figure 4.4.6E).

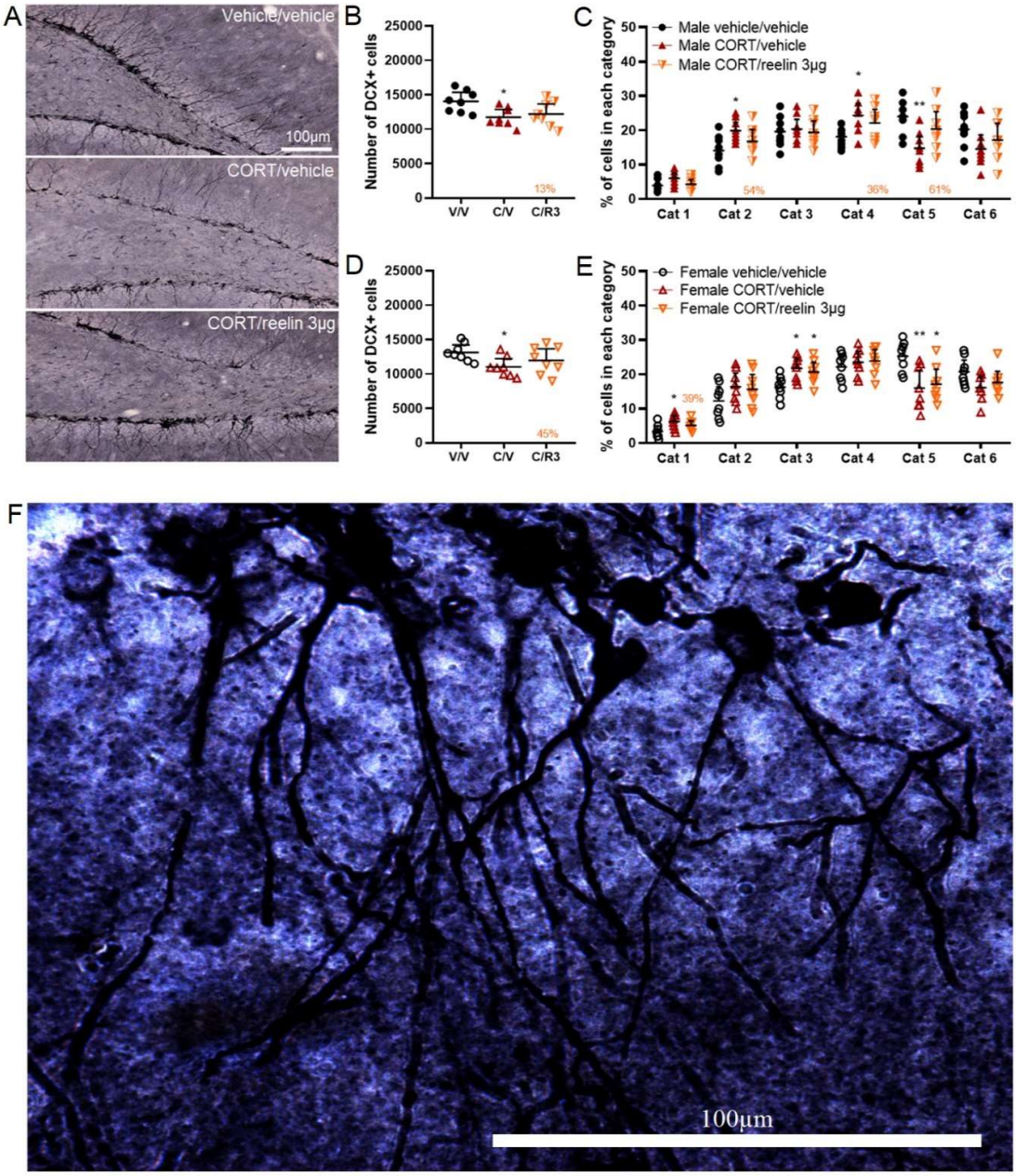


**Figure 4.4.6. The effect of CORT and a single 3µg reelin injection on DCX-IR cells in the SGZ and GCL.** A) Representative photomicrographs of DCX immunoreactivity. CORT significantly decreased the number of DCX-IR cells and slowed the dendritic maturation rate of surviving cells in males (B and C) and females (D and E). Reelin partially recovered dendritic complexity in males and DCX-IR cell counts in females. F) High magnification image of DCX-IR cells. Data are expressed as mean±CI. Percentage of recovery = %. **p*<0.05/***p*<0.01 vs vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

### 4.4.7 GluA1-IR cell counts

I also evaluated the effect of a single reelin injection on GluA1-IR cell numbers because AMPAR transmission is noted to be critical for rapid antidepressant behavioral changes (Zhang et al 2016). I found that CORT decreased the number of GluA1-IR cells in the SGZ for males ($p$=0.025) and females ($p$=0.003), and a single 3µg injection of reelin rescued this deficit by a staggering 159% in males ($p$<0.001) and 60% in females ($p$=0.080; Figure 4.4.7B/C).

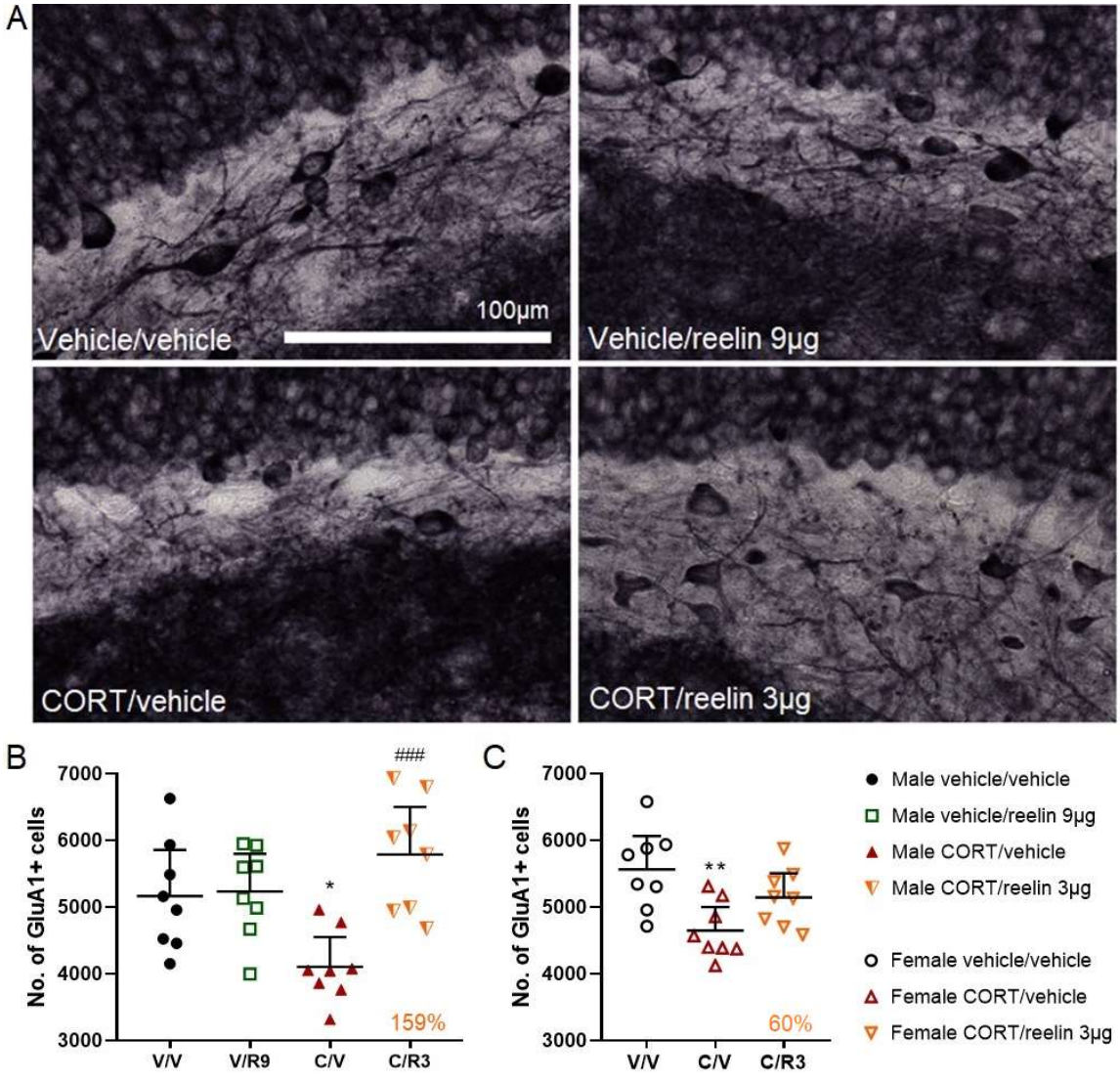


**Figure 4.4.7. The effect of CORT and a single reelin injection on GluA1-IR cells counts in the SGZ.** Representative photomicrographs of GluA1 immunoreactivity in the DG (A). CORT significantly decreased GluA1-IR cell numbers, which was fully recovered by 3µg of reelin in males (B) and partially in females (C). Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05/**$p$<0.01 vs vehicle/vehicle; ###$p$<0.001 vs CORT/vehicle. Figure created with GraphPad Prism and Paint by author.

### 4.4.8 BAX optical density

BAX is inhibited by activation of the PI3K/Akt pathway (see Figure 4.4.8a), which occurs downstream of synaptic reelin signaling and protects from CytC release (Takino et al 2019). Therefore, I evaluated BAX-immunoreactivity (Figure 4.4.8bA) in the DG following CORT and a single 3µg reelin injection. I found that CORT significantly increased the optical density of BAX in the GCL ($p$=0.036), but not when rats were given reelin which only reduced CORT-induced BAX-immunoreactivity by 17% (Figure 4.4.8bB). There was also a main effect of CORT in the ML, and of reelin in the PL, but no individual group differences were observed.

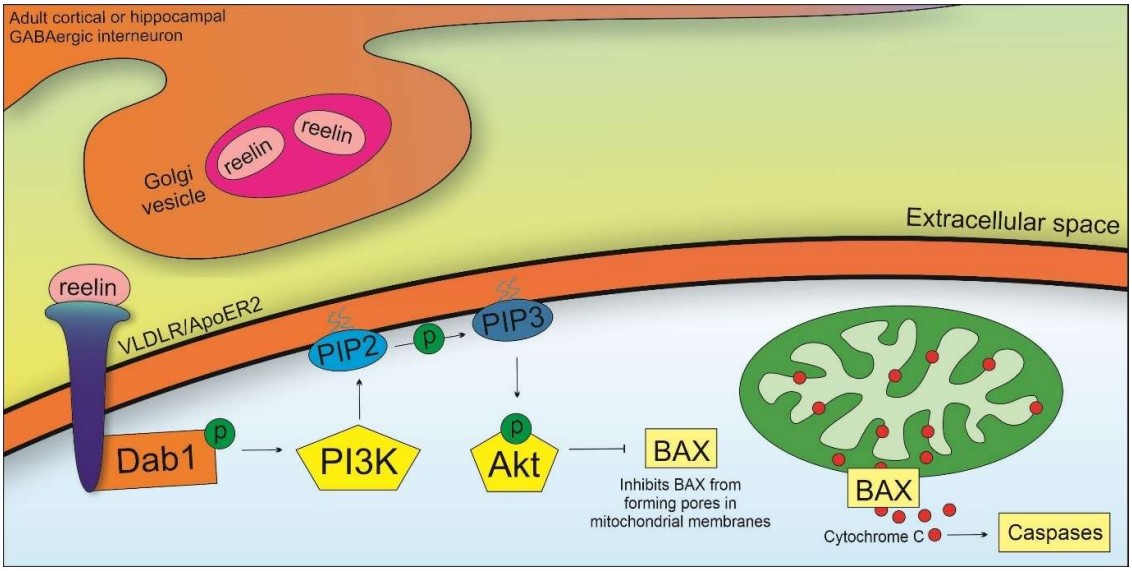


**Figure 4.4.8a. Reelin-PI3K/Akt signaling protects from mitochondrial-related apoptosis.** Reelin-induced Dab1 activity stimulates the PI3K/Akt pathway which inhibits BAX from forming pores in outer mitochondrial membranes. This protects from CytC release into the cytosol, where CytC can facilitate the formation of apoptosomes that in turn activate executioner caspases.

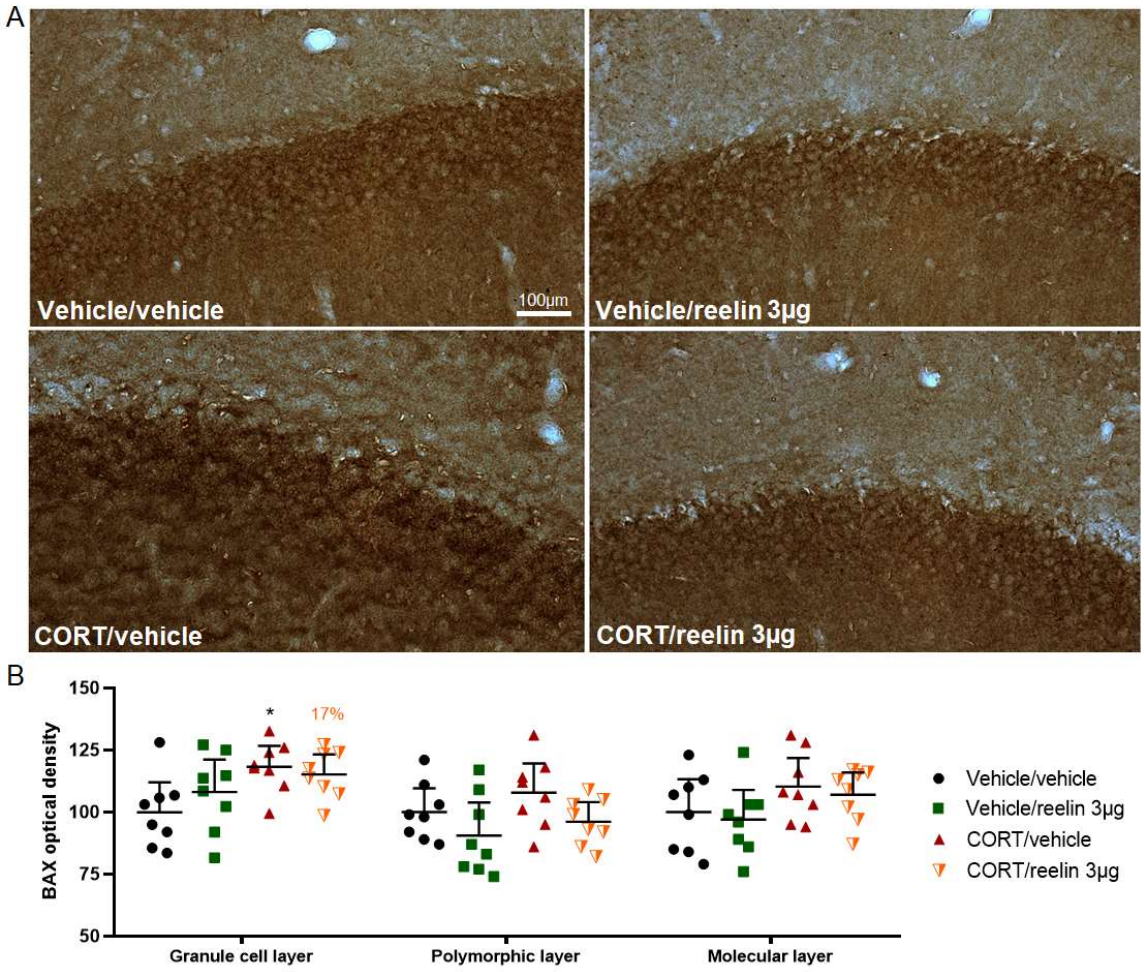


**Figure 4.4.8b. CORT increased the optical density of BAX in the GCL.** A) Representative photomicrographs of GluA1 immunoreactivity in the DG. B) CORT significantly increased the optical density of BAX in the GCL and reelin decreased CORT-induced BAX immunoreactivity by just 17%. Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05 vs vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

### 4.4.9 Cytochrome C optical density and cell counts

Next I analyzed the effects of reelin on CytC expression following CORT, given that stress is associated with increases in hippocampal CytC and reelin signaling may hinder CytC release (Xu et al 2019, Allen et al 2021a). I found that CORT elevated CytC immunoreactivity in the GCL ($p$=0.031) and ML ($p$=0.028) but not in the polymorphic layer ($p$=0.095; Figure 4.4.9B). I also found that CORT increased the number of CytC immunopositive cells in the SGZ ($p$=0.043). Reelin normalized SGZ-CytC cell counts by 87% ($p$=0.093 vs CORT/vehicle; Figure 4.4.9C).

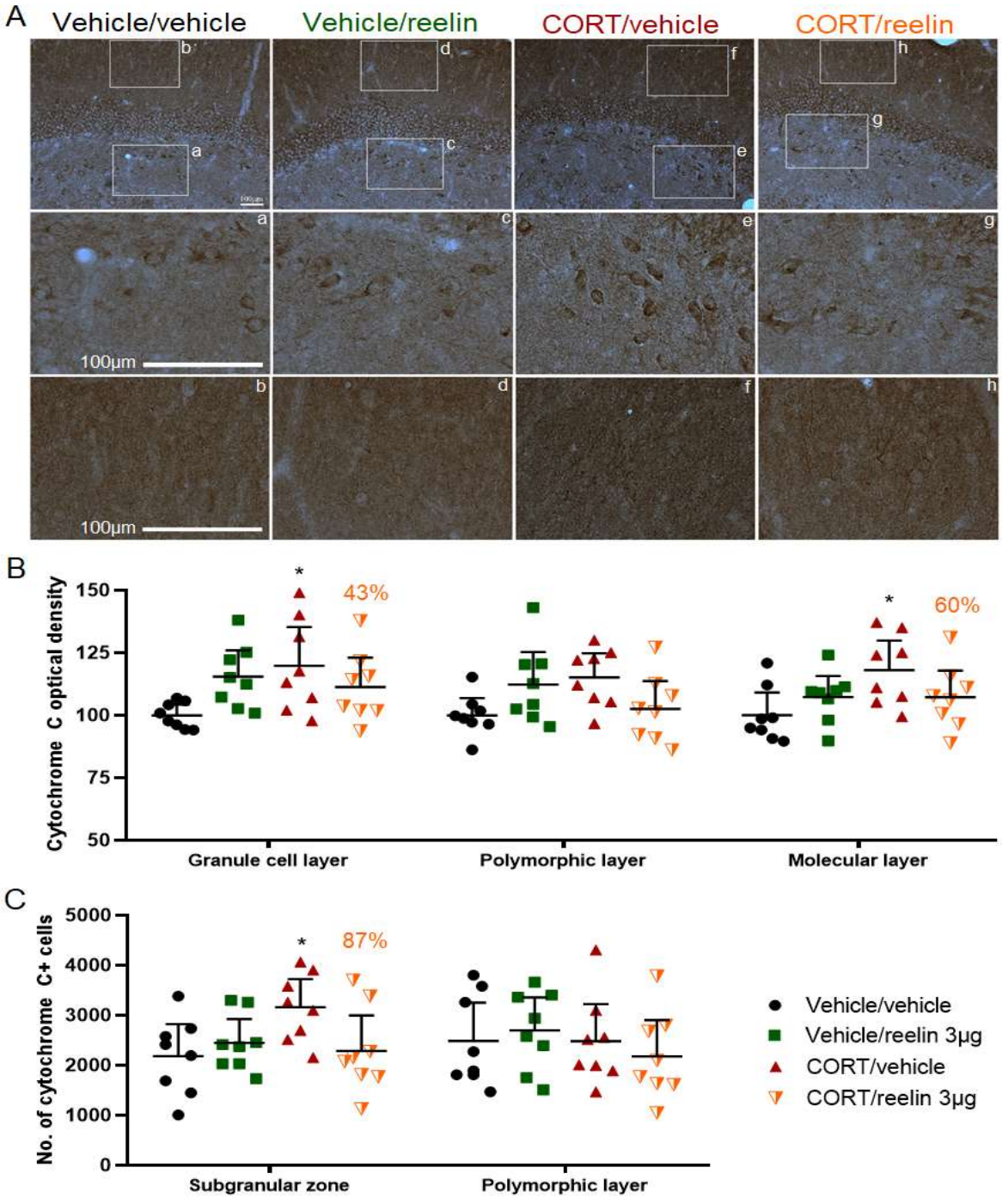


**Figure 4.4.9b. The effect of CORT and a single reelin injection on CytC expression in the DG.** A) Representative photomicrographs of CytC immunoreactivity in the DG. B) CORT significantly increased the optical density of BAX in the GCL and ML which was partially recovered by reelin. C) CORT increased the number of CytC-IR cells in the SGZ which was recovered by reelin by 87%. Data are expressed as mean±CI. Percentage of recovery = %. *$p$<0.05 vs vehicle/vehicle. Figure created with GraphPad Prism and Paint by author.

**Table 4.4.9. Statistical information for the effects of CORT and a single injection of reelin on blood and neurochemical analyses.**

| Measure | Sex | CORT | Reelin | CORT x Reelin | Sex | Sex x CORT | Sex x Reelin |
|---|---|---|---|---|---|---|---|
| SERT cluster number | M | F(1, 28)=6.178, $p$<0.001 | F(1, 28)=0.147, $p$=0.704 | F(1, 28)=0.197, $p$=0.660 | na | | |
| | F | One way: F(2, 21)=0.068, $p$=0.935 | | | | | |
| | M & F | F(1, 49)=5.630, $p$=0.022 | F(1, 49)=0.339, $p$=0.563 | F(1, 49)=0.283, $p$=0.860 | F(1, 49)=1.913, $p$=0.173 | F(1, 49)=1.148, $p$=0.289 | F(1, 49)=0.031, $p$=0.860 |
| SERT cluster size | M | F(1, 28)=8.475, $p$=0.007 | F(1, 28)=2.530, $p$=0.123 | F(1, 28)=1.030, $p$=0.319 | na | | |
| | F | One way: F(2, 21)=4.903, $p$=0.018 | | | | | |
| | M & F | F(1, 49)=10.604, $p$=0.002 | F(1, 49)=7.190, $p$=0.010 | F(1, 49)=1.249, $p$=0.269 | F(1, 49)=11.565, $p$=0.001 | F(1, 49)=0.692, $p$=0.410 | F(1, 49)=0.141, $p$=0.709 |
| Reelin | M | F(1, 62)=21.98, $p$<0.001 | F(1, 62)=1.23, $p$=0.306 | F(1, 62)=0.968, $p$=0.329 | na | | |
| | F | One way: F(2, 21)=4.963, $p$=0.017 | | | | | |
| | M & F | F(1, 83)=15.530, $p$<0.001 | F(1, 83)=0.966, $p$=0.453 | F(1, 83)=4.774, $p$=0.032 | F(1, 83)=3.565, $p$=0.063 | F(1, 83)=0.522, $p$=0.472 | F(1, 83)=0.062, $p$=0.805 |
| DCX cell counts | M | One way: F(2, 22)=4.411, $p$=0.025 | | | na | | |
| | F | One way: F(2, 21)=3.450, $p$=0.051 | | | | | |
| | M & F | F(1, 43)=14.556, $p$<0.001 | F(1, 43)=1.246, $p$=0.271 | na | F(1, 43)=1.657, $p$=0.205 | F(1, 43)=0.000, $p$=0.994 | F(1, 43)=0.362, $p$=0.551 |
| DCX cat 1 | M | One way: F(2, 22)=3.372, $p$=0.053 | | | na | | |
| | F | One way: F(2, 21)=4.749, $p$=0.020 | | | | | |
| | M & F | F(1, 43)=15.082, $p$<0.001 | F(1, 43)=4.987, $p$=0.031 | na | F(1, 43)=0.131, $p$=0.719 | F(1, 43)=0.339, $p$=0.563 | F(1, 43)=0.236, $p$=0.630 |
| DCX cat 2 | M | One way: F(2, 22)=4.594, $p$=0.022 | | | na | | |
| | F | One way: F(2, 21)=1.580, $p$=0.229 | | | | | |
| | M & F | F(1, 43)=10.147, $p$=0.003 | F(1, 43)=1.569, $p$=0.217 | na | F(1, 43)=2.042, $p$=0.160 | F(1, 43)=0.279, $p$=0.600 | F(1, 43)=0.592, $p$=0.446 |
| DCX cat 3 | M | One way: F(2, 22)=0.127, $p$=0.881 | | | na | | |
| | F | One way: F(2, 21)=5.988, $p$=0.009 | | | | | |
| | M & F | F(1, 43)=5.639, *p=0.022* | F(1, 43)=0.635, $p$=0.430 | na | F(1, 43)=0.255, $p$=0.616 | F(1, 43)=3.359, $p$=0.074 | F(1, 43)=0.004, $p$=0.949 |
| DCX cat 4 | M | One way: F(2, 22)=4.784, $p$=0.019 | | | na | | |
| | F | One way: F(2, 21)=0.435, $p$=0.653 | | | | | |
| | M & F | F(1, 43)=7.170, $p$=0.010 | F(1, 43)=0.419, $p$=0.521 | na | F(1, 43)=3.084, $p$=0.086 | F(1, 43)=2.913, $p$=0.095 | F(1, 43)=0.832, $p$=0.367 |
| DCX cat 5 | M | One way: F(2, 22)=6.883, $p$=0.005 | | | na | | |
| | F | One way: F(2, 21)=7.225, $p$=0.004 | | | | | |
| | M & F | F(1, 43)=25.540, $p$<0.001 | F(1, 43)=3.382, $p$=0.073 | na | F(1, 43)=0.126, $p$=0.725 | F(1, 43)=0.000, $p$=0.994 | F(1, 43)=1.497, $p$=0.228 |
| DCX cat 6 | M | One way: F(2, 22)=2.274, $p$=0.127 | | | na | | |
| | F | One way: F(2, 21)=2.832, $p$=0.081 | | | | | |
| | M & F | F(1, 43)=9.469, $p$=0.004 | F(1, 43)=1.472, $p$=0.232 | na | F(1, 43)=0.246, $p$=0.622 | F(1, 43)=0.102, $p$=0.751 | F(1, 43)=0.102, $p$=0.751 |
| GluA1 | M | F(1, 28)=0.941, *p=0.340* | F(1, 28)=11.414, $p$=0.002 | F(1, 28)=9.654, $p$=0.004 | na | | |
| | F | One way: F(2, 21)=7.430, $p$=0.004 | | | | | |

| | M & F | F(1, 49)=19.187, *p*<0.001 | F(1, 49)=12.265, *p<0.001* | na | F(1, 49)=0.024, *p*=0.878 | F(1, 49)=0.102, *p*=0.751 | F(1, 49)=6.391, *p*=0.015 |
|---|---|---|---|---|---|---|---|
| BAX optical density - GCL | M | F(1, 28)=7.952, *p=0.009* | F(1, 28)=0.318, *p*=0.577 | F(1, 28)=1.545, *p*=0.224 | na | | |
| BAX optical density - hilus | | F(1, 28)=2.152, *p=0.153* | F(1, 28)=5.333, *p*=0.029 | F(1, 28)=0.060, *p*=0.809 | | | |
| BAX optical density - ML | | F(1, 28)=2.972, *p=0.096* | F(1, 28)=0.463, *p*=0.502 | F(1, 28)=0.019, *p*=0.893 | | | |
| CytC optical density - GCL | | F(1, 28)=2.678, *p=0.113* | F(1, 28)=0.541, *p*=0.268 | F(1, 28)=6.327, *p*=0.018 | | | |
| CytC optical density - hilus | | F(1, 28)=0.374, *p=0.546* | F(1, 28)=0.001, *p*=0.978 | F(1, 28)=7.944, *p*=0.009 | | | |
| CytC optical density - ML | | F(1, 28)=4.397, *p=0.045* | F(1, 28)=0.176, *p*=0.678 | F(1, 28)=4.500, *p*=0.043 | | | |
| CytC cell counts SGZ | | F(1, 28)=2.735, *p=0.109* | F(1, 28)=1.311, *p*=0.262 | F(1, 28)=5.285, *p*=0.029 | | | |
| CytC cell counts hilus | | F(1, 28)=0.749, *p=0.394* | F(1, 28)=0.023, *p*=0.880 | F(1, 28)=0.707, *p*=0.407 | | | |

### 4.4.10 Correlations

Correlational graphs that depict the relationships between the size of SERT clusters and behavioral and neurochemical alterations are shown in Figure 4.4.10a. I found that larger SERT cluster sizes on peripheral lymphocytes were correlated with more time spent immobile in the FST (r=0.391, *p*=0.003, n=56) and lower reelin-IR cell counts (r=-0.343, *p*=0.010, n=56) when all the groups were included in the analyses. However, there were no significant correlations when the CORT/vehicle or CORT/reelin groups were analyzed individually.

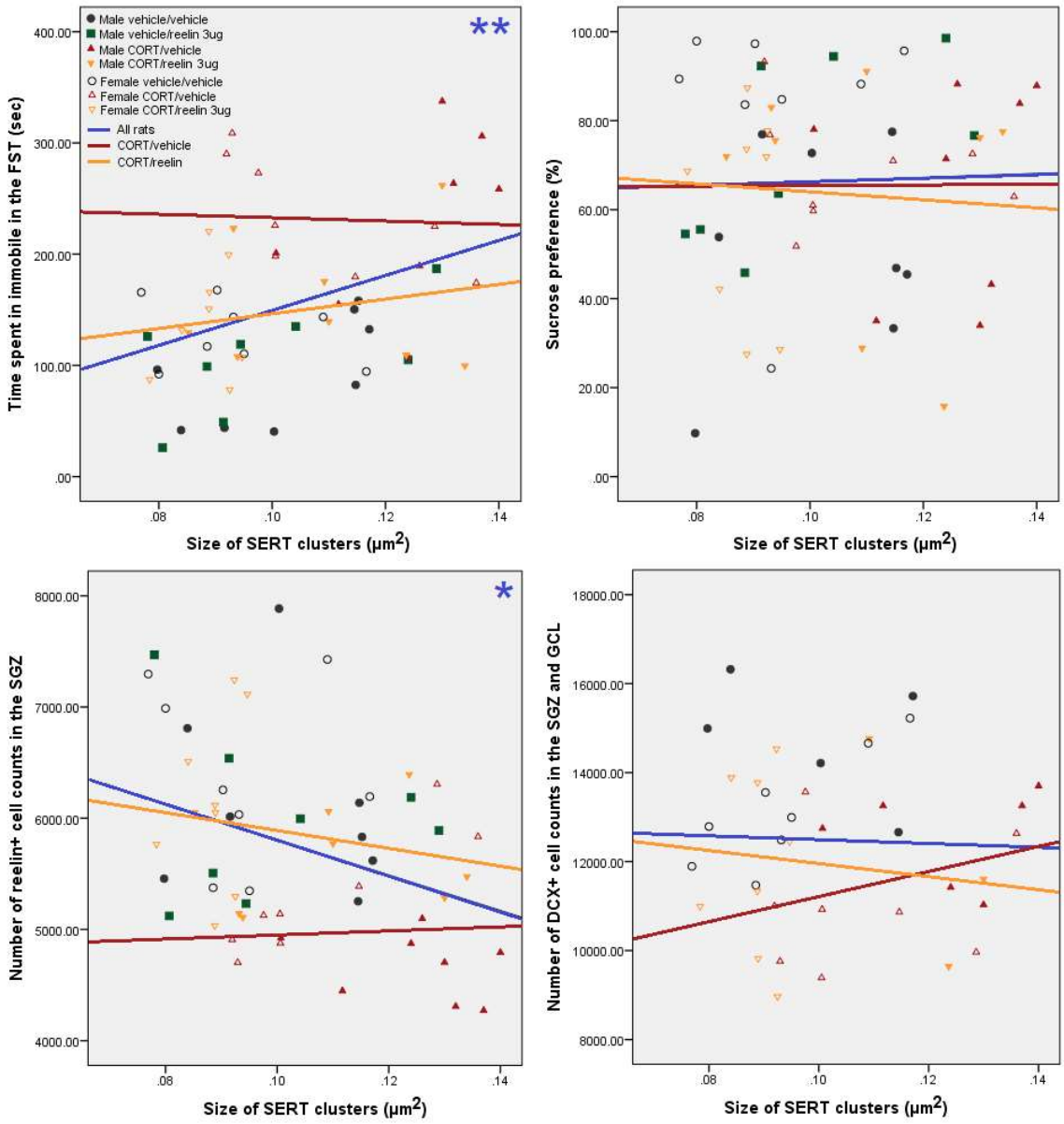


**Figure 4.4.10a. Correlations with SERT MPC.** Significant correlations were observed for SERT cluster size and both immobility times and reelin-IR cell counts when all treatment groups were included in the analyses, which is represented by the blue line (*$p$<0.05/**$p$<0.01). Figure created with SPSS and Paint by author.

Graphs depicting the correlational relationships with reelin cell counts in the SGZ are shown in Figure 4.4.10b. SGZ-reelin correlated negatively with FST-immobility when all the groups were included in the analysis (r=-0.480, $p$<0.001, n=95), and when only the CORT/reelin-treated rats were included (r=-0.365, $p$=0.006, n=55), in whom a reduction in reelin was also associated with heightened CytC-positive cell numbers in the SGZ (r=-0.716, $p$=0.046, n=8).

When only the rats treated with CORT/vehicle were included in the analyses, I saw that the expression of reelin correlated positively with CytC expression in the ML (r=0.854, $p$=0.007, n=8) and GCL (r=0.737, $p$=0.037, n=8), contrary to our hypothesis. No other significant correlations were observed.

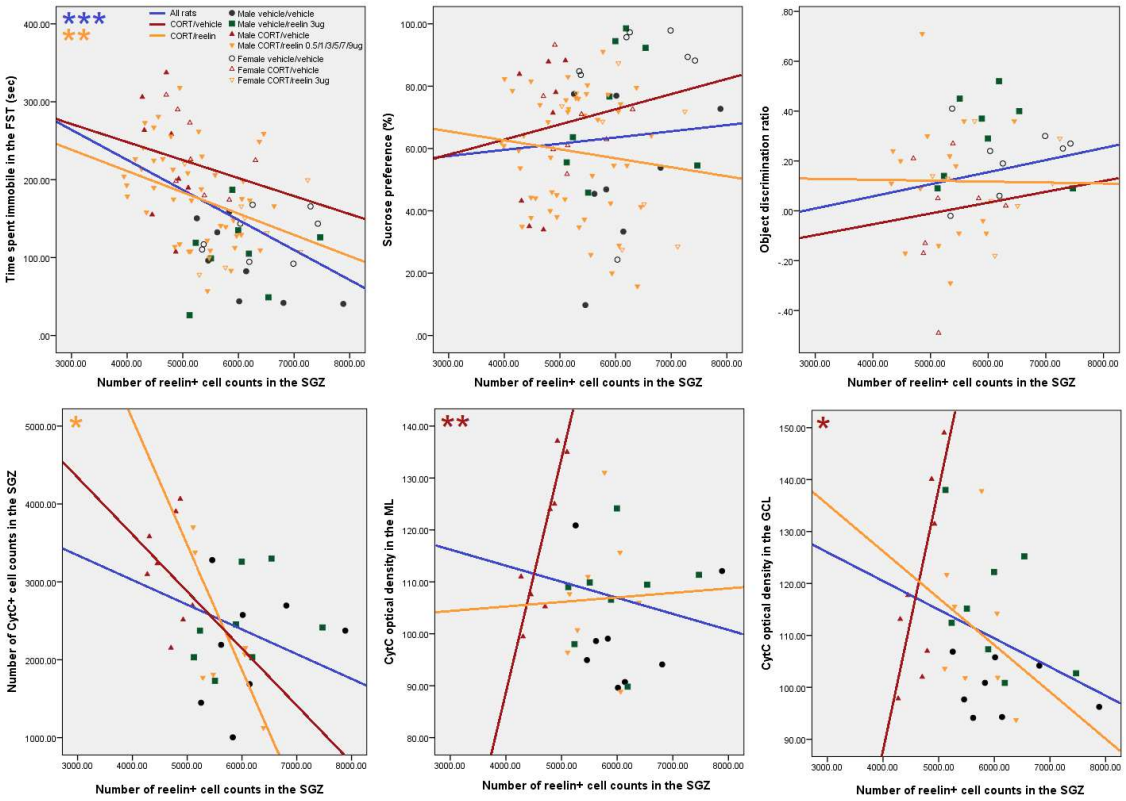


**Figure 4.4.10b. Correlations with the number of reelin-IR cells in the SGZ.** Significant correlations between reelin and behavioral and neurochemical alterations are illustrated by asterisks (*$p$<0.05/**$p$<0.01/***$p$<0.001) that are color-coordinated with the line of best fit when all groups were included in the analyses (blue line), or only the CORT/vehicle (red line) or CORT/reelin rats (orange line). Figure created with SPSS and Paint by author.

The correlational relationships with DCX-IR cell numbers and other behavioral and neurochemical measures are illustrated in Figure 4.4.10c. I found that the number of DCX-IR cells was correlated negatively with time spent immobile in the FST (r=-0.330, $p$=0.021, n=49) and positively with the number of reelin-IR cells (r=0.340, $p$=0.037, n=38) when all treatment groups were included in the analyses. No significant correlations were observed when the groups were analyzed individually.

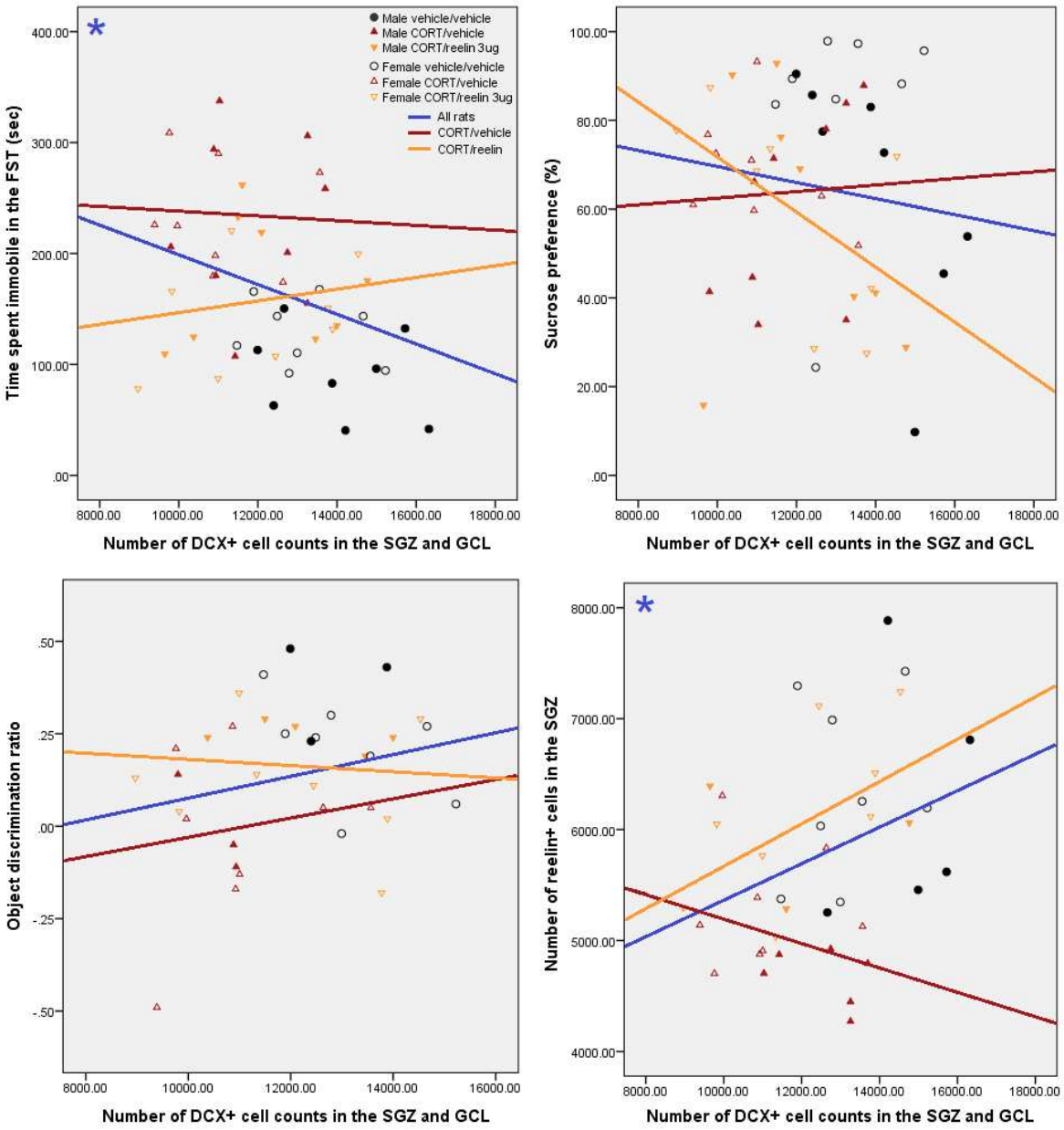


**Figure 4.4.10c. Correlations with the number of DCX-IR cells in the SGZ and GCL.** DCX-IR cell numbers correlated negatively with time spent immobile in the FST and positively with reelin-IR cell counts (*$p$<0.05). Figure created with SPSS and Paint by author.

The correlations regarding GluA1 expression are shown in Figure 4.4.10d. I found that higher GluA1-IR cell counts in the SGZ was associated with a lower time spent immobile in the FST (r=-0.410, $p$=0.002, n=56) and lower reelin levels (r=-0.389, $p$=0.016, n=38).

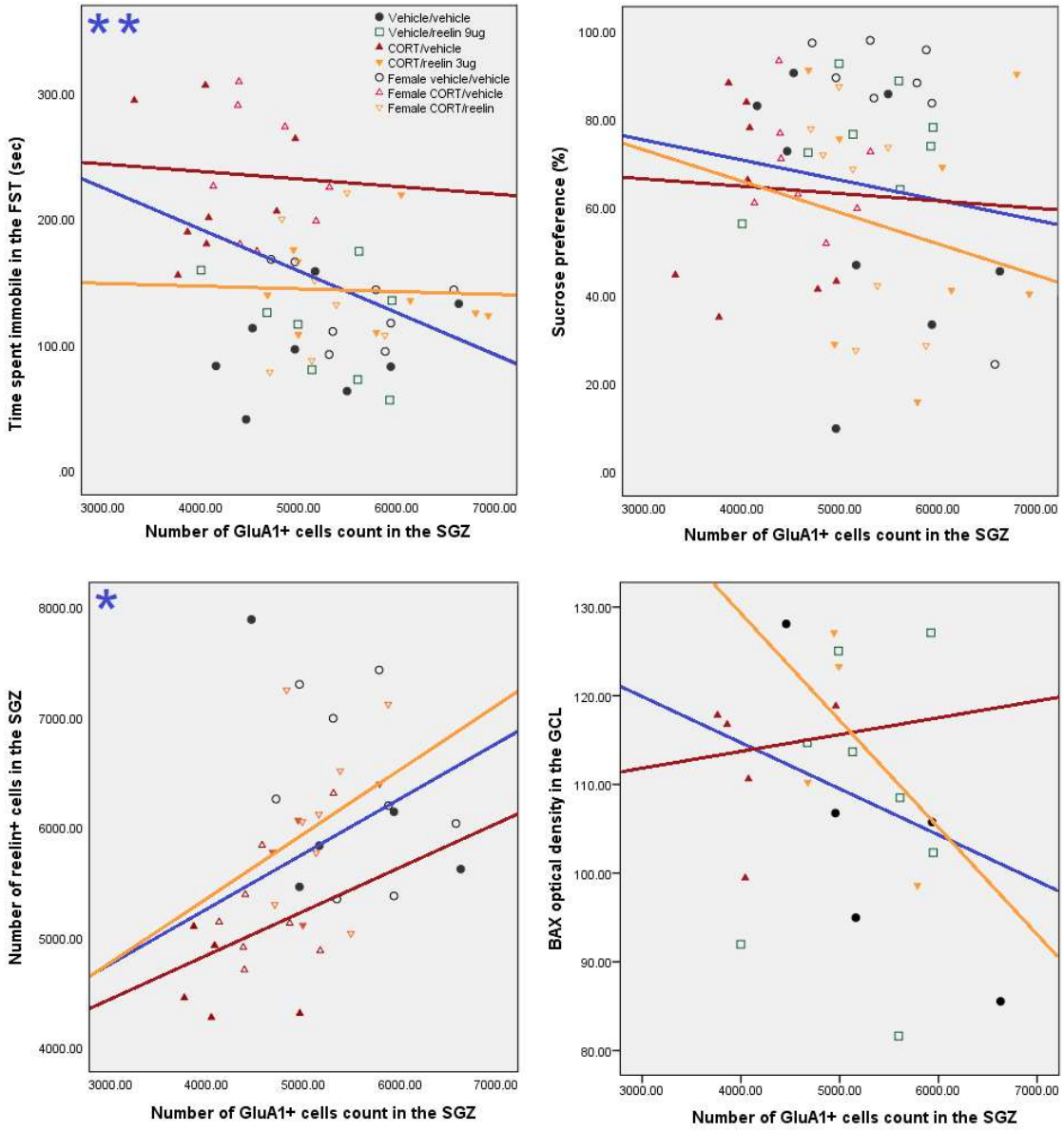


**Figure 4.4.10d. Correlations with the number of GluA1-IR cells in the SGZ.** GluA1-IR cell counts were negatively correlated with FST-immobility (*$p$<0.05). Figure created with SPSS and Paint by author.

I also investigated the relationships between the expression levels of apoptosis-promoting BAX and CytC in the DG with other behavioral and neurochemical measures (Figure 4.4.10e). The only correlation that was significant was that a higher optical density of BAX in the GCL was associated with a larger number of CytC-positive cells in the SGZ when all groups were included in the analysis (r=0.409, $p$=0.047, n=24).

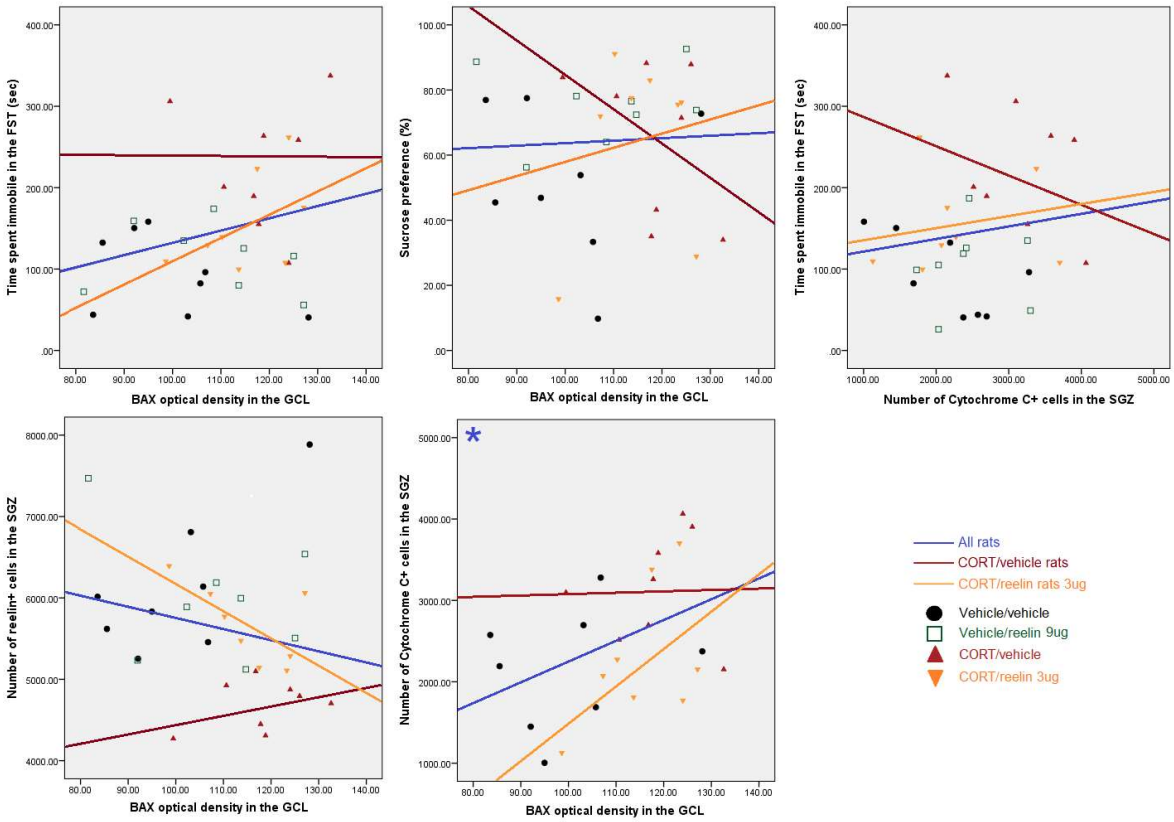


**Figure 4.4.10e. Correlations with BAX and CytC C in the DG.** Significant correlations between reelin and behavioral and neurochemical alterations are illustrated by asterisks (*$p$<0.05/**$p$<0.01/***$p$<0.001) that are color-coordinated with the line of best fit when all groups were included in the analyses (blue line), or only the CORT/vehicle (red line) or CORT/reelin rats (orange line). Figure created with SPSS and Paint by author.

## 4.5 Discussion

Here I provide the first evidence, to our knowledge, that peripheral i.v. reelin can improve behavioral and cognitive deficits induced by chronic stress in a rapid manner, and that these recoveries are associated with an enhancement of reelin and GluA1 expression and a dampening of pro-apoptotic factors in the DG. Furthermore, SERT MPC abnormalities were improved by reelin which may represent a mechanism whereby reelin can indirectly affect central neurotransmission. The rapid reductions in FST-immobility by reelin are unlikely to be explained by the facilitation of neurogenesis in these conditions. The discovery of novel compounds with antidepressant actions is an extremely important avenue of research considering that depression is a very common, devastating condition that is poorly treated (Cipriani et al 2018, Nischal et al 2012, Trivedi et al 2006).

Our laboratory is interested in reelin signaling as an avenue to develop novel fast-acting therapeutics and much work has gone into demonstrating its neuroprotective qualities. For instance: drugs with antidepressant actions raise reelin levels in the rodent hippocampus (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020); *RELN*$^{+/-}$ mice (who express 40-60% of normal reelin levels) are more sensitive to the behavioral changes that are produced by CORT (Lussier et al 2011, Notaras et al 2020, Schroeder et al 2015); an overexpression of reelin protects from the depressogenic effects of stress (Teixeira et al 2011); recombinant reelin can produce fast-acting antidepressant-like effects when infused directly into the hippocampus (Brymer et al 2020); and multiple peripheral i.v. reelin injections lead to similar antidepressant-like effects in males and females (Chapters 2 and 3).

In our laboratory, we model chronic stress by subjecting rats to 3 weeks of daily CORT injections, which consistently increases immobility behavior in the FST (Gregus et al 2005, Johnson et al 2006, Kalynchuk et al 2004, Marks et al 2009, Sterner & Kalynchuk 2010), similarly to that observed in other animals models, such as rodents that are exposed to CUMS (Palucha-Poniewiera et al 2020, Wei et al 2019). While the FST as a measure of depression-like behavior is certainly questionable (Commons et al 2017), one should keep in mind that drugs with antidepressant actions in humans usually decrease FST-immobility in rodents (Kuśmider et al 2007, Takahashi et al 2020), which was our rationale to use it as a screening tool for the putative antidepressant-like actions of exogenous reelin. To evaluate if the effects of peripheral reelin are fast-acting, I first conducted a dose-response analysis whereby male rats were given i.v. reelin (0.5µg, 1µg, 3µg, 5µg, 7µg, and 9µg) 24 hours before the FST. There was a U-shaped dose response in that 3µg produced greater recoveries (55%, $p$=0.003) than lower (0.5, 1µg) and higher (5, 7, 9µg) doses, while having no effect on general locomotor or exploratory activity in several other behavioral tests (see Appendix). Therefore, I decided to evaluate a single 3µg reelin injection in females, which resulted in an even greater attenuation (87%, $p$=0.002) of FST-immobility. Our data suggests that the therapeutic effects of reelin are greater when reelin concentrations are maintained within a target range, bringing neuronal activity to homeostasis, especially since extracellular proteins have long half-lives, a variety of time-dependent effects, and often belong to self-regulating systems (Balmaceda et al 2014, Laham & Gould, 2022). Too little reelin signaling leads to dendritic hypotrophy whereas reelin overexpression leads to dendritic hypertrophy and cell misplacement (Pujadas et al 2010, Bosch et al 2016a).

Cognitive deficits are difficult to treat and are commonly reported in those with depression and other psychiatric disorders that are comorbid with depression (Richardson & Adams 2018). The hippocampal volumetric reductions in depression are thought to contribute (Chung et al 2016, Sawyer et al 2012) given its involvement in learning and memory (Anand & Dhikav 2012). I was motivated to learn whether a single i.v. reelin injection could lessen the cognitive impairments induced by CORT (Lui et al 2017, Zhang et al 2021b) since this was true for intrahippocampal infusions (Brymer et al 2020) but also because bilateral ventricular injections enhanced spatial learning and memory (without influencing exploration or anxiety-like behavior) in wild-type mice (Rogers et al 2011) and recovered associate learning deficits in *RELN*$^{+/-}$ mice (Rogers et al 2013). The OBIP hippocampal-dependent recognition test took place 3 days after the i.v. reelin injection which partially recovered object DR scores in males and females alike. Cognitive deficits have been linked to the hyperphosphorylation of Tau protein by GSK3β in patients with Alzheimer's disease (Toral-Rios et al 2020); perhaps reelin enhances cognition by activating the GSK3β-inhibiting PI3K/Akt pathway, which seems to also delay amyloid-β fibril formation (Beffert et al 2002, Pujadas et al 2014). Amyloid-β molecules aggregate and can become neurotoxic, and they may cause Tau dysfunction (Pulawski et al 2012). However, it could be that reelin facilitates learning and memory by stimulating various forms of neuroplasticity like cell proliferation, the growth of dendrites, and dendritic spine formation (Bosch et al 2016a, Hethorn et al 2015, Jossin & Goffinet 2007, Niu et al 2004). Whether reelin facilitates neuroplasticity directly via central mechanisms or indirectly through peripheral mechanisms is up for debate.

We have found in the past that *RELN*$^{+/-}$ mice and CORT-treated rats have larger SERT clusters in the membrane of blood lymphocytes, as do patients with depression, in whom SERT MPC can be used to predict responsiveness to conventional antidepressants in treatment-naïve patients (Caruncho et al 2019, Rivera-Baltanas et al 2010, 2012, 2015, Romay-Tallon et al 2018). Here I found that the CORT-induced increase in SERT cluster size was partially recovered by a single 3µg injection of reelin in males (66%) and fully in females (136%). This corroborates our previous findings which showed that lymphocytes extracted from CORT-treated rats had similar alteration patterns that were normalized by in vitro reelin incubations, whereas ketamine (10nM, 50nM, and 250nM) had opposite effects (Johnston et al 2020). One can assume that abnormalities in MPC would dysregulate the proper functioning of lymphocytes and lead to a pro-inflammatory phenotype, or other pathophysiological consequences, which can be ameliorated by the activation of reelin receptors (Baitsch et al

2011). In support of this and of the role of inflammation in depression, cytokine concentrations are abnormal in mice that lack reelin (Green-Johnson et al 1995) and anti-inflammatory drugs with antidepressant actions that cannot access the brain from the periphery can increase hippocampal reelin levels (Brymer et al 2018).

It is unknown if reelin can cross the blood-brain barrier, which would have important therapeutic implications, but we have detected reelin-immunoreactivity in some putative transcytosis vesicles in hippocampal stratum-lacunosum brain capillaries (Perez-Costas et al 2015). This represents a possible receptor-mediated mechanism whereby reelin could be transported from the periphery to the brain and vice versa. In any case, we suspect that normalizing SERT MPC would contribute to the alleviation of stress-induced impairments in 5-HTergic and inflammatory signaling. This is because SERT clustering parameters in lipid raft microdomains influences SERT functionality, although the regulatory role of neurotransmitter transporters in the immune system is still unclear (Magnani et al 2004, Robson et al 2017). Interestingly, a group of researchers have provided evidence that antidepressants not only modulate a redistribution of Gα protein in membranous lipid raft domains but that this modulation is critical for G-protein activity and is required for the behavioral changes that antidepressants generate (Allen et al 2007, Czysz et al 2015, Donati & Rasenick 2005, Erb et al 2016, Zhang & Rasenick 2010). Our results are perhaps somewhat unsurprising considering that reelin regulates the clustering of its own receptors (Strasser et al 2004), and has been shown to regulate MPC in synaptosomes (Caruncho et al 2004) and the surface trafficking of glutamatergic NMDARs which are critical for neuroplasticity (Abraham et al 2019, Groc et al 2007). Whether i.v. reelin can rescue CORT-induced alterations in the clustering of other proteins remains unexplored (Romay-Tallon et al 2018).

Our previous work suggests that the restoration of reelin in the SGZ is an important event in the reversal of behavioral deficits (Brymer et al 2018, Johnston et al 2020, Lussier et al 2013a). Here I found that a single i.v. reelin injection partially recovered the number of reelin-IR cells in the SGZ, 3µg recovering cell counts by 69% in males and 79% in females. Interestingly, it was shown recently that the antidepressant-like and synaptic plasticity-enhancing effects of ketamine were abolished when synaptic reelin signaling was interrupted in mice (Kim et al 2021). This could be due to the role that reelin plays in maintaining NMDAR function (Chen et al 2005, Isosaka et al 2006), but reelin is also known to increase the stimulation of AMPARs (Qiu & Weeber 2007, Qiu et al 2006b) which appears to be a requisite trigger for the sustained antidepressant activity of ketamine (Koike & Chaki 2014, Thompson et al 2015, Zanos et al

2016). This appears to also be true for reelin, given that AMPAR antagonist CNQX abolished the fast-acting antidepressant-like effects of intrahippocampally administered reelin (Brymer et al 2020). Therefore, I evaluated the effects of 3µg (the dose that significantly attenuated immobility) on GluA1-IR cell number in the SGZ and found that the CORT-induced decrease was fully recovered by a surprising 159% in males and partially by 60% in females. This finding strongly corroborates the notion that a dramatic increase in glutamatergic neurotransmission underlies, at least to some extent, the behavioral responsiveness to fast-acting antidepressants like ketamine (Koike & Chaki 2014), and could indicate that sex differences in responsivity are related to AMPARs. This seems to also explain why $G_i$-coupled mGluR2/3 antagonists, like LY41495, produce rapid and persistent antidepressant-like effects in rats that are exposed to unpredictable stressors (Dwyer et al 2013, Seo et al 2020, Chu & Hablitz 2000).

Another shared attribute of fast-acting antidepressant compounds that target different receptor families, like reelin, ketamine, LY41495, and psychedelics, is that their increases in neuronal excitability lead to a potentiation of mTOR activity (Brymer et al 2020, Cavalleri et al 2018, Johnston et al 2020, Jossin & Goffinet 2007, Ly et al 2018, Seo et al 2020). Stimulating mTOR assumably augments the insertion of AMPARs into synaptic membranes (Zanos & Gould 2018) and has been identified as a critical mediator of ketamine-induced neuroplasticity and antidepressant responsiveness in animal models (Holubova et al 2016, Li et al 2010). The fact that rapid-acting antidepressants ameliorate the dysfunctional cortico-limbic circuitry that makes one susceptible to MDD supports the neuroplasticity hypothesis of depression (Brymer et al 2020, Kadriu et al 2020b, Thompson et al 2015).

We recently published a report that discussed the possibility that reelin may lead to positive behavioral changes by dampening the occurrence of BAX-mediated mitochondrial-driven apoptosis by activating the mTOR-stimulating PI3K/Akt pathway (Allen et al 2021a). This is because Akt inhibits BAX from forming pores in the outer mitochondrial membrane which would instigate the release of mitochondrial CytC into the cytosol, where CytC can facilitate the formation of executioner caspase-activating apoptosomes (Allen et al 2021a, Chang et al 2003, Jossin & Goffinet 2007, Takino et al 2019). Apoptosis is exacerbated by chronic stress by 1) downregulating glucocorticoid receptors (Gądek-Michalska et al 2013) which form complexes with BAX-inhibiting proteins like Bcl-2 before translocating to the mitochondria in response to CORT (Du et al 2009a), and 2) increasing and decreasing the expression of BAX and Bcl-2, respectively (Juárez-Rojas et al 2015, Xu et al 2019, Zhang et al 2019b). Therefore,

I expected that CORT-exposed rats would express higher levels of BAX and CytC-immunoreactivity and hypothesized that i.v. reelin would, at least partially, lower their expression levels. Indeed, CORT elevated BAX immunoreactivity in the DG which proved significant in the GCL, but a single 3µg reelin injection only lowered expression levels by 17%. CytC-immunoreactivity in the GCL and ML was also heightened by CORT, as well as the number of CytC-positive cells in the proliferative SGZ, and reelin lowered expression levels of CytC in these areas by 43%, 60%, and 87%, respectively.

Given that reelin had little effect on the expression of BAX in these conditions, reelin may normalize CytC-IR cell numbers via different mechanisms, like reducing oxidative events. Reelin sometimes co-expresses with NOS within the hippocampus, for example, and we found that reelin-deficient mice have a lower number of cells that co-express both markers in the DG (Romay-Tallon et al 2010). Curiously, CORT decreases and increases this co-expression in wild-type and *RELN*$^{+/-}$ mice, respectively (Romay-Tallon et al 2015). These findings suggest that stress is more likely to induce apoptosis when reelin levels are low, considering that oxidative stress can promote cell death by increasing the opening probability of the mitochondrial permeability transition pore and by damaging cellular components (Brookes et al 2000, Pizzino et al 2017, Rada & Leto 2008). Another possible mechanism whereby reelin promotes neuroprotection is by restoring the balance between inhibitory and excitatory neurotransmission and reducing NMDAR-mediated excitotoxicity (Brymer et al 2020, Isosaka et al 2006, Qiu & Weeber 2007). Excessive glutamate-triggered cellular damage accelerates oxidative stress, inflammation, and apoptosis, which can be attenuated by activating the PI3K/Akt/mTOR pathway (Nguyen et al 2011, Nowak et al 2019, Sun et al 2019).

Considering that we previously demonstrated AMPAR antagonist CNQX administration precludes the behavioral effects of intrahippocampal reelin without interfering with reelin's effect on neurogenesis (Brymer et al 2020), and that multiple dosages of reelin had little effect on DCX-IR cells in Chapters 2 and 3, I did not expect a single injection of reelin to rescue stress-induced impairments in new-born DCX-IR cells. Indeed, 3µg of reelin partially recovered DCX-IR cell counts in females (45%) but not in males (13%), and dendritic complexity in males but not in females. Findings regarding the role of neurogenesis in depression and antidepressant responsiveness are contradictory, some suggesting that augmenting neurogenesis is necessary for antidepressant behavioral changes (Eliwa et al 2021, Hill et al 2015, Perera et al 2011, Santarelli et al 2003) while others suggest that neurogenesis is of little importance to stress-related behavior and its reversal (Bessa et al 2009, Hanson et al

2011, Surget et al 2008). According to our data, neurogenesis may be relevant for some symptoms of depression, like anhedonia, but an acceleration of neurogenesis and dendritic arborization of dentate granule cells is not likely to explain the quick behavioral changes that reelin can produce.

Taken together, I show that i.v. reelin can attenuate depressive-like behavior by targeting a combination of peripheral and central mechanisms. Further experimentation should elucidate how recombinant reelin influences 1) the MPC of a panel of proteins that are dysregulated by stress, such as reelin's receptors or other neurotransmitter transporters/receptors, and 2) additional markers of apoptosis and oxidative stress. It would also be extremely valuable to define reelin's pharmacokinetic and pharmacodynamic properties and identify promising drug targets along the reelin signaling pathway, which would have substantial clinical implications.

# Chapter 5

## General discussion

## 5.1 Summary of main findings

The central goal of this dissertation was to elucidate whether peripheral intravenous recombinant reelin administration has antidepressant-like properties in the repeated-CORT paradigm of chronic stress. To our knowledge, no other laboratory has utilized i.v. reelin injections as a novel treatment approach in models that are relevant for psychiatric illnesses, or any condition for that matter. Reelin was injected into the lateral tail vein which provided a less invasive methodology to normalize behavioral deficits than intrahippocampal infusions of reelin, which did do so in a rapid manner (Brymer et al 2020).

**Chapter 2**: I first evaluated the effects of multiple dosages of reelin (3 or 5µg given every 5 or 10 days) that were administered to male rats over a three-week period of daily CORT injections. It seemed logical to start with male rats to investigate the antidepressant-like properties of reelin considering that their behavior is thought to be less variable than females who experience hormonal fluctuations associated with the estrous cycle, and we had previously found reelin to be effective in males after intrahippocampal infusions (Brymer et al 2020). I used the FST as a screening tool for antidepressant efficacy since compounds with such activity in MDD patients consistently increase the active coping strategy that stressed rats adopt when subject to inescapable forced swimming (Fenton et al 2015, Hibicke et al 2020, Shepard et al 2018). I found that all but one of the dosages of reelin significantly recovered CORT-induced increases in FST-immobility. As well, i.v. reelin rescued the expression of endogenous reelin in the SGZ which may be critical for the attenuation of behavioral deficits (Brymer et al 2018, Kim et al 2021, Lussier et al 2009, 2013a). I wondered whether the normalization of reelin in the SGZ would enhance adult-born cell proliferation and the dendritic complexity of these immature neurons, but reelin had little effect on neurogenic processes as measured by DCX-IR cells. I found that i.v. reelin did normalize MPC parameters for SERT in blood lymphocytes, which may provide an explanation as to how reelin given peripherally can influence neuronal processes. Considering that all dosages of reelin improved behavioral deficits and only the lowest dosage (3µg every 10 days) rescued reelin expression in the stress regulating PVN, we chose this dosage to evaluate the effects of reelin in females.

**Chapter 3**: I went on to show that deficits in the FST produced by CORT can be rescued by reelin similarly in both males and females. CORT also impaired cognition as ascertained by the OBL (though not significantly), and less so when they were treated with reelin. I confirmed that, like in males, neurogenesis is unlikely to be responsible for the behavioral improvements

observed in these conditions in the female rats. Therefore, I decided to investigate the effects of peripheral reelin on glutamatergic and GABAergic neurotransmission since imbalances in excitatory and inhibitory signaling lead to emotional dysregulation (Brymer et al 2020, Caruncho et al 2004, Krystal et al 2002, Lussier et al 2013b, Thompson et al 2015). As expected, CORT downregulated the expression of $GABA_A$ and AMPA receptors, and increased GluN2B immunoreactivity, but not when the rats were also given i.v. reelin. This corroborates the notion that reelin strengthens hippocampal circuitry (Brymer et al 2020, Caruncho et al 2016), and that modulating excitatory/inhibitory receptors can generate behavioral improvements (Ghosal et al 2020, Kadriu et al 2020b, Pham & Gardier 2019, Seo et al 2020).

**Chapter 4**: I next evaluated if the effects of i.v. reelin are produced in a rapid manner. First, male rats were given a single dose of reelin (either 0.5, 1, 3, 5, 7 or 9µg) after three weeks of CORT, 24 hours before they were placed into the FST. I found that 3µg significantly attenuated FST-immobility, and so I tested this dose in female rats, in whom it also produced quick antidepressant-like effects. Furthermore, cognitive impairments induced by CORT were also improved by reelin. Indeed, the behavioral recoveries in response to reelin were paralleled by increases in SGZ-reelin but occurred independently to enhancements of neurogenesis. Surprisingly, I found that the increase in GluA1-containing AMPARs by reelin in males was two-fold higher after a single injection of reelin compared to when reelin was given every 10 days, but this was not true for females in whom a single reelin injection was weaker than multiple but nevertheless partially recovered GluA1 expression. I was also motivated to see if peripheral reelin could dampen apoptosis in the DG. This is because chronic stress potentiates cell death and reelin activates downstream effectors that signal to arrest pro-apoptotic factors, like BAX, which instigates the release of CytC from mitochondria (Allen et al 2021b). Generally, CORT heightened the expression of BAX and CytC which was dampened by reelin. Considering that a single injection of reelin recovered SERT MPC in lymphocytes partially in males and fully in females, it could be that reelin influences neuronal dynamics by regulating inflammation. After all, activation of reelin receptor ApoER2 induces an anti-inflammatory phenotype in immune cells (Baitsch et al 2011) and inhibiting pro-inflammatory cytokines can increase hippocampal reelin expression (Brymer et al 2018).

Overall, the experiments outlined in this dissertation open the possibility of developing reelin-based therapeutics that could have much faster antidepressant actions than conventional medications. That said, many mechanistic studies are required to further understand how

peripheral reelin orchestrates behavioral and neurochemical responses. The time-course of reelin's actions should also be evaluated further.

### 5.2 I.v. reelin rescues CORT-induced FST-immobility and cognitive deficits

A repeated-CORT injection paradigm is widely used to study the effects of chronic stress on the brain and how compounds can reverse stress-induced alterations that resemble the human condition. Laboratories have shown that several weeks of daily CORT injections (typically between 10-40mg/kg) reliably compromises behavior dose- and time-dependently (Lebedeva 2017, 2020, Lussier et al 2013a). For example, CORT decreases active behaviors in the FST and the preference for sucrose, which is often interpreted as despair- and anhedonia-like behavior, respectively (Brotto et al 2001, Gregus et al 2005, Hill et al 2003, Johnson et al 2006, Kalynchuk et al 2004, Marks et al 2009). Researchers have confidence in this considering that exposing rodents to unpredictable stressors, which elevates plasma CORT levels, also generates similar behavioral disturbances (Culig et al 2017, Monteiro et al 2015, Sequeira-Cordero et al 2019, Willner et al 1987), and the majority of MDD patients have higher levels of cortisol, the human analogue of CORT (Dienes et al 2013, Nandam et al 2020). A decrease in active FST-behavior also occurs independent of changes in muscle tone, which suggests that immobility likely reflects a state of altered mood rather than muscle weakening (Marks et al 2009).

The studies outlined in this dissertation also demonstrate that several weeks of CORT reliably increases FST-immobility, and repeated reelin or a single 3µg injection given 24 hours before the FST significantly reduced immobility behavior. This indicates that the antidepressant-like effects of reelin have a relatively quick onset. These results are in accordance with the literature, considering that: intrahippocampal reelin infusions rapidly rescues FST-immobility (Brymer et al 2020); mice overexpressing reelin are protected from the depressogenic effects of stress, while reelin-deficient mice are more vulnerable (Lussier et al 2011, Teixeira et al 2011); various antidepressants with different mechanisms of action can rescue immobility and reelin expression in the SGZ (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020); and proper reelin signaling may underlie some of the antidepressant actions of ketamine (Kim et al 2021). It would be valuable to compare the effects of reelin alongside other fast-acting antidepressants and determine for how long a single injection of reelin normalizes FST-behaviors, especially since a single injection of reelin had a similar effect on FST-immobility

as multiple injections in males and even more of an effect than multiple injections in females (by 29%). This, and the U-shaped dose-response curve (see Figure 4.4.2A), suggests that the concentration of reelin can be optimized. In other words, greater therapeutic effects are achievable if reelin levels are maintained within a target range that promotes homeostasis in the neuronal environment. Of course, U-shaped curves are common in biology and pharmacology (Laham & Gould, 2022).

Cognitive deficits are also commonly reported in those with depression when evaluated by means that require the hippocampus and PFC, and they are more severe in those with smaller hippocampal volumes (Chung et al 2016, Frodl et al 2006, Kronmüller et al 2009, Richardson & Adams 2018, Rose & Ebmeier 2006). Stress also consistently impairs learning and memory in rodents which is associated with neuronal atrophy in these brain regions (Conrad et al 1996, Diamond et al 1999, Goodman & McIntyre 2017, Kleen et al 2006, Mika et al 2012, Mizoguchi et al 2000). Similarly, chronic CORT treatment disrupts normal object-recognition memory which is associated with neuroplastic insults in the DG (Brymer et al 2018, 2020). The DG, which expresses less reelin in response to stress (Fenton et al 2015), is thought to be involved in pattern separation, or the ability to discriminate between experiences that are highly similar (like the location of your car in the parking lot today vs yesterday) (Ngo et al 2021). Therefore, we aimed to model deficits in cognition induced by stress by evaluating the ability of rodents to recognize novelty in environments that are similar, because they have an innate preference to explore novelty. We had reason to believe that i.v. reelin would counteract the effects of stress since reelin infusions into the brain enhances learning and memory in various rodent models (Brymer et al 2020, Hethorn et al 2015, Rogers et al 2011, 2013). Using the hippocampal-dependent OBL, I found that CORT reduced the amount of time the rats spent exploring an object that was placed in a novel location over an identical object in a familiar location (though not significantly), and multiple dosages of reelin normalized object recognition in both sexes. I later found that a single injection of i.v. reelin partially recovered the significant reduction in novel object exploration generated by CORT in the OBIP task. A small subset of animals was also subjected to a pilot Y-maze test to assess cognition and working memory after CORT and a single injection of reelin (see Appendix). However, based on the data (that lacks statistical robustness) one is unable to make any meaningful interpretations. Our results generally attest to the face and predictive validity of the CORT model and the FST as a screening tool for antidepressant efficacy.

### 5.3 I.v. reelin may influence central processes by regulating SERT MPC

The functions of reelin in the periphery are much less studied than in the brain, but it is thought to influence the development, sustentation, and disease of multiple non-neuronal tissues (Khialeeva & Carpenter 2017). Reelin levels are abnormal in blood plasma from depression, bipolar, and schizophrenia patients (Fatemi et al 2001), where reelin maintains homeostasis and inflammatory responses (Tseng et al 2010, 2014). For instance, mice that are haplo-insufficient for reelin express altered levels of cytokines (Green-Johnson et al 1995) and abnormal MPC in blood lymphocytes (Rivera-Baltanas et al 2010). In fact, we found that the increases in SERT cluster size in lymphocyte membranes of *RELN*$^{+/-}$ mice are also observed in rats that have been treated with CORT and in patients with depression (Rivera-Baltanas et al 2010, 2012, Romay-Tallon et al 2018). Furthermore, improvements in depression scores correspond with the normalization of SERT MPC (Caruncho et al 2019, Rivera-Baltanas et al 2015). CORT also alters the clustering parameters of other important receptors and transporters in lymphocytes, such as the 5-$HT_{2A}$R, which is paralleled in MDD patients and can be used as a putative biomarker of therapeutic efficacy, just like SERT (Rivera-Baltanas et al 2014, Romay-Tallon et al 2018).

We thought that i.v. reelin would normalize the CORT-induced increases in SERT cluster size in lymphocytes, because they express reelin receptors (Suzuki et al 2008); reelin has been shown to regulate the trafficking and clustering of membranous proteins in lipid rafts (Abraham et al 2019, Caruncho et al 2004, Groc et al 2007, Strasser et al 2004); and recombinant reelin reduced SERT cluster size in lymphocytes in vitro (Johnston et al 2020). Indeed, I found that CORT increased the size of SERT clusters in males, and this was normalized by all four dosages of reelin that were tested (3/5µg every 5/10 days). In fact, the size of SERT clusters was negatively correlated with immobility for the CORT/reelin-treated rats. In females, even a single injection of reelin fully rescued (136%) the size of SERT clusters, but the same 3µg dose produced only a partial recovery in males (66%). There was also a correlation between SERT cluster size and the number of reelin-IR cells after a single reelin injection. SERTs are the primary pharmacological target of conventional antidepressants, and their clustering parameters in lipid microdomains provides a mechanism to regulate 5-HT reuptake activity (Magnani et al 2004). 5-HTergic signaling appears to regulate certain immune cell functions, like the activation of T lymphocytes and the secretion of inflammatory mediators by neutrophils (León-Ponte et al 2007, Maes et al 2012), but the roles of neurotransmitters and their transporters/receptors in the immune system are generally unclear (Robson et al 2017).

Nevertheless, mounting evidence seems to suggest that the normal clustering of proteins in membranes is critical for cellular activities. For instance, a group of researchers in Chicago found that antidepressants modulate a redistribution of Gα protein in lipid rafts and argue that it is critical for G-protein activity and the responsiveness to antidepressants (Allen et al 2007, Czysz et al 2015, Donati & Rasenick 2005, Erb et al 2016, Zhang & Rasenick 2010). It would be interesting to compare the effects of conventional antidepressants and reelin on SERT MPC, especially since we previously found that ketamine had the opposite effect of reelin in lymphocytes that were extracted from CORT-treated rats (Johnston et al 2020).

Inflammatory events are a central component in many depressions (Miller et al 2009a, Sperner-Unterweger et al 2014), and cytokines remain one of the best characterized biomarkers for the disorder (Lichtblau et al 2013, Valkanova et al 2013). It is likely that abnormalities in MPC disrupt the normal functioning of lymphocytes, and other immune cells, which could potentiate inflammation. Interestingly, the activation of reelin's receptors has been shown to induce an anti-inflammatory phenotype in other cell types, like macrophages (Baitsch et al 2011). Curiously, the anti-inflammatory drug etanercept, which inhibits TNF-α and reduces FST-immobility, also increases hippocampal reelin without crossing the blood-brain barrier (Brymer et al 2018). Therefore, modulating the activity of immune cells could explain how recombinant reelin protects from a decrease in SGZ-reelin (and other neurochemical alterations), but it likely does not explain reelin's fast-acting antidepressant-like effects considering that patients often take anti-inflammatory agents, but they do not rapidly eliminate depressive symptoms (Iyengar et al 2013, Tyring et al 2006).

The fact that chronic CORT produces alterations in MPC that are matched in human patients provides face validity for the CORT model beyond behavioral and neurochemical alterations and suggests that it could be used as a screening tool for biomarkers (in a bench-to-bedside-back-to bench manner). Potentially, one could eventually develop a panel of biomarkers that could be used to inform the course of clinical treatment and steer outcomes for individual patients. One wonders whether i.v. reelin can rescue the CORT-induced abnormalities in MPC for other important receptors and transporters, such as the DAT, 5-$HT_{2A}$R, β2AR, and GluN2B (Romay-Tallon et al 2018). The link between stress, inflammation, and depression infers that lymphoid organs like the spleen and thymus are implicated in mood disorders (Brymer et al 2019, Maydych 2019). For this reason, our laboratory has been investigating the effects of CORT and reelin on white pulp atrophy in the spleen, and our preliminary results indicate that multiple or singular injections of reelin counteract the CORT-induced decreases in the white

pulp area, which is completely made up of lymphoid tissue. We have only started to scratch the surface of the complex processes that reelin governs in the periphery, and how relevant they are in depression.

### 5.4 CORT-induced decreases in SGZ-reelin are recovered by i.v. reelin

Reelin levels are decreased in the hippocampus of depressed patients (Fatemi 2000, 2011) and CORT-treated rats alike (by about 25%), with longer periods of CORT exposure correlating with larger reductions in reelin-positive cells in the SGZ (Lebedeva et al 2020, Lussier et al 2013a). In addition, ketamine, imipramine, and etanercept, which normalize FST-immobility, protect from decreases in reelin induced by CORT (Brymer et al 2018, Fenton et al 2015, Johnston et al 2020). Similarly, citalopram was shown to counteract the downregulation of hippocampal reelin that was induced by the administration of neurodegenerative kainic acid (Jaako et al 2011). This, as well as the fact that low levels of reelin represent a putative vulnerability factor to the depressogenic effects of stress (Lussier et al 2011, Notaras et al 2020, Schroeder et al 2015), suggests that hippocampal reelin is involved in depression-like behavior.

In this series of experiments, I first evaluated whether multiple dosages of i.v. reelin in male rats could rescue the decreases in reelin that are generated by CORT in the proliferative SGZ. I found that the average percentage of recovery was 72%, but that it reached up to 90% after 5µg was given every 5 days. I then used the lowest dosage that showed to be effective in males (3µg every 10 days) to ascertain how treatment influenced endogenous reelin in females. I found that males and females had similar percentage recoveries that averaged at 76%. Moreover, when all rats were included in the analyses, SGZ-reelin expression was correlated negatively with FST-immobility, and positively with sucrose preference and object DR scores. Afterwards, I evaluated whether a single dose of i.v. reelin in males could counteract the effects of stress on hippocampal reelin and found that partial recoveries were generated. To be more specific, the lower (0.5/1µg) and higher (5/7/9µg) doses of reelin recovered reelin expression by 30-40% and 24-38%, respectively, while the middle 3µg dose (which produced the greatest behavioral recoveries) increased reelin expression by 69%. Therefore, I assessed how 3µg of reelin effected SGZ-reelin in females and found that the CORT-induced reductions were recovered by 79%.

Our results indicate that, like drugs with antidepressant actions, peripheral reelin can increase the number of reelin-expressing cells in the SGZ, even after a single dose. It seems plausible that the restoration of hippocampal reelin is required for behavioral changes. Indeed, the deletion of reelin, reelin receptor ApoER2, or the pharmacological inhibition of their downstream effectors, disrupted the effects driven by ketamine on behavior and synaptic plasticity in the mouse hippocampal CA1 region (Kim et al 2021). This suggests that synaptic reelin signaling may underlie the responsiveness to antidepressant compounds, and there are a variety of possible mechanisms that could explain such a phenomenon. For example, proper reelin signaling might be a key requirement for the antidepressant actions of ketamine due to its regulatory roles over NMDAR subunit composition, trafficking, and activity (Chen et al 2005, Groc et al 2007, Sinagra et al 2005). Reelin also regulates several forms of neuroplasticity, like new-born cell proliferation, dendritic outgrowth, and the formation of dendritic spines and synaptic connections (Beffert et al 2006, Bosch et al 2016a, Niu et al 2004, Niu et al 2008, Pujadas et al 2010, Rogers et al 2013, Teixeira et al 2012, Ventruti et al 2011, Weeber et al 2002). Therefore, a strengthening of hippocampal circuitry follows reelin supplementation and counteracts the depressogenic effects of stress, which has long been known to cause dendritic atrophy in this limbic region (Galea et al 1997, Watanabe et al 1992).

### 5.5 Enhancing neurogenesis is not required for reelin-induced behavioral changes

Human neurogenesis is generally accepted to be a notable phenomenon, which influences learning, memory, and mood (Baptista & Andrade 2018), but neurogenic processes are difficult to analyze in healthy individuals and pathological states for obvious reasons. Post-mortem studies have shown that patients with depression have reduced levels of neurogenesis (Berger et al 2020, Boldrini et al 2012) and that antidepressants can enhance neurogenesis (Chen et al 2001), but these findings are not always replicated (Reif et al 2006). Rates of neurogenesis are also significantly dampened in rodent models for stress-related disorders (Brummelte & Galea 2010b, Brymer et al 2018, Culig et al 2017, Lussier et al 2013a, Sliwowska et al 2010), and antidepressants can rescue these neurogenic deficiencies (Fenton et al 2015) which some argue is integral for therapeutic responsiveness (Perera et al 2011). In line with this, disrupting neurogenesis in mice has been shown to eliminate the behavioral changes driven by antidepressants (Santarelli et al 2003), and the augmentation of neurogenesis by the transgenic inhibition of hippocampal BAX reduces anxiety- and depressive-relevant behaviors (Hill et al

2015). On the other hand, antidepressants have also been shown to normalize behavioral impairments without influencing neurogenesis (Hanson et al 2011), and have been shown to do so even when cytostatic agents are used to arrest neurogenesis (Bessa et al 2009). Additionally, eliminating neurogenesis in mice did not make them more susceptible to the depressogenic effects of unpredictable stress, inferring that deficiencies in neurogenesis do not cause stress-induced behavior (Surget et al 2008). The contradictory findings surrounding the role of neurogenesis in psychiatric disorders begs the question as to whether i.v. reelin augments the integration of adult-born cells into existing hippocampal circuitry after periods of chronic stress.

Reelin is a pleiotropic protein that has been shown to potentiate the dendritic maturation of adult-born cells and the number of synaptic connections in the hippocampus in some conditions (Bosch et al 2016a, Won et al 2006). This has been demonstrated using transgenic mice that overexpress reelin (Pujadas et al 2010) and by inactivating the reelin signaling pathway which impairs neurogenesis (Teixeira et al 2012). We have contributed to this research by showing that the downregulation of reelin in the SGZ is paralleled by decreases in neurogenesis (as measured by DCX-IR cells) and active behaviors in the FST (Lussier et al 2013a), and that neurogenesis can be heightened by intrahippocampal infusions of reelin (Brymer et al 2020). Here, I evaluated the effects of i.v. reelin on DCX-IR new-born cell counts and the dendritic maturation of surviving cells. I tested multiple dosages of reelin in male rats that were treated with CORT and found that it was not effective in rescuing the DCX-IR cell counts or the dendritic complexity of new-born cells (recovery ranges from 17% to 37%). Considering that there are sex differences in depression, neurogenesis, and how stress perturbs neurogenesis (Yagi & Galea 2019, Yagi et al 2020, Yang et al 2017), I investigated how 3µg of reelin given every 10 days effected DCX-IR cells in females, and found that they responded to CORT and reelin in a similar manner than that of males. Despite this, I was motivated to learn whether a single injection of reelin could normalize neurogenic processes, but this did not turn out to be the case.

Our results highlight the unlikelihood that decreases in FST-immobility driven by reelin are due to an enhancement of neurogenesis. That said, this statement could be made with more confidence if one were to carry out additional experiments in which reelin is administered after some sort of neurogenic manipulation. If neurogenesis contributes to the reelin-induced behavioral changes, then eliminating neurogenesis should logically dampen the antidepressant-like actions of reelin. One wonders whether other doses of reelin or administration routes would

facilitate neurogenesis; if the blood-brain barrier is obstructing peripheral reelin from instigating central actions directly; and if reelin would potentiate neurogenesis in other models for depression? One must keep in mind that normal plasma levels of CORT when subject to physiological stress are around 3-5mg/kg (Sandi et al 1996), implying that peripheral reelin may very well rescue neurogenesis in other models for depression (like rodents that express putative genetic vulnerabilities (Overstreet et al 2005) or are exposed to CUMS). It is true that we are giving exceedingly high levels of CORT (40mg/kg per day for three weeks); this is because we aimed to reliably induce FST-immobility to evaluate the antidepressant-like actions of i.v. reelin, instead of relying on the animals natural but more variable response to stressors (Willner 2005). If reelin cannot access the brain from the periphery, then behavioral changes must initially be driven by peripheral actions that would inherently take longer to correct neurogenesis than intrahippocampal infusions (Brymer et al 2020).

Overall, neurogenesis does not seem to be responsible for the antidepressant-like properties of peripheral reelin. Similarly, we previously demonstrated that the antidepressant-like effects of intrahippocampal reelin infusions could be blocked by AMPAR antagonist CNQX, without interfering with neurogenesis (Brymer et al 2020). This provides further evidence that the rapid behavioral changes orchestrated by reelin are independent of neurogenesis, and that AMPARs play an important role, which is also true for rapid antidepressant ketamine (Koike & Chaki 2014, Zhang et al 2016).

### 5.6 The importance of reelin, glutamate, and GABA in depression

Over the last couple of decades, modulating glutamatergic neurotransmission has received considerable attention as a novel treatment approach for those with MDD. This is because the widely used anesthetic ketamine, which blocks the channels of NMDARs, was serendipitously discovered to improve depressive symptoms in hours in patients that were resistant to conventional monoaminergic antidepressants (Berman et al 2000). However, not all NMDAR antagonists have antidepressant actions, which suggests that ketamine may ameliorate depression by influencing other molecular mechanisms (Gould et al 2019, Hillhouse & Porter 2014). Preclinical studies have since demonstrated that ketamine gives rise to a dramatic increase in the activity of AMPARs relative to NMDARs, and that AMPARs are required for the behavioral and neurochemical changes that are occasioned by ketamine (Aleksandrova et al 2017, Koike & Chaki 2014, Zhang et al 2016, Zhou et al 2014). Antidepressants like

fluoxetine and imipramine also upregulate and rely on AMPAR-mediated transmission to generate behavioral changes (Aleksandrova et al 2017, Koike & Chaki 2014), which is also true for excitatory transmission-enhancing $G_i$-coupled mGluR antagonists that do not produce ketamine-like side-effects (Aleksandrova et al 2017, Chu & Hablitz 2000, Dwyer et al 2013, Koike & Chaki 2014, Seo et al 2020, Witkin 2020). Supporting this notion, reductions in GluA1 expression are observed in post-mortem tissue from depressed individuals and stressed rodents in several brain regions that are implicated in mood (Beneyto et al 2007, Bonini et al 2016, Duric et al 2013, Toth et al 2008). Dramatic increases in AMPAR activity promotes TrkB and mTOR signaling, which are also required for fast-acting antidepressant actions because they enhance the insertion of additional AMPARs into the synapse and strengthen excitatory transmission (Cavalleri et al 2018, Li et al 2010, Ly et al 2018, Zanos & Gould 2018, Zhou et al 2014).

Our laboratory has not only shown that intrahippocampal infusions of reelin normalize expression levels of GluA1 in the DG, but that antagonizing AMPARs also abolishes reelin's antidepressant-like actions, and that the putative molecular mechanisms of reelin overlap considerably with those of ketamine (Brymer et al 2020, Johnston et al 2020). This was somewhat expected considering that reelin has been shown to enhance AMPAR-mediated transmission (Qiu et al 2006b); reelin-deficient mice express lower levels of GluA1 (Qiu & Weeber 2007); GluA1-deficient mice exhibit depressive-like phenotypes (Chourbaji et al 2008); and like ketamine, reelin upregulates PI3K/Akt/mTOR activity (Jossin & Goffinet 2007). The modulation of AMPARs by reelin and their ability to quickly change behavior provided a rationale to evaluate the effects of peripheral reelin on GluA1 expression. I found that multiple reelin injections recovered the number of GluA1-IR cells in the SGZ similarly in males and females to control levels, and that GluA1 expression correlated negatively with FST-immobility and positively with sucrose preference (when all treatment groups were included in the analysis). This was true even after a single dose of reelin. This is important because ampakines have dose-dependent antidepressant-like effects (Akinfiresoye & Tizabi 2013, Du et al 2006, Li et al 2001, Lindholm et al 2012) but clinical trials with these compounds did not yield promising results (Arai & Kessler 2007, Bernard et al 2019, Fumagalli et al 2012, Mendez-David et al 2017, Nations et al 2012, Su et al 2009); therefore, reelin supplementation offers a propitious approach to normalize glutamatergic signaling and rapidly improve mood, learning, and memory, all of which are heavily dependent on AMPARs and NMDARs (Krugers et al 2010, Sanderson et al 2009, Sanderson et al 2010). In fact, the behavioral and

neurochemical effects of ketamine are absent in mice when synaptic reelin signaling is blocked, which the authors suggest may be, at least partially, explained by the modulation of NMDARs by reelin (Kim et al 2021).

Reelin regulates NMDAR trafficking, subunit composition, and activity (Bosch et al 2016a, Chen et al 2005, Groc et al 2007). The transient activity of these so-called coincidence-detecting receptors is essential for LTP, but their prolonged activation can lead to excitotoxic events due to the excessive influx of calcium (Monaco et al 2015). GluN2B subunits allow greater amounts of calcium to enter the cell than other subunits, and GluN2B but not GluN2A antagonists abolish NMDAR-driven excitotoxicity in hippocampal slices which highlights their involvement in this phenomena (Preskorn et al 2008, Zhou & Baudry 2006). In fact, deleting GluN2B not only mimics the effects of ketamine, but occludes the antidepressant effects of ketamine in mice (Miller et al 2014). It is interesting then, that chronic CORT exposure increases GluN2B expression in the hippocampus, where reelin can be infused to rescue this deficit (Brymer et al 2020, Calabrese et al 2012). The overexpression of reelin was also found to reduce GluN2B expression in the hippocampus and protect from phenotypes relevant for psychiatric disorders (Teixeira et al 2011). In the present study, when reelin was administered via the lateral tail vein, a partial recovery of GluN2B was observed across the DG, and GluN2B immunoreactivity in the GCL correlated negatively with SGZ-reelin-IR cell counts and DR scores in the OBL (when all treatment groups were included in the analyses). As well as having lower levels of AMPARs, patients with MDD have higher levels of glutamate in circulation and in the brain which one can assume increases excitotoxic events, potentiating inflammation and hippocampal cell atrophy (Hashimoto et al 2007, Mitani et al 2006). This idea is supported by the fact that blocking the reuptake of glutamate by astrocytes brings about anhedonia-like behavior and cognitive deficits in rodents (Bechtholt-Gompf et al 2010). There is evidence that stress increases central glutamate levels by decreasing the expression of $GAD_{65/67}$, which converts glutamate into GABA, indicating that stress also dampens GABAergic signaling (Banasr et al 2017, Hasler et al 2010, Karolewicz et al 2010, Lussier et al 2013b, Ma et al 2016, Tripp et al 2012).

Levels of GABA are indeed lower in the blood, CSF, and brains of depressed patients, and central GABA concentrations correlate positively with hippocampal volume and negatively with anhedonia scores (Abdallah et al 2015, Gabbay et al 2012, Gerner & Hare 1981, Godfrey et al 2018, Hasler et al 2007, Petty & Sherman 1984, Song et al 2012). Reelin is expressed by GABAergic interneurons in the adult hippocampus and cortex, and mice that are deficient in

reelin, which have an enhanced sensitivity to the depressogenic effects of stress, also express reductions in GABA-synthesizing enzymes (Guidotti et al 2000, Liu et al 2001, Notaras et al 2020, Pesold et al 1998). There are reports that show environmental and maternal stress impair GABAergic signaling (Caldji et al 2003, Czéh et al 2018, Otero Losada 1988, Shalaby & Kamal 2009), and our laboratory found that CORT reduces the expression of $GABA_A$Rs in the DG SGZ, and that intrahippocampal infusions of reelin can rescue this deficit (Brymer et al 2020, Lussier et al 2013b). Here, I found that i.v. reelin protects from the stress-induced decreases in SGZ-$GABA_A$Rs similarly in males and females, and that $GABA_A$R-IR cell counts correlated positively with reelin-IR cell counts and sucrose preference, and negatively with FST-immobility and GluN2B immunoreactivity in the GCL (when all treatment groups were included in the analyses). Thus, reelin may augment synapto- and dendrito-genesis and protect from stress by maintaining a neuroprotective environment in which appropriate levels of excitatory and inhibitory transmission minimize excitotoxicity and promote effective signal transduction (Beffert et al 2006, Caruncho et al 2004, Lussier et al 2013b, Niu et al 2008, Rogers et al 2013, Teixeira et al 2012, Weeber et al 2002).

Other compounds with antidepressant actions, like ketamine, also enhance $GABA_A$R activity in the cortex and hippocampus (Wang et al 2017a) and such compounds may rely on $GABA_B$Rs (Rosa et al 2016). Indeed, meta-analyses indicate that GABAergic-enhancing drugs, which are usually used to treat anxiety, also have antidepressant effects that are similar to MAOIs and TCAs (Birkenhäger et al 1995, van Marwijk et al 2012). Interestingly, the neuroactive steroid allopregnanolone – which positively allosterically modulates $GABA_A$Rs – was recently FDA approved for the treatment of post-partum depression (Frieder et al 2019, Wilkinson & Sanacora 2019). The enhancement of GABAergic neurotransmission by reelin could dampen stress-induced phenotypes that are relevant for depression by strengthening the hippocampal connections that inhibit the HPA-axis (Hewitt et al 2009). However, it is clear that both excitatory and inhibitory signaling must be optimized because $GABA_A$R inverse agonists increase neuronal excitability and enhance cognitive ability and learning and memory in animal models (Braudeau et al 2011, Kemp et al 1987, Milić et al 2013). Compounds that negatively allosterically modulate $GABA_A$Rs that contain α5-subunits also produce fast-acting antidepressant-like effects in rodents by enhancing excitatory synaptic strength in the hippocampus with fewer side-effects than ketamine (Fischell et al 2015). Considering that ketamine is psychotomimetic and has a high potential for abuse (Wang et al 2013a), it is necessary to develop novel compounds that modulate excitatory/inhibitory neurotransmission

without antagonizing NMDARs, which would provide a more viable approach to treat depression without the need for supervision. The GluN2B-decreasing and GluA1- and $GABA_AR$-enhancing effects of reelin in these conditions suggest that reelin-based therapeutics would be promising candidate novel fast-acting antidepressants that are free from ketamine-like adverse side-effects.

### 5.7 The importance of reelin and apoptosis in depression

Stress, inflammation, and excitotoxicity are well-documented apoptosis-triggering phenomena that are strongly associated with depression (Allen et al 2018, Dong et al 2009, Fulda et al 2010, Haanen & Vermes 1995). This is evident in that ⅔ of patients with MDD experience hypercortisolemia and have elevated circulating levels of pro-inflammatory cytokines (Brymer et al 2019, Carroll et al 2007, Gądek-Michalska et al 2013). Excessive activation of the HPA-axis causes a downregulation of GRs, which can influence programmed cell death because GRs form complexes with Bcl-2, an anti-apoptotic protein that translocates to the mitochondria and inhibits pro-apoptotic protein BAX from piercing outer mitochondrial membranes (Du et al 2009a). If BAX succeeds in permeabilizing the membrane, calcium and CytC are released which can elevate excitotoxicity and initiate a series of events that lead to cell death; CytC binds to Apaf-1 which facilitates the formation of caspase-9-activating apoptosomes (Allen et al 2021a, McArthur & Kile 2018). Other components of the mitochondria can also escape, like mitochondrial DNA, which is inflammatory (Allen et al 2021a). Chronic stress further potentiates apoptosis by increasing the expression of BAX and decreasing the expression of Bcl-2 (Juárez-Rojas et al 2015, Xu et al 2019, Zhang et al 2019b).

Interestingly, the activity of BAX is hindered by the PI3K/Akt/mTOR pathway which is stimulated downstream of reelin and other compounds with fast-acting antidepressant properties like ketamine, mGluR2/3 antagonists, and 5-HTergic psychedelics (Brymer et al 2020, Johnston et al 2020, Jossin & Goffinet 2007, Kale et al 2018, Li et al 2010, Ly et al 2018, Seo et al 2020, Takino et al 2019). This suggests that dampening programmed cell death could contribute to the behavioral changes that are induced by these drugs, and protect from the volumetric reductions that are observed in MDD patients (Chung et al 2016, Frodl et al 2006, Kronmüller et al 2009, Richardson & Adams 2018, Rose & Ebmeier 2006). This is supported by the fact that transgenic mice that lack *BAX* in neural stem cells are less susceptible to the depressogenic effects of CUMS and CORT treatment (Eliwa et al 2021, Hill et al 2015) and,

on the other hand, Bcl-2-overexpressing mice have much more DCX-IR cells than wild-type mice (Kuhn et al 2005). Therefore, I was motivated to evaluate the effects of peripheral i.v. reelin on the expression of BAX and CytC in the DG. As expected, CORT-treated rats had higher levels of BAX expression in the DG, which was significant in the GCL, but 3µg of reelin only lowered GCL-BAX immunoreactivity by 17%. CORT significantly increased CytC-immunoreactivity in the GCL and ML, and CytC-IR cell counts in the SGZ of the DG, and reelin produced partial and full recoveries of 43%, 60%, and 87%, respectively.

Considering that reelin had modest effects on BAX expression in these conditions, the normalization of CytC-IR cell counts in the SGZ suggests that reelin may decrease apoptosis by regulating other processes. For instance, our laboratory has previously reported that reelin-nNOS co-expressing cells are lower in the DG of reelin-deficient mice, but stress increases this co-expression in those will low reelin levels, whereas stress had the opposite effects in wild-type mice (Romay-Tallon et al 2010, 2015). Oxidative stress can also permeabilize mitochondrial membranes by opening the mitochondrial permeability transition pore and by damaging cellular components (Brookes et al 2000, Pizzino et al 2017, Rada & Leto 2008). Therefore, oxidative stress hinders the production of ATP, which would dramatically impair energy-demanding neuronal functions like signal transduction and the synthesis and delivery of proteins (Allen et al 2021a).

### 5.8 Sex differences in response to CORT and reelin

Lifetime prevalence rates of depression are 20% to 25% for women and 7% to 12% for men (WHO 2002), yet the majority of preclinical research excludes females from experimentation because their behavior is thought to be more variable due to the fluctuation of estrous hormones. Sexual dimorphisms also exist in symptom reporting, treatment responsiveness, suicide rates, and stress reactivity (Goel & Bale 2010, Khan et al 2005, Marcus et al 2008, Park et al 2015b). However, preclinical animal study findings are often divergent from the clinical literature. For instance, female rats often spend less time immobile in the FST than males after periods of CORT exposure (Brotto et al 2001), which raises the issue of extrapolating preclinical findings and the validity of behavioral tests that cannot truly model certain aspects of psychiatric disturbance. We have also found that female CORT-treated rats are more active than males in the FST, and that they are less defensive than males in response to CORT in a predator odor test (Kalynchuk et al 2004). It could be that estrogen and progesterone are

protective from stress in these conditions (Walf & Frye 2006). Male rats were also found to be more susceptible to the depressogenic effects of CUMS (Dalla et al 2005). Sex differences are not always observed, like in the SPT, EPM, or Y-maze (Berger et al 2019, Hill et al 2014). Interestingly, female *RELN*$^{+/-}$ have fewer GRs and less TrkB receptor activation than males, which suggests that they may be more vulnerable to stress when reelin levels are downregulated (Schroeder et al 2018, Hill et al 2013).

In this series of experiments, I first evaluated the effects of multiple dosages of reelin in male rats, and then tested the most effective dosage in females. This provided a way to assess sex differences while also reducing animal suffering. A similar approach was used to evaluate the effects of a single dose of reelin; first, a variety of doses were evaluated in males (0.5, 1, 3, 5, 7, and 9µg) and then the most effective dose was evaluated in females (3µg). I found that females spent more time immobile in the FST than males, but not significantly, and reelin rescued behavioral deficits in the FST similarly for both sexes. Perhaps immobility is affected less by CORT in females because estrous hormones can readily cross the blood-brain barrier, alter GR activity, and have neuroprotective qualities (Stein & Hoffman 2003, Bourke et al 2012). There were also no sex differences in response to CORT and reelin on sucrose preference, time spent in the center of the OFT, anxiety-like behavior in the EPM, or cognitive ability in the OBL or Y-maze (see Appendix). However, CORT-treated females did spend more time in the lit zone of the LDT than males and females travelled further distances in the open-field arena regardless of treatment, which was probably due to differences in bodyweight. No sex differences were found for any neurochemical markers except that a single injection of reelin rescued GluA1-IR cell expression by a massive 159% in males whereas the same dose produced a 60% recovery in females, although levels were similar between the sexes in vehicle/vehicle and CORT/vehicle conditions. A single dose of reelin also fully recovered SERT cluster size in blood lymphocytes in females (by 136%), whereas only a partial recovery was observed for males (66%). Our laboratory does have some preliminary data to suggest that females have lower levels of reelin in the hypothalamic PVN than males, which may contribute to the enhanced reactivity to stress (Seale et al 2004), but CORT downregulated PVN-reelin only in males (in preparation for publication). Furthermore, reelin and oxytocin are co-expressed in some neurons of the PVN and this colocalization was greater in males than females in basal conditions and CORT dampened this co-expression only in males. Sex hormones also influence reelin expression. Indeed, administering high levels of testosterone was shown to decrease reelin levels in the brains of male starlings (Absil et al 2003), perhaps

by regulating the reelin promotor methylation (da Silva et al 2015). Reelin was also increased by exogenous estradiol application to hippocampal slices, but not when estrogen-producing aromatase activity was blocked (Bender et al 2010). More work is needed to uncover if there are sex differences in reelin expression in other brain regions, or if reductions in reelin are similar for both sexes after environmental stress (or in other models that do not rely on supraphysiological levels of CORT to induce behavioral and neurochemical deficiencies).

## 5.9 Limitations

### 5.9.1 The extrapolation of preclinical findings

A major issue with preclinical research is the interpretation and extrapolation of experimental findings, especially when making inferences about disorders that are inherently human. Indeed, humans self-reflect, ruminate, and experience guilt and shame, and evidence of such emotional intelligence in rodents is lacking. Generally, chronic CORT treatment induced a depressive-like phenotype evident by behavioral alterations in the FST and hippocampal-dependent cognitive tests, and neurochemical abnormalities that parallel human phenomena – like reductions in reelin and GluA1 expression and neurogenesis. However, subjecting rodents to the FST to induce learned helplessness behavior has been criticized (Commons et al 2017), but this test has value as a screening tool for antidepressant efficacy regardless of how forced-swimming-induced immobility is interpreted. This is because compounds with antidepressant activity in humans decrease FST-immobility in rodents. Thus, the FST is useful when evaluating the potential antidepressant effects of novel compounds. One could argue, though, that our first-line antidepressant drugs are somewhat ineffective in humans, and so relying on the FST to inform pharmacological decisions is flawed. That said, it should also be kept in mind that conventional monoaminergic-based treatments are less effective and slower at decreasing FST-immobility than ketamine, which can rapidly but transiently normalize immobility, whereas psychedelic drugs normalize immobility behavior for much longer durations of time (Hibicke et al 2020). This is true for human depression as well, providing the FST with strong predictive validity. Therefore, the data outlined in this body of work suggests that reelin may have rapid antidepressant effects in human patients, and perhaps in those with treatment-resistant depression, considering that its downstream molecular mechanisms overlap considerably with those of ketamine and psychedelic drugs which effectively improve mood (Johnston et al 2020, Kadriu et al 2020b, Ly et al 2018). There are many future studies that

should be designed to draw more concrete conclusions regarding the pathological mechanisms of depression and how reelin may bring about symptom relief.

### 5.9.2 Mechanistic studies are necessary to provide more concrete conclusions

Here I demonstrate for the first time that reelin has antidepressant-like effects when administered into the lateral tail vein and that the behavioral changes are associated with the recovery of hippocampal reelin and receptors that bind GABA and glutamate. However, mechanistic studies are required to denote their essentialness for antidepressant responsiveness; that is, we cannot say for certain that these receptors are critical for the antidepressant-like effects of peripheral reelin without manipulating their activity (such as with antagonists). It is true that inhibiting AMPAR activity abolishes the behavioral changes induced by intrahippocampal reelin infusions (Brymer et al 2020) and ketamine (Zhang et al 2016), so this does seem likely. Of course, reelin also signals through the PI3K/Akt/mTOR pathway (Jossin & Goffinet 2007) which increases AMPAR insertion and has been identified as a critical mediator in fast-acting behavioral and neurochemical changes, but this story is perplexing because administration of mTOR inhibitor rapamycin actually prolonged the antidepressant effects of ketamine in humans (Abdallah et al 2020), which is a divergence from the preclinical literature (Cavalleri et al 2018, Li et al 2010, Ly et al 2018, Zanos & Gould 2018, Zhou et al 2014). This may suggest that there are compensatory or feedback mechanisms in play that are manipulatable to optimize therapeutic properties, or, that rapamycin had difficulty accessing the brain, leaving neuronal mTOR unhindered and exerting a peripheral anti-inflammatory response that compliments the antidepressant effects (Liu et al 2019). It would be interesting to evaluate the effects of i.v. reelin alongside an mTOR inhibitor to determine if other reelin downstream mediators, like Rap1 or Dab1-SFK-induced NMDAR regulation, could bring about behavioral changes independent of mTOR (Chen & Leonard 1996, Köhr & Seeburg 1996, Yu et al 1997). On the other hand, mTOR signaling may be necessary for reelin to restore AMPAR and $GABA_AR$ numbers and bring about harmonious neuronal activity. I found that the stress-induced downregulation of $GABA_ARs$ was fully recovered by reelin, so determining their importance in the antidepressant-like effects of i.v. reelin through antagonistic means would also provide interesting data. These experiments further outline the blueprint of neurochemical changes that are relevant for the reversal of depression-relevant behavior, laying

the groundwork for future studies to be based upon, eventually leading to the development of mechanistically novel drugs.

## 5.10 Future directions

### 5.10.1 Does reelin cross the blood-brain barrier?

There is plenty of evidence to suggest that reelin supplementation into the brain enhances several forms of neuroplasticity – like neurogenesis, dendritic outgrowth, and the formation of dendritic spines and synaptic connections – which rescues behavioral, cognitive, and neurochemical deficits in various rodent models (Beffert et al 2006, Bosch et al 2016a, Brymer et al 2020, Hethorn et al 2015, Niu et al 2008, Pujadas et al 2010, Qiu & Weeber 2007, Rogers et al 2013, Teixeira et al 2012, Ventruti et al 2011, Weeber et al 2002). Here I have shown that peripheral reelin can produce similar effects, but we are unsure if reelin generates these changes by directly accessing the brain or indirectly by regulating peripheral systems that influence neurochemistry, such as inflammatory responses. Therefore, it would be very interesting to determine whether full-length reelin or fragments of reelin can cross the blood-brain barrier, which would have important physiological and therapeutic implications. For instance, this would open the possibility of delivering reelin-based compounds through viable and non-invasive administration routes. Interestingly, reelin- and ApoER2-immunoreactivity is detected in endothelial cells in the hippocampal stratum lacunosum-moleculare that line the blood-brain barrier, mainly in putative transcytosis caveolae vesicles, which could represent a receptor-mediated mechanism whereby reelin can access the brain (Perez-Costas et al 2015, Riddell et al 2001). However, reelin is a large protein with very limited diffusion once it is released into the extracellular space, and while reelin may access the brain from periphery, one should also investigate putative peripheral actions with an indirect effect on the CNS that could protect from stress. To evaluate whether reelin can cross the blood-brain barrier, one could inject rats with recombinant reelin that has been tagged with a protein that fluoresces when exposed to certain wavelengths of light. If the fluorescent marker is detected in the brain post-administration, then one could argue that is possesses the ability to directly facilitate neuroplasticity.

### 5.10.2 What is the time-course of the antidepressant-like effects of reelin?

Another interesting future direction is to analyze the time-course of reelin's antidepressant-like effects. Here I have found that 3µg of reelin can significantly attenuate FST-immobility in 24 hours. This makes one wonder if behavioral changes can be achieved in even shorter durations of time; rats could be subjected to the FST and other behavioral assays after say 1, 3, 6, and 12 hours. As well, it would be interesting to evaluate how long the antidepressant-like effects of reelin persist. There is a desperate need to develop compounds that not only work quickly, but that have sustained effects. The scientific community was excited by the discovery that ketamine can change mood in hours, but unfortunately, depression scores are only lowered for around 2 weeks (Berman et al 2000, Domino & Warner 2010, Zarate et al 2006). Ketamine, then, requires continuous administration, which is not ideal considering that it has a high potential for abuse and can damage brain cells with repeated use (Wang et al 2013a). More recently, psychedelic drug-assisted therapy has been shown to produce antidepressant effects that last months to even years after one or two sessions, and they are generally considered to be anti-addictive and physiologically safe (Agin-Liebes et al 2020, Carhart-Harris & Goodwin 2017, Hibicke et al 2020). However, psychedelics come with their own limitations, like their hallucinogenic properties and long-lasting changes in consciousness, which correlate positively with behavioral improvements but complicate their wide-spread clinical use, nonetheless (Griffiths et al 2011, Roseman et al 2018). It would be very interesting to evaluate the temporal effects of reelin by subjecting animals to the FST days, weeks, and months after its administration, and it would be valuable to include a positive control, like ketamine or compounds with more persistent effects. It could be that the antidepressant-like effects of reelin are also persistent and free from the limiting side-effects of other fast-acting therapeutics. A group of researchers in Chicago supported this notion by demonstrating that a bilateral micro-infusion of reelin into the amygdala of mice that were socially isolated for four weeks rescued anxiety-like behavior and aggression 1 month post-infusion (Nin et al 2011b). In fact, the activation of VLDLR and ApoER2 by reelin stimulates similar neuroplasticity-promoting downstream pathways as glutamatergic ketamine and 5-HTergic psychedelics (Johnston et al 2020, Ly et al 2018). Evidence even suggests that the fast-acting antidepressant effects of ketamine rely on reelin signaling to bring about behavioral and neurochemical improvements (Kim et al 2021). These data suggest that reelin signaling could be the common mechanism that antidepressants converge onto to bring about their activity.

### 5.10.3 Does reelin have antidepressant-like effects in other models for depression?

It is important that the antidepressant-like effects of reelin are evaluated in other models for depression, in other species, and using additional behavioral tests. This would shed more light on the neuroprotective qualities of reelin and further verify and extend the data described here, strengthening the prospect of developing reelin-based therapeutics. I found that reelin had little effect on anhedonia-like behavior in these conditions (see Appendix), but it is entirely possible that reelin could rescue such behavior in other models (i.e., that do not raise CORT levels to supraphysiological levels) which may bring about neurochemical abnormalities that are more representative of the human condition. That said, anhedonia is notoriously difficult to treat in patients with MDD as well (McMakin et al 2012, Rubin 2012, Treadway & Zald 2011). CORT also had little effect on anxiety-like behavior in these experiments, so it is unclear if peripheral reelin has anxiolytic-like effects in these conditions, but work from other groups suggests that reelin alleviates anxiety-like behavior in mouse models for long periods of time (Nin et al 2011b). Mice appear to be more sensitive to the anxiety-provoking effects of chronic CORT treatment (see Table 1.10.1b on page 80), which insinuates that mouse models may be advantageous over rat models when evaluating the anxiolytic effects of novel compounds. Anhedonia- and anxiety-like behavior could also be evaluated in other behavioral tests, like the splash test (which can be used to make inferences about grooming behaviors), social interaction tests, place preference paradigms, and self-stimulation tests. Perhaps reelin is more beneficial for some symptoms of depression than others, like cognitive deficits, which remain very difficult to treat with conventional medications (Richardson & Adams 2018). It is true that: reelin-deficient mice have poorer memory performance, including social recognition memory (Iemolo et al 2021, Schroeder et al 2015); a lack of reelin receptor activity is associated with memory impairments (Mulder et al 2004); and intraventricular reelin infusions improve cognitive ability (Hethorn et al 2015, Rogers et al 2011, 2013). This suggests that reelin-based compounds could be a useful synergistic treatment if given alongside conventional antidepressant treatments to combat multiple facets of depressive disorders.

### 5.10.4 Would reelin be beneficial for other psychiatric disorders?

Reelin levels are abnormal in the blood and brains of patients with various psychiatric conditions that have overlapping neurobiological risk factors, including schizophrenia, temporal lobe epilepsy, autism spectrum disorders, Alzheimer's disease, and bipolar disorder

(Fatemi et al 2000, 2001, 2002, Guidotti et al 2000, Impagnatiello et al 1998, Knable et al 2004). Therefore, mice that are deficient in reelin are valuable tools to study psychiatric disturbances. It is true that *RELN*$^{+/-}$ are more sensitive to stress-induced impairments compared to wild-type mice, which is evident by the greater increases in FST-immobility and deficits in spatial and social recognition memory after CORT treatment (Lussier et al 2011, Notaras et al 2020, Schroeder et al 2015). This implies that low levels of reelin represent a vulnerability factor for certain pathologies (Fatemi 2011), or that there may be a threshold of reelin downregulation that occurs before behavioral abnormalities develop (Lebedeva et al 2020). This is corroborated by the fact that reelin-overexpressing mice are protected from behavioral phenotypes that are relevant for schizophrenia and bipolar disorder (Teixeira et al 2011). Therefore, one could argue that reelin promotes stress resiliency and its administration could be beneficial for several conditions.

While I did not see any anxiolytic-like effects of peripheral i.v. reelin in these conditions (although there were also no effects of CORT; see Appendix), other laboratories have reported that reelin supplementation into the hippocampus reduced anxiety-like behavior in the OFT without influencing distance travelled in mice that were exposed to prenatal maternal inflammatory events (Ibi et al 2020). Additionally, mRNA levels of reelin and allopregnanolone were decreased by 35% in the frontal cortex, 55% in the hippocampus, and 65% in the basolateral amygdala after chronic social isolation, which is thought to generate behavioral alterations isomorphic of PTSD (Nin et al 2011b). Interestingly, they found that s.c. allopregnanolone injections rescued social isolation-induced anxiety-like behavior and decreases in reelin and BDNF mRNA expression, and that a single bilateral micro-infusion of reelin into the amygdala abolished anxiety-like behavior and aggression when evaluated 1 month later (Nin et al 2011b). Similarly, mice that lacked the C-terminal of reelin displayed impaired social behaviors (Sakai et al 2016), and reelin-deficient mice are more susceptible to social impairment and reactive to aversive situations after chronic tetrahydrocannabinol consumption compared to wild-type mice (Iemolo et al 2021). Therefore, perhaps the facilitation of synaptic plasticity by reelin would be beneficial for those with anxiety and autism, which are characterized by synaptic defects and social dysfunction (Beffert et al 2005, Hansel 2019, Hill 2004, Justice et al 1977, Yang et al 2016).

The pro-cognitive effects of reelin are well-documented and suggest that reelin could show promise for treating cognitive decline associated with age-related dementias. The depletion of reelin has been noted as an early phenomenon in the pathology of Alzheimer's disease, long

before there is evidence of amyloid-β plaque build-up in the frontal cortex (Herring et al 2012). Reelin seems to delay the formation of amyloid-β plaques by activating the VLDLR/ApoER2 pathway that interacts with soluble amyloid-β species, but reelin can also inhibit GSK3β from hyperphosphorylating Tau via PI3K/Akt, which reduces neurofibrillary tangles that are characteristic of Alzheimer's disease and cognitive decline (Beffert et al 2002, Jossin & Goffinet 2007, Perl 2010, Pujadas et al 2014, Toral-Rios et al 2020). The fact that reelin- and VLDLR/ApoER2-deficient mice have higher levels of phosphorylated Tau compliments this idea (Ohkubo et al 2003), especially since they have poorer memory span and social recognition memory than wild-type mice (Iemolo et al 2021), and their spatial memory is more effected by chronic stress than wild-types (Notaras et al 2020, Schroeder et al 2015). Deficits in hippocampal-dependent cognition tests in various models are also rescued by reelin supplementation into the brain and are linked to increases in GABAergic and glutamatergic signaling (Hethorn et al 2015, Idi et al 2020, Rogers et al 2011, 2013). In general, reelin appears to have multiple neuroprotective properties that improve signal transduction, neuroplasticity, and learning and memory (Shen et al 2001, Toral-Rios et al 2020).

### 5.11 The importance of this work and closing remarks

This work is of the utmost importance, considering that the prevalence rates for depression are exceedingly high, and climbing (Klerman & Weissman 1989, WHO 2002, 2017). However, we have still not developed first-line treatments that are effective or quick to work. It is the unfortunate truth that conventional medications only produce significantly greater therapeutic improvements over placebo in 43% of clinical trials, with the placebo response contributing to 82% of the antidepressant effect (Kirsch 2014). Therefore, these drugs are only slightly more effective than placebos and they only work in around ⅔ of the depressed population (Cipriani et al 2018, Rush et al 2006). This ineffectiveness is paired with a temporal discrepancy between the acute effects of antidepressants and their delayed therapeutic response that does not occur until weeks or months after the regimen start-point, leaving those with severe depression at serious risk given that MDD is a strong predictor of suicide (Cuijpers et al 2014a, Kuo et al 2015, Stahl 2000). Adverse side-effects are also problematic and commonly cause one to discontinue treatment (Garland et al 2009, Nischal et al 2012). This demonstrates the desperate need for antidepressants that work in a larger proportion of patients and at a much faster rate.

While we are beginning to identify molecular mechanisms that bring about rapid antidepressant-like actions – by increasing excitatory transmission in corticolimbic regions (Koike & Chaki 2014 – we have not developed compounds that do this effectively without massively disrupting normal brain functions. For instance, ketamine has a dissociative nature and can cause confusion, it is thought to be addictive, and its chronic use has negative consequences (Wang et al 2013a). Even drugs that specifically increase AMPAR transmission have provided disappointing results in clinical trials besides showing great promise preclinically (Kadriu et al 2021). Reelin is a very promising candidate novel antidepressant compound because it increases AMPAR transmission, regulates NMDARs activity, and normalizes GABAergic tone, as well as rescuing SERT MPC which may contribute to the neurochemical recoveries. Therefore, targeting the reelin signaling pathway, including reelin-cleaving enzymes, reelin receptors, or their downstream mediators like Dab1, may prove to be a valuable novel treatment approach for mood disorders.

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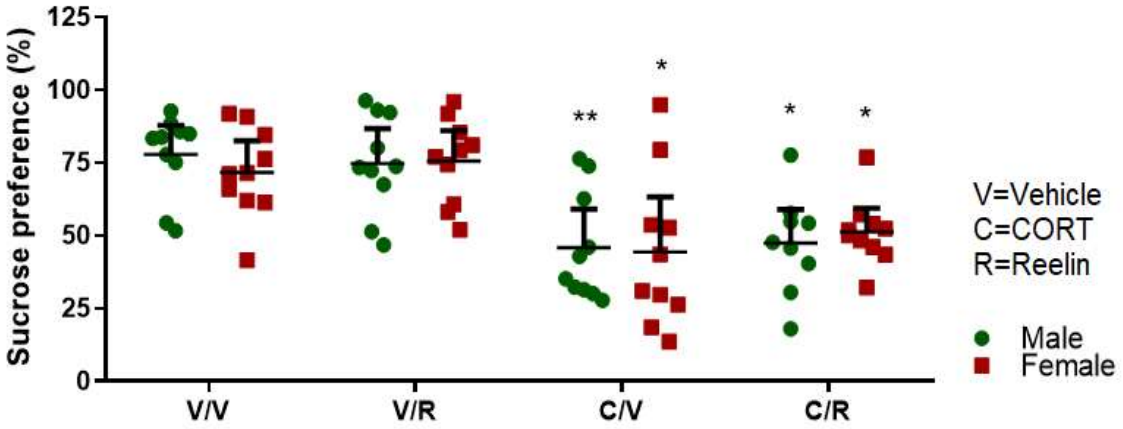
**Appendix A (additional behavioral testing)**

It is difficult to mimic depression in research models considering that animals lack the ability to self-reflect as we do. To make inferences about human conditions, investigators have developed animal behavior tests that are designed to elicit responses that can be measured to make inferences about their mood, such as fear, anxiety, and pleasure. I focused primarily on the FST, and hippocampal-dependent cognition tasks, to ascertain the antidepressant-like effects of i.v. reelin and I acknowledge the limitations related to the use of animal models in mental health research. It is important that reelin is evaluated using additional behavior tests and in other animal models to corroborate these findings.

Anhedonia, or the loss in interest in once pleasurable activities, is a core feature of depression and one that is difficult to assess in a rodent. Anhedonia may look like a patient ignoring phone calls from friends who are at their favorite bar, or no longer going to the gym, if it used to be rewarding. Based on this point, researchers evaluate how rodents respond to rewarding stimuli, like food, sex, or drugs, and paying less interest in the reward could be indicative of anhedonia-like behavior. By measuring the preference for a sweetened solution, I aimed to evaluate the effects of reelin on anhedonia-like behavior, which is often but not always induced by CORT (David et al 2009, Dwivedi et al 2015, Gorzalka et al 2003, Gourley et al 2008, Lebedeva et al 2020). The SPT took place on day 23 (see Figure 3.3.1 on page 131 and Figure 4.3.1 on page 162 for experimental designs) using the following procedure. On day 21 the rats were given 2 bottles of water for 24 hours, one placed at either side of their cage, and they were allowed to drink from them ad libitum. After this, they were given 2 bottles of 1% sucrose solution (200ml each) for another 24 hours. This served to habituate rats to having 2 water bottles and to the sucrose solution, and to determine that a side-preference existed – rats preferring to drink from the bottles that were placed on the right side of the cage (where their bottle was placed for the first 21 days). At 12:00 p.m. on day 23, they were presented with one bottle of 1% sucrose and one bottle of regular drinking water (200ml each) for 24 hours (50% of the animals were given sucrose on the right side and the other 50% on the left side), and after 12 hours the sides of the bottles were switched. See Figure 1.10D on page 74 for visual representation of the SPT procedure. Sucrose and water intake were measured by weighing the bottles each time they were handled, and the percentage of sucrose preference was calculated with the following formula:

$$Sucrose\ preference = sucrose\ consumption \div (sucrose\ consumption + water\ consumption \times 100$$

I found that sucrose preference was unreliably decreased by CORT and reelin was ineffective at recovering sucrose preference. In my second experiment, when male and female rats were given 3µg of reelin every 10 days, I found that all CORT-receiving rats had a decreased preference for sucrose compared to their respective vehicle comparison group ($p \leq 0.043$; see Appendix A Figure 1 and Appendix A Table 1; statistical information for these rats can be found in Appendix A Table 2). However, in my third experiment in which rats received a single injection of reelin, there was no significant effect of either treatment (see Appendix A Figure 2; statistical information for these rats can be found in Appendix A Table 3). This non-replicability represents a limitation of the CORT model and highlights a problem that researchers face when using animal models in general. This is unfortunate because anhedonia is a hallmark symptom of depression and highlights the necessity of evaluating peripheral reelin in other conditions (i.e., environmental stress-based models, behavioral tests, strains, and species).

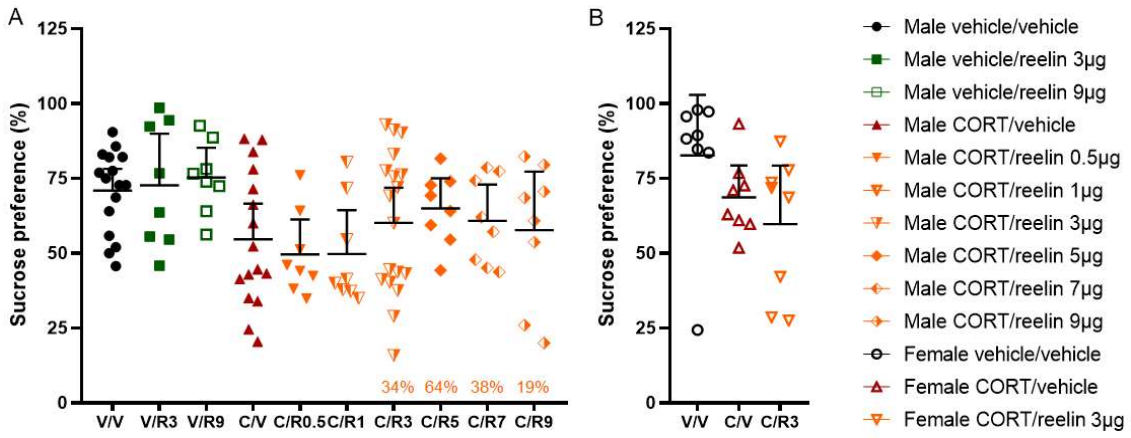



**Appendix A Figure 1. The effect of sex, CORT, and reelin treatment on sucrose preference.** CORT decreased sucrose preference and there were no effects of sex or reelin treatment. Data are expressed as mean±CI. *$p<0.05$/**$p<0.01$ vs vehicle comparison. Figure created with GraphPad Prism by author.

**Appendix A Table 1. The amount of water and sucrose consumption in the SPT.**

| Sex | Treatment (3µg every 10 days) | Water drank (g) | Sucrose drank (g) | Total drank (g) | Sucrose preference (%) |
|---|---|---|---|---|---|
| Male | Vehicle/vehicle | 8.40±1.40 | 31.00±2.53 | 39.40±1.69 | 77.71±4.42 |
| | Vehicle/reelin | 9.04±1.64 | 30.02±3.55 | 39.06±2.60 | 74.61±5.29 |
| | CORT/vehicle | 22.90±4.31 | 18.20±2.68 | 41.10±5.15 | **45.81±5.85 **** |
| | CORT/reelin | 20.60±2.68 | 18.60±2.74 | 39.20±3.04 | **47.39±5.08 *** |
| Female | Vehicle/vehicle | 10.10±1.83 | 28.80±5.83 | 38.90±5.63 | 71.58±4.82 |
| | Vehicle/reelin | 8.90±2.13 | 26.70±2.70 | 35.60±2.99 | 75.47±4.59 |
| | CORT/vehicle | 14.50±2.10 | 11.60±2.20 | 26.10±1.80 | **44.29±8.33 *** |
| | CORT/reelin | 18.90±2.42 | 19.40±2.01 | 38.30±3.84 | **51.17±3.58 *** |

Sucrose preference was calculated using the following formula: *sucrose preference = sucrose drank ÷ (sucrose drank + water drank) × 100.* *$p$<0.05/**$p$<0.01 vs vehicle comparisons.




**Appendix A Figure 2. The effect of CORT and incrementing doses of reelin on sucrose preference.** There were no effects of sex or treatment in males (A) or females (B). Data are expressed as mean±CI. Figure created with GraphPad Prism by author.

We previously found that sucrose preference was unaffected by 21 days of CORT when it was given at a dose of 20mg/kg, but that a sensitization to stress occurred over multiple cycles of CORT treatment that co-occurred with reductions in hippocampal reelin (Lebedeva et al 2020). It is interesting that i.v. reelin had little effect on both sucrose preference and neurogenesis, which suggests that they may be related. In fact, I found that higher levels of neurogenesis strongly correlated with greater sucrose preference when all the females were included ($p$<0.001, N=39, Chapter 3). Supporting this idea, it was recently demonstrated that disrupting neurogenesis causes mice to lose interest in a food reward (Eliwa et al 2021). These findings bring about more questions than answers, like would other doses of reelin or administration

routes improve neurogenesis and thereafter sucrose preference; is the blood-brain barrier obstructing peripheral reelin from instigating central actions directly; and would reelin rescue neurogenesis and sucrose preference in other models for depression? One must keep in mind that normal plasma levels of CORT when subject to physiological stress are around 3-5mg/kg (Sandi et al 1996), implying that peripheral reelin may very well rescue neurogenesis and anhedonia-like behavior in other models for depression (like rodents that express putative genetic vulnerabilities (Overstreet et al 2005) or are exposed to CUMS). It is true that we are giving exceedingly high levels of CORT (40mg/kg per day for three weeks); this is because we aimed to reliably induce FST-immobility to evaluate the antidepressant-like actions of i.v. reelin, instead of relying on the animals natural but more variable response to stressors (Willner 2005). If reelin cannot access the brain from the periphery, then behavioral changes must initially be driven by peripheral actions that would inherently take longer to correct neurogenesis than intrahippocampal infusions of reelin (Brymer et al 2020). Indeed, anhedonia is often the last symptom to be rectified by pharmacotherapy in patients (Rubin 2012), if at all (McMakin et al 2012, Treadway & Zald 2011), which holds up in our CORT-treated rat model.

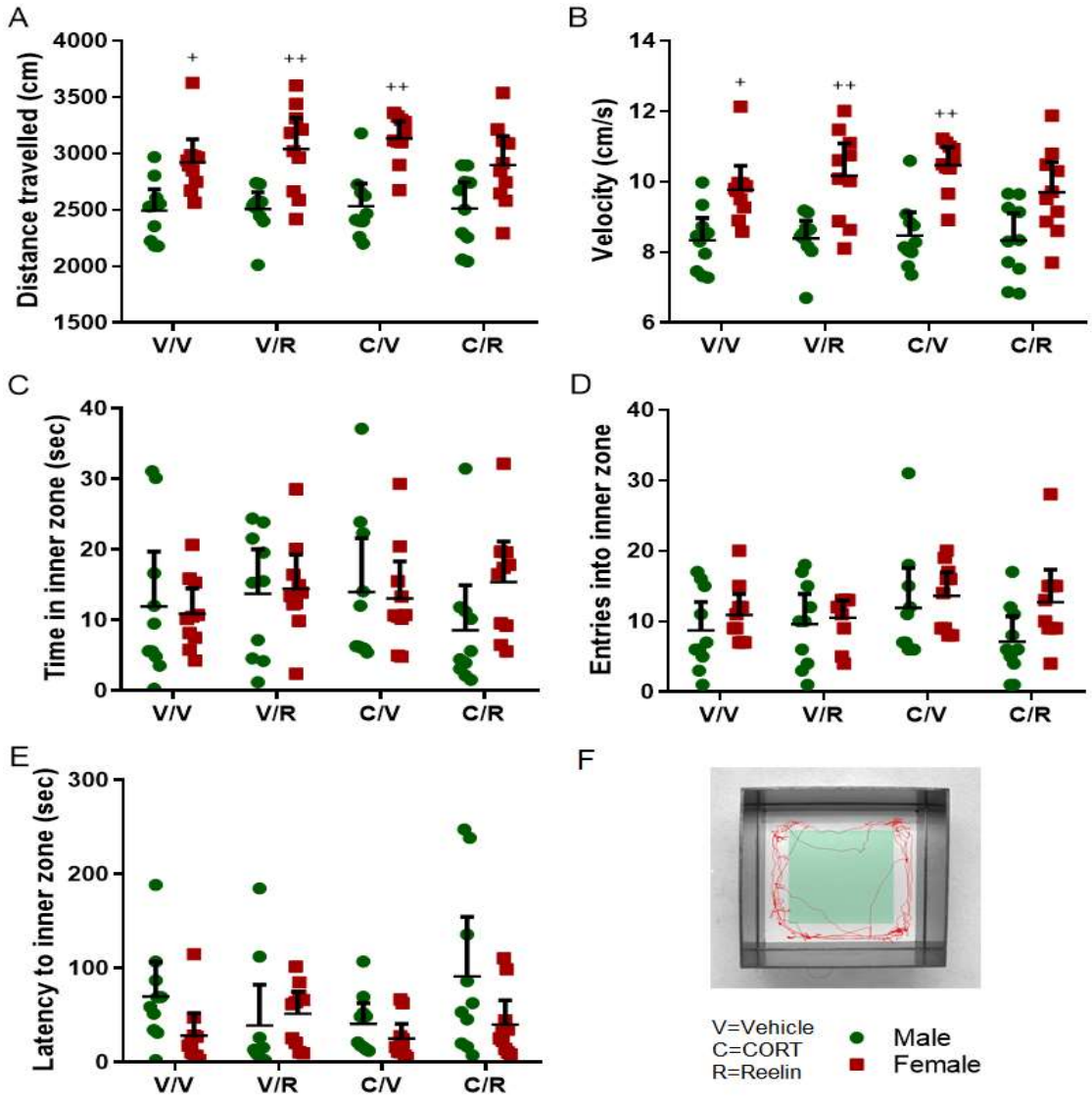
Patients with depression commonly report feelings of anxiety (Breslau et al 1995, Kalin 2020), so I subjected rats to several behavioral tests that are designed to assess how rodents explore a novel environment that can provide indications of anxiety-like behavior, including the OFT, LDT, and EPM.

The OFT was conducted on day 23 in rats that were treated with multiple vehicle or reelin injections (see Figure 3.3.1, page 131). The open-field apparatus was 65cm by 65cm and it had a light blue floor and black 60cm-high walls. I placed the rat into a corner of the maze and analyzed their behavior for 5 minutes. The activity of the rat was recorded by a video camera that was placed above the apparatus and EthoVision® (Noldus Information Technology, Wageningen, The Netherlands) software was used to automatically calculate the distance travelled by the rat, the velocity of its movement, and the amount of time spent in spent in and the number of entries into the inner zone of the arena. Data was calculated with an inner zone equal to 625cm$^2$ (25cm by 25cm) and 2025cm$^2$ (45cm by 45cm; reported here) and similar group differences were observed in each instance. Hot soapy water was used in between trails to wash the arena. The results can be seen in Appendix A Figure 3 below and panel F shows a typical movement path for a given rat. I found that the female vehicle/vehicle rats ($p$=0.038), female vehicle/reelin rats ($p$=0.003), and the female CORT/vehicle rats ($p$=0.001) travelled significantly more than their male comparison groups. Similarly, the female vehicle/vehicle

rats ($p$=0.036), female vehicle/reelin rats ($p$=0.003), and the female CORT/vehicle rats ($p$=0.001) travelled at a greater velocity than their male comparison groups. This is probably related to their lighter bodyweights. There were no significant group differences regarding the time spent in the inner zone, the number of times a rat entered the inner zone, or the latency to enter the inner zone of the open-field arena. Other laboratories have found that mice will avoid the center of the arena when CORT (35µg/ml/day, equivalent to about 5mg/kg/day) is added to their drinking water (Rainer et al 2012), as did Sprague-Dawley rats who were treated identically to ours but placed into a larger arena (Li et al 2017). This suggests that the species, strain of rodent, and apparatus are important for behavioral outcomes considering that we found no effect of CORT on anxiety-like behavior after 3 weeks of daily 20mg/kg or 40mg/kg s.c. injections (Lebedeva et al 2020).

**Appendix A Table 2. Statistical information for the effects of sex, CORT, and multiple reelin injections on behavior.**

| Measure | Sex | CORT | Reelin | CORT × Reelin | Sex × CORT | Sex × Reelin |
|---|---|---|---|---|---|---|
| Sucrose preference | F(1, 72)=0.039, $p$=0.843 | F(1, 72)=52.403, $p$<0.001 | F(1, 72)=0.364, $p$=0.548 | F(1, 72)=0.253, $p$=0.616 | F(1, 72)=0.243, $p$=0.624 | F(1, 72)=0.648, $p$=0.424 |
| OFT-distance travelled | F(1, 72)=53.974, $p$<0.001 | F(1, 72)=0.178, $p$=0.675 | F(1, 72)=0.213, $p$=0.678 | F(1, 72)=2.170, $p$=0.145 | F(1, 72)=0.013, $p$=0.909 | F(1, 72)=0.174, $p$=0.678 |
| OFT-velocity | F(1, 72)=55.557, $p$<0.001 | F(1, 72)=0.118, $p$=0.732 | F(1, 72)=0.275, $p$=0.602 | F(1, 72)=2.382, $p$=0.172 | F(1, 72)=0.033, $p$=0.856 | F(1, 72)=0.103, $p$=0.749 |
| OFT-inner zone time | F(1, 72)=0.546, $p$=0.462 | F(1, 72)=0.00004, $p$=0.995 | F(1, 72)=0.092, $p$=0.762 | F(1, 72)=1.22, $p$=0.273 | F(1, 72)=0.686, $p$=0.410 | F(1, 72)=1.552, $p$=0.217 |
| OFT-inner zone entries | F(1, 72)=4.349, $p$=0.041 | F(1, 72)=1.261, $p$=0.265 | F(1,72)=1.087, $p$=0.301 | F(1, 72)=1.546, $p$=0.218 | F(1, 72)=0.709, $p$=0.402 | F(1, 72)=0.272, $p$=0.604 |
| OFT-latency to inner | F(1, 72)=4.859, $p$=0.031 | F(1, 72)=0.036, $p$=0.851 | F(1, 72)=1.780, $p$=0.186 | F(1, 72)=2.857, $p$=0.955 | F(1, 72)=0.775, $p$=0.388 | F(1, 72)=0.181, $p$=0.671 |

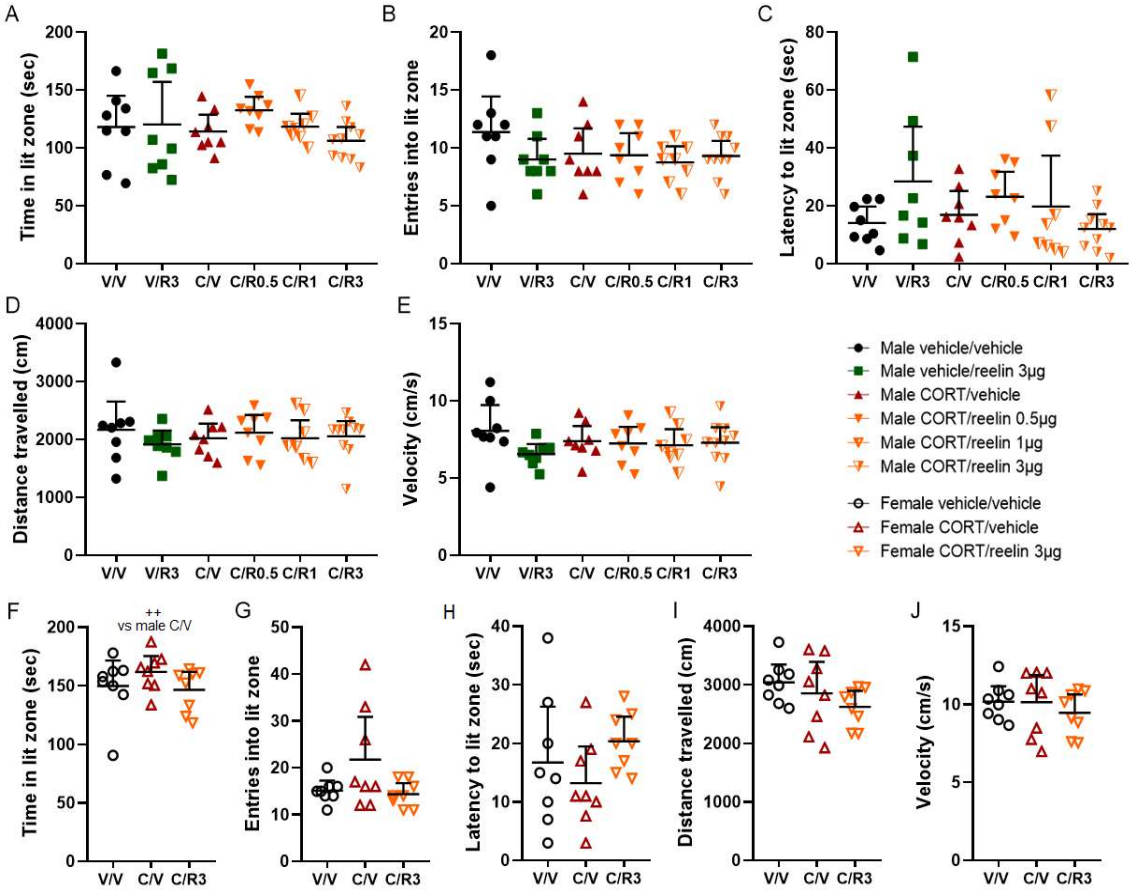



**Appendix A Figure 3. The effect of sex, CORT, and reelin treatment on OFT-behaviors.** A) Distance travelled. B) Velocity. C) Time spent in inner zone. D) Entries into inner zone. E) Latency to enter the inner zone. F) Example of a typical movement path for a given rat. Data are expressed as mean±CI. +$p$<0.05/++$p$<0.01 vs male comparison. Figure created with GraphPad Prism and Paint by author.

Some of the rats that were treated with a single injection of reelin were subjected to the LDT, which is like the OFT, but half of the arena was shaded, and the other half was well-lit, while other rats were subjected to the EPM (and Y-maze; see Figure 4.3.1 for experimental design and Appendix A Table 3 for statistical information).

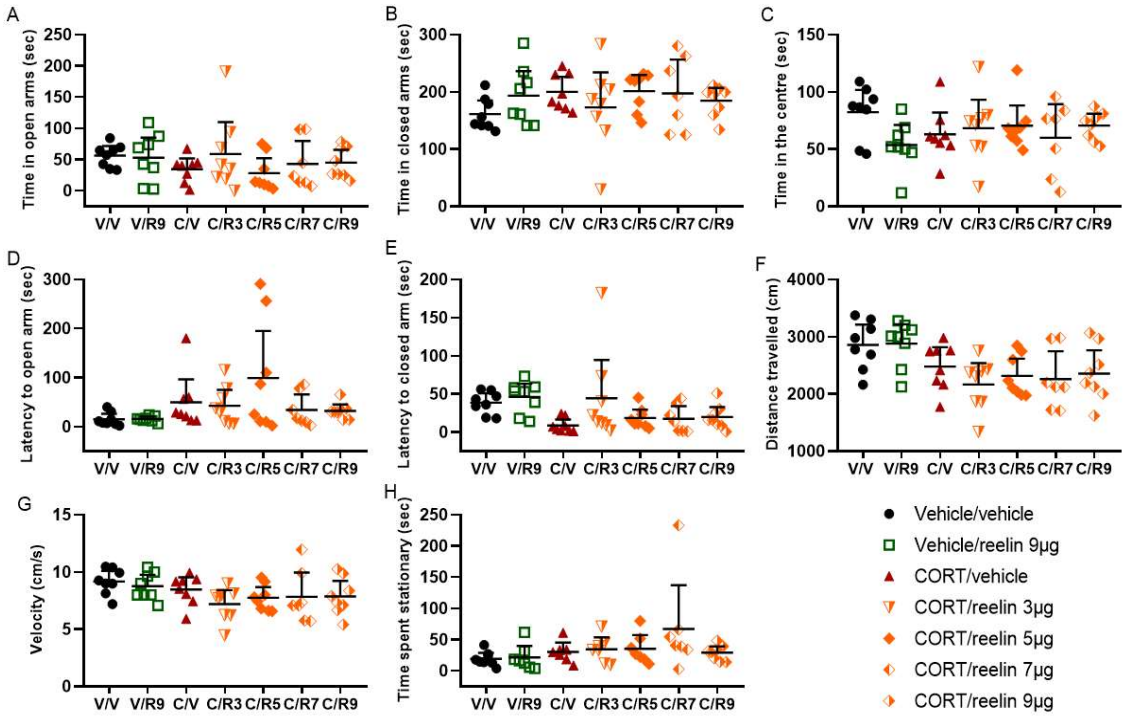
For those that were exposed to the LDT, the procedure was as follows. On day 23, the rats were placed into a corner of the square open-field light/dark arena and were left to explore for 5

minutes while an overhead camera recorded their activity. EthoVision® software was used to calculate the number of entries into the lit half of the arena, as well as the amount of time spent in and the latency to enter the lit half. I found that neither treatment, nor a combination of the two, influenced normal exploratory behavior; there were no group differences in light avoidance which is indicative of anxiety-like behavior (see Appendix A Figure 4). However, I did see that the male CORT/vehicle rats spent less time in the lit zone than the female CORT/vehicle rats ($p<0.01$).




**Appendix A Figure 4. The effect of CORT and a single reelin injection on exploratory behavior in the light/dark test**. There were no group differences in time spent in (A/F), entries into (B/G), or the latency to enter (C/H) the lit zone of the arena or the amount of locomotor activity (D/I) and velocity (E/J), but females did spend more time in the lit zone than males when treated with CORT. Data are expressed as mean±CI. ++$p<0.01$ vs male CORT/vehicle. Figure created with GraphPad Prism by author.

Exposure to the EPM in place of the LDT also occurred on day 23. The EPM involves placing a rat into the center of a "+"-shaped arena for 5 minutes. Two arms opposite one another are open and the other two are enclosed in tall walls, and it is generally thought that an increased time spent in the closed, shaded arms is representative of a more anxious phenotype. Their behavior in the maze was recorded with an overhead video camera and analyzed using EthoVision® software. Our results show that CORT and reelin had no effects on anxiety-like in the EPM, such as time spent in the closed arms, or freezing responses (see Appendix A Figure 5).

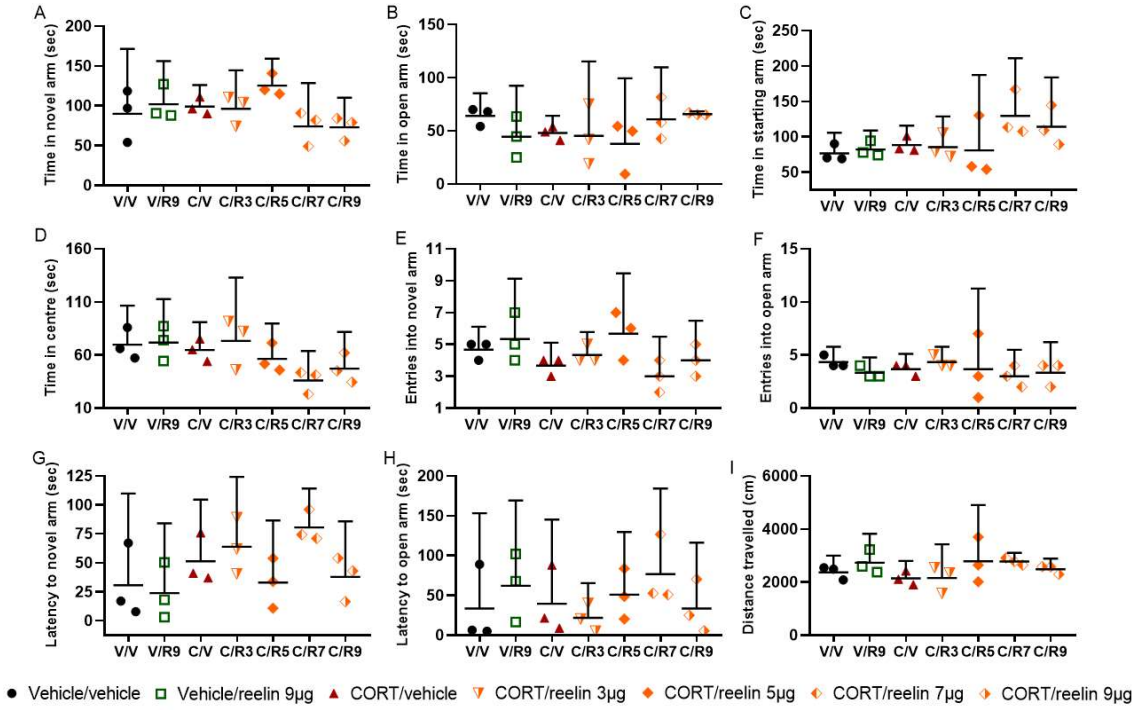



**Appendix A Figure 5. The effect of CORT and a single reelin injection on exploratory behavior in the elevated plus maze.** There was no effect of treatment on time spent in the open or closed arms or on general locomotor activity. A) Time in the open arms. B) Time in the closed arms. C) Time in the center. D) Latency to the open arms. E) Latency to closed arms. F) Distance travelled. G) Velocity. H) Time spent stationary. Data are expressed as mean±CI. Figure created with GraphPad Prism by author.

The inability of CORT to produce anxiety-like behavior in some tests has been witnessed before in these conditions (Gregus et al 2005), but not always (David et al 2009, Kalynchuk et al 2004, Pêgo et al 2008, Skórzewska et al 2006). One could argue that this decreases the face validity of the CORT model, considering that anxiety is commonly reported in those with depression (Kalin 2020). It should be a priority to investigate the effects of reelin in other

models that have stronger construct validity (that do not rely on supraphysiological levels of CORT), like rodents that are subject to CUMS protocols which may develop hedonic- and anxiety-relevant behaviors more consistently (Hesselgrave et al 2021, Strekalova et al 2004, Willner et al 1987, Zhu et al 2014). For example, bilateral micro-infusions of reelin into the amygdala have been shown to attenuate anxiety-like behavior in mice that were socially isolated for four weeks 1 month post-infusion (Nin et al 2011b). In any case, these results indicate that i.v. reelin can restore FST-immobility and cognitive ability independent to any apparent exploratory or anxiety-like behavioral abnormalities or changes in muscle strength (Gregus et al 2005, Marks et al 2009). This is important because exposure to high levels of stress can cause muscle atrophy and dramatic reductions in bodyweight (Allen et al 2010, Hasselgren et al 2010), which should be monitored if subjecting rats to longer CORT-injection periods for ethical purposes. Indeed, over the course of the study reelin did not appear to induce any adverse behavioral or physiological reactions in these conditions, which could represent an advantage over ketamine and other drugs with rapid and long-lasting antidepressant effects that also cause clinically-undesirable reactions (Berman et al 2000, Kadriu et al 2020b), which are detectable in rodent models by behaviors like head weaving and stumbling (Hanks & González-Maeso 2013, McDougall et al 2017).

A subset of animals then went through a hippocampal-dependent spatial memory test that was performed using a Y-shaped arena, based on the innate preference of rodents to explore a novel environment over a familiar one (Holmes et al 2010, Yau et al 2015a). This was done to assess if there were any dramatic behavioral differences that would warrant further experimental attention using the following procedure. Two trials took place on day 24 to assess novel arm discrimination: in the first trial, the rat was placed at the end of the "start" arm facing the center and allowed to explore the maze for 5 minutes with one of the arms (novel arm) blocked. After this, they were returned to their home cage for 2 hours, before being returned to the maze and allowed to explore all three arms for 5 minutes (see Figure 1.10G). Visual cues that could be seen by the rats from within the maze were placed around the room. EthoVision was used to track the animal and calculate the number of entries and time spent in each arm, and less time spent in the novel arm is indicative of hippocampal-dependent spatial memory deficits. I detected no abnormal behavior in the amount of time spent in or entries into any of the Y-maze arms.




**Appendix A Figure 6. The effect of CORT and a single reelin injection on spatial recognition memory in the Y-maze.** There was no effect of treatment on time spent in the novel arm (A), open arm (B), start arm (C), or center (D), or entries into the novel (E) or open arm (F), or latency to novel (G) and open arms (H), as well as distance travelled (I). Data are expressed as mean±CI. Figure created with GraphPad Prism by author.

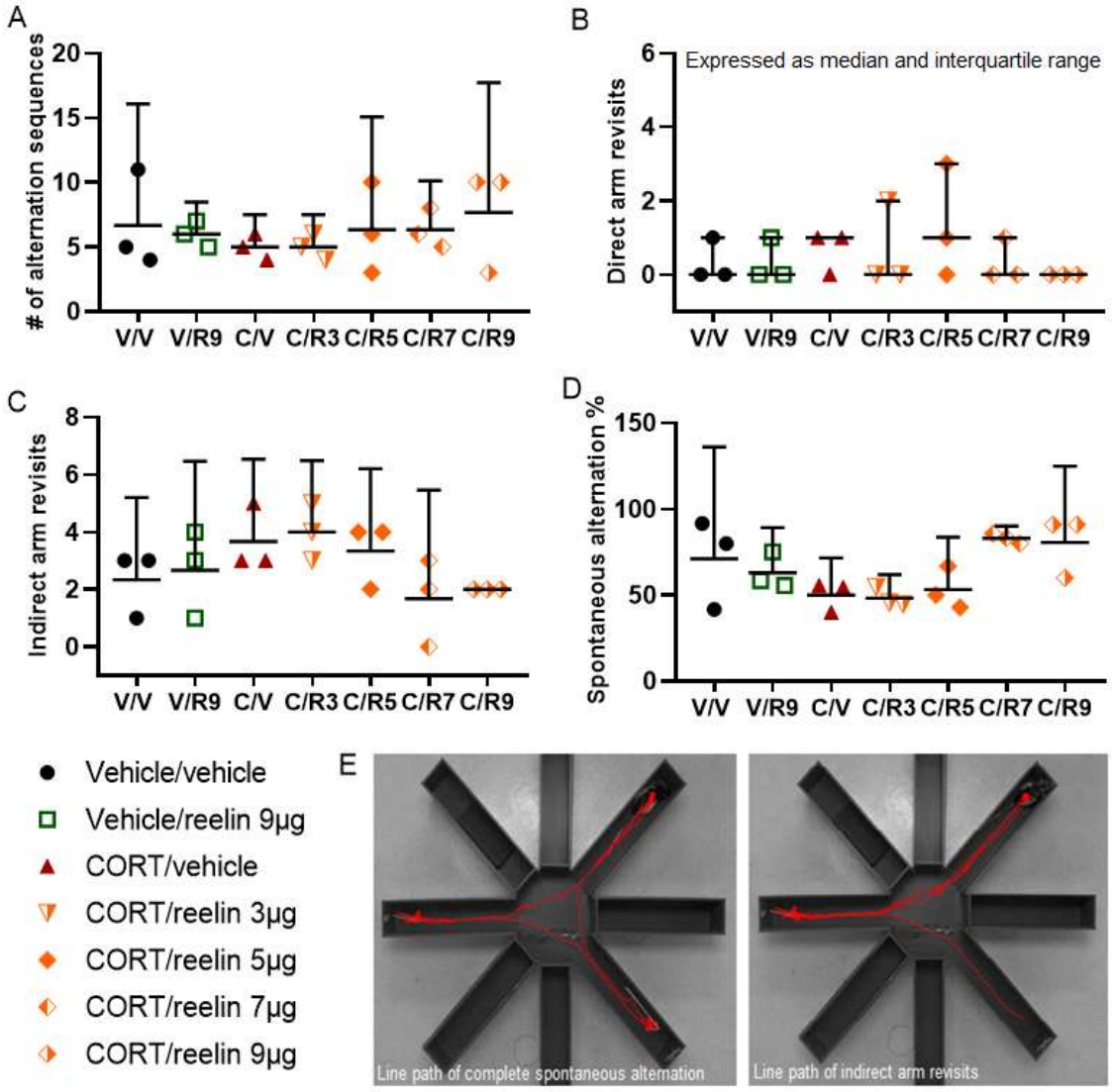
As well as novel arm discrimination, I investigated the effects of CORT and reelin treatment on their spontaneous alternation activity. During trial 2 of the novel arm recognition test, the pattern of arm alternations was recorded and examined to assess spatial working memory. In the following pattern of arm visits, for example, the underlined arms are the start of complete non-repeating 3-arm sequences: Start→Open→Novel→Open→Start→Novel. A Y-maze percentage of spontaneous alternation can be calculated using the following formula:

*YmSponAlt = (number of complete sequences ÷ (total number of alternations – 2) × 100)*

Thus, using the above example of alternations the formula would read *YmSponAlt = (3 / (6 – 2) × 100) = 75%*. Considering that stress impairs rodent cognition, one would assume that stress would decrease the YmSponAlt percentage, or in other words, the number of times they revisited arms directly or indirectly.

I found a significant main effect of reelin on their spontaneous alternation (see Appendix A Figure 7), though no significant group differences were observed with a Tukey *post hoc*. This

result suggests that reelin may affect working memory performance as measured by spontaneous alteration scores, but statistical robustness is lacking. I analyzed direct arm revisits with a non-parametric Kruskal Wallis test considering that the normality of variance ANOVA assumption was not met using the Shapiro-Wilk test of normality ($p$<0.05), but no significant effects were found (k=4.364, $p$=0.628). The effect of reelin should be evaluated in other cognitive-based tests like the MWM and Barnes maze, and in other models for depression, to further validate the pro-cognitive effects of i.v. reelin.




**Appendix A Figure 7. The effect of CORT and a single reelin injection on spatial working memory in the Y-maze.** There were no group differences for the number of complete alternation sequences (A), direct arm revisits (B), or indirect arm revisits (C). There was a significant main effect of reelin on spontaneous alternation but no group differences (D). Data are expressed as mean±CI unless other stated. Figure created with GraphPad Prism and Paint by author.

**Appendix A Table 3. Statistical information for the effects of sex, CORT, and a single reelin injection on behavior.**

| Measure | Sex | CORT | Reelin | CORT x Reelin | Sex | Sex x CORT | Sex x Reelin |
|---|---|---|---|---|---|---|---|
| Sucrose preference | M | F(1, 96)=6.807, *p*=0.011 | F(1, 96)=1.015, *p*=0.420 | F(1, 96)=0.264, *p*=0.768 | Na | | |
| | F | One way: F(2, 21)=2.481, p=0.108 | | | | | |
| | M & F | F(1, 117)=7.425, p=0.007 | F(1, 117)=0.690, p=0.658 | F(1, 117)=0.261, p=0.771 | F(1, 117)=3.959, p=0.049 | F(1, 117)=0.182, p=0.671 | F(1, 117)=1.397, p=0.240 |
| LDT-time in lit zone | M | F(1, 44)=1.072, *p*=0.306 | F(1, 44)=1.507, p=0.226 | F(1, 44)=0.336, p=0.565 | Na | | |
| | F | One way: F(2, 21)=1.207, p=0.319 | | | | | |
| | M & F | F(1, 65)=0.054, p=0.817 | F(1, 65)=1.955, p=0.129 | F(1, 65)=0.375, p=0.542 | F(1, 65)=29.875, p<0.001 | F(1, 65)=0.891, p=0.349 | F(1, 65)=0.201, p=0.656 |
| LDT-entries into lit zone | M | F(1, 44)=0.886, p=0.352 | F(1, 44)=0.913, p=0.443 | F(1, 44)=1.690, p=0.200 | Na | | |
| | F | One way: F(2, 21)=2.696, p=0.073 | | | | | |
| | M & F | F(1, 65)=3.638, p=0.061 | F(1, 65)=3.208, p=0.029 | F(1, 65)=0.543, p=0.464 | F(1, 65)=22.682, p<0.001 | F(1, 65)=7.882, p=0.007 | F(1, 65)=5.912, p=0.018 |
| LDT-latency to lit zone | M | F(1, 44)=1.906, p=0.172 | F(1, 44)=1.014, p=0.396 | F(1, 44)=3.848, p=0.056 | Na | | |
| | F | One way: F(2, 21)=1.466, p=0.254 | | | | | |
| | M & F | F(1, 65)=3.992, p=0.050 | F(1, 65)=2.263, p=0.089 | F(1, 65)=4.877, p=0.031 | F(1, 65)=0.975, p=0.327 | F(1, 65)=0.513, p=0.477 | F(1, 65)=1.923, p=0.170 |
| LDT-distance travelled | M | F(1, 44)=0.002, p=0.969 | F(1, 44)=0.317, p=0.813 | F(1, 44)=1.096, p=0.301 | Na | | |
| | F | One way: F(2, 21)=1.644, p=0.217 | | | | | |
| | M & F | F(1, 65)=0.169, p=0.682 | F(1, 65)=0.838, p=0.478 | F(1, 65)=0.966, p=0.329 | F(1, 65)=37.169, p<0.001 | F(1, 65)=0.020, p=0.888 | F(1, 65)=0.864, p=0.356 |
| LDT-velocity | M | F(1, 44)=0.005, p=0.944 | F(1, 44)=0.986, p=0.408 | F(1, 44)=2.253, p=0.140 | Na | | |
| | F | One way: F(2, 21)=0.493, p=0.618 | | | | | |
| | M & F | F(1, 65)=0.118, p=0.733 | F(1, 65)=1.440, p=0.239 | F(1, 65)=2.003, p=0.162 | F(1, 65)=28.429, p<0.001 | F(1, 65)=0.383, p=0.538 | F(1, 65)=0.338, p=0.563 |
| EPM-open arm time | M | F(1, 46)=1.131, *p*=0.293 | F(1, 46)=0.606, *p*=0.660 | F(1, 46)=0.191, *p*=0.664 | Na | | |
| EPM-closed arm time | | F(1, 48)=0.800, *p*=0.376 | F(1, 48)=0.495, *p*=0.740 | F(1, 48)=2.039, *p*=0.160 | | | |
| EPM-time in centre | | F(1, 46)=0.022, *p*=0.884 | F(1, 46)=0.644, *p*=0.634 | F(1, 46)=5.051, *p*=0.029 | | | |
| EPM-latency to open | | F(1, 48)=1.855, *p*=0.180 | F(1, 48)=2.068, *p*=0.100 | F(1, 48)=0.244, *p*=0.624 | | | |
| EPM-latency to closed | | F(1, 48)=8.580, *p*=0.005 | F(1, 48)=1.996, *p*=0.110 | F(1, 48)=0.032, *p*=0.85 | | | |
| EPM-distance travelled | | F(1, 48)=8.549, *p*=0.005 | F(1, 48)=0.507, *p*=0.731 | F(1, 48)=0.230, *p*=0.634 | | | |
| EPM-velocity | | F(1, 48)=3.568, *p*=0.065 | F(1, 48)=0.645, *p*=0.633 | F(1, 48)=0.286, *p*=0.595 | | | |

| | | | | | |
|---|---|---|---|---|---|
| EPM-time spent stationary | | F(1, 44)=0.605, *p*=0.441 | F(1, 44)=1.682, *p*=0.171 | F(1, 44)=0.017, *p*=0.897 | |
| Y-maze-time in novel | | F(1, 14)=0.689, *p*=0.421 | F(1, 14)=2.784, *p*=0.068 | F(1, 14)=2.583, *p*=0.130 | |
| Y-maze-time in open | | F(1, 14)=0.063, *p*=0.805 | F(1, 14)=0.857, *p*=0.513 | F(1, 14)=3.214, *p*=0.095 | |
| Y-maze-time in start | | F(1, 14)=2.327, *p*=0.149 | F(1, 14)=2.029, *p*=0.145 | F(1, 14)=0.491, *p*=0.495 | |
| Y-maze-time in centre | | F(1, 14)=2.752, *p*=0.119 | F(1, 14)=2.370, *p*=0.101 | F(1, 14)=1.203, *p*=0.291 | |
| Y-maze-distance travelled | | F(1, 14)=0.875, *p*=0.365 | F(1, 14)=1.804, *p*=0.184 | F(1, 14)=0.001, *p*=0.971 | |
| Y-maze-entries into novel | | F(1, 14)=3.728, *p*=0.074 | F(1, 14)=2.811, *p*=0.066 | F(1, 14)=0.076, *p*=0.787 | |
| Y-maze-entries into open | | F(1, 14)=0.179, *p*=0.678 | F(1, 14)=0.552, *p*=0.701 | F(1, 14)=0.179, *p*=0.678 | |
| Y-maze-latency to novel | | F(1, 14)=1.727, *p*=0.210 | F(1, 14)=2.172, *p*=0.125 | F(1, 14)=0.064, *p*=0.804 | |
| Y-maze-latency to open | | F(1, 14)=0.262, *p*=0.617 | F(1, 14)=0.948, *p*=0.466 | F(1, 14)=0.609, *p*=0.448 | |
| Y-maze-complete alternations | | F(1, 14)=0.000, *p*=1.000 | F(1, 14)=0.263, *p*=0.897 | F(1, 14)=1.207, *p*=0.290 | |
| Y-maze-indirect are revisits | | F(1, 14)=0.241, *p*=0.631 | F(1, 14)=1.828, *p*=0.179 | F(1, 14)=2.172, *p*=0.163 | |
| Y-maze-spontaneous alternation | | F(1, 14)=0.045, *p*=0.835 | F(1, 14)=3.256, *p*=0.044 | F(1, 14)=5.714, *p*=0.031 | |

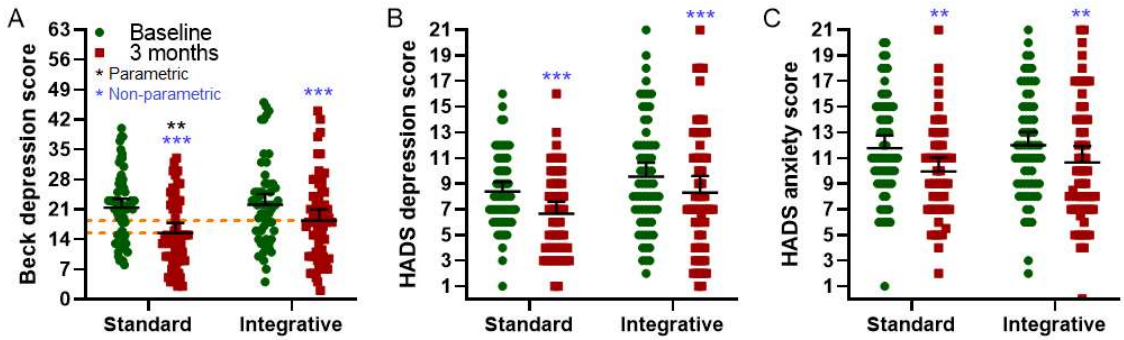
**Appendix B (SERT clustering in blood smears from human depression patients)**

This dissertation provides evidence that reelin normalizes stress-induced increases in the size of SERT clusters on the membrane of blood lymphocytes. This is interesting because depressed patients have larger SERT cluster sizes, much like CORT-treated rats and reelin-deficient mice (Rivera-Baltanas et al 2010, 2012, Romay-Tallon et al 2018). It could be that a lack of plasma reelin results in abnormal MPC parameters considering that reelin regulates membrane protein trafficking and depression patients have lower plasma reelin levels (Fatemi et al 2001, Groc et al 2007, Santana & Marzolo 2017, Strasser et al 2004). Moreover, based on our previous findings that suggest SERT MPC parameters could be used to predict treatment responsiveness to conventional antidepressant pharmacotherapy over an eight-week period, we propose that analyzing the disruption SERT clusters could be used as a putative biomarker of therapeutic outcomes in naïve depression patients (Caruncho et al 2019, Rivera-Baltanas et al 2015, Rivera-Baltanas et al 2012). This suggests that behavioral responsiveness to antidepressants may depend on the normalization of SERT cluster size. Similarly, our team also has preliminary findings that show SERT cluster size predicts treatment responsiveness to repeated transcranial magnetic stimulation (not yet published).

Our group has been receiving blood samples from depression patients from a clinic in Saskatoon, Canada, as part of the Neural Health Project (see www.neuralhealthproject.com for more information). Patients were recruited to receive either a standard conventional antidepressant medication (N=66) or an integrative treatment plan that was designed to include all aspects of the individual's life, with an emphasis on diet, exercise, pharmacotherapy, and the psychotherapeutic relationship between the clinician and patient (N=64). Measures of depression (Beck's inventory and HADS depression) and anxiety (HADS anxiety) were recorded before a patient started their respective treatment and 3 months later. Additional follow up time points are still being analyzed.

Repeated measures ANOVAs were used to determine whether there were effects of time and treatment on each dependent variable. The statistical information can be found in Appendix table 1. Tukey post hoc tests were used to evaluate individual group differences and I found that there was no significant difference between treatment groups in scores of depression/anxiety ($p \geq 0.351$) or SERT MPC parameters ($p \geq 0.526$) at baseline (see Appendix B Figure 1 and 2). Patients in the standard group had significantly lower Beck scores after 3 months of treatment ($p=0.003$) which was not true for the patients in the integrative group

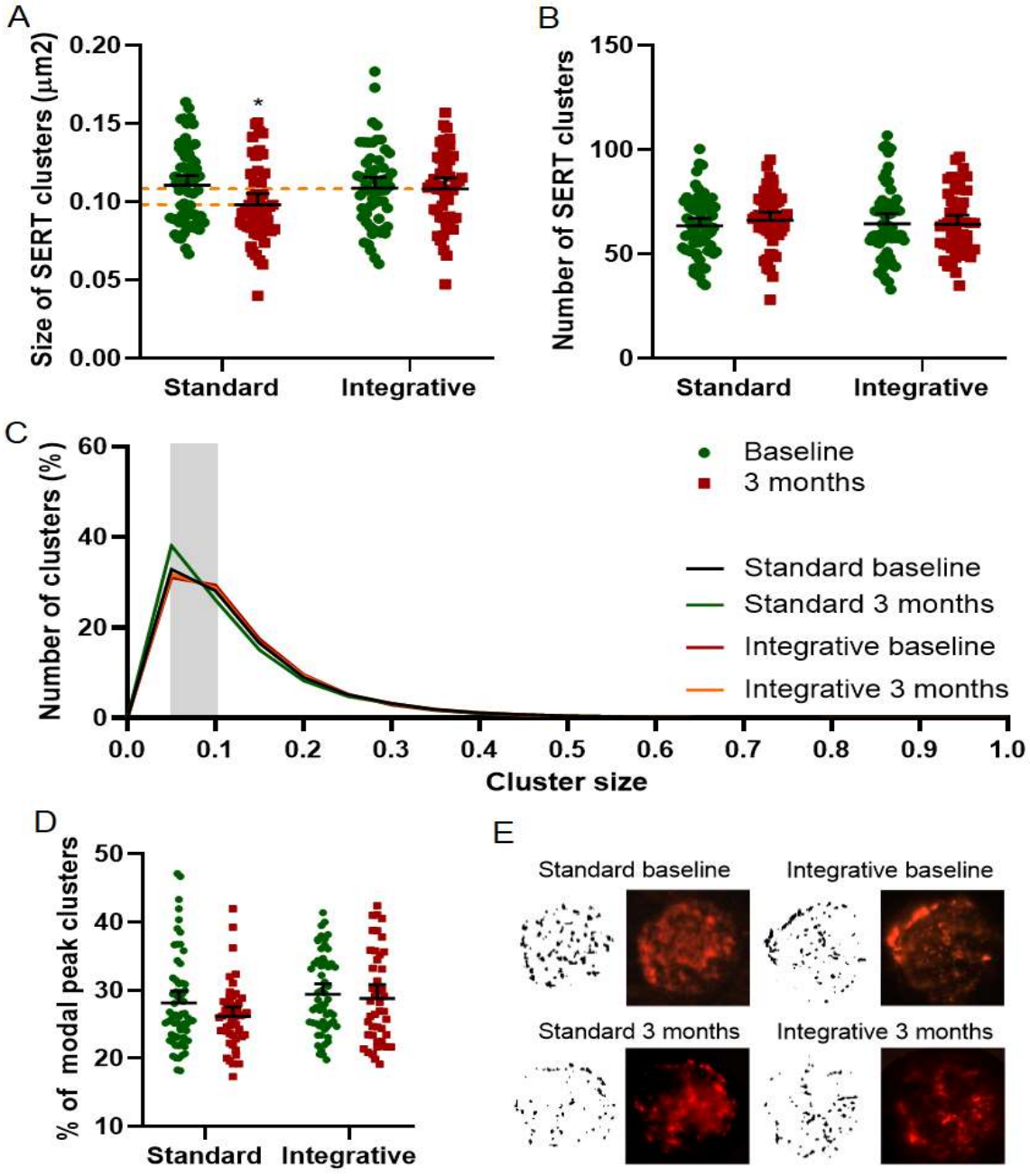
($p$=0.126). There were no significant group differences for HADS depression (a $p$-value of 0.097 was found when comparing those in the standard care group over time and 0.315 for the integrative group) or HADS anxiety (a $p$-value of 0.107 was found when comparing those in the standard care group over time and 0.298 for the integrative group). The scores of standard- and integrative-treated patients were not significantly different from one another after 3 months ($p$>0.05). However, considering that rating scale scores are not continuous variables, I also analyzed the data with non-parametric Friedman's tests followed by Wilcoxon signed ranks paired tests that revealed that standard- (Beck: w=4.97, $p$<0.001; HADS depression: w=3.57, $p$<0.001; HADS anxiety: w=2.81, $p$=0.005) and integrative-treated (Beck: w=4.00, $p$<0.001; HADS depression: w=3.59, $p$<0.001; HADS anxiety: w=3.30, $p$=0.001) patients had significant reductions in depression scores after 3 months. However, Mann Whitney U independent samples tests were used to compare the differences in depression/anxiety scores between treatment groups at baseline (U≤1.29, $p$≥0.196) or at the 3 months timepoint (U≤1.54, $p$≥0.125), which were similar between treatment conditions.




**Appendix B Figure 1. Depression and anxiety rating scale scores for patients at baseline and after 3 months.** There were no group differences at baseline, and only those in the standard treatment group had significant reductions in Beck depressions cores 3 months later (A). There was no significant effect of time or treatment on HADS depression (B) or anxiety (C) scores. Data are expressed as mean±CI. **$p$<0.01/***$p$<0.001 vs baseline comparison. Figure created with GraphPad Prism by author.

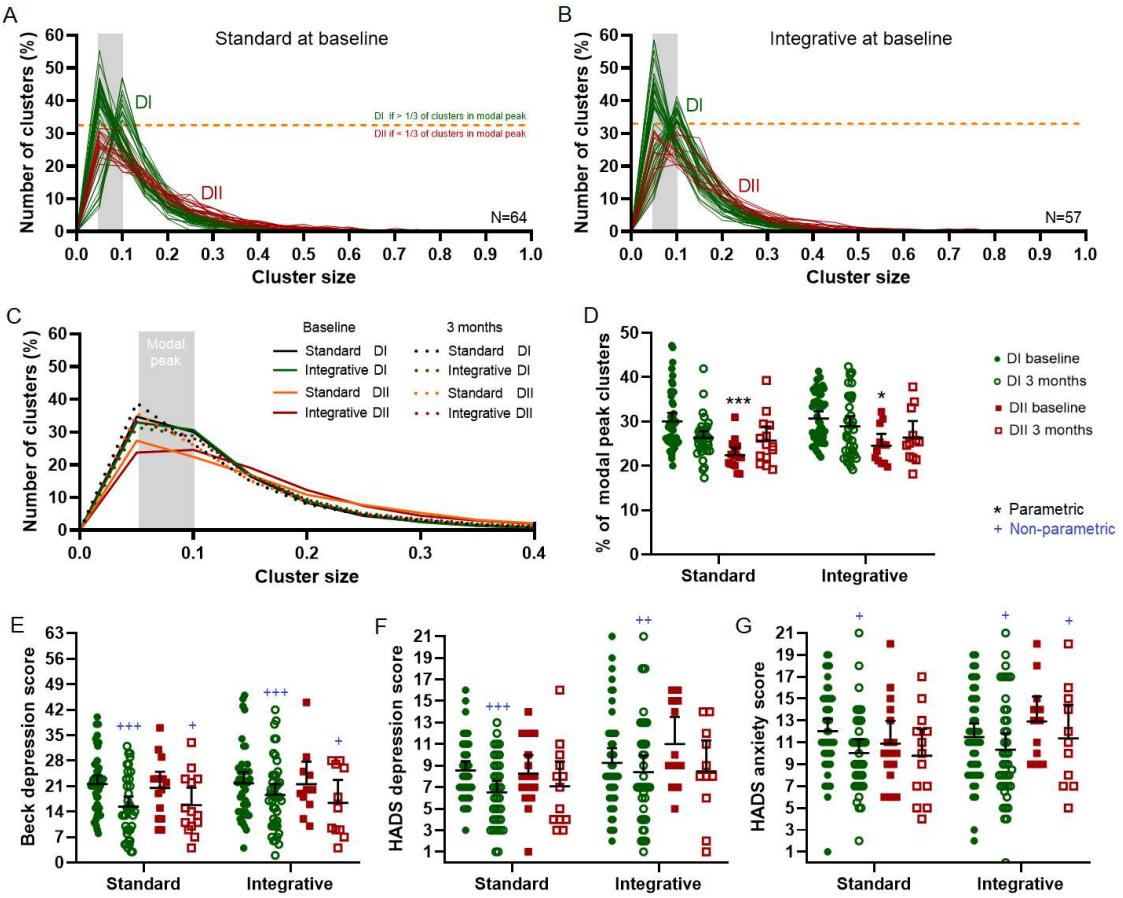
When looking at the effect of time and treatment condition on SERT clustering parameters, I found that there were no group differences in the number of SERT clusters but the size of SERT clusters was significantly reduced after 3 months of standard treatment ($p$=0.045) but not integrative treatment, which corroborates our hypothesis that a reduction in SERT cluster size is associated with greater reductions in depression (Appendix B Figure 2A/B). The shift in SERT cluster size after 3 months of standard treatment is evident in Appendix B Figure 2C

which shows that a larger percentage of clusters were sized in the 0-0.05µm$^2$ range over time. That said, there were no group differences regarding the percentage of model peak SERT clusters (Appendix B Figure 2D).




**Appendix B Figure 2. SERT clustering parameters at baseline and after 3 months.** A) Standard treatment reduced the size of SERT clusters. B) There was no effect of treatment on the number of clusters. C) The average distribution of SERT clusters by size. E) Representative images of SERT MPC in blood lymphocytes and the binary image produced by ImageJ. Data are expressed as mean±CI. *$p$<0.05 vs standard at baseline. Figure created with GraphPad Prism by author.

We have previously shown that plotting the distribution of SERT clusters by size allows one to differentiate 2 subpopulations of patients according to the percentage of clusters within the model peak (Rivera-Baltanas et al 2012). More specifically, we saw that around 75% of patients have ~40% of their SERT clusters in the model peak size range of 0.05-0.1$\mu m^2$ (like controls), while the other 25% had larger clusters with ~25% of them in the modal peak. We referred to the group of patients with a greater percentage of clusters in the model peak as DI and those with larger clusters (fewer within the modal peak) as DII. Here I categorized the patients in a consistent manner, assigning patients to the DII group if under 33% of their clusters fell into the 0.05 to 0.1$\mu m^2$ range (see Appendix B Figure 3A/B). As expected, those in the DII group had significantly less clusters in the modal peak than DI at baseline ($p \leq 0.015$), but not after 3 months of standard or integrative care. I then compared the differences in depression scores between the DI and DII subgroups to evaluate if SERT clustering parameters can be used to predict patient outcomes. With parametric analyses, there were no significant group differences in depression scores between standard and integrative treatment conditions, over time in the same treatment condition, or between DI and DII in the same treatment condition. That said, non-parametric analyses revealed that Beck scores were decreased after 3 months regardless of treatment condition or D-classification ($p \leq 0.038$). Reductions in HADS depression scores with time were similar across DI and DII but came out significant for the standard ($p<0.001$) and integrative ($p=0.002$) DI group. This does seem to suggest that smaller cluster sizes, which is brought about by peripheral reelin, is associated with symptom attenuation. HADS anxiety scores were also significantly decreased by standard ($p=0.012$) and integrative ($p=0.010$) treatment in DI-classed patients and DII-integrative-patients ($p=0.039$). However, there were no group differences between DI or DII.



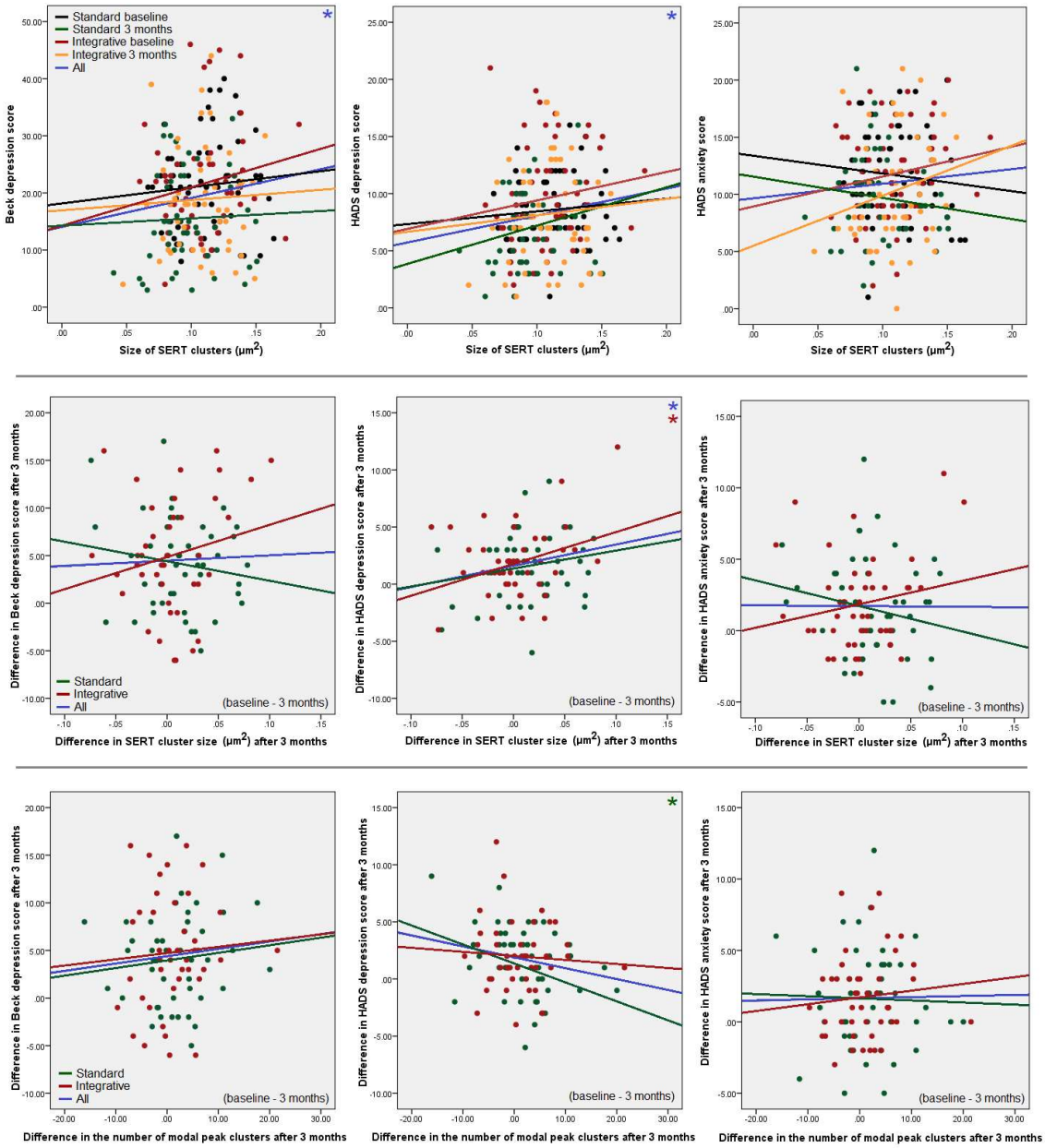

**Appendix B Figure 3. The distribution of SERT clusters predicts treatment responsiveness.** A/B) Two groups of depression patients can be distinguished based on the distribution of SERT cluster size. C/D/E) DI-classed patients had greater reductions in depression scores. F/G) Standard treatment had a larger effect on the distribution of SERT clusters than integrative treatment. Data are expressed as mean±CI. *$p$<0.05/***$p$<0.001 vs same-treatment DI baseline; +$p$<0.05 vs same-treatment baseline. Figure created with GraphPad Prism by author.

Finally, I investigated the correlative relationships between SERT cluster size and scores of depression and anxiety. No significant correlations were found for Beck and HADS depression scores when the groups were analyzed individually, but depressions were more severe in those with larger SERT cluster sizes when the groups were combined (represented by the blue line in Appendix B Figure 4; see Appendix B Table 1 for statistical information). There were no significant correlations for HADS anxiety and SERT cluster size. I also evaluated the relationship between the difference in depression scores (baseline score subtracted from 3-month score) and the difference in SERT cluster size and the number of modal peak clusters (baseline size subtracted from 3-month size). I found that the reduction in HADS depression

scores was correlated with reductions in SERT cluster size in those that received integrative therapy or when both groups were included in the analyses, and with increases in the number of clusters in the modal peak range of 0.05 to 0.1$\mu m^2$ in standard-treated patients.

**Appendix B Table 1. Statistical information for depression/anxiety scores and SERT clustering parameters.**

| Measure | Statistics | Time | Treatment | Time x treatment | DI/DII | DI/DII by time | DI/DII by treatment |
|---|---|---|---|---|---|---|---|
| Beck | Parametric | F(1, 103)=56.88, $p$<0.001 | F(1, 103)=2.21, $p$=0.140 | F(1, 103)=0.004, $p$=0.948 | na | | |
| | Non-parametric | Fr=174.79, $p$<0.001 | | | | | |
| HADS depression | Parametric | F(1, 104)=31.27, $p$<0.001 | F(1, 103)=5.31, $p$=0.023 | F(1, 104)=0.014, $p$=0.908 | | | |
| | Non-parametric | Fr= 161.75, $p$<0.001 | | | | | |
| HADS anxiety | Parametric | F(1, 104)=22.73, $p$<0.00 | F(1, 104)=0.704, $p$=0.403 | F(1, 104)=0.01, $p$=0.916 | | | |
| | Non-parametric | Fr= 165.62, $p$<0.001 | | | | | |
| SERT size | Parametric | F(1, 90)=3.62, $p$=0.060 | F(1, 90)=0.88, $p$=0.352]. | F(1, 90)=2.87, p=0.094 | | | |
| SERT number | | F(1, 90)=0.49, $p$=0.486 | F(1, 90)=0.13, $p$=0.716 | F(1, 90)=0.96, $p$=0.329 | | | |
| % of modal peak clusters | | F(1, 90)=0.614, $p$=0.435 | F(1, 90)=4.213, $p$=0.043 | F(1, 90)=0.614, $p$=0.435 | | | |
| DI/DII % of modal peak clusters | | F(1, 88)=0.142 $p$=0.708 | F(1, 88)=2.186, $p$=0.143 | F(1, 88)=0.531, $p$=0.468 | F(1, 88)=8.092, $p$=0.006 | F(1, 88)=16.182, $p$<0.001 | F(1, 88)=0.000, $p$=0.983 |
| DI/DII Beck | | F(1, 95)=48.928, $p$<0.001 | F(1, 95)=1.171, $p$=0.282 | F(1, 95)=1.716, $p$=0.193 | F(1, 95)=0.188, $p$=0.665 | F(1, 95)=0.439, $p$=0.509 | F(1, 95)=0.045, $p$=0.832 |
| | Non-parametric | Fr=263.789, $p$<0.001 | | | | | |
| DI/DII HADS depression | Parametric | F(1, 97)=24.319, $p$<0.001 | F(1, 97)=5.141, $p$=0.026 | F(1, 97)=0.657, $p$=0.420 | F(1, 97)=0.244, $p$=0.622 | F(1, 97)=0.149, $p$=0.701 | F(1, 97)=0.159, $p$=0.691 |
| | Non-parametric | Fr=247.799, $p$<0.001 | | | | | |
| DI/DII HADS anxiety | Parametric | F(1, 97)=15.801, $p$<0.001 | F(1, 97)=2.139, $p$=0.147 | F(1, 97)=0.042, $p$=0.838 | F(1, 97)=0.035, $p$=0.853 | F(1, 97)=0.056, $p$=0.814 | F(1, 97)=1.648, $p$=0.202 |
| | Non-parametric | Fr=256.820, $p$<0.001 | | | | | |




**Appendix B Figure 4. Correlations between depression/anxiety scores, SERT cluster size, and the number of modal peak clusters.** Significant correlations are indicated by asterisk symbols (*) with color-coordinated lines of best fit (*$p$<0.05). Figure created with SPSS and Paint by author.

**Appendix B Table 2. Correlative statistical information for depression/anxiety scores and SERT cluster size.**

| Absolute scores | | SERT cluster size | |
|---|---|---|---|
| Beck | Baseline standard | r=0.090, *p*=0.489, n=62 | |
| | Baseline integrative | r=0.165, *p*=0.237, n=53 | |
| | 3 months standard | r=0.064, *p*=0.660, n=49 | |
| | 3 months integrative | r=0.043, *p*=0.772, n=49 | |
| | All | r=0.138, *p*=0.044, n=213* | |
| HADS depression | Baseline standard | r=0.096, *p*=0.458, n=62 | |
| | Baseline integrative | r=0.137, *p*=0.309, n=57 | |
| | 3 months standard | r=0.200, *p*=0.174, n=48 | |
| | 3 months integrative | r=0.061, *p*=0.682, n=47 | |
| | All | r=0.153, *p*=0.025, n=214* | |
| HADS anxiety | Baseline standard | r=-0.072, *p*=0.579, n=62 | |
| | Baseline integrative | r=0.154, *p*=0.253, n=57 | |
| | 3 months standard | r=-0.122, *p*=0.408, n=48 | |
| | 3 months integrative | r=0.220, *p*=0.138, n=47 | |
| | All | r=0.078, *p*=0.255, n=219 | |
| **Difference in scores (baseline – 3 months)** | | **Difference in SERT size (baseline – 3 months)** | **Difference in modal peak clusters (baseline – 3 months)** |
| Beck | Standard | r=-0.152, *p*=0.306, n=47 | r=0.124, *p*=0.410, n=46 |
| | Integrative | r=0.221, *p*=0.159, n=42 | r=0.077, *p*=0.633, n=41 |
| | All | r=0.035, *p*=0.748, n=89 | r=0.095, *p*=0.381, n=87 |
| HADS depression | Standard | r=0.168, *p*=0.259, n=47 | r=-0.317, *p*=0.032, n=46* |
| | Integrative | r=0.303, *p*=0.043, n=45 | r=-0.063, *p*=0.685, n=44 |
| | All | r=0.223, *p*=0.035, n=92 | r=-0.204, *p*=0.053, n=90 |
| HADS anxiety | Standard | r=-0.162, *p*=0.277, n=47 | r=-0.034, *p*=0.824, n=46 |
| | Integrative | r=0.190, *p*=0.211, n=45 | r=0.063, *p*=685, n=44 |
| | All | r=-0.007, *p*=0.948, n=92 | r=0.018, *p*=0.864, n=90 |

These data support our previous reports in that SERT cluster sizes are reduced by antidepressant medications, although contrary to what one might expect, this was only true in patients that received 3 months of standard pharmacotherapy and not integrative therapy. This is interesting since those in the standard-treated group had larger reductions in Beck depression scores, suggesting that a relationship exists between the size of SERT clusters and mood disturbance. The distribution of SERT cluster size was normalized after 3 months of treatment, but in these conditions, we did not see patients with a lower number of SERT clusters in the modal peak respond better to pharmacotherapy. That said, we are still analyzing later timepoints which may uncover differences in psychometric scores between standard and integrative care past the 3 months timepoint, where DI and DII classifications may be more predictive. One possible explanation as to why our findings differ between reports could be related to the severity of depression. Here, many of the psychometric scores would be considered mild even at baseline. In addition, previously treatment-naïve patients were recruited whereas the patients in the present study had co-morbidities and were taking different

types of medications. In any case, these data highlight the need for more effective treatments that have superior response rates than conventional medications that work slowly and in a limited number of patients. SERT MPC may predict depression scores in some patients, but a panel of biomarkers should be developed to provide clinicians with more accurate methods to guide treatment outcomes. The therapeutic time-lag that is associated with antidepressants is extremely impractical and identifying patients as responders or non-responders with the use of biomarkers would allow clinicians to deliver personalized treatments which could quicken remission in responders or save treatment-resistant patients months of unnecessary suffering.