The crucial role of reelin in a preclinical model of depression

Josh Allen (MSc)

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University of Saskatchewan
Saskatoon

Supervisors

Dr Hector J. Caruncho

Dr Lisa E. Kalynchuk

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University of Saskatchewan

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Abstract

Depression is a complex psychiatric disorder characterized by a cyclical disease course with repeated episode relapses. Rats treated for 21 days with corticosterone (CORT), a well characterized model of depression, exhibit a depression-like phenotype. Additionally, CORT treated rats have shown a downregulation in reelin. Reelin is a large glycoprotein that has been implicated in many psychiatric illnesses. Reelin is involved in stimulating the growth of dendrites, guiding neuronal migration, and regulating synaptogenesis.

The goal of these experiments was to investigate the involvement of reelin in depression using the CORT model. In the first experiment, reelin downregulation in an animal model of recurrent depression was assessed using chronic and intermittent CORT administration (three cycles, each one followed by a recovery period), and whether there was a correlation between reelin expression in the dentate gyrus (DG) subgranular zone (SGZ) and depression-like behaviour, namely immobility in the forced swim test (FST). In the second experiment, the potential antidepressant effect of reelin (at either 3μg or 5μg either every 5 or 10 days) via lateral tail vein injections on depressive-like behaviour and reelin expression in the SGZ was assessed in the CORT model, and the correlation between reelin downregulation and immobility was again investigated.

CORT produced an increase in depression-like behaviour in both experiments. In the first experiment, FST immobility of rats that received CORT recovered to baseline level after the first cycle, but did not recover after the third. Reelin was downregulated in the SGZ after CORT injections at all time points in the first cycle, and this downregulation was more pronounced in cycle three. As hypothesized, increased FST immobility was negatively correlated with decreased number of reelin-positive cells. When administered reelin, the deleterious CORT-induced behavioural and neurobiological effects were normalised.

These studies provide evidence that repeated and intermittent CORT treatment can be used as an animal model of recurrent depression. Furthermore, they reinforce the idea that reelin downregulation is implicated as an important neurochemical event underlying the depressive-like phenotype, and show for the first time that peripheral reelin has antidepressant or neuroprotective effects in the brain, although the mechanism of action is not yet known.

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Glossary of abbreviations

5HT	5-hydroxytryptamine (serotonin)
5HTT	Serotonin Transporter
5HTTLPR	Serotonin-Transporter-Linked Polymorphic Region
ACTH	Adrenocorticotrophic Hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	Brain-Derived Neurotrophic Factor
CA	Cornu Ammonis
cAMP	Cyclic Adenosine Monophosphate
CMS	Chronic Mild Stress
CNS	Central Nervous System
CORT	Corticosterone
CRF	Corticotropin-Releasing Factor
CSF	Cerebrospinal Fluid
CVS	Chronic Variable Stress
DA	Dopamine
DCX	Doublecortin
DG	Dentate Gyrus
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, 5th Ed.
DST	Dexamethasone Suppression Test
EC	Entorhinal Cortex
ECT	Electroconvulsive Therapy
EPM	Elevated Plus Maze
FST	Forced Swim Test
GABA	*Gamma*-Aminobutyric Acid
GCL	Granule Cell Layer
GWAS	Genome-Wide Association Studies
HPA	Hypothalamic-Pituitary-Adrenal
HPT	Hot Plate Test

IHC	Immunohistochemistry
IL	Interleukin
i.p.	Intraperitoneal
L-E	Long-Evans
MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase-Inhibitor
MDD	Major Depressive Disorder
MDMA	Methylenedioxymethamphetamine
MWM	Morris Water Maze
NA	Noradrenaline
NET	Norepinephrine (Noradrenaline) Transporter
NMDA	N-Methl-D-Asparate
NRI	Noradrenaline Reuptake Inhibitors
NSF	Novelty Suppressed Feeding
OFT	Open Field Test
PVN	Paraventricular Nucleus
RR	Reelin Repeats
s.c.	Subcutaneous
S-D	Sprague-Dawley
SDS	Social Defeat Stress
SEM	Standard Error of the Mean
SERT	Serotonin Transporter
SFKs	Src Family of Non-Receptor Tyrosine Kinases
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

SVZ	Subventricular Zone
TCA	Tricyclic Antidepressant
TFT	Tail Flick Test
TST	Tail Suspension Test
Val^{66}Met	Valine substituted by Methionine in Codon 66
WST	Wire Suspension Test

Specific aims and research goals

The general purpose of this research was to assess the role of reelin in a preclinical model depression. Depression is the most common neuropsychiatric disorder characterized by depressed mood and anhedonia and patients suffering from depression typically experience repeated episode relapses (Kendler et al., 2000), which may be due to the individual becoming more sensitized to stress (Kendler et al., 1999; Morris et al., 2010). To understand the contributing mechanisms leading to the pathology of depression, a large amount of research has focused on the hippocampus. Depressed patients have continuously been found to have a decreased hippocampal volume (Bremner et al., 2000; Sheline et al., 2003; Videbech & Ravnkilde, 2004). Our laboratory has previously shown that chronic CORT treatment over one 21-day cycle decreases the number of reelin-immunopositive cells in the SGZ, where adult neurogenesis occurs, which closely parallels the onset of a depressive-like phenotype (Lussier et al., 2009, 2013a). Furthermore, the CORT-induced decrease in the number of reelin-positive cells was reversed with daily imipramine treatment (Fenton et al., 2015). In addition, heterozygous reeler mice with 50% normal levels of reelin are more vulnerable to the depressogenic effects of CORT treatment, suggesting that reelin could have neuroprotective properties (Lussier et al., 2011). Reelin is necessary for the guiding and positioning of migrating neurons (D'Arcangelo, 2014), the acquisition of LTP, synaptogenesis, and dendritic morphology (Rogers et al., 2013).

In the first experiment outlined in this thesis, the aim was to replicate the episodic nature of depression in an animal model utilising chronic and intermittent CORT injections to assess whether depressive-like behaviour is correlated with a downregulation of reelin-positive cells in the SGZ, and if animals sensitized to stress. The worsening of recovery over subsequent cycles will also be investigated. Only one study to date has been published attempting to mimic

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the cyclical nature of depression using the CORT model in which rats seemed to sensitize to stress (Lebedeva et al., 2017), and so we aim to build on these results adding an additional cycle and using naive animals for behavioural testing. There is a need for future research to study the neurobiological mechanisms of stress sensitization the study previously mentioned demonstrates. Studying recurrent models of depression would allow us to better understand the behavioural and neurobiological alterations produced by such cyclical treatment that may contribute to episode relapse in human patients.

We aimed to answer the following research question: is the decrease in recovery after subsequent cycles of chronic and intermittent CORT treatment paralleled with the downregulation of hippocampal reelin? This led us to hypothesize that subsequent cycles of CORT injections will diminish recovery. That is, depressive-like behaviour will return to baseline levels after the first recovery period, but will not after the third. Furthermore, animals will sensitize to stress and depressive-like behaviour is paralleled with the downregulation of reelin in the SGZ.

Only about 60-70% of depressed patients taking antidepressants currently on the market are responders (Trivedi et al., 2006; Thase et al., 2001; Crown et al., 2002) and therapeutic effects take weeks to develop (Stahl, 2000). Therefore, it is important that newer fast-acting antidepressants become available. To investigate the potential antidepressant properties of the reelin protein, our laboratory infused reelin into the hippocampus and found that FST behaviour was normalised in rats treated with CORT. For this reason, the goal of the experiment outlined in chapter 3 was to assess if peripheral reelin injections into the lateral tail vein would have antidepressant effects in the CORT model of depression. Specifically, if the injection of exogenous reelin reduces the time spent immobile in the FST, and whether it can restore endogenous reelin levels in the SGZ. Our research question asks whether peripheral reelin injections into the lateral tail vein has antidepressant effects in the FST in the CORT model of

depression as well as restore CORT-induced decreases in hippocampal reelin levels. From this question two hypotheses were formulated. The first states that peripheral reelin injections reduces time spent immobile in the FST and restores the downregulation of reelin-positive cells in the SGZ. The second states that all doses of reelin would have antidepressant effects.

Chapter 1

Literature Review

1.1 Depression

Major depressive disorder (MDD) is a mood disorder that causes one to experience ongoing depressed mood and anhedonia which can affect thought, behaviour, emotions and overall well-being (American Psychiatric Association, 2013). Sufferers experience increased physical illness, high mortality rates and impaired social functioning, affecting individuals in educational and occupational settings. Family relationships may suffer and depressed persons may experience suicidal ideation and physically harm themselves. To be diagnosed with depression, one must experience at least 5 of the symptoms listed in table 1.1 nearly every day, most of the day, for at least 2 weeks (American Psychiatric Association, 2013). In addition, there should be no history of manic or hypomanic episodes.

Table 1.1. List of symptoms of depression.

Symptoms	Description
Anhedonia	Loss of interest in once pleasurable activities
Depressed mood	Occurs most days, often irritable and agitated
Weight change	Difference in appetite leading to weight loss/gain
Sleeping problems	Insomnia or hypersomnia, walking during the night
Fatigue	Loss of energy, feeling tired
Feelings of guilt/worthlessness	Low self-esteem, inappropriate feelings of guilt
Aches and pains	No physical basis, associated with stress/anxiety
Difficulty concentrating	Find it hard to think and make decisions
Purposeless activity	Increased purposeless activity, slowed movements
Suicidality	Suicidal thoughts, thoughts of death

Adapted from the Diagnostic and Statistical Manual of mental disorders Fifth Edition (DSM-V) (American Psychiatric Association, 2013).

Depression is the most common psychiatric disorder and incidence rates have risen every year for more than 50 years. It is hard to estimate the number of people who suffer from depression as only half of the population who meet diagnosable criteria pursue treatment. It is estimated that more than 300 million people are sufferers worldwide (World Health

Organisation, 2017). Depression is the "second leading source of disease burden, surpassing cardiovascular diseases, dementia, lung cancer, and diabetes" (Merikangas et al., 2002). By 2020, depression is expected to be the second cause of disability worldwide for individuals of all ages (Lopez-Leon et al., 2008). Women are at least twice as more likely to suffer from depression than men (Nolen-Hoeksema, 2001; Nestler et al., 2002; Parker & Brotchie, 2010). In the United States, depression costs approximately $33 billion per year (Crown et al., 2002). In Canada, a 2012 survey revealed that past-year prevalence rates were 4.7%, which was also found 10 years earlier (Patten et al., 2015). Past-year prevalence rates of MDD ranged from 0.3% in the Czech Republic to 4.5% in Mexico, 5.2% in West Germany and 10% in the United States (Kessler & Bromet, 2013). Most countries have average life-time prevalence rates of around 8-12%. People are most likely to experience depressive episodes around age 35, but there is also a smaller peak in incidence rates at around age 55 (Eaton et al., 1997). The risk of developing depression is increased in the presence of neurological conditions such as stroke, epilepsy and neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (Rickards, 2005). Depression is associated with suicide which is now the third largest cause of death in the western world, with incidence rates highest among individuals aged 15-24 years old (Wong & Licinio, 2001).

Although there are many treatments available for depression, all have limitations. Selective serotonin (5-hydroxytryptamine, 5HT) reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) are the most commonly prescribed treatments, and remission rates with these classes of antidepressants remain low. For example, it has been reported that remission rates are only 30-40% at best, when full compliance of treatment is present (Trivedi et al., 2006; Thase et al., 2001). Crown et al. (2002) stated that at least 20% of patients do not respond satisfactorily to treatment. It has also been said that antidepressants can paradoxically cause suicidal thoughts, along with many other side-effects (Garland et al., 2009; Nischal et al.,

2012); the main reason for discontinuation. Another limitation of current antidepressants is their delayed therapeutic effects. Although the pharmacological effects of SSRIs are exerted in a matter of hours, a therapeutic effect is usually not seen for 3 weeks after chronic treatment (Culig et al., 2017), which could be troublesome in patients with suicidal tendencies (Stahl, 2000). The delayed therapeutic effect is under investigation, but is thought to be a result of the time it takes to increase neurogenesis in the hippocampus.

1.1.1 Aetiology of Depression

The exact pathophysiology underlying the aetiology of depression has not yet been defined, however, several predisposing factors have been recognized. Genotype, for example, has been shown to be an important factor. Although "nature" is said to predispose an individual to depression, it is essentially "nurture" that is accountable for the development of the disorder. Endogenous depression is a term used to describe depression with a familial pattern. Reactive depression, on the other hand, is caused by external factors such as the death of a loved one, divorce or stress in the workplace etc. Psychological stress is often reported prior to the onset of mood disorders, and early adverse life events are associated with the onset of depression later in life. The biological consequences of stress are to activate the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. Successful antidepressant treatment normalises the dysregulation of biological responses in depressed patients and therefore several hypotheses of the aetiology of depression that include chronic stress have been suggested. A combination of one's environment (Shapero et al., 2014; Weiss et al., 1999) and genetics (Sullivan et al., 2000), as well as neurochemical (Delgado, 2000) neuroimmune and neuroendocrine (Gibbons & McHugh, 1962) factors are all elements that may be responsible for the expression of the disorder.

The influence that chronic stress and adverse life events have on the aetiology of depression has been studied extensively. One's ability to cope with stressful situations is important as stress can leave individuals more vulnerable. Adverse life events occurring in early childhood, such as emotional abuse, leaves individuals more susceptible to developing depression later in life (Shapero et al., 2014). Childhood sexual abuse is another early stressor and occurs more often in women than men (Weiss et al., 1999; Cheasty et al., 1998). Neglect, bereavement, physical abuse and being treated worse than siblings are all childhood adversities (Lindert et al., 2014). Adversities in adulthood include losing a job, death of a loved one, harassment, debt etc. In some cases, social factors also play a role in the development of depression. Living in an urban area can increase vulnerability (Weaver et al., 2015), as well as divorce (Schiavone et al., 2015) and low income. Depression has a negative effect on employment status. In a 28-month long study by Fournier et al. (2015), antidepressants and cognitive therapy were shown to increase full-time employment, by 71% and 89% respectively. Social isolation can have a negative effect on emotions. This is also true in animals. Rodents for example find social interactions rewarding and increased HPA activity is associated with social isolation (Weintraub et al., 2010).

Studies have shown that 20-45% of mild depression is due to heritability (Lopez-Leon et al., 2008; Sullivan et al., 2000). First-degree relatives of MDD patients are 3 times more likely to develop the disorder (Lesch, 2004). Researchers investigating genetic loci have had little success identifying specific genes associated with the disorder, as have Genome-Wide Association Studies (GWAS) searching for mutations leading to polymorphisms (Power et al., 2013). However, there are some studies identifying specific polymorphisms involved in MDD, such as the biallelic polymorphism of the promotor region in gene solute carrier family 6 member 4 (*SLC6A4*) which codes for the 5HT transporter (5HTT) - linked to an altered stress response (Aas et al., 2012). 5HTT-linked polymorphic region (5HTTLPR) "is composed of a

44-bp insertion or deletion" which either forms the short-allele variant or long-allele variant (Bondy et al., 2000), the former being associated with mood disorders and suicide (Sen et al., 2004; Priess-Groben & Hyde, 2013; Collier et al., 1996). Other polymorphisms have been associated with depression, such as Val[66]Met (in which a valine is substituted by methionine in codon 66), a polymorphism of a single nucleotide in the *BDNF* gene that codes for brain-derived neurotrophic factor (BDNF). In a study by Goodyer et al. (2010), carriers of Val[66]Met were found to have an increased susceptibility to depression and reduced hippocampal volume (Bueller et al., 2006). Copy number variants (CNV) have also been implicated in depression. It was found that deletion CNV were associated with the disorder, as well as CNV deleting protein coding regions, and that duplicated variants were not (Rucker et al., 2013).

It is not known whether it will ever be possible to identify the specific genes involved in depression as it is polygenic and epistatic, which adds to the complexity of studying the disorder. Genotype and environment effect the aetiology of depression but the extent of the interaction is unclear. Twin studies suggest that the influence of genetics might not be direct, but make one more susceptible or sensitive to environmental risk factors (Rice, 2009).

The monoamine hypothesis of depression was proposed over 50 years ago (Schildkraut, 1965), and has since been supported by quite a bit of supporting evidence. Clinicians in the 1950s observed that depression was induced in patients receiving reserpine (now known to diminish monoamine levels by irreversibly blocking vesicular monoamine transporters) to treat hypertension. It was then found that drugs that block the action of monoamine oxidase (MAO), an enzyme that is increased in depressed patients and responsible for the breakdown of monoamines (Meyer et al., 2009), alleviated depressive symptoms and were named MAO-inhibitors (MAOIs). The monoamines are a group of neurotransmitters including 5HT, noradrenaline (NA), dopamine (DA) and adrenaline. 5HT is involved in many functions in the brain including appetite, mood and sleep, whereas NA is involved in the stress response and

concentration – all usually affected in depressed patients. The theory suggests that depression is caused by a lack of monoamine activity in the brain (Delgado, 2000). Therefore, antidepressants alleviate the symptoms of depression by increasing the synaptic levels of the monoamines, mainly 5HT and NA.

Amongst the first antidepressants on the market were TCAs, imipramine being the first developed, and MAOIs. TCAs work by inhibiting the reuptake of 5HT and NA by blocking the 5HT transporter (SERT) and NA (norepinephrine) transporter (NET), respectively. Extracellular concentrations of 5HT and NA are therefore increased. As the neurotransmitters are not being taken up and remain in the synapse, they can repeatedly stimulate postsynaptic receptors. Originally non-selective MAOIs were introduced, inhibiting both MAO isozymes, MAO-A and MAO-B. MAO-A has a substrate preference for 5HT while MAO-B prefers DA. MAO-A inhibitors are therefore more efficacious in treating depression and produce fewer adverse side-effects. The mechanism of action and therapeutic effects of both TCAs and MAO-A inhibitors support the monoamine theory of depression. Because of this, SSRIs, NA reuptake inhibitors (NRIs) and 5HT–NA reuptake inhibitors (SNRIs) have been developed in more recent times as they are more selective for 5HT and NA transporters, respectively, reducing unwanted side-effects.

Although there is a clear role for 5HT and NA in the aetiology of depression, there are some discrepancies. One such flaw in the theory is that the therapeutic effects of antidepressants takes weeks to develop (Stahl, 2000), even though 5HT and NA concentrations are increased by antidepressant drugs almost instantly. Although SSRIs and SNRIs have greater patient compliance rates, due to fewer side-effects, therapeutic efficacy has not improved compared to TCAs and MAOIs; remission rates remaining under 40% (Trivedi et al., 2006). Although evidence is plentiful in proving antidepressants are efficacious, placebos have been shown to be just as therapeutic (Tollefson et al., 1995; Evans et al., 1997; Goldstein et al., 2002;

Perahia et al., 2006). It is also known that drugs of abuse like cocaine, amphetamine and methylenedioxymethamphetamine (MDMA) inhibit monoamine uptake but do not have therapeutic effects.

Post-mortem brain studies have been carried out to investigate monoamine transporter and receptor alterations in depressed patients (Stockmeier & Rajkowska, 2004). Increased presynaptic $5HT_{1A}$ and postsynaptic $5HT_{2A}$ receptor density has been identified, along with decreased 5HT uptake (Furczyk et al., 2013; Stockmeier, 2003; Zanardi et al., 2001; Sargent et al., 2000; Bligh-Glover et al., 2000). Rivera-Baltanas et al. (2014) assessed $5HT_{2A}$ receptor clustering in peripheral lymphocytes via immunocytochemistry (IHC) and found that receptor size and number was higher in depressed patients. Moreover, antidepressant treatment reduced cluster number and size. In addition, SERT density was decreased in post-mortem examinations of dorsolateral prefrontal and ventral/orbitofrontal cortex from depressed suicide victims' (Mann et al., 2000; Austin et al., 2002). Similarly, alterations were seen in NET and noradrenergic receptor density in post-mortem brains from depressed patients (Klimek et al., 1997).

Several neuronal changes have been found in depressed patients including an enlargement in ventricular volume which is seen in roughly 30-40% of patients (Lauer & Krieg, 1998) as well as decreased frontal cortical (Bremner et al., 2002) and amygdala volumes (Hastings et al., 2004; Drevets, 2000). In the dorsolateral prefrontal cortex, smaller soma sizes in the orbitofrontal cortex and anterior cingulate cortex have been reported, which may be linked to altered neurogenesis (Stockmeier & Rajkowska, 2004). Alterations in the hippocampus have been observed, such as neuronal atrophy and decreased volume (Bremner et al., 2000; Sheline et al., 2003). This is important as the hippocampus is largely involved in the neurogenesis process and is affected by alterations such as hypercortisolemia and altered serotonergic transmission (Mintun et al., 2004; Balu & Lucki, 2009).

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1.1.2 Recurrent depression

Patients with MDD typically experience repeated episode relapses over the course of the illness (Hollon et al., 2006). As the number of episodes experienced increases, patients become more susceptible to another. It is thought that patients who have experienced one episode are about 60% more likely to have another, 70% more likely to then have a third and 90% more likely to have a fourth (Kendler et al., 2000). In addition, patients who have experienced a greater number of episodes appear to have more aggravated symptoms that last a greater duration of time (Karp et al., 2004). The likelihood of experiencing a subsequent episode is positively correlated with earlier age of onset of initial depressive symptoms and symptom severity (Pettit et al., 2006). The residual of depressive symptoms after an episode increases relapse susceptibility (Post, 1992). As over 75% of depressed patients experience recurrent depressive episodes, it is now considered a life-long disease, and around 50% of patients with MDD will experience a recurrent chronic condition in which treatment is needed (Crown et al., 2002).

The kindling hypothesis and stress sensitization hypothesis have been put forward in attempt to explain the relationship between stress and depression. This is because the nature of depression changes over time. Although initial major adverse life events usually provoke the onset of depression, for recurring episodes this is not the case. The kindling hypothesis explains that with increasing episode relapses, subsequent episodes can reoccur spontaneously because they gradually become autonomous of stress (Monroe & Harkness, 2005). This hypothesis has supporting evidence which shows that adverse life events do not always predict depressive episodes (Kendler et al., 2000; Kendler et al., 2001).

The stress sensitization hypothesis suggests that repeated episodes lower the threshold of stress that is needed to cause another episode. Human and animal studies support this

hypothesis. For example, patients who have had an episode report another after minor adverse life events (Morris et al., 2010), and one third of recurrent episodes are brought on by minor or non-causal events (Kendler et al., 1999). In rodents, re-exposure to stressful events after a recovery period resulted in a more rapid onset of anhedonic and depressive symptoms (Remus et al., 2013; Lebedeva et al., 2017), and pre-exposure to stress sensitizes the release of corticosterone following subsequent exposure to stress (Grippo et al., 2007). Similarly, prenatal studies provide evidence that stress at a very early age leads to depression later in life potentially due to sensitization (Morley-Fletcher et al., 2003).

The susceptibility to subsequent episodes or stress sensitization likely correlates with biological changes in the brain. Such changes, for example decreased hippocampal (Bremner et al., 2000, 2002; Sheline et al., 2003) and amygdala (Hastings et al., 2004; Drevets, 2000) volumes after initial episodes increase future-episode vulnerability. Thus, altered brain structure volume correlates with illness duration (Lorenzetti et al., 2009). The neurobiological mechanisms underlying the cyclical nature of depression and episode relapse remain largely unknown. It is therefore important that research, like the experiments outlined in this study, aims to demystify the contributing aetiological factors in recurrent depression.

1.1.3   Hypothalamic-pituitary-adrenal axis dysfunction

The HPA axis forms a major part of the neuroendocrine system that is responsible for the regulation of the stress response and is involved in many biological processes such as the immune system, emotions and mood. The stress response is regulated by a cascade of secretory events, alterations in which are implicated in the aetiology of MDD (Du & Pang, 2015). Neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing factor (CRF) in response to stress which acts on receptors in the anterior pituitary

gland, which then secretes adrenocorticotrophic hormone (ACTH). ACTH in turn stimulates the release of glucocorticoids (e.g. cortisol in humans, corticosterone (CORT) in rodents) by acting on the adrenal cortex. Excessive glucocorticoids suppress the release of CRF through a negative feedback system by acting on the hippocampus, hypothalamus and pituitary.
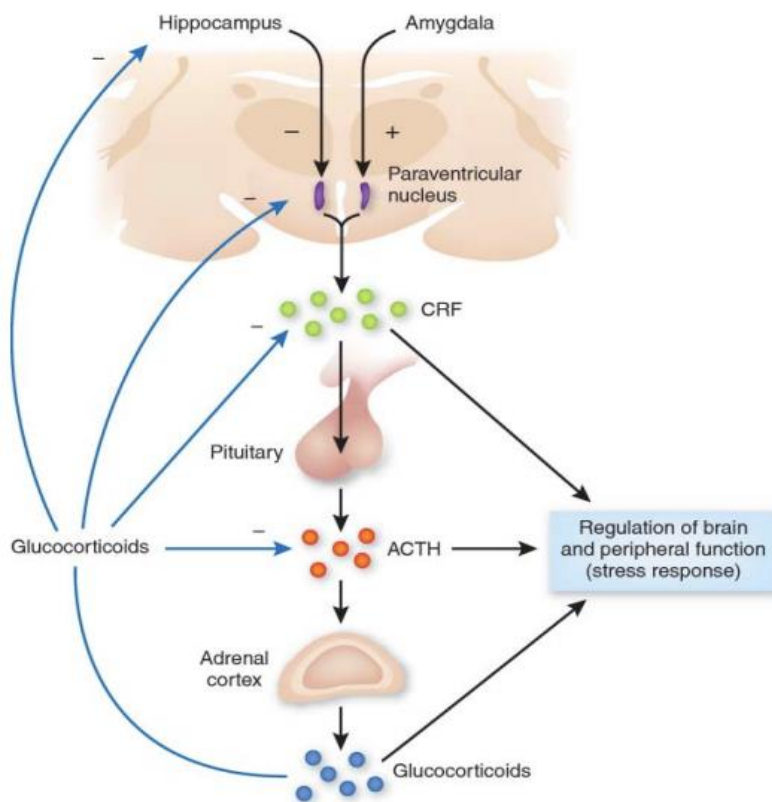
Figure 1.1. The hypothalamic-pituitary-adrenal axis. Adapted from Hyman, 2009.

Increased levels of cortisol were noticed in depressed patients about 50 years ago (Gibbons & McHugh, 1962) - and in more recent psychoneuroendocrinology studies the results have been replicated (Pariante & Lightman, 2008; Varghese & Brown, 2001; Nestler et al., 2002; Heim et al., 2008). Merali et al. (2004) found higher levels of CRF in the cerebrospinal fluid (CSF) of depressed suicide victims in frontal cortical brain regions, as well as in plasma,

compared to controls. Similarly, higher secreted levels of ACTH have been reported in depressed patients (Carroll et al., 2007).

The dexamethasone (synthetic glucocorticoid) suppression test (DST) was once used to help diagnose depression. It assesses adrenal gland function and is now mainly used to diagnose Cushing's syndrome. Dexamethasone binds to glucocorticoid receptors in the pituitary and suppresses the release of ACTH, acting as a negative feedback system. However, an inability of dexamethasone to suppress cortisol secretion has been seen in depressed patients (Yokoyama et al., 2015), most likely due to desensitization of glucocorticoid receptors. Hypercortisolemia is seen in 40-60% of patients not receiving treatment which suggests it is a reliable biomarker for the presence of the disease (Parker et al., 2003). This is important as stress can suppress neurogenesis, cause dendritic atrophy and the loss of excitatory synapses, reduce the effectiveness of neurotrophic factors and cell adhesion molecules, and compromise cell survival and imbalance neurotransmitter systems (Jauregui-Huerta et al., 2010). In rodents, stressful events also cause an increase in CORT levels (Li et al., 2008). Similarly, repeated CORT treatment results in depressive-like behaviour in both mice (David et al., 2009; Murray et al., 2008; Romay-Tallon et al., 2015) and rats (Lebedeva et al., 2017; Gregus et al.,2005; Gorzalka et al., 2003), and will be discussed more in section 1.3.2.

Antidepressants have been shown to regulate the HPA axis (Du & Pang, 2015; Pariante, 2003). For example, restraint-induced CORT and ACTH levels were reduced by chronic but not acute citalopram treatment (Hesketh et al., 2005). With chronic antidepressant treatment, CRF secretion is decreased, and the density in glucocorticoid receptors normalised (Barden, 2004). Thus, it is possible antidepressant treatment stimulates glucocorticoid receptor gene expression.

1.2 Hippocampal function, anatomy and circuitry

The hippocampus is a major structure of the mammalian brain that is part of the limbic system, and receives inputs from most of the cortex and subcortical regions. It plays a key role in long-term potentiation (LTP) and the consolidation of short-term memory to long-term memory by "providing the brain with a spatiotemporal framework within which the various sensory, emotional and cognitive components of an experience are bound together" (Knierim, 2015). The dorsal hippocampus is thought to play a preferential role in learning and memory – spatial, verbal and conceptual, while the ventral hippocampus plays a role in mood (Zhao et al., 2008a) and fear conditioning (Cenquizca & Swanson, 2007). The structure has been in the research spotlight regarding the neurobiological bases of memory ever since 1957, when the famous case of H.M. developed anterograde amnesia after his hippocampus was removed (Squire & Wixted, 2011).

The hippocampus comprises the Cornu Ammonis (CA), the dentate gyrus (DG), the subiculum, presubiculum and parasubiculum (collectively known as the subicular cortex) and the entorhinal cortex (EC) (Amaral & Witter, 1989; Insausti & Amaral, 2012). Anatomists usually refer to the CA as the "hippocampus proper", and use the term hippocampal formation when referring to the hippocampus proper, DG and subiculum collectively.

There are three main subfields of the hippocampus proper; CA1, CA2 and CA3. The stratum oriens is located superficial to the alveus and so is the deepest layer of the CA. Here the basal dendrites of pyramidal neurons from the layer below are found, as well as inhibitory interneurons. Above this layer is the stratum pyramidal layer, which houses the cell bodies of excitatory pyramidal neurons as well as interneurons that project to the other CA subfields. In the CA3 region only, between the stratum pyramidal and the stratum radiatum layers, the stratum lucidum can be found. This is the thinnest strata in which mossy fibres from the DG

course. Next comes the stratum radiatum. Here interneurons are housed which synapse with excitatory and inhibitory neurons, and Schaffer collateral fibres that project from CA3 to CA1. The next layer, the stratum lacunosum is very small and so is usually grouped together with the most superficial layer of the hippocampus, the stratum moleculare. The stratum lacunosum-moleculare, then, houses more Schaffer collateral fibres. In this layer, the pyramidal cell dendrites also form synapses with perforant path fibres (Amaral & Witter, 1989).

The DG is a trilaminate structure of the hippocampus that looks to be wrapped around the end of the CA3 subfield. Like the hippocampus proper, it is made up of strata; the polymorphic layer, the stratum granulosum (also known as the granule cell layer, GCL) and the stratum moleculare (Insausti & Amaral, 2004, 2012). The polymorphic layer that underlies the DG is known as the hilus, or is sometimes referred to as CA4 (Blackstad, 1956). The hilus is plentiful in inhibitory interneurons as well as axons of dentate granule cells that course through this layer to the CA3 region. Excitatory mossy fibre cells are also found here. The GCL is made up of tightly packed excitatory granule cell bodies whose axons span into the hilus and apical dendrites into the molecular layer. The subgranular zone (SGZ) can be found in between the hilus and GCL, a neurogenic site that will discussed in more detail in section 1.2.1. The stratum moleculare, the deepest strata of the DG, houses perforant path fibres that form excitatory synapses with distal apical dendrites of granule cells.
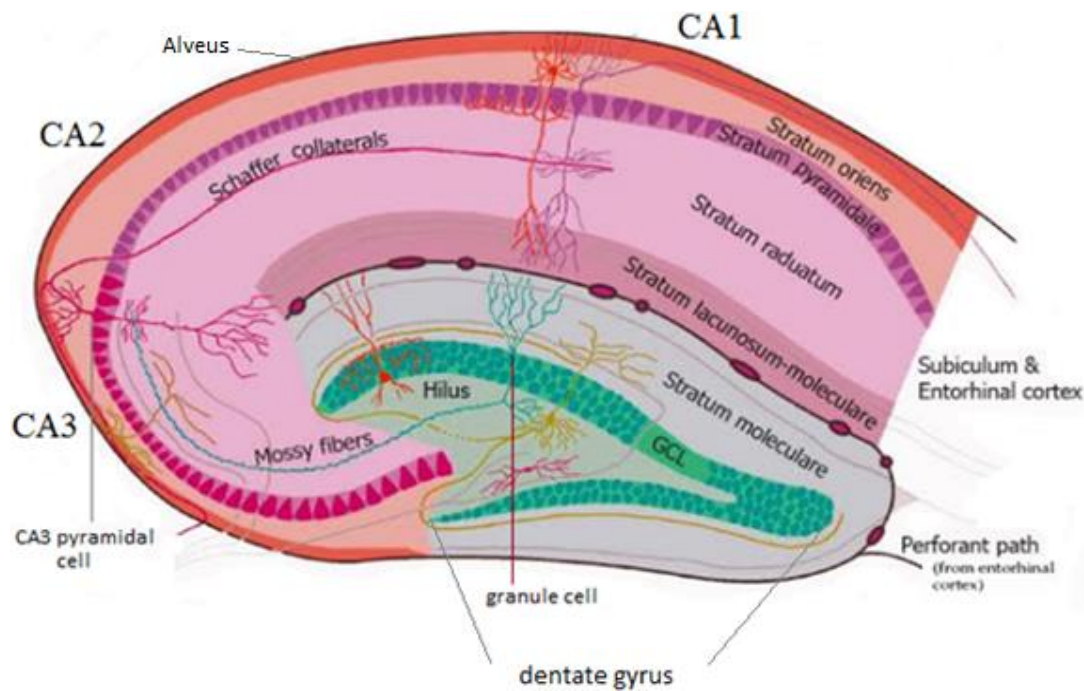
Figure 1.2. Anatomy and circuitry of the rat hippocampus. (Adapted from Lebedeva, 2016).

A unique characteristic of hippocampal circuitry is that it is unidirectional (Amaral et al., 2007). The DG receives input from the EC via the perforant pathway and does not project back. Excitatory granule cells housed in the DG form connections only with cell bodies of CA3 pyramidal cells via mossy fibres. The Schaffer collateral fibres in the CA3 subfield project to the CA1 subfield. The receiving axons of adjacent pyramidal neurons of the CA1 synapse with neurons in the EC and subiculum (Amaral et al., 2007; Amaral & Witter, 1989; Insausti & Amaral, 2004, 2012).

1.2.1 Neurogenesis

Neurogenesis is the birth of new neurons from neural stem cells. It was originally thought to be a developmental process, that neurons are formed before birth and cannot be exchanged. Although it is true that mature neurons cannot divide, research has shown that the

adult brain is capable of regeneration in at least two regions of the brain; the DG SGZ of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. In 1965, Altman and Das were the first to provide evidence that neurons in the DG of the postnatal rat hippocampus had been newly generated. These progenitor cells migrate to the GCL from the SGZ where they mature and integrate with the circuitry already in existence (Schloesser et al., 2014). Later studies supported the idea of adult neurogenesis in rats (Altman, 1969), mice (Kempermann et al., 1997), primates (Gould et al., 1999) and other animals as well as humans (Eriksson et al., 1998; Kukekov et al., 1999). In these studies, the SVZ was identified as another distinct region where progenitor cells divide and differentiate into neurons or glial cells. Most neuroblasts generated here migrate through the rostral migratory stream to the olfactory bulb where they become granule and periglomerular neurons (Zhao et al., 2008a; Ernst & Frisén, 2015). Radial glial cells are the most common type of stem cell found in the mammalian brain and produce neural progenitor cells. There are five stages in the process of neurogenesis; cell proliferation, differentiation, migration, maturation and integration.

Most neurogenesis research focuses on the hippocampus because it is involved in higher cognitive functions such as learning and memory, affective behaviours (Kempermann et al., 2015) and information processing (Ming & Song, 2011). The hippocampus is plentiful in glucocorticoid receptors suggesting that there is a link between the HPA axis and neurogenesis, and that the hippocampus is particularly susceptible to stress-induced impairments (Herman et al., 2005). Furthermore, depressed patients are known to have decreased hippocampal volume (Bremner et al., 2002; Frodl et al., 2006; Sheline et al., 2003, Videbech & Ravnkilde, 2004) which correlates with longer illness duration (Lorenzetti et al., 2009). There is some evidence that this is due to higher cortisol levels in patients with MDD (Pariante et al., 2003; Pariante & Miller, 2001). A study by Abercrombie et al. (2011) found that hippocampal alterations in response to cortisol were greater in females then males,

although volume size has been reported to be smaller in males in depressed patients (Frodl et al., 2002; MacMaster & Kusumakar, 2004; Yang et al., 2017) and unaffected in women (Colle et al., 2017; Kronmüller et al., 2009). This is surprising because women have significantly higher prevalence rates. It is true that the release of excessive glucocorticoids inhibits hippocampal neurogenesis (Nandam et al., 2007). Many preclinical studies have demonstrated a decrease in hippocampal proliferation and new-born cell survival induced by stress (Brummelte & Galea, 2010; Murray et al., 2008; Mayer et al., 2006). Furthermore, Jacobs et al. (2000) reports that when the rat adrenal gland was removed there was an increase in neurogenesis which was reversed with the injection of CORT. On a similar note, genetic ablation of neurogenesis by eliminating neural progenitors resulted in behavioural deficits and a reduction in synaptic plasticity (Saxe et al., 2006).

Chronic antidepressant treatment in rodents increases cell proliferation and the survival of new-born neurons (David et al., 2009; Nandam et al., 2007), as does electroconvulsive therapy (ECT), in the SGZ (Malberg et al., 2000; Jun et al., 2012). This is also seen after running and exposure to an enriched environment (Zhao et al., 2008a). Antidepressants may induce neurogenesis by affecting the levels of two potential mediators of the process, cyclic adenosine monophosphate (cAMP) and BDNF (Vithlani et al., 2013). Increasing neurotrophic signalling would enhance neuroplasticity, whereas mRNA expression of *BDNF* gene may be reduced by increased cortisol levels in the hippocampus. This could interfere with the positive effects typically produced by antidepressants on synaptic plasticity and neurogenesis (Cheeran et al., 2008). Evidence suggests that neurogenesis is involved in the aetiology of depression, but the extent to which this is important remains unclear. With about 700 new neurons added to the human adult hippocampus every day, it is debatable whether a 1.75% turnover of new neurons is sufficient to have a significant role in brain function (Spalding et al., 2013). Several studies have challenged the neurogenesis hypothesis of depression suggesting that it is not the

sole contributor. For example, post-mortem studies have shown no decrease in cell proliferation or cell loss in depressed patients compared to controls (Stockmeier et al., 2004). Though Santarelli et al. (2003) showed that inhibiting hippocampal neurogenesis by x-irradiation reduced the therapeutic effects of antidepressant treatment in mice, ablation of neurogenesis did not cause depressive or anxiety-like states. Exercise is also known to induce neuroplasticity (Knaepen, et al., 2010; Yau et al., 2011) and neurogenesis but inhibiting such activity in humans is not known to cause depressive states.

1.3 Animal models

Animal models are used extensively in research to further understand biological phenomena, in the hope that discoveries made in such models will provide a better understanding of human disease (Fields & Johnston, 2005). Rodents are the most commonly used animal models, mice being used most frequently due to their low-cost, size and rapid reproduction rate. Other commonly used rodents include rats, guinea-pigs, hamsters and gerbils. Truly mimicking disorders, especially ones psychiatric in nature, is impossible in animal models. Animals lack the ability to self-reflect and therefore symptoms that occur solely in humans, for example suicidal ideation, low self-esteem and feelings of worthlessness are impossible to mimic. Therefore, animal models approximate some symptoms that occur to investigate the pathophysiology of the disorder, screen for the therapeutic effects of agents or to elucidate their mechanisms of action.

In 1969, McKinney and Bunney proposed criteria that models had to fulfil to be classed as valid, which were; that the model creates symptoms that can be reasonably compared to human symptoms, that changes in the behaviour within the model can be measured objectively,

that such changes in behaviour can be reversed by treatments used in humans, and that the results of animal studies should be replicated between researchers. In 1990, Willner redefined these conditions into similar criteria he termed face, predictive and construct validity. Face validity refers to the model having similar pathophysiology and phenomenological similarities to the human condition. Predictive validity states the model must respond to drugs used to treat the human condition. To have construct validity a model must have a comparable aetiology. The model/test must measure what it is supposed to measure. The more criteria an animal model satisfies the more reliable the model.

1.3.1 Animal models of depression/anxiety

There are many animal models of depression which allow researchers to investigate the disorder ethically and without potentially confounding variables that would affect research outcomes in humans such as genetics, environment, social status etc. As stated in the previous section, it would be impossible to truly replicate human depression in an animal. Fortunately, when compared to controls, animals that have gone through depressive paradigms do display some similar depression-like phenotypes including changes in weight and appetite, memory and sleep disturbances, despair-like behaviours, impaired cognition, altered psychomotor activity and anhedonia.

Many animal models of depression use stress to induce depressive-like behaviours. Antidepressants are assessed by their ability to normalise these changes. The behavioural effects of such treatments are normally assessed acutely, even though it is known the therapeutic effects of antidepressants in the human condition are only seen after chronic administration. However, stress-based models remain the most frequently used methods to screen for antidepressant properties and are easy to replicate. Genetic-based (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.

Environmental stress alters animal as well as human behaviour. Depressed patients often report stressful situations to be out of their control. For this reason, the learned helplessness model is one of the most widely used. Animals are exposed to uncontrollable and inescapable stressful events such as foot or tail shocks that ultimately lead to the development of depression-like behaviour. Usually the animal is placed in shuttle boxes for one or more days where the learned helplessness is induced. After this period, rats have been shown to lose weight and have altered sleep patterns, altered locomotor activity and develop coat conditions (Seligman et al., 1980). As well, when given the chance to escape rats show an increased escape latency. The learned helplessness model fulfils face and predictive validity – antidepressants and ECT reduce symptoms (Sartorius et al., 2003), whether construct validity is fulfilled is unclear.

Chronic mild stress (CMS) is a model that involves exposing the animal to a series of mild but unpredictable stressors, and is probably the most valid model of depression. This is because the depressive-like state develops over time as it does in humans. Stressors normally include periods of water and food deprivation, adding/changing the number of animals in cages, changes in room temperature, mild shocks and disruption of the 12-hour light-dark cycle for at least 2 weeks. After the stressful period, rodents have shown altered locomotor activity, anhedonia (D'Aquila et al., 1994; Schweizer et al., 2009), decreased sexual behaviour (D'Aquila et al., 1994) and altered sleep patterns (Grønli et al., 2004). There is a lot of variation in results between laboratories, most likely due to the different stressors and husbandry of animals used. Unlike in the learned helplessness model, depressive-like behaviours resulting

from CMS only appear to be reduced by chronic antidepressant treatment (Willner et al., 1987), therefore predictive validity is fulfilled.

Social defeat stress (SDS) is another model of depression that consists of placing (usually) a male rat into a new environment that other conspecific males already occupy each day. The rat will be the intruder of the group and thus usually scrutinized and attacked, resulting in behaviour changes such as altered body weight (Meerlo et al., 1996), hyperactivity (Venzala et al., 2012) decreased social interaction, decreased sexual behaviour and increased drug self-administration including ethanol (Blanchard et al., 2001). Chronic antidepressant treatment reduces the produced behavioural changes (Venzala et al., 2012). Age, gender, the environment and the species used are all variables that may affect the behavioural changes of SDS, and so replication across labs may produce variations in results.

Early life stress has also been studied in animals, and has shown that stress in early life does cause behavioural changes that last until adulthood. In humans, early adverse-life events are known to increase vulnerability to depression, and so early life stress models have construct validity. Stressors include handling the pups at a very early age, separating pups from dams and prenatal stress. Increased fecal boli has been seen in the open field test (OFT) due to early life stress (O'Mahony et al., 2009). Providing a limited amount of nesting material causing stress to the dams resulted in decreased time spent with the pups and decreased amount of time grooming them (Ivy et al., 2008) as well as an increase in the pups' locomotor activity in the OFT.

1.3.2   Exogenous CORT administration as a model of depression

In many animal models, it is hard to control individual differences in response to stressors, psychological or physiological. There is a lot of individual variability in CORT levels in response to adverse stimuli. For this reason, the CORT model is advantageous as it allows researchers to directly examine the influence glucocorticoids may have on depression (Sterner & Kalynchuk, 2010). Although the levels of CORT used are supraphysiological (exceeding plasma concentrations of 5mg/kg), this model avoids the problem of habituation to physical stressors (Sandi et al., 1996). There are several ways to administer CORT, such as in food or water, by pellet implantation, osmotic pump infusion or as our laboratory prefers, subcutaneous injections (s.c.). Typically, rats receive CORT every day for at least 21 consecutive days, though shorter and longer exposures have been used. The CORT model can be viewed as isomorphic, which means that although the disorder may be induced artificially, many of the same symptoms that occur in human depression are produced in the model and they are reversed by antidepressants. Table 1.2 lists some depressive symptoms, how they can be tested in the CORT model, and the result found.

Table 1.2. Comparison of human symptoms of depression with CORT alterations.

Human symptom	Animal Test	CORT alterations	Reference
Despair	FST	↑ immobility time	1, 2, 3, 4, 5, 13, 15, 16, 17, 19
	TST	↓ mobility	6, 17
Anhedonia	SPT	↓ sucrose consumption	11, 13, 14
	NSF	↓ latency to feed	6, 19
Anxiety	OFT	↓ entries & time in centre	19, 20
		↔ distance travelled	4, 5, 19, 20
	EPM	↓ time spent in open arms	2, 7
	LDT	↓ time in light section	2
	POT	↓ contact with aversive stimuli	5
Sociability deficits	SIT	↔ social interaction	4
Spatial learning & memory deficits	MWM	↓ learning	12
Decreased libido	SBT	↓ sexual behaviour	8
Weight gain/loss	Weight	↓ weight	1, 4, 5, 9, 13, 18

Abbreviations: ↑ = increase, ↓ = decrease, ↔ = no difference, EPM = Elevated plus maze, FST = Forced Swim Test, LDT = light/dark test, MWM = Morris Water Maze, NSF = Novelty Supressed Feeding, OFT = Open Field Test, POT = Predator Odour Test, SBT = Sexual Behaviour 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.

There is a growing body of evidence that suggests chronic CORT administration produces reliable depressive-like behaviour in rodents, as well as neurobiological changes. CORT has been shown to increase immobility time in the FST (Brummelte et al., 2006; David et al., 2009; Gourley & Taylor, 2009; Hill et al., 2003; Luo et al., 2017), for example, even after 14 days of 20mg/kg injections (Wróbel et al., 2017). Furthermore, antidepressants have been shown to reduce immobility (Fenton et al., 2015; Rainer et al., 2012). Similarly, mobility time is decreased in the TST in mice (Zhao et al., 2008b) and is also reversed by antidepressant treatment (David et al., 2009). This provides predictive validity. In a study by Marks et al. (2009), the wire suspension test (WST) and OFT were used to assess muscle strength and general locomotor activity, respectively. As no differences were seen in both tests, it suggests that alterations seen in the FST are not due to muscle atrophy or nonspecific decreases in

locomotor activity. Li et al. (2017) also found no differences in locomotor activity in the OFT. Anhedonia, measured by a decrease in sucrose preference is seen after chronic CORT injections (Gourley & Taylor, 2009; Gourley et al., 2008). Although thigmotaxic behaviour (anxiety-like behaviour) is not usually detected in the CORT model (Gregus et al., 2005; Kalynchuk et al., 2004), Li et al. (2017) showed that 21-days of 40mg/kg CORT injections significantly increased the latency to enter the centre of the open field and reduced the time spent in the centre. Similarly, anxiety-like behaviours in the LDT (Murray et al., 2008) and EPM (Luo et al., 2017) are seen. Male rats became more defensive after 21 days of injections, exhibiting more "head-out" behaviour compared to vehicle and female rats, indicative of increased anxiety. They also spent less time with and had less contacts with an aversive stimulus (Kalynchuk et al., 2004). This increases the face validity of the model as anxiety is often comorbid with depression. Sexual behaviour is also altered with exogenous CORT administration (Gorzalka et al., 2001) as well as grooming behaviours (David et al., 2009), indicative of anhedonia. Weight gain is also significantly decreased (Brummelte et al., 2006; Johnson et al., 2006; Gregus et al., 2005). Table 1.3 summarises behavioural differences that are seen in the model considering strain and sex of rodent as well as dose of CORT received, demonstrating face validity.

Table 1.3. Effects of exogenous CORT in behavioural tests.

Test	Strain	Sex	Dose	Finding	Reference
OFT	Wistar	F	20mg/kg s.c.	No difference	3
	L-E	M	40mg/kg s.c.		4
	S-D	M	40mg/kg s.c.	↑thigmotaxic behav.	10
FST	L-E	M	20mg/kg & 40mg/kg s.c.	↑immobility	1
	L-E	M	40mg/kg s.c.		4, 12
	C57BL/6Ntac and CD1 mice	M	35 ug/ml/day or 5 mg/kg/day		8, 14
	Swiss Albino mice	M	40mg/kg s.c.		2
	Wistar	F	20mg/kg s.c.		3
SPT	L-E	M	50/mg/kg s.c.	↓sucrose preference	7
	C57BL/6 mice	M	25-100ug/ml in water		11
TST	C57BL/6Ntac mice	M	35 ug/ml dissolved in water	↓mobility	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	Swiss Albino mice	M	40mg/kg s.c.	↓time spent in open arms	2
NSF	C57BL/6Ntac and CD1 mice	M	35 ug/ml/day or 5 mg/kg/day	↑latency to feed	8, 14
MWM	S-D	M	40mg/kg s.c.	↓time spent in target section	9

Abbreviations: EPM = Elevated Plus Maze, FST = Forced Swim Test, L-E = Long-Evans, LDT = Light/Dark Test, MWM = Morris Water Maze, NSF = Novelty Suppressed Feeding, OFT = Open Field Test, POT = Predator Oder Test, S-D = Sprague-Dawley, 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]David et al., 2009; [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]Rainer et al., 2012.

The effects of CORT are dose and time dependent (Lebedeva et al., 2017). This means that with increasing doses, symptomatology worsens/cell atrophy increases (Yau et al., 2011; Menconi et al., 2008). Johnson et al. (2006) showed that FST immobility increased in rats that received 40mg/kg injections compared to 20mg/kg and 10mg/kg. Similarly, injecting CORT for a greater duration of time results in significantly worse behaviour and neurobiological alterations (Lebedeva et al., 2017; Marks et al., 2015). A single CORT injection produced no effect in the FST (Gregus et al., 2005), nor did treatment for 10 days at a dose of 20mg/kg (Brotto et al., 2001). Rats that received CORT treatment for 21 days, however, exhibited greater immobility in the FST than rats treated for 14 and 7 days (Lussier et al., 2013a).

Chronic exposure to CORT produces a significant change in structural plasticity in the brain, especially in the hippocampus, which is thought to be implicated in the development of a depressive-like phenotype (Sterner & Kalynchuk, 2010). A study by Sousa et al. (2000) found that after 21 days of CORT injections, dendritic length was reduced and stress-induced dendritic regression was observed in granule and CA1 pyramidal cells. Chronic CORT exposure also reduced the percentage of doublecortin (DCX) cells in the SGZ (Lussier et al., 2011). The expression of a glycoprotein called reelin is also downregulated by CORT treatment, which will be discussed in section 1.5.1

The information presented above provides evidence that the CORT model is a reliable tool to study the pathophysiology of depression and the effects of stress on behaviour and the brain. However, it should be noted that the other models should be considered if screening antidepressant efficacy. This is because both CORT (D'Souza et al., 2012) and antidepressants can cause liver damage (De Long & Hardy, 2017; Hsu et al., 2016), and combining CORT and antidepressant treatment at the same time may result in liver morbidity.

1.3.3   Modelling recurrent depression in animals

As mentioned in section 1.1.2, depression is a recurrent disorder (Kendler et al., 2000) where each depressive episode increases one's susceptibility to subsequent episodes. Thus, it is important for researchers to mimic the cyclical nature of depression as closely as possible. It is therefore surprising to find that only two studies, to our knowledge, have attempted to tackle such a problem. First, Remus et al. (2013) aimed to develop an animal model of recurrent depression to study the mechanisms behind increased episode susceptibility after subsequent episodes. To do this, rats were exposed to either 35 days of CMS or left in their home cage (controls). Sucrose preference was measured during this time, in which it slowly declined in

the CMS group. Animals then went through a 20-day recovery period in which they were exposed to no stressors. During this time sucrose preference increased to baseline/control levels. Animals were then re-exposed to CMS for 15 days and a more rapid decrease in the preference for sucrose water was seen in comparison to the initial stress exposure period. The researchers hypothesized that the animals became sensitised to stress, and Lebedeva et al. (2017) found comparable results. In this study rats were subjected to a 21-day period of CORT exposure followed by a 21-day recovery period, followed by another 21-day period of CORT exposure and another recovery period. Results showed that CORT increased the time spent immobile in a dose and time dependent manner. Recovery also diminished; after the initial recovery period immobility time/body weight went down by 53.1% and 42.3% in rats that received 20mg/kg and 40mg/kg, respectively. After the second recovery period, immobility time/body weight only went down by 29.7% and 19.2% in rats that received 20mg/kg and 40mg/kg, respectively.

There is still an enormous amount of research to be done regarding the neurobiological mechanisms underlying recurrent depression. For this reason, a portion of the research in this thesis focuses on replicating a recurrent model of depression using 3 cycles of chronic and intermittent exogenous CORT treatment to study the resulting depressive-like phenotype and neurobiological alterations.

1.4 Behavioural tests used to study depression

To test for depressive-like states, researchers must measure behavioural parameters such as distance travelled, fecal boli, FST immobility and anhedonia. Therefore, tests need to be conducted on the animal model. Different laboratories often report varying results because manually scoring parameters can be very subjective. For example, immobility time in the FST

is subject to different interpretations from person to person of what the threshold is to classify non-movement in the water tank as immobility. However, programs that automatically record behavioural parameters such as distance travelled, entries into and duration spent in zones are being used more often to standardize results.

Behavioural tests have difficulty fulfilling face, predictive and construct validity. This is because interpreting the results and comparing them to human symptoms is sometimes a matter of opinion. Does immobility in the FST indicate learned helplessness? There are often many factors that could affect resulting behaviour - weight, memory, stimuli in the environment, for example. As well, antidepressants are ineffective 30-40% of the time in human depression (Trivedi et al., 2006; Thase et al., 2001; Crown et al., 2002), so relying on antidepressant response to fulfil predictive validity has been criticized. There is not a single test that can capture all depressive-like behaviours and so a battery of tests are usually used to determine the mental state of animals.

As depression is usually comorbid with anxiety, it is understandable that most tests used induce an anxious state. Behavioural tests that induce anxiety include the OFT, EPM, LDT, the marble burying test and novelty induced hypophagia. The OFT is probably the most common behavioural test used to test the emotionality of rodents. It involves placing the animal into a novel, empty, lit up arena and the exploratory behaviour is observed. Thigmotaxic behaviour is indicative of anxiety. Arena shapes and diameters vary across laboratories, as do the protocols and parameters measured. The OFT can be used to screen anxiolytics and antidepressants – successful treatment should decrease thigmotaxic behaviour and fecal boli.

Other common behavioural tests include the SIT, TST (despair), hot plate test (HPT) and tail flick test (TFT – pain response), and the MWM (spatial memory). Home cage

monitoring can also be viewed to interpret behaviour. The FST was utilised in the experiments in this thesis and so will be discussed in further detail.

1.4.1   Forced swim test (FST)

The FST is a behavioural test used to measure despair. There are several paradigms in which the FST can be conducted. A two-day test was originally developed as a behavioural assay for antidepressant efficacy (Porsolt et al., 1978). First, a 15-minute pre-swim test takes place in which the animal will learn of the inescapable nature of the water-filled cylinder in which its placed. The test-day occurs 24 hours later; the animal is again placed in the cylinder for 5 minutes and behaviour scored. The purpose of the test day is to investigate the amount of "despair-like behaviour" acquired by the rats during the pre-swim test. The two-day test has been criticized, however, as it is possible animals spend more time immobile due to adaptive behaviour rather than depressive. Thus, one-day tests are also used, usually for a 10-minute duration. This would eliminate the potential confounding effects of memory (Marks et al, 2009). Parameters usually include the amount of time the animal is immobile (moving just enough to keep afloat), climbing or struggling (attempting to climb the walls/escape from the cylinder) and swimming (any other active behaviour including diving). Increased immobility and fewer attempts to escape the cylinder are indicative of despair-like behaviour. Usually animals initially try to climb the walls of the cylinder, but become more immobile as time goes on. Antidepressants should decrease immobility time. In the experiments outlined in this thesis a one day 10-minute protocol was used, which will be discussed in section 2.2.3.

1.5 Reelin

Reelin is a large extracellular matrix protein that has a wide range of functions in the developing and adult brain. The protein is made up of 3461 amino acids with a molecular mass of 388 kDa and is divided into three sub-domains; an N-terminal, 8 reelin repeats (RR) and a C-terminal (Nakano et al., 2007). It is encoded by the *RELN* gene. The protein gets its name from the abnormal reeler mouse phenotype characterized by a "reeling" gait (Cocito et al., 2016) due to cerebellar hypoplasia. These mutant mice express a homozygous mutation of the *RELN* gene, and the resulting lack of reelin causes cortical neurons to be placed ectopically (Cooper, 2008). Rodents with a disrupted cytoarchitecture in the cerebellum, hippocampus and cortex exhibit tremors, ataxia and impaired motor coordination (D'Arcangelo et al., 1995). The release of reelin depends on its rate of synthesis and not the depolarization of neurons (Lacor et al., 2000).

Reelin was originally thought to be a developmental molecule expressed by Cajal-Retzius cells in the hippocampus and cortex, as well as cerebellar glutamatergic cells (Caruncho et al., 2016) in the external and later the internal granule cell layer after migration occurs (Schiffmann et al., 1997). Howev'/er, it was soon shown that reelin is expressed by *gamma*-aminobutyric acid (GABA) transmitting interneurons (about 50-60%) in the adult hippocampus and cortex as well as glutamatergic cerebellar neurons (D'Arcangelo et al., 1997; Pesold et al., 1998, 1999) which modulate many forms of plasticity, like the formation of dendritic spines, synaptogenesis and acquisition of LTP (Rogers et al., 2013). By governing cell-cell interactions, reelin regulates the positioning of migrating neurons and is essential in lamina formation (D'Arcangelo, 2014). During embryonic corticogenesis, Cajal-Retzius cells synthesize and secrete reelin to orchestrate the typical inside-out development of the six laminae of the cortex (Chameau et al., 2009). Cells that develop early occupy the deep layers

while late-born neurons migrate to the more superficial layers (Chai et al., 2009) . A lack of reelin results in an inverted cortex - an outside-in birth order (Cooper, 2008). For this reason, it has been suggested that reelin acts as a stop signal; maintaining or terminating the cell adhesion of a migrating neuron to the radial glial cell it uses to migrate through the laminae (Qiu et al., 2006).

The region between the third and sixth RR (Nakano et al., 2007) binds with similar affinity to very-low-density-lipoprotein-receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), two receptors of the lipoprotein superfamily (Beffert et al., 2005, 2006; Ranaivoson et al., 2016) . VLDLR is thought to conduct the stop signal, while ApoER2 is important in the migration of late-born neurons and is involved with the acquisition of LTP through interaction with the glutamatergic N-methl-D-asparate (NMDA) receptor (Qiu et al., 2006). There is a link between reelin, Src kinases and NDMA maturation, with reelin controlling the change between NMDA receptor subunit composition (Sinagra et al., 2005). Although several proteins bind reelin and are likely involved in neuronal migration, reelin-α3β1 integrin interactions are crucial. On binding to an α3β1 integrin complex including VLDLR and ApoER2, the integrin-mediated adhesion of the migrating neuron is altered. Furthermore, reelin-α3β1 integrin interactions regulate protein levels of a downstream signalling protein of reelin, Disabled-1 (Dab1), but not its phosphorylation (Dulabon et al., 2000). When reelin binds to VLDLR and ApoER2, the receptors cluster together and activate Src family of non-receptor tyrosine kinases (SFKs); Fyn and Src in turn phosphorylate Dab1 at specific tyrosine residues increasing its activation and inaugurating downstream effects responsible for neuronal migration (Bock & Herz, 2003; Qiu et al., 2006; Chai et al., 2009; D'Arcangelo, 2014; Ranaivoson et al., 2016). When Dab1 is absent, ectopic migration of dentate neuroprogenitor cells and altered dendritic morphology occurs highlighting its importance (Teixeira et al., 2012). Dab1 is upregulated in VLDLR and ApoER2 knockout mice, which have identical phenotypes to reeler and Dab1

deficient mice (Howell et al., 1997) indicating that Dab1, reelin and its receptors function in a common signalling pathway (Dulabon et al., 2000). These knockout mice displayed dysfunctional learning and memory further evidencing VLDLR and ApoER2s role in synaptic plasticity and LTP (Trommsdorff et al., 1999).

Reelin is not only expressed in the CNS, but in the periphery too. It is found in several regions such as lymphatic tissues (Samama & Boehm, 2005), platelets (Tseng et al., 2010) and the kidneys and liver (Smalheiser et al., 2000) from where it is released into blood plasma. However, there is not much known about the role reelin plays in the periphery, or whether it crosses the blood-brain-barrier. A 2015 study by Perez-Costas et al. found that reelin is expressed in brain endothelial cells (mainly in caveolae), which suggests that reelin or its peptides could possibly cross the blood-brain-barrier. Reelin's ability to cross the blood-brain-barrier could have important therapeutic, physiological and pathological implications.

There have been several studies on reelin's involvement in normal brain functioning. For example, Rogers et al. (2013) found that a single reelin injection *in vivo* enhances LTP and synaptic function. It is not known whether the deficits in heterozygous reeler mice that share similarities with a schizophrenic-like phenotype are due to the absence of reelin in development or adulthood (Rogers et al., 2013). In schizophrenic post-mortem brains, a 40% (Guidotti et al., 2000), 50% and 70% reduction in reelin mRNA was seen in the prefrontal cortex, hippocampus and caudate nucleus, respectively (Agam et al., 2013). Similarly, Fatemi et al. (2001) found that serum reelin levels were increased in schizophrenic patients by 49% and in patients with MDD by 34%, but decreased in patients with bipolar disorder by 33%. Prenatal stress, a known predisposing factor in the development of cognitive and emotional disorders, also reduces the expression of reelin-positive cells in cortical layer I (Palacios-García et al., 2015).

1.5.1   Reelin and its implications in depression

Reelin is said to be involved in many neuropsychiatric disorders such as autism, schizophrenia, Alzheimer's disease, and bipolar disorder and a total lack of reelin causes lissencephaly (Fatemi, 2004). Since the functional properties of the reelin protein are impaired in disorders like schizophrenia and bipolar disorder, it is very plausible that reelin downregulation could be implicated in the pathogenesis of depression (Lussier et al., 2011). Tissue from the post-mortem brain of depressed patients provides evidence that reelin levels are decreased in the hippocampus (Knable et al., 2004; Fatemi et al., 2000) .

In a study by Lussier et al. (2009), rats received CORT injections (40mg/kg) once per day for 21 days and a 25% decrease in the number of reelin-positive cells in the SGZ was found. This was later replicated in a study that showed imipramine not only reduced depressive-like behaviour but also restored the number of reelin-positive cells to control levels in both the SGZ and hilus of the hippocampus (Fenton et al., 2015). This is important as neurogenesis occurs in the SGZ, and suggests that stress-induced alterations in GABAergic interneurons that express reelin could alter the process of hippocampal neurogenesis (Caruncho et al., 2016). To investigate this further, CORT was injected for either 7, 14 or 21 days (40mg/kg) and the number of reelin expressing cells in the SGZ, the maturation rate of new-born granule neurons and the onset of depressive-like behaviour recorded (Lussier et al., 2013a). Results showed that after 14 and 21 days, immobility in the FST increased which was paralleled with a decrease in reelin-positive cells. In addition, a decrease in the number of surviving new-born granule cells (measured by DCX-immunopositive cell count) and the complexity in dendritic spines of surviving cells was seen. It could be that reelin downregulation increases depressive-like behaviour by causing a delay in the maturation of new-born cells, and causing the ectopic placement of cells in existing circuitry (Caruncho et al., 2016). With the suppression of Dab1,

adult hippocampal neurogenesis is impaired, which supports this idea. Furthermore, inactivation of the reelin signalling pathway caused abnormal dendrite morphology, and altered migration and formation of circuits (Teixeira et al., 2012) . A reelin deficiency was also shown to increase vulnerability to the depressogenic effects of CORT treatment because heterozygous reeler mice exhibited more exaggerated CORT-induced effects on behaviour and the loss of reelin-positive cells in the SGZ and hilus than wild-type mice (Lussier et al., 2011).

It is known that 5HT receptors and transporters play a role in depression, and for this reason the number of SERT clusters per lymphocyte, the size of SERT clusters per lymphocyte and the percentage of lymphocytes surface occupied by SERT clusters was assessed in homozygous and heterozygous reeler mice (Rivera-Baltanas et al., 2010). Results showed that while the number of SERT clusters per lymphocyte were the same for both wildtype and homozygous reeler mice, the size of clusters and percentage of lymphocytes surface occupied by SERT clusters were lower in wildtype compared to both heterozygous and homozygous reeler mice. 5HT transmission is known to be altered in depressed patients, in which a similar pattern of alterations is seen (Rivera-Baltanas et al., 2012).

Chapter 2

Parallel effects of cyclical corticosterone administration on depression-like behaviour and the downregulation of reelin in the rat hippocampus

Josh Allen[1], Katherina A. Lebedeva[2], Erin Y. Kulhawy[3], Hector J. Caruncho[1], Lisa E.

Kalynchuk[4]

1. College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

2. Department of Psychology, University of Saskatchewan, Saskatoon, SK, Canada

3. Intellectual Property Manager, Innovate, Calgary, AB, Canada

4. College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

2.1 Introduction

Depression is the most common neuropsychiatric disorder characterized by depressed mood and anhedonia. Patients suffering from MDD typically experience repeated episode relapses (Kendler et al., 2000), which may be due to the individual becoming more sensitized to stress (Kendler et al., 1999; Morris et al., 2010). Thus, depression is now considered a life-long disease (Crown et a., 2002). To understand the contributing mechanisms leading to the pathology of depression, a large amount of research has focused on the hippocampus. Depressed patients have continuously been found to have a decreased hippocampal volume (Bremner et al., 2000; Sheline et al., 2003; Videbech & Ravnkilde, 2004), which correlates with longer illness duration (Lorenzetti et al., 2009). Furthermore, numerous preclinical studies show a reduction in dendritic length (Sousa et al., 2000) and decreased cell proliferation and new-born cell survival (Brummelte & Galea, 2010; Murray et al., 2008) after chronic CORT treatment. In addition, these deleterious effects are reversed by antidepressant treatment (David et al., 2009; Nandam et al., 2007; Malberg et al., 2000; Jun et al., 2012).

Our laboratory has previously shown that chronic CORT treatment over one 21-day cycle decreases the number of reelin-immunopositive cells in the SGZ, where adult neurogenesis occurs, which closely parallels the onset of a depressive-like phenotype (Lussier et al., 2009, 2013a; Fenton et al., 2015). In addition, heterozygous reeler mice with 50% normal levels of reelin are more vulnerable to the depressogenic effects of CORT treatment, suggesting that reelin could have neuroprotective properties (Lussier et al., 2011). Reelin is expressed by GABAergic interneurons in the adult hippocampus and is highly involved in the process of neurogenesis (Caruncho et al., 2016; Pesold et al., 1998, 1999). Reelin is necessary for the guiding and positioning of migrating neurons (D'Arcangelo, 2014), the acquisition of LTP,

synaptogenesis, and dendritic morphology (Rogers et al., 2013). Accumulating evidence indicates that reelin is likely involved in the pathogenesis of depression (Caruncho et al., 2016).

Despite the cyclical nature of depression, animal models usually utilize a single bout of stress exposure to develop the depressive-like phenotype. To our knowledge, our laboratory produced the only published study to date which attempted to mimic the cyclical disease course using two cycles of chronic CORT injections interspersed with injection-free (recovery) periods (Lebedeva et al., 2017). Results showed that after 2 cycles, rats became sensitized to stress in the FST in a dose and time dependent manner. The current study aims to replicate this finding with the addition of a third cycle of CORT treatment and recovery period to assess the sensitization of stress and worsening of recovery on re-exposure, while animals remain naïve to behavioural testing. Moreover, whether the development of the depressive like phenotype and worsening of recovery parallels the downregulation of reelin in the SGZ will be assessed. A dose of 20mg/kg was chosen as there were few differences in immobility between the two CORT groups (20mg/kg and 40mg/kg) in the previous study (Lebedeva et al., 2017). As well as this, a smaller dose would be advantages as the longitudinal design of the experiment allows for a severe decrease in weight, and using a higher dose would have likely resulted in the early sacrificing of CORT animals, as guided by ethical protocols. Furthermore, as experimental design does not differ from that conducted by Lebedeva et al. (2017), only time points in a first and third cycle were looked at as they are more informative regarding the research question, allowing us to decrease the number of animals used. We hypothesise that reelin downregulation is paralleled with the development of the depressive-like phenotype, and that recovery will diminish with subsequent cycles of CORT exposure.

2.2 Methods

2.2.1 Animal husbandry

We used 124 male (225-250 grams) Long-Evans rats (Charles River Laboratories, Canada) in this experiment. Rats were 6-8 weeks old at the time of injections. They were housed singly in polypropylene cages and had constant access to food and water, except during behavioural testing procedures. They were maintained on a 12-hour light: dark cycle, lights turning on at 07:00 a.m. The colony room temperature was kept at 21°C. Bedding was changed once a week, and Purina rat chow food topped up at regular intervals. The rats were handled every day for a week prior to injections, and all experimental procedures were done in the light phase of the cycle. A protocol approved by the University of Saskatchewan Committee on Animal Care and Supply was followed.

2.2.2 Experimental procedures

The animals were weight-matched and randomly assigned to one of two treatment groups, repeated and cyclical vehicle (n=61) or CORT (n=63) injections (20mg/kg). The rats were then subdivided into 6 groups which acted as time points (10 days of cycle 1, 21 days of cycle 1, recovery of cycle 1, 10 days of cycle 3, 21 days of cycle 3, recovery of cycle 3) to analyse the effect of cyclical and intermittent CORT administration on behaviour and neurobiology. This type of between-group design was used to avoid repetitive exposure to the FST over time. A cycle consisted of 21 days of injections, and was followed by a 21-day recovery period in which no injections took place. This means that rats in group 21 days of cycle 3 (group 5) went through 21 days of injections, followed by a 21-day recovery period, which was then followed by another 21 days of injections, followed by a 21-day recovery

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period, and finally 21 days of injections (see figure 2.1). The CORT (Steraloids) injections were administered at a dose of 1ml/kg. CORT was suspended in a 0.9% (w/v) sodium chloride and 2% (v/v) Polysorbate-80 (Sigma Aldrich) solution. The body weight of rats was recorded on each day of CORT treatment, and in the end of each recovery period.
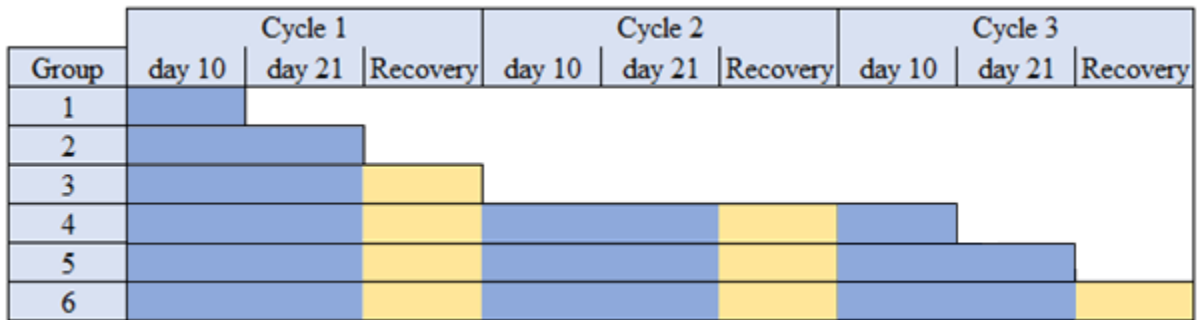
Group	Cycle 1			Cycle 2			Cycle 3		
	day 10	day 21	Recovery	day 10	day 21	Recovery	day 10	day 21	Recovery
1									
2									
3									
4									
5									
6									

Figure 2.1. Representation of animal group time points. Recovery periods were 21 days. The FST took place the day after the last day of injections or at the end of the recovery period. Animals were sacrificed after the FST.

2.2.3   Forced swim test (FST)

The FST took place either after 10 or 21 days of injections (day 11 or day 22 respectively) or at the end of the recovery period, depending on the subgroup, and was employed to assess depression-like behaviour. A one-day protocol was used as it removed the confounding effects that memory could potentially play on a two-day test protocol (Marks et al., 2009). Rats were placed into a rectangular Plexiglas tank that had a width, length and height of 25cm, 25cm and 60cm, respectively, for 10 minutes. After the 10 minutes, rats were taken out of the tank and dried thoroughly with a towel before being returned to the colony room. The water was filled to a height of about 30cm and was at a temperature of 27±2°C. Parameters were manually scored and included time spent "climbing", "swimming" and "immobile". Immobility was defined as moving just enough to keep afloat, swimming was defined as

moving around the cylinder and climbing was defined as attempting to climb the walls of the cylinder – often referred to as struggling behaviour. A higher immobility time is interpreted as learned helplessness, indicative of depressive-like behaviour.

2.2.4 Perfusions and tissue preparation

After behavioural testing took place at each time point, the rats were sacrificed and perfused using 0.1 M phosphate buffer at pH 7.4 followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 (500mls). The brains were then removed and postfixed in 4% (w/v) for 48 hours at a temperature of 4°C. A vibratome (Vibratome 3000, Vibratome Company, St. Louis, MO, USA) was used to section the tissue at a thickness of 50μm, and the resulting brain sections were then kept in a cryoprotectant solution of 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone, and 30% (w/v) sucrose in 0.1 M PBS (pH = 7.4) at -20°C.

2.2.5 Immunohistochemistry

Every 6th section of the hippocampus was used for immunostaining, which was done in a 6-well plate. Reelin-positive cells were visualised using the following immunohistochemical procedure: free-floating tissue was rinsed six times in 0.1 M Tris buffered saline (TBS, pH 7.4). After this, sections went through an antigen-retrieval step in which they were incubated in sodium citrate (pH 6) at 85°C for 30 minutes. After this, sections were incubated in a blocking solution containing 15% (v/v) normal horse serum (NHS), 0.5% triton X-100 and the mouse anti-reelin primary antibody (1:1000, MILLIPORE, MAB5364) in TBS for 24 hours at room temperature. After this, the tissue was incubated in 10% (v/v) H_2O_2 for 30 minutes to

block endogenous peroxide activity. Next, the tissue was incubated at room temperature for 1 hour with a biotinylated horse anti-mouse secondary antibody (1:500 Vector Laboratories, USA, BA2001) diluted in 15% NHS and 0.5% triton in TBS. After this, the tissue was incubated in avidin-biotin complex (ABC, 1:500, Vector Laboratories, USA) for 1 hour at room temperature. Immunolabelling was visualized with 0.02% (w/v) 30-diaminobenozidine (DAB, Sigma Aldrich, St. Louis, MO, PK6100), 0.0078% H_2O_2 diluted in TBS. After approximately 10 minutes, the sections were rinsed three times with TBS to terminate the DAB reaction. The sections were then mounted on Superfrost Plus Microscope glass slides, dehydrated with ethanol and coverslipped using Permount (Fisher Scientific SP15-500).

The number of reelin-positive cells in the SGZ was 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). Five sections per brain were counted, using both hemispheres, at 400x magnification. The cells were counted blind to the treatment group. The number of cells were counted using the following formula: N_{total}: $\Sigma Q^- \times 1$ / ssf $\times$ A(x,y step) / a(frame) $\times$ t/h, where ΣQ^- is the number of counted cells; ssf is the section sampling fraction (1/6); A(x,y step) is the area associated with each x,y movement (5,625μm^2); a(frame) is the area of the counting frame (2,500μm^2); t is the weighted average section thickness; and h is the height of the dissector (12μm). A guard zone of 2μm was used.

2.2.6 Statistical analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 20 (IBM, USA). Data were tested for normality and homogeneity of variance before carrying out appropriate statistical analyses. Main effects and interactions were first analysed using a two-way univariate ANOVA, with time and treatment as independent

variables. Independent samples t-tests were used to compare vehicle and CORT animals at each time point. Pearson's correlation coefficient tests were used to determine the strength of the relationship between time spent immobile and the number of reelin-positive cells. Group differences were considered statistically significant at p<0.05. Data were expressed as means ± standard error of the mean (SEM).

2.3 Results

2.3.1 Immobility behaviour

A two-way ANOVA showed that there was a significant main effect of time [$F_{(5, 112)}=12.574$, p<0.001] and treatment [$F_{(1, 112)}=14.150$, p<0.001] on FST immobility. However, there was no interaction effect of time and treatment [$F_{(1, 112)}=1.502$, p>0.05].

In cycle 1, an independent samples t-test showed that there was no significant difference between vehicle and CORT treated rats in immobility after 10 days of treatment [$t_{(18)}=1.245$, p=0.115]. However, there was a significant effect of treatment after 21 days, in that the CORT rats spent more time immobile than the vehicle rats [$t_{(19)}=2.249$, p=0.019]. After the 21-day recovery period there was no significant difference found between the groups [$t_{(19)}=0.136$, p=0.297].

In the third cycle, the CORT treated rats spent significantly more time immobile than the vehicle rats after both 10 [$t_{(18)}=3.984$, p=0.0005] and 21 [$t_{(18)}=2.946$, p=0.005] days. However, no significant difference was found between the groups after the recovery period [$t_{(20)}=1.009$, p=0.163]. All FST data mentioned above are shown in Figure 2.2A.

To control for the influence of body weight on immobility time, immobility scores were divided by the weight of the rat at the time of testing. The results are as follows:

A two-way ANOVA showed that there was a significant effect of time [$F_{(5, 108)}$=7.815, $p<0.001$] and treatment [$F_{(1, 108)}$ = 32.917, $p<0.001$] on immobility/body weight. However, there was no significant interaction effect of time and treatment [$F_{(5, 108)}$=1.506, $p=0.194$].

In cycle 1, an independent samples t-test showed that there was a significant effect of treatment in immobility/weight scores after 10 days [$t_{(18)}$ = 2.04, $p = 0.05$] and 21 days [$t_{(19)}$ = 2.4, $p = 0.027$], in that the CORT treated rats had higher immobility/weight scores. However, there was no significant effect of treatment after the recovery period on immobility/weight [$t_{(19)}$ = 0.073, $p=0.409$].

In the third cycle, there was a significant effect of treatment after 10 days [$t_{(18)}$ = 4.6, $p<0.001$] and 21 days [$t_{(14)}$ = 3.085, $p=0.008$], as well as after the 21-day recovery period [$t_{(20)}$ = 2.43, $p=0.024$], in that CORT treated rats had greater immobility/weight scores at all time points. These data are shown in Figure 2.2B.
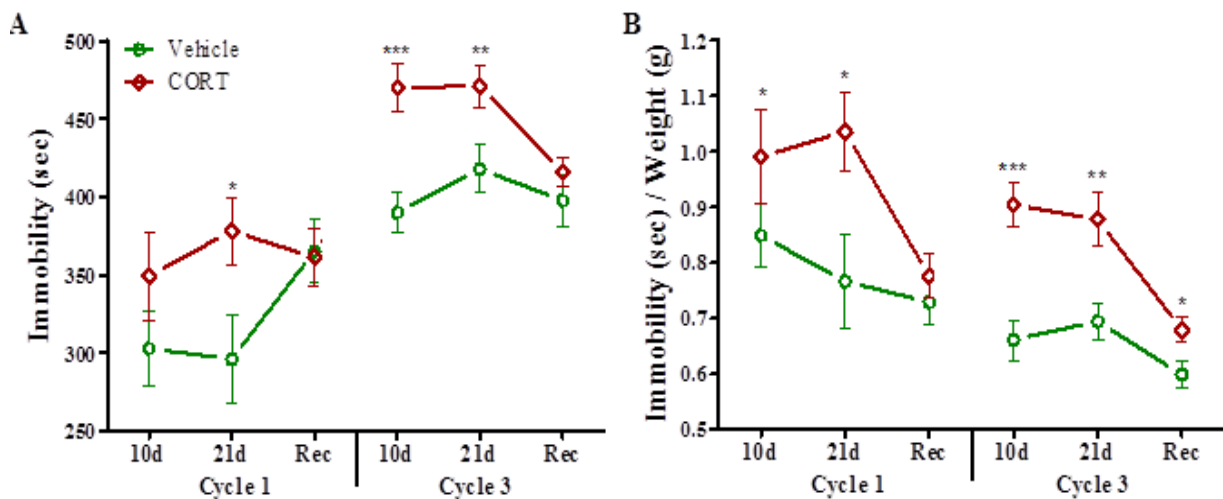

Figure 2.2. Immobility time in the FST. A) Immobility time (sec). B) Immobility time (sec) / body weight (g). Data is presented as mean±SEM. *$p<0.05$, **$p<0.001$, ***$p<0.001$.

The quality of recovery (percent change in immobility time and immobility/body weight) in CORT rats across cycles was evaluated by dividing the mean difference of group 2 (cycle 1 21days) and group 3's score (cycle 1, recovery) by group 2's and multiplying by 100. The analogous calculations were done for cycle 3 and the results displayed in table 2.1.

Table 2.1. % change in recovery over cycles in CORT rats.

	Cycle 1, recovery	Cycle 3, recovery
% change in immobility time	4.4%	11.7%
% change in immobility/body weight	25.1%	22.7%

2.3.2 Reelin expression

Figures 2.3 and 2.4 provide photomicrographs of reelin immunostaining in each of the quantified groups. Only cells located along the SGZ were included in the analysis. A two-way ANOVA showed that there was a significant main effect of time [$F(5, 101) = 2.494$, $p=0.036$] and treatment [$F(1, 101) = 53.486$, $p<0.001$] on the number of reelin-positive cells. However, there was no interaction effect of time and treatment [$F(1, 101) = 0.207$, $p>0.05$].

In cycle 1, an independent samples t-test showed that there was a significant effect of treatment after 10 days [$t(17)=2.874$, $p=0.0105$], 21 days [$t(16)=2.816$, $p=0.0124$] and after the 21-day recovery period [$t(15)=2.585$, $p=0.0207$] (see figure 2.5A).

In cycle 3, there was also a significant effect of treatment after 10 days [$t(18)=3.080$, $p=0.0065$], 21 days [$t(17)=3.127$, $p=0.0061$] and after the 21-day recovery period [$t(18)=3.577$, $p=0.0022$] (see figure 2.5B). There was a 23% CORT-induced reduction in reelin expression after the 1st recovery period, and this reduction grew to 30% after the 3rd recovery period.
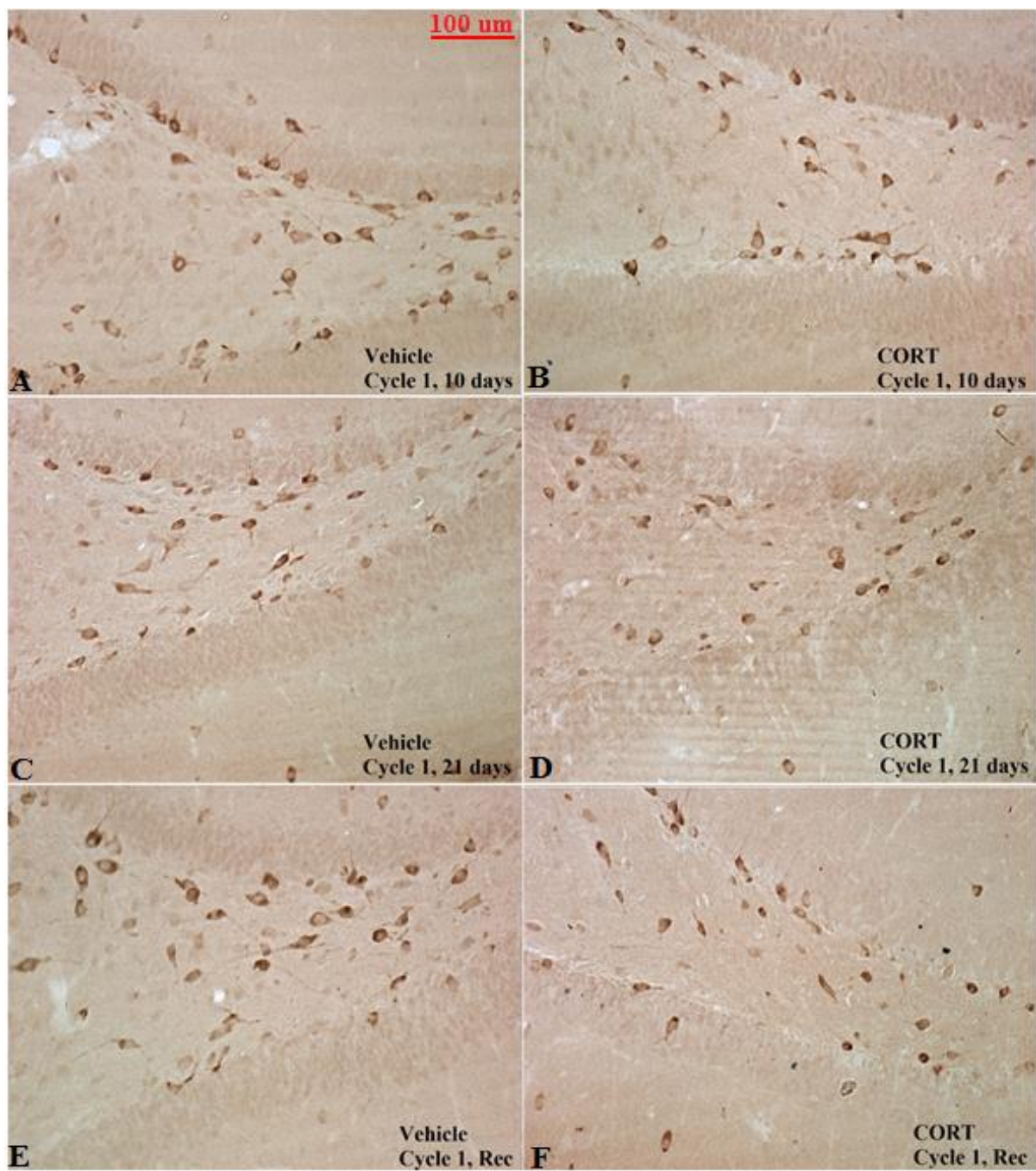
Figure 2.3. Reelin+ cells in the SGZ in cycle 1. A) Vehicle, cycle 1 10 days. B) CORT, cycle 1 10 days. C) Vehicle, cycle 1 21days. D) CORT, cycle 1 21 days. E) Vehicle, cycle 1 recovery. F) CORT, cycle 1 recovery.
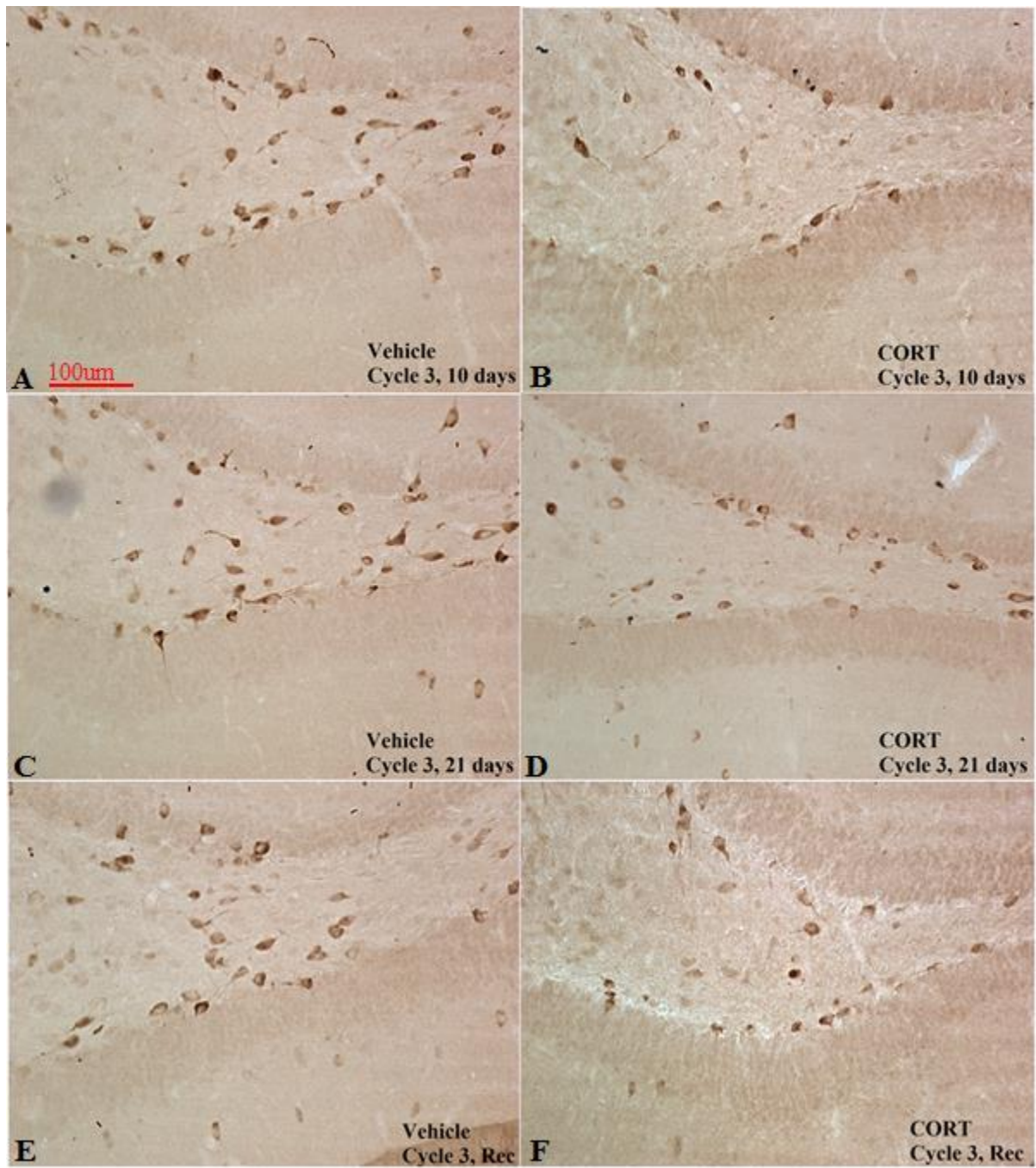
Figure 2.4. Reelin+ cells in the SGZ in cycle 3. A) Vehicle, cycle 3 10 days. B) CORT, cycle 3 10 days. C) Vehicle, cycle 3 21days. D) CORT, cycle 3 21 days. E) Vehicle, cycle 3 recovery. F) CORT, cycle 3 recovery.
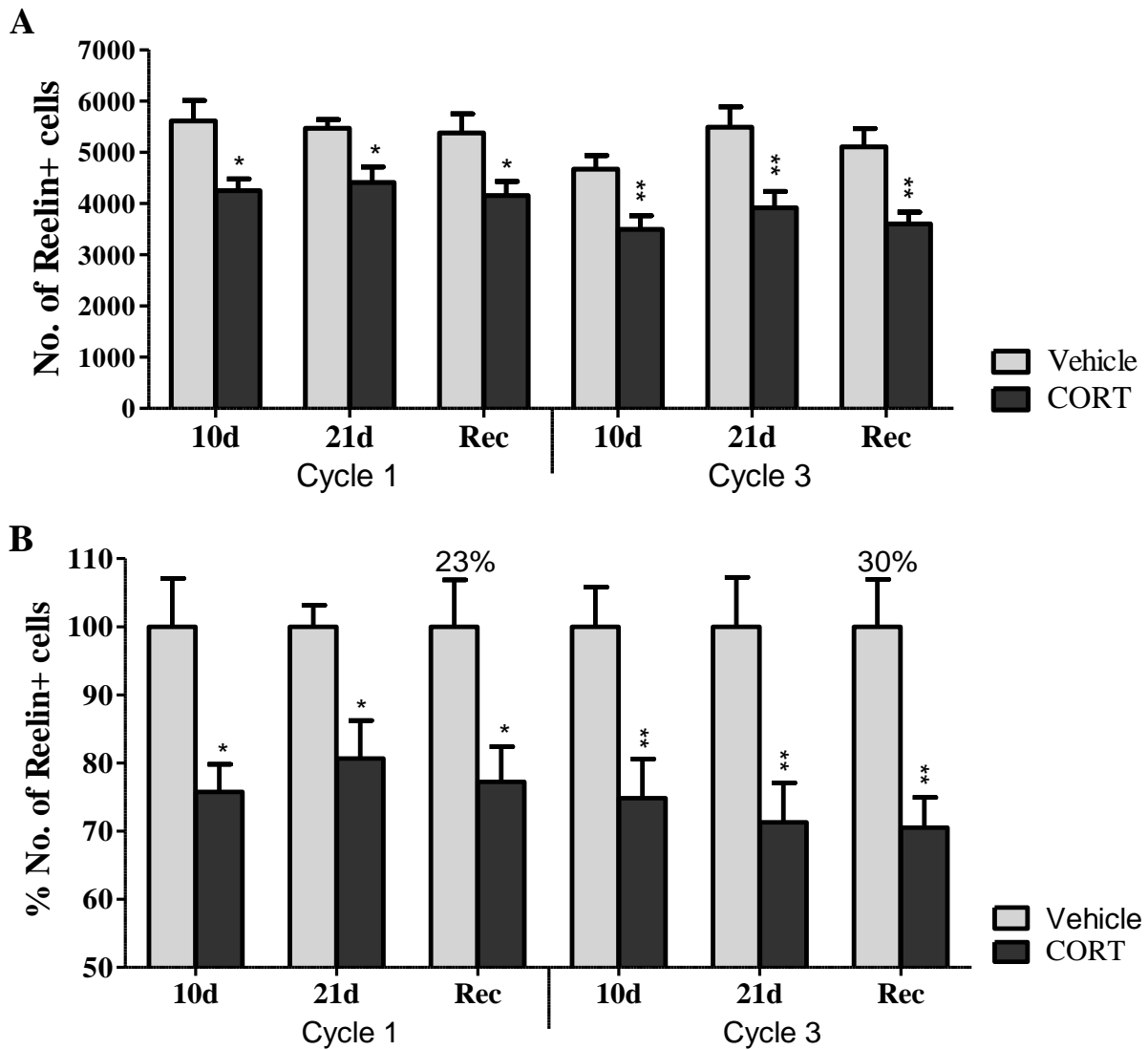
Figure 2.5. Number of reelin+ cells in the SGZ. A) Number of reelin+ cells. B) Percentage of reelin+ cells. CORT reduced reelin-positive cell expression by 23% and 30% in cycle 1 and cycle 3 recovery, respectively. Data is presented as mean±SEM. *p<0.05, **p<0.001 vs CORT.

Table 2.2. Summary of reelin+ cell count in the SGZ over cycles.

Group	Cycle 1			Cycle 3		
	10d (n)	21d (n)	Rec (n)	10d (n)	21d (n)	Rec (n)
Vehicle	5609±399 (10)	5465±173 (8)	5375±371 (9)	4667±271 (10)	5490±397 (9)	5107±355 (10)
CORT	4250±227 (9)	4408±304 (10)	4152±278 (8)	3492±269 (10)	3915±317 (10)	3601±226 (10)
t-test	t(17)=2.847, p=0.0105*	t(16)=2.816, p=0.0124*	t(15)=2.585, p=0.0207*	t(18)=3.080, p=0.006**	t(17)=3.127, p=0.006**	t(18)=3.577, p=0.002**

Data is expressed as mean±SEM. *$p<0.05$, **$p<0.01$.

Table 2.3. CORT-induced changes in immobility and reelin in recovery over cycles.

	Cycle 1, recovery	Cycle 3, recovery
% increase in immobility time	0.27%	4%
% increase in immobility/body weight score	8%	**16%***
% decrease in reelin+ cell count	**23%***	**30%***

$p<0.05$*, $p<0.01$** vs vehicle.

2.3.3 Correlation between immobility and reelin+ cell counts

A Pearson's bivariate correlation coefficient test showed that there is a significant negative correlation between the number of reelin-positive cells and time spent immobile in the FST (r=-0.303, R^2=0.092, p=0.001, n=113) (see figure 2.6A). When immobility is controlled for weight, as was done in previous sections, the Pearson's correlation also shows that there is a significant negative correlation between the number of reelin-positive cells and time spent immobile / weight (r=-0.215, R^2=0.046, p=0.022, n=113) (see figure 2.6B).
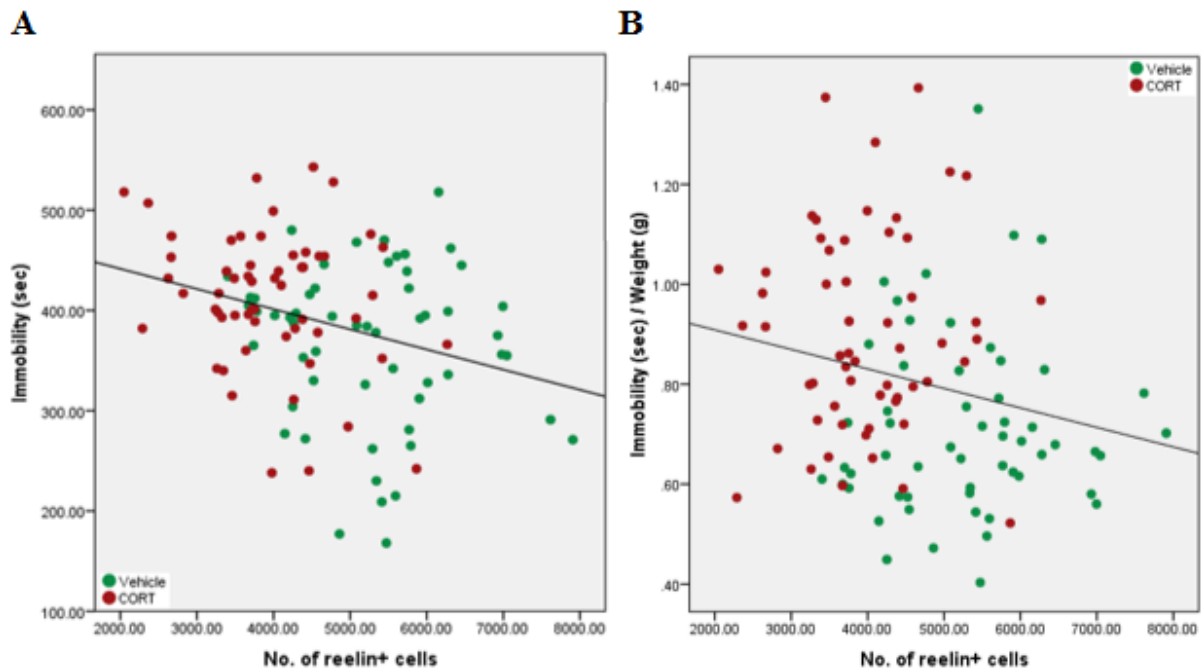

Figure 2.6. Overall relationship between FST immobility and number of reelin+ cells in the SGZ. A) Immobility and reelin+ cells. B) Immobility / weight and reelin+ cells.

When broken down into cycles, a Pearson's correlation also shows that there is a significant negative correlation between the number of reelin-positive cells and time spent

immobile / weight in cycle 1 (r=-0.316, R^2=0.1, p=0.020, n=54) (see figure 2.7A) as well as in cycle 3 (r=-0.331, R^2=0.109, p=0.01, n=59) (see figure 2.7B).
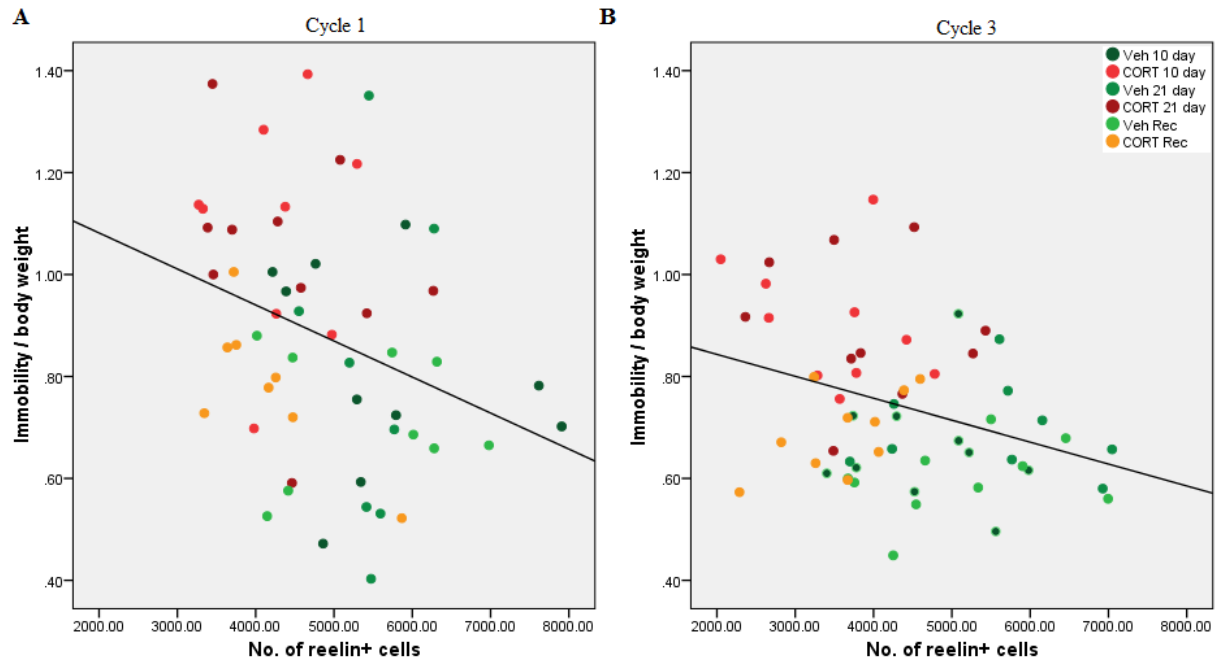

Figure 2.7. Relationship between FST immobility / body weight and number of reelin+ cells in the SGZ over cycles. A) Cycle 1. B) Cycle 3.

As FST behaviour recovered by over 20% in cycle 1 and cycle 3 (see table 2.1) but reelin expression did not after either recovery period, the relationship between the number of reelin-positive cells and FST immobility was again plotted with the recovery time points separated. Results are as follows: in cycle 1 there was a significant negative correlation between the number of reelin-positive cells and time spent immobile / weight in rats that received 10 and 21 days of injections (r=-0.377, R^2=0.142, p=0.021, n=37) (see figure 2.8A) but not after the recovery period (r=-0.357, R^2=0.122, p=0.159, n=17) (figure 2.8B). Similarly, in cycle 3 there was also a significant negative correlation between the number of reelin-positive cells and time spent immobile / weight in rats that received 10 and 21 days of injections (r=-0.468,

$R^2=0.219$, p=0.003, n=39) (figure 2.8C) but not after the recovery period (r=-0.075, $R^2=0.005$, p=0.753, n=20) (figure 2.8D).
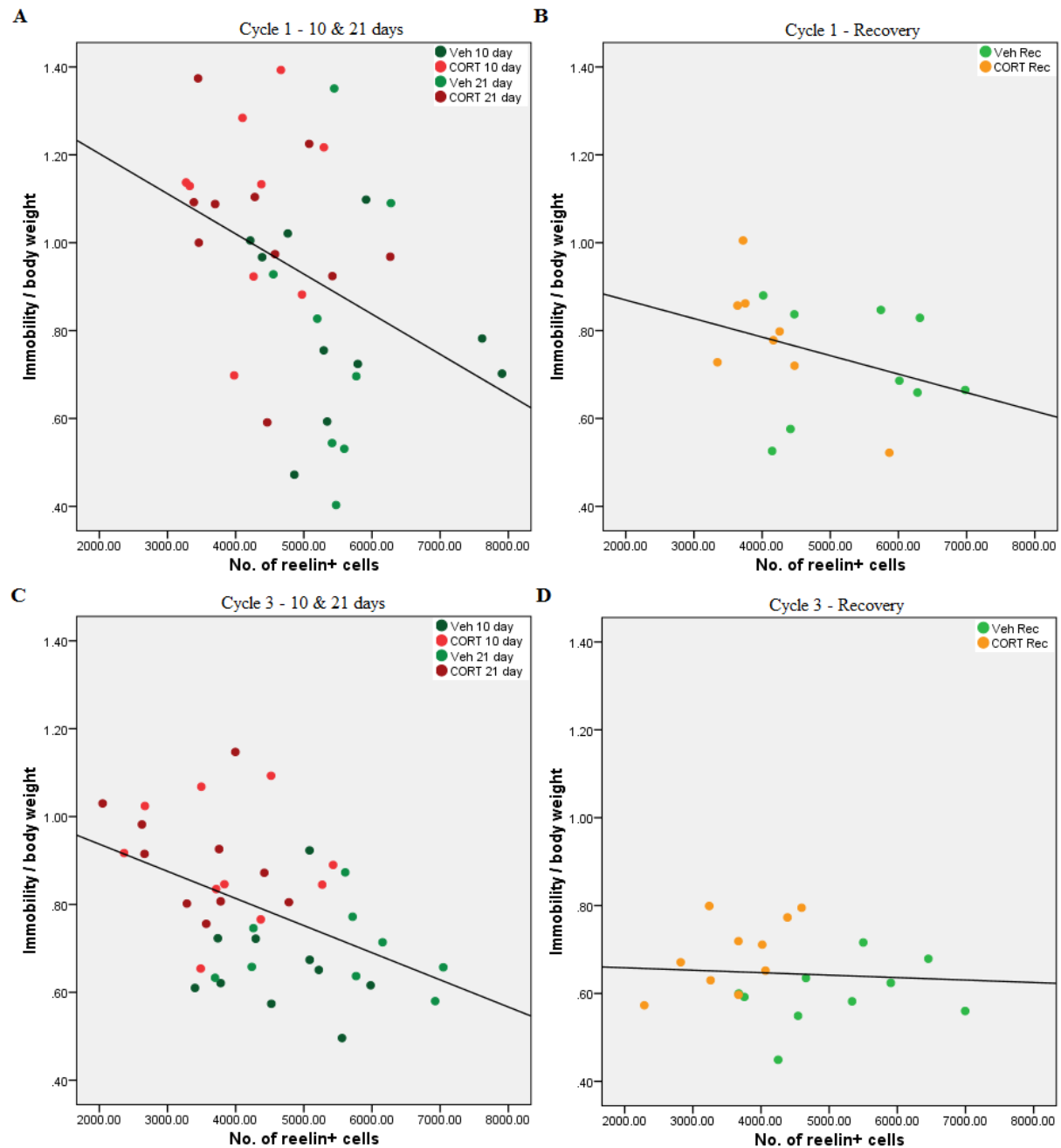

Figure 2.8. Relationship between FST immobility / body weight and number of reelin+ cells in the SGZ separated by time points. A) Cycle 1 - 10 and 21 days. B) Cycle 1 - Recovery. C) Cycle 3 -10 and 21 days. D) Cycle 3 - Recovery.

2.4 Discussion

It is important that animal models of depression are developed to study the neurobiological mechanisms responsible for episode relapse, the presence of residual symptoms, diminished recovery and other characteristics of human depression and their relationship with stress. Research regarding the cyclical nature of depression using animals is scarce, however, some studies have shown that animals can sensitize to stressors (Remus et al., 2013; Lebedeva et al., 2017; Zurita et al., 2000). In these studies, re-exposure to both CMS, CVS and CORT-treatment invoked depressive-like behaviour more rapidly. Our study was novel in using a third cycle of CORT exposure and revealed consistent results; CORT produced time-dependent increases in depressive-like behaviour that was exhibited more rapidly with subsequent cycles. Although 10 days was not enough time to produce significant effects in cycle 1, it did affect behaviour after 10 days in cycle 3. Immobility time was higher in all cycle 3 time points compared to cycle 1 suggesting that symptomatology also exacerbated on re-exposure. This provides better validity for the model as human patients with depression also have aggravated symptomatology when a greater number of episodes are experienced, lasting an increased duration of time (Karp et al., 2004).

It should be noted that immobility time also went up in the vehicle rats over time, suggesting that other factors can influence FST behaviour. Examples of possible confounding variables include stress caused from the saline injections, stress caused from being housed singly, age and body weight. Greater body weight should allow the rat to float with more ease, and therefore since CORT rats are significantly lighter (Lebedeva et al., 2017; Lussier et al., 2013a, 2013b; Gregus et al., 2005) we can assume the increased immobility after CORT treatment is due to a depressive-like state. However, as CORT-induced decreases in weight gain are consistently seen compared to controls (Lebedeva et al., 2017; Lussier et al., 2013a,

2013b; Gregus et al., 2005) it is important to control for the confounding effect weight has on FST immobility nonetheless. We did this by dividing the immobility time by the weight of the rat at the time of behavioural testing. The results show that a difference in immobility is seen after 10 and 21 days of CORT injections, which recovered after the first recovery period. A difference was again seen after 10 and 21 days of cycle 3, as well as after the third recovery period which suggests subsequent cycles diminished recovery. In a similar study, recovery was also diminished after two cycles but this was with a dose of 40mg/kg (Lebedeva et al., 2017) providing further evidence that the depressogenic effects of CORT are dose and time dependent (Johnson et al., 2006; Lussier et al., 2013a). In human depression, partial remission contributes to subsequent episode relapse (Paykel et al., 1995; Fava et al., 2007; Kendler et al., 2000). Residual symptoms are still seen in 32% (Paykel et al., 1995) to 82.3% (Gasto et al., 2003) of remitted patients and is associated with deficits in social functioning (Masai et al., 2016). Residual symptoms and partial remission should be considered when discussing treatment/interventions, and could be factors contributing to the progression of MDD.

High doses of glucocorticoids have catabolic effects on muscle tissue (Tempel & Leibowitz, 1994). However, studies have shown that CORT treatment did not result in differences on general locomotor activity in the OFT (Kalynchuk et al., 2004; Lussier et al., 2011; Gregus et al., 2005), strength in the WST (Marks et al., 2009) or swimming in the MWM (Sousa et al., 2000). Thus, we can assume that increased immobility in the FST is due to a depressive-state and not muscle atrophy or CORT-induced metabolic effects.

Our laboratory has previously reported that the development of the depressive-like phenotype is paralleled by the downregulation of hippocampal reelin (Lussier et al., 2009, 2013a; Fenton et al., 2015). Our data were consistent with these findings. The number of reelin-positive cells was decreased at all time points and was exacerbated in subsequent cycles. More specifically, the 8% to 16% increase in immobility was paralleled with a 23% to 30% decrease

in reelin-positive cells in the SGZ in cycle 1 recovery to cycle 3 recovery, respectively. However, there is also a lack of parallelism in that FST behaviour recovered by 25.1% and 22.7% in cycle 1 and cycle 3 respectively, but the number of reelin-positive cells did not recover at all. The lack of parallelism can be seen when the correlation is plotted with injection period and recovery period time points separately. There is a clear difference in the number of reelin-positive cells, CORT rats being situated further left on graph (figure 2.8B and D) but the correlation with reelin expression and immobility is lost as FST behaviour recovered. The recovery of immobility behaviour could possibly be due to an increase in reelin secretion by the remaining reelin-expressing cells. That is, perhaps CORT resulted in cell death, and the remaining cells secreted enough reelin to recover behaviour in the first cycle, but not after the third when a further decrease in reelin-positive cells is witnessed. This, along with several other studies, indicates that reelin is neuroprotective; a deficiency in reelin increases vulnerability to the depressogenic effects of CORT (Lussier et al., 2011); transgenic mice that display roughly 3 times the normal amount of reelin in the forebrain spend less time immobile in the FST compared to wildtype mice after 21 days of CORT exposure (Teixeira et al., 2011); and reelin-overexpressing mice had similar immobility times to wildtype mice in the absence of CORT indicating that higher reelin levels were only impactful when subjected to adverse environmental conditions. In addition, although not yet published, our laboratory has demonstrated that reelin has antidepressant effects when infused into the hippocampus, or when delivered as a single peripheral injection into the lateral tail vein after 21 days of CORT injections.

The relationship between increased immobility and reelin downregulation was solidified by correlation analyses. There was a negative correlation between immobility and reelin which remained when split into cycles. This strengthens the argument that reelin downregulation as an important neurochemical event underlying the depressive-like

phenotype. Taken together, the results from past studies as well as the current warrant further investigation of reelin's potential antidepressant effects, which is the focus of the next study outlined in this thesis.

Chapter 3

Peripheral reelin injections reverse depression-like behaviour and the downregulation of hippocampal reelin in the CORT model of depression

Josh Allen[1], Raquel Romay-Tallon[1], Milann Mitchell[2], Lisa E. Kalynchuk[3], Hector J.

Caruncho[1]

1. College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

2. Department of Psychology, University of Saskatchewan, Saskatoon, SK, Canada

3. College of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

3.1 Introduction

Depression is the most common psychiatric disorder; incidence rates having risen every year for over 50 years. Over 300 million people are estimated to be sufferers worldwide (World Health Organisation, 2017). However, treatment remains ineffective roughly 30-40% of the time (Trivedi et al., 2006; Thase et al., 2001; Crown et al, 2002). Therapeutic effects also take about 3 weeks to develop and adverse side-effects are common (Stahl, 2000). This is especially a problem in patients with suicidal thoughts. Thus, newer fast-acting antidepressants with fewer side-effects are desperately needed.

There is an abundance of literature showing the involvement of the hippocampus in depression. Once it was found that depressed patients have decreased hippocampal volume (Bremner et al., 2000; Lorenzetti et al., 2009; Sheline et al., 2003; Videbech & Ravnkilde, 2004), researchers began to investigate behavioural and neurobiological alterations resulting from stress-induced hippocampal dysfunction. Preclinical studies revealed that CORT treatment decreased cell proliferation, new-born cell survival (Lussier et al., 2011; Brummelte & Galea, 2010; Murray et al., 2008) and reduced the complexity of dendritic processes (Sousa et al., 2000) in the hippocampus. Moreover, this was reversed by antidepressant treatment (David et al., 2009; Nandam et al., 2007; Malberg et al., 2000; Jun et al., 2012).

Chronic CORT exposure also decreases the levels of hippocampal reelin, a large extracellular matrix protein expressed in the adult hippocampus by GABAergic interneurons (Caruncho et al., 2016). Reelin plays a role in neuronal migration, dendritic morphogenesis, synaptogenesis and the acquisition of LTP (Rogers et al., 2013). Our laboratory showed that CORT administration decreased the expression of reelin-positive cells in the SGZ (Lussier et al., 2009, 2013), which was reversed by the antidepressant drug imipramine (Fenton et al., 2015). Heterozygous reeler mice with 50% normal levels of reelin were also more vulnerable

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to the depressogenic effects of CORT (Lussier et al., 2011), suggesting that reelin could have neuroprotective properties. In heterozygous reeler mice, the size of SERT clusters and percentage of lymphocyte cell surface occupied by SERT clusters were higher than in wildtype mice (Rivera-Baltanas et al., 2010). This is important as 5HT transmission is known to be altered in depressed patients, showing a common observation in human patients and animal models (Rivera-Baltanas et al., 2012). Taking this information into account, it is logical to assume that reelin could have antidepressant effects. Although not yet published, our laboratory infused reelin into the hippocampus after CORT exposure and found that the depressogenic effects were reversed. Based on these results, and the fact that reelin is also expressed in the periphery, we hypothesized that peripheral reelin injections would have similar antidepressant effects. The potential antidepressant effects of reelin when administered peripherally would be of great importance as it would provide a more practical, non-invasive way to possibly treat human patients in the future.

3.2 Methods

3.2.1 Animal husbandry

We used 84 male (236-318 grams) Long-Evans rats (Charles River Laboratories, Canada) in the study. Animal husbandry was the same as described in Chapter 2 section 2.2.1.

3.2.2 Experimental procedures

On arrival to the facility, the rats were given 2 days to habituate. After this, a week of handling took place. During this week, the rats were habituated to a restraining device that was used throughout the injection period to administer the lateral tail vein injections. On day 3, the

restrainer was placed into the cage with the rat for 1 minute. On the 4th, 5th, 6th and 7th day, the rat was placed into the restrainer for 1 minute.

The animals were then randomly assigned to one of two treatment groups; vehicle injections or CORT (Steraloids) injections (40mg/kg). The rats were then subdivided into 10 subgroups; vehicle rats that received either vehicle or reelin injections at 3μg/ml every 10 days, 3μg/ml every 5 days, 5μg/ml every 10 days or 5μg/ml every 5 days, or CORT rats that received either vehicle or reelin injections at 3μg/ml every 10 days, 3μg/ml every 5 days, 5μg/ml every 10 days or 5μg/ml every 5 days (see table 3.1).

Table 3.1. Representation of the subgroups in experiment 2.

			Vehicle	CORT
		Vehicle	n=12	n=12
Reelin	3μg/ml	Every 10 days	n=6	n=6
		Every 5 days	n=8	n=8
	5μg/ml	Every 10 days	n=8	n=8
		Every 5 days	n=8	n=8

The vehicle or CORT injections were given once per day for 21 days between the hours of 08:00 and 11:00 a.m. Rats were weighed each day and injections 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.

Depending on the group, rats either received an injection of vehicle or reelin (R&D Systems, 3820-MR) at a dose of 3μg/ml or 5μg/ml into the lateral tail vein every 5 or 10 days (see figure 3.1).
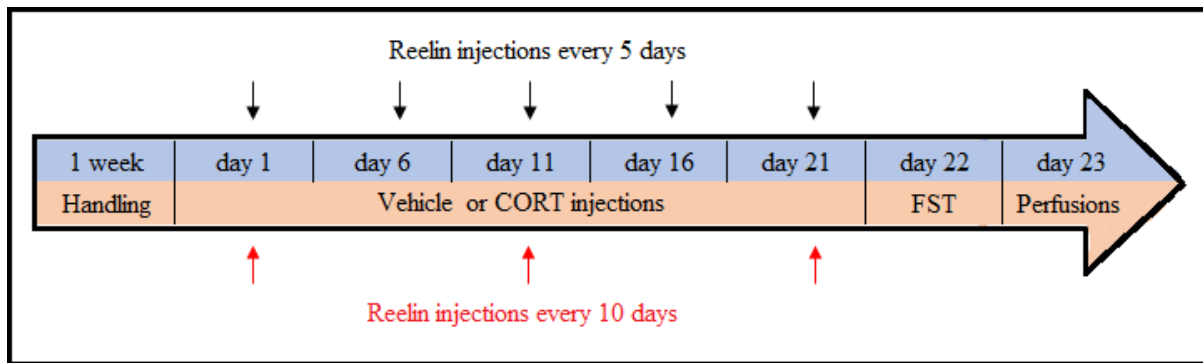
Figure 3.1. Representation of the injection schedule. Vehicle or CORT injections were given once per day for 21 consecutive days. Reelin or vehicle injections were given either every 5 or every 10 days.

3.2.3   Forced swim test (FST)

The FST took place on day 22. The protocol that was used is specified in Chapter 2 section 2.2.3.

3.2.4   Perfusions and tissue preparation

The rats were sacrificed on day 23. First, the rats were anaesthetized with isoflurane, and then perfused transcardially using 0.1 M phosphate buffer (PB, pH 7.4) followed by 4% (w/v) paraformaldehyde in 0.1 M PB (pH 7.4). The brains were then removed and postfixed in 4% paraformaldehyde (w/v) for 48 hours at a temperature of 4°C. A cryostat (Vibratome ULTRAPRO 5000) was used to section the tissue at a thickness of 30μm, which was then kept in a cryoprotectant solution of 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone and 30% (w/v) sucrose in 0.1 M PBS (pH = 7.4) at -20°C.

3.2.5   Immunohistochemistry

Reelin immunohistochemistry and cell counts were done to the protocol specified in Chapter 2.

3.2.6   Statistics

Statistical analyses were carried out using SPSS version 20 (IBM, USA). The data were tested for normality and homogeneity of variance before carrying out appropriate statistical analyses. Two-way ANOVAs were used to analyse behavioural and neurobiological results with vehicle or CORT injections as the first factor and vehicle or reelin injections as the second. A one-way ANOVA was also used to analyse behavioural and neurobiological results with overall treatment as a factor (four groups; vehicle/vehicle, vehicle/CORT, vehicle/reelin and CORT/reelin) when all dose and frequency groups were pooled together or in different subgroups (3µg/ml every 10 days, 3µg/ml every 5 days, 5µg/ml every 10 days and 5µg/ml every 5 days). Post-hoc analyses were performed using the Tukey test if a significant main effect was found. Group differences were considered statistically significant at p<0.05. Data were expressed as means ± SEM.

3.3 Results

3.3.1   Immobility in the FST

When the different dose and frequency subgroups were pooled together to create 4 groups with two treatment factors (vehicle or CORT and vehicle or reelin), a two-way ANOVA can be conducted, which showed that there was a significant main effect of CORT [F(1, 80) =

38.237, p<0.001], reelin [F(1, 80) = 7.170, p=0.0089] and a significant interaction effect of CORT and reelin [F(1, 80) = 6.595, p=0.012] on immobility in the FST.

When combining both treatment factors as one combined treatment (V/V, V/R, C/V, C/R), a one-way ANOVA can be conducted [F(3, 80) = 14.930, p<0.001] along with a Tukey post-hoc test to show which group means differ from each other. The results of the analysis showed that the CORT/vehicle group was significantly different from the vehicle/vehicle (p<0.001), vehicle/reelin (p<0.001) and CORT/reelin (p=0.002) groups. As well, the CORT/reelin group was also significantly different from the vehicle/reelin group (p=0.006) (see figure 3.2).

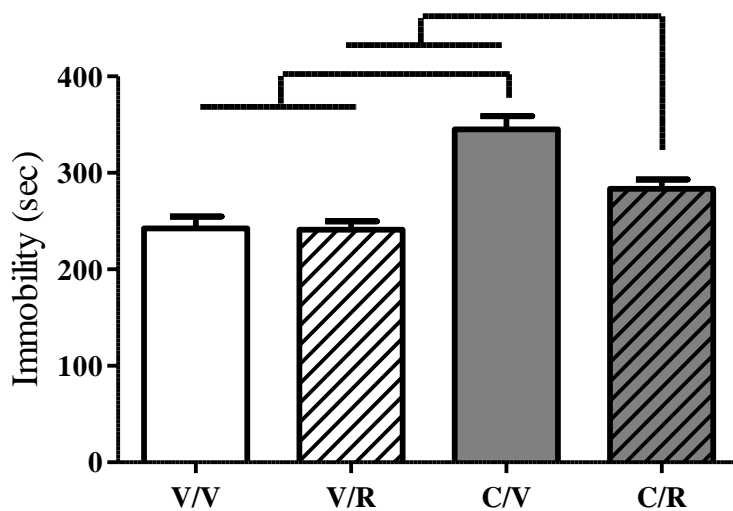**Figure 3.2. Immobility time in the FST.** Data are presented as mean±SEM. Lines represent significantly different groups.

When looking at the dose and frequency subgroups (3µg/ml every 10 days, 3µg/ml every 5 days, 5µg/ml every 10 days, 5µg/ml every 5 days) individually, one-way ANOVAs can be done to investigate group differences using the combined treatment as a factor.

When looking at the 3µg/ml every 10 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [F(3, 32)=11.038, p<0.001]. A Tukey post-hoc test

revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p<0.001$), vehicle/reelin ($p=0.001$) and CORT/reelin ($p=0.02$) groups (see figure 3.3A).

When looking at 3µg/ml every 5 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 36)}=11.357$, $p<0.001$]. A Tukey post-hoc test revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p<0.001$), vehicle/reelin ($p=0.004$) and CORT/reelin ($p=0.09$) groups (see figure 3.3B).

When looking at the 5µg/ml every 10 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 36)}=11.912$, $p<0.001$]. A Tukey post-hoc test revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p<0.001$) and vehicle/reelin ($p<0.001$) groups (see figure 3.3C).

When looking at the 5µg/ml every 5 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 36)}=12.813$, $p<0.001$]. A Tukey post-hoc test revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p<0.001$), vehicle/reelin ($p<0.001$) and CORT/reelin ($p=0.015$) groups (see figure 3.3D).
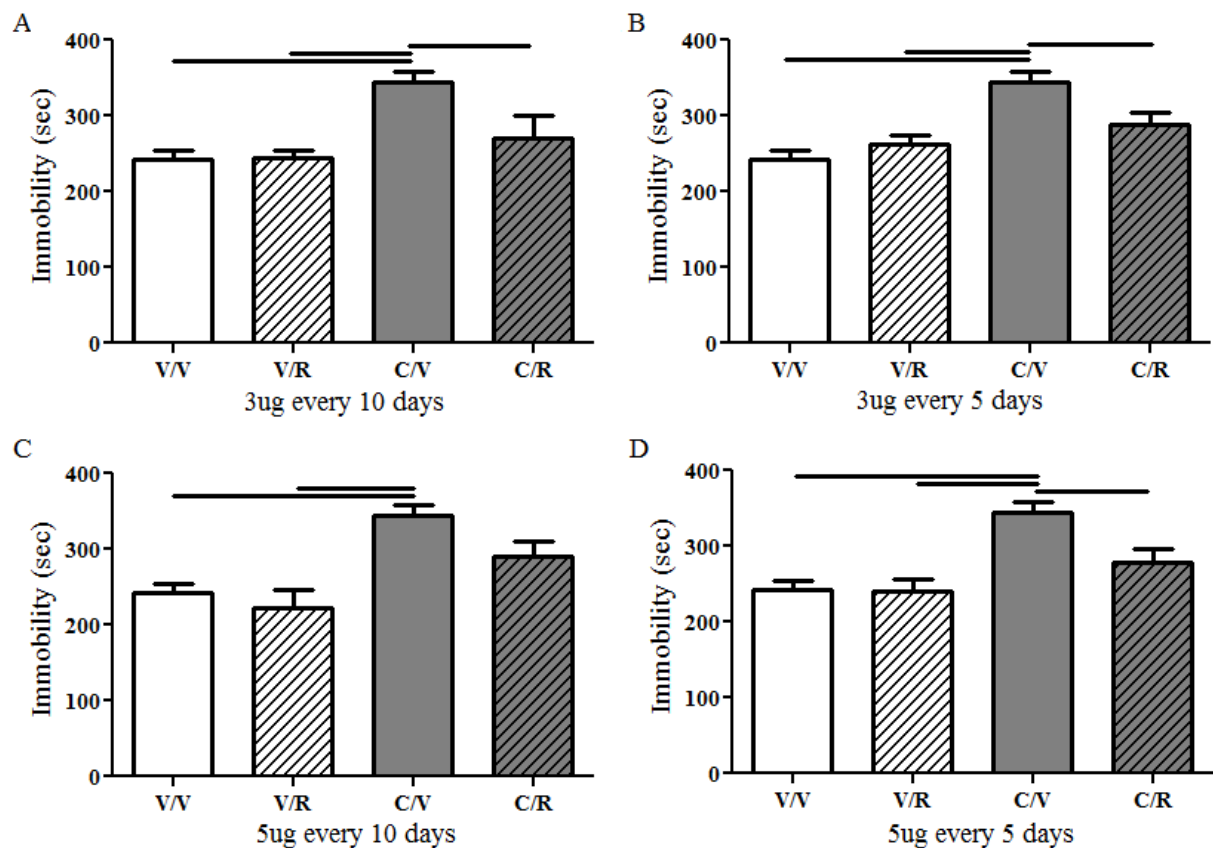
Figure 3.3. Immobility time in the FST in each subgroup. A) 3μg/ml every 10 days. B) 3μg/ml every 5 days. C) 5μg/ml every 10 days. D) 5μg/ml every 5 days. Data is expressed as mean±SEM. Lines represent significantly different groups.

3.3.2 Reelin expression

The reelin data was analysed using the same combination of groups as what was done for the behavioural data. Figure 3.4 provides photomicrographs of reelin immunostaining in each of the quantified subgroups. Only cells located along the SGZ were included in the analysis. The reelin data were analysed using the same combination of groups as what was done for the behavioural data. When the different dose and frequency subgroups are pooled together to create 4 groups with two treatment factors (vehicle or CORT and vehicle or reelin), a two-way ANOVA showed that there was a significant main effect of CORT [$F(1, 74) =$

28.891, p<0.001] and reelin [F(1, 74) = 9.790, p<0.001] on reelin expression, as well as an interaction of CORT and reelin [F(1, 74) = 6.315, p=0.0142].

When combining both treatment factors as one combined treatment (V/V, V/R, C/V, C/R), a one-way ANOVA [F(3, 74) = 12.938, p<0.001] and Tukey post-hoc test can be conducted to determine which group means differ from each other. The results of the analysis showed that the CORT/vehicle group was significantly different from the vehicle/vehicle (p<0.001), vehicle/reelin (p<0.001), and CORT/reelin (p<0.01) groups. There were no other group differences (see figure 3.5).
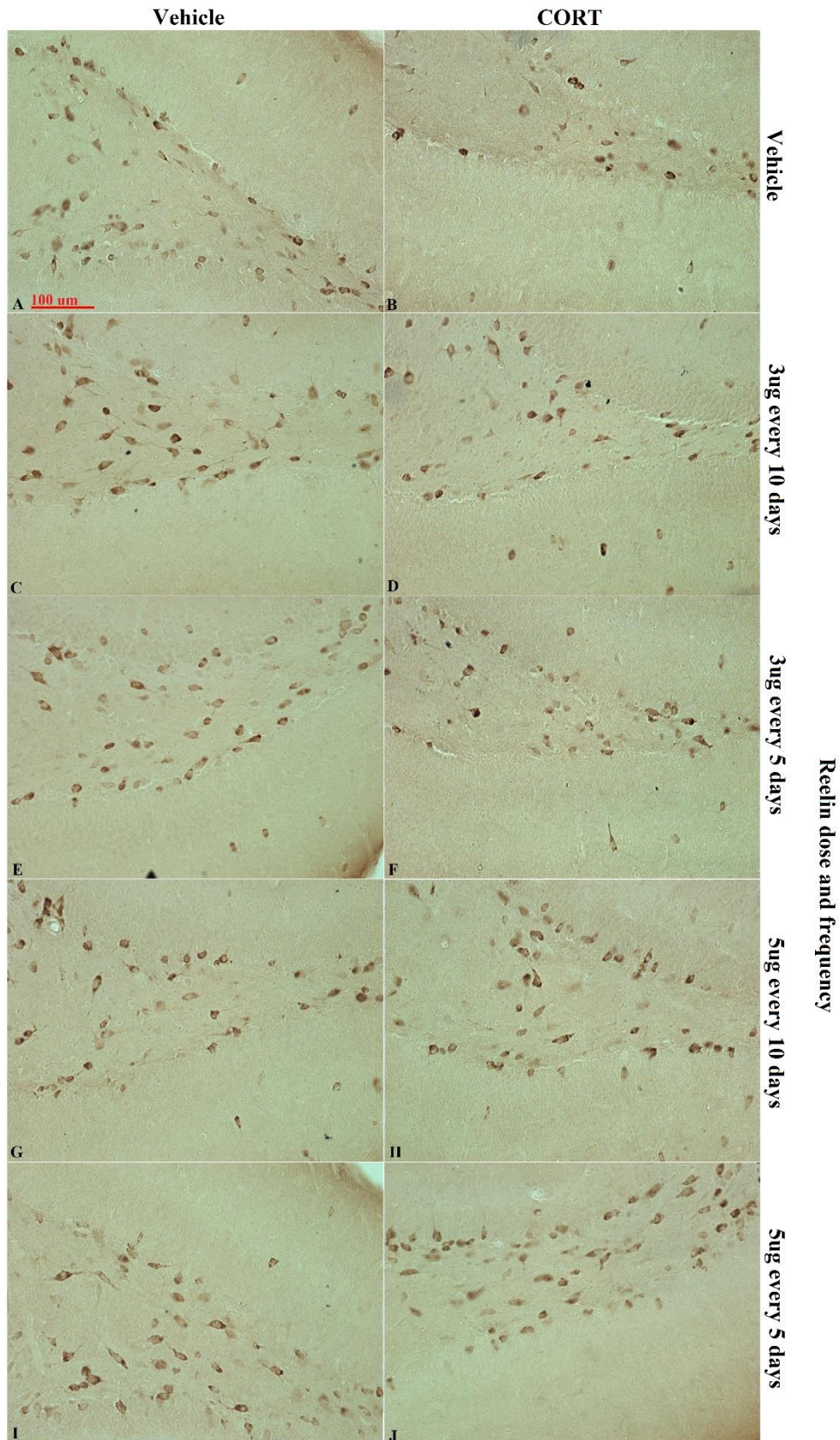
Figure 3.4. Reelin+ cells in the SGZ. A) Vehicle/vehicle; B) CORT/vehicle; C) Vehicle, 3µg/ml every 10 days; D) CORT, 3µg/ml every 10 days; E) Vehicle, 3µg/ml every 5 days; F) CORT, 3µg/ml every5 days; G) Vehicle, 5µg/ml every 10 days; H) CORT, 5µg/ml every 10 days; I) Vehicle, 5µg/ml every 5 days; J) CORT, 5µg/ml every 5 days.
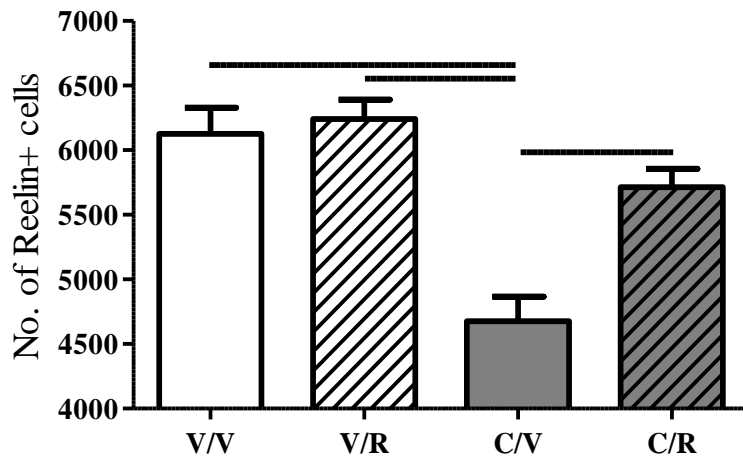
Figure 3.5. Number of reelin+ cells in the SGZ. Data is presented as mean±SEM. Lines represent significantly different groups.

When looking at the dose and frequency subgroups (3μg/ml every 10 days, 3μg/ml every 5 days, 5μg/ml every 10 days, 5μg/ml every 5 days) individually, one-way ANOVAs can be done to investigate the group differences using the combined treatment as a factor.

When looking at the 3μg/ml every 10 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 31)}=13.999$, $p<0.001$]. A Tukey post-hoc test revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p<0.001$), vehicle/reelin ($p<0.001$), and CORT/reelin ($p=0.035$) groups (see figure 3.6A).

Similarly, when looking at 3μg/ml every 5 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 32)}=11.194$, $p<0.001$]. A Tukey post-hoc test revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p<0.001$) and vehicle/reelin ($p<0.001$) groups, but not CORT/reelin (see figure 3.6B).

When looking at the 5μg/ml every 10 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 34)}=12.080$, $p<0.001$]. A Tukey post-hoc revealed that CORT/vehicle was significantly different from vehicle/vehicle ($p<0.001$), vehicle/reelin ($p=0.001$) and CORT/reelin ($p=0.001$) (see figure 3.6C).

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When looking at the 5µg/ml every 5 days subgroup, a one-way ANOVA showed that there was a significant effect of treatment [$F_{(3, 34)}=8.401$, $p<0.001$]. A Tukey post-hoc test revealed that the CORT/vehicle group was significantly different from the vehicle/vehicle ($p=0.001$), vehicle/reelin ($p=0.001$), and CORT/reelin ($p=0.009$) groups (see figure 3.6D).

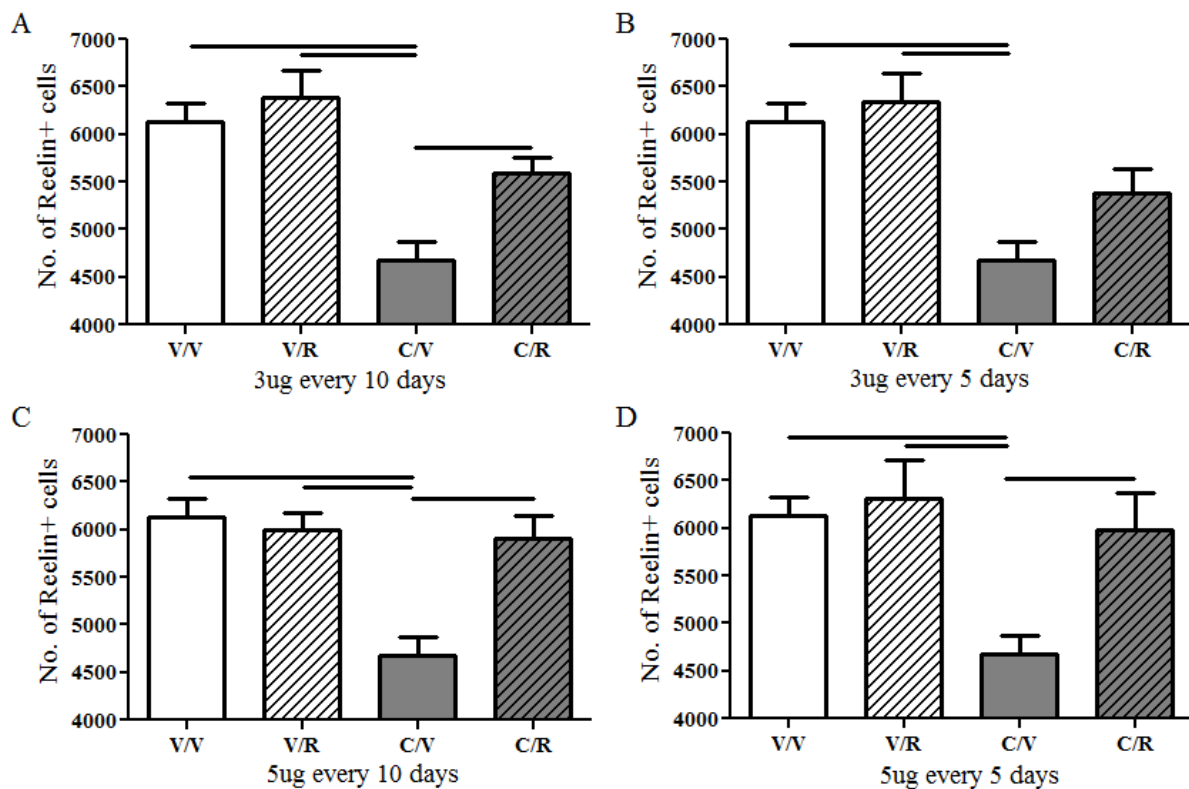

Figure 3.6. Number of reelin+ cells in the SGZ in each subgroup. A) 3µg/ml every 10 days. B) 3µg/ml every 5 days. C) 5µg/ml every 10 days. D) 5µg/ml every 5 days. Data is expressed as mean±SEM. Lines represent significantly different groups.

Table 3.2. Summary of immobility and reelin+ cell counts in each subgroup.

Group	3ug / 10days (n)	3ug / 5 days (n)	5ug / 10days (n)	5ug / 5days (n)	
	Dose / frequency				
Vehicle/vehicle	243±13 (12)	243±13 (12)	243±13 (12)	243±13 (12)	Immobility
	6127±200 (11)	6127±200 (11)	6127±200 (11)	6127±200 (11)	Reelin
Vehicle/reelin	245±9 (6)	263±11 (6)	223±23 (8)	241±15 (8)	Immobility
	6381±289 (6)	6347±300 (4)	5991±188 (7)	6309±402 (7)	Reelin
CORT/vehicle	**345±14**[abc] (12)	**345±14**[abc] (12)	**345±14**[ab] (12)	**345±14**[abc] (12)	Immobility
	4676±191[abc] (12)	**4676±191**[ab] (12)	**4676±191**[abc] (12)	**4676±191**[abc] (12)	Reelin
CORT/reelin	271±29 (6)	289±16 (10)	291±19 (8)	279±17 (8)	Immobility
	5588±164 (6)	5388±248 (9)	5902±249 (8)	5987±379 (8)	Reelin

Data is expressed as mean±SEM. As there is no dose of vehicle/vehicle and CORT/vehicle the value is presented across all subgroups. a=p<0.05 vs vehicle/vehicle, b=p<0.05 vs vehicle/reelin, c=p<0.05 vs CORT/reelin (Tukey post-hoc test).

3.3.3 Correlation between FST immobility and number of reelin+ cells

A Pearson's bivariate correlation coefficient test showed that there is a significant negative correlation between the number of reelin-positive cells and time spent immobile in the FST (r = -0.362, R^2 = 0.131, p = 0.001, n = 78) (see figure 3.7).
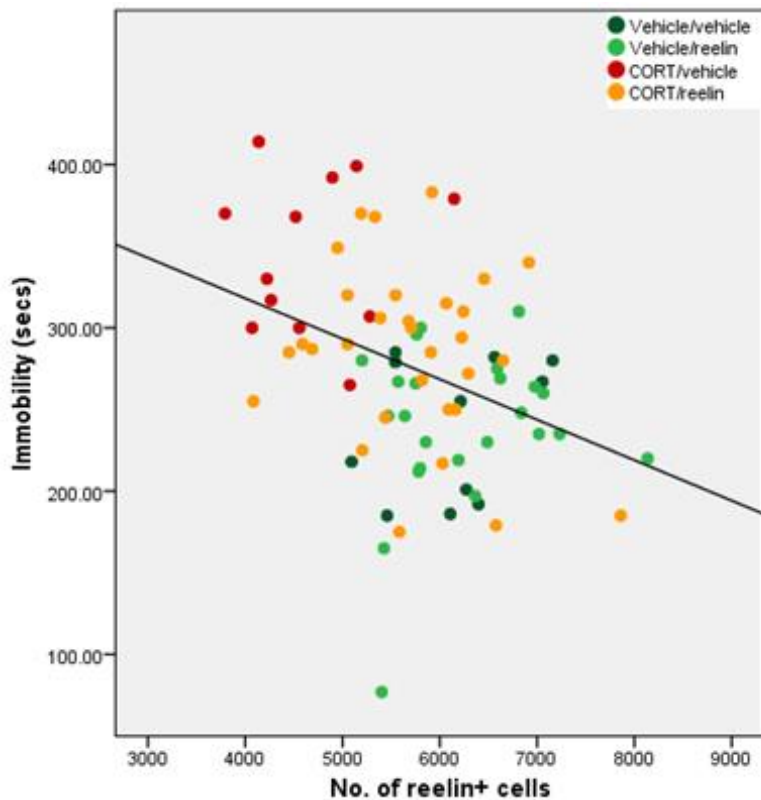
Figure 3.7. Relationship between FST immobility and number of reelin+ cells in the SGZ.

When frequency and dose are taken into account, a Pearson's bivariate correlation showed that there is a significant negative correlation between the number of reelin-positive cells and time spent immobile in all subgroups; 3μg/ml every 10 days (r = -0.428, R^2 = 0.183, p = 0.010, n = 35) (see figure 3.8A), 3μg/ml every 5 days (r = -0.421, R^2 = 0.178, p = 0.010, n = 36) (see figure 3.8B), 5μg/ml every 10 days (r = -0.412, R^2 = 0.170, p = 0.010, n = 38) (see figure 3.8C) and 5μg/ml every 5 days (r = -0.506, R^2 = 0.256, p = 0.001, n = 38) (see figure 3.8D).
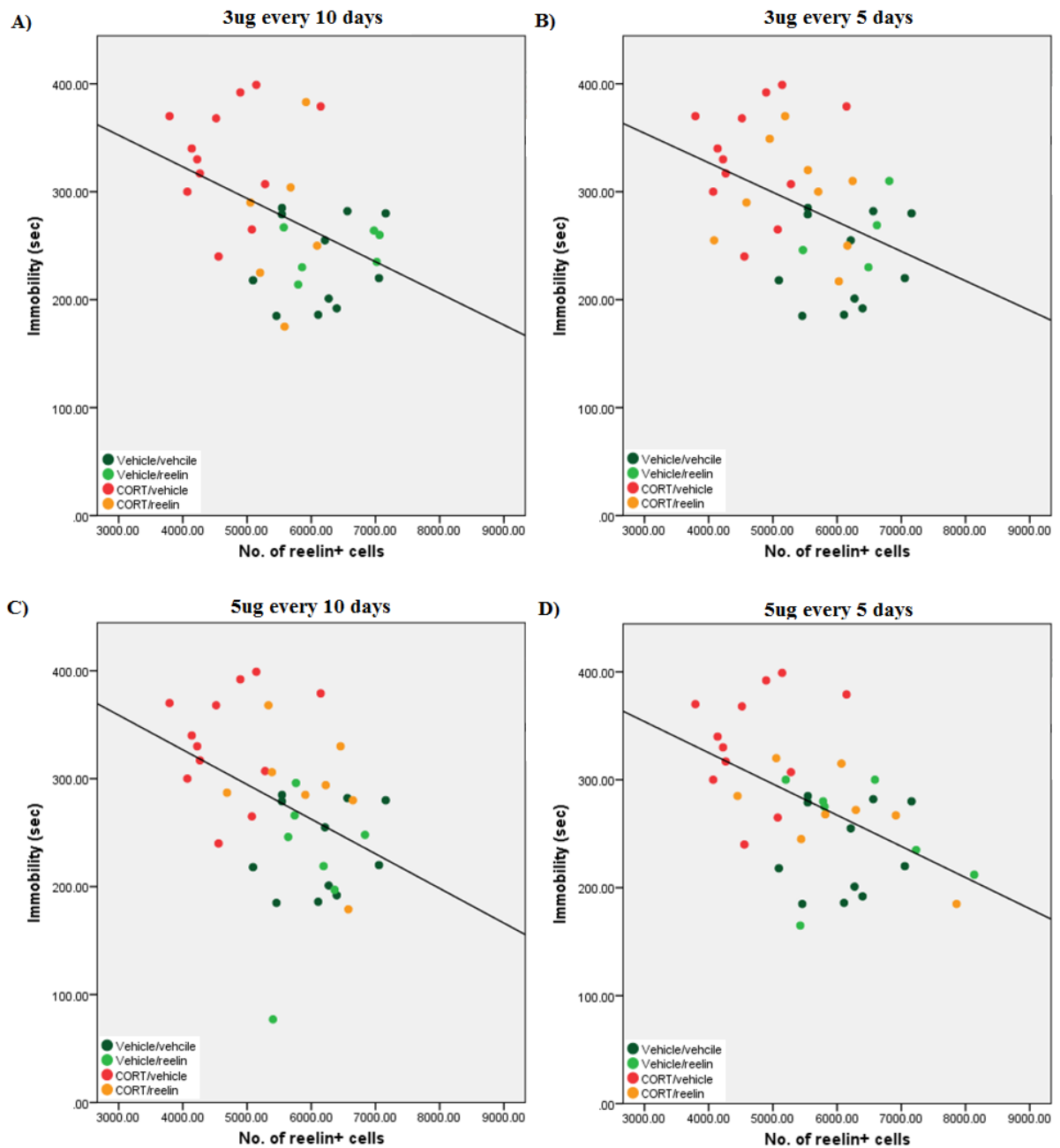
Figure 3.8. Relationship between FST immobility and number of reelin+ cells in the SGZ in each subgroup. A) 3μg/ml every 10 days. B) 3μg/ml every 5 days. C) 5μg/ml every 10 days. D) 5μg/ml every 5 days.

3.4 Discussion

The purpose of this study was to determine the potential antidepressant effects of reelin when it is administered peripherally. The results showed that 21 days of CORT injections increased the time spent immobile in the FST, which is consistent with previous observations (Lebedeva et al., 2017; Luo et al., 2017; Wróbel et al., 2017; Gregus et al., 2005; Zhao et al., 2008b). Furthermore, peripheral reelin injections significantly reduced immobility in the FST to control levels. When looking at the time spent immobile in the different dose/frequency subgroups, it appears all doses except 5μg/ml every 10 days significantly reduced the immobility produced by CORT alone. The is likely a statistical error problem as the trend is similar across all groups (CORT/reelin mean immobility for rats that received 5μg/ml every 10 days was only 2 seconds higher than rats that received 3μg/ml every 5 days). There was no difference between the vehicle/vehicle and vehicle/reelin groups, which indicated that there was no detrimental effect of reelin administration under normal physiological conditions.

Our laboratory previously reported that chronic CORT treatment results in the downregulation of reelin in the SGZ (Lussier et al., 2013a; the previous study in this thesis) as well as the CA1 stratum lacunosum (Lussier et al., 2009) and hilus (Fenton et al., 2015). Furthermore, mice deficient in reelin are more vulnerable to the depressogenic effects of CORT (Lussier et al., 2011). This collection of studies suggests that reelin is neuroprotective and our data supports this idea. All doses of reelin, bar 3μg/ml every 5 days, administered into the lateral tail vein restored the expression of reelin-positive cells in the SGZ in a similar manner to imipramine (Fenton et al., 2015). Again, all doses would likely have significance if the number of animals in groups were increased.

There was a significant correlation between immobility time in the FST and the downregulation of reelin, which was seen across all subgroups. This finding further supports

our idea that the downregulation of reelin plays a role in the development of a depression-like phenotype. To our knowledge, no other researchers have attempted to inject reelin peripherally to determine its potential antidepressant effects. Thus, the mechanism of action by which peripheral reelin exerts its antidepressant effects still needs to be defined. Although not yet published, our laboratory found that infusing reelin directly into the hippocampus decreased FST immobility, but this effect was blocked when an AMPA receptor antagonist was given prior to behavioural testing. It is not yet known if reelin crosses the blood-brain-barrier. If reelin can cross into the brain, peripheral reelin could also exert effects through the activity at the AMPA GluA1 receptor site. However, it is also possible that the effect of peripheral reelin occur through other mechanisms, such as the regulation of the immune system. The immune system is known to be altered in depressed patients raising the possibility that the fast-acting antidepressant effects of reelin could be mediated by the restoration of normal immune functioning. *In vitro* studies support this; when incubated with CORT, SERT clusters are altered on lymphocytes but remain unaltered with the addition of reelin. In addition, heterozygous reeler mice and depressed patients show increased size of SERT clusters and percentage of lymphocytes surface occupied by SERT clusters than wildtype mice (Rivera-Baltanas et al., 2010) and controls (Rivera-Baltanas et al., 2012), respectively. Our laboratory found that when reelin was injected peripherally, the size of SERT clusters on lymphocytes were restored to control levels (not published). The immune system affects the central nervous system (CNS) through cytoknines which maintain normal brain signaling and emotions (Jeon & Kim, 2016) and are known to be altered in depression (Felger & Lotrich, 2013). Proinflammatory cytokines are consistently seen to be increased in depressed patients (Dowlati et al., 2010; Frommberger et al., 1997) and are decreased with antidepressant treatment (Slavich & Irwin, 2014; Smagula et al., 2017; Zhang et al., 2017). In addition, heterozygous reeler mice also express dysregulated levels of cytokines by lymphocytes (Green-Johnson et

al., 1995). Whether reelin crosses the blood-brain-barrier or not still needs to be determined, but it is possible that reelin may exert effects through regulation of pro- or anti-inflammatory peripheral cytokines which are able to access the CNS altering levels of inflammatory mediators such as cyclooxegenase-2, prostaglandin E2, nitric oxide and other cytokines and chemokines (Felger & Lotrich, 2013). Preliminary data from our lab, however, did not find any effect of reelin on anti-inflammatory cytokines interleukin (IL)-13; proinflammatory cytokines IL-1A, IL-1B, IL-4, IL-5, IL-6, IL-12, IL-17A, Il-18, TNFa or VEGF; chemokines LIX, MCP1, MIP2, RANTES, Fractalkine, GMCSF, GCSF or GROKC although further research is needed. Reelin did decrease the CORT-induced increase in levels of chemokines MIP1A and IP-10 in blood and increase anti-inflammatory IL-10. MIP1A is involved in the activation of granulocytes, induces synthesis and release of pro-inflammatory cytokines from fibroblasts and macrophages and promotes cell migration against immune cells (Lee et al., 2000), while IP-10 is involved in chemoattraction of monocytes, T cells, NK cells, dendritic cells and the adhesion of T cells to endothelial cells (Dufour et al., 2002).

To summarise, the CORT-induced depressogenic effects on FST immobility and reelin-positive cells in the SGZ were reversed with peripheral reelin injections. Future studies should go deeper and look at more behavioural and cognitive tests, look at male and female rats and reelin dose-response curves should be conducted.

Chapter 4

General Discussion

4.1 General Discussion

The main purpose of the experiments described in this thesis were to provide further evidence that chronic CORT treatment results in the development of a depression-like phenotype, and that cyclical and intermittent CORT injections can be used as an animal model of recurrent depression in which recovery is diminished with subsequent cycles. In addition, these experiments investigated whether a downregulation of reelin-positive cells in the SGZ parallels the development of the depressive-like behaviour, whether the injection of reelin into the lateral tail vein has antidepressant effects, and normalizes the number of reelin-positive cells in the SGZ. The results strongly support these ideas.

MDD is a common psychiatric disorder characterized by repeated episode relapses. For this reason, it is important that animal models mimic the cyclical nature of the disorder to study the neurobiological mechanisms behind the onset of each episode relapse, and the relationship between episode onset, severity, recovery and stress. A novel aspect of experiment 1 was the use of 3 cycles of CORT treatment interspersed with recovery periods, to closely mimic the cyclical nature of depression. As well, no other research groups have attempted to investigate the neurobiological alterations that follow repeated exposure to CORT injections. There is previous evidence showing that animals sensitize to stress (Remus et al., 2013; Lebedeva et al., 2017; Zurita et al., 2000), and therefore re-exposure to stressful periods would invoke depressive-like behaviour more rapidly. Our results back up the stress sensitization hypothesis. In cycle 1, no difference in FST immobility was observed after 10 days of CORT treatment but it was clear after 21 days of CORT treatment (consistent with previous research using a dosage of 20mg/kg [Brotto et al., 2001; Lebedeva et al., 2017]), which went back to control level after the recovery period. Despite recovering fully, there was a difference after 10 days of a second (Lebedeva et al., 2017) and a third cycle. This tells us that depression-like behaviour was re-

established faster in subsequent cycles, which Remus et al. (2013) and Zurita et al (2000) found with re-exposure to CMS and CVS, respectively. Only half of the injection period of cycle 3 was needed to induce higher immobility times than those seen in cycle 1. Furthermore, the depression-like behaviour was more pronounced in cycle 3 compared to cycle 1, suggesting that symptomatology worsened. Human patients experience similar phenomena; a greater number of episodes correlates with aggravated symptomatology and episode duration (Karp et al., 2004).

CORT treated rats consistently show a significant decrease in weight compared to controls (Lebedeva et al., 2017; Lussier et al., 2013a, 2013b; Gregus et al., 2005). However, as a larger body weight should allow rats to float more effortlessly we can assume the increase in immobility in CORT treated rats is due to a depressive-like state. Nevertheless, it is important to account for this confounding effect as best as possible. This was done by dividing the immobility time in the FST by the weight of the rat at the time of behavioural testing. When body weight is controlled for, a group difference is seen after 10 and 21 days of cycle 1 in that CORT increases immobility at both time points. Immobility then returns to control levels after recovery. This is consistent with what was reported by Lebedeva et al. (2017). After the third recovery period, however, after the 21-day injection-free period. After the third recovery period, however, immobility/body weight scores remained significantly higher in the CORT treated rats. What was once enough time to recover in cycle 1, and assumingly in cycle 2 (as was in Lebedeva et al., 2017), is not sufficient for recovery after 3 cycles. Therefore, as was hypothesized, recovery diminished with subsequent cycles. Increasing the dose to 40mg/kg, however, affected recovery rates in a second cycle (Lebedeva et al., 2017) further evidencing the dose-dependent effects of CORT (Johnson et al., 2006). In patients with depression, the occurrence of partial remission is an important event contributing to episode relapse (Paykel et al., 1995; Fava et al., 2007; Kendler et al., 2000). Furthermore, 32% (Paykel et al., 1995) to

82.3% (Gasto et al., 2003) of remitted patients still show residual symptoms that negatively correlate with levels of social functioning (Masai et al., 2016). Thus, residual symptoms and diminished recovery would be problematic when considering therapeutic intervention. As well as lower inter-episode duration, severity of episodes and increased number of episodes (Kendler et al., 2001), the presence of residual symptoms and partial remission may be important factors in the evolution of depression.

It is important to note that FST immobility increased over time in the vehicle rats which suggests other factors are influencing behaviour, for example age, stress from being housed singly and stress from the saline injections. We have previously seen that CORT treatment does not usually affect general locomotor activity in the OFT (Kalynchuk et al., 2004; Lussier et al., 2011; Gregus et al., 2005), strength in the WST (Marks et al., 2009), or swimming in the MWM (Sousa et al., 2000) indicating that increased FST immobility was not due to catabolic effects on muscle tissue, but rather, that it is indicative of a depression-like state. It would be wise to measure food and water intake in future studies to determine if weight loss is a result of appetite suppression, which could affect strength and be a confounding factor on FST immobility. However, this is probably not the case. Although CORT enhances leptin-induced weight loss (Gemmill et al., 2003; Mishima et al., 2015), our laboratory found that when treated with reelin (outlined in experiment 2), depression-like behaviour was reversed but plasma leptin levels remained the same (not published). An advantage of this study is that rats were naive to behavioural testing which eliminated the confounding effects of memory in the FST, something not done by Lebedeva et al. (2017).

Hippocampal reelin levels are reliably decreased by CORT treatment (Lussier et al., 2009, 2013a; Fenton et al., 2015). A 21% to 25% decrease in reelin expressing cells in the SGZ was seen after 21 days of CORT treatment (Lussier et al., 2009, 2013a) and our data are no different. When controlled for body weight, the CORT-induced 8% increase in immobility was

paralleled with a 23% decrease in reelin-positive cells after cycle 1 recovery which grew to a 16% increase and 30% decrease in immobility and reelin-positive cells, respectively, after cycle 3 recovery. A lack of parallelism between recovery of FST immobility and reelin expression was seen, however. Immobility recovered by over 22% in both cycles while reelin-positive cell expression did not in either. Perhaps CORT resulted in cell death of reelin-expressing neurons, and to compensate for this loss, remaining neurons secreted higher levels of reelin resulting in recovery of behaviour in cycle 1. It is possible as atrophy of cells increased, the compensatory increase in reelin secretion was not sufficient to recover behaviour in cycle 3. A reduction in reelin-positive cells in the SGZ is important as this is one of two neurogenic sites in the adult brain, and similar patterns of hippocampal reelin downregulation are seen in post-mortem depressed patients' tissue (Knable et al., 2004). The question of whether CORT-induced alterations in GABAergic interneurons alters the course of neurogenesis was investigated by Lussier et al. (2013a). They found that CORT decreased the number of surviving new-born cells and dampened dendritic spine complexity. Therefore, reelin downregulation may cause a delay in the maturation of new-born cells, and cause the ectopic placement of cells, resulting in deficits in hippocampal plasticity and depression-like behaviour. Research by Teixeira et al. (2012) supports this idea. They showed that inactivation of the reelin pathway (through Dab1 suppression) caused aberrant migration, altered dendritic morphology and abnormal circuit formation.

The relationship between FST immobility and reelin downregulation was confirmed by correlation coefficient analysis, which showed that higher levels of reelin expression was negatively correlated with lower immobility. The lack of parallelism regarding recovery of immobility but not reelin can also be seen when the relationship between these two variables are plotted over separate time points; the correlation is lost when the recovery groups are plotted separately showing the recovery of behaviour but not reelin expression. These results

support the kindling hypothesis of depression (Post, 1992); the long-lasting downregulation of reelin over each cycle leaves the animal more vulnerable to CORT's depressogenic effects. Reelin expression did not recover, and so less stress might be required to induce more pronounced subsequent helpless behaviour. CORT results in glial and neuronal cell atrophy (Zhang et a., 2015), and so whether the downregulation of reelin is a result of increased cell atrophy or simply neurons no longer expressing the reelin protein should be investigated by staining a marker of cell death.

As over half of patients suffering from depression experience episode relapses (Hollon et al., 2006; Pettit et al., 2006; Solomon et al., 2000), a cyclical model of depression is a valuable research tool; stronger construct and face validity are provided. A downside to this model is that CORT can cause liver damage, and so the screening of antidepressants may lead to liver morbidity.

When full compliance of treatment is present, remission rates are reported to be 30-40% at best (Trivedi et al., 2006; Thase et al., 2001) and treatment is often discontinued due to adverse side-effects. Not only this but therapeutic effects of antidepressant are not seen for about 3 weeks (Stahl, 2000). Thus, it is important that new fast-acting antidepressants are available. In experiment 2, peripheral reelin injections into the lateral tail vein were executed in an attempt to evaluate the potential therapeutic effects of the reelin protein. To date, no one has injected reelin peripherally and knowledge of the functional role reelin plays in the periphery is lacking. Four different doses of reelin were used to get an idea of the therapeutic range; 3μg/ml every 10 days, 3μg/ml every 5 days, 5μg/ml every 10 days and 5μg/ml every 5 days.

Results showed that CORT treated rats had significantly higher immobility scores in the FST. Importantly, reelin injections reversed the CORT-induced deleterious effects to

control levels like other antidepressants (Fenton et al.,2015; Rainer et al., 2012). All doses, except 5μg/ml every 10 days reduced FST immobility to a significant degree. Had more animals been involved in the study, increasing the statistical power, all doses would probably have significantly reversed behaviour.

All doses of reelin restored the number of reelin-positive cells in the SGZ, which is consistent with what Fenton et al. (2015) showed with daily imipramine. The vehicle/reelin rats were indistinguishable from the vehicle/vehicle rats suggesting that there were no adverse effects of reelin under normal physiological conditions. It is therefore arguable that reelin has antidepressant effects and may be neuroprotective. This observation stems from several published and unpublished studies conducted by our laboratory: first, acute (1) and chronic (3) hippocampal reelin infusions (1μg/μl) reversed FST immobility in the CORT model of depression; second, reelin deficient mice are more susceptible to the depressogenic effects of CORT (Lussier et al., 2011); third, a single dose of reelin administered into the lateral tail vein also produced antidepressant effects in the FST, as well as repeated peripheral injections outlined in chapter 3. To further understand the neuroprotective properties reelin may have, an experiment could be designed where reelin is administered on day 1 of CORT injections. You could then observe for how long reelin serves to protect from the CORT-induced deficits.

The mechanisms of action of peripheral reelin are not yet known. As previously mentioned, however, through a series of experiments our laboratory has provided some evidence to suggest AMPA receptors and increased neurogenesis are contributing factors in the antidepressant effects of reelin, at least in the brain. First, a single infusion of reelin into the hippocampus has antidepressant effects in the FST and when examined a week later, the CORT-induced downregulation of DCX was restored. Second, when sacrificed immediately after the FST (24 hours after reelin was infused), DCX downregulation was not restored. This suggests that neurogenesis, per se, is not responsible for the fast-acting antidepressant effects.

Since the CORT-induced downregulation of AMPA receptor subunit GluA1 was restored -- a reduction in the phosphorylation of GluA1 is related to a depressive/anxious phenotype (Rame et al., 2017; Kiselycznyk et al., 2013) -- our group hypothesized that blocking this receptor would abolish the fast-acting antidepressant effects of reelin. On the last day of CORT injections, rats received either an infusion of reelin or reelin + CNQX, which is an AMPA/kainate receptor antagonist. Rats were again sacrificed after the FST and results showed that while reelin alone reversed CORT-induced FST immobility, the reelin-CNQX treated rats showed no behavioural improvement compared to the CORT/vehicle rats. These results suggest that AMPA/GluA1 is a contributing factor in reelin's fact-acting antidepressant effects. It is therefore important to know whether reelin is capable of crossing the blood-brain-barrier as this would have important therapeutic, physiological and pathological implications. AMPA receptors are usually co-expressed with NMDA receptors where they both fundamentally influence processes of cognition, plasticity, LTP, excitotoxicity, neuroprotection and mediate the actions of antidepressants (Fraize et al., 2017; Zarate & Manji, 2008).

Another potential mechanism of action is through the immune system. Mice deficient in reelin show an altered number and size of SERT clusters on blood lymphocytes (Rivera-Baltanas et al., 2010) along the same pattern that is seen in depressed patients (Rivera-Baltanas et al., 2012). In addition, several preliminary data from our lab suggest that reelin may regulate immune functioning. It was found that when incubated with CORT, SERT clusters on lymphocytes are altered, but when incubated with CORT + reelin the alterations were reversed. Moreover, peripheral reelin injections normalized the size of SERT clusters on peripheral lymphocytes, which are typically altered with CORT treatment. It is possible that reelin exerts effects by decreasing levels of peripheral pro-inflammatory cytokines or increasing levels of anti-inflammatory cytokines. Although more research needs to be conducted, our lab did find that reelin lowered levels of chemokines MIP1A and IP-10 in blood which function to activate

eosinophils and basophils, induce synthesis and release pro-inflammatory cytokines (Lee et al., 2000) as well as chemoattract cells and regulate T cell adhesion to endothelial cells (Dufour et al., 2002). Furthermore, anti-inflammatory IL-10 was increased with reelin. Reeler mice also show dysregulated secretion of cytokines by lymphocytes (Green-Johnson et al., 1995), which are commonly used as biomarkers in human depression. The increase in proinflammatory cytokines is consistently seen in depressed patients (Dowlati et al., 2010; Felger & Lotrich, 2013; Frommberger et al., 1997) and are decreased with antidepressant treatment (Slavich & Irwin, 2014; Smagula et al., 2017; Zhang et al., 2017).

4.2 Limitations

There are several limitations that should be pointed out in the studies described in this thesis. First, we shall discuss limitations that arise in chapter 2 - "Parallel effects of cyclical corticosterone administration on depression-like behaviour and the downregulation of reelin in the rat hippocampus". The first and most obvious limitation is the age of the rats. It could be argued that you cannot compare the immobility score or number of reelin expressing cells in animals that differ greatly in age. It is also well-known that rats quickly gain weight, and so older rats will usually be heavier. This will affect the time spent immobile in the FST, hence why immobility time was divided by body weight at the time of testing.

There are also several limitations that arise from the experiment outlined in chapter 3 – "Peripheral reelin injections reverse depression-like behaviour and the downregulation of hippocampal reelin in the CORT model of depression". As previously mentioned, rats were placed into a restraining device to receive the lateral tail vein injections. However, it is impossible to keep the rats in the restrainer for the same amount of time, given that some injections take longer than others. It could be argued that prolonged time in the restrainer and

an increased number of tail-pricks increases the stress response. Saying that, it is important to mention that another group of animals were included in the study to control for the effects of the restrainer – rats that received vehicle injections but were never placed into the restrainer. Although the data for these rats are not published in this thesis, there were no significant differences in immobility time when compared to the vehicle/vehicle rats which suggest the restrainer did not have an effect. Although it appears there was no effect of restrainer, we still cannot say for sure that prolonged time in the restrainer had no effect within groups. In struggling rats, it was sometimes hard to determine whether the full dose of reelin was injected directly into the vein.

4.3 Conclusion

The purpose of the experiments in this thesis was to examine the role of reelin in the pathophysiology of depression. The novel findings presented here are that using three cycles of CORT injections interspersed with recovery periods could be used as an animal model of recurrent depression, with depression-like behaviour exacerbating with subsequent cycles along with diminished recovery. Reelin was downregulated in all time points measured and indeed paralleled the development of the depressive behaviour. This thesis also revealed that peripheral reelin injections have potent antidepressant effects in an animal model of depression.

The data presented in this thesis add to the growing body of evidence that reelin downregulation is involved in the development of the depressive-like phenotype, that it has antidepressant effects peripherally and is neuroprotective. Given that there is a desperate need for newer fast-acting antidepressants, our data are timely and important and may open the door for the development of new drugs for depression with novel mechanisms of action.

4.4 Future work

Future studies should evaluate the downregulation of reelin in other brain areas such as the paraventricular nucleus, the amygdala, the cerebellum and other areas thought to be involved in depression and the stress response.

As mentioned in section 4.2, it is important to control for age in an animal model of recurrent depression. To do this, rats in earlier groups could receive vehicle injections prior to the experimental time point injections. For example, CORT rats that were to be sacrificed after the first recovery period could have already received 21 days of vehicle injections, followed by the recovery period, followed by another 21 days of injections and another recovery period prior to the start of CORT injections. This way, all rats would have been the same age when sacrificed regardless of how many cycles experienced. However, this is a very time consuming and demanding task and may not be feasible.

Regarding peripheral reelin injections, more behavioural tests should be conducted to determine potential antidepressant/anxiolytic properties. For example, one could examine whether reelin influences behaviour in the SPT, anxiety-provoking tests like the EPM or OFT and memory tests such as the MWM.

It would be also interesting to administer different doses of reelin on day 1 of the 21-day CORT injection period to evaluate the potential neuroprotective properties. Behavioural tests could be done on day 7, 14, 21, for example, to see for how long acute reelin treatment served to protect against the CORT-induced depressogenic effects.

As depression is nearly three times more prevalent in women, gender differences need to be explored in both a recurrent model of depression and regarding the effects of peripheral reelin injection in a battery of tests, and on neurobiology.

It would be very valuable to determine the mechanism of action of reelin in the periphery and how it effects brain function. There are many studies that could be conducted. For example, lymphocytes isolated and incubated with reelin after vehicle or CORT injections could be reinjected back into the tail vein of the rat. This would determine if the antidepressant effects occur through mediation of these cells, of which our lab has found some evidence.

Although reelin is present in endothelial cells that make up the blood-brain-barrier (Perez-Costas et al., 2015), whether reelin crosses the blood-brain-barrier or not still needs to be determined. To test this, radioactive markers could be attached to the reelin structure, injected into the tail vein and later post-mortem analysis could detect whether the radioactive markers reached the brain or not. This may also be done by injecting reelin into a homozygous reeler mouse to later see if it is expressed in the port-mortem brain. Since these mice express no reelin, the presence of reelin in the brain must be from the peripheral injection.

As injecting reelin into the tail vein is novel, toxicity studies would need to be done to determine an appropriate dose if considering clinical trials in the future. In the second experiment outlined in this thesis, 2 doses of reelin were given at 2 different frequencies; by the end of the 21-day injection period, reelin-grouped rats received either 9, 15 or 25μg. As outlined in the conclusion, all doses reduced the depressive-like phenotype and restored the expression of reelin in the SGZ. It may be necessary to test increasing doses to investigate potential side-effects. Furthermore, a dose-response study would allow us to develop a therapeutic index; the lowest dosage that would produce behavioural or neurobiological effects, as well as the highest dose before the occurrence of detrimental effects.

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