Examining the Antidepressant Potential of TNF-alpha and Reelin on Associated Behavioural and Neurobiological Markers in a Preclinical Model of Depression

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ABSTRACT

Depression is a debilitating psychiatric disorder affecting approximately 10% of the world's population. Cognitive dysfunction is an often ignored aspect of depression, and predicts patient response to antidepressants. While several classes of antidepressants have been developed for the treatment of depression, over one-third of patients do not respond to conventional antidepressants, and all currently available antidepressants require weeks of continuous administration to achieve therapeutic effects. The delay in the onset of therapeutic effects from the time of taking antidepressants can put patients at risk for suicide. Therefore, there is a need in the literature to develop and identify novel antidepressants that do not work on conventional targets and that have fast-acting properties.

The primary goals of this dissertation were to investigate the pattern of cognitive impairments following CORT treatment and to examine the antidepressant potential of TNF-α receptor inhibition and reelin. To begin, in Chapter 2, I examined the effect of repeated CORT injections on depression-like behaviour using the forced swim test, the object-location, object-in-place, and object-recognition memory tests, and sensorimotor gating using prepulse inhibition. These data suggested that CORT increases depression-like behaviour while simultaneously impairing hippocampal and prefrontal cortex-dependent memory and sensorimotor gating. In Chapter 3, I examined the effects of the TNF-α inhibitor etanercept on CORT induced depression-like behaviour, cognition, and markers of synaptic plasticity through post-mortem analysis of brain tissue. I found that CORT increased depression-like behaviour on the forced swim test and impaired object memory. CORT also impaired several post-mortem markers of hippocampal plasticity, and etanercept restored these measures back to control levels.

In Chapter 4 I examined the antidepressant potential of intrahippocampal reelin infusions and its effects on cognition and markers of synaptic plasticity. I found CORT treatment increased depression-like behaviour on the forced swim test and impaired object memory. Reelin treatment restored both of these measures back to control levels within one day. CORT also decreased markers of hippocampal plasticity, and reelin restored these measures back to control levels. Finally, in Chapter 5, I examined the contribution of AMPA signaling to the antidepressant effects of reelin. I found that infusion of the AMPA antagonist CNQX blocked reelin's antidepressant effects on the forced swim test, without altering CORT's pattern of changes on

markers of hippocampal plasticity. The results of this dissertation reveal novel compounds with antidepressant properties that warrant further investigation into their potential use in humans.

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DEDICATION

I dedicate this collection of work to my wife, Kirstian Gibson.

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δ glutamate receptor beta 1-2

5-HT1A/1B serotonin 1a/b receptors

5-HT2A/2C serotonin 2a/c receptors

ACTH adrenocorticotropin hormone

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA analysis of variance

ApoER2 apolipoprotein E receptor 2

BDNF brain derived neurotrophic factor

BLBP brain lipid binding protein

BrdU 5-bromo-3'-deoxyuridine

BSA bovine serum albumin

CA++ calcium

CA cornu ammonis

CA1.Py pyramidal cell layer of CA1

CA1.SO stratum oriens of CA1

CA1.SR stratum radiatum of CA1

CA3.Py pyramidal cell layer of CA3

CA3.SO stratum oriens of CA3

CA3.SR stratum radiatum of CA3

CA.SL stratum lacunosum of CA3

cAMP cylic AMP

CNQX cyanquixaline

CORT corticosterone

CMS chronic mild stress

CREB cAMP response-element binding

CRH corticotrophin-releasing hormone

CUS chronic unpredictable stress

Dab 1 disabled-1

DAB 3, 3'-diaminobenzidine

DCX doublecortin

DG dentate gyrus

DISC1 disrupted in schizophrenia 1

DR discrimination ratio

DSM-V diagnostic and statistical manual of mental disorders V

DST dexamethasone suppression test

EC entorhinal cortex

EtoH ethanol

FST forced-swim test

GABA gamma-aminobutyric acid

$GABA_A$ gamma-aminobutyric acid alpha

GAD67 glutmic acid decarboxylase 67

GCL granule cell layer

GFAP glial fibrillary acidic protein

GluA1-5 glutamate receptor 1-5

GluK1-5 glutamate receptor kainite 1-5

GluN1-3, A-C glutamate receptor epsilon2 1-3, a-c

GR glucocorticoid receptor

GSK3α glycogen synthase kinase 3α

H2O2 hydrogen peroxide

HPA axis hypothalamic pituitary adrenal axis

hs-CRP high sensitivity C reactive protein

IDO indoleamine 2,3-dioxgenase

IL-1, 6, Interleukin 1, 6

Ikkα inhibitor of kα kinase complex

ir immunoreactive

LDLR low-density lipoprotein receptor

LPS lipopolysaccharide

LTD long term depression

LTP long term potentiation

MAOI's monoamine oxidase inhibitors

ML molecular layer

MLKL mixed lineage kinase domain like protein

mPFC medial PFC

MR mineralocorticoid receptor

mTOR mammalian target of rampomycin

NBQX 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-quinoxaline

NFKB nuclear factor KB

NMDA n-methyl-d-asparate

NMDAR n-methyl-d-asparate receptors

OBIP object-in-place recognition

OBL object-location recognition

OBR object-recognition

OE overexpressing

PI3K phosphatidylinositol-3-kinease

PC perirhinal cortex

PPI prepulse inhibition

Prefrontal cortex (PFC)

PSD-95 post-synpatic density-95

SEM standard error of the mean

SFKs SRC family tyrosine kinases

SGZ subgranular zone

Shh sonic hedhgehog

SNRIs selective norepinephrine reuptake inhibitors

SSRI's selective serotonin reuptake inhibitors

SPT sucrose preference test

sTNF-α soluble TNF-α

TACE TNF-α converting enzyme

TAK1 transforming growth factor-α activated kinase 1

TBS tris buffer solution

TCA's tricyclic antidepressants

TrkB tryosine receptor kinase B

tmTNF-α cell bound surface precursor TNF-α

TNF-α tumor necrosis factor alpha

TNFR1,2 TNF-α receptor 1, 2

TRADD TNF-α associated death domain protein

VEGF vascular endothelial growth factor

VLDL-R very-low densitiy lipoprotein receptor

CHAPTER 1
GENERAL INTRODUCTION

1.1. Thesis Outline

Depression remains the most common psychiatric disorder, effecting approximately 350 million people worldwide (World Health Organization, 2012). In Canada, around 11% of individuals aged 15-24 suffer from depression (Statistics Canada, 2017). Accordingly, depression now ranks as the most common cause of global disability in terms of years lost due to disability. The defining characteristics of depression include anhedonia, a loss of interest in pleasurable activities, and lowered mood (Nestler et al, 2002). Depression is further characterized by alterations in weight, sleep, irritability, thoughts of suicide, and decreased sexual function/interest (Centre for Addiction and Mental Health, 2012). Depression is comorbid with several other disorders, including anxiety (Zimmerman et al., 2013) and in recent years there has been a growing recognition that depression frequently co-occurs with immune system disorders, for example rheumatoid arthritis (Margaratten, Julian, Katz, & Yelin, 2011). These comorbidities can influence the course of the disease, increase risk of relapses, and delay recovery (Hirschfeld, 2001). Therefore, given the prevalence and societal burden of depression, understanding the etiology and developing effective treatments is of paramount importance.

Medications that target the monoergic system remain the primary course of treatment for depressed patients. These medications include monoamine oxidase inhibitors (MOAI's), tricyclic antidepressants (TCA's), and selective serotonin reuptake inhibitors (SSRI's). The serendipitous discovery of the first class of antidepressants targeting the monoamine system lead to the first major theory of depression, the monoamine theory of depression (Schildraut, 1965). This theory posits that depression results from reductions in monoamine systems in limbic regions of the brain, and that restoring monoamine levels back to a non-diseased state will produce an antidepressant effect. Patients generally tolerate antidepressants well, with the most common side-effects including decreased sexual function and weight gain (Clincial Depression Learning Path, 2018). Despite the widespread use of antidepressants, they remain effective in only 30-60% of patients, and many patients become treatment resistant, or become resistant to multiple rounds of antidepressant therapy (Pubmed Health, 2017). Moreover, antidepressants require several weeks of continuous treatment until a therapeutic effect emerges, which is known as the therapeutic lag of antidepressants (Frazer and Benmansour, 2002). Despite the appealing

simplicity of the monoamine hypothesis, it has not gone without criticism, the most prominent being that if monoamine levels are being targeted within hours of taking an antidepressant, then why do therapeutic effects take weeks to occur? This has led to the current favored hypothesis of depression -- the neuroplasticity hypothesis. This hypothesis posits that depression dampens transcriptional regulation of proteins and growth factors which modulate plasticity in the adult brain. This hypothesis dovetails nicely with the therapeutic lag of antidepressants, as these alterations comprise slower-acting processes that can take weeks to occur. Therefore, the high occurrence of non-responders to antidepressants and the time lag it takes for antidepressant effects to occur reinforces the need for novel antidepressants that are not reliant on the monoergic system and that have fast-acting properties. The focus of this dissertation is on experiments investigating novel antidepressants and their effects on behaviour and corresponding neurobiological changes using a well validated preclinical model of depression -- the CORT model of depression.

The first experiment of this dissertation focused on characterizing cognitive deficits as a potential side-effect of repeated corticosterone (CORT) treatment in rats. In the second experiment, I utilized the behavioural tests developed in the first experiment to examine how treatment with the TNF-alpha antagonist etanercept affected CORT-induced alterations in hippocampal-dependent memory and markers of hippocampal plasticity. In the third experiment, I investigated the antidepressant potential of the extracellular matrix protein reelin and its effects on markers of hippocampal dependent memory and markers of hippocampal plasticity. Finally, in the fourth experiment, I investigated a potential mechanism underlying reelin's antidepressant effects and looked at changes in hippocampal neurogenesis, Fos expression, and microglial morphology.

The remaining sections of this chapter will provide an overview of depression, its neurobiology, and the major theories dedicated to explaining its etiology. I will then discuss the stress response and how hippocampal plasticity changes in response to stress. I will then discuss the link between depression and inflammation, and then turn my attention to the role of the extracellular matrix protein reelin and glutamate in depression. Finally, I will conclude this chapter with an overview of remaining unanswered questions in the field of stress and depression research. Chapters 2, 3, 4, and 5 will describe the experimental data addressing the questions

outlined in Chapter 1. Chapter 6 will finish with an overview and discussion of my experimental research, the implications and limitations, and directions for future research.

1.2. Introduction to Depression

Depression is a psychiatric disorder that affects 350 million people worldwide, and 11% of Canadians aged 15-24 (World Health Organization, 2012; Statistics Canada, 2017). Depression is not a new disorder; historical cases of depression have been around for thousands of years. The first written accounts of depression occurred over 4000 years ago in Mesopotamia (Horwitz, Wakefield, & Lorenzo-Luaces, 2016). However, with no understanding of the etiology of the disorder, patients' suffering was attributed to demonic possession. Understanding of depression improved by the time of the Greeks. For example, the father of medicine, Hippocrates, suggested that mental illness was related to the bodily humors (Horwitz, Wakefield, & Lorenzo-Luaces 2016). Hippocrates further classified mental illness into discrete categories, including mania and melancholia (depression). However, further understanding of depression remained stagnant for some time after Hippocrates. The understanding of depression took a significant leap forward when psychology first emerged as a science. At the forefront of this understanding was on one spectrum behaviourism, which believed that human behaviour was shaped by interactions with the environment, and at the other end determinism, which would say that human beings have no choice in what they develop into (e.g., human nature is determined by genes). Finally, the largest leap forward in our modern understanding of depression occurred in the 1950s. A physician treating patients for microbial infections serendipitously discovered that patients taking Iproniazid reported elevated mood. Following this discovery, a flurry of research ensued and it was discovered that Iproniazid was working to inhibit the monoamine oxidase enzyme, increasing the levels of serotonin and dopamine in the brain. This discovery shifted the view of depression to an explanation of arising from a chemical deficiency in the brain, and thus is an illness that requires treatment like any illness of the body.

Although the monoamine hypothesis of depression was revolutionary for its time, it is now generally accepted that depression involves more than deficiencies in monoamines. The currently favored hypothesis of depression is the neuroplasticity hypothesis, which posits that depression involves alterations in gene transcription and regulation, protein synthesis, and growth factors that modulate brain plasticity. This hypothesis nicely explains the downstream signalling cascades that might account for the delayed onset of relief of symptoms from antidepressants.

1.3. Classification of Depression

Depression is characterized by the presence of at least 1 of 2 cardinal symptoms, which must persist for a minimum of 2 weeks (American Psychiatric Association, 2013). The first cardinal symptom is anhedonia. Anhedonia refers to the loss of ability to experience pleasure in what were previously pleasurable activities. For example, a patient who was once a voracious reader may no longer feel like picking up a book. The other cardinal symptom is depressed mood, which includes a mood that is characterized as hopeless, discouraged, or sad (American Psychiatric Association, 2013). These cardinal symptoms are required to persist for nearly all daily activities, for most of the day. In addition to the presence of cardinal symptoms, there must be the presence of 4 out of 9 other symptoms for at least 2 weeks. The first and most frequently reported symptom is significant weight loss or gain, or gain or loss of appetite. Hypersomnia or insomnia is also frequently reported. Insomnia can manifest as trouble falling asleep, frequently waking up during the night, or waking up and not being able to get back to sleep. Not as common as alterations in weight or sleeping patterns include psychomotor agitation or retardation. This includes the inability to sit still or making repetitive movements of the hands (agitation) or talking slower or exaggerated purposeful movements (retardation). In addition to alterations in sleep, fatigue is a common symptom, and the feeling of menial tasks requiring more energy than normal. Feelings of worthlessness or guilt are also a common complaint. This is frequently seen in the act of excessive rumination over past events, particularly past failings. Increasingly recognized are difficulties or impairments in the ability to think, concentrate, or in one's memory. Patients may find that they are more forgetful than they used to be, or that they cannot maintain their focus on a task (partially overlapping with psychomotor agitation). The last symptom is suicidality. Here, patients may have comparatively benign thoughts of death, to thinking about not existing, to formulating specific thoughts about a suicide plan (American Psychiatric Association, 2013). There has been an increasing recognition that depression as currently classified might not be a single homogenous disorder, but rather a complex constellation of separate yet related disorders. This is illustrated by the fact that 2 depressed patients can present with completely different symptoms. For example, patient A can present with weight gain, insomnia, psychomotor retardation, and anhedonia, while patient B can present with weight loss, hypersomnia, psychomotor agitation, and anhedonia. A number of explanations have been put forward to explain this variability, including that subsets of depressed patients

experience neuroinflammation (described later) or glutamate dysregulation (described later) rather than the classically presented monoamine dysfunction. A recent report attempted to identify depression subtypes using magnetic resonance imaging and found 3 unique profiles relating to antidepressant treatment outcomes that correspond with different profiles of brain connectivity (Tokuda, Yoshimoto, Shimizu, Okada, & Takamura et al., 2018). It remains to be seen if the focus of depression research shifts to reflect the growing recognition of the proposed subtypes of depression.

The development of depression typically occurs after puberty, although childhood depression can occur (with a different presentation) (American Psychiatric Association, 2013). Depression is approximately 1.5-3 times more likely to occur in females than males. This increased occurrence of depression in females is attributed to a number of factors, both environmental and biological. Biological factors include differences in hormones, while environmental explanations include societal expectations placed on women. The progression of depression is quite varied, with some patients never experiencing true remission of symptoms (treatment resistant depression), whereas other patients can go years between depressive episodes. A key feature of depression that has begun to be recognized in recent years is the chronicity of the disorder. What this means is that depression is typically not a single time occurrence, but rather comprises multiple episodes over the lifetime, whether it be continuous or sporadic. A major limitation of the depression literature in animal studies is the focus on 1 cycle of depression and not multiple cycles. However, recent reports have begun to explore this question and suggest that the depressed brain becomes more vulnerable to repeated bouts of stress (Lebedeva, Caruncho, & Kalynchuk, 2017). The cyclicity of the disorder underscores the need for the development of more efficacious treatments for the disorder. Once patients undergo antidepressant therapy, recovery typically begins after 1-3 months (Willner, Scheel-Jruger, & Belzung, 2013), with remission occurring after 1 year. However, the course of recovery from depression is significantly decreased in patients whose depressive episode was severe, and in those who have had a high number of depressive episodes previously.

Before depression can be diagnosed, a number of other disorders must first be ruled out. The most common disorder to be mistaken for depression is bipolar disorder, which is differentiated from depression by the occurrence of mania. Another frequently confounding diagnosis is substance abuse disorder. Here, the difference between the two is the presence of a substance

resulting in the depressive episode. A further diagnosis that must be ruled out, while rare, is depression with psychotic features. Ruling out other diagnoses that could be causing depression is key to ensuring appropriate treatment for the patient (American Psychiatric Association, 2013).

1.4. Drugs used to Treat Depression

The first line of defence for treating depression are antidepressant drugs. Currently available antidepressant medications are effective in approximately 60-70% of depressed patients (Willner, Scheel-Jruger, & Belzung, 2013). However, this definition has been criticized for not defining the percentage of patients experiencing remission but instead response rate. When the percentage of patients experiencing remission is considered, the number drops to 45% (Thase, Entsuah, & Rudolph, 2001). This means that upwards of 55% of depressed patients will not experience remission of symptoms, indicating that the underlying pathology initially causing the depression may not be treated by the drug. This stresses the need for more efficacious pharmacological treatments in the management of depression. Antidepressant medications fall into several categories, based on their mechanism of action: MAOI's, TCA's, SSRIs, selective norepinephrine reuptake inhibitors (SNRIs), and atypical antidepressants (Table 1.). Each class of antidepressant medications will be discussed in greater detail below.

MAOI's were the first class of antidepressant compounds to be discovered, and revolutionized the treatment of depression. MAOI's work by inhibiting the enzyme monoamine oxidase, preventing the breakdown of monoamine neurotransmitters, and thereby allowing for higher concentrations of the monoamines (serotonin and norepinephrine) in the brain. While MAOIs were initially the first line of treatment in depression, their use has seen a decrease due to their lack of specificity mechanistically and their unwanted side effects. Some of the most frequently reported side effects include weight gain, fatigue, and hypertension. Other more serious side effects may emerge, including potentially lethal interactions with certain foods (e.g., tyramine) (Santarsieri & Schwartz, 2015). In terms of efficacy, MAOI's are generally reported to be less effective in the treatment of depression than TCA's and SSRI's (Thase, Entsuah, & Rudolph, 2001). Therefore, due to unwanted side effects and lower efficacy than other compounds, MAOI's are largely out of favor for the treatment of depression. However, MAOIs tend to be more efficacious in treating depressed patients with atypical features (Stewart, McGrath, Rabkin, & Quitkin, 1993). TCA's represent the next evolution of depression

Table 1-1. Classes of Antidepressant Medications

Class	Drugs	Common Side Effects
Monoamine Oxidase Inhibitors (MAOIs)	Isocarboxazid Phenelzine Tranylcypromaine Selegiline	Weight gain, sexual dysfunction, hypotension
Triclylic Antidepressants (TCAs)	Imipramine Amitriptylyine Doxepin Desipramine Nortripyline	Weight gain, sedation, tachycardia
Selective Serotonin Reuptake Inhibitors (SSRIs)	Fluoxetine Paraoxetine Sertraline Citalopram Escitalopram	Headaches, sexual dysfunction, weight gain
Selective Norepinephrine Reuptake Inhibitors (SNRIs)	Venlaflaxine Desenlafaxine Duloxetine Levomilnacipran	Nausea, sexual dysfunction, weight gain
Atypical	Bupropion	Headache, weight loss
	Mirtazapine	Sedation, weight gain
	Trazodone	Sedation, nausea
	Vilazodone	Nausea, insomnia
	Vortioxetine	Nausea, dizziness

Modified from (Santarsieri & Schwartz, 2015).

pharmacotherapy, developed in the 70s-80s. TCA's get their name from their chemical 3 ring structure, and work primarily by elevating serotonin and norepinephrine levels through uptake inhibition. They represent an improvement over the MAOI's, and are significantly more effective in the treatment of depression (Thase, Entsuah, & Rudolph, 2001). However, like the MAOI's, TCAs tend to have several undesirable side effects. Most prominent are weight gain, sedation, and tachycardia, an increase in the resting heartbeat. Additionally, due to downstream effects on acetylcholine signalling, patients frequently report dry mouth and blurry vision (Santarsieri & Schwartz, 2015). Like the MAOI's, TCA's have largely fallen out of favor in the treatment of depression due to the widespread use of SSRI's, however TCA's can be beneficial in treatment resistant depression (Santarseiri, 2015).

The 1990s saw the development of the SSRI's and the SNRI's. These compounds constituted a marked improvement over both the MAOI's and the TCA's, largely due to their reduced side effects and higher tolerance from patients. Some of the most common side effects of the newer generation antidepressant compounds include headaches, gastrointestinal irritability, initial anxiety, and sexual dysfunction (Santersieri & Schwartz, 2015). The increased patient adherence to these compounds arises from their selective nature. SSRIs' and SNRI's are *selective* for serotonin or norepinephrine reuptake inhibitors, and do not antagonize acetylcholine signalling like the TCAs. The result is a net increase in serotonin or norepinephrine at neuronal synapses in limbic brain regions. However, criticism in the literature has emerged regarding the over prescription of antidepressants, as 1 in 10 people are prescribed an antidepressant compound in North America (Santersieri, 2015). Although at first glance this number seems excessive, it coincides with the rate of depression observed in the population.

The last group of compounds used to treat depression are the atypical antidepressants. Atypical antidepressants get their name from their mechanism of action. Unlike MAOI's, TCA's, or SSRI's, atypical antidepressants do not act directly on serotonin or norepinephrine, although they may impact these chemicals through downstream mechanisms. One example is mirtazapine, which is classified as a noradrenergic antagonist-specific serotonin antagonist. This compound inhibits norepinephrine alpha-2 autoreceptors and blocks 5-HT2A/2C receptors, allowing more norepinephrine and serotonin in the cortex (Santersieri & Schwartz, 2015). What is notable about the atypical antidepressants is that the majority of these compounds were initially prescribed for other conditions, for example smoking cessation (Roddy, 2004). Due to atypical antidepressants

different mechanism of action, a different panel of side effects are commonly reported than for traditional antidepressants. For example, sedation is the most frequently reported side effect of atypical antidepressants, in addition to nausea. Loss of appetite and weight loss are also commonly seen (Santersieri, 2015). Atypical antidepressants are typically not the first line of treatment in the management of depression, although they can be quite useful due to their sedative effects, which can also have beneficial effects on comorbid anxiety (Golden, Dawkins, & Nicholas, 2009).

An atypical antidepressant medication that has seen increasing research interest is ketamine. Ketamine is a non-competitive antagonist of the NMDA receptor which blocks receptor activation through binding to the phencyclidine site of the complex (Sanacora et al., 2008). Ketamine is remarkable in the fact that a single intravenous infusion can reduce depression symptom severity in as little as 2 hours, and remission can be achieved in 24 hours (Diazgranados et al., 2010; Murrough et al., 2013). Ketamine has also been shown to be efficacious against treatment resistant depression (Cusin et al., 2012). The mechanism behind ketamine's fast-acting antidepressant effects is beyond the scope of this dissertation, however it is hypothesized that this mechanism involves rapid changes in glutamatergic signalling and alterations to neuroplasticity, including brain derived neurotrophic factor (BDNF), which has important effects on long-term potentiation (LTP) (Yoshi & Constantine-Paton, 2010; Korte et al., 1996).

With the exception of ketamine, currently available antidepressant medications take weeks to achieve their therapeutic effects. As levels of serotonin and norepinephrine at the synapse are increased hours after taking an antidepressant, it is argued that there are downstream signalling mechanisms responsible for the actions of antidepressant compounds. For example, serotonin and norepinephrine bind to g-protein coupled receptors. This in turn stimulates cyclic AMP (cAMP), which then alters the activity of protein kinase A, and then interacts with other protein kinases (CaMK), which finally influences the expression of cAMP response-element binding (CREB) (Willner, Scheel-Kruger, & Belzung, 2013). Antidepressants have been demonstrated to elevate CREB expression in the hippocampus following chronic administration (Nibuya, Morinobu, & Duman, 1995). CREB is a member of a family of transcription factors that can influence gene transcription and in turn proteins. One of CREB's most prominent targets is BDNF, which is altered in both human post mortem samples and preclinical models (Duman

& Monteggia, 2006). CREB also influences the activity of vascular endothelial growth factor (VEGF), a member of the neurotrophin family (Willner, 2013). All of these alterations represent long-acting changes, which might in part explain the slow-acting actions of antidepressants. Another view of the slow-acting actions of antidepressants posits that their delayed response is due in part to the brain initially viewing the increased levels of serotonin as toxic. In order to adapt to this, stress-related adaptations occur in the brain, including increased levels of neuronal enzymes, and receptors becoming desensitized and down regulated in order to accommodate this increased level of serotonin (Santersieri & Schwartz, 2015). This process of gene activation can take several weeks to occur, which fits with the timeline for the response to antidepressants. Regardless of the exact mechanism behind the slow-acting actions of antidepressants, it is imperative that antidepressants with fast-acting properties are discovered, particularly for the case of depressed patients with suicidal ideations. A better understanding of the stress response and how this becomes deregulated in the context of chronic stress will contribute towards this understanding.

1.5. The Stress Response

The body's stress response is initiated when an organism encounters a stressor in the environment (be it physical or psychological) that is strong enough to activate the hypothalamic-pituitary-adrenal (HPA) axis. Activation of the HPA axis involves a cascade of neuroendocrine events, which include: 1) corticotrophin-releasing hormone (CRF) is released from neurons in the paraventricular nucleus of the hypothalamus, 2) which travels to the anterior pituitary gland and regulates the transcription of the proopiomelanocortin gene (a member of the corticotrophin peptide family) (Tafet & Nemeroff, 2016), 3) which then triggers the release of adrenocorticotropin hormone (ACTH) into circulation, and 4) ACTH then travels to the adrenal cortex, which stimulates the release of cortisol in humans and corticosterone (CORT) in rodents (Sterner & Kalynchuk, 2010). Upon release, glucocorticoids act on bodily tissues to deal with the stressor by limiting non-essential functions and mobilizing energy. In addition to peripheral effects, glucocorticoids also travel to the brain and exert an inhibitory influence on the periventricular nucleus through a negative feedback loop (Sterner, 2010). Stress receptors in the brain include both mineralocorticoid (MR) and glucocorticoid receptors (GR), which are expressed in the hippocampus, amygdala, and prefrontal cortex, and are the main site of glucocorticoid action. In the hippocampus, glucocorticoids bind to GR, which activate

GABAergic projections located in the paraventricular nucleus of the hypothalamus that inhibit HPA axis activity (Tafet, 2016). Although MR have a high affinity for CORT and are thus activated when circulating CORT levels are low, GR have a low affinity for CORT and are activated when CORT levels are high (Sterner; Willner, Scheel-Kruger, & Belzung, 2013). Tight control of the HPA feedback inhibition mechanism is crucial to maintaining homeostasis. However, when hippocampal GR levels are low, feedback inhibition becomes deregulated, and stimuli that instigate an HPA axis response lead to exaggerated levels of CORT (Sterner).

Activation of the HPA axis under conditions of acute stress is essential for an organism to mount an appropriate behavioural and biological response to the stressor. During an acute stressor, the amygdala activates the HPA axis to ultimately result in the release of glucocorticoids. Acute activation of the HPA axis triggers CRF neurons in the amygdala, initiating a positive feedback loop associated with fear and anger reactions, in addition to catecholaminergic neurons which serve to stimulate arousal and improve cognitive functioning for brief periods (Tafet & Nemeroff, 2016). This activation helps divert energy away from non-essential functions and mobilize the body to effectively deal with the situation. However, when this becomes a problem is under conditions of chronic stress. During chronic stress, the negative feedback mechanism described above fails, resulting in increasing accumulation of glucocorticoids. Excess levels of glucocorticoids are neurotoxic, and over time this toxicity can lead to a loss of hippocampal granule cells and dendritic atrophy in the hippocampus and prefrontal cortex (McEwen & Morrison, 2013; Sapolsky, 2000). Interestingly, the effects seen in the amygdala with chronic stress run in the opposite direction, with dendritic hypertrophy observed (Mitra & Sapolsky, 2008).

This pattern of neuroanatomical alteration aligns with the roles of the hippocampus, prefrontal cortex, and amygdala during the stress response. The function of the hippocampus and prefrontal cortex during stress is to inhibit HPA axis activity, whereas the amygdala serves to heighten HPA axis activity (Feldman, Conforti, & Weidenfeld, 1995). The picture that emerges is that during periods of chronic stress and excessive glucocorticoid release, dendritic hypotrophy and volumetric reductions of the hippocampus and prefrontal cortex accrue, interfering with their ability to inhibit HPA axis activity. Meanwhile, dendritic hypertrophy in the amygdala enhances the HPA axis, leading to even more glucocorticoids being released (Sterner & Kalynchuk, 2010). In addition to the demonstrated effects of prolonged

glucocorticoid release on brain neuroanatomy, glucocorticoid release can lead to immunosuppression, sleep disorders, impaired memory, and depression (Sterner, 2010). With an understanding of how the brain reacts to stress, the link between stress and depression will now be discussed below.

1.6. The Link Between Stress and Depression

Stress is a significant risk factor for the development of depression in human populations. For example, the development of depression is typically preceded by stressful life events (Kessing, Agerbo, & Mortensen, 2003). Relatedly, children with stressful environments while growing up are significantly more likely to develop depression in adulthood than children in non-stressful environments, and have a sensitized HPA axis response (Essex, Klein, Cho, & Kalin, 2002). Half of depressed patients display the hallmarks of a deregulated stress response, hypercortisolemia (Sachar and Baron, 1979), and antidepressant treatment reverses this deregulation (Holsboer, 2001). Furthermore, Cushing's disease, characterized by chronically high circulating levels of glucocorticoids, shares a significant comorbidity with depression (Sonino & Fava, 2002). It is not surprising that elevated levels of CRH are observed in the cerebrospinal fluid of depressed patients, in addition to elevations in urinary free cortisol (Nestler et al., 2002). A way to characterize the link between stress and depression in the clinical population is through the dexamethasone suppression test (DST). In the DST, dexamethasone (a synthetic glucocorticoid analog) is administered and cortisol is later measured. In control patients, dexamethasone suppresses the activity of ACTH and subsequently cortisol secretion through binding to GR's. However, in depressed patients a typical response is elevated cortisol concentration after dexamethasone administration (Dam, Mellerup, & Rafaelsen, 1985). This elevation in cortisol concentration after the DST tends to correlate with poor patient outcome and early relapse (Sterner & Kalynchuk, 2010). What emerges is a consistent pattern of a deregulated stress response which has pathological consequences for the development of depression. However, all of these data are correlational. Animal models can bridge the gap in understanding the link between stress and depression by offering tight control over extraneous variables and allowing the researcher to see the causal effects of stress and its relation to depression.

1.6.1. Animal Models of Stress and Depression

Animal models of depression permit a high level of experimental control over potential confounding variables which allow the researcher to carefully delineate the contributing factors

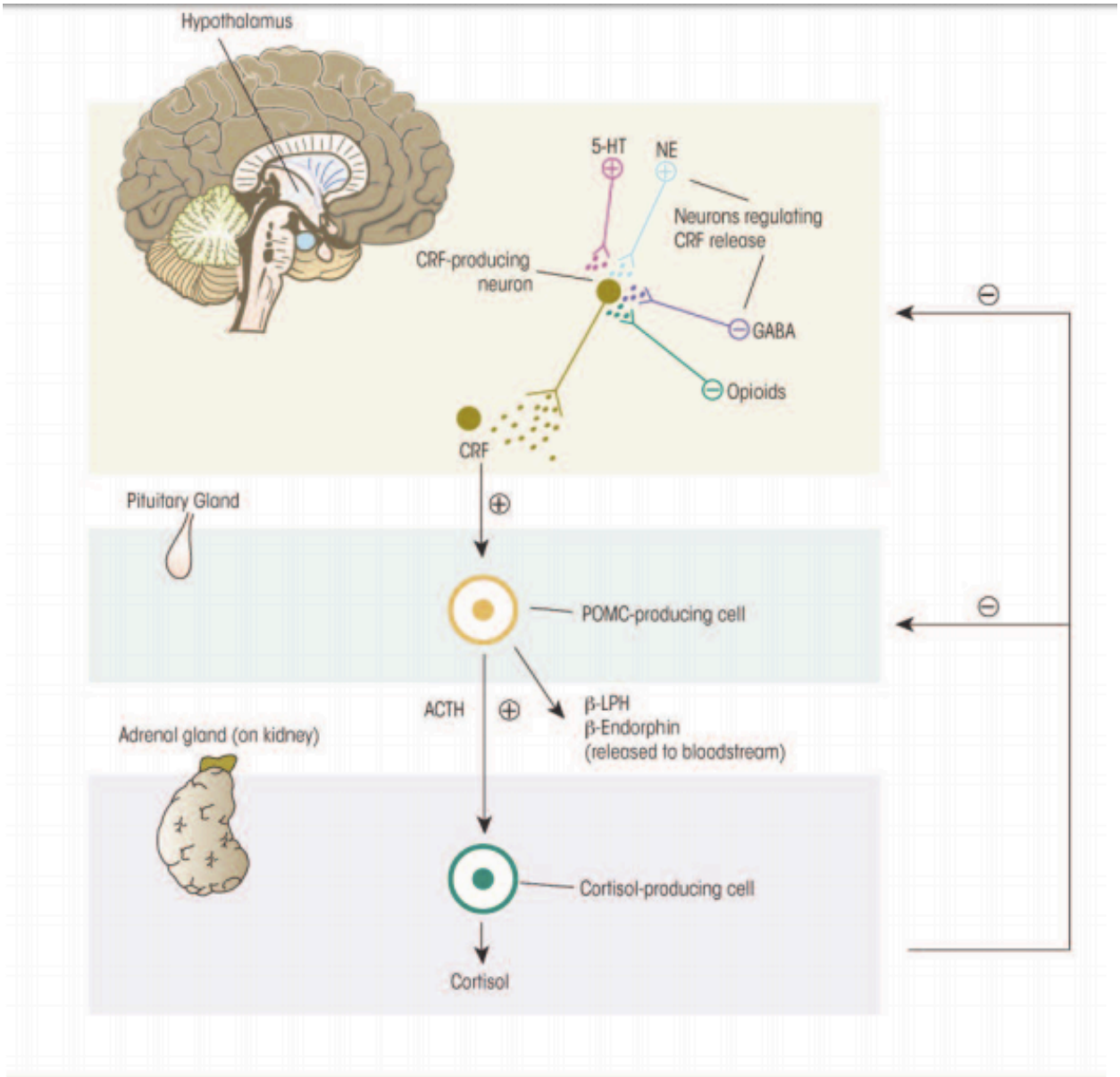

Figure 1-1. The Hypothalamic-Pituitary-Adrenal Axis. When an organism encounters a stressful stimulus the HPA axis becomes activated. Corticotrophin-releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus which then travels to the anterior pituitary gland where it transcribes the proopiomelanocortin gene. This then triggers the release of adrenocorticotrophic hormone (ACTH), which is released into circulation. Upon reaching the adrenal cortex cortisol is secreted in humans or corticosterone in rodents. Glucocorticoids then bind to receptors in the hippocampus which serves as a negative feedback signal for the HPA axis. Figure adapted from (Stephens and Wand, 2012).

to a particular disorder. Several animal models of depression have been developed, including exogenous stress exposure, genetic manipulation, and surgical approaches (e.g., olfactory bulbectomy). However, due to the nature of this dissertation I will only focus on stress models of depression. Stress models of depression focus on the effects of stress on the development of depression-like behaviour in the rodent. Due to the complex nature of depression, an attempt to capitulate the entire disorder cannot be made; rather, specific aspects of the disorder are targeted. And although the ultimate goal for any rodent-derived treatment for depression is to improve patient outcomes, it is simply not feasible (*yet)* to model suicidal behaviour in rodents. Therefore, depression-related behaviours that can be observed in animal models are instead a focus of interest. For example, learned helplessness (despair), or anhedonia are frequently targeted for observation. The following sections will focus on the concept of animal models of depression, common animal models of depression, and ways to measure depression-like behaviour in these models.

Any model of a disorder must meet certain criteria for it to be considered useful. Perhaps the most important aspect is validity. Validity in the case of models of a disorder means that what you are measuring is valid. When designing an animal model of depression, 3 types of validity have to be ensured. First is construct validity (Nester, 2010). Construct validity means that the methods used to create the disorder are representative of those seen in the population. This is clearly seen in animal models of Huntington's disease. As it is known that the diseased form of the huntingtin gene contributes to the disorder, animal models can recreate this mutation and thus achieve construct validity. However, in the case of depression there is no single identified disease-causing genes or proteins. These factors contribute to a difficulty in fully achieving construct validity in animal models of depression. Therefore, researchers must focus in on the most likely contributing factors to the development of depression with the available evidence and manipulate those. As the majority of depressed patients experience some form of a stressful event before the onset of symptoms, stress is frequently targeted in some form. The second consideration for any animal model of depression is face validity. Face validity indicates that the model you are using produces similar alterations to those seen in the human population, particularly similar anatomical and biochemical alterations. For example, depressed human patients typically have reduced hippocampal volume and decreased levels of monoamines. A rodent model of depression would be said to have good face validity if it produced a similar

pattern of neurobiological alterations. The last criteria that should be met is predictive validity. Predictive validity in the case of animal models of depression indicates that treatments in the animal model result in similar responses to those seen in the human population (Nestler). For example, antidepressants should produce similar antidepressant responses in rodents to those seen in humans. While it is unlikely that any current animal model of depression will meet all of the above 3 criteria, the more criteria met the more the researcher can be confident that the model they are using is a good one. Designing animal models of depression that can fulfill all of the above 3 criteria should remain a focus for all researchers.

Once a researcher chooses an animal model of depression, there must be tools available to assess if the model is successfully producing depression-like behaviour. A number of behavioural assays to screen for depression-like behaviour have been developed. Perhaps the most commonly used tool is the forced-swim test (FST). In this task, rodents are placed in a tank filled with water. The tank is high enough that rodents have no opportunity to escape. The rodent's behaviour is recorded by a camera for upwards of 10 minutes. A number of behaviours are then scored for later analysis. The first behaviour is immobility. Immobility refers to the total time the rat spends immobile while in the swimming tank. This is typically measured as the most minimal movements used to keep the rodent's head above water. For example, the rodent might make subtle paw movements while floating in an attempt to keep its head above water. The amount of time spent struggling is also recorded. Struggling is defined as an active behaviour where the rodent is trying to escape from the swimming tank. This is typically manifested as using all paws to scratch at the corners of the tank in an attempt to swim out. The last behaviour scored in the FST is swimming. Swimming is an active behaviour where the rodent swims from point to point in the tank with no apparent goal. The rodent may also dive to the bottom of the tank, which still falls under the category of swimming. Animal models of depression typically result in a clear stratified set of behaviours in the FST. Total immobility time is typically increased, whereas time spent struggling and swimming is decreased. This set of behaviours (increased passive behaviours, decreased active behaviours) is indicative of learned helplessness, or the act of learning that fighting the situation will not help (Lucki, 1997).

Another commonly used measure of depression-like behaviour is the sucrose preference test. Here, animals are trained to drink both unflavored water and sweetened water. The percentage of sucrose solution consumed is reported. Typically, a control rodent will consume

significantly more sucrose than unflavored water, while a stressed animal will not show a preference for the sucrose solution. This is inferred to mean that the animal is experiencing anhedonia, or the loss of ability to experience pleasure in what were previously pleasurable activities (Der-Avakian & Markou, 2012). Importantly, both the sucrose preference test and FST respond to antidepressant therapy, indicating that they are indeed accurately measuring depression-like behaviour in the rodent.

A number of animal models have been developed to study the role of stress in the pathogenesis of depression. These models differ in the *mode* that they employ stress. For example, one of the earliest developed models of depression is repeated restraint stress. Here, a rodent is placed into a plastic restrainer, immobilizing the rat. The rodent is left in the restrainer for several hours per day, and this is done daily for days to weeks. While this model is initially stressful for the rodent, over time the animal becomes habituated to the restrainer and learns that the period of discomfort is only temporary (Table 1-2). Additionally, a further problem with this model is that it often fails to produce depression-like behaviour (Gregus, Wintink, Davis, & Kalynchuk, 2005). Therefore, this model has fallen out of favor in the field of chronic stress, however it is still useful for studying the effects of acute stress. Chronic unpredictable stress (CUS; or chronic mild stress, CMS) represents an improvement in validity over the restraint stress model. In the CUS model, an animal is exposed to several different stressors each day for a period of several weeks. Examples of stressors include cage tilting, lights on during sleeping hours, wet bedding, cage shocks, etc. (Bondi, Rodriguez, Gould, Frazer, & Morilak, 2008). This model is particularly interesting as it attempts to recapitulate the unpredictable nature of stressors that humans encounter in day-to-day life. Several reports have shown this model to successfully induce both depression-like and anxiety behaviours (Yohn & Blendy, 2017; Chakravarty et al., 2013) in rodents.

In contrast to chronic unpredictable stress, which targets the unpredictable nature of stressors in our daily lives, social defeat stress targets the stressors that emerge from our social lives. In this model, a rodent watches a smaller rodent being attacked by a larger "bully" rodent for short periods of time over the course of several days. Alternatively, the rodent will not observe but will instead be placed in the cage with the "bully" rodent. This model is intriguing as it is able to replicate the feeling of defeat that individuals can experience in social interactions. Additionally, this model has the added benefit of stratifying rodents into a group of responders

and non-responders to antidepressant medication, similar to what is observed in the human population, and is therefore effective in creating depression-like behaviour (Wood & Bhatnagar, 2015). However, despite the different ways in which the mode of the stressor is manipulated in the above models, it can be argued that an inherent weakness is that the individual response to the stressor is not being controlled. For example, one animal might perceive stimulus A as highly stressful, whereas the same stimulus might elicit little to no stressful response in another animal. Therefore, a successful animal model of depression should control for the individual variability in response to stress. The repeated CORT model of depression aims to achieve just that.

1.6.2. The CORT Model of Depression

The CORT model of depression seeks to control for the amount of stress experienced by each individual animal. To achieve this, exogenous CORT given at the same dose is administered to each animal over the course of several weeks. Importantly, our lab has shown that the effects of CORT are both time and dose dependent. For example, although 10 mg/kg of CORT starts to increase immobility time in the FST, longer durations of immobility are observed at 20 mg/kg, and still greater effects are seen at 40 mg/kg (Johnson, Fournier, & Kalynchuk, 2006). Additionally, although a single injection of CORT does not alter FST behaviour, increases in immobility time are observed after 7 days of injections, with the largest effects appearing after 21 days. A large number of studies have validated the effectiveness of 21 days of 40 mg/kg of CORT injections in producing depression-like behaviour in the FST (Brummelete, Pawluski, & Galea, 2006; David et al., 2009; Gourley et al., 2008; Gregus et al., 2005; Kalynchuk, Gregus Boudreau, & Perrot-Sinal, 2004; Marks, Founier, & Kalynchuk, 2009; Murray, Smith, & Hutson, 2008; Zhao et al., 2008;), which includes increased immobility time and decreased time spent struggling and swimming. The effects of repeated CORT injections also extend to other behaviours typically seen in the depressed clinical population. For example, repeated CORT injections can impair sexual behaviour (Gorzalka, Hanson, & Hong, 2001; Gorzalka & Hanson, 1998), inhibit grooming behaviours (David et al., 2009), and decrease sucrose intake (David et al., 2009; Gorzalka, Hanson, Harrington, Killam, & Campbell-Meiklejohn, 2003), all of which are indicative of an anhedonic-like phenotype. Furthermore, repeated CORT injections can also produce anxiety-like behaviours in rodents (David et al., 2009; Lee, Shim, Lee, Yang, & Hahm, 2009). This is an important aspect of the CORT model of depression, as in the clinical population

anxiety is frequently comorbid with depression (rates between 20-50%) (Hirschfeld, 2001). This serves to further improve the face validity of the model.

In addition to the behavioural effects produced by repeated CORT injections, accompanying physiological changes also occur. For example, decreases in body weight (Brymer et al., 2018; Coburn-Litvak, Pothakos, Tata, McCloskey, & Anderson, 2003; Fenton et al., 2015; Lussier et al., 2013a) are observed, as well as a dysregulated HPA axis (Johnson et al., 2006), and reduced adrenal weight (Murray et al., 2008). Finally, it is important to note that the CORT-induced behavioural changes described above can be ameliorated by chronic antidepressant treatment (Brymer et al., 2018; Fenton et al., 2015). This suggests that the model possesses predictive validity, as treatments that work in clinically depressed human populations also work in this animal model. Therefore, all of the above observations reinforce the idea that the CORT model of depression is producing effects in line with those observed in humans, and validates its use as a model of depression.

While the CORT model of depression is a useful model to help probe the behavioural and physiological underpinnings of depression, like any model there are drawbacks that limit its utility. The major limitation of the model is the physiological relevancy of the dose used. The standard dosage used in the model is 40 mg/kg, although smaller doses have been used elsewhere (with conflicting results). This dose of CORT is high, and this is reflected in how the animals respond to the drug: decreased body weight and adrenal gland weight. This is in contrast to the normal amount of cortisol or CORT released in response to a stressor, which is significantly less. For example, the mean amount of corticosterone released during stressful events in rodents is usually below a milligram (Thanos et al., 2008). Therefore, the issue that arises when utilizing the CORT model of depression is that of physiological relevancy. This raises the question of whether the behavioural and physiological effects produced by repeated CORT injections are result of the of the animals simply being sick from a supraphysiologic dose of CORT? This does not appear to be the case. Work in our lab has demonstrated that CORT-treated animals do not have alterations in locomotor activity and are not impaired on tests of muscle strength. Although CORT-treated animals do weigh less than their vehicle-treated counterparts, the total amount of food consumed is similar between the two, suggesting that metabolic changes are occurring (Kinlein, Shahanoor, Romeo, & Karatsoreos, 2017). This reinforces the idea that CORT treatment effects on physiology and behaviour are not reliant on making the animals sick.

Table 1-2. Comparison of Animal Models of Depression

Model of Depression	Strengths	Weaknesses
Social Defeat Stress	Produces robust depression-like behaviour and stratifies rats into a group of responders and non-responders	Potential for individual variability in responses to stress
Restraint Stress	Produces depression-like behaviour on a number of behavioural assays	Animals sensitize to the effects of the restrainer
Chronic Unpredictable Stress	Successfully recapitulates the unpredictable nature of stressors seen in the human population	Potential for individual variability in responses to stress
Repeated Corticosterone Injections	Amount of stress each animals receives is carefully controlled	Dose frequently used is not representative of a stressful event in a human

Overall, these results suggest that the 40 mg/kg dose is not so high as to be making the animals sick/weak.

1.7. Neuroanatomy

The temporal and frontal lobes are critically involved in the pathogenesis of depression. The temporal lobes have received special interest in the context of depression as the temporal lobes are the house of the limbic system. The limbic system comprises the hippocampus, amygdala, hypothalamus, and septal area (Rajmohan & Mohandas, 2007) and is involved in the regulation of emotional behaviour. Importantly, the limbic system is known to be compromised by stress, as the hippocampus is demonstrated to decrease in volume and dendritic atrophy is also reported, while the amgydala shows the opposite pattern (Willner, Scheel-Jruger, & Belzung, 2013). While not technically part of the limbic system, prefrontal cortex neurons show dendritic atrophy as well (Radley et al., 2006). Therefore, understanding the neuroanatomy of prefrontal cortex and the hippocampus is critical to an understanding of depression.

1.7.1. The Hippocampal Formation

The hippocampus remains one of the most studied structures in the brain. This is because it is involved in many behaviours, most prominent being its role in learning and memory, and the ease of performing electrophysiological recordings from the area. The hippocampus derived its name for its resemblance to the seahorse, and the structure sits in the temporal lobe of the mammalian brain. The term hippocampus is often used interchangeably with the hippocampal formation, which refers to a series of structures comprised of the hippocampus proper (Cornu Ammonis), dentate gyrus (DG), the subicular cortex (subiculum, presubiculum, and parasubiculum), and the entorhinal cortex (EC) (Amaral & Witter, 1989; Amaral, Dolorfo & Alvarez-royo, 1991).

The hippocampus proper comprises 3 regions: CA1, CA2, and CA3. A fourth region, CA4, has also been proposed. The principal cell layer in all of the subfields of the CA subfields is the stratum pyramidale, made up of excitatory glutamatergic pyramidal neurons. Located superficial to the stratum pyramidale is the stratum oriens, a largely cell-free layer where the dendrites of pyramidal neurons receive innervation from classes of GABAergic interneurons. Located above the CA3 stratum pyramidale layer is the stratum lucidum, an acellular zone occupied by mossy fiber terminals projecting from the DG. The stratum radiatum and stratum lacunosum-moleculare are located immediately adjacent to the stratum lucidum and above the

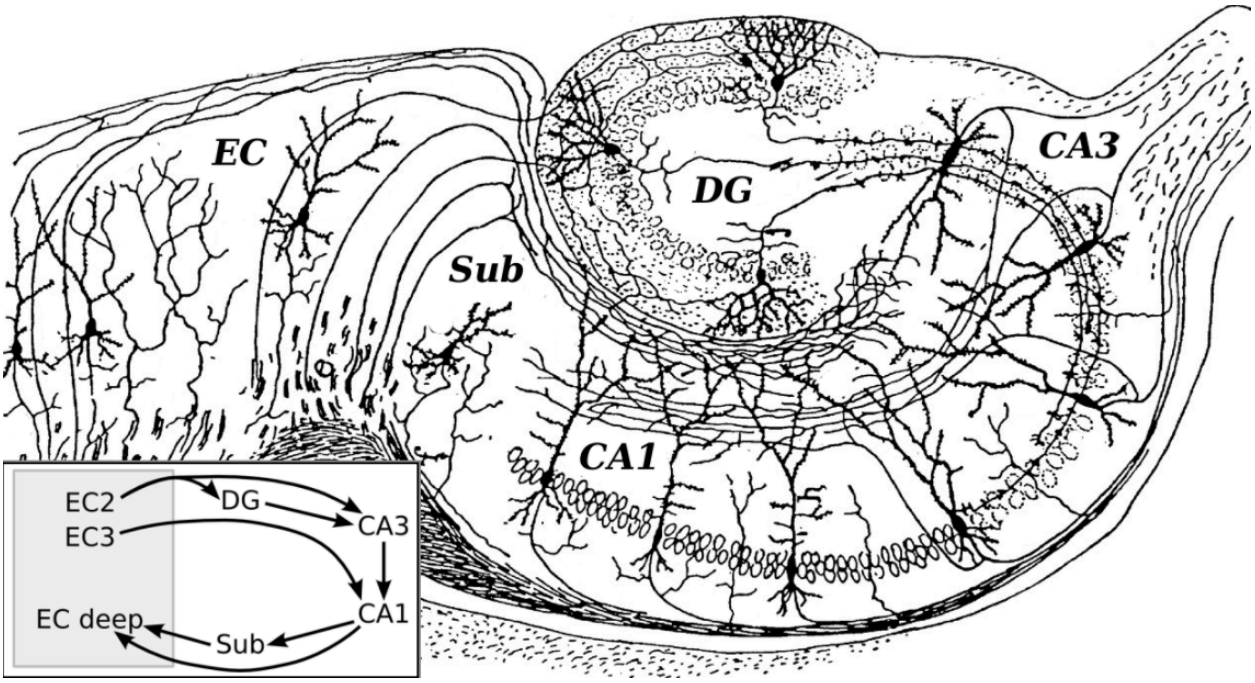

Fig 1-2. Schematic of the rodent hippocampus. The major source of input to the hippocampus starts from the entorhinal cortex. Neurons from the entorhinal cortex project to the dentate gyrus (DG), which give rise to mossy fiberes that synapse onto the pyramidal cells of CA3, which form Shaffer collaterals onto the CA1. The CA1 in turn projects back to the entorhinal cortex and to the subiculum. Adapted from (Garcia Lopez, Garcia-Martin, & Freire, 2010).

stratum pyramidale in layers CA1 and CA2. The stratum radiatum contains the projections of the Schaffer collaterals, the major output of the CA3 to CA1. Finally, the stratum lacunosum-moleculare represents the most peripheral layer in the hippocampus proper, and is the source for extrahippocampal outputs (Amaral & Witter, 1989).

1.7.2. The Dentate Gyrus

The DG serves as the major source of cortical input to the hippocampus. It forms a part of the trisynaptic circuit, one of the most extensively studied circuits in the brain. In the trisynaptic circuit, cortical input goes into the DG from the neurons in the EC, forming the perforant path. From here, neurons give rise to mossy fibers which synapse onto pyramidal neurons in the CA3. Mossy fibers from CA3 then give rise to Schaffer collaterals which synapse onto dendrites of CA1 pyramidal cells. The CA1 then synapses into the subiculum and back to the EC, forming the major source of excitatory output of the hippocampus, and in so doing completing the circuit (Knierim, 2015). The DG is comprised of 3 layers: the stratum granulosum (granule cell layer; GCL), polymorphic layer (hilus), and the stratum moleculare (Amaral & Witter, 1989). An important subregion is the subgranular zone (SGZ), a two-cell wide germinal layer between the hilus and GCL. The SGZ is important as it, along with the subventricular zone, is 1 of only 2 sites of neurogenesis in the adult brain. The GCL forms the principal cell layer in the DG. It is composed of tightly packed excitatory granule neurons which extend processes into the molecular layer (ML) and axons back into the hilus. The GCL is approximately 4 to 8 cells thick, and estimates place roughly 1.2 million neurons in the GCL of a rat (Rapp & Gallagher, 1996). The hilus forms the innermost layer of the DG and contains inhibitory interneurons and excitatory mossy cells. The most common inhibitory interneuron type in the hilus is the pyramidal basket cell, and these cells form inhibitory synaptic contacts onto the proximal dendrites and cell bodies of dentate granule cells (Amaral et al., 2007).

The stratum moleculare is a cell-free layer at the outermost region of the DG and is composed of an inner, middle, and outer subregions. Importantly, it is the recipient of the dendrites of granule cells, pyramidal basket cells, and polymorphic layer cells (Amaral et al., 2007). The projections of the performant path terminate in the middle and outer layer of the stratum moleculare, forming excitatory connections.

1.7.3. The prefrontal cortex

One of the most famous subjects in the annals of psychology is Phineas Cage. Cage was a railway worker who, as a result of an accident, had a tamping iron impaled through his frontal lobes. The consequences of this insult on his behaviour were immediate. Cage became impulsive, did not follow social cues, could not manage money, and became aggressive. It was later that this set of behaviours came to be attributed to the frontal lobes, and fell under the domain of executive functions. While the role and function of the frontal lobes is relatively clear in humans, the question of do rodents possess a prefrontal cortex (PFC) has long been a contentious issue in the field of neuroscience. There are 2 opposing camps in this issue. The first group says that the rodent does not possess a PFC as this structure is uniquely human in its function. For example, they argue that tasks such as reasoning and planning are not ones that a rodent performs. The other group argues that indeed the rodent does have a PFC. Their argument is based off of lesion studies which indicate that lesioning the area of the PFC in the rodent produces behavioural effects similar to those seen in humans. For the purposes of this dissertation, it will be assumed that the rodent does possess an analogous PFC to that seen in humans. The rodent PFC is divided into 2 regions: the lateral and medial PFC (mPFC). The mPFC is further divided into dorsal, medial, and ventral regions. The dorsal mPFC comprises the anterior cingulate cortex, medial the prelimbic cortex, and ventral the infralimbic cortex (Seamans, Lapish, & Durstewitz, 2008). The PFC shares cortical connections with a large number of regions, including the hippocampus, amygdala, entorhinal cortex, perrirhinal cortex, and with premotor and somatosensory cortices. Furthermore, the PFC receives input from the ventral tagmental area and substantia niagra (Seamans, 2008). The mPFC is the only region that possesses direct projections to cholinergic basal forebrain and brainstem nuclei (Seamans). It is with this complex pattern of connections that the PFC is able to modulate many aspects of complex cognitive behaviours. It is therefore perhaps no surprise that Gages' behaviours were so profoundly disrupted.

1.8. The Neurobiology of Depression

Although depression is a complex, heterogeneous disorder, there tend to be clusters of neurobiological alterations observed in both post-mortem and non-invasive imaging studies of depressed patients. The brain structures most commonly implicated in depression include those

involved in emotional behaviours, such as the hippocampus, amygdala, and PFC. Magnetic resonance imaging studies reveal reduced volumes of the anterior cingulate and orbitofrontal

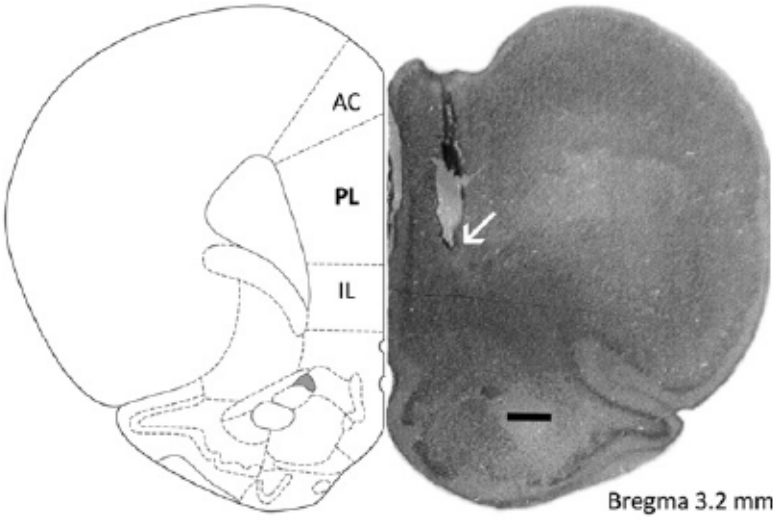

Fig 1-3. Schematic of the rodent mPFC. The anterior cingulate cortex, prelimnic cortex, and infralimbic cortex are all illustrated. Adapted from (Stern, Gazarini, Vanvossen, & Bertoglio, 2013).

cortex of depressed patients (Koolschijin, van Haren, Lensvelt-Mulders, & Kahn, 2009) compared to controls. The dorsolateral PFC shows decreased regional cerebral blood flow and glucose metabolism in depressed patients, which is associated with psychomotor retardation and anhedonia (Drevets, 1998). Moreover, reductions in neuronal and glial density have been observed in the orbitofrontal and dorsolateral PFC of depressed patients during live imaging (Raikowska, Miguel-Hidalgo, & Wei, 1999). Finally, reduced dendritic branching in the PFC is observed in animal models of depression (Lucassen et al., 2014).

Similar to the PFC, the hippocampus shows decreased volume, dendritic retraction, and decreased hippocampal neurogenesis (see section on neurogenesis for a detailed description of this contentious issue in the context of depression; Palazidou, 2012). In contrast to the PFC and hippocampus, which tends to show reductions in volume and activity, the amygdala has been reported to be enlarged in depression, and animal studies show amygdalar dendritic outgrowth (Palazidou). This would indicate that the amygdala is more active in depression, and is therefore most likely contributing to anxiety-like behaviour, which is frequently comorbid with depression. In addition to structural changes observed in depression, there are also changes in brain neurochemistry. Perhaps the most replicated finding in the literature across both preclinical and clinical studies is that both blood and cerebrospinal fluid concentrations of the monoamines are decreased. Post-mortem studies of depressed brains also reveal decreased numbers of the monoamine receptors, for example 5-HT_{1A} and 5-HT_{1B}, located in the hippocampus and PFC (Nautiyal & Hen, 2017). GABA and glutamate, the main drivers of inhibition and excitation in the brain, respectively, have also been implicated in the pathophysiology of depression. Post-mortem depressed brains show deregulated GABA and glutamate homeostasis, in particular alterations to the function and structure of glutamatergic neurons, the dendrites of GABA targeting neurons, astrocytes, and oligodendrocytes (Pabba & Sibille, 2016). Work in our lab has shown that rats subjected to repeated CORT injections show an increase in vesicular glutamate transporter (VGLUT)-2 and a decrease in GABA receptors in the hippocampus (Lussier et al., 2013b), adding further evidence to the idea that depression involves a dysregulation of the balance between GABA and glutamate.

Alterations in neurotropic factors are also seen in depression. Reductions in BDNF are consistently observed in both preclinical and clinical studies. BDNF is widely distributed

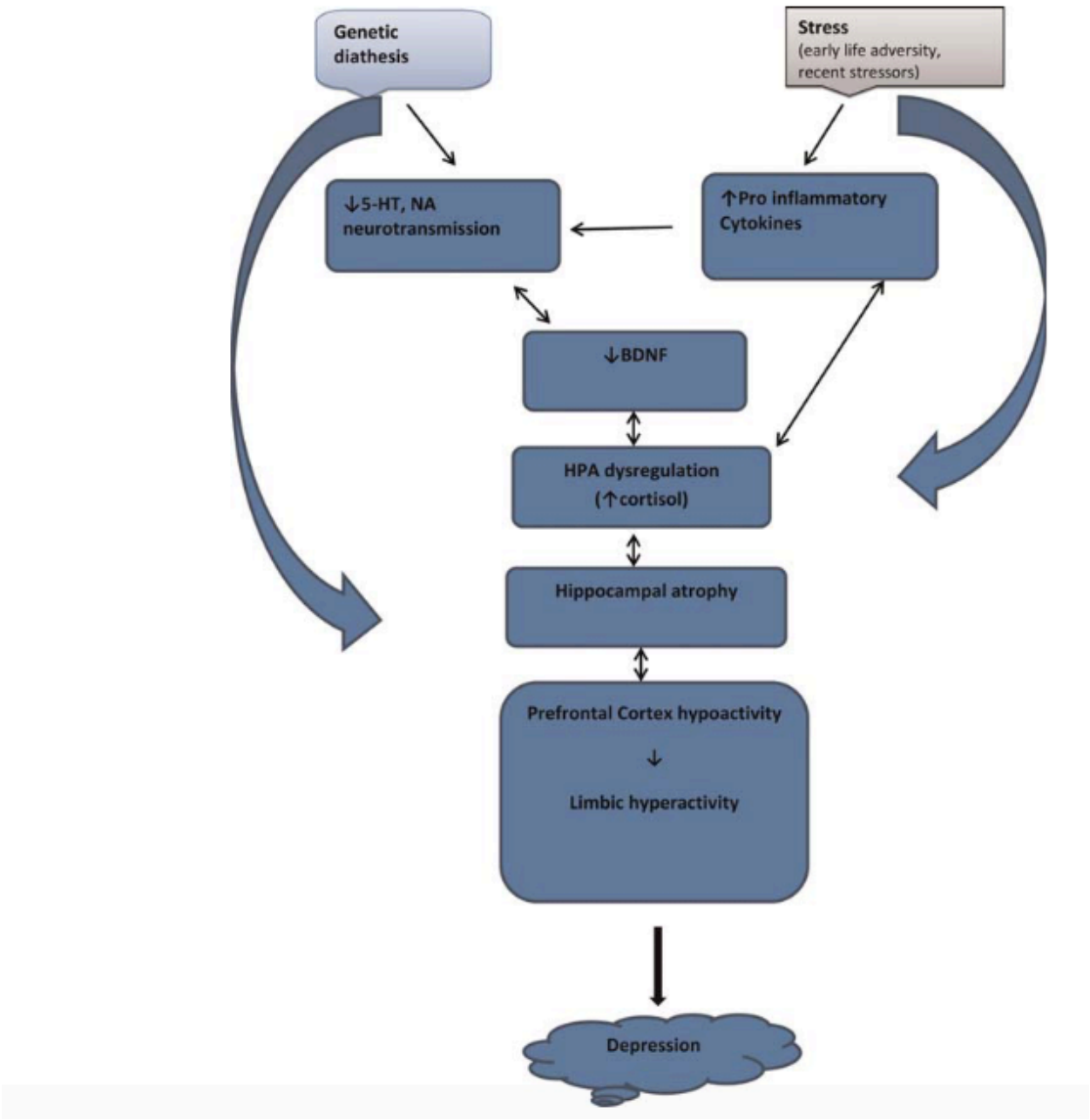

Fig 1-4. Schematic of the proposed neurobiology of depression. Adapted from (Palazidou, 2012).

throughout the hippocampus, and plays important roles in neuronal growth, survival and maturation, and arborisation and synaptic plasticity in the adult brain (Palazidou, 2012). Stress has been shown to suppress BDNF synthesis in the hippocampus, with antidepressant drugs and electroconvulsive therapy increasing BDNF synthesis and signalling in the hippocampus. A negative correlation exists between levels of BDNF and depression symptom severity, with reduced levels of BDNF being associated with increased severity (Palazidou). Therefore, the neurobiology of depression comprises complex patterns of changes in brain structure, at the level of both gross morphology and at the level of proteins and receptors. Given the roles of the hippocampus and prefrontal cortex in cognitive processes, it can be expected that impairments in cognition are a component of depressive symptoms, and this is exactly the case.

1.9. Memory Impairments in Depression

While not consistently recognized as a feature of depression, included in the DSM-V and patient self-reporting are problems in memory and thinking. Cognitive impairment is associated with poor response to treatment, and is observed in over two-thirds of the depressed population (Rock, Rosier, Riedel, & Blackwell, 2014). The most commonly observed deficits in cognition involve alterations in explicitly verbal and visual memory, while performance on implicit memory tasks are spared (Austin, Mitchell, & Goodwin, 2001). A meta-analysis of commonly reported cognitive deficits in depressed patients revealed impairments in executive functioning, memory, and attention. Specifically, in terms of executive function, depressed patients tended to exhibit deficits on spatial working memory tasks, where participants search for tokens while keeping in mind previous token locations (Rock, 2014). In terms of memory, depressed patients exhibited deficits in delayed matching to sample tasks, where participants must remember the visual features of a complex stimulus and select the stimulus they encountered previously from 4 similar targets (Rock). Depressed patients also show deficits in pattern recognition memory, where participants have to correctly identify from 2 choices a previously encountered abstract pattern (Rock). Finally, depressed patients also show deficits in tasks of sustained attention and signal detection. (Rock). Kizilbash, Vanderploeg, and Curtiss (2002) showed significant impairments of immediate recall of new information on the California Verbal Learning Test in depressed veterans. This pattern of cognitive deficits present in depressed patients implicates dysfunction within the hippocampus and PFC broadly.

Several researchers in the depression field argue that it is not cognitive processes impaired in depression, but rather *motivational processes* that are impaired. They argue that depressed patients lack motivation, and it is this lack of motivation that skews the pattern of cognitive alterations observed in the depression literature. For example, Scheurich et al (2008) showed that when controlling for motivation, depressed patients performed at equal or slightly below levels of control patients on several tasks of neurocognitive functioning. This in turn could be correlated with anhedonia/lethargy, as depressed patients might say what is the point of even trying? However, evidence to counter this argument is presented below, and again shifts the focus back to cognitive impairment originating from altered brain function.

An interesting question is what happens to the cognitive performance of depressed patients who are successfully treated and experience remission? Interestingly, Paridiso, Lamberty, Garvey, and Robinson (1997) addressed this question and found that formerly depressed patients still exhibited deficits on set-shifting tasks. In a similar vein, Marcos et al. (1994) showed that recovered depressed patients continue to present with deficits in both immediate memory and delayed recall of visual and verbal material. This is an interesting finding, as it would be expected according to the neuroplasticity hypothesis that once the depressed patient is recovered, impaired plasticity in the brain should be restored. What this suggests is that it is more than likely that most of the cognitive deficits observed in depression are a result of altered brain structure and function (i.e., changes in morphology) rather than simply a lack of motivation. However, more research is required to fully elucidate the mechanism maintaining cognitive dysfunction in depressed patients in remission.

Preclinical models of depression consistently indicate that chronic stress results in impairments in tasks that require functioning of the hippocampus and PFC. For example, deficits in spatial learning and memory are well-validated (Conrad, Galea, Kuroda, & McEwen, 1996; Goodman & McIntyre, 2017; Kleen, Sitomer, Kileen, & Conrad, 2006; Lin et al., 2016; Park et al., 2015). In regards to the PFC, consistent impairments in working memory are observed as a consequence of chronic stress (Diamond, Park, Heman, & rose, 1999; Lee & Goto, 2015; Mika et al., 2012; Mizoguchi et al., 2000). However, it has to be acknowledged that while chronic stress impairs performance on the above measures, acute stress serves to *enhance* both spatial and working memory, likely a result of enhanced physiological arousal.

1.10. Adult Neurogenesis

It was a long-held belief in the neuroscience community that adult neurogenesis, or the birth of new neurons throughout the lifespan, did not occur. Instead, it was believed that the brain was unchanging, and the cyroarchitecture from birth did not alter. This is best illustrated by one of the pioneers of modern neuroanatomy, Santiago Ramon y Cajal:

> "Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, and immutable: everything may die, nothing may be regenerated" (Lie, Song, Colamarino, Ming, & Gage, 2004).

This view of the brain persisted for decades, however it was with a series of pioneering studies that this view came to be challenged. For example, Joseph Altman described neurogenesis occurring in the adult DG and olfactory bulb of adult rats (Altman & Das, 1965). This work was furthered by Michael Kaplan, who demonstrated that the presence of complex environments, such as running wheels and toys, increased the numbers of [^{3}H]-thymidine labelled cells in layer IV of the rat visual cortex (Kaplan, 2001). Lastly, a major change in the acceptance of adult neurogenesis was spearheaded by the development of 5- bromo-3'-deoxyuridine (BrdU). The advantage of BrdU is that it allows for researchers to conduct investigations of neuronal fate, allowing for an age-old debate as to what happens to adult generated neurons to be settled. The advent of new immunohistochemical techniques to detect newly generating neurons lead to the discovery of 2 neurogenic niches in the adult brain: the subventricular zone of the lateral ventricles and the SGZ of the DG. This discovery led the way to important advances in the fields of disease and cognition.

1.10.1. Stages of Neurogenesis

Neurogenesis is more than simply the birth of new neurons. Rather, it is a complex process made up of many different stages that depend on the interplay of intrinsic and extrinsic factors. Adult neurogenesis comprises 5 different stages: 1. Proliferation. 2. Differentiation, 3. Migration, 4. Maturation, and 5. Integration (Kemperman & Gage, 2000). Adult neural stem cells originate from the entire dentate neuroepithilium, and the descendants of these cells relocate to the dorsal hippocampus (Goncalves, Schafer, & Gage, 2016). Within the hippocampal DG, radial glia-like cells represent the pool of neural progenitor cells (NPCs), and these cells occupy the SGZ (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). Radial glial-like cells

express glial fibrillary acidic protein (GFAP), nestin, and brain lipid binding protein (BLBP) (Ming & Song, 2011). These radial glial-like cells divide asymmetrically to produce transiently amplifying progenitor type 2 cells. Type 2 cells are said to represent the transition from glial to a neuronal lineage (Steiner et al., 2006). Type 2 cells can be divided into type 2A and type 2B cells, which differ in their expression of certain biomarkers. For example, type 2A cells express GFAP, nestin, and BLBP, while type 2B cells express nestin, doublecortin (DCX), and PSA-NCAM. Overall, type 2 cells represent cells that have committed to a neuronal fate.

Type 2 cells next give rise to type 3 migratory neuroblasts, representing the population of immature postmitotic neurons that mature into the population of resident granule neurons over the course of weeks to months. Type 3 neuroblasts maintain their expression of DCX while also expressing calretinin. However, as they mature, the expression of DCX and calretinin shifts to a pattern of calbindin and NeuN (Kempermann, Jessberger, Steiner, & Kronenberg, 2004).

1.10.2. Morphological Characteristics of Adult Generated Granule Neurons

It takes approximately 7 weeks for adult generated neurons to reach their final morphological maturity (Kempermann, Song, & Gage, 2015). Immature neurons begin to migrate radially and integrate into the local DG circuitry 1 week after their birth, following an inside-out pattern that is dependent on disrupted in schizophrenia 1 (DISC1) and reelin signalling (Mao et al., 2009). As adult generated granule neurons mature, they begin to extend their apical dendrites first through the GCL and then into the ML. Tracing studies have demonstrated that the first dendrites from these sprouting immature granule cells are detected 16 days post-division (Zhao, Teng, Summers, Ming, & Gage, 2006). Furthermore, approximately 4-10 days post-division these immature granule neurons send axonal projections to pyramidal cells in the CA3 (Zhao, Teng, Summers, Ming, & Gage, 2006). These immature granule cells contain functional GABA and glutamate receptors located on their plasma membrane 4 days post-division. GABA continues to depolarize the cell for 2-3 weeks until the immature granule cells become responsive to glutamate (Kempermann, 2015). After becoming responsive to glutamate, the threshold to induce long-term potentiation (LTP) is decreased in these immature granule cells, biasing input to these new neurons (Marin-Burgin, Mongiat, Pardi, & Schinder, 2012). Finally, as immature granule cells begin to reach a mature stage, their mossy fibers begin to form connections with interneurons dispersed throughout the GCL, hilus, and CA3 (Toni et al., 2008).

1.10.3. Regulation of neurogenesis

As mentioned earlier, hippocampal neurogenesis is a dynamic process regulated by a combination of intrinsic and extrinsic factors. One of the best studied regulators of hippocampal neurogenesis is Notch. Notch is a single-pass transmembrane protein, and is involved in a variety of signalling cascades (Goncalves, Schafer, & Gage, 2016). Inactivation of the Notch pathway component results in an initial increase in hippocampal neurogenesis by causing a premature differentiation of progenitor cells, which eventually leads to a depletion of the progenitor cell pool and a decrease in hippocampal neurogenesis (Ehm et al., 2010). Furthermore, conditional knockout or overexpression of Notch has contrasting effects on dendritic morphology: knockout significantly decreases dendritic arborisation, while overexpression increases dendritic complexity (Breunig, Silbereis, Vaccarino, Sestan, & Rakic, 2007). Hedgehog signalling has also been implicated in the regulation of postnatal hippocampal neurogenesis. Hedgehog signalling is initiated in the brain by sonic hedgehog (Shh) (Goncalves, 2016). Support for the role of Shh in postnatal hippocampal neurogenesis has come from both in vitro and in vivo studies. For example, application of exogenous Shh to cultured neurons promotes progenitor proliferation, while Shh overexpression within the DG increases hippocampal progenitor cell proliferation. Moreover, pharmacological inhibition of Shh in the hippocampus reduces hippocampal cell proliferation (Lai et al., 2003).

Secreted growth factors dynamically regulate postnatal hippocampal neurogenesis. Of these, BDNF is the most widely studied. BDNF expression is highest within the hippocampus, especially in dentate granule cells (Katoh-Semba, Takeuchi, Semba, & Kato, 1997). The regulatory effects of BDNF on hippocampal neurogenesis are best illustrated by studies showing that BDNF infusion into the hippocampus significantly increases neural progenitor proliferation (Scharfman et al., 2005), and others showing heterozygous BDNF mice have reduced survival of neural progenitor cells (Rossi et al., 2006). Furthermore, deletion of the BDNF receptor tyrosine receptor kinase B (TrkB) located on neural progenitor cells reduces hippocampal neurogenesis (Li et al., 2008). It is interesting that antidepressant drugs consistently elevate BDNF expression in the hippocampus, which suggests that a component of antidepressant action involves hippocampal neurogenesis (Zhao, Deng, & Gage, 2008). Extracellular matrix proteins also regulate neurogenesis. Perhaps the best studied of these is reelin. Reelin is expressed in the hippocampus and cerebellum, where it is released by a subset of GABAergic interneurons.

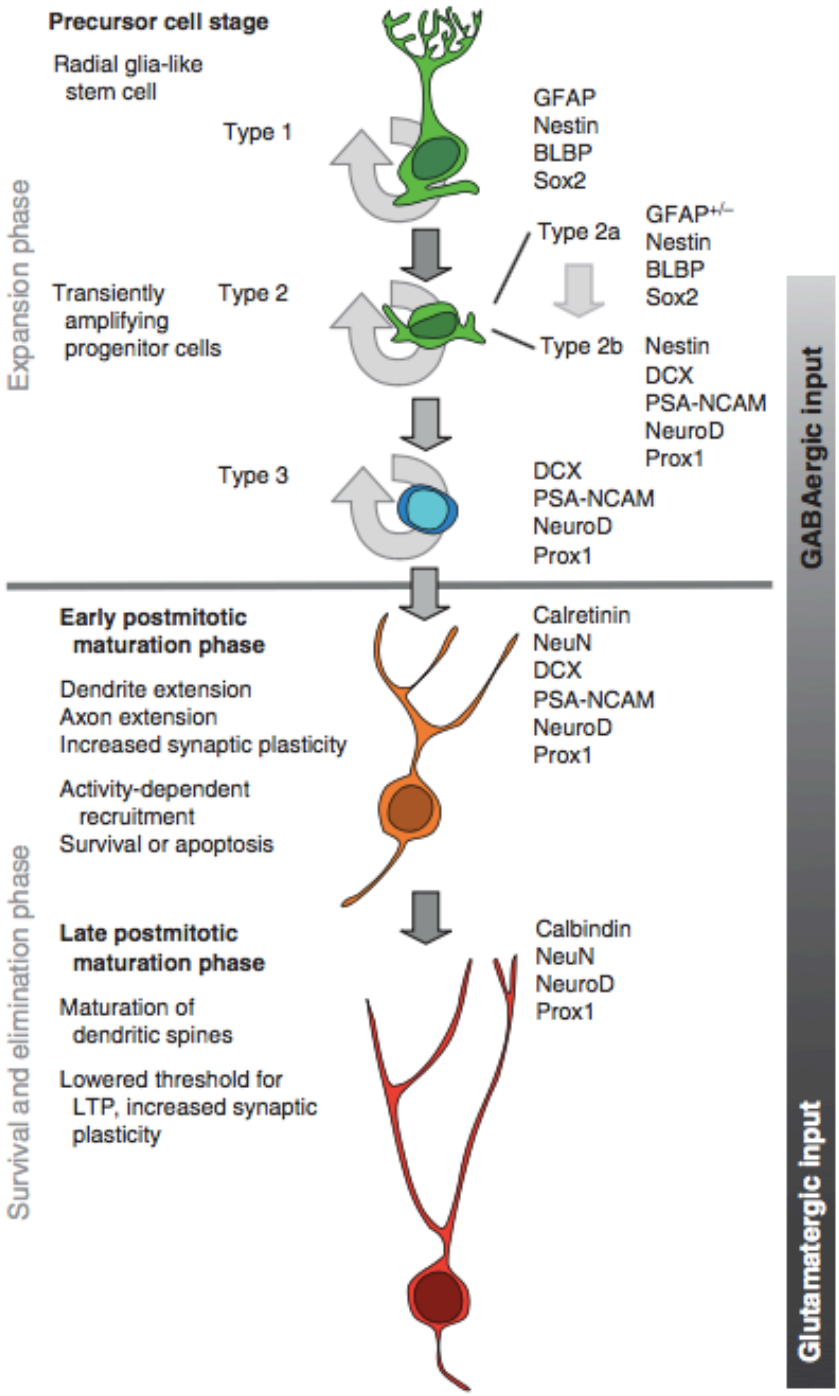

Fig 1-4. The stage of developmental courses in adult hippocampal neurogenesis. Within the hippocampal DG, radial glia-like cells represent the pool of neural progenitor cells (NPCs), and these cells occupy the SGZ (Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). Radial glial-like cells express glial fibrillary acidic protein (GFAP), nestin, and brain lipid binding

protein (BLBP) (Ming & Song, 2011). These radial glial-like cells divide asymmetrically to produce transiently amplifying progenitor type 2 cells. Type 2 cells are said to represent the transition from glial to a neuronal lineage (Steiner et al., 2006). Type 2 cells can be divided into type 2A and type 2B cells, which differ in their expression of certain biomarkers. For example, type 2A cells express GFAP, nestin, and BLBP, while type 2B cells express nestin, doublecortin (DCX), and PSA-NCAM. Overall, type 2 cells represent cells that have committed to a neuronal fate.Type 2 cells next give rise to type 3 migratory neuroblasts, representing the population of immature postmitotic neurons that mature into the population of resident granule neurons over the course of weeks to months. Type 3 neuroblasts maintain their expression of DCX while also expressing calretinin. However, as they mature, the expression of DCX and calretinin shifts to a pattern of calbindin and NeuN. Adapted from (Kempermann, Song, & Gage, 2015).

During neonatal development reelin plays a critical role in cortical lamination, however in the postnatal period its role shifts to one involved in mediating a variety of mechanisms involved in synaptic plasticity. One such role is in the regulation of hippocampal neurogenesis. Pujadas et al. (2010) found that mice overexpressing reelin have a significant increase in DCX-ir granule cells, accompanied by increased complexity in their dendrites. Won et al. (2006) demonstrated that heterozygous reeler mice show a significant reduction in newborn neurons in the DG.

In addition to intrinsic factors, extrinsic factors also play a role in the regulation of neurogenesis. One of the most consistent findings is that environmental enrichment increases the proliferation of neural progenitor cells. Complex environments (access to toys and other interactable objects), and social housing all increase hippocampal neurogenesis (Kempermann, Kuhn, & Gage, 1997). Voluntary running, such as wheel running, is also reported to increase hippocampal neurogenesis (van Praag, Shubert, & Gage, 1999). Finally, antidepressant medication is demonstrated to increase the proliferation of neural progenitor cells. Specifically, tranylcypromine, reboxetine, fluoxetine, and haloperidol have been shown to increase the number of BrdU-labelled progenitor cells in the hippocampus (Malberg, Eisch, Nestler, & Duman, 2000). However, the role of hippocampal neurogenesis in depression and antidepressant action is far from clear, and this issue will be discussed in further detail below.

1.10.4. Neurogenesis in Depression

Animal models of depression repeatedly show that neurogenesis is decreased by exposure to chronic stress (Brummelte & Galea, 2010; David et al., 2009: Nestler et al., 2002; Pham, Nacher, Hof, & McEwen, 2003; Mayer et al., 2006; Murray, Smith, & Hutson, 2008) and that antidepressants and electroconvulsive shock therapy treatment restores this (Czeh et al, 2001; Malberg & Duman, 2003; Mayer, 2006; Murray, 2008; Scott, Wojtowicz, & Burnham, 2000). Work in our lab has shown that the tricyclic antidepressant imipramine and the tumor necrosis factor alpha (TNF-α) inhibitor etanercept restore CORT-induced reductions in hippocampal neurogenesis (Brymer et al., 2018; Fenton et al., 2015). The finding that animal models of depression decrease hippocampal neurogenesis fits nicely with the well-documented hippocampal volume reductions and loss of grey matter seen in depression (Bremner et al., 2000; Grieve, Korganokar, Koslow, Gordon, & Williams, 2013). These findings have led to the proposal of the neurogenesis hypothesis of depression. This hypothesis posits that depression results from a reduction in hippocampal neurogenesis, and that antidepressant medications at

least partially work though an increase in hippocampal neurogenesis. However, this hypothesis has garnered intense criticism from the field, the most vocal being that to date has been little evidence of reductions in hippocampal neurogenesis in post mortem depressed patients. Moreover, there are currently no ways to causally test if neurogenesis is involved in depression in human patients.

Müller et al. (2001) and Reif et al. (2006) both failed to find reductions in proliferating progenitor cells in the hippocampus. Furthermore, it has been demonstrated that antidepressant effects can be achieved without increases in neurogenesis, and ablation of neurogenesis is not sufficient to create a depression-like phenotype (Hanson, Owens, & Nemeroff, 2011; Zhao, Deng, & Gage, 2008). Recently, Sorrels et al. (2018) found that human hippocampal neurogenesis sharply drops from childhood into near undetectable levels in adulthood, boldly suggesting that hippocampal neurogenesis does not occur past childhood. One must approach these findings with caution, as the samples used in the Sorrels et al. study were obtained from epileptic post-mortem tissue. However, counter to this report, Boldrini et al. (2018) report that human hippocampal neurogenesis persists throughout aging, even into the 70th year of life. Moreover, recent reports have found reductions in hippocampal neurogenesis in post-mortem depressed tissue (Boldrini et al., 2012). In an elegant study, Hill et al. (2015) showed that increasing hippocampal neurogenesis through transgenic methods alone is sufficient to create an antidepressant phenotype. Therefore, a complicated picture of the role of hippocampal neurogenesis in depression emerges. It is highly unlikely that hippocampal neurogenesis is absent in humans as Sorrels et al. would suggest, however it is also a possibility that neurogenesis is not as important in humans as it is in rodents. The development of better imaging and staining techniques will inevitably solve the debate as to the true role of hippocampal neurogenesis in the pathogenesis of depression.

1.11. Inflammation and Depression

The notion that inflammation is a contributing factor in the pathogenesis of depression is not a new one. Work in the 1980s's first revealed that some patients with heightened immune activity (e.g., a patient with an intense cold) displayed the hallmark features of depression (i.e., lethargy, depressed mood, anhedonia) (Miller and Raison, 2016). This in turn led to the cytokine sickness hypothesis of depression, which posits that sustained increases in circulating levels of pro-inflammatory cytokines can produce depressive symptoms (Dantzer, 2009). Interestingly,

high circulating levels of the pro-inflammatory cytokine IL-6 are strongly associated with feelings of guilt and suicidal ideation (O'Donovan et al., 2013). If the cytokine sickness hypothesis of depression is correct, then one would expect that disorders characterized by high circulating levels of cytokines would share a high comorbidity with depression, and this turns out to be the case. For example, rheumatoid arthritis, a disorder in which the immune system targets bodily tissues and instigates widespread inflammation, shares a 13-42% comorbidity rate with depression (Margaretten et al., 2011). Furthermore, cancer patients treated with cytokines experience a significant reduction in plasma tryptophan levels that coincides with depression (Capuron et al., 2002). This tryptophan reduction reduces the bioavailability of serotonin, which is a known risk factor for the development of depression according to the monoamine hypothesis. It was later discovered that the culprit for this decrease in plasma tryptophan levels in cancer patients receiving immunotherapy is indoleamine 2,3-dioxygenase (IDO). Activation of IDO decreases tryptophan bioavailability, creating a net decrease in monoamines (Danzter, 2009). Immuno-activation in healthy control subjects creates depression-like behaviour and impairments in cognition (Reichenberg et al., 2001), and serum concentrations of interleukin-6 at 9 years of age is positively correlated with depressive symptomology at 18 years of age (Khandaker et al., 2014). Finally, high circulating levels of cytokines are associated with treatment-resistant depression (Carvalho et al., 2013).

Stress is also known to transiently elevate the expression of pro-inflammatory cytokines. Unsurprisingly, pro-inflammatory cytokines are efficient activators of the hypothalamic-pituitary-adrenal (HPA) axis (Kenis & Maes, 2002), which often becomes dysregulated in depression. However, as not all depressed patients display a dysregulated HPA axis, it is tempting to speculate that elevated levels of pro-inflammatory cytokines occur in a subset of depression patients, possibly those patients who develop symptoms after a period of major life stressors leading to HPA dysfunction.

1.11.1. The role of TNF-α in Depression

TNF-α is a protein that is initially released as a soluble cytokine (sTNF-α) after being enzymatically cleaved by its cell surface bound precursor (tmTNF-α) by TNF-α converting enzyme (TACE) (Bortolato, Carvalho, Soczynska, Perini, & McIntyre, 2015) and is therefore expressed as a transmembrane protein. TNF-α binds to one of two receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNFR1 is activated by soluble and transmembrane

TNF-α, and promotes inflammation and tissue degeneration (Kalliolias & Ivashkiv, 2016). TNFR2's expression is restricted to neurons, endothelial cells, and immune cells, and is involved in mediating cell survival and tissue regeneration (Kalliolias, 2016). sTNF-α possesses a higher affinity for binding with TNFR1. When TNF-α binds to TNFRs, complex 1 is assembled at the plasma membrane and includes the TNF-α associated death domain protein (TRADD) among other complexes resulting in the creation of a scaffolding ubiquitin network (Kalliolias). This scaffolding ubiquitin creates the recruitment and activation of 2 signaling complexes: transforming growth factor (TGF)-α activated kinase 1 (TAK1) complex and the inhibitor of kB (Ikkα) kinase complex (Kalliolias). Activation of TAK1 results in the recruitment of mitogen activated kinase (MAPK) signaling cascades resulting in activation of downstream JUN N-terminal kinase and P38 (Kalliolias). Ikkα activates the canonical nuclear factor KB (NFKB) pathway (Kalliolias). Therefore, the pattern of activation that emerges from induction of complex 1 signaling is one of NFKB and JUN N-terminal kinase and P38 target genes that are responsible for inflammation, cell proliferation and survival, and host defense. In contrast, complex II signaling results in the activation of necroptosis effector mixed lineage kinase domain like protein (MLKL) (Kalliolias) by a RIPK3-dependent mechanism, responsible for TNF-α induced cell death via apoptosis. It is currently unclear what factors lead to the activation of complex I and resultant inflammatory processes or complex II leading to cell death (Kalliolias).

One of the main roles of TNF-α is in maintaining inflammation during times of proinflammatory conditions. During proinflammatory events, TNF-α production is induced by other cytokines (IL-1) and microglia. Once released, TNF-α stimulates the production of other proinflammatory cytokines, including IL-1 and 6, as well as increasing the production of reactive oxygen intermediates, including nitric oxide, which in turn creates a feedback loop of enhanced inflammation (Bortolato, Carvalho, Soczynska, Perrini, & McIntyre, 2015). It is not surprising that increased inflammation as a result of increased TNF-α production results in altered glutamatergic signaling and ecotoxicity. TNF-α upregulates glutaminase in astrocytes, decreases the conversion from glutamate to glutamine, increases the trafficking of NMDA receptors, and promotes long-term depression (LTD) (Bortolato, 2015). Furthermore, TNF-α signaling results in the endocytosis of $GABA_A$ receptors, resulting in an altered balance between inhibition and excitation (Bortolato). Preclinical studies corroborate the role of TNF-α in depression-like

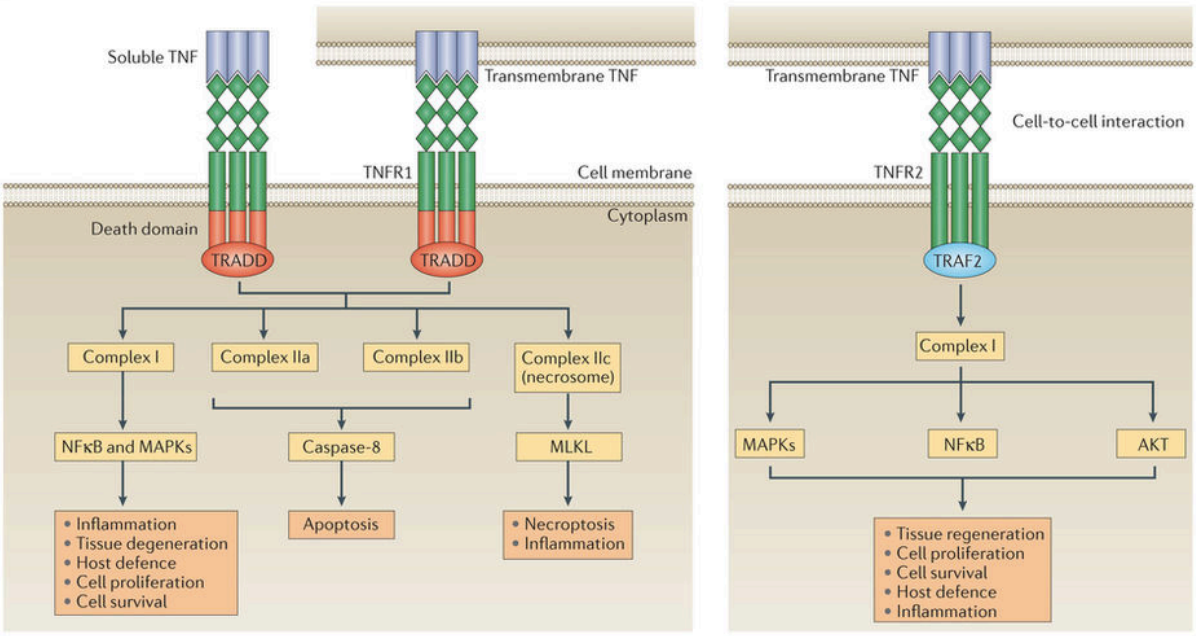

Fig 1-4. Induction of complex 1 signaling is one of NFKB and JUN N-terminal kinase and P38 target genes that are responsible for inflammation, cell proliferation and survival, and host defense. Complex II signaling results in the activation of necroptosis effector mixed lineage kinase domain like protein (MLKL) (Kalliolias) by a RIPK3-dependent mechanism, responsible for TNF-α induced cell death. Adapted from (Kalliolias & Ivashkiv, 2016)

behaviour. Peripheral administration of TNF-α can produce anhedonic behaviour in rodents (van Heesch et al., 2013). Likewise, deletion of TNFR1 or TNFR2 creates an antidepressant phenotype on measures of depression-like behaviour (Simen et al., 2006). Yamada, Iida, and Miyamoto (2000) showed that TNF-α knockout mice display a mild antidepressant phenotype. Along these same lines, administration of the TNF-α inhibitor infliximab during chronic mild stress significantly decreased immobility time in the forced swim test, increased sucrose consumption during the sucrose preference test, and decreased anxiety-like behaviour in the elevated plus maze (Karson et al., 2013).

Given that pro-inflammatory cytokine treatment produces depression-like behaviour and reducing pro-inflammatory cytokine levels alleviates this, the question that arises is whether or not antidepressants can influence inflammation. Interestingly, SSRIs and tricyclic antidepressants are known to reduce levels of TNF-α and other proinflammatory cytokines and increase anti-inflammatory cytokines, including IL-10 (Song et al., 1994). Two potential explanations for how antidepressant drugs might reduce pro-inflammatory cytokine levels have been offered. The first explanation posits that higher levels of activation of serotonergic receptors located on immune cells dynamically regulate the production of pro-and anti-inflammatory cytokines, and this could be influenced by a higher availability of serotonin upon antidepressant treatment, although the pro- or anti- inflammatory effects of peripheral serotonin is still under debate (recently reviewed in Herr et al., 2017; see also Kenis & Maes, 2002). The second explanation sees the increased production of cyclic adenosine monophosphate (cAMP) by antidepressants as a mechanism by which antidepressants might reduce cytokine levels. Specifically, cAMP activates protein kinase A (PKA), increasing the production of cAMP responsive element binding protein (CREB), both of which act to decrease pro-inflammatory cytokine production (Kenis & Maes, 2002). Considerable attention
has been dedicated to the antidepressant potential of ketamine. Ketamine rapidly reverses depression-like behaviour in both clinical and preclinical subjects, in a timeframe of hours (Cusin et al., 2012; Yoshi & Constantine-Paton, 2010). An important question asked within the literature is whether ketamine is altering neuroinflammation? Although this question is relatively recent, it does appear to be the case that at least a component of the antidepressant properties of ketamine involve reductions in neuroinflammation, in particular TNF-α. For example, Wang et al. (2015) have shown that in chronically-stressed rats, the rapid antidepressant effects of

ketamine are accompanied by a reduction of hippocampal TNF-α levels. Moreover, a reduction in depressive symptoms 40 minutes post ketamine infusion in depressed patients has been shown to be correlated with reductions in serum TNF-α levels (Chen et al., 2018). These findings suggest that rapid changes in neuroinflammation, in particular TNF-α, are perhaps one of the mechanisms underlying the antidepressant actions of ketamine.

In terms of clinical studies, a large body of evidence supports the role of TNF-α in depression. Endotoxin administration in control subjects produces an increase in TNF-α in addition to depressed mood and cognitive impairment (Della and Hannestad, 2010). Microarray mRNA studies demonstrate increased expression of tmTNF-α in the prefrontal cortex (PFC) of suicide victims (Pandey et al., 2012). In fact, higher suicidal ideation itself is associated with an increased cytokine profile, including elevated TNF-α (O'Donnovan et al., 2013), and high circulating levels of TNF-α are found in peripheral tissues of suicide victims (Lindqvist et al., 2009). Several lines of evidence support the efficacy of TNF-α inhibitors in the treatment of depression. Patients with rheumatoid arthritis and plaque psoriasis taking prescribed etanercept, which is a TNF-α antagonist, reported significant reductions in depressive symptoms (Kekow et al., 2011; Gelfand et al., 2008). Similarly, patients with Crohns Disease receiving infusions of infliximab experienced significant reductions in depressive symptoms and this decrease was associated with corresponding reductions in proinflammatory cytokines (Guloksuz et al., 2013). Finally, psoriasis patients with comorbid psychiatric conditions report improvement in mood and overall well-being when taking infliximab (Bassukas et al., 2008). Interestingly, inflammation itself is associated with anhedonia, and one of the first symptoms to be alleviated in depressed patients receiving anti-inflammatory compounds is anhedonia (Felger et al., 2016).

Some interesting extensions of these clinical studies have been observed in patients with treatment-resistant depression. Raison et al. (2013) reported that patients with treatment - resistant depression with a high baseline level of inflammation as indicated by elevated high sensitivity C-reactive protein expression responded favorably to infusions of the TNF-α inhibitor infliximab. However, in patients with a low baseline level of high sensitivity C-reactive protein infliximab was not more effective than placebo. This pattern of results suggests that subsets of treatment-resistant patients experience high levels of inflammation, and therapies aimed at reducing inflammation might be particularly effective in these patients. However, other patients may have a different physiological profile such that factors other than inflammation are at play.

This conclusion is consistent with the observation that two depressed patients can present with a different cluster of symptoms. For example, patient A could present with agitation, weight loss, an inability to sleep, and lowered mood, whereas patient B presents with weight gain, excess sleep, psychomotor retardation, and anhedonia. This raises the question (asked elsewhere; see Nestler, 2010) of whether depression is actually part of a constellation of different disorders. It is tempting to suggest that depressed patients with inflammation might represent a subset of depressed patients who require a different course of treatment compared to their non-inflamed counterparts. Kappelmann et al. (2016) conducted a meta-analysis of the effectiveness of anti-cytokine treatments in depression. They found that across 7 double-blind clinical trials involving 1309 subjects, anti-cytokine treatment was generally more efficacious than treatment with placebo. Of interest is the fact that several research groups have reported that currently available non-cytokine antidepressant medications are not more effective than placebo, at least in the treatment of non-severe depression (Fournier et al., 2010; Garland, 2004; Kirsch et al., 2008). Therefore, treatments that target inflammation might represent a more viable approach to the treatment of depression, as the monoamine hypothesis of depression largely does not align with what is currently known about the biological causes of depression. Of the anti-cytokine treatments analyzed, Kapplemann et al. (2016) reported that anti-TNF-α drugs were the most commonly used option. The picture that emerges is that depression is associated with elevations in TNF-α, and treatments aimed at reducing circulating TNF-α produce significant normalization of depressive symptoms. In the next section, we elaborate on some putative mechanisms underlying the antidepressant effect of TNF-α antagonists.

Peripheral injection of TNF-α antagonists (i.e., etanercept) causes a functional decrease in peripheral TNF-α with only an indirect effect on central TNF-α expression, as drugs like etanercept cannot cross the blood brain barrier (Boado et al., 2010). Therefore, it has been assumed that drugs like etanercept are only able to indirectly reduce central inflammation as a consequence of reduced peripheral TNF-α activity (Kerfoot et al., 2006). However, TNF-α per se can cross the blood brain barrier by a receptor mediated mechanism (Pan & Kastin., 2001, 2002; Pan et al., 2006), and when this occurs, it instigates an increase of both TNF-α protein and mRNA by stimulating central expression of TNF-α by microglial cells (Qin et al., 2007; McCoy and Tansey., 2008). Peripheral TNF-α also can stimulate secretion of TNF-α from circumventricular organs and choroid plexus, and TNF-α secreted by these organs can then

induce the activation of microglia and a subsequent increase in TNF-α secretion by microglial cells (Qin et al., 2007; McCoy and Tansey., 2008). As stated, etanercept does not cross the blood brain barrier but it binds peripheral TNF-α, and in doing so, it reduces the effect of peripheral TNF-α in promoting the activation of microglia, which results in decreased secretion of central TNF-α.

High circulating levels of TNF-α are associated with significant reductions in cognitive functioning. Hennesey et al. (2017) showed that systemic TNF-α injections produced deficits on the Morris Water maze and T-maze. High circulating levels of TNF-α were found to be negatively correlated with delayed performance on the Rey Auditory Verbal Learning Test in bipolar patients (Doganavsargil-Baysal et al., 2013). In older adults, post-operative cognitive decline following surgery is common. Terrando et al. (2010) showed that post-operative cognitive decline is accompanied by an increased peripheral production of TNF-α and a subsequent increased production of other proinflammatory cytokines. Sahin et al. (2015), using the chronic mild stress paradigm, investigated the effects of TNF-α inhibition on cognitive functioning and hippocampal BDNF levels. Infliximab administration reversed stress-induced deficits on the Morris water maze and passive avoidance test, and this was accompanied by an increase in hippocampal BDNF levels. Importantly, work in our lab has demonstrated that TNF-α is critically involved in hippocampal dependent memory. Repeated CORT injections impair hippocampal-dependent object-location memory and produce depression-like behaviour, and peripheral etanercept injections restore object-location memory performance to control levels and reverse depression-like behaviour. These results will be discussed in further detail in Chapter 3. From the research described above it is clear that TNF-α is involved in the regulation of cognitive functioning, in particular hippocampal dependent memory. It is currently unclear as to how high circulating levels of TNF-α are impacting cognitive functioning. An interesting hypothesis (and one further detailed in chapter 3) is that repeated corticosterone injections increase TNF-α levels in the periphery, which in turn increases central TNF-α expression. This increased expression of TNF-α reduces hippocampal reelin expression which contributes to cognitive dysfunction. A rescuing of reelin expression by the TNF-α inhibitor etanercept then restores cognitive performance.

1.11. Reelin and its role in Depression

Reelin is an extracellular matrix protein involved in the development of the nervous system and in functions that promote synaptic plasticity. During development, reelin is secreted by cajal retzius cells and cerebellar granule cells, however in the postnatal brain reelin is released by GABAergic interneurons located in the hippocampus and cortex. Reelin is cleaved by metalloproteases at two specific sites, which leads to the generation of 3 large fragments, composed of a N-terminal, central fragment, and a C-terminal. The C terminal by itself can initiate intracellular signaling (Lee & D'Arcangelo, 2016). Reelin is approximately 385 kDA, and is composed of a single peptide, a N-terminal sequence, and a hinge region of 8 reelin repeats containing 350-390 amino acids (Fatemi, 2005). Each of these reelin repeats is itself composed of 2 sub repeats separated by an EGF motif. This reelin protein structure ends with a C-terminus that is composed of 33 amino acids (Fatemi).

Reelin binds to 2 receptors, apolipoportein E receptor 2 (ApoER2), and very-low density lipoprotein receptor (VLDL-R), which belong to the low-density lipoprotein receptor (LDLR) family (Lee & D'Arcangelo, 2016). Reelin binding to these 2 receptors results in their clustering, which itself leads to the dimerization/oligomerization of the adapter protein disabled-1 (Dab-1) through tyrosine phosphorylation. Dab-1 is critical for the transduction of reelin signaling, and it binds to the cytoplasmic tail of both ApoER2 and VLDR-R (Lee, 2016). Once Dab-1 becomes activated the recruitment of SRC family tyrosine kinases (SFKs) to Dab-1 begins. The resulting high concentration of SFKs then triggers a cytosolic kinase cascade (Lee), which includes phosphatidylinositol-3-kinase (PI3K) and the inhibition of glycogen synthase kinase 3α (GSK3α) (Lee). Finally, the recruitment of ubiquitin ligases to now phosphorylated Dab-1 polyubiquitylationates Dab-1, leading to the termination of the signal and both ApoER2 and VLDL-R are recycled back to the membrane (Lee).

The best known functions of reelin are perhaps its role in cortical lamination and brain development. This is best illustrated by the reeler mouse, where a mutation during development leads to decreased levels of reelin in the cerebellum and hippocampus. The brains of these mice fail to display a granule cell layer in the dentate gyrus, have dispersed neurons in multiple cortical layers, have impaired dendrite outgrowth, and have a decreased cerebellar size (Fatemi, 2005). While reelin was initially investigated for its role in development, the finding that reelin is

preferentially expressed by GABAergic interneurons in the adult cortex and hippocampus led to investigations into the role of reelin in postnatal development. These investigations revealed that

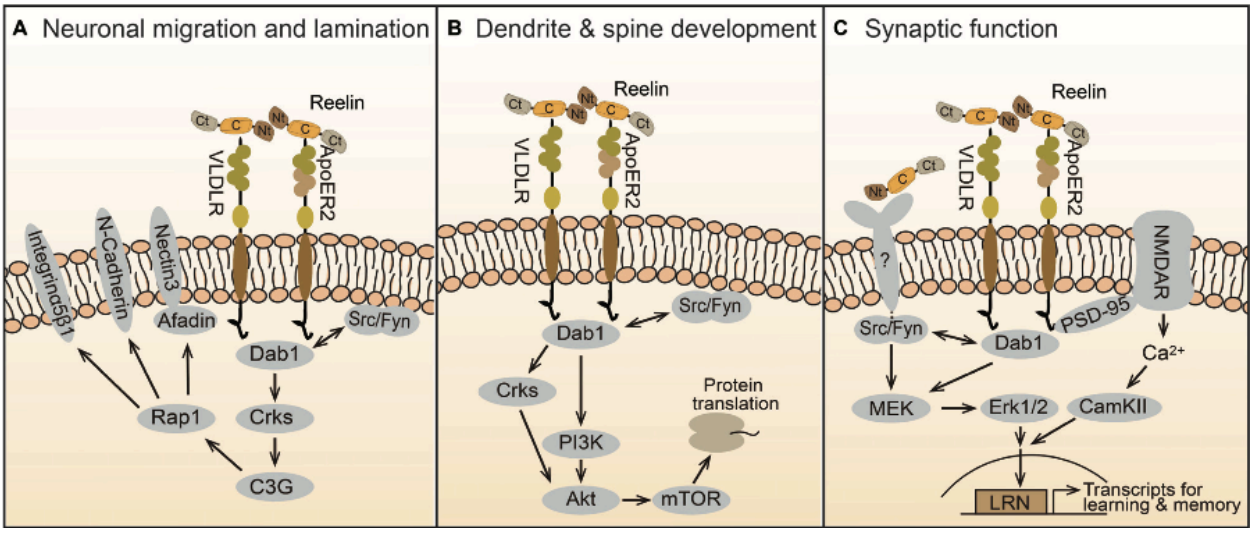

Fig 1-5. Reelin has differential effects on synaptic function and neural development depending on the time in development it is released. During neonatal development, reelin is involved in neuronal migration and lamination. However, after development reelin switches roles to one focused on promoting dendrite and spine development and synaptic function. Adapted from (Lee & D'Arcangelo, 2016)

reelin is critically involved in the control of dendrite and spine development, and synaptic function. It is likely that P13K and akt are the main contributors to the effects of reelin on dendrite development, which are activated by reelin's effects on Dab-1 activity, which in turn activates SFKs. The downstream targets of akt include mammalian target of rampomycin (mTOR), which is shown to be activated by reelin treatment in neuronal cultures and is also involved in reelin's effects on dendrite development (Lee & D'Arcangelo, 2016). This is best illustrated by a report from Jaworski, Spangler, Seeburg, Hoogenraad, and Sheng (2005) which showed that inactivation of either PI3K or mTOR reduced dendritic complexity in neuronal cultures. Moreover, Lim and Walikonis (2008) showed that hepatocyte growth factor increased neurite length and this outgrowth was accompanied by akt activation. Blockade of the PI3K/akt signalling pathway inhibited the hepatocyte growth factor induced increase in neurite outgrowth. These results suggest that the downstream targets of reelin, the PI3K/akt and mTOR pathways, are critically involved in promoting dendritic outgrowth and inhibitors of these pathways impairs this process.

In addition to promoting dendritic outgrowth, reelin is also involved in key aspects of synaptic function. For example, reelin is involved in the promotion of hippocampal long-term potentiation (LTP). This enhancement of hippocampal LTP requires both the ApoER2 and VLDL-R receptors, as ApoER2 and VLDL-R knockout mice display reduced hippocampal LTP (Weeber et al., 2002). These ApoER2 and VLDL-R knockout mice also display deficits in contextual fear conditioning. One way that reelin and its receptor ApoER2 act to enhance LTP is through ApoER2's interaction with NDMAR and PSD-95, which mediates a reelin induced calcium^{++} influx through NMDAR's (Lee & D'Arcangelo, 2016). Reelin also requires the presence of phosphorylated Dab-1 for its enhancement of LTP. For example, Trotter et al. (2013) showed that Dab-1 knockout mice display deficits in LTP and spatial memory, as well as reductions in dendritic spine size. Finally, Rogers et al. (2011) have shown that reelin supplementation enhances hippocampal LTP and learning and memory in naïve mice.

Preclinical studies have implicated deficits in reelin signalling in the pathogenesis of psychiatric disorders, in particular depression. For example, work in our lab has shown that repeated CORT injections significantly reduced the number of reelin positive cells in the SGZ of the hippocampus, while restraint stress had no effect (Lussier et al., 2009). In another study, we

showed that the onset of depression-like behaviour in CORT-treated rats is paralleled by a reduction in reelin positive cells in the SGZ (Lussier et al., 2013a). This suggests that a loss of reelin expression in the hippocampus contributes to depression-like behaviour, and we would expect that animals lacking reelin would show depression-like behaviour. Heterozygous reeler mice, which express 50% of the normal levels of reelin, provide a way to approach this question. Our lab has shown that heterozygous reeler mice, while not spontaneously displaying depression-like behaviour, are in fact more susceptible to the depressogenic effects of CORT injections than are wild type mice. If loss of reelin is a risk factor for the development of depression in animal models, then it might be expected that one mechanism of antidepressants is to enhance hippocampal reelin expression. Work in our lab has shown that the tricyclic antidepressant imipramine restores CORT-induced reductions in hippocampal reelin expression (Fenton et al., 2015). Furthermore, we have shown that the TNF-α inhibitor etanercept can also alleviate CORT-induced reductions in hippocampal reelin expression (Brymer et al., 2018). This finding that etanercept restores reelin expression will be discussed more in Chapter 3. Teixeira et al. (2011) showed through overexpression that reelin prevents the behavioural manifestation of both bipolar disorder and schizophrenia. In terms of clinical studies, Fatemi et al. (2000) found that reelin expression was decreased in the CA4 region of the hippocampus in post-mortem depressed brains. Furthermore, a decrease in reelin and glutamic acid decarboxylase67 (GAD67) was found in the brains of post-mortem schizophrenia and bipolar disorder patients (Guidotti et al., 2000).

A question that remains to be answered is can reelin supplementation reverse the depression-like behaviour induced by repeated CORT treatment? Given that CORT treatment reduces hippocampal reelin expression, it would be hypothesized that reelin supplementation would reverse depression-like behaviour. This question will be addressed in Chapter 4 and 5 of the current dissertation.

In addition to reelin's role in depression-like behaviour, reelin also plays significant roles in learning and memory. For example, as noted above reelin supplementation in naïve mice enhances learning and memory (Rogers et al., 2011). Reelin has been demonstrated to recover synaptic function and spatial learning and memory in a mouse model of Angelman syndrome (Hethorn et al., 2015). Pujadas et al (2014) reported that overexpression of reelin delayed the formation of amyloid beta fibrils and rescued deficits of recognition memory in a mouse model

of Alzheimer's disease. Heterozygous reeler mice display a deficit in contextual fear conditioning as well as LTP (Qiu et al., 2006). Importantly, reelin supplementation in heterozygous reeler mice was shown to recover contextual fear conditioning deficits, suggesting that reelin is critically involved in learning and memory (Rogers et al., 2013). It is likely that the mechanism behind reelin effects on enhancing learning and memory lies in reelin's robust ability to restore hippocampal LTP, as disrupting the reelin signaling pathway disrupts LTP and produces corresponding hippocampal dependent memory deficits. Chapter 4 will explore the ability of reelin supplementation to restore CORT-induced deficits in object location memory, a hippocampal dependent form of memory.

1.13. Glutamate and its role in Depression

Glutamate is the primary excitatory neurotransmitter present in vertebrate nervous systems. While the importance of glutamate was recognized for decades, it was not until the 1970s that glutamate was acknowledged as a neurotransmitter. Glutamate is distributed throughout the central nervous system and spinal cord where it acts to promote rapid neurotransmission. Glutamate is released from vesicles in presynaptic terminals through a CA^{++} mechanism (Hackett & Ueda, 2015). Glutamate plays major roles in brain development, neuronal survival, and axon genesis (Dang & Hashimoto, 2014). Once released, glutamate acts on both ionotropic and metabotropic glutamate receptors. The ionotropic glutamate receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), *N*-Methyl-d-asparate (NMDA), kainate, and δ receptors. Metabotropic glutamate receptors are G protein linked and have seven *trans*-membrane domains including an extracellular N-terminal and an intracellular COOH terminal (Meldrum, 2000). They are composed of 7 subunits, mGluR1-mGluR7. In contrast to the fast-acting actions of ionotropic receptors, metabotropic glutamate receptors are involved in complex, longer-acting signaling cascades.

AMPA receptors are composed of 4 subunits, including GluA1, GluA2, GluA3, and GluA4. All of the GluA1-4 subunits form both homo and heteromers (Traynelis et al., 2010). Kainate subunits include GluK1, GluK2, GluK3, GluK4, and GluK5, and the subunits GluK1-GluK3 form both homo and heteromers (Traynelis, 2010). The NMDA subunits are composed of GluN1, GluN2A-GluN2D, GluN3A, and GluN3B. NMDA receptors are characterized by a voltage sensitive block by magnesium, which is removed upon depolarization (Traynelis). δ subunits include GluD1 and GluD2.

Both AMPA and NMDA receptors are particularly pertinent to the study of depression, due to their well-established roles in learning and memory, stress, and neurotoxicity. AMPARs are responsible for the majority of fast excitatory synaptic transmission in the central nervous system (Krugers, Hoogenraad, & Groc, 2010). AMPARs are first synthesized in the neuronal cell body, and eventually reach their synaptic targets through the motor protein dynein, which aids in the sorting of AMPA into dendrites (Krugers, 2010). Once inside dendrites, AMPARs are trafficked to and from the synapse by exocytotic and endocytic recycling through both intracellular and extracellular receptor pools, and surface diffusion between extrasynaptic and synaptic receptor pools (Krugers).

Contextual learning, where animals learn information regarding a specific context, increases the number GluA subunits in the synapses of hippocampal neurons (Krugers, Hoogenraad, & Groc, 2010), suggesting that learning in the hippocampus is accompanied by corresponding changes in AMAPR trafficking. AMPARs are especially involved in the regulation of spatial learning and memory. For example, GluA1-knockout mice display profound deficits on the Morris water maze task (Sanderson et al., 2010). Interestingly, these same animals display normal acquisition of spatial reference memory tasks. Sanderson et al. (2009) further explored this relationship, showing that GluA1 knockout mice display degraded performance on the novelty preference test if the interval between training and testing was short (< 1 hour) but enhanced performance if the interval was long (<24 hours). To examine the specificity of this spatial memory deficit, Sanderson et al. (2011) examined the ability of GluA1 knockout mice to learn spatial discrimination tasks. Surprisingly, GluA1 knockout mice performed like controls on spatial discrimination tasks, however deficits emerged in purely spatial memory tasks. This pattern of results suggests that perhaps the role of AMPARs in spatial memory is more related to spatial working memory, which does not appear to be affected, and the deficit lies in spatial long-term memory. Given the roles of AMPARs in learning and memory, it is not surprising that disruptions to AMPARs produce alterations in LTP. For example, Selcher, Xu, Hanson, Melenka, and Madison (2012) showed that GluA1 knockout mice displayed reductions in hippocampal LTP in the CA1 region.

Animal models demonstrate that exposure to stress results in alterations in the expression of AMPARs. Caudal et al. (2010) showed that inescapable stress decreases phosphorylation of the AMPA receptor Ser831-GluA1 within both the prefrontal cortex (PFC) and hippocampus,

and decreases phosphorylation in the amygdala (Caudal et al., 2010). Furthermore, stress induces a selective decrease in AMPA receptor mediated synaptic excitation at hippocampal CA1 synapses (Kallarackal et al., 2013). Interestingly, in a recent study by Zanos et al. (2016), the fast-acting antidepressant effects of the ketamine metabolite (2*R,* 6*R*) – HNK were associated with increased phosphorylation of AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors). Along these same lines, Chourbaji et al. (2008) showed that targeted deletion of the GluA1 subunit in mice creates a depression-like phenotype. This pattern of results suggests that ionotropic glutamate receptors are critically involved in the pathogenesis of depression, and inactivation of these receptors results in depression-like behaviour *per se.* The idea of glutamate receptors playing contributive roles in depression will be explored in greater detail in Chapter 5.

NMDA receptors are heterotetramers that contain two GluN1 subunits and two GluN2 or GluN3 subunits, which forms a CA^{++} ion channel. (Vyklicky et al., 2014). NMDA receptor activation requires two molecules of glycine and glutamate (Vyklicky, 2014). The different NMDA subunits are each composed of 4 functional domains: an amino-terminal extracellular domain, a ligand-binding domain, a carboxyl tail domain in the intracellular region, and a transmembrane region (Flores-Soto et al., 2012). Both in vitro and in vivo work show that chronic NMDA receptor activation leads to excitotoxic cell death. Therefore, physiological regulation of NMDA receptor activation is a tightly controlled process.

NMDA receptors are critically involved in the regulation of learning and memory. Constantine-Paton (1994) suggest that the NMDA system is important for the induction of memory formation, but not maintenance. This is best exemplified in studies of learning and memory in humans, where NMDA receptor blockade before learning a task resulted in impaired memory, but NMDA receptor blockade after learning a task had no effect (Hadj Tahar, Blanchet, & Doyon, 2004). Systemic NMDA administration in rats has been shown to have a potentiating effect on cognitive functions, in particular spatial learning (Hlinak & Krejci, 2002), and genetic overexpression achieves the same effect. Along the same lines, mice lacking the NMDA receptor subunit GLUN2A have reduced hippocampal LTP and impaired spatial learning (Sakimura et al., 1995), and mice without NMDA receptors in the hippocampal CA1 present with deficient LTP and profound deficits in both spatial and non-spatial learning (Shimizu, Tang, Rampon, & Tsien, 2000). Unsurprisingly, systemic administration of NMDA receptor antagonists has been shown

to impair performance on tasks reliant on the amygdala and hippocampus, for example acquisition of the Morris water maze (Heale & Harley, 1990). Finally, either controlled or recreational use of the NMDA receptor antagonist ketamine produces cognitive deficits similar to those observed in schizophrenia (Honey et al., 2005). Given that NMDA receptors are involved in learning and memory, it is not surprising that NMDA receptors are also implicated in the pathogenesis of depression.

NMDA receptor systems are increasingly being recognized as a potential therapeutic target in the treatment of depressive symptomology. Olfactory bulbectomy in rodents leads to depression-like behaviour, and this is paralleled by decreased NMDA receptor density in the cerebral cortex and amygdala (Ho et al., 2001). The Flinders Sensitive Line rats, which develop depression spontaneously, exhibit reduced expression of the NMDA receptor GLUN2A subunit in addition to BDNF within the hippocampus (Ryan et al., 2009). Stress is also concomitant with an upregulation of hippocampal GLUN2B expression within the molecular layer (Calabrese et al., 2012; Ciu et al., 2012; Kreutz & Sala, 2012). Importantly, prolonged elevated GLUN2B expression drives excitotoxicity: in vitro work demonstrates that elevated GLUN2B leads to a rapid influx of calcium ions into the cell, trigging an excitotoxic event (Von Engelhardt et al., 2007). This in turn can lead to inflammation or atrophy of the hippocampus, paralleling the reductions in hippocampal volume observed in depressed patients (Bremner et al., 2000). The important distinction here is that while transiently elevated levels of GLUN2B might confer beneficial effects in the nervous system, prolonged elevation of GLUN2B has deleterious consequences on the healthy functioning of the organism. Clinical studies report higher serum levels of glutamate in the blood and cerebrospinal fluid of patients with depression. Interestingly, there is a positive correlation between glutamate levels and the severity of depression, such that increased levels of glutamate correspond with increased depression symptomology (Mitani et al., 2006). Furthermore, the brains of depressed patients reveal increased glutamate levels in the frontal cortex (Hashimoto, Sawa, & Iyo, 2007).

If depression is associated with elevated levels of glutamate, in particular the NMDA receptor, which could correspond to increased excitotoxity, then it would be expected that compounds that reduce glutamate levels would have antidepressant properties. Ketamine, a NMDA receptor antagonist, decreases immobility time in the FST and tail suspension test (Silva, et al., 2010). In humans, a single sub-anesthetic dose of ketamine can result in depression

symptom remission in as little as 2 hours, and the effects persist for as long as 7 days (Diazgranados et al., 2010). AZD6765 and memantine, both members of the NMDA receptor antagonist family, achieve similar antidepressant effects in animal models of depression. However, a problem facing these compounds is that their administration can result in acute effects similar to the symptoms of schizophrenia. Ro25-6891, a compound specific for the GluN2B subunit, significantly decreases immobility time in the FST without producing the unwanted side effects observed with ketamine (Maeng et al., 2008). Therefore, what emerges is that the NMDA receptor and its subunits are consistently elevated in depression, and compounds that act to reduce these levels confer antidepressant effects. The role of NMDA receptors in the CORT model of depression and the impact of reelin on these will be explored in Chapter 4.

1.14. Specific aims and goals

The goal of the research presented in this dissertation was to investigate the antidepressant potential of TNF-α receptor inhibition and reelin on neurobiological and behavioural markers using the CORT model of depression. In order to address these issues, I conducted a series of experiments:

Experiment 1: Repeated CORT administration reliably increases depression-like behaviour on the FST. However, the majority of previous research conducted using the CORT model of depression has either examined changes in depression-like behaviour or on neurobiological markers (e.g., reelin). What has been missing from the literature is an examination of the effects of CORT administration on tests of cognitive functioning. As over two-thirds of depressed patients experience cognitive dysfunction, this is an important gap to be addressed using this model. To examine this issue, I examined the effects of repeated CORT injections (21 days, 40 mg/kg) in male rats on the FST. I then investigated potential alterations in memory performance as a result of CORT exposure, specifically by using the object-recognition, object-location, and object-in-place memory tests. Finally, I also investigated the effects of CORT treatment on the acoustic startle response using prepulse inhibition testing.

Hypothesis: I predicted that repeated CORT injections would increase depression-like behaviour on the FST. I also expected that CORT treatment would impair performance on both the object-location and object-in-place memory tests, but not on the object-recognition test. The rationale for these predictions was that the object-location and object-in-place memory tests are reliant on the hippocampus and PFC, respectively, which show alterations in structural plasticity

in response to stress. However, the perirhinal cortex, which is implicated in performance on the object-recognition memory test, is less involved in depression. Finally, I predicted that repeated CORT injections would produce impairments on prepulse inhibition test, specifically through a reduction in startle response.

Experiment 2: Chronic stress leads to a significant increase in the expression of cytokines in the peripheral and central nervous system. Unsurprisingly, depression is frequently comorbid with autoimmune disorders, for example rheumatoid arthritis (Margaretten et al., 2011). The expression of pro-inflammatory cytokines, which promote inflammation, are increased after chronic stress, whereas the expression of anti-inflammatory cytokines, which act to decrease inflammation, are decreased. In particular, the pro-inflammatory cytokine TNF-α is consistently elevated after chronic stress. Within the nervous system, TNF-α has a variety of functions, including modulating cell recruitment and proliferation, and cell death. Under conditions of stress, TNF-α participates in promoting inflammation through stimulating the production of other pro-inflammatory cytokines, including IL-1 and IL-6. Elevated levels of TNF-α produce a wide range of deleterious effects on physiology, emotional behaviour, and cognition (Bortolato et al., 2015). Inflammation also serves to decrease reelin expression, which is involved in promoting synaptic plasticity and learning and memory. Rats received 21 days of CORT (40 mg/kg) or vehicle injections and half the rats in each group also received semi-weekly injection of the TNF-α inhibitor etanercept (0.8 mg/kg) (Inglis et al., 2005). We then assessed depression-like behaviour using the forced-swim test (FST), cognition using the novel object-location and object- in-place tests, and alterations in hippocampal neurogenesis, reelin, and $GABA_A$ β2/3 receptors using immunohistochemistry.

Hypothesis: I expected that repeated CORT injections would increase depression-like behaviour on the FST, impair both object-location and object-in-place memory, and decrease hippocampal neurogenesis, reelin expression, and $GABA_A$-ir cell numbers. Furthermore, I predicted that etanercept administration would act to reverse the above changes, through an increase in hippocampal reelin expression.

Experiment 3: Repeated CORT injections have been shown to reduce hippocampal reelin expression (Lussier et al., 2013a). Furthermore, post-mortem investigations of depressed brains have shown reduced reelin expression in the CA4 (Fatemi et al., 2000). The antidepressant imipramine has been shown to recover CORT-induced reductions in hippocampal reelin

expression (Fenton et al., 2015). Reelin is an extracellular matrix protein involved in synaptogenesis, promoting contacts onto dendritic spines, neurogenesis, and learning and memory. Therefore, I examined the antidepressant potential of intrahippocampal infusion of exogenous reelin. To address this question, I conducted stereotaxic surgery to implant an indwelling cannula into the dorsal hippocampus of male rats. Rodents then received 21 days of repeated CORT (40 mg/kg) or vehicle injections. A subset of rats received an intrahippocampal reelin infusion (1 μg/μl) on days 7, 14, and 21 of CORT injections, or only on day 21. Rodents then underwent the FST on day 22. A subset of rats were immediately sacrificed after completing the FST, while another subset went on to complete the object-location memory test. We then assessed changes in hippocampal neurogenesis, GluA1 cell counts in the SGZ, GluN2B expression throughout the major subfields of the DG, and changes in $GABA_A$ β2/3 ir cells in the SGZ.

Hypothesis: I predicted that repeated CORT treatment would increase depression-like behaviour on the FST. As reelin expression is decreased by repeated CORT treatment and this change parallels the development of depression-like behaviour, I expected that reelin infusions would significantly reverse depression-like behaviour in the FST. I also predicted that CORT would impair performance on the object-location memory test, and that reelin would rescue this. I expected that CORT would decrease hippocampal neurogenesis, the number of GluA1-ir cells in the SGZ, increase the expression of GluN2B throughout the DG, and decrease the number of $GABA_A$ β2/3-ir cells in the SGZ. As reelin is involved in the regulation of neurogenesis and participates in glutamatergic neurotransmission, I expected that reelin would reverse the above described changes.

Experiment 4: As my previous study (Chapter 4) indicated that reelin has fast-acting antidepressant effects that are accompanied by an increase in the number of GluA1-ir cells in the SGZ, I was interested in examining if the antidepressant effect of reelin requires a contribution of AMPA signaling. To address this question, I conducted stereotaxic surgery to implant an indwelling cannula into the dorsal hippocampus of male rats. Rodents then received 21 days of either repeated CORT (40 mg/kg) or vehicle injections. A subset of rats received a single intrahippocampal reelin infusion (1 μg/μl) on day 21 of injections. Another subset of rats that received reelin also received an intrahippocampal CNQX (AMPA antagonist) infusion (1.25

μg/μl) immediately before the FST. Post-mortem investigation of hippocampal neurogenesis, fos, and microglia morphology was then conducted.

Hypothesis: As intrahippocampal reelin infusions produce a fast-acting antidepressant effect that is accompanied by an increase of GluA1-ir cells in the SGZ, I predicted that infusion of CNQX in reelin-treated animals would abolish reelin's effects on the FST. I further predicted that CNQX would not alter reelin's effects on hippocampal neurogenesis, as not enough time will have elapsed between the reelin and CNQX infusion to produce an effect. In terms of Fos, I predicted that reelin would increase the number of Fos + cells in the SGZ active during the FST, and CNQX would decrease this. Finally, I predicted that reelin would shift the morphology of microglia away from an active phenotype into a more ramified phenotype, with CNQX then reversing this effect.

CHAPTER 2

SELECTIVE DEFICITS IN OBJECT RECOGNITION MEMORY AND PREPULSE INHIBITION PRODUCED BY REPEATED CORTICOSTERONE IN RATS

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Kyle J Brymer was involved in the design of the experiment, conducting all aspects of the experiment, analyzing the data, and writing up the manuscript. Erin Y Fenton was involved in assisting in conducting the experiment. John G Howland was involved in planning the experimental design as well as lending the use of several materials necessary to conduct the experiments listed. Hector J Caruncho and Lisa E Kalynchuk were involved in planning the experimental design and formulating the manuscript. The current chapter was included in the dissertation as the behavioural tests described here (in particular the object-location memory tests) make up key components of the experiments of subsequent chapters in this dissertation.

Abstract

The symptoms of human depression often include cognitive deficits. However, cognitive behaviour is not typically included in the behavioural assessments conducted in preclinical models of depression. For example, it is well known that repeated corticosterone injections in rodents produce depression-like behaviour as measured by the forced swim test, sucrose preference test, and tail suspension tests, but the cognitive impairments produced by corticosterone have not been thoroughly examined. The purpose of this experiment was to assess the effect of repeated corticosterone injections on several versions of object recognition memory and prepulse inhibition, along with the more traditional assessment of depression-like behaviour using the forced swim test. Rats received 21 days of corticosterone (40 mg/kg) or vehicle injections followed by a battery of behavioural tests. Corticosterone decreased body weight and increased immobility in the forced swim test and lowered startle amplitudes and prepulse-facilitation to 30-ms prepulse-pulse intervals. Corticosterone also impaired both object location and object-in-place recognition memory, while sparing performance on object recognition memory. Collectively, our data suggest that CORT produces selective disruptions in prepulse inhibition, object location, and object-in-place recognition memory, and that these cognitive impairments should be taken into account as part of the depressive phenotype produced by chronic stress.

1. Introduction

Depression is a debilitating psychiatric disorder estimated to be the leading cause of disease burden worldwide by 2030 (Willner, Scheel-Kruger, & Belzung, 2013). (Kendler & Gardner, 2010). Exposure to chronic stress is a significant risk factor in the development of depression (Kendler & Gardner, 2010). To better understand the neurobiological bases of depression, preclinical animal models have been developed that expose rodents to extended periods of chronic stress or glucocorticoid exposure followed by an assessment of the resulting changes in behaviour and neurobiology. These models provide the opportunity to relate specific aspects of a depressive phenotype to distinct neurobiological changes, which informs our understanding of the neurobiological consequences of stress and also our insight about the fundamental biological causes of depressive disorders.

Experiments using animal models of depression have demonstrated robust changes in the brain cytoarchitecture in response to stress that mirror some aspects of human depression. For example, both human patients and rodents from preclinical models of depression display reduced hippocampal volume, dendritic atrophy in the hippocampus and prefrontal cortex, and decreased hippocampal neurogenesis manifested as reduced cell proliferation and survival (Jacobs, Praag, & Gage, 2000; Lussuer et al., 2013a; Sahay & Hen, 2007; Synder, Soumier, Brewer, Pickel, & Cameron, 2011). Paralleling the stress-induced alterations in brain structure and function are deficits in cognition. In the clinical literature, depressed patients often show impaired working memory and attention, executive dysfunction, and dampened processing speed and recall of new information(Kizilbash, Vanderploeg, & Curtiss, 2002; Papazacharias & Nardini, 2012). This has not been fully examined in preclinical models, but there is evidence that deficits in the Morris water maze (Warner et al., 2013) and context-object discrimination (Czerniawski, Miyashita, Lewandowski, & Guzowski, 2015) are produced in response to chronic, but not acute, stress. Many of the tasks described above rely on the integrity of the hippocampus, which unsurprisingly is particularly sensitive to the deleterious effects of chronic stress in both humans and rodents (Conrad, 2008).

Work in our lab utilizes the repeated corticosterone (CORT) model of depression to investigate the effects of stress on the hippocampus. The repeated CORT paradigm is a well-validated model of depression involving 21 days of repeated subcutaneous CORT injections (40 mg/kg), which reliably increase depression-like behaviour on the forced swim test (FST) and

sucrose preference test (Fenton et al., 2015; Gregus, Wintink, Davis, & Kalynchuk, 2005; Marks, Fournier, & Kalynchuk, 2009; Sterner & Kalynchuk, 2010), decrease markers of hippocampal plasticity (Lussier et al., 2013a; Fenton et al., 2015), and disrupt the balance between GABA and glutamate within the hippocampus (Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2013b). However, little is known about the effects of CORT injections on cognitive functioning. The sparse work on this topic has focused on low doses of CORT (5 mg/kg CORT; Darcet et al., 2014], CORT given after adrenalectomy [Spanswick & Sutherland, 2010], CORT given acutely [Vargas-Lopez et al., 2015], or after mineralocorticoid overexpression [Ferguson & Sapolsky, 2007], or CORT given via an intrahippocampal infusion [Kelemen, Bahrendt, Born, & Inostroza, 2014]. Therefore, to our knowledge, the effects of repeated (21 days) systemic CORT injections on object memory has not been investigated at a dose (40 mg/kg) that induces depression-like behaviour. Similarly, the effects on prepulse inhibition using the above CORT model have not been investigated. In order to characterize cognitive deficits in the CORT model of depression, we included two well-established behavioural paradigms - prepulse inhibition (PPI) of the acoustic startle response (Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001; Koch, 1999) and three types of object memory testing (Howland, Cazakoff, & Zhang, 2012). Prepulse inhibition is the reduction in acoustic startle response observed when a brief, low intensity stimulus is presented 30-500 ms prior to the presentation of the startle-evoking stimulus (Geyer, Krebs-Thomson, Braff, & Swerdlow, 2001; Koch, 1999). Object-memory tasks involve the rodent encountering a set of objects in a training phase, and then a subsequent testing phase with the objects manipulated in some way (e.g., new location, new object introduced). These tasks were chosen due to their known neurobiological substrates. For example, object location is reliant on the hippocampus (Vogel-Ciemia & Wood, 2014) whereas object-in-place depends on the prefrontal cortex (Barker & Warburton, 2015). In contrast, object recognition memory depends on the perirhinal cortex (Barker & Warburton, 2015). Although deficits in object memory and PPI are well documented in animal models of schizophrenia (Bitanihirwe, Weber, Feldon, & Meyer, 2010b; Howland, Cazakoff, & Zhang, 2012; Ibi et al., 2009; Meyer, Spoerri, Yee, Schwarz, & Feldon, 2008c; Ozawa et al., 2006; Shi, Fatemi, Sidwell, & Patterson, 2003) to our knowledge, this is the first study to systematically investigate the effects of repeated (21 days) CORT injections on rodent performance in object memory and PPI. Given that CORT is known to dampen hippocampal plasticity, we hypothesized that CORT would produce deficits in the

hippocampal version of object memory. We also hypothesized that CORT would produce impairments in the PPI due to the requirement of the hippocampus in this task.

2. Materials and Methods

2.1. Animals

We used 25 male Long-Evans rats purchased from a commercial supplier (Charles River Laboratories, Canada). Rats weighed 225-250g upon arrival and were individually housed in polypropylene cages with the colony room maintained at an ambient temperature of 21 ± 1°C. Both food and water were available ad libitum. All experimental procedures took place during the light phase of the 12:12 light/dark cycle (lights on at 0700). All animals were treated in accordance with an animal use protocol approved by the University of Saskatchewan's Animal Research Ethics Board. Efforts were made to minimize the number of animals used in the study.

2.2. Experimental Procedures

Rats were handled briefly once per day for 7 days prior to the CORT or vehicle injections. Following this acclimatization period, rats were weight-matched into two groups that received either 21 days of CORT injections (CORT group: $n = 13$) or 21 days of vehicle injections (vehicle group: $n = 12$). All injections were administered subcutaneously once per day (between 9:00-2:00 pm). CORT (Steraloids) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 and given at a dose of 40 mg/kg in a volume of 1 ml/kg. Previous work in our laboratory and others has demonstrated that the dose of 40 mg/kg of CORT reliably increases depression-like behaviour in rats (Lussier et al., 2013a; Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004; Johnson, Fournier, & Kalynchuk, 2008; Lebedeva, Caruncho, & Kalynchuk, 2017; Workman et al., 2016; Kott, Mooney-Leber, Shoubah, & Brummelte. 2016; Li et al., 2017).

2.3. Behavioural Testing

All behavioural testing was conducted in a procedures room that was not used for housing the rats or for delivering the CORT/vehicle injections. Behaviour was video-recorded for the FST and object memory tests for offline scoring at a later date. The FST and PPI test were conducted on the 22nd and 23rd day of the experiment, respectively. After the PPI test, the rats received 3 days of CORT or vehicle top up injections, and then three versions of object recognition memory were assessed with 3-day injection intervals in between each test. This was

done to ensure that the effects of CORT on behaviour did not dissipate over time. The experimental design is shown in Figure 1.

2.3.1. FST

The FST was conducted the day after the 21st injection. The FST was modified from the Porsolt Test [3]. Rats were individually placed into a Plexiglas swim tank (25 cm wide x 25 cm long x 60 cm high, 27 ± °C water, 30 cm deep) for 10 minutes. We measured the duration of time each rat spent immobile, struggling, and swimming. An increase in immobility is typically inferred to indicate the presence of a depressive phenotype in rodents (Cryan, Valentino, & Lucki, 2005; Lussier et al., 2013a; Sterner and Kalynchuk, 2010).

2.3.2. PPI

PPI was assessed two days after the final CORT or vehicle injection. We used a protocol that was very similar to previously published methods (Howland, Cazakoff, & Zhang, 2012]). Rats were tested in two standard SR-LAB startle boxes (San Diego Instruments, CA, USA). The startle boxes were washed with 40% EtOH between each set of animals. The PPI session began with a 5-minute acclimatization period, during which a 70-dB background noise was presented, which remained constant for the remainder of the testing. Immediately following the acclimatization period, six pulse alone trials were presented (120 dB, 40ms), which allowed for a baseline startle amplitude to be established before the presentation of the prepulse + pulse trials. Following the pulse-alone trails, 84 trials of three different types were presented in pseudo-random order: pulse-alone (6 trials: 120 dB, 40 ms), prepulse + pulse (6 trials X 3 prepulse intensities X 4 prepulse intervals- discussed below), or no stimulus (6 trials). Prepulse + pulse trials began with the presentation of a 20-ms prepulse of 3, 6, or 12 dB above background noise. Prepulse-pulse time intervals were 30, 50, 80, or 140 milliseconds between the onset of the prepulse and onset of the pulse. Each session concluded with six pulse-alone trials. The intertrial interval randomly varied from 3 to 14 seconds (average 7.5 seconds).

Two measures were calculated for each rat. The first measure, startle amplitude, represents the mean startle response amplitude for each of the six pulse-alone trials, which were presented after the six habituation trials. The second measure, PPI, was calculated by averaging the startle amplitudes for each trial type, and the percent PPI for each prepulse intensity was calculated using the formula: (100-(100xstartle amplitude on prepulse + pulse trials)/ (startle amplitude on pulse alone trials). The average startle amplitude elicited during three blocks of six

pulse alone trails (before, during, and after PPI trails) was compared to measure habituation [Howland, Cazakoff, & Zhang, 2012; Howland, Hannesson, Barnes, & Phillips, 2007; Howland, Hannesson, & Phillips, 2004; Howland, MacKenzie, Yim, Taepavarapruk, & Phillips, 2004b).

2.3.3. Recognition Memory

The recognition memory test procedures were similar to previously published protocols (Howland, Cazakoff, & Zhang, 2012). Testing was conducted in a square open field (60x60x60cm) constructed of white corrugated plastic. One overhead light provided illumination. The object-in-place trials had a black cardboard insert on the northern wall of the arena. Rats received 3 habituation sessions before the first recognition test (object location). The last habitation session occurred 24-48 hours before the first test session. For the remaining tests (object-in-place and object recognition), rats received one habituation session (in pairs) 24-48 hours before the new test. Objects used in all tests were made of porcelain, plastic, or glass and did not exceed 10 cm in height or length. Duplicate copies of objects were used when the same objects were required for both sample and testing phases. Object locations were counterbalanced to eliminate potential side preferences in all tests. In both sample and test phases, objects were placed in the corners of the arena 10 cm from each of the nearest walls and rats were placed in the center of the arena facing the wall opposite the objects. Both the open field and the objects were wiped with 40% EtOH after each session (Howland, Cazakoff, & Zhang, 2012).

2.3.3.1. Object Location Recognition

Three days following PPI testing, rats explored two identical objects for 4 minutes during the sample phase (C1 and C2). The test phase occurred one day later, during which rats explored 2 copies of the sample objects (C3 and C4) for 4 minutes, but with one object moved to a corner location at the front of the box while the other remained in its original position (Fig. 4). Object location memory was inferred when rats spent more time exploring the object in the new location than the one in the original location.

2.3.3.2. Object-in-Place Recognition

Three days following the object location testing, rats were tested in the object-in-place paradigm. During the sample phase, rats explored 4 different objects (D1, E1, F1, G1) located in the four corners of the arena for 5 minutes. Following a 1-hour delay, rats were placed back in

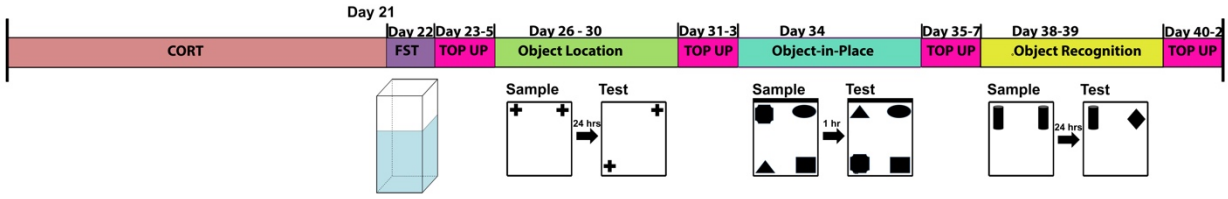

Fig 1. Schematic representation of experimental design used for study. All animals received identical amounts of handling, injections, and behavioural testing. Forced swim test: Rats were placed in a plexiglass swim tank for 10 minutes. Object memory. During object memory testing all animals were placed in a testing chamber made of white corrugated plastic. Object location: Rats explored 2 identical objects during a sample phase followed 24 h later by a testing phase with 2 identical objects but with 1 moved to a new location. Object-in-place: Rats explored 4 different objects in the corners of the box during a sample phase with a black insert on the front wall. 1 h later rats explored 4 of the same objects but with 2 objects in new positions in a test phase. Object recognition: Rats explored 2 duplicate objects during a sample phase. 24 h later rats explored 1 duplicate object and 1 new object during a testing phase.

the arena for the test phase. In the test phase, rats explored 4 identical copies of the objects (D2, E2, F2, G2), however the exploration time was reduced to 3 minutes and the position of 2 of the objects on the same side was switched (i.e., front object became back object and vice-versa), whereas the 2 objects on the other side remained in their original positions. Object-in-place memory was inferred when rats spent more time exploring the pair of objects that switched positions than the objects that remained in their original positions.

2.3.3.3. Novel Object Recognition

Three days following the object-in-place testing, rats were tested for novel object recognition. During the sample phase, rats explored 2 identical objects (A1 and A2) for 4 minutes. The next day, recognition memory for the previously encountered objects was tested during a 4 minute test phase in which subjects explored a copy of the sample object (A3) and a novel object (B1). Object recognition memory was inferred when rats spent more time exploring the novel object.

2.4. Data Analyses

All analyses were conducted using the Statistical Package for Social Sciences (SPSS), version 21 (IBM). Separate one-way ANOVA's were used to test for group differences in body weight and FST behaviour. Corrections for sphericity (Mauchly's Test) were utilized in all repeated measures of variance (ANOVA). Statistical tests were considered significant at $p < .05$. Error bars on all graphs represent the standard error of the mean (SEM).

Prepulse facilitation was observed for the 30-ms interval, whereas prepulse inhibition was observed for the 50, 80, and 140-ms intervals as observed previously (Howland, Cazakoff, & Zhang, 2012). Therefore, data from the 30 millisecond interval were analyzed separately. Startle amplitudes were analyzed with a one-way between-within repeated measures ANOVA (treatment as between-subjects factor; pulse block as repeated measures factor). PPI data were analyzed with a three-way between-within repeated measures ANOVA (treatment as between subjects factor; prepulse intensity and prepulse-pulse interval as repeated measures factor).

Data from the recognition memory tests were scored by an individual blind to treatment according to previously published measures (Howland & Cazakoff, 2010; Howland, Cazakoff, & Zhang, 2012). Briefly, a rat was defined to be actively exploring an object when its nose was directed within 2 cm of an object and either its head or vibrissae were moving, but not when it was standing on top of an object and not directing attention to it. Rats must explore objects for at

least 15 seconds in both the sample and test phases to have reliable recognition memory; therefore, rats with less than 15 seconds of exploration time in either the sample or test phase were eliminated from the study (Bitanihirwe, Weber, Feldon, & Meyer, 2010b). The discrimination ratio (DR) was calculated for the first 2 minutes of the test phase for each rat, as previous reports have reliably demonstrated that the recognition memory tests are most sensitive in the first 1-2 minutes of the test (Bitanihirwe, Weber, Feldon, & Meyer, 2010b; Howland, Cazakoff, & Zhang, 2012). Both total exploration times and the discrimination ratio [(DR)time exploring novel object/location- time exploring familiar object/location/time exploring both objects] were computed for each rat. Any rat with a DR greater than 2 standard deviations above the group mean were removed from the study. Total exploration times during the training and testing phase were analyzed with separate one-way ANOVA's with treatment as a between subject's factor. For test-phase data, the DR was analyzed for each group using separate one-sample *t*-tests (a comparison value of 0 indicates equal exploration of each object (Bitanihirwe, Weber, Feldon, & Meyer, 2010b; Howland & Cazakoff, 2010). Between-group comparisons were made using a one-way ANOVAs.

3. Results

3.1. CORT Decreases body weight

Figure 2 shows the effect of CORT on body weight. Consistent with previous research, CORT had significant effects on body weight. A main effect of both time (F (6, 138) = 165.739, $p < .001$) and treatment was observed (F (1, 23) = 37.81, $p < .001$), with the CORT rats weighing significantly less than the vehicle rats beginning on day 7 and continuing throughout the experiment.

3.2. CORT Increases Immobility in the FST

Figure 2 shows the effect of CORT on FST behaviour. CORT produced clear changes in this test. Specifically, the CORT rats spent significantly more time immobile (F (1, 23) = 67.95, $p < .001$), less time struggling (F (1, 23) = 50.09, $p < .001$), and less time swimming (F (1, 23) = 13.22, $p < .001$) than did the vehicle rats.

3.3. CORT Alters Startle Amplitudes and the 30-ms Prepulse Interval

Figure 3 shows the results of the PPI testing. Panel A focuses on startle amplitudes during the different blocks of pulses. The vehicle rats displayed higher startle amplitudes relative to the CORT rats at all times. This was confirmed by the statistical analyses. A repeated measures

ANOVA revealed a significant main effect of pulse block (F (2, 46) = 34.73, p < .001) and treatment (F (1, 23) = 5.21, p < .05), with no significant pulse block by treatment interaction (F (2, 46) = 1.44, p = .24; Fig. 3).

Next, we then examined the average percent PPI by prepulse-pulse interval (50, 80, and 140 ms) and by prepulse intensity (3, 6, and 12 dB). Both vehicle and CORT-treated rats displayed PPI that depended on intensity (F (2, 46) = 111.65, p < .001) but not interval (F (2, 46) = 1.74, p = .19) or treatment (F (1, 23) = .80, p = .37). The treatment by prepulse interaction was not statistically significant (F (2, 46) = 1.81, p = .17). Data from the 30 millisecon interval revealed a significant main effect of prepulse interval (F (2, 46) = 9.56, p < .001) and treatment (F (1, 23) = 5.55, p < .05), with no significant prepulse interval by treatment interaction (F (2, 46) = 1.48, p = 23; Fig. 3).

3.4. CORT Impairs Object Location Recognition, Object-in-Place Recognition, but not Novel Object Recognition

Figure 4 shows the discrimination ratios for rats in both groups on all three memory tests. Panel A shows the object location testing, panel B shows the object-in-place testing, and panel C shows the object recognition testing.

Analysis of the discrimination ratios during the object location test phase revealed a significant difference between the CORT and vehicle rats. Although the vehicle rats displayed memory that was significantly different from zero (t(11) = 2.57, p = .026), the CORT rats failed to show memory significantly different from zero (t(12) = -1.95, p = .075). Analysis with a one-way ANOVA revealed a significant effect of treatment (F (1, 23) = 10.64, p < .01), with vehicle rats spending significantly more time exploring the object in the novel location than the CORT rats (Fig. 4).

Analysis of the discrimination ratios during the object-in-place recognition memory task revealed a significant difference between the vehicle and CORT rats. Specifically, although the vehicle rats displayed memory significantly different from zero (t(11) = 5.70, p < .001), the CORT rats failed to show memory significantly different from zero (t(12) = -1.49, p = .16). Analysis with a one-way ANOVA revealed a significant effect of treatment, F (1, 23) = 25,42, p<.001, with the vehicle rats spending significantly more time exploring the objects that switched places than the CORT rats (Fig. 4).

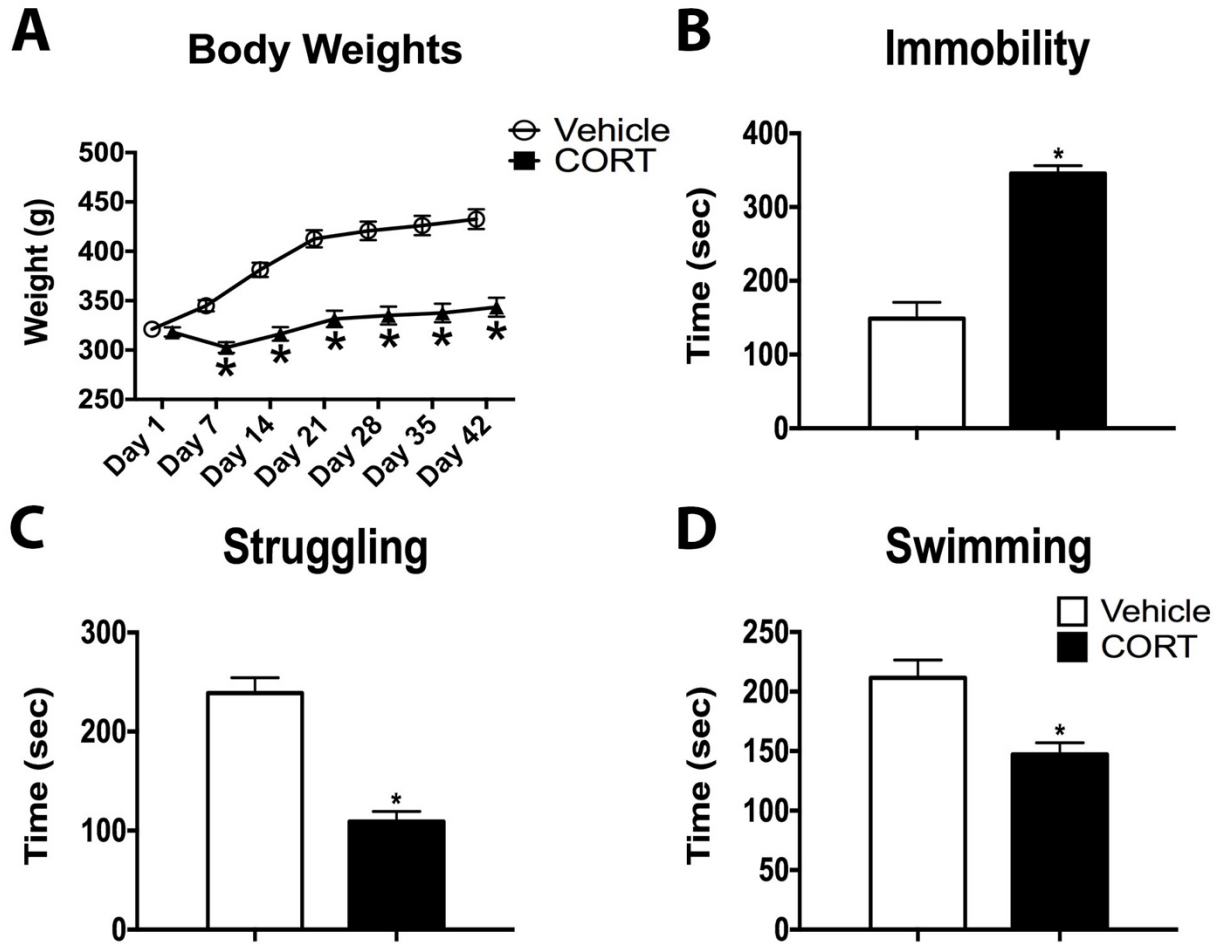

Fig 2. CORT had significant effects on both body weight and depression-like behaviour. Panel A shows the body weight data. CORT-treated rats weighed significantly less than vehicle-treated rats on days 7, 14, 21, 28, 35, and 42 (all p values < .05). Panels B, C, D, and E show the FST behaviour. CORT-treated rats had significantly higher immobility times than vehicle-treated rats (p < .05). CORT-treated rats had significantly lower swimming times than vehicle-treated rats (p < .05). CORT-treated rats had a significantly shorter latency to immobility than vehicle-treated rats (p < .05). CORT-treated rats had significantly lower struggling times than vehicle-treated rats (p < .05). All data are represented as means ± standard error of the mean.

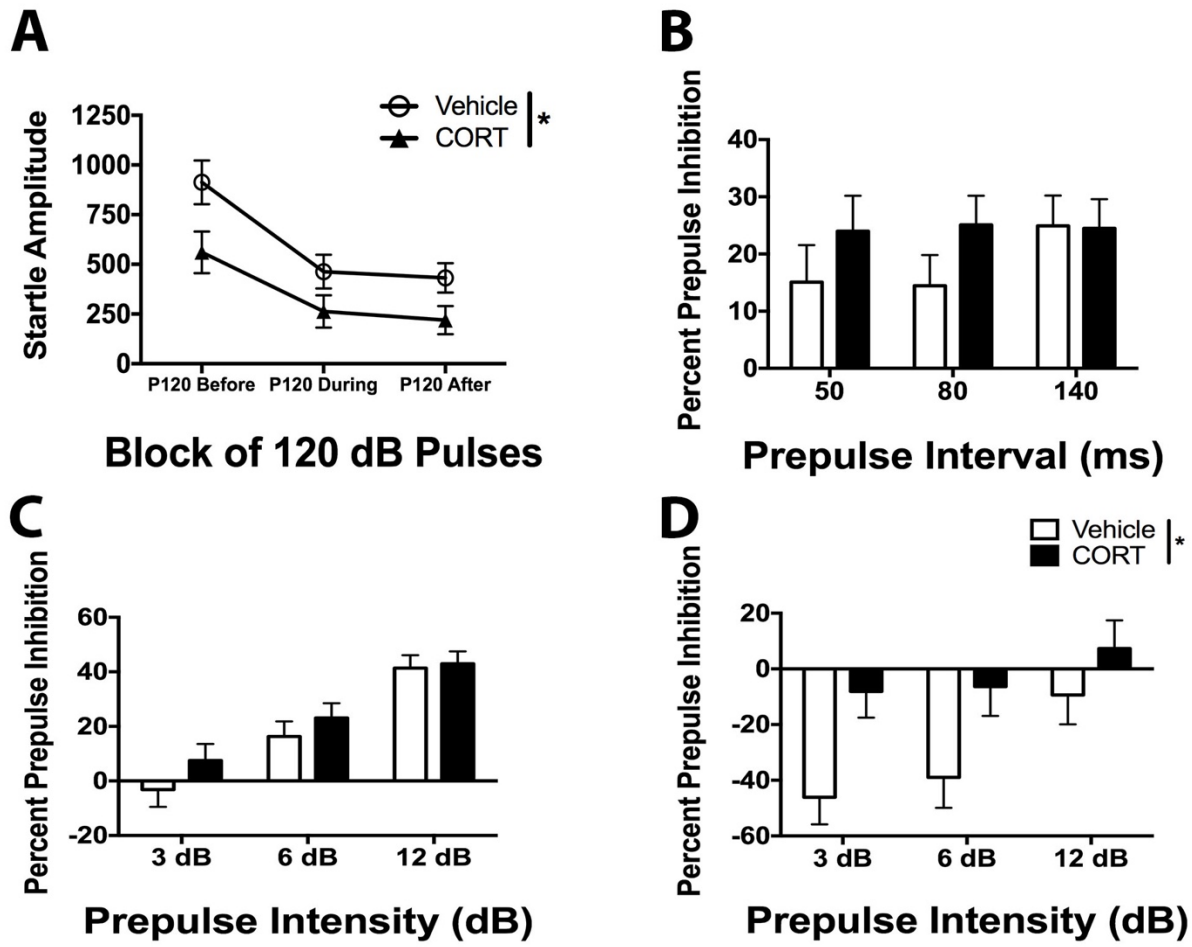

Fig 3. Effects of CORT-treatment on startle amplitudes and prepulse inhibition (PPI) in vehicle and CORT-treated rats. Prepulse-pulse intervals of 50, 80, and 140 ms are shown. Panel A represents the startle amplitudes (arbitrary units) of rats for the pulse-alone trials before, during, and after the PPI trials. Panel B shows percent PPI for each prepulse-pulse interval (50, 80, and 140 ms) for the vehicle and CORT-treated rats. Panel C shows percent PPI for each prepulse intensity (3, 6, and 12 dB above background) averaged for trials with 50, 80, and 140 ms prepulse-pulse intervals. Panel D shows percent PPI for trials with a 30-ms prepulse-pulse interval. Note that a negative PPI score represents an increase in startle responding on trials with a prepulse (3, 6, or 12 dB above background). There was an overall effect of treatment on the 30-ms prepulse-pulse interval. All data are represented as means ± standard error of the mean.

Treatment	Object Location		Object-in-Place		Novel Object Recognition	
	Sample Phase	Test Phase	Sample Phase	Test Phase	Sample Phase	Test Phase
Vehicle	42.52±3.11	33.31±2.19	88.48±4.82	44.02±3.91	54.05±23.34	38.43±13.38
CORT	41.19±2.99	28.61±1.72	79.89±3.74	39.65±2.80	46.59±16.80	30.59±11.45

Table 1. Object recognition, object location, and object-in-place exploration times for vehicle and CORT-treated rats. Total exploration time of objects (seconds ± standard error of the mean) during the sample and test phases of the object recognition, object location, and object-in-place paradigms. The total time for the sample phase is presented, whereas the times for the first 2 minutes is presented for the test phases

Analysis of the discrimination ratios during the object recognition test revealed that both vehicle ($t(11) = 6.30$, $p < .001$) and CORT rats ($t(12) = 6.29$, $p < .001$) displayed memory that was significantly different from zero. Analysis with a one-way ANOVA revealed no effect of treatment (F (1, 23) = .01, p = .92; Fig. 4).

4. Discussion

The results of this experiment make three main points. First, they reveal that CORT has selective effects on object recognition memory. The CORT rats showed impairments on both object location and object-in-place recognition memory, but they had normal performance with object recognition memory. Second, the results showed that prepulse inhibition is selectively effected by CORT. Finally, they confirm that repeated injections of CORT reliably increase depression-like behaviour as measured by the FST (Johnson et al. 2006). This is consistent with previous findings from a number of different labs showing that CORT produces clearly stratified behaviours on the FST.

Work in our lab has demonstrated that the CORT model reliably increases depression-like behaviour on the FST. For example, we have shown that repeated CORT treatment increases time spent immobile and decreases time spent struggling and swimming (Fenton et al., 2015; Lussiet et al., 2013a; Lussier, Romay-Tallon, Kalynchuk, & Caruncho, 2011). Moreover, we have shown that the effects of CORT treatment on depression-like behaviour depend on both the dosage and time. For example, while 7 days of CORT treatment begins to alter FST behaviour, the largest effects are seen with 14 and 21 days of CORT treatment (Lussier et al., 2013a), and in heterozygous reeler mice, 20 mg/kg, but not 5 or 10 mg/kg of CORT, significantly increases depression-like behaviour in the FST (Lussier, Romay-Tallon, Kalynchuk, & Caruncho, 2011). Importantly, these CORT-induced alterations in FST behaviour are not the result of non-specific changes in muscle tone (Marks, Fournier, & Kalynchuk, 2009). Recently, we have also shown that repeating cycles of CORT treatment produces graded effects on FST behaviour (Lebedeva, Caruncho, & Kalynchuk, 2017). Importantly, work in other labs has also shown that CORT treatment produces depression-like behaviour in the FST (Kott, Mooney-Leber, Shoubah, & Brummelte, 2016; Zhao et al., 2008). Therefore, while not a main focus of the current paper, the results from the FST add to the current body of literature and help further establish the validity of the CORT model.

The present study provides the first evidence of selective deficits in object-memory in CORT-treated rats. Specifically, although both vehicle and CORT rats displayed novel object recognition memory, the CORT rats failed to show intact object location recognition and object-in-place recognition. This is evidenced by a negative discrimination ratio during the two tests in the CORT rats. A negative discrimination ratio indicates that a greater time is being spent exploring the familiar objects rather than the novel objects. This points to an inability of CORT rats to recognize new objects in their environment and reference this back to previously encountered information. However, this deficit is specific to location, as novel object recognition memory is still functional in the CORT rats. The argument could be made that the CORT rats were sick, explaining their poor performance on object location recognition and object-in-place recognition. However, as mentioned previously, both the vehicle and CORT rats displayed equal levels of intact novel object recognition memory. Additionally, no significant differences between groups were observed in exploration times during the training and testing phases of the different tasks. Therefore, the CORT rats are still capable of learning, and do not display an inability to explore objects. This points to a fundamental neurobiological underpinning to the results observed.

The neurobiological bases of the object memory tests are well-established. As mentioned previously, the object location memory test typically relies on an intact hippocampus, whereas the object-in-place memory test relies on the prefrontal cortex. In contrast, the novel object recognition memory test relies on the integrity of the perirhinal cortex (Vogel-Ciernia & Wood, 2014). Furthermore, a study by Albasser and colleagues (2011) showed that perirhinal cortex lesions did impair performance on the novel object recognition memory test. However, this deficit was only present with no training trial before the testing phase and with less than a 90-minute delay between the two trials. The protocol used here for the novel object recognition memory test employed both a training phase before the testing and a 24-hour delay between the trials. Winters, Forwood, Cowell, Saksida, and Bussey (2004) reported a double dissociation between the hippocampus and perirhinal cortex in tests of object memory. Specifically, they found that animals with a perirhinal cortex lesion were impaired on the novel object recognition memory task but were spared on the object location memory task, whereas the reverse was found for animals with hippocampal lesions. This suggests that performance on the novel object recognition memory task will be impaired if the perirhinal cortex is damaged due to

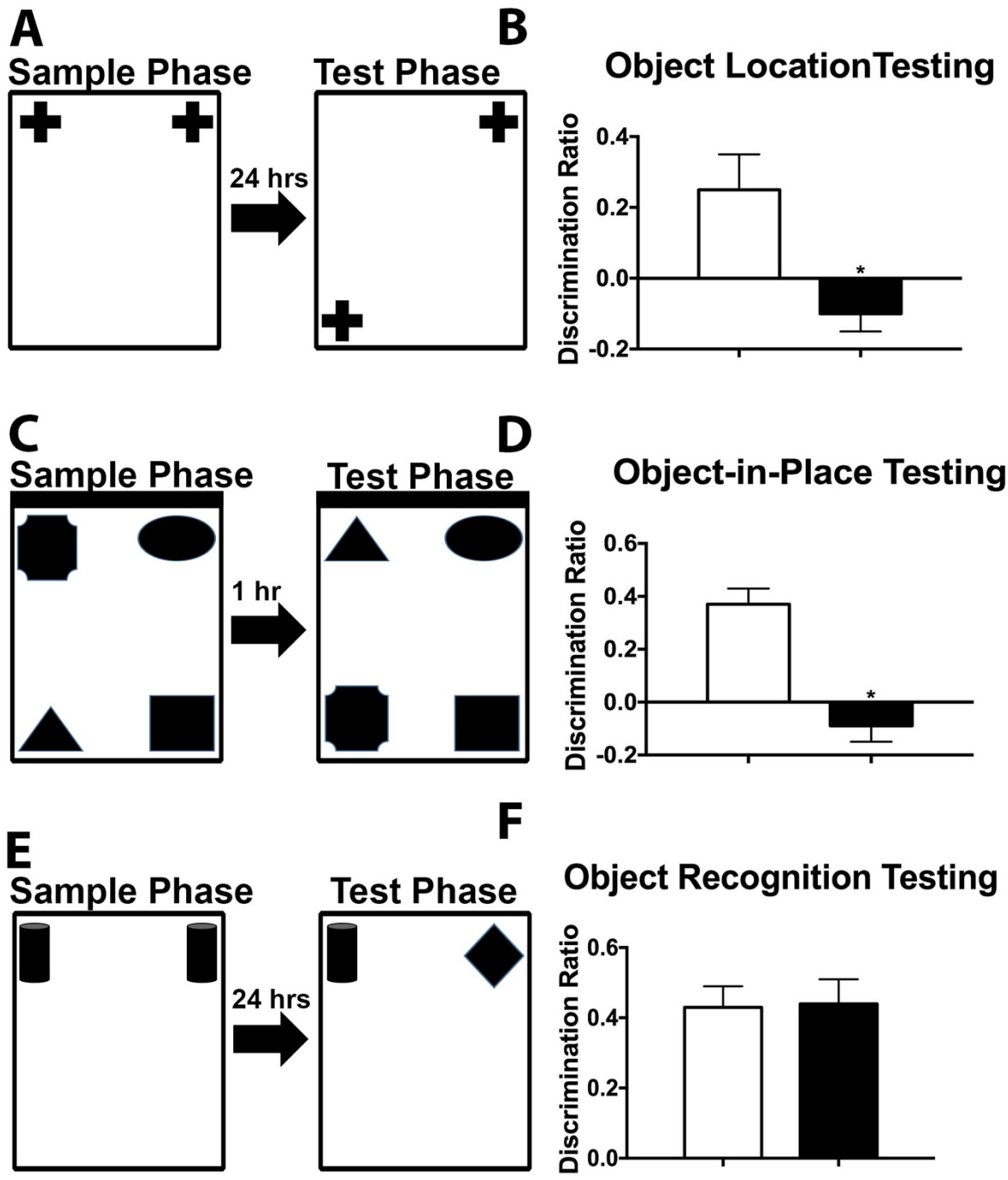

Fig 4. Effects of CORT treatment on the discrimination ratio in the object recognition, object location, and object-in-place recognition memory paradigms. Panel A- CORT-treatment had no effect on novel-object recognition memory. Panel B- CORT-treatment significantly impaired object location recognition memory ($p < .05$). Panel C – CORT-treatment significantly impaired

object-in-place recognition memory ($p < .05$). All data are represented as means ± standard error of the mean.

insult. As the CORT rats in this experiment were not impaired on the object recognition memory task, it appears that the CORT treatment did not damage the perirhinal cortex. However, it cannot be ruled out that the spared performance observed here did not result from our use of a training phase and a 24-hour delay between the training and testing (as was not done in the Albasser and Winters) phases.

The startle amplitudes recorded in this experiment revealed a normal pattern of response, where high startle is observed during the start of PPI, followed by a gradual decline. Additionally, the vehicle rats displayed significantly higher overall startle amplitudes (combined PPI before, during, and after) than did the CORT rats, suggesting that CORT treatment may dampen overall startle amplitudes. However, an alternative explanation for the observed effect is that CORT treatment significantly decreased body weight – the CORT rats weighed significantly less than did the vehicle rats. Therefore, the CORT rats may have startled less simply because they weigh less than the vehicle rats. We observed prepulse facilitation for vehicle, but not CORT rats, for intensities of 3 dB, whereas prepulse inhibition was observed for 6 and 12 dB trials when only considering prepulse intervals of 50, 80, and 140 ms for both groups. However, prepulse intensities of 30 ms resulted in prepulse facilitation in the vehicle rats for 3, 6, and 12 dB intensites, whereas the CORT rats displayed prepulse facilitation for 3 and 6 dB, but not 12 dB (Floresco, Geyer, Gold, & Grace, 2005; Mansbach & Geyer, 1991; Young, Powell, Risbrough, Marston, & Geyer, 2009). In conclusion, CORT treatment altered prepulse inhibition in male rats, however additional work is needed with this model to further validate the effects observed here.

5. Conclusion

In conclusion, the results of this experiment reveal that repeated CORT injections induce a depressive phenotype, with deficits extending to selective impairments in object location recognition, object-in-place recognition, but not novel object recognition. This pattern of results suggests that the normal functioning of the hippocampus and prefrontal cortex is impaired by CORT treatment, however the perrihinal cortex is spared. Although the CORT rats had overall lower startle amplitudes and less pre-pulse facilitation for 30-ms prepulse-pulse intervals than did the vehicle rats, further research is required to validate this finding. The current study adds to the existing literature on depression and extends the CORT model to include memory deficits.

Future studies could examine the ability of antidepressants to reverse the object memory deficits observed here.

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CHAPTER 3

PERIPHERAL ETANERCEPT ADMINSTRATION NORMALIZES BEHAVIOUR, HIPPOCAMPAL NEUROGENESIS, AND HIPPOCAMPAL REELIN AND $GABA_A$ RECEPTOR EXPRESSION IN A PRECLINICAL MODEL OF DEPRESSION

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Kyle J Brymer was involved in the design of the experiment, conducting all aspects of the experiments, analyzing the data, and writing up the manuscript. Erin Y Fenton was involved in assisting in conducting the experiment. Hector J Caruncho and Lisa E Kalynchuk were involved in planning the experimental design and formulating the manuscript. The current chapter was included in the dissertation as the key finding that reelin expression is increased following etanercept treatment subsequently formed the basis of the experimental questions guiding chapters 4 and 5 of the current dissertation.

Abstract

Depression is a serious psychiatric disorder frequently comorbid with autoimmune disorders. Previous work in our lab has demonstrated that repeated corticosterone (CORT) injections in rats reliably increase depression-like behaviour, impair hippocampal- dependent memory, reduce the number and complexity of adult-generated neurons in the dentate gyrus, decrease hippocampal reelin expression, and alter markers of GABAergic function. We hypothesized that peripheral injections of the TNF-α inhibitor etanercept could exert antidepressant effects through a restoration of many of these neurobiological changes. To test this hypothesis, we examined the effect of repeated CORT injections and concurrent injections of etanercept on measures of object-location and object-in-place memory, forced-swim test behaviour, hippocampal neurogenesis, and reelin and GABA β2/3 immunohistochemistry. CORT increased immobility behaviour in the forced swim test and impaired both object-location and object-in-place memory, and these effects were reversed by etanercept. CORT also decreased both the number and complexity of adult-generated neurons, but etanercept restored these measures back to control levels. Finally, CORT decreased the number of reelin and GABA β2/3- ir cells within the subgranular zone of the dentate gyrus, and etanercept restored these to control levels. These novel results demonstrate that peripheral etanercept has antidepressant effects that are accompanied by a restoration of cognitive function, hippocampal neurogenesis, and GABAergic plasticity, and suggest that a normalization of reelin expression in the dentate gyrus could be a key component underlying these novel antidepressant effects.

1. Introduction

There is a strong connection between the immune system and the brain in the context of major depression. Chronic stress induces an inflammatory response, which in the central nervous system includes an increase in pro-inflammatory and a decrease in anti-inflammatory cytokines, in addition to the recruitment of microglia (Campbell, Marriott, Nahmias, & MacQueen, 2004; Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Exposure to chronic stress is a significant risk factor for the development of depression. One of the earliest links between stress and depression was the observation that stressful life events frequently precede the onset of depression in human populations (reviewed in Sterner and Kalynchuk, 2010). Half of depressed patients display the hallmarks of a deregulated stress response, hypercortisolemia (Sachar and Baron, 1979). Moreover, these high circulating levels of cortisol have pathological consequences for healthy brain functioning (Kendler and Gardner, 2010). Studies of post-mortem tissue from patients with depression have revealed dendritic atrophy in the hippocampus and prefrontal cortex, in addition to reductions in hippocampal volume (Bansar, Dwyer, & Duman, 2011; Campbell, Marriott, Nahmias, & MacQueen, 2004). Research using preclinical animal models of depression further corroborate the link between stress and depression. For example, stress significantly increases depression-like and anhedonia behaviour (Sterner, 2010). Stress also produces similar neurobiological alterations as those seen in humans, along waith decreases in hippocampal cell proliferation, survival, and the rate of neuronal maturation (Lussier et al., 2013a). Clinically, patients with autoimmune disorders frequently present with comorbid depression (Andersson et al., 2015) – for example, rheumatoid arthritis patients have a lifetime prevalence rate of 13–42% of developing depression (Margaretten, Julian, Katz, & Yelin, 2011) – and there seems to be a clear bidirectional relationship between depression and autoimmune disorders (Euesden, Danese, Lewis, & Maughan, 2017). Moreover, subsets of depressed patients exhibit the cardinal features of an inflammatory response, most notably an increased expression of pro-inflammatory cytokines in blood and cerebrospinal fluid (Miller, Maletic, & Raison, 2009). Some of the most frequently reported elevated pro-inflammatory cytokines include IL-1, IL-6, and TNF-α (Dowlati et al.,2010), and general alterations in neuroimmune systems represent a key component underlying the neurobiology of both mood and psychotic disorders (Calcia et al., 2016; Rodrigues-Amorim et al., 2017; Wohleb, Franklin, Iwata, & Duman, 2016).

TNF-α is a soluble cytokine with two receptors, the TNF-α receptor 1 and TNF-α

receptor 2. Produced peripherally, TNF-α also acts centrally through activation of toll-like receptor 4 on circumventricular organs and choroid plexus, which in turn activates microglia that then release TNF-α within the brain, with the hippocampus being a key region of expression (McCoy and Tansey, 2008). Within the nervous system, TNF-α has a variety of functions, including modulating cell recruitment and proliferation, and cell death. Under conditions of stress, TNF-α participates in promoting inflammation through stimulating the production of other pro-inflammatory cytokines, including IL-1 and IL-6. Elevated levels of TNF-α produce a wide range of deleterious effects on physiology, emotional behaviour, and cognition (Bortolato, Carvalho, Soczynska, Perini, & McIntyre, 2015). TNF-α also increases excitatory synaptic strength by promoting AMPA receptor expression, while concomitantly decreasing inhibitory synaptic strength by inducing internalization of $GABA_A$ receptors (Stellwagen, Beattie, Seo, & Malenka, 2005). Work in preclinical animal models demonstrates that peripheral administration of TNF-α produces deficits in learning and memory (particularly spatial memory) in addition to anhedonia (Dunn & Swiergiel, 2005; van Heesch, Prins, & Korte-Bouws, 2013). Furthermore, TNF-α knockout mice display an antidepressant-like phenotype (Yamanda, Iida, & Miyamoto, 2000). In an elegant study, Klaus et al. (2016) injected TNF-α peripherally or achieved expression in the hippocampus using viral vectors. Peripheral TNF-α produced anhedonia behaviour and increased fear memory, while brain expression increased anxious behaviour. Increased expression of TNF-α results in a decrease in neurogenesis through apoptotic neuronal death, whereas low levels of TNF-α promote neurogenesis through increased proliferation of neural precursor cells (Fan, Tien, & Zhang, 2011). Finally, peripheral administration of the TNF-α inhibitor etanercept decreases depression-like behaviour in rats subjected to stress paradigms (Bayramgurler, Karson, Ozer, & Utkan, 2013; Krugel, Fischer, Radicke, Sack, & Himmerich, 2013; Sahin et al., 2015). In fact, a recent systematic review and meta-analysis validated the effect of anti –TNF-α drugs in improving depression symptoms, and associated the antidepressant effect with baseline symptom severity (Kappelmann et al., 2016).

In spite of the clinical studies showing antidepressant actions of etanercept, little is known about the mechanisms by which anti –TNF-α drugs exert their antidepressant effects. Thereby, considering that administration of peripheral TNF-α produces anhedonia behaviour and deficits in spatial memory, we were interested in examining if TNF-α inhibition could alleviate stress- induced deficits in depression-like behaviour and measures of cognition.

Work in our lab has demonstrated that prolonged exposure to stress hormones decreases reelin expressing cells in the dentate subgranular zone, and treatment with the tricyclic antidepressant imipramine reverses this effect (Lussier, Caruncho, & Kalynchuk, 2009; Fenton et al., 2015). Reelin is a large extracellular matrix protein primarily expressed by cortical and hippocampal subpopulations of GABAergic interneurons and by cerebellar granule cells (Pesold et al., 1998; Rogers and Weeber, 2008). Within the hippocampus, reelin is involved in a variety of functions, including dendritic spine development and maturation (Niu, Yabut, & D'Arcangelo, 2008; Chameau et al., 2009; Hethorn et al., 2015), hippocampal neurogenesis (Teixeira et al., 2011), promoting and modulating maturation and synaptic plasticity of newborn granule cells (Bosch et al., 2016a), and learning and memory (Beffert et al., 2005). Importantly, there is also evidence of decreased reelin expression in the dentate gyrus in postmortem samples from patients with depression (Fatemi, Earle, & McMenomy, 2000). Interestingly, in a mouse model of aging, a viral-induced infection increased inflammation and reduced the number of reelin expressing interneurons (Doehner and Knuesel, 2010). This suggests that the pattern of behaviours (e.g., depression-like behaviour, impaired cognition) observed under times of increased inflammatory signaling in chronic stress might be related to a loss of hippocampal reelin expression (Caruncho et al., 2016). Therefore, we hypothesized that reducing chronic stress- induced increases in TNF-α would increase reelin expression and normalize behaviour and neurobiological markers. To test this hypothesis, we utilized a repeated corticosterone (CORT) injection model of depression that we and others have shown can produce a robust depressive phenotype (Sterner and Kalynchuk, 2010). Rats received 21 days of CORT or vehicle injections and half the rats in each group also received semi-weekly injection of the TNF-α inhibitor etanercept (Inglis et al., 2005). We then assessed depression-like behaviour using the forced-swim test (FST), cognition using the novel object-location and object- in-place tests, and alterations in hippocampal neurogenesis, reelin, and $GABA_A$ receptors using immunohistochemistry. Our results support our hypothesis and suggest that peripheral administration of a TNF-α antagonist increases hippocampal reelin expression and corresponding neurobiological markers, and normalizes the behavioural phenotype.

2. Materials and Methods

2.1. Animals

We used 24 male adult Long-Evans rats purchased from Charles River (QC, Canada).

The rats were reared in standard conditions by the breeder and weighed 200–250 g at the time of arrival in our lab. Rats were 9 weeks old at the start of the experiment. It is generally accepted that at 9 weeks old rats are in the young adult to adulthood stage (Sengupta, 2013). Rats were individually housed in rectangular polypropylene cages containing standard laboratory bedding with access to food and water *ad libitum.* The rodent colony room was maintained at an ambient temperature of 20 ± 1°C on a 12:12 light-dark cycle (lights on at 7 am). All experimental procedures were in accordance with the guidelines of the Canadian Council and Animal Care and an animal care protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. All efforts were made to minimize the number of rats used in the present study.

2.2. Experimental Procedures

Rat were handled briefly once per day for 7 days prior to the start of experimental manipulations. We weight-matched the rats and randomly assigned them to one of the following four treatment groups: CORT + saline (n = 6), CORT + etanercept (n = 6), vehicle + saline (n = 6), or vehicle + etanercept (n = 6) injections. All CORT and vehicle injections were administered subcutaneously once per day (between 9:00 and 10:00 am) for 21 consecutive days. Etanercept and saline injections were given subcutaneously twice per week (between 2:00 and 3:00 pm) during the 21-day CORT/vehicle injection period. Rats also received 3 days of CORT or vehicle injections and 1 day of etanercept or saline injections in between each behavioural test (forced-swim test, object-location, object-in-place) to maintain the effects of CORT throughout the behavioural testing period. The experimental design is shown in **Figure 1**.

CORT (Steraloids, Newport, RI, United States) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 (Sigma–Aldrich) and given at a dose of 40 mg/kg at a volume of 1 ml/kg. This dose produces reliable and robust increases in depression-like behaviour (Gregus, Wintink, Davis, & Kalynchuk, 2005; Johnson, Fournier, & Kalynchuk, 2006). Etanercept (Immunex, Thousand Oaks, CA, United States) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 and given at a dose of 0.8 mg/kg in a volume of 1 ml/kg. This dose was chosen based on previous studies showing that etanercept can normalize depression-like behaviour in rats (Bayramgurler et al., 2013). Body weight was recorded for each rat during the injection phase of the experiment.

2.3. Behavioural Testing

Behavioural testing was conducted in a procedures room that was not used for any other

aspect of the study. All behaviours were recorded with a digital video camera and stored for offline analyses by researchers blind to the treatment conditions. The FST assessed depression-like behaviour, whereas the novel object location and object-in-place tests assessed recognition memory.

2.3.1. Forced Swim Test

The FST was conducted the day after the final CORT injection. We used a modified version of the Porsolt test, as previously described (Marks et al., 2009). Each rat was individually placed in a Plexiglas swim tank (25 cm diameter × 60 cm high, 27 ± 2°C water, 30 cm deep) for 10 minutes. We measured the duration of time each rat spent immobile, struggling, and swimming.

2.3.2. Object Recognition Memory

Recognition memory was assessed according to previously published protocols (Howland and Cazakoff, 2010; Howland et al., 2012). Object-location (OBL) memory testing took place in a square open-field area (60 × 60 × 60 cm) constructed of white corrugated plastic. Rats received three habituation sessions before the training session. During the first two habituation sessions, rats were brought in pairs and placed in separate arenas for 10 minutes. During the last habituation session, rats were brought in individually and placed in the arena for 10 minutes. Rats were immediately returned to the colony room after each habituation session. The last habitation session occurred 24–48 hours before the first testing session. The objects used in this test were made of porcelain and did not exceed 10 cm in height or length. Duplicate copies of objects were used when the same objects were required for both training and testing phases. Object locations were counterbalanced to eliminate potential side preferences in all tests. In both sample and test phases, objects were placed in the corners of the arena 10 cm from each of the nearest walls and rats were placed in the center of the arena facing the wall opposite the objects. Both the arena and the objects were wiped with 40% EtOH after each session.

The rats were allowed to explore two identical objects for 4 minutes during the sample phase (C1 and C2). Twenty-four hours later, the rats underwent a testing phase, during which they explored two copies of the sample objects (C3 and C4) for 4 minutes, but with one object moved to a corner location at the front of the box while the other maintained its original position. The amount of time each rat spent actively exploring the objects was recorded. A rat received credit for active exploration when its nose was directed within 2 cm of an object and either its

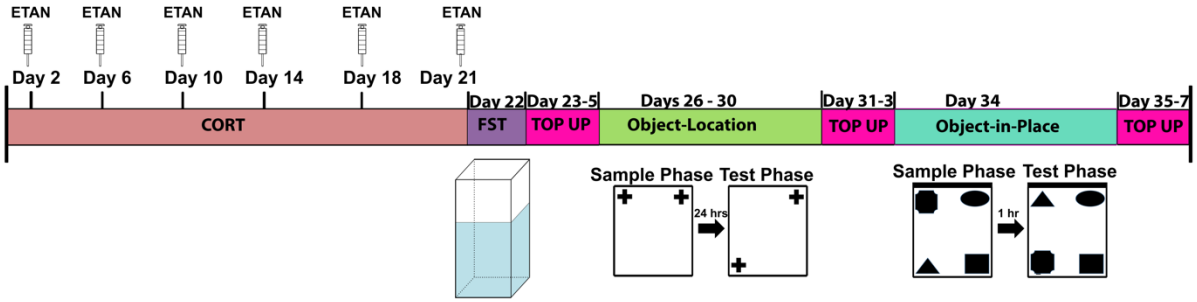


Fig 1. Schematic representation of the experimental design used for the study. All rats received the same amount of handling, injections, and behavioural testing. The injection top up periods in between each behavioural test were implemented to ensure that the effects of CORT did not wane over time.

head or vibrissae were moving, but not when it was standing on top of an object and looking away. Rats were required to explore objects for at least 15 seconds in both the sample and test phases to have reliable recognition memory. A discrimination ratio (DR) was calculated as the time the rat spent exploring the novel orientation subtracted by the time the rat spent exploring the familiar orientation during the first two minutes of the test phase and dividing by two. A positive discrimination ratio indicates intact OBL memory (Howland and Cazakoff, 2010; Howland, Cazakoff, & Zhang, 2012). Three days following object location testing, rats were tested in the object-in-place (OBIP) paradigm. During the sample phase, rats explored 4 different objects (D1, E1, F1, G1) located in the four corners of the arena for 5 minutes. Following a 1-hour delay, rats were placed back in the arena for the test phase. During the test phase, rats again explored 4 identical copies of the objects (D2, E2, F2, G2) for 3 minutes, but in this case the position of 2 of the objects on the same side was switched (i.e., front object becomes the back object and vice-versa). Object-in-place memory is inferred when animals spend more time exploring the pair of objects that switched positions than the objects that remained in their original positions (Howland, 2012). Here a discrimination ratio was again calculated to determine the degree to which rats explored the novel objects. A positive discrimination ratio indicates intact OBIP memory.

2.4. Histology

Rats were sacrificed 3 days after the OBIP memory task. Each rat was deeply anesthetized with 5% isoflurane and then transcardially perfused using physiological saline, followed by ice-cold 4% (w/v) formaldehyde fixative (pH = 7.4). The brains were extracted from the cranial vault and immersed in the same formaldehyde fixative for 48 hours at 4°C. The brains were then sectioned in the coronal plane at 30 μm on a vibrating microtome (VT1200s, Leica Biosystems). Sections were collected and stored at −20°C in a cryoprotectant solution containing 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

2.4.1. Immunohistochemistry

We used a standard immunohistochemistry technique with commercially available antibodies for all of our experiments. Immunostaining was done on free-floating sections in six-well tissue culture plates under gentle agitation. To ensure consistent immunohistochemical processing, we processed all sections for each assay (i.e., DCX, reelin, GABAA

β2/3receptorsubunit) in unison with treatment groups counterbalanced across all tissue plates. To confirm the specificity of our antibodies, we omitted the primary antibody from an additional well of free-floating sections. In the absence of the primary antibody, we were unable to detect any immunoreactive cells. We used doublecortin (DCX) to label immature dentate granule cells, as previously described (Botterill et al., 2015). Brain sections underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85°C for 30 minutes. The sections were then blocked in 5% (v/v) normal goat serum (NGS), 1% (w/v) bovine serum albumin (BSA), and 0.5% (v/v) Triton X-100 in 0.1 M TBS (pH = 7.4), followed by incubation in a rabbit anti-DCX polyclonal primary antibody (1:1000, Cell Signaling Technology) diluted in blocking solution for 24 hours at room temperature. The next day, the sections were treated with 5% (v/v) H_2O_2 in 0.1 M TBS for 30 minutes to block endogenous peroxidase activity. The sections were then incubated for 1 hour in biotinylated goat anti-rabbit secondary antibody (1:500, Vector Laboratories) diluted in 5% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. Sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 0.025% (w/v) DAB, 4.167% $NiSO_4$, and 0.002% (v/v) H_2O_2.

Reelin immunohistochemistry was conducted as previously described (Lussier et al., 2013a). Sections were treated with 0.3% H2O2 in 0.1 M PBS for 30 minutes to block endogenous peroxidase activity. The sections were then blocked in 5% normal horse serum (NHS), 1% (w/v) BSA, and 0.3% (v/v) Triton X-100 in 0.1 M PBS (pH = 7.4), followed by incubation in a mouse anti-reelin primary antibody (1:2000, Vector Labs) diluted in blocking solution for 48 hours at room temperature. Following primary antibody, the sections were then incubated for 2 hours in biotinylated horse anti-mouse secondary antibody (1:500, Vector Labs) diluted in 5% (v/v) NHS, 1% (w/v) BSA, and 0.3% (v/v) Triton X-100 in 0.1 M PBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 h. Sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 0.033% (w/v) DAB and 0.007% (v/v) H_2O_2.

GABAA β2/3 receptor subunit immunohistochemistry was conducted as follows. Sections first underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85°C for 30 minutes. Next, the sections were placed into a blocking solution containing 5% (v/v) NHS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 30 min, and were then

exposed to a monoclonal mouse anti-$GABA_A$ β2/3 receptor primary antibody (clone bd17, 1:1000, Millipore/Sigma) diluted in blocking solution for 48 hours at 4°C. Following this, the sections were treated with 5% (v/v) H_2O_2 in 0.1 M TBS for 30 minutes to block endogenous peroxidase activity. Next, the sections were incubated for 2 hours in biotinylated horse anti-mouse secondary antibody (1:500, Vector Laboratories) diluted in 5% (v/v) NHS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. The sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 4.167% $NiSO_4$ and 0.05% (w/v) glucose oxidase DAB. At the end of each immunohistochemical run, all sections were mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using a series of graded alcohols, cleared in xylene, and cover slipped with Permount mounting medium (Fisher Scientific).

2.4.3. Cell Counting

Immunohistochemical results were quantified as previously described (Lussier et al., 2013a; Botterill et al., 2015). All analyses were conducted by researchers blind to the treatment conditions. Immunostained sections were examined using a Nikon Eclipse E800 microscope with a motorized stage and digital camera (MicroFire, Optronics) connected to a dedicated stereology computer. The dentate SGZ (defined as a two-cell width zone in between the inner granule cell layer and the hilus) and granule cell layer (GCL) were traced at 4 X magnification using a computerized stereology program for DCX-ir cells. However, only the SGZ was traced for reelin and $GABA_A$ β2/3-ir cells (StereoInvestigator, MicroBrightfield, Williston, VT, United States). DCX, reelin, and $GABA_A$ β2/3-ir cells were counted in both the ipsilateral and contralateral hemispheres at 40 x magnification. All counts utilized unbiased stereology using a modified optical fractionator method that excludes cells in focus at the uppermost focal plane to reduce oversampling (Lussier et al., 2013a). The total number of cells was estimated using the following formula: $N_{total} = Q^- \times 1/ssf \times A(x, y\ step) / a(frame) \times t/h$. Q^- represents the number of counted cells, ssf is the section sampling fraction (1 in 12), A(x, y step) is the area associated with each x, y movement (10,000 μm^2), a(frame) is the area of the counting frame (3600 μm^2), t is the weighted average section thickness, and h is the height of the dissector (12 μm) (Lussier et al., 2013a; Botterill et al., 2015). To avoid counting sectioning artifacts, we used a guard zone of 2 μm.

2.4.2.1. Characterization of Immature DCX-ir Neurons

We utilized a dendritic categorization method (Lussier et al., 2013a; Botterill, Brymer, Caruncho, & Kalynchuk, 2015) to determine whether CORT or etanercept altered the dendritic morphology of immature DCX- ir neurons. A meander scan method was used to randomly select 100 DCX-ir cells from each rat. A researcher blind to experimental conditions then assigned the cell to one of six categories of dendritic complexity based on both the presence and extent of apical dendrites (see Figures 5C,D). The proliferative stage encompassed categories one (no process) or two (one small process). Category three (medium process reaching the granule cell layer) and category four (process reaching the molecular layer) comprised the intermediate stage of development. Finally, category five (one major process extending into the molecular layer) and category six (defined dendritic tree with delicate dendritic branching in the granule cell layer) represented mature cell development. Data are presented as the percentage of DCX-ir cells in each of the six categories.

2.5. Statistical Analyses

All statistical analyses were done using IBM's Statistical Package for Social Sciences v24. We used separate one- way ANOVA's to assess the statistical significance of group differences in each measure (i.e., body weight, FST behaviour, OBL, OBIP, DCX, reelin, $GABA_A$ β2/3), and significant main effects were followed by *post hoc* comparisons using Tukey's HSD. The criterion for statistical significance was $p < 0.05$. All graphs depict the mean ± standard error of the mean.

3. Results

3.1 CORT had Significant Effects on body weight

Figure 2 shows fluctuations in body weight in each group during the CORT injections. CORT had significant effects on body weight, consistent with previous findings from our lab (Lussier et al., 2013a,b). We found significant group differences on days 14 [$F(3,23) = 7.414$, $p < 0.01$] and 21 [$F(3,23) = 7.295$, $p < 0.01$]. *Post hoc* analyses revealed that the CORT-treated

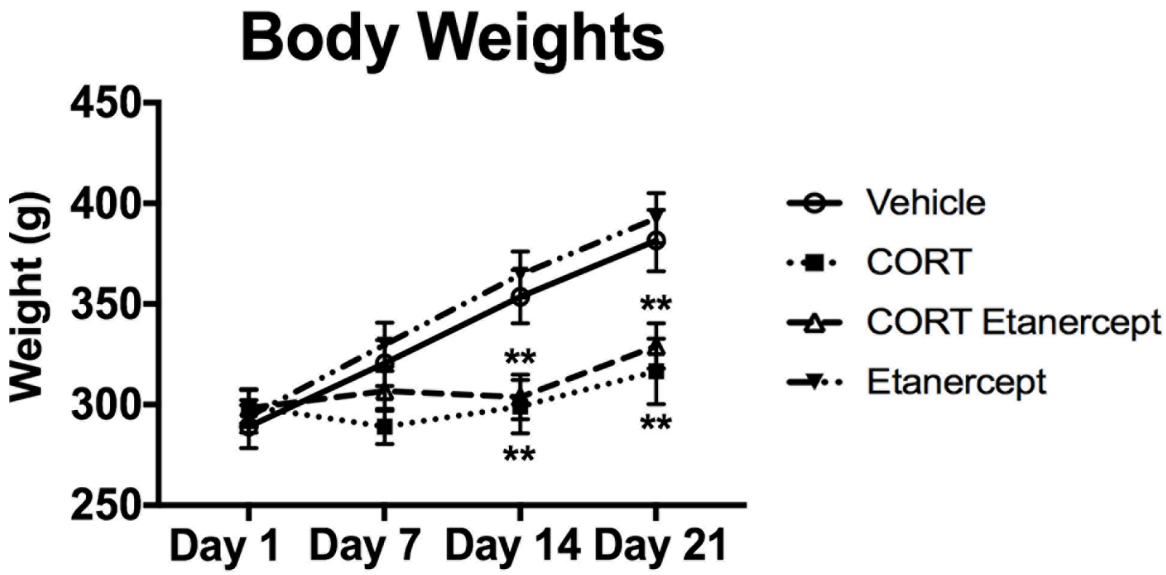


Fig 2. Effect of CORT and etanercept on body weight. The CORT rats weighed significantly less than the vehicle and etanercept rats on days 14 ($p < 0.05$) and days 21 ($p < 0.05$). In addition, the CORT + etanercept rats weighed significantly less than the vehicle or etanercept rats on days 14 ($p < 0.05$) and 21 ($p < 0.05$). All data are represented as means ± standard error of the mean.

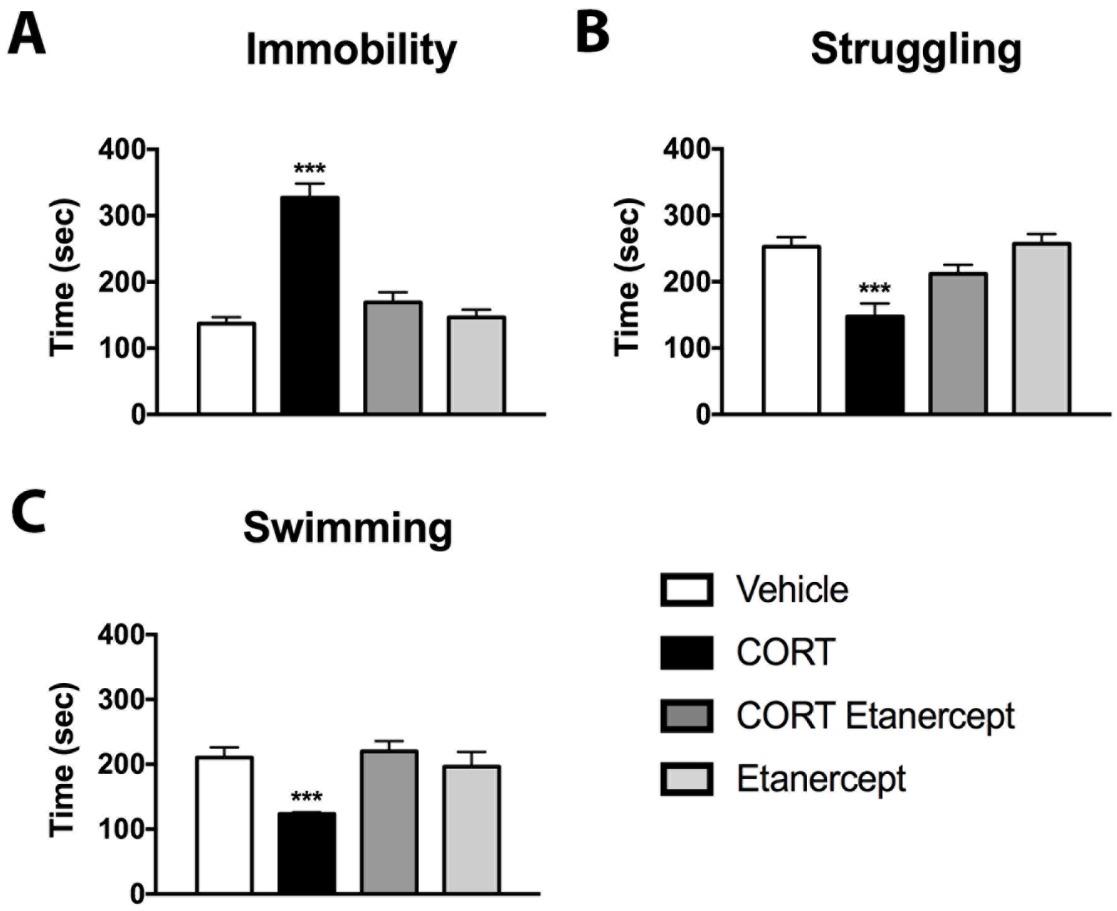


Fig 3. Effect of CORT and etanercept on FST behaviour. **(A)** Shows the effect of treatment on time spent immobile. The CORT rats spent significantly more time immobile than did the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). **(B)** Shows the effect of treatment on time spent struggling. The CORT rats spent significantly less time struggling compared to the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). **(C)** Shows the effect of treatment on time spent swimming. The CORT rats spent significantly less time swimming than did the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). All data are represented as means ± standard error of the mean.

rats weighed significantly less than the vehicle and etanercept rats on day 14 (p-values < 0.05) and day 21 (p-values < 0.05). We also found that the CORT + etanercept rats weighed significantly less than the vehicle and etanercept rats on day 14 (p-values < 0.05) and significantly less than the etanercept rats on day 21 ($p = 0.021$).

3.2. Etanercept Normalizes CORT-Induced Changes in Behaviour on the FST

Figure 3 shows the results of the FST in each group. We found significant group differences in immobility [$F(3,23) = 34.96$, $p < 0.001$], struggling [$F(3,23) = 10.39$, $p < 0.001$], and swimming [$F(3,23) = 7.57$, $p < 0.001$] during the FST. *Post hoc* analyses indicated that the CORT rats spent significantly more time immobile, less time struggling, and less time swimming than the rats in all other groups (p values < 0.05). No other group differences were significant.

3.3. Etanercept Normalizes CORT-Induced Deficits in OBL and OPIP Memory

Figure 4 shows the results of the OBL and OBIP testing in each of the groups. We found significant group differences in both the OBL [$F(3,23) = 5.858$, $p < 0.01$] and OBIP [$F(3,23) = 5.088$, $p < 0.01$] memory tests. *Post hoc* analyses of these main effects revealed that the CORT rats had a lower discrimination ratio than all other groups in the OBL test (p-values < 0.05) and OBIP test (p-values < 0.05). No other group differences were significant.

3.4. Etanercept Restores CORT-Induced Reductions in Hippocampal Neurogenesis

Figure 5 shows the results of our DCX analyses. Figure 5A provides photomicrographs of DCX immunoreactivity in each of the four groups and Figures 5B,D show quantified differences among the groups. Consistent with previous results from our lab (Lussier et al., 2013a; Fenton et al., 2015), we found a significant main effect of group on the total number of DCX- ir cells [$F(3,23) = 20.180$, $p < 0.001$]. *Post hoc* analyses of this main effect revealed that the CORT rats had significantly fewer DCX-ir neurons compared to the vehicle, CORT-etanercept, and etanercept rats (p-values < 0.001). No other group differences were significant. In relation to dendritic complexity, we also found a number of significant group differences. There were significant group differences in the % of neurons in category 1 [$F(3,23) = 7.760$, $p < 0.01$], category 2 [$F(3,23) = 49.504$, $p < 0.001$], category 4 [$F(3,23) = 4.673$, $p < 0.05$], category 5 [$F(3,23) = 16.329$, $p < 0.001$], and category 6 [$F(3,23) = 21.852$, $p < 0.001$]. *Post hoc* analyses showed that the CORT rats had a greater percentage of category 1 cells than did the vehicle ($p = 0.002$) and etanercept rats ($p = 0.003$). The CORT rats also had a greater % of category 2 cells

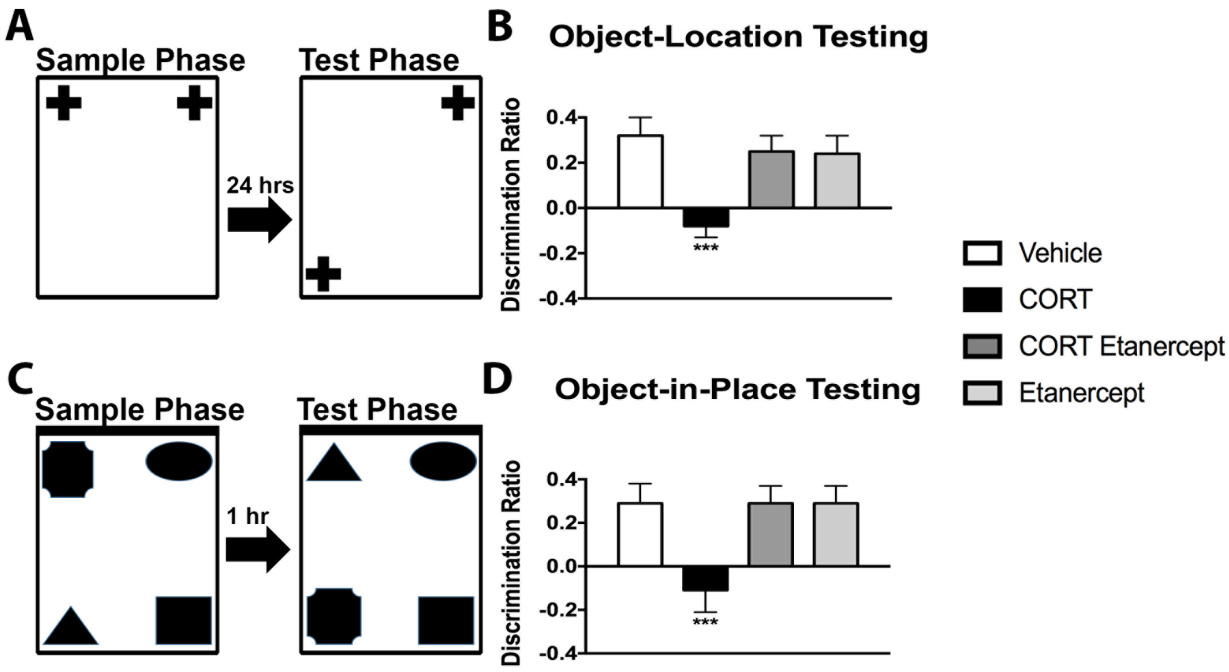


Fig 4. Effect of CORT and etanercept on cognition. **(A)** Shows a schematic of the object location memory test. **(B)** Shows the discrimination ratio in this test for the rats in each group. The CORT rats had a significantly lower discrimination ratio than did the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). **(C)** Shows a schematic of the object-in-place memory test. **(D)** Shows the discrimination ratio in this test displayed by the rats in each group. The CORT rats had a significantly lower discrimination ratio than did the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). All data are represented as means ± standard error of the mean.

than all other groups (p-values < 0.001), while CORT etanercept animals had a greater % of category 2 cells than vehicle rats ($p = 0.042$). CORT rats had a smaller % of category 4 cells than the vehicle rats ($p = 0.009$), and a smaller % of category 5 and category 6 cells than all other groups (p-values < 0.001). In summary, the CORT rats had more DCX-ir cells with less complex dendrites but the CORT + etanercept rats did not differ from vehicle rats in this respect.

3.5 Etanercept Restores CORT-Induced Reductions in Hippocampal Reelin-ir cells

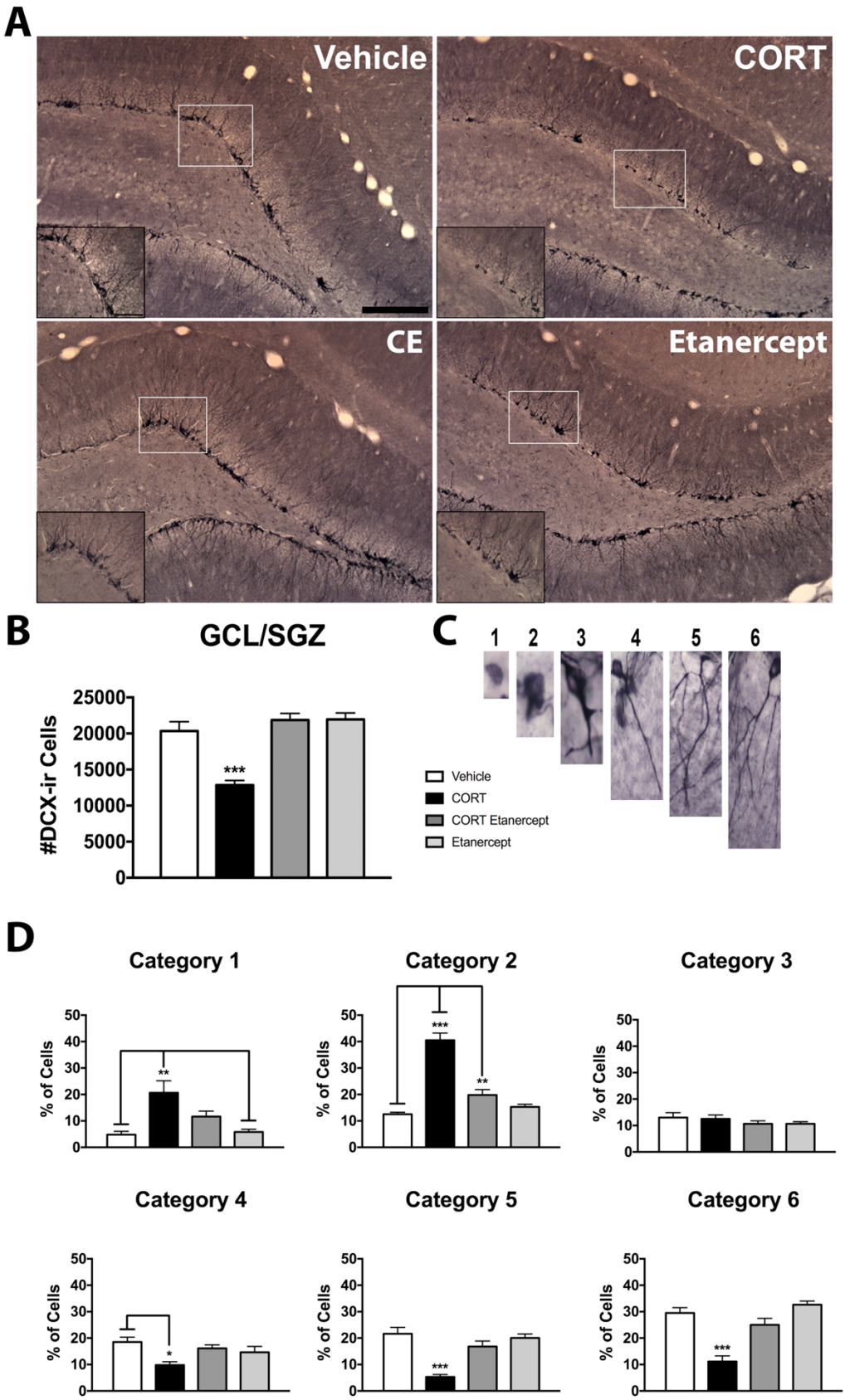
Figure 6 shows the results of the reelin immunohistochemistry. Figure 6A shows the distribution of reelin-ir neurons in the SGZ across the groups and Figure 6B shows the actual reelin- ir cell counts. Our stereological analyses revealed a significant effect of group on the number of reelin-ir cells in the SGZ [$F(3,23) = 14.256$, $p < 0.001$]. *Post hoc* analyses further revealed that the CORT rats had significantly fewer reelin-ir cells than the rats in all other groups (p-values < 0.001). No other group differences were significant.

3.6 Etanercept Restores CORT-Induced Reductions in $GABA_A$ β2/3-ir cells in the SGZ

Figure 7 shows the results of the $GABA_A$ β2/3 immunohistochemistry. Figure 7A provides photomicrographs of $GABA_A$ β2/3 immunoreactivity in each group and Figure 7B provides the results of our stereological cell counts. We found a significant main effect of group on the number of $GABA_A$ β2/3ir cells in the SGZ [$F(3,23) = 4.530$, $p < 0.05$]. *Post hoc* analyses revealed that the CORT rats had significantly fewer $GABA_A$ β2/3-ir cells than the vehicle rats ($p = 0.022$) and CORT + etanercept rats ($p = 0.022$), but not the etanercept only rats ($p = 0.129$). CORT induces internalization of $GABA_A$ receptors in the SGZ (see arrows in Figure 7A) andthis effect was also partially rescued by etanercept.

4. Discussion

The results of this experiment show that prolonged exposure to the stress hormone CORT produces a cluster of effects characterized by increased immobility in the FST, impaired spatial memory on the OBL and OBIP tests, reduced hippocampal neurogenesis, fewer SGZ reelin-ir cells, and dampened $GABA_A$ β2/3 expression levels. Importantly, semi- weekly etanercept given along with CORT prevented all these effects, suggesting that anti-TNF-α drugs could exert their antidepressant effects by facilitating key aspects of hippocampal plasticity. The current experimental paradigm employed the use of individual housing along with CORT injections in adult rats, a protocol that we and others have extensively used in the past to analyze depression-like behaviour (see Sterner and Kalynchuk, 2010, for a review; Gregus, Wintink,


A
Vehicle
CORT
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GCL/SGZ
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25000
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CORT
CORT Etanercept
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D
Category 1
Category 2
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% of Cells
50
40
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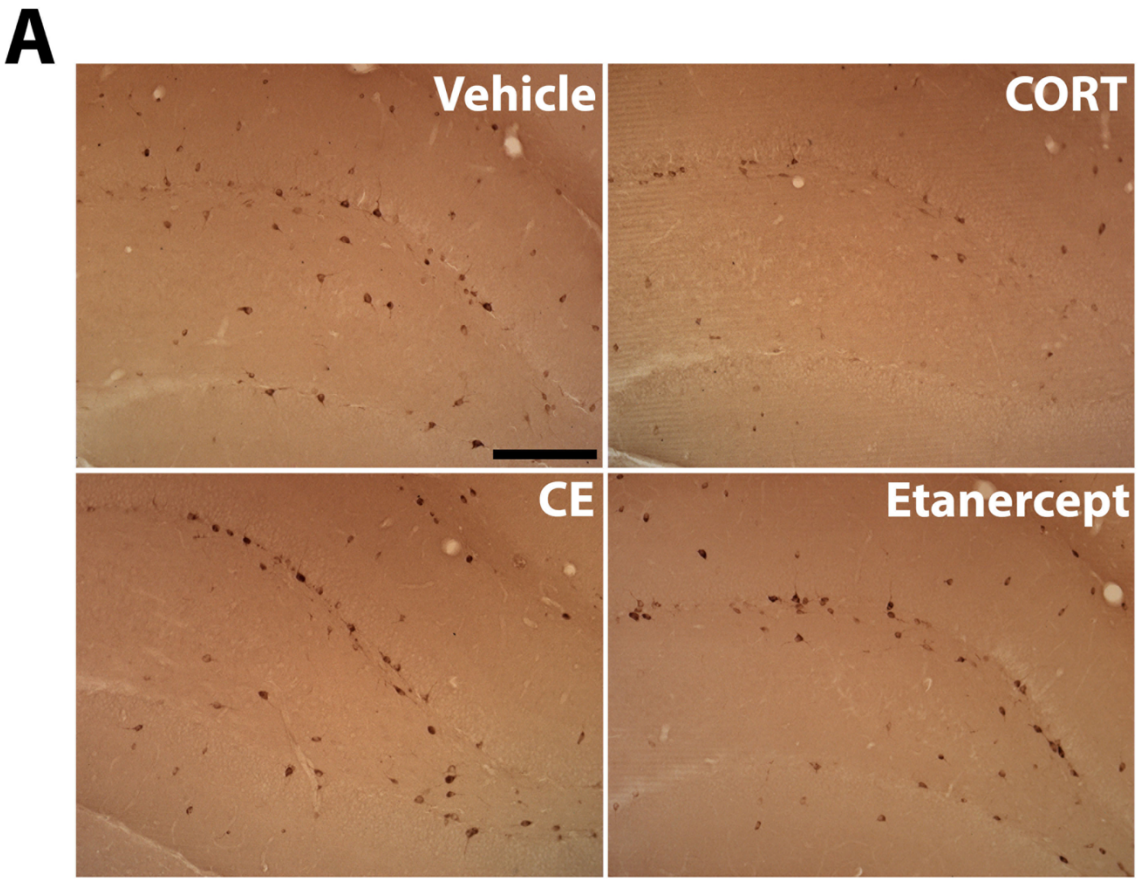
Fig 5. Effect of CORT and etanercept on DCX-ir and dendritic complexity. CORT treatment significantly impaired hippocampal neurogenesis, and etanercept treatment restored this. **(A)** Shows representitive photomicrographs of DCX expression in the dentate gyrus (scale bar = 200 μm). A higher magnification image is pictured in the insets (Scale bar = 50 μm). **(B)** Shows the effect of treatment on the number of DCx-ir cells in the subgranular zone. CORT-treated rats had significantly fewer DCX-ir cells than did the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). **(C)** Shows representative photomicrographs of the six categories of dendritic complexity. **(D)** Shows the quantified categorization of dendritic complexity after analysis of a subset of DCX-ir cells. In general, the CORT rats had a higher percentage of cells in category 1 and category 2 and lower percentage of cells in categories 5 and 6, demonstrating that DCX-ir neurons are less well developed after CORT treatment. All data are represented as means ± standard error of the mean.

Davis, & Kalynchuk, 2005; Johnson, Fournier, & Kalynchuk, 2006; Lussier et al., 2009, 2013a,b; Brummelte and Galea, 2010a,b; Fenton et al., 2015). While it has been noted that social isolation can increase depression-like behaviour in rodents and disrupt the neuroendocrine profile, during postnatal development and weaning period (Lapiz et al., 2003; Leng, Feldon, & Ferger, 2004; Weiss, Pryce, Jongen-Relo, Nan-Bahr, & Feldon, 2004; Shetty and Sadananda, 2017), behavioural alterations related to social isolation in adult rats have been reported only after longer isolation periods than those used in the present study (see Murinova et al., 2017). Furthermore, group housing adult rats can lead to increased cortisol levels, indicative of an increased stress response (Kamakura, Kovalainen, Leppaluoto, Herzig, & Makela, 2016).

Prolonged exposure to CORT in rodents is known to produce a clear behavioural phenotype of depression, characterized by increased immobility in a FST and tail suspension test, decreased preference for sucrose, and decreased interest in sexual behaviour (Marks, Fournier, & Kalynchuk, 2009; Sterner and Kalynchuk, 2010; Lussier et al., 2013a; Fenton et al., 2015). Our FST results are consistent with these previous findings, in that the CORT rats displayed increased immobility and decreased swimming and struggling compared to control rats. Importantly, etanercept reversed the effects of CORT on FST behaviour. The CORT + etanercept displayed significantly less immobility than the CORT rats and in fact, they looked very much like control rats in this test. This was expected, because there have been previous studies that assessed the antidepressant effects of etanercept in the FST. For example, Krugel, Fishcer, Radicke, Sack, and Himmerich (2013) reported that intraperitoneal injections of etanercept can reverse the effects of chronic restraint stress on FST immobility. Adding translational validity to the preclinical observations that TNF-α blockade has antidepressant effects, Raison et al. (2013) observed antidepressant effects of infliximab (a TNF-α inhibitor) in a clinical sample. Specifically, their work demonstrated that infusion of infliximab reduced scores on the Hamilton scale in patients with a high baseline level of inflammation. Furthermore, a recent systematic review and meta-analysis of seven randomized clinical trials demonstrated the antidepressant effectivity of several anti-TNF-α drugs (i.e., adalimumab, etanercept, infliximab, and tocilizumab) (Kappelmann, Lewis, Dantzer, Jones, & Khandaker, 2016). Therefore, our findings and the literature presented here suggest that TNF-α – induced inflammation is critically involved in the pathogenesis of depression, and therapies targeting this inflammation can produce therapeutic benefits. However, the mechanisms by which TNF-α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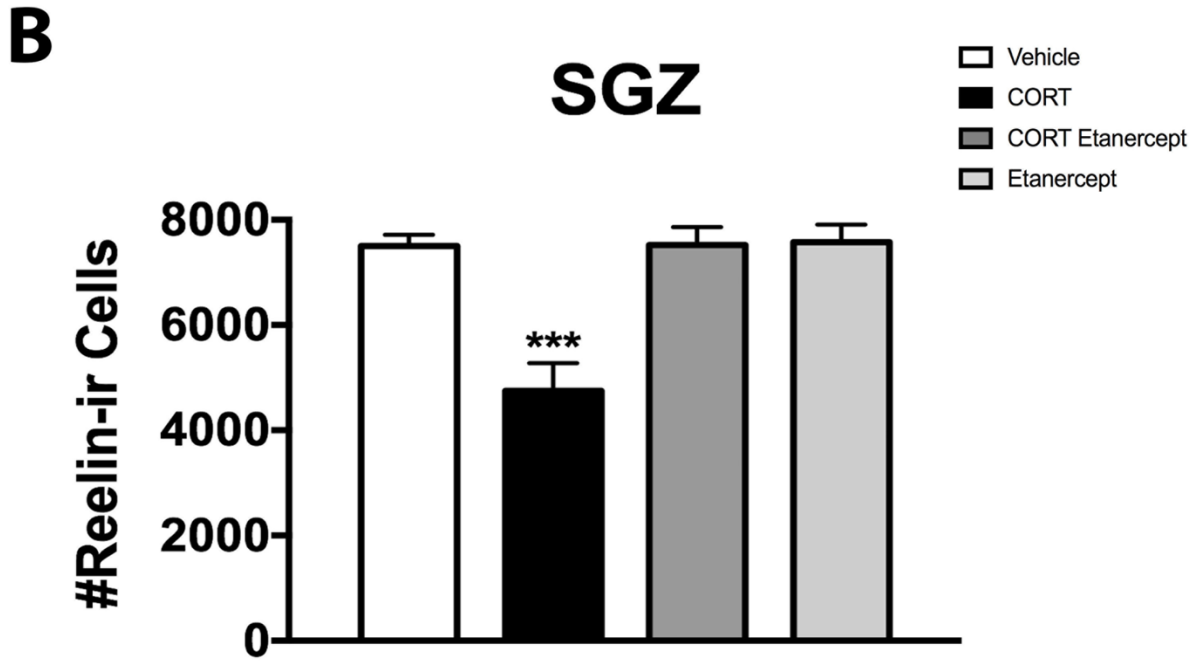
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Fig 6. Effect of CORT and etanercept on the number of reelin-ir neurons. **(A)** Shows representative photomicographs of reelin-ir neurons in the dentate gyrus (scale bar = 200 μm). We focused our attention on reelin-ir cells in the proliferative subgranular zone. **(B)** Shows quantified values of reelin-ir neurons. The CORT rats had significantly fewer reelin-ir cells compared to the vehicle, CORT + etanercept, and etanercept rats ($p < 0.05$). All data are represented as means ± standard error of the mean.

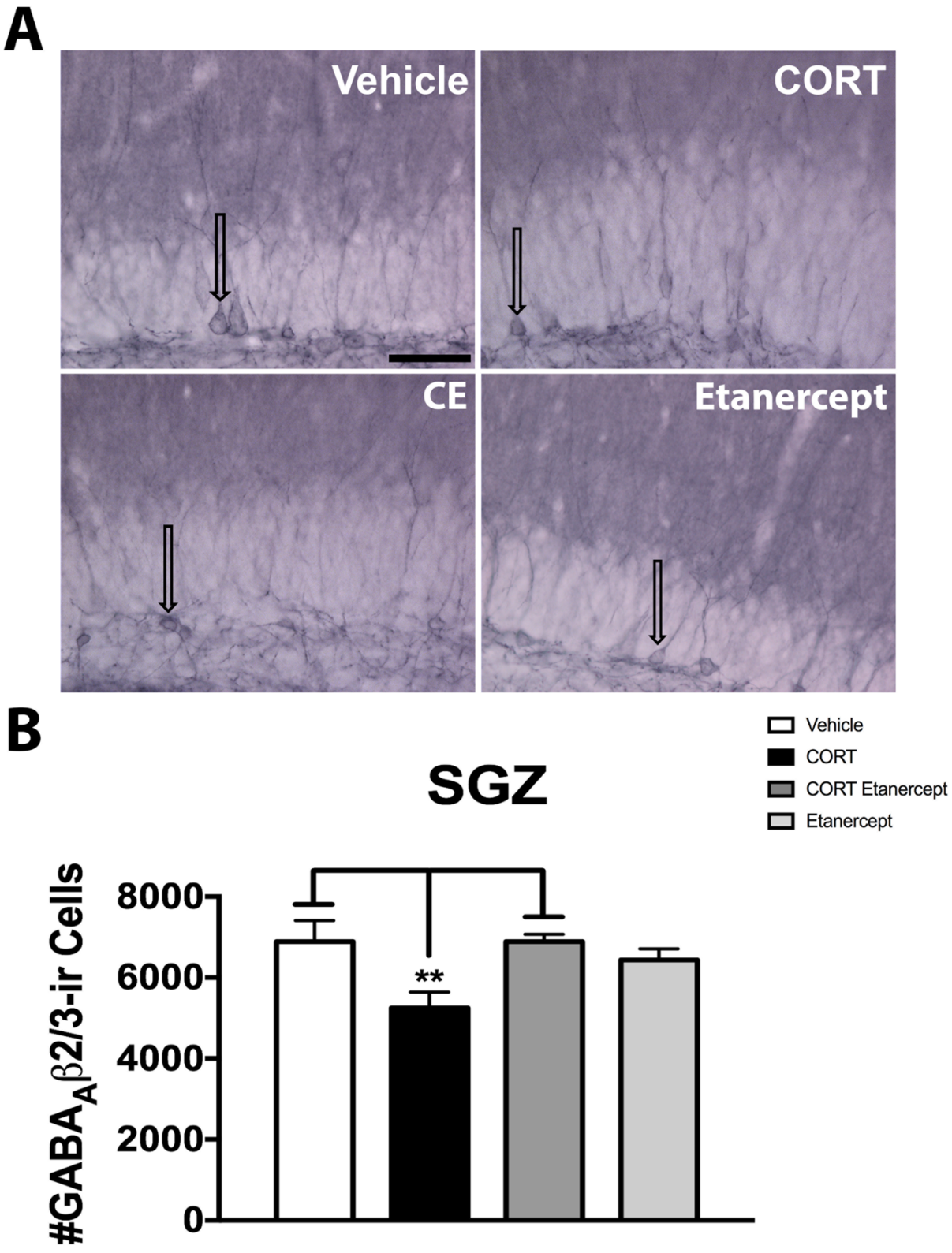

Fig 7. Effect of CORT and etanercept on the number of $GABA_A$ β2/3-ir cells. **(A)** Shows

representative photomicrographs of $GABA_A$ β2/3 expression along the subgranular zone (scale bar = 200 μm). Arrows point to cell bodies showing $GABA_A$ β2/3-ir. Note that in the vehicle rats, $GABA_A$ β2/3 immunostaining appears to be concentrated on the plasma membrane of the cell bodies, whereas after CORT treatment the labeling is more diffuse and can be observed along the cytoplasm indicating receptor internalization. Etanercept partially rescues this effect. **(B)** Shows the quantified number of $GABA_A$ β2/3-ir cells in each group. The CORT rats had significantly fewer $GABA_A$ β2/3-ir cells compared to the vehicle and CORT + etanercept rats ($p < 0.05$). All data are represented as means ± standard error of the mean.

antagonism can produce antidepressant effects are completely unknown.

Chronic stress is known to impair aspects of cognition, particularly hippocampal-mediated spatial memory. For example, deficits in Morris Water maze performance (Warner et al., 2013), fear conditioning (Hoffman, Lorson, Sanabria, Olive, & Conrad, 2014), and OBL memory (Hattiangady et al., 2014) have all been observed after exposure to chronic stress. Inflammation can also impair performance on hippocampal-dependent forms of memory. This has been seen through studies using lipopolysaccharide, where impairments on context-object location discrimination were associated with high levels of proinflammatory cytokines, such as TNF-α (Czerniawski, Miyashita, Lewandowski, & Guzowski, 2015). Chronic restraint stress can also elevate hippocampal TNF-α levels, and this has been linked to impairments in a passive avoidance learning test (Azadbakht, Radahmadi, Jevanmard, & Reisi, 2015). Furthermore, studies of breast-cancer survivors reveal reduced hippocampal volumes and deficits in verbal memory performance that are associated with elevated circulating levels of TNF-α (Kesler et al., 2013). Here, in line with previous research, we report that CORT impairs both OBL and OBIP memory, and that etanercept restores memory performance to control levels. The CORT rats were not able to discriminate the novel object location in either test, whereas rats in all other groups, including the CORT + etanercept rats, performed normally. These findings reinforce the idea that TNF-α plays an important role in hippocampal-dependent memory because the OBL is a hippocampal dependent task, but interestingly, OBIP relies not only on the hippocampus, but also on functional connections between the hippocampus, perirhinal cortex, and medial prefrontal cortex (Barker and Warburton, 2015). These connections require intact NMDA receptor synaptic plasticity (Barker and Warburton, 2008) and it is notable that one action of hippocampal reelin (also restored by etanercept in this experiment) is to regulate the composition and trafficking of NMDA receptor subunits (Bosch et al., 2016b). Therefore, the restoration of reelin –ir cells by etanercept in this experiment could have played a role in the normalization of cognitive behaviour.

One should consider that etanercept is a large molecule that is unable to cross the blood-brain barrier (Boado, Hui, Lu, Zhou, & Pardridge, 2010). Peripheral etanercept seems to be able to indirectly reduce CNS inflammation as a consequence of decreased peripheral inflammatory signals (Kerfoot et al., 2006). A particularly novel finding from this experiment was that peripheral administration of etanercept restored CORT-induced deficits in hippocampal

neurogenesis. Reductions in hippocampal neurogenesis are a well-documented finding in the preclinical stress literature (Sliwowska et al., 2010; Lussier et al., 2013a; Fenton et al., 2015; Levone, Cryan, & O'Leary, 2014). However, the role of hippocampal neurogenesis in depression is a contentious issue, as ablation of hippocampal neurogenesis does not always induce depression- like behaviour (Bessa et al., 2009), and antidepressant effects can be achieved without increases in neurogenesis (Hanson, Owens, & Nemeroff, 2011). Furthermore, studies of post-mortem tissue from depressed patients previously failed to find reductions in hippocampal neurogenesis (Reif et al., 2006); although more recent papers seem to refute that (Boldrini et al., 2012). Interestingly, Hill, Sahay, and Hen (2015) convincingly demonstrated that increasing hippocampal neurogenesis through a transgenic mouse line reversed a CORT- induced depressive phenotype, suggesting that neurogenesis *per se* does play a contributing role in antidepressant responses. Moreover, when considering neurogenesis, one should focus not only on the number of newborn neurons, but also on the maturation of those neurons, including the sprouting of dendritic arborization and integration of new neurons into mature circuits that seem to be dampened in depression and rescued by effective antidepressant treatment (Mateus-Pinherio et al., 2013a,b; Alves et al., 2017; Morais et al., 2017). Importantly, our data indicate that peripheral etanercept administration works in rescuing not only the number of new neurons but also the maturation rate of hippocampal DCX-ir cells, and this could be one mechanism by which etanercept exerts an antidepressant action.

How might peripheral administration of etanercept be acting to normalize CORT-induced deficits in neurogenesis? To our knowledge, this is the first study to report such an effect. It has been shown that peripherally produced TNF-α acts on toll-like receptor 4 on circumventricular organs and choroid plexus, which in turn stimulates microglia to secrete pro-inflammatory cytokines (among them TNF- α), resulting in neurodegenerative events and an increase of apoptotic neuronal death (Bortolato, Carvalho, Soczynska, Perini, & McIntyre, 2015). On the other hand, low levels of TNF-α are known to produce the opposite effect, promoting hippocampal neurogenesis through increased proliferation of neural precursor cells (Fan, Tien, & Zhang, 2011). Therefore, peripheral administration of etanercept likely decreases the expression of peripheral pro-inflammatory cytokines, which indirectly protects against the deleterious effects of CORT. This idea should be further examined in future studies.

Another novel finding from this experiment is that peripheral injections of etanercept

normalize CORT-induced deficits in hippocampal reelin expression. Previous work in our lab has shown that CORT decreases the number of reelin-positive cells in the hippocampus (Lussier et al., 2009, 2013a; Fenton et al., 2015). We believe that the behavioural and neurobiological effects of etanercept observed here are secondary to an increase in hippocampal reelin expression: this idea is supported by our previous observation that heterozygous reeler mice, which express 50% of the normal levels of reelin, are more susceptible to the depressogenic effects of CORT (Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2011), by previous findings showing that the time course for the development of depressive-behaviour in rats coincides with the time course for decreased reelin expression (Lussier, 2013a), that the tricyclic antidepressant imipramine rescues hippocampal reelin levels in a dose-dependent manner (Fenton, 2015), and that oxidative events may be operative in CORT- induced deficits on reelin-ir cells in the dentate subgranular zone (Romay-Tallon et al., 2010, 2015). This leads to the idea that blocking peripheral TNF-α through the administration of etanercept will decrease excitotoxic insults affecting reelin-ir cells in the dentate SGZ, thus restoring the normal release of reelin alongside proliferating neurons in the SGZ, which will facilitate the maturation and integration of immature neurons and rescue the behavioural phenotype (for a more detailed explanation of this hypothesis see Caruncho et al., 2016). Direct support for this idea comes from observations that reelin overexpressing mice have increased neurogenesis (Pujadas et al., 2010) and that exogenous reelin can recover cognitive deficits in mouse models of both Angelman syndrome and Alzheimers Disease (Pujadas et al., 2014; Hethorn et al., 2015). This also dovetails nicely with our finding of that etanercept restores both OBL and OBIP memory, as discussed above.

Finally, we have previously shown that repeated CORT injections reduce $GABA_A$ receptor expression in the hippocampus (Lussier et al., 2013b). Here, we found that CORT decreased the number of $GABA_A$ β2/3-ir cells within the subgranular zone, and induced $GABA_A$ receptor internalization, and etanercept restored this to control levels. TNF-α alters the surface expression of AMPA and $GABA_A$ receptors by increasing AMPA receptor expression and decreasing $GABA_A$ receptors (Stellwagen, Beattie, Seo, & Malenka, 2005), which may contribute to the excitotoxic effects of upregulated TNF-α in a chronic stress paradigm (Bortolato et al., 2015). The normalization of $GABA_A$ receptor expression in SGZ cells by peripheral etanercept may represent a neuroprotective mechanism that is necessary to facilitate SGZ plasticity and restore hippocampal neurogenesis. Indeed, much like reelin, GABA released

from local interneurons exerts a depolarizing effect on immature neurons during the time when they express DCX, promoting neuronal differentiation and maturation (Song, Killeen, & Leonard, 2013). In the absence of GABA, neuronal survival is jeopardized. Therefore, the influence of etanercept on GABA function is likely quite important for its antidepressant effects.

It should also be considered that etanercept is also affecting other body organs: For example, clinical studies in rheumatoid arthritis have indicated that etanercept -prescribed because of its anti-inflammatory activity-, can increase the risk of developing inflammatory bowel disease, lung infections, and pancytopenia (Thavaranjah, Wu, Rhew, Yeldandi, & Kamp, 2009; Santana et al., 2012; O'Toole, Lucci, & Korzenik, 2016), but these side effects of etanercept are not frequently reported and the medication is generally well tolerated.

5. Conclusion

We have shown that peripheral injections of the TNF-α inhibitor etanercept normalize the depressogenic effects of repeated CORT administration, possibly through a restoration of hippocampal reelin expression, hippocampal neurogenesis, and $GABA_A$ β2/3-ir cells in the dentate subgranular zone. These results add to the body of literature suggesting that inflammatory events are a critical component of the pathogenesis of depression and suggest that reelin may be a key link in the relationship between the immune system and brain. Future work should examine whether blocking reelin or its signaling mechanisms can abolish the antidepressant effect of etanercept.

CHAPTER 4

FAST-ACTING ANTIDEPRESSANT EFFECTS OF REELIN IN AN ANIMAL MODEL OF DEPRESSION

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Kyle J Brymer was involved in the design of the experiment, conducting all aspects of the experiment, analyzing the data, and writing up the manuscript. Justin J Botterill, Raquel R Tallon, and Milann A Mitchell were involved in assisting with some aspects of conducting the experiments. Hector J Caruncho and Lisa E Kalynchuk were involved in planning the experimental design and formulating the manuscript. The current chapter was included in the dissertation as the finding that intrahippocampal reelin infusions have fast-acting antidepressant effects is an important finding in the depression and antidepressant field, and formed the basis of chapter 5.

Abstract

We examined the effects of intrahippocampal reelin infusions on measures of depression-like behaviour, cognition, hippocampal neurogenesis, and markers of GABA and glutamate neurotransmission. Stereotaxic surgery was conducted and an indwelling cannula was implanted into the left dorsal hippocampus. Rodents were then administered 21 days of either 40 mg/kg CORT injections or vehicle injections. Reelin was then infused on days 7, 14, and 21(repeated) or only on day 21(single) of corticosterone (CORT) injections. As expected, CORT increased depression-like behaviour in the forced swim test and impaired object-location memory. Notably, these effects were reversed in rats that received a single reelin infusion before behavioural testing. CORT decreased both the number and complexity of adult-generated neurons, and a single reelin infusion increased the number but not complexity of adult-generated neurons, whereas repeated reelin infusions restored both. CORT increased GluN2B immunoreactivity and decreased GluA1-ir cells. Reelin infusions decreased GLUN2B immunoreactivity and increased the number of GLUA1-ir cells to control levels. Finally, CORT decreased the number of $GABA_A$ β2/3-ir cells, and reelin infusions restored this to control levels. These novel results demonstrate that a single intrahippocampal reelin infusion into the dorsal hippocampus has fast acting antidepressant effects, and is accompanied by a restoration of cognitive function, hippocampal neurogenesis, and markers of glutamatergic and GABAergic neurotransmission.

1. Introduction

Depression is a debilitating psychiatric disorder estimated to be the leading cause of disease burden worldwide by 2030 (Willner, Scheel-Jruger, & Belzung, 2013). The majority of antidepressant medications target the monoamine system, however a third of patients do not respond to conventional antidepressant treatment (Raison et al., 2013). Further compounding this issue is that antidepressants take weeks to achieve their therapeutic effects, which presents a problem for depressed patients with suicidal thoughts. In light of this dilemma, novel antidepressants with fast-acting properties are required.

In rodents and humans, chronic stress leads to a significant reduction in reelin-expressing cells in the hippocampus (Lussier et al., 2013a; Fatemi, 2005). For example, work in our lab has demonstrated that repeated corticosterone (CORT) injections reliably reduce the number of reelin-immunoreactive (IR) cells in the subgranular zone (SGZ) of the dentate gyrus (DG). Furthermore, the reduction of reelin cell number becomes more exaggerated with increased doses of CORT (e.g., 20 vs 40 mg/kg) and longer durations of CORT injections (e.g., 14 vs 21 days). (Lussier et al., 2013a; Johnson, Fournier, & Kalynchuk, 2006). Heterozygous reeler mice, which express 50% of the normal levels of reelin, support this observation. In particular, we have shown in a dose-dependent manner that heterozygous reeler mice are more susceptible to repeated CORT injections than wild-type controls. Along the same lines, we recently reported that the tricyclic antidepressant imipramine restored hippocampal reelin levels and reversed depression-like behaviour after CORT treatment, which indicates that restoration of reelin expression coincides with an antidepressant response (Fenton et al., 2015). This led us to hypothesize that reelin could have antidepressant effects in rats subjected to CORT injections.

Alterations or impairments of reelin signaling has been implicated in several psychiatric conditions, including major depression. Importantly, the therapeutic potential of reelin supplementation through genetic overexpression or infusions has been demonstrated across several studies. For example, mesial temporal lobe epilepsy is accompanied by granule cell dispersion and a loss of reelin in the hilus. Remarkably, application of recombinant reelin to hippocampal slice cultures restored the granule cell dispersion to control levels induced by kainite (Orcinha et al, 2016). In line with this, intraventricular injections of exogenous reelin has been shown to recover synaptic function and cognitive deficits in a mouse model of Angelman syndrome (Hethorn et al., 2015). A single intraventricular injection of exogenous reelin was

reported to increase hippocampal CA1 long-term potentiation, dendritic spine density, and spatial learning and memory in otherwise experimentally naïve mice (Rogers et al., 2011). Finally, overexpression of reelin has been found to prevent the behavioural phenotypes of both schizophrenia and bipolar disorder (Teixeira et al, 2011). Taken together, these results demonstrate that reelin has broad effects on cognitive function and can ameliorate many of the structural and functional changes associated with several brain disorders.

In the current study, we were interested in determining whether intrahippocampal reelin infusions produce an antidepressant response -- a question that has yet to be addressed in the literature. To investigate this issue, we used 2 different reelin infusion paradigms across 2 different time points to evaluate the effects of reelin on both behavioural assays and neurobiological markers known to be effected by stress (Fig. 1). We were particularly interested in examining the contribution of GABA and glutamate to the potential antidepressant response of reelin, as previous work has demonstrated that heterozygous reeler mice display a significant reduction in GLUA1 subunit clustering in cultured hippocampal neurons (Qiu and Weeber, 2007), and reelin is released by GABAergic interneurons (Caruncho et al., 2016). As discussed above, based on the findings that supplementation of reelin recovers cognitive function and mood, we tested the hypothesis that intrahippocampal reelin infusions would normalize depression-like behaviour and cognitive dysfunction induced by repeated CORT injections. We also investigated the potential that these changes are accompanied by alterations in GABA and glutamate signaling in the DG, a region we have shown to be susceptible to the depressiogenic effects of CORT (Lussier et al., 2013a).

2. Materials and methods

2.1. Animals

We used 60 adult male Long-Evans rats purchased from Charles River (QC, Canada). The rats weighed 200-250 g at the time of arrival from the breeder. Rats were individually housed in rectangular polypropylene cages containing standard laboratory bedding with access to food and water *ad libitum.* The rodent colony room was maintained at an ambient temperature of 20 ± 1 °C on a 12:12 light-dark cycle (lights on at 7 am). All experimental procedures were in accordance with the guidelines of the Canadian Council and Animal Care and an animal care protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. All efforts were made to minimize the number of rats used in the present study.

2.2. Stereotaxic Surgery

Surgery was conducted as previously described (Botterill, Nogovitsyn, Caruncho, & Kalynchuk, 2016; Botterill, Guskjolen, Marks, Caruncho, & Kalynchuk, 2015b). Briefly, rats were deeply anesthetized with isoflurane (5% initial, 2-2.5% maintenance) and secured into a stereotaxic apparatus using ear bars. Rats were given ketoprofen (Anafen, ketoprofen, 10 mg/kg s.c.) to minimize pain and inflammation. At flat skull position, a single cannula (C313G/spc, Plastics 1, Roanoke, VA, USA) was chronically implanted into the left hemisphere of the dorsal hippocampus using the following coordinates from the rat brain atlas (relative to bregma): -3.5 mm anteroposterior, +2.6 mm mediolateral, -3.1 mm dorsoventral (Paxinos and Watson, 1998). The cannula was secured to the skull with 4 jeweler screws and dental acrylic. We then inserted a dummy cannula into the guide cannula to prevent debris from entering the site during the course of the study. To minimize the risk of post-surgical infection, we administered the antibacterial-antifungal ointment Hibitane (Chlorhedixine acetate B.P. 1% [w/w]) daily around the incision site for at least one week after the surgery.

2.3. Experimental Procedures

2.3.1. CORT and Vehicle Injections

All rats were briefly handled once per day for seven days prior to the start of CORT injections. We then weight-matched the rats and randomly assigned them to one of the following six treatment groups: 21 days of vehicle injections (vehicle, $n = 10$); 21 days of CORT injections (CORT, $n = 10$); vehicle + a single infusion of reelin on day 21 (vehicle reelin single, $n = 10$); CORT + a single infusion of reelin on day 21 (CORT reelin single, $n = 10$); vehicle + 3 infusions of reelin on days 7, 14, and 21 of injections (vehicle reelin repeated, $n = 10$); or CORT + 3 infusions of reelin on days 7, 14, and 21 of injections (CORT reelin repeated, $n = 10$). These two reelin infusion paradigms allowed us to compare and contrast the antidepressant effects of reelin both acutely and repeatedly (Fig. 1).

All CORT and vehicle injections were administered subcutaneously once per day (between 9:00 and 10:00 am). CORT (Steraloids, Newport, RI, USA) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 (Sigma-Aldrich) and given at a dose of 40

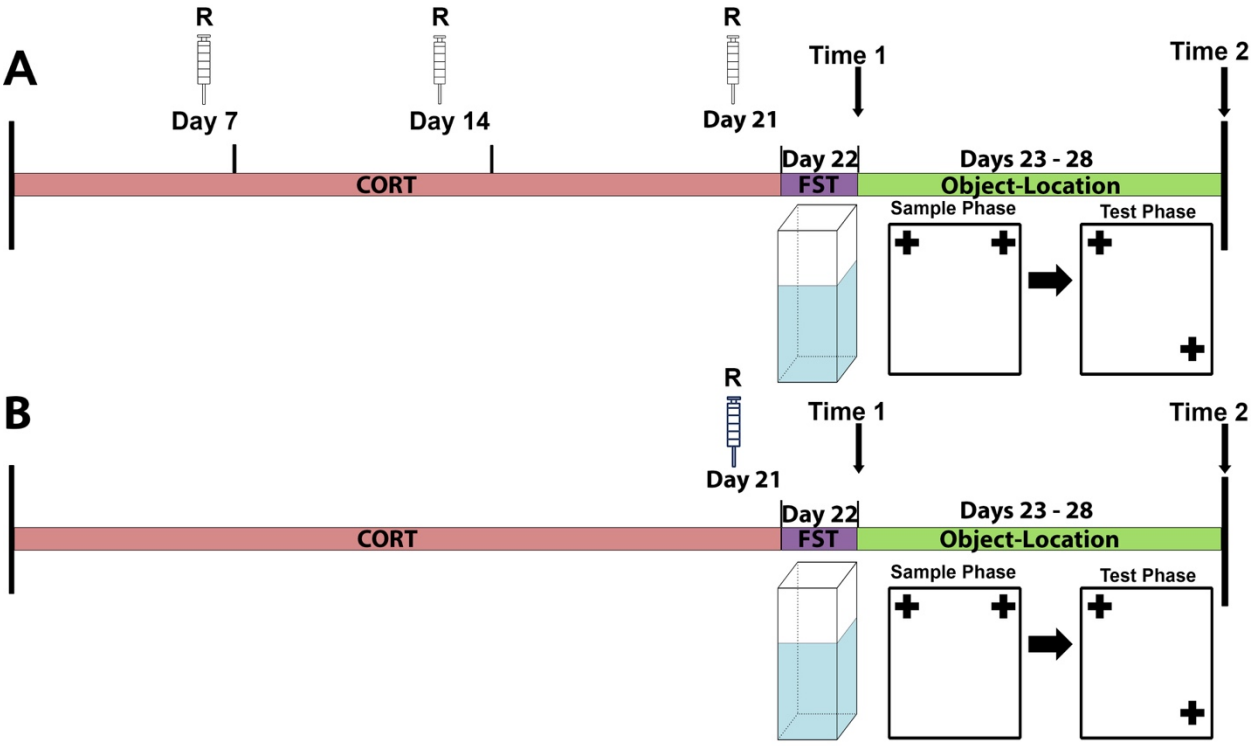

Fig 1. Schematic representation of the experimental design used for the study. Stereotaxic surgery was performed and a chronic indwelling cannula was inserted into the left dorsal hippocampus. Animals were weight-matched and received 21 days of 40mg/kg of corticosterone (CORT) or vehicle injections. A subset of animals received infusions of 1µg/ µl of reelin either on days 7, 14, and 21 of CORT/vehicle injections, or only on day 21. All rats underwent the forced-swim test (FST) on day 21, and a subset of rats were immediately sacrificed (time 1). The remaining rats then underwent the object-location memory paradigm (OBL) from days 23-28, and were then sacrificed (time 2). All experimental groups were evenly distributed throughout both times 1 and 2.

mg/kg in a volume of 1 ml/kg. Our lab has reported that the 40 mg/kg dose reliably increases depression-like behaviour in rats (e.g., Johnson, Fournier, & Kalynchuk, 2006; Lussier et al., 2013a, Fenton et al., 2015). The body weight of each rat was recorded daily for the entire 21 days of CORT or vehicle treatments.

2.3.2. Intrahippocampal Reelin infusions

Recombinant reelin (3820-MR-025/CR; R & D Systems, Minneapolis, MN, USA) was reconstituted immediately prior to infusions to a working concentration of 1 µg per 1µl in 0.1 M PBS (pH = 7.4). Intrahippocampal reelin infusions were administered in a dedicated procedures room for the duration of the experiments. We used a 2 µl Hamilton syringe secured to an infusion pump (PHD 2000; Harvard Apparatus, Holliston, MA, USA). The tip of the Hamilton syringe was inserted into PE-50 tubing that was connected to an infusion needle (C313l/spc, Plastics 1) that extended 1 mm below the base of the guide cannula. The infusion needle was inserted into the guide cannula of each rat and secured to the base of the cannula pedestal. We then infused recombinant reelin directly into the dorsal hippocampus at a rate of 0.5 µl per minute. The infusion needle was left in place for an additional two mins after the infusion to facilitate diffusion. We then slowly removed the infusion needle from the guide cannula.

2.4. Behavioural Testing

The forced-swim test (FST) and object-location (OBL) paradigm were carried out in a dedicated procedures room that was not used for any other aspect of the study. All behaviours were recorded with a digital video camera and stored for offline analyses. The FST assessed depression-like behaviour (Gregus et al., 2005), while the OBL assessed hippocampal-dependent memory (Howland, Cazakoff, & Zhang, 2012).

2.4.1. Forced swim test

The FST was conducted on day 22, the day after the final CORT injection and intrahippocampal reelin infusion (Fig. 1). All rats at both time 1 and 2 completed the FST. We used a modified version of the Porsolt test, as previously described (Lussier et al., 2013a; Gregus, Wintink, Davis, & Kalynchuk, 2005). Each rat was individually placed in a Plexiglas swim tank (25 cm wide x 25 cm long x 60 cm high, 27 ± 2 °C water, 30 cm deep) for 10 minutes. We measured the duration of time each rat spent immobile, struggling, and swimming.

2.4.2. Object Location

Recognition memory test procedures were similar to previously published protocols (Howland, Cazakoff, & Zhang, 2012; Howland and Cazakoff, 2010; Fig.1). Object-location memory testing took place in a square open-field area (60x60x60cm) constructed of white corrugated plastic.

Rats that were not sacrificed at time 1 went on to complete the OBL for time 2 (Fig. 1). Rats received three habituation sessions before the training session. During the first two habituation sessions, rats were brought in pairs and placed in separate arenas for 10 mins. During the last habituation session, rats were brought in individually and placed in the arena for 10 minutes. Rats were immediately returned to the colony room after each habituation session. The last habitation occurred 24-48 hours before the first testing session. Objects used were made of porcelain and did not exceed 10 cm in height or length. Duplicate copies of objects were used when the same objects were required for both training and testing phases. Object locations were counterbalanced to eliminate potential side preference in all tests. In both the sample and test phases, objects were placed in the corners of the arena 10 cm from each of the nearest walls and rats were placed in the center of the arena facing the wall opposite the objects. Both the arena and the objects were wiped with 40% EtOH after each session (Howland, Cazakoff, & Zhang, 2012).

The rats were allowed to explore two identical objects for 4 minutes during the sample phase (C1 and C2). Twenty-four hours later, rats underwent a testing phase, during which they explored two copies of the sample objects (C3 and C4) for 4 minutes, but with one object moved to a corner location at the front of the box while the other maintained its original position. Data were scored by a researcher that was blind to treatment conditions, using previously published measures (Howland, Cazakoff, & Zhang, 2012; Howland & Cazakoff, 2010). Briefly, a rat was defined to be actively exploring an object when its nose was directed within 2 cm of an object and either its head or vibrissae were moving, but not when it was standing on top of an object and not directing attention to it. Rats were required to explore objects for at least 15 seconds in both the sample and test phases to have reliable recognition memory. The discrimination ratio (DR), determined by calculating the time the rat spent exploring the novel orientation subtracted by the time the rat spends exploring the familiar orientation and divided by the two, was calculated for the first 2 min of the test phase for each rat since previous studies have reported

this to be the most sensitive and reliable measure (Howland, Cazakoff, & Zhang, 2012).

2.5. Histology

2.5.1. Perfusions

Rats were sacrificed following the FST at time 1, or upon completing the OBL at time 2 (Fig. 1). Briefly, each rat was deeply anesthetized with 5% isoflurane and then transcardially perfused using room-temperature physiological saline, followed by ice-cold 4% (w/v) formaldehyde fixative (pH = 7.4). The brains were extracted from the cranial vault and immersed in the same formaldehyde fixative for 48 hours at 4 °C. The brains were then sectioned in the coronal plane at 30 μm on a vibrating microtome (VT1200s, Leica Biosystems, Nussloch, Germany). Sections were collected and stored at −20 °C until use in a cryoprotectant solution containing 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

2.5.2 Immunohistochemistry

We used a standard immunohistochemistry technique with widely used and commercially available antibodies for all of our experiments. Immunostaining was done on free-floating sections in six-well tissue culture plates under gentle agitation for every step. To ensure consistent immunohistochemical processing, we processed all sections in unison with treatment groups counterbalanced across all tissue plates. To confirm the specificity of our antibodies, we omitted the primary antibody from an additional well of free-floating sections. In the absence of the primary antibody, we were unable to detect any immunoreactive cells.

Doublecortin immunohistochemistry was run as previously described (Botterill, Brymer, Caruncho, & Kalynchuk, 2015a; Lussier et al., 2013a). Sections underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85 °C for 30 minutes. The sections were then blocked in 5% (v/v) normal goat serum (NGS), 1% (w/v) bovine serum albumin (BSA), and 0.5% (v/v) Triton X-100 in 0.1 M TBS (pH = 7.4), followed by incubation in a rabbit anti-doublecortin (DCX) polyclonal primary antibody (1:1000, catalog #4604S, RRID: AB_561007; Cell Signalling Technologies) diluted in blocking solution for 24 h at room temperature. On the following day, the sections were treated with 5% (v/v) H2O2 in 0.1 M TBS for 30 minutes to block endogenous peroxidase activity. The sections were then incubated for 1 hour in biotinylated goat anti-rabbit secondary antibody (1:500, catalog #BA-1000, RRID: AB_2313606; Vector Laboratories) diluted in 5% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v)

Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. Sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 0.025% (w/v) DAB, 4.167% NiSO4, and 0.002% (v/v) H2O2. The sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using a series of graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

To visualize GLUA1 and GLUN2B staining, sections first underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85 °C for 30 mins. Next, the sections were placed into a blocking solution containing 5% (v/v) normal animal serum, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 30 minutes, and then exposed to either a polyclonal rabbit anti-glutamate receptor 1 (GLUA1; 1:1000, catalog #AB1504, RRID: AB_11212863; Millipore) or a monoclonal mouse anti-GLUN2B primary antibody (1:1000, catalog #75-101, RRID: AB_2232584; NeuroMab) diluted in blocking solution for 48 hours at 4°C. Following this, sections were treated with 5% (v/v) H2O2 in 0.1 M TBS for 30 minutes to block endogenous peroxidase activity. Next, the sections were incubated for 2 hours in either a biotinylated goat anti-rabbit secondary antibody (1:500, catalog #BA-1000, RRID: AB_2313606; Vector Laboratories) or a horse-anti mouse secondary antibody (1:500, catalog #BA-2000. RRID: AB_2313571;Vector Laboratories) diluted in 5% (v/v) normal animal serum, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. The sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 4.167% $NiSO_4$ and 0.05% (w/v) glucose oxidase DAB (Shu, Ju, & Fan, 1988; Botterill et al., 2016). Sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using a series of graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

$GABA_A$ β2/3 receptor subunit immunohistochemistry was conducted as follows. Sections first underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85 °C for 30 min. Next, the sections were placed into a blocking solution containing 5% (v/v) NHS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS for 30 min, and were then exposed to a monoclonal mouse anti-$GABA_A$ β2/3 receptor primary antibody (clone bd17, 1:1000, catalog #MAB341, RRID: AB_2109419; Millipore) diluted in blocking solution for 48 hours at 4°C.

Following this, the sections were treated with 5% (v/v) H_2O_2 in 0.1 M TBS for 30 minutes to block endogenous peroxidase activity. Next, the sections were incubated for 2 hours in biotinylated horse anti-mouse secondary antibody (1:500, catalog #BA-2000. RRID: AB_2313571;Vector Laboratories) diluted in 5% (v/v) NHS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. The sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 4.167% $NiSO_4$ and 0.05% (w/v) glucose oxidase DAB. Sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using a series of graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

2.5.2.1. Quantification of Immunohistochemistry

2.5.2.1.1. Cell Counting

Quantification of immunohistochemistry was conducted as previously described (Botterill, Brymer, Caruncho, & Kalynchuk, 2015a; Lussier et al., 2013a). All analyses were conducted by researchers blind to the treatment conditions. Immunostained sections were examined using a Nikon Eclipse E800 microscope with a motorized stage and digital camera (MicroFire, Optronics, Goleta, CA, USA) connected to a dedicated stereology computer. The dentate subgranular zone (SGZ: defined as a two-cell width zone in between the inner granule cell layer and the hilus) and granule cell layer (GCL) were traced at 4 X magnification using a computerized stereology program for DCX-ir cells. However, only the SGZ was traced for GLUA1-ir cells and $GABA_A$ β2/3-ir cells (StereoInvestigator, MicroBrightfield, Williston, VT, USA). DCX, GLUA1, and $GABA_A$ β2/3-ir cells were counted in both the ipsilateral and contralateral hemispheres at 40 x magnification. All counts utilized unbiased stereology using a modified optical fractionator method that excludes cells in focus at the uppermost focal plane to reduce oversampling (Lussier et al., 2013a). The total number of DCX, GLUA1, and $GABA_A$ β 2/3-ir cells was estimated using the following formula: $N_{total} = \sum Q^{-} \times 1 / ssf \times A(x, y\ step) / a(frame) \times t / h$. $\sum Q^{-}$ represents the number of counted cells, ssf is the section sampling fraction (1 in 12), A(x, y step) is the area associated with each x, y movement (10,000 μm^2), a(frame) is the area of the counting frame (3600 μm^2), t is the weighted average section thickness, and h is the height of the dissector (12 μm) (Botterill 2015a; Fenton et al, 2015; Lussier, 2013a.). To

avoid counting sectioning artifacts, we used a guard zone of 2 μm.

2.5.2.1.2. Characterization of Immature DCX-ir Neurons

We utilized a dendritic categorization method that our laboratory has used in the past (Botterill, Brymer, Caruncho, & Kalynchuk, 2015a; Lussier et al., 2013a) to determine if our intrahippocampal reelin infusions paradigm altered the dendritic morphology of immature DCX-ir neurons. A meander scan method was used to randomly select 100 DCX-ir cells from each rat. A researcher blind to experimental conditions then assigned the cell to one of six complexity categories based on both the presence and extent of apical dendrites (Fig. 3). The proliferative stage encompassed categories one (no process) or two (one small process). Category three (medium process reaching the granule cell layer) and category four (process reaching the molecular layer) make up the intermediate stage of development. Finally, category five (one major process extending into the molecular layer) and category six (defined dendritic tree with delicate dendritic branching in the granule cell layer) represented the postmitotic stage. Data are presented as the percentage of DCX-ir cells in each of the six categories.

2.5.2.1.3. Optical Densitometry

Semiquantitative optical densitometry of GLUN2B immunoreactivity was run on 3 sections per brain with 300 μm between sections using methods previously described by our laboratory (Botterill, Guskjolen, Marks, Caruncho, & Kalynchuk, 2015b). Briefly, we took grayscale photomicrographs on our Nikon E800 microscope with exposure and gain settings held constant for every subject. We then used ImageJ software (V1.46R, National Institutes of Health, Bethesda, MD, USA) to calculate the mean optical density for each region of interest, which calculates the intensity of an immunoreaction product within a traced circle. To control for background staining, the mean optical density of the corpus callosum was subtracted from each region of interest. All values were standardized between white (0) and black (255) and expressed as percentage change from controls.

2.6. Statistical Analyses

All statistical analyses were carried out using IBM's Statistical Package for Social Sciences v24. We determined statistical significance using one-way analysis of variance (ANOVA), and *post hoc* comparisons were made using Tukey HSD when appropriate. The criterion for statistical significance was set at $p < 0.05$. All graphs depict the mean ± standard error of the mean.

3. Results

3.1. CORT had Significant Effects on body Weight with no Supplementary Effects of Reelin

We first sought to determine if intrahippocampal reelin infusions were ameliorating CORT-induced decreases in body weight. We pooled the body weights across both times 1 and 2, as all experimental groups were evenly distributed through both times. Accounting for rats being weight matched to begin the study, significant group differences for body weight were found for days 7 ($F(5, 59) = 7.870$, $p < .001$),14 ($F(5, 59) = 13.778$, $p < .001$), and 21 ($F(5, 59) = 14.436$, $p < .001$). *Post hoc* analyses revealed that the CORT rats weighed significantly less than vehicle and vehicle reelin single rats at day 7 (p values < .05), significantly less than the vehicle, vehicle reelin single, and vehicle reelin repeated rats at day 14 (p values < .05), and significantly less than the vehicle, vehicle reelin single, and vehicle reelin repeated rats at day 21 (p values < .05) (Fig. 2). The CORT reelin single rats weighed significantly less than the vehicle rats on day 7 ($p < .05$), significantly less than the vehicle and vehicle reelin single rats on day 14 (p values < .05), and significantly less than the vehicle, vehicle reelin single, and vehicle reelin repeated rats on day 21 (p values < .05). The CORT reelin repeated rats weighed significantly less than the vehicle and vehicle reelin single rats at day 7 (p values < .05), significantly less than the vehicle, vehicle reelin single, and vehicle reelin repeated rats at day 14 (p values < .05), and significantly less than the vehicle, vehicle reelin single, and vehicle reelin repeated rats at day 21 (p values < .05).

3.2. CORT Increases Depression-like Behaviour on the FST and this is Reversed by Reelin

It is well established that stress increases depression-like behaviour on the FST. As overexpression of reelin has been found to reverse the behavioural phenotypes of bipolar and schizophrenia, we were interested in determining if intrahippocampal reelin infusions could produce an antidepressant effect on the FST. Here, we pooled the FST analyses across both times 1 and 2 as all groups were represented at both time points. We found significant group differences for immobility ($F(5,59) = 18.01$, $p < .001$), struggling ($F(5,59) = 3.94$, $p < .01$), and swimming ($F(5,59) = 9.94$, $p < .001$) (Fig. 1). *Post hoc* analyses revealed that the CORT rats spent significantly more time immobile compared to all other groups (all p values < 0.5), less time struggling than vehicle (p values < .05), cort reelin single, and vehicle reelin repeated, but not vehicle reelin single ($p = .051$) or CORT reelin repeated ($p = .141$), and less time swimming than all other groups (p values < .05). No other significant group differences were observed *(*all p

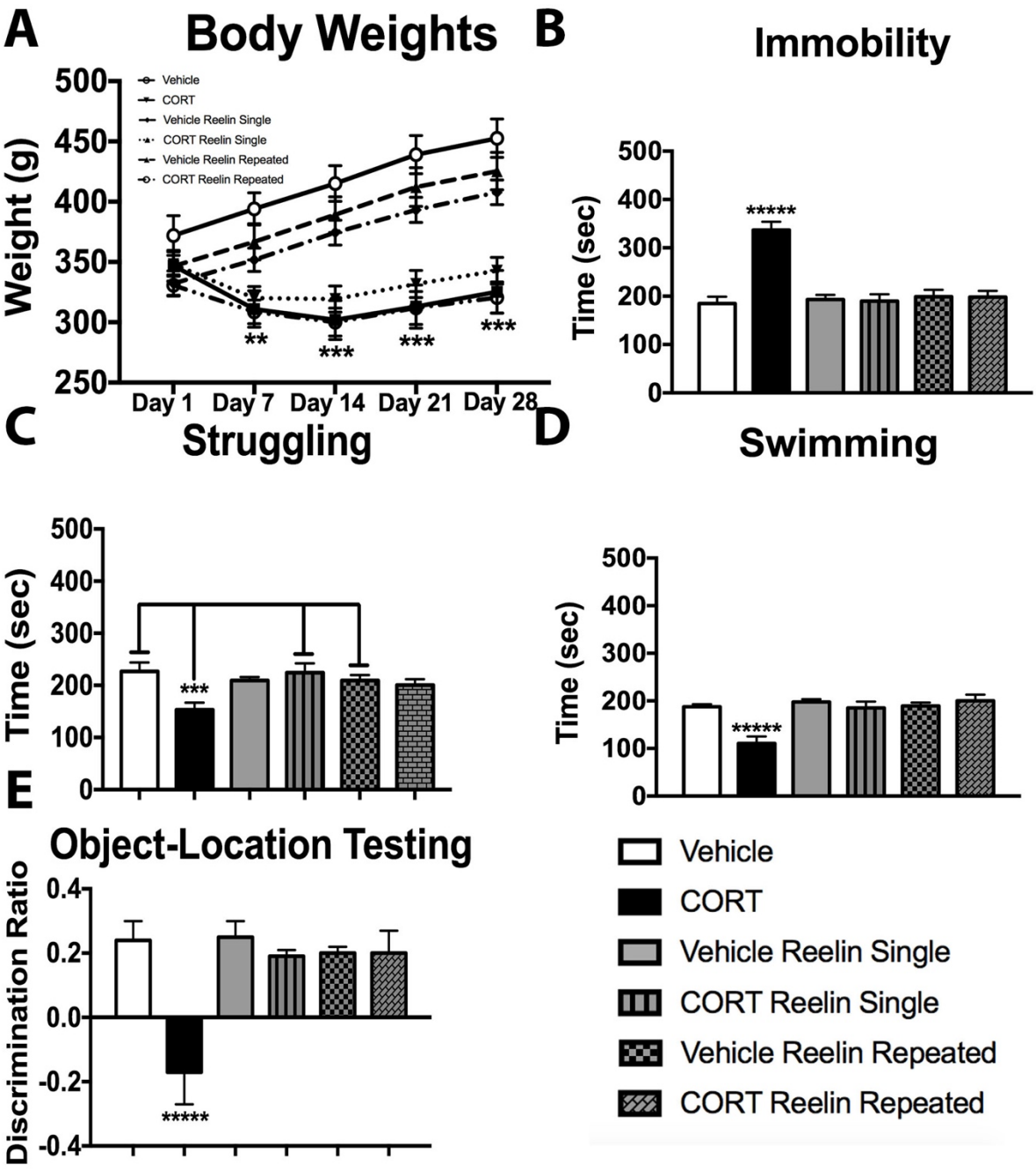

Fig 2. Mean body weight through days 1-21, collapsed across both times 1 and 2. Panel A shows that CORT-treated and CORT reelin repeated rats weighed less than vehicle, vehicle reelin single, and vehicle reelin repeated rats on day 14 ($p < .05$) and 21 ($p < .05$) and significantly less than vehicle and vehicle reelin single rats on day 7 ($p < .05$), and CORT reelin single rats weighed significantly less than vehicle and vehicle reelin single rats on days 7 and 14 ($p < .05$), and significantly less than vehicle, vehicle reelin single, and vehicle reelin repeated rats on day

21 ($p < .05$). Panel B-D; CORT had significant effects on depression-like behaviour, and treatment with reelin reversed this in a fast-acting manner. Data are collapsed across times 1 and 2. Panel B shows the effects of treatment on time spent immobile in the FST. CORT-treated rats spent significantly more time immobile than all other groups ($p < .05$). Panel C shows the effects of treatment on time spent struggling. CORT-treated rats spent significantly less time struggling than all other groups ($p < .05$). Panel D shows the effects of treatment on time spent swimming. The CORT rats spent significantly less time swimming than all other groups ($p < .05$). CORT had significant effects on OBL memory, and reelin restored this. Panel E shows the discrimination ratio for the OBL variant. The CORT rats had a significantly lower discrimination ratio than all other groups ($p < .05$). All data are represented as means ± standard error of the mean.

values > .05) (Fig 2.).

3.3. CORT Impairs Hippocampal-Dependent Memory and Reelin Reverses this Effect

After we demonstrated that intrahippocampal reelin infusions produced an antidepressant effect on the FST, we next sought to determine if reelin could also reverse CORT-induced deficits in cognition at time 2, a finding that we have shown in the past (Brymer et al., 2018). We observed a significant main effect of treatment on object-location performance ($F(5,29) = 7.74$, $p < .001$) (Fig. 2). *Post hoc* analyses revealed that the CORT rats had a significantly lower discrimination ratio than all other groups (all p values < .01) (Fig. 2).

3.4. Intrahippocampal Reelin Infusions Protect Against the Deleterious Effects of CORT on Hippocampal Neurogenesis

As treatment with CORT reliably reduces the number and complexity of immature DCX-ir adult-generated neurons, we were interested in examining if intrahippocampal reelin infusions could reverse this deficit. At time 1, stereological analyses revealed significant group differences in DCX immunostaining in both the ipsilateral ($F(5, 29) = 5.329$, $p < .01$) and contralateral ($F(5, 29) = 4.391$, $p < .01$) SGZ/GCL. *Post hoc* analyses revealed that the CORT rats had significantly fewer DCX-ir cells within the SGZ/GCL in the ipsilateral hemisphere than all other groups (p values < .05) except for the vehicle rats ($p = .148$). Interestingly, *post hoc* analyses revealed that in the contralateral hemisphere, the CORT reelin single rats were not significantly different from the CORT rats ($p = .317$), however the remaining groups were significantly different from CORT (p values < .05). Significant group differences were found when combining the ipsilateral and contralateral hemisphere, ($F(5, 29) = 4.790$, $p < .01$). *Post hoc* tests revealed that the CORT rats had significantly fewer DCX-ir cells than all other groups (p values < .05).

We next examined dendritic complexity. We found significant group differences for categories 1 ($F(5, 29) = 3.966$, $p < .01$), 2 ($F(5, 29) = 18.377$, $p < .001$), 5 ($F(5, 29) = 10.711$, $p < .001$), and 6 ($F(5, 29) = 4.043$, $p < .01$) (Fig. 7). For DCX-ir cells in the proliferative stages of maturation, *post hoc* analyses revealed that the CORT rats had significantly more category 1 cells than the vehicle reelin repeated and CORT reelin repeated rats (p values < .05), but not the vehicle ($p = .187$), vehicle reelin single ($p = .106$), or CORT reelin single rats ($p = .123$). The CORT rats also had significantly more category 2 cells than all other groups (p values < .05) except for the CORT reelin single-treated rats ($p = .103$). For DCX-ir cells in the intermediate stages of development, no significant group differences were found (p values > .05). Finally, for

DCX-ir cells in the post-mitotic stages of maturation, the CORT rats had significantly fewer categories 5 cells than all other groups except for the CORT reelin single rats ($p = .205$), and significantly fewer category 6 cells than the vehicle reelin single, vehicle reelin repeated, and CORT reelin repeated rats (p values $< .05$), but not the vehicle ($p = .051$) or CORT reelin single rats ($p = .507$) (Fig. 4).

Overall, we found that at time 1, 24 hours after the last infusion, a single intrahippocampal reelin infusion increased the number of DCX cells in the ipsilateral hemisphere but did not restore dendritic complexity, while repeated infusions restored both of these measures. We next were interested in determining how this changed by time 2. Stereological analyses revealed significant group differences in both the ipsilateral ($F(5, 29) = 7.958$, $p < .001$) and contralateral ($F(5, 29) = 5.266$, $p < .01$) SGZ/GCL. *Post hoc* analyses revealed that the CORT rats had significantly fewer DCX-ir cells within the SGZ/GCL across both hemispheres than all other groups (all p values $< .05$). Significant group differences were found when combining the ipsilateral and contralateral hemispheres, ($F(5, 29) = 7.381$, $p < .001$. *Post hoc* analyses revealed that the CORT rats had significantly fewer DCX-ir cells than all other groups. Next, to quantify if intrahippocampal reelin infusions influenced the complexity of immature DCX-ir neurons, we examined their dendritic morphology (Fig. 4) We found significant group differences for categories 1 ($F(5, 29) = 98.072$, $p < .001$), 2 ($F(5, 29) = 64.019$, $p < .001$), 4 ($F(5, 29) = 9.359$, $p < .001$), 5 ($F(5, 29) = 10.172$, $p < .001$), and 6 ($F(5, 29) = 20.731$, $p < .001$). For DCX-ir cells in the proliferative stages of maturation, *post hoc* analyses revealed that the CORT rats had significantly more category 1 and 2 cells than all other groups (all p values $< .001$). For DCX-ir cells in the intermediate stages of development, we found that the CORT rats had significantly fewer category 4 cells than all other groups (all p values $< .05$). Finally, for DCX-ir cells in the post-mitotic stages of maturation, the CORT rats had significantly fewer category 5 and 6 cells than all other groups *(*all p values $< .001$) (Fig. 4).

3.5. Treatment with CORT Reduces GLUA1-Positive cells Within the SGZ and Reelin Reverses this

Given that heterozygous reeler mice with only 50% normal levels of reelin have significantly reduced expression of GLUA1 subunit clustering, and that heterozygous reeler mice are more susceptible to the deleterious effects of CORT (Lussier et al., 2011) we examined the number of GLUA1-positive cells in the SGZ. At time 1, stereological analyses revealed

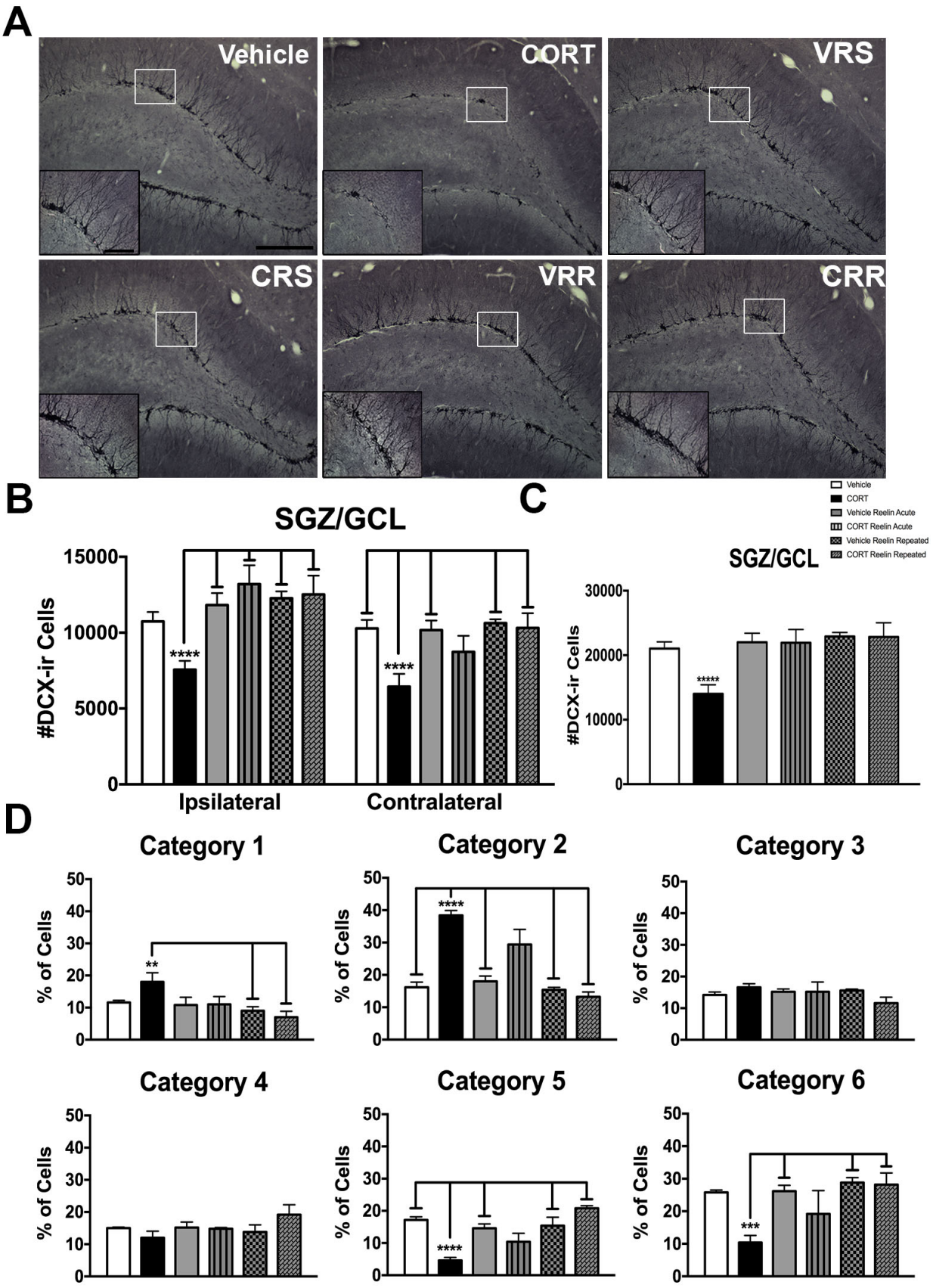
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Fig. 3 Effects of reelin on hippocampal neurogenesis at time 1. CORT had significant effects on hippocampal neurogenesis. Panel A shows representative photomicrographs of doublecortin expression in the granule cell/subgranular zone. Panel B and C shows the effects of treatment on the number of doublecortin-ir cells in the granule cell/subgranular zone. The CORT rats had significantly fewer doublecortin-ir cells in the ipsilateral hemisphere than vehicle reelin single, CORT reelin single, vehicle reelin repeated, and CORT reelin repeated animals ($p < .05$). In the contralateral hemisphere, the CORT rats had significantly fewer doublecortin-ir cells than vehicle, vehicle reelin single, vehicle reelin repeated, and CORT reelin repeated animals ($p < .05$). When combining the ipsilateral and contralateral hemispheres, the CORT rats had significantly fewer DXC-ir cells than all other groups. Panel D shows the quantified categorization of dendritic complexity using doublecortin staining. All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRR = vehicle reelin repeated; CRR = CORT reelin repeated.

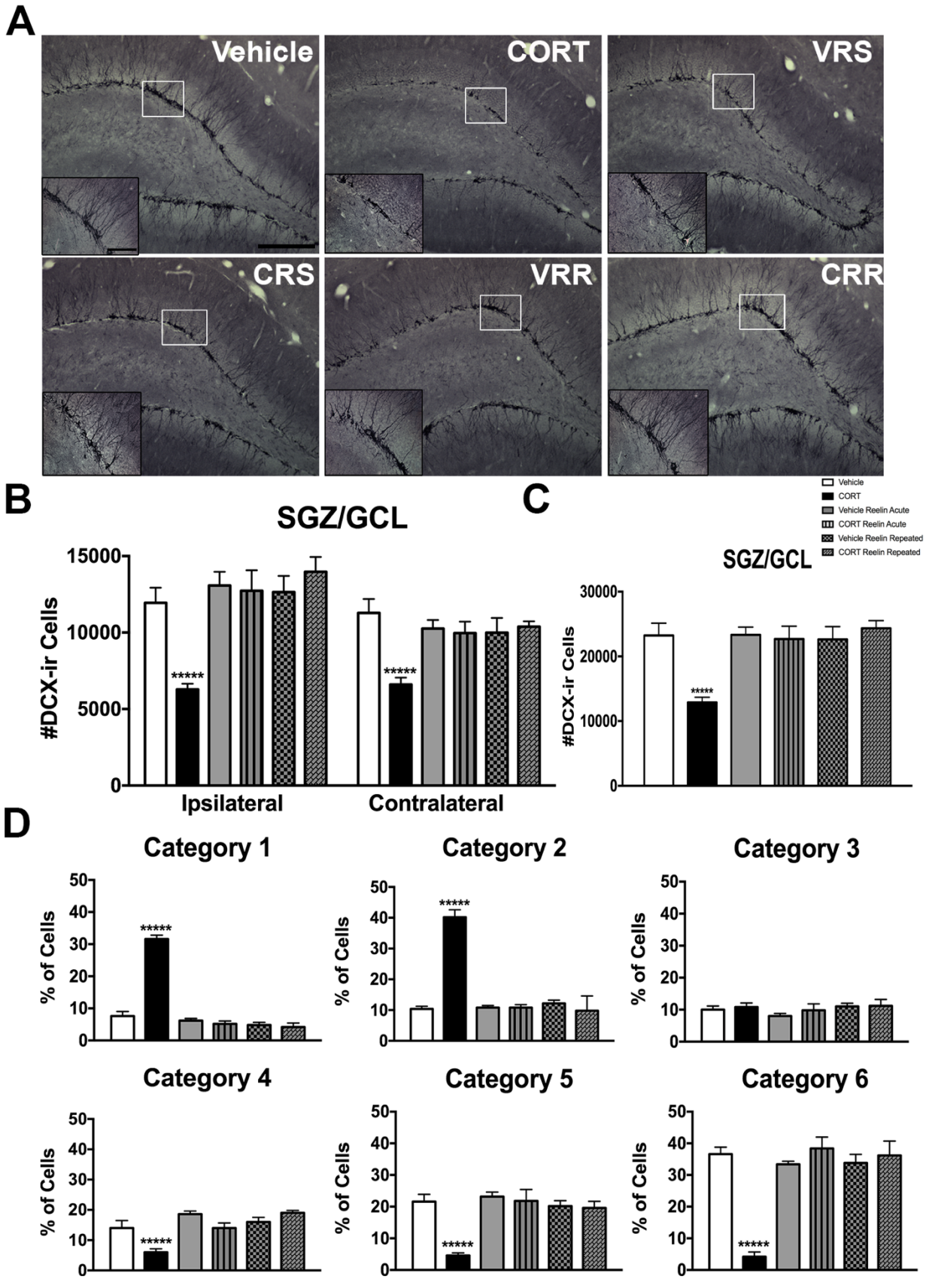
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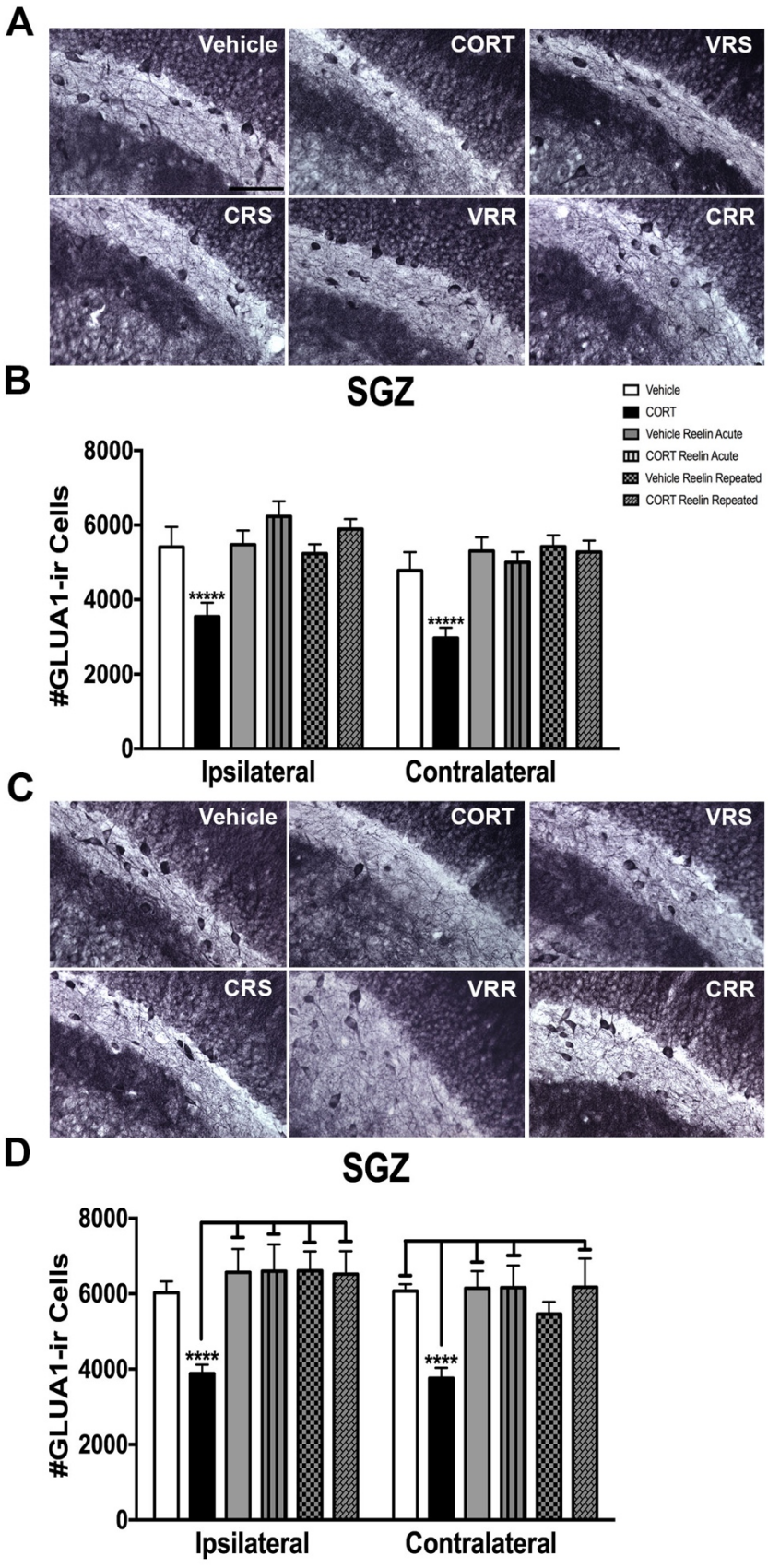
Fig 4. Effects of reelin on hippocampal neurogenesis at time 2. CORT had significant effects on hippocampal neurogenesis. Panel A shows representative photomicrographs of doublecortin expression in the granule cell/subgranular zone. Panel B and C shows the effects of treatment on the number of doublecortin-ir cells in the granule cell/subgranular zone. The CORT rats had significantly fewer doublecortin-ir cells in the ipsilateral hemisphere than all other groups ($p < .05$). In the contralateral hemisphere, the CORT rats had significantly fewer doublecortin-ir cells than all other groups ($p < .05$). When combining the ipsilateral and contralateral hemispheres the CORT rats had significantly fewer DXC-ir cells than all other groups. Panel D shows the quantified categorization of dendritic complexity using doublecortin staining. All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRR = vehicle reelin repeated; CRR = CORT reelin repeated.

significant group differences in both the ipsilateral ($F(5, 29) = 5.949$, $p < .001$) and contralateral hemispheres ($F(5, 29) = 7.473$, $p < .001$) SGZ (Fig. 5). *Post hoc* analyses revealed that the CORT rats had significantly fewer GLUA1-ir cells across both hemispheres compared to all other groups (all *p values* < .05). At time 2, stereological analyses revealed significant main effects in both the ipsilateral ($F(5, 29) = 4.196$, $p < .01$) and contralateral ($F(5, 29) = 4.196$, $p < .01$) SGZ. *Post hoc* analyses revealed that the CORT rats had significantly fewer GLUA1-ir cells in the ipsilateral hemisphere compared to all other groups *(p* values < .05), except for the vehicle rats ($p = .065$), and the CORT rats also had significantly fewer GLUA1-ir cells in the contralateral hemisphere than all other groups (p values < .05), except for the vehicle reelin repeated rats ($p = .444$) (Fig. 5).

3.6. GLUN2B

To investigate if CORT produces alterations in GLUN2B immunoreactivity and if treatment with reelin ameliorates this effect, we conducted semi-quantitative optical densitometry of GLUN2B. At time 1, our densitometry analyses revealed significant group differences in the ipsilateral GCL ($F(5, 29) = 5.436$, $p < .01$), ipsilateral SGZ ($F(5, 29) = 5.618$, $p < .001$), ipsilateral hilus ($F(5, 29) = 4.357$, $p < .01$), ipsilateral molecular layer ($F(5, 29) = 18.430$, $p < .001$), contralateral GCL ($F(5, 29) = 4.924$, $p < .01$), contralateral SGZ ($F(5, 29) = 3.087$, $p < .05$), and contralateral molecular layer ($F(5, 29) = 9.052$, $p < .001$) (Fig. 6). *Post hoc* analyses revealed that the CORT rats had significantly greater GLUN2B immunoreactivity in the ipsilateral GCL than all other groups (p values < .05) except for the CORT reelin repeated rats ($p = .152$). The CORT rats also had greater immunoreactivity in the SGZ than the vehicle and all reelin-treated groups (p values < .05) except for the CORT reelin repeated rats ($p = .063$) and greater immunoreactivity in the hilus than the vehicle reelin single, vehicle reelin repeated, and CORT reelin single ($p < .05$), but not vehicle ($p = .077$) or CORT reelin repeated rats ($p = .113$). Finally, the CORT rats had greater immunoreactivity in the molecular layer than all other groups (all p value*s* < .05).

In the contralateral hemisphere, the CORT rats had significantly greater GLUN2B immunoreactivity in the GCL than all other groups (all p value*s* < .05), greater immunoreactivity in the SGZ than the vehicle and vehicle reelin acute (p values < .05), but not CORT reelin single ($p = .090$), vehicle reelin repeated ($p = .063$), or CORT reelin repeated rats ($p = .233$), and greater immunoreactivity in the molecular layer than all other groups (all p values < .05). No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Ipsilateral
Contralateral

Fig 5. Effects of reelin on GLUA1-ir cells at times 1 and 2. Panel A shows representative photomicrographs of GLUA1 expression in the subgranular zone. Panel B shows the effects of treatment on the number of GLUA1-ir cells. The CORT rats had significantly fewer GLUA1-ir cells than all other groups in the ipsilateral hemisphere ($p < .05$) and contralateral hemisphere ($p < .05$), indicating that reelin is acting rapidly to normalize this deficit. Panel C shows representative photomicrographs of GLUA1 expression in the subgranular zone at time 2. The CORT rats had significantly fewer GLUA1-ir cells in the ipsilateral hemisphere than the vehicle reelin single, CORT reelin single, vehicle reelin repeated, and CORT reelin repeated rats ($p < .05$). In the contralateral hemisphere, the CORT rats had significantly fewer GLUA1-ir cells than vehicle, vehicle reelin single, CORT reelin single, and CORT reelin repeated rats ($p < .05$). All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRR = vehicle reelin repeated; CRR = CORT reelin repeated.

significant group differences were found for the contralateral hilus. We conducted optical densitometry of the sub regions CA3 and CA1, however no significant group differences were found (all p values > .05).

At time 2, our analyses revealed significant group differences in the ipsilateral GCL ($F(5, 29) = 13.434$, $p < .001$), SGZ ($F(5, 29) = 9.858$, $p < .001$), hilus ($F(5, 29) = 11.591$, $p < .001$), molecular layer ($F(5, 29) = 5.828$, $p < .001$) and the contralateral GCL ($F(5, 29) = 15.211$, $p < .001$), SGZ ($F(5, 29) = 10.628$, $p < .001$), hilus ($F(5, 29) = 7.273$, $p < .001$), and molecular layer ($F(5, 29) = 5.281$, $p < .01$) (Fig. 7). *Post hoc* analyses revealed that the CORT rats had greater GLUN2B immunoreactivity within the ipsilateral GCL than all other groups (all p values 05), greater immunoreactivity in the ipsilateral SGZ than all other groups (p values < .05), greater immunoreactivity in the hilus than all other groups (p values < .05), and greater immunoreactivity in the ipsilateral molecular layer than the vehicle reelin acute, vehicle reelin repeated, and CORT reelin repeated rats *(*p values < .05) but not the vehicle ($p = .081$) or CORT reelin single ($p = .636$) rats. In the contralateral hemisphere, the CORT rats had significantly greater GLUN2B immunoreactivity within the GCL than all other groups *(*all p values < .05), greater immunoreactivity in the SGZ than all other groups *(*all p values < .05), greater GLUN2B immunoreactivity in the hilus than all other groups (p values < .05) except for the CORT reelin single-treated rats ($p = .347$), and greater immunoreactivity in the molecular layer than all other groups *(*p values < .05), except for the CORT reelin single rats ($p = .723$). We conducted optical densitometry of the sub regions CA3 and CA1, however no significant group differences were found (all p values > .05).

3.7. Reelin Reduces CORT-Induced Deficits in $GABA_A$ β2/3-ir cells in the SGZ

We have previously found that CORT injections significantly reduce the number of $GABA_A$ β2/3-ir cells in the SGZ (Brymer et al., 2018). As reelin is released by GABAergic interneurons in the hippocampus, we investigated whether intrahippocampal reelin infusions could normalize this effect. At time 1, stereological analyses revealed group differences in the ipsilateral SGZ ($F(5,29) = 5.90$, $p <.001$) and contralateral SGZ ($F(5,29) = 5.45$, $p < .01$). *Post hoc* analyses showed that the CORT rats had significantly fewer $GABA_A$ β2/3-ir cells in the ipsilateral SGZ than all other groups (p values < .05) and significantly fewer $GABA_A$ β2/3-ir cells in the contralateral hemisphere than the vehicle, vehicle reelin repeated, and CORT reelin repeated rats (p values < .05), but not the vehicle reelin single ($p = .068$) or CORT reelin single

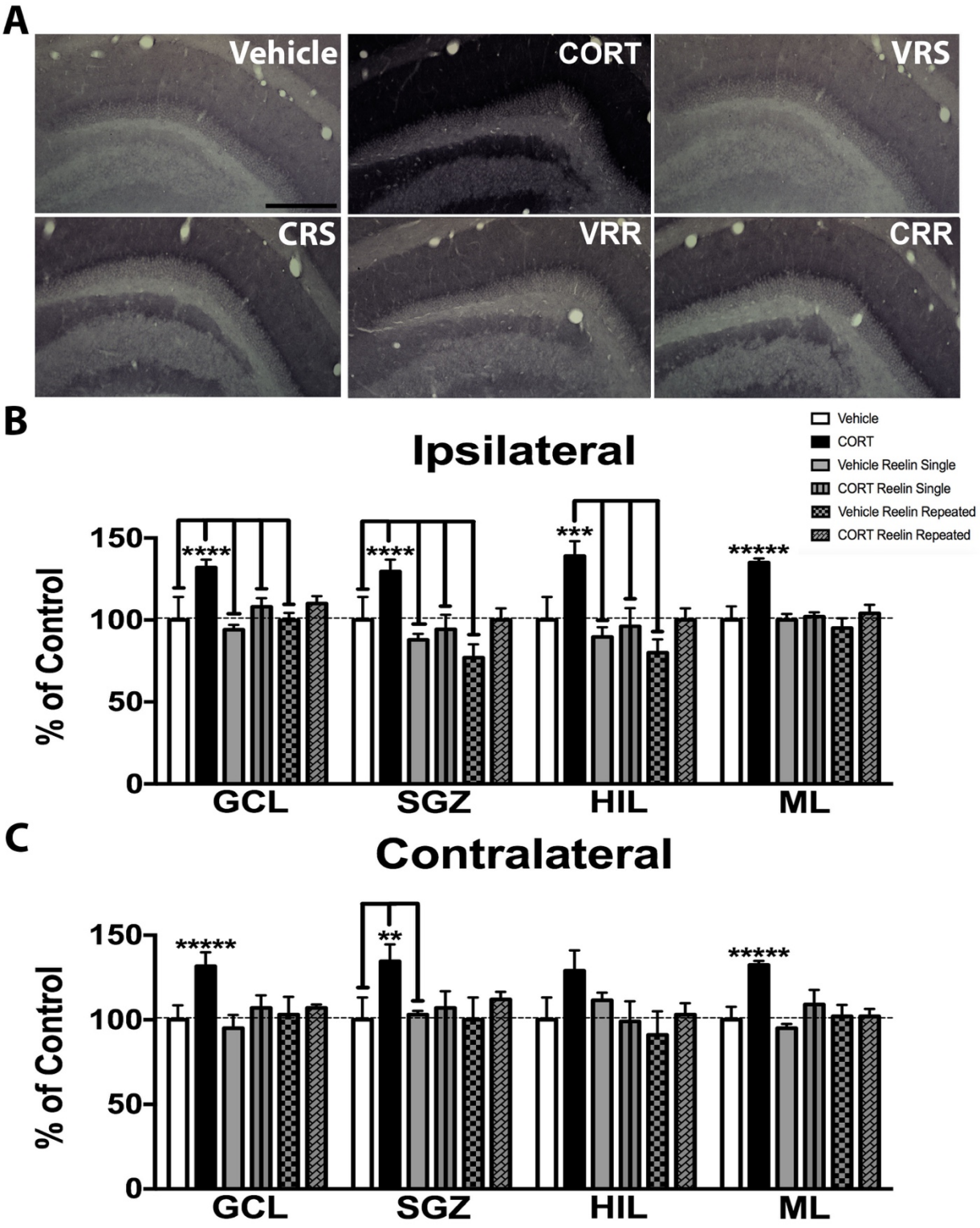
rats (p = .431). At time 2, our analyses revealed significant group differences in the ipsilateral SGZ ($F(5,29) = 3.54$, $p < .05$) but not the contralateral SGZ ($F(5,29) = 1.89$, $p = .133$). *Post hoc* analyses revealed that the CORT rats had significantly fewer $GABA_A$ β2/3-ir cells in the ipsilateral SGZ than the vehicle and CORT reelin single rats (p values < .05), but not the vehicle reelin single (p = .055), vehicle reelin repeated (p = .359) or CORT reelin repeated rats (p = .054) (Fig. 8.)

4. Discussion

Our results show that CORT produced clear behavioural and neurobiological changes across multiple measures. Specifically, CORT increased immobility time in the FST, impaired spatial memory on the OBL test, reduced hippocampal neurogenesis, decreased the number of GLUA1-ir cells in the SGZ, increased GLUN2B immunoreactivity across the DG, and dampened $GABA_A$ α2/3 expression levels. Importantly, intrahippocampal reelin infusions reversed these effects, suggesting that reelin could be exerting its antidepressant effects by facilitating hippocampal plasticity.

Reelin as a fast-acting antidepressant

Previous work in our lab and others has demonstrated that CORT treatment in rats creates a depressive phenotype characterized by increased immobility time in the FST compared to control rats (Lussier et al., 2013a), inhibited sexual behaviour (Gorzalka, Hanson, & Hong, 2001), decreased sucrose preference (David et al., 2009) and increased anxious behaviour in the predator odor test (Kalynchuk, Gregus, Boudrea, & Perrot-Sinal, 2004). We have recently shown that the TNF-α inhibitor etanercept and triclyclic antidepressant imipramine normalize immobility behaviour in the FST (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Fenton et al., 2015). Unexpectedly, our results here show that a single infusion of reelin 24 hours before behavioural testing restored time spent immobile on the FST to control levels. Repeated reelin infusions produced the same effect. What was unexpected about these results was the fasting acting nature of reelin. We had strong reason to believe that reelin would show antidepressant properties, but we did not expect those effects to occur over such a fast timeline. Work in our lab has shown that CORT treatment selectively reduces the number of reelin-positive cells in the SGZ, and the time course for the development of depression-like behaviour coincides with the time course for decreased reelin expression (Lussier et al., 2013a). Furthermore, we have also demonstrated that heterozygous reeler mice, which express 50% of the normal levels of reelin,


A
Vehicle
CORT
VRS
CRS
VRR
CRR
B
Ipsilateral
Vehicle
CORT
Vehicle Reelin Single
CORT Reelin Single
Vehicle Reelin Repeated
CORT Reelin Repeated
% of Control
150
100
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GCL
SGZ
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C
Contralateral
% of Control
150
100
50
0
*****
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*****
GCL
SGZ
HIL
ML

Fig 6. Effects of reelin on GLUN2B immunoreactivity at time 1. Panel A shows representative photomicrographs of GLUN2B expression in the dentate gyrus. Panel B shows the effects of treatment on GLUN2B expression in the ipsilateral hemisphere. CORT treatment increased GLUN2B immunoreactivity throughout the major subfields of the DG, and reelin reversed this. Panel C shows the effects of treatment on GLUN2B expression in the contralateral hemisphere. CORT treatment increased expression of GLUN2B throughout the major subfields of the DG, and reelin reversed this. All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRR = vehicle reelin repeated; CRR = CORT reelin repeated.

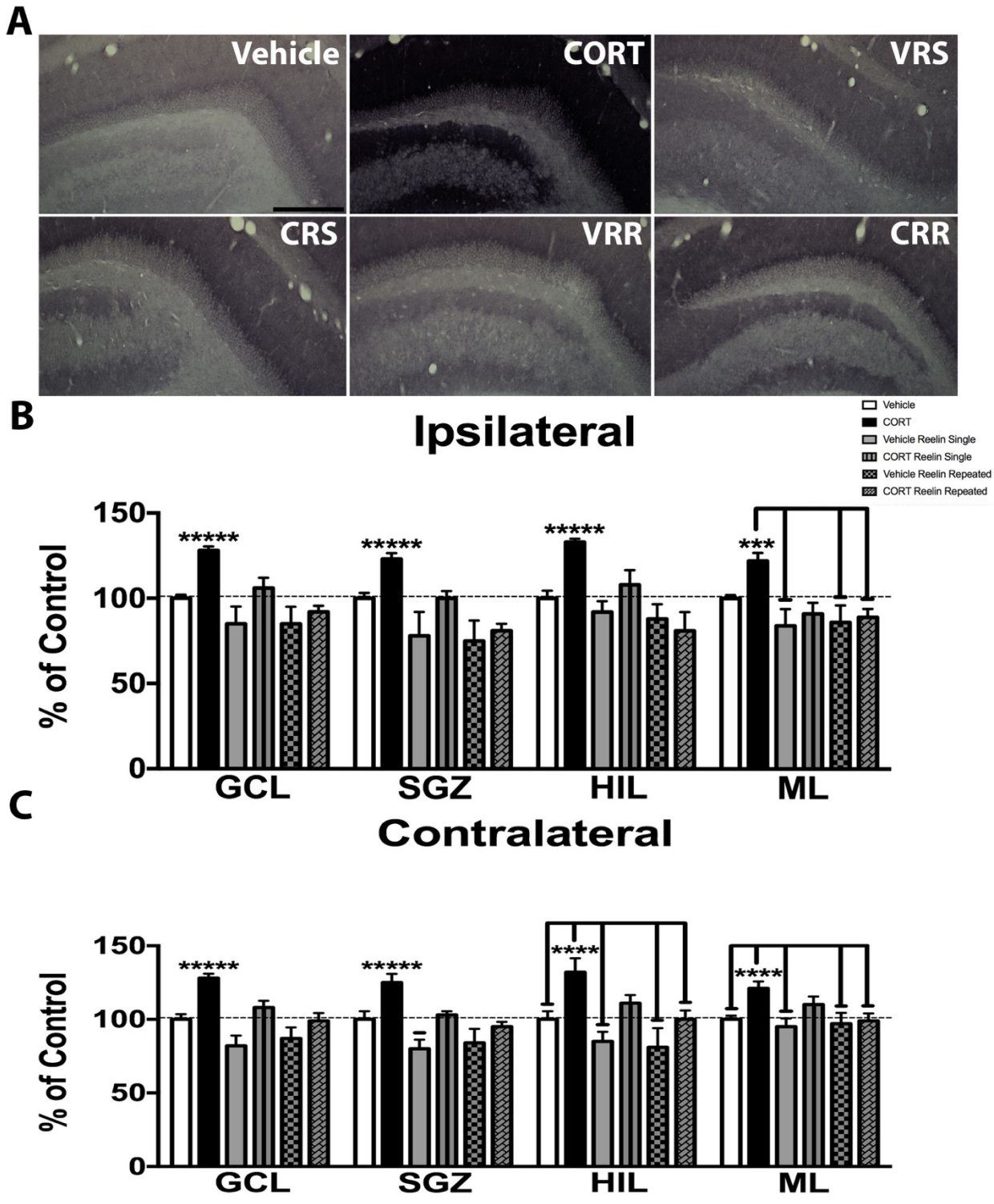

Fig 7. Effects of reelin on GLUN2B immunoreactivity at time 2. Panel A shows representative

photomicrographs of GLUN2B immunoreactivity in the DG. Panel B shows the effects of treatment on GLUN2B immunoreactivity in the ipsilateral hemisphere. CORT treatment increased GLUN2B immunoreactivity throughout the major subfields of the DG, and reelin reversed this. Panel C shows the effects of treatment on GLUN2B immunoreactivity in the contralateral hemisphere. CORT treatment increased GLUN2B immunoreactivity throughout the major subfields of the DG, and reelin reversed this. All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRR = vehicle reelin repeated; CRR = CORT reelin repeated.

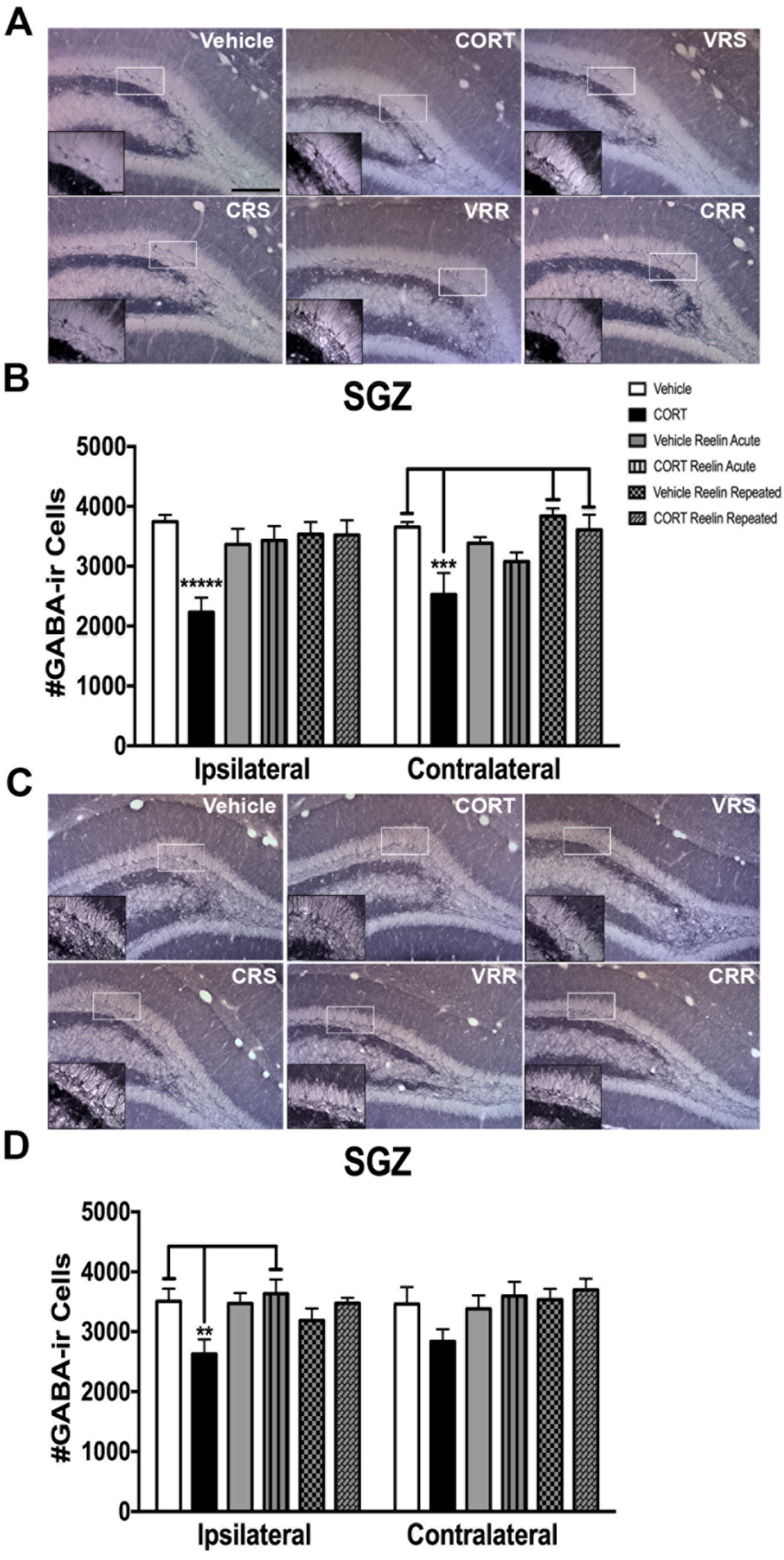
A
Vehicle
CORT
VRS
CRS
VRR
CRR
B
SGZ
Vehicle
CORT
Vehicle Reelin Acute
CORT Reelin Acute
Vehicle Reelin Repeated
CORT Reelin Repeated
#GABA-ir Cells
5000
4000
3000
2000
1000
0
*****
***
Ipsilateral
Contralateral
C
Vehicle
CORT
VRS
CRS
VRR
CRR
D
SGZ
#GABA-ir Cells
5000
4000
3000
2000
1000
0
**
Ipsilateral
Contralateral

Fig 8. Effects of reelin on $GABA_A\beta$ 2/3-ir cells at times 1 and 2. Panel A shows representative photomicrographs of $GABA_A$ β2/3 expression in the dentate gyrus at time 1. Panel B shows the effect of treatment on $GABA_A$ β2/3-ir cells in the subgranular zone. The CORT rats had significantly fewer $GABA_A$ β2/3-ir cells than all other groups in the ipsilateral hemisphere ($p < .05$). In the contralateral hemisphere, the CORT rats had significantly fewer $GABA_A$ β2/3-ir cells than the vehicle, vehicle reelin repeated, and CORT reelin repeated rats ($p < .05$). Panel C shows representative photomicrographs of $GABA_A$ β2/3-ir expression in the dentate gyrus at time 2. Panel D shows the effect of treatment on $GABA_A$ β2/3-ir cells in the subgranular zone. The CORT rats had significantly fewer $GABA_A$ β2/3-ir cells than the vehicle and CORT reelin single rats in the ipsilateral hemisphere ($p < .05$). All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRR = vehicle reelin repeated; CRR = CORT reelin repeated.

are more susceptible to the depressogenic effects of CORT (Lussier et al., 2011). Work in humans confirms this observation, with depressed patients exhibiting a reduction in reelin-positive hippocampal cells (Fatemi, Earle, & McMenomy, 2000). Taken together, our findings and the literature presented here suggest that reelin is critically involved in the pathogenesis of depression, and that increasing hippocampal reelin levels produces fast-acting antidepressant effects. How might reelin be achieving its fast-acting antidepressant effects? Our findings suggest a role for hippocampal neurogenesis or an interaction between glutamate and GABA.

Reelin as a mediator of spatial learning and memory

Chronic stress impairs hippocampal dependent memory, with deficits in fear conditioning and OBL memory observed (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Hoffman, Lorson, Sanabria, Olive, & Conrad, 2014; Hattiangady et al., 2014). Reelin is critically involved in cognition, particularly spatial memory. For example, a single intraventricular injection of reelin in naïve mice has been reported to increase spatial learning and memory (Rogers et al., 2011). Furthermore, reelin supplementation in a mouse model of angelman syndrome recovers deficits in spatial learning and memory (Hethorn et al., 2015). Our findings are in line with previous research, as we report that intrahippocampal reelin infusions restore CORT-induced deficits in OBL memory, with as little as one infusion. This reinforces the idea that reelin is involved in spatial learning and memory, as the OBL is a hippocampal-dependent task. Hippocampal-dependent memory requires NMDA receptor synaptic plasticity (Barker and Warburton, 2008), and hippocampal reelin regulates the composition and trafficking of NMDA receptor subunits (Bosch et al., 2016b). Interestingly, the OBL requires intact NMDA receptor synaptic plasticity within the hippocampus (Barker and Warburton, 2008). Therefore, a restoration of CORT-induced reductions in reelin by intrahippocampal reelin infusions may have played a role in the normalization of cognitive behaviour, and most likely requires a contribution of glutamate.

Reelin and hippocampal neurogenesis

The role of hippocampal neurogenesis in depression is currently unclear, as antidepressant effects can be achieved without increases in neurogenesis (Hanson, Owens, & Nemeroff, 2011) and studies of post-mortem hippocampal tissue from patients with depression have generally failed to find reductions in neurogenesis (Reif et al., 2006). However, Hill et al. (2015) have convincingly shown the causative role that neurogenesis plays in depression-like behaviour. Through a transgenic mouse line, hippocampal neurogenesis was increased, which in

turn rescued a CORT-induced depressive-phenotype. Work in our lab has shown that CORT decreases hippocampal neurogenesis, and the triclyclic antidepressant imipramine restores this (Fenton et al., 2015; Lussier et al., 2013a). Here, we report that CORT decreases both the number and maturation rate of newborn neurons in the DG. A single infusion of reelin 24 hours before sacrifice significantly increases the number of newborn neurons in the ipsilateral, but not contralateral hemisphere, but does not affect the maturation rate of these neurons; however, at time 2, a single infusion of reelin recovers both the number and maturation rate of these newborn neurons. These findings are consistent with those observed in the literature. Pujadas et al. (2010) found that mice overexpressing reelin have a significant increase in DCX-ir granule cells, accompanied by increased complexity in their dendrites. Furthermore, reeler mice show a significant reduction in newborn neurons in the DG (Won et al., 2006). Our results suggest that the fast-acting antidepressant effects of reelin are most likely not directly through an increase of neurogenesis, as this increase in proliferating neurons does not correspond to functional integration and maturation as corroborated by our dendritic complexity analyses. Our findings of reelin on glutamate and GABA dovetail nicely with the effects of reelin on neurogenesis and behaviour.

Reelin and glutamate

Chronic stress disrupts glutamatergic signaling in the hippocampus. This is typically manifested as a decrease in AMPA receptor expression and an increase in NMDA receptor expression. For example, stress has been shown to decrease phosphorylation of the AMPA receptor Ser831-GluA1 within the hippocampus, while increasing hippocampal GLUN2B receptor expression (Ciu, Wu, She, & Liu, 2012; Caudal, Godsil, Mailliet, Bergerot, & Jay, 2010). In line with these findings, we observed that the CORT rats had a significant decrease of GLUA1-ir cells in the SGZ, in addition to an increased expression of GLUN2B immunoreactivity throughout the DG. Interestingly, we found that either single or repeated intrahippocampal reelin infusions across both times 1 and 2 significantly increased the number of GLUA1-ir cells in the SGZ, and decreased GLUN2B immunoreactivity across the DG back to control levels. These findings are similar to those reported elsewhere. For example, application of reelin to cultured hippocampal CA1 neurons elevated AMPAR-mediated synaptic currents (Qiu et al., 2006) and heterozygous reeler mice display a significant reduction in GLUA1 subunit clustering in cultured hippocampal neurons (Qiu and Weeber, 2007). Furthermore,

overexpression of reelin through reelin –OE mice results in a significant reduction of GluN2B in cultured hippocampal neurons (Teixeira et al., 2011), and application of recombinant reelin to cultured hippocampal neurons leads to a significant reduction in both GLUN2B-mediated synaptic currents and duration of GLUN2B subunits within synapses (Groc et al., 2007). Therefore, the picture that emerges is that reelin alters the balance of glutamate by increasing AMPA and therefore hippocampal synaptic plasticity, and decreasing NMDA and excitotoxic events.

Reelin and GABA

Previous work in our lab has shown that repeated CORT injections decrease $GABA_A$ receptor expression in the hippocampus and the number of $GABA_A$ α2/3-ir cells (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2013b). Here, we found that CORT decreased the number of $GABA_A$ α2/3-ir cells within the SGZ at both times 1 and 2. At time 1, we observed that a single intrahippocampal reelin infusion restored this deficit to control levels. Consistent with this finding, GAD67, the key enzyme regulating GABA synthesis, is co-expressed with reelin in GABAergic neurons. Reelin and GABA then bind to postsynaptic receptors distributed throughout dendrites, soma, and axon of pyramidal neurons (Costa et al., 2004). What therefore might emerge is that reelin is restoring the CORT induced $GABA_A$ receptor internalization, creating a neuroprotective mechanism that facilitates SGZ plasticity.

The role of reelin in restoring the balance between GABA and glutamate

For the first time, we have reported here that a single intrahippocampal reelin infusion has fast-acting antidepressant actions accompanied by a restoration of hippocampal neurogenesis, glutamate, and GABA neurotransmission. What might be the mechanism behind the fast-acting antidepressant effects of reelin? We propose that one such pathway might be through a restoration of the CORT-induced imbalance between GABA and glutamate (Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2013b) . Here, we reported that CORT decreased the number of GLUA1-ir cells within the SGZ, increased GLUN2B immunoreactivity throughout the DG, and decreased the number of $GABA_A$ α2/3-ir cells within the SGZ. Remarkably, a single intrahippocampal reelin infusion restored these deficits. These results suggest that reelin is facilitating synaptic plasticity in the SGZ through an uncoupling of GABA and glutamate. By increasing AMPA, decreasing NMDA, and increasing $GABA_A$ activity, reelin would be

promoting SGZ plasticity in a rapid time course, which in turn would have pro-cognitive effects and would act to increase hippocampal neurogenesis. A question that remains to be tested is assessing the contribution of glutamate or GABA to reelin's antidepressant effect, perhaps through animal knockdowns or antagonists.

5. Conclusion

In conclusion, the results provided here are the first to show that reelin has fast-acting antidepressant effects, which are accompanied by an increase in hippocampal neurogenesis, and a restoration of the balance between GABA and glutamate in the hippocampus. It is proposed that reelin's antidepressant actions lie in uncoupling GABA and glutamate. Further work needs to be conducted to determine the extent of the contribution of GABA and glutamate to reelin's antidepressant actions.

CHAPTER 5

CNQX BLOCKS REELIN'S FAST-ACTING ANTIDEPRESSANT EFFECTS IN A PRECLINICAL ANIMAL MODEL OF DEPRESSION

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Kyle J Brymer was involved in the design of the experiment, conducting all aspects of the experiment, analyzing the data, and writing up the manuscript. Kayla A Bannouvong was involved in assisting in conducting the experiments. Hector J Caruncho and Lisa E Kalynchuk were involved in planning the experimental design and formulating the manuscript. The current chapter was included in the dissertation as the finding that the fast-acting antidepressant effects of reelin require AMPA receptor activation is a novel finding and successfully completes the reelin story for the purposes of this dissertation.

Abstract

Depression remains difficult to treat, with a significant proportion of patients not responding to conventional antidepressants. Furthermore, antidepressants take weeks to achieve their therapeutic effects, which exacerbates the need for novel fast-acting antidepressants. Previous work in our lab has shown that a single intrahippocampal infusion of the extracellular matrix protein reelin rapidly reverses depression-like behaviour in the forced swim test (FST), alters hippocampal neurogenesis, and increases the number of GLUA1-ir cells in the subgranular zone. Here, we tested the involvement of AMPA receptors in the fast-acting antidepressant effects of reelin by infusing the AMPA antagonist CNQX after a single intrahippocampal reelin infusion during corticosterone (CORT) treatment. We then assessed changes in the FST, and examined hippocampal neurogenesis, Fos expression, and hilar microglia morphology. CORT injections increased the time spent immobile in the FST, decreased hippocampal neurogenesis, and contributed towards an active microglia phenotype. A single intrahippocampal reelin infusion decreased time spent immobile in the FST, increased the number but not the complexity of doublecortin (DCX)-ir cells, and did not alter the CORT induced microglial phenotype. A single intrahippocampal CNQX infusion following reelin increased the time spent immobile in the FST, increased the number but not the complexity of DCX-ir cells, increased the number of Fos cells active during the FST, and and did not alter the CORT induced microglial phenotype. These novel results demonstrate that CNQX blocks reelin's fast-acting antidepressant effects on the FST without altering reelin's effects on hippocampal neurogenesis or microglia morphology.

1. Introduction

Depression remains difficult to treat with conventional antidepressants, with estimates of treatment resistant depression occurring in 10-50% of depressed patients (Wiles, 2014; Al-Harbi, 2012). As the majority of antidepressants target the monoamine system and a significant proportion of patients do not respond to medication, this suggests that the monoamine system per se is not necessarily a prerequisite for antidepressant action (Duman, 2014; Willner, Scheel-Jruger, & Belzung, 2013, 2013; Blows, 2000). Therefore, there is a need for novel antidepressants that are not reliant on the monoaminergic system.

Both preclinical and human studies implicate impairments in the extracellular matrix protein reelin in the pathogenesis of depression (Lussier et al., 2013a; Fatemi, 2005). For example, work in our lab demonstrates that repeated corticosterone (CORT) injections in rats significantly reduces the number of reelin immunoreactive (IR) cells in the subgranular zone (SGZ) of the hippocampus (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Lussier et al., 2013a). Moreover, heterozygous reeler mice, which express 50% of the normal level of reelin, are more susceptible to the depressogenic effects of CORT (Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2011). Confirming these findings, human post-mortem studies reveal a reduction in reelin-ir cells in the hippocampus of depressed patients (Fatemi, Earle, & McMenomy, 2000). These findings raise the question of whether reelin can produce an antidepressant effect. Our group has data that shows that a single infusion of recombinant reelin into the dorsal hippocampus of CORT-treated rats produces an antidepressant response in the forced-swim test (FST) in as little as 24 hours (Brymer et al., unpublished observations). Moreover, this rapid antidepressant response is accompanied by a restoration of doublecortin (DCX) cell number but not complexity in the SGZ. Interestingly, a single recombinant reelin infusion also increases the number of GLUA1-ir cells in the SGZ to control levels. This finding is particularly interesting, as heterozygous reeler mice display a significant reduction in GLUA1 subunit clustering in cultured hippocampal neurons (Qiu & Weeber, 2007). Therefore, in this experiment we examined whether blocking AMPA receptor activity can abolish the fast-acting antidepressant effect of reelin.

We hypothesized that recombinant reelin infusions into the dorsal hippocampus of CORT-treated rats would reverse depression-like behaviour on the FST. We also hypothesized that infusion of the AMPA antagonist CNQX into the dorsal hippocampus shortly before the FST

would abolish the fast-acting antidepressant effect of reelin. To test these hypotheses, rats underwent stereotaxic surgery to implant an indwelling cannula into the dorsal hippocampus, and then received 21 days of CORT or vehicle injections. We infused recombinant reelin into the dorsal hippocampus as well as CNQX in another subset of rats, and then assessed depression-like behaviour using the FST, and alterations in hippocampal neurogenesis, Fos protein expression, and microglial morphology.

2. Materials and Methods

2.1. Animals

We used 30 adult male Long-Evans rats purchased from Charles River (QC, Canada). The rats weighed 200-250 g at the time of arrival from the breeder. Rats were individually housed in rectangular polypropylene cages containing standard laboratory bedding with access to food and water *ad libitum.* The rodent colony room was maintained at an ambient temperature of 20 ± 1 °C on a 12:12 light-dark cycle (lights on at 7 am). All experimental procedures were in accordance with the guidelines of the Canadian Council and Animal Care and an animal care protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. All efforts were made to minimize the number of rats used in the present study.

2.2. Stereotaxic Surgery

Stereotaxic surgery was conducted as previously described (Botterill, Nogovoystin, Caruncho, & Kalynchuk, 2016; Botterill, Guskjolen, Marks, Caruncho, & Kalynchuk, 2015b). Each rat was deeply anesthetized with isoflurane (5% initial, 2-2.5% maintenance) and then secured into a stereotaxic apparatus using ear bars. Rats were given ketoprofen (Anafen, ketoprofren, 10 mg/kg s.c.) to reduce pain and inflammation. At flat skull position, a single cannula (C313G/spc, Plastics 1, Roanoke, VA, USA) was chronically implanted into the left hemisphere of the dorsal hippocampus using the following coordinates from the rat brain atlas (relative to bregma): -3.5 mm anteroposterior, +2.6 mm mediolateral, -3.1 mm dorsoventral (Paxinos & Watson, 1998). The cannula was secured to the skull with 4 jeweler screws and dental acrylic. A dummy cannula was then inserted into the guide cannula to prevent debris from entering the site during the course of the study. To minimize the risk of post-surgical infection, we administered the antibacterial-antifungal ointment Hibitane (Chlorhedixine acetate B.P. 1% [w/w]) daily around the incision site for at least one week after the surgery.

2.3. Experimental procedures

2.3.1. CORT and Vehicle Injections

All rats were briefly handled once per day for seven days prior to the start of CORT injections. We then weight-matched the rats and randomly assigned them to one of the following six treatment groups: 21 days of vehicle injections (n = 5), 21 days of CORT injections (n = 5); vehicle + a single reelin infusion on day 21 (vehicle reelin single, n = 5); CORT + a single reelin infusion on day 21 (CORT reelin single, n = 5), vehicle + a single reelin infusion on day 21 + an infusion of CNQX on day 22, 30 minutes prior to the FST (vehicle reelin single CNQX, n = 5; and CORT + a single reelin infusion on day 21 + an infusion of CNQX on day 22, 30 minutes prior to the FST (CORT reelin single CNQX, n = 5) (Fig. 1). All CORT and vehicle injections were administered subcutaneously once per day between 9:00 and 10:00 am. CORT (Steraloids, Newport, RI, USA) was suspended in 0.9% physiological saline with 2% (v/v) Tween-80 (Sigma-Aldrich) and given at a dose of 40 mg/kg in a volume of 1 ml/kg. Our lab has reported that the 40 mg/kg dose reliably increases depression-like behaviour in rats (Brymer et al., 2018; Lussier et al., 2013a). The body weight of each rat was recorded daily for the entire 21 days of CORT or vehicle injections.

2.3.2. Intrahippocampal Reelin/CNQX infusions

Intrahippocampal reelin and CNQX infusions were administered in a dedicated procedures room for the duration of the experiments. We used a 2 µl Hamilton syringe secured to an infusion pump (PHD 2000; Harvard Apparatus, Holliston, MA, USA). The tip of the Hamilton syringe was inserted into PE-50 tubing connected to an infusion needle (C313l/spc, Plastics 1) that extended 1 mm below the base of the guide cannula. The infusion needle was inserted into the guide cannula of each rat and secured to the base of the cannula pedestal. Recombinant reelin (3820-MR-025/CR; R & D Systems, Minneapolis, MN, USA) was reconstituted immediately prior to infusions to a working concentration of 1 µg per 1µl in 0.1 M PBS (pH = 7.4). A total of 1µl of reelin was infused at a rate of 0.5µl per minute over 2 minutes into the hippocampus. CNQX (Cat. No. 0190; Tocris Bioscience, Bristol, UK) was reconstituted immediately prior to infusions to a working concentration of 1 µg per 1µl in a solution containing 20% DMSO and 80% saline. A total of 1µl of CNQX was infused at a rate of 0.5µl per minute over 2 minutes. The infusion needle was left in place for an additional two mins after the infusion to facilitate diffusion. We then slowly removed the infusion needle from the guide

cannula.

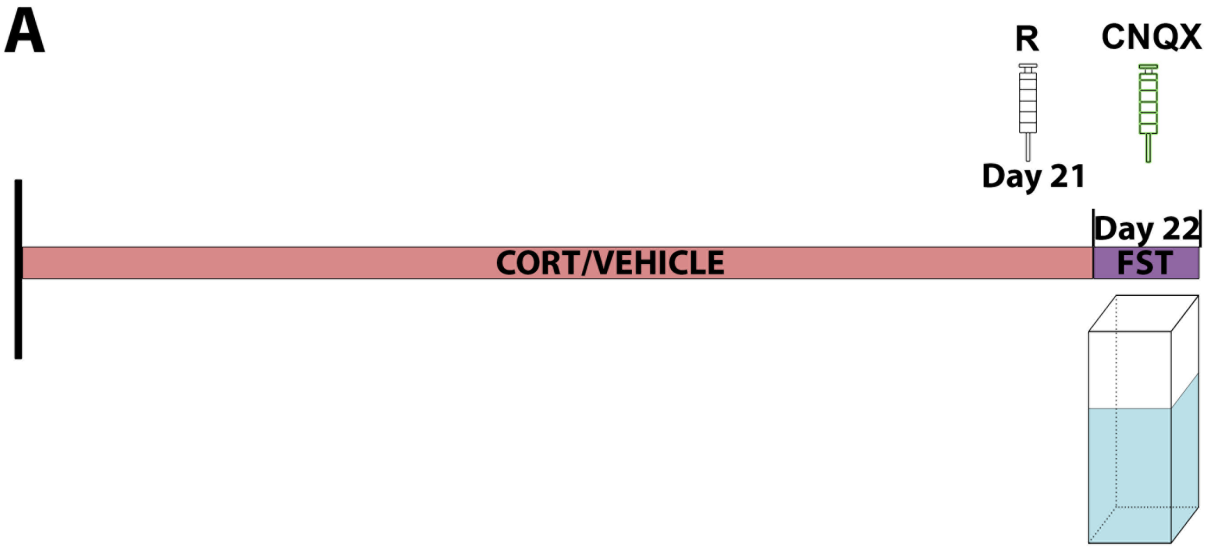


Fig 1. Schematic representation of the experimental design of the study. Animals underwent stereotaxic surgery to implant an indwelling cannula into the left dorsal hippocampus. Animals were weight matched and received 21 days of 40 mg/kg of corticosterone (CORT) or vehicle injections. A subset of animals received a 1µg/µl of reelin on day 21 of CORT/vehicle injections, and a further subset also received a single 1 µg/µl CNQX infusion on day 22, 30 minutes prior to the FST.

2.4. Behavioural Testing

2.4.1. FST

The FST was carried out in a dedicated procedures room that was not used for any other aspect of the study. All behaviours were recorded with a digital video camera and stored for offline analyses. The FST was conducted on day 22, the day after the final CORT injection and intrahippocampal reelin infusion (Fig. 1). We used a modified version of the Porsolt test, as previously described (Lussier et al., 2013a; Gregus, Wintink, Davis, & Kalynchuk, 2005). Each rat was individually placed in a Plexiglas swim tank (25 cm wide x 25 cm long x 60 cm high, 27 ± 2 °C water, 30 cm deep) for 10 minutes. We measured the duration of time each rat spent immobile, struggling, and swimming.

2.5. Histology

2.5.1. Perfusions

Each rat was deeply anesthetized with 5% isoflurane and then transcardially perfused using room-temperature physiological saline, followed by ice-cold 4% (w/v) formaldehyde fixative (pH = 7.4). The brains were extracted from the cranial vault and immersed in the same formaldehyde fixative for 48 hours at 4 °C. The brains were then sectioned in the coronal plane at 30 μm on a vibrating microtome (VT1200s, Leica Biosystems, Nussloch, Germany). Sections were collected and stored at −20 °C until use in a cryoprotectant solution containing 30% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in 0.1 M PBS (pH = 7.4).

2.5.2. Immunohistochemistry

We used a standard immunohistochemistry technique with widely used and commercially available antibodies for all of our assays. Immunostaining was done on free-floating sections in six-well tissue culture plates under gentle agitation for every step. To ensure consistent immunohistochemical processing, we processed all sections in unison with treatment groups counterbalanced across all tissue plates. To confirm the specificity of our antibodies, we omitted the primary antibody from an additional well of free-floating sections. In the absence of the primary antibody, we were unable to detect any immunoreactive cells.

Doublecortin immunohistochemistry was run as previously described (Botterill, Brymer, Caruncho, & Kalynchuk, 2015a; Lussier et al., 2013a). Sections underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85 °C for 30 mins. The sections were then blocked

in 5% (v/v) normal goat serum (NGS), 1% (w/v) bovine serum albumin (BSA), and 0.5% (v/v) Triton X-100 in 0.1 M TBS (pH = 7.4), followed by incubation in a rabbit anti-DCX polyclonal primary antibody (1:1000; Cell Signalling Technologies) diluted in blocking solution for 24 hours at room temperature. On the following day, the sections were treated with 5% (v/v) H_2O_2 in 0.1 M TBS for 30 minutes to block endogenous peroxidase activity. The sections were then incubated for 1 hour in biotinylated goat anti-rabbit secondary antibody (1:500; Vector Laboratories) diluted in 5% (v/v) NGS, 1% (w/v) BSA, and 0.5% (v/v) Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. Sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 0.025% (w/v) DAB, 4.167% $NiSO_4$, and 0.002% (v/v) H_2O_2. The sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using a series of graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

Fos immunohistochemistry was run as previously described (Botterill et al., 2014). Sections were treated with 0.3% H202 in 0.1 M PBS for 30 minutes to block endogenous peroxidase activity. The sections then underwent heat-induced epitope retrieval in sodium citrate buffer (pH = 6.0) at 85°C for 30 minutes. The sections were then blocked in 5% (v/v) normal goat serum (NGS), 1% (w/v) bovine serum albumin (BSA), and 0.3% (v/v) Triton X-100 in 0.1 M TBS (pH = 7.4), followed by incubation in a rabbit anti-Fos polyclonal primary antibody (1:2000; Abcam) diluted in blocking solution for 72 hours at 4°C. After 72 hours, the sections were incubated for 2 hours in biotinylated goat anti-rabbit secondary antibody (1:500; Vector Laboratories) diluted in 5% (v/v) NGS, 1% (w/v) BSA, and 0.3% (v/v) Triton X-100 in 0.1 M TBS, followed by avidin-biotin peroxidase complex (1:500, Vector Laboratories) for 1 hour. Sections were then rinsed with 0.175 M sodium acetate (pH = 6.8) and visualized with 0.025% (w/v) DAB, 4.167% $NiSO_4$, and 0.002% (v/v) H_2O_2. The sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dried overnight, dehydrated using a series of graded alcohols, cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

To examine microglia cell morphology, CD11b immunohistochemistry was conducted. Briefly, free-floating sections were rinsed in 0.1 M TBS (pH = 7.4) and incubated in 1% H202 for 30 min to block endogenous peroxidase activity. The sections were then blocked in a 0.3%

(v/v) Triton X-100 TBS buffer containing 5% (v/v) normal horse serum (NHS) for 1 hour at room temperature, followed by incubation with primary mouse anti-CD11b, clone OX-42, monoclonal antibody diluted in blocking solution (1:200; Millipore, USA) for 48 hours at room temperature. The tissue was then incubated with a biotiylnated secondary antibody (horse anti-mouse; 1:200; Sigma-Aldrich) for 2 hours at room temperature, and then in an avidin-biotin complex (1:500. Vector Laboratories, USA) for 30 minutes at room temperature. Immunolabelling was visualized with 0.05% (w/v) DAB, 4.167% (w/v) nickel ammonium sulphate, and 0.0078% (v/v) H202 diluted in 0.175 M sodium acetate. The sections were then mounted onto glass slides using 0.2 M PB (pH = 7.4), air dryed overnight, dehyrdrated using a series of graded alcohols, cleared in xylens, and coverslipped with Permount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA).

2.5.2.1. Quantification of immunohistochemistry

2.5.2.1.1. DCX cell counting

Quantification of immunolabelling was conducted as previously described (Botterill, Brymer, Caruncho, & Kalynchuk, 2015a; Lussier et al., 2013a). All analyses were performed by researchers blind to the treatment conditions. Immunostained sections were examined using a Nikon Eclipse E800 microscope with a motorized stage and digital camera (MicroFire, Optronics, Goleta, CA, USA) connected to a dedicated stereology computer. The dentate SGZ (defined as a two-cell width zone in between the inner granule cell layer and the hilus) and granule cell layer (GCL) were traced at 4 X magnification using a computerized stereology program for DCX-ir cells (StereoInvestigator, MicroBrightfield, Williston, VT, USA). DCX was counted in both the ipsilateral and contralateral hemispheres at 40 x magnification. All counts utilized unbiased stereology using a modified optical fractionator method that excludes cells in focus at the uppermost focal plane to reduce oversampling (Lussier et al., 2013a). The total number of DCX-ir cells was estimated using the following formula: $N_{total} = \sum Q^{-} \times 1 / ssf \times A(x, y\ step) / a(frame) \times t / h$. $\sum Q^{-}$ represents the number of counted cells, ssf is the section sampling fraction (1 in 12), A(x, y step) is the area associated with each x, y movement (10,000 μm^2), a(frame) is the area of the counting frame (3600 μm^2), t is the weighted average section thickness, and h is the height of the dissector (12 μm) (Botterill, 2015a; Fenton et al, 2015; Lussier et al, 2013a.). To avoid counting sectioning artifacts, we used a guard zone of 2 μm.

2.5.2.1.2. Characterization of immature DCX-ir neurons

We utilized a dendritic categorization method that our laboratory has used in the past (Botterill, Brymer, Caruncho, & Kalynchuk, 2015a; Lussier et al., 2013a) to determine if our intrahippocampal reelin and CNQX infusions paradigm altered the dendritic morphology of immature DCX-ir neurons. A meander scan method was used to randomly select 100 DCX-ir cells from each rat. A researcher blind to the experimental conditions then assigned the cell to one of six complexity categories based on both the presence and extent of apical dendrites (Fig. 3). The proliferative stage encompassed categories one (no process) or two (one small process). Category three (medium process reaching the granule cell layer) and category four (process reaching the molecular layer) made up the intermediate stage of development. Finally, category five (one major process extending into the molecular layer) and category six (defined dendritic tree with delicate dendritic branching in the granule cell layer) represented the postmitotic stage. Data are presented as the percentage of DCX-ir cells in each of the six categories.

2.5.2.1.3. Fos cell counting

Quantification of FOS immunoreactivity was quantified as previously described (Botterill et al., 2014) on a minimum of five sections per brain with 300 μm between sections. Contours were traced at 4 X magnification with a brain atlas serving as a guide (Paxinos & Watson, 1998). Fos-ir cells were counted within these contours at 20 X magnification using a meander scan profile counting method (Knapska & Maren, 2009). We divided the total number of Fos-ir cells in each region by the total area captured within the traced contours (in μm) to determine the density of Fos-ir cells within each traced region for each subject. This density value was then re-expressed as the number of Fos-ir cells per mm^2. Therefore, our reported values reflect the average number of Fos-ir cells within a defined contour within each region (Botterill et al., 2014).

2.5.2.1.4. Morphological analyses of microglia in the hilus

The morphology of randomly selected microglia cells in the hilus was examined using a neuron tracing system (Neurolucida; Microbrightfield) linked to a Nikon microscope. Eight microglial cells per rat (5 rats per group) were randomly selected and individually traced in their entirety on the live image using a 100 X lens with oil immersion. The following criteria were followed for selection of microglial hilar cells: (1) location of the cell body in the hilus; (2) full

staining of the cell body and its processes; (3) most microglial processes unobscured by background staining or by other cells. NeuroExplorer software (Microbrightfield) was used for the quantification of somatic and process morphological parameters. Somatic metrics included cell body and ferret maximum (the longest diameter of the soma). Total process length, number of nodes and ends, average process diameter, and the number of primary processes were all quantified. We conducted a Sholl analysis in which the cell body was placed at the center of concentric circles (with incrementing diamters of 10 μm), and the number of intersections at all radial distances were quantified (Lebedeva, 2017).

2.6 Statistical Analyses

All statistical analyses were carried out using IBM's Statistical Package for Social Sciences v24. We determined statistical significance using one-way analysis of variance (ANOVA), and *post hoc* comparisons were made using Tukey HSD when appropriate. The criterion for statistical significance was set at $p < 0.05$. All graphs depict the mean ± standard error of the mean.

3. Results

3.1. CORT had Significant Effects on body Weight

Figure 2 shows the effects of treatment on body weight. Consistent with previous reports from our lab (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Fenton et al., 2015; Lussier et al., 2013a), CORT had significant effects on body weight. Significant group differences were found on days 7 ($F(5, 29) = 5.251$, $p < .01$), 14 ($F(5, 29) = 10.233$, $p < .001$), and 21 ($F(5, 29) = 11.42$, $p < .001$). *Post hoc* analyses revealed that the CORT rats weighed significantly less than the vehicle rats on day 7 ($p < .05$), significantly less than the vehicle and vehicle reelin single CNQX rats on day 14 (p values < .05), and significantly less than the vehicle, vehicle reelin single, and vehicle reelin single CNQX rats on day 21 (p values < .05). Both the CORT reelin single and CORT reelin single CNQX rats weighed significantly less than the vehicle rats on day 7 (p values < .05), significantly less than the vehicle and vehicle reelin single CNQX rats on day 14 (p values < .05), and significantly less than the vehicle, vehicle reelin single, and vehicle reelin single CNQX rats on day 21 (p values < .05). Finally, the vehicle reelin single rats weighed significantly less than the vehicle rats on day 7 (p value < .05).

3.2. CNQX Abolishes the fast-Acting Antidepressant Effects of Reelin on the FST

Figure 2 shows the effect of treatment on FST behaviour. We found significant group differences for immobility ($F(5, 29) = 12.433$, $p < .001$), struggling ($F(5, 29) = 2.652$, $p < .05$), and swimming ($F(5, 29) = 9.494$, $p < .001$). *Post hoc* analyses revealed that the CORT and CORT reelin single CNQX rats spent significantly more time immobile than all other groups (p values < .05), and the CORT rats spent significantly less time swimming than the vehicle, vehicle reelin single, and CORT reelin single rats (p values < .05). Finally, the CORT reelin single CNQX rats spent significantly less time swimming than the vehicle, vehicle reelin single, , and CORT reelin single rats (p values < .05).

3.3. Reelin and CNQX increase DCX cell counts

Figure 3 shows the results of our DCX analyses. Consistent with previous reports from our lab (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Fenton et al., 2015), we found a significant main effect of treatment on the total number of DCX-ir cells ($F(5, 29) = 3.859$, $p < .05$). *Post hoc* analyses revealed that the CORT rats had significantly fewer DCX-ir cells in the ipsilateral hemisphere than the CORT reelin single and CORT reelin single CNQX rats (p values < .05), but did not differ from the vehicle ($p = .231$), vehicle reelin single ($p = .637$), or vehicle reelin single CNQX rats ($p = .839$). No group differences were found when combining the ipsilateral and contralateral hemispheres (p values > .05).

We also found significant group differences for our dendritic complexity analyses. Significant group differences emerged for the % of cells in categories 1 ($F(5, 29) = 8.054$, $p < .001$), 2 ($F(5, 29) = 67.242$, $p < .001$), 4 ($F(5, 29) = 9.649$, $p < .001$), 5 ($F(5, 29) = 23.314$, $p < .001$), and 6 ($F(5, 29) = 15.193$, $p < .001$). *Post hoc* analyses showed that the CORT and CORT reelin single CNQX rats had significantly more category 1 cells than the vehicle and vehicle reelin single CNQX rats (p values < .05), while the CORT reelin single CNQX rats had significantly more category 1 cells than the vehicle reelin single CNQX rats ($p = .008$). The CORT, CORT reelin single, and CORT reelin single CNQX rats had significantly more category 2 cells than all other groups (p values < .05), and they also had significantly fewer category 5 cells than all other groups (p values < 05). The CORT and CORT reelin single rats had significantly fewer category 6 cells than the vehicle, vehicle reelin single, and vehicle reelin single CNQX rats (p values < .05), and the CORT reelin single CNQX rats had significantly fewer category 6 cells than the vehicle and vehicle reelin CNQX rats (p values < .05).

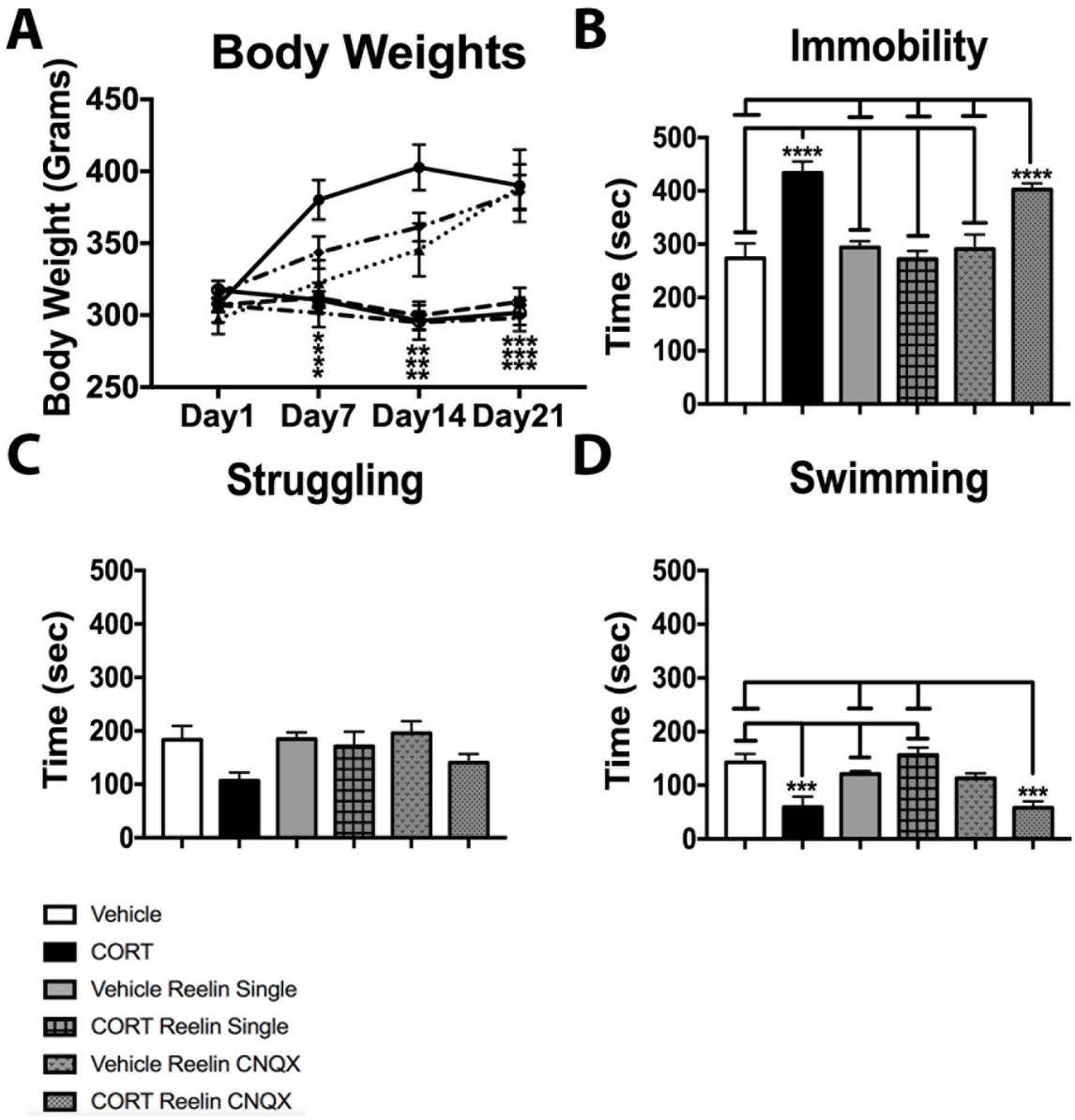


Fig 2. Mean body weight through days 1-21 of CORT/vehicle injections. Treatment with CORT decreased body weight, and reelin and reelin + CNQX did not recover this. Panel B-D; CORT increased depression-like behaviour in the FST, which was reversed by reelin, and CNQX blocked the effects of reelin. The CORT and CORT reelin CNQX rats spent significantly more time immobile than all other groups (panel B) (*p* values < .05). Panel C shows the effect of treatment on time spent struggling. Panel D shows the effect of treatment on time spent swimming. The CORT rats spent significantly less time swimming than the vehicle, vehicle reelin single, and CORT reelin single rats (*p* values < .05), and the CORT reelin CNQX rats

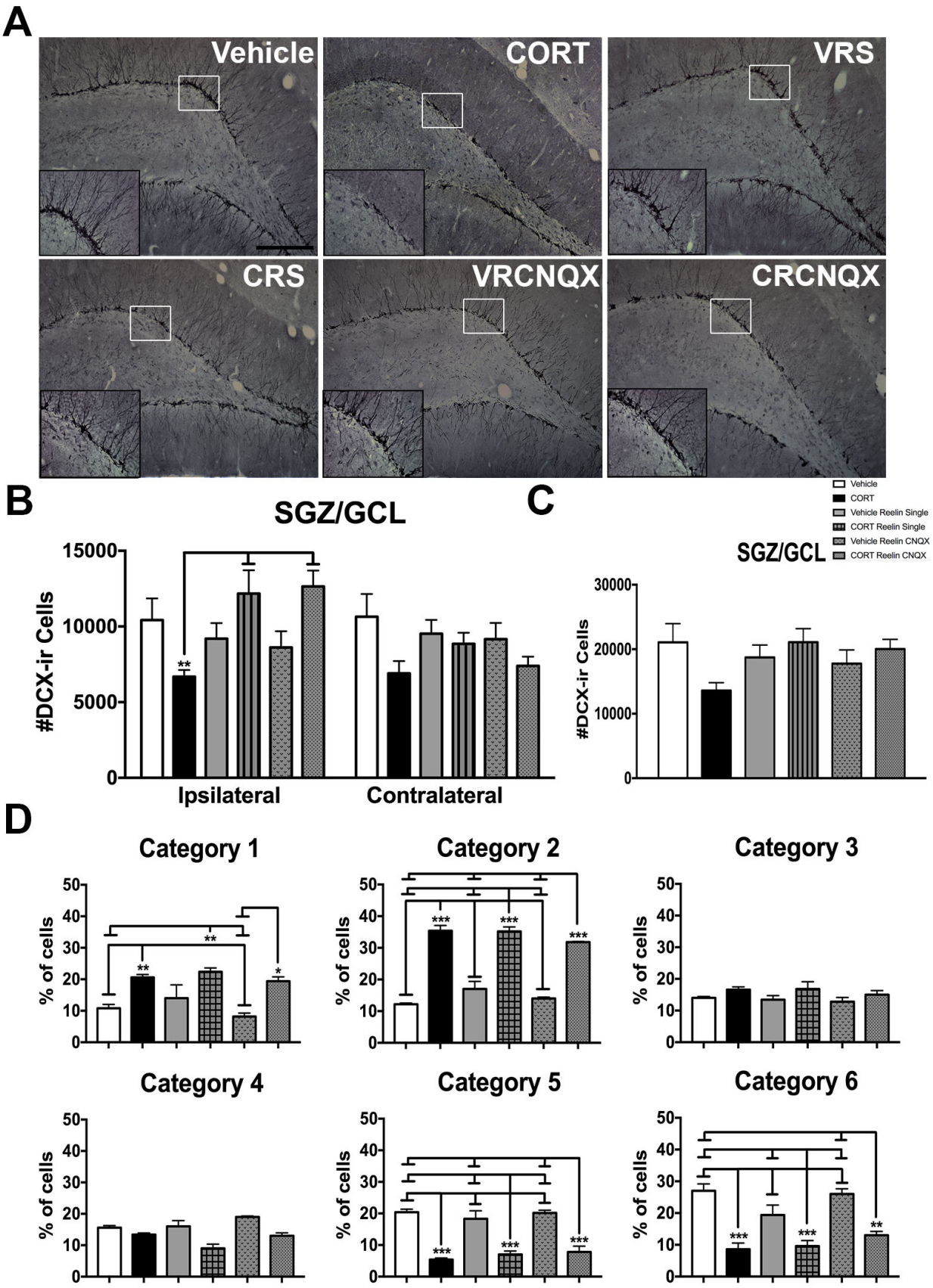
spent significantly less time swimming than the vehicle, vehicle reelin single, and CORT reelin single rats (p values < .05). All data are represented as means ± standard error of the mean. and CORT reelin single rats (p values < .05).

3.4. CNQX Increases the Number of Fos+ cells in the SGZ During the FST

Figure 4 shows the results of our Fos analyses. We found a significant main effect of treatment on the number of Fos-ir cells in both the ipsilateral ($F(5, 29) = 5.341$, $p < .01$) and and contralateral ($F(5, 29) = 3.948$, $p < .01$) hemispheres. *Post hoc* analyses revealed that in the ipsilateral hemisphere, the CORT reelin single CNQX rats had significantly more Fos-ir cells than the vehicle, vehicle reelin single, and CORT reelin single rats (p values < .05), and the vehicle reelin single CNQX rats had significantly more Fos-ir cells than the vehicle reelin single rats ($p = .047$). In the contralateral hemisphere, the vehicle reelin single CNQX rats had significantly more Fos-ir cells than the vehicle reelin single rats ($p = .025$).

3.5. CORT Alters Microglial cell Morphology

Figure 5 shows the results of our microglia morphology analyses. We found a significant main effect of treatment on process length ($F(5, 29) = 9.935$, $p < .001$), process nodes ($F(5, 29) = 8.225$, $p < .001$), process endings ($F(5, 29) = 5.632$, $p < .001$), and process intersections ($F(5, 29) = 6.395$, $p < .001$). *Post hoc* analyses revealed that the vehicle rats had significantly longer process lengths than the CORT, CORT reelin single, and CORT reelin single CNQX rats (p values < .05), the CORT reelin single rats had significantly shorter process lengths than the vehicle reelin single CNQX rats ($p = .008$), and the vehicle reelin single CNQX rats had a significantly longer process length than the CORT reelin single and CORT reelin single CNQX rats (p values < .05). In terms of the number of process nodes, the CORT rats had significantly fewer process nodes than the vehicle reelin single CNQX rats ($p = .003$), the CORT reelin single rats had significantly fewer process nodes than the vehicle, vehicle reelin single, and vehicle reelin single CNQX rats (p values < .05), and the CORT reelin single CNQX rats had significantly fewer process nodes than the vehicle reelin single CNQX rats ($p = .004$). The vehicle reelin single CNQX rats had significantly more process endings than the CORT, CORT reelin single, and CORT reelin single CNQX rats (p values < .05). Finally, the vehicle rats had significantly more process intersections than the CORT, CORT reelin single, and CORT reelin single CNQX rats (p values < .05), and the vehicle reelin single CNQX rats had significantly more process intersections than the CORT reelin single and CORT reelin single CNQX rats (p values < .05).


A
Vehicle
CORT
VRS
CRS
VRCNQX
CRCNQX
B
SGZ/GCL
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15000
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Ipsilateral
Contralateral
C
Vehicle
CORT
Vehicle Reelin Single
CORT Reelin Single
Vehicle Reelin CNQX
CORT Reelin CNQX
SGZ/GCL
#DCX-ir Cells
30000
20000
10000
0
D
Category 1
Category 2
Category 3
Category 4
Category 5
Category 6
% of cells
50
40
30
20
10
0

Fig 3. Effects of intrahippocampal reelin and CNQX-treatment on hippocampal neurogenesis. CORT had significant effects on hippocampal neurogenesis. Panel A shows representative photomicrographs of doublecortin expression in the granule cell/subgranular zone (Scale bar = 200 μm). A higher magnification image is shown in the insets (Scale bar = 50 μm). Panel B and C shows the effects of treatment on the number of doublecortin-ir cells in the granule cell/subgranular zone. Overall, the CORT rats had significantly fewer doublecortin-ir cells than the CORT reelin single and CORT reelin single CNQX rats in the ipsilateral hemisphere (*p* values < .05). Panel D shows the quantified categorization of dendritic complexity using doublecortin staining. Group differences are described in the text. All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRCNQX = vehicle reelin single CNQX; CRCNQX = CORT reelin single CNQX.

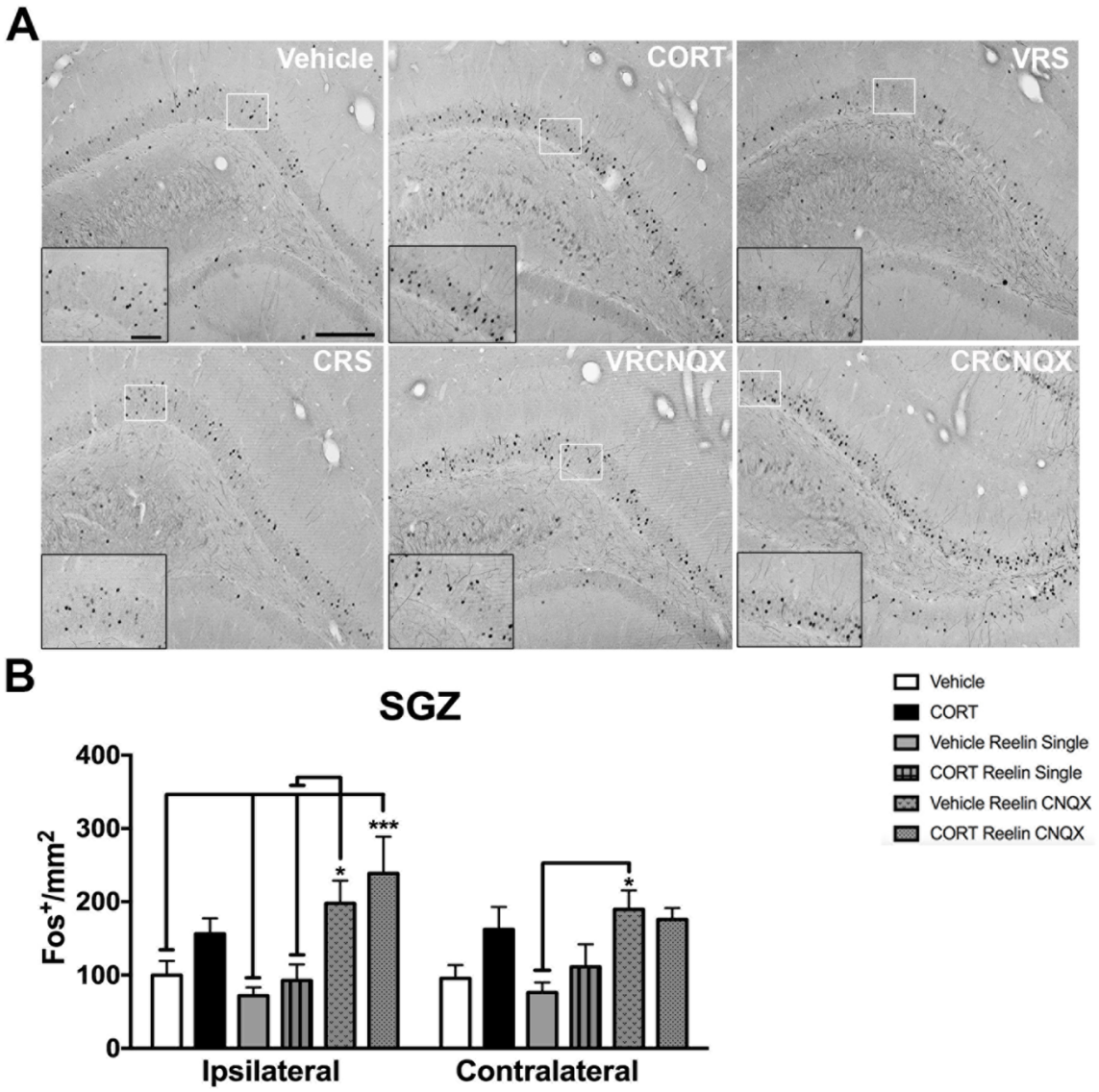

Fig 4. Fos immunoreactivity in the subgranular zone. Panel A shows representative photomicrographs of Fos in the SGZ (Scale bar = 200 μm). A higher magnification image is shown in the inset (Scale bar = 50 μm). Panel B shows the effect of treatment on the number of Fos-ir cells in the SGZ. Overall, the CORT reelin single CNQX rats had significantly more Fos-ir cells in the ipsilateral hemisphere than the vehicle, vehicle reelin, and CORT reelin single rats (p values < .05), and the vehicle reelin single CNQX rats had significantly more Fos-ir cells than the CORT-reelin single rats (p < .05). In the contralateral hemisphere, the vehicle reelin single CNQX rats had significantly more Fos-ir cells than the vehicle reelin single rats (p < .05). All data are represented as means ± standard error of the mean. VRS = vehicle reelin single; CRS =

CORT reelin single; VRCNQX = vehicle reelin single CNQX; CRCNQX = CORT reelin single CNQX.

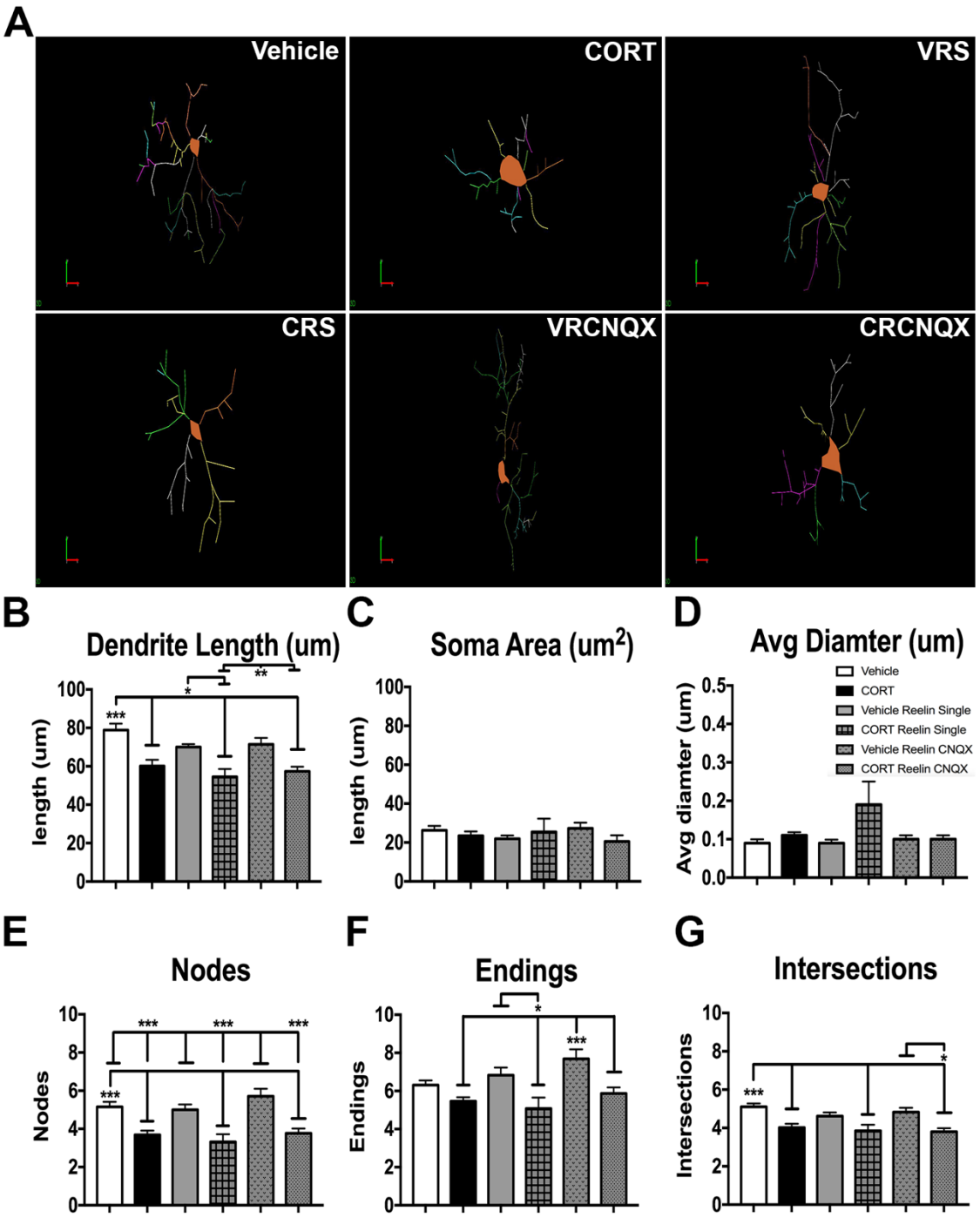

Fig 5. The effect of treatment on microglia cell morphology. Panel A shows representative 3D reconstructions of 1 microglial cell created with Neurolucida software. Panels B-G show the results of our microglia cell morphology analyses. Overall, treatment with CORT decreased the average microgalial cell length, and the numbers of nodes, endings, and processes. Treatment with reelin and reelin + CNQX did not normalize these measures. All data are represented as

means ± standard error of the mean. VRS = vehicle reelin single; CRS = CORT reelin single; VRCNQX = vehicle reelin single CNQX; CRCNQX = CORT reelin single CNQX

single, and vehicle reelin single CNQX rats (p values < .05), and the CORT reelin single CNQX rats had significantly fewer process nodes than the vehicle reelin single CNQX rats (p = .004). The vehicle reelin single CNQX rats had significantly more process endings than the CORT, CORT reelin single, and CORT reelin single CNQX rats (p values < .05). Finally, the vehicle rats had significantly more process intersections than the CORT, CORT reelin single, and CORT reelin single CNQX rats (p values < .05), and the vehicle reelin single CNQX rats had significantly more process intersections than the CORT reelin single and CORT reelin single CNQX rats (p values < .05).

4. Discussion

Our results show that repeated CORT treatment in rats increases immobility time in the FST, decreases hippocampal neurogenesis, and contributes towards an activated migroglia phenotype. A single intrahippocampal reelin infusion reverses the effect of CORT on time spent immobile in the FST, and restores the number DCX-ir cells, but it did not alter the effect of CORT on the complexity of DCX-ir cells or the activated microglial phenotype. Finally, a single intrahippocampal CNQX infusion blocks the effect of reelin on immobility time in the FST, and increases the number of Fos-ir cells active during the FST. These results suggest that CNQX is blocking the fast-acting antidepressant effects of reelin on the FST without altering reelin's effects on hippocampal neurogenesis or microglia morphology.

Treatment with CORT produces clearly stratified behaviours on the FST, characterized by increased immobility time and a decreased time spent swimming (Lebedeva, Caruncho, & Kalynchuk, 2017; Fenton et al., 2015). The results from the current experimental paradigm are consistent with the above observations, as CORT treatment increased time spent immobile and decreased swimming time. Additionally, we observed that a single intrahippocampal reelin infusion reversed the above effects in as little as 24 hours, in line with previous results from our group (Brymer et al., unpublished observations). While it is unexpected that reelin has fast-acting antidepressant effects, it is not without precedent in the literature. For example, reductions in reelin expression in the hippocampus have been observed in post-mortem tissue from patients with depression (Fatemi, Earle, & McMenomy, 2000). The work in human patients parallels those observed in preclinical studies, as CORT treatment reduces the number of reelin-ir cells in the SGZ (Lussier et al., 2013a). Furthermore, we have demonstrated that heterozygous reeler mice are more susceptible to the deleterious effects of CORT than are control animals (Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2011). Work elsewhere has shown that reelin overexpression prevents the behavioural phenotypes of both schizophrenia and bipolar disorder (Teixeira et al., 2011). A particularly novel finding from the current experiment was that an intrahippocampal CNQX infusion in rats treated with CORT and reelin blocked the rapid antidepressant effects of reelin on the FST. This suggests that the fast-acting antidepressant effect requires a contribution from AMPA receptors. The involvement of AMPA in an antidepressant response is consistent with those observed in the literature. For example, the antidepressant effects of zinc in the FST are abolished by application of NBQX, an AMPA

antagonist (Szewczyk, KUubera, & Nowak, 2010). Furthermore, Zanos et al (2016) convincingly showed that the ketamine metabolite (2R,6R)-HNK's fast-acting antidepressant effects were prevented by NBQX. Therefore, the idea that reelin is acting at least in part through AMPA to achieve its antidepressant effects is in line with those observed elsewhere.

Our stereological analyses revealed that CORT treatment decreased both the number and complexity of DCX-ir cells in the GCL/SGZ, consistent with previous reports from our lab (Brymer, Fenton, Kalynchuk, & Caruncho, 2018; Fenton et al., 2015; Lussier et al., 2013a). A single intrahippocampal reelin infusion increased the number but not complexity of DCX-ir cells, and CNQX produced the same effect. The role of hippocampal neurogenesis in the pathogenesis of depression is currently unclear, owing to a number of factors. First, antidepressant effects can be achieved without corresponding increases in neurogenesis (Hanson, Owens, & Nemeroff, 2011). Second, studies of post-mortem tissue from patients with depression have not typically revealed reductions in neurogenesis (Reif et al., 2006). However, more recent post-mortem studies report reductions in hippocampal neurogenesis (Boldrini et al., 2012). Additionally, Hill, Sahay, and Ren (2015) demonstrated that increasing neurogenesis was sufficient to alleviate depression-like behaviour on the FST. This suggests that neurogenesis *per se* plays a contributive role in antidepressant responses.

The finding that a single intrahippocampal reelin infusion and CNQX increases the number of DCX-ir cells in the ipsilateral hemisphere is consistent with those observed in the literature. For example, reeler mice, which express 50% of the normal levels of reelin, have a significant reduction in newborn neurons in the DG (Won et al., 2006), and Pujadas et al. (2010) found that mice overexpressing reelin have a significant increase in DCX-ir granule cells. However, our results show no effects of reelin or CNQX on the complexity of DCX-ir cells, which suggests that these cells are not mature or functionally integrated, therefore they are not likely contributing to the observed antidepressant response of reelin.

Our meander scan analyses revealed a pattern of increased Fos immunoreactivity in CORT treated rats and decreased Fos immunoreactivity in reelin treated rats, however this did not reach statistical significance. On the other hand, CNQX in reelin-treated rats significantly increased Fos immunoreactivity above and beyond that of vehicle and CORT rats. Fos, a member of the immediate early gene family and marker of neuronal activation (Botterill, Guskjolen, Marks, Caruncho, & Kalynchuk, 2015b), is typically expressed after exposure to a

stimulus, as a marker of the cells that were active during the behaviour. It has been reported that chronic stress results in persistent elevations in Fos immunoreactivity in the hippocampus (Lam et al., 2018; Hoffman, Lorson, Sanabria, Olive, & Conrad, 2014; Matsuda et al., 1996) and amygdala (Perrotti et al., 2004) following the FST or fear conditioning. To our knowledge, the work presented here is the first to show the effects of repeated CORT treatment on Fos expression following the FST. Why might CORT and CNQX treatment be increasing Fos expression following the FST? It has been hypothesized that the observed elevations of Fos expression during chronic stress might be a result of increased numbers of cells actively contributing towards a fear memory (Hoffman et al., 2014), suggesting a contribution of the amygdala. While Fos expression in the amygdala was not quantified in the current experiment, based on work presented elsewhere (Hoffman et al., 2014) it would be expected that our treatment would result in increased Fos expression in the amygdala.

To observe changes in microglia morphology in response to stress, we traced a subset of CD11B+ microglia and their processes in the hilus. The CORT rats displayed reduced process length, a reduced ramification of processes (represented by a decrease in the number of branching nodes and ends) and a decrease in the number of process intersections. Interestingly, treatment with reelin and CNQX did not alter these effects, suggesting that activated microglia may not be involved in the depressogenic effects of CORT. An activated microglia phenotype is characterized by reductions in microglia cell ramifications and a retraction of branches (Beynon & Walker, 2012). The morphology of microglia is coupled with their functional properties (Stence, Waite, & Dailey, 2001), and activated microglia release pro-inflammatory cytokines and nitric oxide synthase (Lebedeva, 2017; Liu & Hong, 2003). Elevated levels of pro-inflammatory cytokines are observed under conditions of chronic stress (Brymer, Fenton, Kalynchuk, & Caruncho, 2018) and can lead to a depression-like phenotype (Young, Bruno, & Pomara,, 2014). The fact that we observed antidepressant effects of reelin with an activated microglia phenotype suggests 1 of 2 scenarios: the antidepressant effects of reelin are not directly reliant on a reduction of proinflammatory cytokines, or sufficient time has not elapsed for reelin to alter the microglia morphology into a ramified state. However, as antidepressant effects were observed without corresponding changes in microglia morphology, this suggests that microglia per se are not part of reelin's immediate antidepressant effects.

5. Conclusion

In conclusion, we have shown that repeated CORT increases the time spent immobile in the FST, decreases hippocampal neurogenesis, and contributes towards an activated microglia phenotype. A single intrahippocampal reelin infusion decreases time spent immobile in the FST, increased the number but not complexity of DCX-ir cells, and contributed towards an activated microglia phenotype. Finally, a single intrahippocampal CNQX infusion in reelin-treated rats increases the time spent immobile in the FST, increases the number but not complexity of DCX-ir cells, increases the number of Fos-ir cells in the SGZ, and contributes towards an activated microglia phenotype. The results presented here suggest that the fast-acting antidepressant effects of reelin involve a contribution of AMPA signalling, but neurogenesis and microglia morphology might not be necessary.

CHAPTER 6
GENERAL DISCUSSION

6.1. Summary of the Main Findings

The primary purpose of this dissertation was to investigate the antidepressant potential of TNF-α antagonists and reelin on corresponding neurobiological alterations and behavioural measures in the CORT model of depression. Towards this end, I examined the effects of repeated CORT injections and etanercept and reelin administration on a well-validated measure of depression-like behaviour, object-recognition memory, prepulse inhibition, hippocampal neurogenesis, reelin expression, $GABA_Ab2/3$-ir cells, GluA1-ir cells, GluN2B, Fos, and microglial activation.

In **Chapter 2**, I examined the effect of repeated CORT injections on rodent behaviour in the FST, object-location, object-in-place, object-recognition, and prepulse inhibition. To examine this question, I administered 21 days of repeated CORT injections (40 mg/kg) and then started behavioural testing using the above measures. CORT increased the time spent immobile in the FST and decreased the time spent swimming and struggling. CORT produced a dissociation in object memory, with rodents exhibiting impairments on the object-location and object-in-place memory tests, while performance on the object-recognition memory was spared. Finally, CORT significantly impaired prepulse inhibition, with the CORT rats displaying lower overall startle amplitudes and prepulse inhibition for 30 ms intervals. This pattern of results suggests that CORT treatment is significantly effecting both the hippocampus (object-location) and pre frontal cortex (object-in-place) while sparing integrity of the perirhinal cortex (object-recognition) and disrupting sensorimotor gating. **Chapter 2** provided a paradigm to subsequently study the effects of different classes of putative antidepressants on memory performance in the CORT model.

In **Chapter 3,** I examined the effects of repeated CORT injections and concurrent injections of the TNF-α inhibitor etanercept on the FST, object-location, object-in-place memory, and markers of hippocampal plasticity. To address this question, I administered 21 days of repeated CORT injections (40 mg/kg) and semi-weekly etanercept injections (0.8 mg/kg) followed by behavioural testing and post-mortem analyses of neurobiology. My results revealed that similar to Chapter 2, CORT injections increased the time spent immobile and decreased the time spent swimming and struggling in the FST. Importantly, etanercept reversed this pattern of behaviour, suggesting an antidepressant response. CORT also impaired performance on the

object-location and object-in-place memory tests, with etanercept reversing both of these deficits. Analysis of hippocampal neurogenesis revealed that CORT treatment significantly decreased the number of newborn neurons while also decreasing their dendritic complexity. Etanercept injections significantly increased the number of newborn neurons and their dendritic complexity. Corresponding to past research in our lab, CORT decreased the number of reelin cells in the SGZ, and etanercept reversed this. This finding indicates that the antidepressant effects of etanercept may be through an increase in reelin positive cells, as we have shown that the onset of depression-like behaviour coincides with the loss of reelin cells in the SGZ (Lussier et al., 2013a). Finally, CORT decreased the number of $GABA_A$b2/3-ir cells in the SGZ, and etanercept reversed this. Collectively, my findings indicate that etanercept is acting to reverse depression-like behaviour, reductions in hippocampal neurogenesis, reelin expression, and $GABA_A$ b2/3-ir cells produced by CORT administration. The fact that etanercept increased reelin positive cells was of particular interest, and raises the question of whether reelin could be a novel antidepressant. This question was examined in detail in Chapters 4 and 5.

In **Chapter 4**, I investigated the effect of exogenous reelin application on the FST, object-location memory, and markers of hippocampal plasticity. To examine this question, I conducted stereotaxic surgery to implant an indwelling cannula into the left dorsal hippocampus. I then administered repeated CORT injections (40 mg/kg) for 21 days, and administered intrahippocampal reelin infusions (1 µg/µl) in a repeated (3X) or acute (1X) fashion during the period of CORT injections. Rats were sacrificed immediately after the FST or after completing the object-location memory test. Similar what I reported in Chapters 2 and 3, CORT increased the time spent immobile and decreased the time spent swimming and struggling in the FST. A single reelin infusion significantly decreased time spent immobile in the FST and increased swimming and struggling times at both time points examined. Reelin also reversed CORT-induced impairment on the object-location memory test. Similar to Chapter 3, CORT decreased the number of newborn neurons and their dendritic complexity. Interestingly, a single reelin infusion increased the number of immature neurons in CORT-treated rats after the FST, but did not affect dendritic complexity. Meanwhile, in rats completing the object-location memory test, a single reelin infusion increased both the number of immature neurons and their dendritic complexity. This finding suggests that, as a single infusion of reelin produced an antidepressant response at both time points while neurogenesis was not completely restored at time point 1,

neurogenesis may not be a contributing factor in the antidepressant effects of reelin.

CORT also decreased the number of GluA1-ir cells in the SGZ, and reelin reversed this reduction at both time points. In contrast to GluA1, CORT increased GluN2B immunoreactivity throughout the major subfields of the DG, with reelin acting to normalize this effect. Similar to Chapter 3, CORT decreased the number of $GABA_Ab2/3$-ir cells in the SGZ, and reelin reversed this at both time points. Collectively, my findings indicate that reelin is achieving rapid anti-depressant responses in the FST, which is accompanied by corresponding alterations in markers of hippocampal plasticity. The finding that reelin rapidly increased the number of GluA1-ir cells is of particular interest. Targeted deletion of the GluA1 subunit in mice creates a depression-like phenotype (Chourbaji et al., 2008), and the fast-acting antidepressant effects of the ketamine metabolite (2*R, 6R*) – HNK were associated with increased phosphorylation of AMPARs (Zanos et al., 2016). This suggests that the fast-acting antidepressant effects of reelin may occur through an increase in AMPA receptor activation, in particular GluA1. Chapter 5 will address the question of the role of AMPA in the antidepressant effects produced by reelin.

In **Chapter 5,** I investigated the role of AMPA receptors in the dentate gyrus in the fast-acting antidepressant effects of reelin. I conducted stereotaxic surgery to implant an indwelling cannula into the left dorsal hippocampus. Rats then underwent 21 days of repeated CORT injections (40 mg/kg). A subset of rats received a single intrahippocampal reelin infusion (1 µg/µl) on day 21 of CORT injections. Rats that received reelin also received a single intrahippocampal CNQX infusion (1µg/µl) on day 22, 30 minutes before the FST. Rats then underwent the FST, and were sacrificed immediately after for post-mortem analyses of neurogenesis, Fos, and microglia activation. Similar to what was reported in Chapters 3 and 4, CORT decreased the number of immature neurons and their dendritic complexity. A single reelin application significantly increased the number of surviving neurons, while not altering their dendritic complexity. In a similar vein, CNQX increased the number of surviving newborn neurons without altering their dendritic complexity. This finding further reinforces the point that, while the number of newborn neurons are increased, their dendritic complexity is not in both reelin and reelin CNQX treated rats. This suggests that neurogenesis *per se* is not involved in reelin's effects on the FST, in particular immobility. CORT produced a non-significant increase in the number of Fos positive cells in the SGZ, with reelin causing a non-significant decrease in the number of Fos positive cells. Application of CNQX resulted in a significant increase in the

number of Fos positive cells. Finally, CORT contributed towards an active microglia phenotype, characterized by a retraction of processes. Both reelin and CNQX did not alter the CORT-induced active microglia phenotype.

Chapter 5 was an important conclusion to the current dissertation. Here, we were able to mechanistically show that the fast-acting antidepressant effects of reelin require a contribution of AMPA receptor signaling, as blocking this abolishes reelin's fast-acting antidepressant effects. This is an valuable point to make, as we can say with confidence that we know at least one of the ways that reelin is working. From here, future studies can begin to tease apart the contribution of the different AMPA subunits in relation to their contribution to reelin's antidepressant effects.

The overall results of this dissertation demonstrate the importance of inflammation and hippocampal plasticity in an animal model of depression. Specifically, the experimental data presented here indicates that repeated CORT injections increase depression-like behaviour in the FST, impair both object-location and object-in-place memory while sparing object-recognition memory, and disrupt prepulse inhibition. The TNF-α inhibitor etanercept reverses depression-like behaviour and cognition, while also restoring several markers of hippocampal plasticity, importantly reelin. Reelin application can rapidly reverse CORT-induced increases in depression-like behaviour and deficits in cognition. Furthermore, reelin reverses several neurobiological markers associated with hippocampal plasticity in a rapid manner. The final experiment of my dissertation provides evidence that the fast-acting antidepressant effects of reelin require a contribution of AMPA signaling.

6.2. Behavioural Alterations in the CORT model of Depression

The CORT model is widely recognized for producing similar behavioural alterations as those seen in depression. The hallmarks of depression include anhedonia, lowered mood, and a sense of learned helplessness, or the act of giving up because nothing is going to end up working out. These behaviours are typically examined in the CORT model by assessing alterations in the FST and sucrose preference test after exposure to CORT. These studies have consistently shown that repeated CORT treatment produces a cluster of effects on the FST, characterized by increased time spent immobile, decreased time spent struggling, and decreased time spent swimming (Gregus, Wintink, Davis, & Kalynchuk, 2005; Hill, Brotto, Lee, & Gorzalka, 2003; Johnson, Fournier, & Kalynchuk, 2006; Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004; Marks, Fournier, & Kalynchuk, 2006; Zhao, Shen, Ma, & Du, 2008; Zhao, Xie, Dai, Wang, &

Huang, 2009). In a similar vein, the results of Chapters 2, 3, 4, and 5 support these observations. For example, I have shown that repeated CORT injections consistently increase the time spent immobile, and decrease both the time spent swimming and struggling. These findings are important as they add validity and face value to the CORT model of depression by suggesting that the model consistently measures what it is intended to.

Another focus of behavioural research in the CORT model is on the effects of CORT on anhedonic behaviour. While not a part of the current dissertation, reports have shown that repeated CORT injections increase anhedonia on the sucrose preference test, characterized by a decreased preference for sucrose over unsweetened water (Dwivedi, Roy, Lugli, Rizavi, Zhang, & Smalheiser, 2015; Sturn, Becker, Schroeder, Bikei-Gorzo, & Zimmer, 2015; Lebedeva, 2017). Work in our lab has investigated to what extent the increased immobility time in the FST is due to possible changes in muscle tone. Marks, Fournier, and Kalynchuk (2009) showed that CORT treated rats do not behave differently than vehicle rats on tests of muscle strength, indicating that CORT is not simply decreasing muscle tone and making the animals weak, explaining their increased immobility time.

Compared to the research conducted on measures of depression-like behaviour, there has been less focus on the effects of repeated CORT injections on learning and memory. The little research that is available has been conducted mainly on fear memory. These results consistently show that CORT enhances the acquisition and recall of fear conditioning (Hui et al., 2004; Marks, Fenton, Guskjolen, & Kalynchuk, 2015; Marks & Kalynchuk, 2017; Monsey et al., 2014). However, what has been missing from the literature is the effects of systemic CORT (i.e., 21 days of subcatenous injections) on other types of memory, particularity hippocampal and PFC-dependent memory and tests of sensorimotor gating. The current dissertation aimed to address this gap in the literature by running a series of experiments that examine the effects of repeated CORT injections on tests of hippocampal and PFC-dependent memory and sensorimotor gating.

The findings of the current dissertation show that repeated CORT injections impair performance on both the object-location and object-in-place memory tests, while sparing performance on object-recognition memory. This is an intriguing finding, as it suggests a dissociation between hippocampal and prefrontal cortex-dependent measures of memory and perirhinal cortex memory. Therefore, it might be that the perirhinal cortex is more resilient to the

deleterious effects of glucocorticoids or can resist them longer than can the hippocampus and prefrontal cortex over the course of 21 days of high dose CORT injections. However, it is important to point out that a study by Darcet et al. (2014) reported that CORT impaired performance on the object-recognition memory test. However, this can potentially be explained by their use of a longer CORT exposure period (28 days), their method of delivery (drinking water), and not quantifying the discrimination ratio. The finding that both hippocampal and PFC dependent memory are impaired by CORT treatment are in line with other reports in the literature, which show deficits in both of these areas as a result of chronic stress (Conrad, Galea, Kuroda, & McEwen, 1996; Diamond, Park, Heman, & rose, 1999; Goodman & McIntyre, 2017; Kleen, Sitomer, Kileen, & Conrad, 2006; Lee & Goto, 2015; Mika et al., 2012; Mizoguchi et al., 2000). Importantly, human studies indicate that depressed patients are impaired on tasks that require the hippocampus and PFC (Newcomer, Craft, Hershey, Askins, & Bardgett, 1994; Rose & Ebmeier, 2006). Therefore, the findings of impaired memory after CORT treatment serves to add to the validity of the CORT model as a tool to investigate the behavioural alterations associated with depression.

CORT also produced alterations in prepulse inhibition. Specifically, CORT decreased overall startle amplitudes and increased prepulse inhibition on 30 ms intervals. This is a novel finding, and indicates that CORT treatment is disrupting sensorimotor gating. To date, little to no research has been conducted examining the effects of CORT on prepulse inhibition, suggesting the need of further studies to fully elucidate the effects of CORT on this behaviour. Collectively, my results suggest that object-memory testing and prepulse inhibition are ideally suited to tease apart CORT-induced deficits in memory and sensorimotor gating.

6.3. The role of inflammation and TNF-α in Depression-like Behaviour, Cognition, and Markers of Hippocampal Plasticity

TNF-α is consistently elevated in response to chronic stress in both humans and rodents (Glovatchcka, Ennes, Mayer, & Bradesi, 2012; Halaris et al., 2012; Miller, Maletic, & Raison, 2009; Nievas et al., 2011; Vogelzangs et al., 2012; Yang et al., 2014). In particular, TNF-α expression is significantly increased in the major subfields of the hippocampus, including the DG, CA1, and CA3 (Yang et al., 2015), in addition to the PFC (Couch et al., 2013) following stress. Under conditions that elicit TNF-α release, for example chronic stress, TNF-α is released peripherally and acts on toll-like receptor 4 on circumventricular organs and the choroid plexus,

which in turn stimulates microglia to secrete pro-inflammatory cytokines (among them TNF-α), resulting in neurodegenerative events and an increase of apoptotic neuronal death (Bortolato et al., 2015). High circulating levels of TNF-α are associated with depression-like behaviour, with studies consistently showing elevated levels of TNF-α in depressed patients (Biesmans et al., 2015; Bizik et al., 2014; Jangpangi et al., 2014; Mikova, Yakomova, Bosmans, Kenis, & Maes, 2001). Depressive symptoms in lupus patients are correlated with increased circulating levels of TNF-α (Mak et al., 2013; Postal et al., 2016). Therefore, it follows that if TNF-α is elevated in the serum of depressed patients, then strategies to reduce this will have antidepressant effects, which turns out to be the case. For example, patients with rheumatoid arthritis and plaque psoriasis taking prescribed etanercept report significant reductions in depressive symptoms (Kekow et al., 2011; Gelfand, Kimball, & Mostow, 2008). Moreover, infliximab infusions in Crohns Disease patients significantly reduces depressive symptoms, and this alleviation of symptoms was correlated with decreases in proinflammatory cytokines (Guloksuz, Wichers, & Kenis, 2013). Finally, psoriasis patients with comorbid psychiatric conditions report improvement in mood and overall well-being when taking infliximab (Bassukas, Hyphantis, Gamvroulia, Giatanis, & Mavreas, 2008).

The results of *Chapter 3* are consistent with these findings. Specifically, I found that repeated CORT injections significantly increased the time spent immobile on the FST. In contrast, etanercept injections in CORT treated rats significantly decreased the time spent immobile, suggestive of an antidepressant response. It must be acknowledged that serum TNF-α levels were not collected in this study, and therefore it cannot be conclusively said that TNF *per se* is responsible for the depressogenic and antidepressant effects observed here. However, it is particularly interesting that etanercept administration reversed the CORT-induced depression-like behaviour in light of work done by Raison et al. (2013). Here, Raison et al. showed that infliximab infusion in treatment resistant depression patients alleviated depressive symptoms but only in patients with a high baseline level of inflammation. Therefore, it is likely that proinflammatory cytokines, in particular TNF- α, are elevated as a result of CORT treatment. However, further studies are required to examine the cytokine profile resulting from CORT treatment.

Although TNF-α is most widely studied for its effects on inflammation, autoimmune disorders, and depression, the important role that TNF-α plays in learning and memory is

increasingly being recognized (McIntyre et al., 2015). Indeed, several studies have demonstrated that either administration of TNF-α or genetic manipulation through transgenic animals creates robust alterations in learning and memory. For example, in a mouse model of multiple sclerosis, where animals display upwards of 3 times as much TNF-α as controls in the hippocampus, significant deficits in the expression of fear learning and memory are observed (Habbas et al., 2015). Furthermore, exposing rodents to lipopolysaccharide, a gram negative bacteria which creates a state of inflammation, produces impairments on context-object location discrimination, and this deficit is associated with high levels of proinflammatory cytokines, for example TNF-α (Czerniawski et al., 2015). Chronic restraint stress can also elevate hippocampal TNF-α levels, and this has been linked to impairments in a passive avoidance learning test (Azadbakht et al., 2015). Finally, studies of breast-cancer survivors reveal reduced hippocampal volumes and deficits in verbal memory performance that are associated with elevated circulating levels of TNF-α (Kesler et al., 2013). Taken together, increased levels of TNF-α impair learning and memory, which suggests that lowering these elevated TNF-α levels will correspond to improvements in cognitive functioning. The results of Chapter 3 fit in nicely with these findings. I have shown that repeated CORT injections significantly impair performance on both object-location and object-in-place memory, and that etanercept administration restores both of these measures to control levels. How might etanercept administration be acting to restore deficits in object-location and object-in-place memory? One possibility is an increase in markers of hippocampal plasticity, for example reelin.

TNF-α is involved in the promotion of several markers of hippocampal plasticity. For example, TNF-α dynamically regulates neurogenesis. High circulating levels of TNF-α promote apoptotic neuronal death (Bortolato et al., 2015). On the other hand, low levels of TNF-α are known to produce the opposite effect, promoting hippocampal neurogenesis through increased proliferation of neural precursor cells (Fan et al., 2011). TNF-α alters the surface expression of AMPA and $GABA_A$ receptors by increasing AMPA receptor expression and decreasing $GABA_A$ receptors (Stellwagen et al., 2005), which may contribute to the excitotoxic effects of upregulated TNF-α in a chronic stress paradigm (Bortolato et al., 2015). The normalization of $GABA_A$ receptor expression in SGZ cells by peripheral etanercept may represent a neuroprotective mechanism. There is also preliminary evidence to suggest that TNF-α is involved in reelin signaling. Interestingly, in a mouse model of aging, a viral-induced infection

increased inflammation and reduced the number of reelin-expressing interneurons (Doehner and Knuesel, 2010). The results of Chapter 3 correspond with the pattern of results described above. I found that repeated CORT injections reduced hippocampal neurogenesis, decreased the number of $GABA_A$ b2/3-ir cells in the SGZ, and reduced the number of reelin positive cells in the SGZ. Etanercept administration acted to reverse all of the above described alterations. This suggests that etanercept is acting to restore key aspects of hippocampal plasticity altered by CORT exposure. It is particularly interesting that reelin is involved in the regulation of hippocampal neurogenesis and GABA expression, suggesting that etanercept's increase of hippocampal reelin may be a primary effect driving the increase in markers of hippocampal plasticity and corresponding antidepressant effects.

6.4. The influence of Reelin on Depression-like Behaviour and Cognition

It is widely recognized that chronic stress decreases hippocampal reelin expression in animal models (Lussier et al., 2009; Caruncho et al., 2016; Palacios-Garcia et al., 2015: Brymer et al., 2018). In fact, the timeline for a reduction of reelin-expressing neurons in the hippocampus has been shown to correspond to the development of depression-like behaviour in rodents (Lussier et al., 2013a). For example, work in our lab has investigated the onset of depression-like behaviour and its relation to hippocampal reelin expression. Here, rats were treated with CORT for 21 days, and behaviour on the FST and reelin expression was investigated. It was found that depression-like behaviour starts to manifest on day 14 of CORT treatment. Moreover, reductions in hippocampal reelin expression are also observed after 14 days of treatment, suggesting that the onset of depression-like behaviour corresponds to a reduction in reelin expression. Heterozygous reeler mice, which express 50% of the normal levels of reelin, are more susceptible to the deleterious effects of repeated CORT injections. Reelin overexpression has been shown to prevent the behavioural manifestation of both schizophrenia and bipolar disorder (Teixeira et al., 2011). Recently, Fenton et al. (2015) showed that treatment with the tricyclic antidepressant drug in rodents reverses depression-like behaviour and increases hippocampal reelin expression. Similarly, the results of Chapter 3 show that etanercept administration reverses depression-like behaviour and increases hippocampal reelin expression. This observation is interesting because it suggests that antidepressant responses correspond to an increase in reelin expression. It follows then that increasing reelin expression will convey an antidepressant effect. This idea is consistent with the findings of Chapters 4 and 5. In Chapter 4, I found that intrahippocampal reelin

infusions significantly reversed depression-like behaviour on the FST. Importantly, this antidepressant effect occurred from just a single infusion, 24 hours before behavioural testing. In Chapter 5, I replicated my finding from Chapter 4, again showing that a single intrahippocampal reelin infusion significantly reverses depression-like behaviour in the FST. The finding that reelin has fast-acting antidepressant effects was a novel finding, and raises exciting possibilities about the potential mechanism behind this effect, a topic covered in the next section. Overall, the results described here suggest that chronic stress decreases hippocampal reelin expression, and a part of the action of antidepressants may be to increase reelin expression. My findings are consistent with these observations, as we show that a single intrahippocampal reelin infusion has fast-acting antidepressant effects in a rodent model of depression.

Reelin regulates multiple aspects of synaptic plasticity, and is therefore unsurprisingly involved in promoting learning and memory. Palacios-Garcia et al. (2015) exposed rat pups in utero to prenatal stress and examined reelin expression and cognitive functioning during adulthood of these same rats. Prenatal stress exposure significantly reduced hippocampal reelin expression in adulthood. Furthermore, this deficit in reelin expression was correlated with increased motor activity and impairments in passive avoidance conditioning and the Morris water maze. Interestingly, treadmill exercise in autistic rats has been shown to recover reelin signaling and alleviate autism-induced deficits in spatial learning memory (Seo et al., 2013). Dorsal forebrain reelin-dab1 knockout mice display behavioural abnormalities commonly seen across several common neuropsychiatric disorders, including bipolar and schizophrenia. Specifically, reelin-dab1 knockout mice present with impairments in prepulse inhibition, increased locomotor activity, impaired working memory in the Y-maze and T-maze, and decreased freezing during contextual fear conditioning (Imai et al., 2017). In an intriguing study, Rogers et al. (2011) showed that reelin supplementation in naïve mice enhances cognitive ability, specifically spatial learning and memory, in addition to promoting LTP. If reelin supplementation enhances learning and memory in naïve mice, than it would be expected that reelin supplementation would recover cognitive functioning in animal models of disorders characterized by cognitive deficits, and this turns out to be the case. For example, heterozygous reeler mice present with deficits in prepulse inhibition and contextual fear conditioning. A single invraventricular reelin infusion significantly reversed the above described deficits in prepulse inhibition and contextual fear conditioning (Rogers et al., 2012). Furthermore, intraventricular

exogenous reelin infusions recover synaptic function and cognitive deficits in a mouse model of Angelman syndrome (Hethorn et al., 2015).

Collectively, the findings described above suggest that reductions in reelin signaling impair learning and memory processes, and that reelin supplementation can restore cognitive functioning. The findings from Chapter 4 are consistent with those described here. We have shown that repeated CORT injections impair rodent performance on object-location memory paradigms, and that reelin treatment restores performance back to control levels on this measure. Specifically, in a similar vein to Rogers et al. (2012), we observed that single reelin infusion restores cognitive deficits induced by repeated CORT treatment. However, a potential direction for future research would be to examine if reelin can ameliorate other forms of cognitive impairment induced by the CORT model of depression, for example contextual fear conditioning.

Reelin, as an extracellular matrix protein, plays several key roles in synaptic plasticity. Given reelin's prominent role in development as a regulator of cortical development and lamination, it is not surprising that reelin is involved in the regulation of adult neurogenesis. Pujadas et al. (2010) found that mice overexpressing reelin have a significant increase in the number of DCX-ir granule cells, accompanied by increased complexity in their dendrites. Furthermore, reeler mice show a significant reduction in newborn neurons in the DG (Won et al., 2006). Along these same lines, Teixeira et al. (2012) showed that reelin overexpression accelerates dendritic growth of adult-generated neurons, and inactivation of the reelin signaling pathway impairs adult hippocampal neurogenesis. My findings from Chapters 4 and 5 are in line with those described above. Specifically, I found that CORT decreased both the number and complexity of adult-generated neurons in the GCL/SGZ. Reelin reversed both of these measures, however the time of the infusion determined the effect. When rodents are sacrificed immediately after completing the FST, reelin restores the number but not complexity of DCX cells, however if rodents are sacrificed a week later, then a single reelin infusion restores both the number and complexity of DCX cells. As an antidepressant effect is observed in rats that were sacrificed immediately after completing the FST, this suggests that neurogenesis per se might not be a contributing factor to reelin's antidepressant effects on the FST.

Collectively, my findings indicate that reelin infusions have fast-acting antidepressant effects on the FST and object-location memory test that are accompanied by increases in both the

number and complexity of adult generated neurons in the GCL/SGZ. These findings are in line with those observed in the literature, and serve to further reinforce the point that reelin plays a pivotal role in adult hippocampus neurogenesis. To definitively examine the contribution of neurogenesis to reelin's antidepressant effect, a series of studies could be completed looking at the effects of reelin infusions on depression-like behaviour in rats where neurogenesis is suppressed/ablated. If reelin infusions in this paradigm do not produce an antidepressant effect, than it can conceivably be concluded that reelin's antidepressant effects are dependent on neurogenesis.

6.5. The Importance of Glutamate

The relationship between depression and glutamate is a complex issue. Overall trends about patterns of change in glutamate in depression is difficult, as some receptors are increased while others are decreased. However, when looking specifically at the receptors GluA1 and GluN2B, the relationship becomes clearer. Chronic stress decreases the expression of GluA1, particularly in the hippocampus. Phosphorylation of GluA1 in response to stress decreases in both the hippocampus and mPFC, while increasing in the amygdala (Caudal et al., 2010). At the level of synapses, chronic stress decreases AMPA receptor mediated synaptic excitation at hippocampal CA1 synapses (Kallarackal et al., 2013). In an interesting study, Zhang et al. (2013) showed that the antidepressant and cognitive enhancer tianeptine reduces the surface diffusion of AMPA receptors through a calmodulin-dependent protein kinase II mechanism. This mechanism acts to prevent the CORT-induced dispersal of surface AMPA, restoring LTP. Further reinforcing the role of GluA1 in depression, Zanos et al. (2016) showed that the fast-acting antidepressant effects of ketamine were associated with an increase of GluA1 in the hippocampus. Chourbaji et al. (2008) convincingly showed that targeted deletion of GluA1 in mice leads to a depression-like phenotype, leading support to the glutamate hypothesis of depression, which posits that depression is a disorder characterized by disruptions in glutamatergic signaling. The findings from Chapter 4 are consistent with those observed elsewhere. I found that repeated CORT injections significantly reduced the number of GluA1-ir cells in the SGZ, with reelin treatment increasing this back to control levels. That this effect occurred with a single infusion of reelin suggests that reelin's fast-acting antidepressant effect might involve a contribution of AMPA, and specifically GluA1 signaling.

In contrast to GluA1, which is decreased in depression, expression of GluN2B typically

increases in response to stress. For example, stress is concomitant with an upregulation of hippocampal GLUN2B expression within the molecular layer (Ciu et al., 2012; Kreutz & Sala, 2012). Interestingly, duloxetine, a SNRI, decreased depression-like behaviour and concomitantly decreased Glun2B expression back down to control levels (Calabrese et al., 2012). Prolonged elevation of Glun2B signaling can have damaging effects in the brain, as this signaling drives excitotoxicity --- in vitro work demonstrates that elevated GLUN2B leads to a rapid influx of calcium ions into the cell, trigging an excitotoxic event (Von Engelhardt et al., 2007). Over time, this excitotoxity can lead to inflammation or atrophy of the hippocampus, potentially paralleling the reductions in hippocampal volume observed in depressed patients (Bremner et al., 2000). Similar to the research described above, my findings in Chapter 4 indicate that repeated CORT injections increase GluN2B expression across the major subfields of the DG. Reelin treatment significantly reduced GluN2B expression back down to control levels. The finding of reelin decreasing GluN2B expression back down to control levels is in line with those found elsewhere: Overexpression of reelin through reelin overexpressing mice results in a significant reduction of GluN2B in cultured hippocampal neurons (Teixeira et al., 2011). Therefore, it would appear that reelin is acting to quickly reduce excitotoxic events in the hippocampus, which might be a part of its antidepressant response.

The finding that reelin quickly restored the number of GluA1-ir cells in the SGZ was of particular interest to this dissertation. As mentioned previously, GluA1 knockout mice display depression-like behaviour (Chourbaji et al., 2008), and heterozygous reeler mice display a significant reduction in GLUA1 subunit clustering in cultured hippocampal neurons (Qiu and Weeber, 2007). These findings suggest that GluA1 may be involved in the pathogenesis of depression, and that reelin is involved in GluA1 signaling. In Chapter 5, we therefore hypothesized that the fast-acting antidepressant effects of reelin require a contribution of AMPA, and tested this by infusing the AMPA antagonist CNQX after reelin treatment. Confirming our hypothesis, CNQX blocked reelin's fast-acting antidepressant effects on the FST. Therefore, my findings indicate that reelin's antidepressant response requires the contribution of AMPA receptor signaling. Future studies should investigate the specific AMPA subunits that contribute to reelin's antidepressant response.

6.6. Limitations

6.6.1. Clinical Relevance of the CORT Model of Depression

Animal models of depression have long been viewed with skepticism in the greater scientific field. Criticism of animal models of depression comes in a variety of flavors. First, many depression symptoms are considered to be uniquely human (e.g., suicidal behaviour, guilt) and therefore they cannot be replicated in an animal model. Second, the diagnosis of depression itself has even met criticism, mostly for symptoms involving weight gain or loss, increased or decreased appetite, increased or decreased sleep, which some opponents argue necessitates multiple sub- diagnoses of depression (Nestler & Hyman, 2010). The last major criticism of animal models of depression is that some core aspects that have been modeled (e.g., anhedonia, despair) are still only approximate correlates of the human disorder (Nestler, 2010).

Despite these criticisms, several animal models of depression have been successfully developed that capture several key aspects of the depressive syndrome. The experiments of this dissertation focused on the use of the exogenous CORT administration model. This model has been met with criticism, mainly for the use of high doses of CORT. In this model, high doses (40 mg/kg) are required in order to produce robust alterations in depression-like behaviour in rats within 14 days, though lower doses are effective in mice (Zhao, Xie, Dai, Wang, & Huang, 2009). This is in sharp contrast to the plasma levels of CORT released during stressful encounters in rodents, which range from 2-5 mg/kg. Therefore, it is argued that the dose of CORT used in this model is supraphysiological (Sandi, 1996). While these criticisms are valid, there is a specific rationale for using this dosage of CORT. Mainly, the problem with other animal models of depression, for example restraint stress or chronic unpredictable stress (CUS), is that differences emerge in the amount of stress each animal experiences in response to each of the stressors. The CUS model has also been criticized for being the least reproducible stress model (Willner, 1997). For example, one rodent may not have the same levels of plasma CORT released during a period of cage tilting as opposed to another rodent, thereby raising the issue of individual differences. The CORT model circumvents this issue by ensuring that each animal receives the same amount of CORT each time. Therefore, on a basic level, individual differences are minimized and the effects of glucocorticoids on the brain and behaviour can be more easily teased apart. Therefore, despite the criticisms aimed at the CORT model of depression, it is the authors opinion that these criticisms are offset by greater experimental control of potentially

confounding variables.

6.6.2. Sex Differences

The current dissertation employed the use of male rats. This is an issue with the current body of work, as depression does not only affect males. In fact, women are over 2 times as likely to suffer from depression as are men, and this trend persists across multiple cultures (Angst et al., 2002). Investigating this from a preclinical perspective, female rodents have higher circulating CORT levels and an increased CORT response following stress exposure compared to males (Seale et al., 2004). It is then surprising that females rodents are in fact more resilient to the effects of stress compared to males. For example, CUS exerts more pronounced effects on depression-like behaviour in male rats as compared to female rats (Dalla et al., 2005), and Hill et al. (2004) reported that 20 days of CORT injections failed to increase depression-like behaviour in female rats. Differences also emerge in terms of neurobiology. Although neurogenesis in the dorsal and ventral hippocampus is decreased in male rats in response to stress, neurogenesis is only decreased in the ventral hippocampus of female rats (Brummelte & Galea, 2010). Furthermore, repeated stress failed to produce atrophy of dendrites of pyramidal neurons in the hippocampus of female rats, while in male rats this effect was observed (Galea et al., 1997). These data suggest that females, despite having increased HPA axis activity, possess greater stress resiliency.

A potential explanation of female rodents enhanced stress resiliency might be explained by fluctuations in gonadal hormones. For example, during proestrus (high circulating estrogen levels), female rats display fewer depression and anxiety-like behaviours compared to both male rats and female rats in diestrus (Frye & Walf, 2002). In humans, females are at an increased risk of developing depression during the postpartum period, which is characterized by decreased estradiol levels (Brummelte & Galea, 2010). Finally, higher estrogen levels are associated with increased hippocampal cell proliferation and decreased cell death. Therefore, it appears that fluctuations in gonadal hormones might confer a neuroprotective effect, helping to explain the increased stress resiliency in female rodents. However, more work is needed in female rodents, as female humans are still 2 times more likely to develop depression than are men.

6.7. Future Directions

6.7.1. The Effects of CORT in aged rats

Depression is not restricted to the young. Rates of depression in older adults are similar in

prevalence to the rest of the general population (10.3%; Barua, Ghosh, Kar, & Basilio, 2011). Despite this, little work has been done on the effects of chronic stress in aged rats. The current dissertation used 2-3 month old rats, which corresponds to the young adulthood to adulthood phase in humans. It would be interesting to see the effects of repeated CORT injections in aged rats on measures of depression-like behaviour, for example anhedonia and despair, as it is known that the symptoms of depression in older adults differ from young adults. Older adults typically report greater degrees of aches and pain compared to young adults for example (Barua, 2011). It is well-known that neurogenesis decreases across the lifespan. An intriguing hypothesis would be that aged rats are more susceptible to the deleterious effects of CORT than young rats due to decreased levels of neurogenesis. A way to examine this would be to first determine levels of neurogenesis in aged rodents, and then breed for a line of aged rodents with consistently reduced levels of neurogenesis. These animals could then be exposed to repeated CORT injections to see if they are more susceptible compared to aged rats with higher levels of neurogenesis.

6.7.2. Determining the Timecourse of Reelin's Antidepressant Response

One of the most promising directions for future research generated from this dissertation is to examine the timecourse of reelin's antidepressant response. In Chapter 4, I examined the effects of reelin on neurobiological markers of hippocampal plasticity either 24 hours after the last infusion or 7 days after. However, the FST was only conducted the day after the last infusion, begging the question of what the rodents behaviour would look like on the FST after 7 days. Ketamine, which achieves similar antidepressant effects in rodents and humans, can sustain an antidepressant effect from a single infusion for as long as 2 weeks (Cusin, Hilton, Nirenberg, & Fava, 2012). Therefore, it would be interesting to see if reelin can achieve a similar antidepressant response 2 weeks after the last infusion. To test this hypothesis, a study could be conducted where, similar to Chapters 4 and 5, reelin is infused on day 21 of CORT injections. Then, different groups of rats could then be tested on the FST at day 22, sacrificed, another group only tested on the FST at day 23, sacrificed, and so on. This experimental design would allow for one to determine when reelin's antidepressant response ceases. This would be an interesting area of research to pursue, as the discovery of another compound that can achieve antidepressant effects after a single infusion for upwards of 2 weeks would generate intense interest in the greater scientific community.

6.7.3. Examining the Contribution of Neurogenesis to Reelin's Antidepressant Response

A particularly interesting finding from the current dissertation was the effects of reelin on hippocampal neurogenesis. In Chapters 4 and 5, I found that a single reelin infusion significantly increased the number, but not complexity, of adult-generated neurons in animals sacrificed immediately after the FST. These same animals showed reversals in immobility time on the FST. This suggests that neurogenesis itself might not be important in reversing total immobility time on the FST. However, we were not able to answer this casually. To approach this question, rodents could be used and exposed to irradiation to ablate hippocampal neurogenesis. These same animals could then be given reelin infusions and then tested on the FST. Such a paradigm would allow for one to casually determine if neurogenesis *per se* is required for reelin's fast-acting antidepressant response. Based off the findings from my dissertation, I would hypothesize that ablating hippocampal neurogenesis would not block reelin's antidepressant effects. Such a question is important, as the area of neurogenesis and depression is a contentious issue, with monthly reports finding altered neurogenesis in depression and others not finding any effect.

6.8. Conclusions

The purpose of this dissertation was to use a well-validated animal model of depression to evaluate the antidepressant effects of TNF-α and reelin on associated behavioural and neurobiological markers effected by stress. The main findings from this body of work was that CORT impaired hippocampal and PFC-dependent memory and produced alterations to prepulse inhibition. Moreover, I demonstrated that TNF-α inhibitors exhibited antidepressant effects in an animal model of depression which corresponded to restored cognitive functioning and normalized markers of hippocampal plasticity. Finally, I showed that reelin infusions have fast-acting antidepressant effects in an animal model of depression, accompanied by restored cognitive functioning and normalized markers of hippocampal plasticity. Importantly, I showed that reelin's antidepressant response requires a contribution of AMPA signaling. Collectively, my findings reveal novel compounds with antidepressant properties that warrant further investigation into their potential use in humans.

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